HEPATITIS B ANTIGEN

AND

BLOOD TRANSFUSION

by

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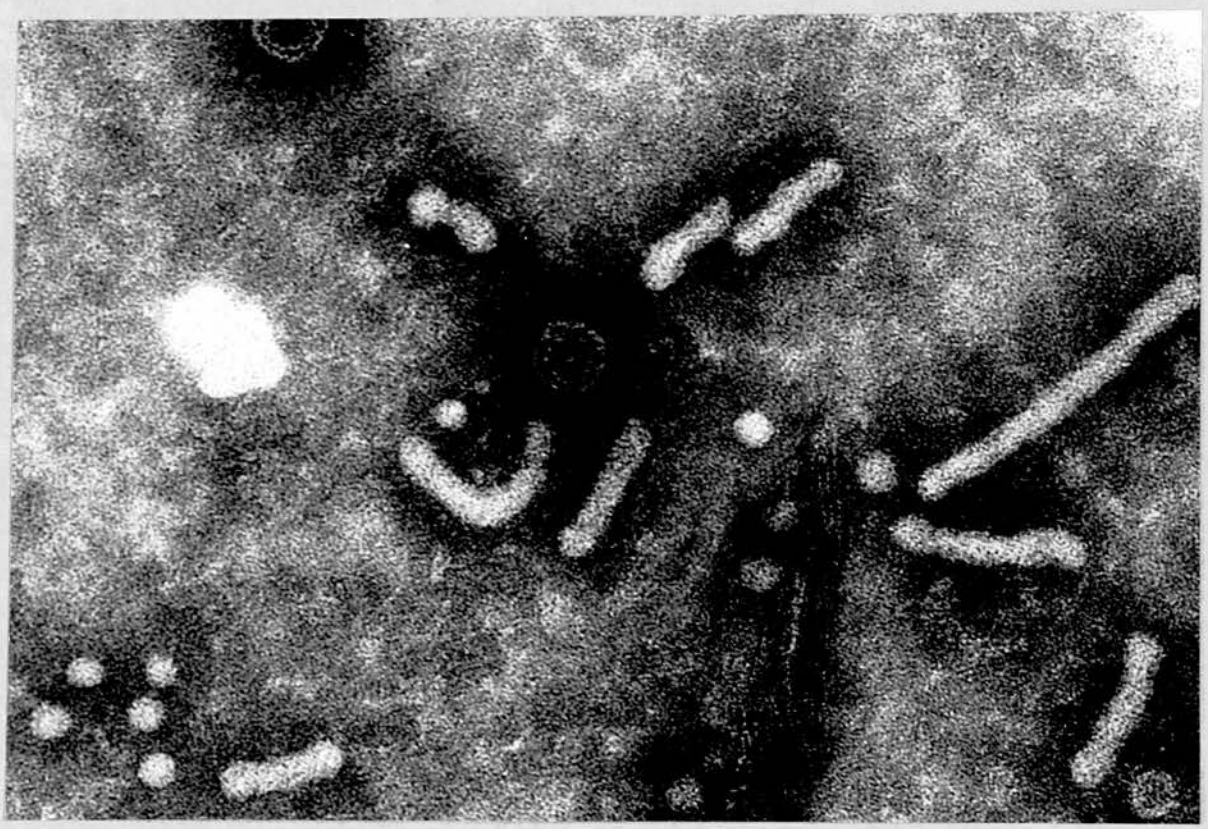

Hepatitis B antigen in serum, showing characteristic small (22nm) spheres, rod like forms, and large spheres (42nm Dane Particles) with central cores. Magnification = 160,000

Declaration

The work reported here was designed and conducted by myself, with the following exceptions:-

1. Production of spherocytes and preparation of reagents in bulk (Part Two, Chapter two and four). In this aspect I was ably assisted by Mr A.D. Watt, Chief Technician, Microbiology Division, South-East of Scotland Blood Transfusion Service.
2. Multicentre evaluation of H.A.I. for donor testing (Part Four, Chapter one). The actual testing was, of necessity, performed by the appropriate technical staff in participating centres.

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Mrs. Nancy Russell has coped admirably with the difficult task of typing the manuscript.

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PUBLICATIONS

(a) Related to thesis

1. Hopkins, R. and Das, P.C. (1973). "A tanned cell haemagglutination test for the detection of hepatitis-associated antigen (Au-Ag) and antibody (anti-Au)". British Journal of Haematology, 25, 619

2. Hopkins, R, and Das, P.C. (1974). "Australia antigen (HB Ag) subtyping by a sensitive tanned cell haemagglutination-inhibition technique". British Journal of Haematology, 27, 501

3. Hopkins, R., Robertson, M., Ross, D., Turnbull, W.M. and Das, P.C. (1975). "Detection of hepatitis B surface antigen (HB_sAg) among blood donors in Scotland: evaluation of a sensitive tanned cell haemagglutination-inhibition test". British Medical Journal, 2, 409

4. Watt, A.D., Robertson, M. and Hopkins, R. (1976). "Bulk preparation of reagents for hepatitis B testing". Journal of Clinical Pathology, 29, 364

(b) Other publications

1. Das, P.C., Hopkins, R., Cash, J.D. and Cumming, R.A. (1971). "Rapid identification of hepatitis-associated antigen and antibody by counter-immunoelectrophoresis". British Journal of Haematology, 21, 673

2. Hopkins, R. and Das, P.C. (1972). "Improved sensitivity of the electrophoresis method by tannic acid for detection of Australia antigen". Journal of Clinical Pathology, 25, 832

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7. Lawton, J.W.M., Hopkins, R, Paterson, I.C. and Das, P.C. (1975). "ad and ay subtypes of hepatitis B antigen in a case of hypogammaglobulinaemia". Journal of Clinical Pathology, 28, 863

SUMMARY

The recognition of Hepatitis B surface antigen (HB_sAg) as a marker of one of the causative agents of posttransfusion hepatitis stimulated a demand for techniques applicable to mass donor testing which would allow blood to be 'cleared' for use within hours of donation. Counter-electrophoresis was the first technique to be widely used for HB_sAg testing within the blood transfusion service but, with the advance of hepatitis testing technology, was soon found to be of insufficient sensitivity. Commercial tests employing the principles of haemagglutination and radioimmunoassay became available, but at a price (particularly radioimmunoassay) which proved prohibitive to many centres. This thesis describes the development and application of a haemagglutination-inhibition (HAI) test for HB_sAg designed specifically to meet the requirements of the blood transfusion service.

The optimum conditions under which fresh human erythrocytes may be tanned and coated with partially purified HB_sAg are determined, and the procedure of testing established using u-bottomed microtitre plates. Sensitivity and specificity are comparable to that of commercial 'third generation' reagents.

To improve reagent economy the test has been 'miniaturised' using Terasaki tissue culture trays. Reagent standardisation has improved by the introduction of glutaraldehyde-fixed spherocytes as HB_sAg carriers, and 'bulk' preparation of reagents, which yielded sufficient for one million tests if conducted in Terasaki trays.

The use of antisera rendered monospecific by adsorption allowed the HAI test to be applied to antigen subtyping, thereby providing useful confirmatory evidence for the presence of HB_sAg in a

positively reacting test serum. The results of a multicentre evaluation of this HAI technique demonstrated that it was an acceptable alternative to commercial third generation HB_sAg tests for use in large scale blood donor testing. Thus, with the appropriate training, the technique can be introduced into most regional transfusion centres, thereby keeping reagent costs to a minimum. Re-investigation of more than one hundred donations implicated in ten cases of posttransfusion hepatitis suggests that if HAI had been used at the time of donation 40 percent of the cases may have been avoided.

Results of an 'on-going' evaluation of the efficacy of hepatitis B immunoglobulin (HB-IgG) in the prevention of hepatitis resulting from accidental exposure to HB_sAg suggest that fractionation of HB_sAb positive plasma provides a product which, by virtue of its high specific antibody content, provides a significant degree of protection against type B hepatitis, provided the 'challenge dose' is small.

The pursuit of a specific theme invariably stimulates new ideas aimed at further improvement. This thesis concludes with a consideration of those lines of research which are considered to be an extrapolation of the present development.

GENERAL INTRODUCTION

I. Viral Hepatitis

Viral hepatitis is the term used to describe acute inflammation of the liver caused by an infectious agent(s) presumed to be a virus. The clinical spectrum of the disease varies from asymptomatic infection through prodromal symptoms of a mild gastrointestinal nature with little or no overt hepatocellular involvement, anicteric hepatitis, hepatitis with jaundice, to acute fulminant hepatitis. Other symptoms such as urticaria, arthritis and polyarteritis nodosa may occur as a result of circulating immune complexes. Complications include chronic hepatitis which may progress to cirrhosis or hepatoma.

During and shortly after the Second World War, volunteer studies indicated two forms of viral hepatitis: infectious hepatitis (IH, virus A) and serum hepatitis (SH, virus B) (MacCallum, McFarlan, Miles, Pollock and Wilson, 1951). A number of attributes are now known to distinguish virus A infection from virus B infection and these are listed in table 1. The most outstanding difference between IH and SH comes from the observation that one does not confer immunity to the other, i.e. the causative agents will not cross-immunise one against the other (Krugman, Giles and Hammond, 1967; Krugman and Giles, 1970). The incubation period of IH is regularly 30-40 days irrespective of oral or parenteral inoculation. On the other hand, that of SH is usually longer, particularly after infection by the oral route (Giles, McCollum, Berndtson, jnr. and Krugman, 1969; Krugman and Giles, 1970). Some patients with a short incubation period are found to possess Hepatitis B Antigen (HB Ag) and it is likely that

TABLE I

Distinguishing Features of Hepatitis A and Hepatitis B

	HEPATITIS A	HEPATITIS B
PROTOTYPE STRAIN	MS 1	MS 2
HEPATITIS B SURFACE ANTIGEN	NO	YES
'FAECAL' ANTIGEN	YES+	UNCERTAIN
INCUBATION PERIOD	30-40 DAYS	20-200 DAYS
ONSET OF ILLNESS	ACUTE	SLOW
RASH, ARTHRALGIA	NO	YES
FEVER	COMMON	RARE
AGE PREVALENCE	6-20 YEARS	20 and OVER **
MORTALITY	0.1-1%	1-37%
RAISED SGOT	3-19 DAYS	35-200 DAYS
RAISED IgM	COMMON	RARE
CHRONIC CARRIER STATE DEVELOPS	UNCERTAIN	NOT UNCOMMON*
EFFECT OF 'NORMAL' IMMUNOGLOBULIN	PROTECTS	VARIABLE***
PARENTERAL TRANSMISSION	RARE	COMMON
ORAL TRANSMISSION	COMMON	RARE

* In the South East Region of Scotland approximately 0.04 percent of healthy blood donors carry HB_sAg for up to 3 years and probably much longer (Hopkins, Robertson, Ross, Turnbull and Das, 1975).

\+ Feinstone, Zapikian and Purcell (1973) described a possible hepatitis A virus in the stool of acutely infected patients. Specific antibody was detected in the convalescent sera of these and other hepatitis A patients.

** In countries where Hepatitis B infection is common (see page 19), the highest prevalence of HB_sAg is found in children 4-8 years old.

*** Depending upon HB_sAb content.

the accelerated development of the illness is due to a high concentration of virus in the challenge dose (Goesser, London, Sutnick, Blumberg and Senior, 1970).

Infectious Hepatitis has been recognised for many centuries, but it has been suggested by Zuckerman (1970) that clinical SH may have a relatively short history. He makes the point that even if HB Ag has been endemic in the population for many years, outbreaks of SH only became apparent after the introduction of large scale parenteral therapy and immunisation procedures and has paralleled the increasing transfusion of blood and use of blood products. It must be borne in mind, however, that it is only within the last few years that we have been able to identify SH as a serologically distinct entity. The origin of HB Ag is uncertain. Havens jnr. (1961) suggested that it represented theemergence of a new strain derived from virus A. This seems unlikely as the causative agents show no antigenic similarities and neither confers immunity against the other (Krugman et al., 1967; Krugman and Giles, 1970). A more acceptable theory is that HB Ag has been with us for a very long time, being maintained in the population by sexual contact and ritual operations involving scarification. With the advent of parenteral therapy, it has emerged as a major complication particularly with regard to the use of blood and blood products.

II. Association of HB Ag with Posttransfusion (Serum) Hepatitis

In 1961, Allison and Blumberg reported that some patients who had received numerous blood transfusions developed precipitating antibodies against serum beta-lipoproteins. During the course of these investigations, an antigen was found in the serum of an Australian Aborigine and was designated 'Australia Antigen' (Au Ag).

It was not for some years that the association with viral hepatitis was discovered (Blumberg, Gerstley, Hungerford, London and Sutnick, 1967; Blumberg, Sutnick and London, 1968). In 1969, Giles et al. presented data to suggest that the antigen was specifically related to long incubation (serum) hepatitis. This discovery was the result of work started a few years earlier at the Willowbrook State School in New York (Krugman et al., 1967).

III. Terminology

As research efforts and publications rapidly increased, new terms were introduced. Australia antigen (Au Ag or Au(i)), serum hepatitis antigen (SH), hepatitis antigen (HA) and hepatitis-associated antigen (HAA) all referred to one and the same thing. This welter of terms and symbols has often proved confusing to clinicians, epidemiologists and laboratory workers. The priority of the term Australia Antigen is acknowledged, but if its association with hepatitis is specific, then the name could be misleading, implying an unusual association with that country. The alternative, hepatitis-associated antigen, could create confusion if other antigen-antibody systems are discovered which prove to be specific for other forms of hepatitis. The terms hepatitis A and hepatitis B were introduced as long ago as 1947 (MacCallum), and it has recently been proposed that the antigen of hepatitis B be referred to as hepatitis B antigen (HB Ag) (World Health Organisation Committee on Viral Hepatitis, 1973).

In the electron microscope, antigen positive sera are frequently characterised by the presence of three morphologic forms (see inside front page), namely 20nm spheres, rod-like structures also 20nm in diameter, and larger 42nm spheres known as Dane particles, some of

which possess an inner electron-dense core. The discovery and subsequent confirmation of the existence of an additional antigenic specificity associated with the Dane particle core (Almeida, Rubenstein and Stott, 1971; Traavik, Kjeldsberg and Siebke, 1973; Brzosko, Madalinski, Krawczynski and Nowaslowski, 1973; Hoofnagle, Gerety and Barker, 1973) and the 'e' system (Magnius and Espmark, 1972; described in detail later) necessitated the adoption of a terminology capable of distinquishing those determinants present on the surface of the antigens' lipoprotein coat and those found in association with the Dane particle core. Hence the term hepatitis B surface antigen (HB_sAg) and hepatitis B core antigen (HB_cAg) are used in this thesis in reference to the surface and core antigens respectively.

As the major portion of this thesis is concerned with the serology of HB_sAg, the physical and chemical properties of the antigen will be considered in detail in the first chapter of Section One.

P A R T O N E

The present position.

SECTION ONE

REVIEW OF INFORMATION CURRENTLY AVAILABLE REGARDING HEPATITIS B SURFACE ANTIGEN

CHAPTER 1

BIOCHEMICAL AND BIOPHYSICAL CHARACTERISTICS

I. Morphology, Stability and Chemical Composition

Bayer, Blumberg and Werner (1968) were first to observe the physical appearance of HB_sAg in the electron microscope. Using gradient-purified material, they were able to identify two types of particle, 20-25nm diameter spheres and rod-like structures 20-25nm diameter, but of varying length. Both types of particle were aggregated by specific antiserum. These findings were soon confirmed by Prince (1968). Almeida, Zuckerman, Taylor and Waterson (1969) observed both types of particle in the serum of a healthy carrier of HB_sAg and in the acute phase sera of two patients with viral hepatitis. In a later publication, cross striations of the rod-like structures, showing a periodicity of 3nm, were noted together with an irregularity in the size of the spherical particles whose diameter varied between 16-25nm (Almeida, 1971). Dane, Cameron and Briggs (1970) reported the presence of large spherical particles, 42nm in diameter (subsequently termed 'Dane Particles'), in multiple serum specimens from three antigen positive patients. These particles were also aggregated by specific antibody and appeared to possess a complex internal structure. Gust, Cross, Kalder and Ferris (1971) and Hirschman, Vernace and Schaffner (1971) soon confirmed the occurrence of Dane particles in antigen positive serum. Almeida (1971) described Dane particles as possessing a double-shelled structure, the outer shell having a diameter of 45nm, and the inner shell, released by the action of a non-ionic detergent, having a diameter of 28nm. Similar particles were found in post-mortem liver

homogenates from serum hepatitis patients (Almeida, Waterson, Trowell and Neale, 1970), while Huang (1971) observed them in the nucleus and occasionally in the cytoplasm of liver cells from renal transplant patients who had developed antigen positive hepatitis.

Hirschman, Schwartz, Vernace, Schaffner and Ganz (1973) studied the structural polymorphism of HB_sAg under different experimental conditions and concluded that the small round particles may be discs of tightly wound helical protein that can stack to form tubes of varying lengths. The interrelationship of the different morphologic forms were investigated under varying conditions of pH, ionic strength and exposure to digestive enzymes and organic denaturing agents. In water and dilute buffer, tubular structures and small round particles predominated. Large 40-80nm, multi-stranded structures with up to four lamellae-like strands appeared in 0.125M phosphate buffer and when the antigen was digested with 0.1 percent chymotrypsin at acid pH, the tubular structures disappeared, leaving small round particles. These findings indicate that the appearance of HB_sAg in serum may be due to equilibria dependent on ionic strength and the concentration of certain polyvalent ions and peptides, including complement. The size distribution of HB_sAg particles was found to alter in the presence of complement and specific antibody but was not affected by complement alone or by inactivated complement and antibody (Hirschman, Kochwa, Rosenfield and Schwarz, 1974).

Hollow particles measuring 15-20nm diameter have been described in one case of acute hepatitis by Ackerman, Cherchel, Valet and Matte (1974), who believed them to be incomplete forms and possible precursors of normal HB_sAg. Muscatello, Bianchi and Pisi (1973) have studied the effect of specific antibody on antigen morphology and

concluded that the detailed definition of the particles becomes less, or is even obscured, in the presence of surface antibody at low concentrations. High antibody concentrations result in clumping of the antigen. Such morphological changes may prove useful in determining the presence of antibody, as non-specific clumping may occur due to the negative staining procedure.

HB_sAg is remarkably stable. It is unchanged morphologically and immunologically, neither does it lose infectivity, after heating at 60°C for one hour, at room temperature for six months, or at minus 20°C for twenty years (Hirschman, Shulman, Barker and Smith, 1969; Barker, Smith, Gehele and Shulman, 1969). Boiling for three minutes does not completely remove antigenicity, since one of thirteen children inoculated with this preparation produced specific antibodies (Krugman and Giles, 1970). Kim and Bissell (1971) found HB_sAg to be unaltered serologically after heat treatment, freeze-thawing, putrefication, acid-alkali digestion and treatment with most hydrolytic enzymes. They did find that subtilisin, subtilopeptidase A and sodium dodecyl sulphate treatment was followed by reduced immunoreactivity. Mazzur, Corbett and Blumberg (1973) found that antigenicity was destroyed by a soluble, heat labile exoenzyme produced during the growth of Pseudomonas aeruginosa and Escherichia coli. This phenomenon may have important epidemiological implications since it is conceivable that HB_sAg is released into the intestine with the bile and immunologically altered by enzymes already there, such that faeces emerge carrying antigen which is no longer identifiable as HB_sAg. Both P. aeruginosa* and E. coli are known bacterial inhabitants of the human gut and the enzymes they release may be equivalent to the 'faecal inhibitory factor' described by Piazza, Di Stasio and De Marco (1971) and Piazza, Di Stasio, Maio and Marzano (1973).

* P. aeruginosa is often present in soil and thus frequently infects the human gut.

Early biochemical investigations (Alter and Blumberg, 1966; Barker et al., 1969; Gerin, Purcell, Hoggan, Holland and Channock, 1969) suggested that the antigen was composed mainly of protein, but possessed a minor lipid moiety. Bouyant density was found to be 1.06-1.30gm./cm^3 in potassium bromide, 1.16gm./cm.^{3} in sucrose, 1.15gm./cm.^{3} in potassium tartrate, with a sedimentation coefficient of 110S in sucrose. Millman, Loeb, Bayer and Blumberg (1970) were unable to detect nucleic acid by spectrophotometry, chemical and radiochemical analysis. The following year, Joswiack, Koscielak, Madalinski, Brzosko and Nowaslowski (1971) reported the presence of RNA in purified, concentrated HB_sAg. Hirschman et al. (1971) reported weak endogenous DNA-synthesising activity which appeared to be dependent on an RNA template, raising the possibility of an RNA-dependent-DNA-polymerase being present in concentrated HB_sAg, similar to the 'reverse transcriptase' associated with some RNA oncogenic viruses (Spiegelman and Schlom, 1972; Gallo, Yang, Smith, Herrera, Ting and Fugioka, 1971). The reaction was, however, stimulated by the addition of poly (dAT) and not by poly (rA)-oligo (dT), as are the known RNA-dependent DNA polymerases (Zuckerman and Howard, 1974); furthermore, this interpretation has not been confirmed by other workers in the hepatitis field.

In 1973, Kaplan, Greenman, Gerin, Purcell and Robinson confirmed the presence of a DNA-polymerase in Dane particle-rich preparations of HB_sAg. They showed that the reaction could be inhibited by actinomycin-D, suggesting that it was associated with a DNA template. Peak activity appeared to be related to Dane particle cores, no DNA-polymerase activity being found in association with the 20nm. spherical particles. The following year, Robinson, Clayton and

Greenman (1974) confirmed and extended these findings by extracting radioactive DNA from labelled Dane particle cores. Electron microscopic examination revealed circular double-stranded DNA molecules approximately 0.78nm in length. Identical circular molecules were obtained when DNA was isolated by a similar procedure from particles that had not undergone a DNA polymerase reaction. The authors suggested that the circular molecules probably serve as a template for the DNA-polymerase reaction carried out by the Dane particle cores. Huang, Mao, Ling and Overby (1975) have reported the presence of free DNA in HB_sAg positive plasma which showed homology with radiolabelled Dane particle DNA by molecular hybridisation. Further work is required to establish conclusively the nature of the DNA polymerase template and to identify the enzyme as viral and not host specific.

Millman, Hutanen, Marino, Bayer and Blumberg (1971) observed that purified HB_sAg reacted with specific antibody but not with antibody to normal human serum components. Treatment of the purified antigen with the non-ionic detergent TWEEN 80 revealed substances which reacted with antibody to human IgG, beta Ia/Ic globulin, beta lipoprotein, transferrin and albumin. In the electron microscope, the treated antigen particles appeared less dense and unfolded, yet retained dimensions similar to those before extraction. One interpretation of these findings is that the components released by detergent extraction are either intricate parts of the macromolecule, or contaminants. Sutnick, Millman, London and Blumberg (1972) showed that the extracted substances differed electrophoretically and immunologically from human serum proteins. A recent study by Burrell (1975) has shown that purified ^{125}I-

labelled 20-25nm. particles produced low affinity immunoprecipitation reactions with antisera to several normal human serum components, which are immunologically distinct from the reaction due to the classical HB_sAg determinants. No evidence was found for the native material in normal serum or liver cells, bo as an integral part of the structure of the 20-25nm particles.

Ultracentrifugation has indicated particles of two molecular weights, 3.6×10^6 and 4.5×10^6 (Dreesman, Hollinger, Suriano, Fujioka, Brunschwig and Melnick, 1972). Antigenic activity was detected in particles having isoelectric points at pH 4 and pH 4.4. Purified ^{125}I-labelled antigen was found to contain six different polypeptides of molecular weights 10,000 to 39,000. The 20-25nm. particles contained increased levels of cysteine amino acid residues (4.8 percent) compared with other animal viruses (0.3 percent to 2 percent), a figure which suggests that sulphydryl groups and/or disulphide bonds may play a major role in maintaining the tertiary structure of the antigenic determinants. Sukeno, Shirachi, Yamaguchi and Ishida (1972) also emphasised the role of disulphide bonds in the structural integrity of HB_sAg by showing that reduction with dithiothreitol caused irreversible loss of structure. In 1973, Dreesman, Hollinger, McCombs and Melnick showed that reduction and alkylation destroyed antigenicity, while reduction alone caused loss of morphological integrity.

Vyas, Williams, Klaus and Bond (1972) obtained an optical density of 3.726 at 280nm using an 0.1 percent solution of protein from purified 20nm. particles. This protein appeared to consist of two polypeptides which contained high proportions of proline, leucine and serine, but only a small amount of tyrosine. Using

optical rotary dispersion and circular dichroism, Sukeno, Shirachi, Shiraishi and Ishida (1972) showed that HB_sAg contained a high concentration of alpha-helices (70 to 80 percent), thus indicating a structural similarity to human lipoproteins. Burrell, Proudfoot, Keen and Marmion (1973) have obtained evidence from periodate treatment and chemical analysis of purified 20nm. particles that significant amounts of carbohydrate are present and that intact carbohydrate is necessary for serological activity. This finding was supported by the work of Howard and Zuckerman (1973) who found that pure HB_sAg, isolated by gel filtration and electrofocusing, was aggregated by concanavalin A. The major phospholipids of HB_sAg have been characterised as phosphatidylcholine, sphingomyelin and lypophosphatydylcholine. In addition, two carbohydrate-containing lipids were observed, one of which appeared to be a non-sialic acid-containing, water soluble glycosphingolipid (Steiner, Heulner and Dreesman, 1974).

II. Antigenic Heterogeneity

Levene and Blumberg (1969) were among the first to discover the existence of antigenic heterogeneity in HB_sAg. They immunised a rabbit with HB_sAg-positive serum from a leukaemic patient, whose serum had given an unusual and inconsistent reaction with specific antisera. The resultant rabbit antiserum, after absorption, had at least one specificity in common with other HB_sAb-containing sera (anti-Au(1)) and one specificity not shared with these. This was demonstrated by the production of a spur in Ouchterlony double diffusion. The rabbit antiserum was designated anti-Au(2). Since anti-Au(1) possessed at least two specificities which they called 'a' and 'c', those of anti-Au(2) they called 'a' and 'b'. In 1970,

Raunio, London, Sutnick, Millman and Blumberg reported the findings of a study in which HB_sAg had been collected from different parts of the world and compared in respect to specificity against a number of antisera. All the antigens appeared to have one specificity in common, but by selection of appropriate reagents, differences could be demonstrated. Kim and Tilles (1971) observed three sub-specificities in combinations 'a', 'ab' and 'abc'. The latter showed electrophoretic mobility equivalent to that of beta-globulin, while 'a' and 'ab' were intermediate between alpha- and beta-2 globulin. The 'b' and 'c' of Kim and Tilles (1971) were not the same as the 'b' and 'c' of Levene and Blumberg (1969). Analysis of the surface antigens of HB_sAg by Le Bouvier (1971) revealed one common specificity 'a' and three additional determinants 'd', 'x' and 'y', which were not shared by all positive sera. It was possible to divide sera into three groups depending upon the possession of 'd' and 'y', i.e. group A (=a+dy-); group D (=ad+y-); group Y (=ay+d-). It is of interest to note that 'x' and 'd' are equivalent to 'b' and 'c' of Kim and Tilles (1971), but are distinct from the 'b' and 'c' of Levene and Blumberg (1969). It has been suggested (Le Bouvier, 1972; Le Bouvier, McCollum, Hierholzer, Irwin, Krugman and Giles, 1972) that 'a', 'd' and 'y' reflect the genotype of the antigen, and that 'x' may be a component of the host which has become integrated into the antigen or firmly bound to its surface. Occasionally, the 'd' and 'y' specificities have been found on the same particle (Nordenfelt and Le Bouvier, 1973/74), the most likely explanation for this being that a dual infection has occurred, resulting in the combination of viral genomes with the formation of a new genome representing both specificities. Recent studies by

Soulier and Courouce-Pauty (1973) suggest a subdivision of group Y into three categories (a^1y,a^2y,a^3y) and of group D into two categories (a^4d,a^5d) with a further group containing a genuine association of D and Y (ady).

In 1972, Bancroft, Mundon and Russell described three immunodiffusion patterns of antigenic reactivity. One showed identity with Y and one with D, while one showed partial identity with both. Absorption with heterologous material showed that the third type of reaction pattern was distinguishing two additional antigenic determinants subsequently designated 'w' and 'r'. This study also suggested that 'w' was more common in the U.S.A., while 'r' predominated in sera from Thailand. Bar-Shany, Edwards, Moseley and Bancroft (1973) confirmed and extended these findings to show that 'w' occurred almost exclusively in the U.S.A., Europe, North Africa and the Middle East. An investigation of antigen subtypes among asymptomatic carriers in Canada (Fienman, Berris, Sinclair, Wrobel, Alter and Holland, 1973) showed that D and Y appeared to be related to country of origin, implying infection at an early age. These findings were confirmed by Mazzur and Blumberg (1973), who studied the determinants 'd', 'y' and 'w' in 31 chronic carriers and concluded that they represented good long term epidemiological markers.

A global pattern of subtype distribution appears to be emerging with zones where there is an excess of one subtype and regions where a mixture of subtypes is common. Mazzur, Burgert and Blumberg (1974) have suggested that a partial explanation of this distribution may be that HB_sAg-positive mothers infect their children who then become carriers of the same subtype. Daughters transmit the antigen to their children and so on through many generations. This mechanism could maintain the subtype brought into a new environment by an

immigrant population. The introduced subtype would not necessarily predominate since there are other mechanisms which produce carriers.

As previously described, treatment of the Dane particles with Tween 80 released an inner 27nm diameter core (Almeida et al., 1971). Specific antibody to this core (HB_cAb) may be found in patients convalescing from antigen positive viral hepatitis, some possessing HB_cAb in the absence of HB_sAg. Such findings, which have been confirmed by a number of other workers (Traavik et al., 1971; Brzosko et al., 1973; Hoofnagle et al., 1973), imply the presence of a further antigenic specificity normally hidden by the stable outer protein coat. Hoofnagle et al. (1973) found HB_cAb in all chronic HB_sAg carriers studied and suggested that it may be a sensitive marker of viral replication even when HB_sAg is not detectable.

Magnius and Espmark (1972) reported the existence of a specificity 'e', distinct from 'a', 'd', 'y', 'w', 'r' and 'x', and possibly related to contagiousness. This antigen was one of a group of precipitating antigens found in HB_sAg-positive sera. The 'e' antigen was frequently present in sera from patients on haemodialysis, and anti-'e' was found in serum specimens from healthy carriers. The presence of 'e' has been confirmed by Nielsen, Dietrichson and Juhl (1974), who suggest that it may be a marker of continued viral replication and abnormal liver function, and therefore of prognostic value.

Neurath, Prince, Lippin and Chen (1973) used immunoabsorbants coated with antibody to Apolipoprotein C to show that HB_sAg particles possess this protein moiety on their surface, and are therefore immunologically related to LP-X, an abnormal plasma lipoprotein detected in the serum of patients with obstructive jaundice. In a

letter to the Editor of Lancet, Goudeau, Houwen and Dankert (1974) have suggested that these results may be due to non-specific adsorption of HB_sAg onto the antibody-coated gel.

There is increasing evidence (Holland, Purcell, Smith and Alter, 1972; Iwarson, Magnius, Lindholm and Lundin, 1973), that in the U.S.A. and Europe group Y is more likely to be associated with clinical hepatitis, while group D is more frequently found in chronic asymptomatic carriers of HB_sAg. In a study of 63 asymptomatic carriers of antigen in Toronto, Fienman et al. (1973) found elevated serum glutamic pyruvic transaminase (SGPT) equally common in 'adr', 'adw' and 'ayw' specificities. Gold, Alter, Holland, Gerin and Purcell (1974) used a modified haemagglutination test to subtype HB_sAb in terms of anti-d and anti-y activity. Blood donors from the U.S.A. and Egypt showed frequencies of antibody that closely parallel the frequency of antigen groups in each population. An interesting result of this study was that antibodies against both 'd' and 'y' were not detected together in any patient, including those who had been repeatedly transfused.

III. Nature of HB Ag

There seems little doubt that HB_sAg is a viral product, while the 'full' Dane particle represents the infectious virion. In the electron microscope HB-Ag exhibits a morphology which is so characteristic as to be of diagnostic value, and possesses the size, pleomorphic symmetry and negative staining characteristics found among other viruses. Furthermore, its close association with type B hepatitis endows it with the epidemiological peculiarities of an infectious agent. The work of Kaplan et al. (1973) and Robinson et al. (1974) provides strong evidence for the association of a DNA-genome with HB_cAg. Early attempts to 'grow' the virus in tissue and organ

culture proved singularly unsuccessful, possibly due to the use of the surface antigen as marker. Subsequent studies suggest that it may be possible to infect cultures with Dane particle-rich material and to show an increase in the amount of core antigen (Panouse-Perrin, Rachman, Courouce-Pauty and Dupuy, 1973). Despite the mounting evidence in favour of the viral nature of the antigen, two features still distinguish it from the majority of other viruses.

1. The amount of DNA associated with HB_cAg (1.6×10^6 daltons) is unlikely to be sufficient to code for the DNA genome; the core and e antigens; in addition to the complex lipoprotein coat that surrounds and presumably protects the core proteins and associated enzymes.

2. The high alpha-helical content of HB_sAg suggests analogy to that of human tissues.

CHAPTER 2

HB_sAg IN BODY FLUIDS

I. Blood

The incidence of HB_sAg in the blood of donors from several countries is listed in table 1(1)2.1.Of those studied, the highest incidence is found in countries near the equator, with the exception of Greenland. The common feature of both polar and tropical populations studied is a poor socio-economic environment with associated overcrowding and poor hygiene, suggesting that this factor is of primary importance concerning the spread of HB_sAg.

In 1969, Lewkonia and Finn reported an increased susceptibility to serum hepatitis among group A blood donors, although subsequent reports have shown there to be no significant relationship between the antigen carrier state and the A, B, O or Rhesus blood groups (Hersch, Goyal, Grubb, Werch and Melnick, 1971; Szmuness, Prince and Cherubin, 1971; Hadzyannis, Papaevangelou and Vissalis, 1973; Vale, Thomas, Hawkes and Kelly, 1974). However, it is now apparent that certain groups of blood donors are associated with a higher risk of HB_sAg transmission than others. These groups include commercial blood donors (Kunin, 1959; Grady and Chalmers, 1964; Cohen and Dougherty, 1968; Walsh, Purcell, Morrow, Chanock and Schmidt, 1970; Cherubin and Prince, 1971), institutionalised blood donors such as prisoners or military personnel (Kliman, Reid, Lilly and Morrison, 1971; Nelson and Cooke, 1971) and blood donors from lower socio-economic communities where overcrowding, poor hygiene, and occasionally drug addiction may be found. Homosexuals

TABLE 1(1)2.I.

HB_sAg Carriers among Blood Donors in Different Populations

Country/ population	Donor source	No. tested	HB_sAg %	Author(s)
Sweden	Paid	11,000	0.18	Iwarson et al., 1972
Norway	Volunteer	3,200	0.16	Solaas, 1970
Denmark	Volunteer	10,000	0.18	Banke et al., 1971
Germany	Volunteer	22,800	0.32	Fiedler, 1971
Holland	Volunteer	10,900	0.09	Brummelhuis, 1970
Scotland	Volunteer	73,800	0.12	Wallace et al., 1972
Switzerland	Volunteer	7,000	0.36	Frey-Wettstein, 1972
Austria	Volunteer	39,700	0.46	Wewalka et al., 1970
Spain	Paid	800	1.0	Guardia et al., 1970
Italy	?	1,800	1.5	Constantino et al., 1970
Yugoslavia	Volunteer	14,400	2.6	Dejanov et al., 1971
Greece	Mixed	5,100	3.3	Hadziyannis et al., 1972
Turkey	?	1,600	3.0	Ertugrul and Say, 1971
Israel	Mixed	10,000	0.9	Bar-Shany et al., 1972
Canada	Volunteer	411,000	0.22	Moore and Path, 1972
U.S.A.	Volunteer	2,596,000	0.10	Dodd et al., 1973
Africa (Nigeria, Kenya, Mali, Senegal, Ivory Coast)	?	8,400	6.6-8.7	Saimot et al., 1972 Francis and Smith, 1971 Bagshave et al., 1971
Australia	Volunteer	34,600	0.11	Nelson and Cooke, 1971
Japan	Paid	5,200	1.0	Okochi and Murakami, 1968
Singapore	?	2,600	3.1	Simons et al., 1972
Papua New Guinea	?	3,500	7.5	Woodfield et al., 1972
Greenland	Not donors	2,900	7.1	Skinhøj et al., 1974
United Kingdom	Volunteer	373,323	0.06	(pooled data from five centres, 1971, Appendix F)

may also constitute a high risk group (Jeffries, James, Jefferiss, MacCleod and Wilcox, 1973). Young adult males show the highest incidence of antigen carriage (Blumberg, Sutnick, London and Melartin, 1972; Sutnick, London, Gerstley, Cronlund and Blumberg, 1968; London, Sutnick and Blumberg, 1969; Mason, Shaw, Harding and Witney, 1972). It could be argued that their greater social mobility makes this group more likely to become infected and to infect others, although it is difficult to see how this principle could operate in all populations studied.

Most of the early data relating to the incidence of HB_sAg were obtained using the relatively insensitive techniques of counter-electrophoresis (C.I.E.O.P.) and immunodiffusion (I.D.) It has been estimated (Chalmers and Alter, 1971; Kliman et al., 1971) that these methods will detect only 20-40 percent of HB_sAg carriers, and evidence is beginning to accumulate which suggests that haemagglutination and radioimmunoassay are capable of detecting circulating antigen at a concentration below the sensitivity of the above techniques (Levy and Hawrisiak, 1972; Geogini jnr., Hollinger, Leduc, Issarescu, George, Blackman, Thayer jnr., 1972; Shaffer, Vyas, Shahed, Chen and Perkins, 1972; Ling, Irace, Decker and Overby, 1973).

Carrera, Bryant and Leonard (1973) have reported HB_sAb and HB_sAg simultaneously in 26 of 10,470 blood donations, suggesting that the presence of antibody may also identify potential antigen carriers. Millman, London, Sutnick and Blumberg (1970) showed that when some HB_sAb positive sera were pelleted, HB_sAg could be detected in the pellet.

The incidence of HB_sAb in most volunteer blood donor populations studied by I.D. or C.I.E.O.P. is similar to that for

HB_sAg; e.g., 0.28 percent in Copenhagen (Banke, Dybkjaer, Nordenfelt and Reinicke, 1971), 0.06 percent in the West of Scotland (Wallace, Milne and Barr, 1972), the principal exception being an incidence of 6.7 percent among volunteer blood donors in New York (Szmuness, Prince, Etling and Pick, 1973). The use of haemagglutination and/or radio-immunoassay techniques lead to an increase in detectable antibody to between 1 percent and 20 percent (Ni, Shoff and Lama, 1972; Alter, Lander and Purcell, 1971). No direct evidence has been presented to implicate HB_sAb-positive blood with HB_sAg-positive jaundice (Gocke and Panick, 1972; Reinicke, Poulson, Banke and Bybkjaer, 1973; Aach, Alter, Hollinger, Holland, Lander, Melnick and Weiler, 1974). However, in a recent letter to Lancet, Renton and Wadsworth (1975) have pointed out that blood containing HB_sAb appears to be four times as infectious as blood negative for antigen and antibody, although this interpretation may not reflect the true situation since their data were derived from an invalid reconstruction of population statistics.

II. Faeces

In 1970, Ferris, Kaldor, Gust and Cross reported a particulate antigen similar in appearance to HB_sAg yet serologically distinct from it, in faecal extracts from cases of HB_sAg-negative hepatitis. These findings were confirmed by Cross, Waugh, Ferris, Gust and Kaldor (1971), who went on to show that the larger particles (40-45 nm) of faecal antigen appeared to possess an antigenic component in common with the Dane particles observed in serum. It was suggested that the faecal antigen represented serum antigen degraded as a result of passage through the intestinal tract. Grob and Jemelka (1971) reported HB_sAg in faeces from eleven cases of sporadic acute hepatitis, whose serum were all HB_sAg positive. Using two-dimensional I.D., Grob and Jemelka (1972) were able to show

that HB_sAg in faeces was immunologically and electrophoretically similar to that in serum. Despite these findings, many workers have been unable to identify HB_sAg in faeces of serum-antigen-positive patients. Moodie, Stannard and Kipps (1974) suggested that this was due to the action of hydrolytic enzymes found in the human gut, capable of degrading the surface lipoprotein of HB_sAg and exposing the inner core of Dane particles which Almeida et al. (1971) have shown to possess their own peculiar antigenicity.

III. Urine

Blainey, Earle, Flewett and Williams (1971) were first to report the presence of HB_sAg in concentrated urine. Addition of specific antiserum resulted in the formation of characteristic immune complexes when viewed by electron microscopy (E.M.). These findings were confirmed by Apostolov, Bauer, Selway, Fox, Dudley and Sherlock (1971) and Trapitzis and Horst (1971), who demonstrated immunological identity between HB_sAg in urine and in serum. Heathcote, Tsianides and Sherlock (1973) detected a substance in the urine of some antigen positive patients, which reacted with HB_sAb by Complement Fixation (C.F.). However, morphologically characteristic HB_sAg could not be seen in the urine by electron microscopy.

