CYTOMEGALOVIRUS INFECTION

IN

RENAL TRANSPLANT RECIPIENTS

Two Volumes (Vol. 1)

by

JOHN DOLAN

A Thesis Presented for the Degree

of Doctor of Philosophy

in

The Faculty of Medicine

at the University of Glasgow

Institute of Virology,
Church Street,
Glasgow
G11 5JR,
Scotland.

July 1989

Dedicated to the staff and patients of the Renal Unit, Western Infirmary, Glasgow who all willingly participated in this project.

Contents

Volume 2

Contents

Acknowledgements

I express my gratitude to the following:-

Professor J.H. Subak-Sharpe for provision of the facilities of the Institute of Virology.

Dr J.D. Briggs for provision of the facilities of the Renal Unit, Western Infirmary, Glasgow.

Dr J.D. Briggs and Dr G. Clements who jointly supervised the project.

All patients and staff of the Renal Unit, Western Infirmary, Glasgow.

Mrs Jean Murray for her friendship, support and encouragement and to whom I shall always be indebted.

Dr R. Crawford, Consultant Haematologist, who kindly helped with the blood transfusion data.

Professor K. Whaley and Mrs J. Veitch of the Immunopathology Department, University of Glasgow who kindly measured IgM-RF levels.

Dr D. Millan who provided Figure 5.

Dr G.D. Murray of the Medical Statistics Unit, Western Infirmary, Glasgow for help with the statistical analysis.

Mr Cockburn of the Haematology Department, Western Infirmary, Glasgow who kindly provided serum samples.

Public Health Laboratory Service of England and Wales for provision of serum from renal donors immediately pretransplant and HCMV strain Davis.

Mr Eric Stewart of Abbott Laboratories for advice and provision of the β_2m RIA kit.

Dr U. Desselberger for advice and useful discussions.

Dr D. McGeoch of the Institute of Virology for the gift of E.coli JM101 and the M13 clone, JR139.

Dr J.D. Oram, Public Health Laboratory Service, Porton Down, Salisbury for provision of vector pEHBI.

Mr J. Aitken of the Institute of Virology for kindly providing particle counts.

The staff working in media and the washroom.

To all my other colleagues who helped in any small way but I am particularly grateful to Cathy Galt and to my friends and colleagues in the Virology Laboratory, Western Infirmary, Glasgow.

Last, but not least, I am particularly grateful to Mrs L. Peedle who conscientiously and efficiently typed this thesis.

The project was supported for 3 years by a grant awarded jointly to Dr J.D. Briggs and Dr G. Clements by the Scottish Hospital Endowments Research Trust. Financial assistance for a further 3 months was provided by the Western Infirmary Kidney Research Fund.

Except where otherwise specified all the results reported in this thesis were obtained by the author's own efforts.

Summary

Evidence of HCMV infection was assessed in a cohort of 47 renal allograft recipients followed prospectively for up to 1 year post transplantation. Serum was obtained from each patient and renal donor immediately pretransplant and tested for HCMV-specific antibody by standard or modified CFT and by Labsystems and NBL HCMV-IgG ELISA. Patients were classified as HCMV-seronegative if the antibody titre by CFT was less than 1:2. Whenever possible, a throat swab, urine and serum samples were taken from each individual immediately pretransplant and every 4 weeks thereafter. HCMV was isolated from specimens provided by 21 renal allograft recipients, 9 with primary HCMV infection and 12 with reinfection and/or reactivation. HCMV shedding usually continued intermittently throughout the first year post transplantation, isolates being obtained from multiple urine samples and/or throat swabs provided by 16 patients.

In each renal allograft recipient, the HCMV isolation data was correlated with the timing of the fourfold or greater rise in HCMV CF antibody titre and/or a significant increase in HCMV-IgM and IgG measured by Labsystems indirect ELISA. There was considerable variation between individual patients in the timing and pattern of the humoral immune response. On or before the day of first HCMV isolation, 12 renal allograft recipients showed a

fourfold or greater rise in HCMV CF antibody titre, 7 were positive for HCMV-IgM and 9 had evidence of a significant increase in the level of HCMV-IgG. These data show that serology alone is of limited value in the detection of active HCMV infection post renal transplantation. Taken overall, the CFT correlated best with HCMV isolation in transplant recipients with primary infection while a significant increase in HCMV-IgG correlated best with HCMV isolation in patients with reinfection and/or reactivation.

Clinical findings were also correlated with HCMV isolation and/or serological data. The small number of renal transplant recipients in this study precluded statistical analysis. However, ★ were more common in patients with primary HCMV infection. Furthermore, there was a close temporal correlation between the onset of symptoms and the HCMV-specific humoral response.

Whenever possible, blood products used to transfuse the cohort of renal allograft recipients were also tested for HCMV CF antibody and the results correlated with the age of the blood donors at the time of donation. Blood donors aged between 20 and 29 years provided 61 blood packs of which 46 (75.4%) were HCMV-seronegative. There was no evidence to suggest that HCMV was transmitted via blood products. Four renal allograft recipients of a kidney from an HCMV-seronegative donor each received HCMV-seropositive blood products but none developed primary HCMV infection post-operatively.

The HCMV serostatus was also available for 31 renal

★ leucopenia, thrombocytopenia, acute rejection, chronic rejection, nephrectomy and pyrexial illness

donors who provided kidneys for 37 renal allograft recipients. All 8 HCMV-seronegative recipients of a kidney from an HCMV-seropositive donor showed evidence of primary HCMV infection after the transplant operation. By contrast, none of the 9 HCMV-seronegative recipients of a kidney from an HCMV-seronegative donor developed HCMV infection throughout the period of study. These data provide further evidence that the donor kidney is the source of HCMV infection in renal transplant recipients.

The ability to detect viral DNA by hybridot assay was also investigated. In preliminary experiments using partially double stranded M13 probes specific for HSV-1, the lower limit of sensitivity was in the ng range. Denaturing agarose gel electrophoresis showed radiolabelled M13 second strand synthesis to be incomplete.

A BamHI subclone of the HindIII E fragment (EHBI) of HCMV AD169 was cloned in the transcription plasmid pGEM2 and ^{32}P-labelled cRNA synthesized. Optimal hybridization conditions were determined empirically. The sensitivity of the RNA-DNA hybridot assay was 8pg of cDNA after 24 hr of autoradiography. In reconstruction experiments, 4.3×10^4 to 3.9×10^5 particles taken from 3 independent stocks of HCMV AD169 and added to 7.2 ml of urine were routinely detected. Radiolabelled cRNA transcripts also detected 65 HCMV isolates from urine samples or throat swabs provided by 18 renal allograft recipients. The sensitivity of the hybridot assay was reduced by urinary DNase. In

reconstruction experiments, 10μg of either human or E.coli DNA added to urine containing HCMV particles or DNA reduced the hybrid signal. Radiolabelled cRNA transcripts did not hybridize to 10μg each of human, E.coli and HSV-1 DNA or 10μg of ribosomal RNA. However, 10μg of HSV-2 DNA gave a hybrid signal equivalent to that obtained with 8pg of vector pGHBI DNA. Four hundred and forty five urine specimens provided by 47 renal allograft recipients throughout the first year post transplantation were tested by RNA-DNA hybridization. When the results were correlated with HCMV isolation, the sensitivity and specificity of the hybridot assay was 25% and 67% respectively.

Abbreviations

A	adenine
ACV	acyclovir
ADCC	antibody-dependent cell-mediated cytotoxicity
ADT	adenosine 5'-diphosphate
ATG	antithymocyte globulin
ATP	adenosine 5'-triphosphate
AIDS	acquired immune deficiency syndrome
β_2m	β_2 microglobulin
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
CCV	channel catfish virus
CF	complement fixing
CFT	complement fixation test
Ci	curie(s)
CIAP	calf intestinal alkaline phosphatase
cm	centimetre(s)
CMV	cytomegalovirus
cpe	cytopathic effect
cpm	counts per minute
CsA	cyclosporin A
CTL	cytotoxic lymphocytes
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
DEA	diethanolamine

DEAFF	detection of early antigen fluorescent foci
DEPC	diethylpyrocarbonate
dGTP	2'-deoxyguanosine-5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	any 2'-deoxyribonucleoside-5'-triphosphate
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
E	early
EBV	Epstein-Barr virus
E.coli	Escherichia coli
EDTA	ethylenediamine tetra-acetic acid
EIU	enzyme immuno units(s)
ELISA	enzyme linked immunosorbent assay
FAT	fluorescent antibody test
fg	femtogram(s)
g	gram(s)
G	guanine
γGT	γ-glutamyltransferase
HCMV	human cytomegalovirus
Helu	human embryo lung
HLA	human leucocyte antigen
hr	hour(s)
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HVS	herpesvirus saimiri
^{125}I	radiolabelled iodine

IE	immediate-early
IFN	interferon
Ig	immunoglobulin
IgM-RF	rheumatoid factor of the IgM class
IHA	indirect haemagglutination
IL	interleukin
IMN	infectious mononucleosis
IPTG	isopropyl-D-thiogalactosidase
kb	kilobase(s)
kd	kilodalton(s)
kg	kilogram(s)
L	late
LB	Luria-Bertani medium
M	molar
MBq	megabecquerels
mCi	millicurie(s)
MCS	multiple cloning site
mg	milligram(s)
MHC	major histocompatibility complex
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
mmol	millimole(s)
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
mu	map unit(s)

MWt	molecular weight
NF1	nuclear factor 1
ng	nanogram(s)
NIEP	non-infectious enveloped particle
NK	natural killer
nm	nanometre(s)
NP40	nonidet P40
O.D.	optical density
OPD	o-phenylenediamine-2HCl
ORF	open reading frame
OSD	optimum sensitizing dose
oz	ounce(s)
^{32}P	radiolabelled phosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG6000	polyethylene glycol 6000
pfu	plaque forming unit(s)
pg	picogram(s)
pmol	picomole(s)
pNNP	para-nitrophenyl phosphate
poly A	polyadenylated
pp	polypeptide
PRV	pseudorabies virus
RF	rheumatoid factor
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	ribonuclease inhibitor

rNTPs	any ribonucleoside-5'-triphosphate
rpm	revolutions per minute
RQ1 DNase	RNase-free DNase
s	second(s)
S	sedimentation coefficient
^{35}S	radiolabelled sulphur
SDS	sodium dodecyl sulphate
SGOT	serum glutamic oxaloacetic transaminase
SGPT	serum glutamic pyruvic transaminase
SRBC	sheep red blood cell
SV40	simian virus 40
syn^+	wild type plaque morphology
T	thymine
TCA	trichloroacetic acid
TEMED	N,N,N',N''-tetramethyl-ethylenediamine
TRIS	tris(hydroxymethyl)aminomethane
U	unit(s)
UTP	uridine 5'-triphosphate
UV	ultraviolet
V	volt(s)
VB	veronal buffer
VBA	veronal buffer containing 0.1% (w/v) bovine serum albumin
v/v	volume/volume
VZV	varicella zoster virus
W	watt(s)
w/v	weight/volume
X-Gal	5-bromo, 4-chloro, 3-indolyl, β-D-galacto-

	pyranoside
μCi	microcurie(s)
μg	microgram(s)
μl	microlitre(s)
μm	micrometre(s)
μmol	micromole(s)

INTRODUCTION

1.1. General properties of human cytomegalovirus

1.1.1 History

The morphological features of HCMV infected cells were first reported many years prior to first isolation of the virus. HCMV characteristically produces cell enlargement with intranuclear inclusions. Such cells were first noted by Jesionek and Kiolemenoglou (1904) and later Ribbert (1904) in a variety of tissues from neonates and infants. Earlier workers regarded the inclusions as protozoan or syphilitic in origin. Subsequently, the similarities between these cells and those infected with HSV (Lipschutz, 1921) and VZV (Tyzzer, 1906) were recognised and led to the consideration of a viral cause.

The recovery of HCMV was first accomplished independently in 3 laboratories in the United States. Smith (1956) isolated the virus from the salivary gland and kidney of 2 moribund infants. Rowe et al.(1956) recovered 3 HCMV strains from surgically removed adenoid tissue of asymptomatic children. The commonly used laboratory adapted strain of HCMV (AD169) originated from these studies. Weller et al. (1957) isolated HCMV from liver biopsy specimens and/or urine samples provided by 3 infants with congenital HCMV disease. HCMV was initially called "salivary gland virus" or "salivary gland inclusion disease virus" (Cole and Kuttner, 1926). Weller et al. (1957) proposed the term "cytomegaloviruses" which is derived from "cytomegalia" originally used by Goodpasture and Talbot

(1921) to indicate the significant enlargement and alteration of infected cells.

The first evidence that HCMV infection was relatively common came from the work of Farber and Wolbach (1932) and in 1965 Klemola and Kaariainen described HCMV as one of the aetiologic agents of mononucleosis.

1.1.2 Morphology

HCMV is a member of a diverse group of viruses belonging to the family herpesviridae (Matthews, 1982) which has 3 subgroups designated alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae whose characteristics are described in Table 1. HCMV belongs to the betaherpesvirinae. Morphologically, HCMV is indistinguishable from other members of the herpesvirus group. The complete virion is 150 to 200nm in diameter, icosahedral in shape and consists of an inner core, a capsid and an envelope (Smith and Rasmussen, 1963; Montplaisir et al., 1972). The inner core is 64nm in diameter and consists of a linear double stranded DNA molecule of approximately 225 kb. The capsid is 110nm in diameter and has 162 capsomeres. The capsid in turn is surrounded by a poorly demarcated area, the tegument. Virions are enveloped by 2 discrete membranes, an inner one derived from the nuclear membrane and an outer one derived from the cytoplasmic membrane of infected cells. When HCMV is propagated in cell cultures 2 additional morphological

complexed helically with protein to form a core.

Table 1 Classification of herpesviruses according to biological properties

Properties	Herpesviruses		
	Alpha-herpes-virinae	Beta-herpes-virinae	Gamma-herpes-virinae
Examples	HSV-1	HCMV	EBV
Length of Cycle	Short	Long	No productive cycle in vitro
Cytopathic Effects	Rapid Extension	Slow Progression	—
Latent Infection	Nerve Ganglia	Numerous Tissues	Lymphoid Tissues

forms are evident. One is an homogenous electron-dense material which accumulates in the cytoplasm, especially in the Golgi region, of infected cells as viral capsids are assembled and enveloped (Ruebner et al., 1964; McGavran and Smith, 1965; Craighead et al., 1972). This dense material "buds" into cytoplasmic tubules similar to HCMV capsids forming circumscribed bodies having a surrounding membrane similar to the viral envelope. Viral nucleocapsids and dense bodies are both enveloped in the Golgi region of infected cells during the same period of the infectious cycle by mechanisms that are morphologically similar (Smith and De Harven, 1973). Nucleocapsids of HCMV are also seen budding from the nuclear membrane but envelopment of dense bodies by the nuclear membrane has not been detected. Dense bodies are of variable size but are usually twice the size of enveloped virions. They lack both a morphologically identifiable nucleocapsid (Smith and De Harven, 1973) and detectable DNA (Sarov and Abady, 1975). However, they contain surface antigenic determinants also detectable on the virions of HCMV as demonstrated by immune electron microscopy (Craighead et al., 1972).

Between 23 (Sarov and Abady, 1975) and 35 (Stinski, 1976) structural polypeptides ranging in MWt from 11 to 290 kd have been described in HCMV virions and dense bodies. Purified dense bodies have structural polypeptides with relative mobilities and molecular weights similar to the structural polypeptides of purified virions. However,in a study by Sarov and Abady (1975) dense bodies were noted to

completely lack 1 of the 23 polypeptides described in the whole virus while 5 others were barely detectable. The major polypeptide of dense bodies has a MWt of 67 kd and comprises 40% of the total protein complement. Stinski (1976) has shown by immunofluorescent studies that HCMV dense bodies do not contain host cellular antigens but are composed solely of protein considered to be specified by the HCMV genome. The biological significance of dense bodies is unknown but they may represent aberrant assembly of virion structural protein (Sarov and Abady, 1975) or envelope and tegument polypeptides (Fiala et al., 1976).

In 1983, Irmiere and Gibson described a third type of virus particle produced in HCMV-infected cells and which they called a non-infectious enveloped particle (NIEP). Although similar in appearance to virions, NIEPs are distinguished from them principally by their lack of DNA and therefore their non-infectivity. The absence of DNA in these particles is also thought to account for their lighter density and altered core appearance. Furthermore, the diameter of the NIEP capsid is 10 to 15% smaller than that of the mature virion. Such NIEPs are produced by all HCMV strains examined but have not been observed in HSV or Old World monkey CMV-infected cells. NIEPs are generally present in lower amounts than virions although HCMV strain AD169 overproduces NIEPs by approximately tenfold. Compared to virions, NIEPs contain between 10 and 30% less of the 74 kd and between 30 and 60% less of the 69 kd

matrix proteins. However, NIEPs contain a 35 kd protein which is not present in virions or dense bodies and which represents between 10 and 15% of the total protein mass (Irmiere and Gibson, 1983; 1985). This protein is thought to be the counterpart of the B-capsid protein of HSV (Gibson and Roizman, 1972; 1974) and CMV strain Colburn (Gibson, 1981b) since they share common properties. Therefore, it is proposed that the 35 kd protein present in NIEPs functions to mediate DNA packaging and/or nucleocapsid envelopment and in the normal course of events is modified or removed in the intact mature virion.

1.1.3 Genomic structure

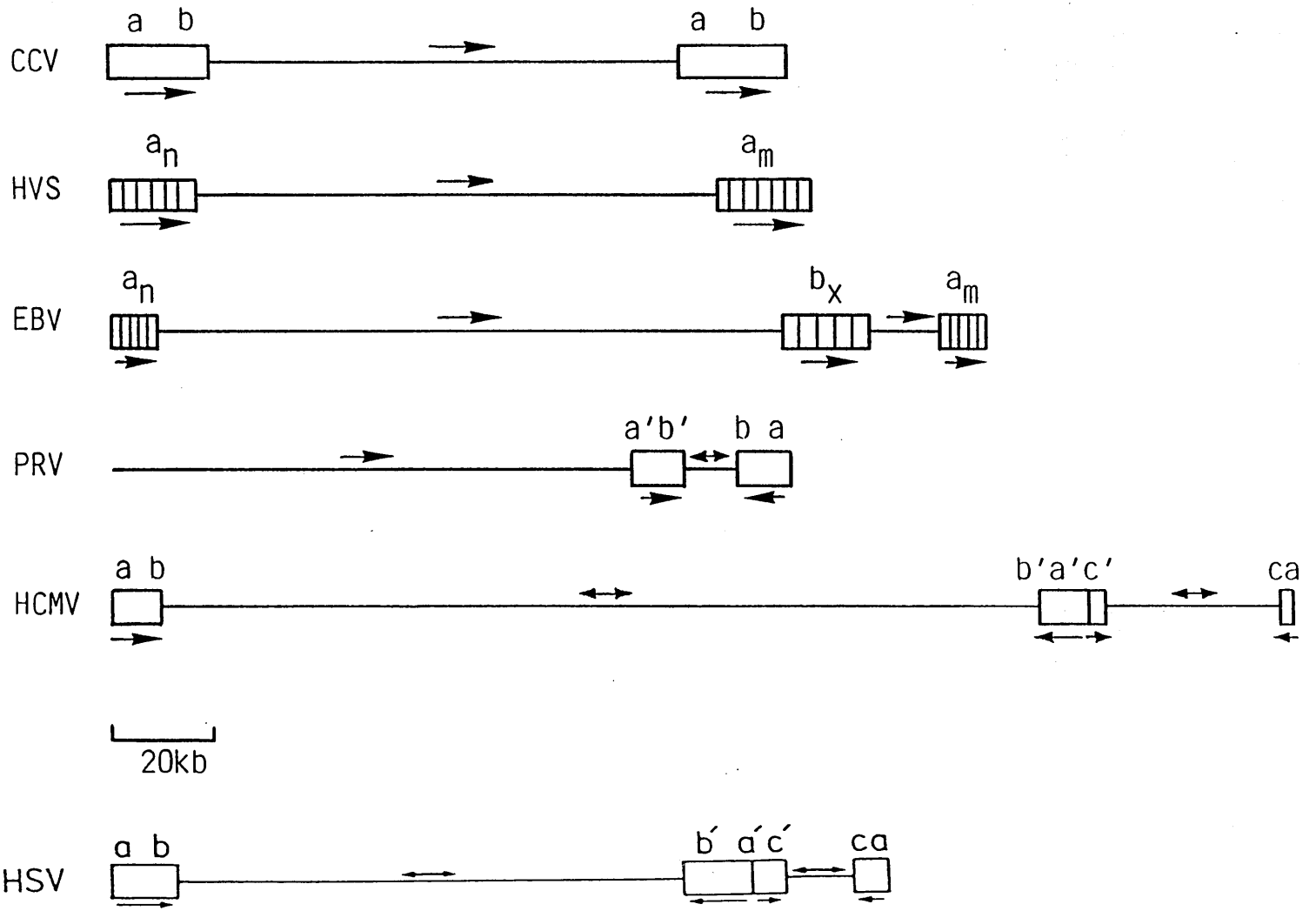
HCMV DNA was first isolated by Crawford and Lee (1964) and then by Huang et al. (1973). Like all herpesvirus DNA it was found to be linear and double stranded. Early reports describing HCMV DNA analysed by cosedimentation with HSV DNA in sucrose gradients (Huang et al., 1973) or by contour measurement (Sarov and Friedman, 1976) indicated that the MWt was 100×10^3 or $107 \pm 2.7 \times 10^3$ kd respectively. Further analysis by electron microscopy demonstrated 2 populations of molecules, a major class of 100×10^3 kd and a minor class of approximately 150×10^3 kd (Kilpatrick and Huang, 1977). In contrast, other workers found that viral DNA molecules of the larger size were more abundant as determined by electron microscopy (De Marchi et al., 1978; Geelen et al., 1978), sedimentation analysis (De Marchi et al., 1978) and reassociation

kinetics (De Marchi et al., 1978). The generally accepted value for the MWt of full length HCMV DNA is 150 x 10^3 kd. This population of larger DNA molecules is infectious (Geelen et al., 1978) whilst the smaller ones are not (Kilpatrick and Huang, 1977). Stinski et al. (1979) have shown that serial high multiplicity passage of HCMV strain Towne resulted in the production of defective cytomegalovirions which contained DNA with a MWt of approximately 100 x 10^3 kd. Additionally, some viral DNA molecules had a MWt of 60 x 10^3 kd. The accumulation of such smaller DNA molecules packaged into virions was associated with a decrease in infectivity but an increase in the particle-to-pfu ratio. It is proposed that deletions, substitutions and duplications are possible mechanisms for the generation of defective CMV DNA. HCMV has a buoyant density of 1.716 to 1.717 g/cm^3 (Huang et al., 1973; Geelen et al., 1978).

An important feature of herpesvirus genomes is that they contain various arrangements of repeat sequences (Figure 1). Demonstration of the existence of repeat sequences within the herpesvirus genomes stems from the work of Sheldrick and Berthelot (1974) on HSV-1 DNA. They performed alkaline denaturation of HSV-1 DNA followed by centrifugation through a neutral pH at high ionic strength in order to obtain single strands of DNA. Examination of such DNA strands by electron microscopy after self-annealing led to the description of 2 types of folding.

Figure 1 Genome structures representing five herpesvirus groups.

Genomes are drawn to scale. Boxed areas represent repetitive sequences and single lines indicate unique segments. The letters a, b and c show the arrangement of sequences within the repeats of each virus group; sequences arranged in inverse orientation relative to a, b and c are denoted by a', b' and c'. Arrows below boxed areas indicate the relative orientations of repeats. Tandemly reiterated sequences within repeats are shown by vertical lines within boxed regions. The number of copies of tandemly reiterated sequences is represented by subscripts (n, m and x) and different subscripts within a group indicate that copy numbers differ between repeats. Segments which invert are shown by double-headed arrows above unique sequences; unique sequences which do not invert are indicated by single-headed arrows. The key to abbreviations is CCV, channel catfish virus; HVS, herpesvirus saimiri; EBV, Epstein-Barr virus; PRV, pseudorabies virus; HCMV, human cytomegalovirus.


CCV
a b
a b
HVS
a_n
a_m
EBV
a_n
b_x
a_m
PRV
a'b'
b a
HCMV
a b
b'a'c'
ca
20kb
HSV
a b
b' a' c'
ca

Form 1 was a single stranded circle closed by a double stranded region linking the circle to a single stranded zone while Form 2 was two single stranded circles connected by a double stranded zone. By measuring the single and double stranded regions, the location of the repeat sequences was identified (Figure 2). La Femina and Hayward (1980) confirmed the presence of such sequences in HCMV DNA by electron microscopic examination of self-annealed single stranded DNA and by blot hybridization with restriction enzyme fragments. They showed that Towne strain HCMV DNA contained an internal, inverted duplication of 7.5% of its length.

From the above it was inferred that the HCMV genome can be divided into 2 regions of unique sequence, a long (U_L) and a short (U_S) one representing approximately 174 kb (82%) and 35 kb (18%) of strain AD169 DNA respectively (Fleckenstein et al., 1982). The junction between the 2 segments is composed of internal repeat (IR) sequences designated IR_L and IR_S.

IR sequences are also present but inverted at both ends of the DNA molecule and are then designated terminal repeats (TR). TR_L is at the end of U_L and TR_S at the end of U_S. The repeat sequences flanking U_L each approximate 11 kb while those at each end of U_S are about 2 kb.

Kilpatrick and Huang (1977) analyzed HCMV DNA which had been partially denatured at a temperature just below that leading to total strand separation. Under these

Figure 2 Structure of the HCMV genome

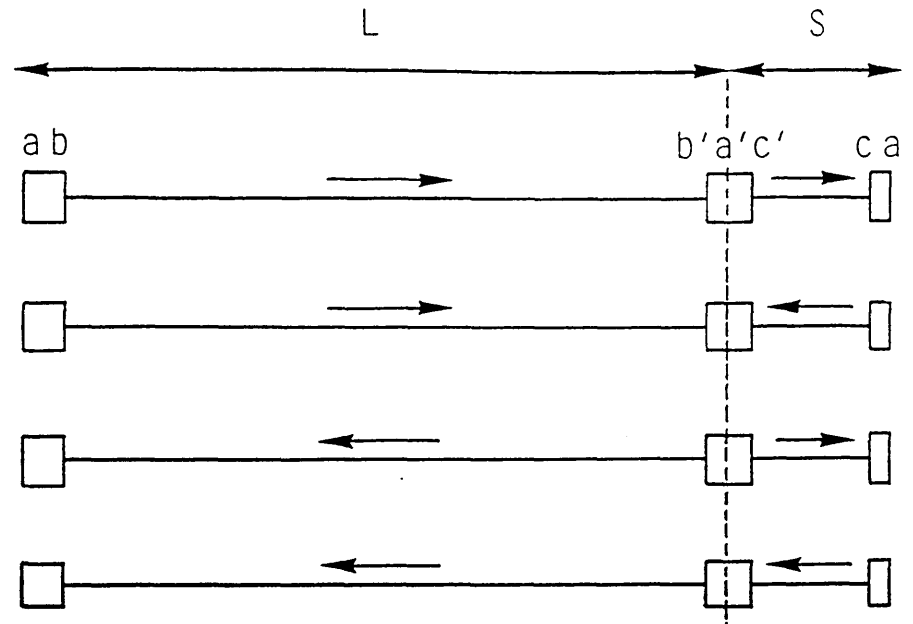
A diagram showing the organization of the HCMV genome. Segments of unique DNA sequence, the long (U_L) and short (U_S), are each flanked by a set of inverted repeats (TR_L/IR_L and TR_S/IR_S respectively). Letters indicate reiterated sequences. Isomerization at the L/S junction results in 4 different conformations.

P : prototype orientation

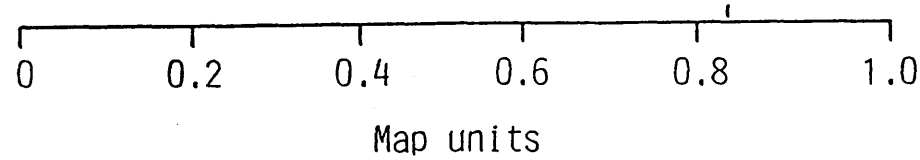
I_S : inversion of the short region

I_L : inversion of the long region

I_{SL} : inversion of the short and long regions.



TR_L	U_L	IR_L	IR_S	U_S	TR_S	
7.0	115	7.0	1.6	23.6	1.6	Megadaltons
10.6	174	10.6	2.4	35.6	2.4	Kb



conditions GC-rich regions of the DNA remain double stranded while AT-rich regions separate into single strands. Four different patterns of these single and double stranded zones were observed. The existence of the 4 isomers of HCMV DNA was confirmed by electron microscopic examination of self-annealed single strands (La Femina and Hayward, 1980) and by blot hybridization with isolated restriction enzyme fragments (De Marchi, 1981; Greenaway et al., 1982; Spector et al., 1982).

Weston and Barrell (1986) have determined the DNA sequence of U_S, IR_S/TR_S and part of U_L of HCMV AD169. Thirty eight open reading frames were identified most of which were preceded by sequences with homology to TATA boxes (Breathnack and Chambon, 1981) and were followed by polyadenylation signals. Twenty four of these open reading frames fell into 5 homologous families which presumably arose by duplication, a process which has not been described for HSV or VZV but has been observed in EBV (Baer et al., 1984). Although the short repeats of HSV, VZV and HCMV are organized in a similar manner, no homologies at the nucleic or amino acid level were noted. Hydrophobicity plots of the predicted protein products of U_S indicated that approximately half had hydrophobic profiles similar to 2 types of known membrane proteins, those with hydrophobic regions at their N and C termini which could be involved in membrane translocation and anchorage and those with several highly hydrophobic possible transmembrane regions.

Transcription of U_S is maximal at late (72 hr) times after infection (McDonough and Spector, 1983). From the sequence data it is difficult to predict the pattern of transcription as long range splicing (Bodescot et al., 1986) may occur. Since some potential open reading frames lack a TATA box it seems likely that splicing is involved in some cases.

At the ends of U_L and U_S are directly reiterated sequences which were demonstrated by Geelen and Westrate (1982) who showed that treatment of HCMV DNA with exonuclease III resulted in the formation of double stranded circular forms. These TR sequences which are designated ab in the L component and ca in the S component of the HCMV genome are also present in inverted orientation at the L/S junction where they are referred to as b'a' and a'c' respectively. The "a" sequence has important functions with respect to providing cis signals for cleaving/packaging DNA (Stow et al., 1983; Spaete and Frenkel, 1985; Spaete and Mocarski, 1985) and inversion of the virus genome during replication (Mocarski et al., 1980; Smiley et al., 1981; Mocarski and Roizman, 1982). Furthermore, the "a" sequences can undergo amplification resulting in several tandem copies which are probably responsible for the size heterogeneity of terminal and junction fragments described by a number of authors (Greenaway et al., 1982; Westrate et al., 1983). A short sequence of DNA homology which Mocarski et al. (1987) describe as the 'herpes pac homology' is highly conserved

amongst a number of herpesviruses (Davison and Wilkie, 1981; Mocarski and Roizman, 1981; Davison, 1984; Matsuo et al., 1984; Albrecht et al., 1985; Bankier et al., 1985; Deiss et al., 1986). The distance from this homologous region to the cleavage site is consistently between 30 and 35 bp in 5 human herpesviruses (Davison and Wilkie, 1981; Davison, 1984; Van den Berg et al., 1984; Mocarski et al., 1987). These findings are consistent with the observations of Spaete and Mocarski (1985) who showed that the HCMV L/S junction could complement HSV-1 packaging signals.

The "a" sequence of HCMV strains AD169 and Towne have been investigated (Tamashiro et al., 1984; Mocarski et al., 1987) and extensive homology demonstrated. However the "a" sequence of Towne strain is not flanked by direct repeats as is that of AD169. Unlike HCMV AD169, all genomic termini of the Towne strain contain at least 1 "a" sequence. Furthermore, the degree of heterogeneity is more complex in the "a" sequence of HCMV AD169. Tamashiro et al. (1984) demonstrated 3 classes of heterogeneity: Class 1 = heterogenous fragments of variable size (0.553, 0.95 or 1.35 kb); Class 2 = tandem duplications of Class 1 fragments; Class 3 = a variable number of 0.2 kb fragments in tandem. Recently, Takekoshi et al. (1987) have described a new HCMV isolate (strain Tanaka) whose L component was composed of 2 subsegments L1 and L2. L2 underwent inversion relative to L1 at high frequency to produce 8 genome isomers. The sequences flanking L2

consisted of inverted repeats unrelated to the "a" sequence.

1.1.4 Replication of HCMV

(a) Normal growth cycle

CMV is highly species specific and therefore human strains will only replicate in cells of human origin. *In vivo*, HCMV can replicate in epithelial (kidney, liver, bile ducts, salivary glands, gut epithelium, pancreas) and endothelial cells while *in vitro* HCMV replicates more efficiently in fibroblasts than epithelial cells (Knowles, 1976; Vonka *et al.*, 1976).

Many light and electron microscopic studies have outlined the course of HCMV infection in permissive cells (Iwasaki *et al.*, 1973; Smith and De Harven 1973, 1974; Albrecht *et al.*, 1980). Within 15 min of inoculation the virus attaches to the cells of the monolayer. Enveloped and non-enveloped virus capsids are detectable in the cytoplasm within minutes of inoculation. The viral capsids migrate to the cell nucleus after which enveloped viral particles are not detectable for approximately 36 to 48 hr, a period called the "viral eclipse". Subsequently, margination of the cell nucleus together with enlargement and rounding of both the nucleus and its cell occur. Novel chromatin structural patterns consisting of bipartite and oblate ellipsoid structures arranged in an orderly fashion along a fibre axis have also been observed (Kierszenbaum and Huang, 1978). The first evidence of new viral assembly is detected in the form of intranuclear and then

* De Marchi (1983a) has reported that cells productively infected with HCMV produced an IE protein which stimulated host cell functions while an E protein subsequently switched off the reaction. One host function which is activated in HCMV-infected cells is thymidine kinase and HCMV may employ this as a mechanism of increasing intracellular thymidine monophosphate concentrations (De Marchi, 1983a). Griffiths and Grundy (1987) have suggested that the continuous activation of host cellular synthesis in cells abortively infected with HCMV may be explained since, in such cells, HCMV genome expression may not proceed as far as the E protein required to inhibit stimulation.

intracytoplasmic viral capsids. Complete virions are first observed extracellularly between 72 and 96 hr after inoculation (St. Jeor and Rapp, 1973a; Michelson-Fiske et al., 1977). Between 5 and 8 days after inoculation cell lysis is observed.

(b) Alteration in host cell functions

HCMV induces the synthesis of cellular DNA in both permissive Helu cells and nonpermissive Vero cells (St. Jeor et al., 1974) although the degree of stimulation of host cell DNA synthesis depends on the physiological state of the infected cells (De Marchi and Kaplan, 1977). St. Jeor et al. (1974) have also shown that HCMV DNA synthesis is not required for the stimulation of host cell DNA synthesis. Indeed, HCMV which has been subjected to low dose UV irradiation shows an enhanced ability to induce host cell DNA synthesis (De Marchi and Kaplan, 1977; Boldough et al., 1978; De Marchi, 1983a) and defective virions produce a growth factor which enhances cellular DNA synthesis (Gonczol and Plotkin, 1984). However in productively infected Helu cells, viral antigens could not be detected by immunofluorescence in those cells which had been stimulated to synthesize cellular DNA (De Marchi and Kaplan, 1976; 1977). These data suggested that the induction of cellular DNA synthesis delayed or inhibited productive HCMV infection.*

HCMV also stimulates host cell RNA synthesis in permissive and nonpermissive cells (Furukawa et al., 1975;

Tanaka et al., 1975). This induction of RNA synthesis precedes viral DNA synthesis by approximately 24 hr and the RNA species synthesized include ribosomal 28S and 18S, and 4S transfer RNA. Tanaka et al. (1975) have suggested that host cell RNA synthesis is induced by a protein or proteins which are synthesized during the early stage of HCMV infection.

In HCMV-infected cells, protein synthesis is detected in 2 peaks, one during the initial 36 hr post infection and the other in the later stage of infection after the onset of viral DNA synthesis. At both stages host cell and viral proteins are synthesized simultaneously although host proteins account for 70 to 90% and 40 to 50% of the total proteins synthesized at early and late times respectively (Stinski, 1977).

The induction of host cell enzymes is selective and has been shown to be mediated through the regulation of transcription (Colberg-Poley and Santomenna, 1988). Poly-ADP ribose synthetase activity increases within 1 hr of inoculation of cells by HCMV and remains high throughout the replication cycle of the virus (Furlini et al., 1984). Between 3 and 6 hr post infection endogenous DNA-dependent RNA polymerase activities increase, RNA polymerase I, II and III being stimulated 6, 16 and 3 times respectively (Tanaka et al., 1975; 1978). At 12 hr post infection cytosol and mitochondrial thymidine kinase activities increase in addition to that of ornithine decarboxylase, the first rate-limiting enzyme in the biosynthesis of

polyamines (Isom, 1979). Synthesis of plasminogen activator, a protease involved in the degeneration of fibrin, transiently increases prior to viral DNA synthesis (Yamanishi and Rapp, 1979). Infection of human fibroblast cells by HCMV also leads to the stimulation of host cell DNA polymerase as well as the induction of a novel virus-specific DNA polymerase (Huang, 1975a; Hirai et al., 1976; Hirai and Watanabe, 1976). Much of the host cell DNA synthesised after HCMV infection is of mitochondrial origin (Furukawa et al., 1976). β-polymerase activity is unaffected by HCMV infection (Hirai and Watanabe, 1976). Weder and Radsak (1983) have also reported an "early" HCMV-induced glycopolypeptide found in association with chromatin preparations. It is suggested that this host-specific molecule may function in DNA replication in infected cells.

1.1.5 Replication of HCMV DNA

There have been few reports describing the replication of HCMV DNA but the similarity in genome structure between HCMV and HSV has tempted authors to propose the replication model for HSV which has been described by Jacob et al. (1979). Therefore, it has been suggested that HCMV replication occurs by the production of concatemers of several viral DNA molecules lined up end to end (La Femina and Hayward, 1983). Indeed, Jean et al. (1978) have observed concatemeric DNA molecules in WI-38 fibroblast

cells lytically infected with HCMV. The genomic localization, sequence and transcription analysis of the HCMV DNA polymerase gene has been reported (Heilbronn et al., 1987; Kouzarides et al., 1987).

1.1.6 Transcription and translation

Like other herpesviruses, the replicative cycle of CMV can be divided into 3 phases, immediate-early (IE), early (E) and late (L) (De Marchi, 1981; Wathen et al., 1981; Wathen and Stinski, 1982; McDonough and Spector, 1983; Jahn et al., 1984). IE transcripts are made in the absence of de novo protein synthesis e.g. in the presence of a cycloheximide block (Wathen and Stinski, 1982; Stinski et al., 1983) suggesting that host factors alone suffice for their expression. Early transcription occurs before the onset of viral DNA synthesis as determined by using phosphonoacetic acid to specifically block viral DNA polymerase (Huang, 1975b). Late transcripts appear after viral DNA synthesis.

(a) Immediate-Early

Major IE transcription of HCMV originates from the U_L region of the viral genome (De Marchi, 1981; Wathen and Stinski, 1982; McDonough and Spector, 1983; Stinski et al., 1983; Jahn et al., 1984; Wilkinson et al., 1984) and predominantly from the HindIII E region in HCMV strain AD169 (McDonough and Spector, 1983; Jahn et al., 1984; Wilkinson et al., 1984). Therefore, although HCMV and HSV

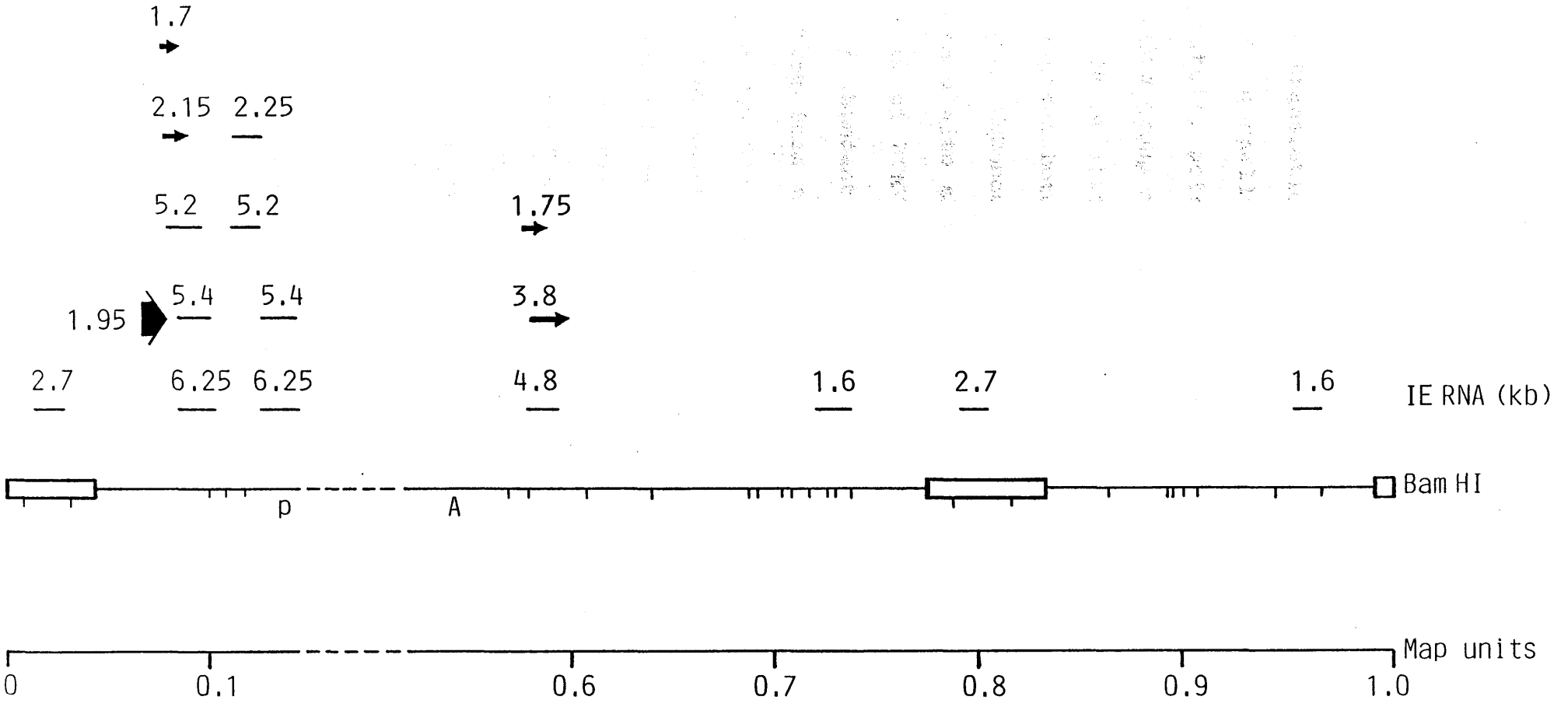
are similar in overall genomic structure, these viruses show major differences in the map positions of their IE genes: While IE transcription of HCMV is limited to a 20 kb segment of U_L, IE transcripts of HSV originate primarily from the inverted repeats of U_S and adjacent U_S segments (Clements et al., 1977; Anderson et al., 1980; Watson et al., 1981). Figure 3 shows the major transcripts of HCMV IE RNAs. In strain AD169, the most abundant IE mRNA is a 1.95 kb transcript transcribed from left to right between 0.0764 and 0.0865 mu in the prototype orientation. The major IE transcripts of HCMV strains Towne and Davis (1.95 kb and 2.2 kb respectively) map in equivalent regions of the genome (De Marchi, 1981; Wathen and Stinski, 1982; De Marchi, 1983b; Stinski et al., 1983). Two other dominant transcripts are synthesized during the IE phase of viral replication in HCMV AD169-infected cells. One (1.7 kb) and the other (2.15 kb) are both initiated between 0.08 and 0.105 mu and in the same orientation as IE 1.9 kb RNA. Stinski et al. (1983) also identified an IE transcriptionally active region (IE-2) immediately downstream of the major IE gene (IE-1) of HCMV Towne strain but the RNA produced differed in both size and orientation from the transcripts observed with strain AD169.

A second transcriptionally active area of HCMV AD169 has been identified between 0.593 and 0.619 mu (Wilkinson et al., 1984) and minor transcripts for the Towne and Davis strains have also been described as originating from equivalent regions of the viral genome (Wathen et al.,

Figure 3 Location of IE transcription on the HCMV genome

A summary showing size, map location and, in some cases, orientation of IE transcripts. The region of the genome omitted from this map, between BamHI P and BamHI A, shows no significant hybridization with cytomegalovirus poly(A)$^+$-RNA.

(From Wilkinson et al., 1984)


1.7
2.15
2.25
5.2
5.2
1.75
1.95
5.4
5.4
3.8
2.7
6.25
6.25
4.8
1.6
2.7
1.6
IE RNA (kb)
Bam HI
p
A
Map units
0
0.1
0.6
0.7
0.8
0.9
1.0

1981; De Marchi, 1983b). Sequence and transcriptional analysis of this region has identified 3 abundant RNAs of 1.65, 1.7 and 3.4 kb (Kouzarides et al., 1988). The putative translation product of the 3.4 kb transcript has features normally associated with membrane bound glycoprotein, potentially the first example within the herpesvirus group of such a protein to be transcribed at IE times. There also appears to be transcription from the repeat regions bordering U_L of strain AD169 (Wilkinson et al., 1984; McDonough et al., 1985). Wilkinson et al. (1984) have also shown that transcription occurs from the U_S region of HCMV AD169 and Weston (1988) detected 4 extremely abundant differentially spliced IE transcripts originating from this region of the virus genome. Five other RNA species transcribed at IE times from the long repeated DNA sequences of strain Towne (Wathen and Stinski, 1982) have not been detected in cells infected with either the Davis (De Marchi, 1983b) or AD169 (Wilkinson et al., 1984) strains of HCMV.

In vitro translation of mRNA selected by hybridization to the most abundantly transcribed region of HCMV shows it to code for a polypeptide with a MWt ranging from 72 to 75 kd depending on the strain (Cameron and Preston, 1981; Gibson, 1981a) and differences in post translation modification (Michelson et al., 1979; Gibson, 1981a; Stinski et al., 1983). However, the major IE polypeptides of strains AD169, Towne and Davis do cross react

immunologically (Goldstein et al., 1982). The major IE polypeptide is found in cells within 1 hr of infection (Stinski, 1978; Blanton and Tevethia, 1981; Stinski et al., 1983). It is a phosphorylated protein (Gibson, 1981a) which accumulates primarily in the nucleus (Tanaka et al., 1979) and is chromatin associated (Stenberg et al., 1984). Both the phosphoprotein and its mRNA reach maximal levels between 0 and 5 hr post infection and decrease to virtually undetectable levels later in infection (Stinski, 1978; Stenberg and Stinski, 1985). The observation that IE proteins of CMV increase chromatin transcriptional activity and alterations in chromatin confirmation within 1 hr of infection (Gibson, 1981a) suggest that the major IE protein may well be a regulatory protein. Munch et al. (1988) have shown that the MCMV IE-1 protein (pp89) is not able to bind DNA directly but pp89-DNA interaction can be mediated by histones. IE-1 and IE-2 proteins independently function as trans-activating factors for E gene viral transcription (Hermiston et al., 1987; Tevethia et al., 1987).

Jahn et al. (1984) have identified a 5 kb IE transcript which is distinguished from other IE transcripts by expression during the IE and L phases of virus replication. Furthermore, this RNA represents an unprecedented case of a large non-coding transcript present in cells that are lytically infected by an animal virus (Plachter et al., 1988). De Marchi (1983b) has also described a 4.8 kb IE RNA transcript confined to the nuclei of infected cells and which is transcribed from IE to L

times after infection. A 2.7 kb RNA transcript has been identified by Wilkinson et al. (1984) at both IE and E times after HCMV AD169 infection of MRC-5 cells but it is not clear whether there was a low level read through of the cycloheximide inhibition resulting in limited early transcription. The IE-2 region of Towne strain HCMV is known to code for a 2.7 kb RNA species which was noted to be most abundant at L times after infection (Stenberg et al., 1985). These data suggest that some IE transcripts are exempt from the general rule of temporal transcription regulation in herpesvirus replication.

The major IE gene of HCMV strains AD169 and Towne have been sequenced (Stenberg et al., 1984; Akrigg et al., 1985). Both genes encode for a spliced RNA molecule containing 4 exon sequences and which is 1736 nucleotides and 1341 nucleotides in strains AD169 and Towne respectively. Both mRNA species have 1 open reading frame which begins within the second exon and extends for 491 amino acids. The predicted MWt of the polypeptide is 64 kd which is between 8 and 11 kd less than that estimated by denaturing polyacrylamide gel electrophoresis. This discrepancy is accounted for by the high density of proline residues at the amino terminal which impart a β-pleated structure and which may be responsible for anomalous migration of the protein during gel electrophoresis.

In HCMV strains AD169 and Towne a promoter-regulatory region has been identified as extending 509 and 465

nucleotides respectively beyond the transcriptional start site (Thomsen et al., 1984; Akrigg et al., 1985). Deletion studies within this region have indicated that CAAT and TATA boxes (Cordon et al., 1980) within 68 bp of the mRNA 5' terminus are required for a minimum level of transcription (Stinski and Roehr, 1985). Although the HCMV IE promoter is a strong promoter (Sinclair,1987), sequences upstream of the CAAT box function to enhance downstream expression of the HCMV IE gene. Indeed, Boshart et al. (1985) have identified a strong transcription enhancer upstream of the transcription initiation site of the major IE gene of HCMV AD169 between nucleotides -524 and -118 relative to the cap site. This regulatory region has a series of repeat sequences referred to as the 16-, 18- and 19- nucleotide repeats which are each reiterated 4 times. The 18 bp repeats have some homology to the enhancer core sequence found in many enhancer elements, including SV40 (Laimins et al., 1982; Weiher et al., 1983). In addition, there is a 21-nucleotide sequence which is repeated twice (Thomsen et al., 1984). A direct correlation has been established between the 18- and 19- nucleotide repeats and the level of downstream expression but the 16-nucleotide sequence has been shown to be unnecessary for the enhancement of downstream transcription (Stinski and Roehr, 1985). It has been suggested that the 19-nucleotide repeat sequences may interact to form a cruciform structure, this secondary conformation facilitating an entry site for some component(s) of the transcriptional machinery (Thomsen et

al., 1984). Indeed, 5 constitutive DNase I hypersensitive sites have been identified at nucleotides -525, -425, -375, -275 and -175 relative to the cap site of the major IE RNA of HCMV strain AD169 (Nelson and Groudine, 1986). Such DNase I-hypersensitive sites have also been found in enhancer elements of eukaryotic genes (Parslow and Granner, 1982) and other viruses (Cremisi, 1981; Schubach and Groudine, 1984) controlling active transcription and are thought to represent structural changes in the chromatin due to complexing with regulatory proteins. Furthermore, Hennighausen and Fleckenstein (1986) have identified 4 high affinity nuclear factor 1 (NF1) binding sites in the promoter-regulatory region of HCMV strain Towne between nucleotides -780 and -610 and another in the first intron at nucleotide +350 with respect to the transcription initiation site of IE-1 RNA. NF1 is a sequence-specific DNA-binding protein which is required for the *in vitro* replication of adenovirus DNA (Nagata *et al.*, 1982; 1983). The NF1 binding sites in the major IE gene of HCMV strain Towne coincide with regions known to be sensitive to DNase in the active gene. However, no NF1 binding sites have been observed in the DNase I-hypersensitive transcription enhancer of the major IE gene (Hennighausen and Fleckenstein, 1986). NF1 binding sites surrounding the major IE promoter of both SCMV strain Colburn and HCMV strain Towne have been reported by Stinski and Roehr (1985) who also demonstrated virus-specific transactivation of the IE-1 promoter-regulatory region in the Towne strain of HCMV.

The major IE protein is also known to regulate its own expression (Stenberg and Stinski, 1985).

(b) Early

The E period of HCMV transcription begins at 8 hr and ends at 27 hr after the onset of HCMV infection. This period extends from the onset of virus coded protein synthesis to the start of virus DNA replication.

McDonough et al. (1985) have identified a single transcription unit in IR_L/TR_L which is active at E times in cells infected with HCMV AD169. Hutchinson et al. (1986) have described the size, temporal expression, gene organization and direction of transcription of the 3 major transcripts from HCMV IR_L. These 3 transcripts in order of their relative abundance are 2.7, 1.2 and 2.0 kb. The genomic sequences encoding the 2.7 kb E transcripts are unspliced and approximately 2.4 kb in size with the corresponding message containing a polyadenylated tail of approximately 300 bp (Greenaway and Wilkinson, 1987). A similar transcript has also been detected in cells during the E phase of infection with HCMV strain Towne (Wathen and Stinski, 1982).

The 2.7 kb E gene promoter upstream of the transcription initiation site lacks the numerous direct and inverted repeat sequences which are present in the corresponding region of the HCMV major IE gene (Akrigg et al., 1985). However 2 sets of imperfect direct repeats, one 9 bases and another 12 bases, each repeated twice have

been observed. Twenty seven nucleotides upstream of the transcription initiation site is a consensus sequence for a TATA box. The best open reading frame correlated with the 2.7 kb E gene encodes a predicted polypeptide of 170 amino acids which has not yet been identified in infected cell extracts. However, the rapid and efficient synthesis of the 2.7 kb transcript during productive infection indicates that it encodes an important viral function.

Hutchinson and Tocci (1986) have investigated a 1.2 kb RNA transcript which is synthesized during E and L times after HCMV infection (McDonough et al., 1985). This transcript maps between 0.792 and 0.797 mu in the long inverted repeat of the HCMV strain Eisenhardt genome. A single large ORF consisting of 254 amino acid codons has been identified within the 1.2 kb mRNA, translation of which would result in the synthesis of a highly basic 30 kd protein. No function yet has been attributed to this protein although the amino acid composition is similar to that of the HCMV 67 kd L protein which is part of the virus tegument (Davis and Huang, 1985). This 67 kd protein has protein kinase activity and binds to DNA. The 30 kd protein may also be a DNA binding protein which would correlate with the temporal expression of the 1.2 kb mRNA which is first detected prior to DNA synthesis and whose expression increases at L times after infection when the number of virus genomes is also increasing (Hutchinson et al., 1986). A viral protein has not yet been related to the 2.0 kb E RNA.

The functional role of most proteins synthesized during the E period of HCMV transcription remains to be elucidated. Some of them play a role in the induction of host cell macromolecular synthesis (Furukawa et al., 1973; 1975; 1976; St. Jeor et al., 1974; Tanaka et al., 1975; Garnett, 1979) and the development of cpe (Furukawa et al., 1975; Albrecht et al., 1983). Viral DNA polymerase and a virus-induced protein kinase also make their appearance during this period (Huang, 1975a; Hirai et al., 1976; Miller and Rapp, 1976; Nishiyama et al., 1983; Michelson et al., 1984). One E protein (ICP 22) described by Mocarski et al. (1988) is released from cells as a soluble protein and is highly immunogenic in both humans and mice.

(c) Late

Late transcription occurs after viral DNA synthesis. At this time more than 90% of the genome is transcribed into stable RNAs which become associated with polyribosomes in various proportions (Chua et al., 1981; De Marchi, 1983b). The proteins coded for by L mRNA are structural proteins and at least 35 have been described (Sarov and Abady, 1975; Fiala et al., 1976; Kim et al., 1976; Stinski, 1976; 1977; 1978; Gupta et al., 1977). Nowak et al. (1984) localized the regions coding for 2 L proteins on the AD169 genome: A 71 kd protein (0.3 to 0.32 mu) and an abundant 65 kd polypeptide. Pande et al. (1984) located the gene coding for a 64 kd structural protein to a 0.5 - 0.51 mu fragment of Towne strain DNA, corresponding to the same

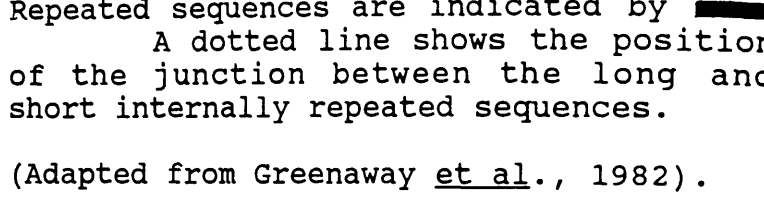
region as that described by Nowak et al. (1984). A DNA binding protein (ICP 36) has also been mapped to a single gene at 0.225 to 0.243 mu on the HCMV strain Towne genome (Mocarski et al., 1985). Expression of many HCMV transcripts is regulated by post transcriptional mechanisms (Wathen and Stinski, 1982; De Marchi, 1983b; Geballe et al., 1986). Even though the HCMV genome is extensively transcribed at E times after infection only a few of these transcripts are present on the polyribosomes or in the cytoplasm while at L times after infection transcripts from all regions of the genome are present on the polysomes or in the cytoplasm. Goins and Stinski (1986) have shown the expression of an HCMV L gene to be post transcriptionally regulated by a 3'-end-processing event involving either the selection of an upstream cleavage and polyadenylation site or the removal of a region of the primary transcript by alternative splicing.

1.1.7 Inter-strain variation

(a) Genomic

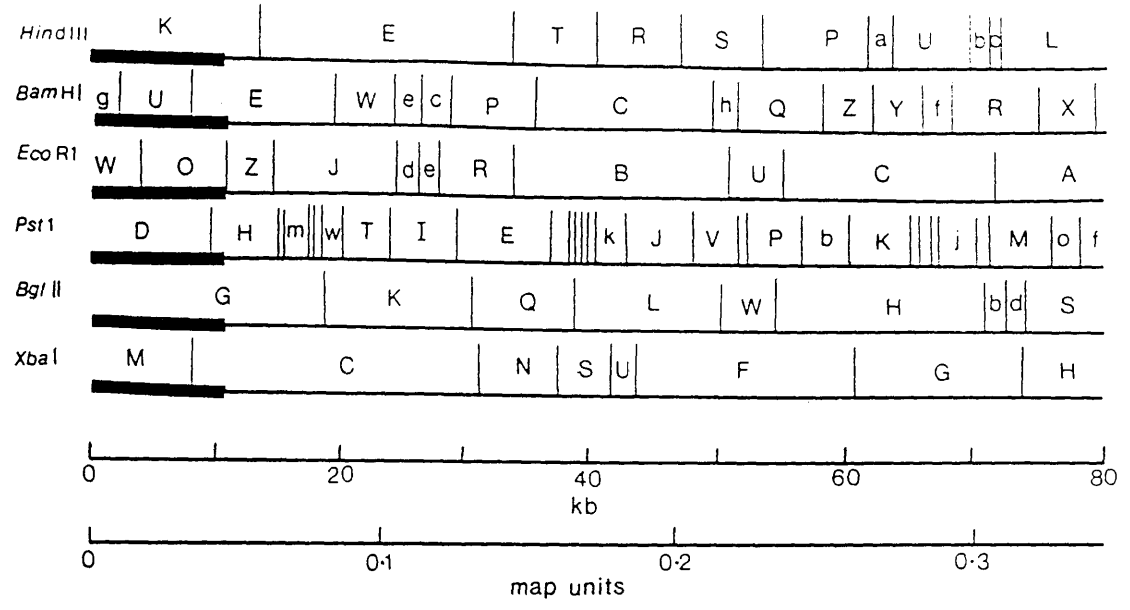
Physical maps of HCMV strain AD169 (Westrate et al., 1980; Fleckenstein et al., 1982; Greenaway et al., 1982; Oram et al., 1982; Spector et al., 1982), Towne (La Femina and Hayward, 1980) and Davis (De Marchi, 1981) have been constructed after digestion with several different endonucleases. Physical maps for the digestion products of HindIII, BamHI, EcoRI, PstI, Bg1II and XbaI of HCMV AD169 are given in Figure 4.

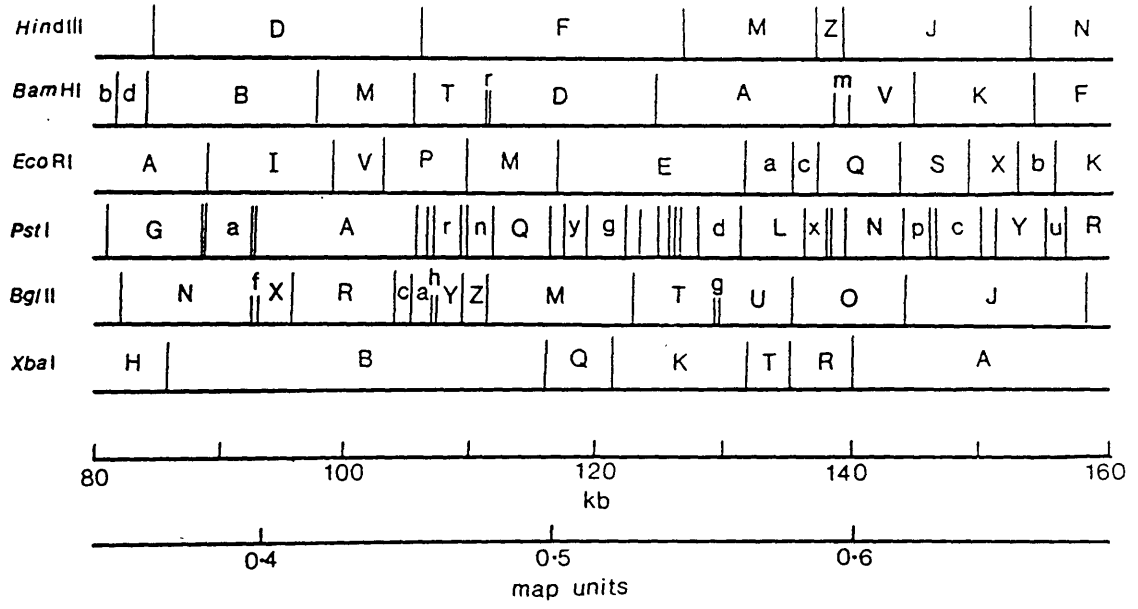
Figure 4 Map of the HCMV genome

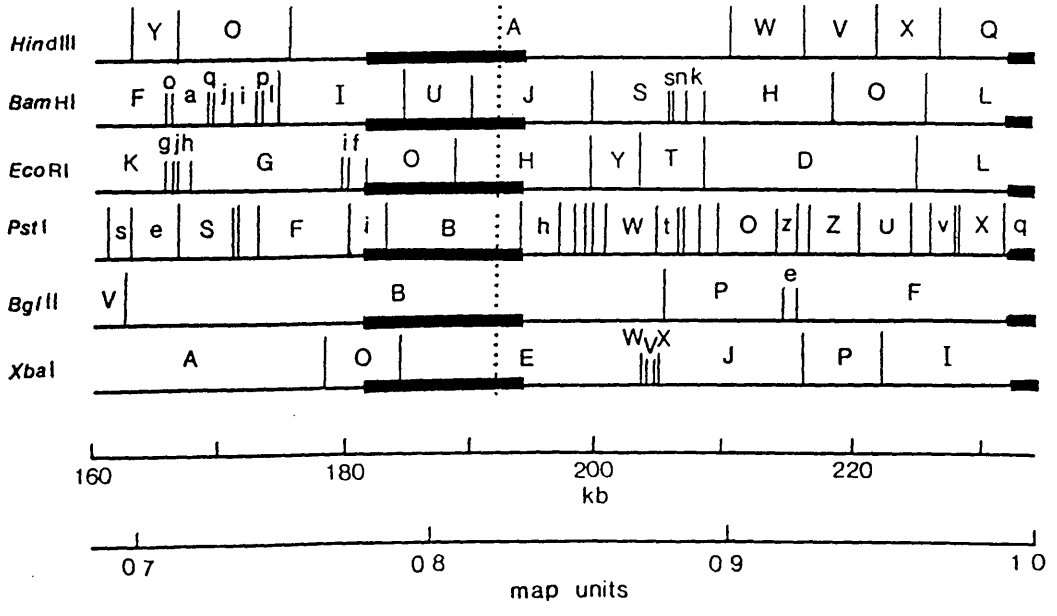


Restriction endonuclease cleavage map of HCMV DNA strain AD169 for the enzymes HindIII, BamHI, PstI, BglII and XbaI. The prototype orientation is shown. Repeated sequences are indicated by ▬ A dotted line shows the position of the junction between the long and short internally repeated sequences.

(Adapted from Greenaway et al., 1982).


HindIII
BamHI
EcoR1
Pst1
Bgl II
Xba I
0
20
40
60
80
kb
0
0·1
0·2
0·3
map units


HindIII
BamHI
EcoRI
Pst I
Bgl II
Xba I
80
100
120
140
160
kb
0·4
0·5
0·6
map units


HindIII
BamHI
EcoRI
Pst I
Bgl II
Xba I
160
180
200
220
kb
0 7
0 8
0 9
1 0
map units

Kilpatrick et al. (1976) compared the restriction enzyme profile of 11 strains of HCMV obtained after digestion with 2 enzymes and found that, although some bands co-migrated, no isolates had identical patterns unless they were epidemiologically related. It is not possible based on co-migration alone to decide whether sequences are homologous or whether co-migrating fragments are located in the same map positions. The same is also true for fragments which do not co-migrate. However, colinearity of HCMV genomes has now been confirmed (La Femina and Hayward, 1980; Westrate et al., 1983; Colimon et al., 1985).

Restriction endonuclease analysis of various HCMV isolates can yield information on the epidemiology of the virus. Huang et al. (1980) were the first to use this technique to study transmission of HCMV from infected mothers to their offspring. Other such studies have included the sexual transmission of HCMV (Handsfield et al., 1985), the reinfection and/or reactivation of HCMV after renal (Chou, 1986) and bone marrow (Drew et al., 1985) transplantation. The same technique has also demonstrated multiple infections in patients with AIDS (Drew et al., 1984; Spector et al., 1984a). HCMV restriction profiles remain stable during in vitro passaging (Doerr et al., 1979; Huang et al., 1980) although some profile modifications have been observed (Furukawa, 1984). Profiles of HCMV DNA isolated repeatedly in vivo

over long periods of time are uniform (Huang et al., 1976; Winston et al., 1985).

Kinetics of DNA-DNA reassociation in liquid medium have demonstrated that HCMVs share at least 80% homology (Kilpatrick et al., 1976). Pritchett (1980) has shown HCMV strains Towne and AD169 to share about 90% sequence homology. Most of the genetic variability and heterogeneity between CMV isolates exists within the repeat sequences (La Femina and Hayward, 1980; Westrate et al., 1983). The HCMV genome shares less than 5% nucleic acid homology with the DNAs of HSV-1, HSV-2, EBV, SCMV and MCMV (Huang and Pagano, 1974). Peden et al. (1982) and Ruger et al. (1984) have shown that AD169 DNA has sequence homologies to cellular DNA.

(b) Antigenic

Antigenic variation between HCMV strains has been studied by neutralization (Weller et al., 1960; Zablotney et al., 1978), complement fixation (Huang et al., 1976), immunodiffusion (Sweet et al., 1985) and immunoprecipitation (Gibson, 1981a; 1983). In 1960, Weller et al. proposed the classification of HCMV into 3 serotypes but more recent studies have not supported this view (Waner and Weller, 1978; Zablotney et al., 1978). The use of anti-HCMV monoclonal antibodies has also failed to permit a serotypic classification of the virus (Pereira et al., 1982b; Rasmussen et al., 1984b).

1.2 Latency

1.2.1 Definition

There are at least 3 mechanisms of viral persistence in the host. One is progressive infection characterized by tissue injury and organ dysfunction e.g. subacute sclerosing panencephalitis caused by measles virus (Horta-Barbosa et al., 1969). Some viruses e.g. HCMV may produce a cytolytic infection which is chronic and subclinical in which organ function is preserved (Ho, 1979). Finally, some viruses may persist in a "silent" state which is referred to as latency. Latent infection differs from acute or persistent infection by the absence of infectious virus but the continued presence of at least 1 other viral attribute (Hudson, 1979). Such a definition, however, depends on the sensitivity and specificity of the test used to define latency. Fluctuations in HCMV CF antibody titre in seropositive individuals has been interpreted as evidence for dynamic interaction between the host and latent virus (Waner et al., 1973).

During the past decade considerable effort has gone into defining experimentally the sites and mechanisms of latency for 2 herpesviruses, HSV and EBV. Stevens and Cook (1971) and Stevens et al. (1972) have demonstrated latent HSV in the spinal and trigeminal ganglia of mice and rabbits infected experimentally. These findings have been confirmed in humans (Bastian et al., 1972; Baringer and Swoveland, 1973; Baringer, 1974). It has been shown that EBV, which causes

infectious mononucleosis (Evans et al., 1968) and is a factor in the aetiology of Burkitt's lymphoma in humans (Zur Hausen et al., 1970; Nonoyama et al., 1973), establishes latent infection in B lymphocytes (Pattengale et al., 1974). EBV persists both by intracellular formation of a closed circular extrachromosomal plasmid and by linear integration of viral nucleic acid sequences into the DNA of host cells (Adams and Lindahl, 1975; Kaschka-Dierich et al., 1977). Following primary infection it is believed that HCMV remains latent in host cells. The evidence for this is described in Section 1.2.2.

1.2.2 Evidence for HCMV latency

The evidence that HCMV establishes latent infection is largely circumstantial. There is a high incidence (over 80%) and lifelong persistence of antibody to HCMV in the absence of detectable virus shedding within the general population (Weller, 1971). Numazaki et al. (1970) showed that there was a progressive increase in the rate of HCMV infection of the uterine cervix throughout the gestational period. All women had pre-existing serum antibody to HCMV and, since no woman seroconverted, it was concluded that cervical HCMV infection resulted from reactivation of latent virus. Patients with rheumatological disorders who receive immunosuppressive drugs are also known to reactivate HCMV from a latent state (Dowling et al., 1976).

Two decades ago Finnish physicians were the first to

document HCMV infections in transfusion recipients (Kaariainen et al., 1966) and HCMV viraemia has been demonstrated by cultivating leucocytes of previously healthy adults, children with congenital infection, patients with heterophil-negative mononucleosis, post perfusion syndrome and a number of immunosuppressive conditions (Diosi et al., 1969; Lang and Hanshaw, 1969; Armstrong et al., 1971; Fiala et al., 1973; Rinaldo et al., 1977). The virus resides in the blood leucocytes in a latent form because it cannot be recovered by cultivation of leucocytes from asymptomatic seropositive healthy donors. However, such cells are capable of transmitting HCMV (Klemola et al., 1969a; Mirkovic et al., 1971; Kane et al., 1975). Indeed, approaches to the prevention of primary HCMV infection have included the exclusive use of blood products from seronegative donors in neonates (Yeager et al., 1981) and recipients of cardiac (Preiksaitis et al., 1983) and bone marrow (Hersman et al., 1982) transplants. Fresh or stored blood or granulocyte transfusions can transmit the infection (Prince et al., 1971; Winston et al., 1980). The risk of transmission of HCMV by blood products is estimated at 2.7% per unit transfused (Armstrong et al., 1976; Singh et al., 1988a), the risk increasing with the number of units (Prince et al., 1971) and the volume (Meyers et al., 1983) of blood received. The quantity of blood required to transmit HCMV is unknown although 50 to 99 ml was sufficient to infect 3 of 18 neonates in a study by Yeager et al. (1981).

HCMV has been increasingly recognized as a cause of frequent and potentially serious infection in recipients of organ grafts. Abundant epidemiologic data indicate that HCMV can be transmitted from donors of transplanted organs to their recipients even though the donor has no evidence of HCMV-related disease and culture of the donated organ reveals no infectious virus. Specifically, when an organ from an HCMV-seropositive donor is transplanted into a seronegative recipient that recipient has a 70 to 80% risk of acquiring HCMV infection (Glenn, 1981). This has been confirmed for recipients of renal (Rubin et al., 1985; Smiley et al., 1985; Weir et al., 1987), cardiac (Chou, 1987; Chou and Norman, 1988; Wreghitt et al., 1988) and hepatic (Rakela et al., 1987; De Hemptinne et al., 1988; Singh et al., 1988a) grafts. Supportive data include the correlation between the incubation period of HCMV and the time interval from transplantation (the postulated inoculation) to the onset of clinical evidence of infection. Restriction endonuclease analysis of HCMV DNA from recipients of transplants from a given individual have indirectly established transmission of HCMV via renal (Wertheim et al., 1983; Chou, 1986) and heart (Chou, 1987) donors. Indeed, infection by HCMV strains from donors has also been demonstrated by restriction endonuclease cleavage patterns of virus isolated from HCMV-seropositive renal allograft recipients (Chou, 1986; Grundy et al., 1987). Not all HCMV-seropositive donors transmit the virus even to seronegative recipients (Warrell et al., 1980; Marker et

al., 1981; Walker et al., 1982). Chou and Norman (1988) have shown that only approximately 60% of HCMV-seropositive renal donors actually transmit the infection. This could be related to differences in immune function in the recipients or to differences in the content or biological properties of latent HCMV in seropositive donors. Using in situ hybridization, Schrier et al. (1985) found that some subjects had a higher content of hybridizable nucleic acid than others perhaps reflecting variation in the amount of latent virus in different persons. HCMV-seronegative recipients of an organ from donors known to be seronegative for HCMV rarely develop HCMV infection (Betts et al., 1975; Ho et al., 1975), nosocomial spread of the virus being uncommon in the context of organ transplantation (Meyers, 1984).

Although bone marrow latently infected with HCMV can transmit the virus (Meyers et al., 1986), the bone marrow donor is not an obviously important source of HCMV since donor serology does not predict the risk of developing HCMV infection after the transplant operation (Meyers et al., 1980; 1983; Hersman et al., 1982). Indeed, HCMV-seropositive bone marrow recipients develop HCMV infections nearly 3 times as frequently as seronegative ones (Miller et al., 1986). However, the acquisition of HCMV from multiple blood products may mask the contribution of the bone marrow donor (Bowden et al., 1985).

1.2.3 Attempts to demonstrate latent HCMV

The cell type in which HCMV persists or remains latent is unknown. There is evidence that lymphocytes may play a role in HCMV infection since individuals with HCMV mononucleosis have atypical lymphocytes and *in vitro* tests reveal depression of lymphocyte functions (Rinaldo *et al.*, 1977; 1980; Carney and Hirsch, 1981). Other authors (Fiala *et al.*, 1977; Howell *et al.*, 1979) have implicated granulocytes as a possible site of HCMV latency. In 1969, Diosi *et al.* reported the recovery of HCMV from cultures of leucocytes provided by 2 of 21 healthy HCMV-seropositive blood donors. However, in further large scale investigations involving 1500 blood donors HCMV was never recovered from leucocyte cultures (Mirkovic *et al.*, 1971; Perham *et al.*, 1971; Kane *et al.*, 1975; Bayer and Tegtmeier, 1976). Furthermore, attempts to cultivate HCMV *in vitro* in primary polymorphonuclear or mononuclear cells have failed to show appreciable virus replication (Rinaldo *et al.*, 1978; Einhorn and Ost, 1984) nor has it been possible to demonstrate HCMV replication in undifferentiated primary human bone marrow cells (Reiser *et al.*, 1986). Skin fibroblasts (Williams *et al.*, 1980), epithelial (Pagano, 1975) and prostatic cells (Rapp *et al.*, 1975) are also possible sites of HCMV latency.

Although there is epidemiologic evidence implicating the transplanted kidney as a source of HCMV infection, cultures of donor kidney specimens have been, with 1 exception, uniformly negative (Pien *et al.*, 1973; Betts *et*

al., 1975; Nankervis, 1976; Howard et al., 1977; Naraqi et al., 1978). The exception, the study by Orsi et al. (1978), reported the isolation of HCMV from the kidney in 8 of 10 renal donors. There is ample evidence that HCMV infects the kidney. HCMV-specific antigen (Payne et al., 1974) and characteristic inclusion bodies (Rifkind et al., 1967) have been identified in glomerular and tubular epithelial cells (Figure 5). HCMV has been isolated from allograft kidneys at biopsy or autopsy (Craighead et al., 1967; Spencer, 1974; Naraqi et al., 1977). Recently, Heieren et al. (1988) have shown HCMV to replicate efficiently in human kidney mesangial cells. The consistent inability to demonstrate latent HCMV in renal allografts by explantation could be explained by the tissue obtained not being representative of the entire organ or the explant procedure not being suitable for the demonstration of latent HCMV. Furthermore, the renal parenchyma may be an uncommon locus of latent infection.

Past attempts to define the site of HCMV latency have been hampered by several factors including the cryptic nature of the infection and the difficulty in detecting low levels of virus in a small percentage of cells. In recent years sensitive molecular techniques have been employed. Therefore, Pagano (1975) has been able to detect HCMV DNA in leucocytes of bone marrow transplant recipients at levels of 2.5 to 19 genome copies per cell. Lymphocytes bearing the OKT4 and OKT8 markers have been shown to

Figure 5 Manifestation of HCMV infection in the kidney

A section taken through a kidney showing

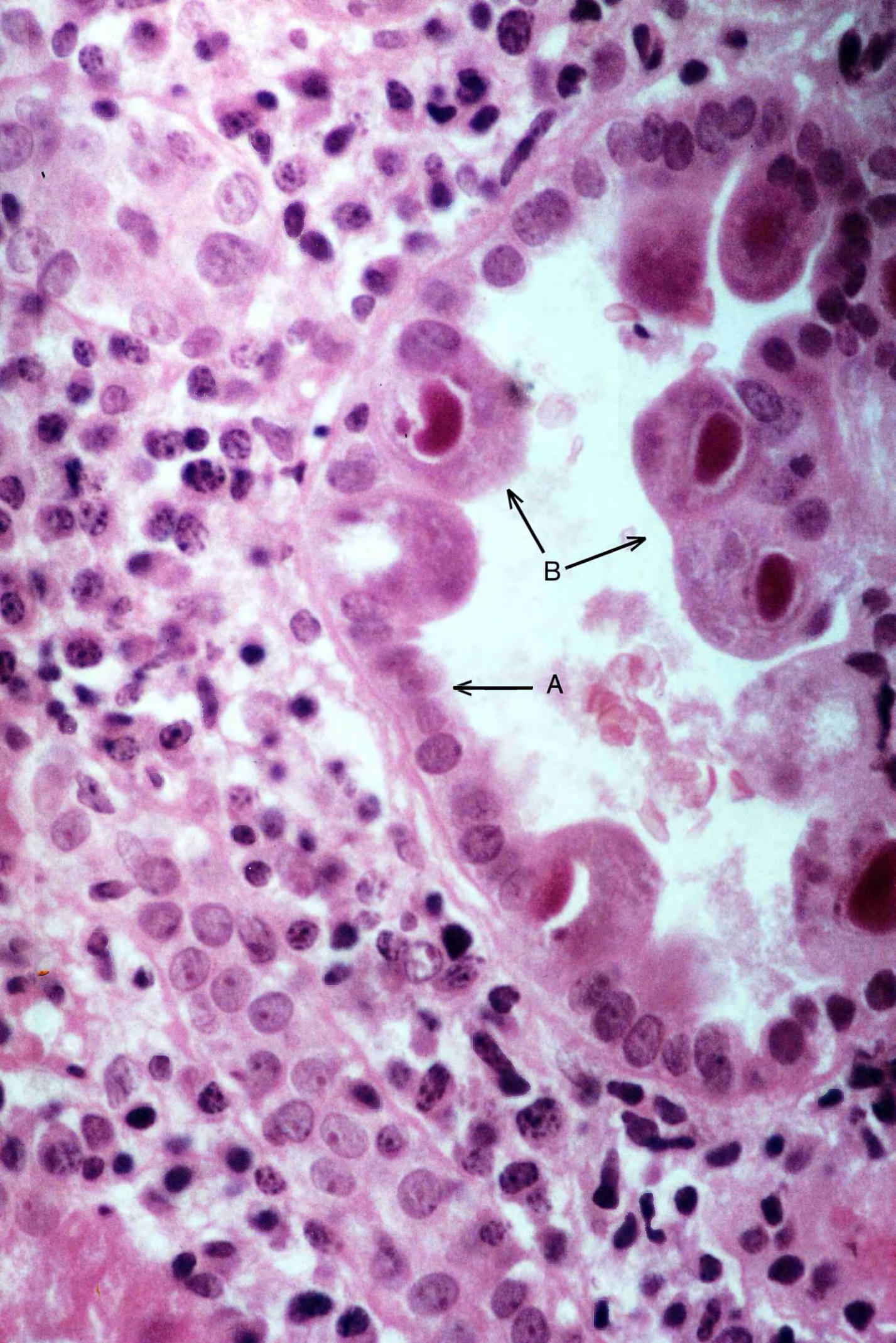
A: Normal tubular epithelium

B: Giant mononuclear tubular cells with characteristic cytomegalic inclusion bodies.

Stain: Haematoxylin and Eosin

Magnification: x 350

The photograph was very kindly provided by my colleague Dr David Millan, Pathology Department, Western Infirmary, Glasgow.


B
A

hybridize specifically to a portion of the HCMV genome which is heavily transcribed during the IE period of infection (Schrier et al., 1985). HCMV-specific sequences have also been identified in a number of locations in the kidney, including cells of the glomerulus and tubular epithelium (Myerson et al., 1984). Less than 1 genome equivalent per cell of HCMV has been demonstrated in 2 lymphoblastoid cell lines (Huang et al., 1978).

1.2.4 Experimental animal models of latent CMV infection

Many animal species e.g. mice, guinea pigs, rats, pigs and monkeys are naturally infected with their own strains of CMV (Weller, 1971) but the murine model of CMV latency has been studied in greatest detail (Olding et al., 1976; Brautigam et al., 1979; Hudson, 1979; Hudson et al., 1979; Cheung et al., 1980). Several variables are apparent including the age of the mice at the time of initial infection, the genetic strain of the mice used, the route of infection and whether live or attenuated virus was inoculated. However, Jordan et al. (1982) have shown that these factors do not directly affect the development of MCMV latency.

Intraperitoneal inoculation of MCMV results in the virus being harboured in a latent phase within peritoneal macrophages (Brautigam et al., 1979). Expression of latent MCMV required activation of the macrophages which contained 5 to 7 viral genome copies per 100 cells. Latent MCMV may persist in other tissues, nucleic acid hybridization having

detected 4.5 and 0.2 genome equivalents per cell of MCMV DNA in cultures of salivary and prostate gland cells respectively (Cheung et al., 1980). Other reports have indicated that MCMV can be reactivated from latently infected spleen cells (Mayo et al., 1977; 1978).

Hamilton and Seaworth (1985) have demonstrated the transmission of latent MCMV in a murine kidney tissue transplantation model. MCMV has also been reported to grow in primary and SV40-transformed kidney cells (Hudson, 1979). Hamilton and Seaworth (1985) have also shown that the transplanted kidney is a more efficient vector of MCMV transmission than transfused blood and that major histocompatibility complex matching and treatment with immunosuppressive drugs favour the reactivation of latent virus from within the renal allograft. Another study by Klotman et al. (1985) has shown that in mice latently infected with 1 strain of MCMV and transplanted with a kidney harbouring latent MCMV of another strain, reactivation of the endogenous recipient strain of MCMV was the source of active infection in 10 of 12 cases but at no time were exogenous and endogenous strains reactivated simultaneously. The donor kidney as the source of latent MCMV in the uninfected recipient was also confirmed. Porter et al. (1985) have shown that MCMV reactivated from renal tissue by explantation has the same restriction enzyme profile as the original infecting strain. Experimental mouse models of cardiac transplantation (Rubin

et al., 1984) and rat models of renal and cardiac transplantation (Bruning et al., 1985; 1988) have been described and confirmed the ability of latent CMV to be transferred to and reactivated in the allograft recipient.

Although animal models have revealed interesting information concerning the reactivation of latent CMV, there are a number of problems including the ability to differentiate when acute or chronic infection ends and latency begins. The small number of animals involved, the relatively brief time intervals used to suggest latency and the lack of firm evidence of latent infection are other areas of difficulty.

1.2.5 In vitro models of CMV latency

A number of in vitro latency systems have been developed. These eliminate the complexities of animal studies and provide a simple reproducible method to examine HCMV latency.

HCMV infection of permissive human diploid fibroblast cells results in the sequentially ordered expression of the virus genome, the generation of progeny virus and eventually cell death. To examine the establishment and maintenance of persistent and latent HCMV infections in vitro it is therefore essential to limit the expression of the virus genome. Various approaches have included the use of inhibitors of virus replication e.g. cytosine arabinoside (Gonczol and Vaczi, 1973), phosphonoformic acid (Gadler and Wahren, 1983) and human leucocyte IFN plus ACV

(Bucher et al., 1983). Other approaches have included the use of cells nonpermissive for HCMV replication (Fioretti et al., 1973) and defective HCMV populations obtained by serial passage at high multiplicity (Stinski et al., 1979). Mocarski and Stinski (1979) established persistent HCMV infections in human fibroblasts by high multiplicity infection and long term tissue culture. Cockley and Rapp (1986) have reported the establishment of an in vitro model in which the HCMV genome can be maintained for an extended period in a latent form by limiting the expression of the virus genome by a combination of IFN-α and ACV followed by incubation at 37^{o}C. Inhibitor removal and temperature shift to 40.5^{o}C resulted in a reduction in the retained HCMV sequences detected by blot hybridization to 0.5 copies of HCMV per haploid cell genome equivalent. Recently, Tanaka et al. (1988) have described the use of indomethacin and tetracaine, prostaglandin synthesis inhibitors, to establish a latently infected human thyroid papillary carcinoma cell line (TPC-1). Such in vitro latency models are likely to provide significant results with respect to the understanding of gene expression in nonpermissive cell lines.

1.2.6 Viral genome

Very little is known about the state of the viral genome during latency. Gadler and Wahren (1983) have provided evidence that HCMV DNA integrates into or is associated with a subset of cell DNA relatively rich in A-T

content. These authors suggest that viral genomes persist in the cells either as a continuous sequence or in a fragmented form. Cockley and Rapp (1986) have shown that only a few unique HCMV genome fragments were detected when virus was incubated at 40.5°C after removal of IFN-α and ACV. The physical state of the HCMV genome may influence the period of reappearance of infectious virus since Tanaka et al. (1987) have shown that reactivation of latent HCMV in vitro requires a much longer period of time than that required for the multiplication of superinfected HCMV.

1.2.7 Cellular factors

Tanaka et al. (1987) have suggested that the blockage of HCMV replication in the TPC-1 cell line during the latent period occurs early in the HCMV replication cycle since IE antigens were synthesized while an appreciable amount of DNA polymerase was not induced. This block may be a cellular function(s) which can be sensitive to temperature (Tanaka et al., 1987), prostaglandin synthesis inhibitors (Tanaka et al., 1988), UV irradiation (Furukawa et al., 1975) and iododeoxyuridine (St. Jeor and Rapp, 1973b). A host cell factor(s) may be required to regulate the viral genome for complete early gene expression or to provide crucial protein(s) for the initiation of viral DNA replication. There have been several reports on the influence of the cell cycle on virus production, yields of HCMV (Furukawa, 1979; Falcieri et al., 1982) and MCMV

(Kramvis and Garnett, 1987) being higher in cells challenged during the S and G-2 phases. A cellular differentiation step is also essential for replication of HCMV in human embryonal carcinoma cells (Gonczol et al., 1984) and human bone marrow cells (Reiser et al., 1986). Boom et al. (1988) have provided evidence that both the expression and activation of the IE enhancer-promoter occur in the absence of viral gene products thereby implying an important role for cellular functions in maintenance of the latent state and in the reactivation process. It is recognized that chromatin organization plays an essential role in the maintenance and establishment of the transcriptional state of cellular genes (Reeves, 1984; Weintraub, 1985). To maintain the repressed state of a gene throughout cell division its repressed conformation must remain intact throughout replication of the gene. This may be mediated by DNA methylation. However, Boom et al. (1987) have shown that the HCMV IE enhancer is refractory to DNA methylation de novo and have speculated that this may provide a molecular basis for the potential of HCMV to become activated even after a long latent period.

1.2.8 Reactivation of latent virus

CMV reactivates in response to a number of stimuli. Explantation techniques may lead to the reactivation of latent virus by removing cells from normal immunological constraints or by changing the cellular replication cycle

resulting in the production of substances vital for reactivation. Support for the latter theory comes from Dutko and Oldstone (1981) who showed that cellular differentiation promoted virus reactivation. In a murine model of cardiac transplantation the trauma of surgery and anaesthesia was itself capable of reactivating MCMV to produce subclinical primary infection (Rubin et al., 1984).

Allogeneic stimuli also promote the reactivation of latent CMV. In vivo, such stimuli include skin grafting (Wu et al., 1975), spleen cell transfer (Mayo et al., 1978) and solid organ and bone marrow transplantation (Craighead, 1969; Rinaldo et al., 1976; Gadler et al., 1982). It has also been postulated that donor leucocytes present in blood transfusions allogeneically stimulate recipient leucocytes thereby reactivating HCMV (Lang, 1972). In vitro allogeneic stimuli which have promoted the reactivation of latent HCMV include co-cultivation of histoincompatible cells (Olding et al., 1975).

Immunosuppressive therapy, which has no in vitro effect, reactivates HCMV from a latent state in vivo. In humans such drugs have included cyclophosphamide and azathioprine (Dowling et al., 1976) while mouse models have used antithymocytic serum (Gardner et al., 1974), antilymphocyte serum and cortisone (Jordan et al., 1977; Shanley et al., 1979) and azathioprine or cyclophosphamide (Mayo et al., 1978). The transient immunosuppressive effect of blood transfusions may itself suffice to

reactivate the recipients' endogenous virus. Recent indirect evidence suggests that reactivation accounts for most post transfusion HCMV infections in seropositive adults (Adler et al., 1985). Transfusion, especially of multiple units of blood, leads to various immunological aberrations including production of cytotoxic antibodies to lymphocytes (Mohankumar et al., 1981), depressed NK cell function (Gascon et al., 1984), abnormal immunoglobulin levels (Ballas et al., 1980) and decreased in vitro T cell responses to foreign antigens (Munn et al., 1981).

1.3 Organ Transplantation

Despite the technical and immunologic advances in recent years, infection remains a major barrier to success in organ transplantation. More than 80% of renal allograft recipients experience at least 1 episode of infection within the first year after the transplant operation and infection remains the major cause of death at all stages post-operatively (Rubin and Tolkoff-Rubin, 1988). A summary of these infections is shown in Figure 6.

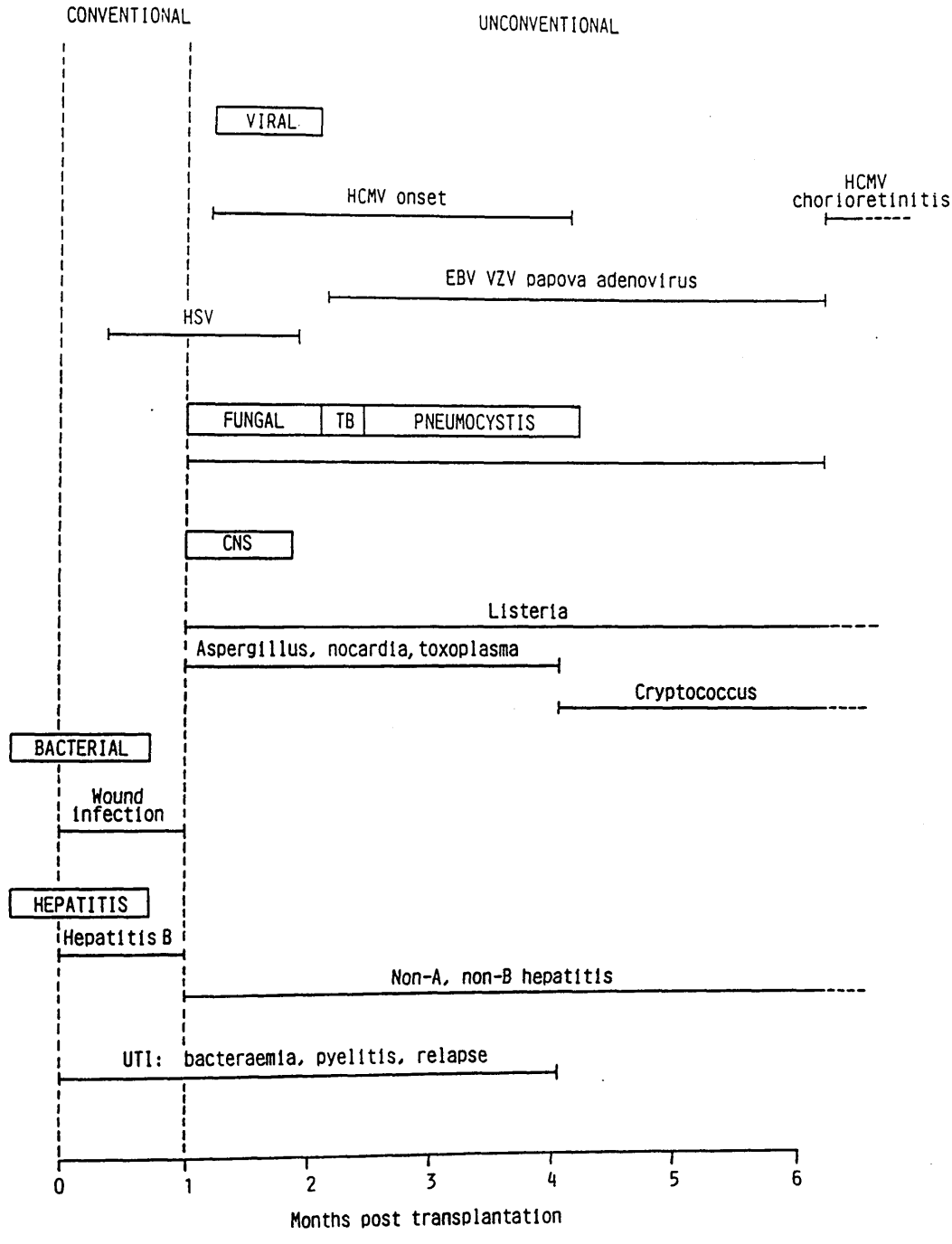
The 5 major herpesviruses (CMV, EBV, HSV-1, HSV-2 and VZV) share 2 characteristics which explain their clinical impact in organ transplant patients: latency and the ability to reactivate as discussed in Section 1.2; cell association and the evasion of neutralizing antibody.

This chapter discusses HCMV, the most important infectious agent

Figure 6 Infections after renal transplantation

The figure shows the timetable for the occurrence of the most frequent infections in renal allograft recipients.

(Modified from Rubin et al., 1981).


CONVENTIONAL
UNCONVENTIONAL
VIRAL
HCMV onset
HCMV chorioretinitis
EBV VZV papova adenovirus
HSV
FUNGAL
TB
PNEUMOCYSTIS
CNS
Listeria
Aspergillus, nocardia, toxoplasma
Cryptococcus
BACTERIAL
Wound infection
HEPATITIS
Hepatitis B
Non-A, non-B hepatitis
UTI: bacteraemia, pyelitis, relapse
0
1
2
3
4
5
6
Months post transplantation

affecting transplant recipients.

1.3.1 Incidence of HCMV infection

HCMV is a ubiquitous agent. Studies indicate that the majority of people in the world experience HCMV infection during their lifetime (Krech, 1973). The prevalence of HCMV rises with increasing age (Rowe et al., 1956) and poor socioeconomic conditions (Lang et al., 1977b). At birth, only 0.5 to 2.5% of neonates have HCMV infection as demonstrated by virus shedding (Larke et al., 1980). However at the end of the first year of life, 8 to 60% of infants in the Western world excrete HCMV (Leinikki et al., 1972). The rate of infection in young adults is approximately 1% per year (Naraqi, 1984). HCMV infections are more common in certain populations including pregnant women (Gold and Nankervis, 1976) and immunocompromised hosts (Ho, 1977). Autopsy and clinical studies indicate that 90% of patients with AIDS develop active HCMV infection during their illness (Reichert et al., 1983).

There are many reports in the literature that examine HCMV in the context of renal transplantation and these are reviewed by Ho (1977), Glenn (1981) and Ho (1982). In these studies up to 60% of seronegative and 90% of seropositive allograft recipients showed evidence of active HCMV infection post-operatively. Using the Mantel-Haenszel test controlling for the site of the study (Fleiss, 1973), Hamilton (1982) has estimated the relative risk of acquiring a secondary HCMV infection to be between 15 and

16 times greater than it is for a primary infection.

After bone marrow transplantation, between 40 and 60% of patients show evidence of active HCMV infection (Neiman et al., 1977; Winston et al., 1979; Meyers et al., 1980). Wreghitt et al. (1988) have reported that 45% of heart transplant recipients experience HCMV infection post-operatively while in a similar study by Dummer et al. (1985) the incidence was 85%. Fifty nine per cent (Singh et al., 1988a) to 77% (Dummer et al., 1983) of adult and 30% of paediatric (Breinig et al., 1987) liver transplant recipients have evidence of active HCMV infection post transplantation.

1.3.2 HCMV-related disease

In renal allograft recipients, the incidence of clinically apparent HCMV infection ranges from 13 to 38%, most of the symptomatic infections occurring in the first few months after the onset of immunosuppressive therapy (Naraqi, 1984).

Pyrexia is the most common feature observed in immunosuppressed patients with symptomatic HCMV infection. This varies in severity and is usually prolonged. Illness most commonly presents with signs of the mononucleosis syndrome (Klemola and Kaariainen, 1965; Klemola et al., 1967; 1969b; 1970). Some combination of the following can also be observed: hepatomegaly, splenomegaly, myalgia and arthralgia (Fiala et al., 1975; Betts et al., 1977; Marker

et al., 1981).

After the mononucleosis syndrome, interstitial pneumonia is the most frequent manifestation of HCMV infection in immunosuppressed patients. It may accompany the mononucleosis syndrome or appear separately. In a study by Hill et al. (1964), 47% of renal allograft recipients whose deaths were complicated by pneumonia showed evidence of pulmonary HCMV infection at post mortem. Between 10 and 50% of bone marrow recipients experience HCMV-associated interstitial pneumonitis of which 60 to 80% prove fatal (Neiman et al., 1973). HCMV pneumonia has also been observed in 44% of cardiac (Stinson et al., 1971) and 30% of hepatic (Fulginiti et al., 1968) transplant recipients. The pneumonia has a variable course and may resolve spontaneously or advance to marked respiratory depression and death.

Clinically apparent hepatitis has been observed in 7 to 16% of renal transplant recipients (Aldrete et al., 1975; Luby et al., 1974; Anuras et al., 1977; Ware et al., 1979). While hepatitis is common it is not usually severe, even in patients who receive other organ transplants (Ten Napel et al., 1984). The causes of hepatic dysfunction after liver transplantation include rejection, ischaemia, vascular thrombosis, bile duct complications, haemolysis and hepatitis (Starzl et al., 1982; Esquivel et al., 1985). Recently, Bronsther et al. (1988) have shown that HCMV hepatitis in liver transplant recipients is a definite clinical entity with specific signs and symptoms,

serological and culture findings and pathological features. A number of authors have reported the onset of chronic hepatic dysfunction during an episode of febrile illness caused by HCMV (Luby et al., 1974; Ware et al., 1975; 1979; Rubin et al., 1977). However, it remains debateable whether HCMV is the sole aetiologic agent of this condition. Indeed, the cause of hepatitis in immunosuppressed patients is often unclear.

Until recently, involvement of the gastrointestinal tract with HCMV has been reported infrequently. Most of the recognized cases have occurred in the colon where the virus is associated with caecal ulceration (Wolfe and Cherry, 1973; Sutherland et al., 1979; Foucar et al., 1981). More recently, the biliary tree has been shown to be a site of HCMV replication (Galloway, 1984). HCMV infection of the gastrointestinal tract has been reported in renal (Franzin et al., 1981; Cohen et al., 1985; Kodama et al., 1985) and bone marrow (Strayer et al., 1981) transplant recipients. The risk of gastrointestinal HCMV infection is 26.5 times greater in patients studied after rather than before liver transplantation (Alexander et al., 1988). Most infections are of little clinical significance but in some cases they are associated with morbidity and increased mortality (Chatterjee et al., 1978; Schooley et al., 1983).

HCMV infection in immunosuppressed patients is associated with chorioretinitis in 4% of cases (Fiala et

al., 1975). HCMV retinitis is usually indicative of disseminated infection and prolonged viraemia may be a factor in the pathogenesis (Pollard et al., 1980). Cessation of immunosuppressive agents may halt progress of the lesions (Egbert et al., 1980).

HCMV infection also predisposes to a number of opportunistic infections the major ones being other viruses of the herpes group and papovaviruses, bacteria e.g. L. monocytogenes, N. asteroides and Legionella; fungi e.g. candida and aspergillus; protozoans e.g. P. carinii, S. stercoralis and T. gondii. Rubin and Tolkoff-Rubin (1988) have recently reviewed this subject.

1.3.3 HCMV and renal allograft rejection

A controversial relationship exists between active HCMV infection and renal transplant dysfunction. Kidney allograft rejection temporally associated with active HCMV infection has been reported by some workers (Rubin et al., 1977; Fryd et al., 1980; Tilg et al., 1987) while others (Bia et al., 1985; Pollak et al., 1987) have failed to confirm this finding. Hamilton (1982) has assessed the available data from 8 transplant centres and shown that renal allograft recipients infected with HCMV have a statistically higher rate of rejection than those patients who remain uninfected. Such data do not prove that HCMV infection provokes or causes allograft rejection, although Mourad et al. (1984) have shown that renal dysfunction abates with the resolution of infection. While Betts et

al. (1977) have demonstrated HCMV infection followed by graft rejection, others (Lopez et al., 1974; Braun et al., 1976) have documented rejection followed by seroconversion and virus isolation.

It has been proposed that HCMV itself incites tissue injury. Interstitial nephritis has been described in neonates (Platt et al., 1985) and in a renal allograft recipient (Cameron et al., 1982) with HCMV infection. Glomerular microthrombi in the absence of an interstitial infiltrate have been observed during symptomatic HCMV infection after renal transplantation (Bia et al., 1985). A distinctive diffuse glomerulopathy has been recognized in renal allograft recipients with clinically apparent HCMV infection and viraemia (Richardson et al., 1981). Immune complexes observed in the circulation of renal allograft recipients with primary HCMV infection (Baldwin et al., 1982) may deposit in the kidney and impair its function (Ozawa and Stewart, 1979).

Enhancement of alloreactivity by concomitant HCMV infection may also be involved in graft rejection. After successful transplantation the MHC antigens of the kidney parenchymal cells are no longer detectable but reappear during rejection (Hayry et al., 1981; Hall et al., 1984). HCMV infection is invariably associated with upregulation of Class II MHC antigens accompanied by evidence of graft rejection (Von Willebrand et al.,1986). Re-expression of MHC antigens may be induced by IFN- in response to virus

infection (Pober et al., 1983; Van Es et al., 1984). HCMV could also induce graft rejection by immunologic recognition of expressed viral IE products in infected kidney cells. The IE-2 gene of HCMV codes for an amino acid sequence also present on the external surface of the HLA-DR β chain (Fujinami et al., 1988). The sequences are sufficiently similar such that an immune response generated against the virus is also observed to react with "self" HLA-DR. The sharing of a microbial epitope with a host "self" epitope from 2 dissimilar proteins has been termed molecular mimicry (Fujinami et al., 1983; Fujinami and Oldstone, 1985) and is sufficiently common that almost 4% of the monoclonal antibodies against viruses also react with host "self" determinants (Srinivasappa et al., 1986).

The available data does not favour reactivation of latent HCMV by alloimmune responses (Miller et al., 1986; Tilg et al., 1987). Indeed, it rather suggests excessive immunosuppression as the main predisposing factor for HCMV infection and is supported by the close correlation between the severity of infection and the intensity of the immunosuppressive regime (Fiala et al., 1975; Glenn, 1981; Rubin et al., 1985). This is discussed in more detail in Section 1.3.4.

1.3.4 Risk factors for HCMV infection and disease

Immunosuppressive therapy and its impact on HCMV-related disease has been reported in detail. The importance of immunosuppressive agents is shown by the

following observations: HCMV infection is seen in almost all patients who are treated with cytotoxic agents regardless of the underlying condition (Duvall et al., 1966; Dowling et al., 1976; Glenn, 1981); renal allograft recipients who do not receive these drugs do not have a high incidence of HCMV infection (Kanich and Craighead, 1966); there is a close temporal relationship between immunosuppressive therapy and the onset of HCMV infection (Fiala et al., 1975); there is a direct correlation between the degree of immunosuppression and the morbidity of infection (Pass et al., 1980). Rubin (1981) has suggested that the net state of immunosuppression is important and if excessive can increase the risk for HCMV infection. Indeed, Preiksaitis et al. (1983) have shown that heart transplant recipients receiving high doses of antithymocyte globulin (ATG), azathioprine and prednisolone experienced more severe HCMV infections than those given low doses.

High doses of corticosteroids (e.g. prednisolone) have been implicated in increasing the incidence of HCMV infections in patients who have received a renal allograft (Velasco et al., 1984). The use of antilymphocytic preparations is well recognized as having a deleterious influence on both the incidence and severity of HCMV infections even when the dosage of coadminstered immunosuppressive therapy is reduced. Indeed, cytoreductive therapy is known to promote HCMV reactivation (Dowling et

al., 1976; Pass et al., 1980; Chou and Norman, 1985) and Tolkoff-Rubin and Rubin (1986) have concluded that adding agents such as ATG is more important in potentiating HCMV disease than is the basic immunosuppressive therapy. Immunosuppressive regimens containing ATG have been associated with a higher frequency of symptomatic HCMV infection in renal transplant patients (Cheeseman et al., 1979; Pass et al., 1980; Canadian Multicentre Transplant Study Group, 1983; Nunan and Banatvala, 1984; Bia et al., 1985). The incidence of HCMV pneumonia in renal allograft recipients has also been related to the use of ATG (Peterson et al., 1983). OKT3, a murine monoclonal antibody directed against lymphocytes, has been shown to increase the risk of disseminated disease in patients with HCMV infection after liver transplantation (Singh et al., 1988b).

Mild primary HCMV infections in renal allograft recipients receiving CsA have been reported (Morris et al., 1983). A lower incidence of HCMV-related disease has been observed in patients receiving CsA than azathioprine perhaps because of the less frequent use of pulse doses of corticosteroids or ATG (Henry et al., 1985; Najarian et al., 1985). However, Bia et al. (1985) have suggested that CsA may lower the risk of HCMV disease independent of its ability to reduce the need for aggressive antilymphocyte therapy. In other studies the incidence of HCMV-related disease in transplant recipients receiving either CsA or azathioprine was equivalent (Dummer et al., 1983; Harris et

al., 1984; Arnfred et al., 1987). Takahashi et al. 1987b) have suggested that the incidence of viral infections in renal transplant patients immunosuppressed with CsA shows a relative increase secondary to a reduction in the frequency of bacterial infections. Although CsA improves graft survival in comparison with other immunosuppressive agents, renal function is often impaired (Opelz, 1988).

In a study by Weir et al. (1987) the major risk for development of HCMV disease was not related to excessive doses of immunotherapy but was due to whether or not an HCMV-seronegative recipient received a kidney from an HCMV-seropositive donor. HCMV-seronegative patients who receive transplants from HCMV-seropositive donors represent the group of renal transplant recipients that is at highest risk for HCMV-related disease (Ho et al., 1975). The same has been confirmed in the context of cardiac (Wreghitt et al., 1988) and liver (Singh et al., 1988a) transplantation. When an HCMV-seropositive patient receives a kidney from an HCMV-seronegative or seropositive donor the incidence of HCMV disease is similar but the latter group have more complications (Smiley et al., 1985). Ringden et al. (1987) have reported a higher incidence of pneumonitis in recipients of bone marrow from HCMV-seropositive donors while others (Grob et al., 1987) have failed to confirm this finding.

The type of transplant operation is an important determinant of HCMV infection. In patients receiving identical prophylactic immunotherapy there is a lower incidence of HCMV infection after renal or pancreatic transplantation than after cardiac or hepatic transplantation (Tilg et al., 1987). Several studies have shown that the most severe form of primary HCMV disease arises when infection is acquired from the donated organ (Ho, 1977; Dummer et al., 1985) than blood products (Wreghitt et al., 1988). Heart and lung transplant recipients are more likely to experience severe or fatal HCMV infections than those receiving a heart transplant alone (Wreghitt et al., 1988) presumably because of the increased dose of virus which may be acquired from 2 organs. Among the known risk factors for HCMV pneumonia in allogeneic bone marrow recipients are age, radiation dose, dose rate and graft-versus-host disease (Meyers et al., 1986; Weiner et al., 1986). Fewer recipients of HLA identical kidneys develop HCMV infection compared to recipients of mismatched kidneys (Pollak et al., 1987). Roenhorst et al. (1985) have reported that recipients of cadaver kidney grafts who are positive for HLA-DRw6 show a significantly increased incidence of HCMV infection and associated clinical symptoms. Pirson et al. (1985) have confirmed an association between HLA-DRw6 and HCMV disease but not infection in renal allograft recipients.

1.3.5 Prophylaxis

(a) Protective matching

Rubin et al. (1979) proposed the concept of "protective matching" of donor-recipient pairs to prevent HCMV disease in recipients of solid organ transplants. Therefore, some transplant centres attempt to match HCMV-seropositive donors with HCMV-seropositive recipients while HCMV-seronegative patients receive organs from HCMV-seronegative donors (May et al., 1978; Burleson et al., 1984). However, not all HCMV-seropositive donors transmit the virus post transplantation (Chou and Norman, 1988). Furthermore, HCMV-seropositive recipients of a kidney from an HCMV-seropositive donor have an overall risk of HCMV disease approximately double that had they received a kidney from an HCMV-seronegative donor (Smiley et al., 1985). Therefore, it would be an unfair disadvantage to HCMV-seropositive patients to only give them kidneys from HCMV-seropositive donors especially in the setting of an inadequate supply of suitable donors.

Several studies have shown that transfusion-associated primary HCMV infection can be prevented if HCMV-seronegative patients are given blood products only from HCMV-seronegative donors (Winston et al., 1980; Adler et al., 1984; Bowden et al., 1986; MacKinnon et al., 1988). Other approaches to the prevention of primary HCMV infection have included the use of leucocyte depleted (Lang et al., 1977a; Verdonck et al., 1985), washed (Williams et al., 1984) and frozen deglycerolized

(Tolkoff-Rubin et al., 1978; Brady et al., 1984) red blood cells.

(b) Passive immunotherapy

In bone marrow transplant recipients there is some evidence that intramuscular [IM] (Meyers et al., 1983) and intravenous [IV] (Condie and O'Reilly, 1984; Winston et al., 1984) HCMV hyperimmune globulin, IV HCMV hyperimmune plasma (Winston et al., 1982) and unselected IV immunoglobulin (Winston et al., 1987) may prevent HCMV disease. The same has also been confirmed in the context of renal transplantation (Snydman et al., 1984; 1987; 1988). However, some workers (Bowden et al., 1986) have failed to show any benefit of passive immunotherapy in reducing the incidence or severity of HCMV infection post transplantation. Indeed, the apparent beneficial effect of immunoglobulin is surprising since severe HCMV-related disease can occur in the presence of high titres of endogenous specific antibody. While passive immune prophylaxis has the advantage of being targeted at high risk patients, blood products may serve as a vehicle for transmitting other infectious agents (Weiland et al., 1986).

(c) Vaccination

In 1976, Plotkin et al. developed a live attenuated vaccine using a virus strain (Towne-125) that was isolated from a child with congenital HCMV infection. Studies in HCMV-seronegative renal transplant candidates found the

virus to be immunogenic but did not prevent HCMV shedding or become latent in the host in a form that could be reactivated following immunosuppression (Lang, 1980; Osborn, 1981). The vaccine has been associated with a lower severity of HCMV disease after renal transplantation (Starr et al., 1981; Plotkin et al., 1984) but there continues to be concern about the safety and efficacy of herpesvirus vaccines in general as well as the oncogenic potential of Towne-125 in particular (Parks and Rapp, 1975; Betts, 1979).

1.3.6 Treatment

(a) Interferon

Although human IFN has been shown to inhibit HCMV replication in vitro (Postic and Dowling, 1977; Rasmussen et al., 1984a), it was of little clinical efficacy when used to treat bone marrow recipients with HCMV infection (Meyers et al., 1982; Wade et al., 1983).

Recent advances in protein engineering have allowed highly purified IFN to be produced. Prophylaxis with leucocyte-derived IFN-α has been shown to prevent severe HCMV infection in renal transplant patients (Cheeseman et al., 1979; Hirsch et al., 1983). However in 1 study, recombinant IFN-α was associated with steroid resistant acute vascular rejection (Kramer et al., 1984). The in vitro antiviral effect of IFN-β (Nakamura et al., 1988) and IFN-γ (Yamamoto et al., 1987) have been reported. IFN-β has also been useful in the treatment of renal allograft

recipients with HCMV pneumonitis (Takahashi et al., 1987a).

(b) Antiviral drugs

Adenine arabinoside [vidarabine] has been used to treat HCMV infection with little or no effect (Ch'ien et al., 1974; Meyers et al., 1982). In clinical trials, ACV used alone or in combination with other antivirals has failed to resolve severe HCMV infections (Wade et al., 1983; Shepp et al., 1984). Although ACV is not therapeutically useful against HCMV the drug does exert a significant prophylactic effect on HCMV shedding and pneumonia (Meyers et al., 1988). Ganciclovir (DHPG) is a new antiviral which, like ACV, is a cogener of deoxyguanosine (Field et al., 1983) but is a more efficient substrate for HCMV-induced deoxypyrimidine kinase than other guanosine analogues (Dolin, 1985). The drug shows excellent activity against HCMV in vitro (Mar et al., 1983; Tyms et al., 1984). Ganciclovir is effective in reducing HCMV shedding (Shepp et al., 1985; Creasy et al., 1986; Erice et al., 1987) and has been successful in treating HCMV pneumonitis (De Hemptinne et al., 1988; Shabtai et al., 1988), retinitis (Jacobson et al., 1988), colitis (Collaborative DHPG Treatment Study Group, 1986) and hepatitis (Harbison et al., 1988). Ganciclovir has no effect on latent virus and immunosuppressed patients may resume HCMV shedding after cessation of therapy (Collaborative DHPG Treatment Study Group, 1986). The main adverse side effect of ganciclovir is reversible

neutropenia. Since the drug is excreted primarily unchanged in the urine, dosage adjustment is required in the event of renal insufficiency. In a murine model, a combination of ganciclovir and immune globulin was found to be more effective in controlling HCMV infection than either modality alone (Wilson et al., 1987). Some authors (Schmidt et al., 1988) have shown a synergistic effect of HCMV immune globulin with ganciclovir in the treatment of HCMV pneumonitis after bone marrow transplantation while others (Aulitzky et al., 1988) have failed to confirm this finding.

1.4 Immunology of HCMV infection

HCMV affects humoral and cell-mediated immune responses in previously healthy as well as immunocompromised hosts. In this chapter the role of each arm of the immune response during HCMV infection will be described although interplay between cellular and humoral immune mechanisms may be more important than either modality alone.

1.4.1 Cell-mediated immunity

(a) Cytotoxic T cells

Cytotoxic T cells (CTL) are non-adherent cells that possess receptors for SRBCs (Quinnan et al., 1981) but not for Fc of IgG (Quinnan et al., 1982; Rook and Quinnan, 1983). In humans, CTL possess surface antigens which are usually OKT3 and OKT8 positive (Reinherz et al., 1979).

Maturation of CTL precursors into effector cells is dependent on lymphokines including IL-1 (Oppenheim et al., 1982), IL-2 (Farrar et al., 1982) and IFN-γ (Reddehase et al., 1982). CTL are typically antigen-specific and are also restricted in activity by antigens of the MHC (Zinkernagel and Welsh, 1976). Three different patterns of specific CTL responses generated against HCMV-infected allogeneic target cells have been described; those restricted by Class 1 HLA-A and/or B loci, Class II HLA-DR or self restricted to lysis of autologous targets (Lindsley et al., 1986).

In both transplant recipients and healthy volunteers, the HCMV-specific cytotoxic response occurs early in infection, either prior to or coincident with clinical features of HCMV infection or shedding and persists until clinical manifestations have resolved (Quinnan et al., 1984a; 1984b). Although lymphocyte proliferation in response to HCMV antigen in vitro correlates poorly with the development or outcome of HCMV infection in bone marrow recipients (Meyers et al.,1980), extensive studies have demonstrated that the development of HCMV-specific CTL responses in vivo correlates closely with recovery (Rook et al., 1984). CTL responses also correlate negatively with graft rejection (Rook et al., 1984).

Mice develop CTL responses that are similar to those seen in humans and are restricted by the MHC Class I antigens, H-2D and K (Quinnan and Manischewitz, 1979).

Nude mice, deficient in T cell responses, are extremely susceptible to MCMV infection (Starr and Allison, 1977). Ho (1980) has shown that adoptive transfer of MCMV-specific CTL protects mice of the same, but not different, H-2 type from fatal MCMV disease.

A subset of T lymphocytes carrying the OKT8 marker ($T8^+$) contains cells with cytotoxic/suppressor function whilst the OKT4 positive T cell subset ($T4^+$) contains lymphocytes with helper as well as cytotoxic activity (Reinherz et al., 1980; Thomas et al., 1980; 1981; Morimoto et al., 1981). Lymphocytes of the $T8^+$ subset have been implicated in recovery from HCMV (Quinnan et al., 1982) and MCMV (Reddehase et al., 1987) infection and exert their antiviral effect in vivo by direct cytolysis of CMV-infected cells (Zinkernagel et al., 1986) or via lymphokines (Lehmann-Grube et al., 1985). Infection by HCMV results in an inverted $T4^+/T8^+$ ratio due to an increase in $T8^+$ cells and a decrease in $T4^+$ cells. This has been confirmed in previously healthy individuals (Carney et al., 1981) and recipients of bone marrow (Persson et al., 1987) and renal (Schooley et al., 1983) transplants. A reduction in the $T4^+/T8^+$ ratio has also been observed in mice infected with MCMV (Doody et al., 1986). The majority of the expanded $T8^+$ population also express HLA-DR (Ia) antigens suggesting a state of activation (Winchester and Kunkel, 1979). In vitro, the $T8^+$ Ia^+ subset is hyporesponsive to mitogens and alloantigens but retains suppressor activity. In

convalescence, the $T4^+/T8^+$ ratio usually reverts to normal and $T8^+$ cells regain full functional activities. However in some allograft recipients, a prolonged T cell subset inversion has been noted and linked to persistent HCMV infection (Rubin et al., 1981a; O'Toole et al., 1986). A reduction in the $T4^+/T8^+$ ratio due to herpesvirus infection in renal transplant patients has also been correlated with a risk of opportunistic superinfection (Schooley et al., 1983). Some authors (Rinaldo et al., 1983; Dummer et al., 1984; Stadtler et al., 1985) have reported that allograft recipients receiving CsA rarely show inversion of the $T4^+/T8^+$ ratio while others (Metselaar et al., 1986; Dafoe et al., 1987) have failed to confirm this finding. The pyrimidinone compound, bropirimine, when administered to MCMV-infected mice was able to restore the $T4^+/T8^+$ ratio (Brideau and Nicholas, 1987).

Although antigens expressed during the E (Rodgers et al., 1987) and L (Forman et al., 1985) phases of MCMV replication are recognized by CTL, non-structural IE proteins serve as the dominant target antigens for the cellular immune system (Reddehase and Koszinowski, 1984). Borysiewics et al. (1983) have also demonstrated HCMV-specific CTL precursors in the peripheral blood of healthy seropositive individuals which recognize antigens expressed in HCMV-infected cells as early as 6 hr post infection. L cells transfected with the IE-1 gene of MCMV are lysed by IE-specific CTL (Koszinowski et al., 1987), the dominant

antigen being the IE-1 polypeptide pp89 (Volkmer et al., 1987). The recognition of non-structural CMV proteins by CTL is not without precedent. Murine CTL can recognise the non-structural large T antigen in SV40-transformed murine cells (Campbell et al., 1983) and the non-structural NS1 protein of influenza virus is also a target for CTL (Benninck et al., 1987). The mechanism by which proteins that lack signal sequences for transport to the plasma membrane and that do not possess transmembrane domains are presented as antigens for CTL is unclear. There is recent evidence for intracellular protein degradation and membrane presentation of peptides in association with Class I gene products (Maryanski et al., 1986; Townsend et al., 1986). Why IE antigens dominate the CTL response to CMV is also unclear since during infection of permissive cells IE gene transcription is transient. However, the infection of permissive embryo fibroblasts in vitro may not necessarily reflect the duration of IE gene expression in other cell types. Some nonpermissive cell lines infected in vitro overproduce the major HCMV IE protein (Jeang et al., 1982) and in vivo-infected lymphocytes from seropositive individuals have been found to selectively express HCMV IE gene products (Schrier et al., 1985).

(b) Natural killer cells

Natural killer (NK) cells are lymphocytes of the type referred to previously as null cells (Herberman and Holden, 1978). They are distinguished from T cells in that they do not form rosettes with SRBCs at 29^{o}C and they lack or have

very low density of T cell surface antigens. NK cells lack surface immunoglobulin differentiating them from B cells and, unlike macrophages, they do not bind to nylon wool columns. NK cells have characteristic cell surface markers including HNK1 [Leu 7] (Abo et al., 1982) and receptors for the Fc portion of IgG (Perussia et al., 1984). Subsets of $HNK1^+$ lymphocytes have also been distinguished on the basis of phenotypic, morphologic and functional criteria (Lanier et al., 1983; Abo et al., 1984). NK cells mediate spontaneous killing of virus-infected and tumour cells without showing conventional immunological specificity or memory and without necessarily sharing HLA antigens with the target cell (Trinchieri and Perussia, 1984). Their action can be augmented by IFN-α and IFN-β (Bandyopadhyay et al., 1987) although in one study long term administration of IFN-α to renal transplant recipients resulted in decreased NK activity (Kelly et al., 1984). Sayers et al. (1986) reported that IFN-γ is a relatively poor stimulator of NK activity against tumour cells while others (Weigent et al., 1983) have shown IFN-γ to enhance NK activity against murine fibroblasts to the same extent as 50-fold higher concentrations of IFN-α or IFN-β. IL-2 also augments NK activity against non-infected and HCMV-infected fibroblasts (Bandyopadhyay et al., 1987) but whether this is (Ortaldo et al., 1984) or is not (Ito et al., 1986) dependent on the induction of IFN-γ remains controversial. NK activity is suppressed by drugs such as

CsA (Introna et al., 1981) or azathioprine and prednisolone (Dupont et al., 1984). HLA-DR$^+$ accessory cells also contribute to NK activity by mechanisms other than the secretion of extracellular IFN (Bandyopadhyay et al., 1986).

The resistance of various strains of mice to the early lethal effect of MCMV infection correlates with the magnitude of the NK cell response (Bancroft et al., 1981; Ohashi et al., 1988). Bukowski et al. (1984) have shown that treatment of mice with antibody to asialo GM1, which selectively depletes NK cell activity in vivo, results in an MCMV infection which is more severe. Suckling mice are protected from lethal MCMV infection by adoptive transfer of NK cells from adult mice (Bukowski et al., 1985). NK cells have been shown to be among the first immune cells to reappear after marrow transplantation (Livnat et al., 1980), usually within the first few months (Dokhelar et al., 1981). Studies in bone marrow transplant recipients have also found increased NK activity associated with improved survival from HCMV infection (Bowden et al., 1987) while others have failed to confirm this finding (Forman et al., 1986). Healthy HCMV-seropositive individuals have persistently raised levels of NK cells in the peripheral blood perhaps reflecting some control in viral dissemination (Gratama et al., 1987). Indeed, a fall in NK activity has been correlated with overwhelming HCMV infection post transplantation (Dokhelar et al., 1981). However during HCMV infection of experimentally infected

healthy volunteers, NK activity followed the CTL response (Quinnan et al., 1984b) and therefore the importance of NK cells in the containment of HCMV infection remains to be determined.

(c) Antibody-dependent cell-mediated cytotoxicity

The effector cells that mediate antibody-dependent cell-mediated cytotoxicity (ADCC) include all leucocytes that possess receptors for the Fc portion of IgG. In human and murine CMV infections, the effector cells that have been found to mediate this effect are similar or identical to NK cells (Quinnan and Manischewitz, 1979; Kirmani et al., 1981). Monocytes and neutrophils may also mediate ADCC (Kohl et al., 1977; Oleski et al., 1977). Certain classes of antibodies are more active than others in ADCC probably because they are more cytophilic and bind to Fc receptors more avidly (Okafor et al., 1974). Like NK cells, ADCC requires direct contact between effector and target cell. ADCC is antigen-specific by virtue of the fact that the effector cells are armed with specific antibodies but the antigens recognized by CMV-specific ADCC are unknown.

(d) Delayed-type hypersensitivity

In contrast to the current state of understanding of the roles of cytotoxic effector cells in defence against HCMV infections, very little is known about delayed-type hypersensitivity (DTH) responses in this respect. However, the effector cells mediating DTH and T cell cytotoxicity are distinct and are subject to different immunoregulatory

controls (Nash and Ashford, 1982). The only currently available method that may be useful for measuring DTH responses to HCMV is by skin test reactivity. This response has been manifest in HCMV-seronegative recipients of the Towne strain vaccine who developed a local reaction at the injection site approximately 10 days after vaccination but not in HCMV-seropositive vaccinees in whom the DTH response may have been actively suppressed (Quinnan et al., 1984b).

1.4.2 Humoral immunity

With the description of T cell-specific antiviral function and the correlation of cytotoxic T cell activity with recovery after HCMV infection, the role of antibody has taken a position of secondary importance in resistance to HCMV. HCMV viraemia and disease occur in the presence of high titres of neutralizing antibody (Fiala et al., 1973). Indeed, renal allograft recipients with primary HCMV infection often show a slow rise in virus-specific neutralizing antibody levels (Craighead, 1969; Pass et al., 1983). HCMV infection may occur in a second offspring of mothers with a previous congenitally infected child, indicating that maternal antibody does not prevent transplacental transmission of HCMV (Embil et al., 1970; Stagno et al., 1973). Furthermore, there is a higher incidence of HCMV infection in organ allograft recipients who are HCMV-seropositive immediately pretransplant than in those who are not (Section 1.3.1).

Some protective role for humoral antibody in HCMV infection is suggested by several observations. Congenitally infected infants born to HCMV-immune mothers have a lower incidence of brain damage and HCMV disease than those born to non-immune mothers (Stagno et al., 1982). Although HCMV-seropositive organ transplant recipients are not protected against HCMV infection they are protected from HCMV-related disease as indicated by the fact that the majority of infections in these patients are asymptomatic (Ho, 1977). Furthermore, there is an association between fatal HCMV infection and an impaired specific antibody response in recipients of renal (Simmons et al., 1974), cardiac (Rasmussen et al., 1982) and bone marrow (Neiman et al., 1977) transplantations. However, it is not clear whether the failure to produce specific antibody is the cause or the result of overwhelming HCMV infection. Furthermore, these data do not prove that antibody is the beneficial component of the immune response. Indeed, the local humoral immune response in transplant recipients with HCMV pneumonitis is not specific to the infecting agent (Milburn et al., 1988). Cytotoxic T cells may be protective and the ability to mount this response may correlate with the ability to mount a humoral immune response.

Antibody production against HCMV is relatively intact in patients receiving immunosuppressive therapy (Rytel and Balay, 1976; Rasmussen et al., 1982) and the detection of

specific antibodies in the different immunoglobulin classes has been described (Van Loon et al., 1985; Nielsen et al., 1986; 1987; 1988). These are discussed in more detail below but their primary function, like all immunoglobulins, is the recognition and binding of specific antigenic determinants followed by secondary effects which may include enhanced phagocytosis, ADCC, or target cell lysis following complement activation.

(a) HCMV-IgM

IgM, a pentamer with 5 disulphide-linked subunits, has a sedimentation coefficient of 19S whereas the individual subunits each have a sedimentation coefficient of 7S [low molecular weight IgM] (Metzger, 1970; McDougal et al., 1975). In acute viral infections, virus-specific IgM antibody is found in the 19S fraction of human serum but 7S IgM antibody has also been noted in patients infected with HCMV (O'Neill et al., 1988b). The significance of this 7S HCMV-IgM antibody remains to be clarified.

Virus-specific IgM antibody in patients serum is usually transient and its presence normally suggests a recent or current infection. Studies of previously healthy individuals have shown HCMV-IgM to persist for up to 3 and 5 months in patients with HCMV mononucleosis (Rasmussen et al., 1982) and in asymptomatic pregnant women (Griffiths et al., 1982a). However in immunosuppressed organ transplant recipients, HCMV-specific IgM antibody may persist for up to 2 years (Nagington, 1971; Kangro et al., 1982;

Sutherland and Briggs, 1983). Studies of pregnant women have also shown that IgM antibody to HCMV generally signifies recent primary infection whereas those women with HCMV reinfection and/or reactivation seldom have this antibody (Stagno et al., 1985). However in organ transplant recipients, HCMV-specific IgM is observed in some individuals with non-primary HCMV infections (Pass et al., 1983) although Wreghitt et al. (1986a) consider high concentrations of HCMV-IgM in the sera of allograft recipients to be diagnostic of primary infection. There is conflicting evidence as to whether high levels of HCMV-IgM in HCMV-seropositive recipients of kidneys from HCMV-seropositive donors represents reinfection by donor virus (Betts and Schmidt, 1981; Chou et al., 1987). However, the presence of HCMV-IgM on the day of transplantation is an index of greater susceptibility to severe recurrent infections at a later date (Tardy et al., 1987).

Complement-fixing IgM (CF-IgM) has been reported for only few viruses including rotavirus (Abe and Inouye, 1979), HSV (Tokumaru, 1966) and HCMV (Booth et al., 1980). HCMV CF-IgM possesses cytolytic activity and is directed against cell surface antigens (Booth and Mohammed, 1988). However, this antibody is detected in only a small proportion of individuals possessing HCMV-IgM and its usefulness in passive immunotherapy remains to be established.

(b) HCMV-IgG

Human immunoglobulin IgG consists of 4 subclasses designated IgG1, IgG2, IgG3 and IgG4 (Grey and Kunkel, 1964; Kunkel et al., 1966). The subclass percentile distribution is usually IgG1, 66%; IgG2, 23%; IgG3, 7%; IgG4, 4% (Yount et al., 1970).

In HCMV infection, as well as in other viral infections, the IgG antibody response appears to be restricted (Beck, 1981; Morell et al., 1983). IgG1 and IgG3 are produced in primary infection and IgG1 is the main responding subclass in patients with reactivation of HCMV. IgG4 specific for HCMV occurs in approximately 30% of individuals with primary infection (Linde et al., 1983). The relative importance of the various subclasses of IgG in recovery from HCMV infection is still not well understood. Recent findings indicate that IgG3 is the first subclass to appear after HCMV infection and shows a higher ratio of IgG concentration to antibody titre than the other subclasses as determined by neutralization and haemagglutination inhibition (HAI) assays (Linde et al., 1983). Furthermore, bone marrow transplant recipients with severe HCMV disease show a concomitant disappearance of virus-specific IgG1 and/or IgG3 (Wahren et al., 1984) perhaps reflecting consumption by antigen or cessation of production leading to more severe illness. The biological roles of IgG1 and IgG3 are not clear (Spiegelberg, 1974; Winkelhake, 1978) but both appear to have neutralizing capacity (Wahren et al., 1984). However, human complement factor Clq binds to

the IgG subclasses in the following order IgG3 > IgG1 > IgG2 > IgG4 (Schumaker et al., 1976) and recently Middeldorp et al. (1986) have reported the presence of cytolytic HCMV-specific CF-IgG in transplant recipients.

It is well appreciated that IgG antibodies can persist in the serum for many years after viral infections, usually being associated with lifelong immunity (Mims, 1982).

(c) HCMV-IgA

Several authors have reported on the occurrence of virus-specific serum antibodies of IgA class in HCMV infections. These can be detected as early as 3 days after the onset of symptoms in 8 of 9 patients with primary HCMV infection (Levy and Sarov, 1980) and in 7 of 8 renal allograft recipients with reinfection and/or reactivation (Sarov et al., 1982). There is considerable individual variation in the duration and magnitude of the HCMV-IgA response (Strand and Hoddevik, 1984). Consequently the results of HCMV-IgA detection may be difficult to interpret. Several authors consider HCMV-IgA as a transient antibody indicative of recent infection or reactivation since serum HCMV-IgA persists longer than specific IgM but declines before specific IgG (Levy and Sarov, 1980). Recently, Morris et al. (1985) have found HCMV-IgA to persist for several years after initial infection perhaps reflecting antigenic boosting by virus reinfection and/or reactivation. Russel et al. (1981) have suggested that serum IgA transports foreign antigens from

the bloodstream into the bile. However, further work is required to determine whether HCMV-specific IgA antibodies in the serum are involved in protecting the host from infection.

(d) Immunogenic proteins

The most direct approach to determining which HCMV proteins elicit the formation of antibodies in man is to use human sera to probe infected cell and virion proteins. This has been done using both immunoprecipitation (Rasmussen et al., 1988) and immunoblotting (Shimokawa et al., 1987) analysis.

At least 3 to 4 polypeptides (MWt: 30, 69 to 70, 72 to 76 and 78 to 80 kd) made before viral DNA synthesis can be precipitated from HCMV-infected cell extracts, predominantly by sera from acutely infected individuals (Blanton and Tevethia, 1981). Immunogenic infected cell proteins made after HCMV DNA synthesis have a MWt ranging from 25-30 to 183 kd (Zaia et al., 1986).

With 1 exception (Anders et al., 1986), immunogenic proteins of purified virions have been studied exclusively by immunoblotting (Landini et al., 1986). The number of polypeptides recognized and the intensity of reaction have been found to correlate closely with serum IgG titres. While IgG reactivity is broad, IgM preferentially binds to a few polypeptides of MWt 34 to 38, 64 to 66 and 150 to 155 kd (Landini et al., 1985).

Of all the polypeptides recognized by human sera, the one of 150 to 155 kd [the phosphorylated matrix protein]

appears to be the most immunogenic (Jahn et al., 1987) and response to this polypeptide persists for years after convalescence when antibody to the other HCMV polypeptides has disappeared (Landini et al., 1985). Another polypeptide of MWt 82 kd may play a role in inducing a protective immune response since renal transplant recipients with antibody to this protein at the time of operation have a lower incidence of HCMV reactivation (Landini et al., 1986). In the majority of HCMV infections there is an intense antibody response to the 64 to 66 kd polypeptides (Zaia et al., 1986) which probably correspond to the lower matrix protein described by Gibson (1983) and which may be the principal component of the CF antigen (Kim et al., 1977). Some immunogenic proteins e.g. the matrix and major capsid proteins are internal structural proteins and are unlikely to be expressed on the viral envelope or the membrane of infected cells. Therefore, the role that antibodies to these proteins might have in the host defence against HCMV is unclear.

Another approach to the study of immunogenic proteins has been to identify those which induce neutralizing antibodies. Characterization of HCMV proteins which compose neutralization epitopes on virions has been done almost exclusively by use of murine monoclonal antibodies (Masuho et al., 1987). HCMV polypeptides identified in this way include a complex of 2 major co-immunoprecipitating polypeptides pp55/130 of 55 and 130 kd with a broad band centred at 92 kd (Rasmussen et al.,

1985). The second polypeptide (pp86) detected by virus neutralizing monoclonal antibody is 86 kd (Rasmussen et al., 1984b). pp86 and pp55/130 are immunologically unrelated (Rasmussen et al., 1988).

For lack of an animal model of HCMV, the effective role of these complexes in inducing neutralizing antibody has been assessed by administering purified proteins (Clark et al., 1984), protein complexes (Gonczol et al., 1986b) or genetically engineered HCMV-protein-vaccinia virus (Cranage et al., 1986) to animals and testing for neutralizing antibody induction.

The mechanism(s) of neutralization of HCMV has been studied by Rundell and Betts (1980; 1981; 1982) but our knowledge remains scant.

1.4.3 Interferon

IFN was first described in 1957 by Isaacs and Lindenmann. It is now known that there are numerous species of IFN. They are classified into 3 types of functionally related proteins designated α, β and γ largely on the basis of their antigenic specificities.

IFN-α is produced mainly by lymphocytes and has over a dozen isolectric forms (Stewart et al., 1977). IFN-β is a glycoprotein of 22 kd (Heine et al., 1980) and is synthesized by fibroblasts in response to virus infection. IFN-γ is a glycoprotein composed of dimers of 20 and 25 kd subunits (Yip et al., 1982) and is produced by T

lymphocytes following non-specific stimulation by mitogens *in vitro* or by sensitized lymphocytes stimulated with specific antigen *in vitro* or *in vivo* (Epstein, 1979).

The mechanism by which IFNs inhibit herpesviruses are poorly understood. NK cells do not appear to be required for the antiviral effect of prophylactically administered IFN in a mouse model of CMV infection (Bukowski *et al.*, 1987). IFN may activate another arm of the host immune response e.g. macrophages (Schultz *et al.*, 1977). Alternatively, IFN may directly inhibit virus replication. Indeed, Stinski *et al.* (1982) have clearly shown IFN-α and -β to inhibit translation of IE HCMV mRNA.

1.4.4 HCMV-induced immunosuppression

HCMV can also cause immunosuppression e.g. depressed *in vitro* lymphocyte proliferative responses (Carney *et al.*, 1983; Roenhorst *et al.*, 1988) and NK activity (Schrier *et al.*, 1986). Monocytes may also be infected by HCMV and their phagocytic activity reduced (Kapasi and Rice, 1986). In other experiments, it has been shown that HCMV can suppress not only T cell proliferation to HCMV but also to HSV and mitogens (Sing and Garnett, 1984). Low passage HCMV isolates are more efficient suppressors than laboratory adapted strains (Schrier and Oldstone, 1986).

The mechanism(s) by which HCMV induces immunosuppression is unknown. However, the demonstration that the monocyte is integral to *in vitro* immunosuppression (Kapasi and Rice, 1986) complements the observation that

only a small percentage of lymphocytes can be shown to express HCMV gene products (Rice et al., 1984; Schrier et al., 1985). Monocytes infected with HCMV in vitro are no longer able to produce IL-1 activity (Rogers et al., 1985). IL-1 is required as a second signal for T cell activation during antigen presentation. Loss of IL-1 activity is now known to be due to release of an inhibitor of IL-1 from HCMV-infected monocytes (Rogers et al., 1985).

1.4.5 Immunological aberrations

A variety of immunologic abnormalities has been observed in association with HCMV infection. These include circulating immune complexes, cold agglutinins, rheumatoid factor (RF), cryoglobulin and monoclonal gammapathy of IgA, IgM and IgG types (Kantor et al., 1970; Vodopick et al., 1974; Stagno et al., 1977). HCMV can also act as a polyclonal B cell activator (Hutt-Fletcher et al., 1983).

1.5 Diagnosis of HCMV infection

The diagnosis of HCMV infection cannot be made clinically but requires laboratory confirmation. Many methods are or have been used and these are discussed in detail below.

1.5.1 Histology

For many years the diagnosis of HCMV infection was made by identifying the characteristic inclusion bodies in histologic sections of autopsy tissues (Smith and Vellios, 1950; Wong and Warner, 1962). More recently, identification of inclusion bodies in tissues obtained by biopsy has been used for making the diagnosis in living patients (Risdon and Turner, 1980). Although histology is a relatively reliable technique its sensitivity is 2 to 6 times less than that of virus isolation (Naraqi, 1984). The specificity of histology for diagnosis of HCMV is high but the intranuclear inclusions can occasionally be confused with those of other herpesviruses or adenovirus (Schumann et al., 1977).

1.5.2 Cytology

Inclusion-bearing cells may be found in urine, saliva, milk, cervical secretion and touch preparations of HCMV-infected tissues. Exfoliative cytology for the detection of HCMV-infected cells in the urinary sediment was used in 1952 and for the first time made it possible to diagnose what proved to be HCMV infection in living patients (Fetterman, 1952). While

the specificity of this method is similar to histology the sensitivity is poor in comparison to virus isolation (Morse and Coleman, 1974). The diagnostic yield can be improved by cytocentrifugation (Schumann et al., 1977). Recently, cytologic examination of alveolar lavage fluid has proved useful in identifying bone marrow transplant recipients with HCMV pneumonitis (Cordonnier et al., 1987).

1.5.3 Electron microscopy

Detection of virus particles in the urine by electron microscopy has been found useful for the rapid diagnosis of HCMV infection in neonates with congenital infection (Stagno et al., 1980). Such patients have a high titre of cell-free virus in the urine. While this method takes only a few hours and so could provide a same-day diagnostic service it is of limited usefulness in the diagnosis of HCMV infection in renal allograft recipients for two reasons: the titre of HCMV found in clinical samples from adults is generally lower than that found in infants (Griffiths, 1987); all 6 human herpesviruses which frequently infect immunosuppressed patients are morphologically indistinguishable (Stagno et al., 1981). Whether the application of specific reagents such as monoclonal antibodies to basic electron microscopy techniques proves useful remains to be determined (Doane and Anderson, 1977).

1.5.4 Viral culture

(a) Collection of specimens

Samples should be collected under sterile conditions and be transported to the laboratory with minimum delay in order to avoid loss in infectivity (Stagno et al., 1981). If storage is required samples should be kept at 4°C but under no circumstances should they be frozen. Freezing of the specimen results in loss of HCMV infectivity (Alford and Britt, 1985). HCMV has been isolated from urine, saliva, throat swabs, circulating leucocytes, cervical secretions, semen, breast milk and tears. Human foreskin and embryonic lung fibroblasts are commercially available and are the only cells that support HCMV replication in vitro.

(b) Detection of HCMV

Virus isolation in conventional tissue culture is the most sensitive method for laboratory diagnosis of active HCMV infections (Goldstein et al., 1982; Sullivan and Hanshaw, 1982). Although this method is sensitive it is hampered by the fact that HCMV grows slowly in cell culture and a considerable period may elapse, often up to 5 weeks, before the typical cpe becomes apparent (Reynolds et al., 1979). Centrifugation of the inoculum on to the cell monolayer enhances the infectivity of HCMV (Hudson et al., 1976). The mechanism is unclear but is time dependent, cells and virus having to be centrifuged during the inoculation phase (Hudson, 1988). Ho (1982) has suggested that centrifugal enhancement of HCMV infectivity relates to

an inherent property of the virus while others (Gleaves et al., 1985; Shuster et al., 1985) explain the phenomenon on the basis of increased HCMV adsorption. Recently, Agha et al. (1988) have proposed that centrifugation overcomes the tendency of negatively charged HCMV particles to be repelled by negatively charged cell membranes.

1.5.5 Tissue immunofluorescence

(a) Cell culture

The rapidity of detection of HCMV-infected culture cells can be significantly increased by using immunofluorescence (Stirk and Griffiths, 1987) and peroxidase-antiperoxidase (Swenson and Kaplan, 1985) staining to identify HCMV-specific proteins. Monoclonal antibodies have been produced that detect IE (Schuster et al., 1985) and L (Sutherland et al., 1987) antigens after 16 hr and 24 to 72 hr post infection respectively. Compared with HCMV isolation, detection of early antigen fluorescent foci (DEAFF) is reported to have a sensitivity of 75 to 100% with a specificity of about 95% (Gleaves et al., 1984; Alpert et al., 1985; Gleaves et al., 1985; Swenson and Kaplan, 1985; Stirk and Griffiths, 1987).

Centrifugation of the inoculum on to indicator cell monolayers has been shown to increase the sensitivity of FAT by 40% (Gleaves et al., 1985). Sonication of urine samples prior to inoculation and testing by FAT increases both the number of fluorescent foci and intensity of

fluorescence of HCMV-infected fibroblasts and decreases non-specific background signal (Agha et al., 1988). The mechanism is unknown but may involve the freeing of virus particles from their β_2m coat (McKeating et al., 1986). The sensitivity of FAT is not increased by pretreatment of cell monolayers with dimethyl sulphoxide or dexamethasone which have been shown to enhance HCMV replication in vitro (Tanaka et al., 1984; Espy et al., 1988).

FAT can be performed in most clinical laboratories and requires minimal technician time and expertise. The reduction in time required by FAT to detect HCMV isolates has significantly reduced the problems associated with specimen contamination and toxicity in cell culture (Howell et al., 1986). However, false negative FAT results may occasionally arise particularly with respect to those specimens containing low titres of HCMV (Stirk and Griffiths, 1987). Furthermore, there is evidence that some strains of HCMV encode proteins not recognized by FAT (Chou and Scott, 1988) but this problem has been resolved by Griffiths et al. (1984) who used a mixture of 7 monoclonal antibodies each directed at a different HCMV protein. Recently, Agha et al. (1988) have shown FAT on inoculated tissue cultures using convalescent human serum to be as sensitive as conventional tube cell culture for the detection of HCMV in clinical specimens.

(b) Clinical specimens

Monoclonal antibodies against HCMV can also be used to stain cells obtained directly from the infected patient

(Volpi et al., 1983; Cordonnier et al., 1987). Although this procedure has the advantage of providing results within a few hours, it is relatively insensitive since it detects only a quarter of bronchoalveolar lavage fluids known to contain HCMV by FAT and/or conventional cell culture (Stirk and Griffiths, 1988). However, direct detection of HCMV antigen in peripheral blood leucocytes by monoclonal antibodies has proved to be as specific as and more sensitive than current virus isolation techniques (Van der Bij et al., 1988).

1.5.6 Enzyme immunoassay for the detection of HCMV antigens

Since the titres of HCMV within urine specimens are relatively high (Stagno et al., 1975), several investigators have attempted to detect the virus by ELISA using monoclonal antibodies. While such assays have given good results with laboratory passaged strains of HCMV, results of poor specificity and sensitivity were obtained when clinical specimens were processed (Yolken and Stopa, 1980; Yolken, 1982).

Recently, McKeating et al. (1986; 1987) have shown that β_2m coats HCMV in body fluids and inhibits the binding of HCMV-specific antibody. Nevertheless, Naqvi and Blair (1986) have been able to detect HCMV antigen by ELISA in 84.47% of clinical samples which yielded the virus in tissue culture.

1.5.7 Serology

A number of serological assays relating to the detection of virus-specific antibodies have been described (Grist et al., 1979). This section discusses the advantages and disadvantages of some of these methods.

(a) Complement fixation test

The CFT for HCMV-specific antibody as described in Section 3.16.2 was the first widely used serological assay for HCMV (Bradstreet and Taylor, 1962). However, the procedure is time consuming and requires considerable expertise to perform. Shorter incubation times have been used but with noticeably inferior sensitivity (Hunt et al., 1984). There are many variables in the test and the end point depends on subjective evaluation. The preparation of potent high-titre CF antigen by alkaline glycine buffer extraction and ultrasonication of infected cells is important for reliable results in the CFT (Booth et al., 1982). Screening at a 1:8 dilution may miss some low positive sera whereas screening at less than 1:8 may give rise to anticomplementary activity (McHugh et al., 1985). Some antibodies do not fix complement and would therefore remain undetected by CFT (Roitt, 1985). Recently, Faix (1985) has shown that antigenic heterogeneity among HCMV strains can result in misclassification of up to 6% of sera as negative for HCMV CF antibody if only a single strain of virus is used as the antigen. However, in a study by Adler and McVoy (1986) the continued use of the AD169 strain of

HCMV as a reference antigen in serological assays is supported.

(b) Indirect haemagglutination test

The IHA test is the simplest of the various techniques and offers the advantages of IgM sensitivity, rapid turnaround times and lack of interference by anticomplementary activity (Faix, 1985). A high incidence of non-specific agglutination can be reduced by the use of glutaraldehyde-fixed human type 0 erythrocytes and careful attention to determining the optimal tannic acid concentration for each cell batch (Yeager, 1979). Some authors have found IHA to be superior to many other commercially available test methods (Phipps et al., 1983; Chung et al., 1984).

(c) Neutralization assays

In the humoral immune response to viruses, the development of neutralization antibodies provides the best information about the state of protection of the individual. However, neutralization tests for HCMV have not been popular because the virus is slow-growing and it is not easy to obtain in a high-titred cell-free form. Methods such as the plaque-reduction neutralization test (Waner et al., 1976) require a 14 day incubation of the plates while microneutralization tests are readable only after 7 to 10 days (Stalder and Ehrensberger, 1980; Rasmussen et al., 1984b). Gonczol et al. (1986a) have now described a simple and reliable microneutralization test that is readable in 24 or 48 hr.

Neutralizing antibodies to HCMV usually appear considerably later than antibodies detectable by other techniques (Spencer and Andersen, 1972; Stalder and Ehrensberger, 1980). While the delayed appearance of neutralizing antibodies may be of little benefit in the serologic diagnosis of acute HCMV infection, the rapid and reliable demonstration of neutralizing antibodies may be important in vaccination studies.

(d) Radioimmunoassay

RIA, a method of detecting antigen-antibody reactions by means of a radiolabelled anti-antibody, is a very specific and sensitive serological method (Knez et al., 1976; Jankowski et al., 1980). However, the preliminary work necessary to establish an RIA is considerable since commercial kits are generally unavailable. The specialized equipment and designated radioisotope facilities required may not be available in all laboratories. RIA is labour intensive and therefore unsuitable as a screening procedure. Furthermore, RIA requires the use of reagents that have a short shelf life. To efficiently detect low levels of specific IgM antibodies by RIA the radiolabel must be fresh. For instance, to detect HCMV-IgM antibodies in cord and adult sera the radiolabelled antibody must be used within 72 hr and 3 to 4 weeks respectively (Griffiths et al., 1982a; 1982b).

(e) Enzyme linked immunosorbent assay

Enzyme immunoassay procedures, first reported in 1971,

were initially used to quantitate antigen and subsequently antibody (Engvall and Perlmann, 1971). ELISA is now firmly established as an alternative to RIA. Instead of using radioactive isotopes, ELISA uses enzymes such as horse radish peroxidase and alkaline phosphatase, for the labelling of antibodies. Each enzyme is detected by its ability to produce an easily recognisable change, quickly and reliably, in a substrate solution. Most tests employ a substrate that develops a strong colouration but there are also substrates which liberate a fluorescent product (Shalev et al., 1980), undergo an increase in temperature (Mattiasson et al., 1977) or generate a burst of chemiluminescent energy (Hornsleth et al., 1988).

While ELISA and RIA techniques are of equal sensitivity and specificity when testing adult sera (Booth et al., 1982; Kangro et al., 1984) the former method does have a number of advantages. These include the stability of the enzyme label and the fact that special facilities are not required for the safe handling and disposal of enzyme-labelled reagents. However some ELISA substrates, such as O-phenylene diamine, are mutagenic (Voogd et al., 1980). ELISA has an objective quantitative end point but there are problems in handling large numbers of sera because of the need to control the substrate reaction.

(f) Latex agglutination

Passive latex agglutination is a simple and rapid method for routine detection of antibody to viral antigens and was first described by Singer and Plotz (1956) for the

detection of RF. The assay is based on the fact that latex particles, when coated with a ligand such as an antigen, form stable colloids. The particles remain in suspension when stored but aggregate when mixed with antibody specific to the antigen.

Latex agglutination is a simple technique requiring little specialized equipment. Reagents are stable and have a relatively long shelf life. Furthermore, the test is rapid since up to 50 sera can be screened within 20 min (Puckett and Davis, 1987). Some centres now use the latex agglutination test for the detection of HCMV antibody in potential organ donors and recipients (Wreghitt, 1988).

1.5.8 Measurement of HCMV-IgM

The potential usefulness of virus-specific IgM class antibodies as a marker of congenital and recently acquired HCMV infections has long been recognized (Weller, 1971). Hence considerable effort has gone into developing a reliable test for HCMV-specific IgM antibodies. Numerous indirect immunoassays utilizing a solid-phase antigen and labelled anti-human IgM indicator antibody have been described including FAT (Hekker et al., 1979), ELISA (Buimovici-Klein et al., 1983) and RIA (Torfason et al., 1981). More recently, antibody-capture assays using enzyme-labelled HCMV antigen (Van Loon et al., 1981) or radiolabelled anti-HCMV antibody [MACRIA] (Sutherland and Briggs, 1983) have been developed.

RIA is a specific and sensitive method for the detection of IgM antibodies to HCMV (Griffiths and Kangro, 1984). ELISA and RIA are of comparable specificity and sensitivity (Demmler et al., 1986). Both assays are more specific than FAT (Griffiths et al., 1982a).

Monitoring of IgM does not usually discriminate between specific and non-specific IgM activities. Some sera contain IgM that attaches non-specifically to the solid phase antigen of the ELISA (Krishna et al., 1980). A further complication is that the majority of sera containing HCMV-specific IgM react with the control antigen (Booth, 1983). Studies on HCMV have shown that much of this non-specific reactivity is due to IgM that attaches to host-cell cytoplasmic antigens. Its effect can be minimised through the use of viral and control antigens prepared from isolated nuclei (Sundqvist and Wahren, 1981) although this does not eliminate the problem of false-positive reactions in sera containing anti-nuclear IgM (Schmitz et al., 1977).

A further complication in all indirect tests for HCMV-IgM is that of false-positive reactions in sera containing RF of the IgM class (IgM-RF) together with virus-specific IgG (Kangro, 1980; Salonen et al., 1980). IgM-RF recognizes the Fc portion of IgG bound to antigen; the attached IgM-RF is then detected by the anti-IgM conjugate. Some 3% of unselected sera have been shown to possess IgM-RF (Roggendorf et al., 1981). To eliminate non-specific IgM activities several methods for removing anti-IgG IgM

from sera have been proposed. These methods include binding of IgG by protein A (Jankowski et al., 1979), serum fractionation (Robertson et al., 1977) and absorption of IgM-RF with IgG-coated latex beads or with heat-aggregated human IgG (Shirodira et al., 1973). Recently, Joassin and Reginster (1986) have shown anti-human IgG hyperimmune serum to be the most efficient method of removing non-specific HCMV-IgM activities. While MACRIA is much less susceptible to reactions involving IgM-RF, it has been shown that spurious results can occur with sera containing heterophile antibody (Morgan-Capner et al., 1983).

1.5.9 Measurement of HCMV-IgG

A variety of serological methods are currently available including FAT (Betts et al., 1976), IHA (Bernstein and Stewart, 1971), CFT (Waner et al., 1973), ELISA (Cheung et al., 1981) and latex agglutination (Beckwith et al., 1985). Recently, antibody capture (Nielsen et al., 1986) and competitive (Wreghitt et al., 1986b) ELISAs have been described.

ELISA and RIA are comparable in sensitivity and some 10 to 100 times more sensitive than CFT and IHA (Booth et al., 1982). While ELISA and RIA provide higher antibody titres than CFT they do not detect substantially more seropositive individuals. Some authors have found IHA to be as sensitive as CFT (Bernstein and Stewart, 1971; Cremer et al., 1978) whereas others (Booth et al., 1982) have

reported IHA titres to be lower and false negative results higher compared to those obtained by CFT. This may be related to HCMV antigenic heterogeneity described by Faix (1985) and discussed in Section 1.5.7(a). Latex agglutination is slightly less sensitive than CFT (Puckett and Davis, 1987), IHA (Shekarchi et al., 1988) and RIA (Barbara et al., 1987). While there is good correlation between CFT and IgG antibody capture ELISA (Nielsen et al., 1986), the competitive ELISA of Wreghitt et al. (1986b) is 5 times more sensitive than CFT and twice as sensitive as indirect ELISA.

The sensitivity of indirect tests for anti-viral IgG is reduced in the presence of IgM-RF. The latter binds non-specifically to the Fc portion of IgG molecules after they have attached to the antigen on the solid phase and hinders the attachment of the anti-IgG conjugate (Salonen et al., 1980).

1.5.10 Novel serological assays

Serological methods that rely on changes in total HCMV-specific antibody levels may be ineffective, particularly in patients whose humoral response is absent or weak. Using immunoblotting techniques, Ashley et al. (1987) and Mirolo et al. (1987) have demonstrated that total antibody levels may remain constant although titres to specific HCMV polypeptides may change during infection. Landini et al. (1986) have identified 2 polypeptides that are consistently and preferentially reactive with HCMV-IgM.

Humoral responses to E and L antigens are useful in differentiating renal allograft recipients with primary HCMV infection from those with reinfection and/or reactivation (O'Neill et al., 1988a). Use of new technology rather than traditional methods may be requisite if HCMV infections are to be evaluated serologically.

1.5.11 Other diagnostic approaches

There is a clear demand for markers which facilitate detection of HCMV and simplify measurements of virus activity in renal transplant recipients. Serum deoxythymidine kinase activity has been used in this respect (Larson et al., 1986). However, this enzyme is also induced by other herpesviruses (Gronowitz et al., 1984).

β_2m, a protein with a MWt of 11.8 kd, is the small invariant chain of human leucocyte antigen (Peterson et al., 1974). Neopterin [7,8-dihydroneopterin triphosphate] is synthesized by macrophages upon stimulation by factors derived from activated T cells (Huber and Troppmair, 1985). Recently, Backmann et al. (1988) have found monitoring of serum β_2m and neopterin levels to be helpful in the early diagnosis of HCMV infections.

1.6 Detection of DNA in clinical specimens

Standard methods for the diagnosis of HCMV depend on either isolating the virus by conventional cell culture or detecting a rise in antibody titre. The slow appearance of cpe in cell culture has limited the usefulness of this method for diagnostic purposes (Richman et al., 1984). Furthermore, tissue culture methods are sometimes impossible due to microbially contaminated specimens or to inactivated non-infectious virus (Schuster et al., 1986). In addition, serological diagnosis in immunosuppressed patients is hampered by the long lag period until an immune response occurs or by a complete lack of response (Augustin et al., 1987).

The speed of diagnosis is becoming more important as the prospect of specific antiviral chemotherapy becomes a reality. This chapter discusses the detection of viral DNA in clinical specimens by nucleic acid hybridization and the polymerase chain reaction (PCR).

1.6.1 Theoretical background of nucleic acid hybridization

Molecular hybridization is based on the tendency of nucleic acids to form double stranded hydrogen-bonded complexes if strands of complementary sequences are incubated under appropriate salt and temperature conditions. The hydrogen bonds between two complementary strands of nucleic acids can be reversibly separated. This can be achieved at neutral pH and low ionic strength by the

application of heat or, in the case of DNA, by treatment with alkali. The temperature at which double stranded DNA or RNA is converted into single strands is defined as the melting temperature (T_m) and is strongly dependent on base composition (Marmur and Doty, 1962), the monovalent cation concentration (Schildkraut and Lifson, 1965) and the concentration of denaturing agents such as formamide, which are often used to reduce the temperature of hybridization (McConaughy et al., 1969). For DNA-DNA

$$T_m = 81.5 + 16.6 \log(Na^+) + 0.41(\%GC) - 0.7(\% \text{ formamide}) - 1.4 (\% \text{ mismatch})$$

(Howley et al., 1979).

Reassociation is the joining together by typical base pairing of two fully-separated complementary sequences. Once reassociation has commenced, zippering describes the formation of successive base pairs. Techniques for measurement of reassociation include the use of hydroxyapatite (Britten et al., 1974), the single strand-specific nuclease [S1] (Smith et al., 1975) and optical methods (Young and Anderson, 1985). The rate at which complementary strands of nucleic acid form stable base-paired duplexes is dependent on a number of factors. From the results of Hutton and Wetmur (1973) it has been well established that the rate of both DNA reannealing and RNA-DNA hybridization is proportional to the square root of the length of the shorter strand. The base composition of nucleic acids has little effect on the rate of DNA-DNA annealing (Wetmur and Davidson, 1968) or RNA-DNA

hybridization (Bishop, 1972). The effect of ionic strength on DNA reannealing was first studied by Wetmur and Davidson (1968) who showed the rate to be strongly dependent on sodium ion concentration at least up to 3.2M. At concentrations up to 0.2M the rate has been shown to be proportional to the cube of the ionic strength (Studier, 1969). The effect of ionic strength on the rate of RNA-DNA hybridization has not been studied in detail but Nygaard and Hall (1964) have demonstrated a 5- to 6-fold increase in the rate of hybridization of phage T2 RNA to T2 DNA by increasing the ionic strength from 0.2 to 1.5M NaCl.

In considering the effect of viscosity on the rate of hybridization it is important to distinguish between microscopic and macroscopic viscosity. Microscopic viscosity refers to the micro-environment around the DNA bases. The macroscopic viscosity is dependent on the presence of polymers (including DNA) which will have no effect on the micro-environment. Thrower and Peacocke (1968) and Subirana and Doty (1966) have reported a decrease in DNA renaturation rates after the microscopic viscosity had been increased with sucrose. Chang et al. (1974) have investigated the effect of macroscopic viscosity on DNA renaturation and shown a 5.7% Ficoll solution to increase the rate of phage T4 DNA reannealing by 50%.

The optimal temperatures for nucleic acid reassociation in aqueous salt solutions lie in the range 60

to 75°C. However, extended incubation at such temperatures can lead to a considerable amount of thermal strand scission. It may be desirable to reduce the hybridization temperature whilst maintaining the stringency of the nucleic acid interaction. This can be achieved by introducing formamide whose effect on the reassociation rate of DNA has been studied by Hutton (1977). Increasing the formamide concentration decreases the optimal DNA renaturation rate by 1.1% per 1% of formamide. Schmeckpeper and Smith (1972) found that the presence of 50% formamide reduced the rate of RNA-DNA hybridization by a factor of 0.25 compared with the reaction rate in an aqueous solution at a similar stringency. Furthermore, the stability of RNA-DNA hybrids is greater in 50% formamide than that of DNA duplexes of similar base composition (Bishop, 1972).

The dependence of DNA reassociation rate on incubation temperature was first studied by Marmur and Doty (1961). They observed that as the temperature was reduced from T_m, the rate of DNA reassociation increased until a maximum was reached at about 25°C below T_m. Further reductions in temperature reduced the reaction rate. A similar dependence for RNA-DNA hybridization has been shown with both reactants free in solution (Bishop, 1972) and with DNA bound to nitrocellulose filters (Birnstiel et al., 1972). However, in contrast to DNA reassociation, the maximum rate of RNA-DNA hybridization is obtained at about 10 to 15°C below the T_m of the hybrids.

1.6.2 Hybridization procedures

(a) Filter hybridization

Single stranded DNA or RNA can be immobilized on nitrocellulose paper under appropriate conditions without losing the ability to form stable duplexes (Gillespie and Spiegelman, 1965; Thomas, 1980). Nitrocellulose filters bind DNA and RNA very efficiently (80$\mu g/cm^2$) but are fragile. Nylon filters are more pliable than nitrocellulose and can be used indefinitely without disintegrating (Anderson and Young, 1985). Although nitrocellulose and nylon filters immobilise nucleic acid, binding by conventional procedures is non-covalent. Therefore, hybrids may dissociate from the filter (Haas et al., 1972). New techniques have been developed for binding of nucleic acid to membranes by UV light induction (Church and Gilbert, 1984). All filters require the nucleic acid to be denatured for binding but there is no immobilisation procedure uniformly applicable to all types of filter. Nitrocellulose filters require high ionic strength for binding of both DNA and RNA (Nagamine et al., 1980).

Hybridization of radioactively-labelled nucleic acids to filter-bound DNA is a three-stage procedure. The filter is first prehybridized in a solution containing heterologous single stranded nucleic acid, SDS and Denhardt's solution (Denhardt, 1966). The purpose of this step is to saturate binding sites on the filter that would otherwise lead to an unacceptable background. The filter

is then incubated in the same solution containing the radioactively-labelled probe for sufficient time to allow hybridization to take place. Even under optimal conditions no more than 80% of a single sequence probe appears in hybrids (Flavell et al., 1974). In practice, for double stranded DNA probes, there is no need to continue hybridization for longer than is necessary to allow the probe in solution to achieve 1-3 times $C_o t$ 1/2, the value required for 50% reassociation and is a product of the concentration of nucleotides (mol. litre^{-1}) and the time in seconds. To determine the number of hours needed to achieve $C_o t$ 1/2 for renaturation of any other probe the appropriate values can be substituted in the equation

$$n = \frac{1}{x} \times \frac{y}{5} \times \frac{2}{10} \times z$$

where

n = number of hours

x = weight of the probe added in μg

y = length of the probe in kb

z = volume of the reaction in ml. (Anderson and Young, 1985)

After hybridization the filters are washed under conditions of temperature and salt concentration such that only specific hybrids are stable. The location of the hybrid molecules is then determined by autoradiography. Nonisotopic nucleic acid probes are described in Section 1.6.4b.

Several developments seek to improve the sensitivity of filter hybridization and bring it to the point where it

may be a realistic diagnostic method. One such approach is sandwich hybridization (Wolf et al., 1986). In the first step, hybridization takes place between the denatured nucleic acid on a filter and unlabelled M13 containing a complementary DNA insert. In the second hybridization, the replicative form of M13 without an insert but radiolabelled with ^{32}P is used. Thus a network of DNA strands forms above the first hybrid increasing the sensitivity of the hybridot assay to less than pg quantities of viral DNA.

(b) In situ hybridization

This methodology allows the detection and localization of nucleic acid sequences within cell and tissue preparations. For example, in situ hybridization can be used to map sequences of interest in condensed chromosomes (Harper et al., 1986), provide analysis of the tissue distribution of transcripts (Singer and Ward, 1982) and enable identification of the site of gene expression (Pardue, 1985). One of the major difficulties with in situ hybridization has proved to be the fixation and pretreatment of the cells to maintain their integrity yet allow access of the probe. The size of the probe is important in this context with small probes, about 70 bp, giving optimal hybridization efficiency (Moench et al., 1985). Pretreatment of slides with poly-L- or D-lysine (Aksamit et al., 1985) or by silanation (Tourtellotte et al., 1987) may be helpful in keeping the specimens intact during the hybridization procedure.

1.6.3 Nucleic acid probes

(a) Nick-translated

The most frequently used methods for uniformly labelling hybridization probes are nick-translation, random priming and *in vitro* transcription using phage polymerases. These are discussed in more detail below.

The process of "nick-translation" utilises DNase I to create single strand nicks in double stranded DNA. The 5'-3' exonuclease and 5'-3' polymerase actions of E.coli DNA polymerase I are then used to remove stretches of single stranded DNA starting at the nicks and replace them with new strands made by the incorporation of labelled deoxyribonucleotides (Rigby *et al.*, 1977). As a result, each nick moves along the DNA strand being repaired in a 5' - 3' direction (nick-translation). Spector *et al.* (1984b) have used such nick-translated DNA probes to detect HCMV in clinical specimens. Advantages and disadvantages of nick-translation are listed in Table 2.

(b) Primer extension

In common with nick-translation, primer extension methods utilize the ability of DNA polymerases to synthesize a new DNA strand complementary to a template strand, starting at a free 3'-hydroxyl. In this case, the latter is provided by a short oligonucleotide primer annealed to the template. The various approaches are described below.

1. Random primers used with Klenow polymerase

Hexanucleotides of random sequence, either derived

Table 2 Advantages and disadvantages of nick-translation.

Advantages

1. Allows control over a variety of parameters, for example, probe size, yield, specific activity, substrate concentration and reaction time.
2. Subcloning is not required.
3. Medium (10^8 to 10^9 cpm/μg) and high (>10^9 cpm/μg) specific activity probes can be produced.
4. Good utilization of label (60 to 70% incorporation).
5. DNA probes allow standard hybridization temperatures to be used.
6. Large amount of probe produced in standard protocols (~1μg), and so a single labelling reaction usually produces sufficient probe for several hybridizations.
7. Ideal for generating microgram quantities of biotinylated probe.
8. Labels circular and linear DNA.
9. Short reaction times possible (60 to 90 min).
10. Leads to very uniform labelling.
11. A variety of radiolabels (^{3}H, ^{35}S and ^{32}P) can be used.
12. More than one labelled nucleotide can be used simultaneously, for example [α-^{32}P]dCTP and [α-^{32}P] dATP.

Particularly suitable for purified DNA available in relatively large amounts, (for example, a plasmid preparation for which the presence of vector sequence in hybridization is unimportant).

Disadvantages

1. Unpredictable results with impure DNA substrates (particularly DNA in agarose).
2. Uniform labelling method - not limited to insert sequence.
3. Large amount of substrate (~1μg) usually required.
4. Temperature and time of reaction require careful control to avoid strand displacement and excision of incorporated label.
5. Requires control of two enzyme activities.
6. DNA-DNA hybrids have lower stability than DNA-RNA and RNA-RNA hybrids.
7. Probe denaturation required.
8. Probe not strand-specific. Strand reannealing of probes may occur.
9. Will not label single stranded DNA.

from DNase I digestion of calf thymus DNA or produced by oligonucleotide synthesis, have been used to prepare labelled copies of both DNA and RNA. Feinberg and Vogelstein (1983) first described this approach for the labelling of DNA fragments to high specific activity. Advantages and disadvantages of random primer labelling with Klenow polymerase are given in Table 3.

2. Unique primers used with Klenow polymerase

High specific activity hybridization probes can be generated using the M13 universal probe primer (Hu and Messing, 1982). The DNA which is to be used as probe is first cloned into one of the bacteriophage M13 vectors. On first infecting bacteria, 100 to 200 copies of the double stranded replicative form of M13 are synthesized but thereafter the synthesis of DNA is asymmetric, only one cDNA strand (+) being produced. This is packaged into phage particles which are then released from the host continuously without lysing it, thus making subsequent purification very simple. Viral inserts are placed in the intergenic M13 sequence which does not affect viral replication and inserts up to 1000 nucleotides are stable on replication of the bacteriophage.

The 13-base sequence of the M13 universal probe primer ($^{5'}$GAAATTGTTATCC$^{3'}$) is complementary to the 5' side of the multiple cloning site (MCS) of the family of M13 vectors and is used to initiate synthesis of the (-) strand from the (+) strand template by the Klenow fragment of E.coli

Table 3 · Advantages and disadvantages of random primer labelling

Advantages

1. High specific activity probes (>10^9cpm/μg).
2. Efficient utilization of label (70 to 80%).
3. Flexible reaction temperature and time (up to overnight).
4. Can label small amounts of DNA.
5. Labels single stranded DNA (and double stranded after denaturation).
6. Labels impure DNA efficiently (minilysate or DNA in agarose).
7. Several labelled nucleotides can be used simultaneously.
8. Subcloning is not required.
9. DNA probes allow standard hybridization temperatures to be used.
10. Leads to uniform labelling.
11. Incorporated label not excised during reaction.
12. Reaction parameters can be controlled (for example, input label, primer concentration) to influence probe size, yield, etc.

Ideal for probe sequence available in small amounts, in impure form (for example, gel-purified insert).

Disadvantages

1. Quantity of probe produced (~70ng); generally sufficient for a single hybridization only
2. Relatively inefficient with circular DNA substrates.
3. Uniform labelling method - not limited to insert sequence.
4. DNA-DNA hybrids have lower stability than DNA-RNA and RNA-RNA hybrids.
5. Denaturation of input DNA required.
6. Probe denaturation required.
7. Probe not strand-specific. Strand reannealing of probes may occur.

DNA polymerase I (Jacobsen et al., 1974). Synthesis of the complementary strand, which can be labelled by incorporation of an [α-^{32}P] deoxyribonucleotide, does not proceed to completion so that the inserted probe sequence remains single stranded. Synthesis of the probe is shown diagrammatically in Figure 7.

One example of an M13 recombinant probe being used diagnostically is for rhinovirus detection in nasal secretions (Al-Nakib et al., 1986). An indirect sandwich hybridization based on M13 probes is described above (Wolf et al., 1986).

3. Unique primers used with reverse transcriptase

Unique primers have been used most frequently in conjunction with reverse transcriptase in two ways. The first is in the technique of primer extension mapping by which a labelled primer is annealed to an RNA molecule and is then extended in order to map the 5'-end of the RNA (Williams and Mason, 1985). The second major approach is more strictly a method of probe production. By annealing an oligo-dT primer to the 3' poly (A) tail of an mRNA molecule, it is possible to synthesize a labelled cDNA in the presence of reverse transcriptase and appropriate nucleotides (Arrand, 1985).

(c) Synthetic oligonucleotide probes

With advances in the technology of synthesizing DNA it has become possible to prepare oligonucleotides of defined sequence at reasonable cost. The success of such an approach depends on the availability and correctness of

Figure 7 M13 partially double stranded DNA probes

A schematic representation of M13 second strand synthesis.

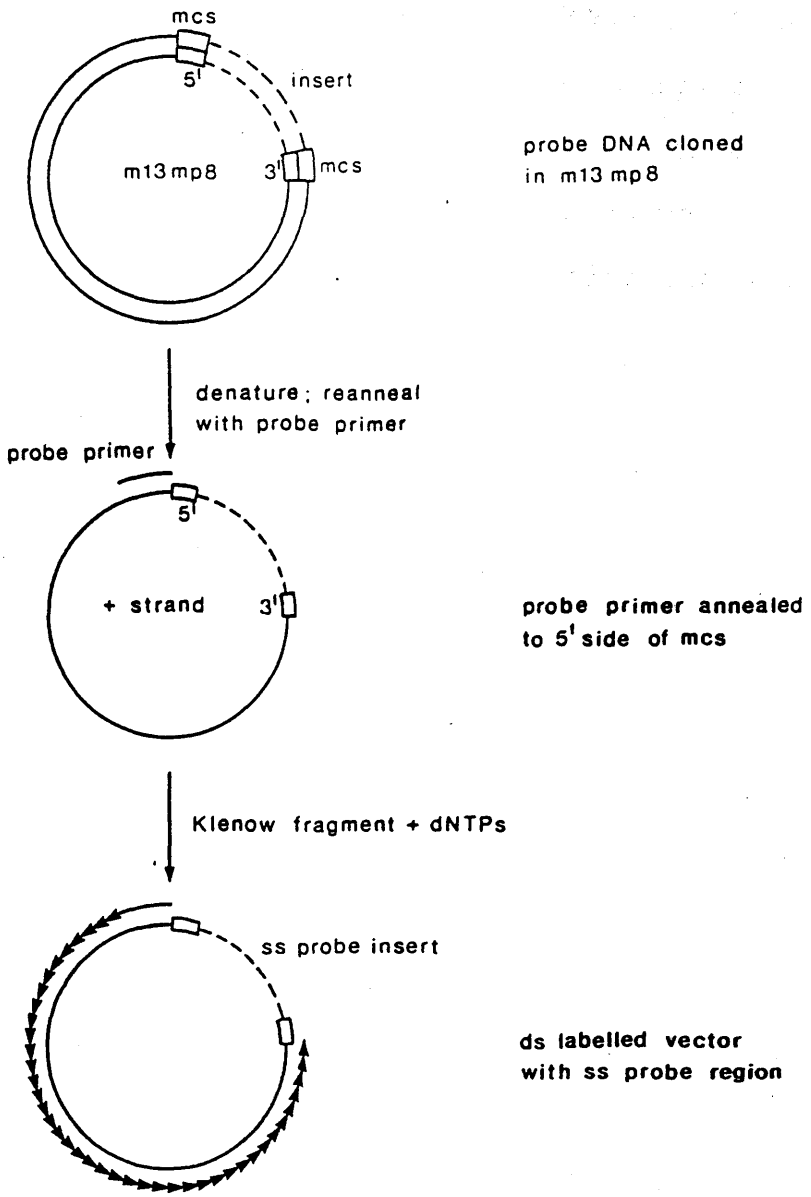
▶▶▶▶▶ : labelled DNA strand

ds : double stranded

ss : single stranded

dNTPs : deoxyribonucleoside triphosphates

MCS : multiple cloning site.


mcs
5'
insert
m13 mp8
3'
mcs
probe DNA cloned
in m13 mp8
denature; reanneal
with probe primer
probe primer
5'
+ strand
3'
probe primer annealed
to 5' side of mcs
Klenow fragment + dNTPs
ss probe insert
ds labelled vector
with ss probe region

sequencing data but has the considerable advantage of eliminating the need for purification and cloning of viral nucleic acids. In addition, absolutely defined sequences, specific for or shared by members of a virus group, can be easily selected. The use of a synthetic oligonucleotide probe in hepatitis B virus (HBV) diagnosis has been described recently (Lin et al., 1987). The sensitivity of hybridization was comparable to that using cloned HBV DNA probes but the time period of hybridization was shortened from 16 to 2 hr.

(d) Methods based on RNA polymerase

RNA polymerases catalyze the synthesis of RNA from ribonucleoside triphosphates (rNTPs) using a DNA template. Historically two main approaches to the production of RNA probes have been adopted. The first involved the use of E.coli RNA polymerase. This enzyme when used in vitro shows very little template and promoter specificity and therefore produces transcripts which have been initiated and terminated more or less at random. The RNA polymerases from a number of bacteriophages including salmonella phage SP6 and coliphages T3, T5, T7 possess a high degree of specificity for their own promoters in vitro (Butler and Chamberlin, 1982; Davanloo et al., 1984).

A number of SP6 and T7 cloning and transcription systems are now available (Green et al., 1983). These generally consist of a pBR322 plasmid into which has been inserted the appropriate RNA polymerase gene and its

promoter. A few bases away from the promoter is a MCS into which a selected fragment of the DNA required as a hybridization probe can be subcloned. Transcription in the presence of [α-^{32}P] rNTP and RNA polymerase proceeds from the promoter through the probe sequence giving rise to an RNA transcript of high specific activity. This is outlined schematically in Figure 8. Up to 10μg of RNA probe may be synthesized from 1μg of template DNA (Melton et al., 1984).

RNA probes have been reported to show higher sensitivity than nick-translated probes in both Northern blots (Melton et al., 1984) and in situ hybridization (Cox et al., 1984). This is most probably due to the inability of the probe to reanneal during hybridization and the greater stability of RNA-RNA and RNA-DNA hybrids compared to DNA-DNA hybrids. A further advantage of RNA probes is that non-specifically bound probe can be removed by treatment with RNase A which is very specific for single stranded RNA (Melton et al., 1984). Other advantages and disadvantages of phage polymerase methods are listed in Table 4.

1.6.4 Labelling of nucleic acid probes

There are 2 important parameters which largely determine the choice of label; resolution and sensitivity. Different applications may require a different balance between these parameters. Other considerations include safe handling of radioisotopes and probe stability. The choice of label is discussed below.

Figure 8 The SP6 polymerase system

▶▶▶▶ : labelled RNA transcripts

rNTPs : ribonucleoside triphosphates

MCS : multiple cloning site

0 : SP6 polymerase

→ → : direction of transcription

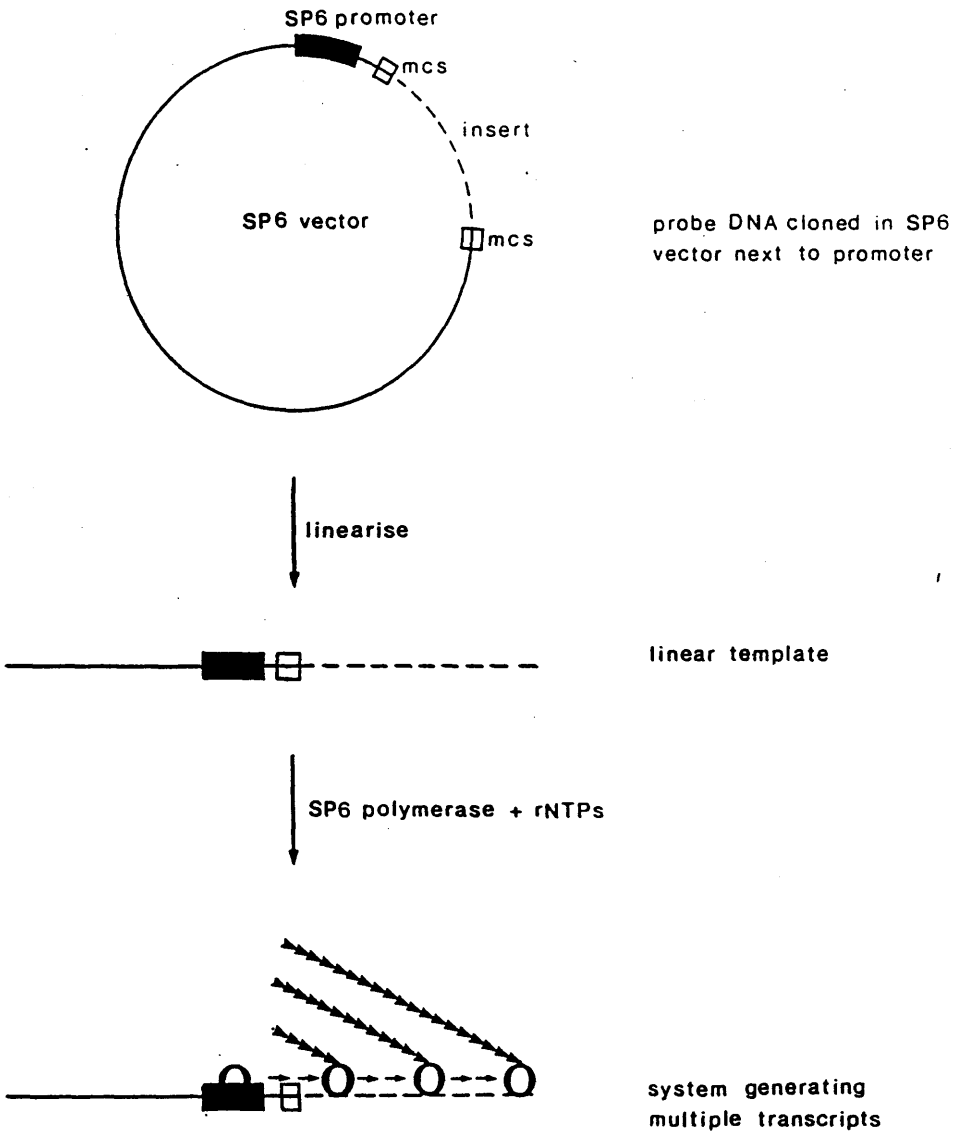

SP6 promoter
mcs
insert
SP6 vector
mcs
probe DNA cloned in SP6 vector next to promoter
linearise
linear template
SP6 polymerase + rNTPs
system generating multiple transcripts

Table 4 Advantages and disadvantages of phage polymerase methods

(a) Radioactive

Traditionally, filter hybridizations have been carried out with radioactively-labelled probes. Indeed, nick-translation with the incorporation of one or more α^{32}P-labelled deoxynucleoside triphosphates into double stranded DNA is often used. As ^{32}P has a half-life of only 14 days, isotopes of longer half-life have been assessed. ^{125}I with a half-life of 60 days may be introduced catalytically into cytosine residues but this alters the T_m of the hybridization reaction and has rarely been used in a diagnostic context (Norval and Bingham, 1987). ^{35}S (half-life 87 days), as the α-^{35}S thiotriphosphate of the nucleotides, has been incorporated into probes by nick-translation. Such probes have been used in the detection of viruses by both filter (Chowdhury et al., 1986) and in situ (Gendelman et al., 1985) hybridization. ^{3}H-labelled probes have been used only for in situ hybridization but exposure times are often in excess of several weeks (Pardue, 1985). Such a lengthy procedure is likely to be of little interest in routine diagnosis.

(b) Non-radioactive

Biotin is a small, water-soluble vitamin which is becoming increasingly popular in the labelling of nucleic acids (Leary et al., 1983). Detection of biotinylated probes after hybridization is generally via avidin, a glycoprotein of MWt 68 kd which has 4 binding sites and extremely high affinity for biotin. Visualization of

Advantages

1. Transcription limited to insert.
2. Efficient utilization of label (80%).
3. High specific activity probes (>10^9cpm/μg).
4. Short reaction time (60 min).
5. No requirement for probe denaturation leading to improved background on nitrocellulose.
6. Probe can be specific for either DNA strand.
7. Template removal possible.
8. Variety of labels (^{32}P, ^{35}S, ^{3}H, biotin) can be used.

Ideal for multiple use probes; avoids contamination with vector sequences.

Disadvantages

1. Subcloning required.
2. Restriction digestion to completion required.
3. RNA probe must be stored correctly to avoid RNase activity.
4. Narrow temperature optima for hybridization for many RNA probes.
5. Difficult to control probe size without limiting transcription.
6. Large amount of template required (1 to 2μg).
7. Small amount of probe produced (~300ng).
8. Premature termination may occur.
9. Template removal may be required.
10. Performance depends on labelling nucleotide.
11. Only a single labelling nucleotide can be used efficiently (except for ^{3}H, where concentration is not limiting).

(a) Radioactive

Traditionally, filter hybridizations have been carried out with radioactively-labelled probes. Indeed, nick-translation with the incorporation of one or more α^{32}P-labelled deoxynucleoside triphosphates into double stranded DNA is often used. As ^{32}P has a half-life of only 14 days, isotopes of longer half-life have been assessed. ^{125}I with a half-life of 60 days may be introduced catalytically into cytosine residues but this alters the T_m of the hybridization reaction and has rarely been used in a diagnostic context (Norval and Bingham, 1987). ^{35}S (half-life 87 days), as the α-^{35}S thiotriphosphate of the nucleotides, has been incorporated into probes by nick-translation. Such probes have been used in the detection of viruses by both filter (Chowdhury et al., 1986) and in situ (Gendelman et al., 1985) hybridization. ^{3}H-labelled probes have been used only for in situ hybridization but exposure times are often in excess of several weeks (Pardue, 1985). Such a lengthy procedure is likely to be of little interest in routine diagnosis.

(b) Non-radioactive

Biotin is a small, water-soluble vitamin which is becoming increasingly popular in the labelling of nucleic acids (Leary et al., 1983). Detection of biotinylated probes after hybridization is generally via avidin, a glycoprotein of MWt 68 kd which has 4 binding sites and extremely high affinity for biotin. Visualization of

biotin-avidin complexes is usually via conjugates of avidin or anti-avidin antibodies with enzymes, fluorescent groups or heavy metals. In practice, streptavidin, an avidin-like protein isolated from the bacterium Streptomyces avidinii, is often used in place of avidin. Its main advantage is a near neutral isoelectric point which leads to a reduction in non-specific binding to DNA thereby minimizing background signal. Presently many studies employ a streptavidin-biotinylated complex containing peroxidase (Przepiorka and Myerson, 1986) or alkaline phosphatase (Rotbart et al., 1988).

There are several advantages of using biotinylated probes. For example, non-toxic materials are employed and there are no problems of inconveniently short half-lives of the label. Detection of hybrids is much faster than for radioactive probes, visualization of hybrids being complete betweem 2 and 4 hr after washing. The major disadvantage of biotin, and of other non-radioactive labels, is low sensitivity in comparison to radioactive labels. Most studies report a sensitivity approximately 5- to 10- fold less than equivalent α^{32}P-labelled probes (McKeating et al., 1985).

The labelling methods described so far have primarily utilized enzymatic reactions to incorporate nucleotides labelled with radioisotopes or non-radioactive reporter molecules e.g. biotin. In the remainder of this section several examples of direct labelling methods which do not use enzymes to incorporate label are outlined briefly. The

aim of such techniques is to attach the label covalently to the nucleic acid probe.

For use in non-radioactive labelling, a photoactivable analogue of biotin has been used for rapid preparation of DNA and RNA hybridization probes (Forster et al., 1985). Upon brief irradiation with visible light, stable linkages are formed with both single and double stranded nucleic acids. Another class of photochemical reagents for investigation of nucleic acid structure and function are the psoralens. These primarily react with pyrimidine bases and can be detected by enzymatic methods (Sheldon et al., 1986).

Peroxidase and alkaline phosphatase have been directly crosslinked to DNA thereby obviating the requirement for a reporter group such as biotin (Jablonski et al., 1986). For example, horseradish peroxidase can be crosslinked to polyethyleneimine with p-benzoquinone and the resulting conjugates covalently linked to DNA using glutaraldehyde (Renz and Kurz, 1984). Other chemical methods include modification of nucleic acids by acetylaminofluorene (Tchen et al., 1984) and 5'-end-labelling of oligodeoxynucleotides with biotin through an aminoalkylphosphoramide linker arm (Kempe et al., 1985).

Whilst direct labelling may offer more convenience in preparation of probes than enzymatic methods, there is little published data on their application and sensitivities.

1.6.5 Clinical application of hybridization probes

Several situations may be envisaged where nucleic acid hybridization might provide valuable information not obtainable by other methods:-

1. Where no infectious virions are present in the clinical sample because of inactivation during specimen collection or transport to the laboratory. The virus may only be found as defective particles or in immune complexes or indeed, only as viral nucleic acid in an integrated or non-integrated form.

2. Where there is contamination of the sample with other microbes.

3. Where there is no in vitro culture system available or the isolation is particularly slow.

4. Where no specific antigenic reagents are available.

5. Where it is necessary to identify a specific cell type, containing virus or viral nucleic acid, within a tissue.

The development of specific, cloned subgenomic fragments of DNA provides new reagents for the detection of HCMV in clinical specimens (Fleckenstein et al., 1982). Several hybridot assays providing results within 24 hr of specimen collection have been established. α^{32}P-labelled nick-translated DNA probes have been shown to detect between 500fg (Augustin et al., 1987) and 100pg (Churchill et al., 1987) of HCMV DNA with a sensitivity of 83% and a specificity of 92% compared with virus isolation (Schuster

et al., 1986). Furthermore, there is a strong correlation between the titre of HCMV in the clinical specimen and the intensity of the hybrid signal (Chou and Merigan, 1983). In vitro synthesized α^{32}P-labelled RNA transcripts specific for HCMV have also been used to detect the virus in urine specimens but the sensitivity of the hybridot assay was equivalent to that when using nick-translated DNA probes (Schuster et al., 1986). Recently an oligo-primed α^{32}P-labelled DNA probe specific for HCMV has been described and shown to have a sensitivity of 86.7% compared with conventional virus isolation (Agha et al., 1988). Nonisotopic HCMV-specific probes have also been assessed. A DNA probe labelled with peroxidase was reported to have a sensitivity of 80pg (Schuster et al., 1986) while hybridization using biotin-labelled probes can detect a minimum of 30pg of HCMV DNA or 3 $\log_{10}$ $TCID_{50}$ of HCMV in urine samples (Buffone et al., 1986; Lurain et al., 1986).

While several groups have reported promising results concerning the detection of HCMV DNA in urine samples there are several problems inherent in the methodology. In addition to the relatively fastidious procedures necessary for the preparation of a specific hybridization probe, sample preparation is also laborious, requiring ultracentrifugation to obtain maximum sensitivity. The use of insert-containing plasmids rather than isolated inserts as hybridization probes may yield false-positive results owing to the presence of homologous bacterial DNA sequences

in the clinical specimens (Ambinder et al., 1986). Another matter of concern is the presence of DNA sequences in the HCMV genome which are complementary to those of human cellular DNA (Ruger et al., 1984). Finally, HCMV DNA cannot be detected in all urine specimens yielding the virus in tissue culture (Spector et al., 1984b).

The detection of HCMV by in situ hybridization in dram vial cell culture (Sorbello et al., 1988), peripheral blood mononuclear cells (Stockl et al., 1988) and formalin fixed paraffin wax embedded specimens (Naoumov et al., 1988) has also been assessed. Although good sensitivity has been reported with biotinylated DNA probes, hybridized DNA complexes were not detected up to 4 days after early nuclear antigens were identified by FAT (Scott et al., 1988; Gleaves et al., 1989). This delay is likely to be related to the sequential events of the HCMV replicative cycle in which early proteins are synthesized before viral DNA replication.

1.6.6 Polymerase chain reaction

Advances in nucleic acid technology during the past few years have yielded practical probe-based assays for diagnosing infectious and genetic diseases. Although the assays are sensitive, cells, tissues, or body fluids often need to be cultured to increase the number of available cells or microorganisms to readily detectable levels.

A novel technique, PCR, has recently been developed for in vitro amplification of DNA or RNA (Saiki et al.,

1988). The principle of the PCR is straightforward, requiring a three-step cycling process: (1) denaturation of double stranded DNA (2) annealing of primers and (3) primer extension. A cycle typically takes between 3 and 5 min and is repeated 20 to 40 times (Mullis and Faloona, 1987). After 30 cycles, a single copy of DNA can be amplified by a factor of 10^6 (Schochetman et al., 1988).

PCR has been used in the detection of HCMV in urine (Demmler et al., 1988) and peripheral blood (Shibata et al., 1988). Using no more than 10μl of specimen, the amplification reaction could be performed within 48 hr and provided a sensitivity and specificity of 93% and 100% respectively compared with virus isolation.

MATERIALS

2.1 Tissue culture cells

The following cells were used during the project:-

Flow 2002, a semicontinuous cell line established from human embryo lung fibroblasts, was purchased from Flow Laboratories Limited, Rickmansworth, England.

BHK21/C13, a continuous cell line established from baby hamster kidney cells by MacPherson and Stoker (1962) and maintained in the Institute of Virology.

Vero, a continuous cell line derived from the kidney of an African green monkey, was provided by the Cytology Laboratory, Institute of Virology.

2.2 Tissue culture media

Flow 2002, BHK21/C13 and Vero cells were grown in the Glasgow modification of Eagles medium (Busby et al., 1964) supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 0.002% (w/v) phenol red.

Foetal calf serum was purchased from Gibco BRL, Paisley, Scotland.

Calf serum and pooled human serum were prepared in the Institute of Virology.

The following composite solutions were used:-

EFX = Eagles medium containing X% (v/v) foetal calf serum

ECX = Eagles medium containing X% (v/v) calf serum

EC1-Pi = Eagles medium containing 1% (v/v) calf serum but lacking orthophosphate.

EHu4 = Eagles medium containing 4% (v/v) pooled human

serum

PBS = Phosphate buffered saline contained 170mM NaCl, 34mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4 in distilled water pH 7.2.

PBS Ca = PBS containing 10% (v/v) calf serum

PBS F = PBS containing 10% (v/v) foetal calf serum.

Trypsin was prepared as a 0.25% solution supplemented with 0.0015% (w/v) phenol red.

Agar overlay medium for HCMV titrations was prepared at 45°C to a final concentration of 0.3% (w/v) Seakem LE agarose, 1 x Eagles medium without phenol red and supplemented with 2.6% (v/v) foetal calf serum.

2.3 Viruses

The following viruses were used during the project:-

HCMV AD169 (Rowe et al., 1956) was provided by the Diagnostic Laboratory, Western Infirmary, Glasgow.

HCMV strain Davis (Weller et al., 1957) was supplied by the Virus Reference Laboratory, Public Health Laboratory Service, Colindale, U.K.

HSV-1 strain 17 syn^+ (HSV-1 17 syn^+) (Brown et al., 1973) was provided by the Institute of Virology.

HSV-2 strain HG52 (HSV-2 HG52) (Timbury, 1971) was supplied by the Institute of Virology.

VZV was provided by the Regional Virus Laboratory, Ruchill Hospital, Glasgow.

2.4 Bacterial strains

Two strains of E.coli were employed:-

JM101 [(lac; pro), sup E, thi$^-$, F'tra D36, pro AB, lac I^q, Z M15] (Messing et al., 1981), the host strain for maintenance of M13 bacteriophage, was a gift from Dr D. McGeoch, Institute of Virology.

DH5 [F^-, end A1, hsd R17 (r^-_k, m^+_k), sup E 44, thi-1, $^-$, rec A1, gyr A96, re 1A1] (Hanahan, 1983), purchased from Gibco BRL, Paisley, Scotland, was used as the host in all recombinant plasmid experiments.

2.5 Bacteriophage

The vector used for the production of partially double stranded DNA probes was derived from M13mp8. The clone, JR139, was a gift from Dr D. McGeoch, Institute of Virology and contained part of HSV-1 gene UL 8.

2.6 Plasmid vectors

Two vectors were used during the project:-

The HindIII E/BamHI (EHBI) subclone in pAT153 was made available by Dr J.D. Oram, PHLS Centre for Applied Microbiology and Research, Porton Down, England.

pGEM2, a transcription plasmid based on the SP6 polymerase system (Melton et al., 1984), was purchased from Promega, Wisconsin, U.S.A. This plasmid contains promoters for both SP6 and T7 polymerase.

2.7 Bacterial culture media

2.7.1 JM101

JM101 were propagated in 2YT broth which was 1.6% (w/v) bactopeptone, 1% (w/v) yeast extract and 0.5% (w/v) NaCl in distilled water sterilized by autoclaving.

Top agarose for plating bacteriophage consisted of LB (1% [w/v] bacto-tryptone, 0.5% [w/v] yeast extract and 170mM NaCl with the pH adjusted to 7.5) supplemented with 0.7% (w/v) agarose (type 1, low EEO) prior to autoclaving.

2.7.2 DH5

DH5 were propagated in LB. Solid media for the growth of colonies were prepared from LB to which was added 1.5% (w/v) bacto-agar. All media were sterilized by autoclaving and then supplemented with ampicillin to a final concentration of 50μg/ml.

2.8 Standard buffer solutions

Denhardt's solution: 0.02% (w/v) Ficoll, 0.02% (w/v), polyvinylpyrollidone, 0.02% (w/v) BSA.

Dye Ficoll: 150mM Na_2HPO_4, 10mM EDTA, 180mM Tris-HCl (pH 7.8), 10% (w/v) Ficoll, 0.05% (w/v) bromophenol blue.

RSB: 10mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl (pH 7.5).

SSC: 150mM NaCl, 15mM Tri-sodium citrate (pH 7.0).

STE: 1mM EDTA (pH 8.0), 100mM NaCl, 10mM Tris-HCl (pH 8.0).

TBE electrophoresis buffer: 89mM Tris-borate, 89mM boric acid, 2mM EDTA.

TBE electroelution buffer: 10.8g Tris-HCl, 2.35g boric acid and 0.37g EDTA in 1 litre of water.

TE: 1mM EDTA, 10mM Tris-HCl.

2.9 Chemicals

M13 hybridization probe primer was purchased from Biolabs, Beverly, MA, U.S.A.

Ammonium persulphate and TEMED were obtained from Biorad Laboratories Ltd., Richmond, CA, U.S.A.

Bactotryptone and yeast extract were supplied by Difco Laboratories, Detroit, Michigan, U.S.A.

Formamide was purchased from Fluka Chemie AG, CH-9470, Buchs.

Seakem LE agarose was obtained from FMC Corporation, Marine Colloids Division, Rockland, U.S.A.

Biotinylated dATP, a 0.16 to 1.77 kb RNA ladder and restriction enzyme (React) buffers were supplied by Gibco BRL, Paisley, Scotland.

Caesium chloride and dimethyl sulphoxide were purchased from Koch-Light Laboratories Ltd., Colinbrook, Bucks., U.K.

BSA, deoxyribonucleotides, Ficoll (average MWt 400000), E. coli DNA, human placental DNA, ribonucleotides and Sephadex G50 were obtained from Pharmacia Ltd., Central Milton Keynes, Bucks, U.K.

Agarose (type 1, low EEO), bacteriophage lambda DNA, bromcresol green, bromophenol blue, diethylpyrocarbonate, DTT, ethidium bromide, IPTG, NP40, salmon sperm DNA, triton X-100, xylene cyanol and X-Gal were provided by Sigma Chemical Company Ltd., Poole, Dorset, U.K.

Ampicillin was purchased from Vestric Ltd., Glasgow.

All other chemicals were obtained from BDH Chemical Company Ltd., Poole, Dorset, U.K. and were of analytical or reagent grade.

2.10 Radiochemicals

All radiochemicals were purchased from Amersham International plc, Bucks, U.K. at the following specificities

5'-[α-^{32}P] dATP)	
5'-[α-^{32}P] dGTP)	3000Ci(111 TBq)/mmol 10 mCi(370MBq)/ml
5'-[α-^{32}P] dCTP)	
5'-[y -^{32}P] ATP	5000Ci(185 TBq)/mmol 10mCi(370MBq)/ml
5'-[α-^{32}P] UTP	800Ci(29.6 TBq)/mmol 20mCi(740 MBq)/ml

^{32}P orthophosphate was carrier free.

2.11 Enzymes

All restriction enzymes, E.coli polymerase I, CIAP, polynucleotide kinase, T4 DNA ligase and Klenow polymerase were purchased from Gibco BRL, Paisley, Scotland.

T7 polymerase and proteinase K were obtained from Boehringer Mannheim, GmbH, West Germany.

RNasin and RQ1 DNase were supplied by Promega, Wisconsin, U.S.A.

DNase (bovine pancreas), lysozyme, RNAse A and RNase T1 were purchased from Sigma Chemical Company Ltd., Poole, Dorset, U.K.

2.12 Complement fixation test reagents

HCMV, HSV (Types 1 and 2) and VZV glycine-extracted freeze-dried CF antigens were purchased from Institute Virion Limited Diagnostic Laboratories, Zurich, Switzerland.

Freeze-dried standard antisera were obtained from the Public Health Laboratory Service, Colindale, U.K.

Complement, a freeze-dried preparation of preserved guinea pig serum, and haemolysin were supplied by Wellcome Reagents Ltd., Beckenham, U.K.

SRBCs in Alsevers solution were purchased from Gibco BRL, Paisley, Scotland.

CFT diluent tablets were obtained from Oxoid Ltd., U.K.

2.13 Enzyme linked immunosorbent assays

The following ELISA systems were used during the project:-

HCMV-specific IgM and IgG purchased from Labsystems Oy, Pulttitie 8, 00880 Helsinki, Finland.

HCMV-specific IgG obtained from Northumbria Biologicals Ltd., Cramlington, U.K.

Rubazyme-M, an ELISA for the detection of IgM antibody to rubella virus in serum, was supplied by Abbott Laboratories, Diagnostic Division, Chicago, U.S.A.

2.14 Other serological assays

Rapitex assays for the detection of RF in serum were purchased from Behring Diagnostics, Hounslow, U.K.

Infectious mononucleosis absorption assays for the detection of heterophile antibodies were obtained from Mercia Diagnostics Ltd., Surrey, U.K.

2.15 Miscellaneous materials

An RIA for the quantitative measurement of β_2m in urine was provided by Abbott Laboratories, Diagnostic Division, Chicago, U.S.A.

Amersham Hyperfilm MP was purchased from Amersham International plc, Bucks., U.K.

Dupont Cronex Lighting Plus intensifying screens were obtained from Dupont, Connecticut, U.S.A.

Hybridization bags and a 96-well dot blot manifold were supplied by Gibco BRL, Paisley, Scotland.

X-Omat S film was provided by Kodak, Herts, U.K.

Cidex was purchased from Lever Industrial, Cheshire, U.K.

Millex 0.45μm filters were obtained from Millipore U.K. Ltd., Middlesex, U.K.

Ecoscint was supplied by National Diagnostics, New Jersey, U.S.A.

Gene Screen Plus TM hybridization transfer membranes were provided by New England Nuclear, Dupont U.K. Ltd., NEN Products Division, Herts, U.K.

Microtitre plates with 96 U-shaped wells, 50mm diameter petri dishes, linbro wells and 25cm^2 and 800ml plastic tissue culture flasks were purchased from Inter Med (Nunc) A/S Nunc, Kamstrupvej 90, Kamstrup, DK-4000, Roskilde, Denmark.

Nitrocellulose membrane filters (0.45μm pore) were obtained from Schleicher and Schuell, D3354, Dassel, West Germany.

2.5cm diameter circles of Whatman No.1 chromatography paper were supplied by Whatman Ltd., Maidstone, U.K.

METHODS

3.1 Tissue Culture

Flow 2002 cells were cultured in 800ml plastic flasks seeded at 2.5 x 10^7 cells in 100ml of EF10. 50mm diameter plastic petri dishes were seeded at a density of 5 x 10^5 cells per dish in 4ml of EF10 and Linbro wells at a density of 5 x 10^4 cells in 1ml of EF10.

BHK21/C13 cells were cultured in 80oz roller bottles seeded at 5 x 10^7 cells in 200ml of EC10. 50mm diameter petri dishes were seeded at a density of 2.0 x 10^6 cells per dish in 4ml of EC10 and Linbro wells at a density of 1.0 x 10^6 cells in 1ml of EC10.

Vero cells were cultured in 800ml plastic flasks seeded at 1.0 x 10^7 cells in 100ml of EF10 and subcultured in Linbro wells at a density of 5.0 x 10^4 in 1ml of EF10.

Cells were grown in an atmosphere of 5% CO_2 in air at 37°C.

All cells were harvested by washing twice with trypsin, incubating at 37°C for a few minutes and resuspending at the required concentration in the appropriate medium.

Routine checks for mycoplasma contamination were performed by the Cytology Laboratory, Institute of Virology.

Sterility checks were routinely performed by plating on to blood agar and incubating at 37°C.

3.2 Virus stocks

3.2.1 HCMV AD169

After removal of the growth medium, subconfluent Flow 2002 cells in an 80oz roller bottle were infected at a moi of 0.01 pfu per cell. The virus was absorbed for 1 hr at 37°C and the cells then overlaid with 50ml of EF5. Approximately 10 days later, when the cpe was complete, the cells were washed once with trypsin, harvested into 50ml of 1x Eagles medium and spun at 2000 rpm for 10 min in an MSE Coolspin centrifuge. The virus preparation was resuspended in 5ml of EF10 containing 10% (v/v) dimethyl sulphoxide and stored in sterile vials at -70°C. Before use, the HCMV stock was sonicated in a Cole Palmer ultrasonic cleaning bath until the solution became clear. Routine sterility checks were performed using blood agar plates. Particle counts were performed by Mr J. Aitken, Electron Microscopy Laboratory, Institute of Virology.

3.2.2 HSV

Ten 80oz bottles containing subconfluent monolayers of BHK21/C13 cells were infected with HSV at a moi of 1:300 pfu per cell. Virus was added in 40ml of EC5 per burler and incubated at 37°C for approximately 4 to 7 days when the cpe was near confluent. Cells were harvested by shaking them into suspension and the contents removed to 250ml glass MSE bottles which were centrifuged at 2000 rpm for 10 min. The pellet was resuspended in 5ml of supernatant, aliquoted and sonicated as described above.

Virus stock was frozen at -70°C until required. Particle counts were performed by Mr J. Aitken, Electron Microscopy Laboratory, Institute of Virology.

3.3 Isolation of virus from urine samples and throat swabs

Samples were transported to the laboratory and inoculated with minimum delay on to semi-confluent monolayers of Flow 2002 cells in 25cm^2 tissue culture bottles. Monolayers were overlaid with 5ml of maintenance medium (EF5) and incubated for a minimum of 8 weeks at 37°C in an atmosphere of 5% CO_2 in air. Cultures were usually examined at weekly intervals for the development of a cpe and then overlaid with fresh maintenance medium. In some bottles the characteristic morphological changes of HCMV were evident. Isolates were subcultured when the cpe was near confluent and identified as HCMV by their ability to grow on Flow 2002 cells but not on Vero cells or on BHK21/C13 cells. Isolates were also confirmed as HCMV by RNA-DNA hybridization (Section 4.4.12). All HSV isolates were confirmed by FAT performed by the staff of the Diagnostic Laboratory, Western Infirmary, Glasgow.

3.4 Virus titration

3.4.1 HCMV

The plaque assay of Wentworth and French (1970) was employed. Serial tenfold dilutions of HCMV were made in PBS containing 2% (v/v) foetal calf serum. 100µl inocula

were added to confluent monolayers of Flow 2002 cells in 50mm plastic petri dishes. After 60 min absorption at 37°C, 5ml of overlay agar medium were added to each plate and the monolayers incubated in a humidified atmosphere of 5% CO_2 in air at a temperature of 37°C. Seven days later a further 5ml of agar overlay medium were added and the incubation continued for 7 more days. Monolayers were then fixed by adding 5ml of Cidex to each plate for a minimum period of 4 hr. Then the agar overlays were discarded and the monolayers stained with Giemsa for 10 min at room temperature. Plaques were counted using a dissecting microscope.

3.4.2 HSV

Serial tenfold dilutions of HSV stock were made in PBS Ca and 100µl inocula added to confluent monolayers of BHK21/C13 cells. The virus was absorbed for 45 min at 37°C after which time 4ml of EHu4 were added. Incubation proceeded for a period of 2 days at 37°C in an humidified atmosphere of 5% CO_2 in air. Monolayers were then stained with Giemsa and plaques counted as described above.

3.5 In vivo labelling of HSV DNA

The method of Lonsdale (1979) was employed.

Confluent BHK21/C13 cells were harvested in EC1-Pi and seeded in Linbro wells at a density of 1 x 10^6 cells in 1ml of EC1-Pi. After overnight incubation at 37°C, the cell monolayers were almost confluent and were then infected

with HSV at a moi of 10 pfu per cell. Incubation was continued for 1 hr at 37°C after which time the medium was replaced by 0.5ml of EC1-Pi containing 50μCi of ^{32}P orthophosphate (carrier free). The infected cells were reincubated at 31°C for 24 to 48 hr.

Cells were harvested by the addition of 0.5ml of 5% (w/v) SDS to each well and mixed with an equal volume of phenol saturated with RSB. The phases were separated by centrifugation at 2000 rpm for 10 min at 4°C. The supernatant was mixed with 2 volumes of ethanol and the DNA precipitated by centrifugation at 2000 rpm for 10 min at 15°C. After the pellet was air dried, it was resuspended in 200μl of sterile distilled water containing 25μg and 5U of boiled RNase A and RNase T1 respectively and incubated at 37°C for a minimum period of 2 hr. Thereafter, 10μl aliquots were spotted on to 2.5cm diameter discs of Whatman No.1 paper. Unbound counts were removed by 3 washes each of 5 min duration in 5% (w/v) TCA at 4°C. The discs were then washed in ethanol, air dried and counted for ^{32}P in Ecoscint in order to equalize counts before restriction enzyme analysis.

3.6 DNA extraction

3.6.1 HSV

The procedure was based on that of Wilkie (1973).

Confluent monolayers of BHK21/C13 cells in 80oz roller bottles were infected as described above. After 3 days of incubation at 31°C, infected cells were detached by

agitation and pelleted by centrifugation at 2000 rpm for 10 min at 4°C. The supernatant containing cell released virus was saved while the cell pellet was resuspended in 5ml of RSB containing 0.5% (w/v) NP40 and held on ice for 10 min to allow lysis of cell membranes and release of nuclei. After centrifugation at 2000 rpm for 10 min at 4°C, the cytoplasmic supernatant was saved and the pellet resuspended in RSB/NP40. The nuclei were re-extracted and the second cytoplasmic supernatant retained. Virus was pelleted by centrifugation of the combined supernatants at 12000 rpm for 2 hr at 4°C in a Sorvall GSA rotor.

The virus pellet was resuspended in 8ml of RSB, sonicated in a Cole Palmer ultrasonic cleaning bath and lysed by the addition of SDS to a final concentration of 0.5% (w/v). The pellet was twice extracted with an equal volume of phenol equilibrated with TE and then an equal volume of chloroform/isoamyl alcohol 24/1 (v/v). The upper aqueous phase was dialysed overnight at 4°C against 0.1 x SSC and the DNA recovered by ethanol precipitation (3 volumes of ethanol and a tenth volume of 3M NaAc) at -20°C overnight.

HSV DNA was purified by isopycnic banding on caesium chloride gradients. Solid caesium chloride was added to the DNA solution to give a refractive index of 1.393. Centrifugation was for 24 hr at 40000 rpm in a Sorvall TV865B vertical rotor at 20°C. The gradients were fractionated by piercing the bottom of the tubes and

collecting 15 drop fractions. 5μl samples of each fraction were assayed by electrophoresis on a 1% (w/v) agarose mini gel containing 0.5μg/ml ethidium bromide and nucleic acids visualised under short wave UV radiation (254nm). Fractions containing HSV DNA were combined, dialysed overnight at 4°C against 0.1 x SSC and stored at -20°C.

3.6.2 Human cellular DNA

Confluent monolayers of Flow 2002 cells grown in an 80oz roller bottle were washed 3 times with 10ml of 1 x SSC: 2% (w/v) SDS and 5M sodium perchlorate added to a final concentration of 1M. The preparation was extracted twice with TE saturated phenol and once with chloroform/isoamyl alcohol 24/1 (v/v). After centrifugation at 2000 rpm for 10 min at 4°C, the upper aqueous layer was made 0.3M sodium acetate and the DNA precipitated in 3 volumes of ethanol at -20°C overnight. The DNA was pelleted at 2000 rpm for 10 min at 15°C, dried in a vacuum chamber and incubated at 37°C in sterile distilled water containing boiled RNase A and proteinase K to a final concentration of 25μg/ml and 20μg/ml respectively. Thereafter, the DNA was phenol extracted as described above and finally dialysed against 0.1 x SSC at 4°C. Stock DNA solutions were stored in aliquots at -20°C.

3.7 Estimation of DNA concentration

The concentration of DNA was estimated by agarose gel electrophoresis in mini gels. Samples of DNA were electrophoresed with known standard concentrations of λ DNA

on 1.0% (w/v) agarose gels in TBE electrophoresis buffer containing 0.5μg/ml ethidium bromide. The DNA was visualized by UV illumination at 254nm and the concentration determined by visual comparison of the sample fluorescence with that of the standards.

A more accurate estimate of the DNA concentration was obtained by optical density (O.D.) measurement at 260nm in quartz microcuvettes in a Perkin Elmer double beam RH spectrophotometer. The concentration was calculated assuming that 1 O.D. unit is equal to 40μg/ml of single stranded DNA. The purity of the DNA could be assessed by calculating the ratio between readings at 260nm and 280nm (O.D. 260/O.D. 280). For pure preparations of DNA this ratio is 1:8.

3.8 Gel electrophoresis

3.8.1 Agarose gel electrophoresis

Horizontal agarose gels (260mm x 160mm) ranging in concentration from 0.5 to 1.2% (w/v) in 1 x TBE electrophoresis buffer were used. The agarose was dissolved by boiling in a microwave oven, cooled to 50°C and 0.5μg/ml ethidium bromide added before pouring. TBE electrophoresis buffer containing 0.5μg/ml ethidium bromide was routinely used. DNA samples with added 0.2 volumes of Dye-Ficoll were subjected to electrophoresis at 2V/cm for 16 hr at room temperature. Gels were photographed under short or long wavelength UV light using Polaroid 665 or 667

film. Gels for autoradiography were dried on to glass plates in an oven at 80°C.

3.8.2 Alkaline agarose gel electrophoresis

0.7% (w/v) agarose in 50mM NaCl, 1mM EDTA was dissolved by boiling in a microwave oven and cooled to 50°C before pouring. Horizontal gels were routinely used. Samples of DNA to be analysed were ethanol precipitated and redissolved in 20µl of alkaline loading buffer (50mM NaOH, 1mM EDTA, 2.5% [w/v] Ficoll, 0.025% [w/v] bromcresol green). Electrophoresis was carried out in a buffer consisting of 30mM NaOH, 2mM EDTA at 2V/cm for 16 hr at 4°C. Prior to autoradiography the gel was soaked in 70% (w/v) TCA at room temperature for 30 min and thereafter placed in a gel drier.

3.8.3 Denaturing polyacrylamide gel for RNA electrophoresis

Vertical urea polyacrylamide gels measuring 265mm x 165mm x 0.35mm were prepared. Polyacrylamide at a concentration of 3.5% (w/v) was made 1.0 x for TBE and 8M for urea from stock solutions of 9.5M urea and 38% (w/v) acrylamide, 2% (w/v) N,N'-methylene bis-acrylamide in 7 x TBE electrophoresis buffer. To a final volume of 30ml were added 300µl of freshly prepared 10% (w/v) ammonium persulphate and 20µl TEMED to initiate polymerization. The gel was cast and allowed to set. 2µl of samples were mixed with 10µl of loading buffer (10M urea, 1mM Na_2EDTA, 17% [v/v] glycerol, 0.6% [w/v] bromophenol blue, 0.6% [w/v]

xylene cyanol), boiled for 5 min, quenched in ice and loaded. Gels were electrophoresed at 40W in 1 x TBE electrophoresis buffer until the bromophenol blue dye reached the bottom of the gel. Radioactive species were detected by autoradiography.

3.9 Restriction enzyme digestion of DNA

All restriction enzyme digests were performed at 37°C for a minimum of 3 hr in the appropriate REact buffer. 1U of the restriction enzyme was used per 1μg of DNA to be cleaved. Reactions were terminated by the addition of 1/6 volume of Dye-Ficoll and the products of digestion separated by gel electrophoresis.

3.10 3'-end-labelling of λ DNA

DNA was digested with the restriction enzyme AvaI using the procedure described in Section 3.9. 1μl each of 5'-[α-^{32}P] dGTP and 5'-[α-^{32}P] dCTP were added and the volume adjusted to 29μl with water. 1μl (10U) of Klenow polymerase was added and the mixture incubated at room temperature for 15 min. Unincorporated radionucleotides were removed by G50 Sephadex chromatography while the radiolabelled DNA was ethanol precipitated and redissolved in 100μl of sterile distilled water.

3.11 5'-end-labelling of the Gibco BRL 0.16-1.77 kb RNA ladder

3.11.1 Dephosphorylation

A reaction mixture consisting of 40μg RNA, 1.38U CIAP, 20μl 10 x CIAP buffer (500mM Tris-HCl [pH 9.0], 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine) was made up to 200μl with DEPC treated water (50μl of DEPC mixed with 20ml of sterile distilled water which is then autoclaved). After incubation at 37^{o}C for 30 min, 160μl water, 40μl 10 x STE and 20μl 10% (w/v) SDS were added, the solution mixed and then heated at 68^{o}C for 15 min. The solution was extracted once with water saturated phenol, twice with phenol/ chloroform 1/1 (v/v), twice with chloroform and 3 times with water saturated ether. Traces of ether were removed by heating at 50^{o}C for 10 min. RNA was precipitated by the addition of a tenth volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of ethanol. After overnight incubation at -20^{o}C, the RNA was pelleted and resuspended in 40μl of DEPC treated water. Finally, the RNA was quantitated by reading the absorbance at A_{260} as described in Section 3.7.

3.11.2 Labelling with polynucleotide kinase

A reaction mixture containing 10μg CIAP treated RNA ladder, 10μl (500mM Tris-HCl [pH 7.5], 100mM $MgCl_2$, 50mM DTT, 1mM spermidine and 1mM Na_2EDTA), 30U polynucleotide kinase and 100μCi [γ - ^{32}P] ATP was prepared. The volume was adjusted to 50μl with DEPC treated water and incubated at 37^{o}C for 30 min. The reaction was stopped by the

addition of 2μl 500mM Na_2EDTA (pH 7.5) and then extracted, once with phenol/chloroform 1/1 (v/v) and once with chloroform. Unincorporated nucleotides were separated by G50 Sephadex chromatography in buffer containing 1mM EDTA, 50mM NaCl, 10mM Tris-HCl, (pH 7.5). Radiolabelled RNA was ethanol precipitated and resuspended in 100μl of DEPC treated water as described in Section 3.11.1.

3.12 M13

3.12.1 Transfection

The method was essentially that of Cohen et al. (1972). E.coli JM101 grown to an O.D. of 0.3 at 630nm was pelleted by centrifugation at 8000 rpm for 5 min at 4°C in a Sorvall SS34 rotor. The cells were resuspended in a half volume of 50mM $CaCl_2$ and left on ice for 20 min. Centrifugation was repeated as before and the cells resuspended in a tenth volume of 50mM $CaCl_2$. 200μl of cells were added to 100μl $CaCl_2$ and 1μg of M13 recombinant DNA (1μg/50μl). The suspension was kept on ice for 45 min followed by incubation at 42°C for 15 min. Thereafter, it was added to 4ml of top agar at 42°C containing 25μl of 100mM IPTG and 25μl of 2% (w/v) X-Gal in dimethylformamide. The mixture was poured directly on to 90mm diameter LB agar plates and incubated overnight at 37°C.

3.12.2 Growth and purification of recombinant M13 clones

Four drops of an overnight culture of JM101 were added to 100ml of 2YT broth which was then dispensed in 1.5ml aliquots. One white recombinant M13 colony was added to each aliquot and incubated for 6 hr at 37°C in a shaking incubator. The broth was transferred to 1.5ml Eppendorf tubes and the cells pelleted by centrifugation at 13000 rpm for 10 min at 15°C. 800µl of supernatant were removed to a fresh Eppendorf tube and bacteriophage precipitated by the addition of 200µl of 20% (w/v) PEG 6000 in 2.5M NaCl at 4°C overnight. Bacteriophage was pelleted by centrifugation at 13000 rpm for 10 min at 15°C, resuspended in 100µl of TE and the DNA extracted once with 50µl TE saturated phenol. After ethanol precipitation, M13 DNA was dissolved in 50µl of sterile distilled water and stored at -20°C.

3.13 Construction of riboprobe pGHBI

3.13.1 Growth and purification of vector pEHBI DNA

The method is based on the alkaline lysis method of Birboim and Doly (1979).

10ml of LB containing 50µg/ml ampicillin were inoculated with 20µl of vector pEHBI seed stock and incubated overnight at 37°C in an orbital shaker. The following morning, this suspension was added to 300ml of LB containing 50µg/ml ampicillin and reincubated overnight as described above. Bacteria were pelleted by centrifugation at 8000 rpm for 5 min at 4°C, washed with 200ml of STE and centrifuged as before. The bacterial pellet was

resuspended in 5ml of Solution 1 (0.5% [w/v] lysozyme in 50mM glucose, 25mM Tris-HCl [pH 8.0], 10mM EDTA) and left at room temperature for 10 min. 10ml of freshly prepared Solution 2 (200mM NaOH, 1% [w/v] SDS) were added, the suspension carefully mixed and left on ice for 10 min until white strands of an SDS/protein/nucleic acid complex were visible. After centrifugation at 15000 rpm for 30 min at 4°C in a Sorvall SS34 rotor, the bacterial pellet was twice extracted with RSB saturated phenol and once with chloroform/isoamyl alcohol 24/1 (v/v). Nucleic acid was ethanol precipitated, vacuum dried and resuspended in 8.5ml of sterile distilled water. Plasmid DNA was purified by centrifugation to equilibrium in caesium chloride - ethidium bromide gradients. Caesium chloride and ethidium bromide at a final concentration of 1g/ml and 500μg/ml respectively were added to the solution of DNA. Centrifugation proceeded at 40000 rpm for 30 hr at 20°C in a Sorvall Ti65 rotor. The supercoiled plasmid DNA was visualized by fluorescence in long wave UV and aspirated by syringe. After the ethidium bromide had been removed by multiple extractions with water saturated butan-2-ol, the DNA was dialysed overnight at 4°C against 0.1 x SSC, ethanol precipitated and redissolved in sterile distilled water to a final concentration of 1mg/ml.

3.13.2 Recovery of HCMV-specific sequences

Cloned HCMV DNA sequences were removed from pEHBI by

double digestion with the restriction enzymes HindIII and EcoRI. Fragments were separated on a 0.6% (w/v) horizontal agarose gel containing 50μg/ml ethidium bromide and visualized by long wave UV illumination. The relevant DNA band was identified, cut from the gel and placed in the "sample well" of an electroelution chamber. After electrophoresis in TBE electroelution buffer at 200V for 3 hr, the insert DNA was removed from the "collection well", ethanol precipitated and resuspended in sterile distilled water at a concentration of 1μg/μl.

3.13.3 Cloning of riboprobe pGHBI

Insert DNA was prepared as described in Section 3.13.2.

Vector pGEM2 DNA was digested with the restriction enzymes EcoRI and HindIII, extracted once with TE saturated phenol, precipitated with an equal volume of isopropanol, air dried and resuspended in sterile distilled water at a concentration of 1μg/μl.

Reactions were performed in 20mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 10mM DTT, 0.6mM ATP in a final volume of 10μl. Ligation of insert and vector DNA was achieved by incubating an approximate tenfold molar excess of insert DNA with 20ng of vector DNA. 1U of T4 DNA ligase was added and the preparation incubated overnight at room temperature.

3.13.4 Transformation of DH5 cells

The ligation reaction was diluted fivefold with sterile distilled water. 1μl of the diluted DNA (approximately 5ng) was added to 20μl of chilled DH5 cells and kept on ice for 30 min after which the cells were incubated at 42°C for 40s and again placed on ice. 80μl of S.O.C. (2% [w/v] bactotryptone, 0.5% [w/v] yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) were added and the preparation incubated for 1 hr at 37°C in an orbital incubator. Cells were streaked on 90mm LB agar plates containing 50μg/ml ampicillin and incubated overnight at 37°C.

3.13.5 Identification of recombinant clones

Single colonies were taken on a toothpick, inoculated into 5ml of LB containing 50μg/ml ampicillin and shaken overnight at 37°C in an orbital shaker. 1.5ml of the suspension was then centrifuged at 13000 rpm for 1 min at 21°C and the bacterial pellet resuspended in 350μl Stets buffer (8% [w/v] sucrose, 0.5% [v/v] Triton X-100, 50mM EDTA [pH 8.0], 10mM Tris-HCl [pH 8.0]) to which were added 25 μl of a freshly prepared solution of 10mg/ml lysozyme. The suspension was vortexed, boiled for 40s and immediately centrifuged at 13000 rpm for 10 min at 21°C. The pellet was discarded. To the supernatant were added an equal volume of isopropanol and a tenth volume of 3M sodium acetate. After overnight storage at -20°C DNA, was concentrated by centrifugation at 13000 rpm for 10 min at

21°C, vacuum dried and resuspended in 50μl of sterile distilled water.

Recombinant clones were identified by agarose gel electrophoresis of DNA fragments generated by restriction enzyme digestion. One of these clones (pGHBI) was grown and purified as described in Section 3.13.1.

3.14 Synthesis of hybridization probes

3.14.1 Radiolabelled M13 DNA

In a standard reaction, a 100 molar excess of M13 hybridization probe primer was added to 50ng of M13 single strand template DNA in a 10μl volume of 50mM NaCl, 10mM Tris-HCl [pH 7.5], 10mM DTT, 10mM $MgCl_2$. The solution was vortexed for 5s, boiled for 3 min and incubated for 30 min at 21°C. 33pmol of 5'-[α-^{32}P] dATP were dried under vacuum and resuspended in the reaction mix. To this were added 5μl of a dNTP solution (150μM dTTP, 150μM dGTP, 150μM dCTP) and 6U of Klenow polymerase. The reaction mix was made up to 20μl with sterile distilled water and then incubated for 6 hr at 15°C. Incorporation of the radiolabelled nucleotide was monitored at hourly intervals by TCA precipitation: 1μl of the reaction mix was diluted in 1ml of sterile distilled water and 10μl spotted on to duplicate 2.5cm discs of Whatman No.1 paper. One disc was washed in 3 changes of 5% (w/v) TCA, rinsed in ethanol and air dried. Washed and unwashed discs were counted for ^{32}P in a liquid scintillation counter and the percentage incorporation

calculated. Unincorporated radionucleotide was removed by passing the reaction mix through a 10ml Sephadex G50 column equilibrated with 0.1 x SSC. Radiolabelled DNA was collected, ethanol precipitated, vacuum dried and resuspended in 8ml of hybridization solution (Section 3.15.3). Probes with efficiencies of 1 to 5 x 10^8 cpm/μg DNA were usually obtained. Sizing of the radiolabelled M13 second strand was by alkaline agarose gel electrophoresis.

3.14.2 Biotin-labelled and unlabelled DNA

Second strand synthesis from the single strand M13 DNA template was also achieved using unlabelled dATP, biotinylated dATP and a 50:50 mixture of these. Reaction conditions were as described in Section 3.14.1 with the exception that 0.5μg of M13 DNA and 350pmol of dATP were used. This higher concentration of M13 DNA allowed direct visualization of the synthesized DNA products by short wave UV illumination after 0.6% (w/v) agarose gel electrophoresis.

3.14.3 Riboprobe pGHBI

10μg of plasmid pGHBI DNA was linearized by digestion with the restriction enzyme EcoRI, extracted once with TE saturated phenol, ethanol precipitated, vacuum dried and resuspended in sterile distilled water at a concentration of 1μg/μl.

1μg of the linearized DNA template was added to 4μl of Gibco BRL 5 x T7 reaction buffer (200mM Tris-HCl [pH 8.0],

40mM $MgCl_2$, 10mM spermidine-HCl, 125mM NaCl) and supplemented with 10mM DTT, 1μl RNasin (2U/μl), 500μM each of ATP, GTP, CTP and 12.5μM each of 5'-[α-^{32}P]-labelled and unlabelled UTP. Finally 22U of T7 RNA polymerase were added. The reaction mix was made up to a final volume of 20μl with DEPC treated water and incubated for a minimum period of 1 hr. Following RNA synthesis, the DNA template was removed by the addition of 1U of RQ1 DNase and incubation for 10 min at 37^{o}C. The solution was made up to 200μl by the addition of 2 x PK buffer (100mM Tris-HCl [pH 8.0], 20mM EDTA, 20mM NaCl, 0.4% [w/v] SDS) and extracted with TE saturated phenol. Unincorporated radionucleotide was removed after passage through a 10ml column of Sephadex G50 equilibrated with 10mM Tris-HCl [pH 7.5], 50mM NaCl and 1mM EDTA [pH 8.0]. Radiolabelled RNA was pooled, ethanol precipitated and resuspended in 8ml of the hybridization solution. Incorporation of the radionucleotide was monitored by TCA precipitation. Usually 5 x 10^7 to 5 x 10^8 cpm/ml of hybridization solution were obtained. Radiolabelled RNA transcripts were subjected to urea/acrylamide gel electrophoresis and their size estimated by reference to ^{32}P-labelled RNA MWt markers.

3.14.4 Nick-translated human DNA

Human DNA probes were labelled internally as described by Rigby et al. (1977).

1μg of DNA was nick-translated in a final volume of

50μl containing 50mM Tris-HCl [pH 7.2], 5mM $MgCl_2$, 1mM DTT, 0.03% (w/v) BSA, 0.05mM dATP, 0.05mM dTTP, 0.7μM each of 5'-[α-^{32}P] dGTP and 5'-[α-^{32}P] dCTP. DNase was added to a final concentration of 0.01μg/ml and the solution vortexed. 5U of E.coli DNA polymerase I were added and the reaction incubated for 1 hr at 15°C.

Incorporation of radiolabelled nucleotides was monitored by TCA precipitation and scintillation counting. Unincorporated radionucleotides were removed from the reaction mix by passage through a 10ml Sephadex G50 column equilibrated with 0.1 x SSC. The radiolabelled DNA probe was ethanol precipitated and resuspended in 200μl of sterile distilled water. Before use, the probe was denatured by boiling, quenched on ice and resuspended in 8ml of hybridization solution. Probes with specific activities of at least 10^8 cpm/μg were used for dot blot hybridization.

3.15 Hybridization

3.15.1 Specimen preparation

10ml of urine were centrifuged at 3000 rpm for 15 min at 4°C and the pellet discarded. The supernatant was transferred to a fresh tube and centrifuged at 10000 rpm for 90 min at 4°C. The supernatant was discarded. To the pellet were added 400μl of lysis solution (3% [w/v] Sarkosyl, 75mM Tris-HCl, 25mM EDTA). 100μl of proteinase K (5mg/ml) were then added and the suspension incubated for 1 hr at 37°C. DNA was extracted once with an equal volume of

TE saturated phenol, ethanol precipitated and resuspended in 50μl of sterile distilled water.

3.15.2 Denaturation of the target DNA

DNA in a volume of 50μl was denatured by the addition of an equal volume of 800mM NaOH and incubated for 10 min at 21^{o}C. After neutralization with 2 volumes of 2M sodium acetate, the DNA was bound to nitrocellulose paper presoaked in 2M sodium acetate using a dot blot manifold. The paper was air dried and baked at 80^{o}C for 2 hr.

Prehybridization and hybridization were carried out in sealed hybridization bags submerged in a water filled plastic box placed in a shaking water bath at the appropriate temperature.

3.15.3 Prehybridization and hybridization conditions

(a) M13 probes

Blots were prehybridized in a solution of 50% (v/v) formamide, 5 x SSC, 5 x Denhardt's buffer, 50mM $NaPO_4$ [pH 6.8], 0.1% (w/v) SDS and 100μg/ml sonicated denatured salmon sperm DNA for 2 to 16 hr at a temperature of 42^{o}C. 80μl of prehybridization solution per cm^2 of nitrocellulose paper was used.

The hybridization solution in which the radiolabelled probe was suspended was essentially the same as the prehybridization solution with the exceptions that the final concentration of $NaPO_4$ (pH 6.8) and SDS were 20mM and

0.2% (w/v) respectively. Hybridization proceeded for 16 hr at 42°C, 50μl of hybridization solution being used per cm^2 of nitrocellulose paper.

(b) Riboprobe pGHBI

The prehybridization and hybridization solutions both consisted of 50% (v/v) formamide, 6 x SSC, 5 x Denhardt's buffer, 0.1% (w/v) SDS and 200μg/ml sonicated denatured salmon sperm DNA. The volumes used were as described for the M13 system. Conditions of hybridization were varied with respect to temperature (40°C, 50°C, 60°C) and time period (14 hr, 38 hr, 62 hr) in order to find the optimum. Standard hybridization conditions are defined as 14 hr at 50°C.

(c) Nick-translated human DNA

The compositions and volumes of the prehybridization and hybridization solutions were as described for riboprobe pGHBI. Prehybridization was performed for 2 to 16 hr at 50°C while hybridization was for 38 hr at 60°C.

3.15.4 Washing

Each blot was washed for 30 min in 2 changes of 1 x SSC, 0.1% (w/v) SDS in a volume of 1 litre at room temperature on a shaking platform. This was followed by 2 further washes, each of 30 min duration, in a 1 litre solution of 0.1 x SSC, 0.1% (w/v) SDS at a temperature of 60°C. Finally each blot was air dried.

3.15.5 Detection of hybrids

Dried blots were autoradiographed by exposure at -70°C to Kodak X-Omat S film sandwiched between 2 Dupont phosphotungstate intensifying screens. In 1 experiment Amersham Hyperfilm MP was assessed.

In the RNA-DNA hybridot assay urine specimens were regarded as negative for HCMV-DNA if no hybrid signal was observed. Urines were reported as positive for HCMV DNA if there was a hybrid signal not present in any of the negative controls with the exception of HSV-2 DNA. In some hybridot assays uninfected control urine showed a hybrid signal equivalent to that observed in some of the clinical specimens. These latter samples were recorded as equivocal for HCMV DNA.

3.16 Complement Fixation Test

VB was used as the diluent throughout and was prepared by dissolving 1 CFT diluent tablet in 100ml of distilled water. VB containing 0.1% (w/v) BSA (VBA) was used to stabilize complement when testing in the absence of other proteins such as antigen or antibody.

A fresh suspension of SRBCs in Alsevers solution was obtained weekly and washed in VB until the supernatant was clear. For volumetric standardization, an aliquot of the SRBC suspension was placed in a haematocrit tube and centrifuged at 3000 rpm for 10 min at 21°C in an MSE benchtop centrifuge. The SRBC suspension was made up to a

concentration of 4% (v/v) in VB.

A freeze-dried preparation of complement (preserved guinea pig serum) was reconstituted with 1ml of distilled water while dilution of this stock solution with 7 volumes of distilled water gave the equivalent of a 1:10 solution of guinea pig serum in isotonic saline. Further dilutions to the desired working strength were made in VB.

Freeze-dried glycine-extracted CF antigen was reconstituted with 1ml of distilled water and VB added to attain final dilutions of 1:8, 1:16 and 1:32 for HCMV, VZV and HSV respectively. Antigens were prepared immediately before use and stored on ice. Control antigens extracted from uninfected cell cultures were similarly prepared.

Freeze-dried standard antisera were reconstituted with 1ml of distilled water and made up to the recommended working dilution with VB.

The test was routinely performed in 96-well microtitre plates with U-shaped wells.

3.16.1 Standardization of complement and haemolytic serum

The haemolytic titre of each new batch of complement was determined by "chessboard" titration against each new batch of lysin.

Serial 1:5 dilutions of complement from 1:60 to 1:288 in VB were prepared. 75μl of VBA were added to each of the haemolytic serum control wells and 50μl to the remaining wells. 25μl of each complement dilution was added to the appropriate well and the microtitre plate incubated

overnight at 4°C. Doubling dilutions of haemolysin from 1:50 to 1:800 in VB were prepared. VB acted as a negative control for haemolysin. To each dilution of haemolysin was added an equal volume of 4% (v/v) washed SRBCs which were then sensitized for 30 min at 37°C. Thereafter, plates were warmed for 30 min at 37°C and 25µl of the sensitized SRBCs were added to the appropriate wells. The plates were incubated for 40 min at 37°C, cells being gently resuspended at 15 min, 30 min and 40 min using a microshaker. SRBCs were allowed to settle at 4°C for a few hours and the plates read. The OSD of haemolytic serum is the dilution giving most lysis with the highest dilution of complement. Since 50% haemolysis is a more sensitive and reproducible index of the haemolytic activity of complement than 100%, 1U of complement (HD_{50}) is the dilution giving 50% lysis with the OSD of haemolysin. In the CFT proper complement is used at $4HD_{50}$.

3.16.2 CFT proper

Sera from each patient were tested in parallel at 2 starting dilutions; 1:2 and 1:8. All sera were diluted in VB.

Prior to use an aliquot of serum was removed from the original sample and incubated for 30 min at 56°C to remove endogenous complement.

25µl of VB were placed in every test well. 25µl of neat serum and 25µl of serum diluted 1:4 in VB were placed

in adjacent wells and diluted from 1:2 to 1:256 and 1:8 to 1:1024 respectively using a hand microdiluter. 25μl of complement 4HD_{50} were added to each test well followed by 25μl of viral antigen at the appropriate dilution. Plates were wrapped in foil and incubated overnight at 4^{o}C. The following morning a 4% (v/v) solution of SRBCs were sensitized by mixing with an equal volume of haemolysin and incubation for a minimum period of 30 min at 37^{o}C (a final mixture of 2% [v/v] cells with OSD of haemolysin). Microtitre plates were warmed for 30 min at 37^{o}C and 25μl of the sensitized SRBCs added to each test well. The plates were reincubated for 40 min at 37^{o}C, cells being gently resuspended at 15 min, 30 min and 40 min using a microshaker. After storage for a few hours at 4^{o}C, the plates were read. The highest dilution of serum in which lysis of SRBCs was limited to 0-25% was taken as its titre.