IV. Saliva and Other Body Fluids

Ward, Borchert, Wright and Kline (1972) detected HB_sAg in saliva in 50 percent of mentally handicapped, institutionalised patients. Antigen positive saliva has been reported by Tanno, Fay and Roncoroni (1972) in cases of acute and chronic hepatitis and postnecrotic cirrhosis, while Kistler, Sonnabend and Krech (1973) were able to detect HB_sAg by I.D. and E.M. in mixed saliva concentrates

from a similar group of patients which included two HB_sAg positive kidney transplant recipients. Antigen has been observed in the saliva and semen of carriers with high serum antigen titres (Heathcote, Cameron and Dane, 1974). Ogra (1973) employed C.I.E.O.P. and immunofluoresence (I.F.) to study patients with icteric and asymptomatic infections. She found antigen in the naso-pharangeal washings of some subjects over a short period. Other workers have reported HB_sAg in synovial fluid (McKenna, O'Brien, Scheinman, Delaney, Pelleochia and Lepore, 1971), amniotic fluid (Matsuda, Tada, Shirachi and Ishida, 1972), gall bladder bile (Serpeau, Mannoni, Dhumlack and Bertheldt, 1971; Bose, Bahu, Hammond and Lubbat, 1971), and sweat (Telatar, Kayhan, Kes and Karacadaq, 1974).

It should be remembered, however, that any biological fluid contaminated with infected blood will appear antigen positive if the test is sufficiently sensitive.

V. Plasma Fractions

The cold alcohol procedure of Cohn, Strong, Hughes, jnr., Mulford, Ashworth, Melin and Taylor (1946) has been most commonly used for industrial scale fractionation of plasma. Several investigators have fractionated HB_sAg-rich plasma and looked for antigen in the resulting fractions (Andrassey, Ritz and Sanwald, 1970; Schroeder and Mozen, 1970; Zuckerman, Taylor, Bird and Russell, 1971). HB_sAg has been detected in all fractions except immune serum globulin, a finding in accordance with the well-recognised non-infectivity of this fraction. Such findings may reflect removal of the antigen by fractionation or more likely, antibody present in the fraction may have masked HB_sAg by complexing with it. In a recent report, Lewis, Maxwell and Brandon (1974)

found concentrates of factor IX to be associated with serum hepatitis in haemophiliacs three times more frequently than concentrates of factor VIII.

CHAPTER 3

HB_sAg LIVER DISEASE

I. Asymptomatic Carriers; Clinical Hepatitis

As early as 1971, Singleton, Merrill, Fitch, Kohler and Rettberg suggested that the majority of HB_sAg positive blood donors might be suffering from some form of underlying liver disease. However, Reinecke, Dybkjaer, Poulsen, Banke, Lylloff and Nordenfelt (1972) reported that none of 24 antigen positive Danish blood donors showed histological evidence of hepatitis. Subsequent studies have shown that some HB_sAg positive donors exhibit histological and/or biochemical evidence of liver dysfunction (Cazal and Robinet-Levy, 1972; Woolfe, Boyes, Dymock, Renton and Stratton, 1972; Papadopoulos, Traianos, Thomakos and Tiniakos, 1973; Anderson, Sun, Berg,and Chang, 1974), and it has been suggested that the best approach to apparently healthy HB_sAg carriers is to observe clinically, with regular biochemical tests of hepatic function as long as no evidence of chronic liver disease occurs, reserving biopsy for those patients with persistent hepatic dysfunction (Griffin, 1973). An increased incidence of Dane particles in the serum of patients with abnormal liver function tests has been reported (Neilsen, Neilsen and Elling, 1973; Moulias, Couceru and Goust, 1973).

The specificity of HB_sAg for serum hepatitis has been confirmed by the Willowbrook experiments of Giles et al. (1969). Initial reports indicate a frequency of HB_sAg in acute hepatitis of 12 percent in Australia (Gust and Lucas, 1971; Ferris, Kaldor and Lucas, 1970; Hawkes, 1970), 33 percent in the U.S.A. (Prince, 1971), 60 percent in

Uganda (Maynard, Sadikali, Anthony and Barker, 1970) and Ghana (Morrow, Sai and Barker, 1971), and 15 percent in London (Farrow, Lamb, Coghill, Lindon, Preece, Zuckerman and Stewart, 1974). From the data currently available, it appears that HB_sAg may be detectable shortly before the onset of acute illness. As the antigen disappears, antibody to the Dane particle core becomes detectable and persists for a relatively short time. Antibody to the surface antigen specificities may not appear for many months, and then possibly only as a result of subsequent challenge (see figure 1(i)3.1. Barker, Peterson, Shulman and Murray (1973) have described primary and secondary antibody responses in adults receiving material known to transmit type B hepatitis. Some of those showing secondary responses subsequently developed disease. It was concluded that primary responses were common after exposure to HB_sAg and that protective immunity frequently but not invariably follows initial exposure.

II. Chronic Liver Disease

Gitnick, Gleich, Schoenfield, Baggenstoss, Sutnick, Blumberg, London and Sommerskill (1969) studied 31 patients with chronic active liver disease and found HB_sAg in 3, all of whom had cirrhosis. These findings led to the speculation that in some patients, a chronic viral infection existed, even in the presence of cirrhosis. Maynard et al. (1970) found HB_sAg in 31 percent of patients in Uganda, as compared with 2 percent in normal controls, and suggested that infection with HB_sAg may be of pathogenic importance in the development of cirrhosis. In 1972, Nowaslowski, Krawczyniski, Brzosko and Madalinski suggested a correlation between hepatocellular damage in cirrhosis and the presence of HB_sAg/HB_sAb immune complexes. Brzosko,

Figure 1(1).3.1.

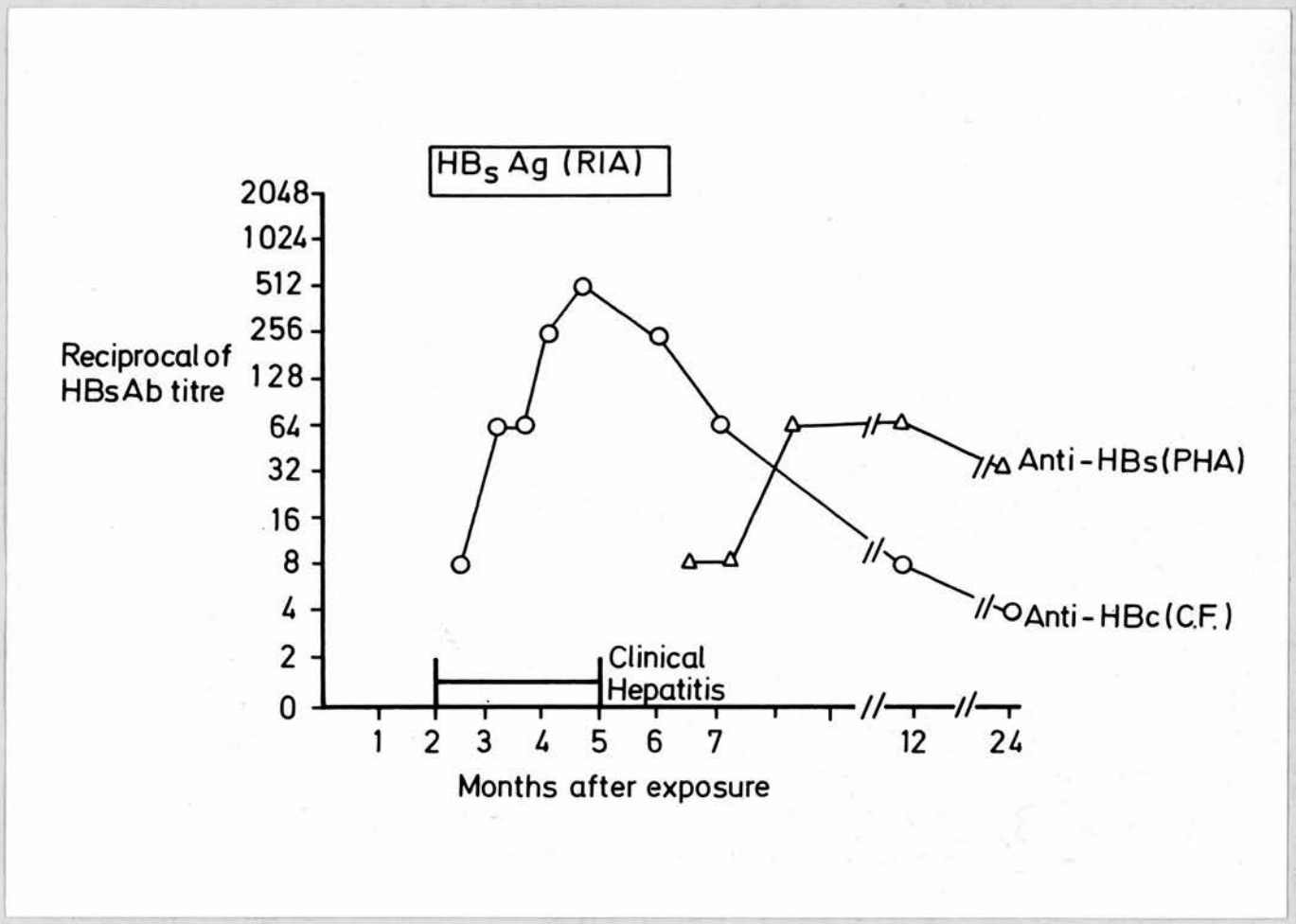

Figure 1(1).3.1. **Serologic responses in a moderately severe case** of type B hepatitis. Antibody to hepatitis B core antigen measured by complement fixation (C.F.), antibody to hepatitis B surface antigen measured by passive haemagglutination (PHA), hepatitis B antigen measured by radioimmunoassay (RIA).

Mikulska, Biedrzycka, Roszkowska, Rudkowski, Rabenda, Oziemska-Losinska and Debski (1973) identified HB_sAg in 69 percent of children with chronic hepatitis, and antibody to [illegible] antigen (HB_cAg) in all of 50 cases studied. More recently, Van Waes, Segers, Van Egmond, Van Nimmen, Barbier, Wieme and Demeulenaere (1974) have reported on a long-term follow-up of 45 patients with chronic hepatitis and 41 patients with cirrhosis. Antigen was present in 42 percent and 49 percent respectively. The clinical, biochemical and histological findings were similar in antigen positive and antigen negative patients. They concluded that when no irreversible lesions existed, the disappearance of antigen was taken as an indication that the disease would resolve.

III. Hepatoma

Reports from many laboratories indicate a higher incidence of HB_sAg in patients with hepatoma than among the general population (Sherlock, Fox, Niazi and Scheuer, 1970; Hadziyannis, Merikas and Afroudakis, 1970; Vogel, Anthony, Natu, Mody and Barker, 1970; Dennison, Peters and Reynolds, 1971; Specht and Noto, 1972), suggesting that hepatoma could be the last stage in a process initiated by acute hepatitis, leading to chronic hepatitis and post-necrotic cirrhosis. Simons (1972) presented evidence to suggest that the use of sensitive techniques will result in a more significant correlation between HB_sAg and hepatoma. Using radio-immunoassay (RIA), Reed, Eddleston, Steen, Williams, Zuckerman, Bowes and Earl (1973) found 23 percent of 38 patients to be HB_sAg positive, the frequency being higher in those born outside Great Britain (66 percent compared with 15 percent). In the absence of conclusive proof of the oncogenic properties of HB_sAg,

it may be that hepatitis B virus is merely a passenger. Nowaslowski (1974) reported that the only cells supporting viral replication within the region of a liver cancer were normal hepatocytes, although this does not exclude the possibility of transformation by the HB Ag genome.

IV. Other Clinical Conditions

A number of symptoms have been described which do not involve the liver and are most probably due to circulating immune complexes. McKenna et al. (1971) have identified HB_sAg in synovial fluid, while joint symptoms have occasionally been reported in acute and chronic hepatitis (Alpert, Isselbacher and Schur, 1971; Buckley, Heizer, Goldfinger and Isselbacher, 1970), and antigen has been found in patients exhibiting polyarteritis nodosa (Baker, Sidel and Kalan, 1971). Similar findings led McCarty and Ormiste (1973) to suggest that an arthritic strain of HB_sAg may exist. The antigen has also been associated with glomerulonephritis and nephrotic syndrome (Coombes, Shory, Borrera, Stastney, Eigenbrodt, Hull and Carter, 1971) and various skin rashes (Alpert et al., 1971). Trepo, Zuckerman, Bird and Prince (1974) have suggested that circulating immune complexes may be responsible for vasculitis or polyarteritis, but do not appear to be pathogenic for the liver. Vos, Grobbelaar and Milner (1973)have presented evidence that the HB_sAg carrier state may predispose some patients to develop renal disease, their evidence being based on the observation that many carriers showed depressed C^3-complement levels and that a relatively high percentage of patients with renal disease carried HB_sAg.

V. HB_sAg Subtypes and Liver Disease

One of the earliest reports implicating a particular antigenic subtype with disease was a publication by Wenzel, Le Bouvier and Beam (1972) relating drug abuse and hepatitis in American marines. All positive sera possessed antigenic specificity "ay". Subsequent reports have shown a predominance of this subtype among drug abusers (Le Bouvier et al., 1972; Iwarson et al., 1973) and in association with serum hepatitis outbreaks in a number of renal dialysis units, including the exceptionally severe outbreak in Edinburgh (Marmion and Tonkin, 1972; Moseley, Edward, Meihaus, Gordon and Redecker, 1972). Gordon, Berberian and Stevenson (1972) were unable to find a correlation between the forms of hepatic injury and the antigenic subtype isolated from both acute and chronic hepatitis patients, while Feinman et al. (1973) found abnormalities of liver function and histology to be equally common in asymptomatic HB_sAg carriers of both 'ad' and 'ay' subtypes in Canada. A number of other studies, however, show that the 'y' specificity is frequently associated with clinical illness, while 'd' specificity is often associated with chronic asymptomatic carriage (Schmidt, Roberto and Lennette, 1972; Holland et al., 1972; Perry and Chaudhary, 1973; Iwarson et al., 1973; Sama, Krishnamurthy and Gurdeep, 1974). In a study relating the degree of liver damage in acute viral hepatitis to the HB_sAg subtype, Nielsen and Le Bouvier (1973) found the 'y' specificity in 79 percent of cases. However, the acute disease was more severe in patients possessing the 'd' specificity as judged by histological and biochemical criteria. A global pattern of subtype distribution seems to be emerging (see chapter 1, II). In America and most Western European countries

where the 'd' specificity predominates among asymptomatic carriers, the 'y' specificity is most frequently identified in clinically apparent hepatitis. It remains to be seen whether the reverse is true in those parts of the world where 'y' is the common specificity among asymptomatic (blood donor) carriers.

CHAPTER 4

TRANSMISSION OF HB_sAg

For many years, it was assumed that serum hepatitis was only transmitted by parenteral routes (Propert, 1938; Beeson, 1943; Turner, Snavely, Grassman, Buchannan and Foster, 1944; Neefe, Stokes, Renhold and Luckens, 1944; Grossman, Stuart and Stokes, 1945; MacFarlane and Chesney, 1944). The discovery of HB_sAg led to renewed interest in the epidemiology of the disease.

I. Parenteral Transmission

In 1967, Ringertz and Zetterberg reported a number of cases among Swedish track-finders. It was suggested that spread of HB_sAg was via bushes contaminated with blood and the communal wash basins shared by competitors, since only eight secondary cases developed from over six hundred index cases, suggesting that transmission was by parenteral means rather than the faecal-oral transmission associated with hepatitis A (infectious hepatitis). The increasing drug abuse among young adults, coupled with a tendency to share syringes, has led to a corresponding increase in the number of cases of infection among such communities (Cherubin, Hargrove and Prince, 1969; Rienicke and Nordenfelt, 1970; Nelson et al., 1971; Szmuness and Prince, 1971; Grady, Bennett, Culhane, Forrest, Iber and the Boston Inter-Hospital Liver Group, 1972), which provide a growing reservoir of potential infection to the community at large. Tattooing has been implicated as a parenteral route for transmission of HB_sAg (Robertson, 1951; Sterner, Agell, Gerzen and Berg, 1971; Gostling, 1971; Scutt, 1972) as have dentists and barbers (Newalka, Gnan, Krassnitski and Passendorfer, 1970; Kohn, 1970; Levin, Maddrey,

Wands and Mendeloff, 1974) and menstrual blood (Mazzur, 1973). Typing and control sera used as standard laboratory reagents have been shown to contain HB_sAg (Ginsberg and Conrad, 1972; Welti, Heal and Miale, 1973).

Attention has been focussed on the possible role of insect vectors in the transmission of HB_sAg. The high prevalence in the tropics (Blumberg, Gerstley, Sutnick, Millman and London, 1970) may depend upon blood-sucking arthropods to ensure a high frequency of exposure during the early years of life when the risk of developing chronic HB_sAg carriage is greatest (Szmuness, Pick and Prince, 1970). Prince, Metselaar, Kafuko, Mukwaya, Ling and Overby (1972) found HB_sAg in 28 out of 187 pools of wild-caught mosquitoes from Kenya and Uganda. In controlled experiments with mosquitoes fed from a carrier, they were able to show that HB_sAg disappeared in parallel with blood meal digestion, being present for at least 90 hours. They could find no evidence of replication inside the mosquito. These findings were subsequently confirmed by Byrom, Davidson, Draper and Zuckerman (1973). A recent report from Greece provides further evidence that mosquitoes may act as vectors for HB_sAg (Papaevangelou and Kourea-Krematinou, 1974). There is only one, as yet unconfirmed, report that some species of mosquito may serve as biological vectors for HB_sAg (Smith, Ogunba and Francis, 1972).

Zebe, Sanwald and Ritz (1972) have reported that transmission of HB_sAg in a dialysis unit outbreak (Barker, Shulman, Murray, Hirschman, Ratner, Diefenbach and Geller, 1970) may have occurred via cockroaches. This possibility was tested experimentally by infecting cockroaches with HB_sAg-positive serum. Antigen remained

detectable for long periods (10-15 days) in both vomitus and haemolymph, suggesting a carrier state rather than just mechanical contamination. It seems likely that other orders of blood-sucking anthropods (ticks, lice, sand-flies, tse-tse flies etc.) may play a similar role, their relative importance depending upon the frequency with which they bite man. This may explain the higher incidence of HB_sAg in the lower socio-economic groups where over-crowding is more likely to occur (Morrow et al., 1971).

II.. Non(inapparent) Parenteral Transmission

It seems unlikely that a viral species should require modern artificial mechanisms of transmission such as blood transfusion and injections for its survival and propagation in nature. The possibility of non-parenteral transmission of HB_sAg is at present being studied in many laboratories. Krugman et al. (1967) were the first to demonstrate direct oral transmission of HB_sAg using the Willowbrook MS2 agent, while other reports indicate that laboratory technicians have become infected by swallowing blood or serum while pipetting specimens from HB_sAg positive dialysis patients (Westwood, Chaudhary and Perry, 1973). Infection with serum by mouth is, however, not equivalent to faecal-oral transmission, and early work with volunteers failed to show infection by the oral route with extracts from the faeces of serum hepatitis patients (Neefe, 1946). This finding may be explained in part by the presence of a "faecal inhibitory factor" present in the human intestine (Piazza et al., 1971), capable of neutralising the antigenicity of HB_sAg. This factor is neither an antibody nor interferon, and appears to be species specific. In 1970, Prince, Hargrove, Szmuness, Cherubin, Fontana and Jeffries presented epidemiologic evidence suggesting

that the major proportion of HB_sAg positive cases of acute hepatitis among urban adults were transmitted non-parenterally. Essentially similar conclusions were drawn by Szmuness and Prince (1971) on the basis of epidemiological analysis of viral hepatitis in Eastern Europe. Subsequently, a number of family studies have suggested that non-parenteral transmission may be a major factor in the home environment (Vas, Spence and Gilmore, 1973; Garibaldi, Hatch, Bisno, Hatch and Gregg, 1972; Dietzman, Madden, Seuer and Dunlop, 1973; Ricci, De Bac and Caramia, 1973; Bruguera, Bosch and Rodes,1973; Bruguera, Bosch, Rodes and Pedrera, 1974), although parenteral transmission can never entirely be ruled out.

Marmion and Tonkin (1972) and Chalmers (1973) have reviewed possible routes of transmission specific for dialysis units. Some of these, e.g. blood transfusion, blood contaminated equipment, "needle sticks"and other types of tissue penetration seem quite obvious. Others such as contamination of conjunctiva or other mucosa, spillage of blood over intact skin, aerosol formation and exposure to contaminated linen have not yet been sufficiently documented. However, the findings of a point prevalence study in 15 American haemodialysis centres indicate that although HB_sAg can be introduced into the dialysis centre by blood transfusion, the role of transfusion in its perpetuation may have been exaggerated (Szmuness, Prince, Grady, Mann, Levine, Freidman, Jacobs, Josephson, Rigot, Shapiro, Stenzel, Suki and Vyas, 1974). The uniformity of antigenic subtypes detectable during outbreaks of dialysis-associated hepatitis would also seem to argue against the predominant role of blood transfusion (Moseley et al., 1972). It appears that other, presumably non-parenteral, routes must exist,

and that they probably outweigh transfusions in relative importance. Data from this study also suggests that the parenteral route is not always responsible for clinical and asymptomatic hepatitis among dialysis centre staff, since after the first six months, the prevalence of hepatitis did not appear to increase with increasing length of employment. Nor did the cumulative presence of HB_sAg and HB_sAb differ among physicians, nurses, technicians and auxillary personnel, suggesting that all personnel are heavily exposed to infection from the very start of employment. Non-parenteral mechanisms of virus transmission among both patients and staff are supported by the results of an environmental surface surveilance study using a swab-rinse assay in conjunction with radioimmunoassay (Favero, Maynard, Petersen, Boyer, Bond, Berquist and Szmuness, 1973). HB_sAg was recovered from such surfaces as gloved hands, needle-clippers, furniture and external parts of dialysis equipment with and without traces of blood contamination, although it is disputable whether such material is infectious in practice.

Venereal transmission of HB_sAg would seem to be a distinct possibility (Vahrman, 1970; Jeffries et al., 1973). Hearsh et al. (1971) found evidence of HB_sAg transmission from antigen positive males to their intimate female contacts. Heathcote and Sherlock(1973) studied the spread of serum hepatitis in London, and concluded that sexual contact with an HB_sAg carrier emerged as the single most important factor. In a later publication, HB_sAg was identified in semen (Heathcote et al., 1974), a finding subsequently confirmed by Linnoman and Goldberg (1974). Heathcote, Gateau and Sherlock (1974) presented evidence that exposure to HB_sAg was more common among sexual partners than among blood relatives. They also found that contacts of carriers

with evidence of liver dysfunction are at greater risk of presumed non-parenteral exposure than are contacts of asymptomatic HB_sAg carriers. Henigst (1973) found a significantly higher incidence of HB_sAg (3 percent) among a group with frequently changing sexual partners, as compared with the normal population (0.4 percent) in Munich. Fulford, Dane, Catterall, Woolf and Denning (1973) have suggested that HB_sAg may be primarily a sexually transmitted agent, and that such well recognised means of transmission as transfusion, the shared needles of drug users and tatooing, could be incidental and may not contribute significantly to the maintenance of the virus in the community. These parenteral routes may, however, carry a greater risk of clinical illness. Conversely, in a study of 449 family contacts from 197 households containing HB_sAg carriers, Szmuness, Prince, Hirsch and Brotman (1973) concluded that sexual transmission does not appear to be of primary importance.

Data emerging from a number of laboratories suggest that non-parenteral transmission may account for the spread of HB_sAg particularly in closed institutions (Kliman et al., 1971; Nelson et al., 1971; Szmuness, Prince, Etling and Pick, 1973; Bryan, Carr and Gregg, 1973; Medhurst, Madhaven and Quinn, 1973). Villarejos, Visona, Gutierrez and Rodriguez (1974) have reported HB_sAg in the saliva of 76 percent of antigen positive acute hepatitis patients during the first three weeks after clinical onset, and in 86 percent of chronic carriers intermittently. The incidence of HB_sAg positive "sneeze samples" from antigenaemic patients were found to be 35 percent. Antibody was found in saliva early in the clinical disease, a feature which may limit infectiousness via this route.

III. Transmission in Pregnancy

In 1954, Stokes, Berk, Malamut, Drake, Barondess, Bashe, Wolman, Farquhar, Bevan, Drummond, Maycock, Capps and Bennett postulated that hepatitis could be transmitted from mother to foetus through the placenta. At that time, however, this concept could not be evaluated due to the lack of a suitable test for hepatitis virus identification. With the discovery of HB_sAg, techniques became available with which to study transmission from mother to neonate.

Does transmission occur before, during or after birth? Krech and Sonnabend (1970) reported a 6 percent incidence of hepatitis in circumstances which suggested transmission during the foetal period or shortly after delivery. Prior to this, London, Di Figlia and Rodgers (1969) were unable to find evidence of transplacental transmission in the case of a young woman who became HB_sAg positive six months before giving birth to an HB_sAg negative baby. Smethick and Go (1970) were also unable to find evidence of transplacental transmission from a study of 2,225 cord sera. Keys, Hobel, Ritman, Oh, Gitnick and Hewitt (1971) suggested that transmission may also occur during passage through the birth canal or by maternal contact in the external environment. More recently, Buchholz, Frosner and Ziegler (1974) found HB_sAg in the cord blood of an infant delivered by Caesarian section, thereby establishing that foetal infection can occur, probably after infection of the placenta. In 1972, Cossart, Hargreaves and March followed the pregnancies of 5 HB_sAg-positive hepatitis patients and three asymptomatic carriers. All babies were HB_sAg negative, suggesting that transplacental transmission did not occur, but two became positive at a later date. The mothers

of both these children remained HB_sAg positive after delivery, while the other mothers had either become HB_sAg negative or had not subsequently looked after their child. It is possible that infection occurred as a result of breast feeding, but these workers were unable to detect HB_sAg in milk, a finding confirmed by London et al. (1969). However, Linneman and Goldberg (1974) have recently detected HB_sAg in the milk of an antigen positive mother. Schwietzer, Wing, McPeak and Spears (1972) found that of 26 women who developed HB_sAg-positive viral hepatitis during late pregnancy, or up to six months after delivery, 19 had HB_sAG positive cord blood, and 10 of their babies became HB_sAg positive. The antigen appeared to cross the placenta in three cases, and neonatal contamination at birth probably occurred in one. The following year, Schwietzer, Moseley, Ashcavi, Edwards and Overby (1973) published results of a transmission study involving infected symptomatic mothers and their offspring. It was concluded that the developmental period when exposure occurred, rather than the clinical status of the mother or the HB_sAg subtype, was the most important factor in determining whether the infant became chronically infected. The highest risk occurred when the antigen was present during the last three months before, or the first two months after delivery. Children of asymptomatic carriers are less likely to become chronically infected than those of mothers with clinical hepatitis. Transfer of some maternal factor such as antibody or anti-viral proteins, in the early gestational period, may lead to resolution of what otherwise could become a persistent infection. These findings have recently been confirmed in Britain by Dr. Yvonne Cossart (1974). Panizon, Rotini and Tomaro (1973) have reported transmission of HB_sAg and HB_sAb from mother to neonate during the later stages of gestation,

resulting in precipitating immune complexes as evidenced by a drop in the immunoglobulin level of the neonate. This passive antibody failed to protect. A follow-up study of 31 infants whose mothers had HB_sAg positive acute hepatitis, while pregnant or in the first two months post partum, revealed that the most common response of the neonate was chronic HB_sAg carriage coupled with histological features of unresolved hepatitis. Occasionally, acute hepatitis developed, but this was followed by rapid healing with removal of the antigen and the appearance of antibody. In a recent study, however, Dupuy, Frommel and Alogille (1975) have shown that fatal HB_sAg hepatitis can result from maternal contamination.

Transfer of HB_sAg from mother to offspring appears to be commonplace and provides a means of perpetuating and expanding the reservoir of infection. Post-natal rather than transplacental (which may occur rarely via a transplacental lesion) or parenteral transmission, would seem to represent the major hazard.

IV. Susceptibility

A genetic susceptibility to infection has been proposed by Blumberg, Friedlander, Woodside, Sutnick and London (1969) in which susceptibility to HB_sAg is inherited as an autosomal recessive trait (see chapter 5). Petrakis (1972) has pointed out that in the above report there is a critical mating between HB_sAg-positive parents which does not support the simple autosomal recessive hypothesis, since only two of seven offspring are HB_sAg positive. It may be that the other five were not exposed. However, Vyas (1974) has provided the first discrete evidence against Blumberg's genetic hypothesis. In a family where both parents were HB_sAg carriers, two of the three children were HB_sAg and HB_sAb negative, while the

remaining child possessed HB_sAb, indicating past exposure. If the recessive hypothesis had been correct, this child would have become a chronic HB_sAg carrier. Results of a recent study in Greenland (Skinhøj, McNair and Anderson, 1974) suggest that variation in genetic susceptibility appears to be of secondary importance since the prevalence of HB_sAg was found to vary 10-fold between different towns despite the existence of very uniform genetic, cultural and climatic conditions. The incidence of 7.1 percent among the normal population is similar to figures obtained from tropical countries and institutions for the mentally retarded (Prince et al., 1970; Blumberg et al., 1970). The common feature of both polar and tropical populations being a poor socio-economic environment.

CHAPTER 5

MECHANISMS OF PATHOGENICITY

When an individual is exposed to HB_sAg, a variety of responses may occur. He may develop antigen in his blood which may persist for a short or long time. He may develop specific antibody or immune complexes. These types of response may or may not be accompanied by clinical evidence of hepatitis. The manner in which a particular individual responds will be the result of an interaction between the host and the infecting agent. The nature of the interaction will be largely determined by the genetic make-up of the host and that of the agent. Blumberg et al. (1967) suggested that the HB_sAg carrier state was the result of an inadequate immune response. He later proposed the following genetic hypothesis (Blumberg et al., 1969); there is a gene HB_sAg^1 which when present in double dose confers susceptibility to persistence of the antigen. That is, individuals homozygous for this gene, when exposed to HB_sAg, become chronic carriers, whereas persons with the alternative genotypes HB_sAg^1/HB_sAg^0 or HB_sAg^0/HB_sAg^0, when exposed are infected only for a short time. Dudley, Fox and Sherlock (1972) and Dudley, O'Shea and Sherlock (1973) put forward the theory that the outcome of infection with HB_sAg may be determined by the competence of the cell-mediated (T-lymphocyte dependent) immune response. They used a leucocyte-migration-inhibition technique to measure cellular immunity to HB_sAg in HB_sAg-positive patients, patients with HB_sAb, and normal controls (Dudley, Guistino and Sherlock, 1972). Cell-mediated immunity, specific for HB_sAg, was only found in patients with a previous history of HB_sAg infection.

These findings together with those of Laiwah (1971), Almeida and Waterson (1969) and Popper and MacKay (1972) are consistent with the view that liver cell injury is not a direct cytopathic effect of HB_sAg but rather a result of the interaction of virus-infected liver cells and the products of the immune response, either antibodies or sensitised lymphocytes. Such a theory would seem to be in conflict with the findings of Reed, Eddleston, Cullens, Williams, Zuckerman, Peters, Williams and Maycock (1973) who infused high-titre antibody-containing plasma into chronic HB_sAg carriers, with no resultant liver function test abnormalities, thus indicating that immune complexes do not, in this instance at least, play a part in hepatocellular damage. Sutnick, Bugbee, London, Loeb, Peyretti, Litwin and Blumberg (1973) showed there to be no difference between the lymphocyte response to phytohaemagglutinin between HB_sAg carriers and normal donors. This led them to suggest that a general immunodeficiency state is not a prerequisite for developing persistent antigenaemia, and that T-lymphocyte abnormality, when present, may be a result of the underlying liver disease rather than a cause.

One obvious way that the virus may damage the liver cell is by causing sufficient functional disturbances to kill it. Alternatively, it might produce chronic hepatitis through some pathogenic immune mechanism:

(1) Cellular material released as a result of viral infection may be immunogenic.

(2) A virus which buds at the cell surface may incorporate cell membrane components which may then become immunogenic by virtue of their association with the virus.

(3) The cell membrane may be altered by incorporation of viral components which might be recognised as foreign.

(4) The continued release of virus particles from the cell surface might lead to a cell-mediated immune reaction or antigen-antibody complexes at the cell surface.

Alberti, Realdi, Tremolada and Cadrobi (1975) have demonstrated HB_sAg on the cell surface in acute but not in chronic hepatitis. Chronic hepatitis is associated principally with lymphocyte infiltration, and as lymphocytes are mediators of the cellular type of immune response, the outcome of infection with HB_sAg may be partially determined by the vigour of the cell-mediated response to the infection. Those with a vigorous cell-mediated response develop acute hepatitis. A few will be overwhelmed by the infection, but the majority recover and clear the antigen. Where there is little or no cell-mediated response, due either to antigen overload or immunosuppression, the virus will continue to proliferate causing little or no damage, and circulating antigen will persist. In the intermediate situations a partial cell-mediated response may occur which is sufficient to cause some liver damage but insufficient to control the growth of the virus. Continuous cycles of virus growth and host response would result in chronic active hepatitis. It is possible that a combination of immune pathogenic responses are in operation, providing an interesting theory which requires further study. Gudat, Bianchi, Sonnabend, Thiel, Aenishaenslin and Stalder (1975) have investigated the appearance of HB_cAg in liver cell nuclei, and HB_sAg in cytoplasm

in order to identify expression patterns which, together with histologic parameters, could be integrated into four reaction types of diagnostic and prognostic importance.

In 1969, Almeida et al. reported the presence of massive immune complexes in the serum of a fatal case of fulminant hepatitis, and suggested a similarity to serum sickness, a condition where severity depends upon the balance between antigen and antibody. This theory appeared to be supported by the finding of HB_sAb or immune complexes in 90 percent of cases of acute serum hepatitis and 100 percent of chronic serum hepatitis patients (Brozosko, Madalinski, Krawczynski, Skwarska and Nowaslowski, 1971). Recently, Madalinski, Sztatchelska-Budkowska and Brzosko (1974) dissociated immune complexes from patients with acute and chronic hepatitis, using DEAE-cellulose chromatography, and showed that the antibodies belonged to the three major classes of immunoglobulins: IgG, IgM and IgA. The deposition of immune complexes in certain sites is probably responsible for non-hepatic complications. Grob, Jemelka and Muller (1971) found that HB_sAg-positive patients with active chronic hepatitis had a decreased component of C3 complement more often than HB_sAg negative patients. Nydegger, Lambert, Gerber and Miescher (1974) used ^{125}I-labelled C1q to detect the presence of immune complexes in HB_sAg carriers. No increased binding of label was found among 18 asymptomatic carriers, but 20 percent to 60 percent of sera from acute transient and chronic persistent hepatitis patients were found to bind Ciq, suggesting the presence of immune complexes. In a recent letter to Lancet, Kater, Schmitz-du Moulin and Borst-Eilers (1974) presented evidence that IgG deposited in the sinusoids of HB_sAg-positive hepatitis patients was either HB_sAb or

HB_cAb, thereby adding support to the hypothesis that immune complexes play a decisive role in the course of serum hepatitis. However, Prince and Trepo (1971) found only a weak correlation between the presence of immune complexes in the serum and liver damage, while Moulias et al. (1973) were unable to find any such association. In the few instances where high titre HB_sAb plasma has been infused into HB_sAg-positive patients or carriers, there has been no evidence of hepatocellular damage directly related to the formation of immune complexes (Lepore, McKenna, Martinez, Stutman, Bonanno and Conklin, 1972; Read et al., 1973).

CHAPTER 6

CONTROL MEASURES

I. Introduction

Control of serum hepatitis at present relies largely on the observance of safety precautions by hospital and laboratory personnel, screening of blood donations and the education of certain sections of the public, particularly drug abusers. Following outbreaks of severe liver disease among hospital and laboratory personnel, stringent safety precautions have been set out (Sutnick, London, Millman, Gerstl y and Blumberg, 1971; Percy-Robb, Proffitt and Whitby, 1970). The most important of these is the provision of adequate facilities for the handling and disposal of potentially infective material, the observance of strict hygiene among staff members, with prohibition of mouth pipetting and the liberal use of effective antiseptics. Renal dialysis units may be classified according to whether they are HB_sAg positive or negative, with appropriate restrictions on patients and staff.

II. Blood Donor Testing

There appears to be disagreement over the effectiveness of blood donor testing in reducing the incidence of posttransfusion hepatitis. A significant reduction has been reported in the East of Scotland (Cameron, 1973, personal communication) and North London (Cleghorn, 1973, personal communication) Regional Transfusion Centres. The most dramatic effect was reported at the Philadelphia General Hospital (U.S.A.) (Senior, Sutnick, Goeser, London, Dahlke and Blumberg, 1974) where the incidence was reduced from 17.9 percent to 5.6 percent by the use of I.D. for testing donor blood. On the other hand, Rienicke,

Banke and Dybkjaer (1973) and Arnt-Hanser and Pyka (1973) both report that systematic removal of HB_sAg-positive blood detected by I.D. and C.I.E.O.P. failed to reduce the incidence of post-transfusion hepatitis. It may be that more sensitive techniques are required before this complication is significantly reduced in all recipient populations. Haemagglutination (HA) and radio-immunoassay (RIA) are capable of detecting HB_sAg below the sensitivity of I.D. and C.I.E.O.P. (Shaffer et al., 1972; Ginsberg, Conrad, Bancroft, Ling and Overby, 1972). Despite the improved sensitivity, Hollinger, Aach, Gitnick, Roche and Melnick (1973) have suggested that the large scale use of RIA would not result in a mjaor reduction in cases of posttransfusion hepatitis, a suggestion which would seem to be borne out by the findings of Jennings, De Pratti, Monroe, Pollock, Watson-Williams and Claus (1973) who compared RIA and C.I.E.O.P. for detection of HB_sAg among blood donors in California, and concluded that RIA would be of little value to Blood Transfusion Services in reducing the incidence of clinical hepatitis following transfusion.

III. Passive Immunisation

Normal gamma globulin (prepared by the fractionation of pooled plasma) does not appear to be effective in the prevention of post-transfusion hepatitis. A field trial conducted in 1945 (Crossman, et al.) and a second trial reported by Mirick, Ward and McCollum (1965) suggested that normal gamma globulin could prevent or modify posttransfusion hepatitis. However, volunteer studies (Drake, Barondess, Bashe, Henle, Henle, Stokes and Pennell, 1953) and a small clinical study (Holland, Rubinson, Morrow and Schmidt, 1966) did not support this conclusion. The results of a

recent co-operative study (Grossman et al., 1970) have indicated that normal gamma globulin is entirely without value in preventing or modifying posttransfusion hepatitis. Preliminary results using gamma globulin prepared by fractionation of plasma known to contain specific HB_sAb demonstrated passive though not necessarily protective immunity in man (Prince, Szmuness, Woods and Grady, 1971; Gocke, 1971; Conrad, Young, Park, Dinger, Gerling, Bickley, Boykins, Tidwell, Petty and Knowles, 1971; Krugman, Giles and Hammond, 1971). Soulier, Blatix, Benamon, Courouce, Amouch and Drouet (1972) at the National Transfusion Centre in Paris have prepared hepatitis B immune globulin and administered it to 27 subjects, 19 of whom had received a transfusion later found to contain HB_sAg, and 8 of whom had been accidentally exposed (needle-sticks) to antigen-containing blood. The hyper-immune globulin was administered between three hours and seven days after exposure. None of the 27 recipients developed jaundice. Follow-up of 18 subjects showed significant transaminase elevations in only 2 subjects and HB_sAg was not detected in any of the 18 cases. It was concluded that passive immunisation appeared to be both effective and innocuous. Despite these encouraging reports, the use of hyper-immune HB_sAb globulin, whether administered to the patient or to the blood prior to transfusion (Katz, Rodrigues and Ward, 1971), must proceed with caution, since in theory its administration could attenuate the disease and lead to an increased risk of producing a chronic carrier state with associated liver disease. Lepore et al. (1972) infused high titre HB_sAb plasma (220-350 mls. per day) for three days into a woman who developed HB_sAg-positive fulminant hepatitis with coma shortly after giving birth. A dramatic clinical

response ensued during which the patients blood became HB_sAg negative and she continued to make a steady and ultimately complete recovery. The following year, Reed et al. (1973) investigated the effect of infusing an immunoglobulin preparation with a high titre of HB_sAb. Six patients with active chronic hepatitis received antibody intravenously. The infusions were well tolerated and only two patients showed evidence of minor and transient immune complex reactions. One patient was HB_sAg negative at the start of the investigation and he was the only one to show antibody, detectable by I.D., after infusion. Where the initial HB_sAg titres were high, little change in titre was noticed, while in two patients with initially low titres, HB_sAg was cleared from the circulation for up to 9 days. No patient showed abnormal liver function test results, suggesting that immune complexes play only a minor role, if any, in causing hepatocellular damage.

IV. Active Immunisation

Krugman and Giles (1973) investigated the effect of active as well as passive immunisation, and reported that both were associated with a protective effect, greater attenuation of the infecting agent and a decrease in the chronic carrier rate. As early as 1971, Krugman, Giles and Hammond produced relative protection by active immunisation with a boiled MS-2 virus preparation. De Gast, Houwen and Nieweg (1973) have shown specific lymphocyte stimulation by heat inactivated HB_sAg, suggesting that vaccination with such a preparation might induce not only humoral but also cell-mediated immunity. Dreesman et al. (1973) have isolated a small polypeptide by acid treatment of reduced non-alkylated HB_sAg. This polypeptide retained antigenic activity and may provide a source of material for development

of a hepatitis B vaccine (see General Discussion). The preparation of active subunits of HB_sAg has also been described by Rao and Vyas (1973) using ultra-centrifugation and chromatography. Such lines of research may lead to the preparation of a synthetic material containing the immunogenic reagent, since there is little doubt that polypeptides and other moieties such as lipoproteins can be attached to a macro-molecular carrier and used for immunisation (Sela, 1966).