Controls comprised (i) anticomplementary activity: serum 1:2 and 1:8 with no antigen tested against 4 and 2 HD_{50} complement (ii) complement (with 2 volumes of VBA replacing antigen and antiserum) $HD_{50}U$ of 4, 2, 1, 0.5 and 0 (cell control) (iii) specific reagent controls: standard positive antiserum titrated from 4 times titre (4T) to 1/4 titre (T/4) against the dilution of antigen used in the test; controls of specific antigen (no antiserum) plus 4 and 2HD_{50} complement and a control of the standard antiserum at the lowest dilution (4T) without antigen plus 4 and 2HD_{50} complement.

Sera with anticomplementary activity were treated by

the addition of 1 volume of freshly reconstituted complement (preserved guinea pig serum) at working dilution to 4 volumes of test serum. This was followed by incubation overnight at 4°C and then for 30 min at 37°C to inactivate residual complement. Sera were diluted 1:8 in VB prior to testing.

Sera were regarded as negative for virus-specific CF antibody if the titre was less than 1:2 in the modified CFT.

3.17 Enzyme linked immunosorbent assays

3.17.1 Labsystems HCMV-specific IgM and IgG

Assays of HCMV-specific IgM and IgG were performed strictly in accord with the manufacturers' protocol. Serum specimens were diluted 1:200 in Labsystems diluent buffer and 100µl dispensed into 2 microtitre wells coated with inactivated AD169 grown in human foreskin fibroblasts and 1 well of uninfected foreskin fibroblasts. Following incubation for 90 min (IgG) or 120 min (IgM) at 37°C the wells were washed 3 times with 200µl of a 1:500 solution of Tween 20 in distilled water. Swine antihuman IgG or IgM coupled to alkaline phosphatase was diluted 1:49 in Labsystems conjugate diluent buffer (10mM PBS, 0.1% [w/v] sodium azide) and 100µl added to each well. After incubation for 2 hr at 37°C followed by another 3 rounds of washing, 100µl of pNNP (2mg/ml in 1.01M DEA, 0.505mM $MgCl_2$, 0.1% [w/v] sodium azide) were added to each well.

The reaction continued for 30 min at 37°C and was stopped by the addition of 100µl of 1M NaOH. The absorbance readings at 410nM and 450nm were recorded. In all experiments an HCMV positive and negative control sera were included. The ELISA value for each serum was calculated using the formula

$$EIU = \frac{(Ass - Arbc) - (Asc - Arbc)}{(Acs - Arbs) - (Acc - Arbc)} \times 100$$

where

EIU = enzyme immuno unit

Ass = average absorbance of the sample in 2 sensitized wells

Arbs = average absorbance of the reagent blank in 2 sensitized wells

Asc = absorbance of the sample in the control well

Arbc = absorbance of the reagent blank in the control well

Acs = average absorbance of the positive control serum in 2 sensitized wells

Acc = absorbance of the positive control serum in the negative control well.

An ELISA IgM value of $\geq$ 40 EIU was regarded as positive and a value of < 20 EIU as negative. Similarly, an ELISA IgG value of $\geq$ 20 EIU was regarded as positive and a value of < 10 EIU as negative. Levels intermediate to the clear positive and negative zones were categorised as equivocal. These values are those recommended by the manufacturer for the interpretation of results obtained.

3.17.2 NBL HCMV-specific IgG

Each test serum was diluted 1:50 in NBL sample diluent

buffer and 100μl added to 1 well coated with HCMV antigen and to 1 uncoated well. After incubation for 30 min at 21^{o}C in a moist atmosphere, the wells were rinsed 4 times with NBL washing solution (saline/Tween 20). 100μl of a solution of swine antihuman IgG coupled to horseradish peroxidase and diluted in NBL conjugate diluent fluid were added to each well. Following incubation for 60 min at room temperature in a moist atmosphere, the wash cycle was repeated. 100μl of the chromogenic substrate (3,3', 5,5' tetramethylbenzidine in citrate/acetate solution) were added to each well and the incubation continued for 30 min at room temperature. The reaction was stopped by adding 100μl of 5M H_2SO_4 and the absorbance reading at 450nM recorded. ELISA values were calculated using the formula

Ac = Ap - An

Ac = corrected absorbance

Ap = absorbance of the sample in the antigen coated well

An = absorbance of the sample in the uncoated well.

In all experiments 3 control sera (high titre positive; low titre positive; negative) were included.

Sera were regarded as positive for HCMV-IgG if the Ac was greater than that produced by the low positive standard serum. Those sera with an Ac between 90 and 100% of that produced by the low positive serum control were regarded as equivocal for HCMV-IgG. Sera classified as negative for HCMV-IgG had an Ac of between 0 and 90% of that produced by the low positive standard serum.

3.17.3 Abbott rubella IgM

Each test serum was diluted 1:10 in Specimen Dilution Buffer and 20μl placed in 2 wells of the ELISA containing 200μl of Specimen Incubation Buffer. Following incubation for 60 min at 45°C, a rubella virus coated bead was added to one of the wells while the other received an uncoated bead. Incubation continued for 90 min at 45°C. Each bead was then washed with 4 to 6ml of deionized water and reincubated for 90 min at 45°C in the presence of 200μl of antihuman IgM conjugated with horseradish peroxidase. The beads were again washed as described above and transferred to an Abbott reaction tube containing 300μl of OPD substrate solution. After incubation for 30 min at 15°C, the reaction was stopped by the addition of 2ml of 1M H_2SO_4 and the absorbance at 492nm recorded. ELISA values were calculated using the formula

$$Ac = Ap - An$$

where Ac = corrected absorbance

Ap = absorbance of the sample in the presence of the rubella virus coated bead

An = absorbance of the sample in the presence of the uncoated bead.

In all experiments 5 control sera (1 high positive; 3 low positive; 1 negative) were included.

Sera were regarded as positive for rubella-IgM if the Ac was greater than that of the average Ac of the 3 low positive controls. Those sera with an Ac of between 90 and 100% of the average Ac of the 3 low positive controls

were regarded as equivocal for rubella-IgM. Sera classified as negative for rubella-IgM had an Ac of between 0 and 90% of the average Ac of the 3 low positive standard sera.

3.18 Other serological assays

3.18.1 Mercia Diagnostics infectious mononucleosis absorption test

25µl of each positive control or test serum were added to a white glass slide and mixed with 25µl of guinea pig antigen. The slides were allowed to stand at room temperature for 30s after which time 25µl of horse cell suspension were added. At 1 min slides were examined for the presence of distinct agglutination. In a second experiment performed in parallel, guinea pig antigen was replaced by an equal volume of ox cell antigen. Sera were regarded as positive for heterophile antibodies if there was agglutination of horse cells in the presence of guinea pig but not ox cell antigen.

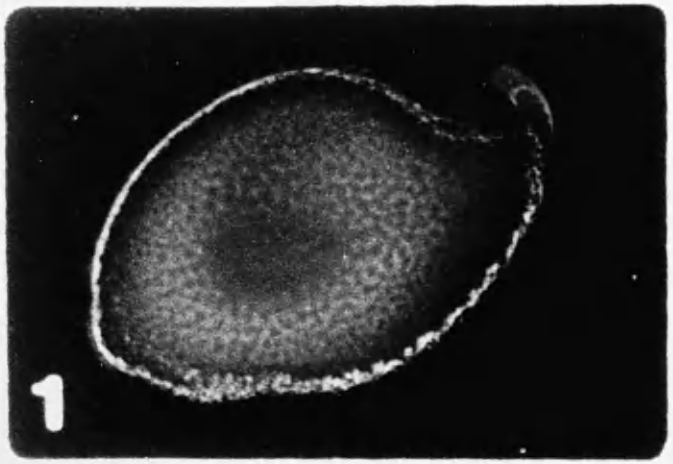
3.18.2 Behring Rapitex RF assay

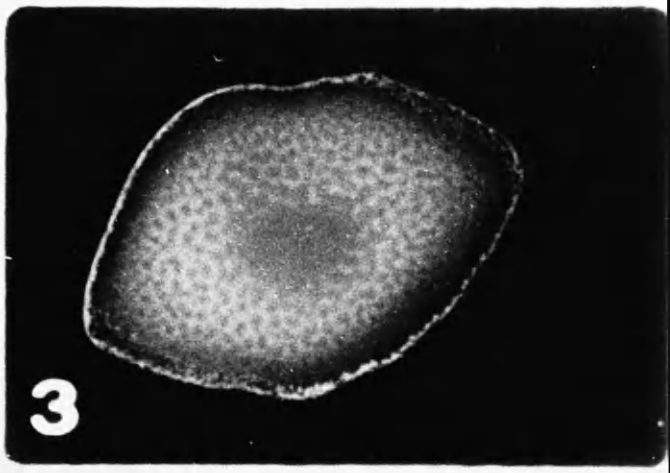
Each test serum was diluted 1:6 with 0.9% (w/v) NaCl. 40µl of this dilution and 40µl of each positive and negative control serum were placed in separate zones of a test plate. 40µl of latex-RF reagent were then added to each zone and the suspension mixed. Marked agglutination indicated the presence of RF. An example is shown in Figure 9.

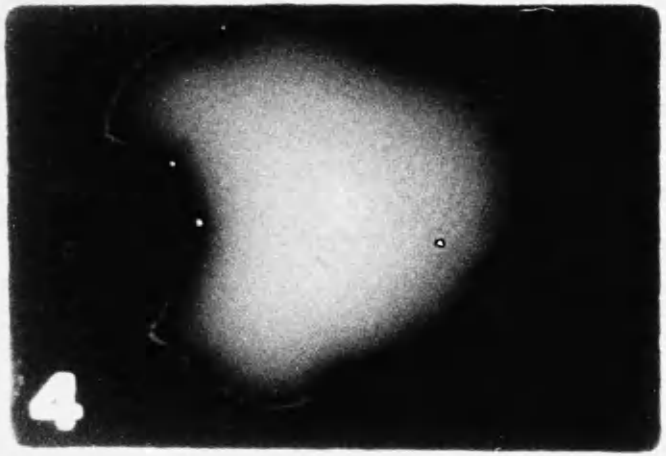
Figure 9 RF latex-slide agglutination test

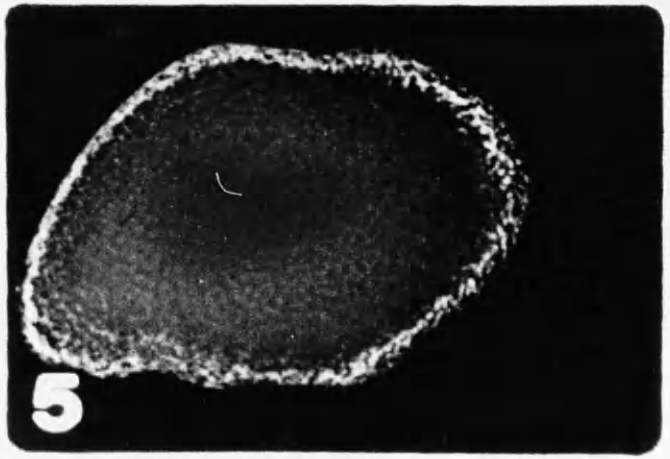
The figure shows the results of a typical latex-slide agglutination test for the presence of RF in serum samples. To each of wells 1-6 were added 40μl of polystyrene particles coated with humanγ globulin. These were mixed with 40μl of positive RF control serum (well 1), 40μl of negative RF control serum (well 2) and 40μl of a 1:6 dilution of each test serum (wells 3-6). Marked agglutination indicates the presence of RF.

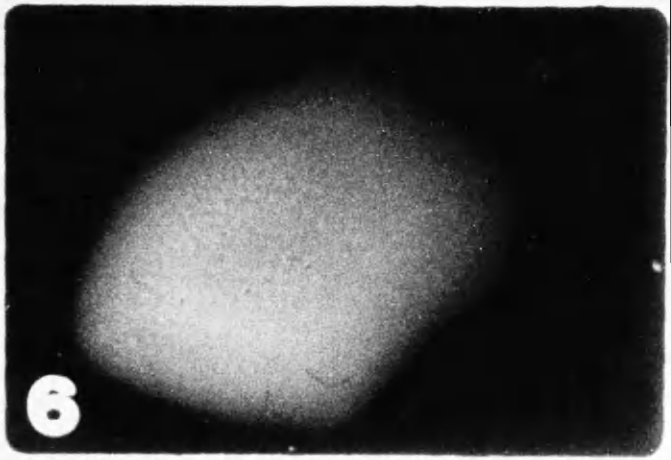
LATEX-REAGENZIEN











BEHRINGWERKE

3.19 Abbott β_2 microglobulin RIA

100μl of each urine specimen were placed in the respective well of a reaction tray containing 200μl of β_2m ^{125}I Reagent solution. One anti-β_2m monoclonal antibody coated bead was introduced into each well which was then incubated for 1 hr at 15^{o}C while rotating at 200 rpm. Beads were then washed twice with 4 to 6ml of deionized water and immediately transferred to an assay tube. The radioactivity in each tube was counted for 1 min in a gamma scintillation counter. In each experiment 6 standards (0, 50, 150, 400, 1000 and 4000μg/litre) and 2 known controls were tested in duplicate. Two assay tubes containing only 200μl of β_2m ^{125}I Reagent solution served as the "total counts" tubes. At the end of the experiment the average cpm of background was subtracted from the average cpm of each standard, control and total (T) count to get the net cpm. For each standard and unknown the percentage of ^{125}I-labelled β_2m bound (B) to the bead (B/T%) was calculated using the formula

$$B/T\% = \frac{\text{net cpm of each standard or unknown}}{\text{net total counts}}$$

The concentration of β_2m in the unknown samples was estimated by reference to a curve of B/T% v standard concentrations of β_2m.

3.20 Renal allograft recipients

3.20.1 Study group

From March 1985 to December 1985 52 patients received kidney transplants at the Western Infirmary, Glasgow. Five patients who suffered acute rejection underwent nephrectomy within 4 weeks of the transplant operation and were therefore omitted from the investigation. The final study group comprised 47 patients with a mean age of 38 years (range 19 to 61). Twenty eight patients were male and 19 female. Forty patients were recipients of a first kidney transplant while 7 received their second. Four patients were recipients of a kidney from a live related donor while the remaining 43 kidneys were of cadaver origin. Each renal allograft recipient is identified by a letter(s) which remained invariable throughout the project.

3.20.2 Immunosuppressive therapy

All patients received a combination of prenisolone and CsA throughout the period of the study. The prednisolone dose was 20mg per day for the first 3 months then reduced to 15mg per day for 3 months followed by 12.5mg per day for 6 months. The CsA dose was 15mg/kg/day for the first few days after the transplant operation with a reduction to around 5 mg/kg/day by 3 months, 4mg/kg/day by 6 months and 2 to 3mg/kg/day by 1 year post transplantation. The CsA dose was adjusted on the basis of whole blood levels measured by RIA. In some patients CsA was replaced by

azathioprine.

3.20.3 Specimens

A throat swab, urine and serum samples were obtained from every patient immediately prior to the transplant operation and subsequently at intervals of 3 to 4 weeks whenever possible. Sera from any 1 renal allograft recipient were always tested in parallel within a single batch of a serological assay. A serum specimen taken from the renal donor immediately prior to nephrectomy was provided by the donating hospital subject to availability. Plasma was separated from an aliquot of blood withdrawn from each transfusion pack, the contents of which had been infused into a renal allograft recipient participating in this project. Information regarding the age of the blood donors and date of donation was provided in confidence by Dr R. Crawford, Consultant, West of Scotland Blood Transfusion Service.

3.20.4 Clinical details

Clinical manifestations in each patient were reported by Dr J.D. Briggs, Consultant Physician who at the time of reporting was unaware of the results of HCMV isolation or the HCMV-specific humoral response. The following clinical parameters were identified.

leucopenia: a decrease in the total number of white cells below 4.0×10^9/litre

thrombocytopenia: a diminution in the total number of blood platelets below 100×10^9/litre

abnormal liver function tests (SGOT level > 35U/litre; SGPT level > 50U/litre; serum γGT > 50U/litre; serum bilirubin > 18μmol/litre)

pyrexia: a temperature of > 38^{o}C for more than 24 hr.

acute rejection: a 10 to 15% rise in the level of serum creatinine which occured within 3 months of the transplant operation or which occured at a later date and resolved.

chronic rejection: a 10 to 15% rise in level of serum creatinine which did not resolve.

nephrectomy: removal of the transplanted kidney.

RESULTS

4.1 Dot blot hybridization using M13 partially double stranded probes

The use of M13 partially double stranded hybridization probes of high specific activity has been described and applied to the selection of M13 clones complementary to the probe from within an M13 shotgun sequence bank (Hu and Messing, 1982). Before using M13 probes for the detection of viral DNA in clinical samples it was necessary to investigate thoroughly the sensitivity and specificity of the M13 hybridization system. HSV-1 17 syn^+ DNA and M13 clones containing HSV-1 17 syn^+ sequences were used for this purpose because of the ease of availability of the latter from within the Institute of Virology.

4.1.1 Sensitivity

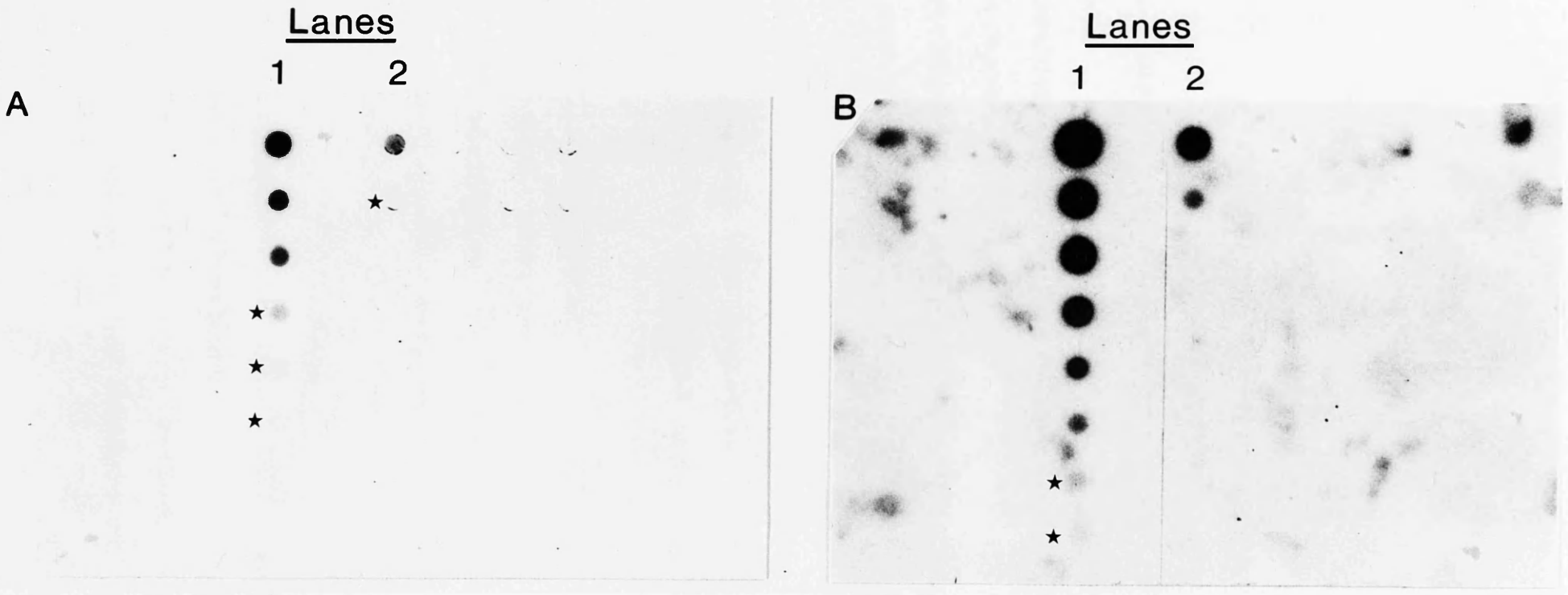
HSV-1 DNA (1μg/20μl) was diluted 1:2 from 500ng to 3.9ng and 1:10 from 50ng to 5pg in distilled water, denatured and bound to a nitrocellulose filter. Both sets of dilutions were simultaneously probed with radiolabelled partially double stranded JR139, an M13 clone containing sequences from gene UL8 of HSV-1. Only 15.6ng and 5ng respectively of HSV-1 DNA could be detected after 20 hr exposure of the nitrocellulose paper to X-ray film (Figure 10A). Exposure for 96 hr increased those signals already obtained after 20 hr of autoradiography and improved the lower limit of detection from 15.6ng to 3.9ng (Figure 10B). Since other authors have been able to detect pg quantities of complementary HCMV DNA using nick-translated (Spector et

Figure 10 Detection of HSV-1 DNA by the M13 probe JR139 after 20 hr (A) and 96 hr (B) of autoradiography

Lane 1: Serial 1:2 dilutions of HSV-1 DNA from 500ng to 3.9ng.

Lane 2: Serial 1:10 dilutions of HSV-1 DNA from 50ng to 5pg.

The probe is 50ng of α^{32}P-labelled partially double stranded JR139 DNA at a specific activity of 1.19×10^9 cpm/μg.


A
Lanes
1
2
B
Lanes
1
2

al., 1984b) and RNA (Schuster et al., 1986) probes, it was decided to try and improve the sensitivity of the M13 hybridization assay.

The integrity of M13 probes depends on the binding of the newly synthesized radiolabelled DNA to the complementary single stranded M13 template. Therefore, it was considered ill advised to attempt to improve the sensitivity of the hybridot assay by increasing the hybridization temperature and risk denaturing the probe. High background signals (Figure 10B) prohibited the use of less stringent washing procedures while increasing the probe concentration to 100ng/ml of hybridization solution failed to improve on the lower limit of detection. Attempts to increase the sensitivity of the M13 hybridization assay were therefore focused on improving the specific activity of the M13 probe.

4.2 M13 reaction conditions

This section describes a number of experiments in which the conditions used to radiolabel the M13 hybridization probe were examined in an attempt to improve the specific activity.

4.2.1 Variation between batches of [α-^{32}P] dATP

In preliminary experiments the incorporation of [α-^{32}P] dATP was seen to vary between batches of the radioactive nucleotide. Two standard M13 reactions were

prepared in parallel and incubated at 37°C. Constituents were identical with the exception that each reaction mix contained [α-^{32}P] dATP from a different batch. One experiment showed the percentage incorporation of the radionucleotide to be 52% at 30 min and by 4 hr 64% of [α-^{32}P] dATP had been incorporated into the second strand of the M13 probe. In the parallel experiment using [α-^{32}P] dATP from another batch, the incorporation of the radionucleotide at the same time points was 17 and 32% respectively. Both experiments were repeated and similar results obtained (Figure 11).

It was concluded that an inhibitor of M13 second strand synthesis was present in some batches of [α-^{32}P] dATP.

4.2.2 Reaction temperature

Two standard M13 reactions were prepared in parallel and incubated at 15 or 37°C. In both experiments incorporation of [α-^{32}P] dATP was monitored at 30 min intervals for 3 or 4 hr. The results are presented in Figure 12. The rate of incorporation of the radionucleotide was higher during the first 90 min of incubation at 37°C but thereafter it reached a plateau. At 15°C, incorporation of [α-^{32}P] dATP continued beyond 2 hr and was still rising after 4 hr of incubation at which time 80% of the radiolabelled nucleotide had been incorporated in contrast to 64% after 4 hr of incubation at 37°C. Subsequent experiments showed that incorporation of [α-^{32}P] dATP did not plateau until after 5 hr of incubation at 15°C. Therefore, in all future experiments the M13 reaction

Figure 11 Time course of incorporation of [α-^{32}P] during M13 second strand synthesis: Variation between batches of the radionucleotide.

The graph shows the percentage incorporation of [α-^{32}P] dATP plotted at 30 min intervals in 2 standard M13 reactions prepared in parallel but with different batches of the radionucleotide □ and △ . The results of a duplicate experiment are also shown (■ and ▲ respectively). Lines are drawn through the average of 2 values at each time point.

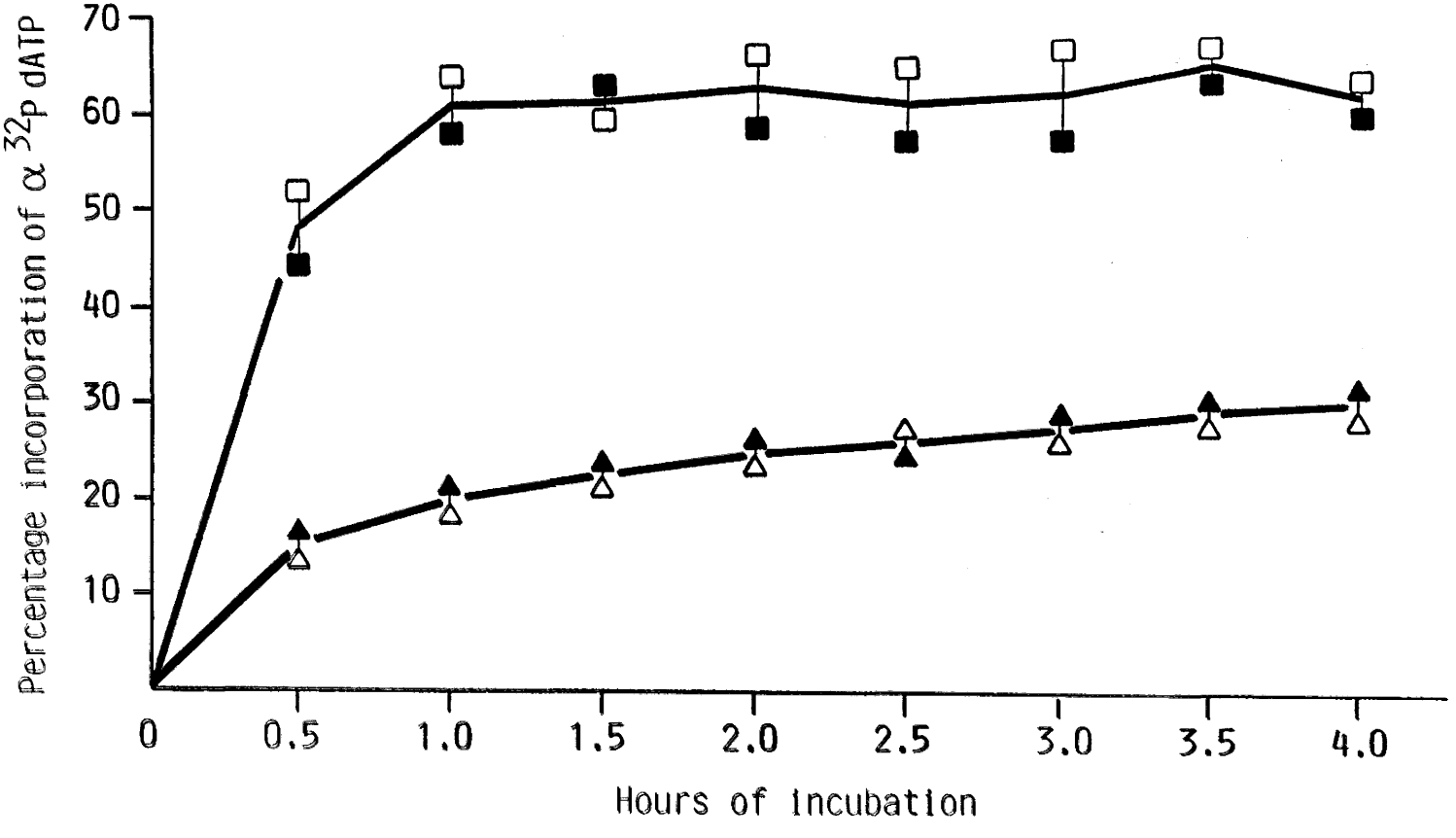
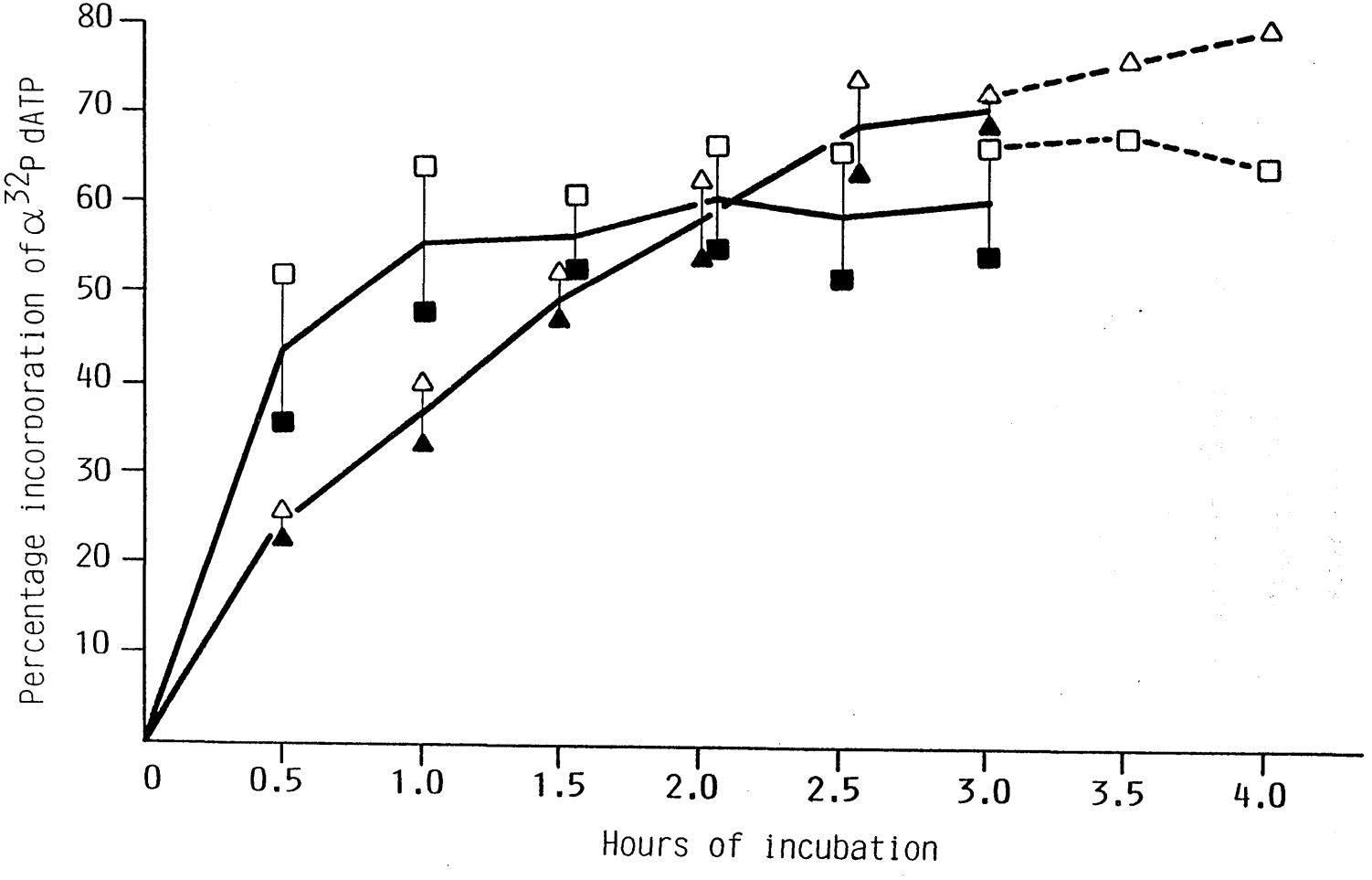

Percentage incorporation of α 32p dATP
70
60
50
40
30
20
10
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Hours of Incubation

Figure 12 Time course of incorporation of [α-^{32}P] during M13 second strand synthesis at 15°C and 37°C.

The graph shows the percentage incorporation of [α-^{32}P] dATP plotted at 30 min intervals in 2 standard M13 reactions prepared in parallel and incubated at 15°C (△) or 37°C (□). The results of a duplicate experiment are also shown (▲ and ■ respectively). Lines are drawn through the average of 2 values at each time point with the exception of 3.5 and 4 hr when the results from only 1 set of experiments (△ and □) were available.


Percentage incorporation of α^{32}P dATP
80
70
60
50
40
30
20
10
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Hours of incubation

mixture was incubated at 15°C for a minimum period of 6 hr.

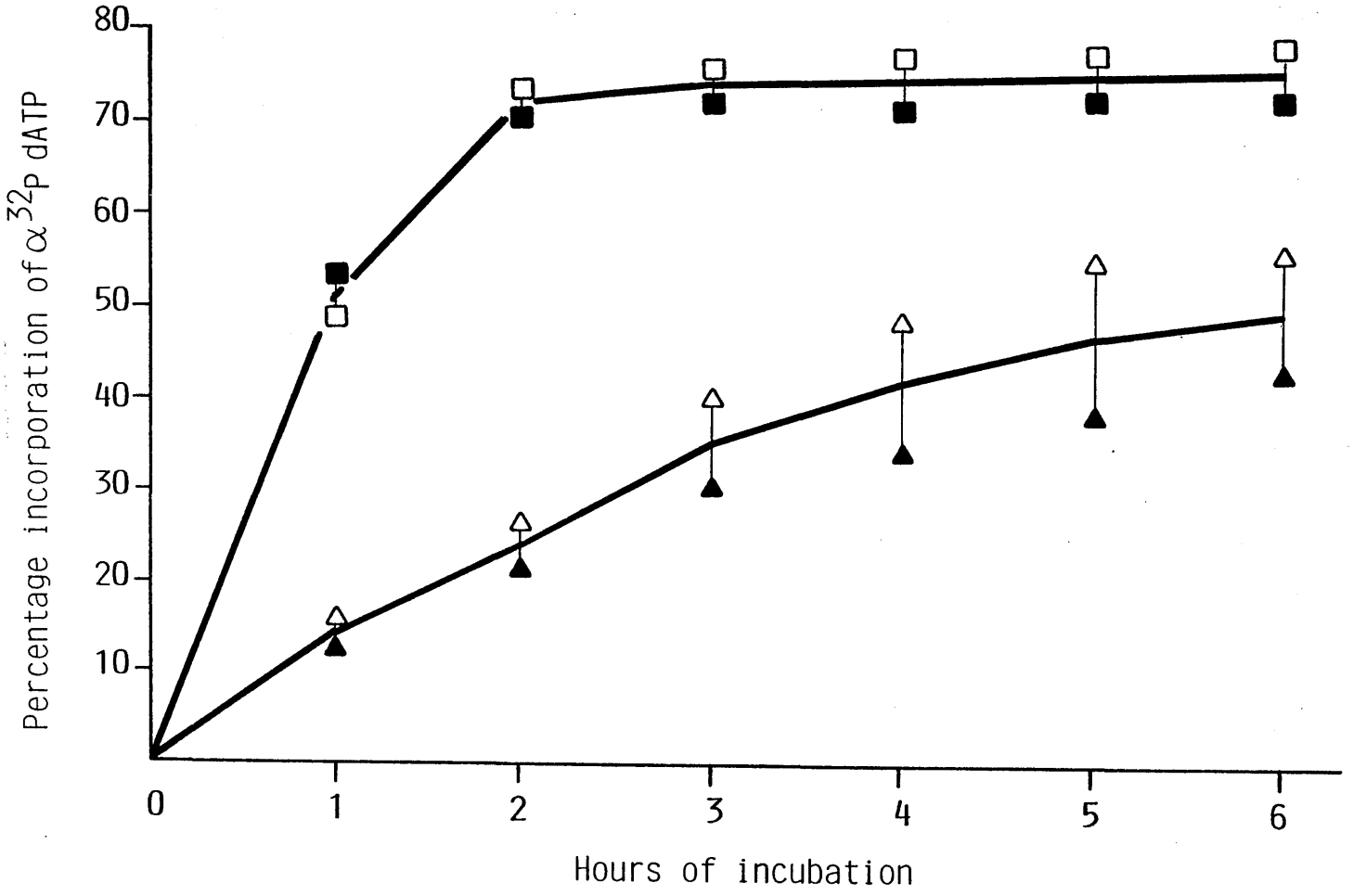
4.2.3 Concentration of Klenow polymerase

An investigation was made into the number of units of Klenow polymerase required to give the most incorporation of [α-^{32}P] dATP. Two standard M13 reactions were prepared in parallel, all constituents being identical except for the number of units of Klenow polymerase. In one reaction containing 1U of the enzyme (1U/μl) the percentage incorporation of [α-^{32}P] dATP at each hour was an average of 39% (range 30.1 to 50.5) lower than that observed in a parallel reaction to which 6U of the enzyme (6U/μl) had been added. The experiment was repeated in parallel and similar results obtained (Figure 13). When the M13 mix contained 12U of Klenow polymerase [6U/μl], the percentage incorporation of [α-^{32}P] dATP at each hour during the first 6 hr of the reaction was an average of 5.5% (range 2.5 to 8.2) higher than in a parallel experiment containing 6U of the enzyme.

Further attempts to increase the specific activity of the M13 hybridization probe were directed at the addition of fresh Klenow polymerase during second strand synthesis. Two standard M13 reactions were prepared in parallel. After 2 and 4 hr of incubation, 6U of Klenow polymerase [6U/μl] were added to one while 1μl of Klenow dilution buffer was added to the other. Monitoring of both reactions showed the percentage incorporation of [α-^{32}P] dATP at each hour to be higher in the former experiment by an average of 7.1% (range 2.6 to 12.2), the difference

Figure 13 Time course of incorporation of [α-^{32}P] dATP during M13 second strand synthesis in the presence of 1 and 6 units of Klenow polymerase

The graph shows the percentage incorporation of [α-^{32}P] dATP plotted at 1 hr intervals in 2 standard M13 reactions containing 1U (△) or 6U (□) of Klenow polymerase. The results of a duplicate experiment are also shown (▲ and ■ respectively). Lines are drawn through the average of 2 values at each time point.


Percentage incorporation of α^{32}P dATP
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
Hours of incubation

increasing with longer incubation times. The addition of fresh Klenow polymerase did not result in a sharp increase in the amount of incorporated radionucleotide (Figure 14).

4.2.4 Concentration of [α-^{32}P] dATP

Higher specific activity M13 hybridization probes were made by increasing the quantity of the radionucleotide available for incorporation. Three standard M13 reactions were prepared in parallel. Constituents were identical with the exception that each reaction contained either 33 or 66 or 132pmol of [α-^{32}P] dATP taken from the same batch. The results of parallel experiments performed on 2 occasions are shown in Figure 15. When 33pmol of the radionucleotide were included in the reaction mix, an average of 73% or 24pmol had been incorporated after 6 hr of incubation. When 66 or 132pmol of [α-^{32}P] dATP were made available for M13 second strand synthesis the average incorporation was 64% and 31% or 42.2pmol and 41.2pmol respectively.

4.2.5 Denaturing agarose gel electrophoresis of radio-labelled M13 reaction products

When preparing M13 probes it is essential for cloned viral DNA to remain single stranded and therefore capable of hybridizing to complementary nucleic acid. Limited M13 second strand synthesis is achieved by restricting the concentration of a nucleotide to an amount which will

Figure 14 Time course of incorporation of [α-^{32}P] ATP during M13 second strand synthesis: Interval addition of Klenow polymerase

The graph shows the percentage incorporation of [α-^{32}P] dATP plotted at 1 hr intervals in 2 standard M13 reactions prepared in parallel. The arrows at 2 and 4 hr represent the addition of 6U of Klenow polymerase [6u/μl] (□) or 1μl of Klenow dilution buffer (△). The results of a duplicate experiment are also shown (■ and ▲ respectively). Lines are drawn through the average of 2 values at each time point with the exception of 6 hr when the results of only 1 set of experiments (□ and △) were available.

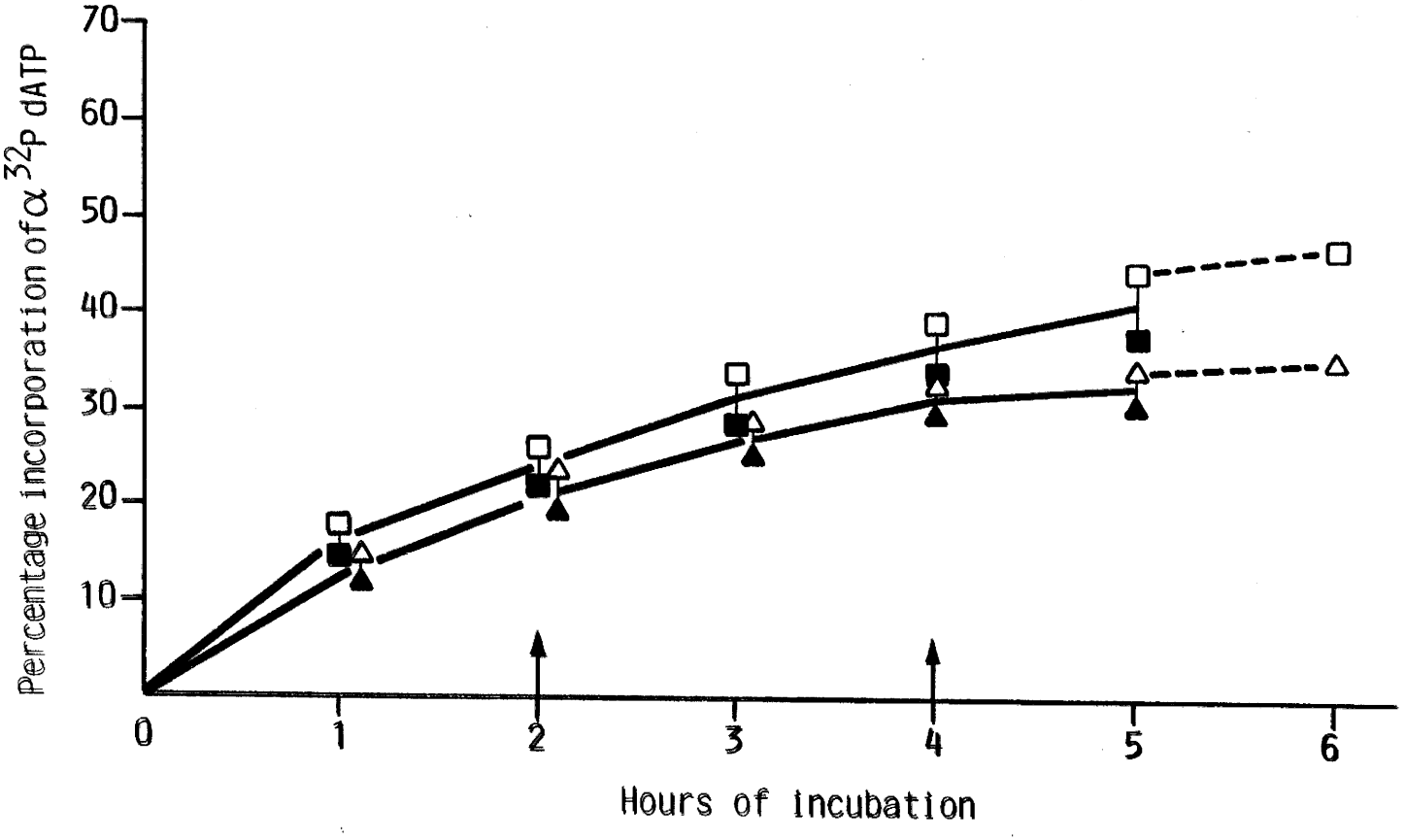
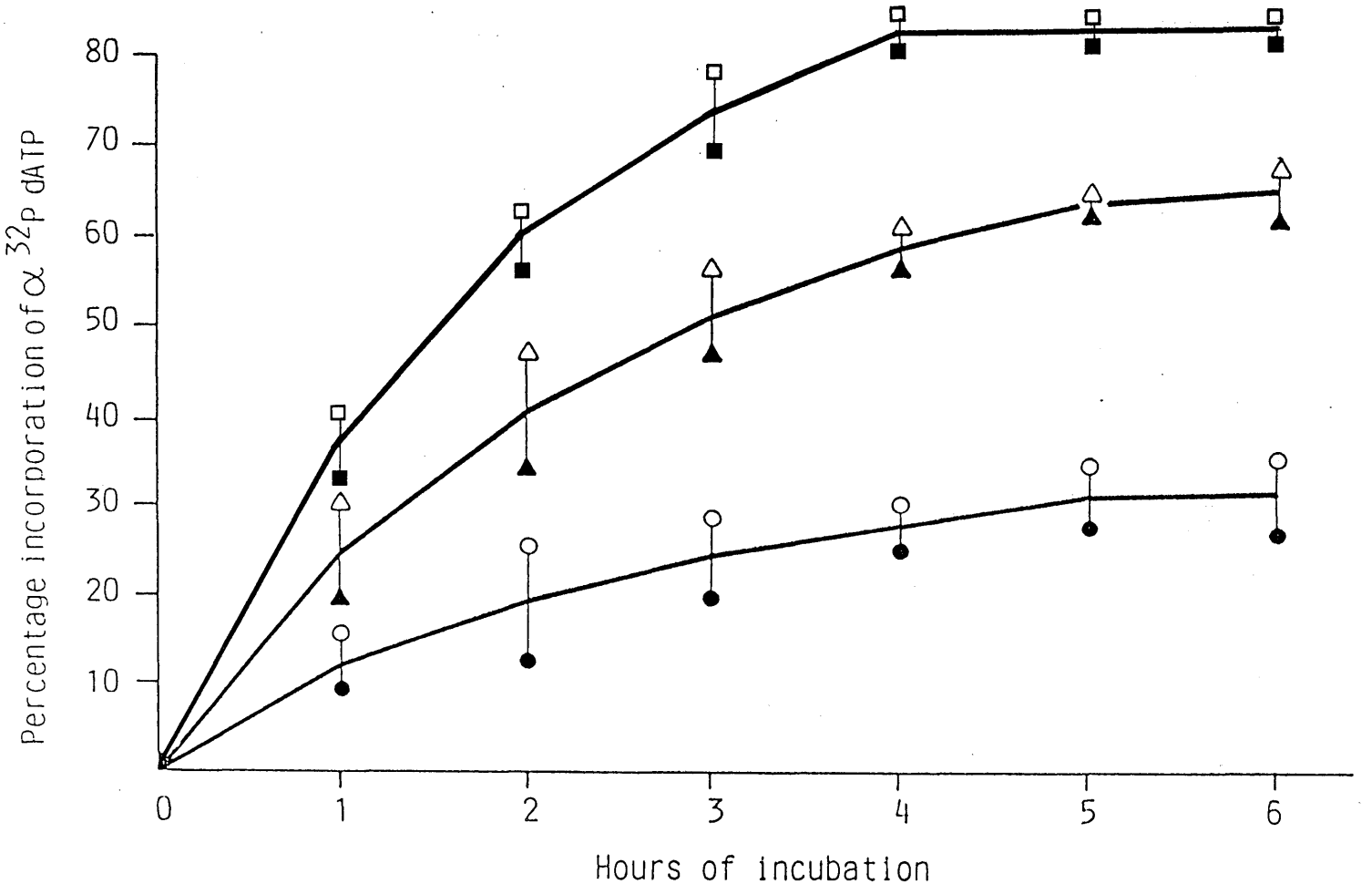

Percentage incorporation of α 32P dATP
70
60
50
40
30
20
10
0
1
2
3
4
5
6
Hours of incubation

Figure 15 Time course of incorporation of [α-^{32}P] dATP during M13 second strand synthesis: Increasing amounts of radionucleotide

The graph shows the percentage incorporation of [α-^{32}P] dATP plotted at 1 hr intervals in 3 standard M13 reactions prepared in parallel but containing either 33pmol (□), 66pmol (△) or 132 pmol (○) of the radionucleotide. The results of a duplicate experiment are also shown (■, ▲ and ● respectively). Lines are drawn through the average of 2 values at each time point.


Percentage incorporation of α 32P dATP
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
Hours of incubation

permit DNA to be synthesized around the M13 template without extending into cloned HSV-specific sequences. For 50ng of M13 DNA (140pmol of nucleotides) this is calculated to be 35pmol of [α-^{32}P] dATP.

The length of the newly synthesized radiolabelled M13 strands can be determined by electrophoresis under denaturing conditions. After autoradiography only the radioactive species but not the unlabelled M13 template is detected and its MWt can be estimated by reference to an appropriate radiolabelled DNA marker. The denaturing system employed here was agarose gel electrophoresis in a sodium hydroxide buffer. A ^{32}P end-labelled AvaI digest of λ DNA served as the MWt marker. Figure 16A shows the radiolabelled species obtained at hourly intervals in a standard M13 reaction during which the percentage incorporation of [α-^{32}P] dATP was also monitored (Figure 16B). While sufficient [α-^{32}P] dATP had been added to the reaction to ensure DNA synthesis around the 7000 bp M13 template, second strand synthesis did not exceed a few hundred bp after 8 hr of incubation at which time 87.1% of the radionucleotide had been incorporated. Increasing the amount of [α-^{32}P] dATP in the M13 reaction to 66pmol with 90.7% incorporation produced similar results. The experiment was repeated on numerous occasions and reproducible results obtained.

Figure 16A

Sodium hydroxide agarose gel electrophoresis of radiolabelled M13 hybridization probes

An autoradiograph showing the ^{32}P-labelled products of a standard M13 reaction after overnight electrophoresis in a sodium hydroxide 0.6% (w/v) agarose gel. The radiolabelled DNA obtained at hourly intervals during the first 8 hr of M13 synthesis is shown in Lanes 1-8 respectively. Percentage incorporation of [α-^{32}P] dATP at equivalent times is given in Figure 16B. Lane 9 is a ^{32}P end-labelled AvaI digest of λ DNA.

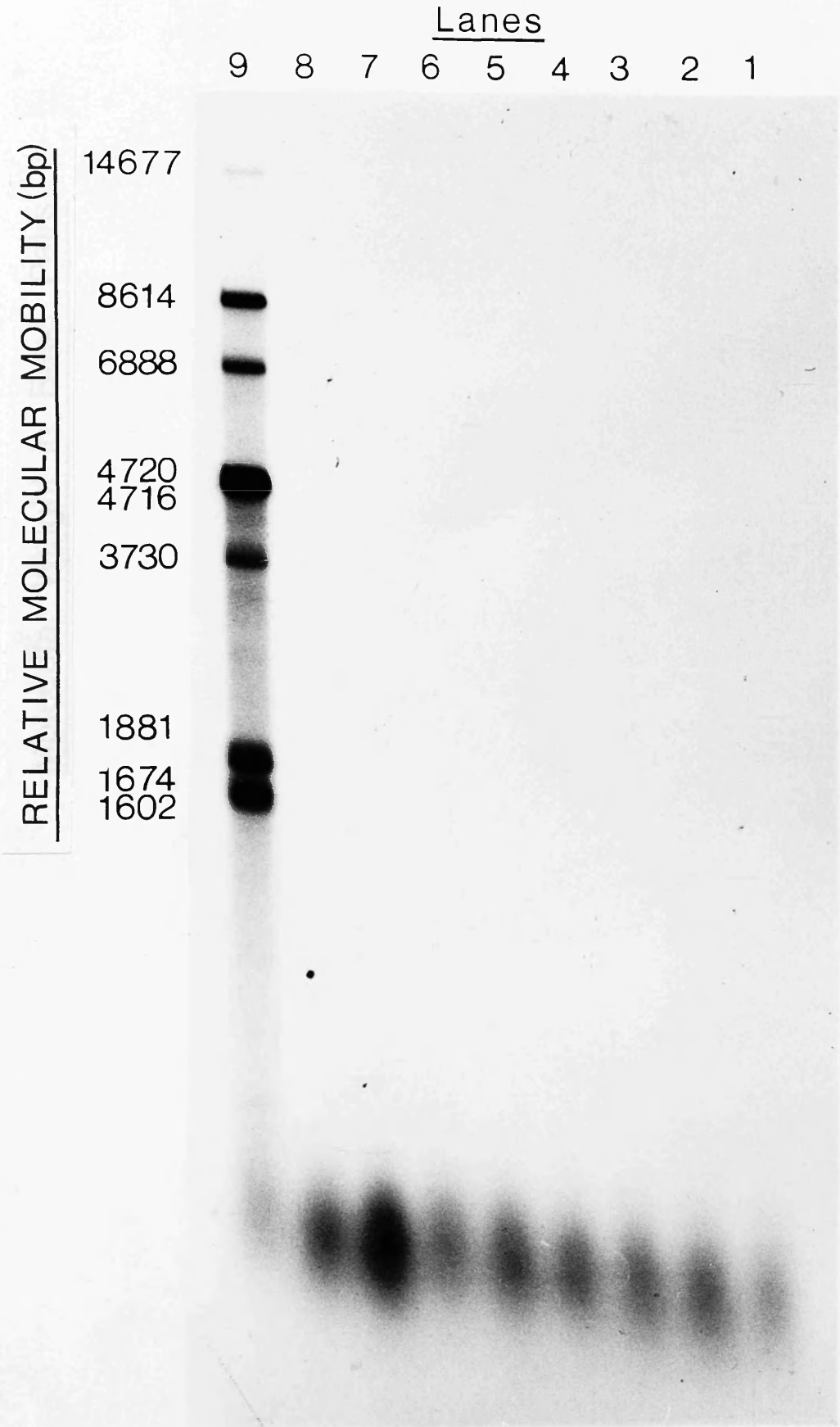
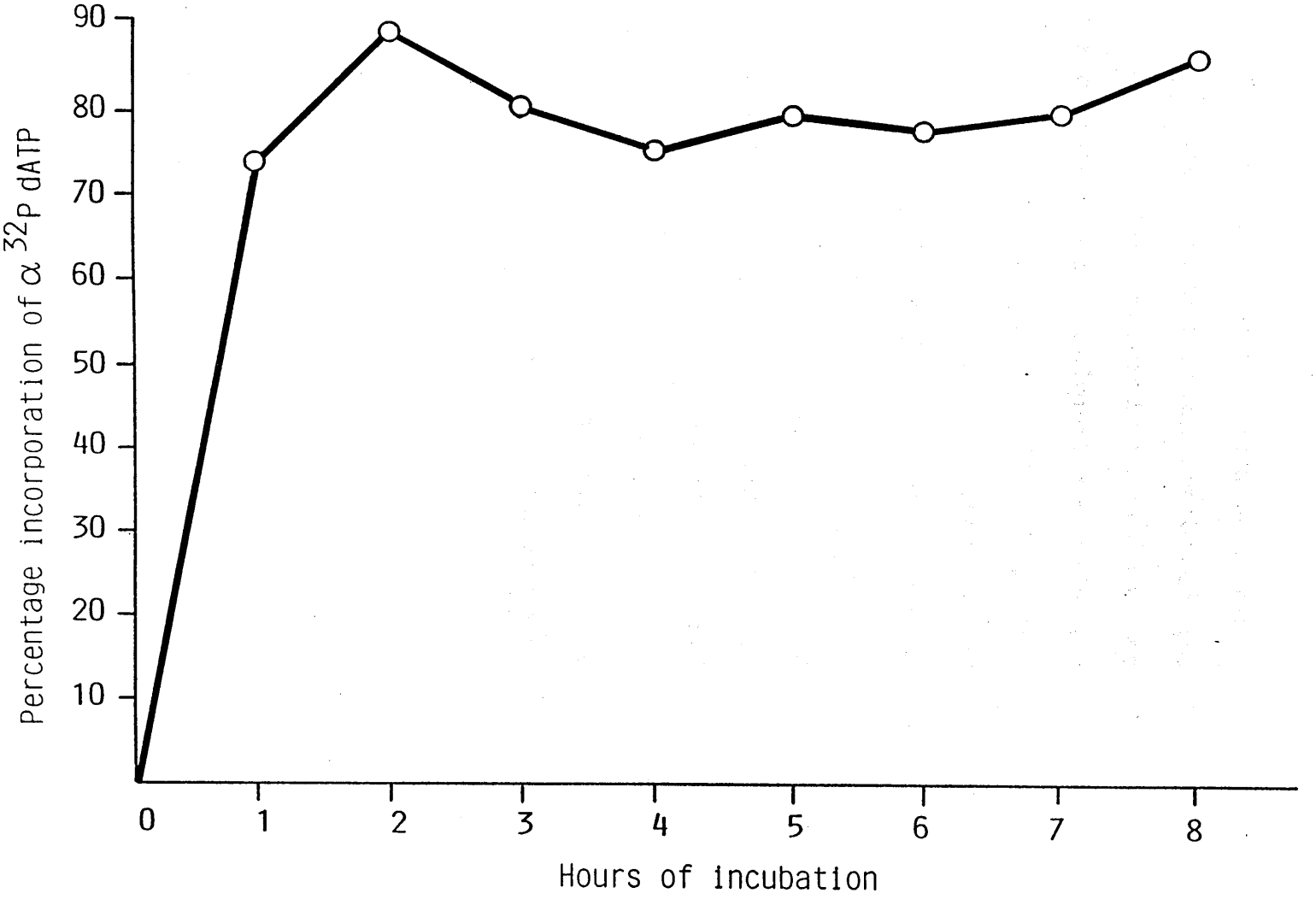

Lanes
9 8 7 6 5 4 3 2 1
RELATIVE MOLECULAR MOBILITY (bp)
14677
8614
6888
4720
4716
3730
1881
1674
1602

Figure 16B Time course of incorporation of [α-^{32}P] dATP during a standard M13 reaction

The graph shows the percentage incorporation of [α-^{32}P] dATP plotted at 1 hr intervals during the first 8 hr of a standard M13 reaction. The radiolabelled M13 DNA products of this reaction are described in Figure 16A.


Percentage incorporation of α 32P dATP
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
Hours of incubation

4.2.6 Non-denaturing agarose gel electrophoresis of radio-labelled M13 reaction products

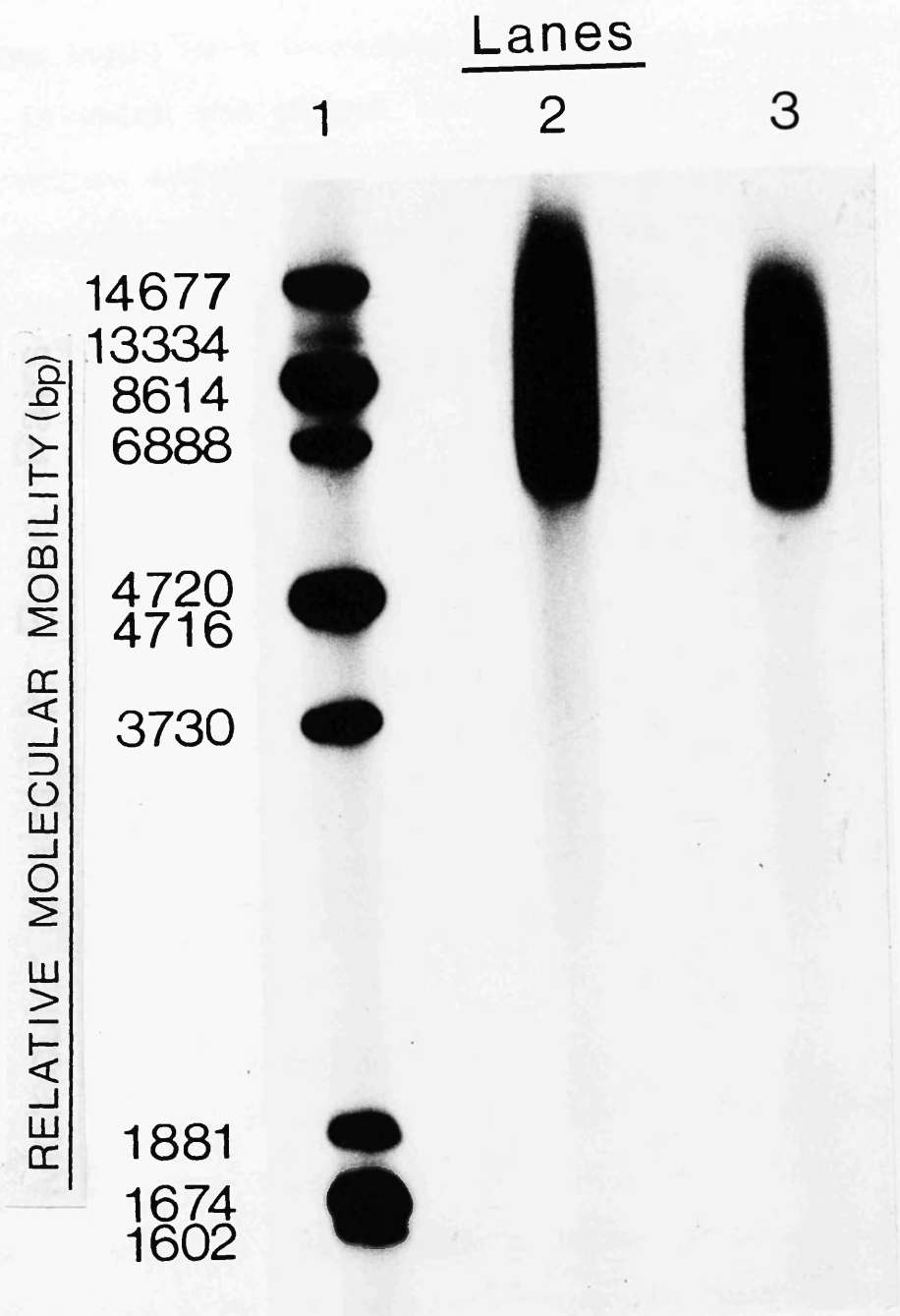
In further experiments 2 standard M13 reactions were prepared in parallel. Constituents were identical with the exception of one reaction which contained 16.5pmol of the radionucleotide and 16.5pmol of unlabelled dATP. The latter experiment was designed to reduce the specific activity of the probe by 50% and possibly increase the stability of the M13 hybrids. After electrophoresis, synthesized radiolabelled M13 DNA not bound to the template would be detected in the same location on the same concentration of agarose gel under both denaturing and non-denaturing conditions. In contrast, if the newly synthesized DNA of a few hundred bp had remained attached to the M13 template then the radiolabelled species would appear to be of higher molecular weight. The results are shown in Figure 17. Both experiments showed the radiolabelled DNA extending from 6000 to 14000 bp but a smear of radioactivity corresponding to a few hundred bp was not observed. Therefore, it was concluded that the newly synthesized M13 DNA had remained annealed to the template.

4.2.7 Use of unlabelled dATP

Second strand synthesis of M13 DNA was further investigated by replacing [α-^{32}P] dATP with unlabelled dATP. Since it would be difficult to detect a 50ng smear

Figure 17 Non-denaturing agarose gel electrophoresis of radiolabelled M13 hybridization probes

Two standard M13 reactions were prepared in parallel, all constituents being identical with the exception that 33pmol of [α-^{32}P] dATP were added to one (Lane 2) while the other received 16.5pmol of [α-^{32}P] dATP plus 16.5pmol of unlabelled dATP (Lane 3). After 8 hr of incubation, radiolabelled M13 DNA was purified from unincorporated [α-^{32}P] dATP and subjected to electrophoresis in a non-denaturing 0.6% (w/v) agarose gel. The autoradiograph of this gel is shown here. Lane 1 is a ^{32}P end-labelled AvaI digest of λ DNA.


Lanes
1
2
3
RELATIVE MOLECULAR MOBILITY (bp)
14677
13334
8614
6888
4720
4716
3730
1881
1674
1602

of DNA in an agarose gel by UV illumination, 500ng of the M13 template was used. All constituents of the reaction were increased proportionately with the exception that 12U of Klenow polymerase [6U/μl] were added. The addition of more enzyme would have increased the final concentration of glycerol in which the enzyme is stored beyond 10% of the reaction volume and risked inhibiting DNA synthesis.

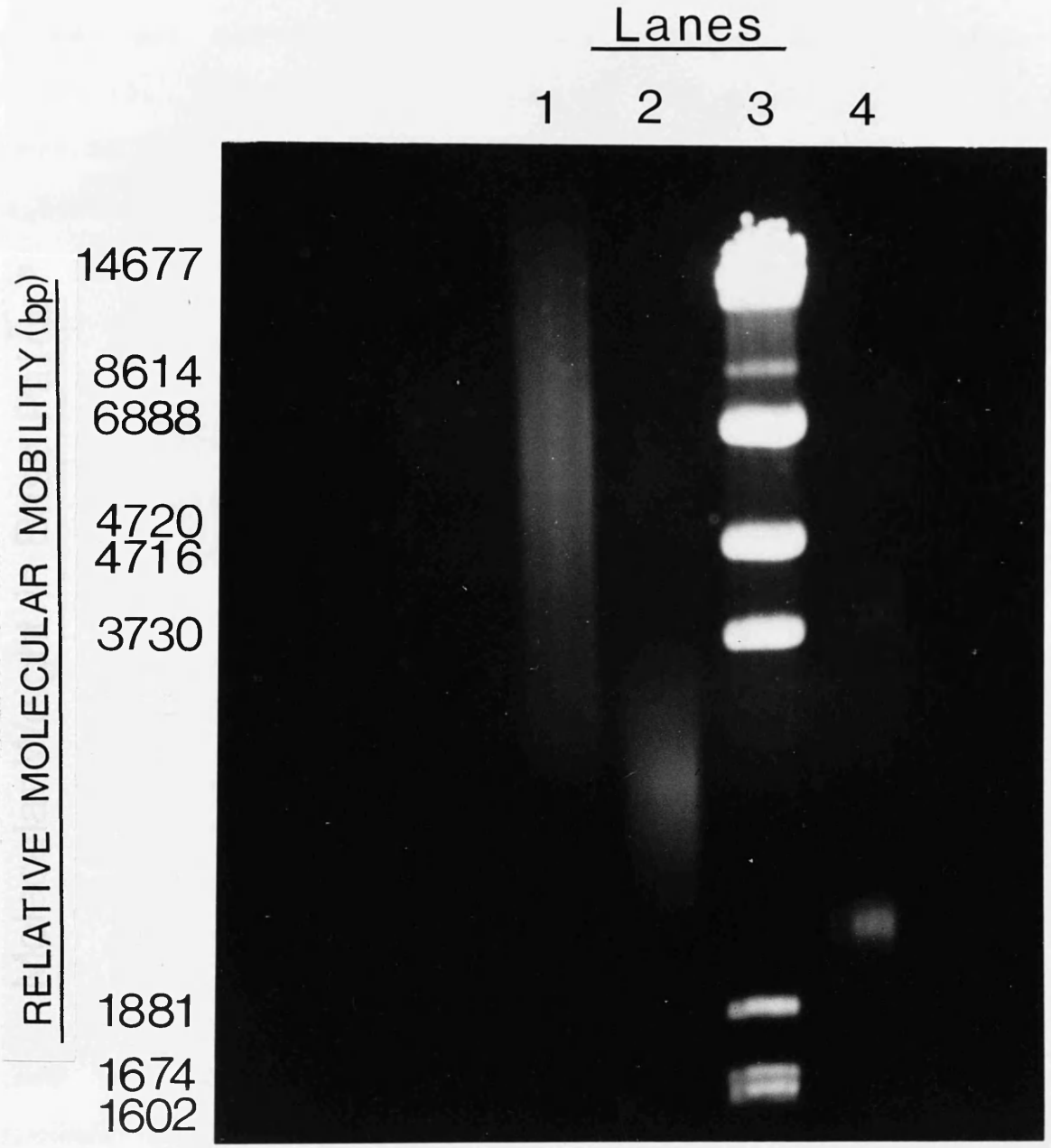
At the start of the experiment most of the M13 template was in a supercoiled form which had an apparent MWt of about 2000 bp (Figure 18). After 1 hr of incubation there was a smear of DNA whose MWt ranged from approximately 2000 to 3000 bp but by 6 hr this had increased to between 3000 and 14000 bp. When compared with the results of Figure 17, Lanes 2 and 3, the results suggest that the partially double stranded DNA products of a standard M13 reaction are of similar size regardless of whether radiolabelled or unlabelled dATP is used as the substrate. Attempts to repeat the experiment in a sodium hydroxide agarose gel were unsuccessful.

4.2.8 Use of biotinylated dATP

Compared to radiolabelled nucleic acid probes, biotinylated ones have the advantages of being less hazardous, having a long shelf life and hybrids can be detected within a few hours since there are no long periods of autoradiography (Arrand, 1985). To investigate the possibility of preparing biotinylated probes, triplicate M13 reactions were prepared in parallel as described in

Figure 18 Non-denaturing agarose gel electrophoresis of unlabelled M13 hybridization probes

Three reaction mixes, each containing 500ng of single strand M13 DNA, were prepared in parallel. Assimilation of standard M13 reaction conditions was achieved by a proportionate increase in the concentration of all reagents with the exception that 12U of Klenow polymerase were used in each experiment. dATP was unlabelled. Reactions were stopped after 0, 1 or 6 hr of incubation and subjected to electrophoresis in a non-denaturing 0.6% (w/v) agarose gel (Lanes 4, 2 and 1 respectively). Lane 3 is an AvaI digest of λ DNA.

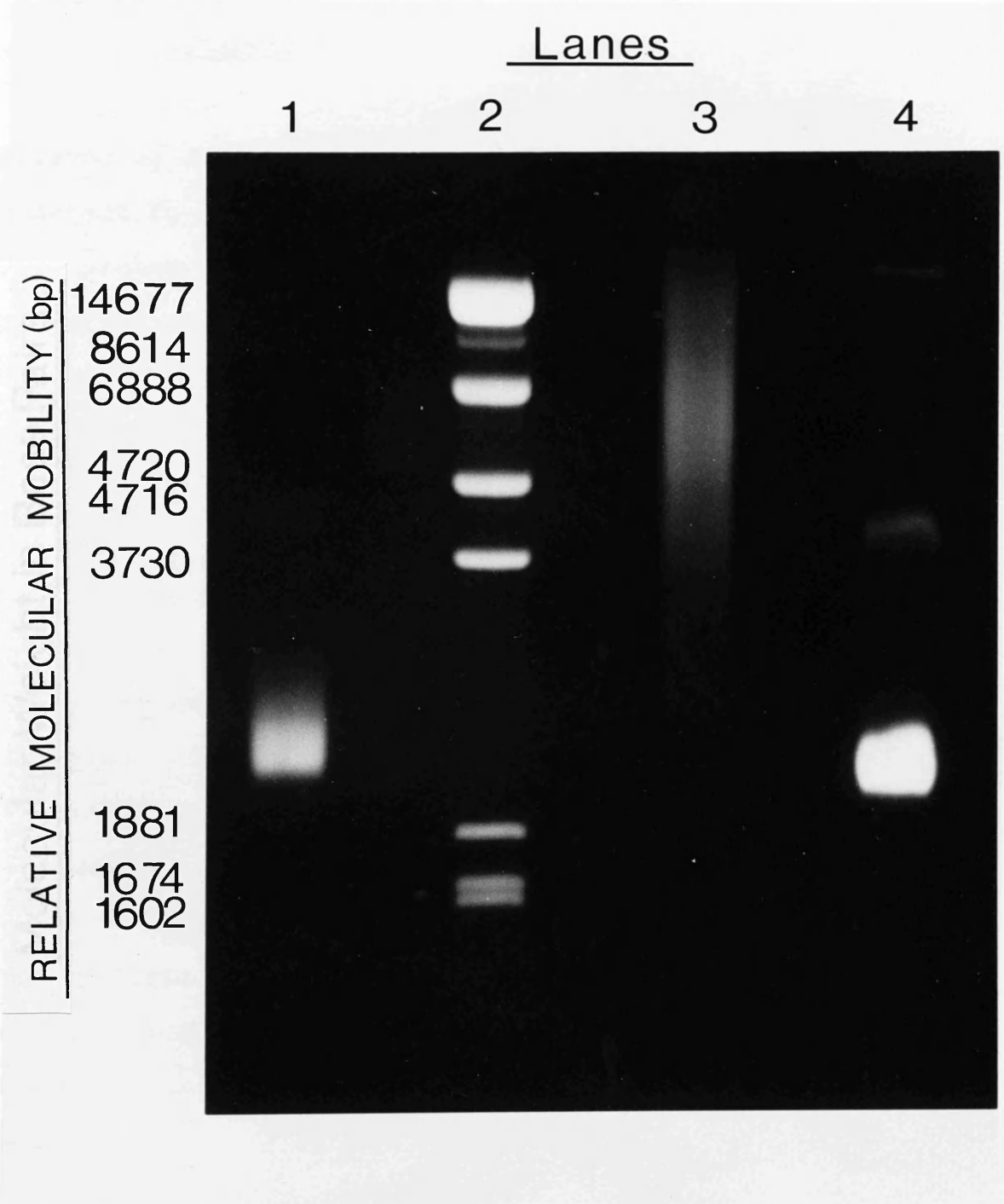

Lanes
1
2
3
4
RELATIVE MOLECULAR MOBILITY (bp)
14677
8614
6888
4720
4716
3730
1881
1674
1602

Section 4.2.7 and the products analysed by non-denaturing agarose gel electrophoresis. The results are shown in Figure 19. When dATP was unlabelled, the partially double stranded M13 DNA had a MWt ranging from approximately 3000 to 14000 nucleotides, thus confirming the result of Figure 18, Lane 1. When unlabelled dATP was replaced by biotinylated dATP, a DNA smear of around 2000 nucleotides was noted at a position equivalent to the single strand M13 template. The latter result suggests that the biotinylated nucleotide had not been incorporated in sufficient quantity to permit synthesis of high MWt products.

In summary, the results of this section show the lower limit of detection of HSV DNA by radiolabelled M13 hybridization probes to be in the ng range. Although it was possible to improve the specific activity of the probes by changing reaction conditions, second strand M13 synthesis was limited to a few hundred nucleotides. The remainder of the 7000 nucleotides M13 template was therefore single stranded and capable of hybridizing to complementary nucleic acid other than HSV-specific sequences. Finally, there was evidence that biotinylated dATP was poorly incorporated during M13 second strand synthesis. Taken together, the results suggest that the M13 hybridot assay would be impractical as a diagnostic test. Therefore, it was decided to concentrate on the riboprobe hybridization system described in the next section.

Figure 19 Non-denaturing agarose gel electrophoresis of biotin-labelled M13 hybridization probes

Three reaction mixes, each containing 500ng of single strand M13 DNA, were prepared in parallel. Assimilation of standard M13 reaction conditions was achieved by a proportionate increase in the concentration of all reagents with the exception that 12U of Klenow polymerase were used in each experiment. The products of each reaction were subjected to overnight electrophoresis in a non-denaturing 0.6% (w/v) agarose gel. One reaction was not allowed to proceed and was therefore the single strand M13 template control (Lane 4). Two other experiments containing an equal concentration of either unlabelled (Lane 3) or biotin-labelled (Lane 1) dATP were stopped after 8 hr of incubation. Lane 2 is an AvaI digest of λ DNA.


Lanes
1
2
3
4
RELATIVE MOLECULAR MOBILITY (bp)
14677
8614
6888
4720
4716
3730
1881
1674
1602

4.3 Riboprobe pGHBI

An increase in hybridization efficiency can be achieved by using single strand RNA probes which are able to detect fg quantities of cDNA (Church and Gilbert, 1984). Such probes are best prepared by using an *in vitro* transcription system in which radiolabelled cRNA specific for probe sequences is synthesized (Melton *et al.*, 1984). This section will describe an HCMV-specific RNA probe and its ability to detect cDNA in a hybridot assay.

4.3.1 Construction

Plasmid pEHBI (a BamHI subclone of the HindIII E region of HCMV AD169 in vector pAT153) was provided by Dr J.D. Oram. Figure 20 shows a HindIII cleavage map of AD 169 and identifies the BamHI subclone (HBI) used in these experiments. This subclone was chosen because it was known not to cross-hybridize to human DNA (Ruger *et al.*, 1984). HCMV-specific sequences were purified and recloned in the transcription plasmid pGEM2 (Figure 21). The new plasmid construct was named pGHBI and is shown diagrammatically in Figure 22.

4.3.2 Linearization

Prior to RNA transcription, vector pGHBI was digested with an appropriate restriction enzyme to linearize the plasmid at a site within the cloned HCMV DNA, distal to the

Figure 20 Location of the BamHI subclone of HCMV AD169 used in the construction of riboprobe pGHBI

Diagrammatic representation of the HCMV genome showing the HindIII cleavage sites and the BamHI subclone from which cRNA probes were transcribed.

Open boxes represent the terminal and internal repeat sequences which bound the long (L) and short (S) unique components of the genome.

Adapted from Oram et al. (1982).

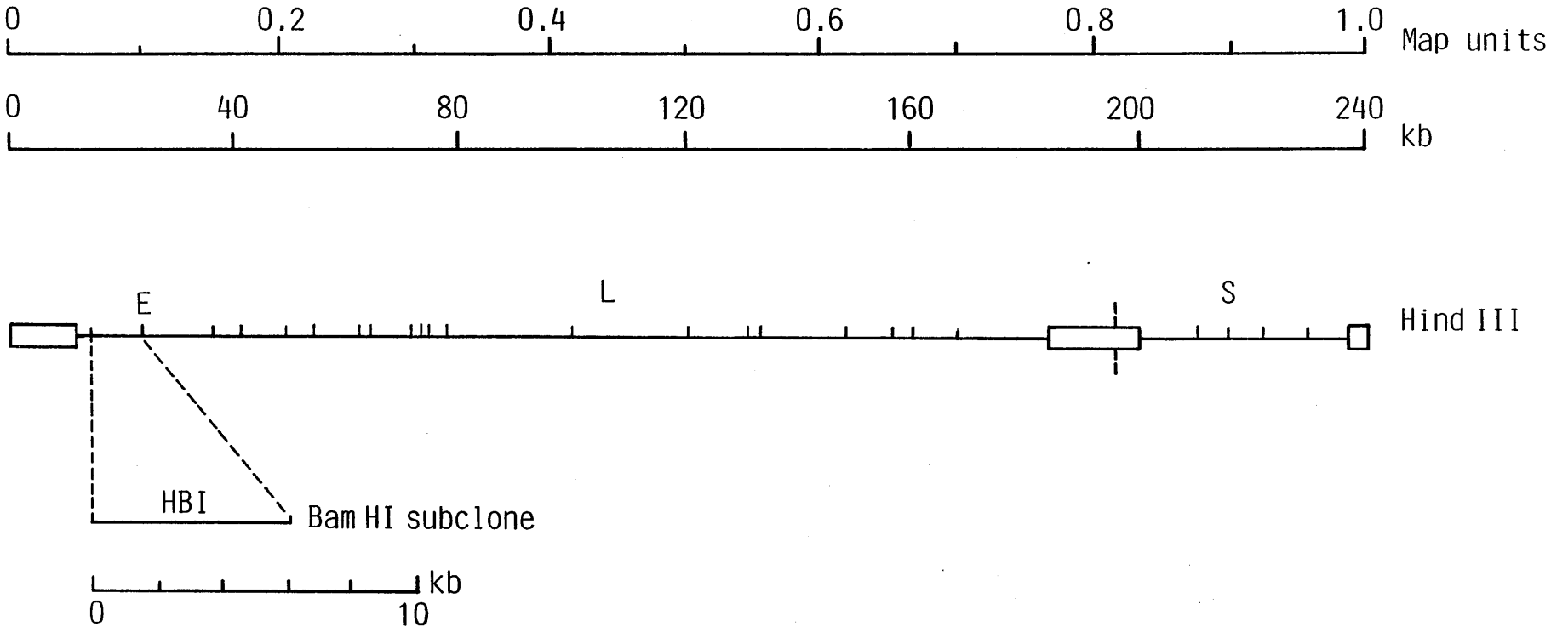

0
0.2
0.4
0.6
0.8
1.0
Map units
0
40
80
120
160
200
240
kb
E
L
S
Hind III
HBI
Bam HI subclone
kb
0
10

Figure 21 Construction of riboprobe pGHBI

Vector pEHBI (a BamHI subclone of the HindIII E fragment of HCMV AD169 cloned in plasmid pAT153) was simultaneously digested with restriction enzymes HindIII and BamHI to separate vector DNA (3657 bp) from HCMV-specific sequences (6100 bp). The latter were purified and recloned in vector pGEM2 to create the riboprobe pGHBI. Double digestion of this new construct with restriction enzymes HindIII and BamHI released vector pGEM2 DNA (2900 bp) from the cloned HCMV DNA fragment.

Restriction enzyme fragments were separated by overnight electrophoresis in a non-denaturing 0.6% (w/v) agarose gel.

Lane 1 : 0.5μg of uncut riboprobe pGHBI

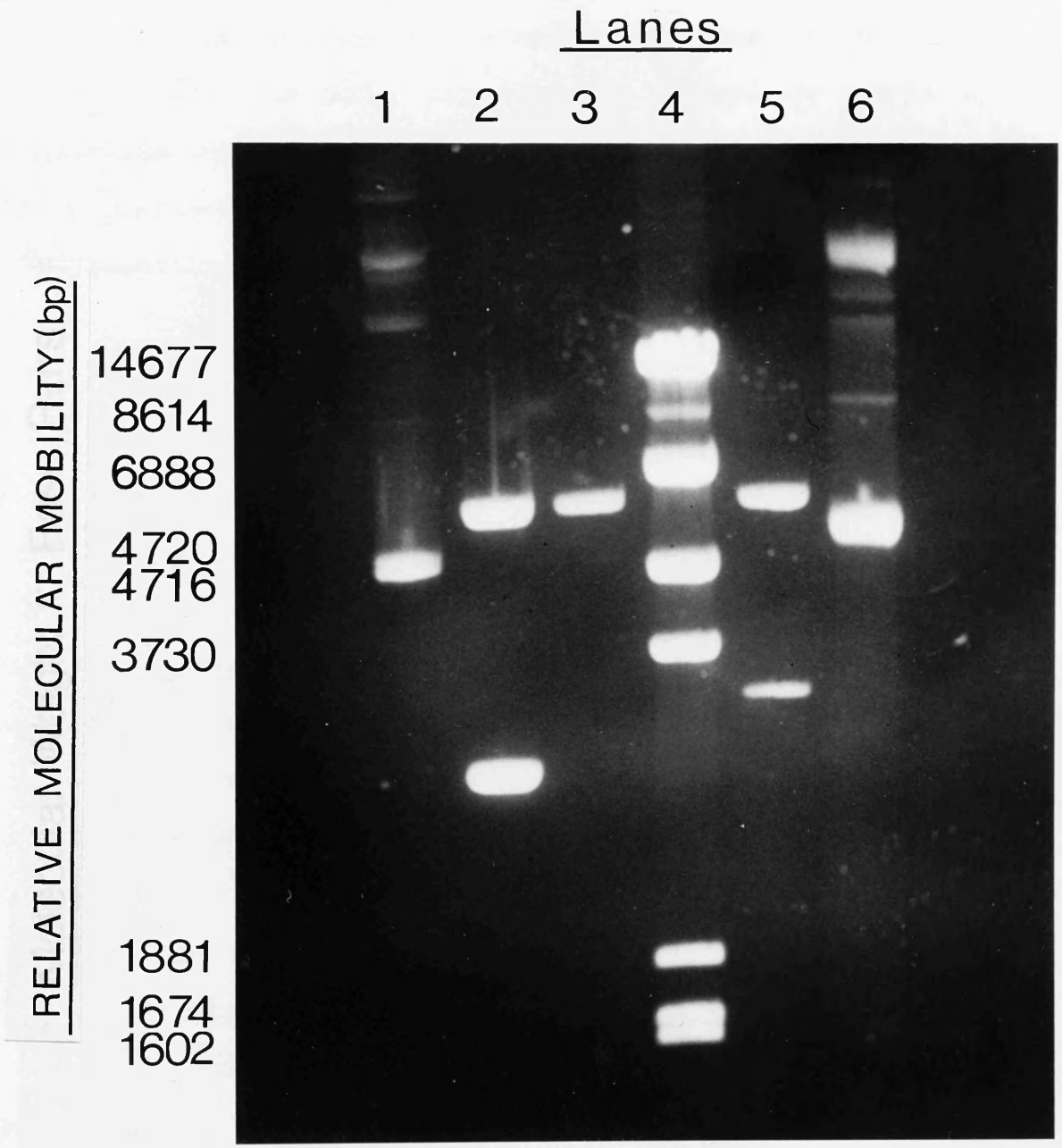
Lane 2 : 1.0μg of riboprobe pGHBI after double digestion with restriction enzymes HindIII and BamHI.

Lane 3 : 0.5μg of the purified HCMV DNA fragment HBI

Lane 4 : AvaI digest of λ DNA

Lane 5 : 0.5μg of vector pEHBI after double digestion with restriction enzymes HindIII and BamHI.

Lane 6 : 0.5μg of uncut vector pEHBI.


Lanes
1
2
3
4
5
6
RELATIVE MOLECULAR MOBILITY (bp)
14677
8614
6888
4720
4716
3730
1881
1674
1602

T7 promoter but proximal to plasmid-specific sequences. This prevents the T7 RNA polymerase transcribing plasmid DNA and therefore only RNA complementary to cloned DNA is synthesized. In all experiments riboprobe pGHBI was linearized by the restriction enzyme EcoRI. The products of digestion are shown in Figure 23 and are depicted diagrammatically in Figure 22.

4.3.3 Transcription Products

Linearization of riboprobe pGHBI with the restriction enzyme EcoRI followed by transcription in the presence of T7 RNA polymerase should give rise to cRNA transcripts with a MWt of 1400 bp (Figure 22). Confirmation of this was sought by subjecting radiolabelled transcripts to urea-acrylamide gel electrophoresis in parallel with RNA of known MWt. The results are shown in Figure 24. Although cRNA with a MWt of 1400 bp was observed, a number of other radiolabelled transcripts of lower MWt were also noted.

4.3.4 Labelling

In 4 experiments performed on separate occasions the percentage incorporation of [α-^{32}P] UTP was monitored at 10 min intervals during the first hour of RNA transcription. The constituents of each reaction were obtained from the same stock solutions with the exception that different batches of radionucleotide were used. Figure 25 shows considerable variation in the percentage incorporation of [α-^{32}P] UTP at the same time point within each reaction.

Figure 22 Schematic representation of riboprobe pGHBI

A figure showing the construction of riboprobe pGHBI and the predicted DNA fragments after digestion with the restriction enzyme EcoRI. Transcription in the presence of T7 RNA polymerase should give rise to cRNA with a MWt of 1400 bp.

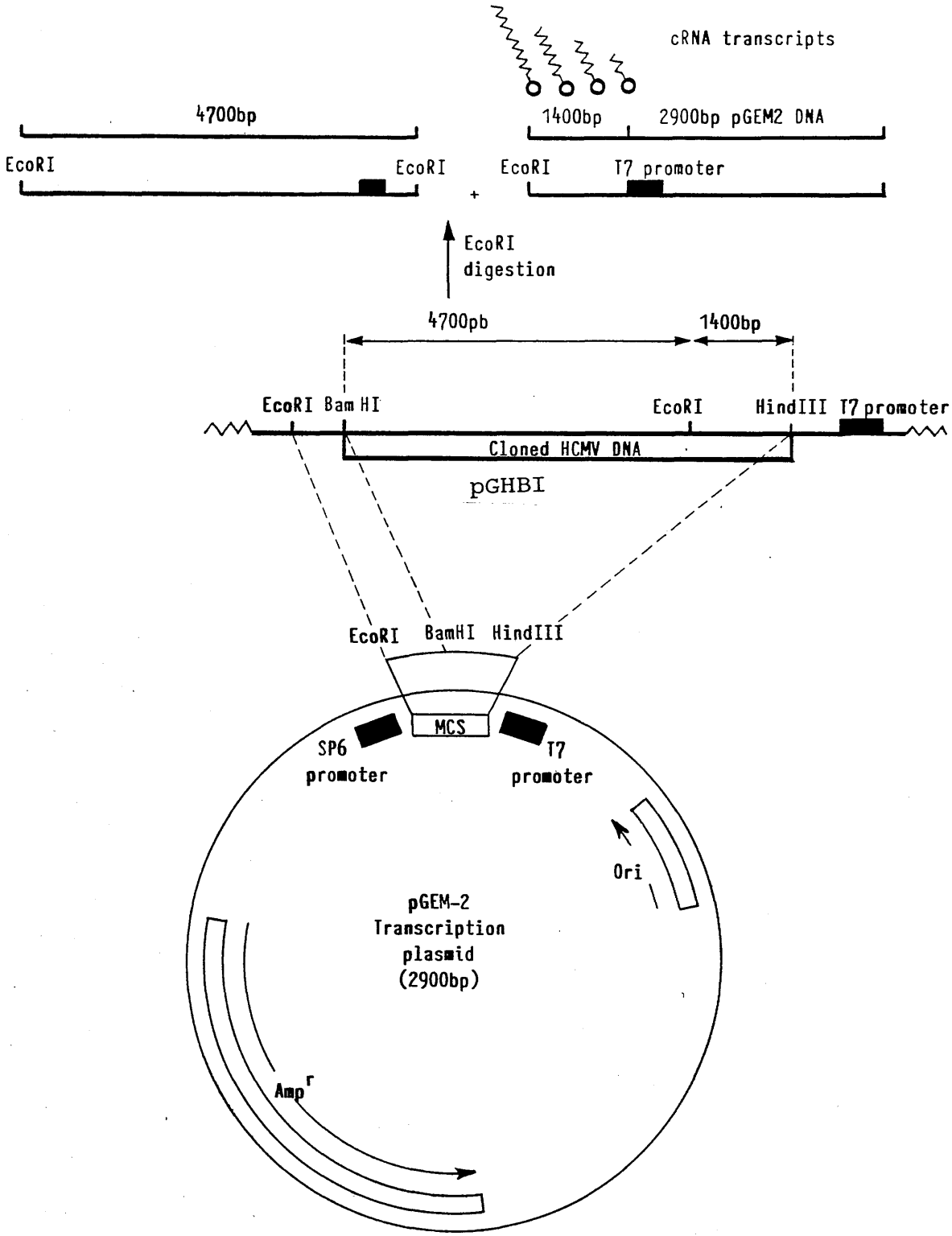

cRNA transcripts
4700bp
1400bp
2900bp pGEM2 DNA
EcoRI
EcoRI
EcoRI
T7 promoter
+
EcoRI
digestion
4700pb
1400bp
EcoRI Bam HI
EcoRI
HindIII T7 promoter
Cloned HCMV DNA
pGHBI
EcoRI BamHI HindIII
MCS
SP6
promoter
T7
promoter
Ori
pGEM-2
Transcription
plasmid
(2900bp)
Amp r

Figure 23 Linearization of riboprobe pGHBI by the restriction enzyme EcoRI

1μg of riboprobe pGHBI was cleaved by the restriction enzyme EcoRI and the DNA fragments separated by electrophoresis in a non-denaturing 0.6% (w/v) agarose gel (Lane 1). An AvaI digest of λ DNA provided the MWt markers (Lane 2).

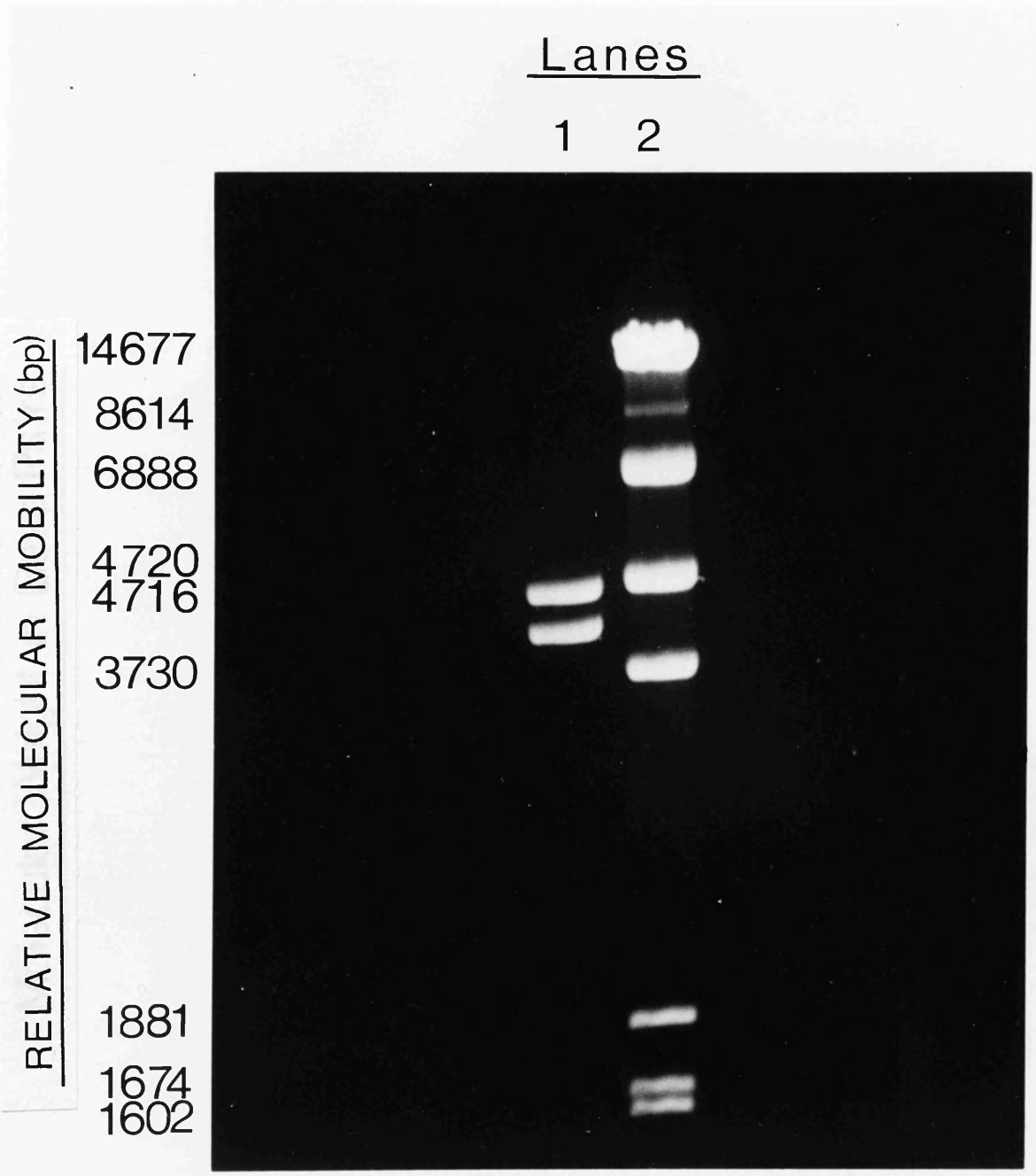

Lanes
1
2
RELATIVE MOLECULAR MOBILITY (bp)
14677
8614
6888
4720
4716
3730
1881
1674
1602

Figure 24 Radiolabelled transcription products of riboprobe pGHBI

An autoradiograph of a 3.5% (w/v) polyacrylamide 8.3M urea gel showing the radiolabelled cRNA transcripts produced after linearization of riboprobe pGHBI by the restriction enzyme EcoRI followed by transcription in the presence of T7 RNA polymerase (Lane 2). A ^{32}P end-labelled 0.16 to 1.77 kb RNA ladder provided the MWt markers (Lane 1).

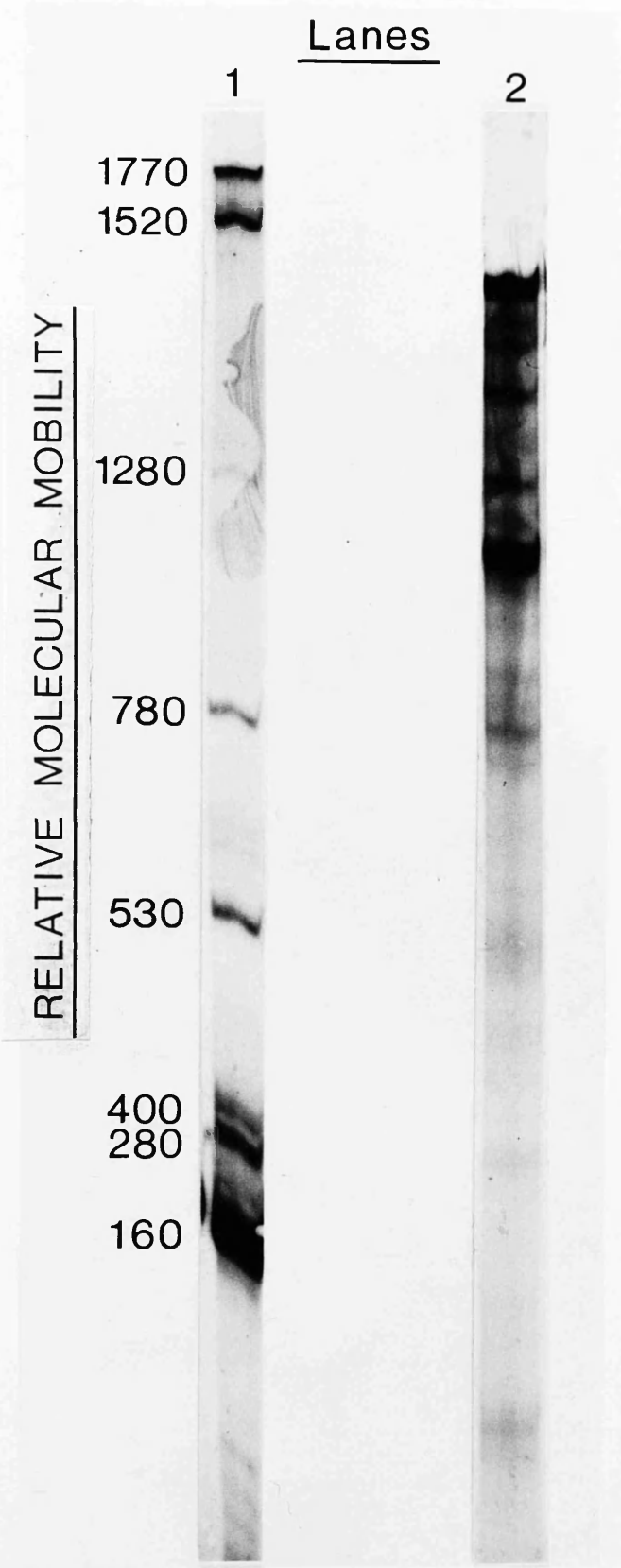
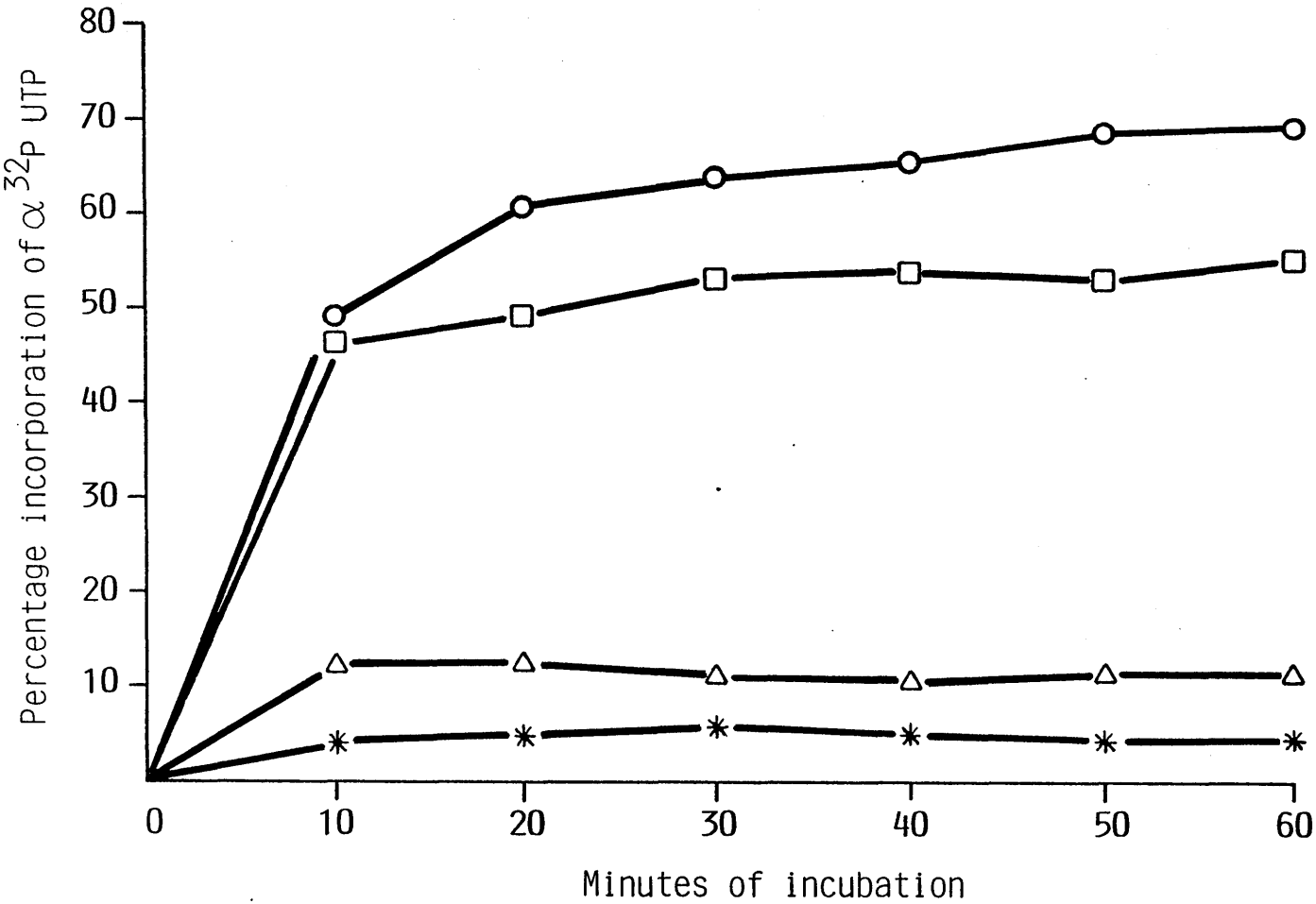

Lanes
1
2
1770
1520
1280
780
530
400
280
160
RELATIVE MOLECULAR MOBILITY

Figure 25 Time course of incorporation of [α-^{32}P]UTP during RNA transcription: Variation between batches of the radionucleotide

Riboprobe pGHBI was linearized with the restriction enzyme EcoRI and transcribed in the presence of T7 RNA polymerase. This graph shows the percentage incorporation of [α-^{32}P] UTP plotted at 10 min intervals during the first hour of RNA transcription. The constituents of each reaction were identical with the exception that [α-^{32}P] UTP was from a different batch (0, □ , Δ and * respectively).


Percentage incorporation of α 32P UTP
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
Minutes of incubation

This in turn would produce probes of differing specific activities.

4.4 RNA-DNA dot blot hybridization

The radiolabelled RNA transcripts described above were used for the detection of complementary vector pEHBI DNA or pGHBI DNA by dot blot hybridization. The specificity of the assay was assessed and attempts made to improve the lower limit of detection. Unless stated otherwise standard conditions of prehybridization, hybridization and washing were employed.

4.4.1 Sensitivity

After 1 hr of autoradiography 800pg of vector pGHBI DNA was routinely detected although in some experiments a faint hybrid signal was noted at 80pg. Similarly, after 24 hr of autoradiography the lower limit of detection of cDNA varied between 80pg and 8pg. Typical autoradiographs are shown in Figure 26.

4.4.2 Hybridization temperature

Three replicate nitrocellulose filters each containing serial 1:10 dilutions of vector pGHBI DNA from 800ng to 80fg were hybridized at a temperature of 40^{o}C or 50^{o}C or 60^{o}C (Figure 27A). Although the lower limit of detection of vector pGHBI DNA was 80pg in each experiment, the hybrid signal observed at each dilution of the target DNA

Figure 26 Sensitivity of the RNA-DNA hybridot assay

The figure shows 2 autoradiographs, one taken after 1 hr and the other after 24 hr exposure of a nitrocellulose filter containing serial 1:10 dilutions of vector pGHBI DNA from 8μg to 800fg. The filter was hybridized with radiolabelled cRNA input counts totalling 9.8×10^7 cpm. Standard prehybridization, hybridization and washing procedures were employed. Asterisks show the positions of hybrid signals noted in the original autoradiographs but which are not obvious in this photograph.

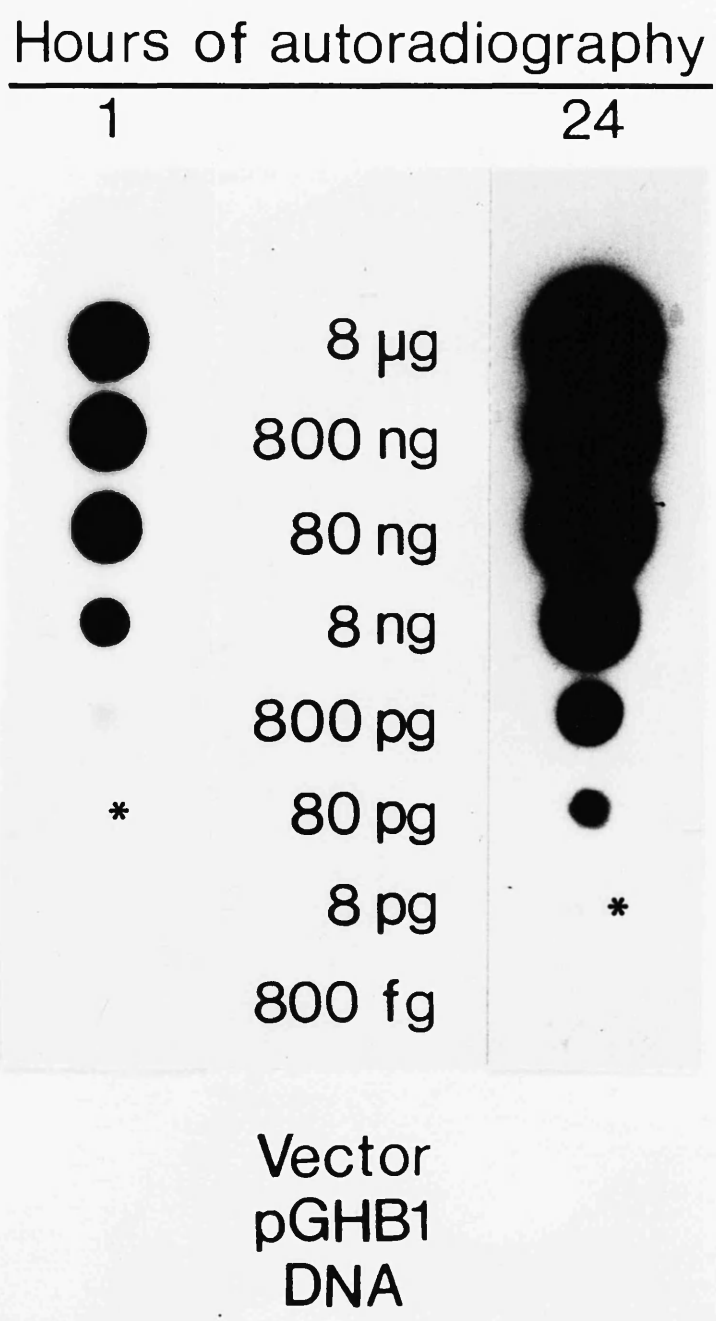
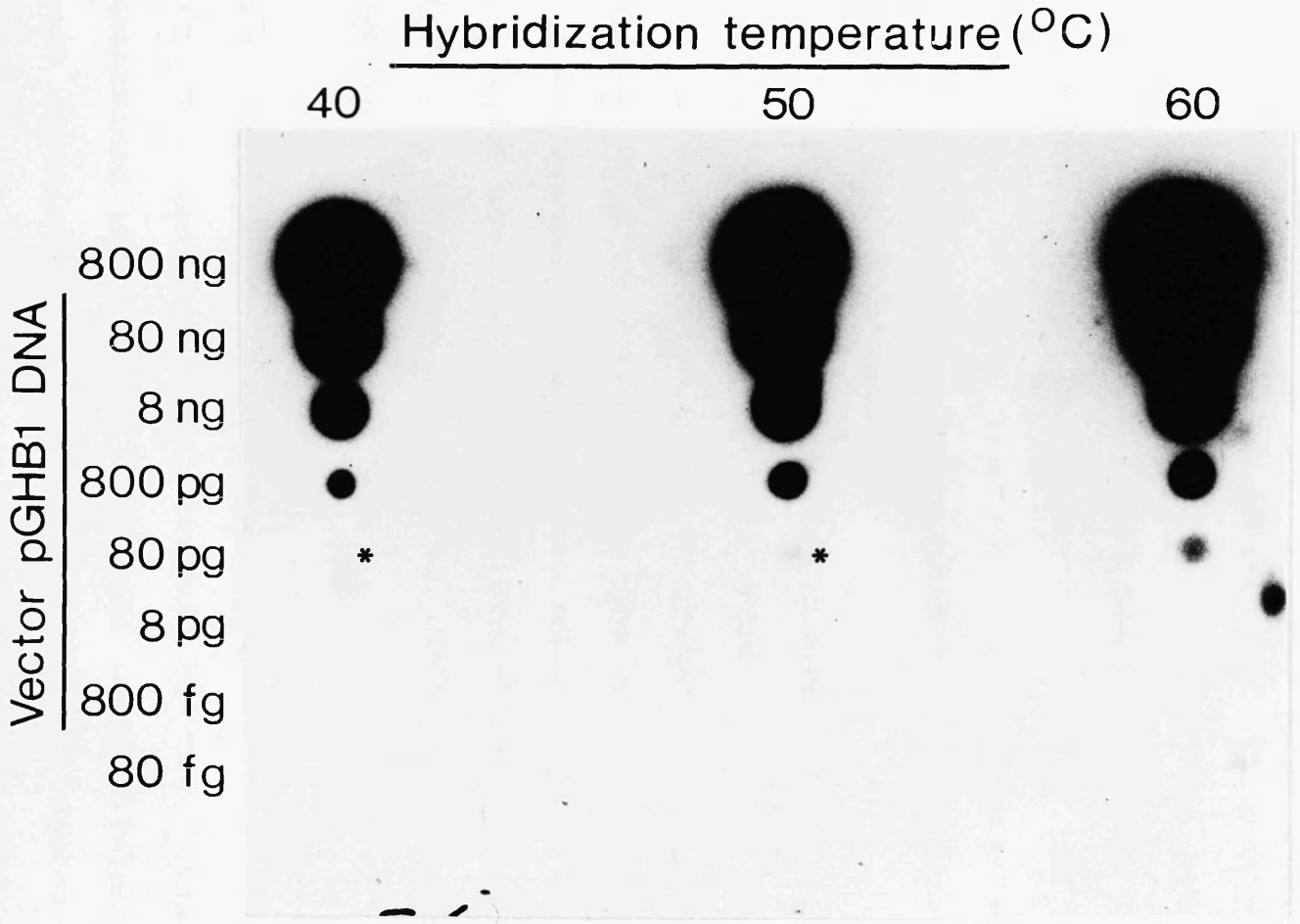

Hours of autoradiography
1
24
8 µg
800 ng
80 ng
8 ng
800 pg
80 pg
8 pg
800 fg
*
*
Vector
pGHB1
DNA

Figure 27A RNA-DNA hybridization: Effect of the hybridization temperature

The figure shows the autoradiograph obtained after 24 hr exposure of 3 replicate nitrocellulose filters each containing serial 1:10 dilutions of vector pGHBI DNA from 800ng to 80fg. One cRNA probe was synthesized and $L\ 10^8$ cpm simultaneously added to each filter which was subsequently hybridized at either 40°C or 50°C or 60°C. Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph. Dots were also counted for ^{32}P in a liquid scintillation counter and the results are shown in Figure 27B.

L input counts totalling


Hybridization temperature (°C)
40
50
60
Vector pGHB1 DNA
800 ng
80 ng
8 ng
800 pg
80 pg
8 pg
800 fg
80 fg
*
*

increased with increasing hybridization temperature. The experiment was repeated and similar results obtained.

Dots were also counted for ^{32}P in a liquid scintillation counter and the results of the aforementioned experiments are shown in Figure 27B. At each dilution of vector pGHBI DNA from 800ng to 800pg the hybrid signal increased as the hybridization temperature was raised from 40 to 60°C. With increasing dilution of the target DNA from 80pg to 80fg the cpm of ^{32}P detected on the nitrocellulose filters were noted to vary independently of the hybridization temperature.

Hybridization at 70°C was unsuccessful on 3 occasions because the nitrocellulose paper became fragile and difficult to manipulate. A nylon filter (Gene Screen Plus), which is more durable, was hybridized at 70°C but the background signal was unacceptably high. Therefore, a decision was made to use nitrocellulose filters at a hybridization temperature of 60°C in all subsequent hybridot assays.

4.4.3 Time period of hybridization

The optimum time period of hybridization was determined empirically. Duplicate nitrocellulose filters, each containing serial 1:10 dilutions of vector pEHBI DNA from 1μg to 100fg, were hybridized at 60°C for either 14 or 62 hr. Other experimental conditions remained standard. Autoradiographs obtained after 63 hr exposure of the nitrocellulose filters are shown in Figure 28A. The hybrid

Figure 27B RNA-DNA hybridization: Effect of the hybridization temperature

After autoradiography, dots were cut from the nitrocellulose filters described in Figure 27A and counted for ^{32}P in a liquid scintillation counter. The cpm of ^{32}P detected at each dilution of vector pGHBI DNA are shown after filter hybridization at either 40°C (○) or 50°C (△) or 60°C (□). The results of duplicate experiments are also shown (● , ▲ and ■ respectively). Lines are drawn through the average of 2 counts at each DNA dilution with the exception of 80pg at 50°C and 8ng at 60°C where the results from only 1 experiment are available (△ and □ respectively).

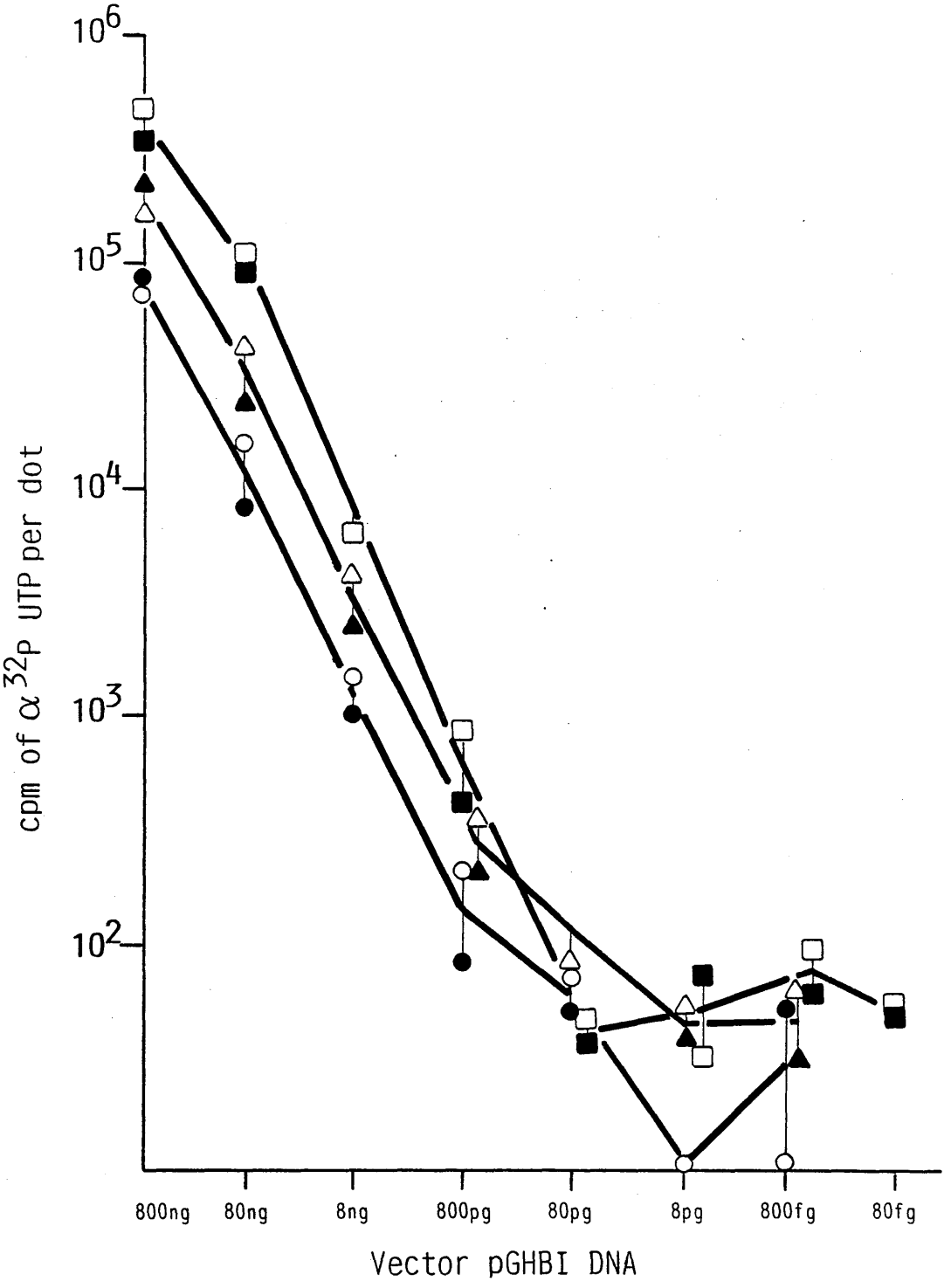
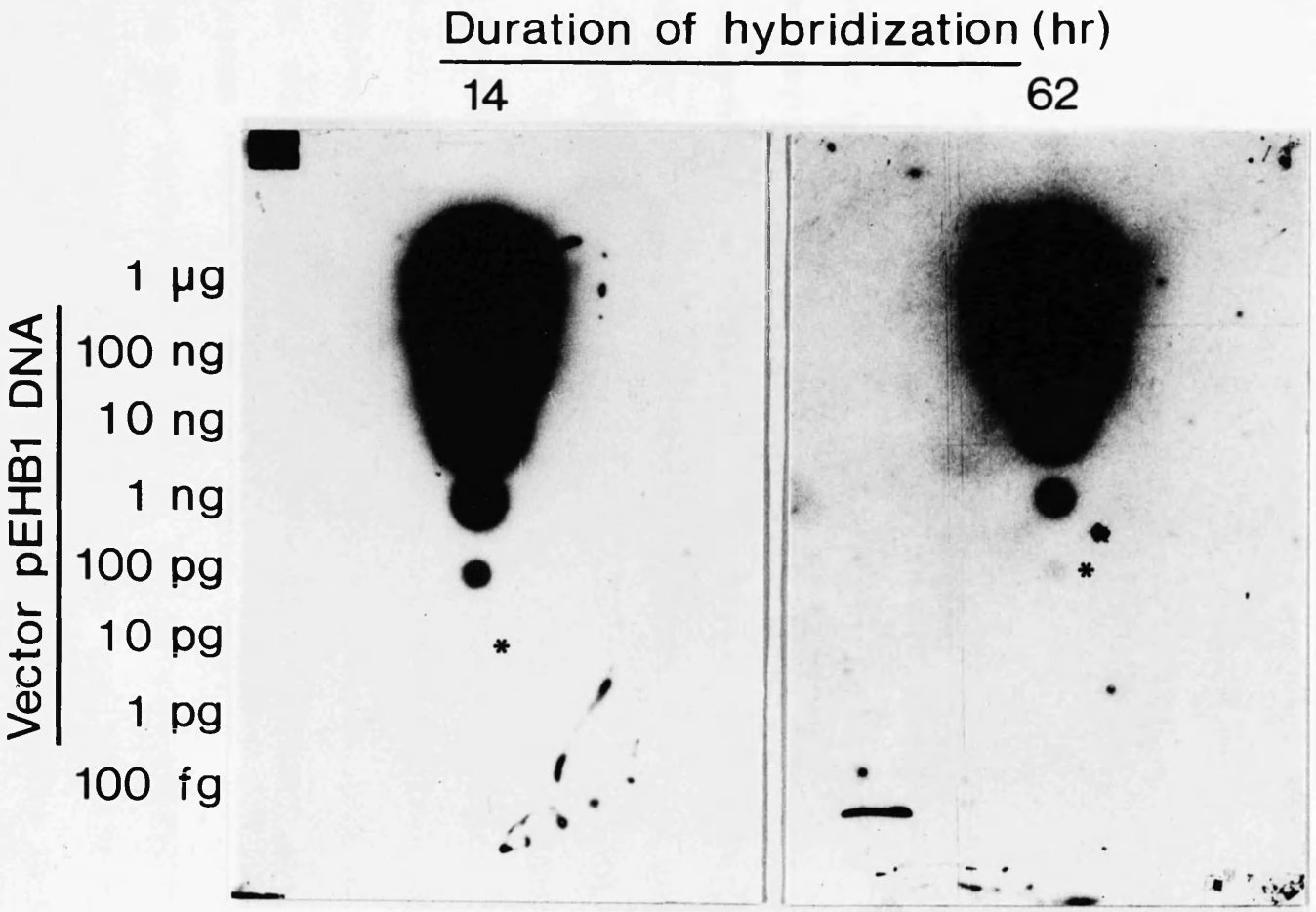

10^6
10^5
10^4
10^3
10^2
cpm of α^{32}P UTP per dot
800ng
80ng
8ng
800pg
80pg
8pg
800fg
80fg
Vector pGHBI DNA

Figure 28A

RNA-DNA hybridization: Variation with the duration of hybridization

The figure shows the autoradiograph obtained after 63 hr exposure of 2 duplicate nitrocellulose filters each containing serial 1:10 dilutions of vector pEHBI DNA from 1μg to 100fg. One cRNA probe was synthesized and 4 x 10^7 cpm simultaneously added to each filter which was subsequently hybridized at 60°C for either 14 hr or 62 hr. Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph. Dots were also counted for ^{32}P in a liquid scintillation counter and the results are shown in Figure 28B.

input counts totalling


Duration of hybridization (hr)
14
62
Vector pEHB1 DNA
1 µg
100 ng
10 ng
1 ng
100 pg
10 pg
1 pg
100 fg

signal observed at each dilution of the target DNA was higher when hybridization was performed for 14 than 62 hr. Furthermore, the lower limit of detection of cDNA was 10pg after 14 hr of hybridization but was 100pg when hybridization was allowed to proceed for 62 hr. The experiment was repeated and similar results obtained.

Dots were also counted for ^{32}P in a liquid scintillation counter and the results of the aforementioned experiments are shown in Figure 28B. The hybrid signals detected at 10ng and 1ng of vector pEHBI DNA were higher after 14 hr of hybridization than after 62 hr. At other dilutions of the target DNA the cpm of ^{32}P detected on each nitrocellulose filter were similar irrespective of the number of hours that the hybridization reaction had been allowed to proceed.

In another experiment, duplicate nitrocellulose filters each containing serial 1:10 dilutions of vector pGHBI DNA from 800ng to 80fg were hybridized at 60^{o}C for either 14 or 38 hr. The autoradiographs obtained after 24 hr exposure of the nitrocellulose filters are shown in Figure 28C. Although the lower limit of detection of cDNA was 8pg in both experiments, the hybrid signal observed at each dilution of the target DNA was marginally improved when hybridization was performed for 14 than 38 hr. These experiments were not repeated. The sensitivity of the hybridot assay was not evaluated at hybridization periods of less than 14 hr but it was decided that it would be

Figure 28B RNA-DNA hybridization: Variation with the duration of hybridization

After autoradiography, dots were cut from the nitrocellulose filters described in Figure 28A and counted for ^{32}P in a liquid scintillation counter. The cpm of ^{32}P detected at each dilution of vector pEHBI DNA are shown after filter hybridization at 60°C for 14 hr (□) and 62 hr (△). The experiment was repeated with a cRNA probe at a specific activity of 1.76×10^7 cpm (■ and ▲ respectively). Lines are drawn through the average of 2 counts at each DNA dilution with the exception of 100fg after 14 hr of hybridization when the result from only 1 experiment (□) is available.

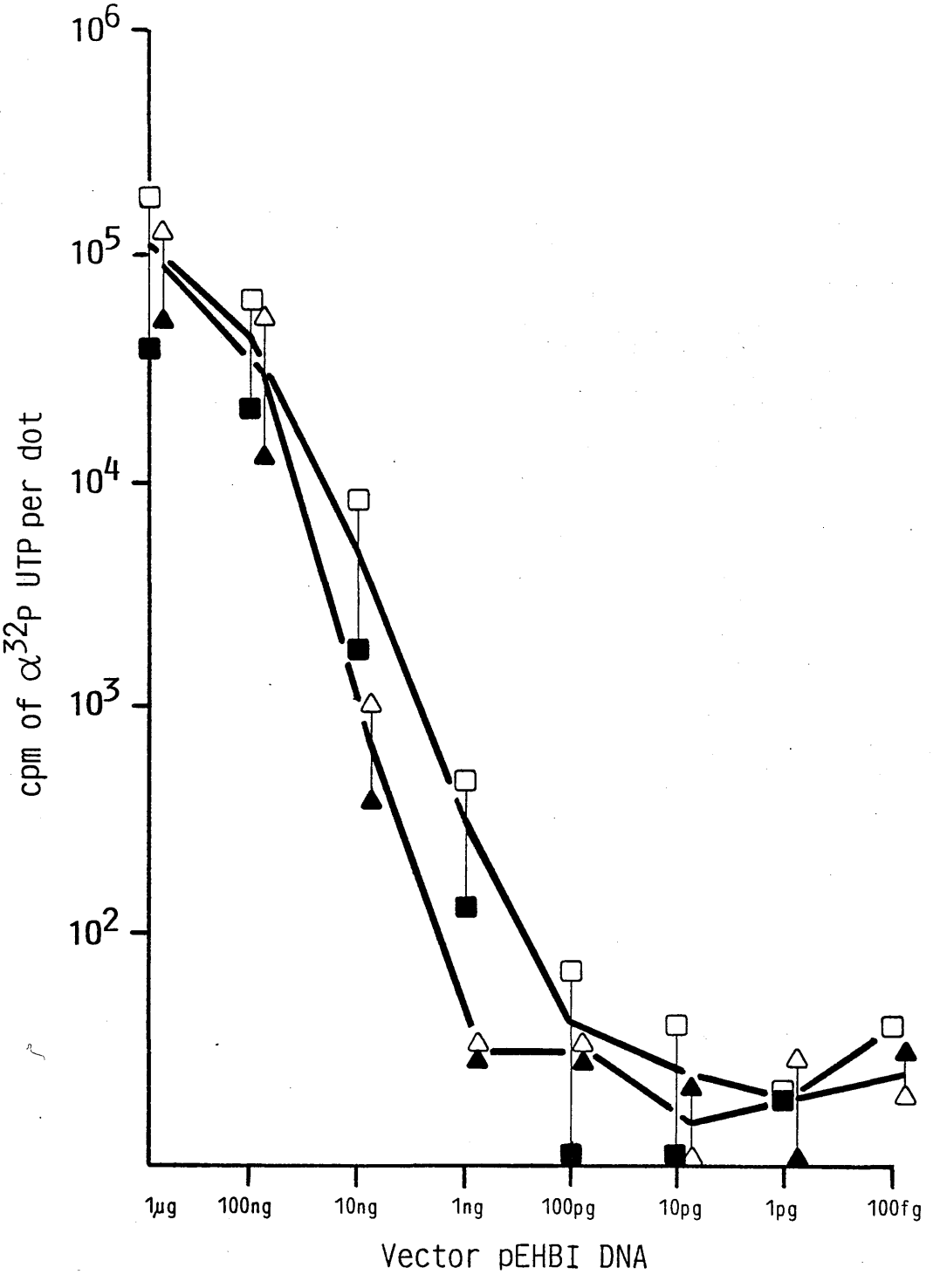
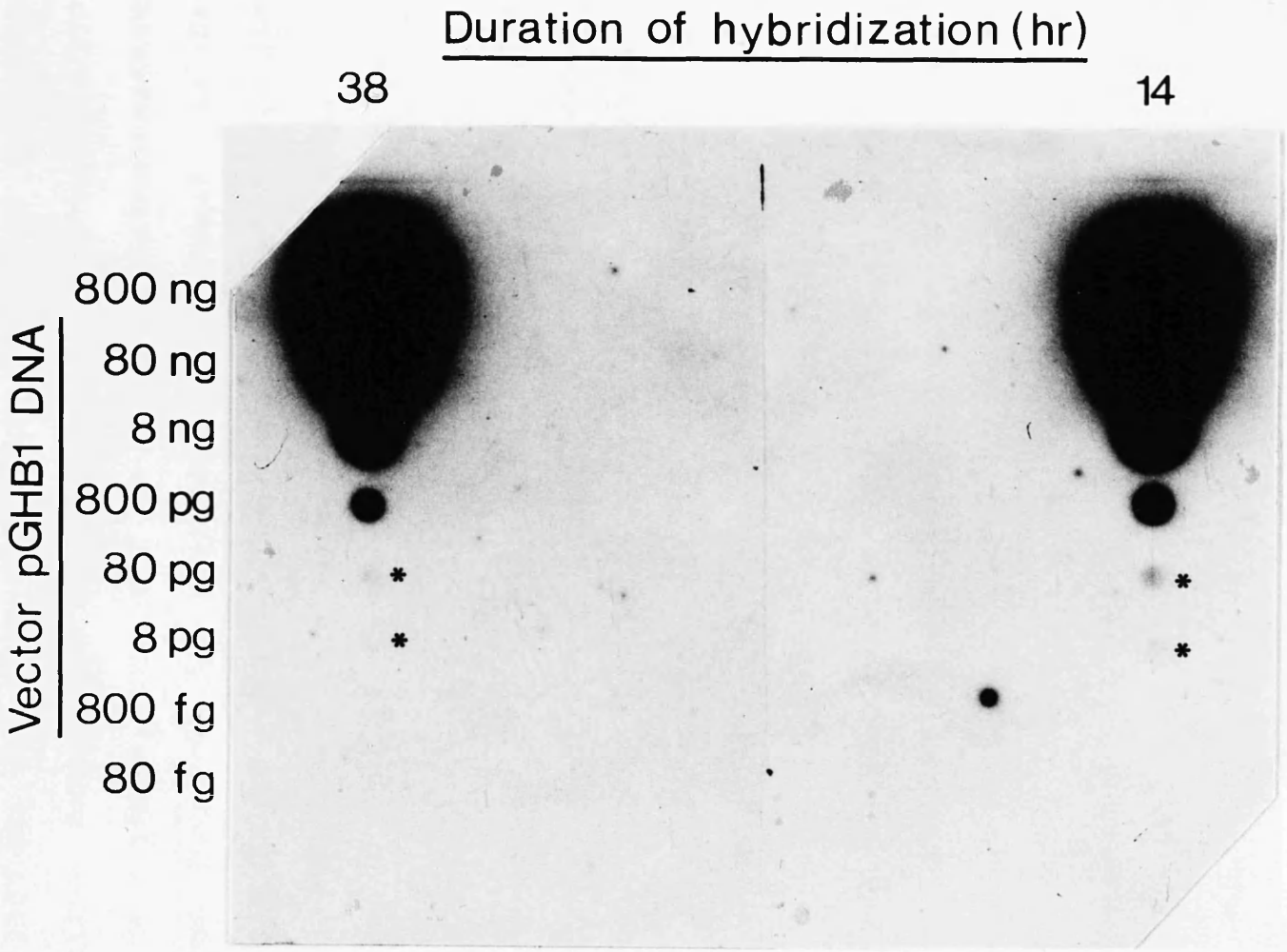

10^6
10^5
10^4
10^3
10^2
cpm of α^{32}p UTP per dot
1µg
100ng
10ng
1ng
100pg
10pg
1pg
100fg
Vector pEHBI DNA

Figure 28C RNA-DNA hybridization: Variation with the duration of hybridization

The figure shows the autoradiograph obtained after 24 hr exposure of duplicate nitrocellulose filters each containing serial 1:10 dilutions of vector pGHBI DNA from 800ng to 80fg. One cRNA probe

was synthesized and $\geq 10^8$ cpm simultaneously added to each filter which was subsequently hybridized at 60°C for either 14 hr or 38 hr. Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph.

≥ input counts totalling


Duration of hybridization (hr)
38
14
Vector pGHB1 DNA
800 ng
80 ng
8 ng
800 pg
80 pg
8 pg
800 fg
80 fg

convenient if all subsequent hybridization reactions were allowed to proceed overnight (approximately 14 hr).

4.4.4 Washing temperature

Attempts to improve the sensitivity of the hybridot assay were now focused on lowering the stringency of the washing procedure (Figure 29). The hybrid signals observed at equivalent dilutions of the target DNA were similar regardless of whether the filter was washed at 40 or 65°C. In both experiments the non-specific background signal was negligible and no evidence of hybridization to 10μg of either E.coli or human DNA could be found. The hybridot assay was repeated using serial 1:10 dilutions of vector pGHBI DNA and confirmation of the results obtained. In all subsequent experiments the nitrocellulose filters were washed in 0.1 x SSC, 0.1% (w/v) SDS at a temperature of 65°C.

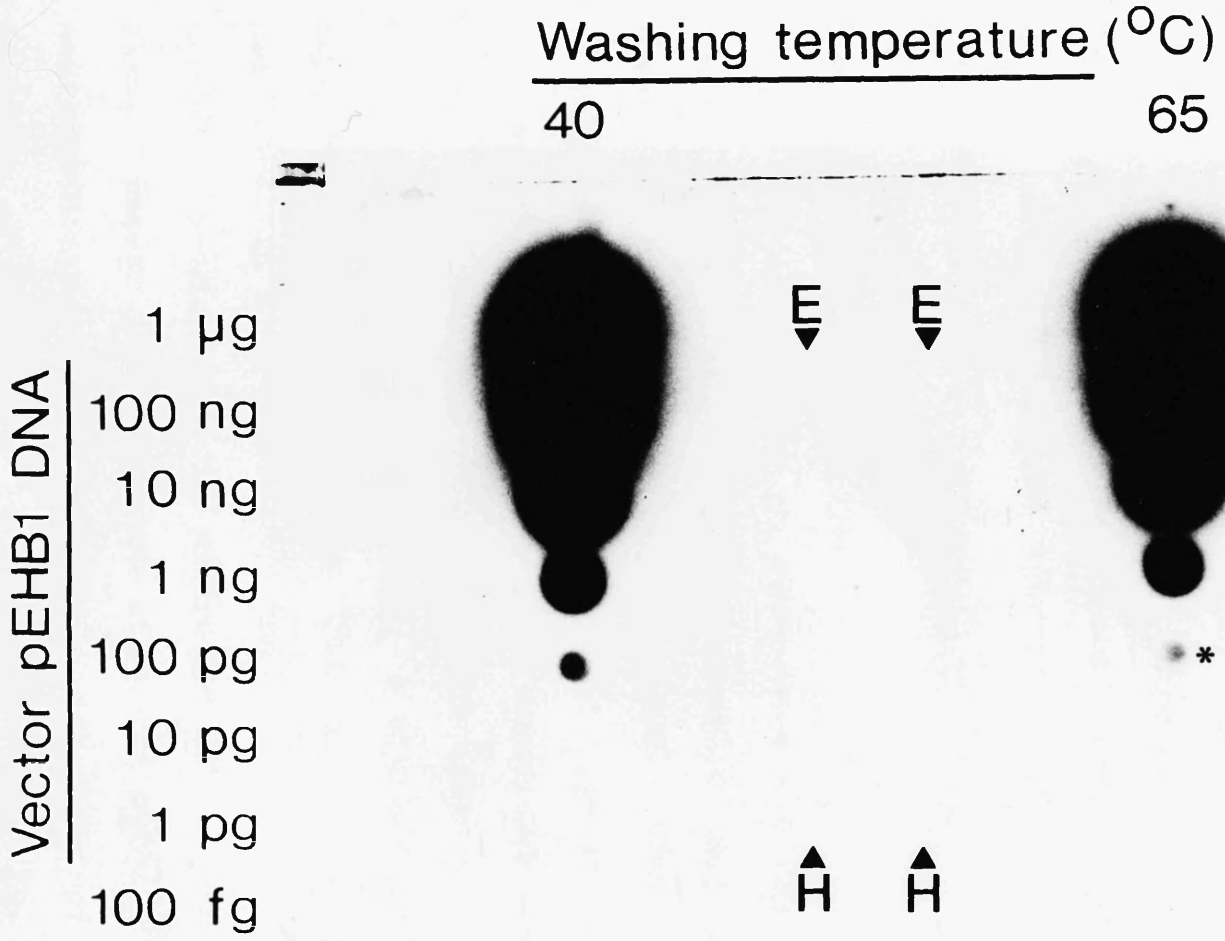
4.4.5 Amersham film (MP)

Further attempts to increase the sensitivity of the hybridot assay were centred on the use of an alternative autoradiography film (Hyperfilm MP, Amersham) which the manufacturers claimed to be more sensitive. Two nitrocellulose filters, each containing serial 1:10 dilutions of vector pEHBI DNA from 1μg to 100fg, were prepared in parallel. One cRNA probe was synthesized and 3 x 10^7 cpm added to both filters which were then hybridized in

input counts totalling

Figure 29 RNA-DNA hybridization: Variation in the washing temperature

A nitrocellulose filter containing 2 sets of serial 1:10 dilutions of vector pEHBI DNA from 1μg to 100fg was prepared. Ten micrograms each of E.coli and human DNA served as negative controls. The filter was hybridized at 60^{o}C for 14 hr with a cRNA probe (input counts totalling of 6.9 x 10^{7} cpm) and then washed twice for 30 min in 1 x SSC, 0.1% (w/v) SDS at 21^{o}C. The nitrocellulose filter was then halved, each half containing 1 set of the DNA dilutions described above together with both negative controls. Both halves of the filter were washed twice for 30 min in 0.1 x SSC, 0.1% (w/v) SDS, one at a temperature of 40^{o}C, the other at 65^{o}C. An autoradiograph obtained after 24 hr exposure of each half of the nitrocellulose filter is shown opposite. Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph. Arrows E and H indicate the positions of 10μg each of E.coli and human DNA respectively.


Washing temperature (°C)
40
65
Vector pEHB1 DNA
1 µg
100 ng
10 ng
1 ng
100 pg
10 pg
1 pg
100 fg
E
E
H
H
*

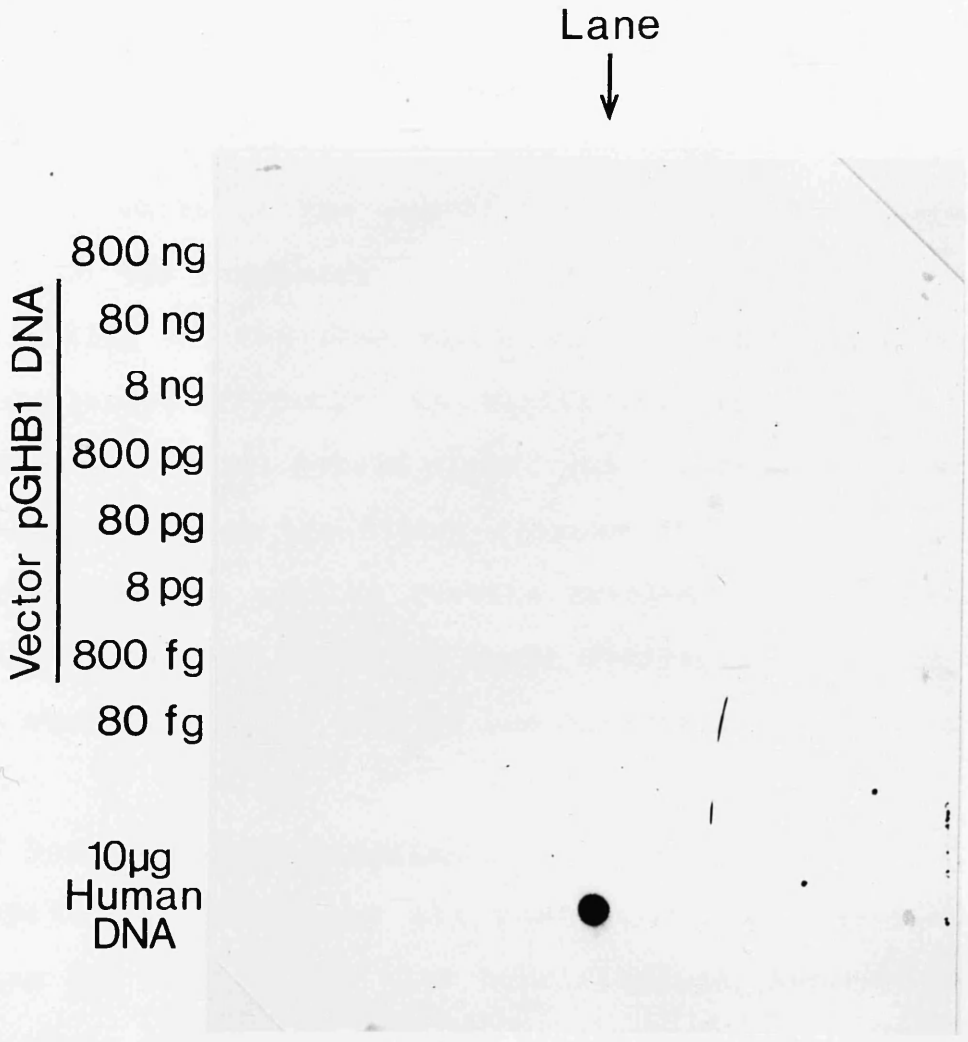
parallel at 60°C. All other experimental conditions remained standard with the exception that the nitrocellulose filters were exposed for 24 hr to either Kodak autoradiography film or Amersham hyperfilm MP. The hybrid signals obtained at equivalent dilutions of the target DNA were similar irrespective of which autoradiography film had been used. Kodak film was chosen for use in all subsequent hybridot assays because it was less expensive.

4.4.6 Detection of human DNA

In the experiment of Section 4.4.4, hybridization between the radiolabelled RNA transcripts and 10μg each of E.coli and human DNA was not observed. Although this may be attributed to assay conditions of high stringency it is also possible that the E.coli and human DNA had not bound to the nitrocellulose paper and were therefore unavailable for hybridization. Therefore, a nitrocellulose filter containing 10μg of human DNA was hybridized with radiolabelled nick-translated human DNA under experimental conditions identical to those of the RNA-DNA hybridot assay. Serial 1:10 dilutions of vector pGHBI DNA served as negative controls. The autoradiograph obtained after 72 hr exposure of the filter is shown in Figure 30. Human DNA was detected but no hybrid signal could be observed at any dilution of the plasmid DNA. Therefore, it was concluded that in the experiment of Section 4.4.4 human DNA had bound

Figure 30 Detection of human DNA

The figure shows an autoradiograph obtained after 72 hr exposure of a nitrocellulose filter which contained 10μg of human DNA and serial 1:10 dilutions of vector pGHBI DNA from 800ng to 80fg. The filter was hybridized with ^{32}P-labelled nick-translated human DNA in conditions identical to those of the RNA-DNA hybridot assay.


Lane
Vector pGHB1 DNA
800 ng
80 ng
8 ng
800 pg
80 pg
8 pg
800 fg
80 fg
10µg
Human
DNA

to the nitrocellulose filter but assay conditions were stringent enough to prevent cross-hybridization to the RNA probe. Furthermore, the inability of the human DNA probe to hybridize to vector pGHBI sequences confirms the lack of homology between human DNA and the BamHI subclone (EHBI) of the HindIII E fragment of HCMV AD169 (Ruger et al., 1984).

4.4.7 Availability of the cRNA probe

Because the target DNA could be loaded on to any one of the 96 wells of the dot blot manifold over an area of 135cm^2 it was necessary to determine if there was equal availability of the cRNA probe at every position on the nitrocellulose filter. At equivalent dilutions of the target DNA an equal hybrid signal was observed irrespective of the position on the filter (Figure 31). The experiment was repeated and similar results obtained. Therefore, it was concluded that there was equal availability of the cRNA probe over the entire area of the nitrocellulose filter.

4.4.8 Reconstruction experiments

Prior to screening all post transplantation urine samples for HCMV by dot blot hybridization, reconstruction experiments were performed to assess the sensitivity and specificity of the hybridot assay when using whole virus preparations. The results obtained after 10 days of autoradiography are shown in Figure 32 and are summarized in Table 5.

Radiolabelled cRNA transcripts were able to detect DNA

Figure 31 RNA-DNA hybridization: Availability of the cRNA probe

Three sets of serial 1:10 dilutions of vector pGHBI DNA from 8μg to 80fg were prepared in parallel and bound to a nitrocellulose filter. One set of dilutions was placed in the middle of the filter while the other 2 were located at either end. The filter was hybridized with a cRNA probe input counts totalling 1.45×10^8 cpm. An autoradiograph obtained after 1 hr exposure of the filter is shown here. Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph.


Lanes
1 2 3 4 5 6 7 8 9 10 11 12
Rows
A
B
C
D
E
F
G
H
*
*

Figure 32 RNA-DNA hybridization: The results of a typical reconstruction experiment

One hundred microlitres of each virus stock was made up to 8ml and serially diluted 1:10 in urine obtained from a patient known to be seronegative for HCMV. The DNA was extracted and bound to a nitrocellulose filter which was then hybridized with a cRNA probe input counts totalling 9.5×10^7 cpm. An autoradiograph obtained after 10 days exposure of the filter is shown here. The location of each positive and negative control is given below.

1A to 1F: Serial 1:10 dilutions of HCMV AD169 (stock V6) from $\leqslant 9 \times 10^6$ to $\leqslant 9 \times 10^2$ particles/7.2 ml.

2A to 2F: Serial 1:10 dilutions of HSV-1 from 1.1×10^8 to 1.1×10^3 particles/7.2 ml.

3A to 3F: Serial 1:10 dilutions of HCMV AD169 (stock V7) from 1.8×10^8 to 1.8×10^3 particles/7.2 ml.

4A to 4F: Serial 1:10 dilutions of HSV-2 from 1.8×10^9 to 1.8×10^4 particles/7.2 ml.

5A to 5F: Serial 1:10 dilutions of HCMV AD169 (stock V8) from 4.3×10^8 to 4.3×10^3 particles/7.2 ml.

6A to 6F: Serial 1:10 dilutions of uninfected Helu cells from 1.8×10^6 to 1.8×10^1 cells/7.2 ml.

12A to 12H: Serial 1:10 dilutions of vector pGHBI DNA from 800ng to 80fg.

Other controls included:-

1μg of HSV-1 DNA	(8A, 8B)
1μg of HSV-2 DNA	(10A, 10B)
10μg of E.coli DNA	(8D, 10D)
10μg of human DNA	(8F, 10F)
10μg of ribosomal RNA	(8H, 10H)

Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph.

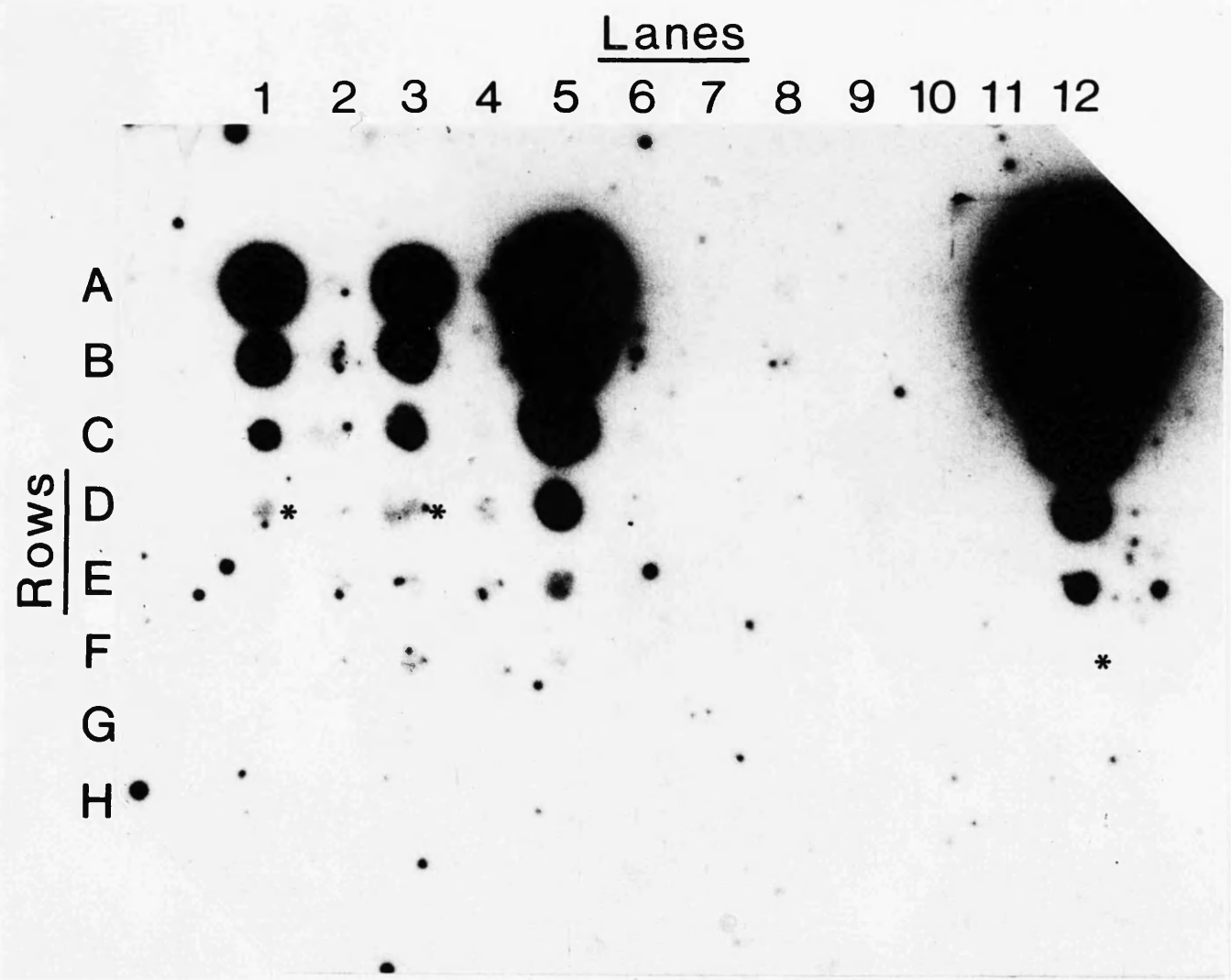

Lanes
1 2 3 4 5 6 7 8 9 10 11 12
Rows
A
B
C
D
E
F
G
H

Table 5 Virus stocks

A table showing the titre and particle counts of all virus stocks used to simulate infected urine samples in reconstruction experiments. Also shown are estimates of the number of viral particles detected by the RNA-DNA hybridot assay after 10 days of autoradiography.

N.A. = not available

N.D. = not detected

* = after 24 hr of autoradiography

Particle counts were performed by Mr J. Aitken, Electron Microscopy Laboratory, Institute of Virology.

Virus	pfu/ml	particles /ml	Number of HCMV particle equivalents of DNA detected by hybridot assay (particles/7.2 ml)
HCMV (stock V5)	2×10^5	4.4×10^9	3.9×10^5*
HCMV (stock V6)	1.3×10^2	$\leqslant 10^8$	$\leqslant 9 \times 10^2$
HCMV (stock V7)	1×10^5	2×10^9	1.8×10^5
HCMV (stock V8)	5×10^4	4.8×10^9	4.3×10^4
HSV-1	5×10^7	1.2×10^9	N.D.
HSV-2	8×10^7	2.0×10^{10}	N.D.
VZV	N.A.	1.4×10^9	N.D.

extracted from all 3 stocks of HCMV AD169. In 2 of these stocks (V7 and V8) the sensitivity of the hybridot assay was similar with 1.8×10^5 and 4.3×10^4 viral particles/ 7.2 ml of urine respectively being detected. In HCMV stock V6 no viral particles were detected by electron microscopy and must therefore have been at a concentration of 10^8/ml or less. Consequently, the lower limit of detection by dot blot hybridization could not have been greater than 9×10^2 viral particles/7.2 ml of urine. No hybrid signal was detected in similar reconstruction experiments using HSV-1 and HSV-2 although background noise was noted in positions 2B, 2E and 4D. DNA extracted from uninfected Flow 2002 cells was never detected by the hybridot assay. In a separate reconstruction experiment, 100μl of a stock of VZV did not hybridize to the cRNA transcripts on 3 occasions of testing (Figure 33).

The ability of the cRNA probe to detect non-infectious HCMV particles was also established. In 2 stocks of HCMV AD169 (V8 and V6) the number of viral particles detected by the hybridot assay was 1 and 2 $\log_{10}$ respectively greater than the number of pfus. This suggests the presence of non-infectious viral particles which contained a DNA core that provided a target for the radiolabelled cRNA transcripts.

The cRNA probe did not hybridize to 10μg of ribosomal RNA or 10μg each of E.coli and human DNA. One microgram of HSV-1 DNA also remained undetectable although 1μg of HSV-2 DNA hybridized to the radiolabelled RNA transcripts to give

Figure 33 RNA-DNA hybridization: VZV reconstruction experiment

One hundred microlitres of a stock of VZV was made up to 7.2ml in urine. The DNA was extracted and then loaded on to a nitrocellulose filter which was hybridized with a cRNA probe input counts totalling 3.57×10^8/cpm. An autoradiograph obtained after 10 days exposure of the nitrocellulose filter is shown here. The location of all positive and negative controls is given below.

1A: 80pg of vector pGHBI DNA

1B to 1G: Serial 1:10 dilutions of HCMV AD169 (stock V8) from 4.32×10^8 to 4.32×10^3 particles/7.2 ml of urine.

1H: 8pg of vector pGHBI DNA

2A: uninfected urine

2B: 10μg of human DNA

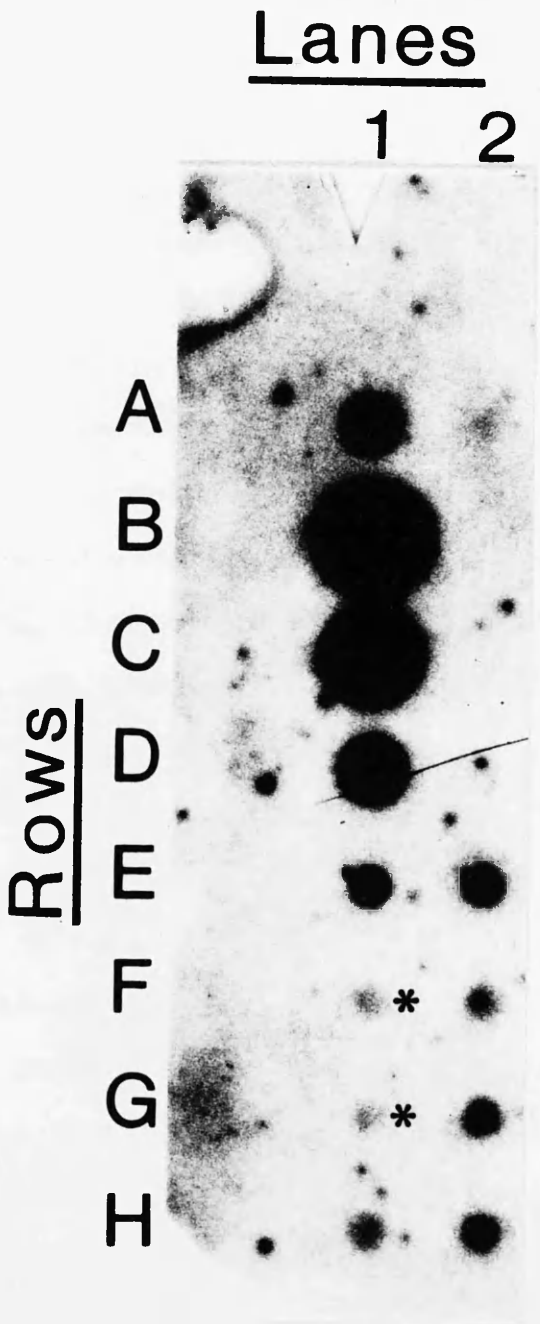
2C: 10μg of E.coli DNA

2D: VZV reconstruction experiment (1.43×10^8 particles in 7.2ml of urine)

2E: 10μg of HSV-2 DNA

2F to 2H: urine samples from a renal allograft recipient.

Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph.


Lanes
1 2
Rows
A
B
C
D
E
F
G
H
*
*

a signal equivalent to that observed with 8pg of vector pGHBI DNA.

In a separate experiment (Figure 34), the RNA transcripts were shown to hybridize to vector pGEM2 DNA, 10ng of the latter giving a hybrid signal equivalent to that observed with 8pg of vector pGHBI DNA.

4.4.9 Precipitation of HCMV from the urine by PEG 6000

An attempt was made to improve the sensitivity of the hybridot assay by adding PEG 6000 to HCMV-infected urine in reconstruction experiments. At equivalent dilutions of HCMV AD169 (stock V5), the hybrid signals observed were similar irrespective of whether PEG 6000 had been added to the urine (Figure 35). Furthermore, the addition of PEG 6000 did not improve the lower limit of detection of HCMV with 3.9×10^5 particles/7.2 ml of urine being detected in reconstruction experiments prepared both in the presence and absence of PEG 6000.

4.4.10 Urinary DNase

The presence of DNase in urine samples may digest any viral DNA available for binding to the nitrocellulose filter and therefore decrease the sensitivity of the hybridot assay. Therefore, reconstruction experiments were prepared and the results of a representative hybridot assay are shown in Figure 36A.

In the absence of an incubation step, the hybrid signals observed were marginally improved when equivalent

Figure 34 RNA-DNA hybridization: Detection of vector pGEM2 DNA

DNA from vectors pGHBI and pGEM2 was serially diluted 1:10 from 800ng to 80fg and from 1μg to 1pg respectively. The DNA was loaded on to a nitrocellulose filter which was hybridized with a cRNA probe input counts totalling 2.8×10^8 cpm. An autoradiograph obtained after 24 hr exposure of the filter is shown here. Asterisks show the position of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph.

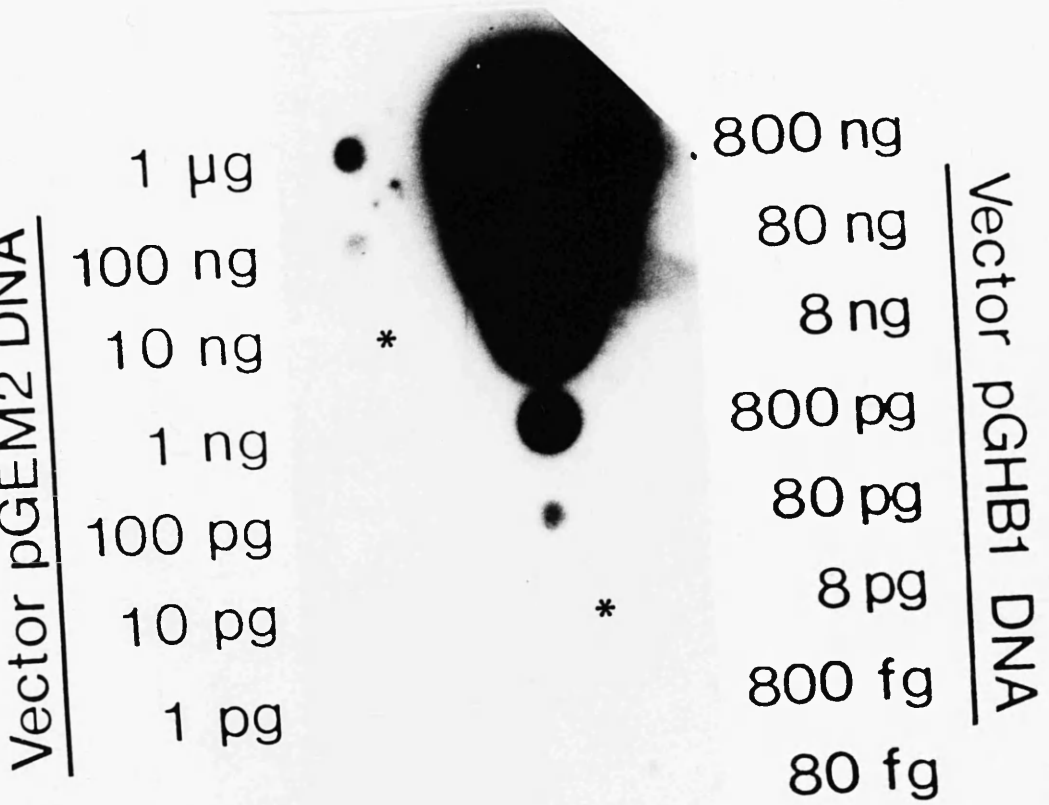

Vector pGEM2 DNA
1 µg
100 ng
10 ng
1 ng
100 pg
10 pg
1 pg
Vector pGHB1 DNA
800 ng
80 ng
8 ng
800 pg
80 pg
8 pg
800 fg
80 fg

Figure 35 RNA-DNA hybridization: PEG 6000 precipitation of HCMV from the urine

HCMV AD169 (stock V5) was serially diluted 1:10 from 3.96×10^7 to 3.96×10^2 particles /7.2 ml of urine. 0.2 volumes of 40% (w/v) PEG 6000 were added and the virus precipitated (Lane 3). A duplicate set of dilutions without PEG 6000 was prepared in parallel (Lane 2). DNA was extracted and loaded on to a nitrocellulose filter which was hybridized with a cRNA probe input counts totalling 10^8 cpm. An autoradiograph obtained after 24 hr exposure of the filter is shown here. Serial 1:10 dilutions of vector pGHBI DNA from 8μg to 800fg provided a set of reference standards (Lane 1). Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph.

+ shows the positions of non-specific background signals.

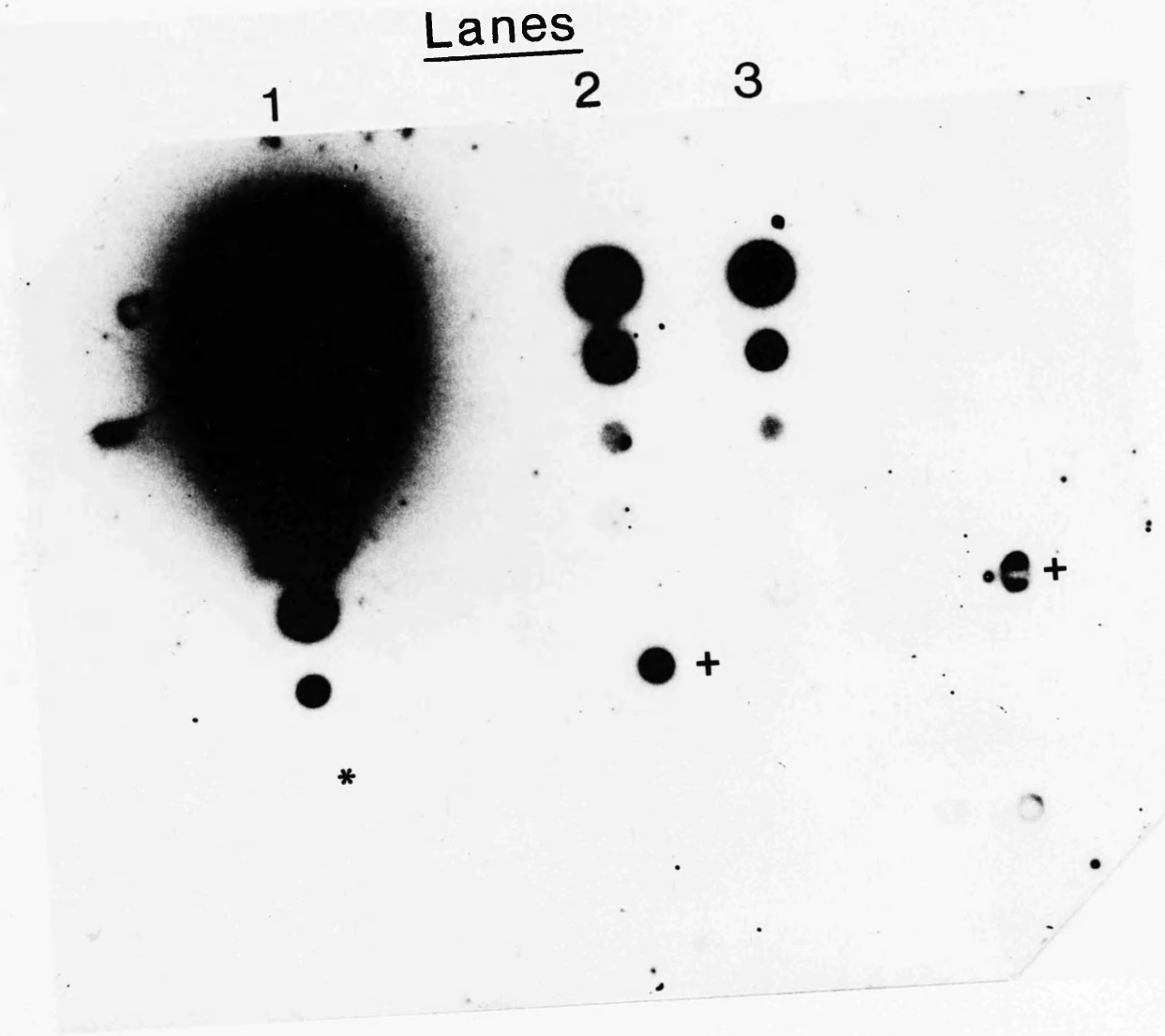
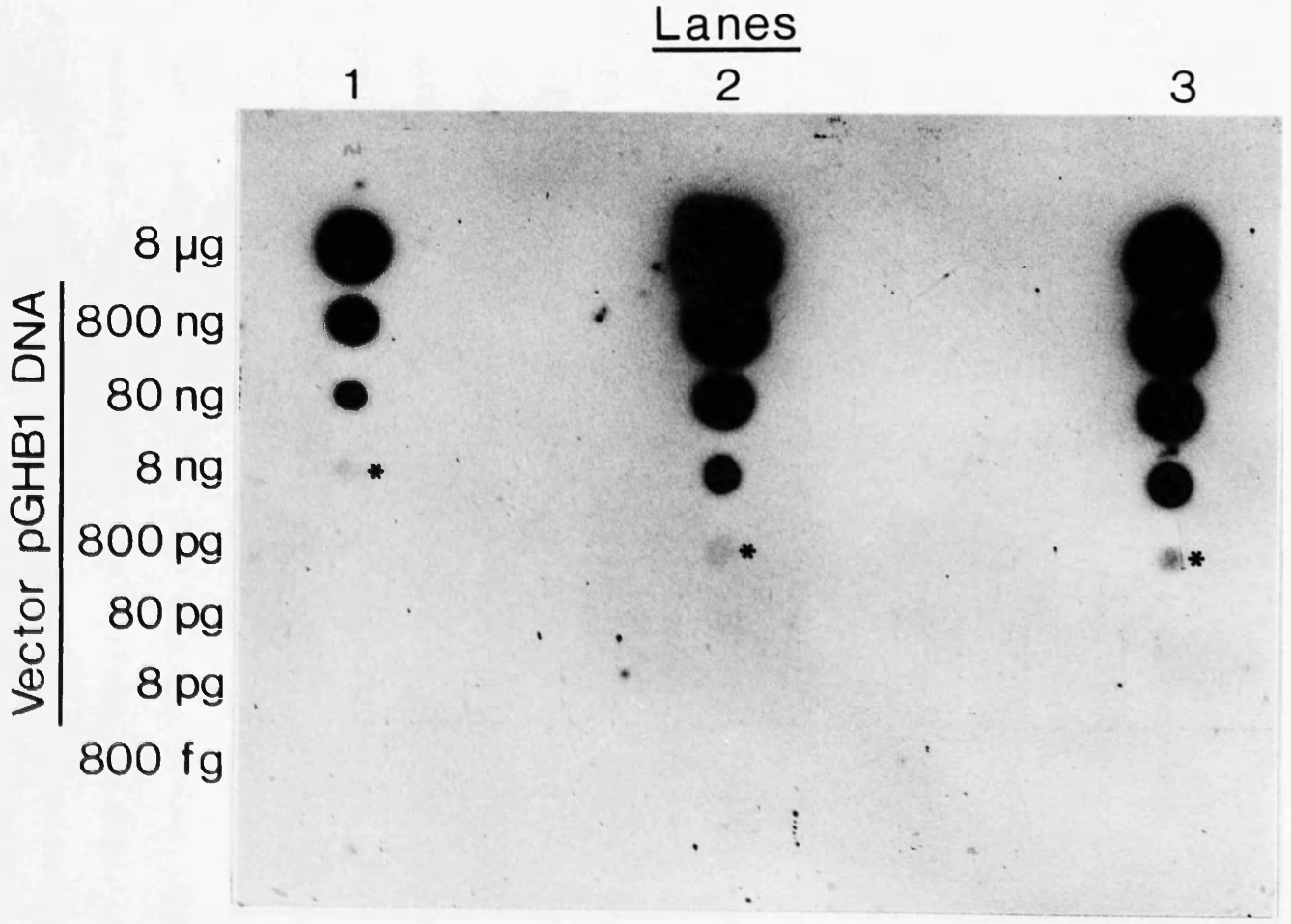

Lanes
1
2
3
+
+
*

Figure 36A RNA-DNA hybridization: Urinary DNase activity

Serial 1:10 dilutions of vector pGHBI DNA from 8μg to 800fg were prepared in urine and incubated overnight at 21^{o}C. The DNA was then denatured and loaded on to a nitrocellulose filter (Lane 1). Controls included replicate dilutions of vector pGHBI DNA in urine (Lane 2) or sterile distilled water (Lane 3) but without a prior incubation step. The filter was hybridized with a radiolabelled cRNA probe and subjected to autoradiography for 1 hr. Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph. Dots were also counted for ^{32}P in a liquid scintillation counter and the results are shown in Figure 36B.


Lanes
1
2
3
Vector pGHB1 DNA
8 µg
800 ng
80 ng
8 ng
800 pg
80 pg
8 pg
800 fg
*
*
*

dilutions of the target DNA were prepared in sterile water than in urine but the lower limit of detection of cDNA was the same in both experiments. However, when vector pGHBI DNA was diluted in urine and incubated overnight at 21°C the hybrid signal at each dilution was reduced compared to the respective controls and the sensitivity of the hybridot assay was decreased by 1 $\log_{10}$.

Dots were also counted for ^{32}P in a liquid scintillation counter and the results of this and a duplicate experiment are shown in Figure 36B. When dilutions of vector pGHBI DNA from 800ng to 800pg were incubated overnight in urine, the hybrid signals detected were less than those observed for equivalent dilutions prepared in sterile water or in urine without a prior incubation step. For all other dilutions of vector pGHBI DNA, the cpm of ^{32}P detected on the nitrocellulose filter were similar at equivalent dilutions of the target DNA irrespective of the experimental conditions.

One hundred microlitres of HCMV AD169 (stock V8) was made up to 8ml and serially diluted 1:10 in urine. After overnight incubation at 37°C, DNA was extracted, denatured and loaded on to a nitrocellulose filter. A duplicate set of dilutions prepared in the absence of an incubation step served as the control. Preincubation of the virus reduced the lower limit of detection by hybridot assay from 4.8 x 10^5 to 4.8 x 10^7 particles/7.2 ml of urine (Figure 37A). This experiment was not repeated.

Figure 36B RNA-DNA hybridization: Urinary DNase activity

After autoradiography, dots were cut from the nitrocellulose filter described in Figure 36A and counted for ^{32}P in a liquid scintillation counter. The graph opposite shows the cpm of ^{32}P detected after dilution of the target DNA in urine followed by overnight incubation at 21°C (o) or after dilution in sterile distilled water (□) or urine without a prior incubation step (△). The results of duplicate experiments are also shown (● , ■ and ▲ respectively). Lines are drawn through the average of 2 counts at each DNA dilution.

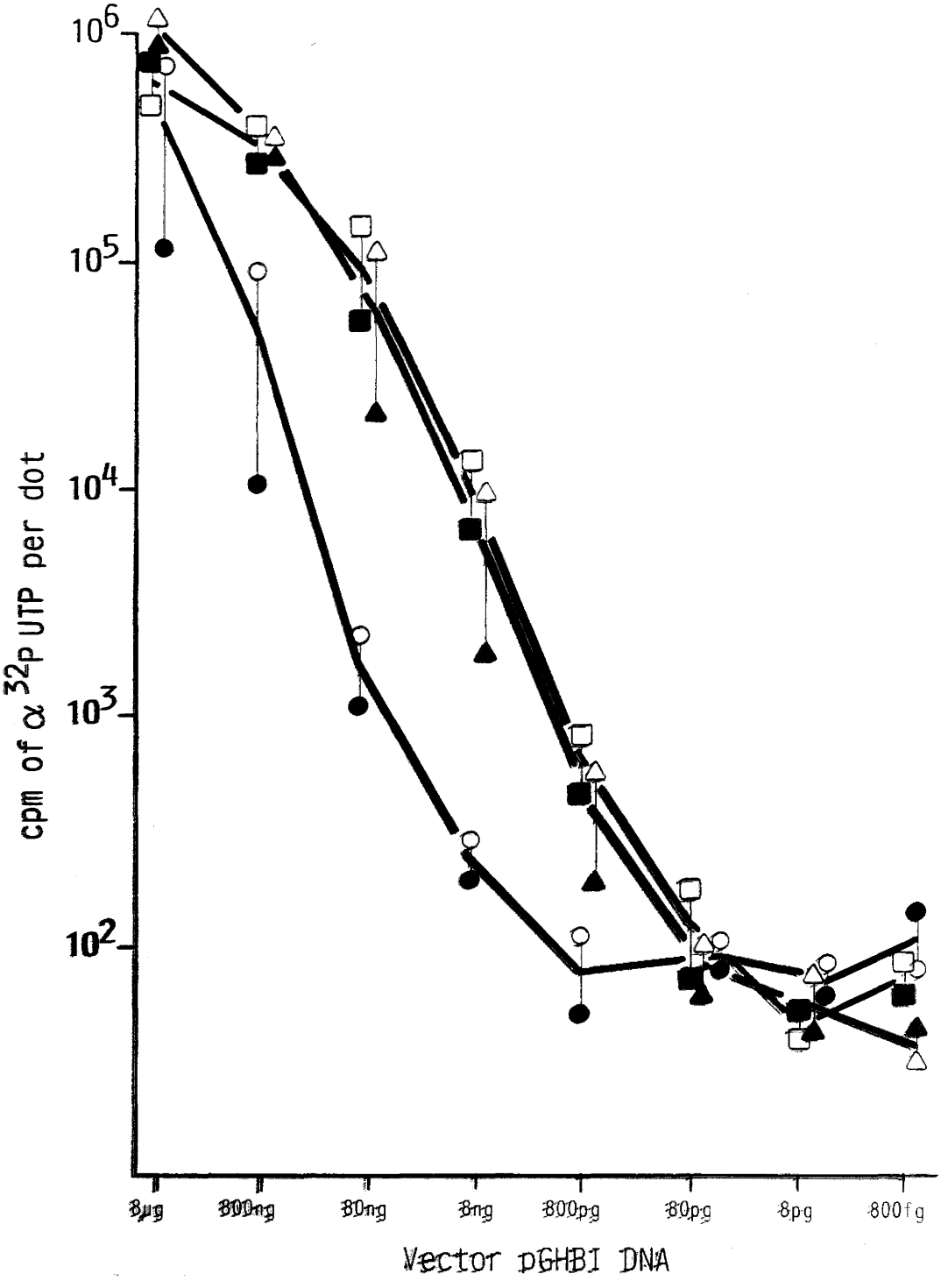

10^6
10^5
10^4
10^3
10^2
cpm of α^{32}p UTP per dot
8µg
800ng
80ng
8ng
800pg
80pg
8pg
800fg
Vector pGHBI DNA

Figure 37A RNA-DNA hybridization: Addition of human and E.coli DNA to HCMV DNA

Serial 1:10 dilutions of vector pGHBI DNA from 800ng to 80fg.

1A to 1H: DNA dilutions alone

3A to 3H: DNA dilutions to each of which were added 10μg of E.coli DNA

5A to 5H: DNA dilutions to each of which were added 10μg of human DNA

Serial 1:10 dilutions of HCMV AD169 (stock V8) from 4.8×10^8 to 4.8×10^3 particles/7.2 ml of urine. The DNA was then extracted and loaded as follows:

7A to 7F: DNA dilutions alone

8A to 8F: DNA dilutions to each of which were added 10μg of E.coli DNA

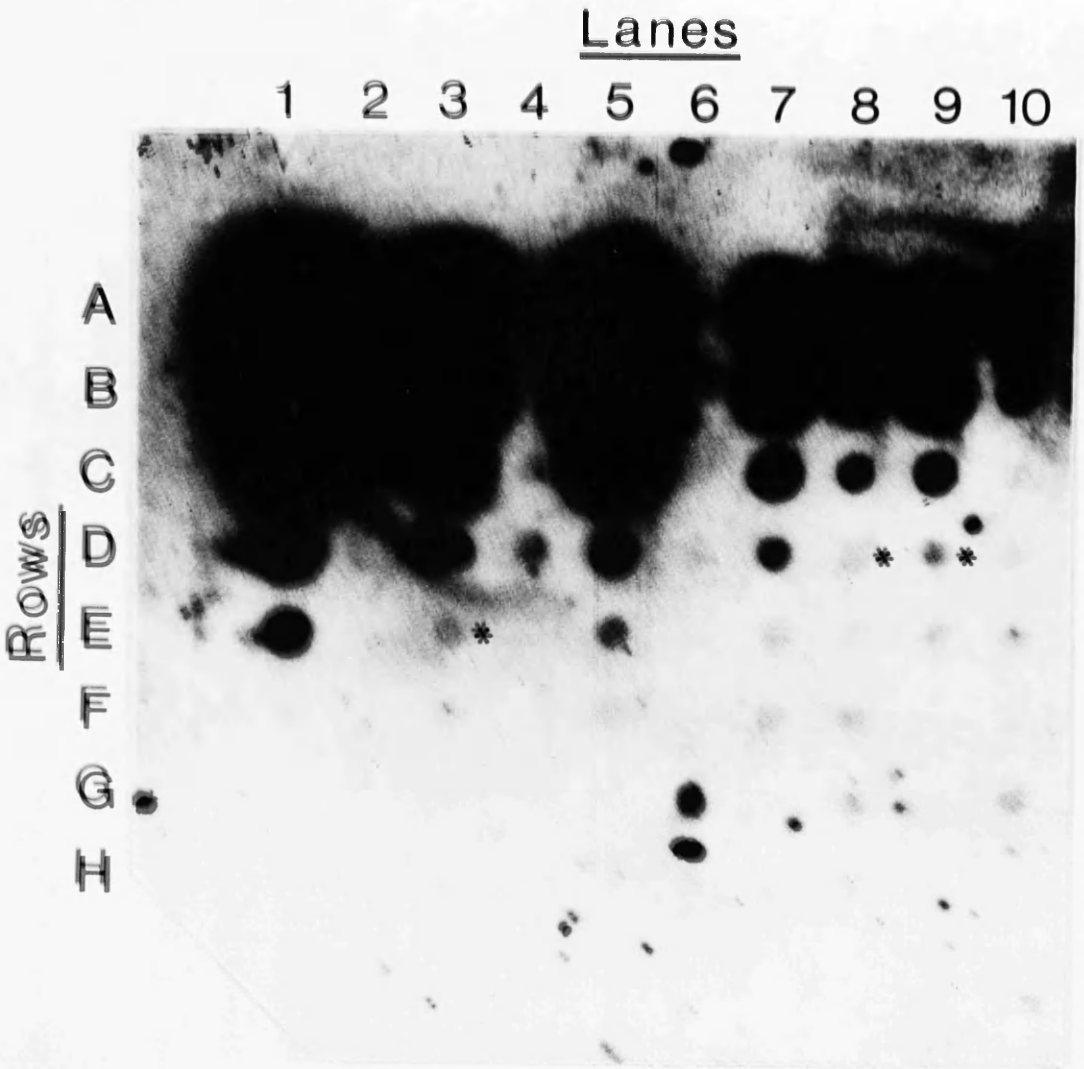
9A to 9F: DNA dilutions to each of which were added 10μg of human DNA

10A to 10F: Serial 1:10 dilutions of HCMV AD169 (stock V8) from 4.8×10^8 to 4.8×10^3 particles/7.2 ml of urine and incubated overnight at 37°C prior to DNA extraction.

The following controls were included:-

7.2ml of normal urine: 4A, 4B
7.2ml of normal urine incubated overnight at 37°C: 10G
10μg of HSV-1 17 syn^+ DNA: 4C
10μg of HSV-2 HG52 DNA: 4D
10μg of human DNA: 6A, 6B
10μg of E.coli DNA: 6C, 6D
10μg of ribosomal RNA: 6E, 6F
100μl of a stock of uninfected Helu cells: 6G, 6H
100μl of a stock of HSV-1 17 syn^+:7G,7H
100μl of a stock of HSV-2 HG52: 8G, 8H
1μg of HSV-1 17 syn^+ DNA: 9G
1μg of HSV-2 HG52 DNA: 9H

Autoradiography was for 3 days.
Asterisks show the positions of hybrid signals noted in the original autoradiograph but which are not obvious in this photograph.


Lanes
1 2 3 4 5 6 7 8 9 10
Rows
A
B
C
D
E
F
G
H

4.4.11 Addition of human and E.coli DNA to HCMV DNA in reconstruction experiments

Human or E.coli DNA extracted from urine specimens may bind to the nitrocellulose filter and decrease the area available for the binding of virus-specific DNA. This in turn would reduce the sensitivity of the hybridot assay. To investigate this possibility reconstruction experiments were performed and the results of a typical hybridot assay are shown in Figure 37A. Although the lower limit of detection of HCMV DNA was not diminished by the addition of human or E.coli DNA, the hybrid signals observed at equivalent dilutions of the target DNA increased in their absence. The addition of human DNA was more effective in reducing the hybrid signal than E.coli DNA.

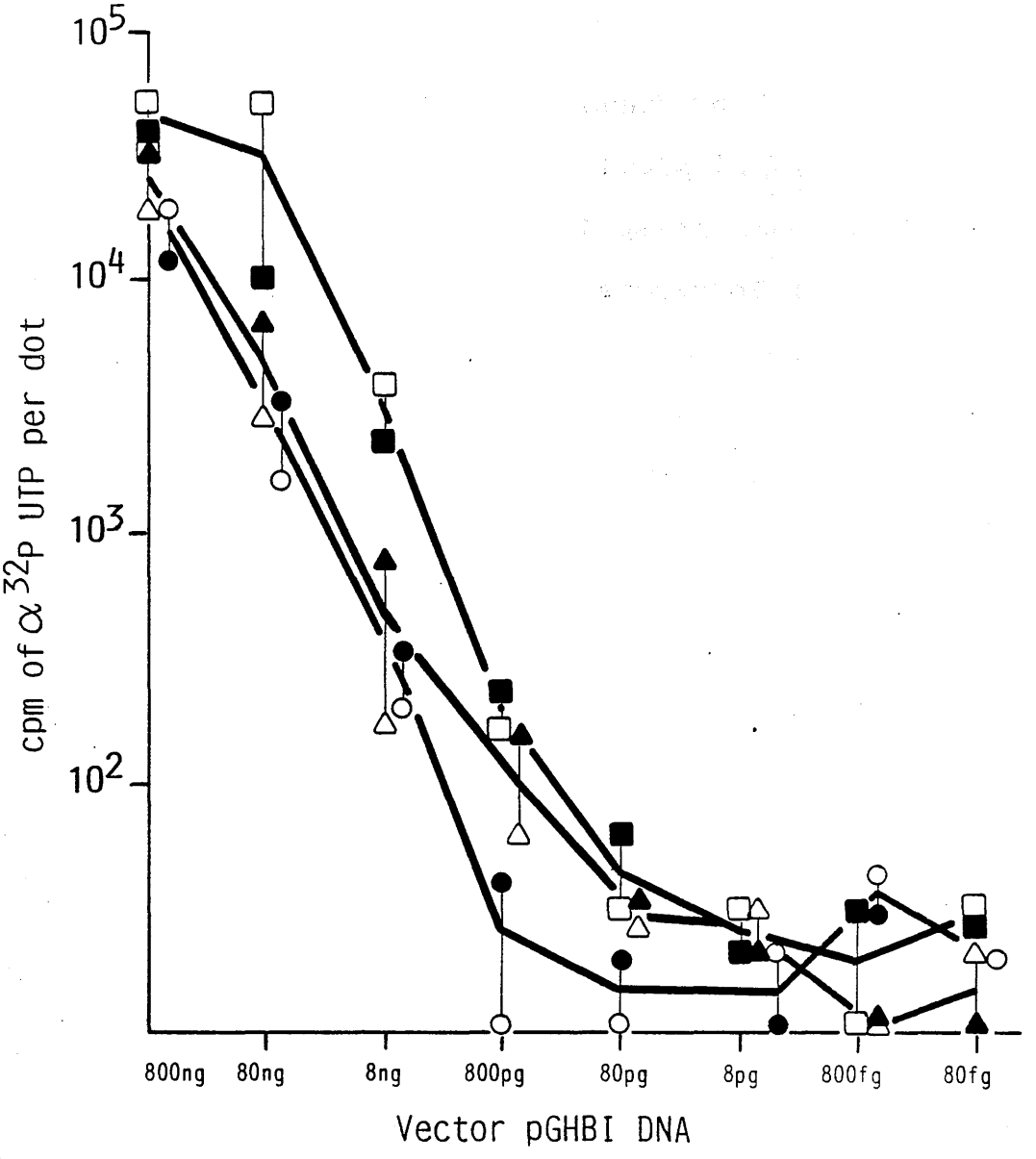
Dots were also counted for ^{32}P and the results of this and a duplicate experiment are shown in Figure 37B. At equivalent dilutions of HCMV DNA ranging from 800ng to 80 pg, the hybrid signals detected were higher in the absence of human and E.coli DNA while the addition of human DNA reduced the hybrid signals below those observed after the addition of E.coli DNA. Between 8pg and 80fg of vector pGHBI DNA the hybrid signals varied independently of the experimental conditions.

4.4.12 Detection of HCMV isolates

An investigation was made into the ability of the radiolabelled cRNA probe to detect HCMV isolates other than strain AD169. Therefore, 100μl were removed from stocks of

Figure 37B RNA-DNA hybridization: Addition of human and E.coli DNA to HCMV DNA

After autoradiography, dots were cut from the nitrocellulose filter described in Figure 37A (Lanes 1A to 1H; 3A to 3H; 5A to 5H) and counted for ^{32}P in a liquid scintillation counter. The graph opposite shows the cpm of ^{32}P detected at each dilution of vector pGHBI DNA after the addition of 10μg of E.coli DNA (△), 10μg of human DNA (o) or no other DNA (□). The results of duplicate experiments are also shown (▲ , ● and ■ respectively). Lines are drawn through the average of 2 counts at each DNA dilution.


10^5
10^4
10^3
10^2
cpm of α^{32}P UTP per dot
800ng
80ng
8ng
800pg
80pg
8pg
800fg
80fg
Vector pGHBI DNA

65 HCMV isolates cultured from urine samples or throat swabs provided by 18 renal allograft recipients (9 with primary HCMV infection and 9 with reinfection and/or reactivation). Each aliquot was added to 7.9ml of urine and the DNA extracted. Positive controls included 100μl of stocks of HCMV strains AD169 and Davis while negative controls were equivalent volumes of stocks of HSV-1 17 syn^+ and HSV-2 HG52 together with 10μg each of human, HSV-1 17 syn^+ and HSV-2 HG52 DNA.

All HCMV isolates were positive in the hybridot assay while the negative controls remained undetectable.

CYTOMEGALOVIRUS INFECTION

IN

RENAL TRANSPLANT RECIPIENTS

Two Volumes (Vol. 2)

by

JOHN DOLAN

A Thesis Presented for the Degree

of Doctor of Philosophy

in

The Faculty of Medicine

at the University of Glasgow

Institute of Virology,
Church Street,
Glasgow
G11 5JR,
Scotland.

July 1989

Volume 2

Contents

4.5 Isolation of HCMV

4.5.1 Specimens submitted for tissue culture

From March 1985 to December 1985, 490 urine samples and 449 throat swabs were collected from 47 renal transplant recipients. Two hundred and nine (42.6%) of the urines and 193 (43%) of the throat swabs were obtained from 22 patients who were seronegative for HCMV immediately prior to the transplant operation. Twenty five renal allograft recipients who were HCMV-seropositive prior to transplantation provided 281 (57.4%) and 256 (57%) of the urines and throat swabs respectively.

Twenty two (4.5%) of the 490 urine samples originally submitted for tissue culture had to be reinoculated on to a newly prepared monolayer of Flow 2002 cells. At the first attempt at virus isolation 4 cultures became contaminated by bacteria and 2 by fungi. A further 16 urine samples from 14 patients caused an unexplained cpe within 3 to 23 days of incubation at 37^{o}C. Only 1 (0.2%) of all throat swabs had to be reinoculated and this was on account of an unexplained cpe which was recorded after 19 days of incubation at 37^{o}C. No attempt was made to identify which virus, if any, with the exception of HCMV and HSV, was responsible for destruction of the cell monolayer on these occasions. When aliquots of these 22 specimens which had been stored at -70^{o}C were reinoculated on to fresh monolayers no cpe was evident.

HSV was isolated from 6 urines and 13 throat swabs (Section 4.9). It was considered unlikely that HCMV could

★ Plaques obtained in tissue culture from a throat swab of patient (c) at 14 weeks post transplantation were lost as a result of bacterial contamination and could only be identified as HCMV on the basis of tissue culture morphology.

also be isolated from these 19 specimens because HSV has a quicker replication cycle which results in destruction of the cell monolayer within a few days of incubation at 37°C.

4.5.2 HCMV isolates

Ninety three viral isolates (83 from urine and 10 from throat swabs) were initially identified as HCMV on the basis of plaque morphology. Of these, 69 (64 from urine and 5 from throat swabs) grew well enough to allow subculturing and positive identification. A further 2 isolates (1 from urine and 1 from a throat swab) were lost as a result of bacterial contamination of the cell monolayer. Attempts to reisolate these 2 viruses from the original specimens which had been stored at -70°C proved to be unsuccessful.

The remaining 22 plaques (18 from urine and 4 from throat swabs) progressed slowly and could only be identified as HCMV by morphology in the original cell monolayer (Figures 39A and B). Ten of these cpes were observed after culture of the urine taken from 7 patients (b,c,d,e,g,h,i) with primary HCMV infection while a further 8 were obtained from the urine of 6 individuals (l,n,o,t,u,w) with reinfection and/or reactivation. The remaining 4 plaques not positively identified as HCMV were cultured from throat swabs provided by 3 patients (d,f,i) each with primary HCMV infection. ★

Of the 9 individuals (a,b,c,d,e,f,g,h,i) with primary

HCMV infection from whom the virus was isolated only 1 (d) consistently shed HCMV which was identified solely on the basis of plaque morphology. In contrast, of the 40 plaques cultured from the urine of patients (a), (b), (c), (e), (f), (g), (h) and (i) only 7 (17.5%) were not positively identified as HCMV, 1 each from patients (b), (e), (g), (h), (i) and 2 from patient (c). All 6 isolates cultured from the urine of patient (f) and both viruses isolated from the throat of patient (c) could be subcultured and identified as HCMV. Six plaques were obtained in tissue culture from throat swabs of patients (a), (c), (f), (i) but 3, one each from patients (c), (f) and (i), failed to progress sufficiently to allow positive identification.

Of the 39 plaques obtained after culturing the urine of 12 patients (l,m,n,o,p,q,r,s,t,u,v,w) with reinfection and/or reactivation, 8 could not be successfully subcultured. These 8 cpes originated from the urine of 6 individuals, one from each of patients (l), (n), (t), (u), and (z) and 2 from each of patients (o) and (w). One of the 3 plaques cultured from the urine of patient (n) and 2 of the 3 cpes originating from the urine of patients (o) and (w) could not be successfully subcultured. Four plaques were isolated from the urine of patient (u) but 1 did not grow well enough to permit positive identification. Throughout the period of study, only 1 urine sample provided by each of patients (l) and (t) yielded a cpe in culture and both were classified as HCMV by plaque morphology. Four patients (m,p,r,v) consistently shed HCMV

which could be subcultured and positively identified.

Plaques were first noted after an average of 26 days in culture [range 7 to 54] (Figure 38A). Of those 22 plaques not positively identified as HCMV, 7 were first noted within 12 to 26 days of incubation while the remaining 15 were initially observed after 29 to 53 days in culture. Four of the 6 isolates cultured from throat swabs were first detected in tissue culture after an incubation period of 31 to 41 days while the remaining 2 viruses showed a cpe after 14 and 25 days of culture respectively. Multiple HCMV isolates obtained from any one individual also differed in the time taken to produce plaques in vitro. For example, of the first 5 isolates cultured from the urine of patient (f), a cpe was initially observed after 26, 7,8, 30 and 9 days of incubation respectively.

No correlation was observed between the isolation of virus which could not be positively identified and the time after transplantation when the corresponding throat swab or urine sample was taken (Figure 38B). For example, of the 8 plaques originating from the urine of patient (h), the only one which failed to subculture was isolated from a sample taken at 33 weeks post transplantation. In contrast, 2 cpes not positively identified as HCMV originated from 2 independent urine samples provided by patient (c) at 4 and 5 weeks respectively after renal transplantation. Five subsequent urines taken from this patient at regular intervals up to 28 weeks after the transplant operation

Figure 38A Timing of HCMV plaque formation

The figure shows the number of days taken for a cpe to be observed in each of 87 cultures at 37°C. Viruses which were subcultured and positively identified as HCMV are denoted by □ or ▨ when isolated from a urine sample or throat swab respectively. Plaques which were classified as HCMV on the basis of morphology in the original cell monolayer are indicated by ■ . Specimens are grouped according to numerical accession order.

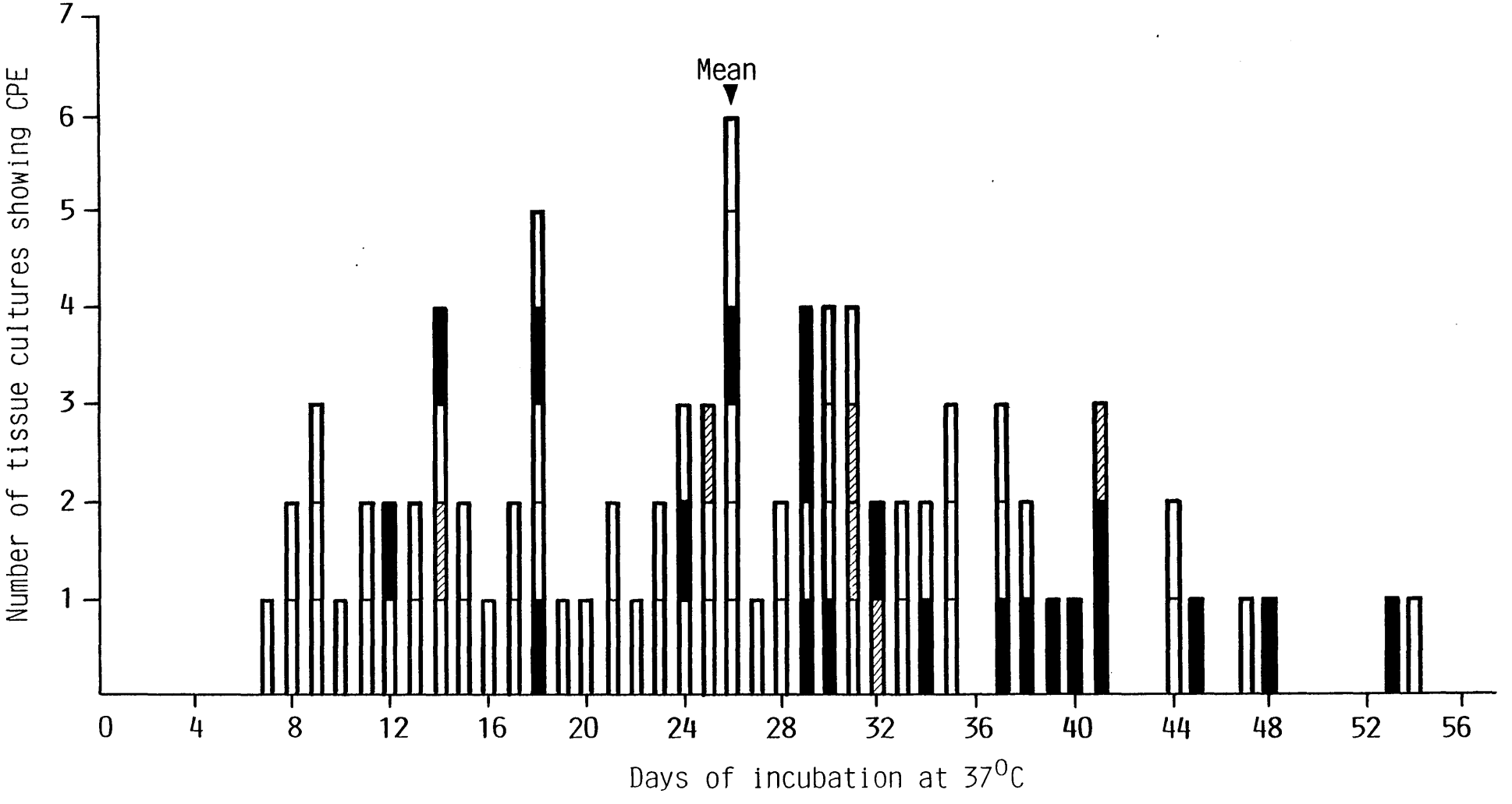

Mean
Number of tissue cultures showing CPE
Days of incubation at 37°C
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
1
2
3
4
5
6
7

Figure 38B Timing of HCMV plaque formation

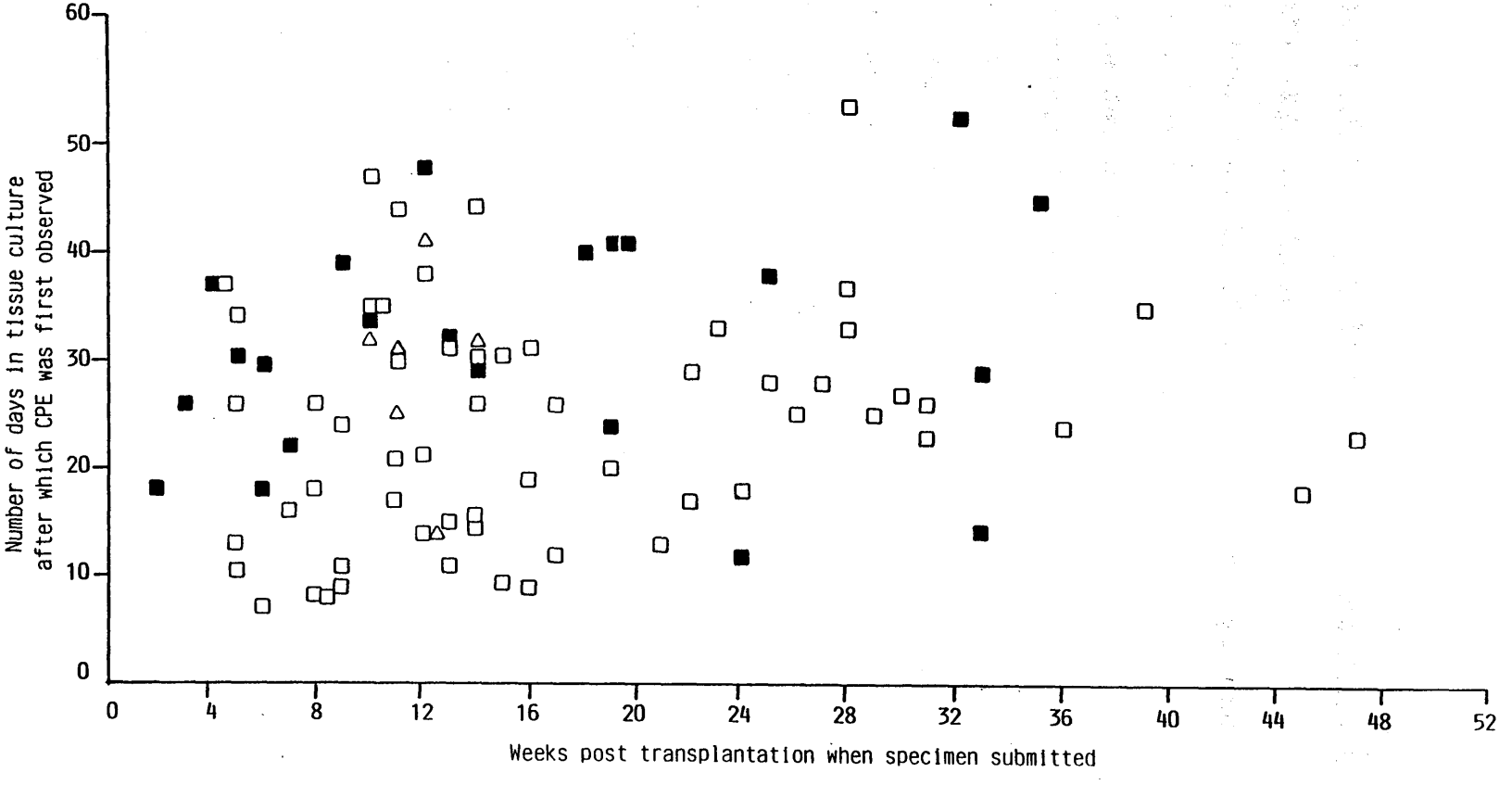
The figure shows the correlation between the number of days in culture at $37^{o}C$ after which cpe was first observed and the weeks post transplantation when the original specimen was submitted.

The following symbols are used:-

□ : A urine yielding a virus positively identified as HCMV

△ : A throat swab yielding a virus positively identified as HCMV

■ : A urine or throat swab which yielded a cpe classified as HCMV only on the basis of plaque morphology in the original cell monolayer.


Number of days in tissue culture after which CPE was first observed
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation when specimen submitted

yielded virus which was subcultured and positively identified as HCMV.

4.5.3 Timing of HCMV shedding post renal transplantation

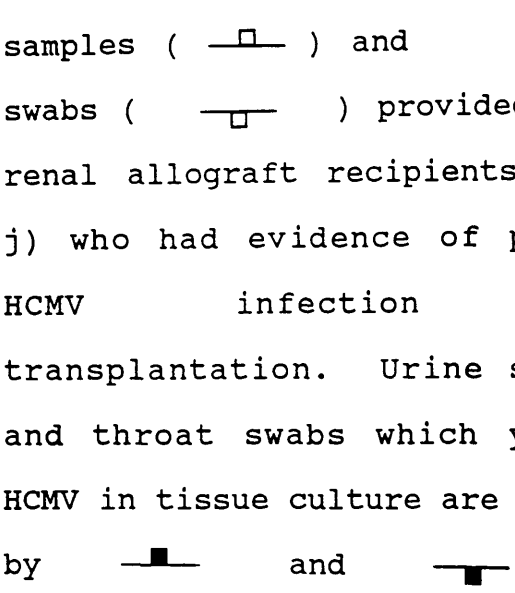
(a) Primary infection

Forty four (40.7%) of 108 urine samples and 8 (7.8%) of 102 throat swabs provided by 11 patients with primary infection yielded HCMV in tissue culture (Figure 39A).

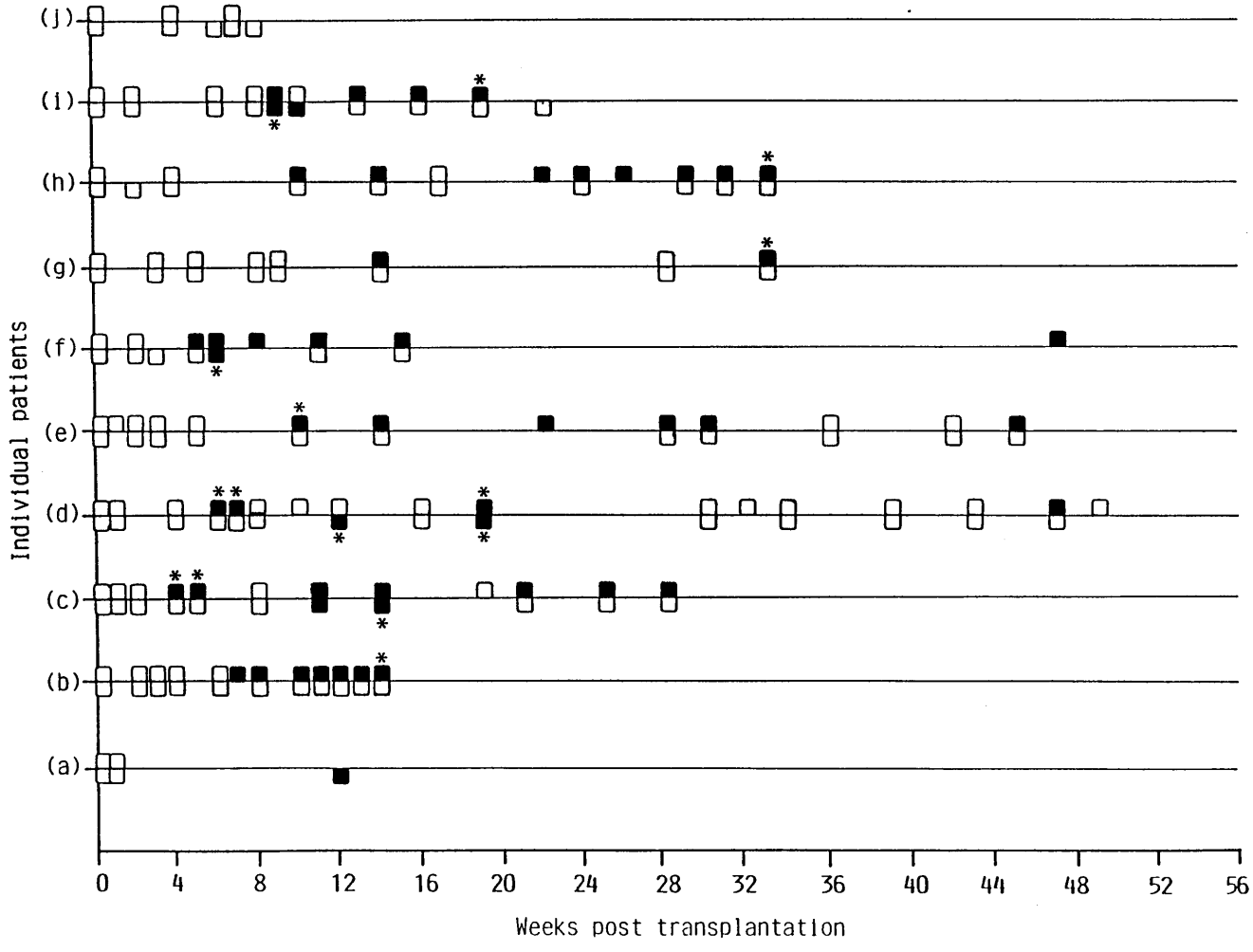
The earliest detection of HCMV shedding was in a urine taken from patient (c) at 4 weeks post transplantation. By 14 weeks after the transplant operation HCMV had been isolated from the urine of 8 renal allograft recipients (b,c,d,e,f,g,h,i). Patients (c), (d), (e), (g), (h), (i) shed HCMV intermittently while virus was consistently isolated from all urine samples provided by patients (b) and (f) on or after the seventh and fifth week respectively post transplantation. Only 2 urine samples were ever obtained from patient (a) who developed acute tubular necrosis and anuria immediately post transplantation. Similarly acute rejection of the renal allograft prevented patient (j) from providing urine samples beyond the seventh week after the transplant operation.

Throat swabs obtained from 5 patients (a,c,d,f,i) between 6 and 12 weeks post transplantation yielded HCMV in tissue culture. HCMV could not be isolated from any of the 20 throat swabs taken beyond the nineteenth week after the transplant operation though 16 of the 26 urine samples obtained during the same period yielded the virus in tissue

Figure 39A HCMV shedding post renal transplantation: Primary infection



The figure shows urine samples (—□—) and throat swabs (—□—) provided by 10 renal allograft recipients (a to j) who had evidence of primary HCMV infection post transplantation. Urine samples and throat swabs which yielded HCMV in tissue culture are denoted by —■— and —■— respectively. Asterisks indicate those cpes which were identified as HCMV only on the basis of plaque morphology in the original cell monolayer. One patient (k) who had serological evidence of primary HCMV infection at 59 weeks post transplantation is omitted from the diagram.


(j)
(i)
(h)
(g)
(f)
(e)
(d)
(c)
(b)
(a)
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
Weeks post transplantation

culture. The onset of HCMV isolation from the throat was coincident with (i) or followed (c,d,f) but in no case preceded excretion of the virus in the urine.

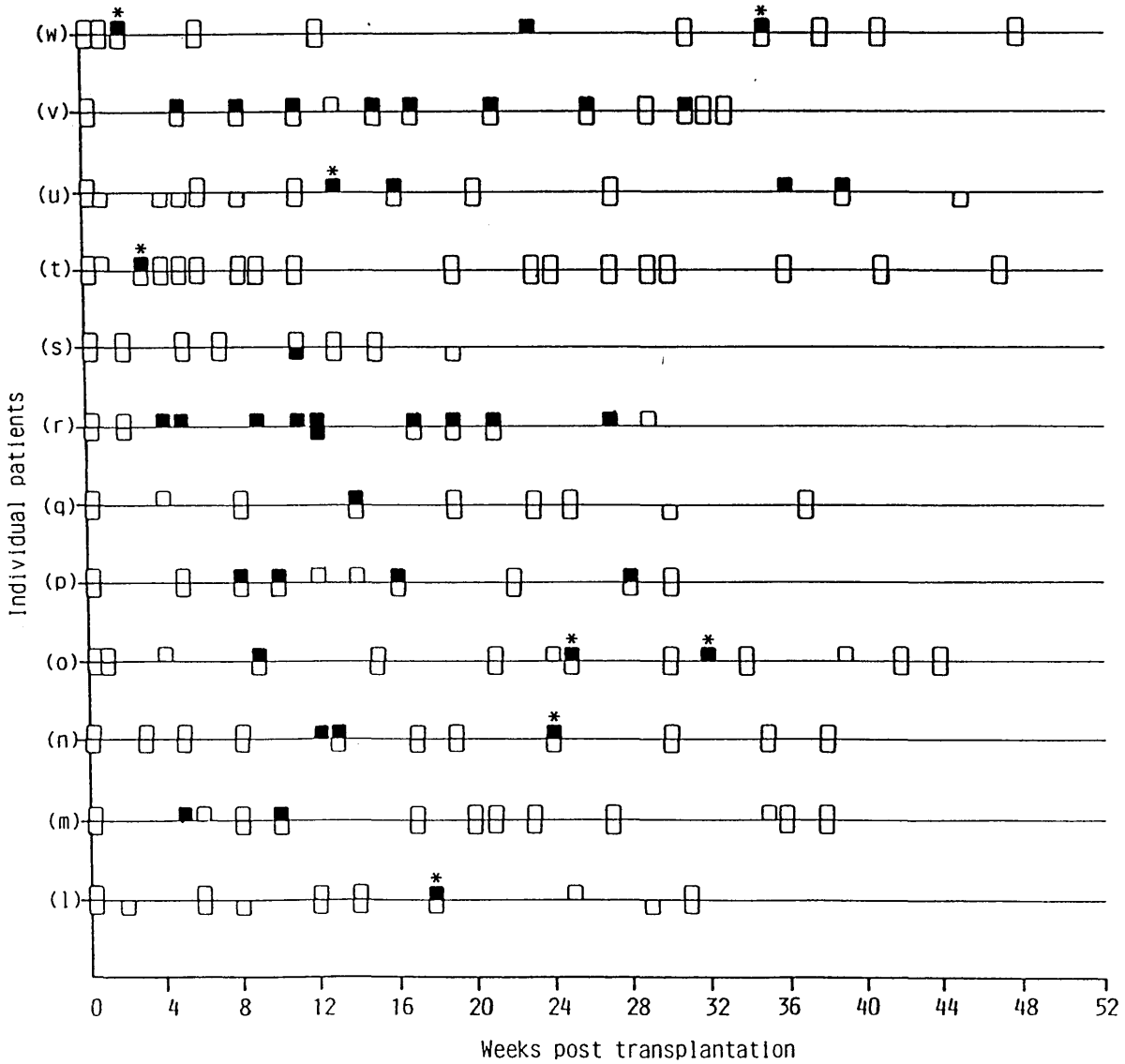
(b) Reinfection and/or reactivation

HCMV was isolated from 12 of the 20 patients in this group (Figure 39B). Virus was first detected in a urine provided by patient (w) 2 weeks after the transplant operation. By 14 weeks, HCMV had been isolated from the urine of 9 other individuals (m,n,o,p,q,r,t,u,v) while HCMV shedding by patient (l) was first noted in a urine sample taken at 18 weeks post transplantation. Ten patients (l,m,n,o,p,q,t,u,v,w) shed HCMV intermittently while there was consistent isolation of virus from urine specimens provided by patient (r) between 4 and 27 weeks after the transplant operation. No urinary isolates were obtained from patient (s) but HCMV was detected in a throat swab taken at 11 weeks post transplantation. HCMV was only isolated from the throat of patient (s) on a single occasion 11 weeks after the transplant operation. HCMV was never cultured from any specimen provided by 9 other individuals who had serological evidence of reinfection and/or reactivation.

HCMV-seropositive patients who receive their kidney from a seronegative donor usually shed endogenous HCMV which has reactivated from a latent state post-operatively. However, HCMV-seropositive renal allograft recipients of kidneys from HCMV-seropositive donors are also at risk of

Figure 39B <u>HCMV shedding post transplantation: Reinfection and/or reactivation</u>

The figure shows urine samples (—□—) and throat swabs (—□—) provided by 12 renal allograft recipients (1 to w) with reinfection and/or reactivation of HCMV and from whom virus was isolated. Urine samples and throat swabs which yielded HCMV in tissue culture are denoted by —■— and —■— respectively. Asterisks indicate those cpes identified as HCMV only on the basis of plaque morphology in the original cell monolayer.


(w)
(v)
(u)
(t)
(s)
(r)
(q)
(p)
(o)
(n)
(m)
(l)
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation

reinfection with another strain of HCMV from within the transplanted kidney (Chou, 1986). In this project the frequency and pattern of virus shedding by each HCMV-seropositive allograft recipient was independent of the HCMV serostatus of the renal donor. Five (l,m,s,t,u) of the 12 patients from whom HCMV was isolated each received their kidney from donors known to be seropositive for HCMV while 4 (n,p,r,v) were each transplanted with a kidney from an HCMV- seronegative donor. Similarly, 4 renal donors who were seropositive for HCMV and 3 who were seronegative provided kidneys for 7 renal allograft recipients with serological evidence of post transplantation HCMV infection but from whom virus was never isolated. No sera were available from those individuals who donated kidneys for 4 other renal transplant recipients including patients (o),(q) and (w).

4.5.4 Urinary β_2 microglobulin levels

Between 10 and 17 weeks post transplantation HCMV was isolated from 16 of the 21 urine samples provided by 8 patients (b,c,d,e,f,g,h,i) with primary HCMV infection (Figure 39A). During the same period 46 urine samples were obtained from 20 transplant recipients with evidence of reinfection and/or reactivation but HCMV was isolated from only 14 (32.5%) urines provided by 7 patients (m,n,p,q,r,u,v) (Figure 39B). An investigation was made into the possibility that low levels of urinary β_2m may have accounted for the inability to isolate HCMV from the

urine of some patients who showed a fourfold or greater rise in HCMV-specific CF antibody after renal transplantation. It was not possible to measure β_2m levels in all urine samples from all renal allograft recipients. Therefore, β_2m was estimated in only 1 urine sample obtained from each of 40 patients (Figure 40A). The β_2m level in each urine was estimated by reference to a standard curve drawn from the results obtained when using known standard controls (Figure 40B). Each urine sample was tested only once but the results of known amounts of β_2m analysed in duplicate confirm that there was good intra-assay reproducibility.

In each group of patients with primary, secondary or no HCMV infection, β_2m levels ranged from $7 \times 10^1 \mu g$/litre to greater than or equal to $4 \times 10^3 \mu g$/litre. Only 3 urines in each of the 3 groups of patients had a β_2m level which was below the limit of normal ($1.6 \times 10^2 \mu g$/litre). Of the 8 patients with primary HCMV infection, virus could be isolated from 7 urine samples in which β_2m was measured and found to range from $7 \times 10^1 \mu g$/litre to greater than or equal to $4 \times 10^3 \mu g$/litre. HCMV was not isolated from the urine of patient (d) at 12 weeks post transplantation when the urinary β_2m level was $8 \times 10^1 \mu g$/litre. Of those patients with reinfection and/or reactivation of HCMV, virus could not be isolated from 13 of the 16 urine samples in which β_2m was measured though only 2, one each from patients (m) and (ss) at 17 and 10 weeks respectively post

Figure 40A Urinary β_2 microglobulin levels post renal transplantation

The figure shows the β_2m level in each of 40 urine samples obtained between 10 and 17 weeks post transplantation. One urine was provided by each of 8 renal allograft recipients (b,c,d,e,f,g,h,i) with primary HCMV infection, 16 (l,m,n,o,p,r,s,t,w,x,y,z,qq,ss,tt,uu) with reinfection and/or reactivation and 16 (aa,bb,cc,dd,ee,ff,gg,hh,ii,jj,kk,ll, mm,nn,oo,pp) with no evidence of active HCMV infection.

The following symbols are used:-

● - virus isolated from this specimen

▲ - virus not isolated from this specimen but other urine samples obtained from the same patient did yield HCMV in tissue culture

O - virus not isolated from this specimen or any other urine samples provided by the same patient.

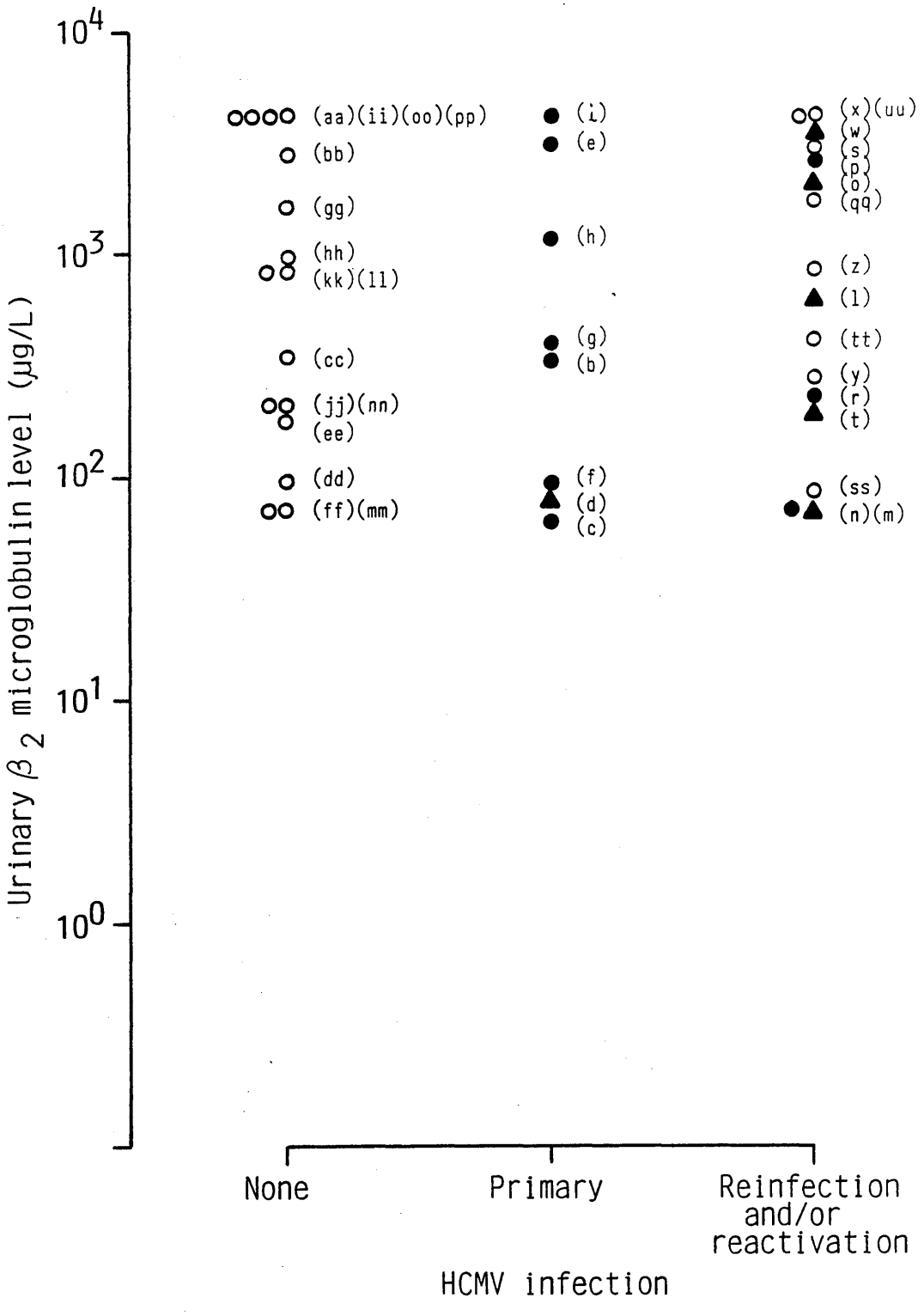
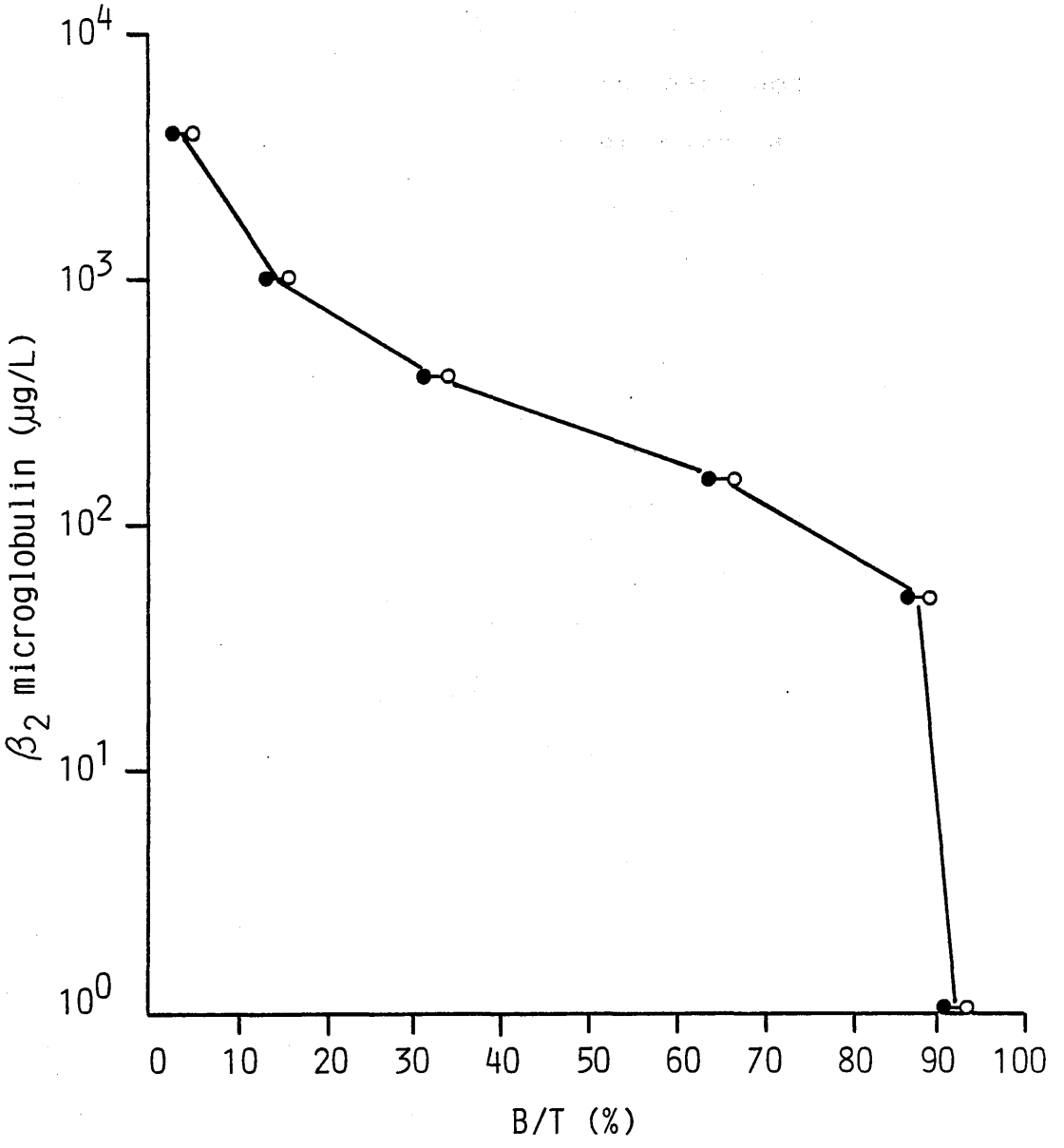

Urinary β_2 microglobulin level (μg/L)
10^4
10^3
10^2
10^1
10^0
(aa)(ii)(oo)(pp)
(bb)
(gg)
(hh)
(kk)(ll)
(cc)
(jj)(nn)
(ee)
(dd)
(ff)(mm)
(i)
(e)
(h)
(g)
(b)
(f)
(d)
(c)
(x)(uu)
(w)
(s)
(p)
(o)
(qq)
(z)
(l)
(tt)
(y)
(r)
(t)
(ss)
(n)(m)
None
Primary
Reinfection and/or reactivation
HCMV infection

Figure 40B β_2 microglobulin calibration curve

Six known concentrations of β_2m ranging from 0 to 4 x 10^3 μg/litre of urine were tested by RIA. The standard curve was obtained by plotting the β_2m concentration of each standard against B/T%, the percentage of the total (T) ^{125}I-labelled β_2m bound (B) to beads coated with anti-β_2m antibody. The results of duplicate experiments (O and ●) performed in parallel are shown and lines are drawn through the average 2 values at each concentration of β_2m. The β_2m concentration in each clinical urine specimen was determined from the curve.


10^4
10^3
10^2
10^1
10^0
β_2 microglobulin (µg/L)
0
10
20
30
40
50
60
70
80
90
100
B/T (%)

transplantation, had a β_2m level below the limit of normal. However, HCMV was isolated from the urine of patient (n) at 13 weeks post transplantation when the level of β_2m was 7 x $10^1\mu$g/litre.

In conclusion therefore, it was not possible to explain on the basis of low urinary β_2m levels the inability to isolate HCMV from the urine of all patients with serological evidence of recent infection.

4.5.5 Correlation between virus isolation and the results of dot blot hybridization

The results of a typical RNA-DNA hybridot assay are shown in Figure 41. Of the 490 urine samples submitted for tissue culture, 445 (91%) were analysed by dot blot hybridization. Twenty (25%) of the 80 urine samples which yielded HCMV in tissue culture were positive by dot blot hybridization, 16 (20%) equivocal and 44 (55%) clearly negative. HCMV could not be isolated from 365 urine samples of which 244 (67%) were negative by dot blot hybridization, 52 (14%) equivocal and 69 (19%) clearly positive. Indeed, of the 94 urine samples obtained from 10 patients who remained seronegative for HCMV throughout the period of study, 66 (70%) were negative by dot blot hybridization but 11 (12%) were equivocal and 17 (18%) clearly positive. HCMV could not be isolated from 79 samples provided by 8 patients who had serological evidence of reinfection and/or reactivation of HCMV. Of these, 52 (66%) were negative, 9 (11%) equivocal and 18 (23%)

Figure 41 A typical RNA-DNA hybridot assay

1B to 1F: Serial 1:10 dilutions of HCMV AD169 (stock V8) from 4.8×10^8 to $4{\cdot}8 \times 10^4$ particles/7.2ml of urine.

12A to 12E: Serial 1:10 dilutions of vector pGHBI DNA from 800pg to 80fg

Additional controls include

7.2ml of normal urine : 2A, 6D, 11G

10μg of human DNA : 2B, 5C, 12F

10μg of E.coli DNA : 2C, 5D, 12G

10μg of ribosomal RNA : 2D, 5E, 12H

10μg of HSV-2 DNA : 2E, 6C, 11A

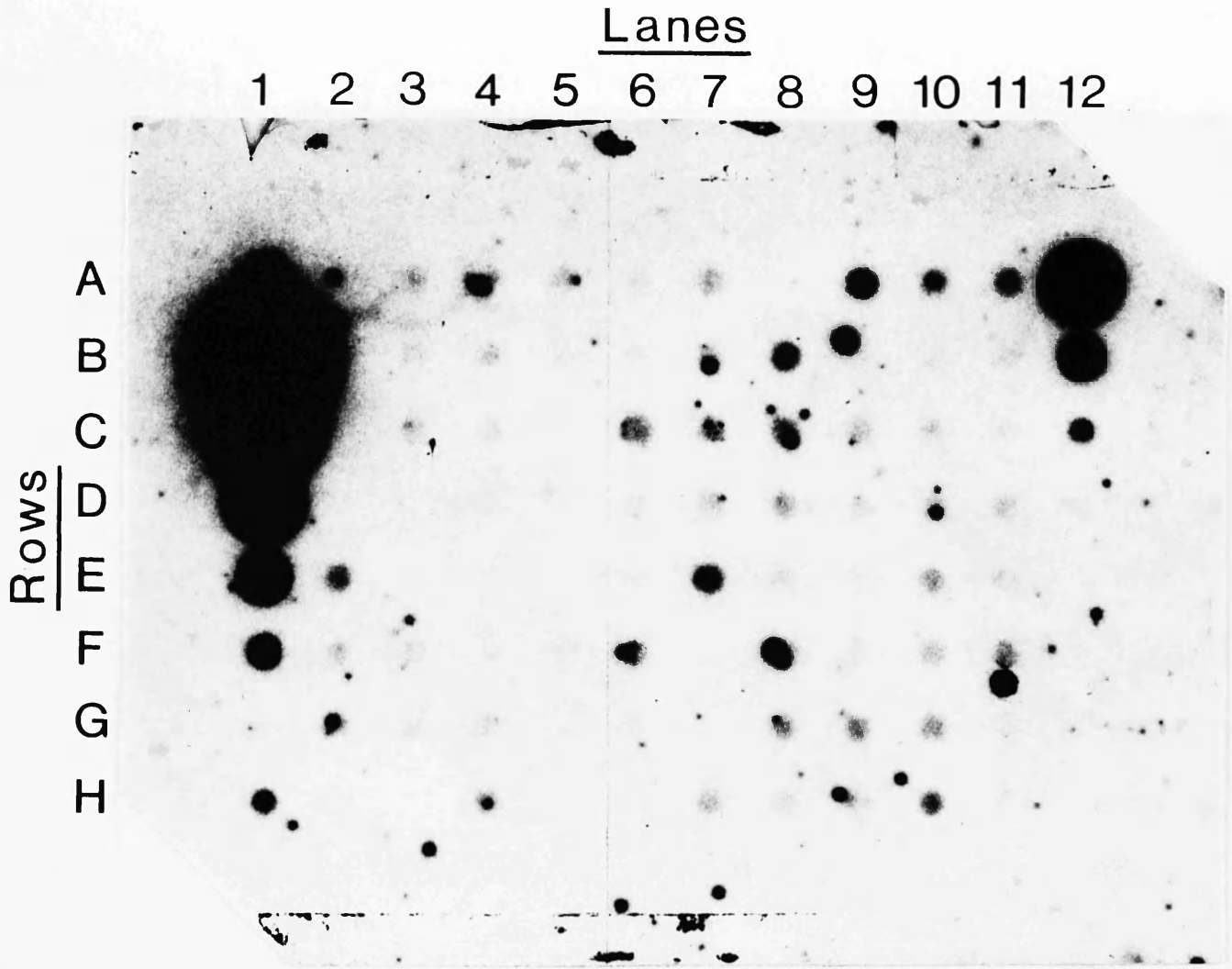
80pg of vector pGHBI DNA: 1A

8pg of vector pGHBI DNA: 1H

800fg of vector pGHBI DNA: 6E

All other positions are occupied by clinical specimens.

Autoradiography was for 10 days.


Lanes
1 2 3 4 5 6 7 8 9 10 11 12
Rows
A
B
C
D
E
F
G
H

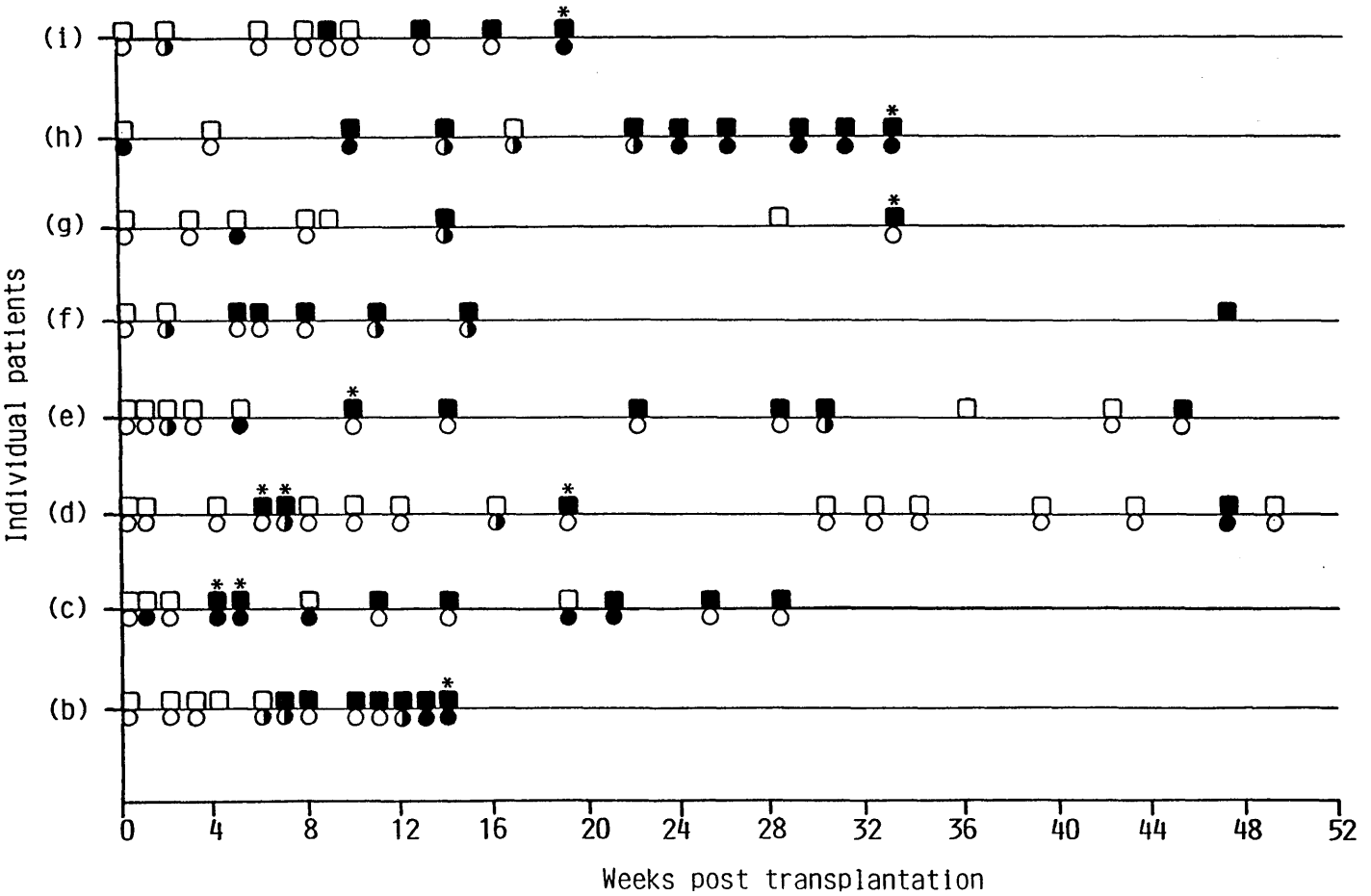
positive by dot blot hybridization.

HSV was isolated from 6 urine specimens provided by 3 renal transplant recipients. Five of these urines obtained from 2 patients were negative for HCMV by dot blot hybridization. Not enough urine was available from the third patient to permit analysis by hybridot assay.

Eighty five urine samples provided by 8 renal allograft recipients (b,c,d,e,f,g,h,i) with primary HCMV infection were tested for HCMV-specific sequences by RNA-DNA hybridization (Figure 42A). HCMV was isolated from 43 of these specimens, 13 (30%) of which were positive for HCMV by hybridot assay while 9 (21%) were equivocal and 21 (49%) were negative. HCMV could not be isolated from 42 other samples of which 6 (14%) were positive, 6 (14%) equivocal and 30 (72%) negative by RNA-DNA hybridization. There was good correlation between the results of virus isolation and those of the hybridot assay in patient (h) but not in patients (b), (c), (d), (e), (f), (g) and (i) who collectively provided 26 urine samples in which HCMV was positively identified by isolation and subculture. Of these, 3 were positive, 6 equivocal and 17 negative by dot blot hybridization. A urine obtained from patient (c) at 1 week after the transplant operation was the first post transplantation urine specimen to be recorded as positive for HCMV by RNA-DNA hybridization although no virus was isolated. Four weeks later 2 other renal allograft recipients (e and g) each provided a urine sample from which HCMV could not be cultured but which were regarded as

Figure 42A Correlation between detection of HCMV in urine samples by tissue culture and RNA-DNA hybridization: Primary HCMV infection

The figure shows those urine samples provided by 8 renal allograft recipients (b,c,d,e,f,g,h,i) with primary HCMV infection. Specimens which yielded HCMV in tissue culture are differentiated from those which did not by the symbols —■— and —□— respectively. Asterisks indicate those cpes which were identified as HCMV only on the basis of plaque morphology in the original cell monolayer. Whenever possible each urine was tested for HCMV DNA by dot blot hybridization using the ^{32}P-labelled cRNA probe described in Sections 4.3 and 4.4. The results of this assay (—○— negative; —◑— equivocal; —●— positive) were correlated with those of HCMV isolation.