V. Transfer Factor

Vyas, Ibrahim, Rao and Likhite (1974) claim to have achieved in vitro transfer of cell-mediated immunity to non-immune cells, as measured by inhibition of leucocyte migration in the presence of HB_sAg, using RNA extracted from the lymphoid tissue of immune guinea pigs. Bearing in mind the immune RNA-mediated transfer of delayed skin reactivity to tuberculin, varidase and monilia antigens (Han, 1973), these findings hold promise for clinical application of systemic transfer of immunity to HB_sAg in man.

VI. Miscellaneous

The work of Tullis, Hinman, Sproul and Nickerson (1970) suggests that the incidence of posttransfusion hepatitis can be greatly reduced by the use of frozen blood. This work has been confirmed by the results of Hayashi, Nakamura and Giorgi (1971) and Carr, De Quesada and Shires (1973). However, Werch, Grey, Hersh and Melnick (1971) found that HB_sAg could still be detected in frozen reconstituted cells if the donor had an unusually high HB_sAg titre. Lo Grippo (1969) has shown that biological products may be effectively sterilised by treatment with beta-propiolactone, particularly if this is combined with exposure to ultraviolet light.

Clinical trials including both active and passive immunisation are still in progress, and until such data have been analysed, prevention of serum hepatitis must depend largely upon the observance of measures that limit the transfer of HB_sAg from one individual to another by any of the proven or implied routes.

CHAPTER 7

ANIMAL MODELS FOR VIRAL HEPATITIS

I. Hepatitis B

A number of workers have found HB_sAg and HB_sAb among apparently naturally infected chimpanzees and gibbons (Maynard, Hartnell and Berquist, 1971; Lichter, 1969), and several subsequent studies have demonstrated the susceptibility of chimpanzees to type B hepatitis (Maynard, Berquist and Krushak, 1972; Barker, Chisari, McGrath, Dalgard, Kirschstein, Almeida, Edgington, Sharpe and Peterson, 1973; Desmyter, Liu, Somer and Mortelmans, 1973). Disease in these animals is generally mild, accompanied by enzyme elevations and with an absence of icterus or significant clinical signs (Maynard et al., 1972; Barker et al., 1973), while serologic events are remarkably similar to those seen in man (Barker, Maynard, Purcell, Hoofnagle, Berquist, London, Gerety and Krushak, 1975).

Areas in which the chimpanzee will prove a useful animal model include preliminary evaluation of the safety and effectiveness of HB-IgG and vaccines, assessment of methods of eliminating HB Ag from blood and blood products, and in the critical evaluation of infectious material for subsequent animal challenge studies. Barker, Almeida, Hoofnagle, Gerety, Jackson and McGrath (1974) have isolated and purified HB_cAg from the liver of an immunosuppressed chimpanzee which died of pneumonia following inoculation with HB_sAg. Chimpanzees may, therefore, become an important if somewhat expensive source of HB_cAg.

II. Hepatitis A

Although transmission of hepatitis A to Patas monkeys was documented by Beaxcroft in 1968 and 1969, the most convincing evidence was obtained by Deinhardt, Holmes, Capps and Popper (1966) who first reported the development of biochemically and histologically typical hepatitis A in Saguinus fuscicollis, Saguinus nigricollis and Saguinus oedipus marmosets. In subsequent experiments Holmes, Wolfe, Deinhardt and Conrad (1971) were able to repeatedly produce hepatitis in marmosets inoculated with acute phase serum from volunteers infected with the MS-1 strain of hepatitis A, but not with normal serum. Mascoli, Ittensohn, Villegas, Arguedas, Provost and Hilleman (1973) infected marmosets using specimens from patients acutely ill with epidemiologically proven hepatitis A in Costa Rica. They were able to produce hepatitis and transmit the agent serially. Convalescent serum (but not pre-exposure or acute phase serum) from patients involved in the original outbreak was found to be protective.

Purcell, Feinstone and Kapikian (1974) used immune electron microscopy to study hepatitis A. Stool extracts from patients infected with the MS-1 strain were reacted with convalescent serum from one of the volunteers. Small 27 nm virus-like particles coated with antibody were visualised in acute phase specimens but not in pre-infection specimens from five out of eight volunteers.

SECTION TWO

TECHNIQUES CURRENTLY AVAILABLE FOR HB_sAg TESTING

Introduction

The need for a simple but sensitive technique for testing large numbers of blood donors on one hand, and a very sensitive yet specific research tool on the other, has led to the development of a number of tests that differ greatly in sensitivity, specificity, simplicity and cost, each possessing its own characteristic advantages and disadvantages. Each of the techniques to be considered has been shown to be sufficiently specific, provided appropriate controls are included. It is proposed to group the various techniques into chapters according to the type of reaction involved.

CHAPTER 1

PRECIPITATION REACTIONS IN AGAROSE

I. Immunodiffusion (I.D.)

This is the technique by which HB_sAg was first detected (Blumberg, 1964), and is still employed in many laboratories because it is simple and specific and provides a useful means of establishing identity. The reactants diffuse through a medium of approximately 1 percent agarose to produce visible precipitation lines where HB_sAg and HB_sAb meet in optimal proportions. Reagents are dispensed, via capillary tubes, into wells cut in the medium. Wells may be replaced by filter paper discs soaked in reagent and placed on the agarose surface (Dequesney and Becker, 1970). Quantitation of HB_sAg is possible by allowing diffusion to occur through a medium into which HB_sAb is incorporated. The distance from the centre of the antigen well to the ring of precipitation is proportional to the antigen concentration. The sensitivity of I.D. may be increased in a number of ways:

1. Pre-filling or topping-up wells (Kim and Tilles, 1971).
2. Concentrating reagents e.g. with lyphogel (Ashcavai and Peters, 1971).
3. Use of an enhancement or reinforcement pattern (testing each serum adjacent to a positive serum) (Schmidt and Lennette, 1972).
4. Use of a closed system of hexagonal wells in conjunction with 8 percent polyethylene glycol has been claimed to increase sensitivity by a factor of 8 to 16-fold (Traavik, Siebke and Kjeldsberg, 1972).

5. Controlled evaporation from the reagent surface, a process termed 'rheophoresis' (Holper and Jambazian, 1972).

Despite the many possible modifications, I.D. remains a slow, insensitive technique uneconomic in terms of reagents. However, because of its ability to produce reactions of identity, it remains a popular method of subtyping both HB_sAg and HB_sAb, as indicated by precipitation 'spurs' (Levene et al., 1969; Gust, 1971; Le Bouvier, 1971; Boenisch and Katz, 1971; Kaplan and Grady, 1971; Mazzur, Falker and Blumberg, 1972; Le Bouvier, 1972; Le Bouvier et al., 1972; Gordon et al., 1972; Magnius and Espmark, 1972; Wenzel et al., 1972; Bancroft et al., 1972).

II. Counter-electrophoresis (C.I.E.O.P.)

This technique replaced I.D. as the most widely used in large scale testing for HB_sAg. Pairs of wells, usually 2 mm. in diameter and 3 mm. apart, are cut in agarose. Test serum (cathodic well) is caused to migrate in an electric field through a suitable medium of diffusion against a stream of HB_sAb (anodic well) migrating in the opposite direction as a result of endosmotic flow. This technique was first applied successfully to the detection of HB_sAg and HB_sAb by Bedarida, Trincheri and Carbonara (1969). Since then a number of modifications have been described (Gocke and Howe, 1970; Passendorfer, Krassnitsky and Wewalka, 1970; Prince and Burke, 1970; Vergani, 1971). The technique has also been modified for simultaneous detection of HB_sAg and HB_sAb by interposing the test sample between an HB_sAb-containing well on one side and an HB_sAg-containing well on the other (Zuckerman and Taylor, 1970). However, this may lead to the crossing-over of one of the reactants, resulting

in the formation of a false positive. A discontinuous buffer system in which buffer in the gel is at a lower ionic strength than that in the electrode vessel is reputed to improve sensitivity (Combridge and Shaw, 1970). Sensitivity may also be improved by diluting reagents in homologous species normal serum (Dreesman, Hollinger and Melnick, 1972) and by staining plates after testing with tannic acid (Hopkins and Das, 1972). Rearrangement of the wells allows counter-electrophoresis to produce reactions of identity and so be applicable to subtyping (Das, Hopkins, Cash and Cumming, 1971). The principle advantage of C.I.E.O.P. over I.D. is speed, since results are available within two hours. Both techniques suffer from the disadvantage of requiring a fixed HB_sAg-HB_sAb ratio and consequently do not take into account the possibility of false negative reactions due to either HB_sAg or HB_sAb in excess (Kohn, 1970). This source of potential errors may be eliminated by using an inhibition reaction, which is also claimed to increase sensitivity (Milner, Dobie and Grobbelaar, 1972). False positive reactions may occur due to the reaction of other antigen-antibody precipitating systems such as anti-ruminant antibody (Alter, Polesky and Holland, 1972; Lama, Ni, Krakur, Greenwalt and Levin, 1974) and lipoprotein isoprecipitins (Verrucci, Blumberg and Morganti, 1970; Blumberg, Hann, London and Yin, 1974). All positive reactions should be confirmed by tests of identity. Holland et al. (1972) have used C.I.E.O.P. for subtyping, using antiserum rendered monospecific by absorption.

CHAPTER 2

AGGLUTINATION REACTIONS

I. Latex Agglutination

Latex agglutination is now an accepted test for the presence of rheumatoid arthritis factor (Singer and Plotz, 1956) and is widely used as a test for human chorionic gonadotrophin in the urine of pregnant women (Graham and Kaush, 1967). In 1971, Leach and Ruck evaluated a commercial latex agglutination test developed by Pfizer Limited for the detection of HB_sAg. Antibody raised in guinea pigs by immunisation with purified HB_sAg was used to coat an 0.5 percent suspension of 0.8 μ, polystyrene latex spheres. The latex was stabilised with bovine serum albumin and preserved with 0.1 percent sodium azide. The preparation appeared to remain stable at $4^{\circ}C$ for at least three months. Test procedure involved 25 ul of test serum being mixed with 25 μl of normal guinea pig serum on a glass slide, before the addition of 25 μl of the antibody-coated latex reagent. The slide was then rocked to and fro for 5 minutes, by which time an HB_sAg-positive serum showed definite agglutination.

Hopkins and Das (1974) have evaluated latex reagents from two commercial sources (Pfizer Limited and Hoechst Limited (rabbit HB_sAb)) and compared them with C.I.E.O.P. for sensitivity and specificity, using sera from blood donors, patients and plasma fractions. Good agreement was obtained when testing blood donations; however, false reactions were observed with both latex preparations among hepatitis patients' sera. The small number of false negative reactions encountered were probably due to the subtype of the

antibody on the latex particles, rather than a lack of sensitivity relative to C.I.E.O.P. False positive reactions were encountered with both kits, but were more frequent with the Hoechst reagent. Such reactions may be caused by a number of factors, such as incomplete clotting of plasma or the presence of rheumatoid factor (Burrell, Dickson, Gerber, McCormick and Marmion, 1972; Ziegenfuss, jnr., 1972). Heating the test serum at 60°C for 10 minutes is claimed to reduce the false positive rate to about 3 percent (Perkins, Perkins and Vyas, 1974). The main advantage of latex agglutination is speed. In their present state of development, however, commercial latex agglutination tests for HB_sAg would seem to be unsuitable for use on patient's sera due to lack of specificity. This drawback may be partially overcome by use of a 'control' latex preparation coated with normal immunoglobulin, although the simultaneous presence of HB_sAg and non-specific agglutinins could not be ruled out.

Malin and Edwards (1972) claim to have improved specificity by coating 0.5 u diameter latex spheres with antibody of human origin. These reagents retained sensitivity for up to eight weeks when stored at 4°C. Stevens, Ziegler and Kelly (1972) described a charcoal particle agglutination-inhibition technique in which antigen was absorbed to particulate charcoal. As with latex agglutination, the HB_sAg-HB_sAb complexes caused macroscopic agglutination. The test appeared capable of detecting 1.5 µgm of HB_sAg protein per ml., making it about 10 times more sensitive than C.I.E.O.P. False positive reactions did occur.

Kachani and Gocke (1973) described an agglutination-flocculation test (AFT) for detection of HB_sAg. This test involved the

simultaneous agglutination and flocculation of HB_sAg by HB_sAb in the presence of 0.5 μ diameter acrylic particles. Preparation of reagents required the sensitisation of acrylic particles with varying concentrations of hyperimmune HB_sAb gamma globulin in borate buffer. Sensitisation was carried out at room temperature and was followed, after 1 hour, by the addition of 5 percent bovine serum albumin. The antibody-coated particles were not washed, as the presence of excess antibody in the fluid phase produced simultaneous flocculation, leading to improved sensitivity. Test sera were heated at 56°C for 10 minutes, then 25 μl. placed into each of six wells in a standard agglutination tray, and 25 μl. of foetal calf serum added to prevent weak non-specific reactions. 25 μl. of bromophenol blue was added to each well before mixing with an applicator stick. Finally, 25 μl. of each of the antibody-coated particle preparations was added to the appropriate wells and the plate mixed on a rotostat for 30 minutes before being read. The authors claimed the technique to be 8-fold more sensitive than RIA and 1000-fold more sensitive than C.I.E.O.P. There are, however, numerous disadvantages which make this test unsuitable for blood donor testing:-

1. Each sample must be tested a number of times, against different concentrations of antibody, to overcome prozone reactions.
2. High-titre rheumatoid sera are a source of false positive reactions.
3. Hyperimmune antibody of high specific activity is crucial for coating the acrylic particles. This would necessitate the immunisation of animals with pure HB_sAg, and require fractionation of the resultant antibody.

4. Sera need to be heated prior to testing to remove a thermolabile inhibitor of agglutination-flocculation which is present in most normal sera.

II. Haemagglutination*(HA) and Haemagglutination-Inhibition (HAI).

In 1969, Juji and Yokahi published details of an HA technique in which formalinised erythrocytes were sensitised with purified HB_sAb and used to detect HB_sAg by direct haemagglutination*. The purified HB_sAb was prepared by a complex procedure involving the formation of immune complexes, which were then dissociated and the HB_sAb isolated by centrifugation and chromatography. Formalinised group 'O' Rh. positive human erythrocytes were tanned and coated with the purified HB_sAb. The sensitised cells were resuspended to 0.5 percent in phosphate buffered saline (P.B.S.), pH 7.2, containing 1 percent normal rabbit serum. Tests were conducted in disposable plates with 'U'-bottomed wells, reagents being added in 25 µl. volumes. After mixing the plates were left overnight at room temperature. This technique was found to be more sensitive than I.D., but unfortunately it was not evaluated for use in mass donor testing due to a high incidence of false positive reactions among the negative controls. It was also found that the sensitised cells deteriorated rapidly if stored at $4^{o}C$.

The following year, Vyas and Shulman (1970) described an HAI test in which fresh human erythrocytes were sensitised with highly purified HB_sAg. These cells were used for measuring HB_sAb

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* sometimes called reverse passive haemagglutination (RPHA).

by direct HA and HB_sAg by an inhibition reaction. Plasma was collected from asymptomatic HB_sAg carriers and the antigen isolated by ultra-centrifugation using density gradients. The purified antigen gave a single band in agarose and, when concentrated 10-fold, gave no precipitation reaction with rabbit antiserum against whole human plasma. Human group 'O' erythrocytes, collected in anticoagulent and washed in saline, were made up to a 40 percent suspension and incubated with purified antigen and chromic chloride, the latter acting as a coupling reagent. A factor affecting sensitivity of the coated cells was found to be the temperature at which sensitisation occurred, this being optimum between 22°C and 41°C (Hawkes, 1973). The sensitised cells were made up to a 0.2 percent suspension in PBS pH 7.3 containing 0.5 percent bovine serum albumin, 0.0025 percent polyvinylpyrolidine (PVP) and Tween 80 at a concentration of 1 in 20,000. The tests were conducted in microtitre plates with 'V'-bottomed wells, using 25 µl. volumes of reagents. The inhibition reaction, used to test for HB_sAg, used 10 units of HB_sAb activity. Sensitivity for HB_sAb detection was approximately 2000 times greater than I.D., while sensitivity for HB_sAg was approximately 120 times that of C.I.E.O.P. The technique initially gained acceptance in many laboratories since its sensitivity compared favourably with C.I.E.O.P. (Shaffer et al., 1972). To achieve reproducible results, Prince et al. (1971) found it necessary to store the sensitised cells in liquid nitrogen until required. Reesink, Duimel and Brummelhuis (1973) also found it necessary to modify the technique in order to obtain a degree of reproducibility. They recommend that the following conditions should be observed:

1. Careful selection of donor cells, as only about 5 percent of donor blood is suitable for sensitisation.
2. Improvement of the chromic chloride coupling, allowing a less pure HB_sAg preparation to be used. Their proposed method of antigen purification involved Freon extraction, absorption and elution from Aerosil, gel filtration and extensive dialysis followed by concentration.
3. The use of a special preserving fluid which allowed the sensitised cells to be stored at 4°C for up to six weeks.

Kwak, Sturgeon and Gitnick (1973) have successfully automated the test using a sampling rate of 60 tests per hours, results being available within 30 minutes. Gold et al. (1974) have applied the test to subtyping HB_sAb in terms of anti-d and anti-y specificity by using a blocking antigen of known subtype.

In an attempt to circumvent the need for density gradient centrifugation, Nelson, Phipps, Watson, Watts and Zwolenski (1973) have extracted HB_sAg from serum with Aerosil 200 and subsequently purified it by column fractionation, pepsin digestion and filtration through a 0.22 μ. millipore filter. Prior to sensitisation the HB_sAg was inactivated by heating at 98°C for one minute. The sensitised cells were found to be unstable unless stored in liquid nitrogen. A cell suspension of 2.5 percent was used in a Technicon autoanalyser, the agglutination pattern being recorded on absorbant paper. Sensitivity for HB_sAg detection was only 4 times greater than C.I.E.O.P., while the sensitivity for HB_sAb detection was 32 times better.

Since publication of the work of Juji and Yokochi (1969), the literature has, until recently, contained no reports concerning the

use of HB_sAb-coated cells for the detection of HB_sAg. In 1973, however, Hirata, Emerick and Boley published details of such a technique using purified guinea pig antibody coupled to double-aldehyde-treated cells. Human type 'O' erythrocytes were stabilised by treatment with pyruvic aldehyde followed by formaldehyde. The stabilised cells were then incubated with a relatively low ionic strength buffer (0.1M phosphate, pH 7.2) containing antibody. Test sera were diluted in phosphate buffer containing gelatin, and tests conducted in 'v'-bottomed microtitre plates. Sensitivity was reported to be considerably greater than C.I.E.O.P. The test has been modified to subtype HB_sAg by coating cells with monospecific antibody prepared with the aid of an immunoabsorbant (Hollinger, Wabi, Dreesman and Melnick, 1973).

At the present time, three commercial companies are offering HB_sAg test kits based on this principle. Table 1(2)2.1. compares the characteristics of each of the products.

Addendum.

In 1969 Avrameas, Taudou and Chuilon (Immunochemistry. 6, 67) published data concerning different methods for coupling antigens to erythrocytes.

TABLE 1(2).2.I.

Comparison of Commercial R.P.H.A. Kits

	Hepatest	Hepanosticon	Auscell
Supplied by	Wellcome	Organon	Abbott
Cell species used	Turkey	Sheep	Human
HB_sAb species used	Horse	Sheep	G.pig.
Test vehicle	u-plate	u-tube	v-plate
Test volume	25 µl.	25 µl.	25 µl.
Incubation time	30-60 mins.	3 hours	3 hours
Final serum dilution	1 in 8	1 in 16	1 in 16
Approx. sensitivity relative to I.D.	1000	400	1000
Approx. cost per test	8-12p.	10p.	16p.

CHAPTER 3

RADIOIMMUNOASSAY (RIA)

I. Introduction

A number of techniques have been described, most of which use a radioactive marker, usually HB_sAg labelled with ^{125}Iodine. HB_sAg is purified by ultra-centrifugation and gel chromatography before being radiolabelled by the chloramine-T method of Hunter and Greenwood (1962). After labelling, it is necessary to separate free label and labelled serum proteins from labelled HB_sAg. This is usually achieved by column chromatography. Most radioimmunoassays are based upon competition for antibody binding sites between standard labelled antigen and any unlabelled antigen which may be present in the serum being tested. The major difference between techniques is the manner in which the immune precipitate of antibody-bound HB_sAg is separated from free HB_sAg after incubation.

II. Double Antibody Precipitation (D.A.-RIA)

The commonest means of separation is the double antibody technique (Aach, Grisham and Parker, 1971; Coller, Millman, Halbherr and Blumberg, 1971; Hollinger, Vorndam and Dreesman, 1971; Lander, Alter and Purcell, 1971). The serum to be tested is incubated with serum containing HB_sAb and labelled HB_sAg. This is followed by the addition of an anti-gamma-globulin to co-precipitate the HB_sAg bound to the first antibody. After incubation the co-precipitate is isolated by centrifugation and counted in an automatic gamma counter. If HB_sAg is present in the test serum it will compete with the labelled HB_sAg for HB_sAb combining sites, thereby reducing the amount of radioactivity in the co-precipitate as compared to simultaneously run negative sera. Detection of circulating HB_sAb involves

incubating the test serum with labelled HB_sAg and then adding an anti-human-IgG. Immune complexes are isolated and counted as for HB_sAg detection. If HB_sAb is present in the test sample, it binds labelled HB_sAg causing a marked increase in radioactivity in the co-precipitate in comparison to control sera. Walsh, Yalow and Berson (1970) used paper chromatoelectrophoresis for the separation of free and antibody bound HB_sAg. This technique takes advantage of the differences in mobility of free and bound HB_sAg in an electric field.

A major disadvantage of D.A.-RIA is the time taken to complete the test, since the standard procedure for detection of the HB_sAg requires 2 to 4 days.

III. Solid Phase (S.P.-RIA)

Attempts to reduce the time factor have led to the development of solid phase techniques (Ling and Overby, 1972; Ginsberg, Bancroft and Conrad, 1972). One such test (marketted by Abbott Laboratories Ltd., under the name AUSRIA-1) involves placing 0.1 ml. of test sample on the bottom of a polypropylene tube coated with purified hyperimmune HB_sAb (usually raised in guinea pigs). The tubes are then capped and incubated for 3 to 16 hours, depending on temperature. The serum sample is aspirated and the bottom of the tube rinsed 5 times with tris-HCl buffer before the addition of 0.1 ml. of ^{125}I-labelled HB_sAb. The tube is then capped and incubated for a further 90 minutes, after which it is once again rinsed 5 times and counted in an automatic gamma counter for 1 minute. For each group of tests 10 control tubes containing normal human serum are included and the standard deviation of the counts of the negative control tubes computed. HB_sAg present in the test serum should complex with the

specific HB_sAb in the tube and not be removed during washing. The labelled antibody should then complex with another site on the bound HB_sAg particle and remain in the tube during subsequent washing, thus raising the radioactivity count above that of the negative control tubes. In theory this technique should be capable of detecting HB_sAg having only two HB_sAb-binding sites available. This may be somewhat optimistic, although in practice the test has proved to be considerably more sensitive than C.I.E.O.P. (Hansson and Johnsson, 1974). Count rates for the negative control tubes are generally less than 0.3 percent of the radioactivity used. A factor of 5 standard deviations from the negative control mean is used to determine whether a serum is positive or negative, i.e. sera having count rates higher than the mean of the negative controls plus 5 standard deviations are considered positive. AUSRIA-1 has recently been superseded by an improved kit (AUSRIA-2) in which the unlabelled HB_sAb is absorbed on to polystyrene beads resulting in an increased surface area and a theoretically improved sensitivity. The solid phase system can be employed for the detection of HB_sAb by using HB_sAg-coated beads in conjunction with labelled HB_sAg (Ginsberg et al., 1972). Purcell, Wong, Alter and Holland (1973) adapted the S.P.-RIA for use with microtitre serological equipment. Polyvinyl 'V'-bottomed microtitre plates serve as the solid phase into each well of which 75 µl. of an appropriate dilution of HB_sAb was added and the plate incubated for 4 hours at 4°C. Residual HB_sAb was aspirated by washing twice with saline and 0.2 ml. of bovine serum albumin added. After a further overnight incubation at 4°C the plates were again washed with saline and the residual fluid aspirated from the wells. The test was performed by adding 25 µl. of sample to the bottom of each well, followed by an overnight incubation at 4°C. The following

day the wells were washed 5 times with saline, and 50 µl. of ^{125}I-labelled HB_sAb added. The plate was then incubated by rocking gently at 37°C for 4 hours. The wells were once again washed with saline before being cut out and placed in the tube of an automatic gamma counter and counted for 4 to 20 minutes each. Results were expressed as the ratio of residual counts in the sample well to the mean residual count of wells that received a sample known to be HB_sAg negative. Radio-labelled HB_sAb was prepared from both human and guinea pig sera. The most sensitive system was obtained using HB_sAb of guinea pig origin, although this system also exhibited many false positive results. When human HB_sAb was used specificity improved but at the expense of sensitivity.

Figenschau and Ulstrup (1974) have described a technique in which Protein-A-carrying Staphylococcus aureus is used as a solid phase anti-gamma globulin reagent. The test is easy to perform, being complete in two hours. The competition principle is used in the test for HB_sAg, while HB_sAb is tested by the direct binding of labelled HB_sAg.

IV. Radioelectrocomplexing

Simons (1973) described a simple, sensitive, rapid and relatively inexpensive radioelectrocomplexing technique which involved complexing radio-labelled HB_sAg or HB_sAb with unlabelled HB_sAg or HB_sAb as a result of counter-directional movement produced during electrophoresis. Microscope slides were covered with 4 ml. of 1 percent agar in 0.05M barbital buffer pH 8.6, and two wells 3 mm. diameter and 4 mm. apart were punched as 4 parallel pairs in the central area of each slide. To detect HB_sAb, 5 µl. of undiluted test serum and labelled HB_sAg were pipetted into the anodic and

cathodic wells respectively. After electrophoresis (10 mA, 200 volts per slide for 90 minutes), the agar was cut into three zones (HB_sAg well zone, inter-well zone and HB_sAb well zone) and transferred to gamma spectrometer tubes. The percentage distribution of label in the three zones was calculated from these values and the radioelectrocomplexing index determined. In the absence of HB_sAb, labelled HB_sAg moved through the inter-well zone and into the HB_sAb well zone. When HB_sAb was present, the labelled HB_sAg was confined to the inter-well zone and the HB_sAg well zone.

CHAPTER 4

MICROSCOPY

I. Electron Microscopy (E.M.)

The technique of negative staining has been used by many workers to study the fine structure of HB_sAg (Bayer et al., 1968; Dane et al., 1970). Direct examination of negatively stained preparations is particularly useful for the demonstration of naturally occurring immune complexes that may prove difficult to identify by rountine serological procedures. The addition of HB_sAb (Immune Electron Microscopy, I.E.M.) is, however, preferable for diagnostic identification of samples in which the three characteristic morphological forms of the HB_sAg are not readily visible. A technique described by Kelen, Hathaway and McCleod (1971) offers a rapid and relatively simple procedure for this purpose. Specimens were diluted 1 in 2 or 1 in 4 and incubated with an equal volume of HB_sAb, then placed on an agar surface. A formvar grid was floated on the surface of the drop. The fluid phase diffused into the agar causing any immune complexes present to be concentrated onto the surface of the grid, which was subsequently stained and viewed. Mayerick and Smith (1973) have described a similar technique using agarose and polyethylene glycol to concentrate the test serum. They claim a sensitivity similar to complement fixation.

While E.M. is essential for studying the morphological characteristics of HB_sAg, it has certain disadvantages, being an elaborate technique requiring specialised personnel and expensive equipment. It has limited application and is time consuming since each sample should be scanned for at least 15 minutes before being recorded as negative. A recent publication by Sama, Benz, Aach,

Hacker and Kaplan (1973) reported 8 false positive samples among patients suffering from primary biliary cirrhosis, and implied that E.M. alone is an unreliable test for HB_sAg.

II. Immunofluorescence (I.F.)

The technique of immunofluorescence has been used to identify HB_sAg in cells from liver, spleen, bone marrow, testis and mesentery (Millman, Zavatone, Gerstley and Blumberg, 1969) from patients with HB_sAg-positive hepatitis. Cells were stained directly with specific HB_sAb conjugated with fluorescine isothiocyanate and the specificity determined by blocking with unconjugated HB_sAb. Similar methods have been used to detect HB_sAg in sections of liver from patients with circulating HB_sAg (Noweslowski, Brzosko, Madalinski and Krawczynski, 1970) and in cultured human liver cells inoculated with HB_sAg-positive serum (Brighton, Taylor and Zuckerman, 1971). The technique is difficult to master, particularly when looking for HB_sAg in mammalian haemopoietic tissues due to their 'sticky' nature. It has the advantage of being able to detect cell-localised HB_sAg, which would be otherwise undetectable except by thin section E.M. or autoradiography. Immunofluorescence is a complex technique involving much washing and is not readily applicable to testing large numbers within a short space of time. Brighton et al. (1971) used the technique to demonstrate HB_sAg in cultured hepatocytes following inoculation, and were able to demonstrate progressive changes spreading from cytoplasm to nucleoli.

CHAPTER 5

MEASUREMENT OF CELL-MEDIATED IMMUNE RESPONSE

I. Lymphocyte Transformation

This test provides a sensitive means of detecting previous infection when specific antibodies are not detectable in the circulation by such sensitive tests as HA and RIA. Lymphocyte transformation was first applied to HB_sAg by Laiwah (1971) using phytohaemagglutinin (PHA), purified protein derivative (PPD), and serum rich in HB_sAg, as transforming agents. Partially purified lymphocytes from 20 ml. of venous blood were resuspended in Eagles medium containing 20 percent normal human serum to give an approximate cell concentration of 6×10^6 cells per ml. All lymphocyte cultures were set up in duplicate, each tube containing lymphocyte suspension, Eagles medium, penicillin and streptomycin. Nothing further was added to the control cultures. Each test was stimulated with (a) PHA, (b) PPD, (c) HB_sAg. All tubes were incubated for 120 hours at $37^{\circ}C$. Assessment of transformation was measured by uptake of ^{14}C-labelled thymidine, added 18 hours before harvesting (i.e. after 102 hours incubation), determined in a scintillation counter. In a recent publication, Laiwah, Chaudhari and Anderson (1973) have shown that purified HB_sAg is equally as good a stimulant as HB_sAg-rich serum in both lymphocyte transformation and leucocyte migration inhibition. Pettigrew, Goudie, Russell and Chaudhari (1972) were able to confirm the findings of Laiwah (1971), but noted that all HB_sAg-rich sera were not equally effective in stimulating the transformation of sensitised lymphocytes. They found evidence of cell-mediated immunity in all eleven cases of chronic alcoholic liver disease studied; this leads them to suggest that HB_sAg may be an aetiological factor in the development of alcoholic cirrhosis.

II. Leucocyte Migration Inhibition

This technique may, in common with lymphocyte transformation, be used to assay the state of cell-mediated immune responsiveness of an individual to HB_sAg. The test described by Laiwah et al. (1973) is a modification of that reported earlier by Sorborg and Bendixen (1967). Leucocytes at a concentration of approximately 1.2×10^8 cells per ml. were mixed with either normal (control) serum or HB_sAg-rich serum and incubated at 37°C for 90 minutes. Capillary tubes of 50 µl. capacity were then filled with the incubated cell suspension and one end sealed. They were spun at 900 g. for 5 minutes and the packed cell layers separated by breaking the capillary tubes just below the cell-fluid interphase. The stubs containing the cells were immediately laid on the floor of migration chambers containing Eagles medium. After incubation at 37°C for 18 hours the area of cell migration was magnified by a projection microscope and measured by planimetry. The effect of the stimulating agent on cell migration was expressed as the Migration Index (M.I.), where

$$M.I. = \frac{\text{mean migration area of stimulated cultures}}{\text{mean migration area of control cultures}}$$

Ibrahim, Vyas and Perkins (1975) have used this technique in conjunction with HA and RIA to study the immune response to HB_sAg in normal persons, HB_sAg carriers, acute HB_sAg-positive hepatitis patients and convalescent hepatitis B patients. Their findings suggest that HB_sAg is eliminated rapidly by a cell-mediated immune response, detectable for a limited period, followed by an HB_sAb response in relatively few patients more than 3 months after the clearance of circulating HB_sAg.

CHAPTER 6

MISCELLANEOUS TECHNIQUES

I. Complement Fixation (C.F.)

The serum to be tested is mixed with complement and HB_sAb (or HB_sAg). If an antigen-antibody reaction occurs complement will be 'fixed' as demonstrated by testing for residual complement. A number of procedures are available, although the micro-technique has been most widely used for HB_sAg testing (Purcell, Holland, Walsh, Wong, Morrow and Chanock, 1969; Shulman and Barker, 1969; Taylor, 1970). Overnight incubation at 4°C provides a more sensitive but often less specific test, while incubation at 37°C for only 60 minutes results in a less sensitive but more specific reaction. The test for HB_sAg has been automated (Sturgeon, Kwak, and Gitnick, 1971; Kwak, Gitnick and Sturgeon, 1973). Sera containing high concentrations of HB_sAg may not react at low dilutions due to a prozone phenomenon, therefore test sera should be assayed over a range of dilutions. HB_sAg and HB_sAb may be present in the same specimen as immune complexes (Almeida et al., 1969), a situation which may result in anti-complementary activity (Shulman and Barker, 1969). Ross and Pringle (1971) have found that heating the test serum at 85°C for 1 hour may liberate HB_sAg, HB_sAb being destroyed at this temperature. C.F. is slightly more sensitive than C.I.E.O.P. for detection of HB_sAg, but approximately equivalent for the detection of HB_sAb.

II. Immune Adherence Haemagglutination (I.A.H.)

The phenomenon of immune adherence was first described by Laveran and Mesnic (1901) and was based on the observation that complexes of antigen, antibody and complement adhere to primate

erythrocytes. An I.A.H. assay for HB_sAg has been described by Mayumi, Okochi and Nishioka (1971), and is reputed to be 100 times more sensitive than C.F., but only 10 times more sensitive for the detection of HB_sAb (Nishioka, 1972). The major disadvantage of this technique is that large prozones are observed when the HB_sAg titre is high, and for this reason it is necessary to test sera over a wide range of dilutions. Conversely, if the HB_sAg concentration is low the presence of normal serum complement C^3 inactivator may inhibit the reaction, causing a false negative result.

III. Platelet Aggregation

The interaction of HB_sAg-HB_sAb immune complexes on the surface of blood platelets results in their aggregation under certain conditions, with a consequent change in their settling patterns (Melartin, Myllyla and Penttinen, 1970). Unfortunately, individual batches of platelets vary in their sensitivity to immune complexes, thus application of this principle for large scale HB_sAg testing is not recommended. False reactions due to non-immunological factors may also occur.

SECTION THREE

AIMS OF PROJECT

I. Introduction

While 'third generation' assay techniques, such as R.P.H.A., HAI and RIA, are beginning to be seriously evaluated by some Blood Transfusion Centres, many still rely on C.I.E.O.P. to detect HB_sAg carriers among their populations. The short-comings of C.I.E.O.P. have already been mentioned (Part One, Section Two, Chapter 1, II), and the advantages and disadvantages of R.P.H.A., HAI and RIA are discussed in Part One (Section Two, Chapter 3). It is hoped that this project will provide a simple, sensitive, rapid and economic test for HB_sAg suitable for adoption by any Blood Transfusion Centre.

II. General Aim

To improve HB_sAg testing facilities within the Blood Transfusion Service, and at the same time avoid the necessity of purchasing large quantities of expensive commercial reagents.

III. Specific Aims

1. Development of a simple, sensitive and economic HB_sAg test suitable for assaying large numbers of blood donations in a relatively short time, thereby causing the minimum of delay to fresh blood required for transfusion and/or processing.

2. Evaluation of the above test in terms of sensitivity and specificity by (a) comparison with R.P.H.A. and RIA using established HB_sAg panels, and (b) comparison with C.I.E.O.P. in determining the incidence of HB_sAg and HB_sAb in the blood donor population of the South-East Region of Scotland.

3. Application of the test to HB_sAg subtyping, thereby providing essential confirmatory and useful epidemiological information.

4. Modification of the test for detection of Hepatitis B core antibody (HB_cAb).

5. Re-investigation of C.I.E.O.P.-negative donations implicated in posttransfusion hepatitis.

6. Follow-up of recipients of hyperimmune HB_sAb gamma globulin given as a preventative measure in cases of accidental (usually needle-stick) exposure for HB_sAg.

P A R T T W O

Basic developments.

I. Introduction

The association of HB_sAg with posttransfusion hepatitis stimulated demand for a rapid means of testing blood donations prior to use. At one time, C.I.E.O.P. fulfilled the requirement admirably since it used inexpensive equipment, was easy to perform and allowed fresh blood to be used within hours of donation, an important consideration concerning the availability of labile products such as cryoprecipitate and platelets. As the technology of HB_sAg testing became more sophisticated and sensitive, the inadequacies of C.I.E.O.P. became apparent. Commercial R.P.H.A. and RIA tests are available but at a price which may prove prohibitive to some centres. Direct haemagglutination tests suffer nonspecific false positive reactions, while RIA introduces the additional hazard of isotope handling, is time-consuming and requires expensive counting equipment

The decision to undertake the development of a haemagglutination test designed specifically for the requirements of the Blood Transfusion Service was guided by the following considerations:

1. Haemagglutination (HA) and haemagglutination-inhibition (HAI) are sensitive techniques which have been in use in one form or another for many years within the Blood Transfusion Service. They are, on the whole, simple to perform and easy to read, requiring no sophisticated or expensive equipment.

2. Basic reagents (erythrocytes, HB_sAg, HB_sAb, normal serum) are in abundant supply at most centres as a result of current C.I.E.O.P. testing.

3. The stable nature of 'fixed' erythrocytes could lead to standardisation of testing within and between centres if reagents are prepared in bulk. This would be much more difficult with a test such as RIA where labelling and standardisation must be performed at relatively short intervals.

4. There is every possibility of eventual automation, since many Blood Transfusion Centres are gaining experience in the use of autoanalysers for various routine blood bank assays.

A basic concept for tanning and sensitisation of erythrocytes will be outlined and followed by a series of experiments designed to determine the optimal conditions of a number of variables. The resultant modified procedure will then be described in detail and compared for sensitivity and specificity with current techniques.

II. Brief Background to Erythrocyte Sensitisation

Red blood cells are convenient, passive carriers of antigen (Hirata and Stashak, 1965; Sindo and Wakakura, 1952), acting as markers which allow agglutination by specific antibody to be observed with the naked eye. If the cells are tanned before being sensitised with antigen they are more readily agglutinated by specific antibody (Shiari, 1964). Treatment with tannic acid increases the instability of the cells causing a normally non-agglutinating reaction to result in agglutination (Pirofsky, Cordova and Imel, 1962). Once treated with tannic acid at the right concentration the cells will exhibit agglutination settling patterns. The tendency to agglutinate may be balanced by adding a stabilising agent such as normal serum so that autoagglutination is just cancelled out. The cells are thus in a very sensitive state, ready to agglutinate in the presence of a very small amount of specific antibody. This form of passive

haemagglutination is quite distinct from the active haemagglutination seen with Influenza Virus (Francis, 1947), where the virus alone is responsible for causing certain types of erythrocytes to agglutinate, since it actively absorbs onto receptor sites on the erythrocyte surface.

Felton, Francis and Scott (1961) used passive haemagglutination to study Herpes Simplex Virus. They showed that pH and tannic acid concentration were critical, while the age of the antigen and the temperature and time of sensitisation were more flexible, allowing some diversity of performance and application. A few years earlier, Scott, Felton and Barney (1957) had shown that the concentration of antigen used for sensitisation had a direct effect upon the HA titre of specific antibody, the titre being reduced when lesser concentrations of antigen were used.

III. Initial (Starting) Procedure for Preparation of HB_sAg-coated Human Erythrocytes

1. HB_sAg (C.I.E.O.P.-positive) serum was treated with beta-propiolactone and ultra-violet light in an attempt at inactivation.

2. The antigen was pelleted by centrifugation (120,000 g. av. for 5 hours at 20°C), washed and resuspended in 1/20th of the original volume, heated at 60°C for 30 minutes and stored at -20°C until required.

3. Ten mls. of human group O rhesus negative blood was collected in anticoagulant and allowed to stand overnight at 4°C.

4. The blood was washed three times in physiological saline and made up to a 6.6 percent suspension in phosphate buffered saline (P.B.S.) pH 7.2 and an equal volume of 1 in 10,000 tannic acid was added before incubating at 37°C for 15 minutes.

5. The tanned cells were washed in P.B.S. pH 6.4 and re-suspended to a 4 percent suspension in the same buffer prior to adding HB_sAg in a ratio of 4:1 (v/v; cells; HB_sAg). The mixture was then incubated at room temperature (approx. 20°C) for 60 minutes.

6. The coated cells were washed three times in stabilising buffer (P.B.S. pH 7.2 containing 2 percent normal human serum) and allowed to stand overnight at 4°C as a 1 percent cell suspension.

IV. Initial Test Procedures

HB_sAb was tested by direct haemagglutination of antigen coated cells, while HB_sAg was tested by an inhibition reaction. Both reactions were performed in disposable u-bottomed microtitre plates using 25 µl. volumes of reagent. Positive reactions suggestive of HB_sAb were confirmed by (a) retesting with pooled-plasma-coated cells and (b) demonstrating that activity could be inhibited by HB_sAg but not normal serum. Positive reactions suggestive of HB_sAg were confirmed by testing the inhibitory effect of specific antibody compared with normal serum.

V. Experiments to Determine the Optimum Conditions for Tanning, Sensitisation and Performance of the Test

1. Effect of tannic acid concentration on sensitivity

Different concentrations of freshly prepared tannic acid (M & B batch no. 06743) in P.B.S. (Appendix A) pH 7.2 were used to tan erythrocytes in a 37°C water bath for 15 minutes. The effect upon sensitivity of the HA test is shown in table 2.V.1. At a 1 in 5,000 concentration cell lysis occurred, while a 1 in 50,000 concentration proved too weak as evidenced by a decline in the ability to detect HB_sAb. Tannic acid at a concentration of 1 in 10,000 proved optimal. It is suggested that this type of experiment be

repeated for different brands of tannic acid, and indeed each batch, since experience has shown that considerable variation exists (the B.D.H. product was satisfactory at 1 in 5,000, while MERC tannic acid appeared to be unsatisfactory at any concentration).

Table 2.V.1. Effect of tannic acid concentration on sensitivity

Tannic acid concentration	Reciprocal of HB_sAb titre (H.M.)
1 in 5,000	cell lysis
1 in 10,000	4,096
1 in 20,000	1,024
1 in 50,000	8

2. Effect of HB_sAg concentration (for cell coating) on sensitivity

Partially purified HB_sAg was prepared as previously described. The final pellet was resuspended in 1/20th its original volume and serially diluted in PBS pH 6.4. Each dilution was titrated using latex agglutination (Pfizer batch no. L13/3) and C.I.E.O.P. (using antiserum T2160C supplied by Hoechst Ltd.) Each HB_sAg dilution served to sensitise an aliquot of cells, the results of which are given in table 2.V.2. Optimal sensitivity was observed using the dilution of partially purified antigen containing 128-256 latex units (4-8 C.I.E.O.P. units) of HB_sAg activity. If more HB_sAg was coated onto the cells autoagglutination ensued, while reduction of HB_sAg on the cell surface reduced sensitivity.

Table 2.V.2 Effect of HB_sAg concentration (for cell coating) on sensitivity

Reciprocal of HB_sAg dilution	Reciprocal of HB_sAg titre (latex units)	Reciprocal of HB_sAg titre (CIEOP units)	Reciprocal of HB_sAb titre (T2160C)	Negative control
1	512	16		auto-agglutination
2	256	8	20,480	good
4	128	4	20,480	good
8	64	2	5,120	good
16	32	-	1,280	good
32	16	-	40	good
64	8	-	-	good
128	4	-	-	good

3. Effect of temperature and time of sensitisation on sensitivity

Aliquots of tanned cells were incubated with optimal concentrations of HB_sAg at four different temperatures each for a period of one hour. Further aliquots of cells were then sensitised for varying times at optimum temperature. Tables 2.V.3a and 2.V.3b present data indicating that sensitisation is most efficient over the temperature range 20°C-37°C, while at that temperature maximum HB_sAg coating of the cells was obtained after only 60 minutes. Longer sensitisation times did not improve sensitivity.

Table 2.V.3a Effect of temperature of sensitisation on sensitivity

Temperature °C for 1 hour	Reciprocal of HB_sAb titre (H.M.)
4	400
20	4,000
37	4,000
60	1,000

Table 2.V.3b Effect of time of sensitisation on sensitivity

Time (minutes)	Reciprocal of HB_sAb titre (H.M.)
5	200
15	400
30	1,000
60	4,000
90	4,000
120	4,000

4. Effect of purity of HB_sAg used for cell coating on sensitivity

Four aliquots of HB_sAg were prepared and adjusted to contain optimal concentrations of antigen.

(a) HB_sAg in normal serum.

(b) Partially purified HB_sAg prepared as just described.

(c) Partially purified HB_sAg prepared by polyethylene glycol extraction and filtration through Sephadex G200 (Appendix B).

(d) Purified HB_sAg prepared by density gradient centrifugation (Appendix C) and normally used for radiolabelling.

HB_sAg from each preparation was used to coat tanned cells. Coating unpurified HB_sAg resulted in autoagglutination of the sensitised cells(table 2.V.4)possibly due to the excess of protein resulting from the presence of normal serum. Preparations (b) and (c) showed optimal sensitisation, while the highly purified HB_sAg exhibited a much reduced sensitivity. An interesting observation was that within a few days of sensitisation, cells coated with (c) and (d) had lost most of their activity, presumably as a result of HB_sAg eluting from the cell surface, suggesting that over-purification is to be avoided. It may be that a certain amount of non-specific protein is required to allow a stable attachment between antigen and cell, excess leading to autoagglutination.

Table 2.V.4. Effect of purity of HB_sAg used for cell sensitisation on sensitivity

HB_sAg preparation	Reciprocal of HB_sAb titre (T2160C)
(a) unpurified	autoagglutination
(b) pelleted/washed	20,680
(c) polyethylene glycol/G200	20,680
(d) density gradient centrifugation	320

5. Effect of sensitised cell concentration on sensitivity

Sensitised cells were made up to different concentrations in stabilising buffer and used to titrate an HB_sAb-positive serum. Table 2.V.5. shows that the 0.75 percent cell suspension combined good sensitivity with ease of reading. Increasing the cell concentration reduced sensitivity, while using fewer cells resulted in poor readability.

Table 2.V.5. Effect of sensitised cell concentration on sensitivity

Cell concentration (percent)	Reciprocal of HB_sAb titre (T2160C)	Readability
2	5,120	good
1	20,480	good
0.75	40,960	good
0.5	40,960	good
0.25	unreadable	unreadable

6. Effect of using serum or plasma as test sample

Two blood samples were taken from each of five accredited donors (i.e. donors of long standing, where previous donations have not been implicated in posttransfusion hepatitis). One sample was allowed to clot in order to obtain natural serum, while the second sample was collected in anticoagulant in order to obtain plasma. Each serum and plasma sample was then serially diluted and tested against coated cells. The results in table 2.V.6 indicate that even in the absence of HB_sAb the use of plasma could lead to non-specific false positive reactions. Such reactions were not evident even with high concentrations of serum.

Table 2.V.6. Effect of using plasma or serum as test sample

Accredited donors	Reciprocal of serum/plasma dilution 2	4	8	16	32	64	128	256
1.plasma	+	+	+	-	-	-	-	-
serum	-	-	-	-	-	-	-	-
2.plasma	+	+	+	±	-	-	-	-
serum	-	-	-	-	-	-	-	-
3.plasma	+	+	+	+	-	-	-	-
serum	-	-	-	-	-	-	-	-
4.plasma	+	+	+	+	-	-	-	-
serum	-	-	-	-	-	-	-	-
5.plasma	+	+	±	-	-	-	-	-
serum	-	-	-	-	-	-	-	-

\+ positive agglutination
\- negative agglutination
± weak agglutination

7. Effect of collecting fresh blood in different anti-coagulants on the suitability of cells for sensitisation

Fresh group 'O' rhesus negative blood from the same donor was collected in four different anticoagulants, stored at 4°C overnight, tanned and sensitised with HB_sAg. Only blood collected in lithium-heparin was found to be unsuitable as evidenced by autoagglutination of the sensitised cells (table 2.V.7).

Table 2.V.7. Effect of collecting fresh blood in different anti-coagulants on suitability of cells for sensitisation

Anticoagulant	Reciprocal of HB_sAb titre (T2160)
Heparin	32,000
A.C.D.	32,000
E.D.T.A.	32,000
Lithium-heparin	autoagglutination

A.C.D. = acid citrate dextrose
E.D.T.A. = ethylene diamine tetra acetic acid

8. Variation among different batches of HB_sAg sensitised cells

Serum from 10 different HB_sAg carriers (8 adw, 2 ayw) were prepared for cell sensitisation and each preparation used to sensitise an aliquot of cells from each of three cell donors. The data in table 2.V.8 suggests that the techniques for HB_sAg preparation, cell tanning and sensitisation are sufficiently reproducible to be able to produce a standardised reagent. The prozone type of phenomenon observed with preparation 5 remains unexplained. However, this could be overcome by mixing with any of the other preparations.

Table 2.V.8. Variation among different batches of HB_sAg sensitised cells

Batch no.	HB_sAg source	HB_sAg subtype	Donor cell batch	Reciprocal of HB_sAb titre (H.M.) 10	20	40	80	160	320	640	1280	2560	5120	10240
1	Patient	ayw	a	+	+	+	+	+	+	+	+	-	-	-
			b	+	+	+	+	+	+	+	+	-	-	-
			c	+	+	+	+	+	+	+	+	-	-	-
2	Donor	ayw	a	+	+	+	+	+	+	+	+	-	-	-
			b	+	+	+	+	+	+	+	+	-	-	-
			c	+	+	+	+	+	+	+	+	-	-	-
3	Donor	adw	a	+	+	+	+	+	+	+	+	+	+	-
			b	+	+	+	+	+	+	+	+	+	+	-
			c	+	+	+	+	+	+	+	+	+	+	-
4	Donor	adw	a	+	+	+	+	+	+	+	+	+	+	-
			b	+	+	+	+	+	+	+	+	+	+	-
			c	+	+	+	+	+	+	+	+	+	+	-
5	Donor	adw	a	-	-	-	+	+	+	+	+	+	+	-
			b	-	-	±	+	+	+	+	+	+	+	-
			c	-	-	-	+	+	+	+	+	+	+	-
6	Donor	adw	a	+	+	+	+	+	+	+	+	+	+	-
			b	+	+	+	+	+	+	+	+	+	+	-
			c	+	+	+	+	+	+	+	+	+	+	-
7	Donor	adw	a	+	+	+	+	+	+	+	+	+	±	-
			b	+	+	+	+	+	+	+	+	+	+	-
			c	+	+	+	+	+	+	+	+	+	+	-
8	Donor	adw	a	+	+	+	+	+	+	+	+	+	+	-
			b	+	+	+	+	+	+	+	+	+	+	-
			c	+	+	+	+	+	+	+	+	+	+	-
9	Donor	adw	a	+	+	+	+	+	+	+	+	+	-	-
			b	+	+	+	+	+	+	+	+	+	-	-
			c	+	+	+	+	+	+	+	+	+	-	-
10	Donor	adw	a	+	+	+	+	+	+	+	+	+	-	-
			b	+	+	+	+	+	+	+	+	+	-	-
			c	+	+	+	+	+	+	+	+	±	±	-

\+ = positive agglutination
\- = negative agglutination
± = weak agglutination

9. Effect of HB_sAb concentration on HAI sensitivity

An HB_sAb-positive serum was titrated and the last dilution to show complete agglutination was considered to represent 1 HA unit of HB_sAb activity (i.e. a doubling dilution back would contain 2 HA units of activity etc.) The endpoint of an HB_sAg titration was determined using 2, 4, 8 and 16 units of HA activity. Table 2.V.9 indicates that the sensitivity of the inhibition reaction is increased as the amount of HB_sAb decreases. However, there comes a point at which the dilution effect of adding test serum and cells results in premature collapsing of the agglutination pattern leading to false HB_sAg positive results.

Table 2.V.9. Effect of HB_sAb concentration on HAI sensitivity

HA units of HB_sAb activity	Reciprocal of HB_sAg titre (B.D.4)
2	premature collapsing
4	1,000
8	800
16	200

10. Effect of pre-incubation time on HAI sensitivity

HB_sAg positive serum was titrated by pre-incubating with 4 HA units of HB_sAb activity at 37°C for varying time intervals prior to the addition of sensitised cells. Table 2.V.10 shows that maximum inhibition of HB_sAb under these conditions occurs after 30 minutes. Further pre-incubation did not enhance sensitivity.

Table 2.V.10. Effect of pre-incubation time on HAI sensitivity

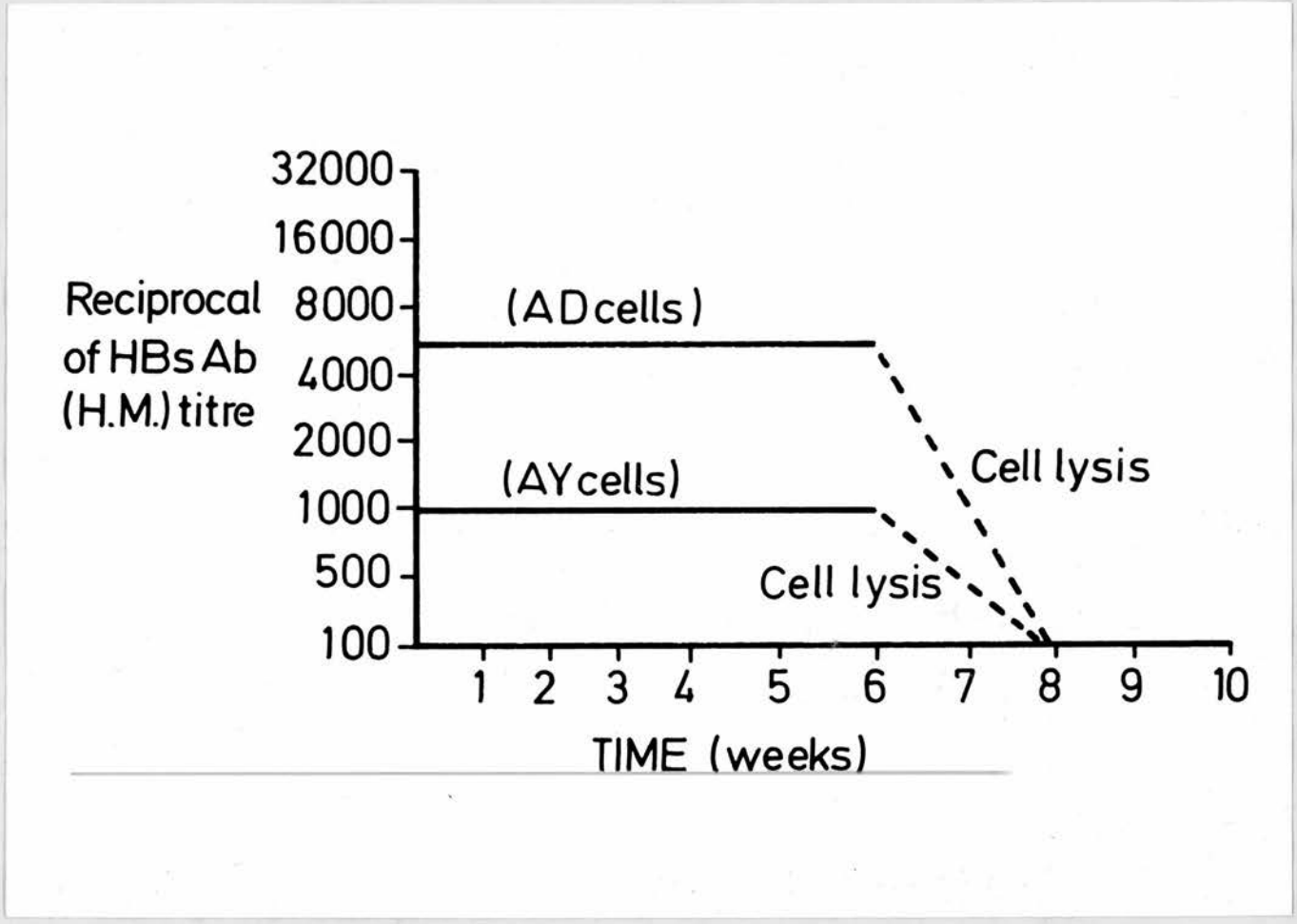
Pre-incubation time	Reciprocal of HB_sAg titre (B.D.4)
0	100
15 mins.	800
30 mins.	2,000
60 mins.	2,000
120 mins.	2,000
240 mins.(RT)	2,000
overnight ($4^{\circ}C$)	2,000

R.T. = room temperature

11. Effect of storage (at $4^{\circ}C$) on sensitivity

Freshly prepared sensitised cells were kept at $4^{\circ}C$ until cell lysis was apparent (approximately 7.5 weeks). At intervals during storage the cells were titrated against an HB_sAb-positive serum. Some cells were frozen in liquid nitrogen in the presence of dimethylsulphoxide (DMSO) to prevent the formation of disruptive ice crystals. At $4^{\circ}C$ the cells retained sensitivity for approximately 6 weeks after which rapid deterioration was observed due to cell lysis caused by ageing (Fig.2.V.11). Storage in liquid nitrogen proved unsuitable as approximately 80 percent of the cells showed lysis during subsequent washing to remove DMSO.

Figure 2.V.11. Effect of storage (at 4°C) on sensitivity



VI. Final (Detailed) Procedure for Preparation of HB_sAg-sensitised Human Erythrocytes

In the light of data obtained from the preceding experiments, the following procedure was adopted for preparation of HB_sAg, tanning and sensitisation of cells.

1. Preparation of HB_sAg

HB_sAg was obtained by bleeding a carrier into a dry bottle and allowing the blood to clot. HB_sAg-positive serum was recovered and treated with betapropiolactone and ultra-violet light

(Appendix D) according to the method of Lo Grippo and Hartmen (1958) and Lo Grippo (1973). The treated serum was then spun at 120,000 g. (av) for 5 hours on an M.S.E. Superspeed 75 centrifuge using an 8 x 25 ml. titanium angle head rotor. The resultant pellet was resuspended overnight in 2 mls. physiological saline followed by gentle mixing with a pasteur pipette before being transferred to a new centrifuge tube and made up to 16 mls. with saline. The suspension was then re-pelleted as before and the second pellet resuspended in 0.5 mls. PBS pH 6.4. The concentration of HB_sAg was then adjusted to 128-256 latex units by addition of PBS pH 6.4, whereupon the preparation was heated to 60°C for 30 minutes, then divided into 0.5 ml. aliquots and stored at -20°C until required.

2. Tanning and sensitisation

Human group O rhesus negative venous blood was collected in A.C.D. and allowed to stand overnight at 4°C. The following day 3 mls. were washed three times in 12 mls. of sterile physiological saline (M.S.E. Minor bench centrifuge no. 3 for 5 minutes.) 0.08 mls. of packed, washed cells were resuspended in 1.25 mls. PBS pH 7.2 (6.6 percent cell suspension) and incubated with an equal volume of freshly prepared 1 in 10,000 tannic acid (M & B batch no. 06743) prepared in the same buffer. The mixture was incubated in a 37°C waterbath for 15 minutes, shaking gently every few minutes to ensure thorough mixing. The tanned cells were pelleted (M.S.E. Minor bench centrifuge no. 3 for 1 minute) and washed once in 2.5 mls. PBS pH 6.4 before being resuspended in 2 mls. PBS pH 6.4 (i.e. 4 percent cell suspension). 0.5 mls. prepared HB_sAg was added to the tanned cells (i.e. 4 volumes cells:1 volume HB_sAg) and the sensitising

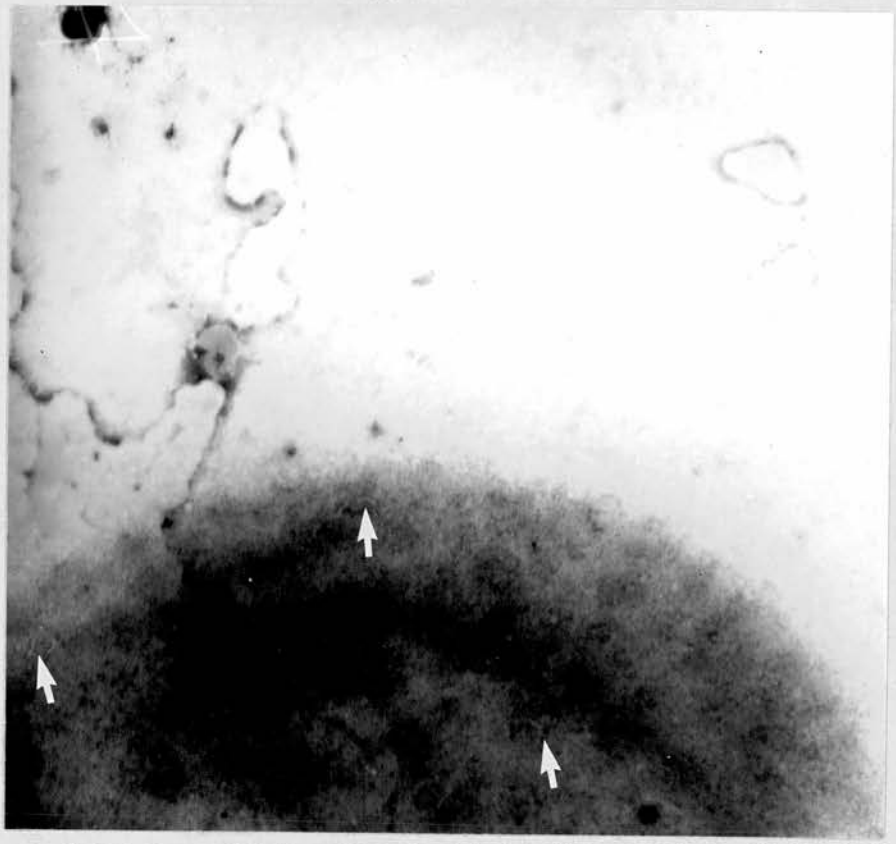
mixture incubated for 60 minutes at room temperature (approximately 22°C) using either a Luckham rotostat or a Mattburn mixer to ensure thorough mixing. The sensitised cells were washed three times in 3 mls. of cell diluent (PBS pH 7.2 containing 2 percent normal human serum), made up to a 1 percent cell suspension and left at 4°C overnight. Next morning, the supernatant was replaced with fresh cell diluent and the cells stored at 4°C until required. Photograph 2.vi.1 shows HB_sAg absorbed onto the surface of a red cell. Cells from the same source were sensitised with a 1 in 125 dilution of pooled human plasma and used as 'control' reagents.

VII. Final Test Procedure

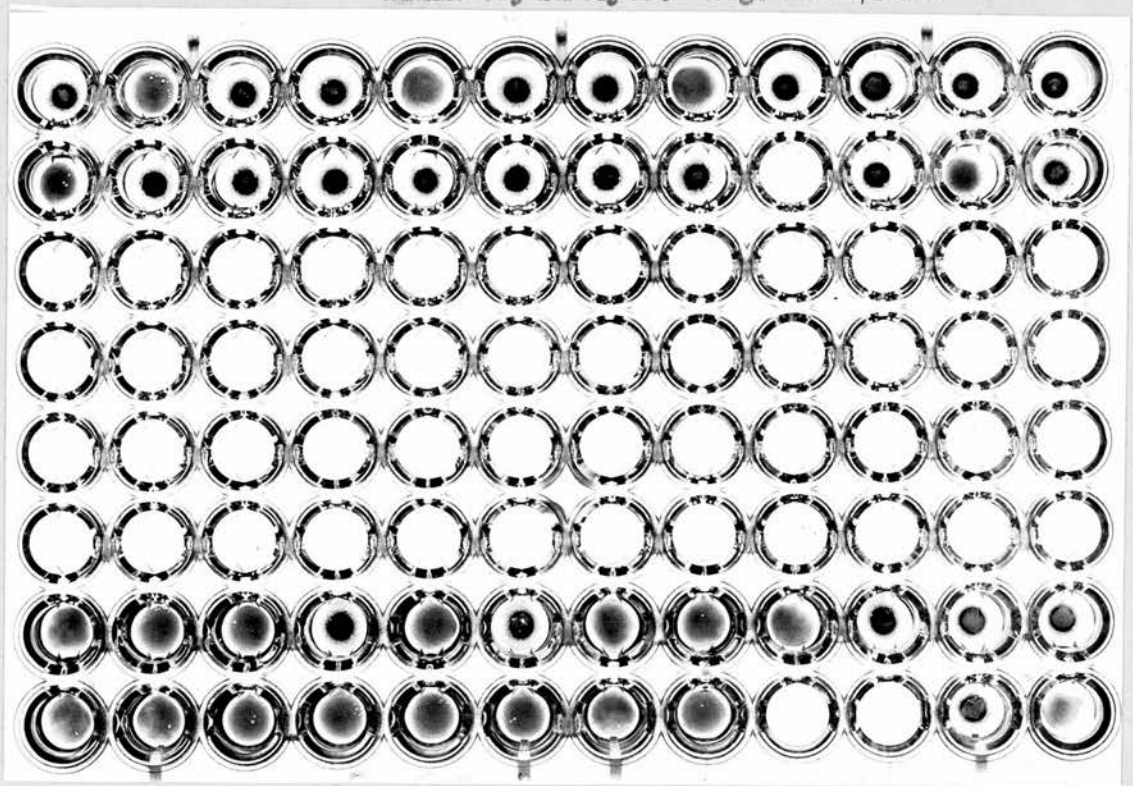
Tests were performed in disposable u-bottomed microtitre plates (Flow Laboratories) into which reagents were added in 25 ul. volumes using a pasteur pipette. Serum specimens were diluted to 1 in 10 in PBS pH 7.2 prior to testing.

(a) Direct haemagglutination test for HB_sAb

25 µl. of test serum dilution (1 in 10) was pipetted into a well and 25 µl. of 0.75 percent HB_sAg-sensitised cells added. Each plate contained a positive control (antiserum + sensitised cells) and a negative control (normal serum + sensitised cells). The plate was gently shaken to ensure thorough mixing and incubated for 60 minutes in a 37°C incubator. The presence of detectable HB_sAb was characterised by a smooth matt of agglutinated cells covering the bottom of the well, while a compact button of cells indicated that no HB_sAb could be detected (photograph 2.vi.4). Positive reactions were confirmed by retesting with plasma-sensitised 'control' cells, and by showing that activity could be inhibited by HB_sAg but not normal serum.



Photograph 2.VI.1. HB_sAg adsorbed onto the surface of tanned human erythrocyte. Mag. = 25,000



Photograph 2.VII.1. Haemagglutination settling patterns in u-bottomed wells.

(b) Haemagglutination-inhibition test for HB_sAg

An HB_sAb-positive serum was serially diluted in PBS pH 7.2 containing 10 percent normal human serum and the end-point (i.e. the lowest concentration producing complete agglutination) determined. This was defined as one haemagglutinating unit (1 HA unit). To test for HB_sAg, 25 µl. of test serum dilution were mixed with 25 µl. of HB_sAb containing 4 HA units of activity for 30 minutes at 37°C. 25 µl. of HB_sAg-sensitised cells were then added and after gentle mixing, the plate was re-incubated for a further 60 minutes. HB_sAg in the test sample would neutralise the 4 HA units of antibody activity and cause the sensitised cells to fall as a negative button. Absence of HB_sAg in the test serum would leave the agglutinating ability of the 4 HA units intact, leading to a positive agglutination pattern. A positive control (antiserum + HB_sAg + sensitised cells) and a negative control (antiserum + normal serum + sensitised cells) were included in each plate. Positive reactions were confirmed by demonstrating the ability of the sample to reduce the titre of HB_sAb as compared with normal serum.

VIII. Sensitivity of HA and HAI

The ability of the inhibition reaction to detect HB_sAg (pool of subtypes adw/ayw) was compared with I.D., C.I.E.O.P. (Hopkins and Das, 1972), latex agglutination (Leach and Ruck, 1971), using two commercial reagents (Pfizer Limited: Hoechst Limited), I.E.M. (Kelen et al., 1971), RIA-SP (Ausria-I) using reagents from Abbott Laboratories Limited (Kent) and RIA-DA based on the test described by Lander et al. (1971) and performed by the Virus Diagnostic Laboratory at Edinburgh University Medical School.

The sensitivity of the direct haemagglutination test for HB_sAb was assessed by comparing the 'end-point titre' of a known positive serum as determined by HA and C.I.E.O.P.

Figure 2.VIII.a indicates that HAI exhibits a degree of sensitivity similar to both RIA techniques, being more sensitive than I.D., C.I.E.O.P., latex agglutination and I.E.M. The direct HA test is capable of detecting very low levels of HB_sAb compared with C.I.E.O.P. (figure 2.VIII.b).

Figure 2.VIII.a. Relative sensitivity of HAI

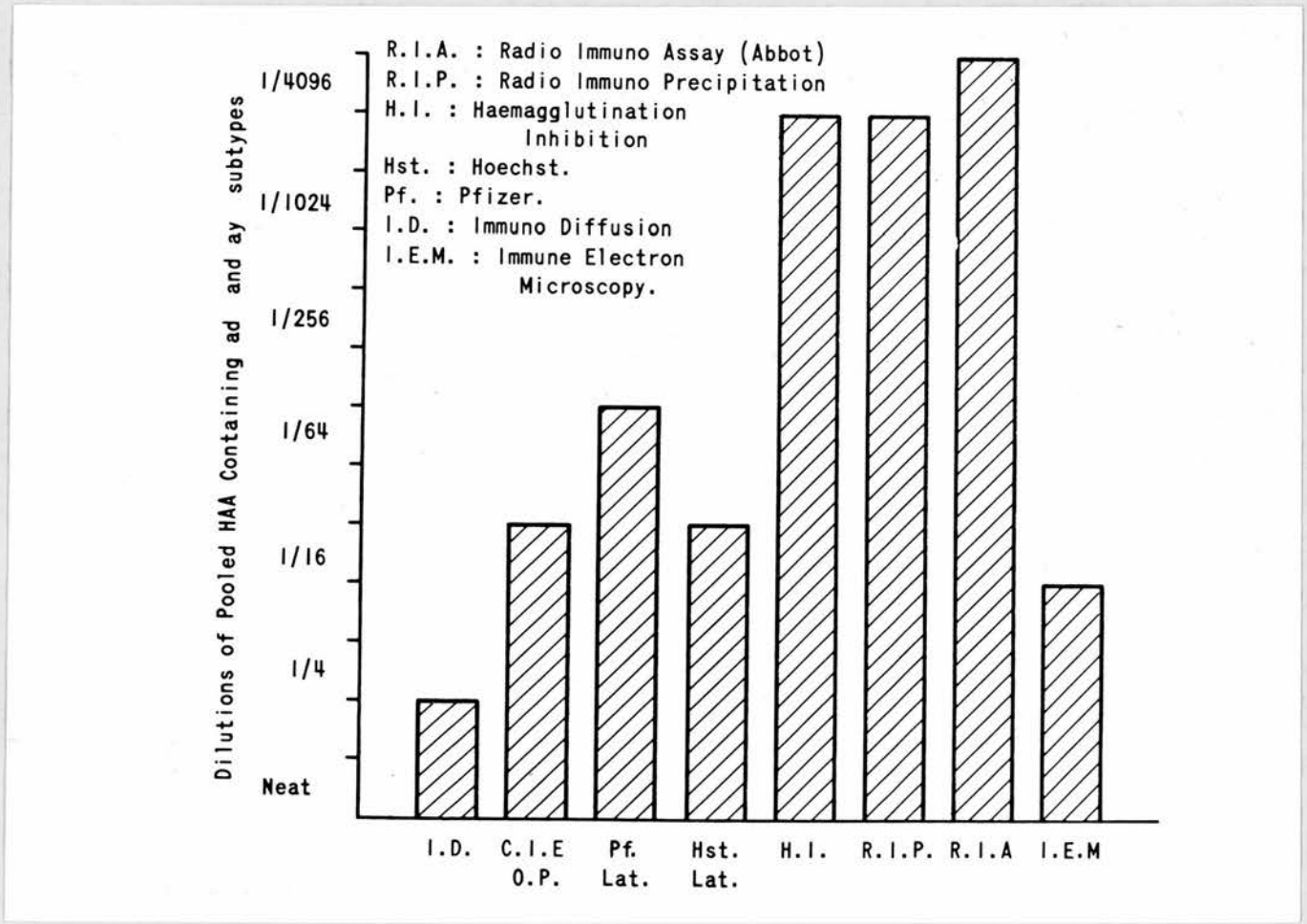
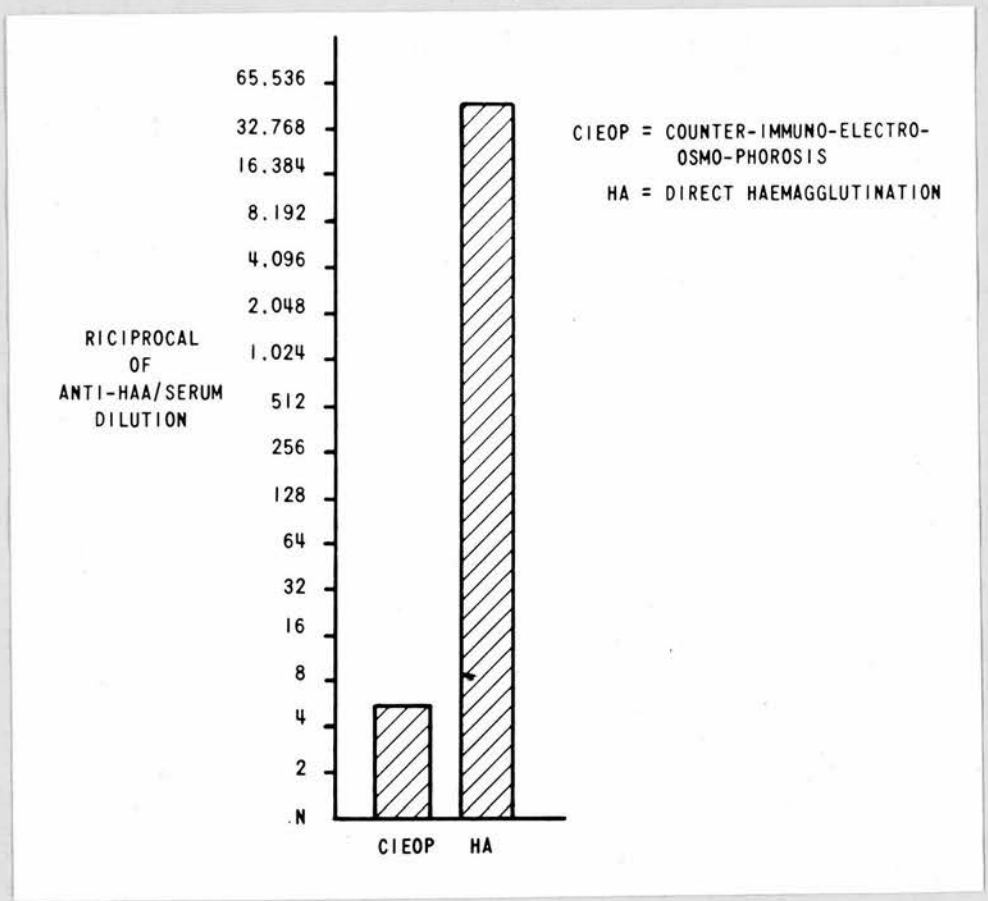


Figure 2.VIII.b. Relative sensitivity of HA



IX. Specificity of HAI and HA

The specificity of HAI was tested by coding HB_sAg-positive and HB_sAg-negative sera and distributing them randomly among the test sera.

Figure 2.IX.a shows the results obtained when HB_sAg-positive sera were coded, mixed with HB_sAg-negative sera and tested by a variety of techniques. HAI detected all positive samples as did C.I.E.O.P. and RIA, and gave no false positive reactions, while one batch of latex, I.D. and I.E.M. failed to detect several of the positive sera, and latex reagents from both commercial sources gave false positive reactions.

Table 2.IX.b confirms the specificity of HA and HAI by showing complete agreement with C.I.E.O.P. when sera from selected blood donors and dialysis patients were tested for antigen and antibody. In two patients, low levels of antibody capable of agglutinating HB_sAg-sensitised cells were in fact due to reactions against serum protein(s) as demonstrated by the agglutination of plasma-sensitised cells. This antibody did not inhibit the reactivity of HB_sAg.

Figure 2.IX.a. Results obtained when HB_sAg-positive and HB_sAg-negative sera were coded and tested by a variety of techniques

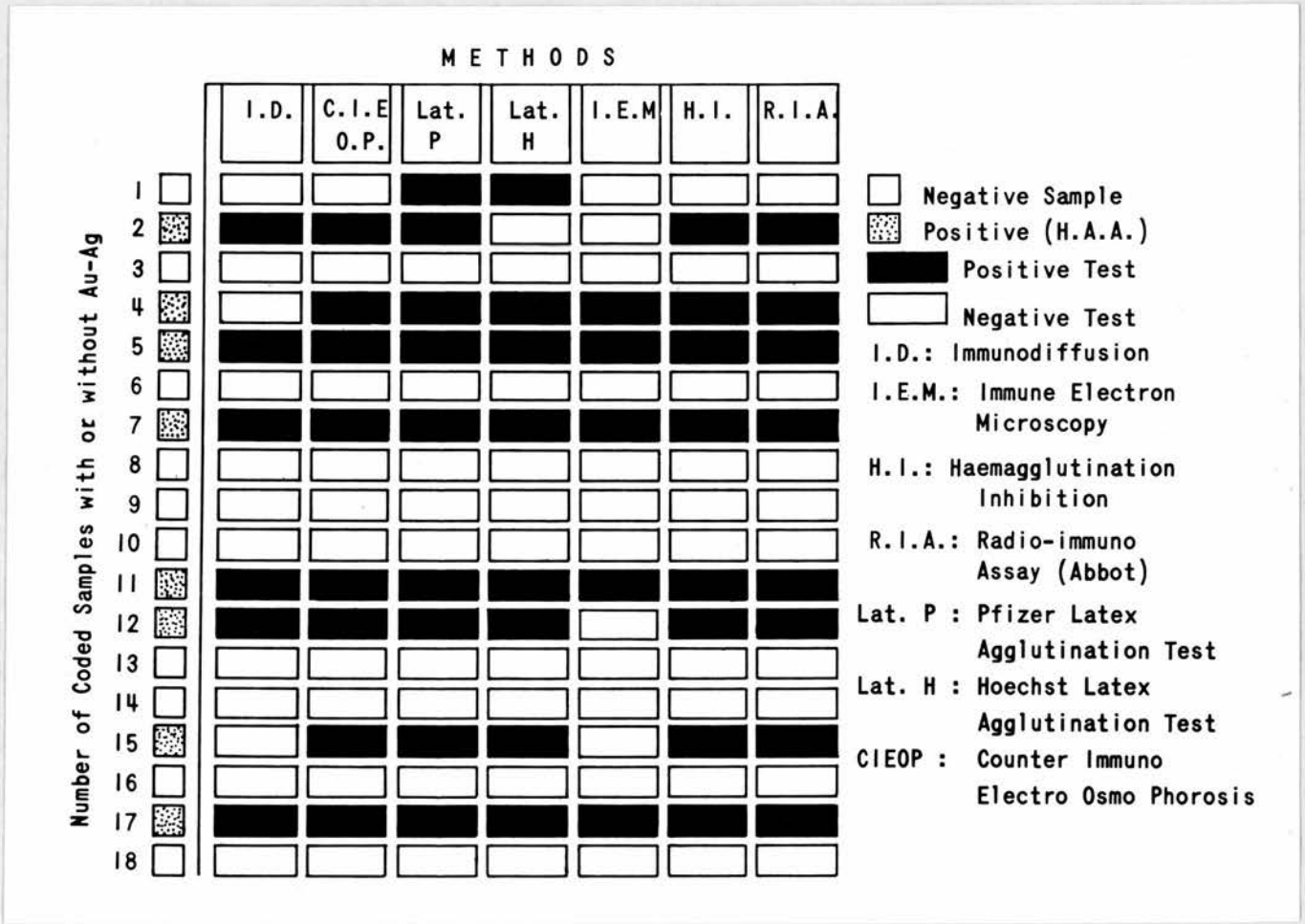


Table 2.IX.b. Results obtained when sera from selected blood donors and dialysis patients were tested for HB_sAg and HB_sAb by HAI, HA and C.I.E.O.P.

	No. tested	C.I.E.O.P.		HAI/HA		
		HB_sAb	HB_sAg	HB_sAb	HB_sAg	'Plasma reaction'
Donors	76	4	7	4	7	0
Dialysis patients	14	2	0	2	0	2*

* Different samples from those possessing HB_sAb

X. Discussion

The agglutination of particulate antigen by its specific antibody is probably the simplest way of estimating the quantity of that antibody in serum. Some soluble antigens can be coated onto inert materials which then react as if they themselves possessed the specificity of the antigen. Red blood cells are convenient passive carriers of antigens since it is possible to coat almost any antigen onto their complex surface, thus providing a sensitive indicator of specific antibody activity. Correctly sensitised cells lend themselves to the observation of settling patterns, e.g. in the wells of perspex trays or in glass tubes.

Many antigens may be effectively coated onto red cells merely by exposing the cells to antigen for a period of time, but sensitivity of the resultant cell is greatly improved if their agglutinability has been increased by previous tanning (Boyden, 1951). To avoid difficulties due to the presence in the test sera of heterophile agglutinins, it is sensible to use homologous species cells wherever possible. The haemagglutination test described in this thesis was designed to test human serum for HB_sAb and HB_sAg, and therefore uses human group O rhesus negative erythrocytes, partly because they are easily obtainable, but mainly because there is little chance of a test serum possessing antibody to either the blood group or rhesus determinants.

There is, of course, the possibility that if antigen coated cells are kell positive, the presence of anti-kell in the test serum could lead to haemagglutination in the absence of an HB_sAg-HB_sAb reaction. This possibility is, however, remote since a saline-reacting anti-kell is uncommon and tannic acid treatment would tend to reduce such activity by altering the cell surface. The use of

fixed cells would further reduce the likelihood of such misleading reactions. It is also unlikely that antibodies to the HL-A system would lead to misinterpretation in hepatitis B testing since such antibodies, even if present are not haemagglutinating.