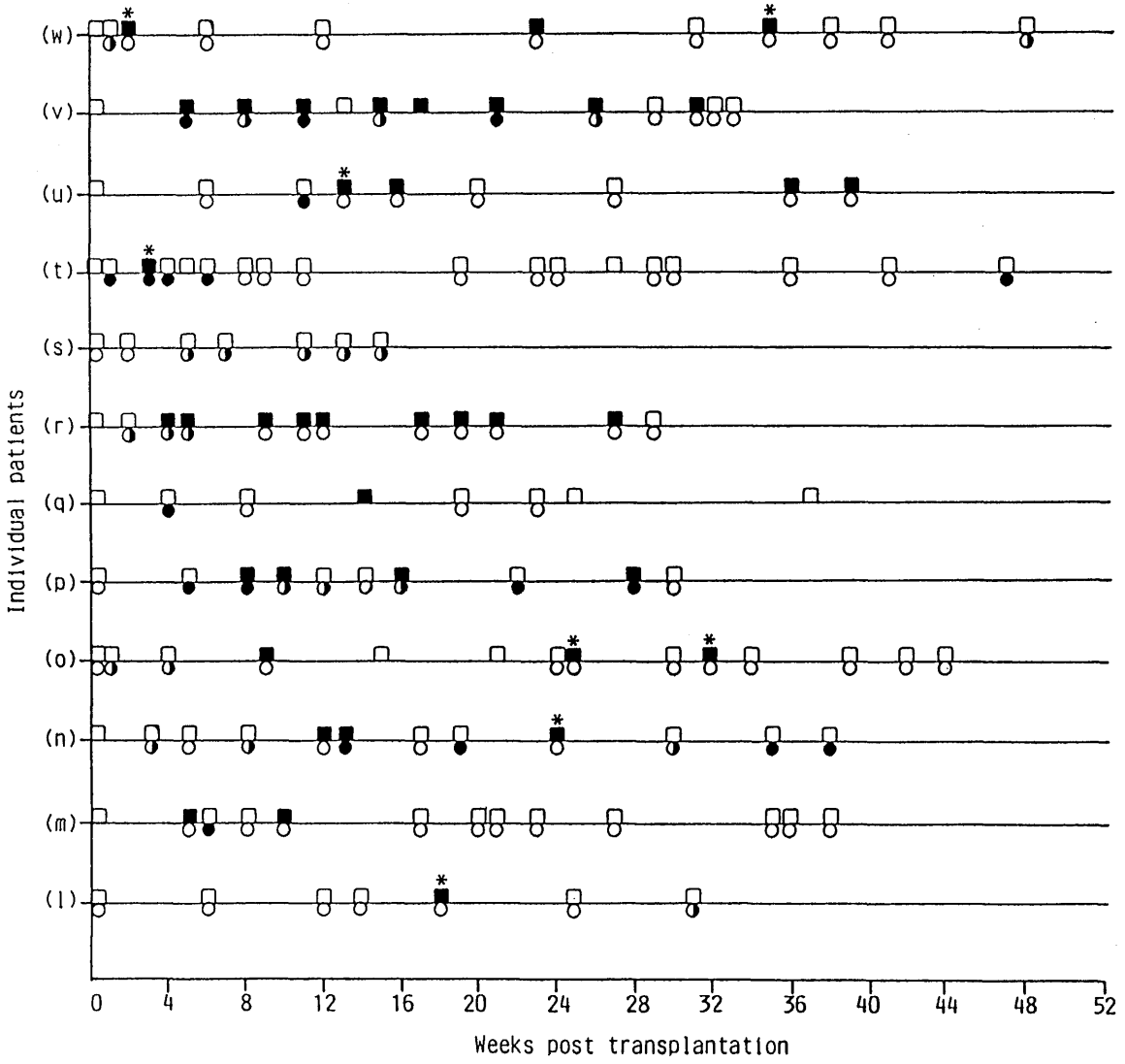

(i)
(h)
(g)
(f)
(e)
(d)
(c)
(b)
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation

positive for the virus by hybridot assay. In patients (b), (d) and (i), the first urine to be classified as positive for HCMV by dot blot hybridization was taken at 13, 47 and 19 weeks respectively post transplantation. No urine provided by patient (f) was ever positive for HCMV by RNA-DNA hybridization although 2 of the 5 specimens which yielded the virus in tissue culture were equivocal. A urine obtained from patient (h) immediately pretransplant appeared positive for HCMV in the hybridot assay while a specimen taken 4 weeks later was negative. In patients (b), (d) and (i), the onset of HCMV isolation preceded the first detection of the virus by RNA-DNA hybridization by 6, 41 and 10 weeks respectively, while in patients (c), (e) and (g) a positive result in the hybridot assay was recorded between 3 and 9 weeks prior to the onset of HCMV isolation.

Also tested for HCMV by hybridot assay were 117 urine specimens obtained from 12 renal allograft recipients (l, m,n,o,p,q,r,s,t,u,v,w) with reinfection and/or reactivation of HCMV who shed the virus post-operatively (Figure 42B). HCMV was isolated and positively identified in 29 of these urines of which 6 (21%) were positive, 7 (24%) equivocal and 16 (55%) negative by RNA-DNA hybridization. Eight other urinary isolates were identified as HCMV on the basis of plaque morphology in the original cell monolayer. When the respective urine samples were tested in the hybridot assay only 1, that provided by patient (t) at 3 weeks post

Figure 42B Correlation between detection of HCMV in urine samples by tissue culture and RNA-DNA hybridization: Reinfection and/or reactivation

The figure shows those urine samples provided by 12 renal allograft recipients (l,m,n,o,p,q,r,s,t,u,v,w) with reinfection and/or reactivation of HCMV who shed the virus post-operatively. Urinary isolates were obtained from all patients with the exception of (s) from whom HCMV was isolated in a throat swab taken at 11 weeks after the transplant operation. Urine specimens which yielded HCMV in tissue culture are differentiated from those which did not by the symbols —■— and —□— respectively. Asterisks indicate those cpes which were identified as HCMV only on the basis of plaque morphology in the original cell monolayer. Whenever possible, all specimens were tested for HCMV DNA by dot blot hybridization using the ^{32}P-labelled cRNA probe described in Sections 4.3 and 4.4. The results of this assay (—○— negative; —◑— equivocal; —●— positive) are correlated with those of HCMV isolation.


(w)
(v)
(u)
(t)
(s)
(r)
(q)
(p)
(o)
(n)
(m)
(l)
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation

transplantation, was positive. Of the 80 urine samples which did not yield HCMV in tissue culture, 12 (15%) were positive by dot blot hybridization while 15 (19%) were equivocal and 53 (66%) negative. RNA-DNA hybridization could not positively detect HCMV-specific sequences in any urine obtained from patients (l), (o), (r) and (w) throughout the post transplant period although 16 HCMV isolates were identified in tissue culture. Furthermore, in these 4 renal allograft recipients virus was isolated from only 2 of 8 other urine specimens which were equivocal for HCMV by dot blot hybridization. Patients (m), (q) and (u) provided 1 urine sample at 6, 4 and 11 weeks respectively post transplantation each of which was positive for HCMV in the hybridot assay but none yielded the virus in tissue culture. In patients (n), (p), (q), (t) and (v), the first post transplantation urine specimen to be classified as positive for HCMV by RNA-DNA hybridization was taken at 13, 5, 4, 1 and 5 weeks respectively after the transplant operation but virus was isolated from only 2 specimens, 1 from each of patients (n) and (v). The onset of HCMV isolation in patients (m) and (n) preceded a positive reaction in the hybridot assay by 1 week while patients (p), (q), (t) and (u) first showed evidence of a positive reaction by dot hybridization in urine samples taken between 2 and 10 weeks prior to first isolation of HCMV. In patient (v), the onset of HCMV isolation from the urine at 5 weeks post transplantation coincided with the first positive result in the hybridot assay. No urinary isolates

were obtained from patient (s) who shed HCMV in the throat at 11 weeks after the transplant operation and who remained equivocal for HCMV by RNA-DNA hybridization in 5 urine specimens provided between 5 and 15 weeks post-operatively.

4.6 HCMV-specific CF antibody titres

4.6.1 Specificity

Prior to the measurement of the HCMV CF antibody titre in all serum samples, an investigation was made into the possibility of cross reaction between HCMV CF antigen and HSV or VZV antigens. Therefore, a number of sera were simultaneously tested for CF antibody to HCMV, HSV and VZV, but no cross reaction was noted (Figure 43).

4.6.2 Timing of the fourfold or greater rise in HCMV CF antibody titre post renal transplantation

Ten (a,b,c,d,e,f,g,h,i,j) of the 11 renal allograft recipients with primary HCMV infection showed a fourfold or greater rise in the HCMV CF antibody titre between 3 and 14 weeks post transplantation while 1 patient (k) had a eightfold rise in the HCMV CF antibody titre between 40 and 59 weeks after the transplant operation (Figure 44).

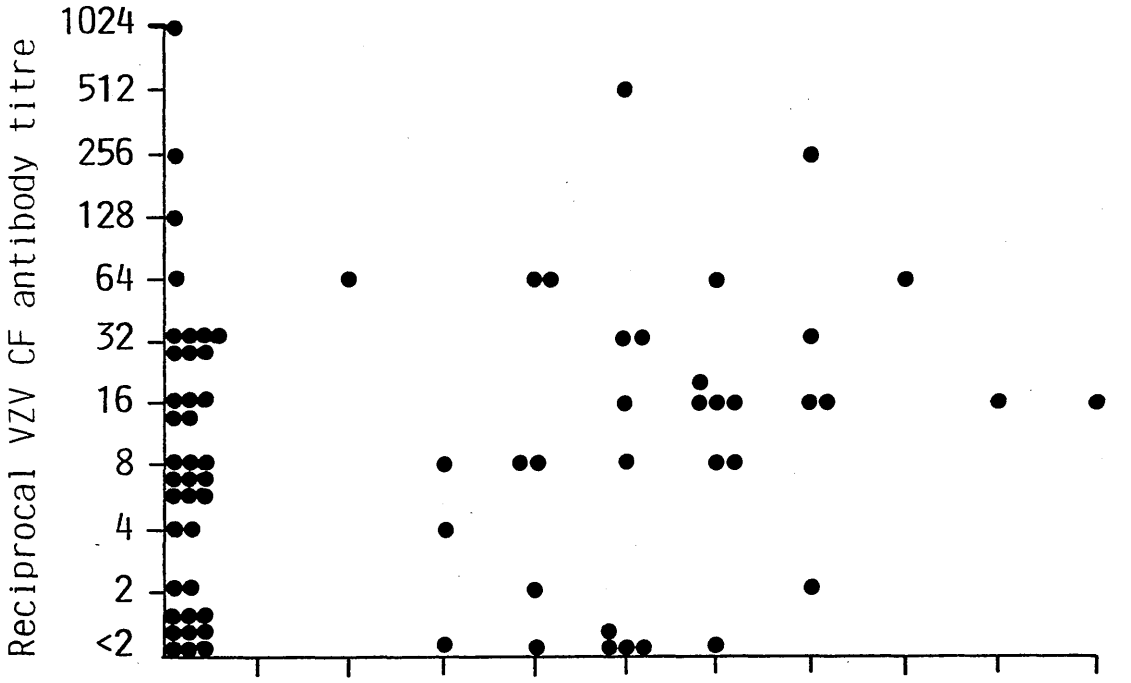
Of the 18 patients with reinfection and/or reactivation of HCMV who had a fourfold or greater rise in the HCMV CF antibody titre, virus was isolated from 10 (l,m,n,o,p,q,r,s,t,u). No significant difference was noted between shedders and non-shedders of HCMV in the timing of

Figure 43 Correlation between the HCMV, HSV and VZV CF antibody titres

Figure A shows the correlation between the HCMV and HSV CF antibody titres in 75 sera, 1 each of which was provided by 28 renal donors and 47 renal allograft recipients immediately pretransplant. Seventy three of these sera were also assayed for VZV CF antibody and the titres are correlated with those of HCMV in Figure B. All tests were performed in parallel using identical reagents with the exception of the respective CF antigens.

	Spearman rank correlation
Figure A	0.293 ($p > 0.01$)
Figure B	0.139 ($p > 0.01$)

Spearman rank correlations were kindly provided by Dr G.D. Murray, Medical Statistics Unit, Western Infirmary, Glasgow.


Reciprocal VZV CF antibody titre
1024
512
256
128
64
32
16
8
4
2
<2

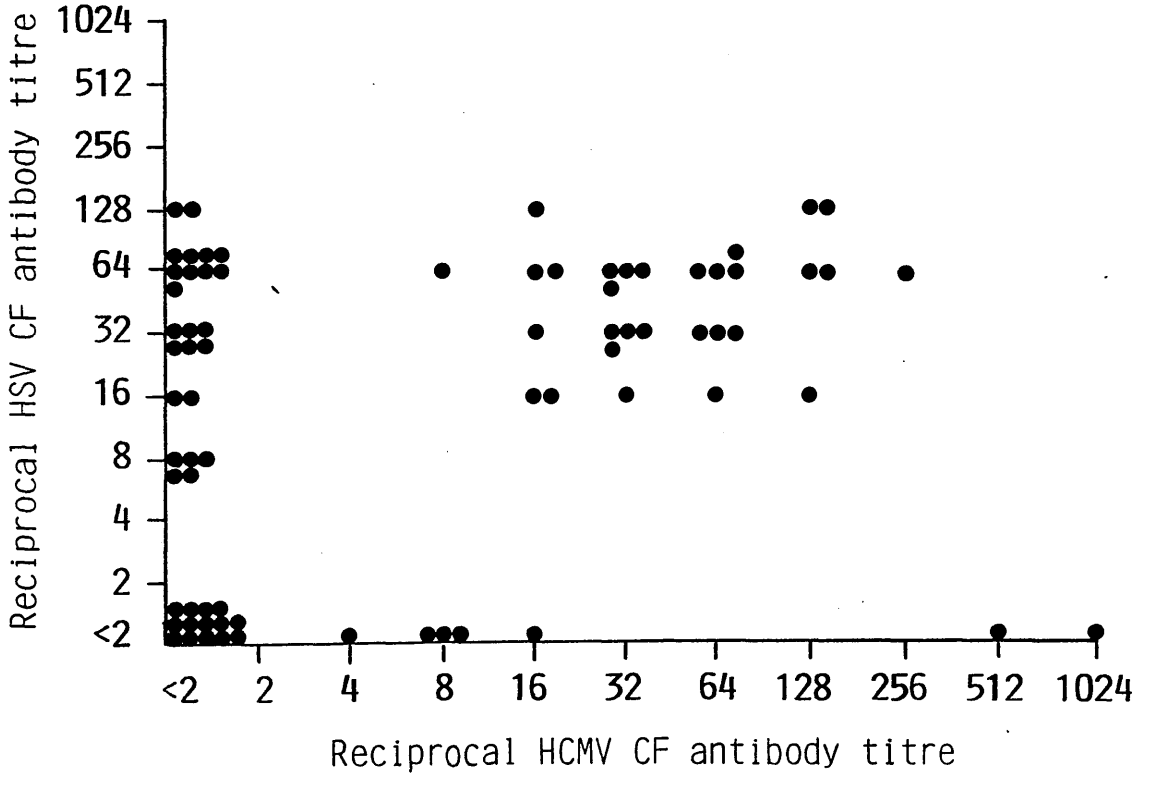
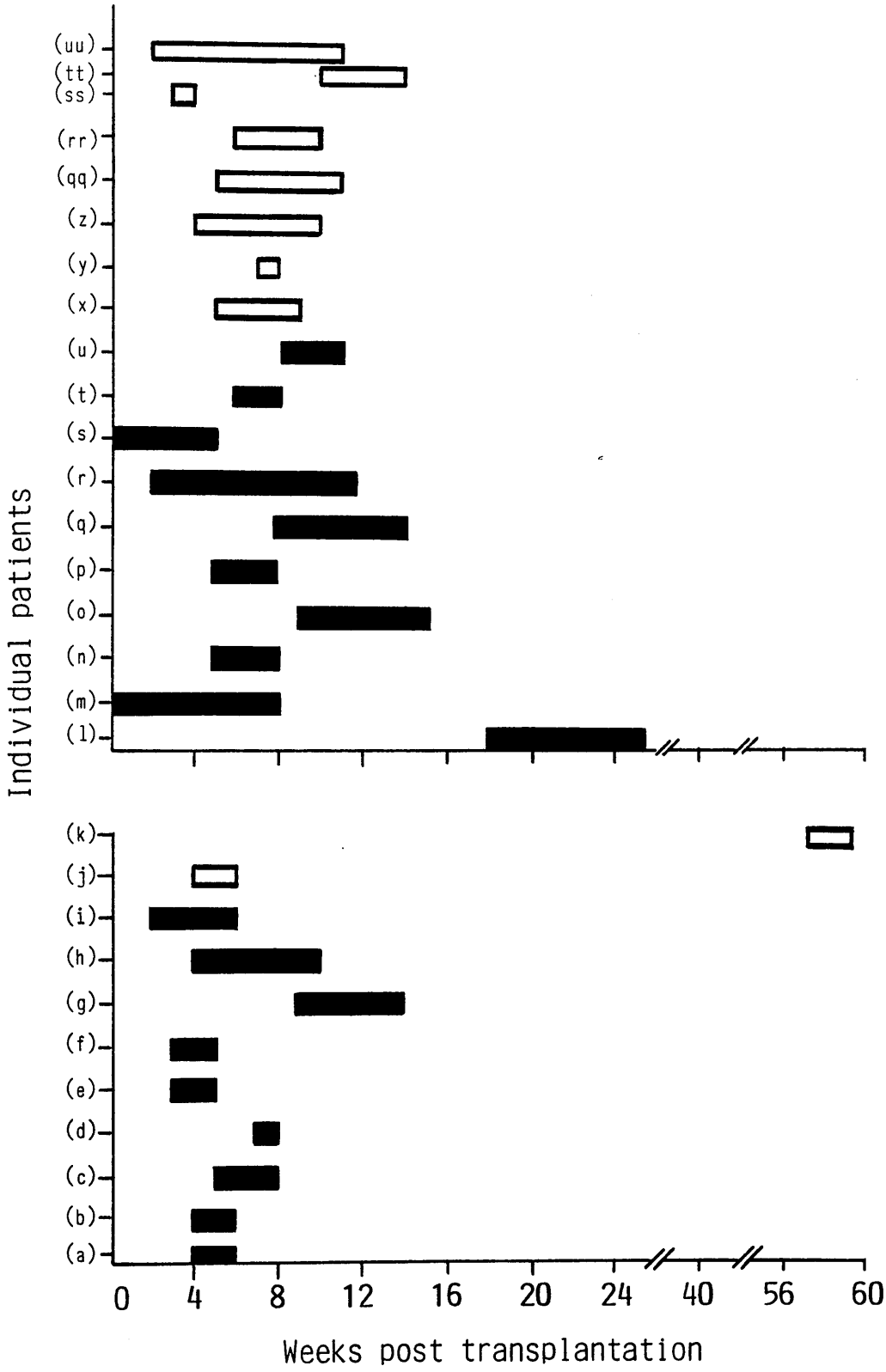

Reciprocal HSV CF antibody titre
1024
512
256
128
64
32
16
8
4
2
<2
<2
2
4
8
16
32
64
128
256
512
1024
Reciprocal HCMV CF antibody titre

Figure 44 Timing of the fourfold or greater rise in HCMV CF antibody titre after renal transplantation

The figure shows the timing of the fourfold or greater rise in HCMV CF antibody titre in 11 patients (a to k) with primary infection and 18 (l,m,n,o,p, q,r,s,t,u,x,y,z,qq,rr,ss,tt,uu) with reinfection and/or reactivation of HCMV after renal transplantation. Each bar identifies the time interval between consecutive serum samples the last of which showed the fourfold or greater rise in HCMV CF antibody titre. ■ and □ differentiate patients from whom virus was isolated and not isolated respectively.


Individual patients
(uu)
(tt)
(ss)
(rr)
(qq)
(z)
(y)
(x)
(u)
(t)
(s)
(r)
(q)
(p)
(o)
(n)
(m)
(l)
(k)
(j)
(i)
(h)
(g)
(f)
(e)
(d)
(c)
(b)
(a)
0
4
8
12
16
20
24
40
56
60
Weeks post transplantation

this rise in CF antibody titre post renal transplantation. In 17 renal allograft recipients with reinfection and/or reactivation of HCMV (m,n,o,p,q,r,s,t,u,x,y,z, aa, bb, cc, dd, ee), the fourfold or greater rise in HCMV CF antibody titre was recorded up to 16 weeks after the transplant operation and in 1 individual (l) between 18 and 25 weeks post transplantation.

4.6.3 Changing levels of HCMV CF antibody post renal transplantation

Figure 45 shows the post transplantation HCMV CF antibody titres in each of 10 patients (a,b,c,d,e,f,g,h,i,j) with primary infection. The rise in HCMV CF antibody titres was very abrupt, 1 patient showing an increase in titre from less than 1:2 to greater than or equal to 1:1024 over a 2 week period. However, once the CF antibody titre had reached a plateau it was maintained with minimal fluctuation for at least between 35 and 53 weeks after the transplant operation in the 9 patients (b,c,d,e,f,g,h,i,j) with primary HCMV infection whose CF antibody levels were regularly monitored for this period of time.

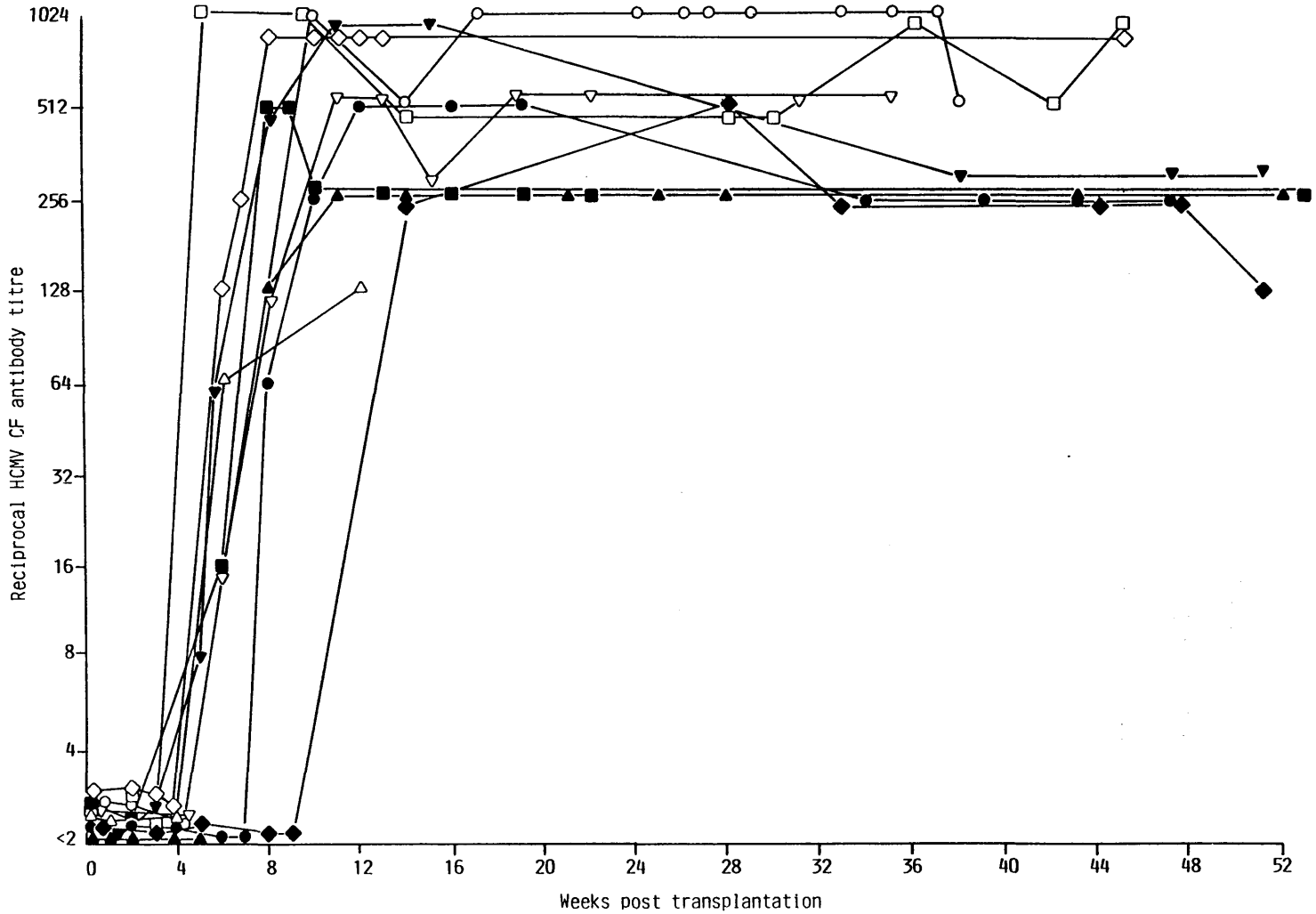
4.7 Post transplantation levels of HCMV-IgG measured by Labsystems ELISA

4.7.1 Reproducibility

To assess inter-assay variation in the measurement of HCMV-specific IgG, 191 serum samples were each subjected to

Figure 45 Post transplantation HCMV CF antibody titres in patients with primary infection

The figure shows the post transplantation HCMV CF antibody titres in each of 10 renal allograft recipients (a to j) with primary HCMV infection. Patient (k) who showed an eightfold increase in HCMV CF antibody at 59 weeks post transplantation is omitted from the diagram.


1024
512
256
128
64
32
16
8
4
<2
Reciprocal HCMV CF antibody titre
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation

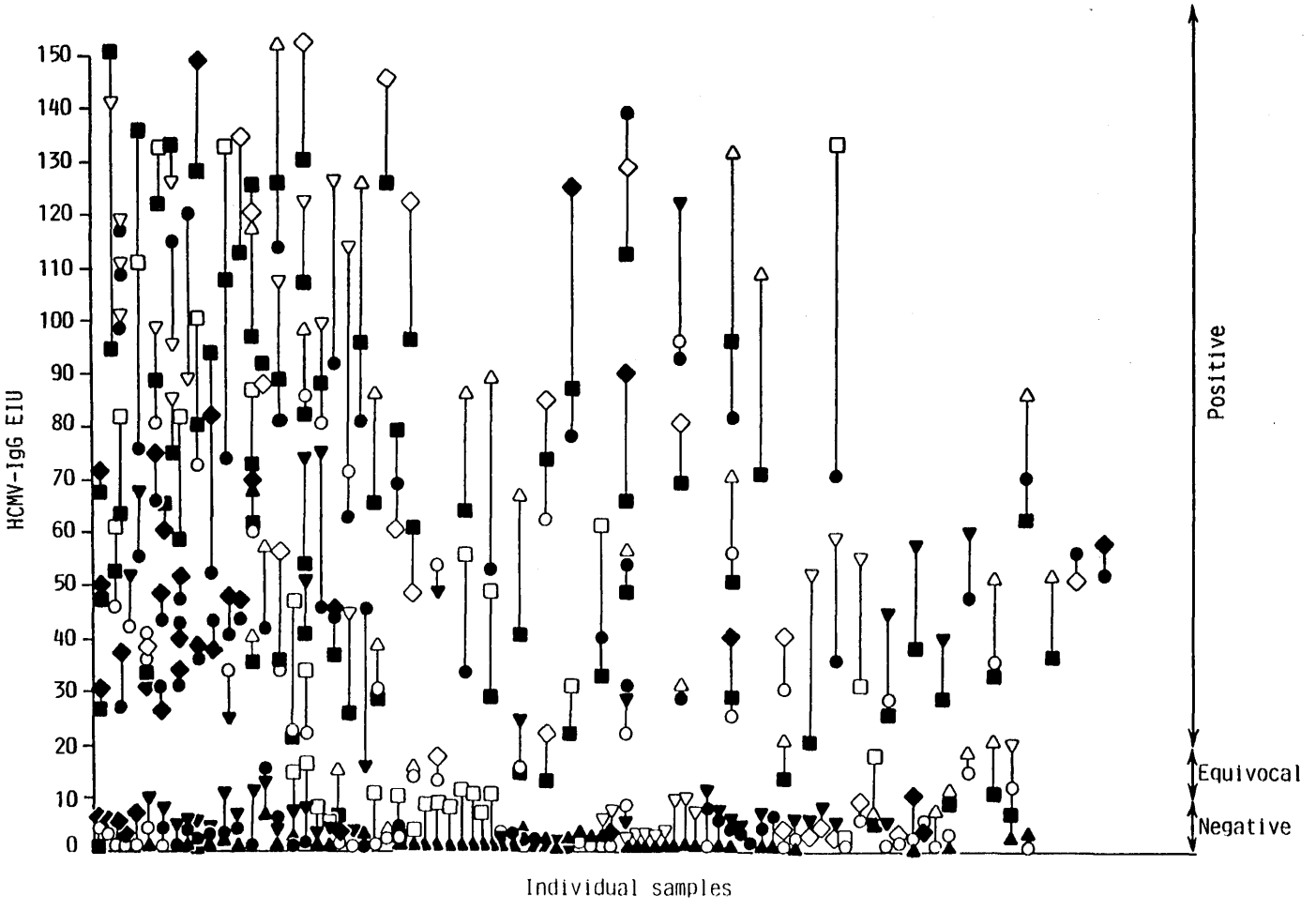
testing in 2 batches of the Labsystems ELISA. The results are shown in Figure 46.

On reanalysis of 79 sera initially classified as negative for HCMV-IgG, 1 serum was found to be positive while 18 sera each had an HCMV-IgG antibody level in the equivocal zone. The remaining 60 sera were confirmed to be negative for HCMV-IgG. When 109 sera which were regarded as positive for HCMV-IgG were retested, the HCMV serostatus was confirmed in 104 while the other 5 sera were each found to have an HCMV-IgG level in the equivocal zone. Confirmation of the antibody level was obtained in each of the 3 sera initially classified as equivocal for HCMV-IgG.

Taken overall, the average variation in the ELISA value of the 191 sera was 13.2 EIU (range 0 to 62.0). Of the 80 sera in which HCMV-IgG was negative or equivocal, the ELISA value varied by an average of 5.96 EIU (range 0 to 16.7). The average variation in the ELISA value of 111 sera classified as positive for HCMV-IgG was 18.4 EIU (range 0.1 to 62.0).

Figure 46 Reproducibility of HCMV-IgG levels measured by Labsystems ELISA

One hundred and ninety one sera provided by 47 renal allograft recipients and 7 renal donors were tested for HCMV-IgG by Labsystems ELISA. The HCMV-IgG level in each serum is denoted by a symbol unique to a particular batch of the ELISA (○, ●, △, ▲, ◇, ◆, ▽, ▼ and ■ respectively). The variation in the level of HCMV-IgG within each serum tested in 2 batches of the ELISA is represented by a solid vertical line.


HCMV-IgG EIU
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Positive
Equivocal
Negative
Individual samples

4.7.2 Correlation between HCMV-IgG levels measured by Labsystems ELISA and HSV and VZV CF antibody titres

An investigation was made into the possibility of HSV and VZV antibodies being detected by the Labsystems HCMV-IgG ELISA. Sera obtained immediately pretransplant from each of 47 renal allograft recipients and 26 renal donors were tested in parallel for HSV and VZV CF antibodies as described in Section 4.6.1. Titres were correlated with HCMV-IgG levels measured by Labsystems ELISA and the results are shown in Figure 47. No correlation was observed between HCMV-IgG levels and HSV or VZV CF antibody titres.

4.7.3 Correlation between HCMV-IgG levels measured by Labsystems ELISA and HCMV CF antibody titres

The sera described in Section 4.7.2 were also tested in parallel for HCMV CF antibody and the results correlated with the HCMV-IgG level (Figure 48).

There was a wide scatter in HCMV-IgG levels amongst sera with equivalent HCMV CF antibody titres of > 1:8. However, HCMV-IgG levels measured by Labsystems ELISA and HCMV CF antibody titres correlated significantly overall, the Spearman rank correlation being 0.891 ($p < 0.01$).

All 38 sera with an HCMV CF antibody titre of less than 1:2 were negative for HCMV-IgG on the first occasion of testing. Of the 22 sera which were reanalysed, 18 were confirmed to be negative for HCMV-IgG while 4 others had an HCMV-IgG level ranging from 10.3 to 11.9 EIU. However,

Figure 47 Correlation between HCMV-IgG levels measured by Labsystems ELISA and HSV and VZV CF antibody titres

Seventy three sera provided by 26 renal donors and 47 renal allograft recipients immediately pretransplant were tested in parallel for HSV and VZV CF antibody. All reagents were identical with the exception of the respective CF antigen. Results are correlated with the level of HCMV-IgG measured in various batches of the Labsystems ELISA (○ ● △ ▲ ◇ ◆ ▽ ▼ and ■ respectively).

HCMV-IgG EIU with	Spearman rank correlation
HSV CF antibody titre	0.289 ($p > 0.01$)
VZV CF antibody titre	0.135 ($p > 0.01$)

Spearman rank correlations were kindly provided by Dr G.D. Murray, Medical Statistics Unit, Western Infirmary, Glasgow.

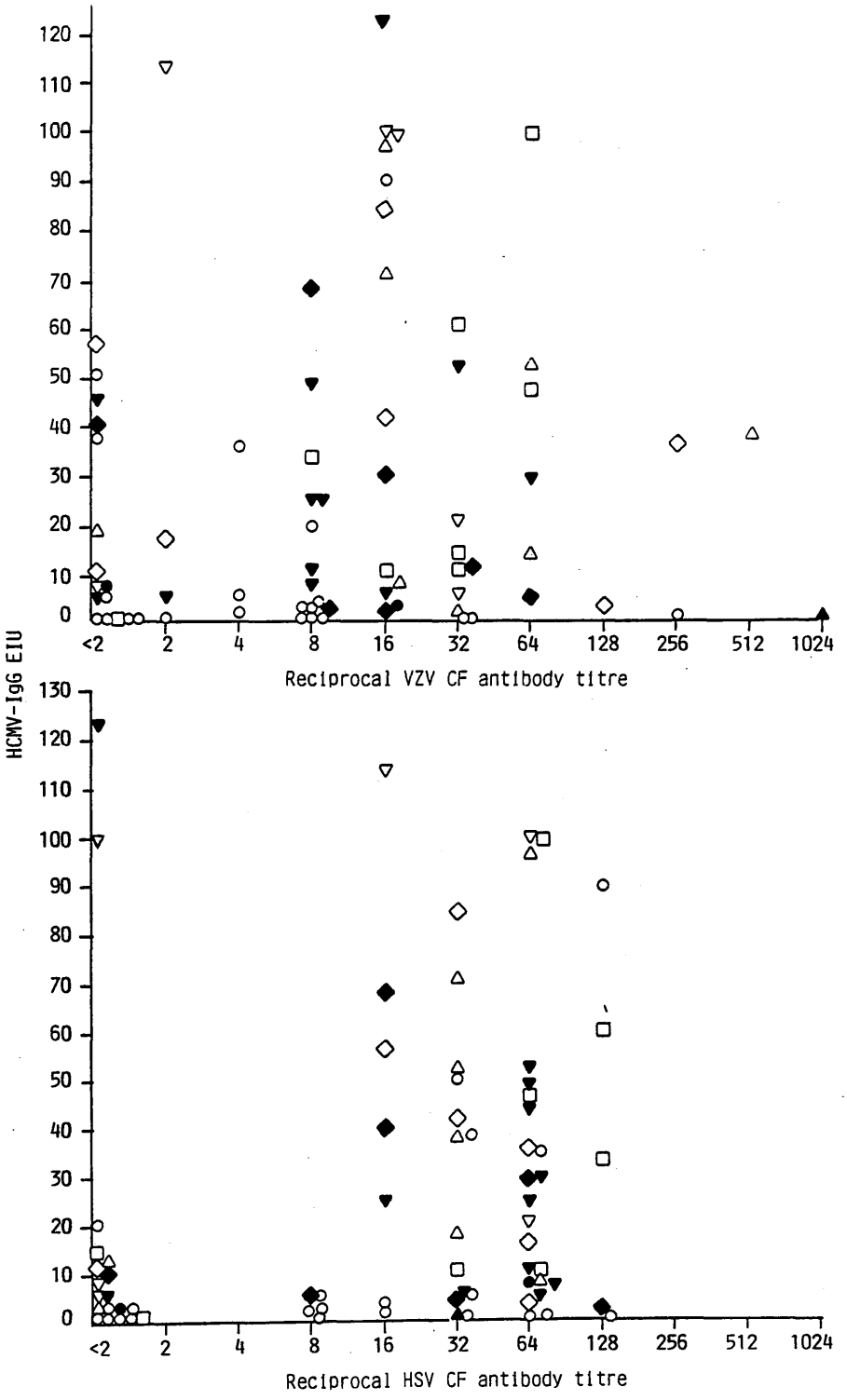
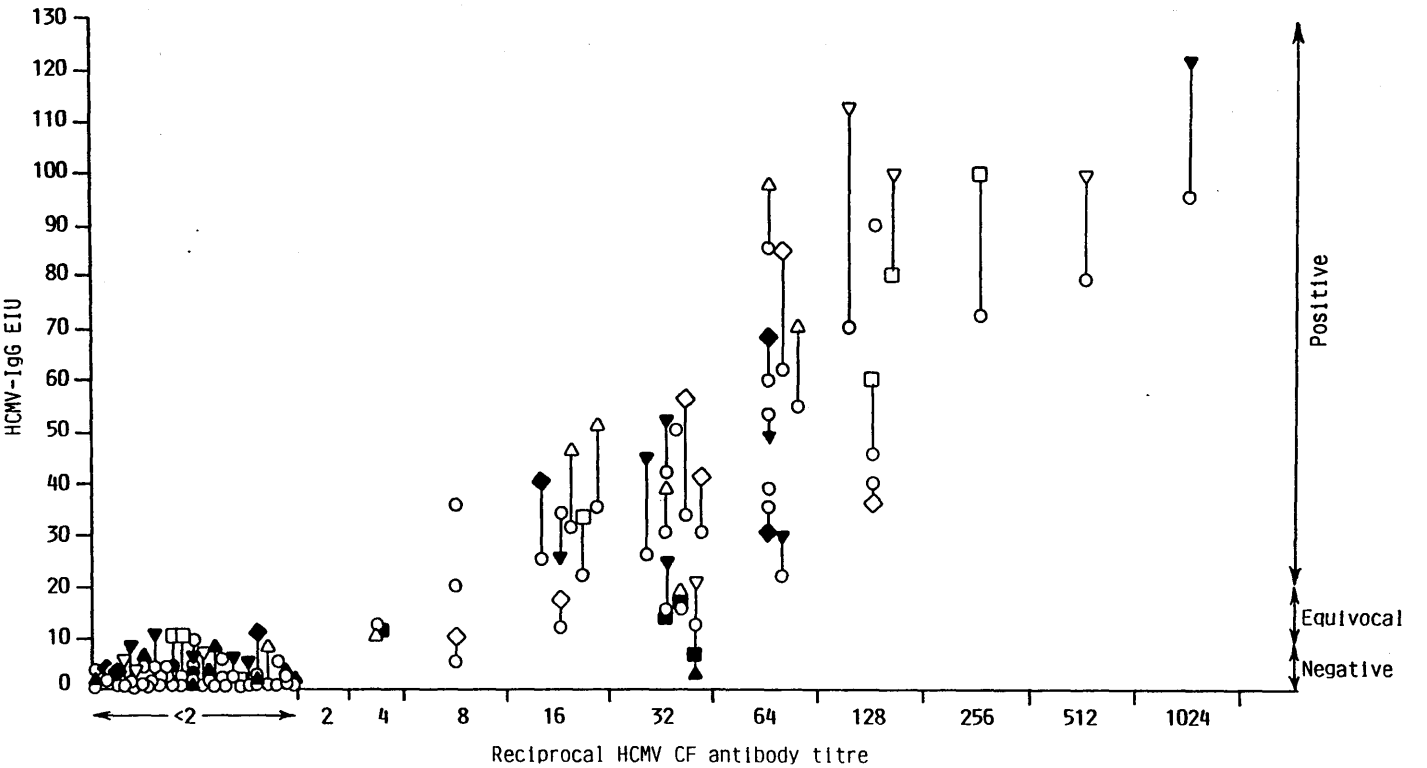

HCMV-IgG EIU
120
110
100
90
80
70
60
50
40
30
20
10
0
<2
2
4
8
16
32
64
128
256
512
1024
Reciprocal VZV CF antibody titre
130
120
110
100
90
80
70
60
50
40
30
20
10
0
<2
2
4
8
16
32
64
128
256
512
1024
Reciprocal HSV CF antibody titre

Figure 48 Correlation between the level of HCMV-IgG measured by Labsystems ELISA and the respective HCMV CF antibody titre

Sera provided by each of 26 renal donors and 47 renal allograft recipients immediately pretransplant were tested in parallel for HCMV CF antibody. Titres were correlated with the respective level of HCMV-IgG measured by Labsystems ELISA. The HCMV-IgG level in each serum is denoted by a symbol unique to a particular batch of the ELISA (○, ●, △, ▲, ◇, ◆, ▽, ▼ and ■ respectively). Fifty four sera were each tested in 2 batches of the ELISA and the variation in the level of HCMV-IgG is represented by a solid vertical line. A further 19 sera were tested for HCMV-IgG on only 1 occasion. Spearman rank correlation = 0.891 ($p < 0.01$).

The Spearman rank correlation was kindly provided by Dr G.D. Murray, Medical Statistics Unit, Western Infirmary, Glasgow.


HCMV-IgG EIU
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Positive
Equivocal
Negative
<2
2
4
8
16
32
64
128
256
512
1024
Reciprocal HCMV CF antibody titre

when these 4 sera were retested for a third time all were found to be negative for HCMV-IgG.

A total of 35 sera, one each of which was provided by 10 renal donors and 25 renal allograft recipients immediately pretransplant, were confirmed to have HCMV CF antibody titres ranging from 1:4 to greater than or equal to 1:1024. Of these, 31 sera were positive and 4 equivocal when first tested for HCMV-IgG by Labsystems ELISA. Reanalysis confirmed 29 sera to be positive for HCMV-IgG while 5 had levels ranging from 13.2 to 15.8 EIU and 1 serum was negative for HCMV-IgG. Two of the 5 sera regarded as equivocal for HCMV-IgG were also equivocal on the first occasion of testing and subsequent analysis. A third serum from a renal donor who provided a kidney for patient (s) had an HCMV CF antibody titre of 1:16 on 2 occasions of testing and was equivocal for HCMV-IgG in 2 batches of the ELISA. Sera obtained from each of patients (y) and (ss) immediately before the transplant operation had reproducible HCMV CF antibody titres of 1:32 and were also positive for HCMV-IgG when first tested by ELISA. On the second occasion of testing, the HCMV-IgG level in the serum provided by patient (y) was 15.8 EIU and retesting confirmed it to be equivocal for HCMV-IgG. The pretransplant serum obtained from patient (ss) was retested for HCMV-IgG on 3 more occasions and was considered to be equivocal once and negative twice. A sixth serum sample with an HCMV CF antibody titre of 1:8 was initially

regarded as equivocal for HCMV-IgG but when retested the HCMV-IgG level was found to be 6.23 EIU.

4.7.4 Correlation between the HCMV-IgG serostatus determined by NBL ELISA and the HCMV CF antibody titre

Seventy two of the 73 sera whose HCMV antibody levels were determined by CFT were also analyzed for HCMV-IgG by NBL ELISA. Sixty four sera were each tested in 2 different batches of the ELISA while 7 were examined only once. A serum obtained from patient (uu) immediately pretransplant was overlooked and not analysed by NBL ELISA.

Of those sera with an HCMV CF antibody titre of less than 1:2 which were provided by 16 renal donors and 22 renal allograft recipients immediately before the transplant operation, all 38 were confirmed to be negative for HCMV-IgG by NBL ELISA.

Ten renal donors were consistently positive for HCMV CF antibody and 7 of these were also positive for HCMV-IgG in the NBL ELISA. Sera obtained from 3 other renal donors had reproducible HCMV CF antibody titres ranging from 1:8 to 1:16 but were considered to be negative for HCMV-IgG when tested in 2 different batches of the NBL ELISA.

Sera provided by 24 renal allograft recipients immediately before the transplant operation were positive for HCMV CF antibody. Twenty two of these sera were confirmed positive and 2 negative for HCMV-IgG on 2 occasions of testing in varied batches of the NBL ELISA.

4.7.5 Analysis of 7 sera with uncertain levels of HCMV antibody

Of the 73 sera whose HCMV CF antibody titres were correlated with HCMV-IgG levels measured by Labsystems ELISA, 72 were also examined by the NBL HCMV-IgG ELISA. Concordant results were obtained for 65 sera, 27 being seropositive and 38 seronegative by each method of analysis. Discordant results were found in 7 sera which are described in more detail below.

Three renal donors each provided sera which were positive for HCMV CF antibody but negative for HCMV-IgG when tested in 2 batches of the NBL ELISA. On reanalysis by Labsystems ELISA, 1 serum with an HCMV CF antibody titre of 1:16 was consistently positive for HCMV-IgG while another serum with an HCMV CF antibody titre of 1:8 had HCMV-IgG levels of 6.2 and 10.2 EIU on each occasion of testing. Both these sera were obtained from renal donors whose kidneys were transplanted into 2 recipients each of whom developed a primary HCMV infection post-operatively. Such findings are suggestive of both renal donors having prior exposure to the virus. Serum obtained from a third renal donor had an HCMV CF antibody titre of 1:16 but was equivocal for HCMV-IgG in each of 3 batches of the Labsystems ELISA. This renal donor provided a kidney for patient (s) whose pretransplantation HCMV serostatus is discussed below.

Four other sera, one each of which was provided by patients (l), (s), (y) and (ss) immediately prior to renal

transplantation, also had uncertain levels of HCMV-specific antibody. The sera obtained from patients (l) and (y) each had a reproducible HCMV CF antibody titre of 1:32 and were positive for HCMV-IgG when twice tested by NBL ELISA. Reanalysis by Labsystems ELISA found the serum from patient (l) to be equivocal for HCMV-IgG while the serum from patient (y) had an HCMV-IgG level of 14.9, 15.8 and 25.0 EIU on each occasion of testing. Patient (s) provided a serum with an HCMV CF antibody titre of 1:4 which was negative for HCMV-IgG in 2 batches of the NBL ELISA and equivocal on 3 occasions of testing by Labsystems HCMV-IgG ELISA. The pretransplant serum from patient (ss) had a reproducible HCMV CF antibody titre of 1:32 but was negative for HCMV-IgG when twice analysed by NBL ELISA. Reexamination in 4 batches of the Labsystems ELISA found the HCMV-IgG level to be 3.5, 7.2, 13.2 and 21.8 EIU on each occasion of testing.

As discussed in Section 4.5.3(b), there was consistent inability to isolate HCMV from the urine of some renal allograft recipients who showed evidence of an HCMV-specific humoral response after the transplant operation. This group of renal transplant recipients, which includes patients (s) (y) and (ss), were generally considered to have reinfection and/or reactivation of HCMV rather than primary infection. Indeed, the post transplantation HCMV-IgG response in each of patients (s) and (y) was typical of reinfection and/or reactivation of the virus with levels

falling by the end of the first year after the transplant operation (Figure 59B). Patient (ss) would have been most unlikely to have developed a primary HCMV infection because the transplanted kidney was donated by an individual confirmed to be seronegative for HCMV. Furthermore, this patient's post transplantation HCMV-IgM response was negligible and atypical of primary infection (Figure 59B). In patient (l), HCMV was isolated from only 1 urine sample and this together with the absence of rising levels of HCMV-IgM and IgG after the transplant operation were features consistent with reinfection and/or reactivation of virus (Figure 59B).

4.7.6 Kinetics of the post transplantation HCMV-IgG response

Ten renal allograft recipients (a,b,c,d,e,f,g,h,i,j) with primary HCMV infection each showed an increase in HCMV-IgG during the first year post transplantation while an HCMV-IgG response in patient (k) was first noted in a serum obtained at 69 weeks post transplantation (Figure 49A). Similarly, 18 (m,n,o,p,q,r,s,t,u,v,w,x,y,z,qq,rr,ss, uu) of the 20 renal allograft recipients with reinfection and/or reactivation of HCMV showed a significant increase in HCMV-IgG which peaked between 8 and 14 weeks after the transplant operation (Figure 49B).

Because of the large number of sera involved it was not possible to retest all sera from all patients. Therefore, confirmation of the post transplantation HCMV-

Figure 49A Post transplantation levels of HCMV-IgG measured by Labsystems ELISA: Primary infection

The figure shows the levels of HCMV-IgG between 0 and 76 weeks post transplantation in each of 11 renal allograft recipients (a to k) with primary HCMV infection.

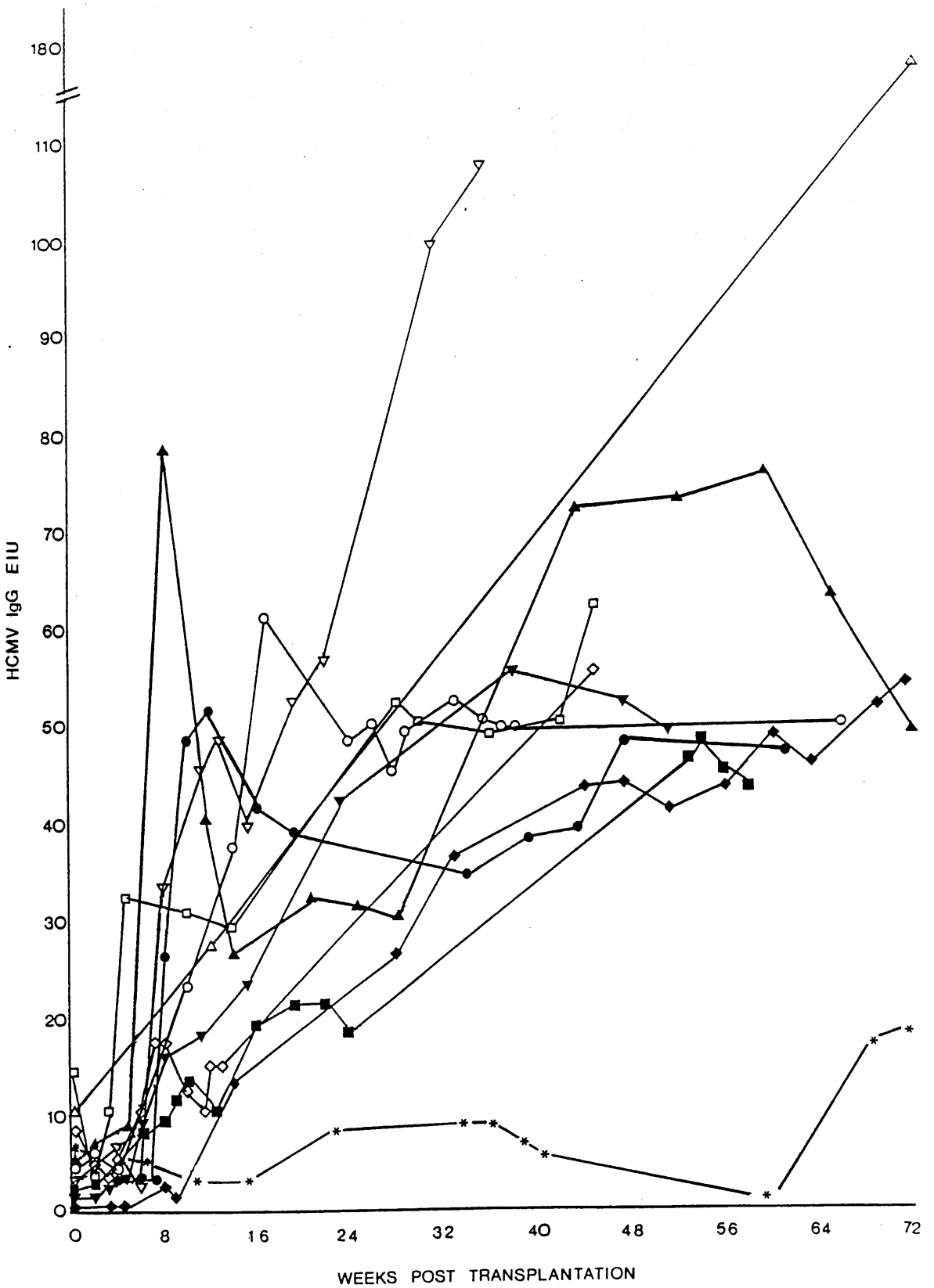
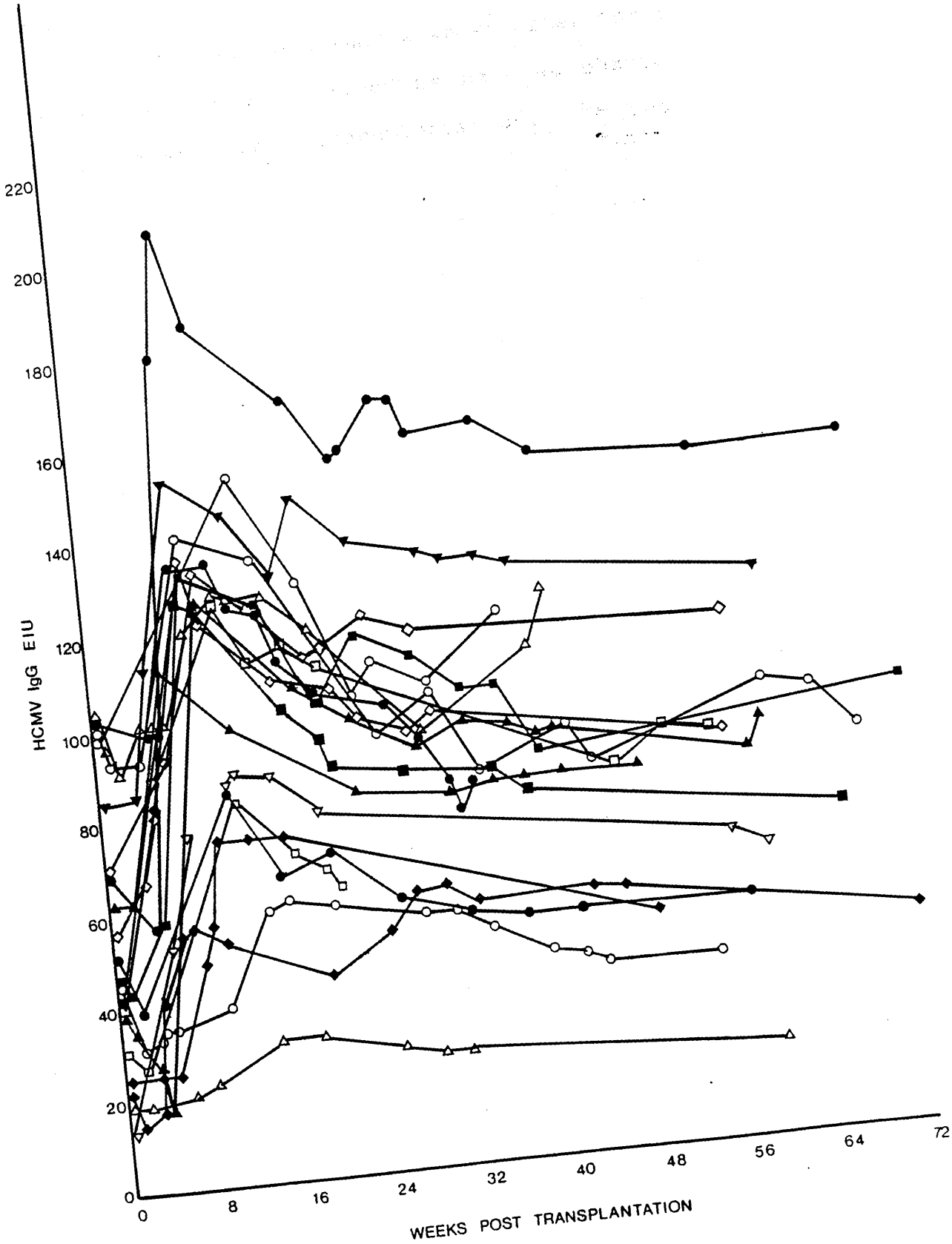

180
110
100
90
80
70
60
50
40
30
20
10
0
HCMV IgG EIU
0
8
16
24
32
40
48
56
64
72
WEEKS POST TRANSPLANTATION

Figure 49B Post transplantation levels of HCMV-IgG measured by Labsystems ELISA: Reinfection and/or reactivation

The figure shows the levels of HCMV-IgG between 0 and 77 weeks post transplantation in each of 20 renal allograft recipients (l,m,n,o,p,q,r,s,t, u,v,w,x,y,z,qq,rr,ss,tt,uu) with reinfection and/or reactivation of HCMV.


HCMV IgG EIU
0
20
40
60
80
100
120
140
160
180
200
220
0
8
16
24
32
40
48
56
64
72
WEEKS POST TRANSPLANTATION

IgG response in patients with primary and secondary HCMV infection was obtained by reanalysis of 2 sera taken from each patient, 1 between 6 and 14 weeks after the transplant operation and another obtained as near as possible to the fifty second week post transplantation. To reduce error attributable to inter-assay variation, sera were retested in parallel within the same batch of the HCMV-IgG ELISA. Whenever possible, levels of HCMV-IgG were compared with those previously recorded in another batch of the ELISA (Figures 50A, B and C).

(a) Primary HCMV infection

In 10 patients (a,b,c,d,e,f,g,h,i,j) the level of HCMV-IgG between 8 and 14 weeks after the transplant operation was an average of 22.3 EIU (range 4.0 to 73.1) higher than observed immediately pretransplant. HCMV was isolated from all patients with the exception of 1 (j) whose HCMV-IgG level had increased by an average of 32.2 EIU (range 25.2 to 39.2) by the eleventh week post transplantation. Between 35 and 53 weeks after the transplant operation, sera were available from 9 patients (b,c,d,e,f,g,h,i,j). By this time post transplantation, levels of HCMV-IgG had risen above the pretransplant value by an average of 51.8 EIU (range 29.1 to 101.7) and in patient (j) by 94.9 EIU (range 88.1 to 101.7). In 8 (b,c, d,e,f,g,h,j) of the 9 patients for whom sufficient data are available, there was an average increase in HCMV-IgG of 28.9 EIU (range - 3.2 to 62.7) from between 6 and 14 weeks

Figure 50A Change in post transplantation levels of HCMV-IgG measured by Labsystems ELISA

Between 8 and 14 weeks post transplantation, a serum sample was taken from each of 10 renal allograft recipients (a,b,c,d,e,f,g,h,i,j) with primary HCMV infection while 20 patients (l,m,n,o,p,q,r,s,t,u,v,w,x,y,z,qq,rr,ss, tt,uu) with reinfection and/or reactivation provided a serum sample between 6 and 14 weeks after the transplant operation. Serum was obtained between 11 and 17 weeks post transplantation from each of 11 renal allograft recipients (bb,cc,dd,ee,ff,gg, ii,jj,kk,mm,oo) with no evidence of active HCMV infection. All sera were tested for HCMV-IgG by Labsystems ELISA. The magnitude of the HCMV-IgG response in each patient was then calculated by reference to the pretransplant HCMV-IgG level, a negative value indicating a fall in HCMV-IgG. Sera were initially tested in 1 of 9 different batches of the Labsystems ELISA (● / O) and whenever possible retested in parallel within a single batch of the ELISA (■ / □). The variation in the magnitude of the HCMV-IgG response when measured in 2 batches of the Labsystems ELISA is represented by a solid vertical line. (● / ■) or (O / □) differentiate sera obtained from those renal allograft recipients who shed or did not shed HCMV respectively. Within each group of patients, $\bar{A}$ indicates the average change in the level of HCMV-IgG above that recorded immediately pretransplant. Five renal allograft recipients with no evidence of active HCMV infection and patient (k) who first showed serological evidence of HCMV infection at 59 weeks post transplantation were omitted from this correlation.

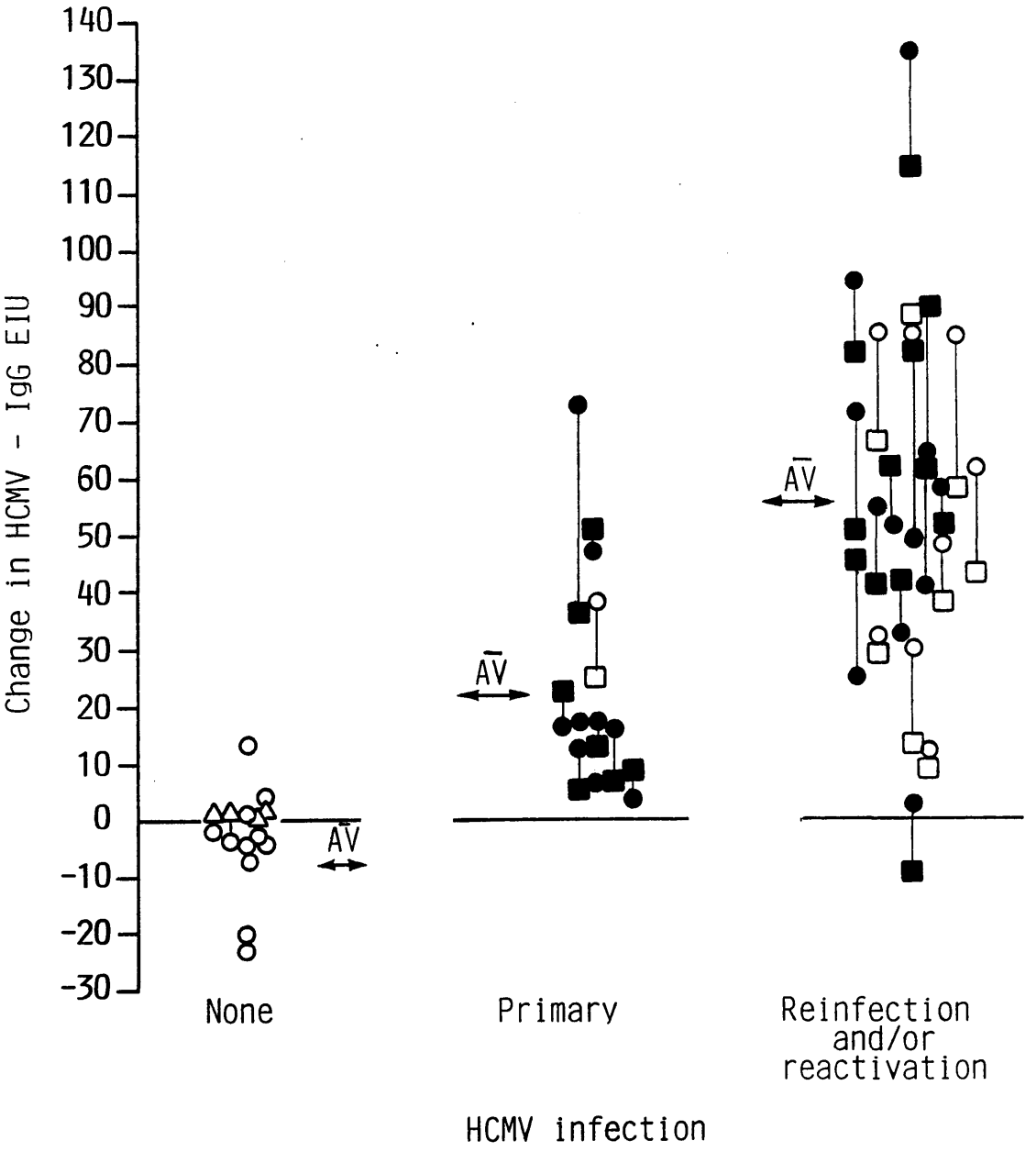

Change in HCMV - IgG EIU
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
$\bar{\Delta V}$
$\bar{\Delta V}$
$\bar{\Delta V}$
None
Primary
Reinfection and/or reactivation
HCMV infection

Figure 50B Change in post transplantation levels of HCMV-IgG measured by Labsystems ELISA

Nine renal allograft recipients (b,c,d,e,f,g,h,i,j) with primary HCMV infection each provided a serum sample between 35 and 53 weeks after the transplant operation. A serum sample was also obtained between 21 and 60 weeks post transplantation from all 20 patients (l,m,n,o,p,q,r,s,t,u,v,w,x,y,z, qq,rr,ss,tt,uu) with reinfection and/or reactivation. Between 31 and 61 weeks post transplantation, serum was taken from 11 (bb,cc,dd,ee,ff,gg,ii,jj,kk,mm, oo) of the 16 renal allograft recipients with no evidence of active HCMV infection. The procedure for testing and reporting the HCMV-IgG level in each serum was as described in Figure 50A.

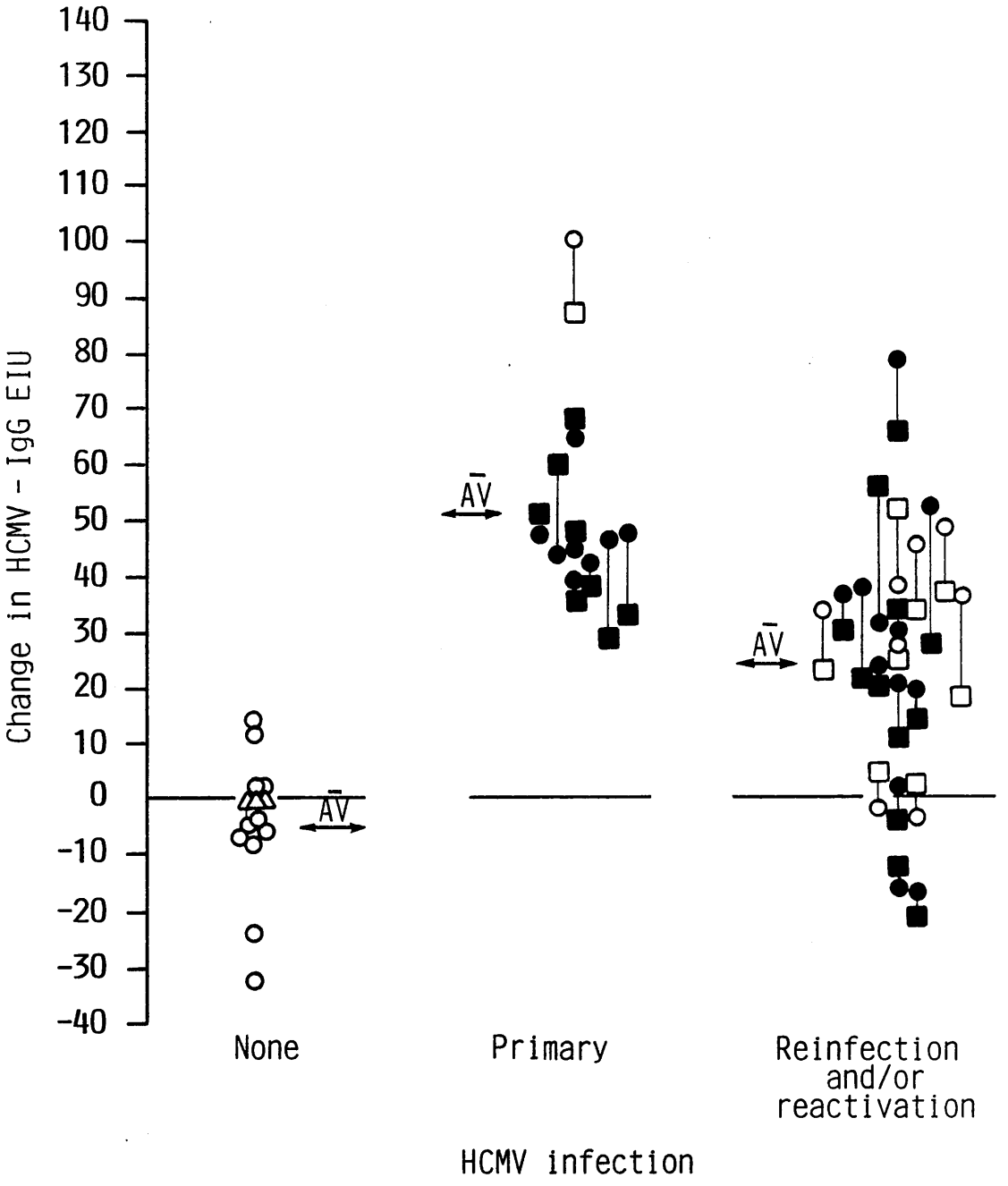
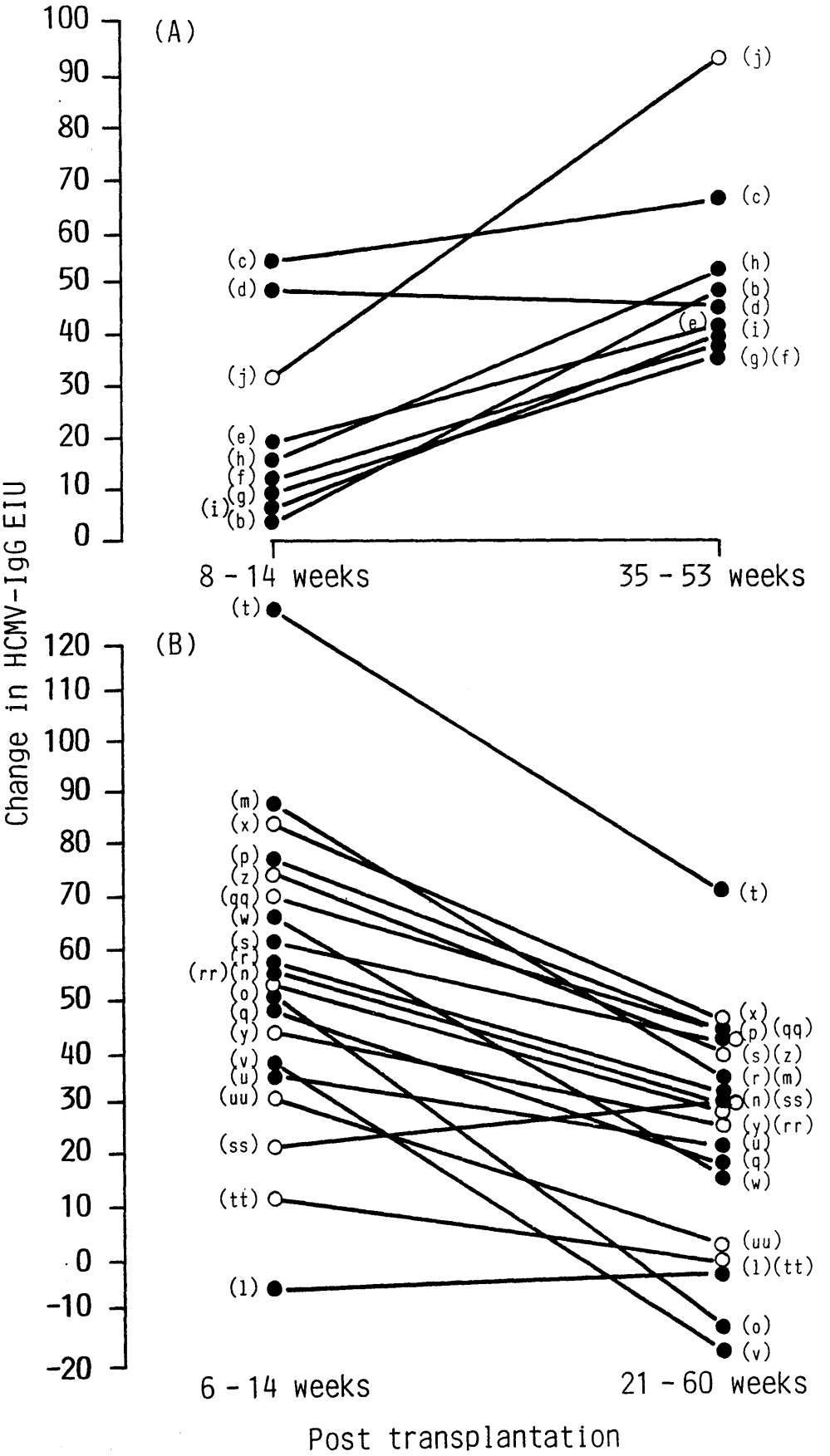

140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
Change in HCMV - IgG EIU
AV
AV
AV
None
Primary
Reinfection and/or reactivation
HCMV infection

Figure 50C Change in post transplantation levels of HCMV-IgG measured by Labsystems ELISA

Nine renal allograft recipients (b,c,d, e,f,g,h,i,j) with primary HCMV infection each provided 2 serum samples, 1 between 8 and 14 weeks and another between 35 and 53 weeks post transplantation. Similarly 2 sera, 1 taken between 6 and 14 weeks and another between 21 and 60 weeks after the transplant operation, were obtained from each of 20 patients (l,m,n,o,p,q,r,s,t,u,v,w,x,y,z,qq,rr,ss, tt,uu) with reinfection and/or reactivation. The HCMV-IgG level in each serum was measured in 2 batches of the Labsystems ELISA and an average value calculated. Only that serum provided by patient (i) at 13 weeks post transplantation was tested in a single batch of the ELISA. The difference between the average HCMV-IgG level in the 2 sera taken from each patient is indicated by a solid line ● and ○ differentiate those renal allograft recipients from whom HCMV was isolated and not isolated respectively.


(A)
100
90
80
70
60
50
40
30
20
10
0
(c)
(d)
(j)
(e)
(h)
(f)
(g)
(i)
(b)
(j)
(c)
(h)
(b)
(d)
(e)
(i)
(g)(f)
8 - 14 weeks
35 - 53 weeks
(B)
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
(t)
(m)
(x)
(p)
(z)
(qq)
(w)
(s)
(r)
(rr)
(n)
(o)
(q)
(y)
(v)
(u)
(uu)
(ss)
(tt)
(l)
(t)
(x)
(p)(qq)
(s)(z)
(r)(m)
(n)(ss)
(y)(rr)
(u)
(q)
(w)
(uu)
(l)(tt)
(o)
(v)
6 - 14 weeks
21 - 60 weeks
Change in HCMV-IgG EIU
Post transplantation

up to the end of the first year after renal transplantation, a negative value indicating a fall in IgG.

Between 9 and 44 weeks after the transplant operation, patient (g) showed a progressive increase in HCMV-IgG from 1.3 to 43.6 EIU, a level which was maintained with minimal fluctuation for at least 51 weeks post transplantation (Figure 59A). The HCMV-IgG response in patient (c) peaked at 78.7 EIU 8 weeks after the transplant operation and then declined to 25.8 EIU by 14 weeks post transplantation. Thereafter, the HCMV-IgG level did not change significantly until it increased to 72.2 EIU at 43 weeks after the transplant operation. The next recorded serum sample taken from this patient at 52 weeks post transplantation showed an HCMV-IgG level of 73.7 EIU. In patient (d), there was a gradual increase in the level of HCMV-IgG from 3.23 to 51.3 EIU between 7 and 12 weeks post transplantation. The HCMV-IgG level then decreased to 37.5 EIU at 19 weeks after the transplant operation but did not fluctuate significantly thereafter. In patient (e), the level of HCMV-IgG increased by 22.7 EIU between 3 and 5 weeks post transplantation and did not alter significantly until 28 weeks after the transplant operation when the level of HCMV-IgG had increased by 23.2 EIU beyond that recorded in the preceding serum sample taken at 14 weeks post transplantation. Thereafter the HCMV-IgG response did not change significantly. HCMV-IgG in patient (j) gradually increased from 6.25 to 47.6 EIU between 4 and 13 weeks post

transplantation and did not fluctuate significantly until an HCMV-IgG level of 99.4 EIU was noted at 31 weeks after the transplant operation. The last recorded serum sample taken from this patient at 35 weeks post transplantation showed the HCMV-IgG level to be 108.0 EIU. In patients (a), (b), (f) and (i), there was a gap of 63,32,23 and 29 weeks respectively between 2 consecutive serum samples and therefore it was not possible to comment on the pattern of the HCMV-IgG response in these renal allograft recipients although in each patient the level of HCMV-IgG had increased during the period of observation.

(b) Reinfection and/or reactivation

Eighteen patients (m,n,o,p,q,r,s,t,u,v,w,x,y,z,qq,rr, ss, uu) showed a significant increase in HCMV-IgG with the highest levels being recorded in sera obtained between 6 and 14 weeks after the transplant operation. By this time post transplantation, HCMV-IgG had increased by an average of 55.2 EIU (range -2.9 to 126.2). Between 21 and 60 weeks after the transplant operation, the HCMV-IgG level was an average of 24.2 EIU (range -19.3 to 72.5) higher than that observed immediately pretransplant although in 3 patients (l,tt,uu) HCMV-IgG had approximated pretransplant levels while in 2 others (o and v) HCMV-IgG showed an average fall of 14.5 and 19.3 EIU respectively below that recorded immediately pretransplant.

From between 6 and 14 weeks to between 21 and 60 weeks post transplantation, HCMV-IgG decreased by an average of 31.0 EIU (range -6.6 to 66.2), 1 patient (ss) showing an

increase in HCMV-IgG of 6.6 EIU (range 3.6 to 9.6).

Change in HCMV-IgG levels post transplantation could not differentiate those renal allograft recipients who shed HCMV from those who did not. Within 6 to 14 weeks after the transplant operation the HCMV-IgG level had increased by an average of 58.7 EIU (range -8.3 to 137.1) in the 12 patients (l,m,n,o,p,q,r,s,t,u,v,w) from whom the virus was isolated. During the same period, 8 patients (x,y,z,qq,rr, ss, tt, uu) from whom HCMV was never isolated showed an average increase in HCMV-IgG of 49.6 EIU (range 10.8 to 87.7) above that recorded immediately pretransplant. Between 21 and 60 weeks post transplantation, the HCMV-IgG level was an average of 22.5 EIU (range -21.5 to 79.7) or 26.5 EIU (range -4.5 to 49.2) above the pretransplant value in those renal allograft recipients who shed or did not shed HCMV respectively. In the 12 patients from whom virus was isolated, the level of HCMV-IgG decreased by an average of 36.4 EIU (range 0.85 to 66.2) from between 6 and 14 weeks to between 21 and 60 weeks post transplantation. During the same period, the HCMV-IgG level decreased by an average of 22.9 EIU (range -6.6 to 39.5) in the 8 patients from whom HCMV was never isolated.

The HCMV serological status of the renal donors did not influence the HCMV-IgG response in those renal allograft recipients with reinfection and/or reactivation of the virus. Between 6 and 14 weeks after the transplant operation, the 8 patients (l,m,s,t,x,y,u,qq) who each

received their kidney from donors known to be seropositive for HCMV showed an average increase in HCMV-IgG of 63.7 EIU (range -5.35 to 126.2) above that recorded immediately pretransplant. During the same period, HCMV-IgG had risen above the pretransplant level by an average of 49.1 EIU (range 11.7 to 77.2) in the 8 renal allograft recipients (n,p,r,v,z,rr,ss,tt) who were each transplanted with a kidney from an HCMV-seronegative donor. Between 21 and 60 weeks post transplantation, the level of HCMV-IgG was an average of 35.1 EIU (range -2.7 to 72.5) or 22.6 EIU (range -19.3 to 44.5) higher than that observed immediately pretransplant in those patients who received their kidney from donors known to be seropositive or seronegative for HCMV respectively. Levels of HCMV-IgG decreased by an average of 29.0 EIU (range 0.85 to 55.2) from between 6 and 14 weeks to between 21 and 60 weeks post transplantation in the 8 patients who were each transplanted with a kidney from an HCMV-seropositive donor while during the same period HCMV-IgG fell by an average of 26.2 EIU (range -6.6 to 57.8) in the 8 renal allograft recipients who each received their kidney from donors known to be seronegative for HCMV. The donor serological status was not available for 4 other renal allograft recipients (o,q,w,uu) who showed evidence of reinfection and/or reactivation.

Figure 59B shows the post transplantation levels of HCMV-IgG in each of the 20 renal allograft recipients with reinfection and/or reactivation. Eighteen patients (m,n,o, p,q,r,s,t,u,v,w,x,y,z,qq,rr,ss,uu) developed a significant

HCMV-IgG response the onset of which was first recorded up to 14 weeks after the transplant operation. A serum provided by patient (ss) at 4 weeks post transplantation was the first to show a significant increase in HCMV-IgG. Within 8 weeks of the transplant operation, the HCMV-IgG level had significantly increased in 10 other renal allograft recipients (m,n,p,s,t,v,w,y,qq,rr). By the twelfth week post transplantation, a further 6 patients (o,r,u,x,z,uu) had developed a significant increase in HCMV-IgG the precise onset of which is uncertain because of the time interval between consecutive serum samples. No serum was available from patient (q) until 14 weeks post transplantation when the HCMV-IgG level had increased by 35.6 EIU.

HCMV-IgG levels in patients (m,n,o,p,q,r,s,t,u,v,w,x, y,z,qq,rr,ss,uu) peaked between 6 and 14 weeks post transplantation and declined gradually thereafter. In patient (z), the HCMV-IgG level decreased by 21.5 EIU below the pretransplant level before showing a significant increase at 9 weeks after the transplant operation. Pretransplant levels of HCMV-IgG were approximated at 25, 23, 27, 26 and 32 weeks post transplantation in patients (o), (q), (u), (v) and (uu) respectively. At 32 weeks after the transplant operation, the HCMV-IgG level in patient (v) fell by 23.1 EIU below that recorded immediately pretransplant. However, sera taken from patients (m,n,p,r,s,t,w,x,y,z,qq, rr,ss) between 21 and 77 weeks

post transplantation continued to show a significant increase in HCMV-IgG ranging from 21.5 to 79.7 EIU above the pretransplant level.

(c) No active HCMV infection

In the 11 renal allograft recipients (bb,cc,dd,ee,ff, gg,ii,jj,kk,mm,oo) for whom sufficient data are available, the level of HCMV-IgG in sera obtained between 11 and 17 weeks post transplantation was an average of 3.89 EIU (range -14.1 to 22.7) lower than that recorded immediately pretransplant. By 31 to 61 weeks after the transplant operation, HCMV-IgG had fallen by an average of 4.75 EIU (range -14.7 to 32.6) below the pretransplant level. In patient (kk) who was seropositive for HCMV prior to renal transplantation, virus-specific IgG dropped below the pretransplant level by 20.6 and 32.6 EIU at 13 and 55 weeks respectively post transplantation. Patient (oo), who was also seropositive for HCMV pre-operatively, provided sera at 16 and 51 weeks after the transplant operation when the HCMV-IgG level was noted to be 22.7 and 23.6 EIU respectively below that recorded immediately pretransplant.

4.8 Post transplantation levels of HCMV-IgM measured by Labsystems ELISA

4.8.1 Specificity

HCMV-IgM levels in the sera of 14 renal donors and 47 renal allograft recipients immediately pretransplant were compared with the respective HSV and VZV CF antibody titres measured in parallel as described in Section 4.6.1.

Fourteen renal donors whose HCMV CF antibody titres were less than 1:2 were not tested for HCMV-IgM. Insufficient serum prevented the measurement of HCMV-IgM in 1 donor who was seropositive HCMV. One other donor was tested for HCMV-IgM and CF antibodies to HCMV and HSV but not VZV. The results are shown in Figure 51.

All of the 61 sera tested were negative for HCMV-IgM, the maximum recorded level being 19 EIU in a serum sample whose HSV and VZV CF antibody titres were 1:64 and 1:16 respectively. No correlation was observed between the CF antibody titres and the respective HCMV-IgM level.

4.8.2 Cross reaction with heterophile agglutinins

In this study, pretransplant sera from 44 of the 47 renal allograft recipients were tested for heterophile agglutinins and all were found to be negative . Of the 3 patients whose pretransplant sera were not tested for heterophile antibodies, 1 (aa) remained seronegative for HCMV throughout the period of observation and 2 (a,j) had evidence of primary HCMV infection.

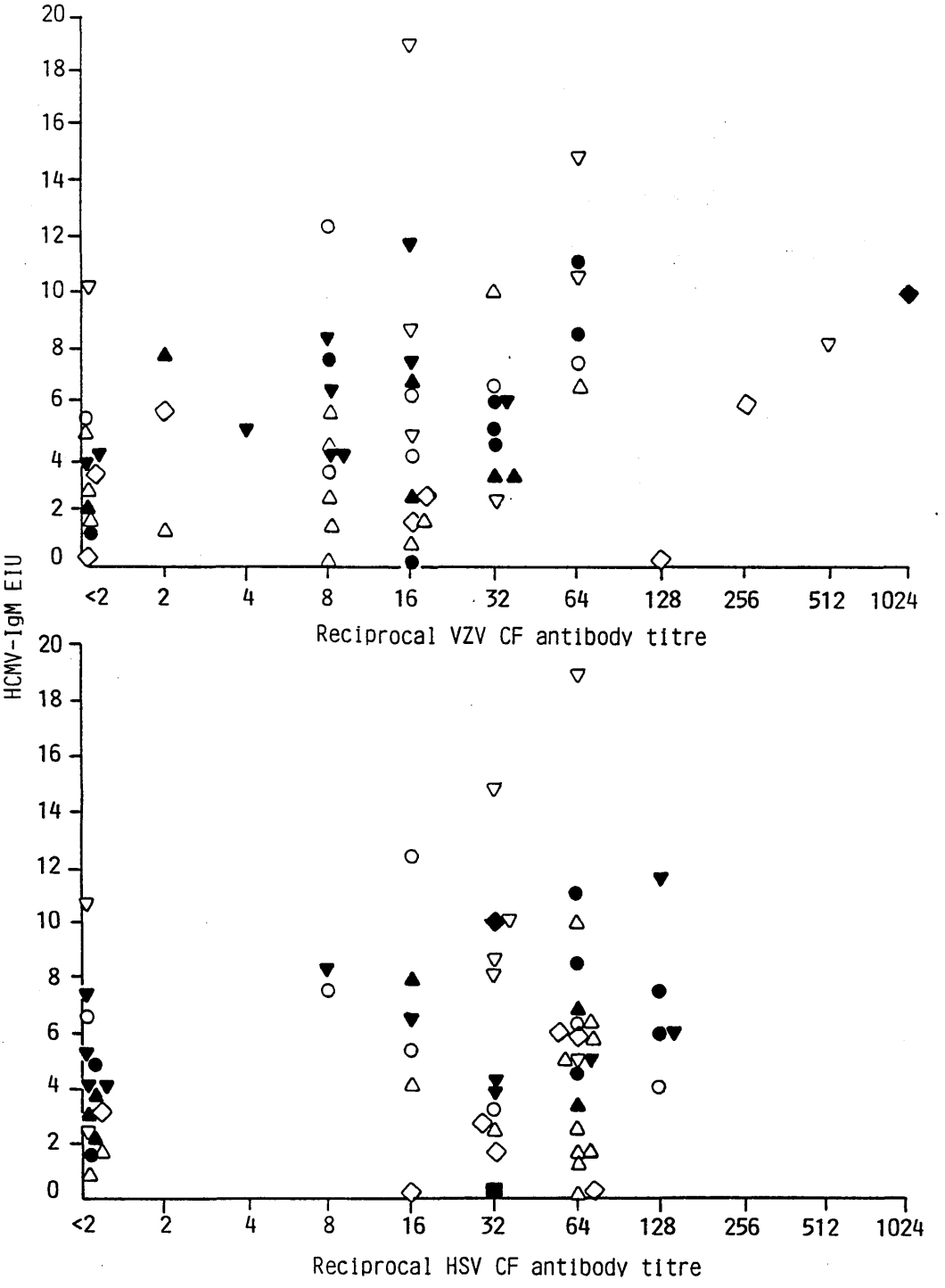
An investigation was also made into the possibility that post transplantation levels of HCMV-IgM measured by Labsystems ELISA may be unduly elevated as a result of cross reaction with heterotypic antibodies. In each patient with a positive or equivocal HCMV-IgM response only 1 serum, that with the highest HCMV-IgM level post transplantation, was tested and found to be negative for heterotypic antibody.

Figure 51 Correlation between HCMV-IgM levels measured by Labsystems ELISA and the respective HSV and VZV CF antibody titres

Sixty sera provided by 13 renal donors and 47 renal allograft recipients immediately pretransplant were tested in parallel for HSV and VZV CF antibody. All reagents were identical with the exception of the respective CF antigen. Serum taken from another renal donor was tested for HSV but not VZV CF antibody. Results are correlated with the respective HCMV-IgM levels measured in various batches of the Labsystems ELISA (○ ● △ ▲ ◇ ◆ ▽ ▼ ■).

HCMV-IgM EIU with	Spearman rank correlation
HSV CF antibody titre	0.161 ($p > 0.01$)
VZV CF antibody titre	0.147 ($p > 0.01$)

Spearman rank correlations were kindly provided by Dr G.D. Murray, Medical Statistics Unit, Western Infirmary, Glasgow.


HCMV-IgM EIU
20
18
16
14
12
10
8
6
4
2
0
<2
2
4
8
16
32
64
128
256
512
1024
Reciprocal VZV CF antibody titre
Reciprocal HSV CF antibody titre

4.8.3 Correlation between HCMV-IgM levels measured by Labsystems ELISA and IgM-RF titres

In this study, 74 sera from 21 renal allograft recipients (a,c,d,e,f,h,i,j,n,q,r,s,v,w,x,y,z,qq,rr,tt,uu) were each tested for RF by latex-slide agglutination and 27 (38%) from 7 patients (c,d,f,i,j,v,tt) were positive. A further 2 sera, one each from patients (i) and (z), were equivocal for RF. Because this is a qualitative test and non-specific reactions can occur due to, for example, hyperlipidaemia, a quantitative assessment of IgM-RF levels was undertaken. The number of samples precluded the testing of IgM-RF in all HCMV-IgM positive sera and therefore only 44 of the 74 sera positive for HCMV-IgM were submitted for measurement of the IgM-RF level. The 44 sera were provided by 20 renal allograft recipients (a,c,d,e,f,h,i,j,n,q,r,s,v,w,x,y,z,qq,tt,uu) and, whenever possible, samples showing the highest level of HCMV-IgM within each individual were chosen. Only 2 sera from 1 patient (d) had levels of IgM-RF higher than the normal limit. Of the 43 sera tested for RF by latex-slide agglutination and IgM-RF by ELISA, 24 (54%) from 16 individuals (a,c,e,f,h,i,n,q,r,s,w,x,y,z,qq,uu) were considered to be negative by both methods of analysis. A further 18 sera (37%) from 6 renal allograft recipients (c,d,i,j,v,tt) were found to be positive for RF by latex-slide agglutination but only 2 (4.6%) from patient (d) had levels of IgM-RF above the upper limit of normal. One serum from patient (i) which was equivocal for RF by latex-

slide agglutination had an IgM-RF level of 175 U/ml.

The temporal relationship between changes in post transplantation levels of IgM-RF and HCMV-IgM was assessed in each of 8 patients, 3 (c,d,i) with primary HCMV infection, 3 (r,t,qq) with reinfection and/or reactivation and 2 (cc,mm) who remained seronegative for HCMV throughout the post transplant period. All sera obtained from these 8 renal allograft recipients were simultaneously tested for IgM-RF and HCMV-IgM thereby allowing inter- and intra-patient comparisons to be made between antibody levels. Furthermore, patients (c) and (t) were transplanted on the same day with a kidney obtained from the same renal donor. The same is also true for patients (i and qq) and (r and mm).

In patients (c) and (d), a significant increase in IgM-RF levels had occurred by the eighth week after the transplant operation, no such rise being evident in previous samples obtained up to 5 and 7 weeks respectively post transplantation (Figure 52). IgM-RF levels in patients (c) and (d) continued to increase until 11 and 10 weeks respectively post transplantation after which they decreased, approximating pretransplant levels at 52 and 39 weeks respectively after the transplant operation. Patient (c) also showed rising levels of IgM-RF with a maximum titre of 310 U/ml being recorded in a serum provided at 8 weeks post transplantation. By 10 weeks after the transplant operation IgM-RF had decreased to 175 U/ml, a

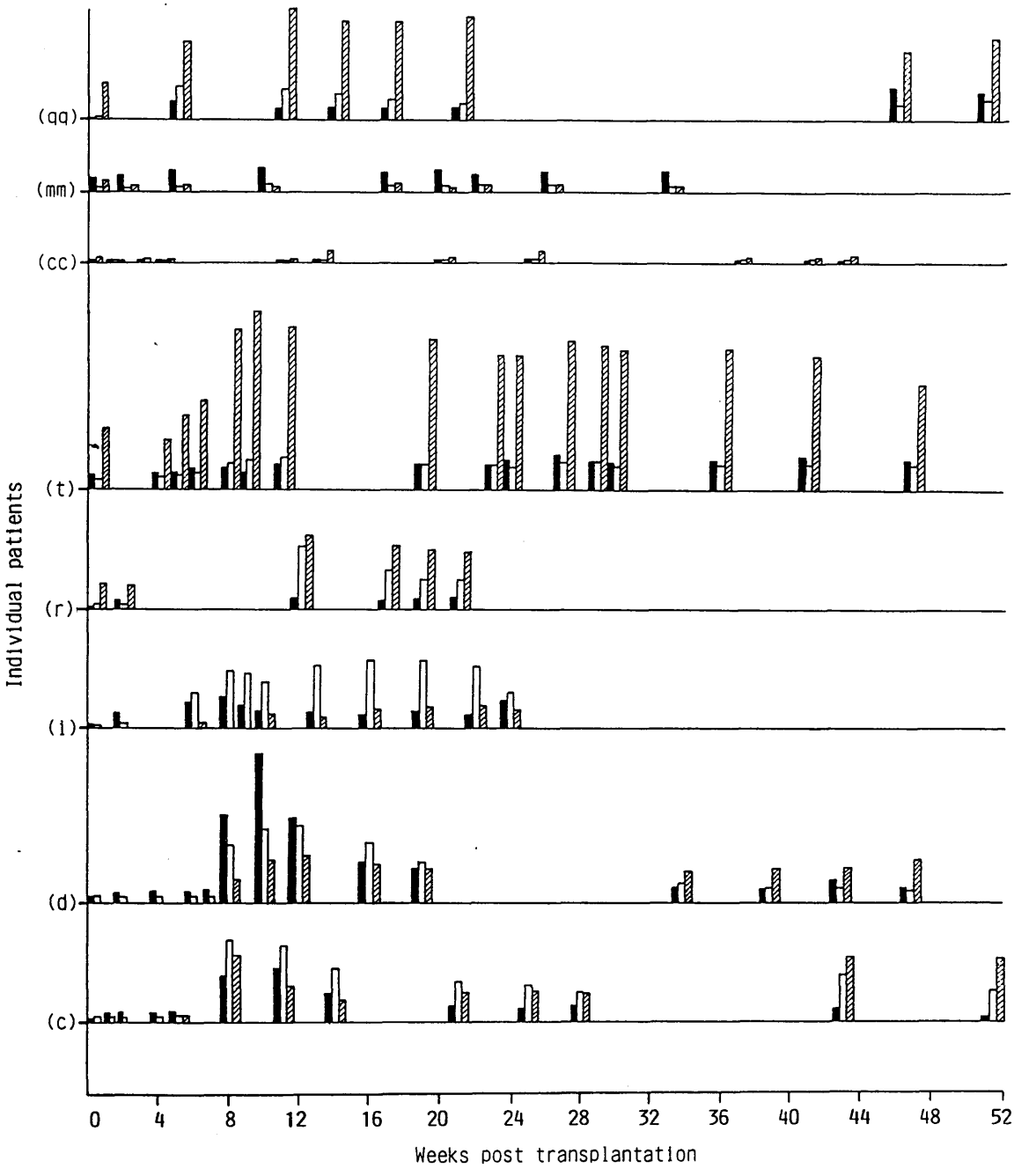
Figure 52 Correlation between post transplantation levels of HCMV-IgM, HCMV-IgG and IgM-RF

The figure shows the post transplantation levels of HCMV-IgM (□) and HCMV-IgG (▨) in each of 8 renal allograft recipients, 3 (c,d,i) with primary HCMV infection, 3 (r,t,qq) with reinfection and/or reactivation and 2 (cc,mm) with no evidence of active HCMV infection. Results are correlated with IgM-RF levels (■). The following scales are used:-

□ : 20 EIU of HCMV-IgM

▨ : 20 EIU of HCMV-IgG

▌ : 200U/ml of IgM-RF


(qq)
(mm)
(cc)
(t)
(r)
(i)
(d)
(c)
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation

level which was maintained with minimal fluctuation for at least 24 weeks post transplantation.

Of the 3 patients with reinfection and/or reactivation of HCMV, patient (qq) showed a significant increase in IgM-RF from less than 80 U/ml immediately pretransplant to 205 U/ml at 5 weeks after the transplant operation. Thereafter, an IgM-RF level of approximately 120 U/ml was maintained from 11 to 21 weeks post transplantation while sera obtained at 46 and 59 weeks after the transplant operation showed the IgM-RF level to be 350 and 275 U/ml respectively. In patient (t), only minor fluctuations in post transplantation levels of IgM-RF were recorded while sera from patient (r) were unavailable between 2 and 12 weeks after the transplant operation during which time a significant increase in the IgM-RF level may have occurred.

Neither of the 2 patients (cc,mm) who remained seronegative for HCMV showed any significant increase in IgM-RF throughout the post transplant period.

Synchronous changes in post transplantation levels of IgM-RF and HCMV-IgM were noted in each of patients (c), (d) and (qq) but not in patient (r) whose IgM-RF levels did not alter significantly throughout the post transplant period while levels of HCMV-IgM had increased and subsequently declined. Between 6 and 10 weeks after the transplant operation, IgM-RF and HCMV-IgM levels in patient (i) were seen to increase and then decrease in parallel. Thereafter, no significant change in the HCMV-IgM level was noted until a decrease of 29.5 EIU was recorded in a serum

taken at 24 weeks post transplantation. During the same period IgM-RF levels did not change significantly.

Post transplantation levels of IgM-RF and HCMV-IgM measured by ELISA did not correlate with those of HCMV-IgG. For example, throughout the post transplant period, patients (i) and (qq) had equivalent levels of IgM-RF but HCMV-IgM was consistently higher in patient (i) who had lower levels of HCMV-IgG than those found in patient (qq).

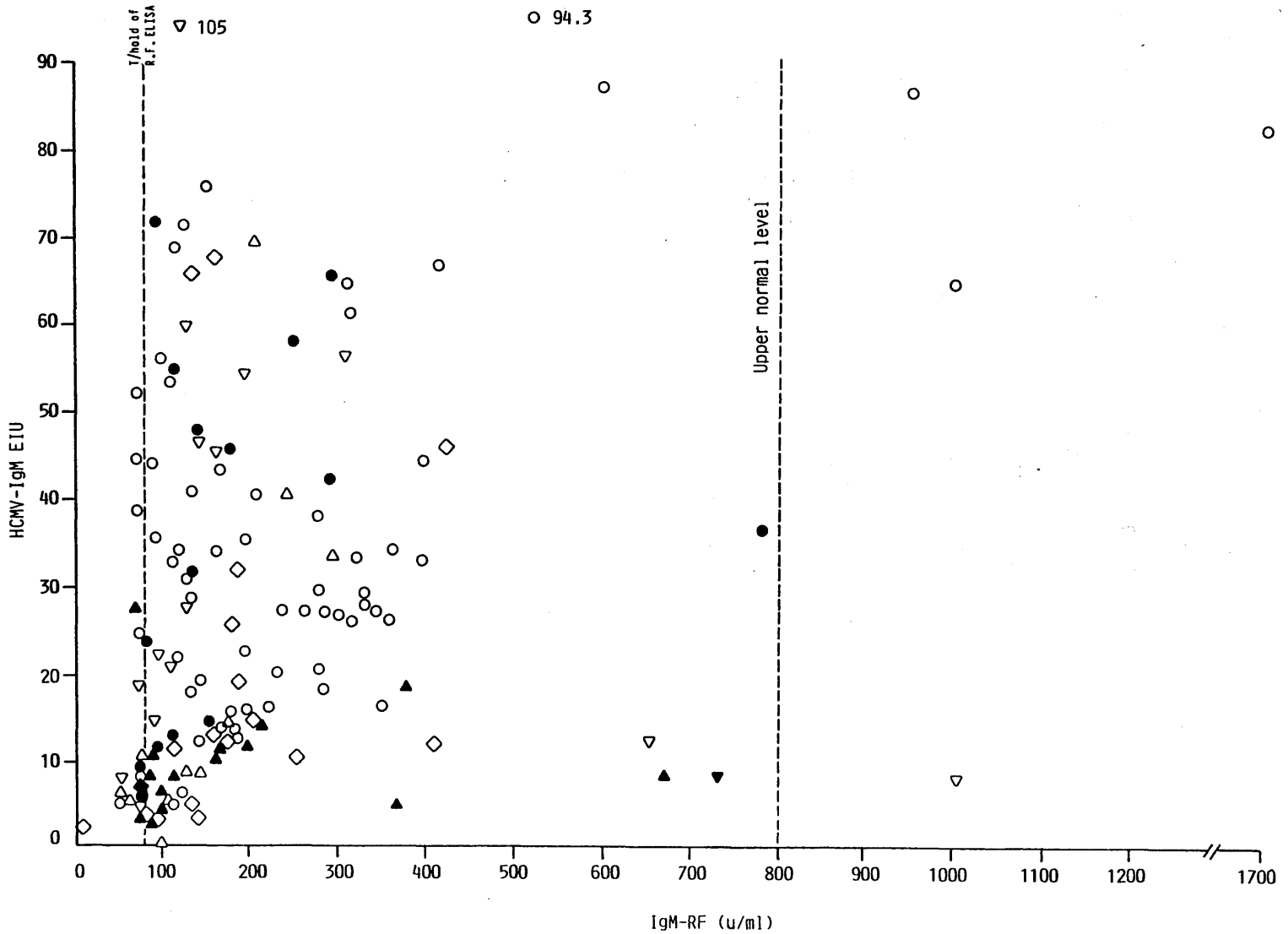
In order to establish whether IgM-RF was complexing with HCMV-IgG and producing false positive results in the HCMV-IgM ELISA, 136 sera provided by 33 renal allograft recipients were each analysed for IgM-RF and the results correlated with the respective HCMV-IgM level. The results are shown in Figure 53. No correlation was observed between levels of IgM-RF and those of HCMV-IgM. For example, in all sera whose IgM-RF titre fell between 100 and 200U/ml, HCMV-IgM levels ranged from 3.5 to 75.4 EIU. Furthermore, in 4 sera considered to be negative for HCMV-IgM (range 7.66 to 12.2 EIU), IgM-RF levels ranged from 650 to 1000 EIU.

In a further attempt to determine whether IgM-RF present in serum samples would produce false positive reactions in the HCMV-IgM ELISA, reconstruction experiments were performed in which a known number of units of polyclonal RF were added to aliquots of a serum whose IgM-RF, HCMV-IgM and HCMV-IgG levels were < 80 U/ml, 6.08 EIU and 61.0 EIU respectively. This serum was selected because

Figure 53 Correlation between post transplantation levels of HCMV-IgM and IgM-RF

In each of 136 sera provided by 33 renal allograft recipients the HCMV-IgM level measured by Labsystems ELISA was correlated with the respective IgM-RF titre. Whenever possible a minimum of 3 sera were obtained from each renal allograft recipient, one immediately pretransplant, another containing the highest recorded level of HCMV-IgM and a third taken near the end of the first year after renal transplantation. All sera were positive for HCMV-IgG by Labsystems ELISA. HCMV-IgM levels were measured in any 1 of 9 different batches of the Labsystems ELISA (O, ● , △ , ▲ , ◇ , ◆ , ▽ , ▼ and ■ respectively).

IgM-RF levels were kindly provided by Mrs J. Veitch and Professor K. Whaley, Immunopathology Department, University of Glasgow.


105
94.3
T/hold of R.F. ELISA
Upper normal level
HCMV-IgM EIU
IgM-RF (u/ml)
0
10
20
30
40
50
60
70
80
90
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1700

it was the only one known to be positive for HCMV-IgG but negative for HCMV-IgM and had undetectable (<80 U/ml) levels of IgM-RF. All sera were negative for HCMV-IgM and there was no correlation between the concentration of the polyclonal RF and the level of HCMV-IgM (Figure 54). For each serum tested the intra-assay reproducibility in the level of HCMV-IgM was very good, the average variation in antibody levels being 2.28 EIU (range 0 to 6.81).

4.8.4 Correlation betweem HCMV-IgM levels measured by Labsystems ELISA and rubella-specific IgM levels measured by Abbott ELISA

RF causing false positive reactions in the Labsystems indirect HCMV-IgM ELISA may also be expected to produce falsely elevated IgM levels to any other infectious agent when similarly tested by indirect ELISA.

No correlation was noted between the HCMV-IgM level measured by Labsystems ELISA and the corresponding level of rubella-IgM tested by Abbott ELISA (Figure 55). Thirty seven (97.3%) of the sera assayed were clearly negative for rubella-IgM although the HCMV-IgM level ranged from 1.51 to 105.1 EIU. The remaining serum, that from patient (b) at 13 weeks post transplantation, had a level of rubella-IgM equivalent to the low positive control while the HCMV-IgM level was 38.3 EIU and no RF was detected by latex-slide agglutination.

Figure 54 HCMV-IgM levels in serum to which polyclonal RF had been added

A serum with an IgM-RF, HCMV-IgM and HCMV-IgG level of < 80 U/ml, 6.08 EIU and 61.0 EIU respectively was divided into aliquots. Three preparations of polyclonal RF at a starting concentration of 15000 U/ml (●), 4400 U/ml (▲) or 2600 U/ml (■) were added to separate aliquots so that the final concentration of RF ranged from 2000 to 31 U/ml, 800 to 27 U/ml or 500 to 31 U/ml respectively. These ranges in the concentration were chosen because they reflected those found in sera provided by the renal transplant recipients. All reconstructed sera were tested in parallel and on 2 occasions within a single batch of the Labsystems HCMV-IgM ELISA. The intra-assay variation in the level of HCMV-IgM within each reconstructed serum is represented by a solid vertical line.

IgM-RF levels were kindly provided by Mrs J. Veitch and Professor K. Whaley, Immunopathology Department, University of Glasgow.

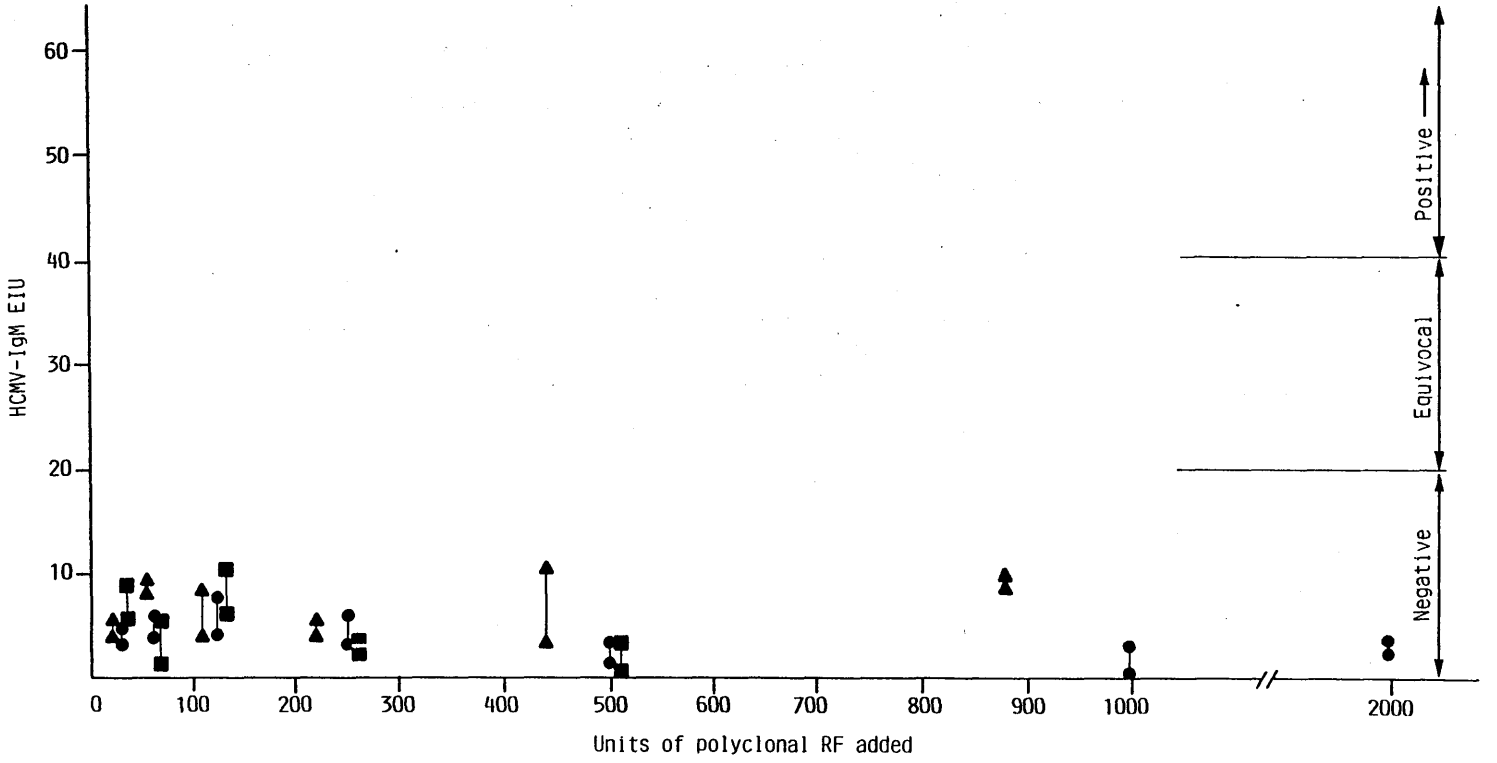
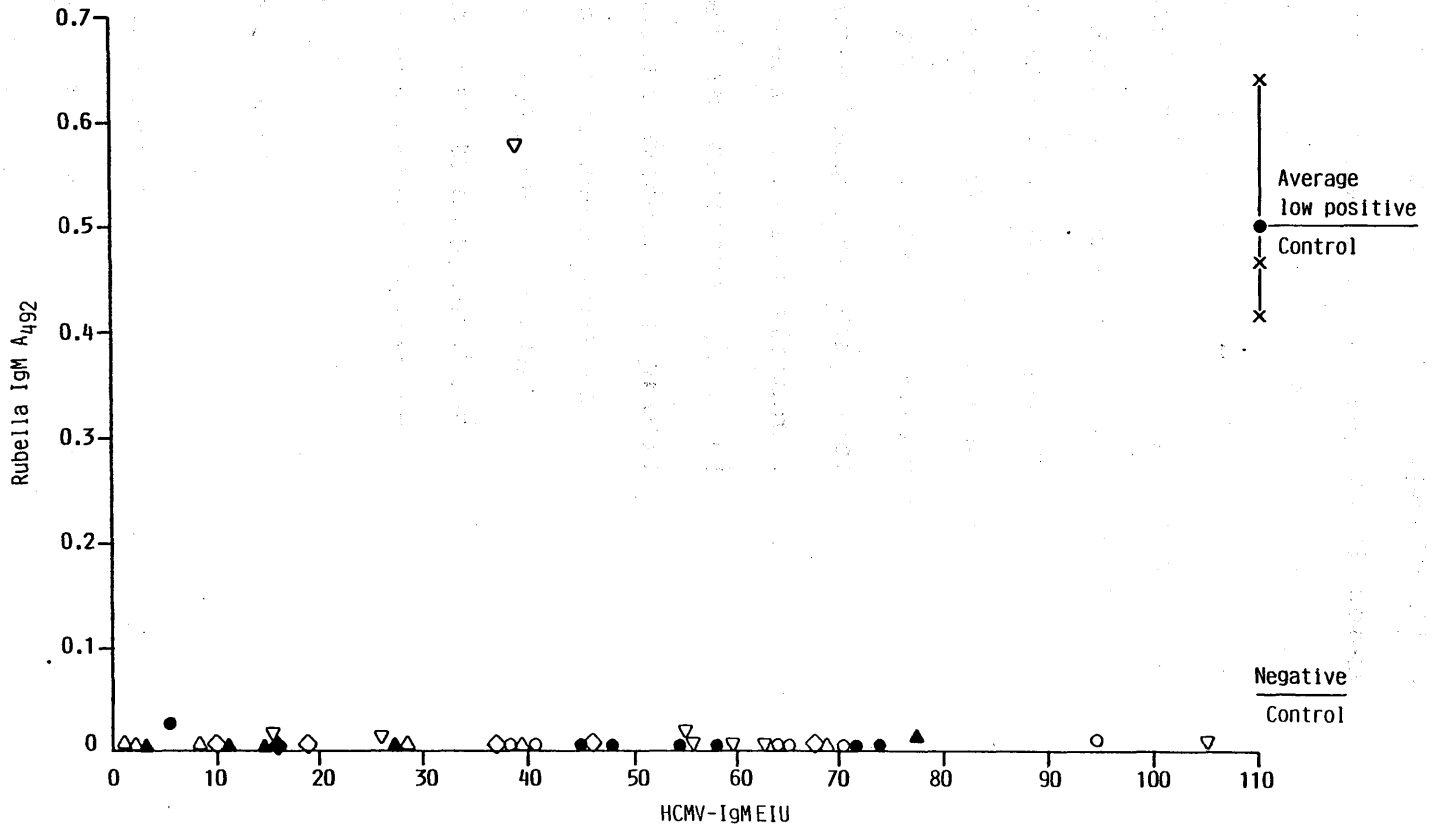

HCMV-IgM EIU
60
50
40
30
20
10
0
100
200
300
400
500
600
700
800
900
1000
2000
Units of polyclonal RF added
Positive
Equivocal
Negative

Figure 55 Correlation between HCMV-IgM levels measured by Labsystems ELISA and rubella-IgM levels tested by Abbott ELISA

A post transplantation serum sample was obtained from each of 38 renal allograft recipients, 11 (a,b,c,d, e,f,g,h,i,j,k) with primary HCMV infection, 19 (l,m,n,o,p,q,r,s,t,u,v,w,x, y,z,qq,rr,ss,uu) with reinfection and/or reactivation and 8 (bb,cc,dd,ee,ff,ii, kk,oo) with no active HCMV infection. Whenever possible the serum showing the highest recorded level of HCMV-IgM within each patient was chosen. All sera were tested on 1 occasion within a single batch of the Abbott indirect rubella-IgM ELISA and the results correlated with HCMV-IgM levels measured in any 1 of 9 different batches of the Labsystems indirect ELISA (O, ● , △ , ▲ , ◇ , ◆ , ▽ , ▼ and ■ respectively).


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Rubella IgM A_{492}
0
10
20
30
40
50
60
70
80
90
100
110
HCMV-IgM EIU
Average
low positive
Control
Negative
Control

4.8.5 Kinetics of the post transplantation HCMV-IgM response

(a) Primary HCMV infection (Figures 56A and 59A)

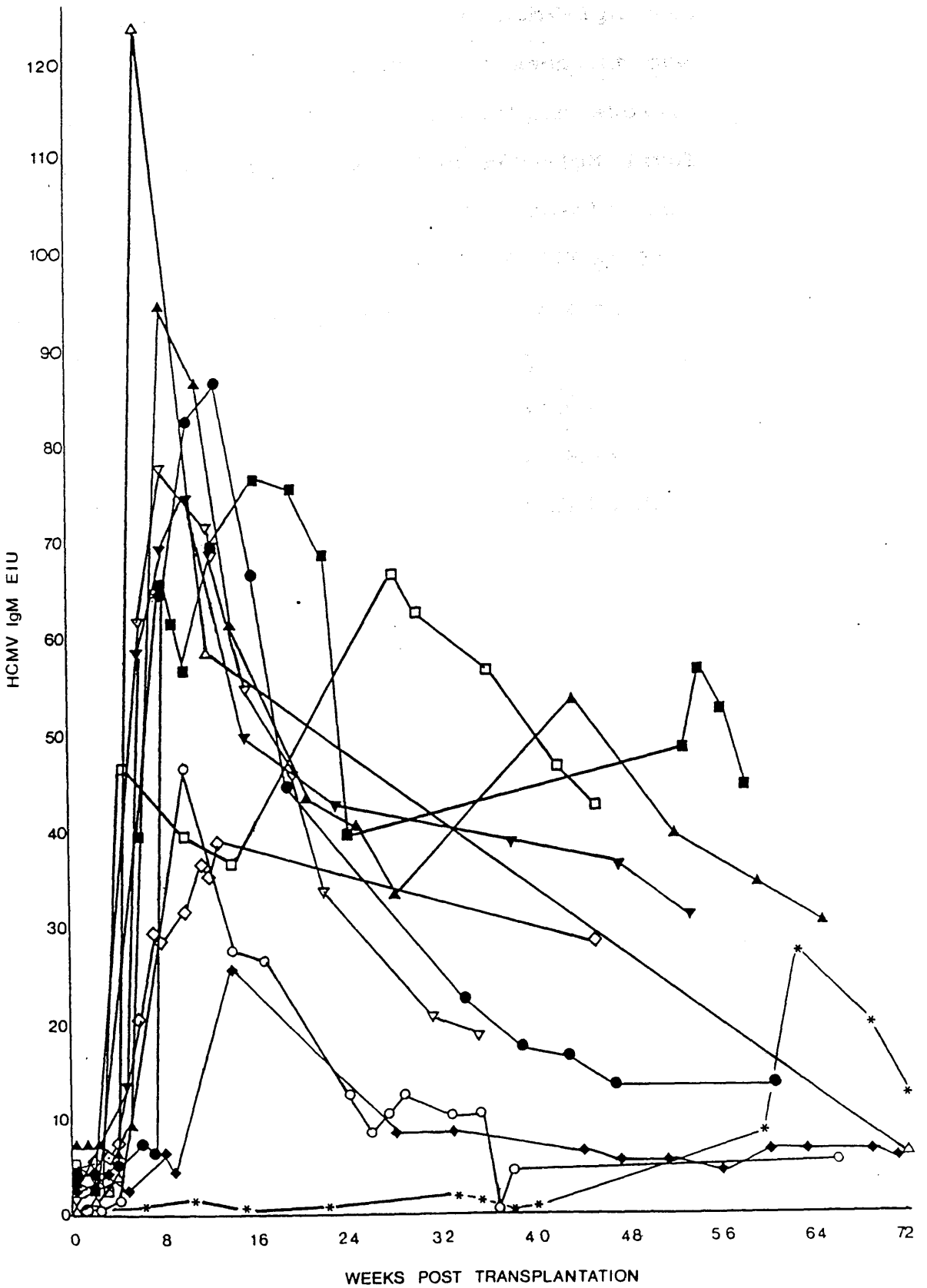
The earliest serum to show a significant increase in HCMV-IgM was taken from patient (e) at 5 weeks after the transplant operation. By 6 weeks post transplantation the HCMV-IgM level had risen significantly in 3 other renal allograft recipients (a,f,j) while patients (c,d,i) showed a significant HCMV-IgM response which was first recorded at 8 weeks after the transplant operation. The last renal allograft recipient (h) to show a significant increase in HCMV-IgM did so in a serum provided at 10 weeks post transplantation although no sera were available during the preceding 6 weeks. A significant HCMV-IgM response was never observed in patients (b), (g) and (k) who first showed serological evidence of primary HCMV infection in a serum obtained at 59 weeks post transplantation.