It is generally assumed that tannic acid acts upon cells in such a way as to cause them to take up protein antigens. However, this may not be its main function since cells will adsorb antigen and become agglutinable without the aid of tannic acid (Hirata and Stashak, 1965; Sindo and Wakakura, 1952) though the sensitivity of such preparations is usually low. The quantity of protein antigen attached to the cell increases after tannic acid treatment (Shioiri, 1964) but its chief function appears to be to increase the instability of the cells so making a normally non-agglutinating reaction result in agglutination. In this respect, the role of tannic acid may be analogous to that of some enzymes, for it has been observed that the red blood cells of some cold-blooded animals may absorb bacterial antigens but are not agglutinated by the homologous antibody unless they have been altered in some way by the action of proteolytic enzymes (Neter, Cohen, Westphal and Lüderitz, 1959). Once treated with tannic acid at the right concentration, red cells will show agglutination settling patterns. This tendency to agglutinate may be balanced by adding normal serum as a stabiliser (Atkin, 1909) so that it is just cancelled out. The coated cells are thus in a very sensitive state ready to agglutinate in the presence of a very small amount of antibody, hence the great sensitivity of the test. The greater the concentration of tannic acid used, the greater the agglutinability of the cells. However, they must not be made so agglutinable that they cannot be stabilised. Some batches of cells may be abnormally sensitive to the action of tannic acid, and to avoid autoagglutination or cell lysis,

it is advisable to carry out tests using different concentrations of tannic acid for each 'source' of cells. Tanning is usually carried out at 37°C (George and Vaughan, 1962; Stavitsky, 1954) but appears to be effective in the cold (Shioiri, 1964) or at room temperature (Boyden and Sorkin, 1955). A period of approximately 15 minutes is considered sufficient for the reaction. There is evidence that it is completed very rapidly, increased time of exposure having little effect on the sensitivity of the cells produced (Herbert, 1967). After tanning a single wash was found sifficient to remove excess reagent.

In order to successfully coat HB_sAg onto tanned human red cells, it was necessary to adhere closely to predetermined criteria relating to antigen concentration and degree of purity. Of particular interest was the finding that optimal coating required partially purified (pelleted, washed) HB_sAg. The autoagglutination observed when neat HB_sAg-containing serum was used probably reflects the influence of excess non-specific protein. At the other extreme, the poor sensitivity and rapid deterioration in activity of cells coated with highly purified HB_sAg suggests that a certain amount of non-specific protein is required to facilitate and stabilise the adsorption of HB_sAg onto the tanned red cell surface.

The apparent absence of marked variation among different sensitised cell batches avoids dependence upon a select group of red cell donors and reflects the inherent stability of the technique. As is the case with many haemagglutination tests, serum as opposed to plasma is the preferred test material. In this particular technique, blood collected in anticoagulent may be converted to a useable serum by the addition of commercial bovine thrombin, while serum prepared by recalcification invariably leads to autoagglutination of the HB_sAg-coated cells.

The relatively high sensitivity of the HA and HAI tests should improve efficiency of HB_sAb and HB_sAg detectability and lead to a greater economy of specific antiserum for antigen testing. Despite the inherent specificity of an inhibition reaction, experience in a variety of other biological systems, including hormone estimation where the tanned cell haemagglutination system has been used extensively, has shown that non-specific inhibition can occur despite the use of specific antibody (Stavitsky and Ingraham, 1964). However, the specificity of the present HAI test seems to be acceptable

It is probable that a proportion of the normal, apparently healthy population may show positive reactions with HB_sAg-coated cells due to low levels of antibody directed against or cross-reacting with plasma proteins. Brumelhuis (1971, personal communication) found 13 such reactions in 30,000 samples tested by the Netherlands Red Cross Transfusion Service using I.D. Multi-transfused patients are likely to produce such reactions due to the development of iso-precipitins (Blumberg, 1964; Langenhuysen, 1971). The use of highly purified HB_sAg for cell coating may overcome this problem, but as has already been shown, this is not practicable. Therefore, sera showing agglutination with HB_sAg-coated cells should be retested against 'control' cells coated with pooled human plasma, when a specific HB_sAb will show no agglutination.

Antigen may also be attached to red cells by means of a chemical bond. Jandl and Simmons (1957) reported a method of coupling proteins to red cells by the use of metallic cations. They found the most useful of them to be chromium. Vyas and Shulman (1970) adopted this principle to couple HB_sAg to red cells using chromic chloride. The logistics of application of this technique for large scale testing are not practicable due to the high degree of purity of HB_sAg required (Vyas et al., 1972) and the great variability observed between different

batches of cells (Reesink and Duimiel, 1973). Furthermore, it is desirable to use ~~treated~~ (inactivated ?) HB_sAg for coating cells which will subsequently be used as a routine test reagent. In our experience, the outcome of coupling betaprone-ultra-violet light-irradiated HB_sAg to human erythrocytes by chromic chloride has proved unreliable.

It is hoped that the tanned cell HA/HAI technique described will be a step toward fulfilling the current HB_sAg/HB_sAb testing requirements of the Blood Transfusion Service.

PART THREE

MODIFICATIONS OF BASIC TEST

CHAPTER 1

SUBTYPING BY HAI

I. Introduction

The foregoing has resulted in a simple, efficient and economic test for HB_sAg and HB_sAb. Despite the apparent specificity of the inhibition principle, it is of utmost importance to obtain confirmatory data on any sample giving an HB_sAg positive result. One could recommend that a sample be shown to be positive by at least one other technique. Such a principle may have been acceptable when the commonly used tests were I.D. and C.I.E.O.P. where it was a relatively simple matter to show identity with known positive controls (Das et al., 1971). With 'third generation' methodology, however, a situation may arise where a sample is positive only by HAI, RPHA or RIA, the latter two being particularly prone to false positive reactions. In such circumstances, the ability to identify a positively reacting sample as belonging to one of the established subtypes would provide valuable confirmatory information.

II. Preparation of Monospecific Antisera

A number of HB_sAb positive sera were tested by C.I.E.O.P. against HB_sAg previously subtyped by Dr. G.L. Le Bouvier (Yale University, U.S.A.) One antiserum reacted only with HB_sAg possessing the 'd' antigenic determinant, suggesting the presence of anti-d (and presumably anti-a) in the antibody positive serum. Anti-a and anti-w were neutralised by concentrating the antiserum five times with lyphogel (Gelman) and incubating with different concentrations of HB_sAg (ay) at room temperature for one hour followed by $4^{\circ}C$ overnight. Most of the resulting immune complex was removed by centrifugation at 20,000 r.p.m. (Sorval RC 2B) for one hour. The supernatant was tested

for the presence of anti-d by direct haemagglutination using HB_sAg (ad) coated cells (figure 3.1.II.a). The absence of anti-ay activity was demonstrated by the absence of agglutination of HB_sAg (ay) coated cells (table 3.1.II.b).

Figure 3.1.II.a. Titration of HB_sAb (anti-d) following adsorption with different concentrations of HB_sAg (ay)

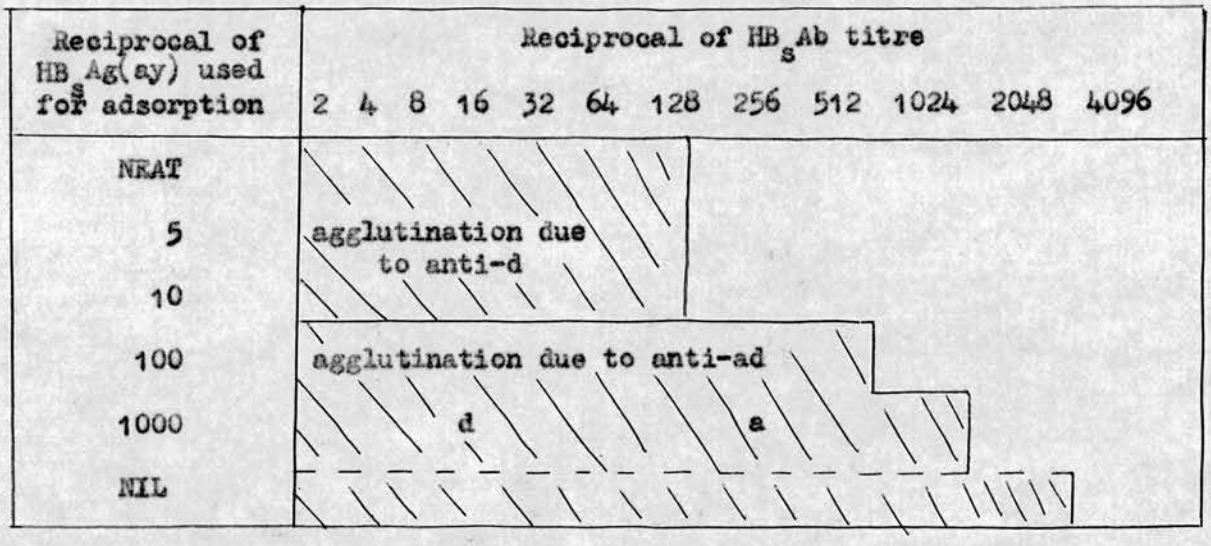


Table 3.1.II.b. Confirmation of monospecific HB_sAb (anti-d) following adsorption with HB_sAg (ay)

	HB_sAg coated cells	
	ad	ay
HB_sAb (anti-ad) adsorbed with HB_sAg (ay)	+	-
Unabsorbed	+	+

\+ agglutination - no agglutination

Anti-y was prepared in the same way by adsorbing HB_sAb (anti-ay) with HB_sAg (ad).

III. Procedure for Subtyping HB_sAg for Determinants d and y

Monospecific antibody was used in conjunction with cells coated with the appropriate HB_sAg subtype. For example, anti-d was used in conjunction with HB_sAg (ad) coated cells. Absence of agglutination was indicative of 'd' in the test sample, while the presence of agglutination suggested that the anti-d had not been neutralised and the test sample did not, therefore, possess the d determinant. As d and y are mutually exclusive, confirmation was obtained by using anti-y and HB_sAg (ay) coated cells to test for the y determinant, which if present, neutralised the anti-y and caused the HB_sAg (ay) coated cells not to be agglutinated.

The reliability of subtyping by HAI was investigated by typing HB_sAg positive sera from the following different sources: (i) National Institutes of Health, Bethesda, U.S.A., (ii) American Red Cross and (iii) Local (blood donor) carriers, and patients involved in the dialysis-associated hepatitis outbreak in Edinburgh in 1969. Sera from (i) and (ii) had been subtyped at source by I.D., while sera from (iii) had been subtyped by Dr. G.L. Le Bouvier (Yale University, Connecticut, U.S.A.), also using I.D. All sera were double-coded and interspersed with negative sera prior to subtyping.

IV. Results

Complete agreement was found between the subtyping data obtained by HAI and I.D. (table 3.1.IV).

Table 3.1.IV. Comparison of HB_sAg subtyping by HAI and I.D.

Sample	Origin	I.D.	HAI
208	National Institutes of Health	y	y
209		y	y
213		d	d
217		y	y
228		d	d
1001	American Red Cross	d	d
1018		d	d
1006		d	d
2002		y	y
2005		y	y
BD-2-Edin	Local(donor)carrier	y	y
BD-7-Edin	" " "	d	d
BD-9-Edin	" " "	d	d
H226	Dialysis patient	y	y
G.C.	" "	y	y

V. Discussion

At the time of writing, most HB_sAg subtyping is performed by I.D. or C.I.E.O.P. Unfortunately, both techniques are wasteful of precious monospecific typing antisera and are relatively insensitive, being unable to subtype sera found positive only by the more sensitive tests such as RPHA, RIA and HAI.

Application of HAI to HB_sAg subtyping permits rapid (within 2 hours) identification of the d and y determinants, with a sensitivity similar to that of other 'third generation' tests, and therefore provides essential confirmatory information relatively quickly.

The discovery of at least two mutually exclusive but relatively common determinants of the HB_sAg system clearly implies that techniques and reagents used for blood donor testing should be capable of detecting both subtypes with more or less equal sensitivity.

Subtyping of HB_sAg and HB_sAb provides an important epidemeological tool to probe the relationship between subtype and disease.

By adopting the same approach used for d/y subtyping, the HAI test has been applied successfully to the w/r system. Furthermore, the HAI test may be used to subtype HB_sAb by using HB_sAg positive sera of known subtype.

CHAPTER 2

PREPARATION OF HB_sAg-COATED, GLUTERALDEHYDE-FIXED SPHEROCYTES

I. Introduction

Since the shelf life of fresh HB_sAg-coated erythrocytes is only four to six weeks routine blood donor testing (for example, 500 tests per day) would necessitate reagent preparation at least once per month. The interval between preparation of subsequent batches of reagent could be increased if fixed erythrocytes were used. Furthermore, if large scale production were possible, reagent standardisation would also be improved.

Fixation of erythrocytes renders them mechanically more robust as evidenced by the absence of lysis in distilled water, and by an increased shelf life. Das (1970) reported that the storage life of fibrinogen-coated sheep erythrocytes was extended from one month to more than five months as a result of prior fixation in formaldehyde. In the same year, Hoq and Das (1970) published details of a study concerning the preparation of human erythrocytes for the assay of human serum fibrinogen degradation products. Part of this study involved a comparison of formaldehyde, pyruvic aldehyde and gluteraldehyde as cell fixatives. Results indicated a high incidence of autoagglutination among formaldehyde and pyruvic aldehyde-fixed cell batches, while none of seventeen batches of gluteraldehyde-fixed cells were so affected.

It seemed logical, therefore, to investigate the use of gluteraldehyde for the fixation of human group O rhesus negative erythrocytes prior to tanning and coating with HB_sAg. Unfortunately, three out of five cell batches fixed in gluteraldehyde according to the method

of Hoq and Das (1970) and subsequently tanned and sensitised with HB_sAg resulted in autoagglutination. Such a high incidence of unusable reagent was clearly unacceptable. Tanning and sensitisation of 'fresh' cell batches seldom resulted in autoagglutination, so it was decided to modify the method of glutaraldehyde fixation by using only as much fixative as would provide an acceptably robust cell.

As standardisation of reagents should theoretically be enhanced by a high degree of cell uniformity it was also decided to introduce a form of 'selection' by washing the fresh erythrocytes in a controlled hypotonic medium to 'select' the more robust among them.

II(a). Preparation of Spherocytes

One unit (approximately 450 ml.) of human group O rhesus negative blood not more than three weeks old and collected in anti-coagulant (citrate phosphate dextrose) was obtained from the blood bank.

The molarity of phosphate buffer pH 7.2(Appendix E) required for selection of the appropriate cells was determined by serially diluting isotonic (0.2M) buffer in deionised water. 0.1 ml. of fresh, well-mixed blood was added to 2 ml. of each dilution of buffer in a test tube. The cells were evenly suspended and allowed to stand at room temperature for 60 minutes. The suspension showing at least 50 percent haemolysis was arbitrarily considered optimum. This usually occurred within the range 0.045M to 0.030M but was determined for each unit of blood being spherocyted. The remainder of the blood unit was then mixed with the optimum hypotonic concentration of buffer in the ratio one volume of blood to four volumes of hypotonic buffer. The bulk volume was divided into 200 ml. aliquots for ease

of handling. After 60 minutes at room temperature the aliquots were centrifuged at 1500 r.p.m. for 15 minutes at 10°C on an M.S.E. 6L centrifuge and the supernatant (showing considerable haemalysis) was replaced with fresh buffer. This procedure was repeated until only a minimum of haemolysis could be observed. At this point the remaining cells accounted for approximately half of those originally present. These erythrocytes were considered to be uniform since they represented the most robust of the original cell population. Wet film microscopy showed them to have lost their characteristic biconcave appearance and become rounded or 'spherocyted'. They were thus subsequently referred to as spherocytes.

II(b). Fixation of Spherocytes

One aliquot of spherocytes was divided into smaller quantities (approximately 25 ml.) to each of which gluteraldehyde (Koch-Light, 25 percent) was added to give the following gluteraldehyde concentrations: 1 in 100, 1 in 200, 1 in 400, 1 in 800, 1 in 1600, 1 in 3200 and 1 in 6400. After thorough mixing the spherocyte aliquots containing fixative were left at room temperature overnight. The following morning spherocytes from each aliquot were examined microscopically and subjected to a 'water resistance' test which involved adding 25 µl. of 'settled' glutaraldehyde-treated spherocytes to 2 ml. deionised water, when properly fixed spherocytes resisted haemolysis. Dilutions of glutaraldehyde up to 1 in 1600 produced acceptable fixation, although dilutions up to 1 in 400 tended to distort the shape of the spherocytes, causing them to revert to biconcave discs.

The remaining aliquots of fresh spherocytes were fixed in the appropriate concentration of glutaraldehyde (approximately 0.02 per cent)

as above and stored at 4°C in a total volume of 200 ml. in deionised water containing 0.1 percent sodium azide.

III. Tanning and Sensitisation of Glutaraldehyde-fixed Spherocytes

The procedure was exactly that used for fresh cells, except that the concentration of tannic acid was halved.

IV. Performance and Storage of HB_sAg-coated, Glutaraldehyde-fixed Spherocytes

Two batches of glutaraldehyde-fixed spherocytes were each divided into five aliquots and coated with HB_sAg (adyw) as previously described. After stabilisation, the sensitivity of the sensitised fixed spherocytes was determined by titration of a standard HB_sAb positive control. This titration was repeated at approximately one month intervals for one year, during which time each of the ten aliquots was stored at 4°C.

Table 3.2.IV.a shows that spherocyting and subsequent fixation in weak glutaraldehyde solution has no adverse effect on HB_sAg coating. Furthermore, table 3.2.IV.b shows that glutaraldehyde-fixed HB_sAg-coated spherocytes may be stored at 4°C for up to one year without appreciable loss of sensitivity.

Table 3.2.IV.a. Comparative sensitivity of HB_sAg-coated fresh human erythrocytes and HB_sAg-coated glutaraldehyde fixed spherocytes

HB_sAg coated cells	Reciprocal of HB_sAg (GSA-2-73) titre
fresh erythrocytes	8000
fixed spherocytes 1a	16000
b	16000
c	8000
d	16000
e	8000
fixed spherocytes 2a	16000
b	16000
c	16000
d	16000
e	8000

Table 3.2.IV.b. Comparative storage life (at 4°C) of HB_sAg coated fresh human erythrocytes and HB_sAg coated glutaraldehyde fixed spherocytes

HB_sAg coated cells	Time (in months)												
	0	1	2	3	4	5	6	7	8	9	10	11	12
fresh erythrocytes	+	+	- (lysis)										
fixed spherocytes	+	+	+	+	+	+	+	+	+	+	+	+	+

\+ denotes acceptable sensitivity

V. Discussion

Gluteraldehyde-fixed spherocytes prepared from reasonably uniform human group O rhesus negative erythrocytes appear to be stable carriers of HB_sAg that provide a sensitive indicator of specific antibody. Osmosis provides a useful means of selecting those cells most suited to fixation and subsequent HB_sAg coating. The desired level of osmotic effect will vary somewhat according to the particular sample of erythrocytes being treated, but a medium having an osmolality between 100 m.mol. and 200 m.mol. should be suitable. This is conveniently produced in an aqueous solution of sodium and potassium phosphate with a molarity between 0.07M and 0.15M. Under such conditions, the less physically robust cells will undergo haemolysis while the more physically robust are converted to spherocytes. While considerable variation is possible, a recovery of spherocytes of the order of 20 percent to 50 percent of the original erythrocyte population is preferable. A plurality of treatments may be required to obtain a suitable level of spherocyte conversion, the number of treatments depending to some extent upon the ratio of erythrocytes to medium. Using the 1 to 4 ratio previously described, it is usual to perform at least four treatments.

Fixation of spherocytes may be possible with agents such as alcohol or acetic acid. However, the class of fixatives comprising the aldehydes were of general interest in this project, and in particular those derived from dicarboxylic acids (for example, gluteraldehyde). A suitable concentration of gluteraldehyde, leading to fixation in 12 to 72 hours, was between 0.06 percent to 0.08 percent of the stock solution (Koch Light, 25 percent). The resultant fixed spherocytes were conveniently stored at $4^{\circ}C$ in

deionised water containing an antibacterial agent (0.1 percent sodium azide) until required for coating with HB_sAg.

The finding that glutaraldehyde-fixed spherocytes may be stably coated with HB_sAg opened the way for large scale preparation of a highly standardised HB_sAg test reagent, particularly as the test procedure may be modified to provide maximum economy of reagent utilisation.

CHAPTER 3

MINITURISATION OF TEST PROCEDURE

I. Introduction

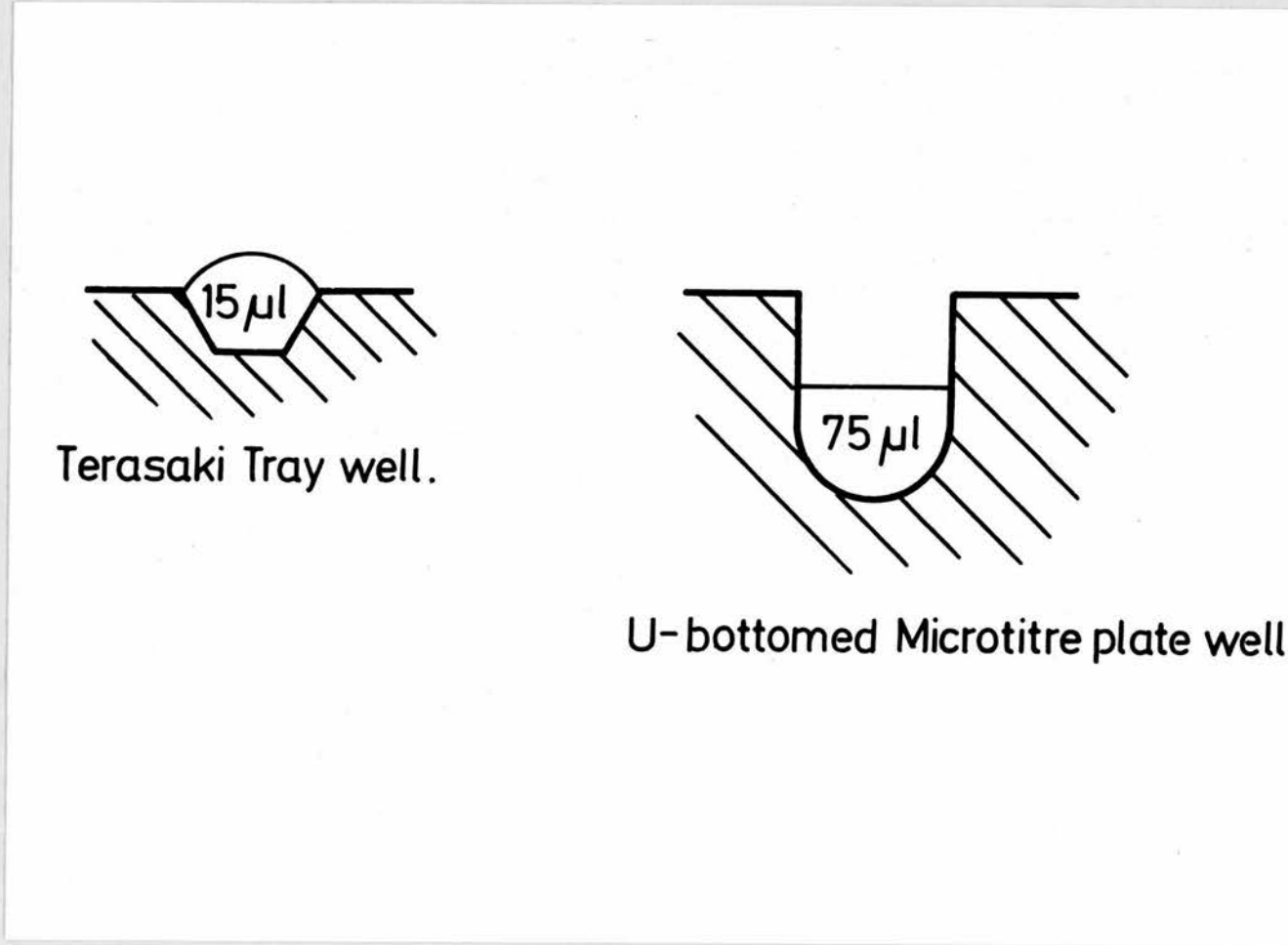
In its present form the HAI test for HB_sAg is simple to set up and to read. Its reliability is such that it may be used with confidence for large scale blood donor testing. The South-East of Scotland Regional Transfusion Centre (at Edinburgh Royal Infirmary) currently tests about 5,000 blood donations per month for HB_sAb and HB_sAg. This is equivalent in terms of reagent usage to 10,000 tests per month. The monthly requirement of HB_sAg-coated, glutaraldehyde-fixed spherocytes equals 10,000 x 25 µl. = 250,000 µl. or 250 mls. of working suspension (0.75 percent). If 50 mls. of neat HB_sAg positive serum yields approximately 3 ml. of HB_sAg suitable for spherocyte coating, and 0.5 mls. of such an HB_sAg preparation results in sufficient coated spherocytes for 400 tests (therefore 50 mls. neat HB_sAg positive serum provides 2,400 tests), then 10,000 tests would require $\frac{10,000}{2,400}$ x 50 = 200 ml. HB_sAg positive serum to be processed. For the purpose of reagent standardisation, it is advisable to prepare larger quantities of reagent, say sufficient for one million tests, and correspondingly more HB_sAg positive plasma would require to be processed. One million tests would necessitate the processing of $\frac{1,000,000}{10,000}$ x 200 = 20 litres of strongly HB_sAg positive serum, clearly a mammoth undertaking for any Regional Blood Transfusion laboratory quite apart from the hazards involved in the handling of so much potentially dangerous material. Clearly, there is a case for investigating the possibility of a more economic usage of reagents. It is the purpose of this chapter

to investigate the possibility of reducing the volume and concentration of reagents required for HB_sAg testing by HAI.

II. Materials, Methods and Results

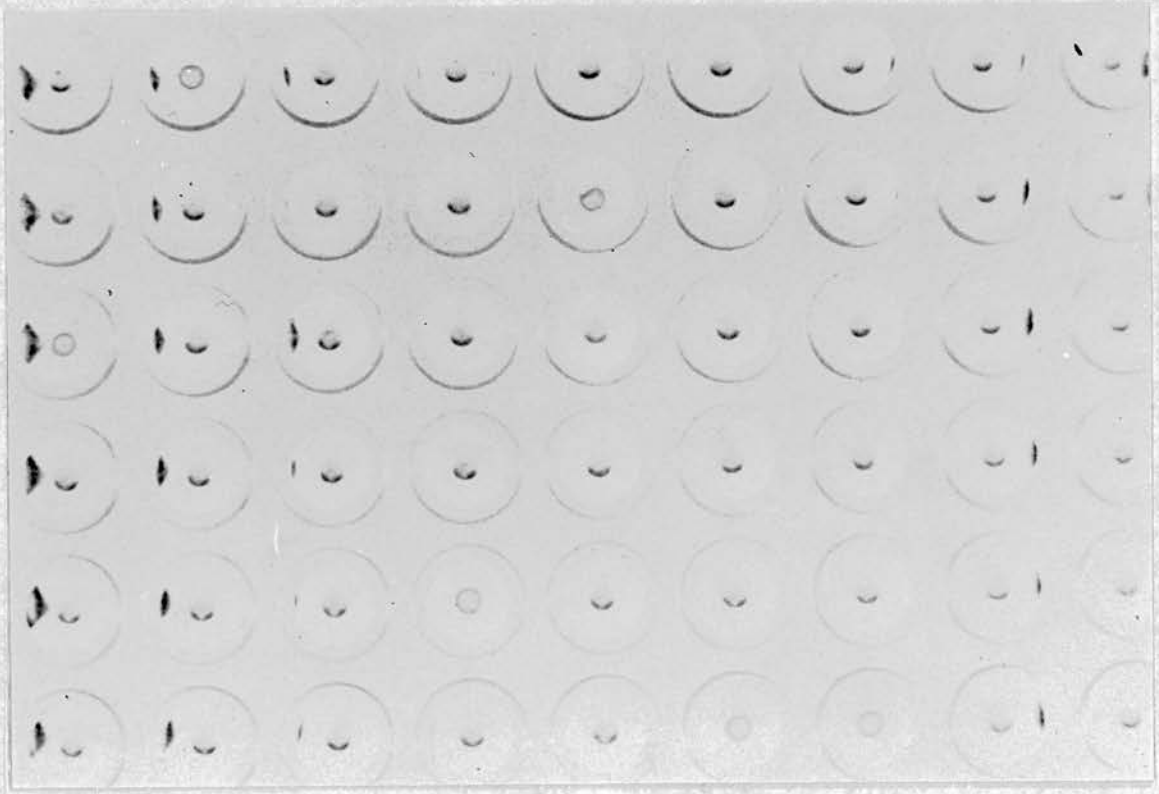
Terasaki microtest tissue culture trays (Bio-cult) are admirably suited for use in a miniturised version of the HAI test. Each tray measures 82 mm. by 57 mm. and contains 60 x 15 µl. wells which have a bevelled side and a flat base. Diagram 3.3.II.a shows the cross-sectional view of a Terasaki tray as compared to a 'U' bottomed microlitre plate.

Diagram 3.3.II.a. Diagramatic comparison of Terasaki tray well and microlitre plate (u-bottomed) well



Test procedure is essentially the same as previously described for the 'u' bottomed microlitre plate except that 5 µl. volumes of reagents are added using an Eppendorf 5 ul. pipette with disposable tip. During incubation (30 minutes at 37°C) the cells settle to form a continuous circle around the periphery of the base of the well, at which point positive and negative agglutination patterns are indistinguishable. The tray is tilted, carefully to avoid undue vibration, to an angle of 40° and left for approximately 15 minutes. In those wells where no agglutination has occurred, the cells gradually slide down the sides of the bottom of the well, while in those wells where agglutination is present, they remain as a complete circle of agglutination around the base of the well (diagram 3.3.II.b).

Diagram 3.3.II.b. Cell settling patterns in Terasaki trays



The distinction between positive and negative may be enhanced by centrifuging the plates (Griffin & George bench centrifuge with serological head, 60 seconds at No. 2 setting) prior to placing them at an angle.

Further economies are possible if the concentration of sensitised cells is reduced. The optimum cell concentration for use in the miniturised (Terasaki tray) system was found by titrating an HB_sAb positive serum using different concentrations of sensitised cells. The results shown in table 3.3.II.c. indicate that a 0.25 percent cell suspension provides the most acceptable results in terms of sensitivity and ease of reading.

Table 3.3.II.c. Effect of HB_sAg coated spherocyte concentration on sensitivity and readability of the Terasaki tray HAI test system

HB_sAg coated cell concentration	Reciprocal of HB_sAb titre (T2160C)	Readability
2 percent	-	too many cells
1 percent	-	too many cells
0.5 percent	20,480	good
0.25 percent	40,960	good
0.125 percent	40,960	poor
0.05 percent	-	not visible

III. Discussion

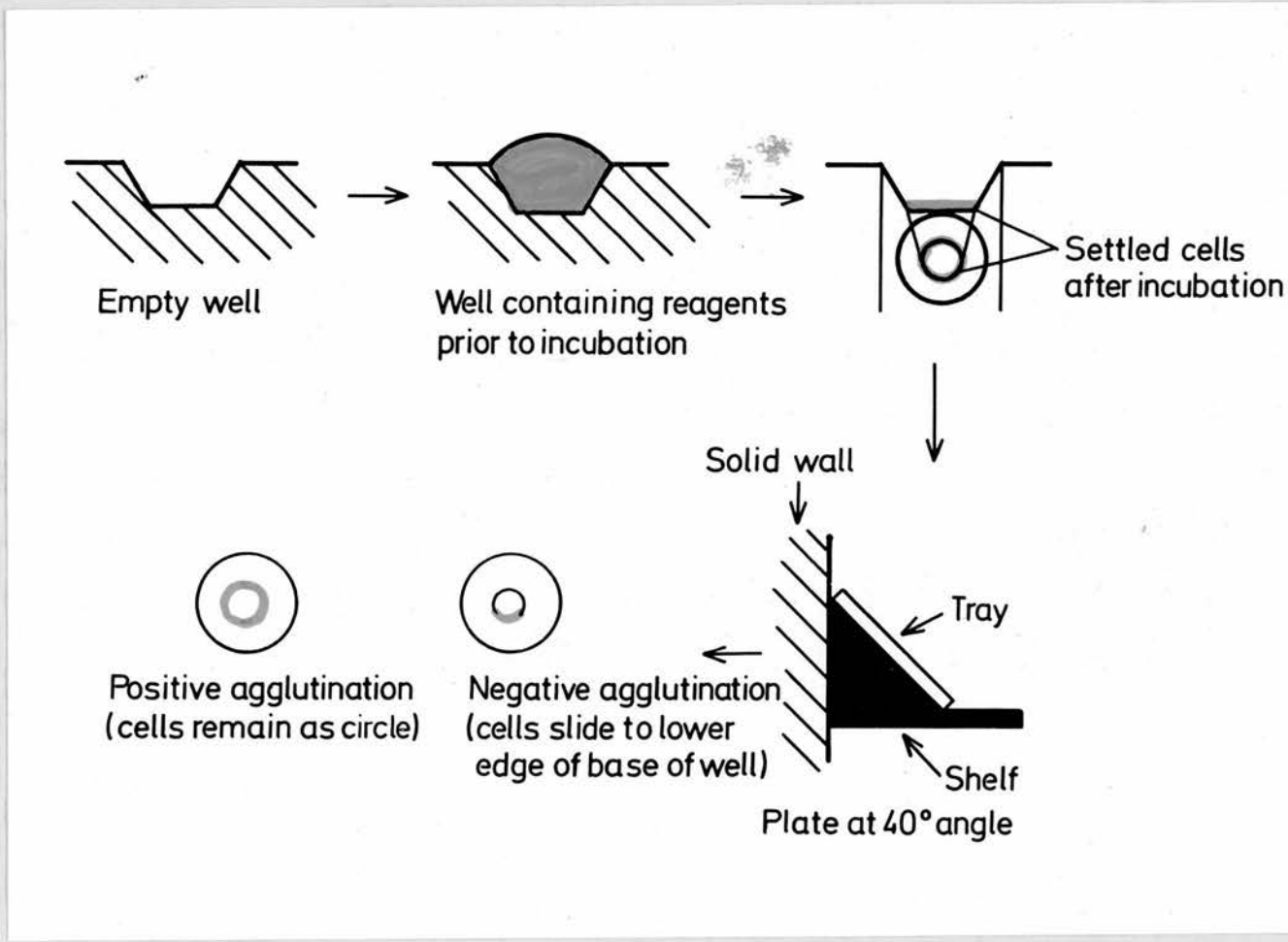
As a test vehicle the Terasaki tray allows a reduction in the volume of each reagent from 25 ul to 5 ul. This is an important economy not only in terms of HB_sAg-coated gluteraldehyde-fixed spherocytes but also of test serum which may not be readily available

in the quantities necessary for confirmatory and/or subtyping tests using other techniques. Reduction in volume combined with the use of only a 0.25 percent spherocyte suspension leads to an overall economy of the order of fifteen-fold. In practical terms, this means that if, as has already been stated, 20 litres of neat HB_sAg positive serum requires processing to provide sufficient reagents for one million tests by the microlitre plate method, the same number of tests could be performed by the miniturised (Terasaki tray) method as a result of processing only $\frac{20}{15}$ = 1.3 litres of neat serum. Logistics such as these place bulk reagent preparation easily within the capability of many Blood Transfusion Laboratories.

Economics of this magnitude are seldom achieved without sacrifice, and in this instance the robustness of the test appears to suffer. The miniturised method is particularly vulnerable to rough handling and/or vibration once it has been placed at the 40° angle. Fortunately, this problem is easily overcome by using an angled shelf bracketed to a solid vibration-free wall (diagr. 3.3.III). This arrangement also has the advantage of introducing a degree of uniformity to each test since the angle is kept constant. If the angle is much greater than 40° agglutinated cells will collapse prematurely, while a smaller angle will cause slower cell settling.

It was also found that a 1 in 5 dilution of test serum could be used.

Diagram 3.4. Wall-mounted shelf recommended for Terasaki tray system



CHAPTER 4

BULK PREPARATION OF HB_sAg-COATED, GLUTARALDEHYDE-FIXED SPHEROCYTES

I. Introduction

Large-scale preparation and storage of a reagent used for serological testing is very important for standardisation and economy, particularly if that reagent is sufficiently stable to survive transportation to and subsequent storage in other laboratories. Unfortunately, the highly sensitive 'third generation' HB_sAg test reagents are only available to the majority of Blood Transfusion Centres on a commercial basis, the cost ranging from 10 new pence per test (Hepatest- R.P.H.A., Wellcome Reagents, if bought in bulk) to 50 new pence per test or more, apparently depending upon geographical location (Ausria-II, RIA Abbott Laboratories). Experimental data suggest that HB_sAg-coated, gluteraldehyde-fixed spherocytes are a stable, sensitive and specific reagent well suited to testing large numbers of blood donors.

This chapter describes the bulk preparation of HB_sAg-coated, gluteraldehyde-fixed spherocytes in a quantity sufficient for approximately one million tests (if the miniaturised test system is employed).

II. Tanning, Sensitisation and Stabilisation of Sufficient Reagent for One Million Tests

Sixteen mls. of packed glutaraldehyde-fixed spherocytes were washed twice with 500 ml. volumes of physiological saline (MRC bottle spun at 1,000 r.p.m. for 5 minutes on MSE 6L centrifuge at room temperature). The cells were resuspended in 500 ml. of tannic acid (M. & B., 1 in 60,000 dilution in PBS pH 7.2 previously heated to 37°C)

and left in a 37°C waterbath for 15 minutes with occasional mixing. The tanned cells were spun down as before, washed twice with 500 ml. PBS pH 6.4 and finally resuspended in 400 ml. of the same buffer. Twenty ml. HB_sAg, prepared as previously described and containing both the 'd' and 'y' determinants, was added and the spherocyte-antigen suspension mixed continuously at room temperature (see discussion). After 20 hours, a 1 ml. aliquot was removed, stabilised (as previously described) and used to titrate a known standard HB_sAb-containing serum. Sampling was repeated at approximately 20 hour intervals until a satisfactory sensitivity was achieved, when the bulk of the reagent was removed from the mixer and the spherocytes recovered by centrifugation as before. The sensitised spherocytes were then stabilised by resuspension and mixing for 4 hours at room temperature in 500 ml. PBS pH 7.2 containing 2 percent normal human serum to give a cell suspension of approximately 3.0 percent (or ten times working concentration). Storage of these cells overnight at 4°C was followed by a repitition of the stabilising procedure. Thereafter, the 3.0 percent suspension was dispersed into 50 ml. aliquots and stored at 4°C until required, when each aliquot (or part thereof) could be diluted ten-fold with PBS pH 7.2 containing 2 percent normal human serum to achieve working concentration.

III. Discussion

The ability to prepare a large batch of stable HB_sAg test reagents in a Blood Transfusion Centre provides the basis for uniformity of donor HB_sAg testing and represents a considerable saving in both cash (one million Hepatests = £80,000, while one million AUSRIA - II = £500,000) and time, and allows third generation

testing efficiency to be made available at a fraction of the cost of purchasing commercial reagents.

Bulk preparation seems to result in reagents which are every bit as sensitive and stable as those produced in much smaller 'experimental' quantities, since transportation over thousands of miles via air freight appears to have no adverse effect even when cells are maintained in the liquid state.

If sampling of the sensitising mixture suggests that a satisfactory sensitivity is not being achieved, it is possible to add more HB_sAg. However, care must be taken in this respect since an excess of antigen will lead to autoagglutination, presumably resulting from overcoating of the fixed spherocytes. It is well to bear in mind that stabilisation of 'samples' requires overnight at $4^{\circ}C$, during which time the bulk reagent is still sensitising. Experience of bulk reagent preparation suggests that sensitivity plateaus after about 48 hours if no further HB_sAg is added.

An interesting observation was that cell sensitisation proved successful when mixing with a mechanical turntable, but unsuccessful when mixing with a magnetic stirrer and plastic-coated follower. Adsorbtion of HB_sAg onto the follower has been eliminated, but the electrochemical or electrophysical possibilities for such a failure have yet to be investigated.

Addendum

The work reported in this chapter and in chapter 2 (IIa and IIb) was undertaken in collaboration with Mr. A.D. Watt (Chief Technician, Microbiology Unit, Blood Transfusion Service, Royal Infirmary, Edinburgh) and has been included in this thesis to emphasise the potential of the technique developed in Part Two.

CHAPTER 5

REAGENT SAFETY

I. Introduction

Reagents used routinely should be made as safe as possible for the protection of staff members who are in daily contact with them. This is particularly important when a known pathogen, such as HB Ag, is involved. General viral inactivating procedures, such as treatment with betapropiolactone and ultra-violet irradiation (La Grippo, Hayashi and Saeed, 1971), are necessary precautionary measures, but until a valid test of infectivity becomes available, the effectiveness of such measures for the inactivation of HB Ag must remain questionable.

Gamma irradiation from a cobalt source is a recognised method of virus inactivation for which some data is available (Ginosa, 1968). Unfortunately, the reagent to be irradiated should be in the lyophilised state to prevent the generation of excess heat.

Pasteurisation (60°C for 10 hours) is considered by some to be an effective means of inactivating HB Ag (Mozen, Schroeder and Cabasso, 1972) based upon the hepatitis-free history of human albumin prepared by Cohn fractionation.