In patient (a), the HCMV-IgM level increased from 6.5 EIU at 4 weeks post transplantation to 123 EIU by the sixth week after the transplant operation. The level of HCMV-IgM then decreased to 58.1 EIU at 12 weeks post transplantation while the next available serum taken 66 weeks later had an HCMV-IgM level of 12.4 EIU.

Patient (c) showed a significant HCMV-IgM response of 94.3 EIU in a serum provided at 8 weeks post transplantation. By 21 weeks after the transplant operation, HCMV-IgM had dropped to a level of 43.4 EIU which was then maintained with minimal fluctuation until at

Figure 56A Post transplantation levels of HCMV-IgM measured by Labsystems ELISA: Primary infection

The figure shows the levels of HCMV-IgM between 0 and 76 weeks post transplantation in each of 11 renal allograft recipients (a to k) with primary HCMV infection.


HCMV IgM EIU
120
110
100
90
80
70
60
50
40
30
20
10
0
0
8
16
24
32
40
48
56
64
72
WEEKS POST TRANSPLANTATION

sera were obtained during the preceding 6 weeks. Thereafter, HCMV-IgM gradually decreased to a level of 12.1 EIU by 24 weeks after the transplant operation.

Patient (i) first showed an HCMV-IgM response of 39.7 EIU in a serum provided at 6 weeks post transplantation. Two weeks later HCMV-IgM had increased to a level of 64.6 EIU which was maintained with minimal variation until 22 weeks after the transplant operation. A serum taken at 24 weeks post transplantation showed the HCMV-IgM level to be 39.2 EIU. The next available serum was obtained after an interval of 29 weeks when the HCMV-IgM level was 47.8 EIU.

In patient (j), HCMV-IgM increased from 3.86 EIU at 4 weeks post transplantation to 61.5 EIU by the sixth week after the transplant operation. The HCMV-IgM response continued to rise, reaching a level of 77.5 EIU two weeks later. Thereafter, the HCMV-IgM level gradually decreased and was noted to be 18.5 EIU in the last serum obtained from this patient at 38 weeks post transplantation.

Two other patients (b,g) with primary HCMV infection never showed evidence of a significant HCMV-IgM response. In patient (b), HCMV-IgM increased from 6.4 to 20.2 EIU between 4 and 6 weeks respectively post transplantation. By the seventh week after the transplant operation, HCMV-IgM had risen to a level of 29.8 EIU which was maintained with minimal fluctuation for at least a further 6 weeks. No other sera were available until 45 weeks post transplantation when the HCMV-IgM level was 29.6 EIU. A serum taken from patient (g) at 9 weeks after the

transplant operation had an HCMV-IgM level of 4.34 EIU. The next serum was obtained 5 weeks later when the HCMV-IgM level had increased to 26.0 EIU. There was an interval of 14 weeks until the next serum sample which showed the HCMV-IgM level to be 8.07 EIU. In all subsequent sera the HCMV-IgM level never exceeded 7.86 EIU.

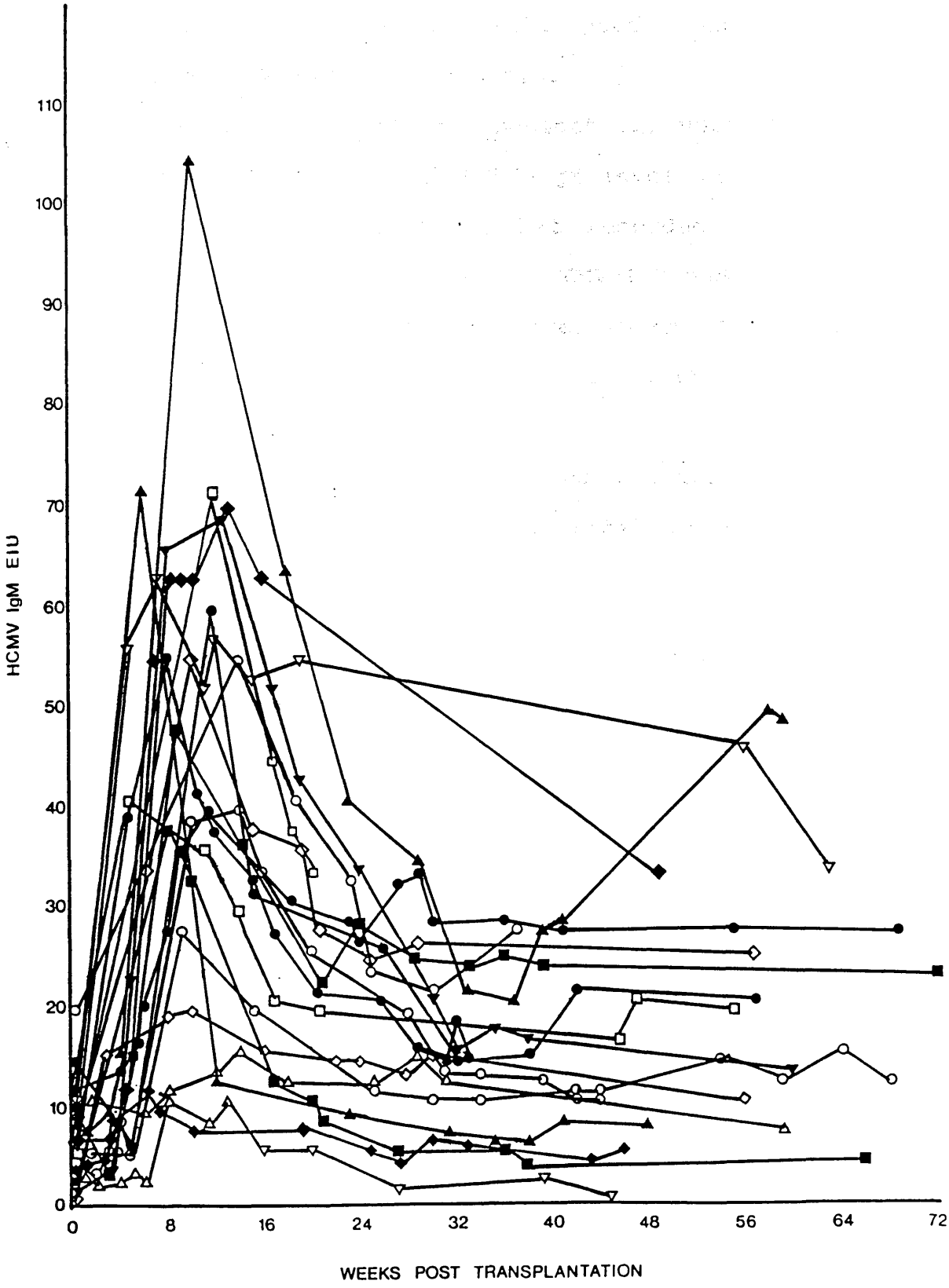
(b) Reinfection and/or reactivation (Figures 56B and 59B)

Thirteen renal allograft recipients (n,q,r,s,v,w,x,y, z,qq,rr,tt,uu) developed a significant HCMV-IgM response after the transplant operation, while 7 (l,m,o,p,t,u,ss) did not. The earliest sera to be regarded as positive for HCMV-IgM were taken from patients (s) and (qq) at 5 weeks post transplantation. By the eighth week after the transplant operation 4 other patients (n,v,w,y) had evidence of a significant increase in HCMV-IgM while patients (r), (x), (z), (rr) and (uu) became positive for HCMV-IgM at 12, 9, 10, 10 and 11 weeks respectively post transplantation. The last patients (q and tt) to show a significant HCMV-IgM response did so in sera provided at 14 weeks after the transplant operation.

In patient (n), HCMV-IgM increased from 3.74 EIU at 3 weeks post transplantation to 22.4 EIU at 5 weeks after the transplant operation. The HCMV-IgM response continued and reached a peak of 68.0 EIU at 13 weeks post transplantation. Thereafter, the HCMV-IgM level gradually decreased so that by 32 weeks after the transplant

Figure 56B Post transplantation levels of HCMV-IgM measured by Labsystems ELISA: Reinfection and/or reactivation

The figure shows the levels of HCMV-IgM between 0 and 77 weeks post transplantation in each of 20 renal allograft recipients (l,m,n,o,p,q,r,s,t, u,v,w,x,y,z,qq,rr,ss,tt,uu) with rein-infection and/or reactivation of HCMV.


HCMV IgM EIU
0
10
20
30
40
50
60
70
80
90
100
110
0
8
16
24
32
40
48
56
64
72
WEEKS POST TRANSPLANTATION

operation it had fallen to 15.8 EIU. This level was maintained for at least a further 6 weeks while the next available serum taken at 60 weeks post transplantation showed an HCMV-IgM level of 13.6 EIU.

No sera were received from patient (q) until 14 weeks post transplantation when the HCMV-IgM level was 54.6 EIU, an increase of 35.6 EIU above that recorded immediately pretransplant. Eleven weeks later, HCMV-IgM had dropped to 24.0 EIU and it remained at this level in the 2 subsequent sera obtained at 30 and 37 weeks after the transplant operation.

Patient (r) first provided a post transplant serum sample 2 weeks after receiving the renal allograft. At this time the HCMV-IgM level was 4.22 EIU but it then increased being 71.1 EIU in the next available serum taken 10 weeks later. The HCMV-IgM level then gradually decreased and was 32.7 EIU in the final serum to be obtained from this patient at 21 weeks post-operatively.

An HCMV-IgM response of 44.8 EIU was recorded in a serum provided by patient (s) at 5 weeks after the transplant operation. Thereafter the HCMV-IgM level did not change significantly in 5 other sera obtained up to 19 weeks post transplantation. No serum was then available until 56 weeks after the transplant operation when the HCMV-IgM level was 45.2 EIU.

In patient (v), HCMV-IgM increased from 11.1 EIU immediately pretransplant to 54.5 EIU at 8 weeks post-operatively. Thereafter, HCMV-IgM, gradually decreased to a

level of 20.4 EIU at 21 weeks after the transplant operation. This level was then maintained until at least 33 weeks post transplantation after which no other specimens were available for analysis.

Between 2 and 6 weeks post transplantation, the HCMV-IgM level in patient (w) increased from 8.02 to 71.7 EIU. The next serum was provided 6 weeks later when the HCMV-IgM level had decreased to 13.3 EIU. In all subsequent sera obtained up to 48 weeks post-operatively, the HCMV-IgM level never exceeded 8.75 EIU.

Patient (x) showed an HCMV-IgM level of 3.1 EIU in a serum taken 1 week after the transplant operation while sera obtained at 5 and 9 weeks post transplantation had an HCMV-IgM level of 14.3 and 47.6 EIU respectively. By 21 weeks after the transplant operation, HCMV-IgM had fallen to a level of 22.8 EIU which did not change significantly in 5 subsequent sera taken up to 39 weeks post-operatively. An HCMV-IgM level of 22.8 EIU was observed in the next serum provided by this patient at 76 weeks after the transplant operation.

The HCMV-IgM response in patient (y) gradually increased from 4.35 EIU at 3 weeks post transplantation to 63.0 EIU at 8 weeks after the transplant operation. This level was then maintained with minimal variation until at least 16 weeks post-operatively. The next available serum taken 33 weeks later showed the HCMV-IgM level to be 33.3 EIU.

Between 3 and 10 weeks after the transplant operation, the HCMV-IgM level in patient (z) increased from 9.1 to 105.1 EIU. By 29 weeks post transplantation, HCMV-IgM had decreased to a level of 24.1 EIU which was equivalent to that observed in 4 other sera obtained up to 41 weeks after the transplant operation. There was an interval of 17 weeks until the next serum sample which had an HCMV-IgM level of 48.6 EIU.

Patient (qq) first provided a post transplantation serum sample 5 weeks after receiving the renal allograft. At this time the HCMV-IgM level was 40.6 EIU. A serum taken 6 weeks later showed the HCMV-IgM level to be 34.3 EIU. By 21 weeks post transplantation, HCMV-IgM had dropped to a level of 19.3 EIU which did not change significantly in the 2 subsequent sera obtained from this patient at 46 and 51 weeks after the transplant operation.

In patient (rr), the post transplantation HCMV-IgM response gradually increased and reached a maximum recorded level of 55.0 EIU at 10 weeks after the transplant operation. Thereafter, the HCMV-IgM level gradually decreased and was 24.0 EIU at 25 weeks post transplantation. Four weeks later the HCMV-IgM level was 24.8 EIU while the next recorded serum taken at 57 weeks after the transplant operation had an HCMV-IgM level of 24.2 EIU.

Between 5 and 10 weeks post transplantation, the HCMV-IgM level in patient (tt) had increased from 4.73 to 37.5 EIU. A serum taken 4 weeks later showed the HCMV-IgM level

to be 40.1 EIU. By 31 weeks after the transplant operation, the HCMV-IgM level had decreased to 13.2 EIU. In 5 subsequent sera obtained up to 54 weeks post-operatively, the HCMV-IgM level never exceeded 14.3 EIU.

An HCMV-IgM level of 5.38 EIU was recorded in a serum taken from patient (uu) at 2 weeks post transplantation. The next serum sample was obtained 11 weeks after the transplant operation when the HCMV-IgM level was 59.6 EIU. Twenty one weeks later, HCMV-IgM had dropped to a level of 14.0 EIU which was maintained with minimal variation in 3 other sera provided at 38, 42 and 57 weeks post-operatively.

In patients (l), (p), (u) and (ss), the HCMV-IgM level never exceeded 15.3, 18.9, 10.6 and 11.7 EIU respectively. The highest recorded level of HCMV-IgM in patient (m) was 37.8 EIU in a serum taken at 8 weeks after the transplant operation although no sera were available during the intervening period. Nine weeks later, HCMV-IgM had decreased to a level of 12.9 EIU which did not change significantly in the 6 subsequent sera obtained up to 66 weeks post-operatively. Between 4 and 15 weeks after the transplant operation, only 3 sera were provided by patient (o), the highest HCMV-IgM level being 27.3 EIU in a serum taken at 9 weeks post transplantation. At 25 weeks after the transplant operation the HCMV-IgM level was 11.9 EIU. Five other sera obtained between 30 and 59 weeks post transplantation showed an average HCMV-IgM level of 10.5

EIU (range 9.5 to 11.5). In patient (t), the HCMV-IgM response gradually increased from 13.1 EIU at 4 weeks after the transplant operation to 38.4 EIU at 11 weeks post transplantation. No serum was then available until 8 weeks later when HCMV-IgM had decreased to a level of 29.5 EIU which was equivalent to that observed in 9 other sera obtained between 23 and 55 weeks post-operatively.

In conclusion therefore, no difference was observed between the pattern of the HCMV-IgM response in patients (l,m,n,o,p,q,r,s,t,u,v,w) who shed HCMV and those who did not (x,y,z,qq,rr,ss,tt,uu). Similarly, the HCMV-IgM response could not differentiate renal allograft recipients (l,m,s,t,x,y,u,qq) who each received their kidney from an HCMV-seropositive donor from those (n,p,r,v,z,rr,ss,tt) who were each transplanted with a kidney from an HCMV-seronegative donor. The donor serological status was not available for 4 other renal allograft recipients (o,q,w,uu).

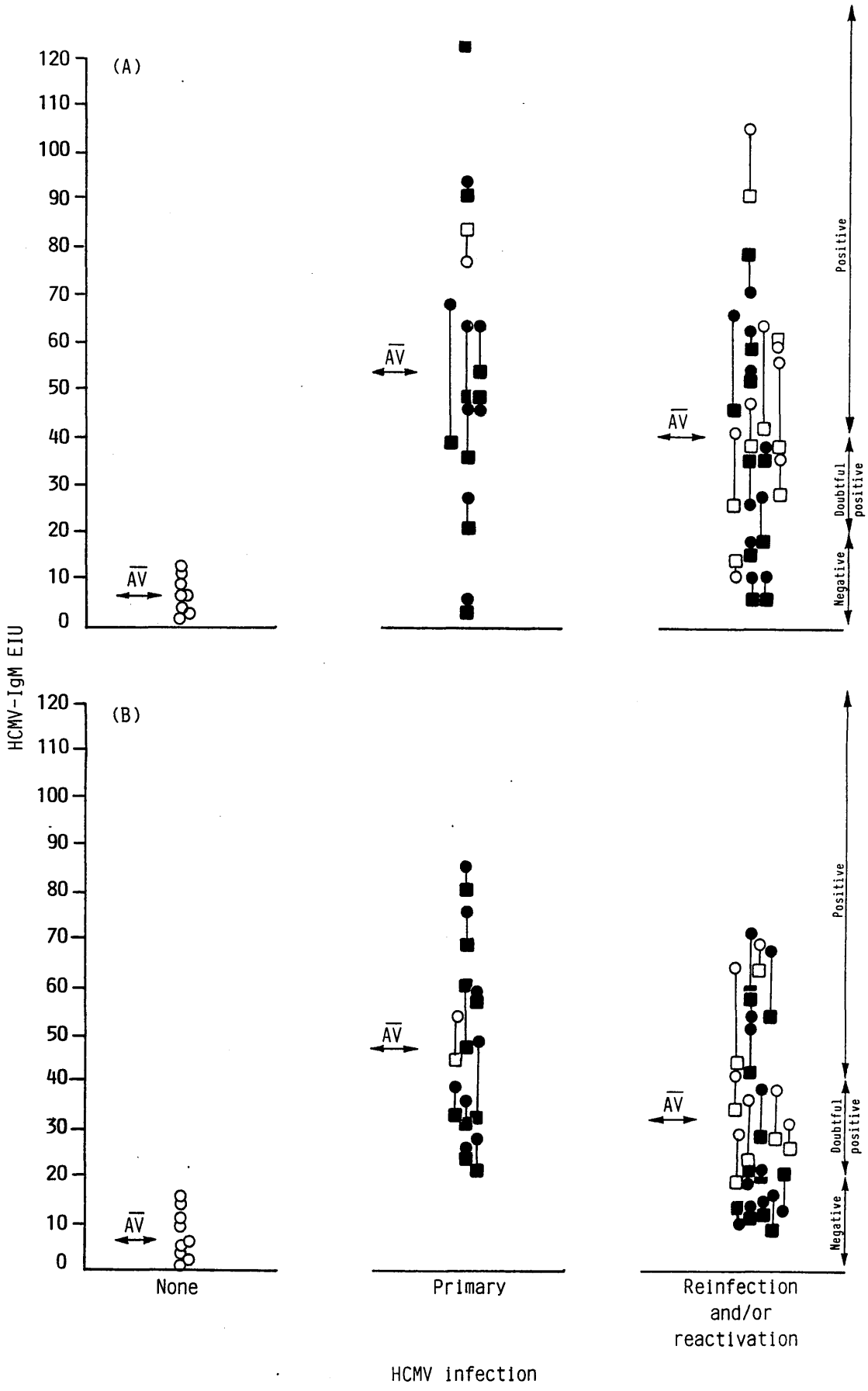
4.8.6 Comparison between HCMV-IgM levels in primary infection and reinfection and/or reactivation

Some authors have suggested that post transplantation HCMV-IgM levels may be significantly higher in patients with primary infection than in those with reinfection and/or reactivation of HCMV (Wreghitt et al., 1986). Therefore, a comparison was made between HCMV-IgM levels in both these groups of patients during the course of this project. To avoid inter-assay variation when comparing HCMV-IgM levels in sera obtained from different patients it is necessary to reanalyse such samples in parallel within 1 batch of the ELISA. Because of the large number of sera involved it was impossible to retest all samples from all patients in this project. Therefore, the comparison between HCMV-IgM levels in patients with primary HCMV infection and those with reinfection and/or reactivation was based on the simultaneous testing of 2 sera from each individual, 1 taken between 5 and 11 weeks and another between 9 and 18 weeks post transplantation. Examination of the serological response has already shown that the HCMV-IgM levels in such sera would represent the highest recorded in 29 of the 30 patients with HCMV infection during the first year after the transplant operation. Results were compared with those already obtained in another batch of the ELISA (Figure 57).

A comparison between HCMV-IgM levels in patients with primary HCMV infection and those with reinfection and/or reactivation of the virus found no obvious difference in samples obtained from these individuals between 5 and 11

Figure 57 Comparison between HCMV-IgM levels in renal allograft recipients with primary HCMV infection, reinfection and/or reactivation or no active HCMV infection

Between 5 and 11 weeks after the transplant operation, a serum was obtained from each of 10 renal allograft recipients (a,b,c,d,e,f,g,h,i,j) with primary HCMV infection, 18 (l,m,n,o,p,s,t,u,v,w,x,y,z,qq,rr,ss,tt,uu) with reinfection and/of reactivation and 8 (bb,cc,dd,ff,ii,kk,mm,oo) with no evidence of active HCMV infection (Figure A). With the exception of patient (ss), a serum was also provided by each of the aforementioned renal allograft recipients between 12 and 18 weeks post transplantation (Figure B). Patient (ee) who showed no evidence of active HCMV infection and patients (q) and (r) who developed reinfection and/or reactivation of HCMV after the transplant operation also provided a serum between 12 and 18 weeks but not between 5 and 11 weeks post transplantation. Sera were initially tested in 1 of 9 different batches of the ELISA (● / ○) and were retested in parallel within a single batch of the ELISA (■ / □). The variation in the magnitude of the HCMV-IgM response when measured in 2 batches of the Labsystems ELISA is represented by a solid vertical line. (● / ■) or (○/ □) differentiate sera obtained from those renal allograft recipients who shed or did not shed HCMV respectively. Within each group of patients ◄AV► shows the average change in the HCMV-IgM level above that recorded immediately pretransplant. Seven renal allograft recipients (aa,gg,hh,jj,ll,nn, pp) with no evidence of active HCMV infection and patient (k) who first showed serological evidence of primary HCMV infection at 59 weeks post transplantation were omitted from this experiment.


(A)
(B)
HCMV-IgM EIU
120
110
100
90
80
70
60
50
40
30
20
10
0
AV
Positive
Doubtful positive
Negative
None
Primary
Reinfection and/or reactivation
HCMV infection

weeks or 12 and 18 weeks post renal transplantation. In 10 patients (a,b,c,d,e,f,g,h,i,j) with primary HCMV infection, HCMV-IgM had risen above the pretransplant level by an average of 54.9 EIU (range 3.9 to 123.0) between 5 and 11 weeks after the transplant operation. During the same period HCMV-IgM levels were an average of 41.7 EIU (range 5.7 to 105.1) higher than the pretransplant level in 18 patients (l,m,n,o,p,s,t,u,v,w,x,y,z,qq,rr,ss,tt,uu) with reinfection and/or reactivation of HCMV for whom sufficient data was available. Between 12 and 18 weeks post transplantation, HCMV had risen above the pretransplant level by an average of 47.8 EIU (range 20.6 to 86.3) and 33.8 EIU (range 8.5 to 71.1) in the 10 patients with primary HCMV infection and 19 (l,m,n,o,p,q,r,s,t,u,v,w,x,y, z,qq,rr,tt,uu) with reinfection and/or reactivation of the virus respectively.

For each serum there was good reproducibility in the HCMV-IgM ELISA value. A serum sample obtained between 5 and 11 weeks post transplantation from each of 9 patients (b,c,d,e,f,g,h,i,j) with primary HCMV infection was tested in 2 batches of the ELISA and HCMV-IgM levels found to vary by an average of 9.73 EIU (range 1.8 to 30.5). Similar examination of a serum obtained between 12 and 18 weeks after the transplant operation from each of 10 patients (a,b,c,d,e,f,g,h,i,j) with primary HCMV infection showed the HCMV-IgM level in any 1 sample to vary by an average of 7.08 EIU (range 0.4 to 15.9). With regard to those

patients with reinfection and/or reactivation of HCMV, the average variation between HCMV-IgM levels measured in 2 batches of the ELISA was 8.35 EIU (range 0.9 to 20.8) for 18 sera or 7.51 EIU (range 0.4 to 21.1) for 19 sera, each obtained from a different renal allograft recipient.

Post transplantation HCMV-IgM levels did not differentiate those patients with reinfection and/or reactivation of HCMV who shed virus from those who did not. In 10 (l,m,n,o,p,s,t,u,v,w) of the 12 individuals (l,m,n,o, p,q,r,s,t,u,v,w) from whom HCMV was isolated and sufficient data available, the highest recorded HCMV-IgM level between 5 and 11 weeks post transplantation ranged from 5.7 to 79.7 EIU with an average of 37.2 EIU. During the same period the highest recorded HCMV-IgM level in 8 patients (x,y,z,qq,rr,ss,tt,uu) from whom HCMV was never isolated ranged from 11.7 to 105.1 EIU with an average of 47.2 EIU. Similarly, between 12 and 18 weeks after the transplant operation, the highest recorded HCMV-IgM level ranged from 8.5 to 71.1 EIU (an average of 30.9 EIU) in the 12 patients (l,m,n,o,p,q,r,s,t,u,v,w) who shed HCMV and from 17.8 to 69.1 EIU (an average of 38.7 EIU) in 7 (x, y,z,qq,rr,tt,uu) of the 8 patients who did not shed HCMV after renal transplantation and for whom sufficient data was available.

Eight renal allograft recipients (l,m,s,t,u,x,y,qq) with reinfection and/or reactivation of HCMV each received their kidney from an HCMV-seropositive donor while 8 other patients (n,p,r,v,z,rr,ss,tt) who were each transplanted with a kidney from an HCMV-seronegative donor most likely

experienced reactivation of endogenous virus. Both groups of patients could not be differentiated on the basis of the HCMV-IgM response, the highest recorded levels between 5 and 11 weeks and between 12 and 18 weeks post transplantation being 33.3 EIU (range 8.1 to 60.9) and 29.9 EIU (range 11.6 to 66.3) respectively in those renal allograft recipients who each received a kidney from an HCMV-seropositive donor while sera obtained from patients (n,p,v,z,rr,ss,tt) between 5 and 11 weeks after the transplant operation and from patients (n,p,r,v,z,rr,tt) between 12 and 18 weeks post transplantation showed an average HCMV-IgM level of 45.2 EIU (range 12.6 to 97.7) and 40.8 EIU (range 13.7 to 65.4) respectively.

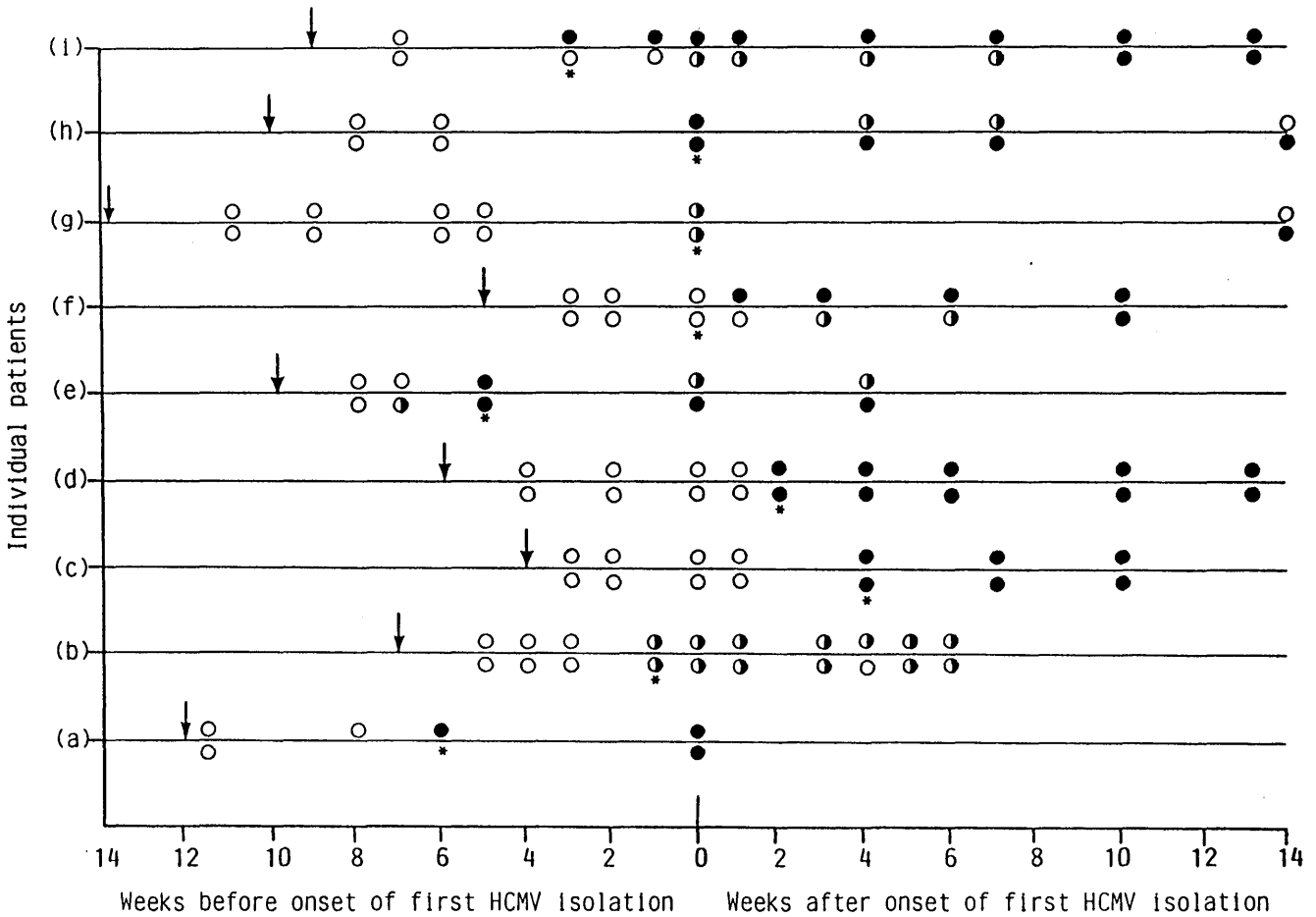
4.9 Overall correlation between HCMV-specific IgM, IgG and CF antibody levels and the detection of HCMV by tissue culture and RNA-DNA hybridization

4.9.1 Primary HCMV infection

HCMV was isolated from 9 (a,b,c,d,e,f,g,h,i) of the 11 renal allograft recipients with primary HCMV infection all of whom showed a fourfold or greater rise in the HCMV CF antibody titre (Figure 58A). In patients (a), (b), (e) and (i), this rise was recorded between 1 and 6 weeks prior to the onset of HCMV isolation while the virus was first isolated from patients (c) and (d) at 4 and 2 weeks respectively before a fourfold rise in HCMV CF antibody titre was observed. In patients (f), (g) and (h), HCMV

Figure 58A <u>Correlation between the onset of HCMV isolation, the fourfold or greater rise in HCMV CF antibody titre and HCMV-IgM and IgG levels measured by Labsystems ELISA: Primary HCMV infection</u>

In each of 9 renal allograft recipients (a,b,c,d,e,f,g,h,i) with primary HCMV infection who shed the virus post-operatively, the onset of HCMV isolation (Time 0) has been correlated with HCMV-IgM levels (—○— negative; —◐— equivocal; —●— positive) and HCMV-IgG levels (—○— negative; —◐— equivocal; —●— positive) measured by Labsystems ELISA. Results are correlated with the timing of the fourfold or greater rise in HCMV CF antibody titre (*) and the renal transplant operation (↓).


Individual patients
(i)
(h)
(g)
(f)
(e)
(d)
(c)
(b)
(a)
14 12 10 8 6 4 2 0 2 4 6 8 10 12 14
Weeks before onset of first HCMV isolation
Weeks after onset of first HCMV isolation

isolation and the fourfold or greater rise in virus-specific CF antibody titre were coincident.

When the fourfold or greater rise in HCMV CF antibody titre was first detected, patients (a,c,d,e,h,i) were positive for HCMV-IgM while (b) and (g) were equivocal and (f) negative. Four patients (c,d,e,h) were positive, 2 (b,g) were equivocal and 2 (f,i) were negative for HCMV-IgG when the fourfold or greater rise in HCMV CF antibody titre was first recorded. The serum obtained from patient (a) which showed a fourfold rise in HCMV CF antibody titre was not tested for HCMV-IgG. Virus was never isolated from 2 other renal allograft recipients (j and k) with primary HCMV infection. Patient (j) was positive and (k) equivocal for HCMV-IgM when the fourfold rise in CF antibody titre was first observed while both patients were seronegative for HCMV-IgG at this time. In no case were HCMV-IgM or IgG levels positive prior to the fourfold or greater rise in the virus-specific CF antibody titre.

Patients (a), (e) and (i) were positive for HCMV-IgM at 6, 5 and 3 weeks respectively prior to the onset of virus isolation. At the time of initial HCMV isolation, 3 renal allograft recipients (a,h,i) were positive for HCMV-IgM while patients (b), (e) and (g) were equivocal. Patient (b) remained equivocal for HCMV-IgM until at least 6 weeks after the onset of HCMV isolation. A serum taken from patient (g) at 14 weeks after initial HCMV isolation was negative for HCMV-IgM although no specimens were available during the intervening period. Patients (c), (d)

and (f) who were negative for HCMV-IgM on the day of first HCMV isolation became positive between 1 and 4 weeks later.

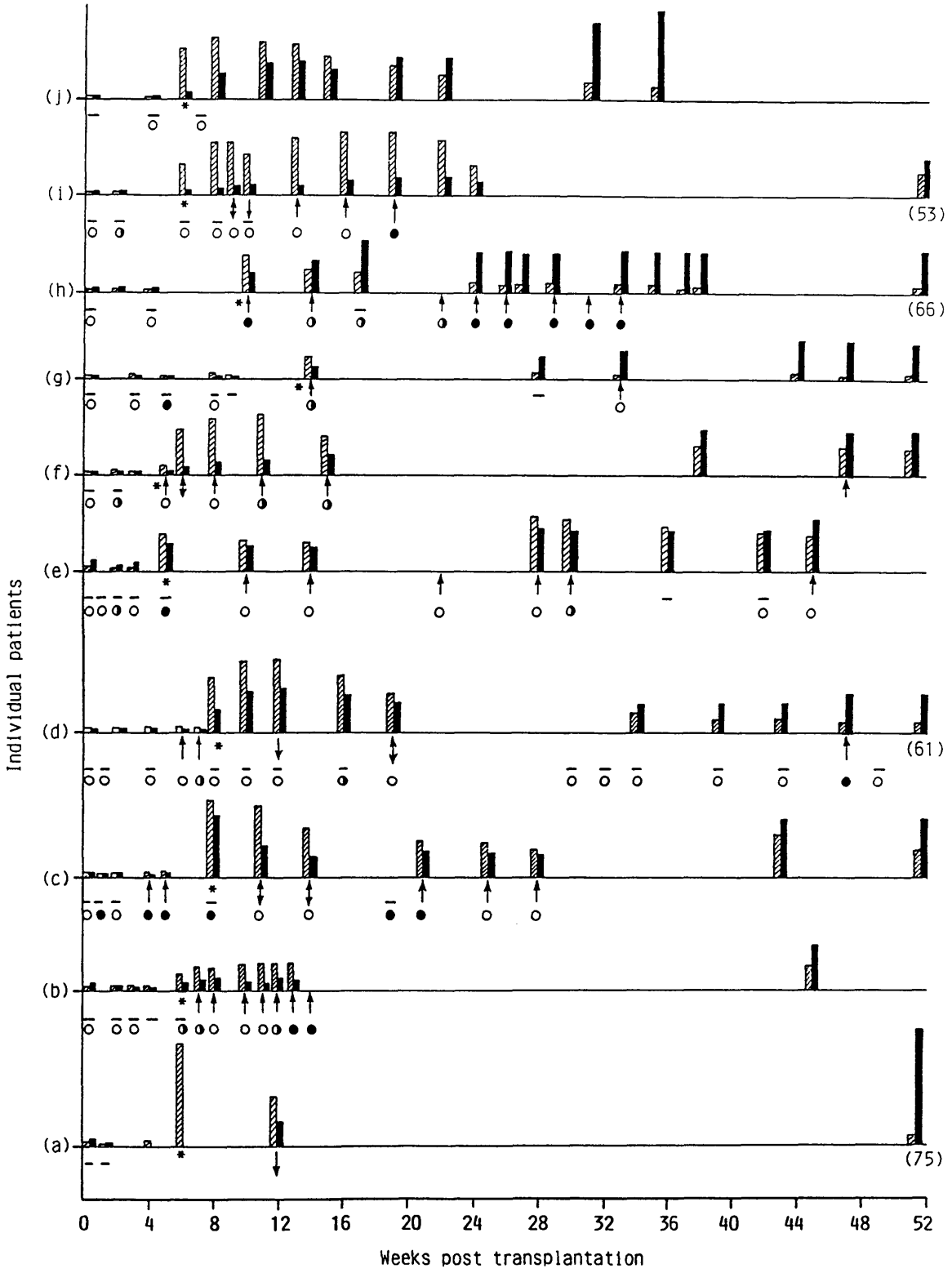
Only 1 renal allograft recipient (e) was positive for HCMV-IgG prior to the onset of HCMV isolation while on the day of initial HCMV isolation 3 patients (a,e,h) were positive for HCMV-IgG. Patients (b), (g) and (i) were equivocal for HCMV-IgG when HCMV was first isolated but patient (i) became positive 10 weeks later. Patient (b) remained equivocal for HCMV-IgG in sera taken up to 6 weeks after initial HCMV isolation while insufficient data are available to draw conclusions about the timing of seroconversion in patient (g) who was noted to be positive for HCMV-IgG 14 weeks after HCMV was first isolated. The 3 renal allograft recipients (c,d,f) who were negative for HCMV-IgG at the onset of virus isolation became positive between 2 and 10 weeks later.

The following data is shown in Figure 59A.

When a fourfold or greater rise in HCMV CF antibody titre was first recorded, urine specimens submitted simultaneously by each of patients (c), (e) and (h) were positive by hybridot assay while those taken from patients (b) and (g) were equivocal and those from patients (d), (f) and (i) were negative. Urine samples provided by patients (c) and (g) were positive by RNA-DNA hybridization at 7 and 9 weeks respectively prior to the fourfold or greater rise in HCMV CF antibody titre while in patients (b), (d) and (i) this rise preceded a positive result in the hybridot

Figure 59A Post transplantation levels of HCMV-IgM and IgG measured by Labsystems ELISA: Primary infection

The figure shows the post transplantation levels of HCMV-IgM (▨ = 20 EIU) and HCMV-IgG (▮ = 20 EIU) measured by Labsystems ELISA in each of 10 renal allograft recipients (a,b,c,d,e,f,g,h,i, j) with primary HCMV infection. The timing of the fourfold or greater rise in HCMV CF antibody titre is shown by *. Urine samples and/or throat swabs which did not yield HCMV in tissue culture are indicated by — . Symbols ↑ and ↓ differentiate those HCMV isolates obtained from urine samples and throat swabs respectively. Simultaneous isolation of HCMV from a urine sample and throat swab is indicated by ↕ . The results of the RNA-DNA hybridot assay (O negative; ◑ equivocal; ● positive) are also shown. Patient (k) who showed serological evidence of primary HCMV infection at 59 weeks post transplantation is omitted from the diagram. Figures within brackets indicate the number of weeks post transplantation when that serum was obtained.


(j)
(i)
(53)
(h)
(66)
(g)
(f)
(e)
(d)
(61)
(c)
(b)
(a)
(75)
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation

assay by 7, 39 and 13 weeks respectively. In patients (e) and (h), the fourfold or greater rise in HCMV CF antibody titre and the first positive result by hybridot assay were coincident.

When an increase in HCMV-IgM of greater than 40 EIU was first recorded, urine specimens submitted by each of patients (c), (e) and (h) were positive by hybridot assay while those taken from patients (d), (f) and (i) were negative. Patient (c) became positive for HCMV-IgM at 8 weeks post transplantation, 7 weeks after providing a urine sample which was the first to be classified as positive by RNA-DNA hybridization. Patients (d) and (i) showed a significant HCMV-IgM response 39 and 13 weeks respectively prior to the urine becoming positive by dot blot hybridization. The first recorded positive HCMV-IgM level and hybridot assay in each of patients (e) and (h) were coincident. No serum from patient (b) was ever positive for HCMV-IgM although 2 urines provided at 13 and 14 weeks after the transplant operation were positive by hybridot assay. The highest recorded level of HCMV-IgM in patient (g) was 26.0 EIU in a serum taken at 14 weeks post transplantation, 9 weeks after providing a urine sample, the only one from this patient found positive by RNA-DNA hybridization.

Urine obtained from both patients (d) and (g) was negative while that provided by each of patients (c), (e), (h) and (i) was positive by RNA-DNA hybridization when an HCMV-IgG level of greater than 20 EIU was first recorded.

Patient (f) became positive for HCMV-IgG at 15 weeks after the transplant operation when a urine sample was equivocal by hybridot assay. A positive HCMV-IgG level was first detected in patient (c) 7 weeks after a urine specimen had been found positive by dot blot hybridization. A urine sample submitted by patient (g) 5 weeks after the transplant operation was positive by hybridot assay while a serum taken simultaneously had an HCMV-IgG level of 0.2 EIU. Consecutive sera taken at 8, 9 and 14 weeks post transplantation showed the HCMV-IgG level to be 2.8, 1.3 and 14.3 EIU respectively. The only urine provided by patient (d) which was positive by RNA-DNA hybridization was taken 47 weeks after the transplant operation, 39 weeks after the onset of a significant HCMV-IgG response. The first urine specimen taken from patient (b) and shown to be positive by RNA-DNA hybridization was provided at 13 weeks post transplantation when the HCMV-IgG level was 15.5 EIU. The next available serum was obtained 32 weeks later when the HCMV-IgG level was 56.4 EIU but no urine specimen was available for analysis.

Only 2 urine samples obtained from patient (j), one at 4 weeks and the other at 7 weeks after the transplant operation, were tested by dot blot hybridization and both were negative while a serum provided at 6 weeks post transplantation showed an eightfold increase in the HCMV CF antibody titre and a 57.6 EIU increase in the level of HCMV-IgM. Patient (j) became positive for HCMV-IgG 8 weeks

after the transplant operation. Insufficient data is available for patient (a) to allow a correlation to be made between the results of the hybridot assay and the humoral immune response.

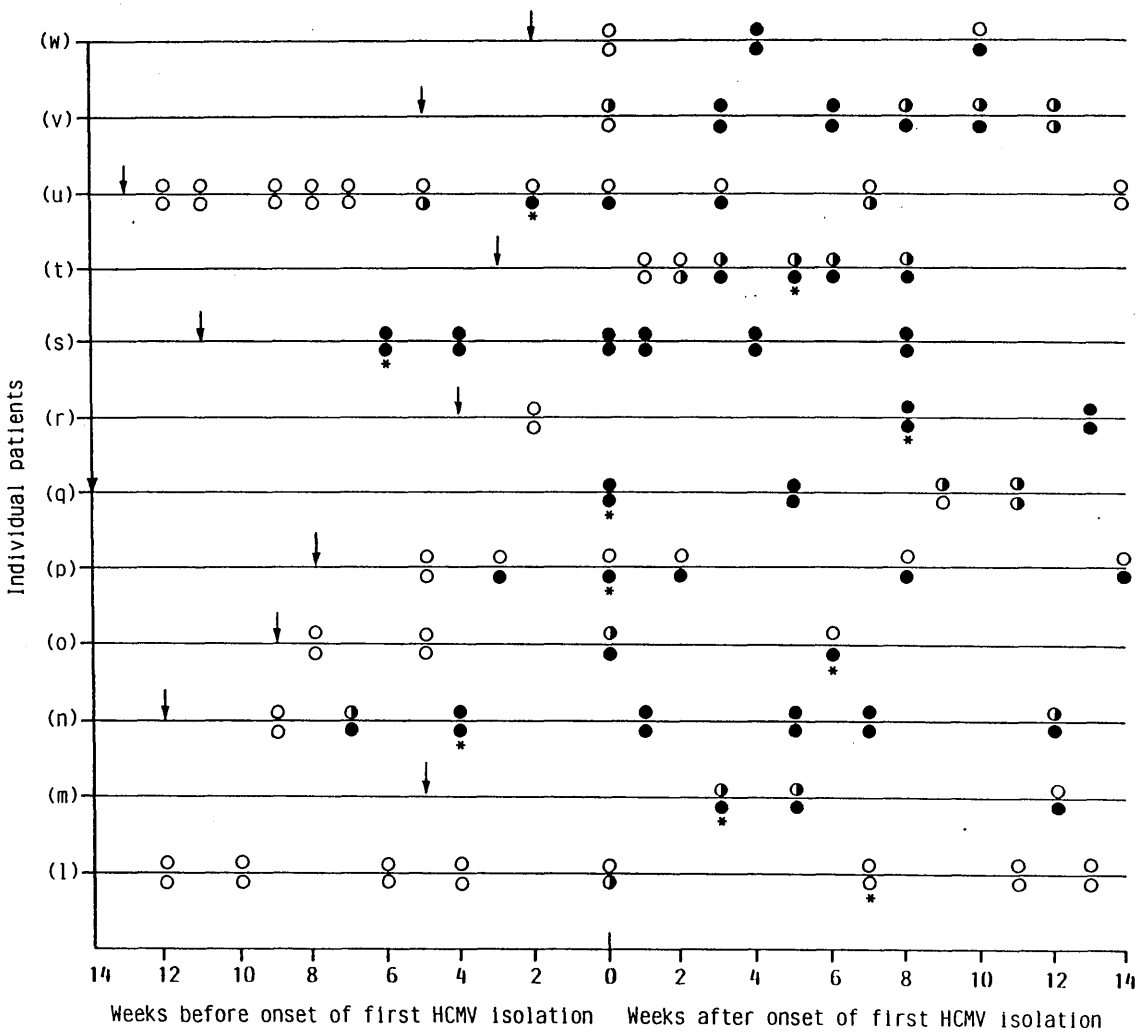
4.9.2 Reinfection and/or reactivation

Ten (l,m,n,o,p,q,r,s,t,u) of the 12 patients from whom HCMV was isolated showed a fourfold or greater rise in HCMV CF antibody titre (Figure 58B). In patients (n), (s) and (u), this rise was recorded between 2 and 6 weeks prior to the onset of virus isolation while HCMV was first isolated from patients (l), (o) and (t) at 7, 6 and 5 weeks respectively before a fourfold or greater rise in virus-specific CF antibody titre was observed. Patients (p) and (q) showed a fourfold or greater rise in HCMV CF antibody titre coincident with the onset of HCMV isolation while in patients (m) and (r) it was not possible to accurately relate initial virus isolation to the rise in CF antibody titre.

When the fourfold or greater rise in HCMV CF antibody titre was recorded, patients (n), (q), (r) and (s) were positive for HCMV-IgM while (m) and (t) were equivocal and (l), (o), (p) and (u) negative. No renal allograft recipient was positive for HCMV-IgM prior to showing a fourfold or greater rise in HCMV CF antibody titre. Nine patients (m,n,o,p,q,r,s,t,u) had evidence of a fourfold or greater rise in HCMV CF antibody titre coincident with a 20 EIU or greater increase in HCMV-IgG although in patients

Figure 58B Correlation between the onset of HCMV isolation, the fourfold or greater rise in HCMV CF antibody titre and HCMV-IgM and IgG levels measured by Labsystems ELISA: Re-infection and/or reactivation of HCMV

In each of 12 renal allograft recipients (l,m,n,o,p,q,r,s,t,u,v,w) with reinfection and/or reactivation of HCMV who shed the virus post-operatively, the onset of HCMV isolation (Time 0) has been correlated with HCMV-IgM levels (—○— negative; —◐— equivocal; —●— positive) an increase in HCMV-IgG of 0 to 9 EIU (—○—), 10 to 19 EIU (—◐—) and 20 EIU or more (—●—) measured by Labsystems ELISA. Results are correlated with the timing of the fourfold or greater rise in HCMV CF antibody titre (*) and the renal transplant operation (↓). Patient (l) received a renal allograft 18 weeks prior to the onset of HCMV isolation.


(w)
(v)
(u)
(t)
(s)
(r)
(q)
(p)
(o)
(n)
(m)
(l)
Individual patients
14 12 10 8 6 4 2 0 2 4 6 8 10 12 14
Weeks before onset of first HCMV isolation
Weeks after onset of first HCMV isolation

(n), (o), (p) and (t) a significant increase in HCMV-IgG was first recorded in a serum taken at 5, 6, 3 and 5 weeks respectively before the fourfold or greater rise in HCMV CF antibody titre was observed. In patient (l), the highest recorded increase in HCMV-IgG was 11.4 EIU in a serum taken 7 weeks before that showing the fourfold rise in HCMV CF antibody titre but no sera were submitted in the intervening period.

HCMV was never isolated from 8 other renal allograft recipients (x,y,z,qq,rr,ss,tt,uu) with reinfection and/or reactivation. Patients (y) and (qq) showed a significant increase in HCMV-IgM at 1 and 6 weeks respectively before the fourfold or greater rise in HCMV CF antibody titre was detected while patients (x,z,rr,tt,uu) first became positive for HCMV-IgM coincidentally with the fourfold or greater rise in virus-specific CF antibody titre. Patient (ss) remained negative for HCMV-IgM throughout the period of study. When the fourfold or greater rise in HCMV CF antibody titre was first recorded, patients (x), (y), (z), (rr), (ss) and (uu) showed a 20 EIU or greater increase in HCMV-IgG. A significant HCMV-IgG response was noted in a serum taken from patient (qq) 6 weeks before a sixteenfold rise in HCMV CF antibody titre was detected. The highest recorded increase in HCMV-IgG in patient (tt) was 14.9 EIU in a serum provided 2 weeks after that showing a fourfold rise in virus-specific CF antibody titre.

Patients (n) and (s) were positive for HCMV-IgM at 4

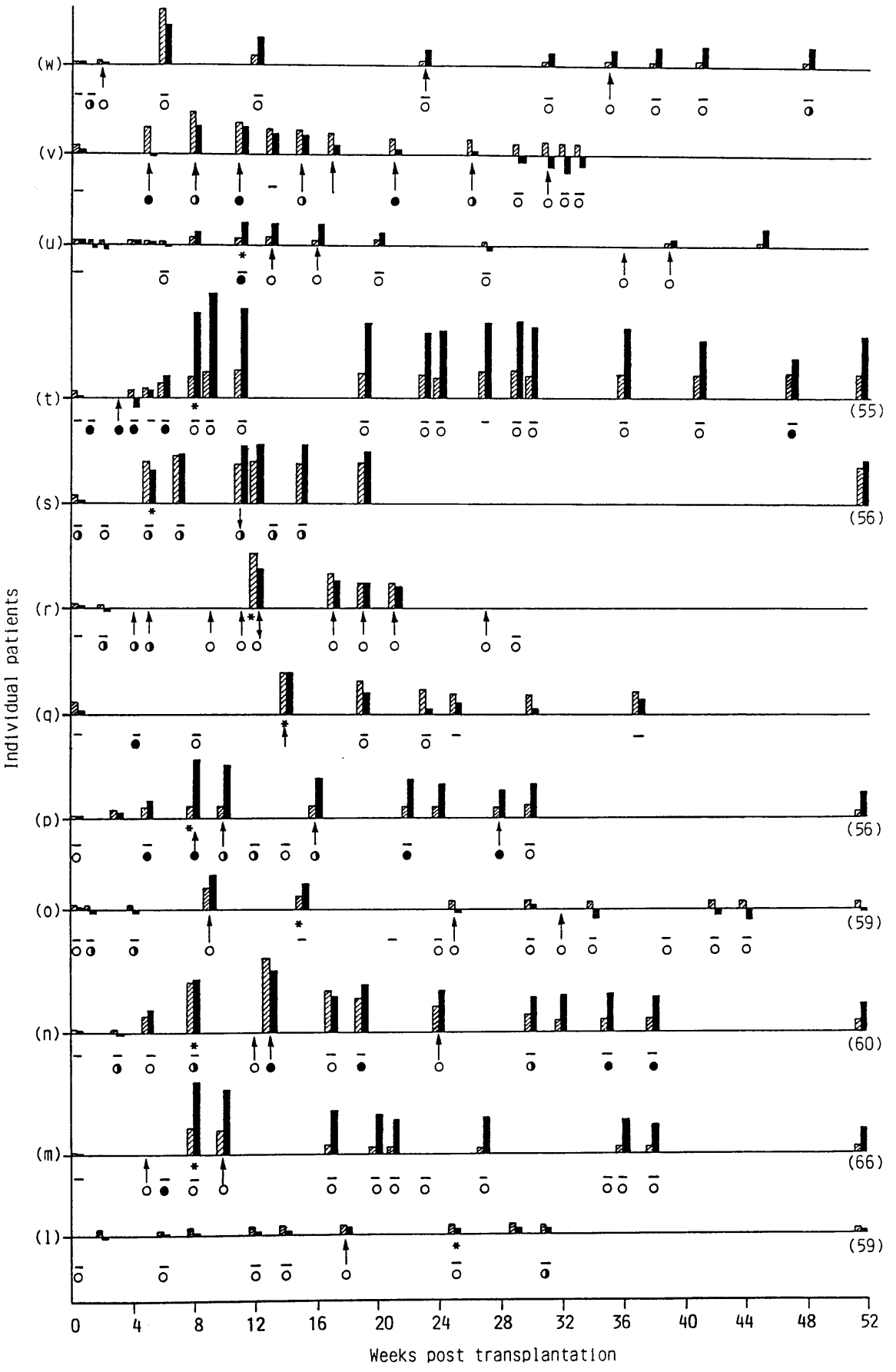
and 6 weeks respectively prior to the onset of virus isolation. At the time of initial HCMV isolation, 2 renal allograft recipients (q and s) were positive for HCMV-IgM, while (o) and (v) were equivocal and (l), (p), (u) and (w) negative. Patients (v) and (w) became positive for HCMV-IgM at 3 and 4 weeks respectively after the onset of virus isolation while (l), (p) and (u) remained negative throughout. No serological data are available for patients (m), (r) and (t) at the time of first HCMV isolation but from subsequent results patient (t) must have been negative for HCMV-IgM at that time. Four renal allograft recipients (n,p,s,u) developed a 20 EIU or greater rise in HCMV-IgG between 2 and 7 weeks prior to the onset of HCMV isolation. On the day of first virus isolation, HCMV-IgG had increased by 20 EIU or more in patients (o), (p), (q), (s) and (u), between 10 and 19 EIU in patient (l) and between 0 and 9 EIU in patients (v) and (w). Patients (t), (v) and (w) showed a significant rise in HCMV-IgG at 3, 3 and 4 weeks respectively after the onset of first HCMV isolation while the HCMV-IgG level in patient (l) never increased by more than 11.4 EIU above that recorded immediately pretransplant.

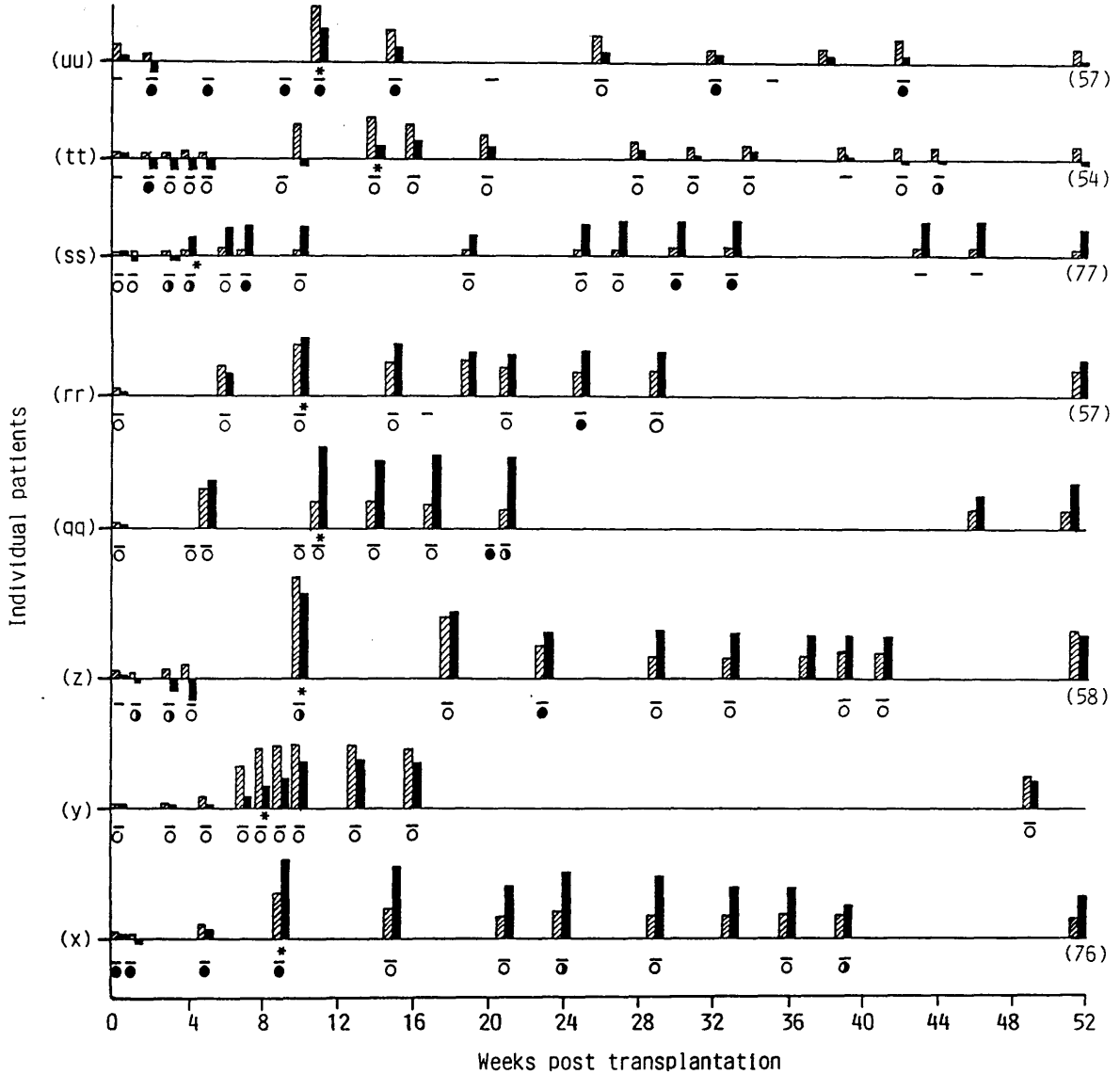
The following data are shown in Figure 59B.

When a fourfold or greater rise in HCMV CF antibody titre was first recorded, urine specimens submitted simultaneously by each of patients (p), (u), (x) and (uu) were positive by hybridot assay while those taken from patients (n), (s), (z) and (ss) were equivocal and those

Figure 59B Post transplantation levels of HCMV-IgM and IgG measured by Labsystems ELISA: Reinfection and/or reactivation

The figure shows the post transplantation levels of HCMV-IgM (▨ = 20 EIU) and HCMV-IgG (▮ = 20 EIU) measured by Labsystems ELISA in each of 20 renal allograft recipients(l,m,n,o,p,q,r,s,t,u, v,w,x,y,z,qq,rr,ss,tt,uu) with reinfection and/or reactivation of HCMV. The timing of the fourfold or greater rise in HCMV CF antibody titre is shown by *. Urine samples and/or throat swabs which did not yield HCMV in tissue culture are indicated by — . Symbols ↑ and ↓ differentiate those HCMV isolates obtained from urine samples and throat swabs respectively. Simultaneous isolation of HCMV from a urine sample and throat swab is indicated by ↕ . The results of the RNA-DNA hybridot assay (○ negative; ◑ equivocal; ● positive) are also shown. Figures within brackets indicate the number of weeks post transplantation when that serum was obtained.


(w)
(v)
(u)
(t)
(55)
(s)
(56)
(r)
(q)
(p)
(56)
(o)
(59)
(n)
(60)
(m)
(66)
(l)
(59)
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation


(uu)
(57)
(tt)
(54)
(ss)
(77)
(rr)
(57)
(qq)
(z)
(58)
(y)
(x)
(76)
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation

from patients (l), (m), (r), (t), (y), (qq), (rr) and (tt) were negative. Urine obtained from each of patients (o) and (q) at the time of the fourfold or greater rise in HCMV CF antibody titre was insufficient for testing by RNA-DNA hybridization. Urine specimens provided by patients (m), (p), (q), (t), (x), (tt) and (uu) were positive by dot blot hybridization at 2,3,10,7,9,12 and 9 weeks respectively prior to the fourfold or greater rise in HCMV CF antibody titre while in patients (n), (z), (qq), (rr) and (ss) this rise preceded a positive result in the hybridot assay by 5, 13, 9, 15 and 3 weeks respectively. In patient (u), the fourfold or greater rise in HCMV CF antibody titre and first positive result by RNA-DNA hybridization were coincident. Five other renal allograft recipients (l,o,r, s,y) who showed a fourfold or greater rise in HCMV CF antibody titre never provided urine specimens that were positive by hybridot assay. Of the 2 renal allograft recipients who never showed a fourfold or greater rise in HCMV CF antibody titre, patient (v) submitted urine specimens which were positive by dot blot hybridization at 5, 11 and 21 weeks post transplantation. No urine sample provided by patient (w) was positive by hybridot assay.

When an increase in HCMV-IgM of greater than 40 EIU was first recorded, urine specimens submitted by each of patients (x) and (uu) were positive by hybridot assay while those taken from patients (n), (s), (v) and (z) were equivocal and those from patients (r), (w), (y), (qq), (rr)

and (tt) were negative. Insufficient urine for testing by dot blot hybridization was provided by patient (q) at 14 weeks post transplantation when a significant increase in HCMV-IgM was first noted. Transplant recipients (n), (z), (qq) and (rr) showed a significant increase in HCMV-IgM at 5, 13, 15 and 15 weeks respectively prior to providing a urine sample that was positive by RNA-DNA hybridization while in patients (q), (v), (x), (tt) and (uu), a urine sample was positive by hybridot assay at 10, 3, 9, 12 and 9 weeks respectively prior to a significant increase in HCMV-IgM. No urine specimen submitted by patients (r), (s), (w) and (y) was ever positive by dot blot hybridization although post transplantation levels of HCMV-IgM exceeded 40 EIU. HCMV-IgM levels in patients (m), (p), (t), (u) and (ss) remained equivocal throughout although each renal allograft recipient provided at least 1 urine specimen that was positive by RNA-DNA hybridization. In patients (l) and (o), HCMV-IgM levels never exceeded 15.3 and 27.3 EIU respectively and all urine samples remained negative or equivocal by hybridot assay.

When a significant increase in HCMV-IgG was first recorded, urine specimens provided by each of patients (p), (t), (u), (x) and (uu) were positive by dot blot hybridization while those obtained from patients (s), (v), (z) and (ss) were equivocal and those from patients (m), (n), (o), (r), (w), (y), (qq) and (rr) were negative. Insufficient urine for testing by RNA-DNA hybridization was taken from patient (q) at 14 weeks post transplantation

when the HCMV-IgG level had increased by 55.0 EIU. Transplant recipients (n), (z), (qq), (rr) and (ss) showed a significant increase in HCMV-IgG at 8, 13, 15 , 19 and 3 weeks respectively prior to providing a urine specimen which was positive by hybridot assay while in patients (m), (t), (v), (x) and (uu) the urine was positive by dot blot hybridization at 2, 5, 3, 9 and 9 weeks respectively prior to a significant increase in HCMV-IgG. In each of patients (p) and (u) a significant increase in HCMV-IgG and first positive result by hybridot assay were coincident. No urine specimen submitted by patients (o), (r), (s), (w) and (y) was ever positive by RNA-DNA hybridization although significant increases in HCMV-IgG were observed. In patient (l), the highest recorded increase in HCMV-IgG was 11.4 EIU and none of the 7 urines provided were positive by dot blot hybridization. No significant increase in HCMV-IgG was noted in any serum obtained from patient (tt) but 1 urine sample, that taken at 2 weeks post transplantation, was positive by hybridot assay.

4.10 Blood donors

4.10.1 HCMV serostatus

The HCMV serostatus was determined in 127 individuals who donated blood subsequently transfused into 37 of the 47 renal allograft recipients involved in this study. Forty eight (38%) of the blood donors were seropositive and 79 (62%) seronegative for HCMV by the modified CFT. HCMV-IgM levels were determined in each of 33 HCMV-seropositive blood donations provided by 15 individuals and all were found to be negative.

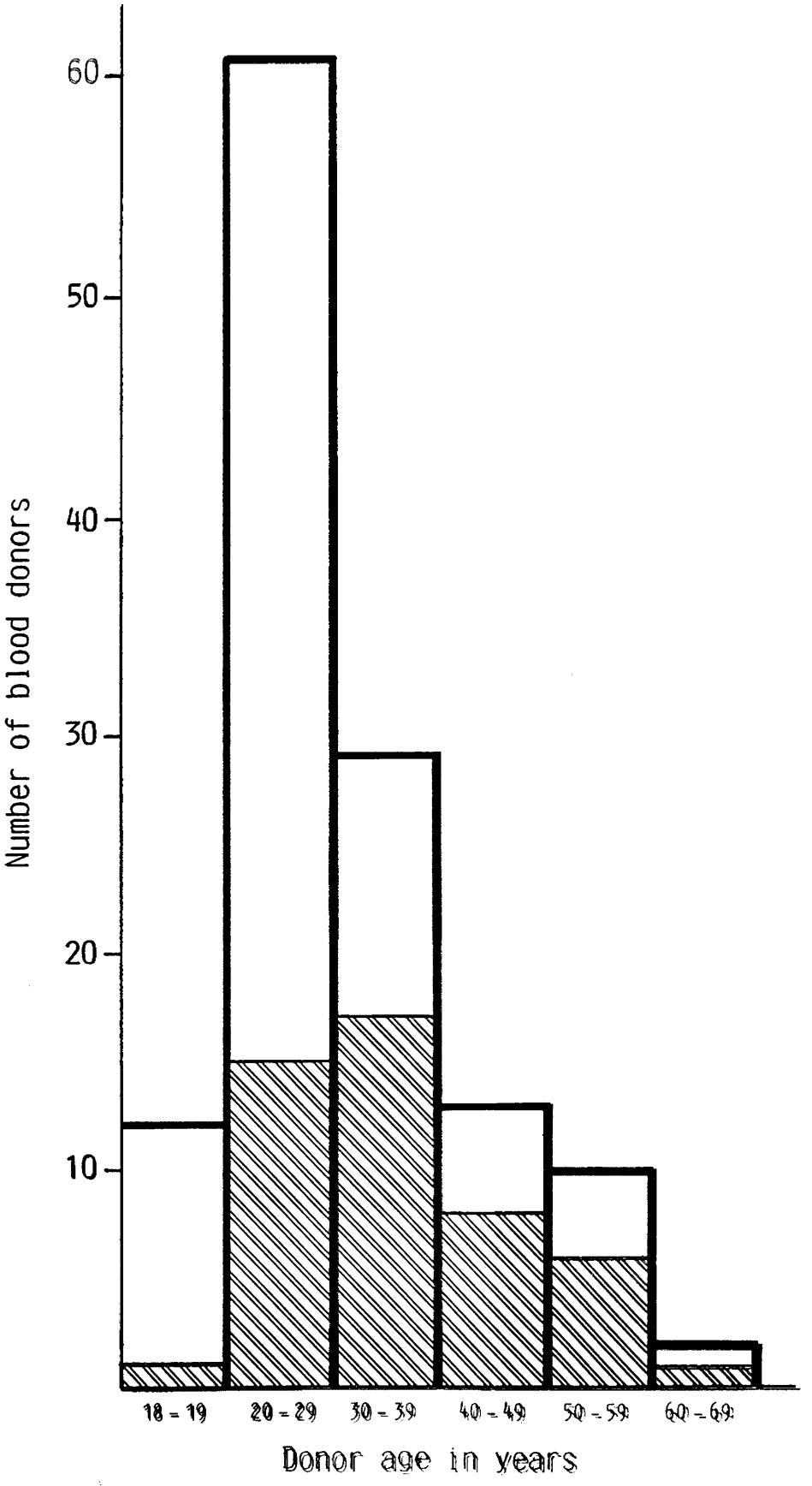
4.10.2 Age groups

The majority of the blood donations were received from individuals aged between 20 and 29 years of age who provided 61 blood packs comprising 48% of the total (Figure 60). With increasing age the number of donors gradually declined so that only 1 blood donation was received from each of 2 individuals aged between 60 and 69 years.

One of 12 blood donations provided by individuals aged 18 or 19 years was seropositive for HCMV. Thereafter, the proportion of blood packs which was HCMV-seropositive increased from 15/61 (24%) of those obtained from people aged betweeen 20 and 29 years to 17/29 (59%) of those provided by donors aged 30 to 39 years. Of the 25 blood donations received from individuals aged 40 years and over, 15 (60%) were HCMV-seropositive.

Figure 60 Age of blood donors

The 127 blood donors who provided blood for 37 renal allograft recipients are grouped according to age in years (18 and 19, 20 to 29, 30 to 39, up to and including 60 to 69). The total number of blood donors in each age group is shown while the hatched area indicates that proportion of donors found to be seropositive for HCMV.


60
50
40
30
20
10
Number of blood donors
18 - 19
20 - 29
30 - 39
40 - 49
50 - 59
60 - 69
Donor age in years

4.10.3 Transmission of HCMV

Four patients (aa,ff,gg,hh) who were seronegative for HCMV immediately pretransplant and who each received a kidney from an HCMV-seronegative renal donor were each transfused with 1 unit of blood provided by individuals who were seropositive for HCMV. All blood had been stored at 4°C for up to 3 weeks. In every case the serostatus was confirmed by CFT and Labsystems HCMV-IgG ELISA. Throughout the period of the study none of the 4 renal allograft recipients described above showed any evidence of primary HCMV infection.

4.11 HSV infections in renal allograft recipients

The CFT was used to determine the HSV serostatus of the 47 renal allograft recipients involved in this study and 35 (74%) were found to be seropositive immediately before the transplant operation. There was evidence of post transplantation HSV infection in 24 patients (d,h,i,l, m,n,o,p,r,v,w,x,y,z,bb,cc,ff,jj,kk,nn,qq,rr,ss,tt) all of whom were seropositive immediately pretransplant and must therefore have developed reinfection and/or reactivation of the virus. No renal allograft recipient sustained a primary HSV infection after the transplant operation.

4.11.1 Isolation of HSV

Three hundred and seventy two urine samples and 337 throat swabs were obtained from the 35 transplant recipients who were seropositive for HSV immediately

pretransplant. Six urine samples (2%) from 3 patients (y, jj,ss) yielded HSV in tissue culture while the virus was isolated from 13 throat swabs (4%) provided by 9 renal allograft recipients (l,m,r,w,y,cc,ff,jj,nn).

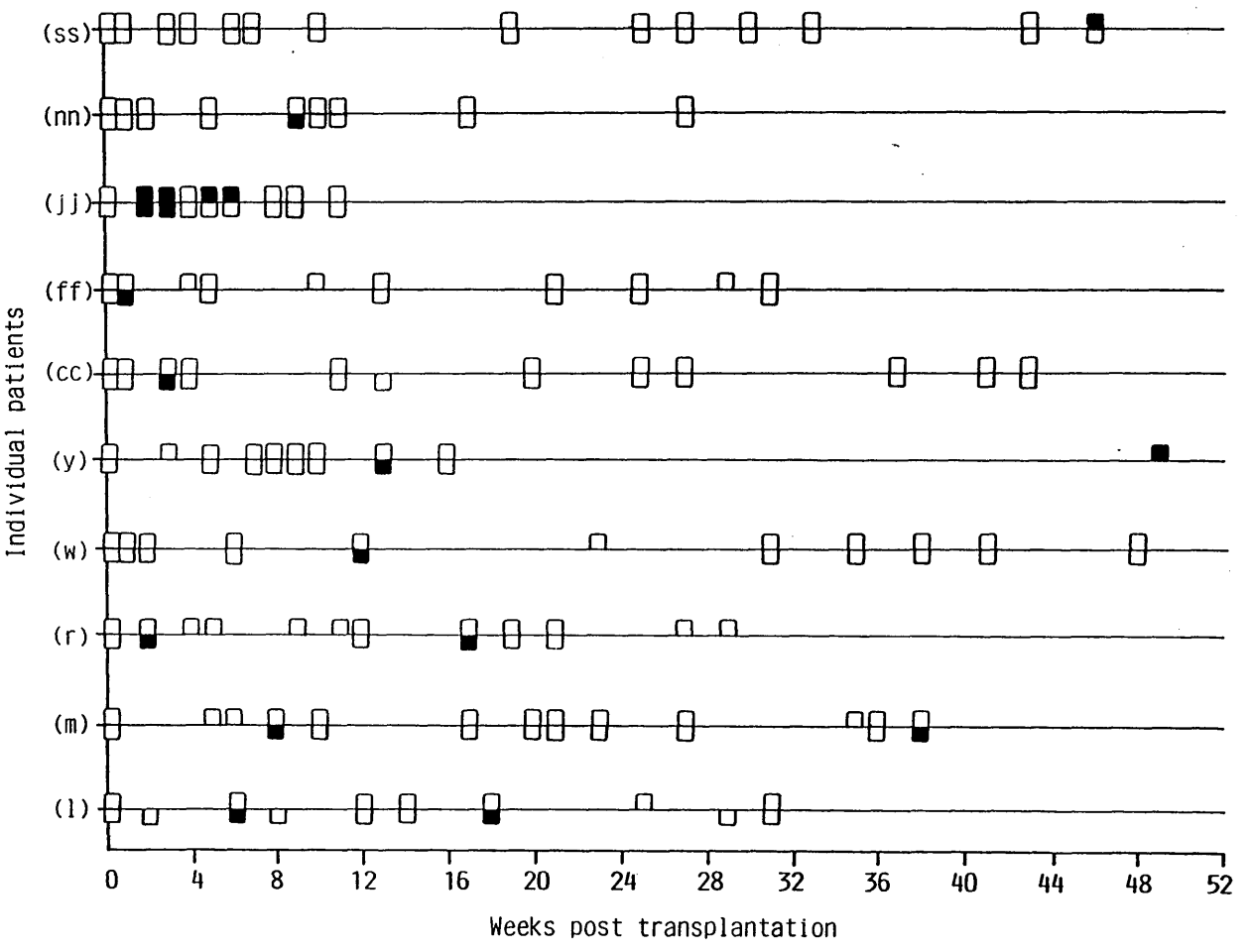
Isolation of HSV from the urine was first recorded in a sample provided by patient (jj) at 2 weeks after the transplant operation (Figure 61). Other urine specimens obtained from the same patient at 3,5 and 6 weeks post transplantation also yielded HSV in tissue culture while a urine sample provided at 4 weeks after the transplant operation was free of the virus. Patients (y) and (ss) also shed HSV in the urine, virus being isolated from specimens taken at 49 and 46 weeks respectively post transplantation.

The first throat swab to yield HSV in tissue culture was obtained from patient (ff) at 1 week after the transplant operation. By 13 weeks post transplantation, HSV had been isolated from 10 throat swabs, 1 from each of patients (l), (m), (r), (w), (y), (cc), (ff), (nn) and 2 from patient (jj). A throat swab taken from each of patients (l) and (r) at 18 and 17 weeks respectively after renal transplantation also yielded HSV in tissue culture while the final HSV isolate was harvested from a throat swab provided by patient (m) at 38 weeks post transplantation.

Five patients (w,y,cc,ff,nn) shed HSV from the throat on only 1 occasion while 4 other renal allograft recipients

Figure 61 HSV shedding after renal transplantation

The figure shows urine samples (—□—) and throat swabs (—┬—) provided by 10 renal allograft recipients (l,m,r,w,y,cc,ff,jj,nn,ss) who shed HSV post-operatively. Urine samples and throat swabs which yielded HSV in tissue culture are differentiated by —■— and —┬— respectively.


(ss)
(nn)
(jj)
(ff)
(cc)
(y)
(w)
(r)
(m)
(l)
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks post transplantation

(l,m,r,j) each provided 2 independent throat swabs from which the virus was isolated. HSV was harvested from 2 consecutive throat swabs taken from patient (jj) at 2 and 3 weeks respectively post transplantation. The pattern of HSV shedding from the throat of patients (l), (m) and (r) was intermittent with an interval of 12, 30 and 15 weeks respectively between isolates, no virus being isolated from throat swabs provided within the intervening period.

HSV was isolated from both the urine and throat of 2 renal allograft recipients. In patient (y), isolation of HSV from the throat preceded that from the urine by 36 weeks while HSV was cultured from urine samples and throat swabs provided by patient (jj) at 2 and 3 weeks after the transplant operation.

Restriction enzyme analysis of HSV strains isolated from patients (l), (m), (y) and (jj) confirmed the urinary isolates to be Type 2 while those cultured from throat swabs were Type 1 (Figures 62A and B). Both HSV-1 isolates shed from the throat of patient (m) at 8 and 38 weeks post transplantation had an identical DNA profile after digestion with restriction enzymes EcoRI and BamHI. Restriction enzyme analysis also showed the virus isolated from each of the throat swabs provided by patient (jj) at 3, 5 and 6 weeks after the transplant operation to belong to the same strain of HSV-1. Furthermore, at 2 and 3 weeks post transplantation, patient (jj) also shed HSV-2 in the urine, both isolates having an identical DNA profile after digestion with restriction enzymes XbaI and BamHI.

Figure 62A Autoradiographs of BamHI digests of HSV DNA labelled in vivo with ^{32}P

All isolates were obtained from patient (jj).

Lane	Source	Weeks Post Transplantation
2	Throat swab	2
3	Urine	3
4	Throat swab	3
5	Urine	5
6	Urine	6

Lanes 1 and 7 show the DNA profiles of HSV-1 17 syn^+ and HSV-2 HG52 respectively.

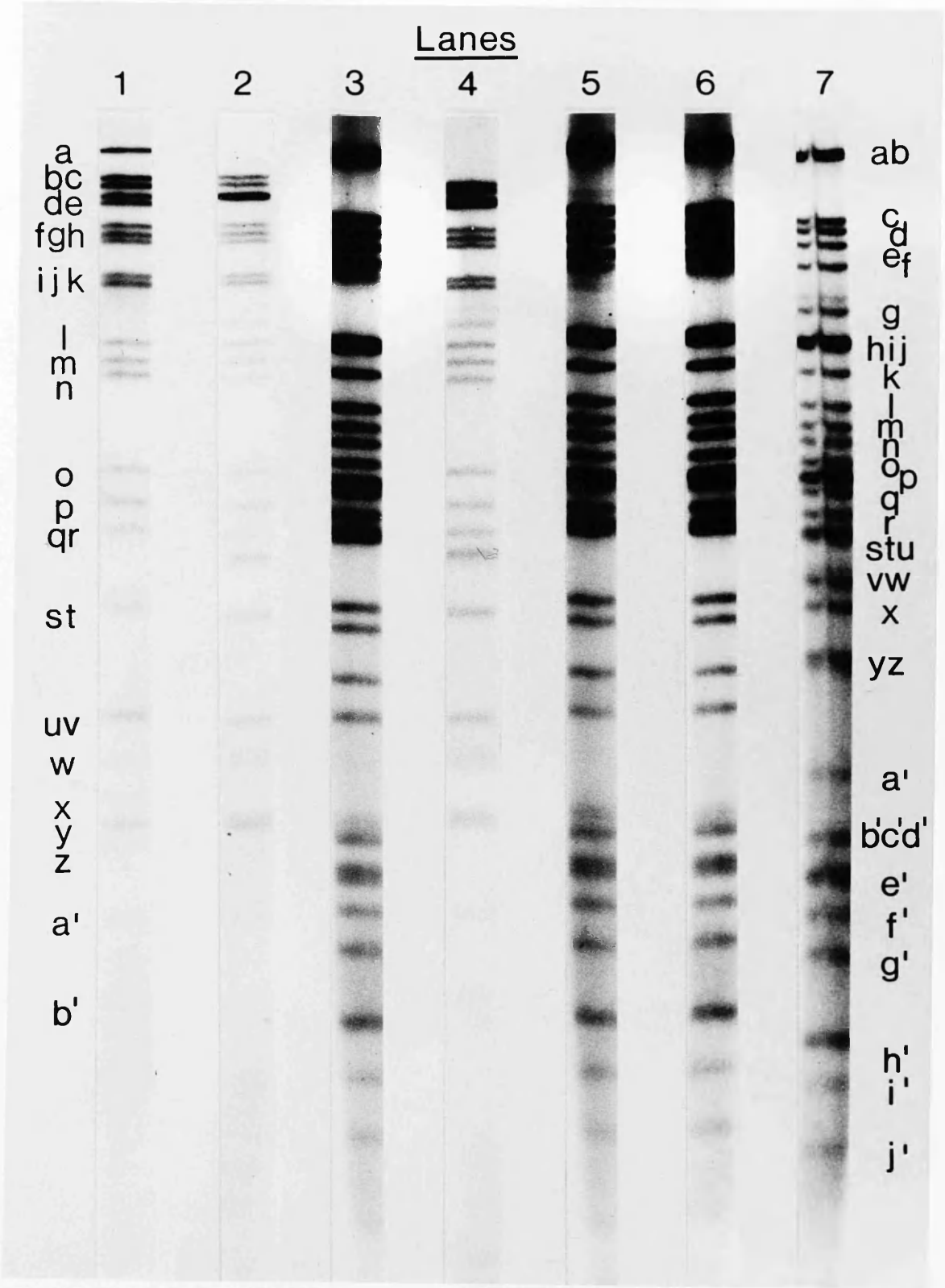
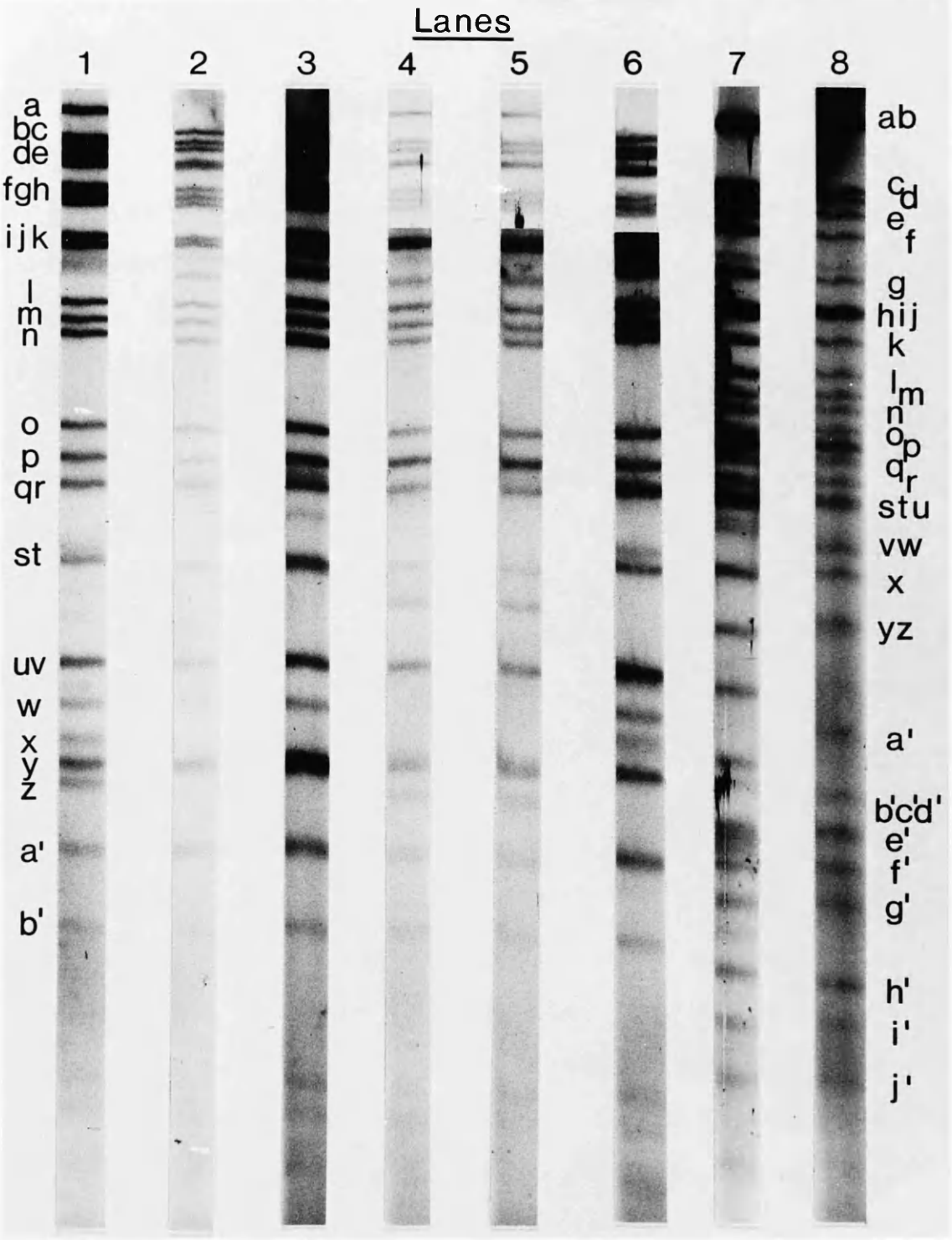

Lanes
1
2
3
4
5
6
7
a
bc
de
fgh
ijk
l
m
n
o
p
qr
st
uv
w
x
y
z
a'
b'
ab
c
d
e
f
g
hij
k
l
m
n
o
p
q
r
stu
vw
x
yz
a'
b'c'd'
e'
f'
g'
h'
i'
j'

Figure 62B Autoradiographs of BamHI digests of HSV DNA labelled in vivo with ^{32}P

Lane	Patient	Source	Weeks Post Transplantation
2	m	Throat swab	8
3	m	Throat swab	38
4	l	Throat swab	6
5	l	Throat swab	18
6	y	Throat swab	13
7	y	Urine	49

Lanes 1 and 8 show the DNA profiles of HSV-1 17 syn^+ and HSV-2 HG52 respectively.


Lanes
1
2
3
4
5
6
7
8
a
bc
de
fgh
ijk
l
m
n
o
p
qr
st
uv
w
x
y
z
a'
b'
ab
c
d
e
f
g
hij
k
l
m
n
o
p
q
r
stu
vw
x
yz
a'
b'
c'
d'
e'
f'
g'
h'
i'
j'

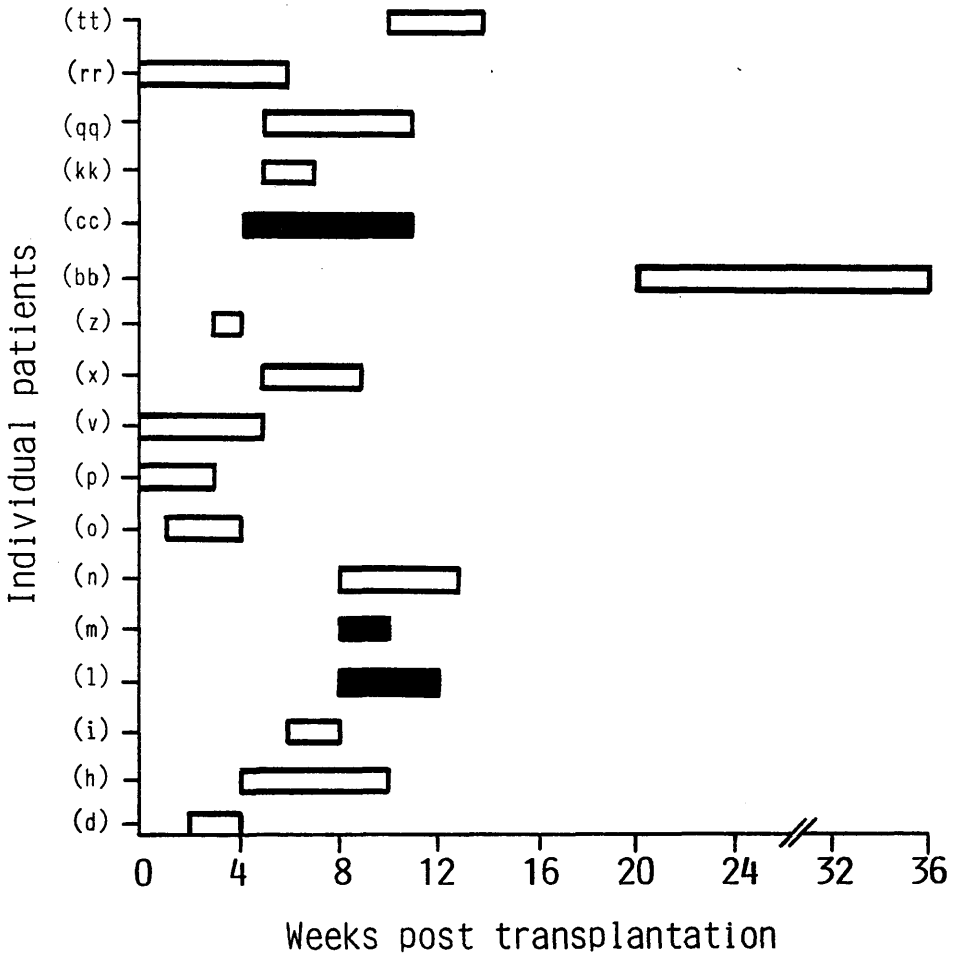
4.11.2 Timing of the fourfold or greater rise in HSV CF antibody titre post renal transplantation

Sixteen renal allograft recipients showed a fourfold or greater rise in the HSV CF antibody titre after renal transplantation (Figure 63). In patients (d), (o), (p) and (z), this rise occurred within the first 4 weeks after the transplant operation. By 12 weeks post transplantation, a further 10 renal allograft recipients (h,i,l,m,v,x,cc,kk,qq rr) had evidence of a fourfold or greater rise in the HSV CF antibody titre. Patient (n) had an eightfold rise in the HSV CF antibody titre between 8 and 13 weeks post-operatively while in patient (tt) the fourfold rise in HSV CF antibody titre occurred between the tenth and fourteenth week after the transplant operation. The last renal allograft recipient (bb) to show a fourfold rise in HSV CF antibody titre did so in a serum taken at 36 weeks post transplantation, the CF titre in the preceding serum taken at 20 weeks being the same as that immediately pretransplant.

Three renal allograft recipients shed HSV and had a significant increase in the HSV CF antibody titre. Patients (l) and (m) showed an eightfold rise in the HSV CF antibody titre between 8 and 12 weeks and between 8 and 10 weeks respectively after the transplant operation while patient (cc) had a sixteenfold increase in the HSV CF antibody titre between the fourth and eleventh week post transplantation.

Figure 63 Timing of the fourfold or greater rise in HSV CF antibody titre after renal transplantation

The figure shows the timing of the fourfold or greater rise in HSV CF antibody titre in each of 17 renal allograft recipients (d,h,i,l,m,n,o,p,v,x, z,bb,cc,kk,qq,rr,tt) with reinfection and/or reactivation of HSV after the transplant operation. Each bar identifies the time interval between 2 consecutive serum samples the last of which showed the fourfold or greater rise in HSV CF antibody titre. ■ and □ differentiate patients from whom virus was isolated and not isolated respectively.


(tt)
(rr)
(qq)
(kk)
(cc)
(bb)
(z)
(x)
(v)
(p)
(o)
(n)
(m)
(l)
(i)
(h)
(d)
Individual patients
0
4
8
12
16
20
24
32
36
Weeks post transplantation

Thirteen renal allograft recipients demonstrated a fourfold or greater rise in CF antibody titre to both HSV and HCMV. In 6 patients (d,l,o,p,z,rr), the rise in HSV CF antibody titre preceded that of HCMV while in 3 other renal transplant recipients (i, m,n) the fourfold or greater rise in HCMV CF antibody titre preceded that of HSV. Sera taken from each of patients (h), (x), (qq) and (tt) at 10, 9, 11 and 14 weeks respectively post transplantation showed a fourfold or greater rise in CF antibody titre to both HSV and HCMV.

4.11.3 Correlation between HSV isolation and the fourfold or greater rise in HSV CF antibody titre

HSV was isolated from throat swabs provided by 3 renal allograft recipients (l,m,cc) who were known to have a fourfold or greater rise in HSV CF antibody titre. On the day of first HSV isolation none of these 3 patients showed a significant increase in the HSV CF antibody titre above that recorded immediately pretransplant (Figure 64). In patient (l), there was a fourfold rise in HSV CF antibody titre between 2 and 6 weeks after the onset of HSV isolation while patient (cc) had evidence of a sixteenfold increase in the HSV CF antibody titre which must have occurred between 1 and 8 weeks after HSV was first isolated. Patient (m) showed a fourfold rise in HSV CF antibody titre which was recorded in a serum provided at 2 weeks after initial isolation of HSV from a throat swab.

4.11.4 Transmission of HSV by renal donors

An investigation was made into the possibility that neurones in parasympathetic ganglia within the transplanted kidneys may have harboured latent HSV which subsequently reactivated and infected the renal allograft recipients. The HSV serostatus immediately pretransplant was known for 34 transplant recipients and their respective 26 renal donors, 6 of whom provided both their kidneys. Of the 19 HSV-seropositive patients who each received their kidney from an HSV-seropositive donor, 16 (d,i,m,n,r,v,x,y,z,ff, jj,kk,qq,rr,ss,tt) developed HSV infection after renal transplantation while 3 (p,bb,cc) of the 6 (c,p,t,aa,bb,cc) HSV-seropositive recipients of a kidney from an HSV-seronegative donor had evidence of post transplantation HSV infection which was most likely due to reactivation of the virus. None of the 9 renal allograft recipients who were seronegative for HSV immediately pretransplant subsequently developed HSV infection though 5 of them (k,s,gg,ii,mm) had each been transplanted with a kidney whose donor was seropositive for HSV.

4.12 Clinical details

4.12.1 Source of HCMV infection

Many studies have shown the renal allograft to be one source of post transplantation HCMV infection and these observations have been confirmed during the course of this project. The HCMV serostatus was available for 31 donors

who provided kidneys for 37 renal allograft recipients, 12 kidneys being received from 6 cadaveric donors. Sera from 28 of the renal donors were tested for antibody to HCMV by the modified CFT while the HCMV serostatus of the other 3 donors was determined in the donating hospital by FAT or RIA.

Post transplantation HCMV infection developed in all 8 HCMV-seronegative renal allograft recipients (a,b,c,d,f,i,j k) whose kidney donors were seropositive for HCMV. By contrast, no evidence of HCMV infection was ever found in the 9 HCMV-seronegative patients (aa,cc,ff,gg,hh,ii,jj,mm, pp) who each received a kidney from a donor known to be seronegative for HCMV. The 8 individuals (l,m,s,t,u,x,y, qq) who were seropositive for HCMV immediately pretransplant and whose kidney donors were also HCMV-seropositive all showed evidence of HCMV infection after the transplant operation. Of the 12 patients (n,p,r,v,z, bb,dd,kk,ll,rr,ss,tt) who were also seropositive for HCMV prior to renal transplantation but who each received a kidney from an HCMV-seronegative donor, 8 (n,p,r,v,z,rr,ss, tt) developed HCMV infection which was assumed to be reactivation of latent virus.

Maintenance of the renal allograft was shown to be unnecessary for the development of post transplantation HCMV and HSV infection. One patient suffered acute rejection and underwent nephrectomy 8 days after the transplant operation. Although no post transplantation urine samples or throat swabs were submitted for tissue

culture, serum specimens (taken between 4 and 9 weeks after the transplant operation), showed a fourfold rise in HCMV and HSV CF antibody titres, no such increases being observed in a preceding serum obtained at 2 weeks post transplantation.

4.12.2 Symptoms

Table 6 summarizes the frequency of symptoms in patients with primary HCMV infection, reinfection and/or reactivation or no evidence of active HCMV infection. Only 1 (9.1%) of the 11 renal allograft recipients with primary HCMV infection remained asymptomatic while 5 (25%) of the 20 patients with reinfection and/or reactivation were free of symptoms. Of the 16 transplant recipients who showed no evidence of active HCMV infection, 9 (56%) were symptomatic. Symptoms developed in 3 of the 6 renal allograft recipients with secondary HSV infection in the absence of HCMV infection. Not all patients developed all symptoms and within each group of patients the timing of the onset and duration of symptoms was variable (Figure 65). However, a clustering of symptoms was evident within the first few months after the transplant operation and again at the onset of the second year post-operatively.

(a) Primary HCMV infection (Figure 65A)

In patients (a), (c), (d), (e), (g), (h), (i) and (j) there was a close temporal relationship between the timing of the fourfold or greater rise in HCMV CF antibody titre

Table 6 The incidence of clinical findings

The frequency of leucopenia, thrombocytopenia, acute and chronic rejection, nephrectomy, pyrexial illness and abnormal liver function tests is shown in each of 11 renal allograft recipients with primary HCMV infection, 20 with reinfection and/or reactivation and 16 with no evidence of active HCMV infection. Also included in the Table is the frequency of symptoms in 6 patients in whom evidence of active HSV but not HCMV infection was found.

Infection	Number of Patients	Leucopenia	Thrombo-cytopenia	Acute Reject-ion	Chronic Rejection	Nephrect-omy	Pyrexial Illness	Abnormal Liver Function Tests	None
Primary CMV	11	6 (54%)	4 (36%)	6 (54%)	4 (36%)	4 (36%)	4 (36%)	5 (45%)	1 (9%)
Secondary CMV	20	5 (25%)	1 (5%)	9 (45%)	3 (15%)	2 (10%)	1 (5%)	10 (50%)	5 (25%)
No CMV	16	1 (6%)	1 (6%)	5 (31%)	2 (12%)	3 (18%)	1 (6%)	4 (25%)	7 (43%)
Primary or Secondary CMV	31	11 (35%)	5 (16%)	15 (48%)	8 (25%)	6 (19%)	5 (16%)	15 (48%)	6 (19%)
Secondary HSV No CMV	6	0 (0%)	1 (16%)	2 (33%)	0 (0%)	1 (16%)	1 (16%)	1 (16%)	3 (50%)

Numbers considered too small to permit meaningful statistical analysis (Professor J.H. Subak-Sharpe and Dr G.D. Murray, personal communication).

Figure 65A Clinical findings: Primary HCMV infection

The figure shows the timing of symptoms in each of 11 renal allograft recipients (a to k) with primary HCMV infection. The following symbols are used:-

 : leucopenia

 : thrombocytopenia

 : pyrexial illness

 : acute rejection

 : chronic rejection

 : abnormal liver function tests. In those cases where only 1 or 2 liver enzymes are significantly increased these are identified by the appropriate abbreviation.

Fn : nephrectomy at n weeks post transplantation

 : onset of azathioprine therapy

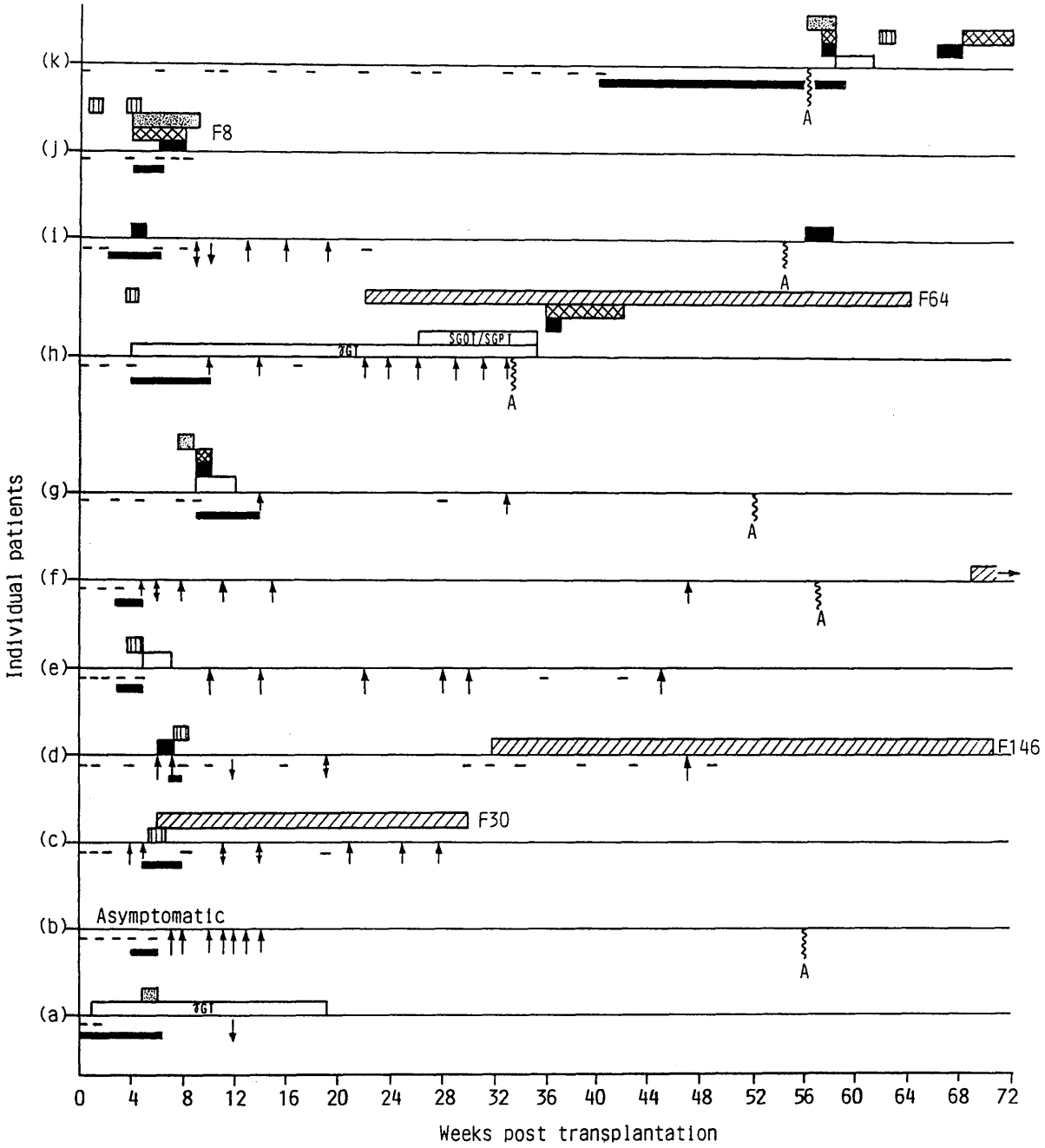
 : HCMV isolated from a urine sample

 : HCMV isolated from a throat swab

 : simultaneous isolation of HCMV from a urine sample and a throat swab

– : no viral isolates.

A solid line below the line of the patient indicates the time interval between consecutive sera, the last of which showed a fourfold or greater rise in HCMV CF antibody titre.


(k)
(j)
(i)
(h)
(g)
(f)
(e)
(d)
(c)
(b)
(a)
F8
F64
F146
F30
Asymptomatic
SGOT/SGPT
γGT
γGT
A
Individual patients
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
Weeks post transplantation

and the onset of symptoms. The time interval between consecutive serum samples from each patient often precluded an accurate correlation from being made between the humoral immune response and the onset of symptoms but in patient (d) an episode of leucopenia and in patient (g) a pyrexial illness clearly preceded the fourfold or greater rise in HCMV CF antibody titre by at least 1 week.

Urine samples and/or throat swabs obtained from patients (a), (e), (g), (h) and (j) at the onset of symptoms failed to yield HCMV in culture while in patient (d) an episode of leucopenia and first isolation of HCMV were coincident. In 5 renal allograft recipients (a,e,g,h,i), the onset of symptoms preceded first isolation of HCMV by between 5 and 11 weeks. Patients (e), (g) and (i) continued to shed HCMV from the urine and/or throat 14 to 38 weeks after all symptoms had resolved. Throughout the period of observation patient (b) remained asymptomatic although HCMV was consistently isolated from urine specimens provided between 7 and 14 weeks after the transplant operation. Symptoms were initially noted in patient (k) at 56 weeks post transplantation but insufficient data are available to allow an accurate temporal correlation to be made either with virus isolation or the humoral immune response. Of the 6 renal allograft recipients who received azathioprine, 4 (f,h,i,k) developed symptoms which were first recorded between 0 and 12 weeks after the onset of therapy.

Acute graft rejection was noted in 6 renal transplant recipients (c,d,e,h,j,k). Urine obtained from patients (d), (h) and (j) at the time of acute renal failure did not yield HCMV in culture. Patients (c) and (d) both shed HCMV in urine taken 1 and 2 weeks prior to the onset of acute rejection which in patient (c) proceeded to chronic rejection and nephrectomy at 30 weeks post transplantation. The onset of HCMV shedding in the urine of patients (e) and (h) followed an episode of acute rejection by 6 weeks but no specimens were submitted for culture during the intervening period. Chronic rejection was first observed in patients (c), (d), (f) and (h) at 2, 24, 64 and 12 weeks respectively after initial HCMV isolation. During the period of chronic rejection, 5 of the 7 urine specimens submitted by patient (c) and all 6 of those provided by patient (h) yielded HCMV in culture while virus was isolated from only 1 of 6 urine samples obtained from patient (d). No specimens were provided by patient (f) during the episode of chronic rejection. Nephrectomy was undertaken in patients (c), (d), (h) and (j) at 30, 146, 64 and 8 weeks respectively after the transplant operation.

(b) Reinfection and/or reactivation (Figures 65B and C)

Of those patients who shed HCMV post-operatively, (l), (n), (o), (p), (q), (r), (s) and (t) remained asymptomatic when a fourfold or greater rise in HCMV CF antibody titre was detected. A close temporal relationship was observed between a thirty two fold increase in HCMV CF antibody

Figure 65B Clinical findings: Reinfection and/or reactivation of HCMV

The figure shows the timing of symptoms in each of 12 renal allograft recipients (l,m,n,o,p,q,r,s,t,u,v,w) with reinfection and/or reactivation of HCMV and from whom the virus was isolated. The following symbols are used:-

 : leucopenia

 : thrombocytopenia

 : pyrexial illness

 : acute rejection

 : chronic rejection

 : abnormal liver function tests. In those cases where only 1 or 2 liver enzymes are significantly increased these are identified by the appropriate abbreviation.

Fn : nephrectomy at n weeks post transplantation

 : onset of azathioprine therapy

 : HCMV isolated from a urine sample

 : HCMV isolated from a throat swab

 : simultaneous isolation of HCMV from a urine sample and a throat swab

– : no viral isolates.

A solid line below the line of the patient indicates the time interval between consecutive sera, the last of which showed a fourfold or greater rise in HCMV CF antibody titre.

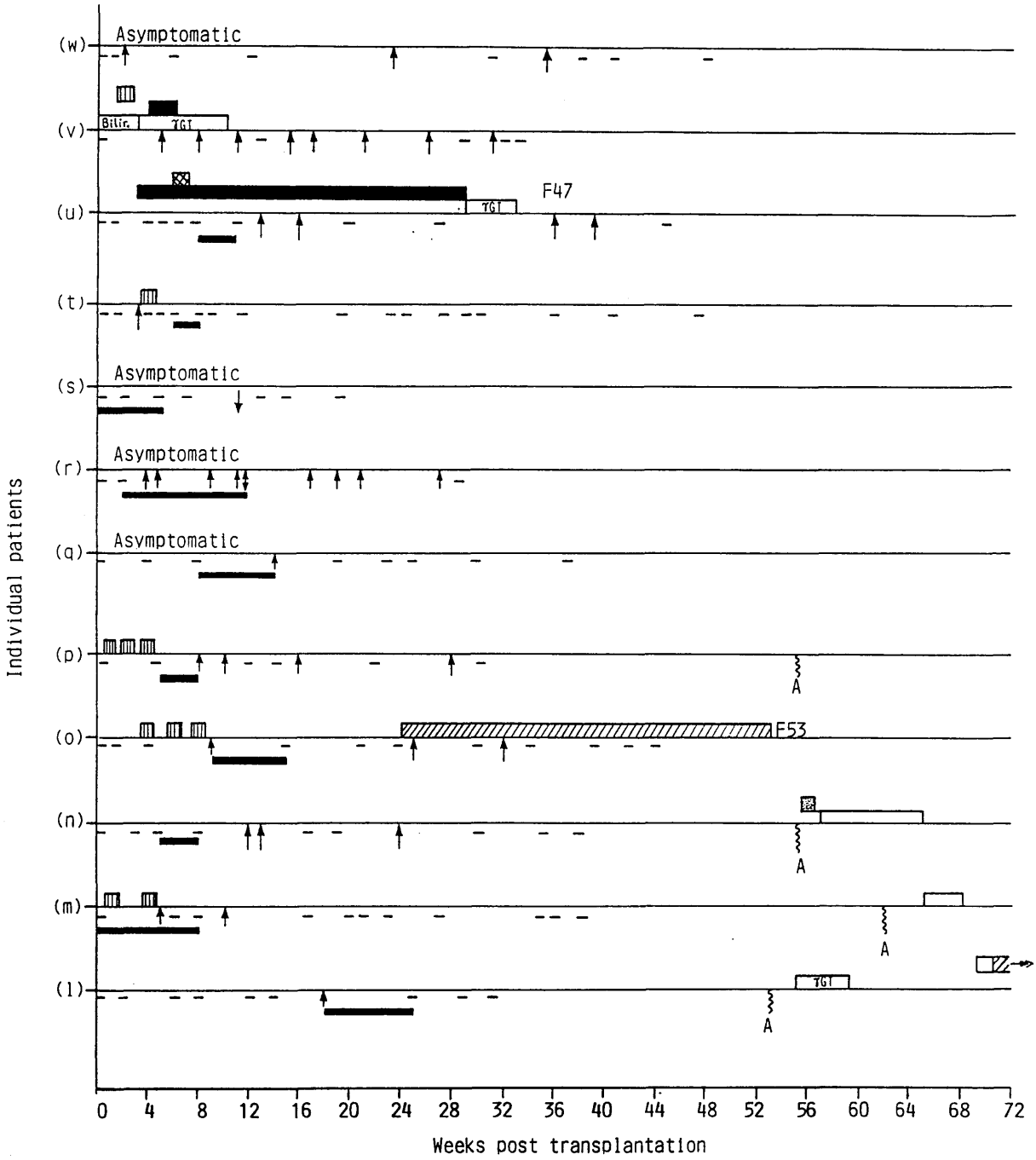

Individual patients
(w)
Asymptomatic
(v)
Bilir.
TGI
(u)
F47
TGI
(t)
(s)
Asymptomatic
(r)
Asymptomatic
(q)
Asymptomatic
(p)
A
(o)
F53
(n)
A
(m)
A
(l)
TGI
A
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
Weeks post transplantation

Figure 65C Clinical findings: Reinfection and/or reactivation of HCMV

The figure shows the timing of symptoms in each of 8 renal allograft recipients (x,y,z,qq,rr,ss,tt,uu) with reinfection and/or reactivation of HCMV but from whom the virus was never isolated. The following symbols are used:-

 : leucopenia

 : thrombocytopenia

 : pyrexial illness

 : acute rejection

 : chronic rejection

 : abnormal liver function tests. In those cases where only 1 or 2 liver enzymes are significantly increased these are identified by the appropriate abbreviation.

Fn : nephrectomy at n weeks post transplantation


A : onset of azathioprine therapy

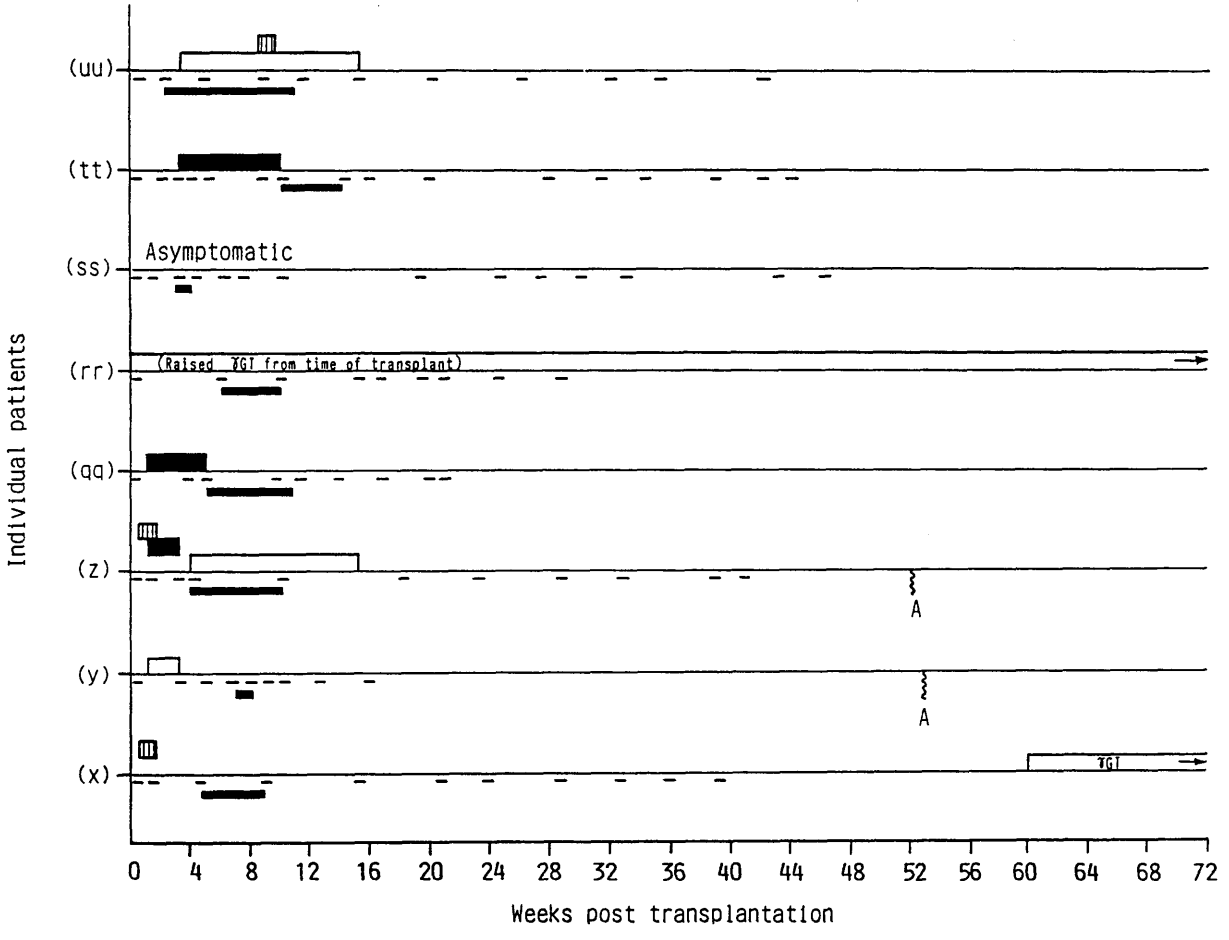
 : HCMV isolated from a urine sample

 : HCMV isolated from a throat swab

 : simultaneous isolation of HCMV from a urine sample and a throat swab

– : no viral isolates.

A solid line below the line of the patient indicates the time interval between consecutive sera, the last of which showed a fourfold or greater rise in HCMV CF antibody titre.


Individual patients
(uu)
(tt)
(ss)
Asymptomatic
(rr)
(Raised ɣGT from time of transplant)
(qq)
(z)
A
(y)
A
(x)
ɣGT
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Weeks post transplantation

titre and acute rejection in patient (m) while in patients (o), (p) and (t) acute rejection clearly preceded the fourfold or greater rise in the HCMV CF antibody titre by at least 2 to 5 weeks. Patient (u) showed a fourfold rise in HCMV CF antibody titre in a serum provided at 8 and 4 weeks after the onset of leucopenia and thrombocytopenia respectively. Of the 2 renal allograft recipients in whom a fourfold or greater rise in HCMV CF antibody titre was never detected, patient (w) remained asymptomatic throughout while patient (v) had episodes of acute rejection, leucopenia and abnormal liver function tests during the first 10 weeks after the transplant operation.

Patients (l), (n), (q), (r), (s) and (w) remained free of symptoms during the first year post transplantation although HCMV was frequently isolated from urine samples and/or throat swabs. In patient (u), leucopenia and thrombocytopenia were first recorded at 10 and 6 weeks respectively prior to the onset of HCMV isolation while a raised level of serum γGT was observed between 16 and 20 weeks after first virus isolation. Patient (v), from whose urine HCMV was isolated on 8 occasions between 5 and 31 weeks post transplantation, was symptomatic as described in the previous paragraph. Of the 4 transplant recipients who received azathioprine, 3 (l,m,n) developed abnormal liver function tests between 2 and 3 weeks after the onset of therapy. In patient (n), a pyrexial illness was noted 1 week after commencing azathioprine while in patient (l) acute rejection progressing to chronic rejection was

reported 16 weeks after starting azathioprine.

Five renal allograft recipients (m,o,p,t,v) experienced 1 or more episodes of acute rejection during the first year after the transplant operation and these were all recorded within the first 8 weeks post transplantation. Urine taken from patients (o) and (t) at the onset of acute rejection failed to yield HCMV in tissue culture while virus was isolated from urine provided by 4 renal allograft recipients (m,o,p,v) between 3 and 7 weeks after the onset of acute rejection. In patient (t), the only urine to yield HCMV in culture was taken 1 week before an episode of acute rejection. After acute rejection had resolved, patients (m), (p) and (t) remained asymptomatic while patient (o) proceeded to chronic rejection and graft failure at 53 weeks post transplantation. During this episode of chronic rejection, HCMV was isolated from 2 of the 8 urine samples provided. Nephrectomy was undertaken in 2 of this group of recipients (o and u) at 53 and 47 weeks respectively after the transplant operation.

Of the 8 patients (x,y,z,qq,rr,ss,tt,uu) with reinfection and/or reactivation of HCMV but from whom the virus was never isolated, 1 (ss) remained asymptomatic throughout the period of observation. Five renal allograft recipients (x,y,z,rr,uu) had abnormal liver function tests. Patient (rr) showed a raised serum γGT level from the onset of renal transplantation while in patient (y) abnormal liver function tests were first observed at least

6 weeks prior to the fourfold rise in the HCMV CF antibody titre. In patients (z) and (uu), there was a close temporal relationship between the fourfold or greater rise in HCMV CF antibody titre and the onset of abnormal liver function tests. A raised level of serum γGT in patient (x) which was initially observed at 60 weeks post transplantation persisted up to 3 years after the transplant operation. The onset of leucopenia in each of patients (z), (qq) and (tt) clearly preceded the fourfold or greater rise in the HCMV CF antibody titre by a minimum of 3, 4 and 7 weeks respectively. Neither of the 2 renal allograft recipients (y and z) who received azathioprine developed symptoms after receiving therapy. Acute rejection was noted in patients (x) and (z) 1 week after the transplant operation and at least 4 and 3 weeks respectively before the fourfold or greater rise in the HCMV CF antibody titre. In patient (uu), there was an episode of acute rejection 9 weeks after the transplant operation but it was not possible to accurately relate this to the timing of the fourfold rise in the HCMV CF antibody titre. None of the patients in this group experienced chronic rejection or underwent nephrectomy.