II. Pasteurisation of HB_sAg-coated, Glutaraldehyde-fixed Spherocytes

A 3 percent suspension of HB_sAg-coated glutaraldehyde-fixed spherocytes was divided into 8 x 1 ml. aliquots which were allowed to stand in a 60°C waterbath for periods ranging from 0 to 24 hours. At the appropriate times, an aliquot was removed and stored at 4°C. After removal of the final aliquot (24 hours), each was diluted ten

times (with PBS pH 7.2 containing 2 percent normal human serum) to a working concentration of 0.3 percent and used to titrate a standard HB_sAb-positive serum. Table 3.5.II indicates that this reagent may be maintained at a temperature of 60°C for at least 16 hours before significant reduction in sensitivity occurs.

Table 3.5.II. Effect of heating HB_sAg-coated glutaraldehyde-fixed spherocytes at 60°C for various times

Time (hours) at 60°C	Reciprocal of HB_sAb titre
0	40,960
2	40,960
4	40,960
8	40,960
10	40,960
16	40,960
20	40,960
24	5,120

III. Gamma Irradiation of Pasteurised HB_sAg-coated, Glutaraldehyde-fixed Spherocytes

HB_sAg-coated, glutaraldehyde-fixed spherocytes were adjusted to a 3 percent suspension in PBS pH 7.2 and divided into 1 ml. aliquots in clean glass screw cap tubes (Flow Laboratories). The contents of each tube was 'shell frozen' in liquid nitrogen and lyophilised on a Virtis dryer. The lyophilised reagent was stored at room temperature. Twelve tubes containing lyophilised reagent were exposed to 2.5 megarads of gamma irradiation from a cobalt source (by kind permission of Ethicon Limited).

Lyophilised and lyophilised-irradiated reagents were reconstituted by adding 1 ml. PBS pH 7.2 containing 2 percent normal human serum to the tube, allowing to stand for 5 minutes and adding a further 9 mls., resulting in 10 mls. of working concentration. Titration of a standard HB_sAb positive serum showed the sensitivity of both lyophilised and lyophilised-irradiated reagents had been reduced by one or two doubling dilutions.

IV. Discussion

RPHA tests for HB_sAg have to some extent allayed fears concerning reagent infectivity by using cells coated with specific antibody presumed to be free of HB_sAg. Unfortunately, such reagents are expensive to produce, unstable once reconstituted and subject to non-specific agglutination due to the presence of impurities or unwanted specificities among the immunoglobulins used for coating. The HAI test, on the other hand, exhibits a high degree of specificity and is capable of equal or greater sensitivity than RPHA. Reagents are relatively inexpensive to produce in large quantities and are extremely stable, as further evidenced by their ability to withstand pasteurisation, lyophilisation and gamma irradiation. HB_sAg-coated reagents do, however, carry an increased infectivity risk and for this reason reagents were submitted to the procedures detailed above.

Pasteurisation of 'bulk' reagents presented no additional problems and appeared to leave sensitivity as unaffected as in the pilot experiments. The reduced titre obtained after 24 hours at 60°C could have resulted from elution of HB_sAg from the spherocyte surface or deterioration of HB_sAg antigenic sites. These possibilities were not investigated further. Subsequent batches of reagents were treated at 60°C for 16-18 hours (conveniently

overnight). Experience has shown that their stability remains unimpaired over the following months of storage at 4°C. Similarly, gamma irradiation appears to have little effect upon subsequent reagent stability whether stored in the lyophilised or liquid state. The slight loss of sensitivity following reconstitution is disappointing, more so since the best settling patterns were obtained using a 1 in 10 dilution of test serum (pre-lyophilisation reagents could be used with a 1 in 5 dilution of test serum). Lyophilisation appears to be responsible as the sensitivity of pre- and post-irradiated lyophilised reagents was identical. It may be that the forces acting upon the 'frozen' reagents during 'drying' caused the less tightly bound antigen particles to be dislodged from the spherocyte surface.

CHAPTER 6

COMPLEXED REVERSE PASSIVE HAEMAGGLUTINATION (C.R.P.H.A.)

I. Introduction

In a recent publication (Technical Report Series No. 570, 1975), the World Health Organisation have recommended that RPHA be used for routine blood donor (HB_sAg) testing. Three such tests are commercially available and were briefly compared in Part One (section 2, chapter 2) of this thesis. RPHA has the advantage of being a simple one-step test, with a sensitivity between C.I.E.O.P. and RIA, which may be read within 1 to 3 hours. Unfortunately, the commercial product is expensive and unstable once reconstituted, and the purified animal hyperimmune globulin used leads to occasional false positive reactions. The long and complex procedure of raising antisera in animals, purifying it and finally adsorbing it onto a suitable carrier places RPHA reagent preparation beyond the scope of most Blood Transfusion Service laboratories.

The addition of one simple step to the procedure for preparation of HB_sAg-coated, gluteraldehyde-fixed spherocytes enables these reagents to be used in a one-step RPHA test for HB_sAg.

II. Antibody Overcoating of HB_sAg-coated, Gluteraldehyde-fixed Spherocytes

An aliquot (5 mls. of 3 percent suspension) of HB_sAg (adyw)-coated gluteraldehyde-fixed, stabilised spherocytes was spun gently on a bench centrifuge and the supernatant removed. The packed cells were resuspended in 20 mls. of neat human serum known to contain a high (HA titre $> \frac{1}{1000}$) level of HB_sAb (it is preferable that the antiserum be active against all the HB_sAg determinants

coated onto the spherocyte surface, and it may be necessary to mix antisera in order to achieve such broad-spectrum activity), and an equal volume of PBS pH 7.2 added. The container was then attached to a mechanical (Mattburn mixer) mixer and the HB_sAg-coated spherocyte-antiserum suspension mixed at room temperature for approximately 72 hours. (It is essential that the mixing be continuous in order to prevent the formation of immune aggregates). The antigen-antibody coated spherocytes were then washed three times in PBS pH 7.2, adjusted to working strength (approximately 0.3 percent), and allowed to stand at 4°C for a period of 4 to 5 days, after which they were ready for use. The presence of HB_sAg-HB_sAb immune complexes on the spherocyte surface led to the adoption of the term complexed reverse passive haemagglutination (C.R.P.H.A.)

III. <u>C.R.P.H.A. Test for HB_sAg</u>

Test sera were diluted 1 in 5 in PBS pH 7.2 as for HAI. 10 µl. of the test serum dilution was placed into the well of a Terasaki tray and 5 µl. of C.R.P.H.A. reagent (0.3 percent suspension) added. The tray was gently shaken to enhance mixing, and floated on the surface of a 37°C waterbath for 1 hour, by which time the spherocytes had 'settled' to form a circle round the base of the well. The tray was then gently placed at an angle of 40 to 50° as for HAI and read after 5 to 15 minutes or when the negative controls (10 µl. 1 in 5 normal serum + 5 µl. C.R.P.H.A. reagent) were down. The presence of detectable HB_sAg in the test sera was evidenced by a complete circle of agglutination, while in the absence of an antigen-antibody reaction, the spherocytes slide down to the lower part of the base of the well.

IV. Comparison of C.R.P.H.A. Sensitivity with other Third Generation Tests for HB_sAg

(a) Titration sensitivity

Doubling dilutions of two HB_sAg positive sera (one adw and one ayw) tested by C.R.P.H.A., HAI, RPHA (Hepatest), and RIA (Ausria-2).

(b) Panel studies

C.R.P.H.A., HAI and RIA were used to test two well documented panels (B and C) supplied by the Central Public Health Laboratory (C.P.H.L.) and two lesser known panels provided by the West of Scotland Regional Transfusion Centre (Law Hospital) and the Natal Blood Transfusion Centre (Durban, South Africa).

V. Results

Titration study results(table3.6.V.a)indicate that C.R.P.H.A. performed as described is as sensitive as the commercial RPHA (Hepatest), but less sensitive than HAI or RIA (Ausria-2).

Table 3.6.V.a.Comparative titration-sensitivity of C.R.P.H.A., RPHA, HAI and RIA

Test	Reciprocal of HB_sAg(adw) titre	Reciprocal of HB_sAg(ayw) titre
C.R.P.H.A.	2,000	2,000
HAI	8,000	16,000
RPHA (Hepatest	2,000	4,000
RIA (Ausria-2)	16,000	8,000

Results obtained from C.P.H.L. panels B and C (tables 3.6.V.b and 3.6.V.c) suggest that the sensitivity of C.R.P.M.A. implied by the titration studies is borne out when sera containing various concentrations of HB_sAg are tested under code. Results obtained from testing the Natal B.T.S. panel (table 3.6.V.d) and the West of Scotland B.T.S. panel (table 3.6.V.e) further confirm that the sensitivity of C.R.P.H.A. is comparable with established 'third generation' tests, although results obtained with samples No. 20 and 27 in the Natal panel and samples No. 21 and 26 in the West of Scotland panel suggest that the C.R.P.H.A. reagents are vulnerable to occasional false positive as well as false negative (sample 15, table 3.6.V.e) reactions as is the case with commercial RPHA reagents. This aspect of their performance requires further investigation.

VI. Discussion

Continuous mechanical mixing of HB_sAg-coated, glutaraldehyde-fixed, stabilised spherocytes in an environment of neat serum containing an excess of specific antibody results, after 2 to 4 days, in a suspension of HB_sAg-coated spherocytes which are 'overcoated' with specific antibody but are not agglutinated (presumably because no HB_s antigenic sites remain exposed to react with antibody overcoated onto other antigen-coated spherocytes). However, these antibody overcoated spherocytes will agglutinate if introduced into a test medium possessing free antigen.

Preliminary observations suggest that C.R.P.H.A. reagents are extremely stable, presumably because they utilise the HB_sAg-coated spherocyte as a carrier to which antibody is linked by a stable antigen-antibody interaction, a feature which removes the necessity

of preparing and passively coating purified HB_sAb which results in a rather unstable cell-antibody bond.

Provided a reasonable balance of anti-d and anti-y is maintained in the overcoating procedure, sensitivity of C.R.P.H.A. is equivalent to any of the commercial RPHA tests, while pilot experiments suggest that certain modifications of test procedure may further increase sensitivity.

The simplicity and economy inherent in the preparation of HAI reagents applies equally to C.R.P.H.A., since antibody overcoating requires only one simple additional step. If after the first overcoating, free antigenic sites remain, the overcoating procedure may be repeated as often as is necessary (provided the specificity of HB_sAb and HB_sAg correspond).

C.R.P.H.A. reagents also possess the potential for HB_sAg and HB_sAb subtyping, although this has not been investigated in any detail. For example, if ady coated spherocytes are overcoated with anti-ad, then they will be agglutinated by a and d antigenic determinants in test serum, but will also be agglutinated if anti-y is present, and vice versa.

Table 3.6.V.b.i Results of testing C.P.H.L. Panel B by C.R.P.H.A., HAI and RIA.

Sample	HB_sAg Test		
	C.R.P.H.A.	HAI	RIA (AUSRIA-2)
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+
13	+	+	+
14	+	+	+
15	+	+	+
16	+	+	+
17	+	+	+
18	+	+	+
19	-	-	-
20	+	+	+

Table 3.6.V.c. **Result of testing C.P.H.L. Panel C by C.R.P.H.A., HAI and RIA**

Sample	HB_sAg Test		
	C.R.P.H.A.	HAI	RIA (AUSRIA-2)
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	-	-	-
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+
13	-	-	-
14	-	-	-
15	+	+	+
16	+	+	+
17	+	+	+
18	+	+	+
19	+	+	+
20	-	-	-

Table 3.6.V.d. **Results of testing panel provided by the Natal Blood Transfusion Service (Durban, South Africa) using C.R.P.H.A., HAI and RIA**

Sample	HB_sAg Test		
	C.R.P.H.A.	HAI	RIA (AUSRIA-2)
1	-	-	-
2	+	+	+
3	-	-	-
4	+	+	+
5	+	+	+
6	-	-	-
7	-	-	-
8	+	+	+
9	+	+	+
10	+	+	+
11	-	-	-
12	-	-	-
13	+	+	+
14	+	+	+
15	-	-	-
16	+	+	+
17	-	-	-
18	+	+	+
19	-	-	-
20	+	-	-
21	+	+	+
22	+	+	+
23	-	-	-
24	+	+	+
25	-	-	-
26	-	-	-
27	+	-	-
28	-	-	-
29	+	+	+
30	+	+	+
31	-	-	-
32	+	+	+
33	+	+	+
34	+	+	+
35	-	-	-
36	-	-	-
37	-	-	-
38	-	-	-

Table 3.6.V.e. Results of testing panel provided by the West of Scotland Blood Transfusion Service using C.R.P.H.A., HAI and RIA

Sample	HB_sAg Test		
	C.R.P.H.A.	HAI	RIA (AUSRIA-2)
1	-	+	-
2	+	+	+
3	+	+	+
4	-	-	-
5	+	+	+
6	+	+	+
7	+	+	+
8	-	-	-
9	+	+	+
10	+	+	+
11	-	-	-
12	+	+	+
13	+	+	+
14	-	+	-
15	+	+	+
16	-	-	-
17	+	+	+
18	+	+	+
19	+	+	+
20	+	+	+
21	+	-	-
22	+	+	+
23	-	-	-
24	+	+	+
25	-	-	-
26	+	-	-
27	-	-	-
28	-	-	-
29	+	+	+
30	-	-	-
31	+	+	+
32	+	+	+
33	-	-	-
34	+	+	+
35	-	-	-
36	-	+	-

PART FOUR

APPLICATIONS

CHAPTER 1

EVALUATION OF HAI FOR DETECTION OF HB_sAg AMONG BLOOD DONORS

I. Introduction

Despite the rejection of blood donations found to possess HB_sAg by C.I.E.O.P., hepatitis continues to complicate the therapeutic use of blood and blood products (Reinicke et al., 1973). More sensitive serological tests for HB_sAg may further reduce the incidence of posttransfusion hepatitis but are unlikely to eliminate it completely because of the possible infectivity of blood containing antibody to the Dane particle core in the absence of detectable HB_sAg (Hoofnagle, Gerety, Li and Barker, 1974) and the possible existence of a hepatitis C virus (Prince, Brotman, Grady, Kuhns, Hazzi, Levine and Millman, 1974) - quite apart from other possible causes such as hepatitis A virus and other microbial agents (Zuckerman, 1970).

'Third generation' tests for HB_sAg using the principles of haemagglutination and radioimmunoassay are now commercially available and reports suggest that these kits can detect carriers of HB_sAg too weak to be positive by C.I.E.O.P. (Ling and Overby, 1972; Cayzer, Dane, Cameron and Denning, 1974). Unfortunately, the effective use of such commercial reagents in the blood transfusion service will prove expensive and only time will tell whether such expense is justified.

Data reported in Part Two (VIII and IX) of this thesis suggests that the HAI test possesses the characteristics of accepted "third generation" technology. The potential of HAI for use in mass blood donor testing was investigated by conducting a multi-centre evaluation involving over 70,000 donations.

II. Materials and Methods

Three Scottish Regional Transfusion Centres were involved in the evaluation study: the South-East of Scotland Regional Transfusion Centre, Edinburgh, the East of Scotland Regional Transfusion Centre, Dundee, and the North of Scotland Regional Transfusion Centre, Inverness. The initial part of the study involved the use of fresh human erythrocytes coated with HB_sAg. Antigen was prepared centrally at Edinburgh, but cell sensitisation and subsequent reagent standardisation were performed at each centre independently. However, with the implimentation of bulk production of fixed spherocytes, it became possible to supply each centre with reagents from the same batch, thereby improving standardisation considerably. The miniturised (Terasaki tray) version of HAI was performed at each centre in parallel with C.I.E.O.P. 4086 of the 44,053 donations tested at Edinburgh were also tested with the 'Hepatest' (Wellcome Reagents) R.P.H.A. kit. Positive HAI reactions were confirmed by subtyping.

III. Results

The overall incidence of HB_sAg among donors at the three centres taking part was 0.04 percent. HAI detected seven HB_sAg carriers missed by C.I.E.O.P. (table 4.1.III). Five of these were 'new' donors and two were donors whose previous donation had been implicated, among others, in posttransfusion hepatitis. Each centre found at least one additional positive by HAI. Among the 4,086 donations tested using 'Hepatest', three HB_sAg carriers were found, but these were positive by HAI and C.I.E.O.P. In addition, the commercial RPHA tests gave 20 positive reactions which were subsequently shown to be nonspecific.

Table 4.1.III. **Incidence of HB_sAg among blood donors tested at three centres**

	No.donations tested	No.HAI pos.	No.CIEOP pos.
Edinburgh	44,053	18	14
Inverness	16,371	4	2
Dundee	9,800	4	3
Total	70,224	26 (0.04%)	19 (0.03%)

IV. Discussion

Recent reports have highlighted the inadequacies of C.I.E.O.P. for detecting HB_sAg in blood donations (Reinicke et al., 1973; Ling and Overby, 1972; Cayzer et al., 1974). The American Red Cross seem to have opted for RIA, while Regional Transfusion Centres in the U.K. appear to favour RPHA. Three commercial RPHA assays are currently available and have been reviewed in Part One (section two, chapter 2) of this thesis. The cheapest of these retails at approximately 10 pence per test if bought in bulk. All three tests are susceptible to a small number of false positive reactions, these being resolved by absorption-titration experiments.

HAI has a sensitivity for HB_sAg detection comparable with other third generation techniques and, being an inhibition reaction, has an inherent specificity not found in RPHA tests. The use of monospecific antibody (prepared by absorption) allows HAI to be used to subtype antigen-positive sera, providing an essential confirmatory step.

The cost of HAI is negligible due to the availability of raw material, the considerable expertise in haemagglutination technology within the blood transfusion service and the simplicity of reagent preparation combined with the small quantities (5 µl.) needed for testing.

The prime criterion in evaluating a new screening test such as this must be the number of additional antigen carriers detected in comparison with current techniques. The results indicate that HAI is capable of detecting donations containing HB_sAg in concentrations below the sensitivity of C.I.E.O.P.*

HAI may prove an acceptable alternative to commercial third generation HB_sAg tests for use in large scale blood donor testing. It seems that with appropriate training, the technique may be readily introduced into most regional transfusion centres, thereby keeping reagent costs to a minimum.

* HAI detects 50-100 ngm HB_sAg protein per ml.
CIEOP detects approximately 5000 ngm HB_sAg protein per ml.

CHAPTER 2

PREVALENCE OF HB_sAg SUBTYPES AMONG (BLOOD DONOR) CARRIERS IN SOUTH-EAST SCOTLAND AND THEIR ASSOCIATION WITH ABNORMAL LIVER FUNCTION

I. Introduction

The 'd' determinant of HB_sAg appears to predominate among apparently healthy blood donors in Western Europe and the U.S.A. (Mazzur et al., 1974), while the 'y' determinant is frequently associated with clinical evidence of HB_sAg positive hepatitis in these reagions (Wenzel et al., 1972; Le Bouvier et al., 1972; Moseley et al., 1972). Some workers have been unable to find such a distinct association between the HB_sAg subtype and liver damage (Gordon et al., 1972; Feinman et al., 1973). On the basis of subtype distribution among acute and chronic cases of type B hepatitis, it has been suggested that infection with the d determinant is more likely to result in mild illness progressing to the chronic HB_sAg carrier state, whereas infection with the y determinant seems likely to result in a severe, acute, but self-limiting illness (Holland et al., 1972; Dodd et al., 1973; Zuckerman, Hacker and Aach, 1974).

HB_sAg (blood donor) carriers were subtyped for determinants a, d, y, w and r by HAI and liver function data (serum glutamic pyruvic transaminase, SGPT; serum glutamic oxaloacetic transaminase, SGOT) was obtained, where possible, in an attempt to correlate HB_sAg subtype with abnormal liver function in the South-East of Scotland.

II. Materials and Methods

HAI subtyping was performed as described in Part Three (chapter 1) of this thesis. Liver function tests (SGPT and SGOT) were performed

in the Department of Clinical Chemistry at the Edinburgh Royal Infirmary. Eighty apparently healthy blood donors found to carry HB_sAg were subtyped. Thirty gave regular (three-monthly) blood samples for HB_sAg and liver function determination.

III. Results

As expected, the 'd' determinant was found to predominate among HB_sAg (blood donor) carriers in the South-East of Scotland (table 4.2.III.a). All samples possessed both the 'a' and 'w' determinants in addition to either 'd' or 'y'.

Table 4.2.III.a. Incidence of HB_sAg subtypes among apparently healthy HB_sAg carriers in South-East Scotland

No. subtyped	No.adw	No.ayw	No.adr	No.ayr
80	64 (80%)	16	0	0

Of the thirty carriers who presented regularly for follow-up study, only four exhibited persistent abnormal liver function, while two more showed occasional slight SGPT and SGOT elevations (table 4.2.III.b). Of these 6, one carried HB_sAg of subtype ayw.

Table 4.2.III.b. Incidence of abnormal liver function among apparently healthy HB_sAg carriers in South-East Scotland

	Liver Function	
	Normal	Abnormal
adw (26)	21	5
ayw (4)	3	1
Total (30)	24	6

IV. Statistical Analysis

In order to test the (Null) hypothesis that there is no significant difference between the adw and ayw groups in relation to liver function other than would be expected to occur by chance, Fisher's exact test was applied as follows (nomenclature according to Geigy Scientific Tables):

	Normal	Abnormal	
adw	x_1	$N_1 - x_1$	N_1
ayw	x_2	$N_2 - x_2$	N_2
	X	$N - X$	N

From significance limits for four-fold tables; the first table was rearranged so that N_1 N_2 and x_1 $(N_1 - x_1)$, that is:

	Abnormal	Normal	
adw	1	3	4
ayw	5	21	26
	6	24	30

N	N_1	x_1	$2\alpha=0.20$	$2\alpha=0.10$	$2\alpha=0.05$	$2\alpha=0.02$	$2\alpha=0.01$	$2\alpha=0.002$
30	4	1	. - 20	. - 22	. - 24	. - 25	. - 26	. - 27

Therefore the Null hypothesis cannot be rejected, that is, there is no significant relationship between adw and ayw subtypes and liver function.

Fisher's exact test was applied because the figures in certain boxes were too small to apply χ^2 analysis.

V. Discussion

The predominance of the adw configuration of HB_sAg determinants (80 percent) among apparently healthy blood donors in the South-East of Scotland is in agreement with previous studies covering Western Europe and the U.S.A. (Mazzur et al., 1974). If the thirty HB_sAg carriers followed for liver function studies are representative of other apparently healthy HB_sAg carriers in this region, it would appear that approximately one in five of such carriers are experiencing a mild chronic liver disorder. Statistical evaluation of the present study suggests that among apparently healthy carriers of HB_sAg persistent liver dysfunction is not significantly associated with either the d or y antigenic determinant. This is in conflict with the frequent association of the y determinant in more severe 'clinical' cases of HB_sAg positive hepatitis (Wenzel et al., 1972; Le Bouvier et al., 1972; Moseley et al., 1972).

Following the discovery of the 'e' antigen system of HB_sAg by Magnius and Espmark (1972) and its possible association with chronic liver dysfunction (Feinman, Berris, Sinclair, Wrobel, Murphy and Maynard, 1975), the incidence of e and anti-e among apparently

healthy HB_sAg (blood donor) carriers in this region was investigated using the I.D. technique. At the time of writing, 22 HB_sAg carriers have been tested. Three were e-antigen positive, 16 possessed e-antibody, while neither e-antigen nor e-antibody could be found in 6. The three e-antigen positive carriers were the only ones to exhibit persistent elevations of SGOT and SGPT, suggesting that in apparently healthy HB_sAg carriers, the e antigen may provide a better marker of possible chronic hepatitis than the HB_sAg subtype. There was no correlation between presence of e-antigen and the d or y determinant.

CHAPTER 3

RE-INVESTIGATION OF BLOOD DONATIONS IMPLICATED IN POSTTRANSFUSION HEPATITIS

I. Introduction, Materials and Methods

Data presented in Chapter 1 of this part of the thesis clearly indicates that HAI is capable of detecting HB_sAg carriers who would be missed by C.I.E.O.P. Since not all recipients of HB_sAg positive blood develop antigenaemia and/or hepatitis (Gocke and Kavey, 1969), it is important to establish whether or not those carriers found to be HAI positive but C.I.E.O.P. negative are likely to be infective. From mid-1973 the South-East of Scotland Regional Blood Transfusion Service has stored 0.5 ml. aliquots of serum at -20°C from all donations for a period of six months. Since that time eight patients with suspected posttransfusion (viral) hepatitis have been reported to the Blood Transfusion Centre, and it has been possible to investigate each case by retesting the original C.I.E.O.P. negative donations using HAI, RIA (Ausria I) and RPHA (Hepatest). In addition, two suspected posttransfusion hepatitis cases were referred from Manchester Blood Transfusion Centre for similar investigation, although in this case the donations actually tested were obtained at a return visit of the donors and were not therefore truly representative of the original donations.

II. Results and Comments

The retesting of more than 100 donations implicated in eight cases of posttransfusion hepatitis in Edinburgh showed that in two cases an HB_sAg positive unit of blood had been transfused which was not detected during initial testing by C.I.E.O.P. (table 4.3.II).

Table 4.3.II. Re-investigation of donors implicated in posttransfusion hepatitis

Patient	No. of donations involved	Counter-electrophoresis	Ausria 1	EHAI	Turkey cells
Vei.$^{E}_{+}$	8	All neg.	All neg.	All neg.	All neg.
McG.$^{E}_{+}$	14	"	"	"	"
Moc.$^{E}_{-}$	18	"	1 Pos.	1 Pos.	1 Pos.
Lil.$^{E}_{+}$	18	"	All neg.	All neg.	All neg.
New.$^{E}_{+}$	32	"	"	"	"
Lid.$^{E}_{+}$	14	"	1 Pos.	1 Pos.	1 Pos.
Gow.$^{E}_{-}$	3	"	All neg.	All neg.	All neg.
Pea.$^{E}_{-}$	10	"	"	"	"
Far.$^{M}_{+}$	4	"	1 Pos.	1 Pos.	"
Ali.^{M}n.t.	17	"	All neg.	1 Pos.	1 Pos.

E = Edinburgh
M = Manchester
\+ = HB_sAg Positive
\- = HB_sAg Negative
n.t. = not tested

Re-investigation of the two cases from Manchester revealed a positive HAI result from each though, interestingly, one was missed by RPHA and the other by RIA. The positive unit transfused in case 'Far.' was subtyped by HAI as adw, while that transfused in case 'Ali.' was found to be ayw, suggesting that the commercial RIA test used may be more sensitive for the detection of adw than ayw. A similar RIA-bias has been described by Vanderweld, Mahmood, Goffin, Porter Megson and Cossart (1974).

It appears that HAI is capable of detecting C.I.E.O.P.-negative HB_sAg carriers which can produce hepatitis if transfused into a susceptible recipient. Had HAI been used at the time of donation, it is probable that 40 percent of the posttransfusion hepatitis cases investigated here would have been avoided. Whether the four remaining cases in which all donations were nagative by HAI, RPHA and RIA and the recipient was HB_sAg negative were in fact due to HB Ag is unclear, particularly in the face of circumstantial evidence of a hepatitis C virus(es) (Prince et al., 1974). Unfortunately, convalescent sera were not available to test for the appearance of HB_sAb.

CHAPTER 4

EFFECTIVENESS OF HEPATITIS B IMMUNOGLOBULIN IN THE PREVENTION OF HEPATITIS RESULTING FROM ACCIDENTAL EXPOSURE TO HB_sAg

I. Introduction

Normal immunoglobulin (N-IgG) prepared from the pooled plasma of unselected blood donors may prevent or modify infectious (type A) hepatitis. There are, however, conflicting reports regarding its efficacy in relation to serum (type B) hepatitis (Grossman et al., 1945; McCallum, 1965; Drake et al., 1953; Holland et al., 1966). During the recent virulent outbreak of dialysis-associated type B hepatitis in Edinburgh (Marmion and Tonkin, 1972) N-IgG exhibited no apparent protective effect. On the other hand, the use of hyper-immune hepatitis B globulin (HB-IgG) in France and the U.S.A. was associated with a definite protective effect which was particularly evident if administered soon after exposure to infective material (Krugman et al., 1971; Soulier et al., 1972). Soulier et al. (1972) followed 18 recipients of HB-IgG who had been exposed to HB_sAg by transfusion of antigen positive blood or by accidental parenteral exposure. A rise in transaminase levels occurred in two patients, but none developed HB_s antigenaemia, neither did passive immunisation appear to favour the development of an HB_sAg carrier state as had been feared by some workers (Prince et al., 1971).

Large clinical trials are currently being conducted in the U.K. and U.S.A. to provide further data regarding the safety and efficacy of HB-IgG, meanwhile this chapter is designed to provide an insight into the use of HB-IgG by considering the initial findings of an 'on-going' study at the South-East of Scotland Regional Blood

Transfusion Centre, Edinburgh. The HB-IgG used in this study was prepared at the Liberton Protein Fractionation Centre nearby. Its serologic (HB_sAb) status was compared with similar preparations from other centres.

II. Materials and Methods

(a) HB-IgG preparations

Since 1971 three batches of HB-IgG have been prepared from pooled donations known to possess specific HB_sAb by C.I.E.O.P. In each preparation the starting pool and the final product were exhaustively investigated for the presence of HB_sAg or immune complexes. HB-IgG was supplied as a 10 percent solution of 1 gm. or 0.5 gm. doses for intramuscular use. Similar preparations were obtained from Paris, Amsterdam and the Australian Commonwealth Serum Laboratory (C.S.L.) for comparison of serologic activity with our local product.

(b) Laboratory tests

Tests for HB_sAg were by HAI and RIA (Ausria-2). The amount of HB_sAb activity was estimated by direct HA titration, while E.M. was used to look for HB_sAg-HB_sAb immune complexes. Follow-up liver functions tests consisted of measuring serum bilirubin and transaminase (SGOT and SGPT) levels.

(c) Experimental protocol

0.5 - 1.0 gm. HB-IgG was given within 3 to 5 days following accidental exposure (needle-stick or non-parenteral exposure) to HB_sAg. Prior to treatment the accident victim was tested for HB_sAb and HB_sAg to (i) avoid administering HB-IgG to an individual already carrying high concentrations of HB_sAb or HB_sAg and

thereby avoid wasting HB-IgG by unnecessary innoculation, and (2) determine base level of HB_sAb (if any) in the victim prior to treatment. Serum samples were obtained 3 to 4 days after HB-IgG administration and, where possible, at fortnightly intervals for the first three months, then at monthly intervals for the next three months. Each accident was categorised as 'high' or 'low' risk depending upon the relevant circumstances. Twenty-two high risk cases of direct parenteral innoculation with HB_sAg positive blood resulted from either 'needle sticks' or cuts with broken glass. A further twenty-three low risk cases occurred as a result of accident situations in which infected blood was splashed onto large areas of unprotected skin, swallowed as a result of mouth to mouth resuscitation, or parenteral innoculation with blood from a patient who had previously been HB_sAg positive but who was HB_sAg negative at the time of the accident. During the follow-up, a record was kept of any other treatment the HB-IgG recipient received.

III. Results

(a) Assessment of HB-IgG preparations

HB-IgG prepared in Amsterdam, Edinburgh, Paris and the Commonwealth Serum Laboratory (Australia) were compared for HB_sAb activity by HA. All preparations exhibited a high concentration of HB_sAb with slight variations within and between centres (table 4.4.III.a).

Table 4.4.III.a. Titration of different preparations of HB-IgG

Origin	Batch No.	Reciprocal of HA titre
Edinburgh	GHB/1	6,000
	GHB/2	512,000
	GHB/3	128,000
Paris	91C	6,000
	82	128,000
	61	128,000
Amsterdam	1	12,000
	2	1,000
	3	4,000
Australia	2	64,000
	3	8,000
	4	32,000
	5	16,000

(b) Assessment of response to HB-IgG

Of the 45 recipients of HB-IgG only 22 were available for comprehensive follow-up (Table 4.4.III.b). None developed HB_s-antigenaemia and serum bilirubin levels remained normal. Four HB-IgG recipients (BL, McD, EG and JA) exhibited abnormal transaminase levels. BL had chronic hepatitis with persistently abnormal liver function prior to inclusion in the HB-IgG study, suggesting that the elevated transaminase values were unrelated to recent HB_sAg exposure. Patients McD and JA were receiving anti-tuberculosis therapy and suffering from glandular fever respectively. However, the liver function data

Table 4.4.III.b. **Follow-up of accident victims receiving HB-IgG.**

Patient	Occupation	Accident category	HB-IgG Dose/ batch	Follow-up period (weeks)	Peak SGPT/SGOT/ HB_sAb titre at time (weeks)	Reciprocal of peak HB_sAb titre at time (weeks)	Time (weeks) of last HB_sAb pos. sample
SK	Nurse	LR	1 gm/ GHB2	28	- / 5/ -	256/0-4	17
HL	"	HR	"	32	26/27/ -	128/2	12
WD	Dr.	"	"	28	18/32/ -	256/0-3	8
BL	Nurse	LR	"	16	59/82/*	16/1	3
ML	"	"	"	32	19/20/ -	256/2	9
B	Dr.	"	"	48	28/24/ -	128/1	2
IR	Nurse	"	"	32	35/30/ -	32/4	12
MoD	"	"	"	32	46/15/8**	128/2	18
PN	"	"	"	32	21/16/ -	64/4	11
HT	Dr.	"	"	28	17/17/ -	4/0-4	4
GB	Nurse	HR	"	24	-/17/ -	128/1	10
BW	"	"	"	32	4/12/ -	128/3	12
EG	Techn.	"	***	72	66/ -/17	16/1	14
GG	Dr.	"	1 gm/ GHB2	24	26/24/ -	32/0-5	10
PP	"	LR	"	32	17/29/ -	8000****	-
GS	Techn.	"	"	12	-/ -/ -	20/3	5
GY	Dr.	"	"	20	34/27/ -	128/7	1
BN	"	HR	"	8	35/33/ -	64/2	8
TH	Nurse	"	0.5 gm/ GHB3	16	38/35/ -	32/1	2
[illegible]	Dr.						
BK	Dr.	LR	"	20	38/26/ -	16/1	20
FQ	"	"	"	24	16/22/ -	-/-	-
JA	Nurse	HR	"	28	110/80/4	40/0-1****	-

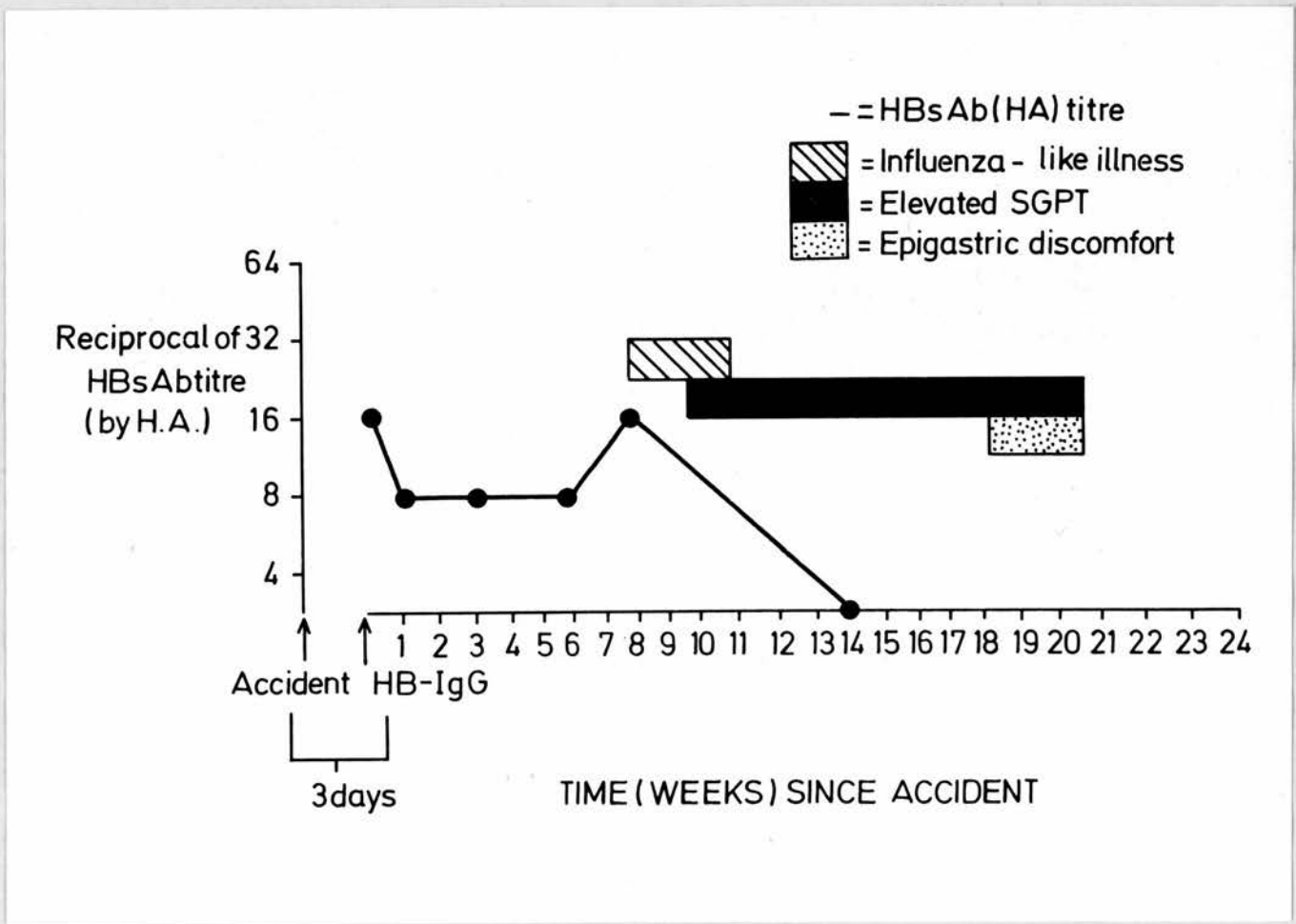
LR = low risk; HR = high risk; * chronic hepatitis; ** receiving anti-T.B. drugs;

- = no data available; *** = HB-IgG prepared at Lister Institute (Elstree);

**** pre-existing HB_sAb.

relating to patient EG (figure 4.4.III.c) may be related to his accidental exposure to HB_sAg. EG is a medical laboratory technician who cut his hand on a broken centrifuge tube containing HB_sAg (ayw) positive blood from a renal unit patient implicated in two fatalities during the 1969-1970 dialysis-associated epidemic in Edinburgh. There was little doubt, therefore, regarding the infectivity and virulence of the innoculum. EG received 20 ml. N-IgG immediately after the accident, the following day and five weeks later. He also received 1 gm. of HB-IgG (supplied by Lister Institute, Elstree) three days after the accident. An influenza-like

Figure 4.4.III.c. Post-HB-IgG follow-up of patient EG.



illness followed by abdominal pain developed nine weeks later with epigastric discomfort 20 weeks post innoculation, the latter being preceded by a moderate but persistent SGPT elevation lasting eleven weeks. The circulating HB_sAb titre rose prior to the prodromal symptoms but no HB_sAg was detected throughout the follow-up period. EG has remained well since then.

In all but one of the patients followed the HB-IgG administered contained sufficient activity for HB_sAb to be detectable (suggesting that antibody was in excess) for an average of 11 weeks. The highest passive HB_sAb titre usually occurred between 1 to 4 weeks post HB-IgG administration, a feature which may reflect the cumulative effect of the slow release of antibody from the site of intramuscular innoculation into the circulation. Those receiving 1 gm. of GHB 2 usually exhibited higher circulating HB_sAb levels than those receiving 0.5 gm. of GHB 3. Although one would expect this to be the case, the data cannot be compared directly since initial quality control studies on the HB-IgG preparations suggest that the activity of GHB 2 was greater than that of GHB 3.

As anticipated, the recipients of HB-IgG were all medical, nursing or technical staff, and as such it was interesting to note that among this high exposure risk group, 9 percent showed evidence of previous exposure to HB_sAg, representing ten times the incidence of HB_sAb than among healthy blood donors in the same region.

IV. Discussion

The distinction between 'high risk' and 'low risk' exposures to HB_sAg is difficult if not impossible to make (not all the six staff members who died in the 1969-1970 dialysis-associated outbreak in Edinburgh were obviously parenterally exposed). The type of

accidental involvement and the source of the innoculum should be carefully analysed in each case. Some of the subjects in the present study did not report actual parenteral innoculation, but were given HB-IgG because of the circumstances surrounding their exposure (e.g. mouth to mouth resuscitation of a neonate immediately after delivery of an antigen positive mother). In such cases assessment of the prophylactic value of HB-IgG may prove difficult.

None of the HB-IgG recipients showed evidence of immediate or delayed reaction to the injection. Most had detectable circulating HB_sAb for an average of eleven weeks after innoculation, confirming the findings of Szmuness, Prince, Goodman, Enrich, Pick and Ansari (1974). The reason for the rapid disappearance of antibody in BL, B, TH and FQ is not clear. None had pre-existing, anti-globulin (GM, Inv) to the administered HB-IgG. BL had chronic hepatitis possibly of autoimmune origin which may have accelerated HB_sAb removal by virtue of abnormal protein metabolism, but the other three were clinically and biochemically normal. Since immuno-globulins are suicidal proteins, being catabolised in the performance of their function, it could be argued that accelerated clearance of HB-IgG may reflect the presence of HB_sAg in or on the tissues. Millman et al. (1969) reported the occurrence of HB_sAg in the hepatocytes of serum negative patients and demonstrated cellular hypersensitivity to HB_sAg in nearly 60 percent of a group of HB_sAg negative chronic hepatitis patients. Further investigations regarding the occasional rapid clearance of HB-IgG are clearly merited.