Clinical virologic correlations were also unable to distinguish the 8 renal allograft recipients (l,m,s,t,u,x, y,qq) who each received a kidney from an HCMV-seropositive donor and at risk of reinfection by a donor strain of virus from the 8 patients (n,p,r,v,z,rr,ss,tt) who each received their kidney from an HCMV-seronegative donor and most

likely experienced reactivation of endogenous virus.

(c) No active HCMV infection (Figure 65D)

Of the 16 renal allograft recipients in whom there was no evidence of active HCMV infection, 8 (aa,cc,ff,hh,ii,kk, mm,pp) developed symptoms. A pyrexial illness was noted in patient (ff) at 1 week post transplantation while leucopenia of 1 week duration was first recorded in patient (ii) at 21 weeks after the transplant operation. Abnormal liver function tests were observed in 4 renal allograft recipients (hh, ii, kk, pp). In patients (hh) and (ii), abnormal liver function tests were initially recorded at 1 and 7 weeks respectively post transplantation. Abnormal liver function tests in patient (kk) were first noted at 52 weeks after the transplant operation coincident with the onset of azathioprine therapy while in patient (pp) abnormal liver function tests were observed from 69 weeks post transplantation. Two (ii, kk) of the 4 renal transplant recipients who received azathioprine developed symptoms very soon after receiving therapy. Episodes of acute rejection occurring at intervals of 1 to 53 weeks after the transplant operation were recorded in 5 renal allograft recipients (aa,cc,ff,ii,mm). In 2 patients (aa and ii), acute rejection proceeded to chronic rejection and nephrectomy at 33 and 20 weeks respectively post transplantation. Sixty seven weeks after receiving a renal allograft, patient (cc) suffered graft failure and subsequent nephrectomy on account of CsA toxicity.

Figure 65D Clinical findings: No active HCMV infection

The figure shows the timing of symptoms in each of 8 renal allograft recipients (aa,cc,ff,hh,ii,kk,mm,pp) with no evidence of active HCMV infection. The following symbols are used:-

 : leucopenia

 : thrombocytopenia

 : pyrexial illness

 : acute rejection

 : chronic rejection

 : abnormal liver function tests. In those cases where only 1 or 2 liver enzymes are significantly increased these are identified by the appropriate abbreviation.

Fn : nephrectomy at n weeks post transplantation

 : onset of azathioprine therapy

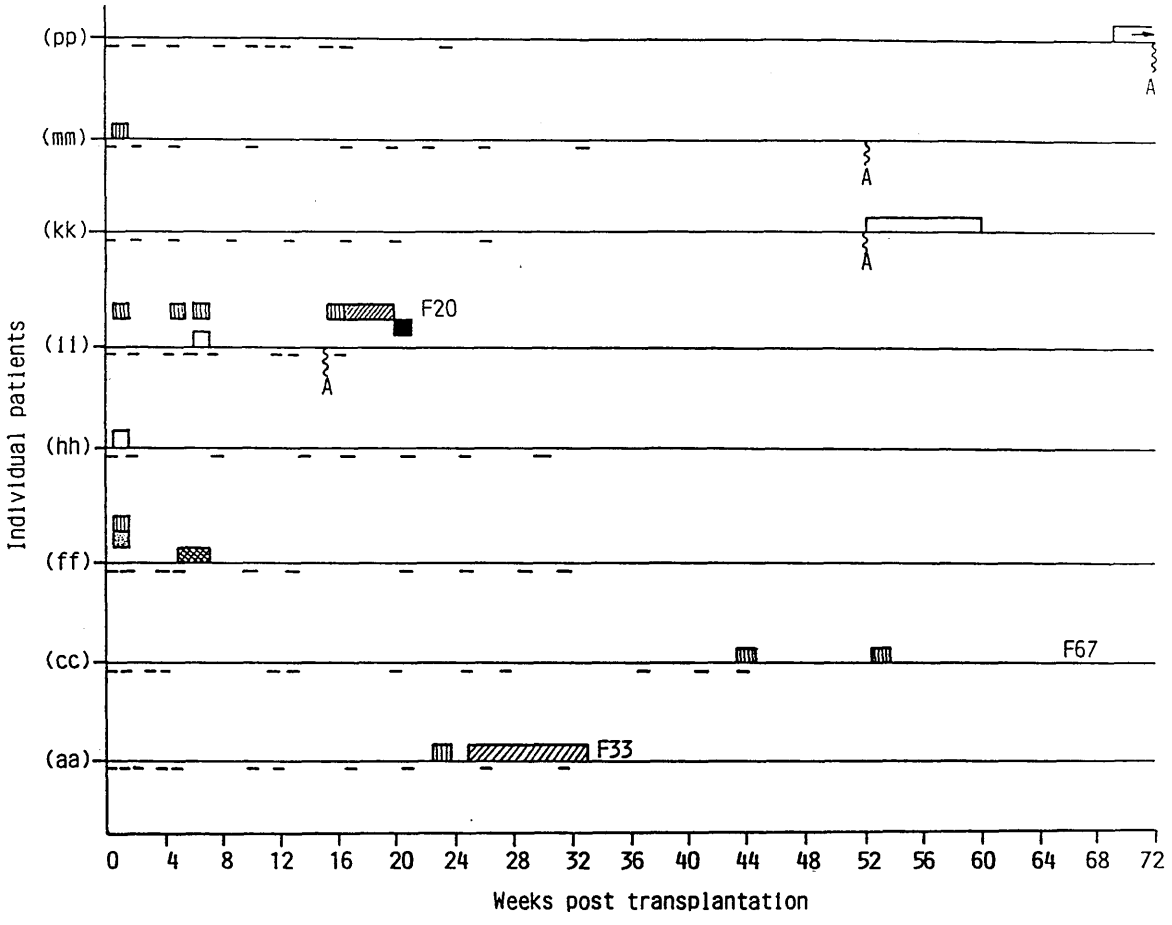
 : HCMV isolated from a urine sample

 : HCMV isolated from a throat swab

 : simultaneous isolation of HCMV from a urine sample and a throat swab

– : no viral isolates.

A solid line below the line of the patient indicates the time interval between consecutive sera, the last of which showed a fourfold or greater rise in HCMV CF antibody titre.


(pp)
(mm)
(kk)
(ii)
(hh)
(ff)
(cc)
(aa)
A
F20
F67
F33
Individual patients
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72
Weeks post transplantation

DISCUSSION

5.1 Incidence of HCMV infection

In this study 31 (66%) of the 47 renal allograft recipients developed HCMV infection on the basis of serology and/or virus isolation. Eleven (35%) were primary HCMV infections while 20 (65%) were secondary, either reinfection and/or reactivation. This incidence is in accord with previous studies which have shown that between 43% and 92% of renal transplant patients develop HCMV infection post-operatively (Glenn, 1981).

5.2 HCMV isolation

Only 6 (1.2%) of the 490 urine samples and none of the 449 throat swabs submitted for tissue culture had to be reinoculated on to a newly prepared monolayer of Flow 2002 cells after the original culture had become contaminated. This low frequency of contamination is accounted for by the filtration of all specimens prior to inoculation of the cell monolayer. Specimen filtration may also have depleted the number of epithelial cells containing infectious virus. The isolation data reported in this study may therefore underestimate the frequency of HCMV shedding. However, a pilot study using unfiltered specimens resulted in an unacceptably high level of culture contamination.

In cultures showing a cpe, plaques developed an average of 26 days (range 7 to 54) after inoculation of the cell monolayer. Many investigators dispose of apparently uninfected cultures after 3 to 4 weeks of incubation (Lui et al., 1987). However, this study shows that 38 (40.8%) of

the 93 cpes identified as HCMV were first observed more than 4 weeks after specimen inoculation. This has important implications when considering the effect of antiviral therapy since premature disposal of cultures may underestimate HCMV shedding. The data shown here confirm that incubation may need to be continued for at least up to 8 weeks after specimen inoculation before any clinical sample can be reported as negative for HCMV (Sorbello et al., 1988). However, a laboratory dealing with large numbers of specimens for the routine screening of HCMV has to maintain a balance between achieving the best possible sensitivity at the most effective cost both in monetary and labour terms.

Twenty two specimens yielded a cpe that progressed slowly and could only be identified as HCMV on the basis of plaque morphology. Such plaques were isolated from both urine samples and throat swabs obtained at intervals throughout the period of study and from patients with primary HCMV infection or reinfection and/or reactivation. Specimens taken from any one individual also varied in the time taken to show a cpe. Renal allograft recipients with primary infection usually only shed 1 strain of HCMV (Wertheim et al., 1983). Therefore, the inability of some plaques to progress in tissue culture cannot always be explained on the basis of inter-strain variation. Some urine samples may have been toxic to the cell monolayer and inhibited HCMV replication. Alternatively, the condition

of some cell monolayers may not have favoured HCMV infection. Indeed, embryonic lung fibroblasts may vary by as much as tenfold in their susceptibility to HCMV infection (Stirk and Griffiths, 1987). When dealing with large numbers of specimens it is not always possible to maintain subconfluent monolayers of cells which may occasionally have overgrown and therefore provided sub-optimal conditions for viral replication (Tanaka et al., 1985). Rapid diagnostic tests such as the hybridot assay do not require virus replication and should theoretically detect HCMV irrespective of its ability to grow in culture. Furthermore, highly specific probes are available that do not cross react with contaminating bacterial DNA. DEAFF can detect HCMV after 6 hr of incubation in tissue culture but does not rely on a cpe to become apparent. This method should theoretically also detect infectious virus prior to the cell monolayer being overgrown with bacterial or fungal contaminants.

The usefulness of new procedures in detecting HCMV in clinical specimens is commonly assessed by reference to virus isolation and some assays have been shown to be highly specific and sensitive. The study reported here shows that some patients with serological evidence of active HCMV infection provided urine samples that failed to yield HCMV in tissue culture. These specimens may nevertheless have contained HCMV that could not be detected by conventional techniques. Whether new rapid diagnostic procedures can detect such virus particles remains unclear.

However, a prospective study correlating the HCMV-specific immune response and virus isolation with DEAFF or hybridot assay may permit a more accurate assessment of the sensitivity of these new techniques. In all reported studies to date isolation of HCMV has been, albeit sometimes slightly, more sensitive, than other methods of virus detection. However, positive results by DEAFF or hybridot assay in the absence of HCMV isolation should not simply be disregarded as spurious reactions without taking into account other parameters of infection such as serology or symptoms.

HCMV was isolated more frequently from urine specimens provided by patients with primary HCMV infection than those with reinfection and/or reactivation. Of the 108 urine samples obtained from 11 renal allograft recipients with primary HCMV infection, 44 (40.7%) yielded the virus in tissue culture. However, HCMV was cultured from only 39 (17.5%) of the 222 urine specimens taken from 20 patients with reinfection and/or reactivation. Pre-existing humoral and cellular immunity in HCMV-seropositive transplant recipients most likely limited viral dissemination. However, the immune response could not entirely contain replication and spread of the virus since HCMV was isolated from urine samples obtained up to 47 or 39 weeks post transplantation in patients with primary HCMV infection or reinfection and/or reactivation respectively. Furthermore, there was no obvious difference in the time interval

between transplantation and first detection of HCMV shedding in allograft recipients who were HCMV-seronegative or -seropositive immediately pretransplant. HCMV is highly cell associated and therefore may escape circulating antibodies while cell-mediated immunity is compromised by immunosuppressive therapy (Tamura et al., 1980).

HCMV was isolated regularly but not always consistently from 9 of the 10 patients with primary infection who showed an HCMV-specific humoral response during the first year after renal transplantation. The pattern of HCMV isolation in renal allograft recipients with reinfection and/or reactivation was more varied: urine samples provided by 1 patient consistently yielded HCMV in tissue culture while specimens taken from others did so intermittently or not at all. This may be related to non-homogenous distribution of the virus in specimens. From the variable nature of HCMV isolation it is clear that serial urine samples must be submitted for tissue culture and that a single negative culture does not exclude active HCMV infection.

The pattern of HCMV isolation may reflect the ability of the immune system to contain the infection. However, virus isolation could not be correlated with immunosuppressive therapy. CsA is excreted primarily unchanged in the urine but HCMV isolation could not be related to deterioration in renal function when increased blood levels of CsA and further immunosuppression may favour virus dissemination. In any event, blood levels of

CsA are monitored regularly and maintained within the therapeutic range. HCMV is known to remain latent in the host after primary infection but not all individuals may harbour HCMV in a form that can be reactivated. HCMV-seropositive renal transplant recipients who receive a kidney from an HCMV-seropositive donor may be reinfected with HCMV of donor origin. In this project, restriction endonuclease analysis of HCMV isolates was not undertaken but the pattern of virus isolation could not be related to donor HCMV serostatus. Indeed, of the 8 renal allograft recipients with reinfection and/or reactivation of HCMV who each received a kidney from an HCMV-seropositive donor, 3 provided urine samples from which virus was never isolated. β_2m coats HCMV particles present in clinical samples and assists in the binding of the virus to receptors present on the target cell (McKeating et al., 1987). Based on a limited analysis of a single urine specimen taken from each of 40 renal transplant patients, only 3 had a β_2m level below the limit of normal. No correlation was noted between the concentration of β_2m and virus isolation.

Of the 306 throat swabs submitted for tissue culture by all patients with serological evidence of active HCMV infection, only 10 (3.2%) yielded HCMV in culture. Isolation of HCMV from throat swabs was coincident with or followed but in no case preceded isolation of the virus from the urine. HCMV was isolated from the throat but not the urine obtained from 2 renal transplant patients, 1 with

primary infection and the other with reinfection and/or reactivation. Therefore, in most cases adequate information on HCMV shedding following renal transplantation can be gained by culturing urine samples alone. In only exceptional cases (e.g. anuria) would additional diagnostic information become available from throat swabs.

5.3 Serology

5.3.1 CFT

The CFT used in this project for the detection of antibodies to HCMV was specific and did not cross react with HSV or VZV. The modified CFT proved to be more sensitive than the standard test. One patient, who immediately pretransplant had an HCMV antibody titre of 1:4 in the modified CFT, must have been seropositive for HCMV but would have been regarded as seronegative by the standard assay. In 17 of the 18 renal allograft recipients who showed a fourfold or greater rise in the HCMV CF antibody titre, this rise was first recorded between 3 and 16 weeks post transplantation. The final patient to show a fourfold rise in HCMV CF antibody titre did so between 18 and 25 weeks after the transplant operation. In all 18 renal allograft recipients, the rise in HCMV CF antibody titre was maintained with minimal fluctuation until at least between 35 and 53 weeks post-operatively. This is in part attributable to the use of glycine-extracted antigen which is known to improve the reproducibility of the CFT for HCMV antibody (Pereira et al., 1982a). The results

also show that in most patients, paired sera submitted for CFT beyond the first 4 months post transplantation are unlikely to yield clinically useful data. Indeed, by this time after the transplant operation the HCMV CF antibody titre may have reached a plateau and any preceding rise in titre may go undetected. Early detection of a fourfold or greater rise in HCMV CF antibody titre is facilitated by the provision of a serum immediately pretransplant which can be tested in parallel with sera obtained at intervals throughout the first year after the transplant operation. The timing of the fourfold or greater rise in HCMV CF antibody titre could not distinguish between patients with primary HCMV infection and those with reinfection and/or reactivation nor could it distinguish patients from whom virus was or was not isolated. However, this may be attributed to the frequency of sampling. Sera obtained at weekly intervals may have been more useful in this respect.

Many studies have reported on the insensitivity of the CFT (Booth et al., 1982). However in this project, all 11 renal allograft recipients with primary HCMV infection and 18 of the 20 patients with reinfection and/or reactivation showed a fourfold or greater rise in the HCMV CF antibody titre. This rise was first recorded prior to or coincident with the onset of HCMV isolation in 7 of the 9 transplant recipients with primary infection and 5 of the 8 patients with reinfection and/or reactivation who shed the virus post-operatively and for whom sufficient data are available. Furthermore, a fourfold or greater rise in the

HCMV CF antibody titre was detected in 1 patient with primary HCMV infection when a significant increase in HCMV-IgM but not IgG was noted. The results of this project also show that in a small proportion of renal allograft recipients (3/31 [9.6%] in this study) a fourfold or greater rise in HCMV CF antibody titre may also be detected when changes in HCMV-IgM and IgG levels are negative or equivocal. Therefore, the CFT may be a useful adjunct to the battery of investigations in the diagnosis of HCMV infection in renal allograft recipients particularly in those with primary infection. However, the data also shows that 2 patients with reinfection and/or reactivation of HCMV who shed the virus post-operatively did not show a fourfold or greater rise in HCMV CF antibody titre. This may be related to strain heterogeneity described by Faix (1985). Therefore, the inability to detect a significant increase in HCMV CF antibody levels in some renal transplant recipients does not exclude active infection. Indeed, some authors have reported significant increases in HCMV-IgM levels prior to a fourfold or greater rise in the HCMV CF antibody titre (Tardy et al., 1987).

The CFT also proved to be more reliable than HCMV-IgG ELISA in determining the HCMV serostatus of renal donors and recipients immediately pretransplant. Of 73 sera tested by both methods, concordant results were obtained in 67 (91.7%). By retrospective analysis of the pattern of HCMV isolation, the clinical findings and careful

examination of donor/recipient pairings, it was considered that the HCMV CF antibody titre more accurately reflected the HCMV serostatus in 7 sera with uncertain HCMV-IgG levels. Although measurement of HCMV-IgG levels by ELISA may be a practical solution to quickly determining donor and recipient HCMV serostatus immediately pretransplant, the results of this project show that all sera should be retested by CFT at the earliest opportunity. The CFT is itself impractical as a rapid diagnostic test on account of the overnight incubation step. Recently, a latex method for the rapid detection of HCMV antibody has been shown to be useful in determining donor and recipient serostatus immediately pretransplant (Wreghitt, 1988). However, time did not permit an assessment to be made of the usefulness of the latex test in determining the HCMV serostatus in those sera with uncertain HCMV-IgG levels.

5.3.2 HCMV-IgG

Labsystems ELISA was routinely used to determine HCMV-IgG levels in all sera. The ELISA was specific and did not cross react with antibodies to HSV and VZV. However, considerable variation was noted in the HCMV-IgG level in some sera when tested in different batches of the ELISA. This is probably attributable to batch to batch variation in reagents and the amount of antigen bound to wells of the ELISA. Operator error in, for example, pipetting may also contribute. The variability in estimated levels of HCMV-IgG emphasizes the need to retest all sera within a single

batch of the ELISA when comparing antibody levels in a number of sera taken from 1 or more individuals.

Although there was good overall correlation between HCMV-IgG levels and HCMV CF antibody titres, there was considerable variation between HCMV-IgG levels in sera with the same titre of HCMV CF antibody. This confirms the findings of other investigators (Nielsen et al., 1986). Different serological assays most likely measure different antibodies. Indeed, IgG subclasses fix complement in the order IgG3 > IgG1 > IgG2 > IgG4 but all may be equally detected by ELISA. Furthermore, renal allograft recipients may vary in the subtype and epitope specificity of antibodies produced.

HCMV-IgG ELISAs produced by different manufacturers may also give conflicting data. In this project, 6 (8.3%) of 72 sera tested by both Labsystems and NBL ELISA yielded disconcordant results. This may reflect differences between assays in the antigens, reagents, serum dilutions and incubation times used. Unfortunately, reference sera against which the sensitivity and specificity of an ELISA can be compared are as yet unavailable. All disconcordant results in this study were from sera subsequently found to be positive for HCMV by CFT and of these 2 were provided by renal donors. Confusion as to the HCMV serostatus of kidney donors and recipients may result in an HCMV-seronegative transplant patient receiving a kidney from a donor thought to be seronegative for HCMV but who is in fact seropositive. Allograft recipients in this category

may not be considered as being at high risk of developing HCMV infection and prophylactic therapy may not be instigated.

Early diagnosis of HCMV infection by measuring virus-specific IgG proved disappointing in the 11 renal transplant patients with primary infection. A significant increase in HCMV-IgG was noted in 3 patients on or before the onset of virus isolation and in 4 transplant recipients prior to or coincident with a fourfold or greater rise in the HCMV CF antibody titre. However, in patients with reinfection and/or reactivation measurement of HCMV-IgG levels by ELISA proved useful. Of 10 renal allograft recipients who shed HCMV post-operatively and from whom sufficient data are available, 6 showed a significant increase in HCMV-IgG on or before the onset of virus isolation. Seven of the 20 patients with serological evidence of reinfection and/or reactivation had evidence of a significant increase in HCMV-IgG prior to a fourfold or greater rise in the HCMV CF antibody titre while in 10 other patients the fourfold or greater rise in HCMV CF antibody titre and significant increase in HCMV-IgG were coincident. Furthermore, 2 renal transplant recipients with reinfection and/or reactivation of HCMV showed a significant increase in HCMV-IgG but not CF antibody post-operatively. These data clearly show the usefulness of the HCMV-IgG ELISA in the early detection of HCMV infection in renal allograft recipients with reinfection and/or

reactivation. This probably reflects the ability of this group of patients to mount an early anamnestic response by clonal expansion of circulating memory lymphocytes which are absent in HCMV-seronegative recipients. CF antibody may appear later in the course of HCMV infection than IgG of a subclass which mediates other in vivo functions including neutralization or ADCC and which is not detected by CFT. The magnitude of the HCMV-IgG response in transplant recipients with reinfection and/or reactivation could not distinguish patients from whom HCMV was or was not isolated. However, the immune response is influenced by a variety of factors including the host's age and HLA type. Therefore, unless large numbers of individuals are studied, it may be difficult to correlate antibody levels with other parameters of HCMV infection. The magnitude of the HCMV-IgG response was also unable to distinguish HCMV-seropositive individuals who received their kidney from HCMV-seronegative donors and most likely to experience reactivation of endogenous virus from those who received their kidney from HCMV-seropositive donors and also at risk of reinfection by donor virus. However, not all renal allografts transmit HCMV post-operatively.

In patients with primary infection, HCMV-IgG levels gradually increased between 6 and 14 weeks to the end of the first year post transplantation while during the same period HCMV-IgG levels decreased in 19 of the 20 patients with reinfection and/or reactivation. In the absence of a

serum taken immediately pretransplant the pattern of the HCMV-IgG response may be helpful in distinguishing renal allograft recipients with primary HCMV infection from those with reinfection and/or reactivation. Patients with reinfection and/or reactivation of HCMV who provide paired sera beyond the first 3 months post transplantation may show static or falling levels of HCMV-IgG. This could be interpreted as resolution of infection. However, the isolation data reported here shows that virus shedding may persist and antiviral therapy should not be discontinued. These findings provide further evidence as to the inability of the humoral immune response to contain HCMV infection in some renal transplant recipients.

5.3.3 HCMV-IgM

Labsystems ELISA was routinely used for the determination of HCMV-IgM levels in all sera. In some studies, macroglobulins specific for HCMV have been shown to cross react with other herpesvirus antigens (Hanshaw et al., 1972). The findings in this study are consistent with those of other investigators (Rasmussen et al., 1982) who found no cross reaction between HCMV-IgM and HSV or VZV antigens.

Heterotypic IgM antibody responses have been reported in as many as 30% of patients with IMN and polyclonal stimulation of B lymphocytes by EBV seems to be the most likely explanation (Schmitz, 1982). In this project, none of the 44 patients examined had evidence of heterophile

antibodies immediately pretransplant. Time did not permit the testing for heterophile agglutinins in all sera from all patients. However, in each renal allograft recipient with an HCMV-IgM response that serum showing the highest recorded level of HCMV-IgM was negative for heterotypic antibody.

HCMV-IgM may be falsely positive in the presence of IgM-RF which is an IgM antibody to IgG circulating in the blood of patients with certain collagen vascular disorders. IgM-RF may complex with HCMV-specific IgG and adsorb to the specific antigen of the ELISA. It may then cross react with conjugated anti-IgM and produce false-positive results. The data reported here show that in renal allograft recipients with increasing levels of IgM-RF during the first few months post-operatively, there was a close temporal relationship between the HCMV-IgM and IgM-RF response. This probably reflects the on-going interaction between HCMV and the immune system. Many investigators pretreat all sera to remove IgM-RF prior to testing for HCMV-IgM (Stagno et al., 1985). However, the results of this project show that there was no obvious correlation between levels of IgM-RF and HCMV-IgM in 136 sera submitted by 33 patients. Furthermore, only 4 of these sera had an IgM-RF level above the upper limit of normal. If IgM-RF caused false-positive reactions in sera tested by Labsystems ELISA it may also be expected to yield falsely elevated IgM levels to any other virus when tested by

indirect ELISA. However, sera with equivocal or positive levels of HCMV-IgM were clearly negative when tested for rubella-IgM. In reconstruction experiments, IgM-RF added to an HCMV-IgG positive but HCMV-IgM and IgM-RF negative serum did not raise the level of HCMV-IgM when tested by Labsystems indirect ELISA. These data clearly show that IgM-RF does not interact with the Labsystems ELISA and create falsely elevated HCMV-IgM levels. Salonen et al. (1980) have reported similar findings using another HCMV-IgM ELISA. It may be that the use of a sensitive assay allows the virus-specific IgM to be diluted beyond the concentration of IgM-RF thus eliminating the potential for interference. Indeed, attempts to remove IgM-RF may also deplete HCMV-IgM and produce false negative results. Some studies have used a latex-slide agglutination method to identify RF positive sera (Rasmussen et al., 1982). However, this is a qualitative test and non-specific reactions may occur due to, for example, hyperlipidaemia (Hechemy and Michaelson, 1984). In this project the specificity of the RF latex assay was 40% in comparison to measurement of IgM-RF by ELISA. Therefore, testing by latex-slide agglutination alone vastly overestimates the number of IgM-RF positive sera.

Considerable effort has gone into developing a reliable assay for HCMV-specific IgM antibodies and numerous immunoassays have been reported. Although such methods may be of use in the diagnosis of acute HCMV infection, particularly in immunosuppressed patients who

are at considerable risk of HCMV-induced disease, little is known about the temporal relationship between the onset of HCMV isolation and HCMV-IgM levels. An HCMV-IgM response may be detected prior to virus isolation (Tardy et al., 1987). In this project, of the 9 patients with primary HCMV infection from whom the virus was isolated, 4 showed evidence of an HCMV-IgM response at or before the onset of HCMV isolation. Only 3 of 12 renal allograft recipients with reinfection and/or reactivation had elevated HCMV-IgM levels at or before virus isolation. Similar findings have been reported by Sutherland and Briggs (1983) who used a MACRIA assay and showed HCMV isolation from 2 renal transplant recipients at 13 and 23 days prior to a significant increase in HCMV-IgM. The results of this project also show that some patients have a transient increase in HCMV-IgM and therefore the inability to detect this antibody does not exclude active HCMV infection. This confirms the findings of Kangro et al. (1982). Some renal allograft recipients have been noted to maintain high levels of HCMV-IgM throughout the first year post transplantation both in this study and in others (Pass et al., 1983). Therefore, a significantly increased HCMV-IgM level is not necessarily diagnostic of acute infection.

The results of this project confirm other reports that renal allograft recipients with reinfection and/or reactivation are able to mount an HCMV-IgM response post transplantation (Kangro et al., 1982). Some investigators

have reported significantly higher levels of HCMV-IgM in renal transplant patients with primary HCMV infection than in those with reinfection and/or reactivation (Wreghitt et al., 1986a). However, the results of this project do not concur with these findings. Inter-assay variation may account for this discrepancy. Furthermore, unless sera from a number of individuals are tested within a single batch of any serological assay, as in this project, it may be difficult to compare HCMV-IgM levels between groups of patients. Some authors may also have pretreated certain sera to remove RF and inadvertently reduced the concentration of HCMV-IgM. Sutherland and Briggs (1983) have proposed that an HCMV-IgM response in renal allograft recipients with reinfection and/or reactivation of HCMV is due to reinfection by a new HCMV strain, presumably from the donated kidney. In this project, the highest recorded levels of HCMV-IgM in HCMV-seropositive patients who received a kidney from an HCMV-seronegative donor were not obviously different from those in HCMV-seropositive recipients of a kidney from an HCMV-seropositive donor. However, restriction endonuclease analysis of HCMV isolates was not undertaken and the number of patients studied was small.

In conclusion therefore, the results of this project show that measurement of HCMV-specific CF, IgM and IgG antibodies in each individual patient is required to establish the presence of an active infection at the earliest opportunity. In a number of cases HCMV isolation

precedes a detectable host humoral response. This temporal advantage may be lost because the cpe evolves slowly. Therefore, rapid diagnostic methods such as DEAFF have an important role in the early diagnosis of HCMV infection. The usefulness of nucleic acid probes in this respect remains to be determined.

5.4 Dot blot hybridization

5.4.1 M13 probes

Although M13 probes have been successfully employed in the selection of complementary clones from within a shotgun sequencing bank (Hu and Messing, 1982), the M13 hybridization system described here proved to be disappointing. The sensitivity of the assay did not extend beyond the ng range although nick-translated (Spector et al., 1984b) and RNA (Schuster et al., 1986) probes have been reported to detect pg quantities of cDNA. This may have been attributable to the low concentration (50ng) of M13 DNA in 8 ml of hybridization solution. Background noise also proved to be a problem. There was marked variation in the concentration of [α-^{32}P] dATP from within different batches of the radionucleotide, a problem which could not be resolved after discussion with the manufacturers. The newly synthesized radiolabelled DNA strand remained attached to the M13 template but did not extend beyond a few hundred bp. This could not be explained on the basis of insufficient substrate. Second strand M13 synthesis was theoretically limited to within a few bp of cloned viral sequences by restricting [α-^{32}P] dATP to 33pmol per 50ng of template DNA. However, increasing [α-^{32}P] dATP to 66pmol per 50ng of template DNA did not achieve radiolabelled M13 second strands of higher MWt. Consequently, most of the M13 template remained single stranded and able to hybridize with cDNA other than cloned viral-specific sequences. Replacing [α-^{32}P] dATP by

biotinylated dATP was also unsuccessful in efficiently labelling M13 DNA possibly due to a steric hindrance effect of the biotin molecule attached to the nucleotide. However, many authors have successfully used biotinylated probes to detect cDNA (Leary et al., 1983).

5.4.2 Riboprobe pGHBI

The BamHI subclone of the HindIII E region of HCMV AD169 was successfully cloned in the transcription plasmid pGEM2 which contains promoters for both T7 and SP6 polymerase. In all experiments T7 polymerase was used, the resultant transcripts having the same sense as mRNA originating from the same fragment of the HCMV genome. The magnitude of any specific hybrid signal is then proportional to the amount of cDNA bound to the nitrocellulose filter and to the number of HCMV particles from which the DNA was extracted. After urea-polyacrylamide gel electrophoresis, radiolabelled transcription products of predicted MWt 1400 bp were observed in addition to a number of transcripts of lower MWt. These latter transcription products may represent incompletely synthesized cRNA or breakdown products of full length radiolabelled transcripts.

In the hybridot assay, radiolabelled cRNA transcripts routinely detected 8 and/or 80pg of vector pGHBI DNA and 10 and/or 100pg of vector pEHBI DNA after 24 hr of autoradiography. This level of sensitivity is in accord

with the findings of Melton et al. (1984) although Church and Gilbert (1984) have been able to detect fg quantities of cDNA using a single stranded RNA probe. Background noise was negligible probably reflecting the reduced tendency of single stranded RNA to bind to nitrocellulose paper. The hybrid signals observed at each dilution of target pGHBI DNA from 800ng to 80pg increased as the hybridization temperature was raised from 40 to 60°C. This phenomenon probably reflects increased diffusion of available cRNA probe to filter bound sequences with increasing hybridization temperature. At 70°C the nitrocellulose filter became too fragile to manipulate. Nylon filters which are stable at 70°C were tested in the hybridot assay but background noise was unacceptably high and time did not permit reappraisal of hybridization conditions. When the period of hybridization was extended from 14 to 62 hr, the hybrid signal detected at each dilution of the target DNA diminished and the lower limit of detection was reduced by 1 $\log_{10}$. This is probably accounted for by sequences leaching off the filter and gradual degradation of the radiolabelled probe. Hybrid signals obtained after hybridization for 38 hr compared to 14 hr were only marginally reduced. The sensitivity of the hybridot assay was not evaluated at hybridization periods of less than 14 hr since it was convenient if all hybridization reactions were allowed to proceed overnight (approximately 14 hr).

The specificity of the hybridot assay was established. There was no cross reactivity with 10μg of human DNA thus confirming the findings of Ruger et al. (1984). 10μg each of HSV-1 and E.coli DNA were also negative by dot blot hybridization but 10μg of HSV-2 HG52 DNA showed a hybrid signal equivalent to that obtained with 8pg of vector pGHBI DNA. However, it was not considered necessary to increase the stringency of the hybridot assay since HSV-2 in clinical samples can be readily detected by culture in BHK21/C13 cells. Furthermore, it would be most unlikely to extract microgram amounts of HSV-2 DNA from the urine of a renal transplant recipient as this would require the virus to be present in excess of 10^9 particles/ml. Contrary to other findings that RNA transcripts may react non-specifically with ribosomal RNA (Cova et al., 1988), the cRNA probe used in this project did not detect 10μg of ribosomal RNA. This could be attributed to the preparative procedure involving denaturation by sodium hydroxide which would degrade RNA (Anderson and Young, 1985). The radiolabelled cRNA transcripts also hybridized to vector pGEM2 DNA, 10ng of this plasmid DNA giving a hybrid signal equivalent to that observed with 8pg of vector pGHBI DNA. This cross hybridization was probably attributable to identical leader sequences present in both vectors. However, it was considered unlikely that vector pGEM2, recently constructed for in vitro use, would be found in any urine specimen.

The sensitivity of the hybridot assay was also established. Radiolabelled cRNA transcripts consistently detected between 4.3×10^4 to 3.9×10^5 particles taken from any one of 3 stocks of HCMV AD169 and added to 7.2 ml of urine. These findings are in accord with those of Chou and Merigan (1983). Dot blot hybridization was more sensitive than electron microscopy in the detection of HCMV. One stock of HCMV AD169 in which no virus particles were observed by electron microscopy had a titre of 1.3×10^2 pfu/ml and was also positive by hybridot assay. Furthermore, electron microscopy cannot distinguish between members of the herpesvirus group whilst the cRNA probe did not cross react with HSV-1 or VZV. One other advantage of the hybridot assay was the ability to detect non-infectious particles which presumably contained a DNA core that provided a target for the radiolabelled cRNA transcripts. Other diagnostic procedures such as DEAFF only detect HCMV which can replicate in a cell monolayer and express viral antigens.

Compared with HCMV isolation, the results of the hybridot assay were disappointing with a specificity of 67% and a sensitivity of 25% when 445 urine specimens were tested for HCMV DNA. Sixty eight urine specimens could not be clearly identified as either positive or negative by RNA-DNA hybridization. Therefore, the RNA-DNA hybridot assay described here was clearly less sensitive and specific than that described by other authors whose radiolabelled cRNA transcripts originated from the HindIII

L fragment of HCMV AD169 (Schuster et al., 1986). This poor performance cannot be explained on the basis of genetic heterogeneity between viral isolates since the cRNA probe detected HCMV strains AD169 and Davis in addition to 65 other independent isolates cultured from urine samples or throat swabs provided by 18 patients. Restriction endonuclease analysis of HCMV isolates was not undertaken but it is unlikely that any 2 individuals would shed identical HCMV strains. However, the cRNA probe was generated from the major IE gene of HCMV which does show heterogeneity between isolates (Chandler and McDougall, 1986). Urinary DNase activity was observed and noted to reduce the sensitivity of the hybridot assay by 2 $\log_{10}$ in reconstruction experiments. However, most viral DNA in clinical specimens is likely to be encapsidated. When HCMV particles were incubated overnight at 37^{o}C the sensitivity of the hybridot assay was also reduced by 2 $\log_{10}$. Urinary proteases may have degraded the viral capsid protein and exposed HCMV nucleic acid to DNase. The lower limit of detection of cDNA was also reduced when HCMV DNA was added to 10μg of either human or E.coli DNA prior to binding to the nitrocellulose filter. Therefore, DNA extracted from human epithelial cells or bacteria present in urine samples may also compete for available space on the nitrocellulose filter whose binding capacity was 8μg of DNA. Smaller DNA molecules bind more efficiently to nitrocellulose paper than those of a higher MWt and

therefore it is difficult to explain why human DNA reduced the sensitivity of the hybridot assay more effectively than E.coli DNA. It is possible that human DNA was fragmented during the preparative procedure. The poor performance of the hybridot assay may also be explained on the basis that all urine specimens had been stored at -70°C for up to 3 years prior to testing by RNA-DNA hybridization. Long periods of storage in urine may have degraded the virus particles and/or DNA although Gibson et al. (1985) have been able to detect polyomavirus DNA in urine specimens which had been stored at 4°C for up to 7 years. Attempts to increase the sensitivity of the hybridot assay by using PEG 6000 to aid precipitation of HCMV, by using alternative autoradiography film and by reducing the stringency of the procedure for washing the nitrocellulose filters after hybridization all proved to be unsuccessful.

Most reports investigating the usefulness of dot blot hybridization in the rapid diagnosis of HCMV infections have involved testing 1 or a few urine specimens taken from individuals with a variety of clinical conditions. No group has yet prospectively followed a cohort of renal allograft recipients and correlated the results of virus isolation and the HCMV-specific humoral response with the results of a hybridot assay. The study reported here shows virus isolation to be more sensitive than RNA-DNA hybridization in detecting urinary HCMV shedding post renal transplantation. Nine (45%) of the 20 renal allograft recipients from whom HCMV was isolated submitted urine

samples found to be positive by hybridot assay on or before the day of first HCMV isolation. Only 13 (43%) of the 30 patients who showed an HCMV-specific humoral response provided urine specimens which were positive by dot blot hybridization on or before the day when a significant increase in either HCMV-IgM, IgG or CF antibody was first recorded. However, the significance of a positive result in the hybridot assay is unclear since 10 patients who remained HCMV-seronegative throughout the period of study provided 17 urine samples which were clearly positive by RNA-DNA hybridization. Such hybrid signals must have been false-positives and may be attributed to cross reaction with DNA of another virus, possibly human polyomavirus (e.g. BK virus) which is shed in high titre in the urine of some renal allograft recipients post-operatively (Gardner et al., 1984). With few exceptions however, positive results in the hybridot assay were spurious and atypical of any pattern of virus shedding. Nevertheless, it is possible to rehybridize the filters with a probe specific for another virus. Radiolabelled cRNA may have been trapped on the nitrocellulose filter by impurities originating from the urine specimens, but the latter were routinely phenol extracted and ethanol precipitated. Finally, false positive signals may have resulted from incomplete digestion of the DNA template after RNA transcription (Melton et al., 1984). False positive hybrid signals may be reduced by incorporating RNase into the washing solution.

This enzyme would digest cRNA probe not wholly complementary to the target DNA but time did not permit a detailed assessment of this approach.

Some studies correlate the sensitivity of HCMV-specific probes with virus isolation from urine submitted by individuals who usually shed HCMV in high titre e.g. congenitally infected neonates. However,it is important to take account of the population whose specimens have been tested by dot blot hybridization. Renal allograft recipients are less heavily immunosuppressed than some other transplant patients and experience less severe HCMV infection. This is probably attributable to a more effective immune response and increased clearing of circulating virus. Therefore, the majority of renal allograft recipients may not shed HCMV in an amount which exceeds the lower limit of sensitivity of the hybridot assay. Recently, Schuster et al. (1986) have described an RNA-DNA hybridot assay which has a sensitivity of 83% when used to detect HCMV shedding by patients, including renal allograft recipients, with a variety of clinical disorders. The sensitivity of the RNA-DNA hybridot assay described in this project may be improved by increasing the volume of urine submitted for testing but this may create difficulties in terms of storage and processing. Furthermore, renal allograft recipients whose kidney function is already compromised may not be able to provide large quantities of urine especially within the first few months after the transplant operation when the risk of HCMV

infection is at its greatest. An alternative approach would be to use PCR to amplify DNA sequences from a small volume of urine.

5.5 Source of HCMV infection

The results of this project clearly show that the donor kidney most likely transmitted HCMV infection in renal allograft recipients and confirms the findings of others (Chou, 1986). All 8 HCMV-seronegative recipients of a kidney from an HCMV-seropositive donor developed primary HCMV infection while no evidence of infection was ever found in any of the 9 HCMV-seronegative recipients of a kidney from an HCMV-seronegative donor. Some studies have reported on the inability of some kidneys from HCMV-seropositive donors to transmit the infection even to HCMV-seronegative recipients (Chou and Norman, 1988). However, this may simply reflect the inability to detect active HCMV infection in some patients. Restriction endonuclease analysis of HCMV isolates was not undertaken and therefore it is not possible to comment on the frequency of HCMV reinfection in HCMV-seropositive recipients of a kidney from an HCMV-seropositive donor.

In this study there was no evidence of HCMV transmission by blood products but the number of patients studied was small. The risk of HCMV infection via blood products increases with the number of units transfused. However, few renal allograft recipients in this project

received large numbers of transfusions. Furthermore, all blood products had been stored at 4°C for up to 3 weeks thus decreasing the risk of HCMV transmission even further. Finally, the majority of blood donors were aged between 19 and 29 years and, as expected, were mostly seronegative for HCMV.

5.6 HSV infections

Twenty four (51%) of the 47 renal allograft recipients had evidence of post transplantation HSV infection all of which were reinfection and/or reactivation. These data confirm the findings of Ho (1977) who has reported that most HSV infections in renal allograft recipients are due to reactivation of latent virus.

Isolation of HSV was less frequent than that of HCMV with only 6 (1.2%) of 490 urine samples and 13 (2.8%) of 449 throat swabs yielding the virus in tissue culture. Unlike HCMV, HSV can establish latent infection in the trigeminal ganglia. Therefore, it is not surprising that HSV was more commonly isolated from throat swabs than urine samples. Isolation of HSV from urine was only attained in female transplant recipients and most likely represents contamination of these specimens by virus shed from the genital tract. HSV isolation was more common during the first 3 months post-operatively when immunosuppressive therapy is maximal. However in some renal allograft recipients, HSV was first isolated many months after the transplant operation and may represent random shedding

which occurs in healthy individuals in response to a variety of stimuli. Unlike most cases of HCMV infection, isolation of HSV was transient and probably reflects the ability of the immune response to contain HSV infection. Restriction endonuclease analysis of virus isolates showed that only HSV-1 was ever isolated from throat swabs while only HSV-2 was shed in the urine. This contrasts with the findings in HCMV infection when 1 strain is commonly isolated from many sites (Grundy et al., 1987). In this project there was no evidence of reinfection by exogenous strains of HSV, all viruses isolated from either urine samples or throat swabs of any 1 individual having the same restriction enzyme profile. Transmission of HSV by the renal allograft has recently been reported by some workers (Koneru et al., 1988) but these findings could not be confirmed here. However, not all renal allografts may harbour HSV in a form that can be readily reactivated as in the case of HCMV.

Of the 16 renal transplant recipients with serological evidence of active HSV infection, only 3 submitted specimens from which the virus was isolated. While this may be explained on the basis of efficient clearing of HSV by the immune response it is also possible that infrequent sampling may have overlooked transient or intermittent shedding. However, it is interesting to note that a fourfold or greater rise in HSV CF antibody titre was never observed in sera taken from 7 patients who shed the virus

post-operatively. In 3 other renal allograft recipients, a fourfold or greater rise in HSV CF antibody titre was first recorded in sera provided some weeks after initial HSV isolation. These data provide further evidence on the importance of virus isolation in the early diagnosis of infection and underline the insensitivity of the CFT in some cases.

5.7 Clinical findings

Unfortunately, the small number of patients in this study precluded any statistical correlations to be made between HCMV infection and clinical findings. However, symptoms were more common in those patients with HCMV infection and, with the exception of abnormal liver function tests, symptoms occurred more frequently in renal allograft recipients with primary HCMV infection. This confirms the findings of others and provides further evidence as to the ability of the immune response to abrogate the effects of HCMV infection (Ho et al., 1975). Not all patients developed all symptoms. This may be attributable to genetic susceptibility to HCMV infection and disease but the small number of patients in this study did not permit a correlation to be made with HLA status. Furthermore, some strains of HCMV may be more pathogenic than others.

In this study the onset of symptoms was often associated with a humoral immune response thus confirming the findings of Kangro et al. (1982). However, this

temporal association cannot be taken as evidence that HCMV infection is responsible for the clinical findings. In some patients the onset of symptoms often preceded by a number of weeks first detection of HCMV infection by serology and/or virus isolation. Similar findings have been reported by Pass et al. (1983). Such data suggest that symptoms suggestive of HCMV infection should be treated with the appropriate antiviral therapy even in the absence of laboratory evidence of active infection. By contrast, some renal allograft recipients showed evidence of active HCMV infection many weeks prior to the onset of symptoms while in others HCMV shedding continued after all symptoms had resolved. In such cases, symptoms may be entirely related to infection by another virus. Alternatively, symptoms may only develop when HCMV infection occurs concomitantly with another factor(s) such as high doses of immunosuppressive therapy which promotes HCMV dissemination. Some renal allograft recipients with HCMV infection remained asymptomatic throughout. The ability to identify this group of patients immediately pretransplant would permit prophylactic therapy to be targeted at those transplant recipients at highest risk of HCMV-induced disease.

As in previous reports, graft rejection and nephrectomy were more common in patients with primary HCMV infection but a cause and effect relationship is difficult to establish. Indeed, some renal allograft recipients who

remained seronegative for HCMV throughout the period of study showed episodes of acute rejection within the first few months post transplantation.

Recent reports have suggested that post transplantation HCMV-IgM levels are prognostic of clinical outcome (Wreghitt et al., 1986). In this project only 1 patient with primary HCMV infection remained asymptomatic. Interesting HCMV-IgM levels were equivocal by Labsystems ELISA. However, HCMV-IgM levels varied irrespective of clinical findings in patients with reinfection and/or reactivation when pre-existing immunity may have abrogated the effects of HCMV infection.

Renal transplant recipients at the Western Infirmary, Glasgow routinely have their immunosuppressive therapy changed from CsA to azathioprine at the end of the first year post transplantation. CsA is nephrotoxic. Indeed, in one renal allograft recipient CsA adversely affected renal function and nephrectomy was undertaken. Azathioprine was well tolerated although some patients developed abnormal liver function tests. However, these data cannot be compared with those from other centres which use azathioprine as the primary immunosuppressive agent from the onset of the transplant operation. One patient with primary HCMV infection developed symptoms shortly after the onset of azathioprine therapy. Unfortunately, virus isolation data was unavailable and the time interval between consecutive sera did not permit the timing of the humoral immune response to be determined precisely.

Nevertheless, it is interesting to speculate that the immunosuppressive effect of azathioprine but not CsA induced HCMV reactivation. If this is indeed the case some transplant recipients may not benefit from azathioprine therapy.

Future Prospects

This project has provided information concerning the temporal correlation between the onset of HCMV infection and the humoral immune response in renal transplant recipients during the first year post-operatively. However, a study confined to the first few months post transplantation may yield more detailed information concerning how frequently and for how long HCMV isolation precedes the host humoral immune response. Such an investigation could also incorporate a study of the cell-mediated immune system.

A number of sera have been collected during the course of this project from patients whose virus isolation data and clinical features are now known. Such sera provide a good opportunity to assess the sensitivity and specificity of new serological assays. The host humoral immune response to specific HCMV antigens could also be assessed and the results correlated with early diagnosis of and recovery from HCMV infection.

Restriction enzyme analysis of HCMV isolates obtained during the course of this project was not undertaken. However, it would be interesting to study the DNA profiles of viruses isolated from individual patients throughout the first year post transplantation and to establish if only 1 HCMV strain is ever shed by 1 renal allograft recipient. The clinical details could possibly also be correlated with

the restriction enzyme profiles to establish if some HCMV isolates are potentially more pathogenic than others.

Further work requires to be done on the detection of HCMV in clinical specimens by dot blot hybridization. The sensitivity of the hybridot assay when using fresh rather than stored specimens needs to be assessed. The use of PCR to increase the concentration of HCMV-specific sequences in clinical specimens may also improve the sensitivity of the hybridization technique. The origin of the false-positive hybrid signals in the RNA-DNA hybridot assay also needs further investigation. Re-hybridization of the nitrocellulose filters with a specific probe may yield information concerning which virus, if any, contained sequences which cross-hybridized with those of the cRNA probe used in this project. However, if the false-positive hybrid signals were solely attributable to non-specific binding then an alternative HCMV cRNA probe and/or improved washing procedures may be requisite.

BIBLIOGRAPHY

ABE, Y. and INOUYE, S. (1979). Complement-fixing immunoglobulin M antibody response in patients with infantile gastroenteritis. J. Clin. Microbiol. 9, 284-287.

ABO, T., COOPER, M.D. and BALCH, C.M. (1982). Postnatal expansion of the natural killer and killer cell population in humans identified by the monoclonal HNK-1 antibody. J. Exp. Med. 155, 321-326.

ABO, T., MILLER, C.A. and BALCH, C.M. (1984). Characterization of human granular lymphocyte subpopulations expressing HNK-1 (Leu-7) and Leu-11 antigens in the blood and lymphoid tissues from fetuses, neonates and adults. Eur. J. Immunol. 14, 616-623.

ADAMS, A. and LINDAHL, T. (1975). Epstein-Barr virus genomes with properties of circular DNA molecules in carrier cells. Proc. Natl. Acad. Sci. USA 72, 1477-1481.

ADLER, S.P., BAGGETT, J. and McVOY, M. (1985). Transfusion-associated cytomegalovirus infections in seropositive cardiac surgery patients. Lancet 2, 743-746.

ADLER, S.P., LAWRENCE, L.T., BAGGETT, J., BIRO, V. and SHARP, D.E. (1984). Prevention of transfusion-associated cytomegalovirus infection in very low-birthweight infants using frozen blood and donors seronegative for cytomegalovirus. Transfusion 24, 333-335.

ADLER, S.P. and McVOY, M. (1986). Detection of cytomegalovirus antibody by enzyme immunoassay and lack of evidence for an effect resulting from strain heterogeneity. J. Clin. Microbiol. 24, 870-872.

AGHA, S.A., COLEMAN, J.C., MAHMOUD, L.A., ABD-ELAAL, A.M. and SELWYN, S. (1988). New and sensitive standard cell culture technique for the detection of cytomegalovirus in clinical specimens. J. Med. Virol. 26, 85-92.

AKRIGG, A., WILKINSON, G.W.G. and ORAM, J.D. (1985). The structure of the major immediate early gene of human cytomegalovirus strain AD169. Virus Res. 2, 107-121.

AKSAMIT, A.J., MOURRAIN, P., SEVER, J.L. and MAJOR, E.C. (1985). Progressive multifocal leukoencephalopathy investigation of three cases using *in situ* hybridization with JC virus biotinylated DNA probe. Ann. Neurol. 18, 490-496.

ALBRECHT, M., DARAI, G. and FLUGEL, R.M. (1985). Analysis of the genome termini of tupaia herpesvirus DNA by restriction mapping and nucleotide sequencing. J. Virol. 56, 466-474.

ALBRECHT, T., CAVALLO, T., COLE, N.L. and GRAVES, K. (1980). Cytomegalovirus: Development and progression of cytopathic effects in human cell culture. Lab. Invest. 42, 1-7.

ALBRECHT, T., SPEELMAN, D.J. and STEINSLAND, O.S. (1983). Similarities between cytomegalovirus-induced cell rounding and contraction of smooth muscle cells. Life Sci. 32, 2273-2278.

ALDRETE, J.S., STERLIN, W.A., HATHAWAY, B.M., MORGAN, J.M. and DIETHELM, A.G. (1975). Gastrointestinal and hepatic complications affecting patients with renal allografts. Am. J. Surg. 129, 115-124.

ALEXANDER, J.A., CUELLAR, R.E., FADDEN, R.J., GENOVESE, J.J., GAVALER, J.S. and VAN THIEL, D.H. (1988). Cytomegalovirus infection of the upper gastrointestinal trace before and after liver transplantation. Transplantation 46, 378-382.

ALFORD, C.A.Jr. and BRITT, W.J. (1985). Cytomegalovirus. In: Virology. Eds. B.N. Fields, D.M. Knipe, R.M. Chanock, J.L. Melnick, B. Roizman and R.E. Shope, pp 629-660. Published by Raven Press, New York.

AL-NAKIB, W., STANWAY, G., FORSYTH, M., HUGHES, P.J., ALMOND, J.W. and TYRRELL, D.A.J. (1986). Detection of human rhinoviruses and their molecular relationship using cDNA probes. J. Med. Virol. 20, 289-296.

ALPERT, G., MAZERON, M.C., COLIMAN, R. and PLOTKIN, S. (1985). Rapid detection of human cytomegalovirus in the urine of humans. J. Infect. Dis. 152, 631-633.

AMBINDER, R.F., CHARACHE, P., STAAL, S., WRIGHT, P., FORMAN, M., HAYWARD, S.D. and HAYWARD, G.S. (1986). The vector homology problem in diagnostic nucleic acid hybridization of clinical specimens. J. Clin. Microbiol. 24, 16-20.

ANDERS, D.G., IRMIERE, A. and GIBSON, W. (1986). Identification and characterization of a major early cytomegalovirus DNA binding protein. J. Virol. 58, 253-262.

ANDERSON, K.P., COSTA, R.H., HOLLAND, L.E. and WAGNER, E.K. (1980). Characterization of herpes simplex virus type 1 RNA present in the absence of de novo protein synthesis. J. Virol. 34, 9-27.

ANDERSON, M.L.M. and YOUNG, B.D. (1985). Quantitative filter hybridisation. In: Nucleic acid hybridisation. A practical approach. Eds. B.D. Hames and S.J. Higgins, pp 73-111. Published by IRL Press, Oxford.

ANURAS, S., PIROS, J., BENNEY, W.W., FORKER, E.L., COLVILLE, D.S. and CORRY, R.J. (1977). Liver disease in renal transplant recipients. Arch. Intern. Med. 137, 42-48.

ARMSTRONG, D., MATHEW, E. and STEGER, L. (1971). Post-transfusion cytomegaloviraemia and persistence of cytomegalovirus in blood. Infect. Immun. 3, 159-163.

ARMSTRONG, J.A., TARR, G.C., YOUNGBLOOD, L.A., DOWLING, J.N., SASLOW, A.R., LUCAS, J.P. and HO, M. (1976). Cytomegalovirus infection in children undergoing open-heart surgery. Yale J. Biol. Med. 49, 83-91.

ARNFRED, J., NIELSEN, C.M., SPENCER, E.S. and ANDERSEN, H.K. (1987). A prospective study on infection with cytomegalovirus in renal allograft recipients immunosuppressed with cyclosporine A and low dose prednisolone. Scand. J. Infect. Dis. 19, 297-392.

ARRAND, J.E. (1985). Preparation of nucleic acid probes. In: Nucleic acid hybridisation. A practical approach. Eds. B.D. Hames and S.J. Higgins, pp 17-45. Published by IRL Press, Oxford.

ASHLEY, R., MERTZ, G.J. and COREY, L. (1987). Detection of asymptomatic herpes simplex virus infections after vaccination. J. Virol. 61, 264-268.

AUGUSTIN, S., POPOW-KRAUPP, T., HEINZ, F.X. and KUNZ, C. (1987). Problems in detection of cytomegalovirus in urine samples by dot blot hybridization. J. Clin. Microbiol. 25, 1973-1977.

AULITZKY, W.E., TILG, H., NIEDERWIESER, D., HACKL, M., MEISTER, B. and HUBER, C. Ganciclovir and hyperimmunoglobulin for treating cytomegalovirus infection in bone marrow transplant recipients. J. Infect. Dis. 158, 488-489.

BACKMAN, L., RINGDEN, O. and BJORKHEM, I. (1988). Monitoring of serum β_2 microglobulin and neopterin levels in renal transplant recipients: Increased values during impaired renal function and cytomegalovirus infection. Transplant. Proc. 20, 410-412.

BAER, R., BANKIER, A.T., BIGGIN, M.D., DEININGER, P.L., FARRELL, P.J., GIBSON, T.J., HATFULL, G., HUDSON, G.S., SATCHWELL, S.C., SEGUIN, C., TUFFNELL, P.S. and BARRELL, B.G. (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature 310, 207-211.

BALDWIN, W.M., VAN ES, A., VALENTIJN, R.M., VAN GEMERT, G.W., DAHA, M.R. and VAN ES, L.A. (1982). Increased IgM and IgM immune complex-like material in the circulation of renal transplant recipients with primary cytomegalovirus infections. Clin. exp. Immunol. 50, 515-524.

BALLAS, S.K., BURKA, E.R., LEWIS, C.N. and KRASNOW, S.H. (1980). Serum immunoglobulin levels in patients having sickle cell syndromes. Am. J. Clin. Pathol. 73, 394-396.

BANCROFT, G.J., SHELLAM, G.R. and CHALMER, J.E. (1981). Genetic influences on augmentation of natural killer (NK) cells during murine cytomegalovirus infection: Correlation with patterns of resistance. J. Immunol. 126, 988-994.

BANDYOPADHYAY, S., MILLER, D.S., MATSUMOTO-KOBAYASHI, M., CLARK, S.C. and STARR, S.E. (1987). Effects of interferons and interleukin 2 on natural killing of cytomegalovirus-infected fibroblasts. Clin. exp. Immunol. 67, 372-382.

BANDYOPADHYAY, S., PERUSSIA, B., TRINCHIERI, G., MILLER, D.S. and STARR, S.E. (1986). Requirement for HLA-DR$^+$ accessory cells in natural killing of cytomegalovirus-infected fibroblasts. J. Exp. Med. 164, 180-195.

BANKIER, A.T., DIETRICH, W., BAER, R., BARRELL, B.G., COLBERE-GARAPIN, F., FLECKENSTEIN, B. and BODEMER, W. (1985). Terminal repetitive sequences in herpesvirus saimiri virion DNA. J. Virol. 55, 133-139.

BARBARA, J.A.J., MOULSDALE, H., BROWN, S., GRIFFITHS, P.D., BERRY, N.J. and CONTRERAS, M. (1987). A modified latex agglutination test for anti-CMV, suitable for pretransfusion screening. J. Clin. Pathol. 40, 115-116.

BARINGER, J.R. (1974). Recovery of herpes simplex virus from human sacral ganglions. N. Engl. J. Med. 291, 828-830.

BARINGER, J.R. and SWOVELAND, P. (1973). Recovery of herpes simplex virus from human trigeminal ganglions. N. Engl. J. Med. 288, 648-650.

BASTIAN, F.O., RABSON, A.S., YEE, C.L. and TRALKA, T.S. (1972). Herpesvirus hominis. Isolation from human trigeminal ganglion. Science 178, 306-307.

BAYER, W.L. and TEGTMEIER, G.E. (1976). The blood donor: Detection and magnitude of cytomegalovirus carrier states and the prevalence of cytomegalovirus antibody. Yale J. Biol. Med. 49, 5-12.

BECK, O.E. (1981). Distribution of virus antibody activity among human IgG subclasses. Clin. exp. Immunol. 43, 626-632.

BECKWITH, D.G., HALSTEAD, D.C., ALPAUGH, K., SCHWEDER, A., BLOUNT-FRONEFIELD, D.A. and TOTH, K. (1985). Comparison of a latex agglutination test with five other methods for determining the presence of antibody against cytomegalovirus. J. Clin. Microbiol. 21, 328-331.

BENNINCK, J.R., YEWDELL, J.W., SMITH, G.L. and MOSS, B. (1987). Anti-influenza virus cytotoxic T lymphocytes recognize the three viral polymerases and a nonstructural protein: Responsiveness to individual viral antigens is major histocompatibility complex controlled. J. Virol. 61, 1098-1102.

BERNSTEIN, M.T. and STEWART, J.A. (1971). Indirect haemagglutination test for detection of antibodies to cytomegalovirus. Appl. Microbiol. 21, 84-89.

BETTS, R.F. (1979). Cytomegalovirus vaccine in renal transplants [Editorial]. Ann. Intern. Med. 91, 780-782.

BETTS, R.F., FREEMAN, R.B., DOUGLAS, R.G.Jr., TALLEY, T.E. and RUNDELL, B. (1975). Transmission of cytomegalovirus infection with renal allograft. Kidney Int. 8, 385-392.

BETTS, R.F., FREEMAN, R.B., DOUGLAS, R.G.Jr. and TALLEY, T.E. (1977). Clinical manifestations of renal allograft derived primary cytomegalovirus infection. Am. J. Dis. Child. 131, 759-763.

BETTS, R.F., GEORGE, S.D., RUNDELL, B.B., FREEMAN, R.B. and DOUGLAS, R.G. (1976). Comparative activity of immunofluorescent antibody in cytomegalovirus infection. J. Clin. Microbiol. 4, 151-156.

BETTS, R.F. and SCHMIDT, S. (1981). Cytolytic IgM antibody to cytomegalovirus in primary cytomegalovirus infection in humans. J. Infect. Dis. 143, 821-826.

BIA, M.J., ANDIMAN, W., GAUDIO, K., KLIGER, A., SIEGEL, N., SMITH, D. and FLYE, W. (1985). Effect of treatment with cyclosporine versus azathioprine on incidence and severity of cytomegalovirus infection posttransplantation. Transplantation 40, 610-614.

BIRNBOIM, H.C. and DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513-1523.

BIRNSTIEL, M.L., SELLS, B.H. and PURDOM, I.F. (1972). Kinetic complexity of RNA molecules. J. Mol. Biol. 63, 21-39.

BISHOP, J.O. (1972). Molecular hybridization of ribonucleic acid with a large excess of deoxyribonucleic acid. Biochem. J. 126, 171-185.

BLANTON, R.A. and TEVETHIA, M.J. (1981). Immunoprecipitation of virus-specific immediate-early and early polypeptides from cells lytically infected with human cytomegalovirus strain AD169. Virology 112, 262-273.

BODESCOT, M., BRISON, O. and PERRICAUDET, M. (1986). An Epstein-Barr virus transcription unit is at least 84 kilobases long. Nucleic Acids Res. 14, 2611-2620.

BOLDOGH, I., GONCZOL, L., GARTNER, L. and VACZI, G. (1978). Stimulation of host DNA synthesis and induction of early antigens by ultraviolet-light irradiated human cytomegalovirus. Arch. Virol. 58, 289-299.

BOOM, R., GEELEN, J.L., SOL, C.J., MINNAAR, R.P. and VAN DER NOORDAA, J. (1987). Resistance to methylation de novo of the human cytomegalovirus immediate early enhancer in a model for virus latency and reactivation in vitro. J. gen. Virol. 68, 2839-2852.

BOOM, R., SOL, C.J.A., MINNAAR, R.P., GEELEN, J.L.M.C., RAPP, A.K. and VAN DER NOORDAA, J. (1988). Induction of gene expression under human cytomegalovirus immediate early enhancer-promoter control by inhibition of protein synthesis is cell cycle-dependent. J. gen. Virol. 69, 1179-1193.

BOOTH, J.C. (1983). The use of the enzyme-linked immunosorbent assay (ELISA) technique in clinical virology. In: Recent advances in clinical virology. Ed. A.P. Waterson, pp 73-98. Published by Churchill Livingstone, London.

BOOTH, J.C., HANNINGTON, G., BAKIR, T.M.F., STERN, H., KANGRO, H., GRIFFITHS, P.D. and HEATH, R.B. (1982). Comparison of enzyme-linked immunosorbent assay, radioimmunoassay, complement fixation, anticomplement immunofluorescence and passive haemagglutination techniques for detecting cytomegalovirus IgG antibody. J. Clin. Pathol. 35, 1345-1348.

BOOTH, J.C., HANNINGTON, G., TRYHORN, Y. and STERN, H. (1980). Cytomegalovirus complement-fixing IgM antibody. J. Med. Virol. 5, 183-193.

BOOTH, J.C. and MOHAMMED, H.S. (1988). Complement-mediated cytolytic activity associated with cytomegalovirus complement-fixing IgM antibody. J. Med. Virol. 24, 351-359.

BORYSIEWICS, L.K., MORRIS, S., PAGE, J.D. and SISSONS, J.E.P. (1983). Human cytomegalovirus-specific cytotoxic T lymphocytes: Requirements for *in vitro* generation and specificity. Eur. J. Immunol. 13, 804-809.

BOSHART, M., WEBER, F., JAHN, G., DORSCH-HASLER, K., FLECKENSTEIN, B. and SCHAFFNER, W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell 41, 521-530.

BOWDEN, R.A., DAY, L.M., AMOS, D.E. and MEYERS, J.D. (1987). Natural cytotoxic activity against cytomegalovirus-infected target cells following marrow transplantation. Transplantation 44, 504-508.

BOWDEN, R.A., SAYERS, M., FLOURNOY, N., NEWTON, B., BANAJI, M., THOMAS, E.D. and MEYERS, J.D. (1986). Cytomegalovirus immune globulin and seronegative blood products to prevent primary cytomegalovirus infection after marrow transplantation. N. Engl. J. Med. 314, 1006-1010.

BOWDEN, R.A., SAYERS, M., McIVER, J., LESZCYNSKI, J., FLOURNOY, N., THOMAS, E.D. and MEYERS, J.D. (1985). Comparative trial of intravenous cytomegalovirus globulin and seronegative blood products for the prevention of primary cytomegalovirus infection following marrow transplant. Clin. Res. 33, 395A. (abstract).

BRADSTREET, C.M.P. and TAYLOR, C.E.D. (1962). Technique of complement-fixation test applicable to the diagnosis of virus disease. Monthly Bulletin of the Ministry of Health and Public Health Laboratory Service 21, 96-104.

BRADY, M.T., MILAM, J.D., ANDERSON, D.C., HAWKINS, E.P., SPEER, M.E., SEAVY, D., BIJOU, H. and YOW, M.D. (1984). Use of deglycerolized red blood cells to prevent post-transfusion infection with cytomegalovirus in neonates. J. Infect. Dis. 150, 334-339.

BRAUN, W.E., NANKERVIS, G., BANOWSKY, L.H., PROTIVA, D., BIEKERT, E. and McHEMRY, M.C. (1976). A prospective study of cytomegalovirus infections in 75 renal allograft recipients. Proc. clin. Dial. Transplant Forum 6, 8-12.

BRAUTIGAM, A.R., DUTKO, F.J., OLDING, L.B. and OLDSTONE, M.B.A. (1979). Pathogenesis of murine cytomegalovirus infection: The macrophage as a permissive cell for cytomegalovirus infection, replication, and latency. J. gen. Virol. 44, 349-359.

BREATHNACK, R. and CHAMBON, P. (1981). Organisation and expression of eukaryotic split genes coding for proteins. Ann. Rev. Biochem. 50, 349-384.

BREINIG, M.K., ZITELLI, B., STARZL, T.E. and HO, M. (1987). Epstein-Barr virus, cytomegalovirus, and other viral infections in children after liver transplantation. J. Infect. Dis. 156, 273-279.

BRIDEAU, R.J. and NICHOLAS (WOLCOTT), J.A. (1987). Pyrimidinone (bropirimine) mediated alteration of T lymphocyte subsets during murine cytomegalovirus infection. Clin. exp. Immunol. 68, 613-620.

BRITTEN, R.J., GRAHAM, D.E. and NEUFIELD, B.R. (1974). In: Methods in Enzymology. Eds. L. Grossman and K. Moldave, p 363. Published by Academic Press, London.

BRONSTHER, O., MAKOWKA, L., JAFFE, R., DEMETRIS, A.J., BREINIG, M.K., HO, M., ESQUIVEL, C.O., GORDON, R.D., IWATSUKI, S., TZAKIS, A., MARSH, J.W.Jr., MAZZAFERRO, V., THIEL, D.V. and STARZL, T.E. (1988). Occurrence of cytomegalovirus hepatitis in liver transplant patients. J. Med. Virol. 24, 423-434.

BROWN, S.M., RITCHIE, D.A. and SUBAK-SHARPE, J.H. (1973). Genetic studies with herpes simplex virus type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. J. gen. Virol. 18, 329-346.

BRUNING, J.H., BRUGGEMAN, C.A., VAN BOVEN, C.P.A. and VAN BREDA-VRIESMAN, P.J.C. (1985). Passive transfer of CMV by cardiac and renal organ transplants in a rat model. Transplantation 41, 695-698.

BRUNING, J.H., BRUGGEMAN, C.A. and VAN BREDA-VRIESMAN, P.J.C. (1988). The transfer of cytomegalovirus infection in rats by latently infected renal allografts, and the role of various immunosuppressive regimens in virus reactivation. Transplantation 46, 623-624.

BUCHER, L.J., WIGDAHL, B. and RAPP, F. (1983). Maintenance of human cytomegalovirus genome in human diploid fibroblast cells. Virology 130, 269-271.

BUFFONE, G.J., SCHIMBOR, C.M., DEMMLER, G.J., WILSON, D.R. and DARLINGTON, G.J. (1986). Detection of cytomegalovirus in urine by nonisotopic DNA hybridization. J. Infect. Dis. 154, 163-166.

BUIMOVICI-KLEIN, E., TINKER, M.K., O'BEIRNE, A.J., LANGE, M. and COOPER, Z. (1983). IgM detection by ELISA in the diagnosis of cytomegalovirus infections in homosexual and heterosexual immunosuppressed patients. Arch. Virol. 78, 203-212.

BUKOWSKI, J.F., McINTYRE, K.W., YANG, H. and WELSH, R.M. (1987). Natural killer cells are not required for interferon-mediated prophylaxis against vaccinia or murine cytomegalovirus infections. J. gen. Virol. 68, 2219-2222.

BUKOWSKI, J.F., WARNER, J.F., DENNERT, G. and WELSH, R.M. (1985). Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. J. Exp. Med. 161, 40-52.

BUKOWSKI, J.F., WODA, B.A. and WELSH, R.M. (1984). Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. J. Virol. 52, 119-128.

BURLESON, R.L., LAMBERSO, H.V., HUBBEL, C. and BURLESON, A. (1984). Prevention of primary CMV infection in renal transplant patients by antibody screening of donors and recipients. In: CMV: Pathogenesis and prevention of human infection. Eds. S.A. Plotkin, S. Michelson, J.S. Pagano and F. Rapp, p 379. Published by Liss, New York.

BUSBY, D.W.G., HOUSE, W. and MacDONALD, J.R. (1964). Virological Techniques. Churchill Press, London.

BUTLER, E.T. and CHAMBERLIN, M.J. (1982). Bacteriophage SP6-specific RNA polymerase. I. Isolation and characterization of the enzyme. J. Biol. Chem. 257, 5772-5778.

CAMERON, J., RIGBY, R.J., VAN DETH, A.G. and PETRIE, J.J. B. (1982). Severe tubulo-interstitial disease in a renal allograft due to cytomegalovirus infection. Clin. Nephrol. 18, 321-325.

CAMERON, J.M. and PRESTON, C.M. (1981). Comparison of the immediate early polypeptides of human cytomegalovirus isolates. J. gen. Virol. 54, 421-424.

CAMPBELL, A.E., FOLEY, F.L. and TEVETHIA, S.S. (1983). Demonstration of multiple antigenic sites of the SV40 transplantation rejection antigen by using cytotoxic T lymphocyte clones. J. Immunol. 130, 490-492.

CANADIAN MULTICENTRE TRANSPLANT STUDY GROUP. (1983). A randomized clinical trial of cyclosporine in cadaveric renal transplantation. N. Engl. J. Med. 309, 809-815.

CARNEY, W.P. and HIRSCH, M.S. (1981). Mechanisms of immunosuppression in cytomegalovirus mononucleosis. II. Virus-monocyte interactions. J. Infect. Dis. 144, 47-54.

CARNEY, W.P., IACOVIELLO, V. and HIRSCH, M.S. (1983). Functional properties of T lymphocytes and other subsets in cytomegalovirus mononucleosis. J. Immunol. 130, 390-393.

CARNEY, W.P., RUBIN, R.H., HOFFMAN, R.A., HANSEN, W.P., HEALEY, K. and HIRSCH, M.S. (1981). Analysis of T-lymphocyte subsets in cytomegalovirus mononucleosis. J. Immunol. 126, 2114-2116.

CHANDLER, S.H. and McDOUGALL, J.K. (1986). Comparison of restriction site polymorphisms among clinical isolates and laboratory strains of human cytomegalovirus. J. gen. Virol. 67, 2179-2192.

CHANG, C-T., HAIN, T.C., HUTTON, J.R. and WETMUR, J.G. (1974). Effects of microscopic and macroscopic viscosity on the rate of renaturation of DNA. Biopolymers 13, 1847-1858.

CHATTERJEE, S., FIALA, M., WEINER, J., STEWART, J., STACEY, B. and WARNER, N. (1978). Primary cytomegalovirus and opportunistic infections. J. Am. Med. Assoc. 240, 2446-2449.

CHEESEMAN, S.H., RUBIN, R.H., STEWART, J.A., TOLKOFF-RUBIN, N.E., COSIMI, A.B., CANTELL, K., GILBERT, J., WINKLE, S., HERRIN, J.T., BLACK, P.H., RUSSELL, P.S. and HIRSCH, M.S. (1979). Controlled clinical trials of prophylactic human-leukocyte interferon in renal transplantation. N. Engl. J. Med. 300, 1345-1349.

CHUA, C.C., CARTER, T.H. and ST. JEOR, S. (1981). Transcription of the human cytomegalovirus genome in productively infected cells. J. gen. Virol. 56, 1-11.

CHUNG, A., CHANG, W., LEUNG, M.K., OFOSU, F.A. and MOORE, B.P.L. (1984). Assessment of methods for cytomegalovirus antibody testing of blood donations. Vox Sang 47, 343-347.

CHURCH, G.M. and GILBERT, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA 81, 1991-1995.

CHURCHILL, M.A., ZAIA, J.A., FORMAN, S.J., SHEIBANI, K., AZUMI, N. and BLUME, K.G. (1987). Quantitation of human cytomegalovirus DNA in lungs from bone marrow transplant recipients with interstitial pneumonia. J. Infect. Dis. 155, 501-509.

CLARK, B.R., ZAIA, J.A., BALCE-DIRECTO, L. and TING, Y.P. (1984). Isolation and partial characterization of a 64000 D glycoprotein of human cytomegalovirus. J. Virol. 49, 279-282.

CLEMENTS, J.B., WATSON, R.J. and WILKIE, N.M. (1977). Temporal regulation of herpes simplex virus type 1 transcription: Location of transcripts on the viral genome. Cell 12, 275-285.

COCKLEY, K.D. and RAPP, F. (1986). Analysis of long-term human cytomegalovirus latency in vitro. Intervirology 26, 129-139.

COHEN, E.B., KOMOROWSKI, R.A., KAUFFMAN, M. and ADAMS, M. (1985). Unexpectedly high incidence of cytomegalovirus infection in apparent peptic ulcers in renal transplant recipients. Surgery 97, 606-612.

COHEN, S.N., CHONG, A.C.Y. and HSU, L. (1972). Non-chromosomal antibiotic resistance in bacteria: Genetic transformation by R factor DNA. Proc. Natl. Acad. Sci. USA 69, 2110-2114.

COLBERG-POLEY, A.M. and SANTOMENNA, L.D. (1988). Selective induction of chromosomal gene expression by human cytomegalovirus. Virology 166, 217-228.

COLE, R. and KUTTNER A.G. (1926). A filtrable virus present in the submaxillary glands of guinea pigs. J. Exp. Med. 44, 855-873.

COLIMON, R., SAMOGYI, T., BERTRAND, C. and MICHELSON, S. (1985). Demonstration of the colinearity of human cytomegalovirus genomes and construction of restriction maps of unknown isolates using cloned subgenomic fragments. J. gen Virol. 66, 2183-2198.

CHUA, C.C., CARTER, T.H. and ST. JEOR, S. (1981). Transcription of the human cytomegalovirus genome in productively infected cells. J. gen. Virol. 56, 1-11.

CHUNG, A., CHANG, W., LEUNG, M.K., OFOSU, F.A. and MOORE, B.P.L. (1984). Assessment of methods for cytomegalovirus antibody testing of blood donations. Vox Sang 47, 343-347.

CHURCH, G.M. and GILBERT, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA 81, 1991-1995.

CHURCHILL, M.A., ZAIA, J.A., FORMAN, S.J., SHEIBANI, K., AZUMI, N. and BLUME, K.G. (1987). Quantitation of human cytomegalovirus DNA in lungs from bone marrow transplant recipients with interstitial pneumonia. J. Infect. Dis. 155, 501-509.

CLARK, B.R., ZAIA, J.A., BALCE-DIRECTO, L. and TING, Y.P. (1984). Isolation and partial characterization of a 64000 D glycoprotein of human cytomegalovirus. J. Virol. 49, 279-282.

CLEMENTS, J.B., WATSON, R.J. and WILKIE, N.M. (1977). Temporal regulation of herpes simplex virus type 1 transcription: Location of transcripts on the viral genome. Cell 12, 275-285.

COCKLEY, K.D. and RAPP, F. (1986). Analysis of long-term human cytomegalovirus latency in vitro. Intervirology 26, 129-139.

COHEN, E.B., KOMOROWSKI, R.A., KAUFFMAN, M. and ADAMS, M. (1985). Unexpectedly high incidence of cytomegalovirus infection in apparent peptic ulcers in renal transplant recipients. Surgery 97, 606-612.

COHEN, S.N., CHONG, A.C.Y. and HSU, L. (1972). Non-chromosomal antibiotic resistance in bacteria: Genetic transformation by R factor DNA. Proc. Natl. Acad. Sci. USA 69, 2110-2114.

COLBERG-POLEY, A.M. and SANTOMENNA, L.D. (1988). Selective induction of chromosomal gene expression by human cytomegalovirus. Virology 166, 217-228.

COLE, R. and KUTTNER A.G. (1926). A filtrable virus present in the submaxillary glands of guinea pigs. J. Exp. Med. 44, 855-873.

COLIMON, R., SAMOGYI, T., BERTRAND, C. and MICHELSON, S. (1985). Demonstration of the colinearity of human cytomegalovirus genomes and construction of restriction maps of unknown isolates using cloned subgenomic fragments. J. gen Virol. 66, 2183-2198.

COLLABORATIVE DHPG TREATMENT STUDY GROUP.(1986). Treatment of serious cytomegalovirus infections with 9-(1,3-dihydroxy-2-propoxymethyl)guanine in patients with AIDS and other immunodeficiencies. N. Engl. J. Med. 314, 801-805.

CONDIE, R.M. and O'REILLY, R.J. (1984). Prevention of cytomegalovirus infection by prophylaxis with an intravenous, hyperimmune, native, unmodified cytomegalovirus globulin: Randomized trial in bone marrow transplant recipients. Am. J. Med. 76, 134-141.

CORDON, J., WASYLYK, B., BUCHWALDER, A., SASSONE-CORSI, P., KEDINGER, C. and CHAMBON, P. (1980). Promoter sequences of eukaryotic protein-coding genes. Science 209, 1406-1414.

CORDONNIER, C., ESCUDIER, E., NICOLAS, J-C., FLEURY, J., DEFORGES, L., INGRAND, D., BRICOUT, F. and BERNAUDIN, J-F. (1987). Evaluation of three assays on alveolar lavage fluid in the diagnosis of cytomegalovirus pneumonitis after bone marrow transplantation. J. Infect. Dis. 155, 495-500.

COVA, L., KOPECKA, H., AYMARD, M. and GIRARD, M. (1988). Use of cRNA probes for the detection of enteroviruses by molecular hybridization. J. Med. Virol. 24, 11-18.

COX, K.H., DELEON, D.V., ANGERER, L.M. and ANGERER, R.C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. Dev. Biol. 101, 485-502.

CRAIGHEAD, J.E. (1969). Immunologic response to cytomegalovirus infection in renal allograft recipients. Am. J. Epidemiol. 90, 506-513.

CRAIGHEAD, J.E., HANSHAW, J.B. and CARPENTER, C.B. (1967). CMV infection after renal allotransplantation. J. Am. Med. Assoc. 201, 725-728.

CRAIGHEAD, J.E., KANICH, R.E. and ALMEIDA, J.D. (1972). Nonviral microbodies with viral antigenicity produced in cytomegalovirus-infected cells. J. Virol. 10, 766-775.

CRANAGE, M.P., KOUZARIDES, T., BANKIER, A.T., SATCHWELL, S., WESTON, K., TOMLINSON, P., BARRELL, B., HART, H., BELL, S.E., MINSON, A.C. and SMITH, G.L. (1986). Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralising antibodies via its expression in recombinant vaccinia. Embo J. 5, 3057-3063.

CRAWFORD, L.V. and LEE, A.J. (1964). The nucleic acid of human cytomegalovirus. Virology 23, 105-107.

CREASY, T.S., FLOWER, A.J.E. and VEITCH, P.S. (1986). Life-threatening cytomegalovirus infection treated with dihydroproproxymethylguanine. Lancet 1, 675.

CREMER, N.E., HOFFMAN, M. and LENNETTE, E.H. (1978). Analysis of antibody assay methods and classes of viral antibodies in serodiagnosis of cytomegalovirus infection. J. Clin. Microbiol. 8, 153-159.

CREMISI, C. (1981). The appearance of DNase I hypersensitive sites at the 5' end of the late SV40 genes is correlated with the transcriptional switch. Nucleic Acids Res. 9, 5949-5964.

DAFOE, D.C., STOOLMAN, L.M., CAMPBELL, D.A.Jr., LORBER, M.I., WASKERWITZ, J. and TURCOTTE, J.G. (1987). T cell subset patterns in cyclosporine-treated renal transplant recipients with primary cytomegalovirus disease. Transplantation 43, 452-454.

DAVANLOO, P., ROSENBERG, A.H., DUNN, J.J. and STUDIER, F.W. (1984). Cloning and expression of the gene for bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Soc. USA 81, 2035-2039.

DAVIS, M.G. and HUANG, E-S. (1985). Nucleotide sequence of a human cytomegalovirus DNA fragment encoding a 67-kilodalton phosphorylated viral protein. J. Virol. 56, 7-11.

DAVISON, A.J. (1984). Structure of the genome termini of varicella-zoster virus. J. gen. Virol. 65, 1969-1977.

DAVISON, A.J. and WILKIE, N.M. (1981). Nucleotide sequences of the joint between the L and S segments of herpes simplex virus types 1 and 2. J. gen. Virol. 55, 315-331.

DE HEMPTINNE, B., LAMY, M.E., SALIZZONI, M., CORNU, C., MOSTIN, J., FEVERY, J., DE GROOTE, V. and OTTE, J.B. (1988). Successful treatment of cytomegalovirus disease with 9-(1,3-dihydroxy-2-propoxymethyl guanine). Transplant. Proc. 20 (Suppl. 1), 652-655.

DEISS, L.P., CHOU, J. and FRENKEL, N. (1986). Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. J. Virol. 59, 605-618.

DE MARCHI, J.M. (1981). Human cytomegalovirus DNA: Restriction enzyme cleavage maps and map locations for immediate-early, early and late RNAs. Virology 114, 23-38.

DE MARCHI, J.M. (1983a). Correlation between stimulation of host cell DNA synthesis by human cytomegalovirus and lack of expression of a subset of early virus genes. Virology 129, 274-286.

DE MARCHI, J.M. (1983b). Post-transcriptional control of human cytomegalovirus gene expression. Virology 124, 390-402.

DE MARCHI, J.M., BLANKENSHIP, M.L., BROWN, G.D. and KAPLAN, A.S. (1978). Size and complexity of human cytomegalovirus DNA. Virology 89, 643-646.

DE MARCHI, J.M. and KAPLAN, A.S. (1976). Replication of human cytomegalovirus DNA: Lack of dependence on cell DNA synthesis. J. Virol. 18, 1063-1070.

DE MARCHI, J.M. and KAPLAN, A.S. (1977). Physiological state of human embryonic lung cells affects their response to human cytomegalovirus. J. Virol. 23, 126-132.

DEMMLER, G.J., BUFFONE, G.J., SCHIMBOR, C.M. and MAY, R.A. (1988). Detection of cytomegalovirus in urine from newborns by using polymerase chain reaction DNA amplification. J. Infect. Dis. 158, 1177-1184.

DEMMLER, G.J., SIX, H.R., HURST, S.M. and YOW, M.D. (1986). Enzyme-linked immunosorbent assay for the detection of IgM-class antibodies to cytomegalovirus. J. Infect. Dis. 153, 1152-1155.

DENHARDT, D.T. (1966). A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. 23, 641-646.

DIOSI, P., MOLDOVAN, E. and TOMESCU, N. (1969). Latent cytomegalovirus infection in blood donors. Br. Med. J. 4, 660-662.

DOANE, F.W. and ANDERSON, N. (1977). Electron microscopic and immune electron microscopic procedures for diagnosis of viral infections. In: Comparative diagnosis of viral diseases. II. Eds. E. Kurstak and C. Kurstak, pp 505-539. Published by Academic Press, New York.

DOERR, H.W., KUNZLER, A. and SCHMIDTZ, H. (1979). Cytomegalovirus strain differentiation by DNA restriction analysis. Oncology 36, 245-247.

DOKHELAR, M-C., WIELS, J., LIPINSKI, M., TETAUD, C., DEVERGIE, A., GLUCKMAN, E. and TURSZ, T. (1981). Natural killer cell activity in human bone marrow recipients. Early reappearance of peripheral natural killer activity in graft-versus-host disease. Transplantation 31, 61-65.

DOLIN, R. (1985). Antiviral chemotherapy and chemoprophylaxis. Science 227, 1296-1303.

DOODY, D.P., WILSON, E.J., MEDEARIS, D.N. and RUBIN, R.H. (1986). Changes in the phenotype of T-cell subset determinants following murine cytomegalovirus infection. Clin. Immunol. Immunopathol. 40, 466-475.

DOWLING, J.N., SASLOW, A.R., ARMSTRONG, J.A. and HO, M. (1976). Cytomegalovirus infection in patients receiving immunosuppressive therapy for rheumatologic disorders. J. Infect. Dis. 133, 399-408.

DREW, J.W., HUANG, E-S., MILLER, M.J., LIN, C.H., HO, W.G., GALE, R.P. and CHAMPLIN, R.E. (1985). Molecular epidemiology of cytomegalovirus infections associated with bone marrow transplantation. Ann. Intern. Med. 102, 16-20.

DREW, W.L., SWEET, E.S., MINER, R.C. and MOCARSKI, E.S. (1984). Multiple infections by cytomegalovirus in patients with acquired immunodeficiency syndrome: Documentation by Southern blot hybridization. J. Infect. Dis. 150, 952-953.

DUMMER, J.S., HARDY, A., POORSATTAR, A. and HO, M. (1983). Early infections in kidney, heart and liver transplant recipients on cyclosporine. Transplantation 36, 259-267.

DUMMER, J.S., HO, M., RABIN, B., GRIFFITH, B.P., HARDESTY, R.L. and BAHNSON, H.T. (1984). The effect of cytomegalovirus and Epstein-Barr virus infection on T lymphocyte subsets in cardiac transplant patients on cyclosporine. Transplantation 38, 433-435.

DUMMER, J.S., WHITE, L.T., HO, M., GRIFFITH, B.P., HARDESTY, R.L. and BAHNSON, H.T. (1985). Morbidity of cytomegalovirus infection in recipients of heart or heart-lung transplants who received cyclosporine. J. Infect. Dis. 152, 1182-1191.

DUPONT, E., VANDERCRUYS, M. and WYBRAN, J. (1984). Deficient natural killer function in patients receiving immunosuppressive drugs: Analysis at the cellular level. Cell. Immunol. 88, 85-95.

DUTKO, F.J. and OLDSTONE, M.B.A. (1981).Cytomegalovirus causes a latent infection in undifferentiated cells and is activated by induction of cell differentiation. J. Exp. Med. 154, 1636-1651.

DUVALL, C.P., CASAZZA, A.R., GRIMLEY, P.M., CARBONE, P.P. and ROWE, W.P. (1966). Recovery of cytomegalovirus from adults with neoplastic disease. Ann. Intern. Med. 64, 531-541.

EGBERT, P.R., POLLARD, R.B., GALLAGHER, J.B. and MERIGAN, T.C. (1980). Cytomegalovirus retinitis in immunosuppressed hosts. II. Ocular manifestations. Ann. Intern. Med. 93, 664-670.

EINHORN, L. and OST, A. (1984). Cytomegalovirus infection of human blood cells. J. Infect. Dis. 149, 207-214.

EMBIL, J.A., OZERE, R.L. and HALDANE, E.V. (1970). Congenital cytomegalovirus infection in two siblings from consecutive pregnancies. J. Paediatr. 77, 417-421.

ENGVALL, E. and PERLMANN, P. (1971). Enzyme linked immunosorbent assay. ELISA. III. Quantitation of specific antibodies by enzyme-linked anti-immunoglobulin in antigen coated tubes. J. Immunol. 109, 129-135.

EPSTEIN, L.B. (1979). The comparative biology of immune and classical interferons. In: Biology of the lymphokines. Eds. S. Cohen, E. Pick and J.J. Oppenheim, pp 443-514. Published by Academic Press, New York.

ERICE, A., JORDAN, M.C., CHACE, B.A., FLETCHER, C., CHINNOCK, B.J. and BALFOUR, H.H.Jr.(1987). Ganciclovir treatment of cytomegalovirus disease in transplant recipients and other immunocompromised hosts. J. Am. Med. Assoc. 257, 3082-3087.

ESPY, M.J., WOLD, A.D., ILSTRUP, D.M. and SMITH, T.F. (1988). Effect of treatment of shell vial cell cultures with dimethyl sulfoxide and dexamethasone for detection of cytomegalovirus. J. Clin. Microbiol.26, 1091-1093.

ESQUIVEL, C.O., JAFFE, R., GORDON, R.D., IWATSUKI, S., SHAW, B.W.Jr. and STARZL, T.E. (1985). Liver rejection and its differentiation from other causes of graft dysfunction. Seminars in Liver Disease 5, 369-374.

EVANS, A.S., NIEDERMAN, J.C. and McCOLLUM, R.W. (1968). Seroepidemiologic studies of infectious mononucleosis with EB virus. N.Engl. J. Med. 279, 1121-1127.

FAIX, R.G. (1985). Cytomegalovirus antigenic heterogeneity can cause false-negative results in indirect haemagglutination and complement fixation antibody assays. J. Clin. Microbiol. 22, 768-771.

FALCIERI, E., MUSIANI, M., ZERBINI, M. and CARPI, C. (1982). Human cytomegalovirus cycle in synchronized cells: Electron microscope study. Microbiologica 5, 43-47.

FARBER, S. and WOLBACH, S.B. (1932). Intranuclear and cytoplasmic inclusions ("protozoan-like bodies") in the salivary glands and other organs of infants. Am. J. Pathol. Child. 8, 123-126.

FARRAR, J.J., BENJAMIN, W.R., HILFIKER, M.L., HOWARD, M., FARRAR, W.L. and FULLER-FARRAR, J. (1982). The biochemistry, biology and role of interleukin-2 in the induction of cytotoxic T cell and antibody-forming B cell responses. Immunol. Rev. 63, 129-135.

FEINBERG, A.P. and VOGELSTEIN, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragements to high specific activity. Anal. Biochem. 132, 6-13.

FETTERMAN, G.H. (1952). A new laboratory aid in the clinical diagnosis of inclusion disease of infancy. Am. J. Clin. Pathol. 22, 424.

FIALA, M., CHATTERJEE, S.N., CARSON, S., POOLSAWAT, S., HEINER, D.C., SAXON, A. and GUZE, L.B. (1977). Cytomegalovirus retinitis secondary to chronic viremia in phagocytic leukocytes. Am. J. Ophthalmol. 84, 567-573.

FIALA, M., EDMONDSON, L. and GUZE, L.B. (1973). Simplified method for isolation of cytomegalovirus and demonstration of frequent viremia in renal transplant patients. Proc. Soc. Exp. Biol. Med. 144, 871-875.

FIALA, M., HONESS, R.W., HEINER, D.C., HEINER, J.W.Jr., MURNAME, J., WALLACE, R. and GUZE, L.B. (1976). Cytomegalovirus proteins. I. Polypeptides of virions and dense bodies. J. Virol. 19, 243-254.

FIALA, M., PAYNE, J.E., BERNE, T.V., MOORE, T.C., HENLE, W., MONTGOMERIE, J.Z., CHATTERJEE, S.N. and GUZE, L.B. (1975). Epidemiology of cytomegalovirus infection after transplantation and immunosuppression. J. Infect. Dis. 132, 421-433.

FIELD, A.K., DAVIES, M.E., DE WITT, C., PERRY, H.C., LIOU, R., GERMERSHAUSEN, J., KARKAS, J.D., ASHTON, W.T., JOHNSTON, D.B.R. and TOLMAN, R.L. (1983). 9-(2-hydroxy-1-[hydroxymethyl]ethoxy)methylguanine: A selective inhibitor of herpes group virus replication. Proc. Natl. Acad. Sci. USA 80, 4139-4143.

FIORETTI, A., FURUKAWA, T., SANTOLI, D. and PLOTKIN, S. (1973). Nonproductive infection of guinea pig cells with human cytomegalovirus. J. Virol. 11, 998-1003.

FLAVELL, R.A., BIRFELDER, E.J., SANDERS, J.P.M. and BORST, P. (1974). DNA-DNA hybridization on nitrocellulose filters. I. General considerations and non-ideal kinetics. Eur. J. Biochem. 47, 535-543.

FLECKENSTEIN, B., MULLER, B. and COLLINS, I. (1982). Cloning of the complete cytomegalovirus genome in cosmids. Gene 18, 39-46.

FLEISS, J.L. (1973). Statistical method for rates and proportions, pp 109-129. Published by Wiley, New York.

FORMAN, S.J., ZAIA, J.A., CLARK, B.R., WRIGHT, C.L., MILLS, B.J., POTTATHIL, R., RACKLIN, B.C., GALLAGHER, M.T., WETTE, K. and BLUME, K.G. (1985). A 64,000 dalton matrix protein of human cytomegalovirus induces *in vitro* immune responses similar to those of whole virus antigen. J. Immunol. 134, 3391-3395.

FORMAN, S.J., ZAIA, J.A., WRIGHT, C., GALLAGHER, M.T. and BLUME, K.G. (1986). Increased leu-7-positive T lymphocytes during cytomegalovirus infection following allogeneic bone marrow transplantation for hematologic malignancies. Transplantation 41, 268-271.

FORSTER, A.C., McINNES, J.L., SKINGLE, D.C. and SYMONS, R.H. (1985). Non-radioactive hybridization probes prepared by chemical labelling of DNA and RNA with a novel reagent, photobiotin. Nucleic Acids Res. 13, 745-761.

FOUCAR, E., MUKAI, K., FOUCAR, K., SUTHERLAND, D.E.R. and VAN BUREN, C.T. (1981). Colon ulceration in lethal cytomegalovirus infection. Am. J. Clin. Pathol. 76, 788-801.

FRANZIN, G., MUOLO, A. and GRIMINELLI, T. (1981). Cytomegalovirus inclusions in the gastroduodenal mucosa of patients after renal transplantation. Gut 22, 698-701.

FRYD, D.S., PETERSON, P.K., FERGUSON, R.M., SIMMONS, R.L., BALFOUR, H.H.Jr. and NAJARIAN, J.S. (1980). Cytomegalovirus as a risk factor in renal transplantation. Transplantation 30, 436-439.

FUJINAMI, R.S., NELSON, J.A., WALKER, L. and OLDSTONE, M.B.A. (1988). Sequence homology and immunologic cross-reactivity of human cytomegalovirus with HLA-DR β chain: A means for graft rejection and immunosuppression. J. Virol. 62, 100-105.

FUJINAMI, R.S. and OLDSTONE, M.B.A. (1985). Amino acid homology between the encephalitogenic site of myelin basic protein and virus: Mechanism for autoimmunity. Science 230, 1043-1045.

FUJINAMI, R.S., OLDSTONE, M.B.A., WROBLEWSKA, Z., FRANKEL, M.E. and KOPROWSKI, H. (1983). Molecular mimicry in virus infection: Cross reaction in measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. Proc. Natl. Acad. Sci. USA 80, 2346-2350.

FULGINITI, V.A., SCRIBNER, R., GROTH, C.G., PUTNAM, C.W., BRETTSCHNEIDER, L., GILBERT, S., PORTER, K.A. and STARZL, T.E. (1968). Infections in recipients of liver homografts. N. Engl. J. Med. 279, 619-626.

FURLINI, G., RIPALTI, A., LANDINI, M.P. and LA PLACA, M. (1984). Increased levels of ADP ribosylation during cytomegalovirus replication in human embryo fibroblasts. Microbiologica 7, 279-285.

FURUKAWA, T. (1979). Cell cycle-dependent chronic infection of human cytomegalovirus in human osteogenic sarcoma cells. J. gen. Virol. 45, 81-88.

FURUKAWA, T. (1984). A variant of human cytomegalovirus derived from a persistently infected culture. Virology 137, 191-194.

FURUKAWA, T., FIORETTI, A. and PLOTKIN, S.A. (1973). Growth characteristics of cytomegalovirus in human fibroblasts with demonstration of protein synthesis early in viral replication. J. Virol. 11, 991-997.

FURUKAWA, T., SAKUMA, S. and PLOTKIN, S.A. (1976). Human cytomegalovirus infection of WI-38 cells stimulates mitochondrial DNA synthesis. Nature 262, 414-416.

FURUKAWA, T., TANAKA, S. and PLOTKIN, S.A. (1975). Stimulation of macromolecular synthesis in guinea pig cells by human cytomegalovirus. Proc. Soc. Exp. Biol. Med. 148, 1249-1251.

GADLER, H., TILLEGARD, A. and GROTH, C-G. (1982). Studies of cytomegalovirus infection in renal allograft recipients. I. Virus isolation. Scand. J. Infect. Dis. 14, 81-87.

GADLER, H. and WAHREN, B. (1983). Association between virus and cell DNA during latent cytomegalovirus infection in vitro. Arch. Virol. 75, 291-298.

GALLOWAY, P.G. (1984). Widespread cytomegalovirus infection involving the gastrointestinal tract, biliary tree, and gallbladder in an immunocompromised patient. Gastroenterology 87, 1407.

GARDNER, M.B., OFFICER, J.E., PARKER, J., ESTES, J.D. and RONGEY, R.W. (1974). Induction of disseminated virulent cytomegalovirus infection by immunosuppression of naturally chronically infected wild mice. Infect. Immun. 10, 966-969.

GARDNER, S.D., MacKENZIE, E.F.D., SMITH, C. and PORTER, A.A. (1984). Prospective study of the human polyomaviruses BK and JC and cytomegalovirus in renal transplant recipients. J. Clin. Pathol. 37, 578-586.

GARNETT, H.M. (1979). The early effects of human cytomegalovirus infection on macromolecular synthesis in human embryonic fibroblasts. Arch. Virol. 60, 147-151.

GASCON, P., ZOUMBOS, N.C. and YOUNG, N.S. (1984). Immunologic abnormalities in patients receiving multiple blood transfusions. Ann. Intern. Med. 100, 173-177.

GEBALLE, A.P., LEACH, F.S. and MOCARSKI, E.S. (1986). Regulation of cytomegalovirus late gene expression: γ genes are controlled by posttranscriptional events. J. Virol. 57, 864-874.

GEELEN, J.L.M.C., WALIG, C., WERTHEIM, P. and VAN DER NOORDAA, J. (1978). Human cytomegalovirus DNA. I. Molecular weight and infectivity. J. Virol. 26, 813-816.

GEELEN, J.L.M.C. and WESTRATE, M.W. (1982). Organization of the human cytomegalovirus genome. In: Herpesvirus DNA. Ed. Y. Becker. Published by Martinus Nijhoff.

GENDELMAN, H.E., MOENCH, T.R., NARAYAN, O., GRAFFIN, D.E. and CLEMENTS, J.E. (1985). A double labeling technique for performing immunocytochemistry and *in situ* hybridization in virus infected cell cultures and tissues. J. Virol. Methods 11, 93-103.

GIBSON, P.E., GARDNER, S.D. and PORTER, A.A. (1985). Detection of human polyomavirus DNA in urine specimens by hybridot assay. Arch. Virol. 84, 233-240.

GIBSON, W. (1981a). Immediate-early protein of human cytomegalovirus strains AD169, Davis, and Towne differ in electrophoretic mobility. Virology 112, 350-354.

GIBSON, W. (1981b). Structural and non-structural proteins of strain Colburn cytomegalovirus. Virology 111, 516-537.

GIBSON, W. (1983). Protein counterparts of human and simian cytomegaloviruses. Virology 128, 391-406.

GIBSON, W. and ROIZMAN, B. (1972). Proteins specified by herpes simplex virus. VII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. 10, 1044-1052.

GIBSON, W. and ROIZMAN, B. (1974). Proteins specified by herpes simplex virus. X. Staining and radiolabelling properties of B capsid and virion proteins in polyacrylamide gels. J. Virol. 13, 155-165.

GILLESPIE, D. and SPIEGELMAN, S. (1965). A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. 12, 829-842.

GLEAVES, C.A., HURSH, D.A., RICE, D.H. and MEYERS, J.D. (1989). Detection of cytomegalovirus from clinical specimens in centrifugation culture by *in situ* DNA hybridization and monoclonal antibody staining. J. Clin. Microbiol. 27, 21-23.

GLEAVES, C.A., SMITH, T.F., SHUSTER, E.A. and PEARSON, G.R. (1984). Rapid detection of cytomegalovirus in MRC-5 cells inoculated with urine specimens by using low-speed centrifugation and monoclonal antibody to an early antigen. J. Clin. Microbiol. 19, 917-919.

GLEAVES, C.A., SMITH, T.F., SHUSTER, E.A. and PEARSON, G.R. (1985). Comparison of standard tube and shell vial cell culture techniques for the detection of cytomegalovirus in clinical specimens. J. Clin. Microbiol. 21, 217-221.

GLENN, J. (1981). Cytomegalovirus infections following renal transplantation. Rev. Infect. Dis. 3, 1151-1178.

GOINS, W.F. and STINSKI, M.F. (1986). Expression of a human cytomegalovirus late gene is posttranscriptionally regulated by a 3'-end-processing event occurring exclusively late after infection. Mol. Cell. Biol. 6, 4202-4213.

GOLD, E. and NANKERVIS, G.A. (1976). Cytomegalovirus. In: Viral infections of humans. Ed. A.S. Evans, pp 143-161. Published by Plenum Press, New York.

GOLDSTEIN, L.C., McDOUGALL, J.K., HACKMAN, R.C., MEYERS, J.D., THOMAS, E.D. and NOWINSKI, R.C. (1982). Monoclonal antibodies to cytomegalovirus: Rapid identification of clinical isolates and preliminary use in diagnosis of cytomegalovirus pneumonia. Infect. Immun. 38, 273-281.

GONCZOL, E., ANDREWS, P.W. and PLOTKIN, S.A. (1984). Cytomegalovirus replicates in differentiated but not in undifferentiated human embryonal carcinoma cells. Science 224, 159-161.

GONCZOL, E., FURLINI, G., IANACONE, J. and PLOTKIN, S.A. (1986a). A rapid microneutralization assay for cytomegalovirus. J. Virol. Methods 14, 37-41.

GONCZOL, E., HUDECZ, F., IANACONE, J., DIETZSCHOLD, B., STARR, S. and PLOTKIN, S.A. (1986b). Immune response to isolated human cytomegalovirus envelope proteins. J. Virol. 58, 661-664.

GONCZOL, E. and PLOTKIN, S.A. (1984). Cells infected with human cytomegalovirus release a factor(s) that stimulates cell DNA synthesis. J. gen. Virol. 65, 1833-1837.

GONCZOL, E. and VACZI, L. (1973). Cytomegalovirus latency in cultured human cells. J. gen. Virol. 18, 143-151.

GOODPASTURE, E.W. and TALBOT, F.B. (1921). Concerning the nature of "proteozoan-like" cells in certain lesions of infancy. Am. J. Dis. Child. 21, 415-421.

GRATAMA, J.W., KARDOL, M., NAIPAL, A.M.I.H., SLATS, J., DEN OUDEN, A., STIJNEN, T., D'AMARO, J., THE, T.H. and BRUNING, J.W. (1987). The influence of cytomegalovirus carrier status on lymphocyte subsets and natural immunity. Clin. exp. Immunol. 68, 16-24.

GREEN, M.R., MANIATIS, T. and MELTON, D.A. (1983). Human β-globin pre-mRNA synthesized in vitro is accurately spliced in Xenopus oocyte nuclei. Cell 32, 681-694.

GREENAWAY, P.J., ORAM, J.D., DOWNING, R.G. and PATEL, K. (1982). Human cytomegalovirus DNA: BamHI, EcoRI and PstI restriction endonuclease maps. Gene 18, 355-360.

GREENAWAY, P.J. and WILKINSON, G.W.G. (1987). Nucleotide sequence of the most abundantly transcribed early gene of human cytomegalovirus strain AD169. Virus Res. 7, 17-31.

GREY, H.M. and KUNKEL, H.G. (1964). H chain subgroups of myeloma proteins and normal 7S gammaglobulins. J. Exp. Med. 120, 253-266.

GRIFFITHS, P.D. (1987). Cytomegalovirus. In: Principles and practice of clinical virology. Eds. A.J. Zuckerman, J.E. Banatvala and J.R. Pattison, pp 75-109. Published by John Wiley & Sons, Chichester.

★ GRIFFITHS, P.D. and KANGRO, H.O. (1984). A user's guide to the indirect solid-phase radioimmunoassay for the detection of cytomegalovirus-specific IgM antibodies. J. Virol. Methods 8, 271-282.

GRIFFITHS, P.D., STAGNO, S., PASS, R.F., SMITH, R.J. and ALFORD, C.A.Jr. (1982a). Infection with cytomegalovirus during pregnancy: Specific IgM antibodies as a marker of recent primary infection. J. Infect. Dis. 145, 647-653.

GRIFFITHS, P.D., STAGNO, S., PASS, R.F., SMITH, R.J. and ALFORD, C.A.Jr. (1982b). Congenital cytomegalovirus infection: Diagnostic and prognostic significance of the detection of specific IgM antibodies in cord serum. Pediatrics 69, 544-549.

GRIFFITHS, P.D., STIRK, P.R., GANCZAKOWSKI, M., PANJWANI, D.D., BALL, M.G., BLACKLOCK, H.A. and PRENTICE, H.G. (1984). Rapid diagnosis of cytomegalovirus infection in immunocompromised patients by detection of early antigen fluorescent foci. Lancet 2, 1242-1245.

★ GRIFFITHS, P.D. and GRUNDY, J.E. (1987). Molecular biology and immunology of cytomegalovirus. Biochem. J. 241, 313-324.

GRIST, N.R., BELL, E.J., FOLLETT, E.A.C. and URQUHART, G.E.D. (1979). Diagnostic methods in clinical virology. Published by Blackwell Scientific Publications, Oxford.

GROB, J.P., GRUNDY, J.E., PRENTICE, H.G., GRIFFITHS, P.D., HOFFBRAND, A.V., HUGHES, M.D., TATE, T., WIMPERIS, J.Z. and BRENNER, M.K. (1987). Immune donors can protect marrow-transplant recipients from severe cytomegalovirus infections. Lancet 1, 774-776.

GRONOWITZ, J.S., KALLANDER, C.F.R., DIDERHOLM, H., HAGBERG, H. and PETTERSSON, U. (1984). Application of an *in vitro* assay for serum thymidine kinase: Results on viral disease and malignancies in humans. Int. J. Cancer 33, 5-12.

GRUNDY, J.E., SUPER, M., LUI, S., SWENY, P. and GRIFFITHS, P.D. (1987). The source of cytomegalovirus infection in seropositive renal allograft recipients is frequently the donor kidney. Transplant. Proc. 19, 2126-2128.

GUPTA, P., St. JEOR, S. and RAPP, F. (1977). Comparison of the polypeptides of several strains of human cytomegalovirus. J. gen. Virol. 34, 447-454.

HAAS, M., VOGT, M. and DULBECCO, R. (1972). Loss of simian virus 40 DNA-RNA hybrids from nitrocellulose membranes: Implications for the study of virus-host DNA interactions. Proc. Natl. Acad. Sci. USA 69, 2160-2164.

HALL, B., DUGGIN, G.G., PHILIPS, J., BISHOP, G.A., HORVATH, J.S. and TILLER, D.J. (1984). Increased expression of HLA DR antigens on renal tubular cells in renal transplants: Relevance to the rejection response. Lancet 2, 247-251.

HAMILTON, J.D. (1982). Cytomegalovirus and immunity. In: Monographs in virology. Ed. J.L. Meinick, Vol. 12. Published by Karger-Verlag, Basil.

HAMILTON, J.D. and SEAWORTH, B.J. (1985). Transmission of latent cytomegalovirus in a murine kidney tissue transplantation model. Transplantation 39, 290-296.

HANAHAN, D. (1983). Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166, 557-580.

HANDSFIELD, H.H., CHANDLER, S.H., CAINE, V.A., MEYERS, J.D., CORRY, L., MEDEIROS, E. and McDOUGALL,. J.K. (1985). Cytomegalovirus infection in sex partners: Evidence for sexual transmission. J. Infect. Dis. 151, 344-348.

HANSHAW, J.B., NIEDERMAN, J.C. and CHESSIN, L.N. (1972). Cytomegalovirus macroglobulin in cell-associated herpesvirus infections. J. Infect. Dis. 125, 304-306.

HARBISON, M.A., DE GIROLAMI, P.C., JENKINS, R.L. and HAMMER, S.M. (1988). Ganciclovir therapy of severe cytomegalovirus infections in solid-organ transplant recipients. Transplantation 46, 82-88.

HARPER, M.E., MARSELLE, L.M., GALLO, R.C. and WONG-STAAL, F. (1986). Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in situ hybridization. Proc. Natl. Acad. Sci. USA 83, 772-776.

HARRIS, K.R., SAEED, A.A., DIGARD, N.J., WHITEFORD, K., GEOGHEGAN, T.A., LEE, H.A. and SLAPAK, M. (1984). Cytomegalovirus titers in kidney transplant donor and recipient: Influence of cyclosporine-A. Transplant.Proc. 16, 31-33.

HAYRY, P., VON WILLEBRAND, E., AHONEN, J. and EKLUND, B. (1981). Are transplantation antigens stable constituents on human renal allografts? Proc. Eur. Dial. Transplant Assoc. 18, 367-375.

HECHEMY, K.E. and MICHAELSON, E.E. (1984). Latex particle assays in laboratory medicine, part II. Lab. Manage. 22, 26-35.

HEIEREN, M.H., VAN DER WOUDE, F.J. and BALFOUR, H.H.Jr. (1988). Cytomegalovirus replicates efficiently in human kidney mesangial cells. Proc. Natl. Acad. Sci. USA 85, 1642-1646.

HEILBRONN, R., JAHN, G., BURKLE, A., FREESE, U-K., FLECKENSTEIN, B. and ZUR HAUSEN, H. (1987). Genomic localization, sequence analysis, and transcription of the putative human cytomegalovirus DNA polymerase gene. J. Virol. 61, 119-124.

HEINE, J.W., DE LEY, M., VAN DAMME, J., BILIAU, A. and DE SOMER, P. (1980). Human fibroblast interferon purified to homogeneity by a two-step procedure. Ann. N.Y. Acad. Sci. 350, 364-373.

HEKKER, A.C., BRAND-SAATHOF, B., VIS, J. and MEIJERS, R.C. (1979). Indirect immunofluorescence test for detection of IgM antibodies to cytomegalovirus. J. Infect. Dis. 140, 596-600.

HENNIGHAUSEN, L. and FLECKENSTEIN, B. (1986). Nuclear factor 1 interacts with five DNA elements in the promoter region of the human cytomegalovirus major immediate early gene. EMBO J. 5, 1367-1371.

HENRY, M.L., SOMMER, B.G. and FERGUSON, R.M. (1985). The impact of cyclosporine compared with azathioprine for cadaveric renal transplant immunosuppression. Transplant. Proc. 17, 1244-1246.

HERBERMAN, R.B. and HOLDEN, H.T. (1978). Natural cell-mediated immunity. Adv. Cancer Res. 27, 305-377.

HERMISTON, T.W., MALONE, C.L., WITTE, P.R. and STINSKI, M.F. (1987). Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. J. Virol. 61, 3214-3221.

HERSMAN, J., MEYERS, J.D., THOMAS, E.D., BUCKNER, C.D. and CLIFT, R. (1982). The effect of granulocyte transfusions upon the incidence of cytomegalovirus infection after allogeneic marrow transplantation. Ann. Intern. Med. 96, 149-152.

HILL, R.B.Jr., ROWLANDS, D.T. and RIFKIND, D. (1964). Infectious pulmonary disease in patients receiving immunosuppressive therapy for organ transplantation. N. Engl. J. Med. 271, 1021-1027.

HIRAI, K., FURUKAWA, T. and PLOTKIN, S.A. (1976). Induction of DNA polymerase in WI-38 and guinea pig cells infected with human cytomegalovirus (HCMV). Virology 70, 251-255.

HIRAI, K. and WATANABE, Y. (1976). Induction of α-type DNA polymerases in human cytomegalovirus-infected WI-38 cells. Biochim. Biophys. Acta 447, 328-339.

HIRSCH, M.S., SCHOOLEY, R.T., COSIMI, A.B., RUSSELL, P.S., DELMONICO, F.L., TOLKOFF-RUBIN, N.E., HERRIN, J.T., CANTELL, K., FARRELL, M.L., ROTA, T.R. and RUBIN, R.H. (1983). Effect of interferon-alpha on cytomegalovirus reactivation syndromes in renal-transplant recipients. N. Engl. J. Med. 308, 1489-1493.

HO, M. (1977). Virus infections after transplantation in man. Arch. Virol. 55, 1-24.

HO, M. (1979). Cytomegalovirus. In: Principles and practice of infectious diseases. Eds. G.L. Mandell, R.G. Douglas and J.E. Bennett, pp 1307-1323. Published by Wiley Medical Publications, Chichester.

HO, M. (1980). Role of specific cytotoxic lymphocytes in cellular immunity against murine cytomegalovirus. Infect. Immun. 27, 767-776.

HO, M. (1982). Cytomegalovirus: Biology and Infection, p 226. Published by Plenum Medical Books, New York.

HO, M., SUWANSIRIKUL, S., DOWLING, J.N., YOUNGBLOOD, L.A. and ARMSTRONG, J.A. (1975). The transplanted kidney as a source of cytomegalovirus infection. N. Engl. J. Med. 293, 1109-1112.

HORNSLETH, A., AAEN, K. and GUNDESTRUP, M. (1988). Detection of respiratory syncytial virus and rotavirus by enhanced chemiluminescence enzyme-linked immunosorbent assay. J. Clin. Microbiol. 26, 630-635.

HORTA-BARBOSA, L., FUCILLO, D.A., SEVER, J.L. and ZEMAN, W. (1969). Subacute sclerosing panencephalitis: Isolation of measles virus from a brain biopsy. Nature 221, 974-976.

HOWARD, R.J., KALIS, J.M., BALFOUR, H.H.Jr., MARKER, S.M., SIMMONS, R.L. and NAJARIAN, J.S. (1977). Viral infections in kidney donors and recipients. A prospective study. Transplant. Proc. 9, 113-116.

HOWELL, C.L., MILLER, M.J. and BRUCKNER, D.A. (1986). Elimination of toxicity and enhanced cytomegalovirus detection in cell cultures inoculated with semen from patients with acquired immunodeficiency syndrome. J. Clin. Microbiol. 24, 657-660.

HOWELL, C.L., MILLER, M.J. and MARTIN, W.J. (1979). Comparison of rates of virus isolation from leukocyte populations separated from blood by conventional and Ficoll-Pacque/Macrodex methods. J. Clin. Microbiol. 10, 533-537.

HOWLEY, P.M., ISRAEL, M.A., LAW, M.F. and MARTIN, M.A. (1979). A rapid method for detecting and mapping homology between heterologous DNAs. J. Biol. Chem. 254, 4876-4883.

HU, N. and MESSING, J. (1982). The making of strand-specific M13 probes. Gene 17, 271-277.

HUANG, E-S. (1975a). Human cytomegalovirus. III. Virus-induced DNA polymerase. J. Virol. 16, 298-310.

HUANG, E-S. (1975b). Human cytomegalovirus. IV. Specific inhibition of virus-induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. J. Virol. 16, 1560-1565.

HUANG, E-S., ALFORD, C.A., REYNOLDS, D.W., STAGNO, S. and PASS, R.F. (1980). Molecular epidemiology of cytomegalovirus infection in women and their infants. N. Engl. J. Med. 303, 958-962.

HUANG, E-S., CHEN, S.T. and PAGANO, J.S. (1973). Human cytomegalovirus. I. Purification and characterization of viral DNA. J. Virol. 12, 1473-1481.

HUANG, E-S., KILPATRICK, B.A., HUANG, Y.T. and PAGANO, J.S. (1976). Detection of human cytomegalovirus and analysis of strain variation. Yale J. Biol. Med. 49, 29-43.

HUANG, E-S., LEYRITZ, M., MENEZES, J. and JONCAS, J.H. (1978). Persistence of both human cytomegalovirus and Epstein-Barr virus genomes in two human lymphoblastoid cell lines. J. gen. Virol. 40, 519-529.

HUANG, E-S. and PAGANO, J.S. (1974). Human cytomegalovirus. II. Lack of relatedness to DNA of herpes simplex virus 1 and 2, Epstein-Barr virus and non-human strains of cytomegalovirus. J. Virol. 13, 642-645.

HUBER, C. and TROPPMAIR, J. (1985). In: Biochemical and clinical aspects of pteridines. Eds. Curtis, Wachter and Pfleider, p 279. Published by Walter de Gruyter, Berlin.

HUDSON, J.B. (1979). The murine cytomegalovirus as a model for the study of viral pathogenesis and persistent infections. Arch. Virol. 62, 1-29.

HUDSON, J.B. (1988). Further studies on the mechanism of centrifugal enhancement of cytomegalovirus infectivity. J. Virol. Methods 19, 97-108.

HUDSON, J.B., CHANTLER, J.K., LOH, L., MISRA, V. and MULLER, M.T. (1979). Model systems for analysis of latent cytomegalovirus infections. Can. J. Microbiol. 25, 245-253.

HUDSON, J.B., MISRA, V. and MOSMANN, T.R. (1976). Cytomegalovirus infectivity: Analysis of the phenomenon of centrifugal enhancement of infectivity. Virology 72, 235-243.