Several batches of N-IgG, Rhesus anti-D and vaccinia immuno-globulin were tested for HB_sAb. No activity was found in the few anti-D or anti-Vaccinia preparations tested, and only one of the

27 N-IgG preparations exhibited an HB_sAb titre of greater than 1/500 by HA, most showing a titre of less than 1/10. It would seem, therefore, that the fractionation of HB_sAb positive plasma produces a product which, by virtue of its high HB_sAb content, provides a significant degree of protection against type B hepatitis as a result of accidental exposure involving a relatively small challenge dose. The presence of detectable circulating HB_sAb for some weeks after HB-IgG administration suggests antibody excess and infers that any circulating HB_sAg has been complexed and, hopefully, neutralised. It seems logical that HB-IgG should be administered as soon as possible after exposure (except where HB_sAb is already present in high concentration or where the victim is already found to be an HB_sAg carrier) for maximum efficacy before the virus gains the protection of the (hepatic) cells in which it replicates.

The HB-IgG preparations used in this study possessed specific antibody to both the d and y determinants of HB_sAg. It seems logical that the protective value of the product will be dependent in part upon the presence of antibody to all the HB_sAg determinants associated with the infecting virus. The HB_sAb activity of GHB 2 and GHB 3 was similar to that of HB-IgG prepared at other centres. Variation between batches is a feature requiring further assessment. HB-IgG is currently prescribed by volume or weight, whereas it may prove more beneficial to the recipient if doses were to be standardised in terms of biological activity. The feasibility of expressing the antibody activity directly is supported by Ginsberg et al. (1972) who observed a correlation between antibody titre and apparent protection. It is felt that the present study demonstrates the prophylactic value of HB-IgG which in turn emphasises the need to standardise such preparations on a world-wide basis.

PART FIVE

FINAL DISCUSSION AND CONSIDERATION OF FUTURE LINES OF RESEARCH

FINAL DISCUSSION

I. Identification of HB_sAg

Recognition of the significance of the presence of HB_sAg in blood has led to a proliferation of laboratory techniques over the past five years, many of which have been reviewed in Part One (Section Two) of this thesis. It is now established beyond doubt that 'third generation' tests (RIA, RPHA, HAI) are superior to C.I.E.O.P. for the detection of HB_sAg among blood donors and patients, increasing the number of detectable carriers (blood donor) by up to 50 percent (Koretz, Klehs, Ritman, Damus and Gitnick, 1973; Vandervelde et al., 1974; Hopkins, Robertson, Ross, Turnbull and Das, 1975; Wallace, Barr and Milne, 1975; Polesky and Taswell, 1975; Dodd, Ni, Malin and Greenwalt, 1975). Data presented in Part Four (chapters 1 and 3) suggests that the higher sensitivity of HAI, RPHA and RIA could result in a worthwhile reduction of the number of cases of posttransfusion type B hepatitis.

As one with some experience in the use of most of the newer HB_sAg assays, I find the most endearing quality of RIA to be its objectivity. Unfortunately, this is offset by the high cost of reagents and counting equipment, quite apart from the hazards of handling radioactive isotopes. Many laboratories, particularly those responsible for blood donor testing, are reluctant to use RIA because of cost and the time required for completion of the test. Among those who have evaluated some form of RIA a popular choice has been the sandwich solid phase kit (SP-RIA-AUSRIA) available from Abbott Laboratories (see Part One, Section 2, chapter 3). The initial version of the test (AUSRIA-I) utilised HB_sAb-coated tubes and required a total incubation time of 21 hours. This was replaced by a modified system (AUSRIA-2) in which the reactive surface area

was increased and the amount of non-specific 'background' isotope binding decreased by using an HB_sAb-coated bead, leading to a slight improvement in both sensitivity and specificity. Kinetic studies based on the AUSRIA test (Prince and Jass, 1974) revealed a marked effect of incubation temperature and time upon sensitivity, being enhanced at incubation temperatures up to 50°C, but declined if kept beyond eight hours at this temperature. Such findings may be explained by (1) the increasing speed of diffusion of HB_sAg towards bound HB_sAb attached to the solid phase, (2) reduction in the background count, and (3) partial denaturation of HB_sAg at higher temperatures leading to the exposure of additional antigenic sites.

To achieve an acceptable compromise providing good sensitivity in a reasonable time, instructions with the AUSRIA-2 kit suggest that serum samples be incubated by floating on a 45°C waterbath initially for 2-4 hours, followed by a 1 hour incubation with labelled antibody.

For those laboratories with facilities for liquid scintillation but not direct gamma counting, Jordan, Spiehler, Haendiges and Helman (1974) have described a method of solubilising the tube-bound radiolabelled (AUSRIA) complex with glacial acetic acid suitable for subsequent liquid scintillation counting. It is claimed the method avoids high background, chemiluminescence and quench.

Duimel and Brummelhuis (1973) have used voluminous m-diazobenzyloxymethylene cellulose as a carrier of HB_sAb in a competitive SP-RIA also requiring highly purified ^{125}I-labelled HB_sAg. The presence of either HB_sAg or HB_sAb in the test sample results in a decrease in the amounts of bound tracer (separated by centrifugation) compared with a negative control serum. Differentiation between

HB_sAg and HB_sAb necessitated a sandwich SP-RIA, although they point out that this may be accomplished by a single competitive SP-RIA test if a mixture of immunoadsorbants (insolubilized HB_sAg and HB_sAb) are used.

Hollinger, Werch and Melnick (1974) compared the effectiveness of SP-RIA (AUSRIA-1) with that of double antibody radioimmunoassay (DA-RIA) as screening tests for reducing the incidence of post-transfusion hepatitis B. Seven out of eight susceptible recipients who received DA-RIA positive blood subsequently developed clinical hepatitis B or showed serologic evidence of exposure to HB_sAg. In contrast, SP-RIA failed to implicate a positive donor in five of these, including two in whom clinical hepatitis B developed. Since both tests were of comparable sensitivity for HB_sAg detection, it is interesting to postulate that DA-RIA was recognising additional antigenic determinants (HB_cAg?) which were not identified by SP-RIA. This situation could result from the use of low pH (pH 2.4) to dissociate antigen-antibody complexes in the preparation of purified HB_sAg for raising HB_sAb for use in the DA-RIA. It is known that such treatment may disrupt the outer coat of the Dane particle, exposing the inner core (Hollinger et al., 1974). Antiserum produced in this way could contain HB_cAb in addition to HB_sAb and provide the capability for detecting an additional set of antigen-antibody reactions. At the time of writing DA-RIA kits are not commercially available.

The alternative to RIA is some form of haemagglutination. The best RPHA reagents achieve a sensitivity similar to RIA, are simple to use since they require no special equipment, and are considerably cheaper to purchase (Part One, section 2, chapter 2).

Hepatest (Wellcome Reagents) appears to be the RPHA of choice in the U.K., while 'Hepanosticon' (Organon) is attracting considerable interest on the continent (Reesink et al., 1973; Schuurs and Kacaki, 1974; Hadziyannis, 1974) and Abbott Laboratories are supplying 'Ausced' mainly in the U.S.A. (Germain, Sturdivant and Rightsel, 1973). Hepatest has been evaluated by a number of blood transfusion centres in Britain (Cayzer et al., 1974; Barbara, Denning, Clegham, Dane and Briggs, 1975) and is currently being used by many centres in England and Wales.

It is to be expected that extremely sensitive tests will be prone to occasional false positive reactions. However, recent data suggests that RPHA tests, particularly using animal erythrocytes as antibody 'carriers', may be subject to a higher than expected incidence of false reactions in certain racial groups (Chrystie, Islam and Banatvala, 1974; Wilcox, 1975). Shattock and Smith (1975) compared Hepatest and Hepanosticon with I.D. using heterogenous serum samples derived mainly from patients. While concluding that both RPHA tests satisfied most of the requirements for mass screening they did point out that batch variation in sensitivity does occur. Another RPHA test (Raphadex B) has recently been described by Prince, Ikram, Chicot, Wright, Vnek, Neurath, Lippin and Swiss (1975). This uses immunochemically purified chimpanzee HB_sAb bound to stabilised human erythrocytes. The test has equivalent sensitivity to RIA (AUSRIA-1) and detected a similar number of HB_sAg-containing specimens during screening of volunteer blood donors. Unfortunately, there appear to be differences in sensitivity between different reagent batches (Prince, 1975, personal communication).

In parts Two and Four of this thesis, evidence has been presented that the HAI test described possesses both the sensitivity and specificity required of a third generation HB_sAg technique. Reagent stability combined with the relative ease of large scale preparation results in a highly standardised test system which may be readily introduced into most blood transfusion centres at a cost considerably below that of any comparable commercial reagent.

An interesting new development has recently come to light regarding a haemagglutination procedure with very high sensitivity for the detection of HB_sAg (Pert and Verch, 1975). Fresh human erythrocytes were coated with HB_sAb and used to test for the presence of HB_sAg. Treatment of the reagent with cobra venom factor resulted in inhibition, and there was a marked loss of sensitivity with cells pretreated with formalin or chromic chloride, suggesting that C3 and some type of reactive sites on the cells are required. Sensitivity is claimed to be several orders of magnitude higher than other related tests, and the authors go as far as to suggest that this may be sufficient to detect HB_sAg if present in virtually any blood sample. Reproducible discrimination between positive and negative settling patterns is often difficult for the untrained observer, but it is hoped to overcome this problem by partially automating the technique (Pert, 1975, personal communication).

Detection of circulating antibody to the core of the Dane particle (HB_cAb) may provide a further means of identifying blood donors capable of transmitting hepatitis type B, since most individuals who are chronic carriers of HB_sAg have circulating HB_cAb (Purcell, Gerin, Almeida and Holland, 1973/4). Development of serological techniques for the detection of HB_cAb have been

hindered by the limited availability of the antigen. Free HB_cAg has not yet been found in the serum of either acutely or chronically infected individuals (probably because it becomes heavily coated with antibody). Chronic HB_sAg carriers could theoretically provide a source of HB_cAg, particularly if only those whose sera was rich in Dane particles were chosen. Unfortunately, this would involve processing large quantities of potentially infectious material (Fauval, Babiuk, Sheaff and Spence, 1975), a prospect which would not appeal to many laboratories even if they possessed the necessary equipment. Alternatively, HB_cAg may often be extracted from the nuclei of infected liver tissue (Barker et al., 1974; Maupas, Werner, Larouze, Millman, London, O'Connell and Blumberg, 1975) care being taken to remove free antibody before HB_cAg is exposed.

Despite the application of RIA to detection of HB_cAg and HB_cAb (Purcell et al., 1973/4; Greenman, Robinson and Vyas, 1975) complement fixation (C.F.) has been used extensively to characterise the HB_cAb response in patients with hepatitis B (Hoofnagle, Gerety and Barker, 1974). HB_cAb is frequently detectable while the surface antigen is still present during acute infection. The titre of HB_cAb generally falls to low levels after recovery, but high HB_cAb titres are routinely found in persistent HB_sAg carriers. Preliminary investigation of the possibility that HB_cAb might serve as a marker of chronic infection with hepatitis B virus in some individuals without detectable HB_sAg has revealed a high prevalence of HB_cAb in donors (without HB_sAg) who were implicated in cases of hepatitis following blood transfusion.

The DNA polymerase reaction can be used to radiolabel Dane particle cores for a specific and sensitive DA-RIA for HB_cAb (Robinson, 1975). DNA polymerase activity appears early in the

period of HB_sAg reactivity (corresponding to peak viral replication) and disappears at about the same time as HB_sAg (Krugman, Hoofnagle, Gerety, Kaplan and Gerin, 1974). Quantitation of DNA polymerase may provide a useful approach in distinguishing the degree of infectivity of HB_sAg positive sera.

II. Subtypes of HB_sAg

The ability to subtype a weakly reacting test sample provides important confirmation of the specificity of the original HB_sAg positive reaction. As detailed in Part Three(chapter 1), determinants a, d, y, w and r are readily definable by HAI. As 'new' HB_sAg (virus-coded) reactivities are discovered the value of a sensitive but simple subtyping test will become even more apparent. Since the information in Part One (Section 1, chapter 1, II) was compiled, some new HB_sAg determinants have been reported or rumoured, for example 'g', 'n', 'q' and 't' (Le Bouvier and Williams, 1975). It must be shown that each new postulant antigen is distinct, associated with the surface of HB_sAg particles, and coded for by the HB-Ag viral genome. Among the newly postulated determinants most data is available regarding 't', a peculiar feature of which is its variable physical behaviour, being overt in association with the adw phenotype, cryptic in association with ayw, and apparently absent from ayr.

Though apparently not attached to the HB_sAg particle surface, the e antigen, first described by Magnius and Espmark (1972), has generated considerable interest in recent months as a possible indicator of liver damage in HB_sAg carriers. e-antigen or specific e-antibody can be detected in the three-to-five-times concentrated serum of most HB_sAg carriers by I.D. Most investigators agree that

e-antigen is associated with active and usually continuing liver disease and may prove a particularly useful prognostic aid, while e-antibody appears to be significantly associated with normal or only mildly abnormal liver function (El Sheikh, Woolf, Galbraith, Eddleston, Dymock and Williams, 1975; Eleftheriou, Thomas, Heathcote and Sherlock, 1975; Feinman et al., 1975). My own investigations (as yet unpublished) agree with these findings, since the majority of apparently healthy HB_sAg (blood donor) carriers in the South-East of Scotland are typable for the e-system by I.D. provided test serum is concentrated three to five times with lyphogel. To date the only carriers to exhibit e-antigen are those with persistently elevated transaminase values, representing about 10 percent of carriers tested for the e-system. Since both e-antigen and DNA polymerase appear to be associated with viral replication, infectivity, abnormal liver function and the presence of Dane particles, it is tempting to hypothesise that they may represent the same product.

III. Immunisation against Hepatitis B

Although HB_sAg is distributed throughout the world, there are certain 'high risk' groups who would benefit from some form of active or passive protection. These include patients receiving blood transfusions, drug abusers and health-care personnel such as surgeons, physicians, dentists, nurses, laboratory technicians and blood bank workers. Haemodialysis patients and staff, children of HB_sAg positive mothers, institutionalised patients and persons visiting or living in certain tropical areas where sanitation is poor are also considered to be at risk.

Progress towards the development of a vaccine against hepatitis B has been hampered by the very limited success obtained in growing the virus in culture and the difficulty of obtaining a suitable animal model. Diluted and heat-treated whole serum (Krugman et al., 1971) may be regarded as an inactivated 'vaccine', but it is a very crude way of inducing immunity and it is improbable that such material will be licensed for general use. Isolated coat protein challenges the body's immune mechanism in the same way as the whole infectious agent and the possibility of using purified HB_sAg free of nucleic acid, and therefore not infective, appears attractive. However, such an approach may be precluded by contaminating host protein which may include various pre-existing structures of the liver cell and may thus induce undesirable immunological reactions (Popper and Mackay, 1972). Subunits of HB_sAg, in the form of small polypeptides, are considered by some (Zuckerman and Howard, 1975) to possess a greater potential as possible immunogens. In a recent report by Dreesman, Chairez, Suarez, Hollinger, Courtney and Melnick (1975), five purified polypeptides were isolated from HB_sAg types adw and ayw and injected into guinea pigs. Antibody to each HB_sAg type was measured by RIA. Although not all the five elicited detectable antibody response, it was concluded that subunits free of antigenically cross-reacting host components and which do not contain infectious viral nucleic acid may yield an effective hepatitis B vaccine. Another approach could be the development of a synthetic peptide, which, when coupled to a macromolecular carrier, could serve as a suitable immunogen. Successful transmission of hepatitis B virus to chimpanzees (Barker et al., 1973) may provide a means of monitoring vaccines when they are developed.

Results of studies on passive immunisation using pooled human immunoglobulin have been inconsistent, probably because most preparations contained little or no HB_sAb. However, the evidence in Part Four (chapter 4) suggests that immunoglobulin with a high titre of HB_sAb (HB-IgG) prepared from plasma of selected donors could offer protection in certain cases. Since the early work of Prince et al. (1971), Gocke (1971), Conrad et al. (1971), Krugman et al. (1971) and Soulier et al. (1972), the protective effect of HB-IgG has been studied on a variety of 'high risk' groups. Szmuness et al. (1974) compared the protective effects of HB-IgG and normal immune globulin (passive haemagglutination titre = 1/16) on children admitted to three institutions and concluded that both preparations were effective in preventing or modifying non-parenterally transmitted hepatitis in an endemic setting during a 1.5 to two year follow-up period. Kohler, Dubois, Merrill and Bowes (1974) found HB-IgG to be effective, compared to no treatment, in preventing neonatal hepatitis B infection in babies born to HB_sAg positive mothers, and Redeker, Mosley, Gocke, McKee and Pollack (1975) obtained evidence that HB-IgG appeared effective in suppressing not only disease, but infection itself in spouses of patients with acute type B hepatitis, compared with normal immune globulin.

The majority of reports concerning the effectiveness of HB-IgG have been with regard to prevention of type B hepatitis in dialysis units and in individuals accidentally exposed to HB_sAg as a result of needle-stick-type accidents. Half as many cases of hepatitis B developed among patients treated with HB-IgG as compared with the group receiving normal globulin during a clinical trial

in two haemodialysis centres in New Jersey (Szmuness, Prince, Hoofnagle, Ribot and Jacobs, 1975), while in a double blind study reported by Desmyter, Bradbourne, Vermylen, Daneels and Boelaert (1975) HB-IgG significantly protected haemodialysis patients against development of HB_s antigenaemia, compared to control patients receiving normal gamma globulin. Results of a large multicentre study (Prince, Szmuness, Mann, Vyas, Grady, Shapiro, Suki, Freidman and Stenzel, 1975) in which haemodialysis patients and staff were given either high, intermediate or low titre HB-IgG suggest that high titre material may be most protective particularly among patients.

Preliminary evaluation of patients who received prophylactic HB-IgG following accidental needle-stick exposure to HB_sAg indicated that HB-IgG significantly reduced the frequency of both clinical and subclinical hepatitis during the first 3 to 4 months after injection (Seeff, Zimmerman, Wright, Felsher, Finkelstein, Garcia-Pont, Greenlee, Dietz, Hamilton, Koff, Leevy, Kiernan, Tamburro, Schiff, Vlahcevic, Zemel, Zimman and Nath, 1975). However, less than 10 percent of the recipients had detectable HB_sAb at the sixth month after injection, suggesting that HB-IgG might need to be given every 3 to 4 months to continually exposed individuals. This is in agreement with my own findings reported in Part Four (chapter 4).

HB-IgG is administered to 'high risk' groups in the hope that circulating HB_sAb will prevent the hepatitis B virus (should it enter the blood) from reaching its target organ and replicating. The logic of this seems to be borne out by the findings of De la Concha, Ortiz, Hernandez-Guio and Hernando (1975) who found immunity to the disease to be directly dependent on the titre of circulating HB_sAb.

Given that high titre HB-IgG appears to be initially more protective than normal gamma globulin in certain circumstances, the question of when and where it should be used is not as clear-cut as might be supposed. As follow-up is extended there are indications that normal gamma globulin is more effective than HB-IgG in inducing longer-lasting passive-active immunity to HB_sAg. Grady and Lee (1975) found that 12 months after injection more recipients of normal gamma globulin possessed HB_sAb than in a group receiving high titre HB-IgG, suggesting that the latter treatment may inhibit active stimulation. A similar finding was noted by Seeff et al. (1975) with over 30 percent of normal gamma globulin recipients possessing HB_sAb after six months, compared with only 8 percent of the HB-IgG group. This is an important point because the need to stimulate active immunity must be one of the considerations in the decision to use HB-IgG or normal gamma globulin.

On the basis of available data, it is difficult to decide upon specific guidelines for the use of either product, or indeed the frequency of administration. Theoretically immunoprophylaxis need not be given to persons who already have circulating HB_sAb. The main requirement for HB-IgG would seem to be in cases of accidental HB_sAg inoculation where one should aim for maximum immediate protection and be less concerned with long term immunity. HB-IgG does not seem to be indicated in the prophylaxis of transfusion-associated hepatitis since approximately 90 percent of such complications are not now caused by hepatitis B virus (Prince et al., 1974). Posttransfusion (type B) hepatitis should continue to be prevented mainly by using the least amount of blood possible, using all-volunteer blood and screening donors by the most sensitive

assays available. Such preventative measures should have a much greater potential impact than prophylactic gamma globulin.

It is difficult to know whether immunoprophylaxis should be given to persons who are repeatedly exposed to HB Ag, and if so, whether it should be in the form of HB-IgG or normal gamma globulin. Experimental hepatitis B vaccines are being prepared but several years will be required before they are accepted as safe and effective. In the interim, the use of HB-IgG or normal gamma globulin will depend upon the availability of the former and upon their relative ability to induce passive-active immunity.

IV. <u>The Way Ahead</u> **(future research arising from work reported in this thesis).**

At the time of writing the haemagglutination-inhibition test for detection of HB_sAg, described in this thesis, is already proving its worth in three Scottish Regional Blood Transfusion Centres and the Iranian National Blood Transfusion Service, while laboratories as far afield as South Africa and the U.S.A. are evaluating reagents prepared in Edinburgh. However successful a technique may appear, there is invariably room for improvement. Automation of HB_sAg testing may be desirable in centres required to test very large numbers of donations daily, and to this end I am pleased to report that Mr. John Lockyer at the Blood Transfusion Centre in Bristol is employing HB_sAg-coated, gluteraldehyde-fixed spherocytes prepared in Edinburgh to develop a fully automated test procedure using the Technicon autoanalyser. Although many laboratories may not consider automation of HB_sAg testing worthwhile, the fact remains that the main weakness of the present (manual) HAI test is its lack of

objectivity. Automation at the point of reading, possibly in the form of an optical scanning device in conjunction with a numerical scale, would be desirable provided sensitivity did not suffer.

The discovery that HB_sAg-coated gluteraldehyde-fixed spherocytes could be 'overcoated' with specific antibody and subsequently used for a direct RPHA-type test for HB_sAg has meant that extremely stable RPHA reagents could be available to any laboratory capable of preparing HB_sAg-coated, gluteraldehyde-fixed spherocytes. Not only do overcoated cells constitute a more stable reagent than any of the commercial RPHA kits (which are extremely unstable when reconstituted), but the fact that stabilised HB_sAg-coated spherocytes 'select' specific HB_sAb from the environment infers that specificity should also be improved. These are features of the overcoated spherocytes which are in the process of investigation.

The work of Purcell et al. (1973/4) concerning the association of HB_cAb with HB_sAg carriage and possible infectivity suggests that a simple direct haemagglutination test for HB_cAb could prove useful. To this end, one aim of current research is the extraction of HB_cAg from serum and/or infected liver for subsequent spherocyte coating. Preliminary experiments using a crude HB_cAg-HB_sAg preparation suggest that it may be possible to coat HB_cAg in the same way as HB_sAg, although this may require de-purifying the 'purified' HB_cAg with normal serum prior to coating.

Perhaps the most exciting prospect looming ahead of virologists working in the hepatitis field is that of soon identifying other posttransfusion hepatitis viruses. Their existence seems certain since with the

introduction of 'third generation' HB_sAg testing most posttransfusion (viral) hepatitis cases are negative for HB_sAg and subsequently show no evidence of antibody response to either surface or core components (Prince, et al., 1974; Feinstone, Kapikian, Purcell, Alter and Holland, 1975; Alter, Purcell, Holland, Feinstone, Morrow and Moritsugu, 1975; Galbraith, Portmann, Eddleston and Williams, 1975; Villarejos, Visona, Eduarte, Provost and Hilleman, 1975). In order to eliminate hepatitis A virus (HA-Ag) as the causative agent of non-B hepatitis, I have obtained antiserum to the MS-1 strain of HA-Ag from the National Institutes of Health (Bethesda, U.S.A.) which I am currently using to attempt to identify the causative agent of 'epidemiological' hepatitis A outbreaks in Edinburgh. When sufficient of the causative (virus) agent has been isolated from pre- and acute phase stool extracts, it is hoped to attempt spherocyte coating. Meanwhile electron microscopy and immune adherence haemagglutination appear to be identifying a virus-like particle serologically related to MS-1.

APPENDICES

APPENDIX A

Phosphate Buffered Saline (P.B.S.)

Stock Solutions.

1. Disodium hydrogen orthophosphate (Na_2PHO_4, B.D.H., analar grade, molecular weight = 177.99) - a 0.15 molar solution was prepared by dissolving 53.397 gms. in 2000 ml. of sterile distilled water. Two grams of sodium azide was also added to prevent microbial growth.

2. Potassium dihydrogen orthophosphate ($K\ H_2PO_4$, B.D.H., analar grade, molecular weight = 136.09) - a 0.15 molar solution was prepared by dissolving 40.827 gm. in 2000 ml. sterile distilled water. Two grams sodium azide was also added (see above).

3. Sodium chloride (NaCl, B.D.H., analar grade, molecular weight = 58.44) - a 0.15 molar solution was prepared by dissolving 17.532 gm. in 2000 ml. sterile distilled water. Sodium azide was added as above.

Stock solutions were stored at 4°C or room temperature.

P.B.S. pH 6.4 was prepared by mixing the following volumes of stock solutions:-

Na_2HPO_4	280 ml.
$K\ H_2\ PO_4$	520 ml.
NaCl	800 ml.
tot al volume =	1600 ml.

P.B.S. pH 7.2 was prepared by mixing the following volumes of stock solutions:-

Na_2HPO_4	715 ml.
$K\ H_2PO_4$	285 ml.
NaCl	1000 ml.
total volume =	2000 ml.

Working solutions were kept at 4°C.

APPENDIX B

Preparation of HB_sAg from serum by extraction with polyethylene glycol (P.E.G.)

Twenty-five ml. HB_sAg positive serum was centrifuged at 120,000 g. and the pellet resuspended to the original volume in P.B.S. pH 6.4. The pH was adjusted to 4.6, and 2 gm. P.E.G. 6000 were added. After stirring for 10 minutes, the mixture was centrifuged at 1500 g. for 10 minutes, the supernatant discarded and the precipitate resuspended in 25 ml. of sterile distilled water. The P.E.G. was subsequently removed by filtration through Sephadex G200. Fractions possessing HB_sAg activity were pooled, concentrated ten times using an Amicon cell, dialysed against P.B.S. pH 6.4 and adjusted to contain 128 latex units of activity.

APPENDIX C

Preparation of HB_sAg from serum by density gradient centrifugation

HB_sAg positive serum was centrifuged at 120,000 g. and the pellet resuspended to 1/20 of its original volume in P.B.S. pH 6.4. One ml. of this concentrate was layered onto a 4 ml. caesium chloride (CsCl, B.D.H. analar grade, molecular weight = 168.36) density gradient (2 gm. CsCl dissolved in 4 ml. sterile distilled water). The gradient was then centrifuged at 130,000 g. for 24 hours at $4^{\circ}C$, after which 0.2 ml. fractions were collected from the bottom of the tube. The refractive index of each fraction was measured and the density determined from tables. Fractions having a density between 1.1948 - 1.2502 $g./cm^3$ were pooled, dialysed against P.B.S. pH 6.4 and re-gradiented. Those fractions possessing most HB_sAg (as determined by immune electron microscopy) were pooled, dialysed against P.B.S. pH 6.4 and adjusted to contain 128 latex units of HB_sAg activity.

APPENDIX D

Treatment of HB_sAg positive serum with betapropiolactone (B.P.L.) and ultra-violet light (U.V.L.)

B.P.L.* was diluted 1 in 10 in sterile distilled water. This 10 percent solution was further diluted 1 in 10 (to give final B.P.L. concentration of 17 percent) in saline bicarbonate solution**. (Abovepprocedures should be carried out at $0^{\circ}C$ to $4^{\circ}C$).

One volume of 1 percent B.P.L. solution was mixed with two volumes of HB_sAg-positive serum for 2 hours at $37^{\circ}C$ and left overnight at $4^{\circ}C$.

The serum B.P.L. mixture was then decanted into plastic petri dishes (just sufficient to cover bottom of dish) and exposed to U.V.L. in a Luckham chamber for 2 hours.

*B.P.L. (Betaprone: Fellows and Testagar) should be stored at minus $15^{\circ}C$ to minus $20^{\circ}C$ when not in use, and care should be taken to avoid inhalation of fumes.

**Saline bicarbonate solution - The following reagents were dissolved in 100 ml. sterile distilled water containing 1 mgm./ml. sodium azide;

1.68 gm. $NaHCO_3$ (B.D.H., analar grade, mol.wt.84.01)
0.90 gm. NaCl (" " " " " 58.44)
1 mgm. phenol (M & B, detached crystals, mol. wt. 94.11)

APPENDIX E

Phosphate Buffer pH 7.2

Stock solutions consisted of:

A. 0.2M Na_2HPO_4 (B.D.H., analar grade)
B. 0.02M K H_2PO_4 (B.D.H., analar grade)

prepared by dissolving in sterile distilled water containing 0.1 percent (w/v) sodium azide.

Working solutions were prepared by dissolving stock solutions in sterile distilled water containing 0.1 percent (w/v) sodium azide.

APPENDIX F

Summary of HB_sAg testing data from five English Blood Transfusion Centres.

SUMMARY OF RESULTS OF TESTS FOR AUSTRALIA ANTIGEN AND ANTIBODY IN DONORS RECEIVED FROM RTCs UP TO SEPTEMBER 1972

	Number	Antigen Positive	Antibody Positive
, SHEFFIELD (1) (April - October 1971; first six months)			
ll donations	56,057	41 (1: 1367)	19 (1: 2950)
eneral public	54,557	38 (1: 1462)	17 (1: 3208)
niversity etc.	1,222	1 (1: 1222)	1 (1: 1222)
risons etc.	278	2 (1: 139)	1 (1: 278)
(2) (November 1971 - April 1972; second six months)			
ll donations	51,594	15 (1: 3439)	2 (1: 25,797)
eneral Public	50,005	13 (1: 3846)	2 (1: 25,002)
niversity etc.	1,163	0	0
risons etc.	426	2 (1: 213)	0
. TOOTING (1) (February - May 1972; first four months)			
ll donations	71,794	49 (1: 1465)	128 (1: 561)
eneral public	66,022	38 (1: 1737)	98 (1: 674)
niversity etc.	1,751	1 (1: 1751)	6 (1: 292)
rmed Forces	1,451	0	2 (1: 726)
risons etc.	570	2 (1: 285)	8 (1: 71)
ew donors	2,000	8 (1: 250)	14 (1: 143)
(2) (June - August 1972			
ll donations	56,877	50 (1: 1138)	1117 (1: 486)
eneral public	49,207	30 (1: 1640)	84 (1: 586)
niversity etc.	729	0	0
rmed forces	0	0	0
risons etc.	1,004	6 (1: 67)	10 (1: 100)
. BRISTOL (November 1971 - May 1972)			
ll donations	66,799	42 (1: 1590)	40 (1: 1670)
eneral public	61,044	34 (1: 1801)	32 (1: 1908)
niversity etc.	2,618	2 (1: 1309)	2 (1: 1309)
rmed Forces	2,164	4 (1: 541)	5 (1: 433)
risons etc.	973	2 (1: 486)	1 (1: 973)
. CAMBRIDGE (January 1971 - February 1972)			
ll donations	23,390	16 (1: 1462)	12 (1: 1941)
eneral public	19,438	13 (1: 1494)	10 (1: 1944)
niversity etc.	1,527	1 (1: 1527)	0
rmed Forces	1,449	0	0
risons etc.	976	2 (1: 488)	2 (1: 488)
. WESSEX (October 1971 - June 1972)			
ll donations	46,752	18 (1: 2597)	-
eneral public	42,675	9 (1: 4742)	-
rmed Forces	2,401	3 (1: 800)	-
risons etc.	1,676	6 (1: 279)	-

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The incidence and significance of hepatitis B (surface) antibody in a burns unit

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Summary

The incidence of hepatitis B (surface) antigen and its specific antibody among patients and staff of a burns unit were investigated using a sensitive haemagglutination test. Comparison with appropriate age- and sex-matched controls showed a higher incidence of specific antibody among both patients and staff within the unit. No antigen was detected.

INTRODUCTION

SEVERE burns (in excess of 40 per cent total body surface) are associated with profound alterations in plasma volume during the first few hours (Kukral and Shoemaker, 1970). Specific therapy frequently employs the transfusion of plasma during the initial resuscitation period (Muir and Barcley, 1973), followed by whole blood for those with more than 20 per cent full-thickness burns (Sanders, 1974). Further transfusion may be required during subsequent skin grafting.

Hepatitis B (serum hepatitis) has emerged as a significant complication following the transfusion of blood and blood products, and it has been estimated that 25–60 per cent of recipients of hepatitis B (surface) antigen (HB_s-Ag)-positive blood develop antigenaemia, and in many it is subsequently associated with hepatitis (Gocke and Kavey, 1969). Each unit of dried plasma is prepared from a pool of 10 donations, and since a severely burned adult may receive as many as 12 units of plasma during the initial period of resuscitation, the maximum total exposure might be as high as 120 blood donations for plasma alone. The outcome of a challenge with HB_s-Ag in the burned patient may be influenced by the transient immune deficiency state which follows major thermal injury (Rapaport et al., 1968; Munster et al., 1970; Daniels et al., 1971; Mahler and Batchelor, 1971) since a high incidence of HB_s-Ag has been found in association with conditions characterized by underlying immune deficiency (Blumberg et al., 1967).

The following article describes the results of a study designed to detect HB_s-Ag and HB_s-Ab in the sera of 118 burned patients who received plasma and whole blood prior to the introduction of routine HB_s-Ag blood-donation testing in 1971.

PATIENTS AND METHODS

Sera from 118 patients previously treated in the burns unit and 26 staff (medical and nursing) were collected and stored at −20°C until tested for HB_s-Ag and HB_s-Ab. The controls consisted of 118 age- and sex-matched, healthy blood donors being tested for the first time and with no history of blood transfusion, and 25 members of the medical and nursing staff from a general surgical ward in the same hospital. A haemagglutination technique (Hopkins and Das, 1973) was used to test the sera for the presence of HB_s-Ag and HB_s-Ab. All specimens were coded and their origin not revealed to the person responsible for testing. Sera giving positive reactions were confirmed and subtyped by a modification of this technique (Hopkins and Das,

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1974). Serum glutamic pyruvic transaminase (SGPT) values were also determined on most samples.

RESULTS

The overall results are summarized in *Table I* and show that HB_s-Ag was not detected in any of the patients, appropriate matched controls, or members of the burn unit staff. HB_s-Ab, however, was present in 6·9 per cent of the patients but in none of the control blood-donor population.

Table I. Incidence of HB_s-Ag and HB_s-Ab amongst patients and staff of a burns unit and their respective controls

	No. tested	*No. HB_s-Ag*	*No. HB_s-Ab-Pos*	
Patients	118	0	8	(6·9)†
Controls	118	0	0	(0)
Staff	26	0	2*	(7·6)
Controls	23	0	1**	(4·3)

† Figures in parentheses represent percentages
* Titres were 1/80 and 1/40; only anti-a was detectable when subtyped
** Titre was 1/640 and subtyped as anti-ad

This difference was statistically significant at the 1 per cent level (McNamar test). In the staff of the burns unit the incidence of HB_s-Ab was 7·6 per cent compared to 4·3 per cent in a similar group working in a general surgical unit. The difference between these two was not significant.

Further details of the HB_s-Ab-positive patients are summarized in *Table II*. With the exception of 1 patient, the HB_s-Ag titre was low. In 5 patients it was so low that it was not possible to subtype. One of these patients had a slightly abnormal elevation of SGPT.

DISCUSSION

Since February, 1974 all blood donations in the South-east region of Scotland have been tested for HB_s-Ab and HB_s-Ag by haemagglutination and haemagglutination-inhibition respectively (Hopkins and Das, 1973). Current evidence, using this technology on 44,053 donations, shows an HB_s-Ab incidence of 0·7 per cent which compares with 0·04 per cent as determined by counter-electrophoresis (CIEOP) in the same laboratory during the previous 12 months (Hopkins et al., 1975). If this frequency (0·7 per cent) is taken as a baseline for the regional population, then the incidence of HB_s-Ab in burned patients, following treatment, is 9·8 times greater. This difference was highly significant. In a recent publication Payne et al. (1974) found that the combined incidence of HB_s-Ab in patients admitted to medical and surgical wards of a general hospital was almost 3 times greater than that of the local blood donor population. It seems likely, therefore, that burned patients are exposed to HB_s-Ag to a considerably greater extent than most other groups of hospitalized patients. This is hardly surprising in view of the fact that resuscitation and subsequent plastic surgery may expose a burned individual to more than 100 different blood donors and that the immune status of these patients at the time of exposure could be abnormal.

Within the group of HB_s-Ab-positive patients no correlation was found between the degree of exposure (number transfusions given), the time elapsed since exposure, and the HB_s-Ab titre. In all but one sample (patient no. 6) the amount of antibody was too low to be detected by CIEOP, a feature which may account for the fact that in 5 of these patients only the anti-a specificity

Table II. Some details of HB_s-Ab-positive patients

Patient no.	*Percentage burn*	*Time (yrs) between treatment*	HB_s-Ab *titre*	*Subtype*	*SGPT*
6	22	15	4000	ay	3
7	31	2	10	a	2
15	25	2	10	a	10
33	18	2	80	a	N.T.
90	22	5	40	a	6
104	30	10	40	a	7
106	20	16	160	ad	17
115	8	2	80	ad	1

could be identified. SGPT values were only slightly elevated in one of the antibody-positive patients.

The incidence of HB_s-Ab among the surgical and nursing staff of the burns unit was found to be 10·8 times greater than in the regional blood-donor population and almost twice that of comparable staff in a general surgical ward of the same hospital. Because the numbers in this part of the study are small, it is not possible to come to firm conclusions, but it does suggest that the high risk seen in the burned patients is likely to be transferred to the staff. Such a conclusion is supported by the observation that in haemodialysis units 1 or 2 members of staff suffer some exposure for every 2 patients (Editorial, British Medical Journal, 1972).

CONCLUSION

The patients studied in this series were transfused with blood and plasma prior to routine HB_s-Ag testing of blood donations, and thus the clinical significance of the results so obtained should not be interpreted in light of existing practices. Further studies are in hand to ascertain the incidence of HB_s-Ab in burned patients managed with blood and plasma which has been HB_s-Ag tested. The data do suggest, however, that burned patients may represent a population at especially high risk and that this risk is transferred to their attending staff. Burned patients may constitute a particularly sensitive hospital population on which the quality of HB_s-Ag testing by the Blood Transfusion Service can be readily assessed.

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ad and *ay* subtypes of hepatitis B antigen in a case of hypogammaglobulinaemia

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SYNOPSIS We describe the finding of d and y specificities of hepatitis B surface antigen (HB_sAg) in a case of hypogammaglobulinaemia of the 'common variable' type treated with fresh frozen plasma infusions. Absorption studies show that the two specificities are on separate particles, suggesting dual infection. It raises important questions regarding the relationship between HB_sAg persistence and the immune status of the carrier.

Levine and Blumberg (1969) first suggested that HB_sAg could be separated into subtypes with a shared group specificity designated a. The principal subtype specificities have since been defined and designated d and y (le Bouvier, 1971) and w and r (Bancroft *et al*, 1972). These pairs of specificities seem to represent two allelomorphic systems, the four phenotypes being adw (D), adr, ayw (Y), and ayr. The last is exceedingly rare in a European population. Increasingly, hepatitis reference laboratories are able to test for d and y specificities using monospecific antisera. As a rule, the two common phenotypes, D and Y, are mutually exclusive. The finding of d and y specificities in the same serum is rare. It is even less common to find both d and y on separate particles, and the few reported cases were all on long-term haemodialysis (van Kooten-Kok Doorschodt *et al*, 1972; Soulier and Couroucé-Pauty, 1973). In our present report immunoabsorption studies indicate that the two specificities reside on separate particles, suggesting dual infection.

To the best of our knowledge there are no previous reports of this finding in hypogammaglobulinaemia. Such patients, if treated with fresh frozen plasma infusions, may be at particular risk of becoming hepatitis B virus (HBV) carriers.

Case Report

A 57-year-old Caucasian man had had recurrent respiratory infections since adolescence. In 1957, aged 39 years, he was investigated for recurrent cough and purulent sputum. The bronchogram was normal and a diagnosis of chronic suppurative bronchitis was made. In 1962 he was admitted to hospital with bilateral bronchopneumonia and found to have a serum γ-globulin level of 0·2 g/l. Regular therapy with pooled normal human γ-globulin was begun and he was much improved subsequently. After four years he began to have anaphylactic reactions to the γ-globulin and treatment was discontinued. Despite continuous prophylactic antibiotic therapy he deteriorated and in 1969, after an attempt at desensitization, γ-globulin therapy was resumed. Further anaphylactic reactions ensued and in September 1971 he was started on infusions of fresh frozen plasma, initially one per week and later on every second week, along with continuous oral ampicillin. In March 1972 he suffered an episode of general malaise, anorexia, and upper abdominal pain. He was not jaundiced and his serum bilirubin, SGOT, SGPT, and LDH levels were normal, but the serum alkaline phosphatase was raised at 263 IU/l. A liver biopsy was not done and serum was not tested for HB_sAg. Subsequently the alkaline phosphatase returned to normal. In September 1973, plasma infusions were reduced to three-weekly intervals and on this regimen he has since remained reasonably well.

In June 1974 his immune status was fully investigated, with the following results:

Peripheral blood:

Absolute lymphocyte and granulocyte counts were normal.