HUNT, A.F., ALLEN, D.L., BROWN, R.L., ROBB, B.A., PUCKETT, A.Y. and ENTWISTLE, C.C. (1984). Comparative trials of six methods for the detection of CMV antibody in blood donors. J. Clin. Pathol. 37, 95-97.

HUTCHINSON, N.I., SONDERMEYER, R. and TOCCI, M.J. (1986). Organization and expression of the major genes from the long inverted repeat of the human cytomegalovirus genome. Virology 155, 160-171.

HUTCHINSON, N.I. and TOCCI, M.J. (1986). Characterization of a major early gene from the human cytomegalovirus long inverted repeat; Predicted amino acid sequence of a 30-kDa protein encoded by the 1.2 Kb mRNA. Virology 155, 172-182.

HUTT-FLETCHER, L.M., BALACHANDRAN, N. and ELKINS, M.H. (1983). B cell activation by cytomegalovirus. J. Exp. Med. 158, 2171-2176.

HUTTON, J.R. (1977). Renaturation kinetics and thermal stability of DNA in aqueous solutions of formamide and urea. Nucleic Acids Res. 4, 3537-3555.

HUTTON, J.R. and WETMUR, J.G. (1973). Renaturation of bacteriophage 0X174 DNA-RNA hybrid: RNA length effect and nucleation rate constant. J. Mol. Biol. 77, 495-500.

INTRONA, M., ALLAVENA, P., SPREAFICO, F. and MANTOVANI, A. (1981). Inhibition of human natural killer activity by cyclosporin A. Transplantation 31, 113-116.

IRMIERE, A. and GIBSON, W. (1983). Isolation and characterization of a noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus. Virology 130, 118-133.

IRMIERE, A. and GIBSON, W. (1985). Isolation of human cytomegalovirus intranuclear capsids, characterization of their protein constituents, and demonstration that the B-capsid assembly protein is also abundant in noninfectious enveloped particles. J. Virol. 56, 277-283.

ISAACS, A. and LINDENMANN, J. (1957). Virus interference. I. The interferon. Proc. R. Soc. London Ser. B. 147, 258-267.

ISOM, H.C. (1979). Stimulation of ornithine decarboxylase by human cytomegalovirus. J. gen. Virol. 42, 265-278.

ITO, M., BANDYOPADHYAY, S., MATSUMOTO-KOBAYASHI, M., CLARK, S.C., MILLER, D. and STARR, S.E. (1986). Interleukin 2 enhances natural killing of varicella-zoster virus-infected targets. Clin. exp. Immunol. 65, 182-189.

IWASAKI, Y., FURUKAWA, T., PLOTKIN, S. and KOPROWSKI, A. (1973). Ultrastructural study on the sequence of human cytomegalovirus infection in human diploid cells. Arch. ges. virusforsch. 40, 311-324.

JABLONSKI, E., MOOMAW, E.W., TULLIS, R.H. and RUTH, J.L. (1986). Preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probes. Nucleic Acids Res. 14, 6115-6128.

JACOB, R.L., MORSE, L.S. and ROIZMAN, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J. Virol. 29, 448-457.

JACOBSEN, H., KLENOW, H. and OVERGAARD-HANSEN, K. (1974). The N-terminal amino-acid sequences of DNA polymerase I from Escherichia coli and of the large and the small fragments obtained by a limited proteolysis. Eur. J. Biochem. 45, 623-627.

JACOBSON, M.A., O'DONNELL, J.J., BRODIE, H.R., WOFSY, C. and MILLS, J. (1988). Randomized prospective trial of ganciclovir maintenance therapy for cytomegalovirus retinitis. J. Med. Virol. 25, 339-349.

JAHN, G., KNUST, E., SCHMOLLA, H., SARRE, T., NELSON, J.A., McDOUGALL, J.K. and FLECKENSTEIN, B. (1984). Predominant immediate-early transcripts of human cytomegalovirus AD169. J. Virol. 49, 363-370.

JAHN, G., SCHOLL, V.C., TRAUPE, B. and FLECKENSTEIN, B. (1987). The two major structural phosphoproteins (pp65 and pp150) of human cytomegalovirus and their antigenic properties. J. gen. Virol. 68, 1327-1337.

JANKOWSKI, M.A., GUT, W., NAWROCKA, E. and KANTOCH, M. (1980). Detection and differentiation of cytomegalovirus antibodies by radioimmunoassay. J. Virol. Methods 1, 133-138.

JANKOWSKI, M.A., GUT, W.L., SWITALSKI, L., IMBS, D. and KANTOCH, M. (1979). IgM fluorescence antibodies in sera of pregnant women exposed to rubella. Arch. Virol. 60, 123-130.

JEAN, J-H., YOSHIMURA, N., FURUKAWA, T. and PLOTKIN, S.A. (1978). Intracellular forms of the parental human cytomegalovirus genome at early stages of the infective process. Virology 86, 281-286.

JEANG, K.T., CHIN, G. and HAYWARD, G.S. (1982). Characterization of cytomegalovirus immediate early genes. I. Nonpermissive rodent cells overproduce tk IE 94K protein from CMV (Colburn). Virology 121, 393-403.

* KAARIAINEN, L., KLEMOLA, E. and PALOHEIMO, J. (1966). Rise of cytomegalovirus antibodies in infectious mononucleosis-like syndrome after transfusion. Br. Med. J. 1, 1270-1272.

JESIONEK, A. and KIOLEMENOGLOU, B. (1904). Uber einen Befund von protozoenartigen Gebilden in den Organen eines heriditarluctischen Fotus. Munch. Med. Wochenschr. 51, 1905-1907.

JOASSIN, L. and REGINSTER, M. (1986). Elimination of nonspecific cytomegalovirus immunoglobulin M activities in the enzyme-linked immunosorbent assay by using anti-human immunoglobulin G. J. Clin. Microbiol. 23, 576-581.

JORDAN, M.C., SHANLEY, J.D. and STEVENS, J.G. (1977). Immunosuppression reactivates and disseminates latent murine cytomegalovirus. J. gen. Virol. 37, 419-423.

JORDAN, M.C., TAKAGI, J.L. and STEVENS, J.G. (1982). Activation of latent murine cytomegalovirus in vivo and in vitro: A pathogenetic role for acute infection. J. Infect. Dis. 145, 699-705.

*

KANE, R.C., ROUSSEAU, W.E., NOBLE, G.R., TEGTMEIER, G.E., WULFF, H., HERNDON, H.B., CHIN, T.D.Y. and BAYER, W.L. (1975). Cytomegalovirus infection in a volunteer blood donor population. Infect. Immun. 11, 719-723.

KANGRO, H.O. (1980). Evaluation of a radioimmunoassay for IgM-class antibodies against cytomegalovirus. J. Exp. Pathol. 61, 512-520.

KANGRO, H.O., BOOTH, J.C., BAKIR, T.M.F., TRYHORN, Y. and SUTHERLAND, S. (1984). Detection of IgM antibodies against cytomegalovirus: Comparison of two radioimmunoassays, enzyme-linked immunosorbent assay and immunofluorescent antibody test. J. Med. Virol. 14, 73-80.

KANGRO, H.O., GRIFFITHS, P.D., HUBER, T.J. and HEATH, R.B. (1982). Specific IgM class antibody production following infection with cytomegalovirus. J. Med. Virol. 10, 203-212.

KANICH, R.E. and CRAIGHEAD, J.E. (1966). Cytomegalovirus infection and cytomegalic inclusion disease in renal homotransplant recipients. Am. J. Med. 40, 874-882.

KANTOR, G.L., GOLDBERG, L.S., JOHNSON, B.L., DERECHIN, M.M. and BARNETT, E.V. (1970). Immunological abnormalities induced by postperfusion cytomegalovirus infection. Ann. Intern. Med. 73, 553-558.

KAPASI, K. and RICE, G.P.A. (1986). Rule of the monocyte in cytomegalovirus-mediated immunosuppression in vitro. J. Infect. Dis. 154, 881-884.

KASCHKA-DIERICH, C., FALK, L., BJURSELL, G., ADAMS, A. and LINDAHL, T. (1977). Human lymphoblastoid cell lines derived from individuals without lymphoproliferative disease contain the same latent forms of Epstein-Barr virus DNA as those found in tumor cells. Int. J. Cancer 20, 173-180.

KELLY, A.P., SCHOOLEY, R.T., RUBIN, R.H. and HIRSCH, M.S. (1984). Effect of interferon alpha on natural killer cell cytotoxicity in kidney transplant recipients. Clin. Immunol. Immunopathol. 32, 20-30.

KEMPE, T., SUNDQUIST, W.I., CHOW, F. and HU, S-L. (1985). Chemical and enzymatic biotin-labeling of oligodeoxyribonucleotides. Nucleic Acids Res. 13, 45-57.

KIERSZENBAUM, A.L. and HUANG, E-S. (1978). Chromatin pattern consisting of repeating bipartite structures in WI-38 cells infected with human cytomegalovirus. J. Virol. 28, 661-664.

KILPATRICK, B.A. and HUANG, E-S. (1977). Human cytomegalovirus genome: partial denaturation map and organization of genome sequences. J. Virol. 24, 261-276.

KILPATRICK, B.A., HUANG, E-S. and PAGANO, J.S. (1976). Analysis of cytomegalovirus genomes with restriction endonucleases HindIII and EcoRI. J. Virol. 18, 1095-1105.

KIM, K.S., MOON, H.M., SAPIENZA, V.J. and CARP, R.I. (1977). Complement fixing antigen of human cytomegalovirus. J. Infect. Dis. 135, 281-288.

KIM, K.S., SAPIENZA, V.J., CARP, R.I. and MOON, H.M. (1976). Analysis of structural polypeptides of purified human cytomegalovirus. J. Virol. 20, 604-611.

KIRMANI, N., GINN, R.K., MITTAL, K.K., MANISCHEWITZ, J.F. and QUINNAN, G.V. (1981). Cytomegalovirus-specific cytotoxicity mediated by non-T lymphocytes from peripheral blood of normal volunteers. Infect. Immun. 34, 441-447.

KLEMOLA, E. and KAARIAINEN, L. (1965). Cytomegalovirus as a possible cause of a disease resembling infectious mononucleosis. Br. Med. J. 2, 1099-1102.

KLEMOLA, E., KAARIAINEN, L., VON ESSEN, R., HALTIA, K., KOIVUNIEMI, A. and VON BONSDORFF, C.H. (1967). Further studies on cytomegalovirus mononucleosis in previously healthy individuals. Acta. Med. Scand. 182, 311-322.

KLEMOLA, E., VON ESSEN, R., HENLE, G. and HENLE, W. (1970). Infectious-mononucleosis-like disease with negative heterophil agglutination test. Clinical features in relation to Epstein-Barr virus and cytomegalovirus antibodies. J. Infect. Dis. 121, 608-614.

KLEMOLA, E., VON ESSEN, R., PALOHEIMO, J. and FURUHJELM, U. (1969a). Cytomegalovirus antibodies in donors of fresh blood to patients submitted to open-heart surgery. Scand. J. Infect. Dis. 1, 137-140.

KLEMOLA, E., VON ESSEN, R., WAGER, O., HALTIA, K., KOIVUNIEMI, A. and SALMI, I. (1969b). Cytomegalovirus mononucleosis in previously healthy individuals. Ann. Intern. Med. 71, 11-19.

KLOTMAN, M.E., STARNES, D. and HAMILTON, J.D. (1985). The source of murine cytomegalovirus in mice receiving kidney allografts. J. Infect. Dis. 152, 1192-1196.

KNEZ, V., STEWART, J.A. and ZIEGLER, D.W. (1976). Cytomegalovirus specific IgM and IgG response in humans studied by radioimmunoassay. J. Immunol. 117, 2006-2013.

KNOWLES, W.A. (1976). In vitro cultivation of human cytomegalovirus in thyroid epithelial cells. Arch. Virol. 50, 119-124.

KODAMA, T., FUKUDA, T., OMORI, Y. and OKA, T. (1985). Gastroduodenal cytomegalovirus infection after renal transplantation: Fiberscopic observations. Endoscopy 17, 157-164.

KOHL, S., STARR, S.E., OLESKE, J.M., SHORE, S.L., ASHMAN, R.B. and NAHMIAS, A.J. (1977). Human monocyte-macrophage-mediated antibody-dependent cytotoxicity to herpes simplex virus infected cells. J. Immunol. 118, 729-735.

KONERU, B., TZAKIS, A.G., DE PUYDT, L.E., DEMETRIS, A.J., ARMSTRONG, J.A., DUMMER, J.S. and STARZL, T.E. (1988). Transmission of fatal herpes simplex infection through renal transplantation. Transplantation 45, 653-656.

KOSZINOWSKI, U.H., REDDEHASE, M.J., KEIL, G.M. and SCHICKEDANZ, J. (1987). Host immune response to cytomegalovirus: Products of transfected viral immediate-early genes are recognized by cloned cytolytic T lymphocytes. J. Virol. 61, 2054-2058.

KOUZARIDES, T., BANKIER, A.T., SATCHWELL, S.C., PREDDY, E. and BARRELL, B.G. (1988). An immediate early gene of human cytomegalovirus encodes a potential membrane glycoprotein. Virology 165, 151-164.

KOUZARIDES, T., BANKIER, A.T., SATCHWELL, S.C., WESTON, K., TOMLINSON, P. and BARRELL, B.G. (1987). Sequence and transcription analysis of the human cytomegalovirus DNA polymerase gene. J. Virol. 61, 125-133.

KRAMER, P., TEN KATE, F.W.J., BIJNEN, A.B., JEEKEL, J. and WEIMAR, W. (1984). Recombinant leucocyte interferon A induces steroid-resistant acute vascular rejection episodes in renal transplant recipients. Lancet 1, 989-990.

KRAMVIS, A. and GARNETT, H.M. (1987). Infection of Vervet monkey bone marrow stromal cells with simian cytomegalovirus. J. Infect. Dis. 156, 645-648.

KRECH, U. (1973). Complement-fixing antibodies against cytomegalovirus in different parts of the world. Bull. WHO 49, 103-106.

KRISHNA, R.V., MEURMAN, O.H., ZIEGLER, T. and KRECH, U.H. (1980). Solid-phase enzyme immunoassay for determination of antibodies to cytomegalovirus. J. Clin. Microbiol. 12, 46-51.

KUNKEL, H.G., YOUNT, W.J. and LITWIN, S.D. (1966). Genetically determined antigen of the Ne subgroup of gammaglobulin. Detection by precipitin analysis. Science, 154, 1041-1043.

LA FEMINA, R.L. and HAWYARD, G.S. (1980). Structural organization of the DNA molecules from human cytomegalovirus. In: Animal virus genetics. Eds. B.N. Fields and R. Jaenisch, pp 39-55. Published by Academic Press, New York.

LA FEMINA, R.L. and HAYWARD, G.S. (1983). Replicative forms of human cytomegalovirus DNA with joined termini are found in permissively infected human cells but not in non-permissive Balb/c-3T3 mouse cells. J. gen. Virol. 64, 373-389.

LAIMINS, L.A., KHOURY, G., GORMAN, C., HOWARD, B. and GRUSS, P. (1982). Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA 79, 6453-6457.

LANDINI, M.P., MIROLO, G., COPPOLECCHIA, P., RE, M.C. and LA PLACA, M. (1986). Serum antibodies to individual cytomegalovirus structural polypeptides in renal transplant recipients during viral infection. Microbiol. Immunol. 30, 683-694.

LANDINI, M.P., RE, M.C., MIROLO, G., BALDASSARRI, B. and LA PLACA, M. (1985). Human immune response to cytomegalovirus structural polypeptides studied by immunoblotting. J. Med. Virol. 17, 303-311.

LANG, D.J. (1972). Cytomegalovirus infections in organ transplantation and post-transfusion: An hypothesis. Arch. ges. Virusforsch. 37, 365-377.

LANG, D.J. (1980). Cytomegalovirus immunization: Status, prospects and problems. Rev. Infect. Dis. 23, 449-458.

LANG, D.J., EBERT, P.A., RODGERS, B.M., BOGGESS, H.P. and RIXSE, R.S. (1977a). Reduction of postperfusion cytomegalovirus infections following the use of leukocyte depleted blood. Transfusion 17, 391-395.

LANG, D.J., GARRUTO, R.M. and GAJDUSEK, D.C. (1977b). Early acquisition of cytomegalovirus and Epstein-Barr virus antibody in several isolated Melanesian populations. Am. J. Epidemiol. 105, 480-487.

LANG, D.J. and HANSHAW, J.B. (1969). Cytomegalovirus infection and the post perfusion syndrome: Recognition of primary infections in four patients. N. Engl. J. Med. 280, 1145-1149.

LANIER, L.L., LE, A.M., PHILLIPS, J.H., WARNER, N.L. and BABCOCK, G.F. (1983). Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J. Immunol. 131, 1789-1796.

LARKE, R.P.B., WHEATLEY, E., SAIGAL, S. and CHERNESKY, M.A. (1980). Congenital cytomegalovirus infection in an urban Canadian community. J. Infect. Dis. 142, 647-653.

LARSON, A., FRODIN, L., TUFVESON, G., LARSSON, E., KALLANDER, C.F.R. and GRONOWITZ, J.S. (1986). Deoxythymidine kinase, a possible marker for monitoring activity of cytomegalovirus infection after renal transplantation. Scand. J. Urol. Nephrol. 20, 75-76.

LEARY, J.J., BRIGATI, D.J. and WARD, D.C. (1983). Rapid and sensitive colorimetric method for visualizing biotin-labelled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. Proc. Natl. Acad. Sci. USA 80, 4045-4049.

LEHMANN-GRUBE, F., ASSMANN, U., LOLIGER, G., MOSKOPHIDIS, D. and LOHLER, J. (1985). Mechanism of recovery from acute virus infection. I. Role of T lymphocytes in the clearance of lymphocytic choriomeningitis virus from spleens of mice. J. Immunol. 134, 608-615.

LEINIKKI, R., HEINONEN, K. and PETTAY, O. (1972). Incidence of cytomegalovirus infections in early childhood. Scand. J. Infect. Dis. 4, 1-5.

LEVY, E. and SAROV, I. (1980). Determination of IgA antibodies to human cytomegalovirus by enzyme-linked immunosorbent assay (ELISA). J. Med. Virol. 6, 249-257.

LIN, H.J., WU, P-C. and LAI, C-L. (1987). An oligonucleotide probe for the detection of hepatitis B virus DNA in serum. J. Virol. Methods 15, 139-149.

LINDE, G.A., HAMMARSTROM, L., PERSSON, M.A.A., SMITH, C.I.E., SUNDQVIST, V-A. and WAHREN, B. (1983). Virus-specific antibody activity of different subclasses of immunoglobulins G and A in cytomegalovirus infections. Infect. Immunol. 42, 237-244.

LINDSLEY, M.D., TORPEY, D.J. and RINALDO, C.R.Jr. (1986). HLA-DR-restricted cytotoxicity of cytomegalovirus-infected monocytes mediated by leu-3-positive T cells. J. Immunol. 136, 3045-3051.

LIPSCHUTZ, B. (1921). Untersuchungen uber die Atiologie der Krankheiten der Herpesgruppe (Herpes zoster, Herpes genitalis, Herpes febrilis). Arch. Dermatol. Syphilol. (Berl.) 136, 428-482.

LIVNAT, S., SEIGNEURET, M., STORB, R. and PRENTICE, R.L. (1980). Analysis of cytotoxic effector cell function in patients with leukemia or aplastic anemia before and after marrow transplantation. J. Immunol. 124, 481-490.

LONSDALE, D.M. (1979). A rapid technique for distinguishing herpes simplex virus type 1 from type 2 by restriction enzyme technology. Lancet 1, 849-852.

LOPEZ, C., SIMMONS, R.L., MAUER, S.M., NAJARIAN, J.S. and GOOD, R.A. (1974). Association of renal allograft rejection with virus infections. Am. J. Med. 56, 280-289.

LUBY, J.P., BRUNETT, W., HULL, A.R., WARE, A.J., SHOREY, J.W. and PETERS, P.C. (1974). Relationship between cytomegalovirus and hepatic function abnormalities in the period after renal transplant. J. Infect. Dis. 129, 511-518.

LUI, S.F., SWENY, P., GRUNDY, J.E., BLAXILL, A., GRIFFITHS, P.D., MOORHEAD, J.F. and FERNANDO, O.N. (1987). The clinical application of a rapid diagnostic test for the detection of cytomegalovirus infection in renal transplant recipients. Transplant. Proc. 19, 2129-2130.

LURAIN, N.S., THOMPSON, K.D. and FARRAND, S.K. (1986). Rapid detection of cytomegalovirus in clinical specimens by using biotinylated DNA probes and analysis of cross-reactivity with herpes simplex virus. J. Clin. Microbiol. 24, 724-730.

McCONAUGHY, J.L., LAIRD, C.D. and McCARTHY, B.J. (1969). Nucleic acid reassociation in formamide. Biochemistry 8, 3289-3295.

McDONOUGH, S.H. and SPECTOR, D.H. (1983). Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. Virology 125, 31-46.

McDONOUGH, S.H., STAPRANS, S.I. and SPECTOR, D.H. (1985). Analysis of the major transcripts encoded by the long repeat of human cytomegalovirus strain AD169. J. Virol. 53, 711-718.

McDOUGAL, J.S., HARISDANGKUL, V. and CHRISTIAN, C.L. (1975). Naturally-occurring low molecular weight IgM in patients with rheumatoid arthritis, systemic lupus erythematosus and macroglobulinemia. J. Immunol. 115, 223-229.

McGAVRAN, M.H. and SMITH, M.G. (1965). Ultrastructural cytochemical and microscopical observations on cytomegalovirus (salivary gland virus) infection of human cells in tissue culture. Exp. Mol. Pathol. 4, 1-10.

McHUGH, T.M., CASAVANT, C.H., WILBER, J.C. and STITES, D.P. (1985). Comparison of six methods for the detection of antibody to cytomegalovirus. J. Clin. Microbiol. 22, 1014-1019.

McKEATING, J.A., AL-NAKIB, W., GREENAWAY, P.J. and GRIFFITHS, P.D. (1985). Detection of cytomegalovirus by DNA-DNA hybridization employing probes labelled with ^{32}-phosphorus or biotin. J. Virol. Methods 11, 207-216.

McKEATING, J.A., GRIFFITHS, P.D. and GRUNDY, J.E. (1987). Cytomegalovirus in urine specimens has host β_2 microglobulin bound to the viral envelope: A mechanism for evading the host immune response? J. gen. Virol. 68, 785-792.

McKEATING, J.A., GRUNDY, J.E., VARGHESE, Z. and GRIFFITHS, P.D. (1986). Detection of cytomegalovirus by ELISA in urine samples is inhibited by β_2-microglobulin. J. Med. Virol. 18, 341-348.

MacKINNON, S., BURNETT, A.K., CRAWFORD, R.J., CAMERON, S., LEASK, B.G.S. and SOMMERVILLE, R.G. (1988). Seronegative blood products prevent primary cytomegalovirus infection after bone marrow transplantation. J. Clin. Pathol. 41, 948-950.

MacPHERSON, I.A. and STOKER, M.G.P. (1962). Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence. Virology 16, 147-151.

MAR, E., CHENG, Y. and HUANG, E. (1983). Effects of 9-(1,3-dihydroxy-2-propoxymethyl)guanine on human cytomegalovirus replication in vitro. Antimicrob. Agents Chemother. 24, 518-521.

MARKER, S.C., HOWARD, R.J., SIMMONS, R.L., KALIS, J.M., CONNELLY, D.P., NAJARIAN, J.S. and BALFOUR, H.H.Jr. (1981). Cytomegalovirus infection: A quantitative prospective study of 320 consecutive renal transplants. Surgery 89, 660-671.

MARMUR, J. and DOTY, P. (1961). Thermal renaturation of deoxyribonucleic acids. J. Mol. Biol. 3, 585-594.

MARMUR, J. and DOTY, P. (1962). Determination of the base composition of DNA from its thermal denaturation temperature. J. Mol. Biol. 5, 109-118.

MARYANSKI, J.L., PALA, P., CORRADIN, G., JORDAN, B.R. and CEROTTINI, J-C. (1986). H-2 restricted cytolytic T cells specific for H2A can recognize a synthetic H2A peptide. Nature 324, 578-582.

MASUHO, Y., MATSUMOTO, Y., SUGANO, T., FUJINAGA, S. and MINAMISHIMA, Y. (1987). Human monoclonal antibodies neutralizing human cytomegalovirus. J. gen. Virol. 68, 1457-1461.

MATSUO, T., HELLER, M., PETTI, L., OSHIRO, E. and KIEFF, E. (1984). Persistence of the entire Epstein-Barr virus genome integrated into human lymphocyte DNA. Science 226, 1322-1324.

MATTHEWS, R.E.F. (1982). Classification and nomenclature of viruses. Intervirology 17, 47-51.

MATTIASSON, B., BORREBAECK, C., SANDFRIDSON, B. and MOSBACH, K. (1977). Thermometric enzyme-linked immunosorbent assay: TELISA. Biochim. Biophys. Acta 483, 221-227.

MAY, A.G., BETTS, R.F., FREEMAN, R.B. and ANDRUS, C.H. (1978). An analysis of cytomegalovirus infection and HLA antigen matching on the outcome of renal transplantation. Ann. Surg. 187, 110-117.

MAYO, D.R., ARMSTRONG, J.A. and HO, M. (1977). Reactivation of murine cytomegalovirus by cyclophosphamide. Nature 267, 721-723.

MAYO, D., ARMSTRONG, J.A. and HO, M. (1978). Activation of latent murine cytomegalovirus infection: Cocultivation, cell transfer, and the effect of immunosuppression. J. Infect. Dis. 138, 890-896.

MELTON, D.A., KRIEG, P.A., REBAGLIATI, M.R., MANIATIS, T., ZINN, K. and GREEN, M.R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. 12, 7035-7056.

MESSING, J., CREA, R. and SEEBURG, P.H. (1981). A system for shotgun DNA sequencing. Nucleic Acids Res. 9, 309-321.

METSELAAR, H.J., ROTHBARTH, P.H., WENTING, G.J., VAESSEN, L.B., MASUREL, N., JEEKEL, J. and WEIMAR, W. (1986). Mononuclear subsets during cytomegalovirus disease in renal transplant recipients treated with cyclosporine and rabbit antithymocyte globulin. J. Med. Virol. 19, 95-100.

METZGER, H. (1970). Structure and function of M macroglobulins. Adv. Immunol. 12, 57-116.

MEYERS, J.D. (1984). Cytomegalovirus infection following marrow transplantation: Risk, treatment and prevention. In: Pathogenesis and prevention of human cytomegalobirus infection. Ed. S.A. Plotkin, pp 101-120. Published by Alan R. Liss, New York.

MEYERS, J.D., FLOURNOY, N. and THOMAS, E.D. (1980). Cytomegalovirus infection and specific cell-mediated immunity after marrow transplant. J. Infect. Dis. 142, 816-824.

MEYERS, J.D., FLOURNOY, N. and THOMAS, E.D. (1986). Risk factors for cytomegalovirus infection after human marrow transplantation. J. Infect. Dis. 153, 478-488.

MEYERS, J.D., LESZCZYNSKI, J., ZAIA, J.A., FLOURNOY, N., NEWTON, B., SNYDMAN, D.R., WRIGHT, G.G., LEVIN, M.J. and THOMAS, E.D. (1983). Prevention of cytomegalovirus infection by cytomegalovirus immune globulin after marrow transplantation. Ann. Intern. Med. 98, 442-446.

MEYERS, J.D., McGUFFIN, R.W., BRYSON, Y.J., CANTELL, K. and THOMAS, E.D. (1982). Treatment of cytomegalovirus pneumonia after marrow transplant with combined vidarabine and human leukocyte interferon. J. Infect. Dis. 146, 80-84.

MEYERS, J.D., REED, E.C., SHEPP, D.H., THORNQUIST, M., DANDLIKER, P.S., VICARY, C.A., FLOURNOY, N., KIRK, L.E., KERSEY, J.H., THOMAS, E.D. and BALFOUR, H.H.Jr. (1988). Acyclovir for prevention of cytomegalovirus infection and disease after allogeneic marrow transplantation. N. Eng. J. Med. 318, 70-75.

MICHELSON, S., HORODNICEANU, F., KRESS, M. and TARDY-PANIT, M. (1979). Human cytomegalovirus induced immediate early antigens: Analysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis after immunoprecipitation. J. Virol. 32, 259-267.

MICHELSON, S., TARDY-PANIT, M. and BARZU, O. (1984). Properties of a human cytomegalovirus induced protein kinase. Virology 134, 259-268.

MICHELSON-FISKE, S., ARNOULT, J. and FEBVRE, H. (1977). Growth of cytomegalovirus at supra-optimal temperatures. J. gen. Virol. 36, 437-447.

MIDDELDORP, J.M., JONGSMA, J. and THE, T.H. (1986). Killing of human cytomegalovirus-infected fibroblasts by antiviral antibody and complement. J. Infect. Dis. 153, 48-55.

MILBURN, H.J., GRUNDY, J.E., DU BOIS, R.M., PRENTICE, H.G. and GRIFFITHS, P.D. (1988). Is the measurement of virus-specific antibody in the lungs of transplant recipients with cytomegalovirus pneumonitis of diagnostic or prognostic value? J. Med. Virol. 26, 197-206.

MILLER, R.L. and RAPP, F. (1976). Distinguishing cytomegalovirus, mycoplasma and cellular DNA polymerase. J. Virol. 20, 564-569.

MILLER, W., FLYNN, P., McCULLOUGH, J., BALFOUR, H.H.Jr., GOLDMAN, A., HAAKE, R., McGLAVE, P., RAMSAY, N. and KERSEY, J. (1986). Cytomegalovirus infection after bone marrow transplantation: An association with acute graft-v-host disease. Blood 67, 1162-1167.

MIMS, C.A. (1982). The pathogenesis of infectious disease, pp 110-134. Published by Academic Press, London.

MIRKOVIC, R., WERCH, J., SOUTH, M.A. and BENYESH-MELNICK, M. (1971). Incidence of cytomegaloviraemia in blood bank donors and in infants with congenital cytomegalic inclusion disease. Infect. Immun. 3, 45-50.

MIROLO, G., BALDASSARRI, B., RIPALTI, A., RE, M.C., CLEMENTI, M., MANZIN, A. and LANDINI, M.P. (1987). Antibody response to individual cytomegalovirus structural proteins in different groups of subjects. Eur. J. Clin. Microbiol. 6, 207-210.

MOCARSKI, E.S., LIU, A.C. and SPAETE, R.R. (1987). Structure and variability of the a sequence in the genome of human cytomegalovirus (Towne strain). J. gen. Virol. 68, 2223-2230.

MOCARSKI, E.S., PEREIRA, L. and McCORMICK, A.L. (1988). Human cytomegalovirus ICP22, the product of the HWLF1 reading frame, is an early nuclear protein that is released from cells. J. gen. Virol. 69, 2613-2621.

MOCARSKI, E.S., PEREIRA, L. and MICHAEL, N. (1985). Precise localization of genes on large animal virus genomes: Use of gt 11 and monoclonal antibodies to map the gene for a cytomegalovirus protein family. Proc. Natl. Acad. Sci. USA 82, 1266-1270.

MOCARSKI, E.S., POST, L.E. and ROIZMAN, B. (1980). Molecular engineering of the herpes simplex virus genome: Insertion of a second L-S junction into the genome causes additional genome inversions. Cell 22, 243-255.

MOCARSKI, E.S. and ROIZMAN, B. (1981). Site-specific inversion sequence of herpes simplex virus genome: Domain and structural features. Proc. Natl. Acad. Sci. USA 78, 7047-7051.

MOCARSKI, E.S. and ROIZMAN, B. (1982). Structure and role of the herpes simplex DNA termini in inversion, circularization and generation of virion DNA. Cell 31, 89-97.

MOCARSKI, E.S. and STINSKI, M.F. (1979). Persistence of the cytomegalovirus genome in human cells. J. Virol. 31, 761-775.

MOENCH, T.R., GENDELMAN, H.E., CLEMENTS, J.E., NARAYAN, O. and GRIFFIN, D.E. (1985). Efficiency of *in situ* hybridization as a function of probe size and fixation method. J. Virol. Methods 11, 119-130.

MOHANKUMAR, T., ELLIS, T.M., DAYAL, H., DU VALL, C., MENDEZ-PICON, G. and LEE, H.M. (1981). Potentiating effect of HLA matching and blood transfusion on renal allograft survival. Transplantation 32, 244-247.

MONTPLAISIR, S., BELLONCIK, S., LEDUCK, W.P., ONJI, P.A., MARTINEARU, B. and KRUSTAK, E. (1972). Electron microscopy in the rapid diagnosis of cytomegalovirus: Ultrastructural observation and comparison of methods of diagnosis. J. Infect. Dis. 125, 533-538.

MORELL, A., ROTH-WICKY, B. and SKVARIL, F. (1983). Immunoglobulin G subclass restriction of antibodies against hepatitis B surface antigen. Infect. Immunol. 39, 565-568.

MORGAN-CAPNER, P., TEDDER, R.S. and MACE, J.E. (1983). Rubella-specific IgM reactivity in sera from cases of infectious mononucleosis. J. Hyg. 90, 407-413.

MORIMOTO, C., REINHERZ, E.L. and SCHLOSSMAN, S.F. (1981). Regulation of *in vitro* primary anti-DNP production by functional subsets of T lymphocytes in man. J. Immunol. 127, 69-78.

MORRIS, G.E., COLEMAN, R.M., BEST, J.M., BENETATO, B.B. and NAHMIAS, A.J. (1985). Persistence of serum IgA antibodies to herpes simplex, varicella-zoster, cytomegalovirus, and rubella virus detected by enzyme-linked immunosorbent assays. J. Med. Virol. 16, 343-349.

MORRIS, P.J., FRENCH, M.E., DUNNILL, M.S., HUNNISETT, A.G.W., TING, A., THOMPSON, J.F. and WOOD, R.F.M. (1983). A controlled trial of cyclosporine in renal transplantation with conversion to azathioprine and prednisolone after three months. Transplantation 36, 273-277.

MORSE, A.R. and COLEMAN, D.V. (1974). An evaluation of cytology in the diagnosis of herpes simplex virus infection and cytomegalovirus infection of the cervix uteri. Br. J. Obstet. Gynaecol. 81, 393-398.

MOURAD, G., CHONG, G., POULIQUEN, M., RUIZ, G., MANDEN, J. and MION, C. (1984). Withdrawal of azathioprine and antilymphocyte globulins in kidney transplant recipients with cytomegalovirus. Transplant. Proc. 16, 1313-1315.

MULLIS, K.B. and FALOONA, F.A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol. 155, 335-350.

MUNCH, K., KEIL, G.M., MESSERLE, M. and KOSZINOWSKI, U.H. (1988). Interaction of the 89K murine cytomegalovirus immediate-early protein with core histones. Virology 163, 405-412.

MUNN, C.G., MARKENSON, A.L., KAPADIA, A. and DE SOUSA, M. (1981). Impaired T-cell mitogen responses in some patients with the thalassaemia intermedia. Thymus 3, 119-128.

MYERSON, D., HACKMAN, R.C., NELSON, J.A., WARD, D.C. and McDOUGALL, J.R. (1984). Widespread presence of histologically occult cytomegalovirus. Human Pathol. 15, 430-439.

NAGAMINE, Y., SENTENAC, A. and FROMAGEOT, P. (1980). Selective blotting of restriction DNA fragments on nitrocellulose membranes at low salt concentrations. Nucleic Acids Res. 8, 2453-2460.

NAGATA, K., GUGGENHEIMER, R.A., ENOMOTO, T., LICHY, J.H. and HURWITZ, J. (1982). Adenovirus DNA replication in vitro: Identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. USA 79, 6438-6442.

NAGATA, K., GUGGENHEIMER, R.A. and HURWITZ, J. (1983). Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA 80, 6177-6181.

NAGINGTON, J. (1971). Cytomegalovirus antibody production in renal transplant patients. J. Hyg. 69, 645-660.

NAJARIAN, J.S., FRYD, D.S., STRAND, M., CANAFAX, D.M., ASCHER, N.L., PAYNE, W.D., SIMMONS, R.L. and SUTHERLAND, D.E.R. (1985). A single institution, randomized prospective trial of cyclosporine versus azathioprine-antilymphocyte globulin for immunosuppression in renal allograft recipients. Ann. Surg. 201, 142-157.

NAKAMURU, K., EIZURU, Y. and MINAMISHIMA, Y. (1988). Effect of natural human interferon-β on the replication of human cytomegalovirus. J. Med. Virol. 26, 363-373.

NANKERVIS, G.A. (1976). Comments on CMV infections in renal transplant patients. Yale J. Biol. Med. 49, 27-28.

NAOUMOV, N.V., ALEXANDER, G.J.M., EDDLESTON, A.L.W.F. and WILLIAMS, R. (1988). In situ hybridisation in formalin fixed, paraffin wax embedded liver specimens: Method for detecting human and viral DNA using biotinylated probes. J. Clin. Pathol. 41, 793-798.

NAQVI, S.H. and BLAIR, L.L. (1986). Detection of cytomegalovirus antigen and antibodies in the urine of small infants and children. J. Med. Virol. 18, 139-147.

NARAQI, S. (1984). Cytomegaloviruses. In: Textbook of human virology. Ed. R.B. Belshe, pp 887-927. Published by PSG Publishing Company, Inc., Massachusetts.

NARAQI, S., JACKSON, G.G., JONASSON, O. and RUBENIS, M. (1978). Search for latent cytomegalovirus in renal allografts. Infect. Immun. 19, 699-703.

NARAQI, S., JACKSON, G.G., JONASSON, O. and YAMASHIROYA, H.M. (1977). Prevalence, incidence and source of infections with herpesviruses in patients with renal allografts. J. Infect. Dis. 136, 531-540.

NASH, A.A. and ASHFORD, N.P.N. (1982). Split T-cell tolerance in herpes simplex virus-infected mice and its implication for anti-viral immunity. Immunology 45, 761-767.

NEIMAN, P.E., REEVES, W., RAY, G., FLOURNOY, N., LERNER, K.G., SALE, G.E. and THOMAS, E.D. (1977). A prospective analysis of interstitial pneumonia and opportunistic viral infection among recipients of allogeneic bone marrow grafts. J. Infect. Dis. 136, 754-767.

NEIMAN, P., WASSERMAN, P.B., WENTWORTH, B.B., KAO, G.F., LERNER, K.G., STORB, R., BUCKNER, C.D., CLIFT, R.A., FEFER, A., FASS, L., GLUCKSBERG, H. and THOMAS, E.D. (1973). Interstitial pneumonia and cytomegalovirus infection as complications of human marrow transplantation. Transplantation 15, 478-485.

NELSON, J.A. and GROUDINE, M. (1986). Transcriptional regulation of the human cytomegalovirus major immediate-early gene is associated with induction of DNase I-hypersensitive sites. Mol. Cell. Biol. 6, 452-461.

NIELSEN, S.L., RONHOLM, E., SORENSEN, I. and ANDERSEN, H.K. (1986). Detection of immunoglobulin G antibodies to cytomegalovirus antigens by antibody-capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. 24, 998-1003.

NIELSEN, S.L., RONHOLM, E., SORENSEN, I., JAEGER, P. and ANDERSEN, H.K. (1987). Improvement of serological diagnosis of neonatal cytomegalovirus infection by simultaneously testing for specific immunoglobulins E and M by antibody-capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. 25, 1406-1410.

NIELSEN, S.L., SORENSEN, I. and ANDERSEN, H.K. (1988). Kinetics of specific immunoglobulins M, E, A, and G in congenital, primary, and secondary cytomegalovirus infection studied by antibody-capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. 26, 654-661.

NISHIYAMA, Y., MAENO, K. and YOSHIDA, S. (1983). Characterization of human cytomegalovirus-induced DNA polymerase and the associated 3'- to -5', exonuclease. Virology 124, 221-231.

NONOYAMA, M., HUANG, C.H., PAGANO, J.S., KLEIN, G. and SINGH, S. (1973). DNA of Epstein-Barr virus detected in tissue of Burkitt's lymphoma and nasopharyngeal carcinoma. Proc. Natl. Acad. Sci. USA 70, 3265-3268.

NORVAL, M. and BINGHAM, R.W. (1987). Advances in the use of nucleic acid probes in diagnosis of viral diseases of man. Arch. Virol. 97, 151-165.

NOWAK, B., GMEINER, A., SARNOW, P., LEVINE, A.J. and FLECKENSTEIN, B. (1984). Physical mapping of human cytomegalovirus genes: Identification of DNA sequences coding for a virion phosphoprotein of 71 kDa and a viral 65 kDa polypeptide. Virology 134, 91-102.

NUMAZAKI, Y., YANO, N., MORIZUKA, T., TAKAI, S. and ISHIDA, N. (1970). Primary infection with human cytomegalovirus: Virus isolation from healthy infants and pregnant women. Am. J. Epidemiol. 91, 410-417.

NUNAN, T.O. and BANATVALA, J.E. (1984). Cytomegalovirus infections in renal transplant recipients. Br. Med. J. 288, 1477-1478.

NYGAARD, A.P. and HALL, B.D. (1964). Formation and properties of RNA-DNA complexes. J. Mol. Biol. 9, 125-142.

OHASHI, T., NAWA, Y., MINAMISHIMA, Y. and OWHASHI, M. (1988). Susceptibility of W/W^{V} mice to murine cytomegalovirus infection. Arch. Virol. 101, 237-246.

OKAFOR, G.O., TURNER, M.W. and HAY, F.C. (1974). Localization of monocyte binding site of human immunoglobulin G. Nature 248, 228-230.

OLDING, L.B., JENSEN, F.C. and OLDSTONE, M.B.A. (1975). Pathogenesis of cytomegalovirus infection. I. Activation of virus from bone marrow-derived lymphocytes by *in vitro* allogenic reaction. J. Exp. Med. 141, 561-572.

OLDING, L.B., KINGSBURY, D.T. and OLDSTONE, M.B.A. (1976). Pathogenesis of cytomegalovirus infection. Distribution of viral products, immune complexes and autoimmunity during latent murine infection. J. gen. Virol. 33, 267-280.

OLESKI, J.M., ASHMAN, R.B., KOHL, S., SHORE, S.S., STARR, S.E., WOOD, P. and NAHMIAS, A.J. (1977). Human polymorphonuclear leukocytes as mediators of antibody dependent cellular cytotoxicity to herpes simplex virus-infected cells. Clin. exp. Immunol. 27, 446-453.

O'NEILL, H.J., SHIRODARIA, P.V., CONNOLLY, J.H., SIMPSON, D.I.H. and McGEOWN, M.G. (1988a). Cytomegalovirus-specific antibody responses in renal transplant patients with primary and recurrent CMV infections. J. Med. Virol. 24, 461-470.

O'NEILL, H.J., SHIRODARIA, P.V. and SIMPSON, D.M. (1988b). Low and high molecular weight cytomegalovirus-specific immunoglobulin M antibody in renal transplant patients with cytomegalovirus infections. J. Med. Virol. 24, 445-451.

OPELZ, G. (1988). Comparison of immunosuppressive protocols in renal transplantation: A multicenter view. Transplant. Proc. 20 (Suppl. 8), 31-36.

OPPENHEIM, J.J., STADLER, B.M., SIRAGANIAN, R.P., MAGE, M. and MATHIESON, B. (1982). Lymphokines: Their role in lymphocyte responses. Federation Proc. 41, 257-262.

ORAM, J.D., DOWNING, R.G., AKRIGG, A., DOLLERY, A.A., DUGGLEBY, C.J., WILKINSON, G.W.G. and GREENAWAY, P.J. (1982). Use of recombinant plasmids to investigate the structure of the human cytomegalovirus genome. J. gen. Virol. 59, 111-129.

ORSI, E.V., HOWARD, J.L., BATURAY, N., ENDE, N., RIBOT, S. and ESLAMI, H. (1978). High incidence of virus isolation from donor and recipient tissues associated with renal transplantation. Nature 272, 372-373.

ORTALDO, J.R., MASON, A.T., GERARD, J.P., HENDERSON, L.E., FARRAR, W., HOPKINS, R.F., HERBERMAN, R.B. and RABIN, H. (1984). Effects of natural and recombinant IL-2 on regulation of IFN production and natural killer activity: Lack of involvement of the Tac antigen for these immunoregulatory effects. J. Immunol. 133, 779-783.

OSBORN, J.E. (1981). Cytomegalovirus: Pathogenicity, immunology and vaccine initiative. J. Infect. Dis. 143, 618-630.

O'TOOLE, C.M., GRAY, J.J., MAHER, P. and WREGHITT, T.G. (1986). Persistent excretion of cytomegalovirus in heart transplant patients correlates with inversion of the ratio of T helper/T suppressor-cytotoxic cells. J. Infect. Dis. 153, 1160-1162.

OZAWA, T. and STEWART, J.A. (1979). Immune-complex glomerulonephritis associated with cytomegalovirus infection. Am. J. Clin. Pathol. 72, 103-107.

PAGANO, J.S. (1975). Diseases and mechanisms of persistent DNA virus infection: Latency and cellular transformation. J. Infect. Dis. 132, 209-233.

PANDE, H., BAAK, S.W., RIGGS, A.D., CLARK, B.R., SHIVELY, J.E. and ZAIA, J.A. (1984). Cloning and physical mapping of a gene fragment coding for a 64-kilodalton major late antigen of human cytomegalovirus. Proc. Natl. Acad. Sci. USA 81, 4965-4969.

PARDUE, M.L. (1985). In situ hybridisation. In: Nucleic acid hybridisation. A practical approach. Eds. B.D. Hames and S.J. Higgins, pp 179-202. Published by IRL Press, Oxford.

PARKS, W.P. and RAPP, F. (1975). Prospects for herpes virus vaccination - safety and efficacy considerations. Prog. Med. Virol. 21, 188-206.

PARSLOW, T.G. and GRANNER, D.K. (1982). Chromatin changes accompany immunoglobulin kappa gene activation: A potential control region within the gene. Nature 299, 449-451.

PASS, R.F., GRIFFITHS, P.D. and AUGUST, A.M. (1983). Antibody response to cytomegalovirus after renal transplantation: Comparison of patients with primary and recurrent infections. J. Infect. Dis. 147, 40-46.

PASS, R.F., WHITLEY, R.J., DIETHELM, A.G., WHELCHEL, J.D., REYNOLDS, D.W. and ALFORD, C.A. (1980). Cytomegalovirus infection in patients with renal transplants: Potentiation by antithymocyte globulin and an incompatible graft. J. Infect. Dis. 142, 9-17.

PATTENGALE, P.K., SMITH, R.W. and GERBER, P. (1974). B-cell characteristics of human peripheral and cord blood lymphocytes transformed by Epstein-Barr virus. J. Natl. Cancer Inst. 52, 1081-1086.

PAYNE, J.E., FIALA, M., SPENCER, M., CHATTERJEE, S.N. and BERNE, T.V. (1974). Cytomegalovirus antigen-antibody complexes in biopsy specimens in renal allograft rejection. Surg. Forum 25, 273-275.

PEDEN, K., MOUNTS, P. and HAYWARD, G. (1982). Homology between mammalian cell DNA sequences and human herpesvirus genomes detected by a hybridization procedure with high complexity probe. Cell 31, 71-80.

PEREIRA, L., HOFFMAN, M. and CREMER, N. (1982a). Electrophoretic analysis of polypeptides immune precipitated from cytomegalovirus-infected cell extracts by human sera. Infect. Immun. 36, 933-942.

PEREIRA, L., HOFFMAN, M., GALLO, D. and CREMER, N.(1982b). Monoclonal antibodies to human cytomegalovirus: Three surface membrane proteins with unique immunological and electrophoretic properties specify cross-reactive determinants. Infect. Immun. 36, 924-932.

PERHAM, T.G.M., CAUL, E.O., CONWAY, P.J. and MOTT, M.G. (1971). Cytomegalovirus infection in blood donors - a prospective study. Br. J. Haematol. 20, 307-320.

PERSSON, U., MYRENFORS, P., RINGDEN, O., SUNDBERG, B., LARSSON, P., GUNNAR, S. and JOHANSSON, O. (1987). T lymphocyte subpopulations in bone-marrow-transplanted patients in relation to graft-versus-host disease and cytomegalovirus-induced infection. Transplantation 43, 663-668.

PERUSSIA, B., TRINCHIERI, G., JACKSON, A., WARNER, N.L., FAUST, J., RUMPOLD, H., KRAFT, D. and LANIER, L.L. (1984). The Fc receptor for IgG on human natural killer cells: Phenotypic, functional, and comparative studies with monoclonal antibodies. J. Immunol. 133, 180-189.

PETERSON, P.A., RASK, L. and LINDBLAD, J.B. (1974). Highly purified papain-solubilized HLA-antigens contain β_2-microglobulin. Proc. Natl. Acad. Sci. USA 71, 35-39.

PETERSON, P.K., BALFOUR, H.H.Jr., FRYD, D.S., FERGUSON, R., KRONENBERG, R. and SIMMONS, R.L. (1983). Risk factors in the development of cytomegalovirus-related pneumonia in renal transplant recipients. J. Infect. Dis. 148, 1121.

PHIPPS, P.H., GREGOIRE, L., ROSSIER, E. and PERRY, E. (1983). Comparison of five methods of cytomegalovirus antibody screening of blood donors. J. Clin. Microbiol. 18, 1296-1300.

PIEN, F.D., SMITH, T.F., ANDERSON, C.F., WEBEL, M.L. and TASWELL, H.F. (1973). Herpesviruses in renal transplant patients. Transplantation 16, 489-495.

PIRSON, Y., LECLERCQ, B. and SQUIFFLET, J.P. (1985). Risk factors for cytomegalovirus infection and disease after renal transplantation. Br. Med. J. 291, 1576.

PLACHTER, B., TRAUPE, B., ALBRECHT, J. and JAHN, G. (1988). Abundant 5kb RNA of human cytomegalovirus without a major translational reading frame. J. gen. Virol. 69, 2251-2266.

PLATT, J.L., SIBLEY, R.K. and MICHAEL, A.F. (1985). Interstitial nephritis associated with cytomegalovirus infection. Kidney Int. 28, 550-552.

PLOTKIN, S.A., FARQUHAR, J. and HORNBERGER, E. (1976). Clinical trials of immunization with the towne 125 strain of human cytomegalovirus. J. Infect. Dis. 134, 470-475.

PLOTKIN, S.A., SMILEY, M.L., FRIEDMAN, H.M., STARR, S.E., FLEISHER, G.R., WLODAVER, C., DAFOE, D.C., FRIEDMAN, A.D., GROSSMAN, R.A. and BARKER, C.F. (1984). Towne-vaccine-induced prevention of cytomegalovirus disease after renal transplants. Lancet 1, 528-530.

POBER, J.S., GIMBRONE, M.A.Jr., COTRAN, R.S., REISS, C.S., BURAKOFF, S.J., FIERS, W. and AULT, K.A. (1983). Ia expression by vascular endothelium is inducible by activated T cells and by human gamma interferon. J. Exp. Med. 157, 1339-1353.

POLLAK, R., BARBER, P.L., MOZES, M.F. and PRUSAK, B.F. (1987). Cytomegalovirus as a risk factor in living-related renal transplantation. Ann. Surg. 205, 302-304.

POLLARD, R.B., EGBERT, P.R., GALLAGHER, J.G. and MERIGAN, T.C. (1980). Cytomegalovirus retinitis in immunosuppressed hosts. I. Natural history and effects of treatment with adenine arabinoside. Ann. Intern. Med. 93, 655-664.

PORTER, K.R., STARNES, D.M. and HAMILTON, J.D. (1985). Reactivation of latent murine cytomegalovirus from kidney. Kidney Int. 28, 922-925.

POSTIC, B. and DOWLING, J.N. (1977). Susceptibility of clinical isolates of cytomegalovirus to human interferon. Antimicrob. Agents Chemother. 11, 656-672.

PREIKSAITIS, J.K., ROSNO, S., GRUMET, C. and MERIGAN, T.C. (1983). Infections due to herpesviruses in cardiac transplant recipients: Role of the donor heart and immunosuppressive therapy. J. Infect. Dis. 147, 974-981.

PRINCE, A.M., SZMUNESS, W., MILLIAN, S.J. and DAVID, D.S. (1971). A serologic study of cytomegalovirus infections associated with blood transfusions. N. Engl. J. Med. 284, 1125-1131.

PRITCHETT, R.F. (1980). DNA nucleotide sequence heterogeneity between the Towne and AD-169 strains of cytomegalovirus. J. Virol. 36, 152-161.

PRZEPIORKA, D. and MYERSON, D. (1986). A single-step silver enhancement method permitting rapid diagnosis of cytomegalovirus infection in formalin-fixed, paraffin-embedded tissue sections by in situ hybridization and immunoperoxidase detection. J. Histochem. Cytochem. 34, 1731-1734.

PUCKETT, A. and DAVIS, J.E. (1987). Cytomegalovirus (CMV) antibody screening in blood donors: Modification of new latex agglutination test compared with two standard methods. J. Clin. Pathol. 40, 581-583.

QUINNAN, G.V., BURNS, W.H., KIRMANI, N., ROOK, A.H., MANISCHEWITZ, J., JACKSON, L., SANTOS, G.W. and SARAL, R. (1984a). HLA-restricted cytotoxic T lymphocytes are an important defense mechanism in cytomegalovirus infections. Rev. Infect. Dis. 6, 156-162.

QUINNAN, G.V., DELERY, M., ROOK, A.H., FREDERICK, W.R., EPSTEIN, J.S., MANISCHEWITZ, J.F., JACKSON, L., RAMSEY, K.M., MITTAL, K., PLOTKIN, S.A. and HILLEMAN, M.R. (1984b). Comparative virulence and immunogenicity of the Towne strain and a nonattenuated strain of cytomegalovirus. Ann. Intern. Med. 101, 478-483.

QUINNAN, G.V., KIRMANI, N., ESBER, E., SARAL, R., MANISCHEWITZ, J.F., ROGERS, J.L., ROOK, A.H., SANTOS, G.W. and BURNS, W.J. (1981). HLA-restricted cytotoxic lymphocyte responses to cytomegalovirus infection of bone marrow transplant recipients. J. Immunol. 126, 2036-2041.

QUINNAN, G.V., KIRMANI, N., ROOK, A.H., MANISCHEWITZ, J.F., JACKSON, L., MORESCHI, G., SANTOS, G.W., SARAL, R. and BURNS, W.J. (1982). HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. N. Engl. J. Med. 307, 7-13.

QUINNAN, G.V. and MANISCHEWITZ, J.F. (1979). The role of natural killer cells and antibody-dependent cell-mediated cytotoxicity during murine cytomegalovirus infection. J. Exp. Med. 150, 1549-1554.

RAKELA, J., WIESNER, R.H., TASWELL, H.F., HERMANS, P.E., SMITH, T.F., PERKINS, J.D. and KROM, R.A.F. (1987). Incidence of cytomegalovirus infection and its relationship to donor-recipient serologic status in liver transplantation. Transplant. Proc. 19, 2399-2402.

RAPP, F., GEDER, L., MURASKO, D., LAUSCH, R., LADDA, R., HUANG, E-S. and WEBBER, M.M. (1975). Long-term persistence of cytomegalovirus genome in cultured human cells of prostatic origin. J. Virol. 16, 982-990.

RASMUSSEN, L.E., CHEN, P.T. and MERIGAN, T.C. (1984a). Comparison of antiviral activities of cloned and native human interferons against herpes simplex virus types 1 and 2 and human cytomegalovirus. Antimicrob. Agents Chemother. 26, 599-600.

RASMUSSEN, L., KELSALL, D., NELSON, R., CARNEY, W., HIRSCH, M., WINSTON, D., PREIKSAITIS, J. and MERIGAN, T.C. (1982). Virus-specific IgG and IgM antibodies in normal and immunocompromised subjects infected with cytomegalovirus. J. Infect. Dis. 145, 191-199.

RASMUSSEN, L., MULLENAX, J., NELSON, R. and MERIGAN, T.C. (1985). Viral polypeptides detected by a complement-dependent neutralizing murine monoclonal antibody to human cytomegalovirus. J. Virol. 55, 274-280.

RASMUSSEN, L.E., NELSON, R.M., KELSALL, D.C. and MERIGAN, T.C. (1984b). Murine monoclonal antibody to a single protein neutralizes the infectivity of human cytomegalovirus. Proc. Natl. Acad. Sci. USA 81, 876-880.

RASMUSSEN, L., NELSON, M., NEFF, M. and MERIGAN, T.C. Jr. (1988). Characterization of two different human cytomegalovirus glycoproteins which are targets for virus neutralizing antibody. Virology 163, 308-318.

REDDEHASE, M.J. and KOSZINOWSKI, U.H. (1984). Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection. Nature 312, 369-371.

REDDEHASE, M.J., MUTTER, W., MUNCH, K., BUHRING, H-J. and KOSZINOWSKI, U.H. (1987). CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. J. Virol. 61, 3102-3108.

REDDEHASE, M.J., SUESSMUTH, W., MOYERS, C., FALK, W. and DROEGE, W. (1982). Interleukin 2 is not sufficient as helper component for the activation of cytotoxic T lymphocytes by synergizes with a late helper effect that is provided by irradiated I-region-incompatible stimulator cells. J. Immunol. 128, 61-68.

REEVES, R. (1984). Transcriptionally active chromatin. Biochim. Biophys. Acta 782, 343-393.

REICHERT, C.M., O'LEARY, T.J., LEVENS, D.L., SIMRELL, C.R. and MACHER, A.M. (1983). Autopsy pathology in the acquired immune deficiency syndrome. Am. J. Pathol. 112, 357-382.

REINHERZ, E.L., KUNG, P.C., BREARD, J.M., GOLDSTEIN, G. and SCHLOSSMAN, S.F. (1980). T cell requirements for generation of helper factor(s) in man: Analysis of the subsets involved. J. Immunol. 124, 1883-1887.

REINHERZ, E.L., KUNG, P.C., GOLDSTEIN, G. and SCHLOSSMAN, S.F. (1979). Separation of functional subsets of human T cells by a monoclonal antibody. Proc. Natl. Acad. Sci. USA 76, 4061-4065.

REISER, H., KUHN, J., DOERR, H.W., KIRCHNER, H., MUNK, K. and BRAUN, R. (1986). Human cytomegalovirus replicates in primary human bone marrow cells. J. gen. Virol. 67, 2595-2604.

RENZ, M. and KURZ, C. (1984). A colorimetric method for DNA hybridization. Nucleic Acids Res. 12, 3435-3444.

REYNOLDS, D.W., STAGNO, S. and ALFORD, C.A. (1979). Laboratory diagnosis of cytomegalovirus infections. In: Diagnostic procedures for viral, rickettsial and chlamydial infections. Eds. E.H. Lennette and N.J. Schmidt, pp 399-439. Published by the American Public Health Association Inc.

RIBBERT, D. (1904). Uber protozoenartige Zellen in der Niere eines syphilitischen Neugeborenen und in der Parotis von Kindern. Zentralbl. Allg. Pathol. 15, 945-948.

RICE, G.P.A., SCHRIER, R.D. and OLDSTONE, M.B.A. (1984). Cytomegalovirus infects human lymphocytes and monocytes: Virus expression is restricted to immediate-early gene products. Proc. Natl. Acad. Sci. USA 81, 6134-6138.

RICHARDSON, W.P., COLVIN, R.B., CHEESEMAN, S.H., TOLKOFF-RUBIN, N.E., HERRIN, J.T., COSIMI, A.B., COLLINS, A.B., HIRSCH, M.S., McCLUSKEY, R.T., RUSSELL, P.S. and RUBIN, R.H. (1981). Glomerulopathy associated with cytomegalovirus viremia in renal allografts. N. Engl. J. Med. 305, 57-63.

RICHMAN, D.D., CLEVELAND, P.H., REDFIELD, D.C., OXMAN, M.N. and WAHL, G.M. (1984). Rapid viral diagnosis. J. Infect. Dis. 149, 298-310.

RIFKIND, D., GOODMAN, N. and HILL, R.B.Jr. (1967). The clinical significance of CMV infection in renal transplant recipients. Ann. Intern. Med. 66, 1116-1128.

RIGBY, P.W., DIECKMANN, M., RHODES, C. and BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113, 237-251.

RINALDO, C.R.Jr., BLACK, P.H. and HIRSCH, M.S. (1977). Interaction of cytomegalovirus with leukocytes from patients with mononucleosis due to cytomegalovirus. J. Infect. Dis. 136, 667-678.

RINALDO, C.R.Jr., CARNEY, W.P., RICHTER, B.S., BLACK, P.H. and HIRSCH, M.S. (1980). Mechanisms of immunosuppression in cytomegalovirus mononucleosis. J. Infect. Dis. 141, 488-495.

RINALDO, C.R.Jr, HAMOUDI, W.H., DE BIASIO, R.L., RABIN, B., HAKALA, T.R. and LIEBERT, M. (1983). Cellular immune response and cytomegalovirus infection in renal transplant recipients receiving cyclosporine. Transplant. Proc. 15, 2775-2778.

RINALDO, C.R.Jr., HIRSCH, M.S. and BLACK, P.H. (1976). Activation of latent viruses following bone marrow transplantation. Transplant. Proc. 8, 669-672.

RINALDO, C.R.Jr., RICHTER, B.S., BLACK, P.H., CALLERY, R., CHESS, L. and HIRSCH, M.S. (1978). Replication of herpes simplex virus and cytomegalovirus in human leukocytes. J. Immunol. 120, 130-136.

RINGDEN, O., LONNQVIST, B. and SUNDBERG, B. (1987). Cytomegalovirus immune bone-marrow donors and interstitial pneumonitis. Lancet 2, 105-106.

RISDON, R.A. and TURNER, D.R. (1980). Atlas of renal pathology. Published by MTP Press, England.

ROBERTSON, P.W., KERTESZ, V. and CLOONAN, M.J. (1977). Elimination of false-positive cytomegalovirus immunoglobulin M-fluorescent-antibody ractions with immunoglobulin M serum fractions. J. Clin. Microbiol. 6, 174-175.

RODGERS, B., BORYSIEWICZ, L., MUNDIN, J., GRAHAM, S. and SISSONS, P. (1987). Immunoaffinity purification of a 72K early antigen of human cytomegalovirus: Analysis of humoral and cell-mediated immunity to the purified polypeptide. J. gen. Virol. 68, 2371-2378.

ROENHORST, H.W., KALLENBERG, C.G.M. and THE, T.H. (1988). The cellular immune response to cell-associated and cell-free cytomegalovirus (CMV) antigens after primary CMV-infection in non-immunocompromised hosts: Development and maintenance of CMV-latency and its influence on immunocompetence. Clin. exp. Immunol. 74, 326-332.

ROENHORST, H.W., TEGZESS, A.M., BEELEN, J.M., MIDDELDORP, J.M. and THE, T.H. (1985). HLA-DRw6 as a risk factor for active cytomegalovirus but not for herpes simplex virus infection after renal allograft transplantation. Br. Med. J. 291, 619-622.

ROGERS, B.C., SCOTT, D.M., MUNDIN, J. and SISSONS, J.G.P. (1985). Monocyte-derived inhibitor of interleukin 1 induced by human cytomegalovirus. J. Virol. 55, 527-532.

ROGGENDORF, M., HEINZ, F., DEINHARDT, F. and KUNZ, C. (1981). Serological diagnosis of acute tick-borne encephalitis by demonstration of antibodies of the IgM class. J. Med. Virol. 7, 41-50.

ROITT, I.M. (1985). Essential immunology. Published by Blackwell Scientific Publications, London.

ROOK, A.H. and QUINNAN, G.V. (1983). Cell mediated immunity to human cytomegalovirus. In: Human immunity to viruses. Ed. F.E. Ennis, pp 241-246. Published by Academic Press, New York.

ROOK, A.H., QUINNAN, G.V., FREDERICK, W.F., MANISCHEWITZ, J.F., JACKSON, L., KIRKANI, N., DANTZLER, T., LEE, B.B. and COURIER, C.B. (1984). Importance of cytotoxic lymphocytes during cytomegalovirus infection of renal transplant recipients. Am. J. Med. 76, 385-392.

ROTBART, H.A., EASTMAN, P.S., RUTH, J.L., HIRATA, K.K. and LEVIN, M.J. (1988). Nonisotopic oligomeric probes for the human enteroviruses. J. Clin. Microbiol. 26, 2669-2671.

ROWE, W.P., HARTLEY, J.W., WATERMAN, S., TURNER, H.C. and HUEBNER, R.J. (1956). Cytopathogenic agent resembling human salivary gland virus recovered from tissue cultures of human adenoids. Proc. Soc. Exp. Biol. Med. 92, 418-424.

RUBIN, R.H. (1981). Infection in the renal transplant patient. In: Clinical approach to infection in the compromised host. Eds. R.H. Rubin and L.S. Young. Published by Plenum Press, New York.

RUBIN, R.H., CARNEY, W.P., SCHOOLEY, R.T., COLVIN, R.B., BURTON, R.C., HOFFMAN, R.A., HANSEN, W.P., COSIMI, A.B., RUSSELL, P.S. and HIRSCH, M.S. (1981a). The effect of infection on T lymphocyte sub-populations: A preliminary report. Int. J. Immunopharmacol. 3, 307-312.

RUBIN, R.H., COSIMI, A.B., TOLKOFF-RUBIN, N.E., RUSSELL, P.S. and HIRSCH, M.S. (1977). Infectious disease syndromes attributable to cytomegalovirus and their significance among renal transplant recipients. Transplantation 24, 458-464.

RUBIN, R.H., RUSSELL, P.S., LEVIN, M. and COHEN, C. (1979). Summary of a workshop on cytomegalovirus infections during organ transplantation. J. Infect. Dis. 139, 728-734.

RUBIN, R.H. and TOLKOFF-RUBIN, N.E. (1988). Opportunistic infections in renal allograft recipients. Transplant. Proc. 20, (Suppl. 8), 12-18.

RUBIN, R.H., TOLKOFF-RUBIN, N.E., OLIVER, D., ROTA, T.R., HAMILTON, J., BETTS, R.F., PASS, R.F., HILLIS, W., SZMUNESS, W., FARRELL, M.L. and HIRSCH, M.S. (1985). Multicenter seroepidemiologic study of the impact of cytomegalovirus infection on renal transplantation. Transplantation 40, 243-249.

RUBIN, R.H., WILSON, E.J., BARRETT, L.V. and MEDEARIS, D.N. (1984). Primary cytomegalovirus infection following cardiac transplantation in a murine model. Transplantation 37, 306-310.

RUBIN, R.H., WOLFSON, J.S., COSIMI, A.B. and TOLKOFF-RUBIN, N.E. (1981b). Infection in the renal transplant recipient. Am. J. Med. 70, 405-411.

RUEBNER, B.H., MIYAI, K., SLUSSER, R.J., WEDEMEYER, P. and MEDEARIS, D.N. (1964). Mouse cytomegalovirus infection. An electron microscopic study of hepatic parenchymal cells. Am. J. Pathol. 44, 799-821.

RUGER, R., BORNKAMM, G.W. and FLECKENSTEIN, B. (1984). Human cytomegalovirus DNA sequences with homologies to the cellular genome. J. gen. Virol. 65, 1351-1364.

RUNDELL, B.B. and BETTS, R.F. (1980). Physical properties of cytomegalovirus immune complexes prepared with IgG neutralizing antibody, anti-IgG and complement. J. Immunol. 124, 337-342.

RUNDELL, B.B. and BETTS, R.F. (1981). Interaction of cytomegalovirus immune complexes with host cells. Infect. Immun. 3, 658-665.

RUNDELL, B.B. and BETTS, R.F. (1982). Neutralization and sensitization of cytomegalovirus by IgG antibody, anti-IgG antibody and complement. J. Med. Virol. 10, 109-118.

RUSSELL, M.W., BROWN, T.A. and MESTECKY, J. (1981). Role of serum IgA. Hepatobiliary transport of circulating antigen. J. Exp. Med. 153, 968-976.

RYTEL, M.W. and BALAY, J. (1976). Cytomegalovirus and immunity in renal allograft recipients: Assessment of the competence of humoral immunity. Infect. Immun. 13, 1633-1637.

SAIKI, R.K., GELFAND, D.H., STOFFEL, S., SCHARF, S.J., HIGUCHI, R., HORN, G.T., MULLIS, K.B. and ERLICH, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487-491.

SALONEN, E.M., VAHERI, A., SUNI, J. and WAGER, O. (1980). Rheumatoid factor in acute viral infections: Interference with determination of IgM, IgG and IgA antibodies in an enzyme immunoassay. J. Infect. Dis. 142, 250-255.

SAROV, I. and ABADY, I. (1975). The morphogenesis of human cytomegalovirus. Isolation and polypeptide characterization of cytomegalovirions and dense bodies. Virology 66, 464-473.

SAROV, I. and FRIEDMAN, A. (1976). Electron microscopy of human cytomegalovirus DNA. Arch. Virol. 50, 343-347.

SAROV, I., LEVY, E., AYMARD, M., CHARDONNET, Y., BOSSHARD, S., REVILLARD, J.P., FRIEDMAN, M., NORD, E., GREIFF, M. and HAIKIN, H. (1982). Detection of virus-specific IgA antibodies in serum of kidney transplant patients with recurrent cytomegalovirus infection by enzymeimmuno and radioimmunoassay techniques. Clin. exp. Immunol. 48, 321-328.

SAYERS, T.J., MASON, A.T. and ORTALDO, J.R. (1986). Regulation of human natural killer cell activity by interferon-y: lack of a role in interleukin 2-mediated augmentation. J. Immunol. 136, 2176-2180.

SCHILDKRAUT, C.L. and LIFSON, S. (1965). Dependence of the melting temperature of DNA on salt concentration. Biopolymers 3, 195-208.

SCHMECKPEPER, B.J. and SMITH, K.D. (1972). Use of formamide in nucleic acid reassociation. Biochemistry 11, 1319-1326.

SCHMIDT, G.M., KOVACS, A., ZAIA, J.A., HORAK, D.A., BLUME, K.G., NADEMANEE, A.P., O'DONNELL, M.R., SNYDER, D.S. and FORMAN, S.J. (1988). Ganciclovir/immunoglobulin combination therapy for the treatment of human cytomegalovirus-associated interstitial pneumonia in bone marrow allograft recipients. Transplantation 46, 905-907.

SCHMITZ, H. (1982). Detection of immunoglobulin M antibody to Epstein-Barr virus by use of an enzyme-labelled antigen. J. Clin. Microbiol. 16, 361-366.

SCHMITZ, H., DOERR, H-W., KAMPA, D. and VOGT, A. (1977). Solid-phase enzyme immunoassay for immunoglobulin M antibodies to cytomegalovirus. J. Clin. Microbiol. 5, 629-634.

SCHOCHETMAN, G., OU, C-Y. and JONES, W.K. (1988). Polymerase chain reaction. J. Infect. Dis. 158, 1154-1157.

SCHOOLEY, R.T., HIRSCH, M.S., COLVIN, R.B., COSIMI, A.B., TOLKOFF-RUBIN, N.E., McCLUSKY, R.T., BURTON, R.C., RUSSELL, P.S., HERRIN, J.T., DELMONICO, F.L., GIORGI, J.V., HENLE, W. and RUBIN, R.H. (1983). Association of herpesvirus infections with T-lymphocyte-subset alterations, glomerulopathy, and opportunistic infections after renal transplantation. N. Engl. J. Med. 308, 307-313.

SCHRIER, R.D., NELSON, J.A. and OLDSTONE, M.B.A. (1985). Detection of human cytomegalovirus in peripheral blood lymphocytes in a natural infection. Science 230, 1048-1051.

SCHRIER, R.D. and OLDSTONE, M.B.A. (1986). Recent clinical isolates of cytomegalovirus suppress human cytomegalovirus-specific human leukocyte antigen-restricted cytotoxic T-lymphocyte activity. J. Virol. 59, 127-131.

SCHRIER, R.D., RICE, G.P.A. and OLDSTONE, M.B.A. (1986). Suppression of natural killer cell activity and T cell proliferation by fresh isolates of human cytomegalovirus. J. Infect. Dis. 153, 1084-1901.

SCHUBACH, W. and GROUDINE, M. (1984). Alteration of c-myc chromatin structure by avian leukosis virus integration. Nature 307, 702-708.

SCHULTZ, R.M., PAPAMETHEAKIS, J.D. and CHIRAGOS, M.A. (1977). Interferon: The ultimate inducer of macrophage activation by polyanions. Science 197, 674-675.

SCHUMAKER, V.N., CALCOTT, M.A., SPIEGELBERG, H.L. and MULLER-EBERHARD, H.J. (1976). Ultracentrifuge studies of the binding of IgG of different subclasses to the C1q subunit of the first component of complement. Biochemistry 15, 5175-5181.

SCHUMANN, G.B., BERRING, S. and HILL, R.B. (1977). Use of the cytocentrifuge for the detection of cytomegalovirus inclusions in the urine of renal allograft patients. A case report. Acta Cytol. 21, 168-172.

SCHUSTER, V., MATZ, B., WIEGAND, H., TRAUB, B., KAMPA, D. and NEUMANN-HAEFELIN, D. (1986). Detection of human cytomegalovirus in urine by DNA-DNA and RNA-DNA hybridization. J. Infect. Dis. 154, 309-314.

SCOTT, A.A., WALKER, K.A., HENNIGAR, L.M., WILLIAMS, C.H., MANOS, J.P. and GANSLER, T. (1988). Detection of cytomegalovirus in shell vial cultures by using a DNA probe and early nuclear antigen monoclonal antibody. J. Clin. Microbiol. 26, 1895-1897.

SHABTAI, M., LUFT, B., WALTZER, W.C., ANAISE, D. and RAPAPORT, F.T. (1988). Massive cytomegalovirus pneumonia and myocarditis in a renal transplant recipient: Successful treatment with DHPG. Transplant. Proc. 20, 562-563.

SHALEV, A., GREENBERG, A.H. and McALPINE, P.J. (1980). Detection of attograms of antigen by a high-sensitivity enzyme-linked immunosorbent assay (HS-ELISA) using a fluorogenic substrate. J. Immunol. Methods 38, 125-139.

SHANLEY, J.D., JORDAN, M.C., COOK, M.L. and STEVENS, J.G. (1979). Pathogenesis of reactivated latent murine cytomegalovirus infection. Am. J. Path. 95, 67-80.

SHEKARCHI, I.C., FUCCILLO, D.A., SEVER, J.L. and MADDEN, D.M. (1988). Avidin-biotin latex agglutination assay for detection of antibodies to viral antigens. J. Clin. Microbiol. 26, 954-956.

SHELDON, E.L., KELLOGG, D.E., WATSON, R., LEVENSON, C.H. and ERLICH, H.A. (1986). Use of nonisotopic M13 probes for genetic analysis: Application to HLA class II loci. Proc. Natl. Acad. Sci. USA 83, 9085-9089.

SHELDRICK, P. and BERTHELOT, N. (1974). Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. 39, 667-678.

SHEPP, D.H., DANDLIKER, P.S., DE MIRANDA, P., BURNETTE, T.C., CEDERBERG, D.M., KIRK, L.E. and MEYERS, J.D. (1985). Activity of 9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine in the treatment of cytomegalovirus pneumonia. Ann. Intern. Med. 103, 368-373.

SHEPP, D.H., NEWTON, B.A. and MEYERS, J.D. (1984). Intravenous lymphoblastoid interferon and acyclovir for treatment of cytomegaloviral pneumonia. J. Infect. Dis. 150, 776-777.

SHIBATA, D., MARTIN, W.J., APPLEMAN, M.D., CAUSEY, D.M., LEEDOM, J.M. and ARNHEIM, N. (1988). Detection of cytomegalovirus DNA in peripheral blood of patients infected with human immunodeficiency virus. J. Infect. Dis. 158, 1185-1192.

SHIMOKAWA, K., MURAYAMA, T., ISHIDA, S. and FURUKAWA, T. (1987). Analysis by immunoblotting of human cytomegalovirus antibody in sera of renal transplant recipient. J. Med. Virol. 21, 339-346.

SHIRODIRA, D.V., FRASER, K.B. and STANFORD, F. (1973). Secondary fluorescent staining of virus antigens by rheumatoid factor and fluorescein-conjugated anti IgM. Ann. Rheum. Dis. 32, 53-67.

SHUSTER, E.A., BENEKE, J.S., TEGTMEIER, G.E., PEARSON, G.R., GLEAVES, C.A., WULD, A.D. and SMITH, T.F. (1985). Monoclonal antibody for rapid laboratory detection of cytomegalovirus infections: Characterization and diagnostic application. Mayo Clinic Proc.60, 577-586.

SIMMONS, R.L., LOPEZ, C., BALFOUR, H.H.Jr., KALIS, J., RATAZZI, L.C. and NAJARIAN, J.S. (1974). Cytomegalovirus: Clinical virological correlations in renal transplant recipients. Ann. Surg. 180, 623-634.

SINCLAIR, J.H. (1987). The human cytomegalovirus immediate early gene promoter is a strong promoter in cultured Drosophila melanogaster cells. Nucleic Acid Res. 15, 2392.

SING, G.K. and GARNETT, H.M. (1984). The effects of human cytomegalovirus challenge in vitro on subpopulations of T cells from seronegative donors. J. Med. Virol. 14, 363-371.

SINGER, J.M. and PLOTZ, C.M. (1956). The latex fixation test. I. Application to the serologic diagnosis of rheumatoid arthritis. Am. J. Med. 21, 888-892.

SINGER, R.H. and WARD, D.C. (1982). Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinated nucleotide analog. Proc. Natl. Acad. Sci. USA 79, 7331-7335.

SINGH, N., DUMMER, J.S., KUSNE, S., BREINIG, M.K., ARMSTRONG, J.A., MAKOWKA, L., STARZL, T.E. and HO, M. (1988a). Infections with cytomegalovirus and other herpesviruses in 121 liver transplant recipients: Transmission by donated organ and the effect of OKT3 antibodies. J. Infect. Dis. 158, 124-131.

SINGH, N., DUMMER, J.S., KUSNE, S., MAKOWKA, L., STARZL, T.E. and HO, M. (1988b). Impact of OKT3 therapy on cytomegalovirus and herpes simplex virus infections after liver transplantation. Transplant. Proc. 20 (Suppl. 1), 661-662.

SMILEY, J.R., FONG, B.S. and LEUNG, W-C. (1981). Construction of a double-jointed herpes simplex viral DNA molecule: Inverted repeats are required for segment inversion, and direct repeats promote deletions. Virology 113, 345-362.

SMILEY, M.L., WLODAVER, C.G., GROSSMAN, R.A., BARKER, C.F., PERLOFF, L.J., TUSTIN, N.B., STARR, S.E., PLOTKIN, S.A. and FRIEDMAN, H.M. (1985). The role of pretransplant immunity in protection from cytomegalovirus disease following renal transplantation. Transplantation 40, 157-161.

SMITH, J.D. and DE HARVEN, E. (1973). Herpes simplex virus and human cytomegalovirus replication in WI-38 cells. I. Sequence of viral replication. J. Virol. 12, 919-930.

SMITH, J.D. and DE HARVEN, E. (1974). Herpes simplex virus and human cytomegalovirus replication in WI-38 cells. II. An ultrastructural study of viral penetration. J. Virol. 14, 945-956.

SMITH, K.O. and RASMUSSEN, L. (1963). Morphology of cytomegalovirus (salivary gland virus). J. Bacteriol. 85, 1319-1325.

SMITH, M.G. (1956). Propagation in tissue culture of cytopathogenic virus from human salivary gland virus (SGV) disease. Proc. Soc. Exp. Biol. Med. 92, 424-430.

SMITH, M.G. and VELLIOS, F. (1950). Inclusion disease or generalized salivary gland virus infection. Arch. Pathol. 50, 862-884.

SMITH, M.J., BRITTEN, R.J. and DAVIDSON, E.H. (1975). Studies on nucleic acid reassociation kinetics: Reactivity of single-stranded tails in DNA-DNA renaturation. Proc. Natl. Acad. Sci. USA 72, 4805-4809.

SNYDMAN, D.R., McIVER, J., LESZCZYNSKI, J., CHO, S.I., WERNER, B.G., BERARDI, V.P., LO GERFO, F., HEINZE-LACEY, B. and GRADY, G.F. (1984). A pilot trial of a novel cytomegalovirus immune globulin in renal transplant recipients. Transplantation 38, 553-557.

SNYDMAN, D.R., WERNER, B.G., HEINZE-LACEY, B., BERARDI, V.P., TILNEY, N.L., KIRKMAN, R.L., MILFORD, E.L., CHO, S.I., BUSH, H.L.Jr., LEVEY, A.S., STROM, T.B., CARPENTER, C.B., LEVEY, R.H., HARMON, W.E., ZIMMERMAN, C.E., SHAPIRO, M.E., STEINMAN, T., LO GERFO, F., IDELSON, B., SCHROTER, G.P.J., LEVIN, M.J., McIVER, J., LESZCZYNSKI, J. and GRADY, G.F. (1987). Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients. N. Eng. J. Med. 317, 1049-1054.

SNYDMAN, D.R., WERNER, B.G., TILNEY, N.L., KIRKMAN, R.L., MILFORD, E.L., CHO, S.I., BUSH, H.L.Jr., LEVEY, A.S., STROM, T.B., CARPENTER, C.B., BERARDI, V.P., LEVEY, R.H., HARMON, W.E., ZIMMERMAN, C.E., TENNY, A., HEINZE-LACEY, B., SHAPIRO, M.E., STEINMAN, T., LO GERFO, F., IDELSON, B., McIVER, J., LESZCZYNSKI, J. and GRADY, G.F. (1988). A further analysis of primary cytomegalovirus disease prevention in renal transplant recipients with a cytomegalovirus immune globulin: Interim comparison of a randomized and an open-label trial. Transplant. Proc. 20 (Suppl. 8), 24-30.

SORBELLO, A.F., ELMENDORF, S.L., McSHARRY, J.J., VENEZIA, R.A. and ECHOLS, R.M. (1988). Rapid detection of cytomegalovirus by fluorescent monoclonal antibody staining and *in situ* hybridization in a dram vial cell culture system. J. Clin. Microbiol. 26, 1111-1114.

SPAETE, R.R. and FRENKEL, N. (1985). The herpes simplex virus amplicon: Analysis of cis-acting replication functions. Proc. Natl. Acad. Sci. USA 82, 694-698.

SPAETE, R.R. and MOCARSKI, E.S. (1985). The a sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes. J. Virol. 54, 817-824.

SPECTOR, D.H., HOCK, J.L. and TAMASHIRO, J.C. (1982). Cleavage maps for human cytomegalovirus DNA strain AD-169 for restriction endonucleases EcoRI, BglII and HindIII. J. Virol. 42, 558-582.

SPECTOR, S.A., HIRATA, K.K. and NEWMAN, T.R. (1984a). Identification of multiple cytomegalovirus strains in homosexual men with acquired immunodeficiency syndrome. J. Infect. Dis. 150, 953-956.

SPECTOR, S.A., RUA, J.A., SPECTOR, D.H. and McMILLAN, R. (1984b). Detection of human cytomegalovirus in clinical specimens by DNA-DNA hybridization. J. Infect. Dis. 150, 121-126.

SPENCER, E.S. (1974). Clinical aspects of CMV infection in kidney graft recipients. Scand. J. Infect. Dis. 6, 315-323.

SPENCER, E.S. and ANDERSEN, H.K. (1972). The development of immunofluorescent antibodies as compared with complement-fixing and virus-neutralizing antibodies in human cytomegalovirus infection. Scand. J. Infect. Dis. 4, 109-112.

SPIEGELBERG, H. (1974). Biological activities of immunoglobulins of different classes and subclasses. Adv. Immunol. 19, 259-294.

SRINIVASAPPA, J., SAEGUSA, J., PRABHAKAR, B.S., GENTRY, M.K., BUCHMEIER, M.J., WIKTOR, T.J., KOPROWSKI, H., OLDSTONE, M.B.A. and NOTKINS, A.L. (1986). Molecular mimicry: Frequency of reactivity of monoclonal antiviral antibodies with normal tissues. J. Virol. 57, 397-401.

STADTLER, J., KOLLER, C., HAMMER, C., WEBER, B., LAND, W., CASTRO, L.A. and BRENDEL, W. (1985). Monitoring of viral infections after renal transplantation by fine needle aspiration biopsy and monoclonal antibodies. Transplant. Proc. 15, 168-170.

STAGNO, S., PASS, R.F., DWORSKY, M.E., HENDERSON, R.E., MOORE, E.G., WALTON, P.D. and ALFORD, C.A. (1982). Congenital cytomegalovirus infection. The relative importance of primary and recurrent maternal infection. N. Engl. J. Med. 306, 945-949.

STAGNO, S., PASS, R.F., REYNOLDS, D.W. and ALFORD, C.A. (1981). Diagnosis of cytomegalovirus infections. In: The human herpesviruses. Eds. A. Nahmias, W. Dowdle and R. Schinazi, pp 363-373. Published by Elsevier, New York.

STAGNO, S., PASS, R.F., REYNOLDS, D.W., MOORE, M.A., NAHMIAS, A.J. and ALFORD, C.A. (1980). Comparative study of diagnostic procedures for congenital cytomegalovirus infection. Pediatrics 65, 251-257.

STAGNO, S., REYNOLDS, D.W., LAKEMAN, A., CHARAMELLA, L.J. and ALFORD, C.A. (1973). Congenital cytomegalovirus infection: Consecutive occurrence due to viruses with similar antigenic compositions. Pediatrics 52, 788-794.

STAGNO, S., REYNOLDS, D.W., TSIANTOS, A., FUCCILLO, D.A., LONG, W. and ALFORD, C.A. (1975). Comparative serial virologic and serologic studies of symptomatic and subclinical congenitally and natally acquired cytomegalovirus infections. J. Infect. Dis. 132, 568-577.

STAGNO, S., TINKER, M.K., ELROD, C., FUCCILLO, D.A., CLOUD, G. and O'BEIRNE, A.J. (1985). Immunoglobulin M antibodies detected by enzyme-linked immunosorbent assay and radioimmunoassay in the diagnosis of cytomegalovirus infections in pregnant women and newborn infants. J. Clin. Microbiol. 21, 930-935.

STAGNO, S., VOLANAKIS, J.E., REYNOLDS, D.W., STROUD, R. and ALFORD, C.A. (1977). Immune complexes in congenital and natal cytomegalovirus infections of man. J. Clin. Invest. 60, 838-845.

STALDER, H. and EHRENSBERGER, A. (1980). Microneutralization of cytomegalovirus. J. Infect. Dis. 142, 102-105.

STARR, S.E., GLAZER, J.P., FRIEDMAN, H.M., FARQUHAR, J.D. and PLOTKIN, S.A. (1981). Specific cellular and humoral immunity after immunization with live Towne strain cytomegalovirus vaccine. J. Infect. Dis. 143, 585-589.

STARR, W.E. and ALLISON, A.C. (1977). Role of T lymphocytes in recovery from murine cytomegalovirus infection. Infect. Immun. 17, 458-462.

STARZL, T.E., IWATSKUI, S., VAN THIEL, D.H., GARTNER, J.C., ZITELLI, B.J., MALATACK, J.J., SCHADE, R.R. and SHAW, B.W.Jr. (1982). Evolution of liver transplantation. Hepatology 2, 614-636.

STENBERG, R.M. and STINSKI, M.F. (1985). Autoregulation of the human cytomegalovirus major immediate-early gene. J. Virol. 56, 676-682.

STENBERG, R.M., THOMSEN, D.R. and STINSKI, M.F. (1984). Structural analysis of the major immediate early gene of human cytomegalovirus. J. Virol. 49, 190-199.

STENBERG, R.M., WITTE, P.R. and STINSKI, M.F. (1985). Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. 56, 665-675.

STEVENS, J.G. and COOK, M.L. (1971). Latent herpes simplex virus in spinal ganglia of mice. Science 173, 843-845.

STEVENS, J.G., NESBURN, A.B. and COOK, M.L. (1972). Latent herpes simplex virus from trigeminal ganglia of rabbits with recurrent eye infection. Nature (New Biol.) 235, 216-217.

STEWART, W.E., LIN, L.S., WIRANOWSKA-STEWART, M. and CANTELL, K. (1977). Elimination of size and change heterogeneities of human leukocyte interferon by chemical cleavage. Proc. Natl. Acad. Sci. USA 74, 4200-4204.

STINSKI, M.F. (1976). Human cytomegalovirus: Glycoproteins associated with virions and dense bodies. J. Virol. 19, 594-609.

STINSKI, M.F. (1977). Synthesis of proteins and glycoproteins in cells infected with human cytomegalovirus. J. Virol. 23, 751-767.

STINSKI, M.F. (1978). Sequence of protein synthesis in cells infected by human cytomegalovirus: Early and late virus-induced polypeptides. J. Virol. 26, 686-701.

STINSKI, M.F., MOCARSKI, E.S. and THOMSEN, D.R. (1979). DNA of human cytomegalovirus: Size heterogeneity and defectiveness resulting from serial undiluted passage. J. Virol. 31, 231-239.

STINSKI, M.F. and ROEHR, T.J. (1985). Activation of the major immediate early gene of human cytomegalovirus by cis-acting elements in the promoter-regulatory sequence and by virus-specific trans-acting components. J. Virol. 55, 431-441.

STINSKI, M.F., THOMSEN, D.R. and RODRIGUEZ, J.E. (1982). Synthesis of human cytomegalovirus-specified RNA and protein in interferon-treated cells at early times after infection. J. gen. Virol. 60, 261-270.

STINSKI, M.F., THOMSEN, D.R., STENBERG, R.M. and GOLDSTEIN, L.C. (1983). Organization and expression of the immediate early genes of human cytomegalovirus. J. Virol. 46, 1-14.

STINSON, E.B., BIEBER, C.P., GRIEPP, R.B., CLARK, D.A., SHUMWAY, N.E. and REMINGTON, J.S. (1971). Infectious complications after cardiac transplantation in man. Ann. Intern. Med. 74, 22-36.

STIRK, P.R. and GRIFFITHS, P.D. (1987). Use of monoclonal antibodies for the diagnosis of cytomegalovirus infection by the detection of early antigen fluorescent foci (DEAFF) in cell culture. J. Med. Virol. 21, 329-337.

STIRK, P.R. and GRIFFITHS, P.D. (1988). Comparative sensitivity of three methods for the diagnosis of cytomegalovirus lung infection. J. Virol. Methods 20, 133-142.

ST JEOR, S.C., ALBRECHT, T.B., FUNK, F.D. and RAPP, F. (1974). Stimulation of cellular DNA synthesis by human cytomegalovirus. J. Virol. 13, 353-362.

ST JEOR, S. and RAPP, F. (1973a). Cytomegalovirus: Conversion of nonpermissive cells to a permissive state for virus replication. Science 181, 1060-1061.

ST JEOR, S. and RAPP, F. (1973b). Cytomegalovirus replication in cells pre-treated with 5-iodo-2'-deoxyuridine. J. Virol. 11, 986-990.

STOCKL, E., POPOW-KRAUPP, T., HEINZ, F.X., MUHLBACHER, F., BALCKE, P. and KUNZ, C. (1988). Potential of *in situ* hybridization for early diagnosis of productive cytomegalovirus infection. J. Clin. Microbiol. 26, 2536-2540.

STOW, N.D., McMONAGLE, E.C. and DAVISON, A.J. (1983). Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. Nucleic Acids Res. 11, 8205-8220.

STRAND, O.A. and HODDEVIK, G.M. (1984). The diagnostic significance of specific serum IgA detection in cytomegalovirus infections. Arch. Virol. 82, 173-180.

STRAYER, D.S., PHILLIPS, G.B., BARKER, K.H., WINOKUC, T. and DESCHRYVER-KECSKEMETI, K. (1981). Gastric cytomegalovirus infection in bone marrow transplant patients. Cancer 48, 1478-1483.

STUDIER, F.W. (1969). Effects of the conformation of single-stranded DNA on renaturation and aggregation. J. Mol. Biol. 41, 199-209.

SUBIRANA, J.A. and DOTY, P. (1966). Kinetics of renaturation of denatured DNA. I. Spectrophotometric results. Biopolymers 4, 171-187.

SULLIVAN, J.L. and HANSHAW, J.B. (1982). Human cytomegalovirus infections. In: Human herpesvirus infections: Clinical aspects. Eds. R. Glaser and T. Gotlieb-Stematsky, pp 57-83. Published by Marcel Dekker, New York.

SUNDQVIST, V-A. and WAHREN, B. (1981). An interchangeable ELISA for cytomegalovirus antigen and antibody. J. Virol. Methods 3, 301-312.

SUTHERLAND, D.E.R., CHAN, F.Y., FOUCAR, E., SIMMONS, R.L., HOWARD, R.J. and NAJARIAN, J.S. (1979). The bleeding cecal ulcer in transplant patients. Surgery 86, 386-398.

SUTHERLAND, S. and BRIGGS, J.D. (1983). The detection of antibodies to cytomegalovirus in the sera of renal transplant patients by an IgM antibody capture assay. J. Med. Virol. 11, 147-159.

SUTHERLAND, S., CRAWFORD, D.H., WILSON, S.A., MORGAN, B., AZIM, T. and HUEHNS, E.R. (1987). Production and characterisation of a human monoclonal antibody to cytomegalovirus and its use in an early nuclear fluorescence assay. J. Med. Virol. 22, 245-255.

SWEET, G.H., BRYANT, S.A., TEGTMEIER, G.E., BENEKE, J.S. and BAYER, W.L. (1985). Early and late antigens of human cytomegalovirus: Electroimmunodiffusion assay of numbers, relationships, and reactivities with donor sera. J. Med. Virol. 15, 137-148.

SWENSON, P.D. and KAPLAN, M.H. (1985). Rapid detection of cytomegalovirus in cell culture by indirect immunoperoxidase staining with monoclonal antibody to an early nuclear antigen. J. Clin. Microbiol. 21, 669-673.

TAKAHASHI, K., TERAOKA, S., YAGISAWA, T., FUCHINOUE, S., HONDA, H., TOMA, H., AGISHI, T. and OTA, K. (1987a). Effect of human interferon-β on life-threatening viral pneumonitis in kidney transplant recipients. Transplant. Proc. 19, 4089-4095.

TAKAHASHI, K., YAGISAWA, T., TERAOKA, S., TOMA, H., AGISHI, T., OTA, K. and KOBAYASHI, S. (1987b). Viral infections in kidney transplant patients immunosuppressed with cyclosporine. Transplant. Proc. 19, 2142-2149.

TAKEKOSHI, M., IHARA, S., TANAKA, S., MAEDA-TAKEKOSHI, F. and WATANABE, Y. (1987). A new human cytomegalovirus isolate has an invertible subsegment within its L component producing eight genome isomers. J. gen. Virol. 68, 765-776.

TAMASHIRO, J.C., FILPULA, D., FRIEDMANN, T. and SPECTOR, D.H. (1984). Structure of the heterogenous L-S region of human cytomegalovirus strain AD169 DNA. J. Virol. 52, 541-548.

TAMURA, T., CHIBA, S., CHIBA, Y. and NAKAO, T. (1980). Virus excretion and neutralizing antibody response in saliva in human cytomegalovirus infection. Infect. Immun. 29, 842-845.

TANAKA, J., KAMIYA, S., OGURA, T., SATO, H., OGURA, H. and HATANO, M. (1985). Effect of dimethyl sulfoxide on interaction of human cytomegalovirus with host cell: Conversion of a nonproductive state of cell to a productive state for virus replication. Virology 146, 165-176.

TANAKA, J., OGURA, T., KAMIYA, S., SATO, H., YASHIE, T., OGURA, H. and HATANO, M. (1984). Enhanced replication of human cytomegalovirus in human fibroblasts treated with dexamethasone. J. gen. Virol. 65, 1759-1767.

TANAKA, J., OGURA, T., LIDA, H., SATO, H. and HATANO, M. (1988). Inhibitors of prostaglandin synthesis inhibit growth of human cytomegalovirus and reactivation of latent virus in a productively and latently infected human cell line. Virology 163, 205-208.

TANAKA, J., OGURA, T., SATO, H. and HATANO, M. (1987). Establishment and biological characterization of an *in vitro* human cytomegalovirus latency model. Viorology 161, 62-72.

TANAKA, S., FURUKAWA, T. and PLOTKIN, S.A. (1975). Human cytomegalovirus stimulates host cell RNA synthesis. J. Virol. 15, 297-304.

TANAKA, S., IHARA, S. and WATANABE, Y. (1978). Human cytomegalovirus induces DNA-dependent RNA polymerases in human diploid cells. Virology 89, 179-185.

TANAKA, S., OTSUKA, M., IHARA, S., MAEDA, F. and WATANABE, Y. (1979). Introduction of pre-early nuclear antigen(s) in HEL cells infected with human cytomegalovirus. Microbiol. Immunol. 23, 263-271.

TARDY, J.C., POUTEIL-NOBLE, C., TOURAINE, J.L. and AYMARD, M. (1987). Prognostic value of anti-cytomegalovirus IgM in kidney graft recipients. Transplant. Proc. 19, 4066-4067.

TCHEN, P., FUCHS, R.P.P., SAGE, E. and LENG, M. (1984). Chemically modified nucleic acids as immunodetectable probes in hybridization experiments. Proc. Natl. Acad. Sci. USA 81, 3466-3470.

TEN NAPEL, C.H.H., HOUTHOFF, J.F. and THE, T.H. (1984). Cytomegalovirus hepatitis in normal and immune compromised hosts. Liver 4, 184-194.

TEVETHIA, M.J., SPECTOR, S.J., LEISURE, K.M. and STINSKI, M.F. (1987). Participation of two human cytomegalovirus immediate early gene regions in transcriptional activation of adenovirus promoters. Virology, 161, 276-285.

THOMAS, P. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77, 5201-5205.

THOMAS, Y., SOSMAN, J., IRIGOYEN, O., FRIEDMAN, S.M., KUNG, P.C., GOLDSTEIN, G. and CHESS, L. (1980). Functional analysis of human T cell subsets defined by monoclonal antibodies. I. Collaborative T-T interactions in the immunoregulation of B cell differentiation. J. Immunol. 125, 2402-2408.

THOMAS, Y., SOSMAN, J., ROGOZINSKI, L., IRIGOYEN, O., KUNG, P.C., GOLDSTEIN, G. and CHESS, L. (1981). Functional analysis of human T cell subsets defined by monoclonal antibodies. III. Regulation of helper factor production by T cell subsets. J. Immunol. 126, 1948-1951.

THOMSEN, D.R., STENBERG, R.M., GOINS, W.F. and STINSKI, M.F. (1984). Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA 81, 659-663.

THROWER, K.J. and PEACOCKE, A.R. (1968). Kinetics and spectrophotometric studies on the renaturation of deoxyribonucleic acid. Biochem. J. 109, 543-557.

TILG, H., MARGREITER, R., SCRIBA, M., MARTH, C., NIEDERWIESER, D., AULITZKY, W., SPIELBERGER, M., WACHTER, H. and HUBER, C. (1987). Clinical presentation of CMV infection in solid organ transplant recipients and its impact on graft rejection and neopterin excretion. Clin. Transplant. 1, 37-43.

TIMBURY, M.C. (1971). Temperature-sensitive mutants of herpes simplex virus type 2. J. gen. Virol. 13, 373-376.

TOKUMARU, T. (1966). A possible role of A-immunoglobulin in herpes simplex virus infection in man. J. Immunol. 97, 248-259.

TOLKOFF-RUBIN, N.E. and RUBIN, R.H. (1986). The impact of cyclosporine therapy on the occurrence of infection in the renal transplant recipient. Transplant. Proc. 18, 168-173.

TOLKOFF-RUBIN, N.E., RUBIN, R.H., KELLER, E.E., BAKER, G.P., STEWART, J.A. and HIRSCH, M.S. (1978). Cytomegalovirus infection in dialysis patients and personnel. Ann. Intern. Med. 89, 625-628.

TORFASON, E.G., KALLANDER, C. and HALONEN, P. (1981). Solid-phase radioimmunoassay of serum IgG, IgM, and IgA antibodies to cytomegalovirus. J. Med. Virol. 7, 85-96.

TOURTELLOTTE, W.W., VERITY, A.N., SCHMID, P., MARTINEZ, S. and SHAPSHAK, P. (1987). Covalent binding of formalin fixed paraffin embedded brain tissue sections to glass slides suitable for in situ hybridization. J. Virol. Methods 15, 87-100.

TOWNSEND, A.R.M., ROTHBARD, J., GOTCH, F.M., BAHADUR, G., WRAITH, D. and McMICHAEL, A.J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell 44, 959-968.

TRINCHIERI, G. and PERUSSIA, B. (1984). Human natural killer cells: Biological and pathological aspects. Lab. Invest. 50, 489-513.

TYMS, A.S., DAVIS, J.M., JEFFRIES, D.J. and MEYERS, J.D. (1984). BW B759U, an analogue of acyclovir, inhibits human cytomegalovirus in vitro [Letter]. Lancet 2, 924-925.

TYZZER, E.E. (1906). The histology of the skin lesions in varicella. J. Med. Res. 14, 361-392.

VAN DEN BERG, F.M., VAN GOYEN, A.J.J., VOLKERS, H. and WALBOOMERS, J.M.M. (1984). Heterogeneity in subregions of the terminal repeats of herpes simplex virus type 2 DNA. Intervirology 21, 96-103.

VAN DER BIJ, W., TORENSMA, R., VAN SON, W.J., ANEMA, J., SCHIRM, J., TEGZESS, A.M. and THE, T.H. (1988). Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leucocytes. J. Med. Virol. 25, 179-188.

VAN ES, A., BALDWIN, W.M., OLJANS, P.J., TANKE, H.J., PLOEM, J.S. and VAN ES, L.A. (1984). Expression of HLA-DR on T lymphocytes following renal transplantation, and association with graft-rejection episodes and cytomegalovirus infection. Transplantation 37, 65-69.

VAN LOON, A.M., HEESSEN, F.W.A., VAN DER LOGT, J.T.M. and VAN DER VEEN, J. (1981). Direct enzyme-linked immunosorbent assay that uses peroxidase-labelled antigen for determination of immunoglobulin M antibody to cytomegalovirus. J. Clin. Microbiol. 13, 416-422.

VAN LOON, A.M., VAN DER LOGT, J.T.M., HEESSEN, F.W.A. and VAN DER VEEN, J. (1985). Quantitation of immunoglobulin E antibody to cytomegalovirus by antibody capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. 21, 558-561.

VELASCO, N., CATTO, G.R.D., EDWARD, N., ENGESET, J. and MOFFAT, M.A.J. (1984). The effect of the dosage of steroids on the incidence of cytomegalovirus infections in renal transplant recipients. J. Infect. 9, 69-78.

VERDONCK, L.F., MIDDELDORP, J.M., KREEFT, H.A.J.G., THE, T.H., HEKKER, A. and DE GAST, G.C. (1985). Primary cytomegalovirus infection and its prevention after autologous bone marrow transplantation. Transplantation 39, 455-457.

VODOPICK, H., CHASKES, S.J., SOLOMON, A. and STEWART, J.A. (1974). Transient monoclonal gammapathy associated with cytomegalovirus infection. Blood 44, 189-195.

VOLKMER, H., BERTHOLET, C., JONJIC, S., WITTEK, R. and KOSZINOWSKI, U.H. (1987). Cytolytic T lymphocyte recognition of the murine cytomegalovirus nonstructural immediate-early protein pp89 expressed by recombinant vaccinia virus. J. Exp. Med. 166, 668-677.

VOLPI, A., WHITLEY, R.J., CEBALLOS, R., STAGNO, S. and PEREIRA, L. (1983). Rapid diagnosis of pneumonia due to cytomegalovirus with specific monoclonal antibodies. J. Infect. Dis. 147, 1119-1120.

VONKA, V., ANISIMOVA, E. and MACEK, M. (1976). Replication of cytomegalovirus in human epitheliod diploid cell line. Arch. Virol. 52, 283-296.

VON WILLEBRAND, E., PETTERSSON, E., AHONEN, J. and HAYRY, P. (1986). CMV infection, class II antigen expression, and human kidney allograft rejection. Transplantation 42, 364-367.

VOOGD, C.E., VAN DER STEL, J.J. and JACOBS, J.J.J.A.A. (1980). On the mutagenic action of some enzyme immunoassay substrates. J. Immunol. Methods 36, 55-61.

WADE, J.C., McGUFFIN, R.W., SPRINGMEYER, S.C., NEWTON, B., SINGER,J.W. and MEYERS, J.D. (1983). Treatment of cytomegalovirus pneumonia with high-dose acyclovir and human leukocyte interferon. J. Infect. Dis. 148, 557-562.

WAHREN, B., LINDE, A., SUNDQVIST, V-A., LJUNGMAN, P., LONNQVIST, B. and RINGDEN, O. (1984). IgG-subclass-specific CMV reactivity in bone marrow transplant recipients. Transplantation 38, 479-483.

WALKER, D.P., LONGSON, M., MALLICK, N.P. and JOHNSON, R.W. (1982). A prospective study of cytomegalovirus and herpes simplex virus disease in renal transplant patients. J. Clin. Pathol. 35, 1190-1193.

WANER, J.L. and WELLER, T.H. (1978). Analysis of antigenic diversity among human cytomegaloviruses by kinetic neutralization tests with high-titred rabbit antisera. Infect. Immun. 21, 151-157.

WANER, J.L., WELLER, T.H. and KEVY, S.V. (1973). Patterns of a cytomegaloviral complement fixing antibody activity. A longitudinal study of blood donors. J. Infect. Dis. 127, 538-543.

WANER, J.L., WELLER, T.H. and STEWART, J.A. (1976). In: Manual of clinical immunology. Eds. N.E. Rose and H. Friedman, pp 423-427. Published by the American Society for Microbiology, Washington.

WARE, A.J., LUBY, J.P., EIGENBRODT, E.H., LONG, D.L. and HULL, A.R. (1975). Spectrum of liver disease in renal transplant recipients. Gastroenterology 68, 755-764.

WARE, A.J., LUBY, J.P., HOLLINGER, B., EIGENBRODT, E.H., CUTHBERT, J.A., ATKINS, C.R., SHOREY, J., HULL, A.R. and COMBES, B. (1979). Etiology of liver disease in renal-transplant patients. Ann. Intern. Med. 91, 364-371.

WARRELL, M.J., CHINN, I., MORRIS, P.J. and TOBIN, J.O. (1980). The effects of viral infections on renal transplants and their recipients. Quart. J. Med. 49, 219-231.

WATHEN, M.W. and STINSKI, M.F. (1982). Temporal patterns of human cytomegalovirus transcription: Mapping the viral RNAs synthesized at immediate early, early and late times after infection. J. Virol. 41, 462-477.

WATHEN, M.W., THOMSEN, D.R. and STINSKI, M.F. (1981). Temporal regulation of human cytomegalovirus transcription at immediate early and early times after infection. J. Virol. 38, 446-459.

WATSON, R.J., SULLIVAN, M. and VANDE WOUDE, G.F. (1981). Structures of two spliced herpes simplex virus type 1 immediate-early mRNA's which map at the junctions of the unique and reiterated regions of the virus DNA S component. J. Virol. 37, 431-444.

WEDER, D. and RADSAK, K.D. (1983). Induction of a host-specific chromatin-associated glycopolypeptide by human cytomegalovirus. J. gen. Virol. 64, 2749-2761.

WEIGENT, D.A., LANGFORD, M.P., FLEISCHMANN, W.R. and STANTON, G.J. (1983). Potentiation of lymphocyte natural killing by mixtures of alpha or beta interferon with recombinant gamma interferon. Infect. Immun. 40, 35-38.

WEIHER, H., KONIG, M. and GRUSS, P. (1983). Multiple point mutations affecting the simian virus 40 enhancer. Science 219, 626-631.

WEILAND, O., MATTSSON, L. and GLAUMANN, H. (1986). Non-A, non-B hepatitis after intravenous gammaglobulin. Lancet 1, 976-977.

WEINER, R.S., BORTIN, M.M., GALE, R.P., GLUCKMAN, E., KAY, H.E.M., KOLB, H-J., HARTZ, A.J. and RIMM, A.A. (1986). Interstitial pneumonitis after bone marrow transplantation. Assessment of risk factors. Ann. Intern. Med. 104, 168-175.

WEINTRAUB, H. (1985). Assembly and propagation of repressed and derepressed chromosomal states. Cell 42, 705-711.

WEIR, M.R., IRWIN, B.C., MATERS, A.W., GENEMANS, G., SHEN, S.Y., CHARACHE, P. and WILLIAMS, G.M. (1987). Morbid outcome of cytomegalovirus-negative transplant recipients receiving cytomegalovirus-positive kidneys. Transplant. Proc. 19, 2137-2141.

WELLER, T.H. (1971). The cytomegaloviruses, ubiquitous agents with protean clinical manifestations. N. Engl. J. Med. 285, 203-214; 267-274.

WELLER, T.H., HANSHAW, J.B. and SCOTT, D.E. (1960). Serological differentiation of viruses responsible for cytomegalic inclusion disease. Virology 12, 130-132.

WELLER, T.H., MACACULEY, J.E., CRAIG, J.M. and WIRTH, P. (1957). Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion disease. Proc. Soc. Exp. Biol. Med. 94, 4-12.

WENTWORTH, B.B. and FRENCH, L. (1970). Plaque assay of cytomegalovirus strains of human origin. Proc. Soc. Exp. Biol. Med. 135, 253-259.

WERTHEIM, P., BUURMAN, C., GEELEN, J. and VAN DER NOORDAA, J. (1983). Transmission of cytomegalovirus by renal allograft demonstrated by restriction enzyme analysis. Lancet 1, 980-981.

WESTON, K. (1988). An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. Virology 162, 406-416.

WESTON, K. and BARRELL, B.G. (1986). Sequence of the short unique region, short repeats and part of the long repeats of human cytomegalovirus. J. Mol. Biol. 192, 177-208.

WESTRATE, M.W., GEELEN, J.L.M.C., WERTHEIM, P.M.E. and VAN DER NOORDAA, J.V.D. (1983). Comparison of the physical maps of the DNAs of two cytomegalovirus strains. J. gen. Virol. 64, 47-55.

WESTRATE, M.W., GEELEN, J.L.M.C. and VAN DER NOORDAA, J. (1980). Human cytomegalovirus DNA: Physical maps for the restriction endonucleases BglII, HindIII, and XbaI. J. gen. Virol. 49, 1-21.

WETMUR, J.G. and DAVIDSON, N. (1968). Kinetics of renaturation of DNA. J. Mol. Biol. 31, 349-370.

WILKIE, N.M. (1973). The synthesis and substructure of herpesvirus DNA: The distribution of alkali-labile single strand interruptions in HSV-1 DNA. J. gen. Virol. 21, 453-467.

WILKINSON, G.W.G., AKRIGG, A. and GREENAWAY, P.J. (1984). Transcription of the immediate early genes of human cytomegalovirus strain AD169. Virus Res. 1, 101-116.

WILLIAMS, A.E., McDONALD, M., LUBAN, N.L.C., SACHER, R., MIKESELL, J., HOFFMAN-PANETTA, K., WILLIAMS, K. and DODD, R.Y. (1984). Low incidence of neonatal post-transfusion cytomegalovirus infection associated with the use of washed red blood cell products (Abstract). Transfusion 24, 430.

WILLIAMS, J.G. and MASON, P.J. (1985). Hybridisation in the analysis of RNA. In: Nucleic Acid hybridisation. A practical approach. Eds. B.D. Hames and S.J. Higgins, pp 139-160. Published by IRL Press, Oxford.

WILLIAMS, L., BLAKESLEE, J.R.Jr., BOLDOGH, I. and HUANG, E-S. (1980). Detection of cytomegalovirus genomes in human skin fibroblasts by DNA hybridisation. J. gen. Virol. 51, 435-438.

WILSON, E.J., MEDEARIS, D.N.Jr., HANSEN, L.A. and RUBIN, R.H. (1987). 9-(1,3-dihydroxy-2-propoxymethyl)guanine prevents death but not immunity in murine cytomegalovirus-infected normal and immunosuppressed BALB/c mice. Antimicrob. Agents Chemother. 31, 1017-1020.

WINCHESTER, R.J. and KUNKEL, H.G. (1979). The human Ia system. Adv. Immunol. 28, 221-249.

WINKELHAKE, J.L. (1978). Immunoglobulin structure and effector functions. Immunochemistry 15, 695-714.

WINSTON, D.J., GALE, R.P., MEYER, D.V. and YOUNG, L.S. (1979). Infectious complications of human bone marrow transplantation. Medicine 58, 1-31.

WINSTON, D.J., HO, W.G., HOWELL, C.L., MILLER, M.J., MICKEY, R., MARTIN, W.J., LIN, C.H. and GALE, R.P. (1980). Cytomegalovirus infections associated with leukocyte transfusions. Ann. Intern. Med. 93, 671-675.

WINSTON, D.J., HO, W.G., LIN, C.H., BARTONI, K., BUDINGER, M.D., GALE, R.P. and CHAMPLIN, R.E. (1987). Intravenous immune globulin for prevention of cytomegalovirus infection and interstitial pneumonia after bone marrow transplantation. Ann. Intern. Med. 106, 12-18.

WINSTON, D.J., HO, W.G., LIN, C.H., BUDINGER, M.D., CHAMPLIN, R.E. and GALE, R.P. (1984). Intravenous immunoglobulin for modification of cytomegalovirus infections associated with bone marrow transplantation: Preliminary results of a controlled trial. Am. J. Med. 76, 128-133.

WINSTON, D.J., HUANG, E-S., MILLER, M.J., LIN, C.H., HO, W.G., GALE, R.P. and CHAMPLIN, R.E. (1985). Molecular epidemiology of cytomegalovirus infections associated with bone marrow transplantation. Ann. Intern. Med. 102, 16-20.

WINSTON, D.J., POLLARD, R.B., HO, W.G., GALLAGHER, J.G., RASSMUSSEN, L.E., HUANG, S.N., LIU, C.H., GOSSETT, T.G., MERIGAN, T.C. and GALE, R.P. (1982). Cytomegalovirus immune plasma in bone marrow transplant recipients. Ann. Intern. Med. 97, 11-18.

WOLF, H., LESER, U., HAUS, M., GU, S.Y. and PATHMANATHAN, R. (1986). Sandwich nucleic acid hybridization: A method with a universally usable labelled probe for various specific tests. J. Virol. Methods 13, 1-8.

WOLFE, B.M. and CHERRY, J.D. (1973). Hemorrhage from cecal ulcers of cytomegalovirus infection. Ann. Surg. 177, 490-494.

WONG, T.W. and WARNER, N.E. (1962). Cytomegalic inclusion disease in adults: Report of 14 cases with review of literature. Arch. Pathol. 74, 403-422.

WREGHITT, T. (1988). Assessment of tests for detecting cytomegalovirus antibody in transplant donors and recipients. Transplant. Proc. 20, 778.

WREGHITT, T.G., GRAY, J.J. and CHANDLER, C. (1986a). Prognostic value of cytomegalovirus IgM antibody in transplant recipients. Lancet 1, 1157-1158.

WREGHITT, T.G., HAKIM, M., GRAY, J.J., KUCIA, S., WALLWORK, J. and ENGLISH, T.A.H. (1988). Cytomegalovirus infections in heart and heart and lung transplant recipients. J. Clin. Pathol. 41, 660-667.

WREGHITT, T.G., HICKS, J., GRAY, J.J. and O'CONNOR, C. (1986b). Development of a competitive enzyme-linked immunosorbent assay for detecting cytomegalovirus antibody. J. Med. Virol. 18, 119-129.

WU, B.C., DOWLING, J.N., ARMSTRONG, J.A. and HO, M. (1975). Enhancement of mouse cytomegalovirus infection during host-versus-graft reaction. Science 190, 56-58.

YAMAMOTO, Y., SHIMOKATA, K., MAENO, K. and NISHIYAMA, Y. (1987). Effect of recombinant human interferon-γ against human cytomegalovirus. Arch. Virol. 94, 323-329.

YAMANISHI, K. and RAPP, F. (1979). Production of plasminogen activator by human and hamster cells infected with human cytomegalovirus. J. Virol. 31, 415-419.

YEAGER, A.S. (1979). Improved indirect haemagglutination test for cytomegalovirus using human O erythrocytes in lysine. J. Clin. Microbiol. 10, 64-68.

YEAGER, A.S., GRUMET, F.C., HAFLEIGH, E.B., ARVIN, A.M., BRADLEY, J.S. and PROBER, C.G. (1981). Prevention of transfusion-acquired cytomegalovirus infections in newborn infants. J. Paediatr. 98, 281-287.

YIP, Y.K., BARROWELOUGH, B.S., URBAN, C. and VILCEK, J. (1982). Purification of two subspecies of human gamma (immune) interferon. Proc. Natl. Acad. Sci. USA 79, 1820-1824.

YOLKEN, R.H. (1982). Enzyme immunoassays for the detection of infectious antigens in body fluids: Current limitations and future prospects. Rev. Infect. Dis. 4, 35-68.

YOLKEN, R.H. and STOPA, P.J. (1980). Comparison of seven enzyme immunoassay systems for measurement of cytomegalovirus. J. Clin. Microbiol. 11, 546-551.

YOUNG, B.D. and ANDERSON, M.L.M. (1985). Quantitative analysis of solution hybridisation. In: Nucleic acid hybridisation. A practical approach. Eds. B.D. Hames and S.J. Higgins, pp 47-71. Published by IRL Press, Oxford.

YOUNT, W.J., HONG, R., SELIGMAN, M., GOOD, R. and KUNKEL, H.G. (1970). Imbalances of gamma globulin subgroups and gene defects in patients with primary hypogammaglobulinemia. J. Clin. Invest. 49, 1957-1966.

ZABLOTNEY, S.L., WENTWORTH, B.B. and ALEXANDER, E.R. (1978). Antigenic relatedness of 17 strains of human cytomegalovirus. Am. J. Epidemiol. 107, 336-343.

ZAIA, J.A., FORMAN, S.J., TING, Y.P., VANDERWAL-URBINA, E. and BLUME, K.G. (1986). Polypeptide-specific antibody response to human cytomegalovirus after infection in bone marrow transplant recipients. J. Infect. Dis. 153, 780-787.

ZINKERNAGEL, R.M., HAENSELER, E., LEIST, T., CERNY, A., HENGARTNER, H. and ALTHAGE, A. (1986). T cell-mediated hepatitis in mice infected with lymphocytic choriomeningitis virus. Liver cell destruction by H-2 class I-restricted virus-specific cytotoxic T cells as a physiological correlate of the ^{51}Cr-release assay? J. Exp. Med. 164, 1075-1092.

ZINKERNAGEL, R.M. and WELSH, R.M. (1976). H-2 compatibility requirement for virus-specific T-cell-mediated effector functions in vivo. J. Immunol. 117, 1495-2001.

ZUR HAUSEN, H., SCHULTE-HOLTHAUSEN, H., KLEIN, G., HENLE, W., HENLE, G., CLIFFORD, P. and SANTESSON, L. (1970). EBV-DNA in biopsies of Burkitt tumors and anaplastic carcinomas of the nasopharynx. Nature 228, 1056-1058.