Serum immunoglobulins:

IgG 30 IU/ml, IgA 23 IU/ml, IgM 28 IU/ml (2½ weeks after plasma infusion).

Serum complement:

C3 and C4 levels normal.

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Red cell serology:
Direct and indirect antiglobulin tests and screen for anti-erythrocyte antibodies negative
Iso-agglutinin titres:
Iso-agglutinins not detectable (red cells, group O)
Phagocyte function:
Phagocytosis and intracellular killing of *Staphylococcus aureus* by blood leucocytes, stimulated NBT test, and leucocyte chemotaxis (migration through 3μ Millipore filter), all normal
Lymphocyte function:
Stimulation of blood lymphocytes (incorporation of 3H-thymidine into DNA) by mitogens (phytohaemagglutinin, conconavolin A, and poke-weed mitogen) and by pooled allogeneic cells, all normal
Blood lymphocyte subpopulations:
The T cell count (E rosettes) was normal; B cells (EAC rosettes) 6% (normal 10-20%)
Skin testing:
Positive delayed hypersensitivity response to mumps antigen; no response to PPD and Candida
Antibody response to tetanus toxoid:
No antibody detected (passive haemagglutination technique) before and 14 days after immunization

These results confirmed hypogammaglobulinaemia and demonstrated intact cell-mediated immunity, both phagocytic and lymphoid.

In September 1974 routine testing in a diagnostic laboratory (Regional Virus Laboratory, City Hospital, Edinburgh) showed that the patient's serum was positive for HB$_s$Ag (by counter-immunoelectrophoresis, CIEP). At this time the liver was not enlarged and liver function tests (serum bilirubin, GOT, GPT, and alkaline phosphatase) were normal.

HB$_s$Ag TESTING AND SUBTYPING

Methods used in our laboratory to confirm HB$_s$Ag positivity were haemagglutination inhibition (HAI), as previously described by Hopkins and Das (1973), haemagglutination (turkey cell HA kit, Wellcome Reagents Ltd), and radioimmunoassay (Austria I RIA kit, Abbot Labs, Ltd). To identify and titre d and y specificities, monospecific antisera were used in the HAI assay as previously described (Hopkins and Das, 1974).

IMMUNOABSORPTION

In order to determine whether d and y were associated with the same or separate particles, 0·5 ml of a 1 in 5 serum dilution was incubated with an equal volume of monospecific anti-y serum[1] at 37°C for 1 hour, and at 4°C for 16 hours. The mixture was then centrifuged at 30 000 × g for 60 minutes to pellet immune complexes. The supernatant and pellet were tested for a, d, and y antigenic specificities by HAI and the supernatant for the presence of anti-y antibody by direct haemagglutination.

ELECTRON MICROSCOPY

Serum was centrifuged at 120 000 × g for 4 hours and the pellet washed once in saline and reconstituted to 1/20th of the original volume. One drop of this suspension was mixed with an equal volume of 2% phosphotungstic acid (pH 6·8) and absorbed onto a collodion-coated copper grid (400 mesh) for examination in the electron microscope (Hitachi 12A).

Results

HB$_s$Ag SUBTYPES

HB$_s$Ag positivity was confirmed in our laboratory by HAI, HA, and RIA. Both d and y subspecificities were detectable in the serum to titres of 1 in 200 and 1 in 100 respectively (HAI method).

The results of immunoabsorption against monospecific anti-y are summarized in the table. Anti-y was present in the supernatant, showing that antibody was present in excess. Therefore apparent absence of y specificity in the pellet was presumably because all y determinant combining sites were occupied by anti-y. No y antigen was detectable in the supernatant while d subspecificity remained at the original titre (1:20, equivalent to 1:200 of the undiluted serum), indicating that d bearing particles had not complexed with anti-y, that is, d and y subspecificities were on separate particles. The reciprocal absorption (using monospecific anti-d) was performed on a serum sample taken at a different time; d subspecificity was removed, leaving y undiminished in the supernatant at a titre of 1:1000. Because specificity a is common to all subtypes, the

	Titres		
Antigen Specificity	*Serum*[2] *(1:10 Dilution) before Absorption*	*Supernatant after Absorption*	*Pellet*
a	1/1600	1/1000	1/200
d	1/20	1/20	Negative
y	1/10	Negative	Negative

Table *Immunoabsorption with monospecific anti-y*[1]

[1]Monospecific anti-y antiserum prepared by affinity chromatography
[2]Titres performed on whole serum: figures corrected for 1:10 dilution incurred during absorption
Reciprocal absorption of a separate serum sample using monospecific anti-d antiserum removed d antigen leaving y antigen undiminished (see text).

[1]Prepared by affinity chromatography. Antiserum containing anti-ayw activity was processed on an activated Sepharose 4B column to which was coupled HB$_s$Ag of specificity adw, removing anti-aw activity.

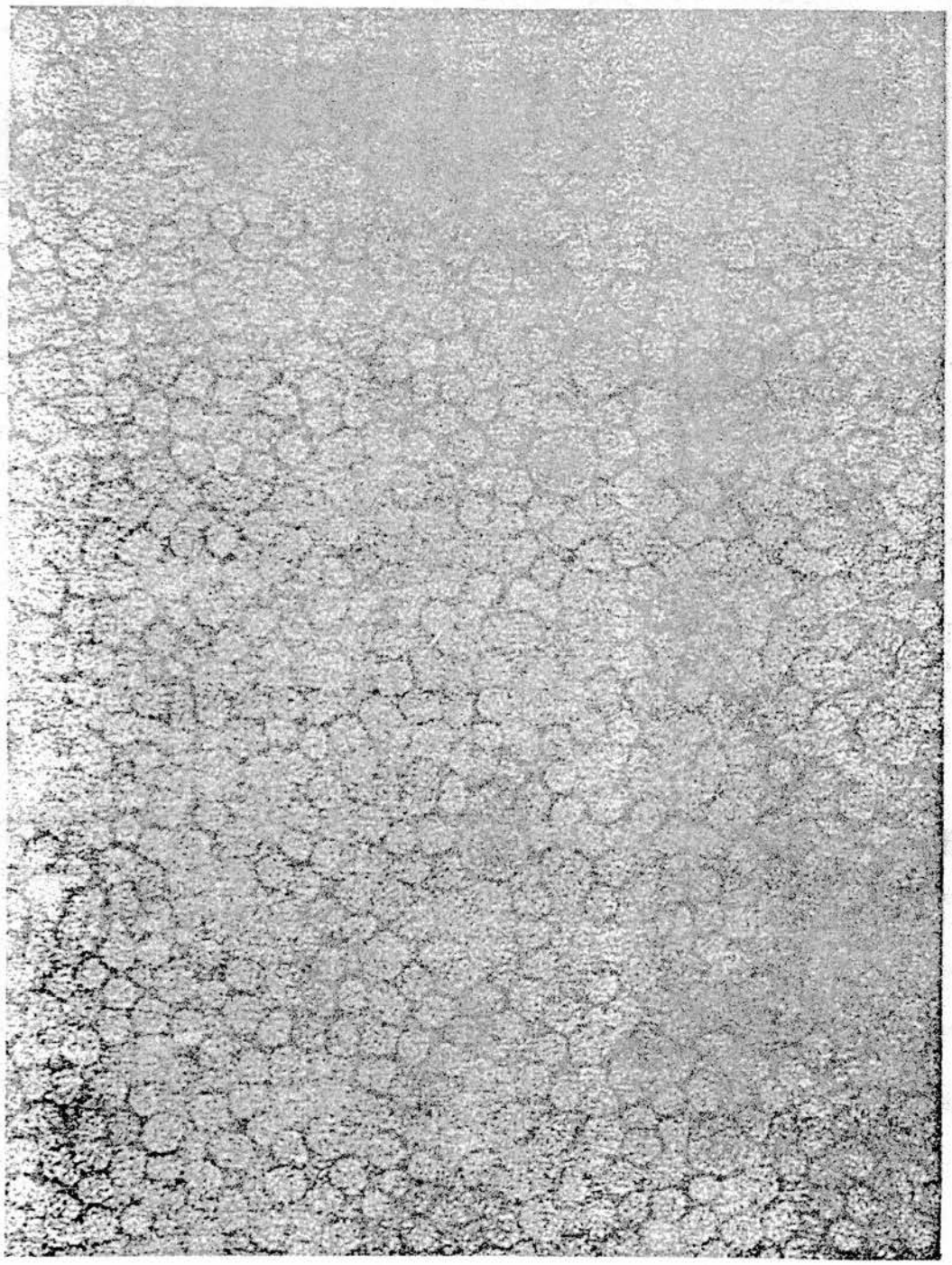

Figure *Electron micrograph (× 210 000) showing 20nm spherical and rod-like structures of HB_sAg and 42nm Dane particles*

presence of a in both the pellet and supernatant provided good supporting evidence that we were dealing with two distinct populations of HB_sAg particles.

ELECTRON MICROSCOPY

EM studies (figure) showed the characteristic spherical and rod-like structures, 20 nm in diameter, as well as numerous 42 nm Dane particles, believed to represent the complete infectious virion.

Review of Patient's Sera and Plasma Donors

Retrospective investigation of a serum sample of 7 February 1973 showed the presence of both HB_sAg specificities (at the same titres as in 1974), but a sample of 2 December 1970 was negative. We were unable to locate any serum samples taken between these dates. From the beginning of plasma infusions on 7 September 1971 until 7 February 1973 he had received 72 donations of plasma. Screening of all blood donations for HB_sAg by the CIEP method was begun in September 1971. Initially the patient received four donations of plasma that had not been tested. The remaining 68 donations were HB_sAg negative by the relatively insensitive CIEP test. None of the 72 donors involved has subsequently been found to be positive by CIEP or by the more sensitive HAI technique which has been in routine use since the beginning of 1974.

Review of Other Hypogammaglobulinaemic Patients

Recognizing the possibility that other similar patients may have become HB_sAg carriers, we tested all the hypogammaglobulinaemic patients known to us in the Edinburgh area. There were nine such patients in all; seven had received γ-globulin therapy at some stage, and one had been receiving fresh frozen plasma infusions for two and a half years. All were negative for HB_sAg by the HAI method.

Discussion

Generally speaking the d and y specificities of HB_sAg are mutually exclusive, and it is exceptional to find both specificities in the same serum. There are three reports where d and y specificities may have been present on separate particles in patients on haemodialysis. Ling *et al* (1973) reported two such cases indicated by the results of radioimmunoassay, but cross absorption studies were not done. Van Kooten Kok-Doorschodt *et al* (1972) described a case and Soulier and Couroucé-Pauty (1973) reported two further cases where d and y subspecificities could be separated by absorption. The absorption studies which we describe clearly indicate that the *ad* and *ay* subtypes were present as distinct populations. The simplest and most likely explanation is that the patient underwent dual infection from two positive donors. Unfortunately, serum samples were not available to show whether the two subtypes appeared sequentially. Therefore there is the small possibility that he was infected by one donor who carried both subtypes.

It is now widely believed that the immune response to HBV determines the nature and degree of tissue damage and whether the subject develops the carrier state. The HBV *per se* is probably not cytocidal to hepatocytes (Hand and Finlayson, 1973) but cell damage results primarily from a cell-mediated response of the delayed hypersensitivity type (directed against the virus or altered cell components). This is supported by several lines of evidence. If cellular immune responses are heightened by giving transfer factor in chronic active hepatitis (HB_sAg positive), then there is a transient rise in serum transaminase levels (Shulman *et al*, 1974). It has been repeatedly shown, using leucocyte-migration inhibition and lymphocyte transformation tests, that clinical disease is associated with a positive *in vitro* response to HB_sAg, while in the chronic carrier there is a negative response (Dudley *et al*, 1972; Laiwah *et al*, 1973).

Specific antibody alone is protective if present before infection (Sutnick *et al*, 1972) or when given prophylactically after accidental inoculation (Das and Hopkins, 1974). Antibody may also mediate cellular cytotoxicity to liver cells in chronic active hepatitis (Eddleston and Williams, 1974). In established HBV infection the cell mediated immune response would seem to act in conjunction with circulating antibody to eliminate the virus; that is, cytotoxic T or K cells cause lysis of infected hepatocytes, and the liberated viral particles are then neutralized by circulating antibody. Thus it can be postulated that both the cellular and humoral arms of the immune response must be impaired (or inadequate) to allow the development of the HB_sAg carrier state with little or no liver damage. In the case we describe here, no deficiency in T cell function could be detected; however, it is possible that a specific defect existed which was not detectable by the screening tests used, none of which was specific for HB_sAg.

There is ample epidemiological evidence indicating that impaired immune function predisposes to the carrier status. There is a relatively high incidence of HB_sAg carriers among institutionalized cases of Down's syndrome (Sutnick *et al*, 1968), chronic renal disease on haemodialysis (Turner and White,

1969), transfused lymphocytic leukaemia (Sutnick *et al*, 1970), and lepromatous leprosy (Blumberg and Melartin, 1970); in all these conditions cellular immune responses or both cellular and humoral responses are impaired. Tolerance induction may be important, and this is a plausible explanation for the high incidence of carriers in infants exposed to the HB virus in neonatal or prenatal life, reported by Schweitzer *et al* (1972).

It is well recognized that generally there is no increased susceptibility to viral infections in hypogammaglobulinaemia, where cell mediated immunity and interferon production are intact. However, absence of specific antibody would increase the susceptibility to any virus which gains entry via the blood stream. This seems to be so in the case of inoculated HBV (see above). Moreover, established immunity to one subtype may confer immunity to infection by the other subtype, by virtue of antibody against common specificities, provided that the challenge dose of virus is not overwhelming. From these considerations we conclude that hypogammaglobulinaemic patients are more susceptible to HBV infection and to reinfection by the other subtype.

It is noteworthy that we failed to identify the infective donor(s) despite retrospective checking of every plasma donation against the records of HB_sAg positive donors. The majority of the people who gave the original 72 donations of plasma would have been tested by CIEP or HAI at subsequent donations and none has proved positive. Nevertheless our finding of both d and y subspecificities in this case of hypogammaglobulinaemia indicates the strong possibility of double infection from plasma infusions. Patients with hypogammaglobulinaemia who receive only pooled human γ-globulin are exposed to a negligible risk, but those who develop severe reactions to γ-globulin and require plasma infusions may be at particular risk of becoming HBV carriers. We recommend that all hypogammaglobulinaemic patients having regular fresh frozen plasma should be monitored closely for HB_sAg.

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Request for reprint to P.C.D. at B.T.S.

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Australia Antigen (HB-Ag) Subtyping by a Sensitive Tanned Cell Haemagglutination-Inhibition Technique

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Summary. The tanned cell haemagglutination-inhibition technique was used to subtype Hepatitis B antigen (HB-Ag) sera according to their D (ad+, y−) and Y (ay+, d−) specificity. The results were in agreement with those obtained by workers in the United States and Sweden using immunodiffusion. The majority (35 out of 39 tested) of HB-Ag blood donors detected during the last 2 yr in South-East Scotland were found to possess the D subtype. The Hepatitis B antibody subtype content of two preparations of immunoglobulin prepared for clinical use has also been studied.

Analysis of the surface antigens of Hepatitis B antigen (HB-Ag) have revealed one common specificity 'a' and three additional determinants, 'd', 'y' and 'x' (Le Bouvier, 1971). Specificities a, d and y appear to reflect the genotype of the antigen, while x may be a component of the host which has become integrated into the particle or firmly attached to its surface (Le Bouvier, 1972; Le Bouvier *et al*, 1972). It is now possible to divide HB-Ag positive material into at least two subtypes, i.e. 'Set D' (ad+, y−) or 'Set Y' (ay+, d−). A third subtype may be included within a miscellaneous collection of HB-Ag samples which do not fall into these categories and designated 'Set A' (a+, dy−). Most, and perhaps all, of these are more likely to reflect the inability of immunodiffusion to detect very low levels of d and y. Two additional antigenic determinants designated 'w' and 'r' have recently been described (Bancroft *et al*, 1972) in a study in which w predominated in the Western hemisphere, and r characterized HB-Ag of Eastern origin. HB-Ag appears to breed true in the sense that a given specificity gives rise to HB-Ag of the same specificity in the recipient (Krugman & Giles, 1970). This makes it possible to trace the spread of both subtypes independently through the community. Iwarson *et al* (1973) have reported evidence for a change in the dominant subtype associated with HB-Ag positive clinical hepatitis in Sweden, from D to Y over a period of 20 yr. A number of other recent publications suggest that subtype D is often found in the chronic asymptomatic carrier (Holland *et al*, 1972; Wenzel *et al*, 1972). The Y subtype has also been associated with outbreaks of serum hepatitis in renal dialysis units, including the exceptionally severe outbreak in Edinburgh (Marmion & Tonkin, 1972). Recently Nielsen & Le Bouvier (1973) have studied the relationship between HB-Ag subtypes and the degree of liver damage in acute viral hepatitis. They too identified subtype Y in the majority of cases, but surprisingly found that when acute disease did occur in association with

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subtype D, it was usually more severe than that associated with Y. Immunodiffusion (ID) was the first technique to be used for the detection of HB-Ag (Blumberg, 1964) and remains a useful means of subtyping (Gust, 1971). Counter-electrophoresis (CIEOP) employing antisera rendered monospecific by absorption has also been applied to subtyping (Holland *et al*, 1972), and solid phase radioimmunoassay (RIA) promises to provide a sensitive means of subtyping HB-Ag not detectable by ID or CIEOP (Ginsberg *et al*, 1972).

Prince *et al* (1972) were the first to apply haemagglutination inhibition (HAI) to the subtyping of HB-Ag, but the chromic chloride technique used (Vyas & Shulman, 1970) has proved unsuitable for mass donor screening. We report the application of a simple, rapid and sensitive tanned cell HAI (Hopkins & Das, 1973) to the subtyping of HB-Ag derived from local blood donors, dialysis patients and sera from standard panels supplied by the National Institute of Health and the American Red Cross.

MATERIALS AND METHODS

A number of sera containing Hepatitis B antibody (HB-Ab) were tested by ID and CIEOP against previously subtyped HB-Ag sera. It was apparent that one of the antisera reacted strongly with antigen of subtype D, but weakly, if at all, with antigen of subtype Y, suggesting a predominance of anti-a and anti-d. Anti-a was removed by absorption with HB-Ag of subtype Y by concentrating the antiserum five times with lyphogel and incubating with different concentrations of Y at room temperature for 1 hr, followed by overnight incubation at 4°C. Immune complexes were removed by centrifugation at 20 000 rpm (Sorval RC2B) for 1 hr and the supernatant tested for the presence of anti-d by direct haemagglutination (HA) with D-sensitized cells (Fig 1). Removal of anti-a activity was indicated by agglutination of D-sensitized cells, but not of Y-sensitized cells (Table I). Anti-y was prepared in the same way by absorbing the appropriate HB-Ab with HB-Ag of subtype D.

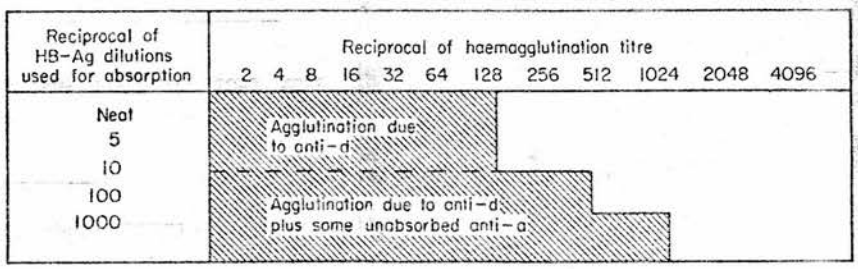

FIG 1. Titration of HB-Ab (anti-ad) after absorption with different concentrations of HB-Ag of specificity Y.

HB-Ag was subtyped by HAI (Hopkins & Das, 1973) using anti-d and D-sensitized cells. Absence of agglutination was indicative of HB-Ag specificity d while the presence of agglutination showed that anti-d had not been neutralized by the antigen, which may possess specificity y. This was confirmed by retesting with anti-y antibody when a true Y antigen would not allow agglutination of Y-sensitized cells.

TABLE I. Confirmation of monospecific HB-Ab following absorption

Antisera	*Cells sensitized with HB-Ag of subtype*	
	D	*Y*
Monospecific anti-d (prepared by absorption)	+	–

The reliability of the technique was tested by subtyping HB-Ag positive sera from three different sources: five sera from a standard panel supplied by the National Institute of Health, Bethesda, U.S.A., and five sera from a panel supplied by the American Red Cross. These sera had previously been subtyped at source; three sera from healthy carriers detected during routine donor screening by CIEOP at the Blood Transfusion Centre, Royal Infirmary, Edinburgh, and two sera from dialysis patients involved in the Edinburgh outbreak of 1969–70, which had already been subtyped (Dr G. Le Bouvier, Yale University Medical School, Connecticut, U.S.A.) using ID. The sera from all three sources were double coded and an equal number of HB-Ag negative sera included, before being subtyped by HAI.

For the past 2 yr all blood donated in the South-East Region of Scotland has been tested for HB-Ag by CIEOP. During this period 39 HB-Ag positive sera have been identified. In order to determine the prevalence of each HB-Ag subtype in the local donor population these sera were investigated by HAI using monospecific HB-Ab as described above. Most of these sera had already been subtyped by Dr Le Bouvier (Yale University Medical School, Connecticut, U.S.A.) or by Dr L. Magnuis (National Bacteriological Laboratory, Stockholm) using ID.

The technique has also been used to titrate the anti-ad and anti-ay specificity in the hyperimmune (HB-Ab) gammaglobulin (HB-Ab, IgG) prepared at the Protein Fractionation Centre, Royal Infirmary, Edinburgh, and used prophylactically in cases of accidental exposure to HB-Ag.

RESULTS

The results of subtyping HB-Ag positive sera by HAI and ID are given in Table II. Complete agreement was found between the two techniques. The prevalence of D and Y amongst HB-Ag positive blood donors in South-East Scotland is shown in Table III. The majority appear to possess HB-Ag of subtype D. Table IV shows the differences in reactivity to two batches of HB-Ab, IgG prepared at the Protein Fractionation Centre, Royal Infirmary, Edinburgh.

DISCUSSION

At the present time most HB-Ag is subtyped by ID or CIEOP. Kim & Tillies (1971) were first to note the greater negative charge associated with the Y subtype. This was subsequently confirmed by P. E. Gibson & Y. E. Cossart (personal communication, 1973) who showed that

TABLE II. Comparison of subtyping by HAI and ID of HB-Ag positive sera from three different sources

Sample	*Origin*	*Immunodiffusion*	*Haemagglutination inhibition*
208	National Institutes of Health, Bethesda, U.S.A.	Y	Y
209		Y	Y
213		D	D
217		Y	Y
228		D	D
1001	American Red Cross	D	D
1018		D	D
1006		D	D
2002		Y	Y
2005		Y	Y
BD-2-Edin	Local HB-Ag positive blood donors	Y	Y
BD-7-Edin		D	D
BD-9-Edin		D	D
H226	HB-Ag positive dialysis patients	Y	Y
GC		Y	Y

TABLE III. Prevalence of D and Y subtypes of HB-Ag as determined by haemagglutination-inhibition amongst antigen positive blood donors in South-East Scotland

No. of carriers	*No. of donors with D subtype* (%)	*No. of donors with Y subtype* (%)
39	35 (89.7)	4 (10.3)

TABLE IV. Haemagglutination titre of HB-Ab IgG prepared at the Protein Fractionation Centre, Edinburgh

Batches of gamma-globulin	*Reciprocal titre of cells sensitized with HB-Ag of subtype D and subtype Y*	
	D	*Y*
Gamma-G(2)/H_2	32000	512000
Gamma-G(3)/H_3	32000	64000

HB-Ag of subtype Y migrated further than D in immunoelectrophoresis. Because of their limited sensitivity, ID and CIEOP are wasteful of precious monospecific typing antisera and are unable to subtype HB-Ag detected by more sensitive means (Shaffer *et al*, 1972; Ling & Overby, 1972). Solid phase RIA offers a means by which such sera may be subtyped. Unfortunately, this technique seems prone to give false positive results with certain sera (Sgouris, 1973; Prince *et al*, 1973) requires expensive counting equipment and specially trained personnel to handle radioactive material. At the time of writing, solid phase RIA subtyping kits are not commercially available. HAI (Hopkins & Das, 1973), on the other hand, is a simple technique using human 'O' Rh negative erythrocytes sensitized with beta-propiolactone/UVL-treated HB-Ag. It has overcome many of the problems associated with the chromic chloride technique. The application described here permits a rapid (within 2 hr) differentiation of D and Y subtypes of HB-Ag with a sensitivity similar to that of RIA. The discovery of at least two subtypes of HB-Ag clearly implies that techniques and reagents used for routine testing of blood donors and hepatitis patients should be capable of detecting both subtypes. The apparent predominance of D amongst healthy HB-Ag positive blood donors in South-East Scotland is in agreement with other Western European donor populations (Iwarson *et al*, 1973; Holland *et al*, 1972).

Subtyping of HB-Ag is an important epidemiological tool in view of the possible relationship between subtype and disease. The prophylactic use of HB-Ab, IgG in 'accident' circumstances may be effective, but as a therapeutic measure in the treatment of disease requires further investigation. Its efficacy may be enhanced by using HB-Ab, IgG of the appropriate subtype specificity.

Addendum

Since acceptance of this paper for publication the authors have received further confirmation of the subtypes relating to the antigen-positive blood donors detected locally. Results obtained from Dr Overby (Abbott Laboratories) using radioimmunoassay show complete agreement with those obtained by HAI. Dr Overby has also reported that each of the donor-carriers tested possessed the antigenic determinant w by immunodiffusion.

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A Tanned Cell Haemagglutination Test for the Detection of Hepatitis-Associated-Antigen (Au-Ag) and Antibody (Anti-Au)

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Summary. A tanned cell haemagglutination technique using inactivated Au-Ag to sensitize human 'O' Rh negative erythrocytes is described. The method may be used to detect Au-Ag by haemagglutination inhibition (HI) and anti-Au by direct haemagglutination (HA). Sensitivity and specificity are demonstrated and its application to mass donor screening is considered.

Although not all post-transfusion jaundice is caused by hepatitis B virus, transmission of hepatitis B is one of the most serious complications of the use of blood and blood products. This situation is likely to persist until all donated blood is screened for Australia antigen (synonyms: Au-Ag, HAA, HB-Ag) by a rapid, specific and sensitive test system. The techniques currently used in most Transfusion Services are counter electrophoresis (CIEOP) (Prince & Burke, 1970), which lacks sensitivity, and latex agglutination (Leach & Ruck, 1971), which is both rapid and sensitive, but lacks specificity (Hopkins & Das, 1973; Ziegenfuss, 1972; Burrell *et al*, 1972). The radio-immuno techniques (Lander *et al*, 1971; Ling & Overby, 1972) are extremely sensitive research tools, but are costly and not yet applicable to the rapid mass screening demanded by the Blood Transfusion Service.

Most laboratories have experience of some form of haemagglutination, and many have found it amenable to automation. Juji & Yokochi (1969) described a haemagglutination test using formalinized erythrocytes coated with anti-Au (synonyms: anti-HAA, Hb-Ab). Unfortunately they observed non-specific agglutination, and a tendency for sensitized cells to lyse within a few days. Vyas & Shulman (1970) published details of a haemagglutination technique using chromic chloride as a coupling agent. This very sensitive technique has gained acceptance in many research laboratories since it compares favourably with CIEOP (Shaffer *et al*, 1972). When considering the logistics of its application to routine donor screening, limitations have arisen due to the highly purified antigen required (Vyas *et al*, 1972), and great variability observed between different batches of cells (Reesink & Duimel, 1972). Furthermore, it is desirable to use inactivated antigen for coating erythrocytes which will subsequently be used as a routine test reagent. In our experience the outcome of coupling inactivated Au-Ag to human erythrocytes by chromic chloride has proved very unpredictable.

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This communication describes a tanned cell haemagglutination technique, incorporating inactivated antigen, which we believe goes a long way towards filling the Au-Ag screening requirements of the Blood Transfusion Service.

MATERIALS AND METHODS

Standard Sera

Known Au-Ag positive sera, used as controls, were derived from panels obtained from the NIH (Bethesda), American National Red Cross, Blood Research Laboratory, and apparently healthy carriers found locally. Sera from six healthy males (39–64 yr), repeatedly tested serologically and electron microscopically, were used as negative controls. Known specific anti-Au capable of reacting with both ad^+ and ay^+ antigens (Le Bouvier, 1972), were used in the inhibition reaction to detect Au-Ag.

Test Material

Samples were obtained from selected donors, known contacts of Au-Ag, renal dialysis patients and a subject involved in an 'accident' with Au-Ag positive blood, who subsequently received hyper-immune anti-Au IgG prepared by the Scottish National Plasma Fractionation Centre, Edinburgh. Au-Ag positive and Au-Ag negative normal sera were coded and included among the test samples.

Phosphate Buffered Saline (PBS)

This was prepared from 0.15 M Na_2HPO_4, 0.15 M KH_2PO_4 and 0.15 M NaCl. Two batches were made, one adjusted to pH 6.4 and the other to pH 7.2. Cell diluent consisting of PBS, pH 7.2, containing 1% normal rabbit serum (previously inactivated at 56°C for 25 min and absorbed with washed packed red cells from the batch being sensitized) was used to suspend the sensitized cells. Diluting Fluid (D.F.) consisting of 50% normal human serum in PBS 7.2 was used to make serial dilutions for end-point titrations. Diluting media were stored at 4°C.

Preparation of Antigen

Au-Ag positive serum collected from apparently healthy carriers was inactivated using β-propiolactone and ultraviolet light according to Lo Grippo (Lo Grippo & Hartman, 1958; Lo Grippo *et al*, 1971). The inactivated material was pelleted by centrifugation at 120 000 *g* for 4 hr in a M.S.E. Superspeed 65 centrifuge, using an 8 × 25 ml angle head, washed twice in sterile physiological saline and finally resuspended in a small volume of PBS 6.4 and adjusted to give an end point titre of 1/256 by latex agglutination (Pfizer Ltd, Kent, Batch No. L13/4; Leach & Ruck, 1971), or a titre of 1/4 by CIEOP (Das *et al*, 1971), against a commercial anti-Au serum (Batch T2160C) supplied by Hoechst Pharmaceuticals (Hounslow). Au-Ag, thus prepared and still containing some serum proteins was stored at −20°C until required, when it was thawed and heated at 60°C for 30 min prior to sensitization.

Tanning and Sensitization Procedure

Human 'O' Rh negative blood was collected in heparin, washed three times in physiological saline and packed by centrifuging at 800 *g* for 5 min. The packed cells were then resuspended

in PBS, pH 7.2, to give a suspension of 6.6% (v/v) to which was added an equal volume of 1:10 000 tannic acid (w/v) freshly prepared in the same buffer. The mixture was incubated in a 37°C water bath for 15 min, shaking gently every 3 min. The tanned cells were washed once in an equal volume of PBS, pH 6.4, and resuspended in the same buffer to a 4% cell suspension, which was divided into two aliquots. Au-Ag was added to one aliquot in the ratio of 1 volume Au-Ag to 4 volumes of cells, mixed and incubated for 1 hr at room temperature (20°C) on a rotostat (Luckham Ltd, Sussex) to ensure thorough mixing. The remaining aliquot of cells was sensitized in the same way, with 1/125 dilution (in PBS, pH 6.4) of pooled normal human plasma (Au-Ag and anti-Au negative) for use as a plasma sensitized cell control. The sensitized cells were washed three times in equal volumes of cell diluent then made up to a 1.5% suspension in the same diluent and left at 4°C overnight. Next morning the supernatant was replaced with fresh cell diluent and the cells stored at 4°C in small aliquots.

Test Procedure

Two systems were employed. One used disposable U-bottomed microtitre plates (Flow Laboratories, Irvine) into which reagents were dispensed as drops from a pasteur pipette—referred to as the microtitre system (M.T.S.). In the other system Terasaki microtest tissue culture trays with lids (Bio Cult Laboratories, Paisley) were used. Reagents were dispensed in 5 μl volumes using an Eppendorf pipette with disposable tips (Alderman and Co. Ltd, London). This was designated the mini-microtitre system (M.M.T.S.). In M.T.S. 0.75% sensitized cells were used, while a similar degree of sensitivity was obtained in M.M.T.S. with 0.3% sensitized cells.

(i) *Microtitre system.* For detection of anti-Au by direct haemagglutination (HA), the test serum was diluted $\frac{1}{2}$–$\frac{1}{4}$ in PBS 7.2 and mixed with an equal volume of sensitized cells. The plate was covered to diminish evaporation, and incubated at 37°C for 1 hr. Antibody-containing wells showed positive agglutination characterized by a smooth 'mat' of cells, while absence of antibody was characterized by a 'button' of cells at the bottom of the well (Fig 1). Once confirmed, the test serum was further diluted and the end point titre, the lowest concentration of antiserum producing complete agglutination, was obtained and defined as 1 HA unit. Controls included (a) virus control = sensitized cells + cell diluent, (b) positive control = sensitized cells + known anti-Au and (c) negative control = sensitized cells + normal serum.

Au-Ag was detected by a two-stage haemagglutination inhibition reaction (HI). The test serum was diluted $\frac{1}{2}$–$\frac{1}{4}$ in PBS 7.2 and mixed with an equal volume of standard anti-Au containing 4 HA units of antibody activity and the plate incubated at 37°C for 30 min. Sensitized cells were then added, the plate mixed and reincubated at 37°C for a further hour. Agglutination indicated that no antigen was present. Controls included (a) positive control = anti-Au + known Au-Ag + sensitized cells, and (b) negative control = anti-Au + normal serum + sensitized cells.

(ii) *Mini-microtitre system.* For detection of anti-Au, test serum was diluted 1/2 in PBS 7.2 and added to an equal volume of sensitized cells, mixed on a rotostat at room temperature (20°C) for 15 min and then transferred to a 37°C incubator for a further 15 min. The tray was then spun for 10–15 s at No. 1 speed (0–25 *g*) in a bench

centrifuge equipped with a serological head (Griffin and George, Middlesex). Finally the tray was inclined at an angle of 45–50 degrees, at room temperature and read against a light background at 5 min intervals until control reactions were complete. If no agglutination occurs the cells slide down to the lower part of the base of the wells (Fig 2).

For detection of Au-Ag, the test serum was diluted 1/2 in PBS 7.2 mixed with an equal volume of anti-Au containing 4 HA units and incubated for 1 hr at 37°C. Sensitized cells (5 μl) were then added to each well, and the two-phase incubation procedure repeated. The tray was centrifuged and read as described. Controls for HA and HI were the same as for M.T.S.

Confirmation

Positive reactions for anti-Au were confirmed by (a) retesting the sample with plasma sensitized cells and (b) using the test sera as anti-Au in an inhibition reaction with known Au-Ag positive controls and normal sera.

Positive Au-Ag reactions were confirmed by the ability of the sample to inhibit the titre of known anti-Au sera, when compared with normal serum.

Determination of Sensitivity

The ability of HI to detect Au-Ag was compared with immuno diffusion (ID), electrophoresis (Das *et al*, 1971; Hopkins & Das, 1972), latex agglutination (Leach & Ruck, 1971) using two commercial reagents (Pfizer Ltd, and Hoechst Pharmaceuticals), immune electron microscopy (Kelen *et al*, 1971), radio-immunoassay (RIA) using reagents from Abbot Laboratories (Kent), and radio-immuno precipitation (RIP) performed at the Bacteriology Department, Edinburgh University Medical School.

The sensitivity of HA was measured by comparing Anti-Au titres with CIEOP and also by its ability to detect circulating anti-Au after administration of hyperimmune gamma-globulin to 'accident cases'.

Specificity

This was investigated by coding known Au-Ag positive and Au-Ag negative sera and distributing them randomly among the test samples. All positive results were repeated for confirmation.

RESULTS

Fig 3 shows partially purified Au-Ag prior to sensitization, while Fig 4 shows a sensitized cell with Au-Ag bound to its surface. No free Au-Ag can be seen in the supernatant surrounding the cell.

Pooled Au-Ag containing both ad^+ and ay^+ subtypes was titred and compared with a variety of other techniques (Fig 5). It is clear that the haemagglutination-inhibition method has a similar degree of sensitivity to the radio-immuno methods, and is considerably more sensitive than most of the techniques commonly used in routine laboratories for blood donor or patient screening.

The direct haemagglutination test is capable of detecting very low levels of anti-Au as compared with CIEOP (Fig 6). This was further evidenced by its successful application in

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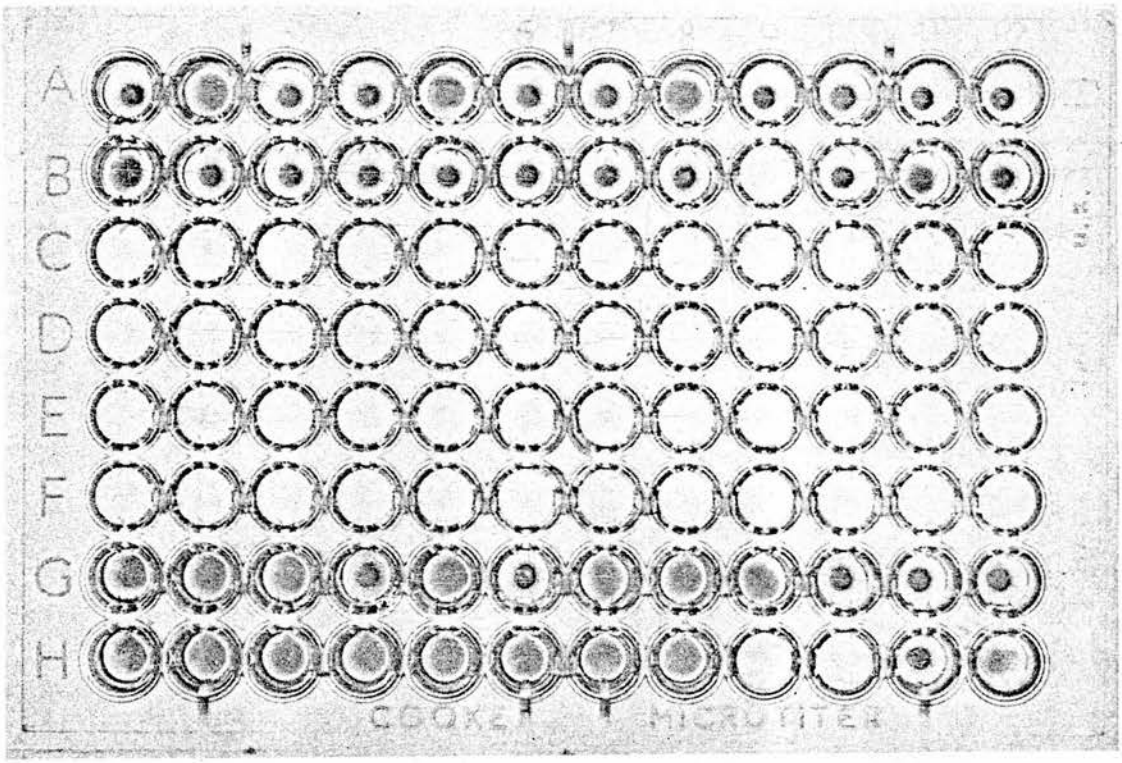

FIG 1. Appearance of positive and negative haemagglutination patterns in microtitre system.

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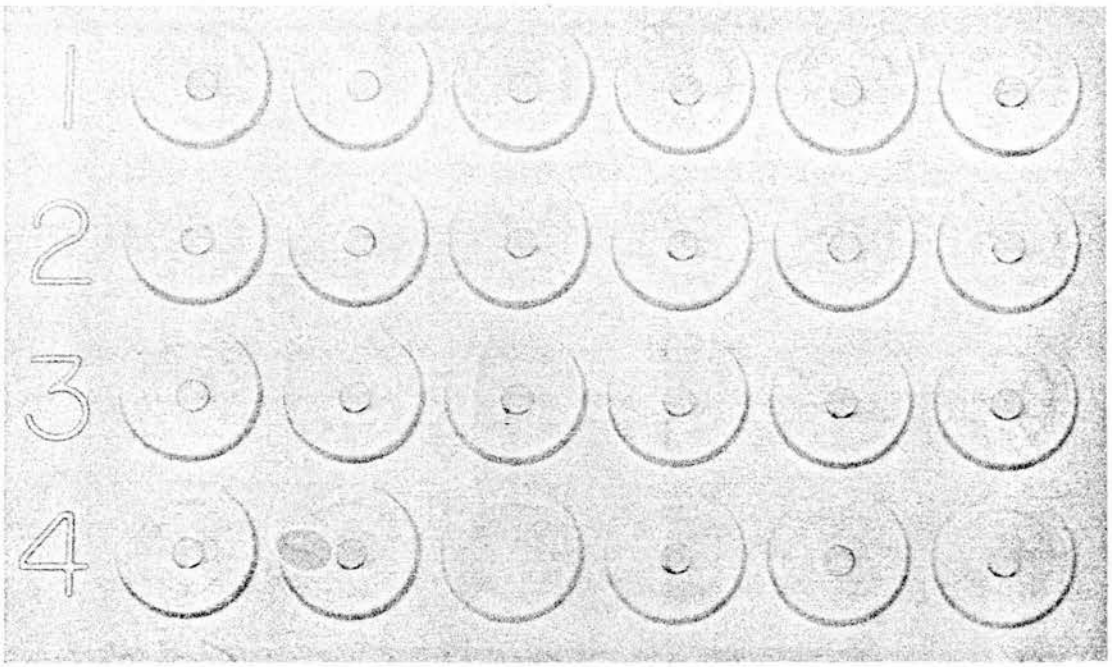

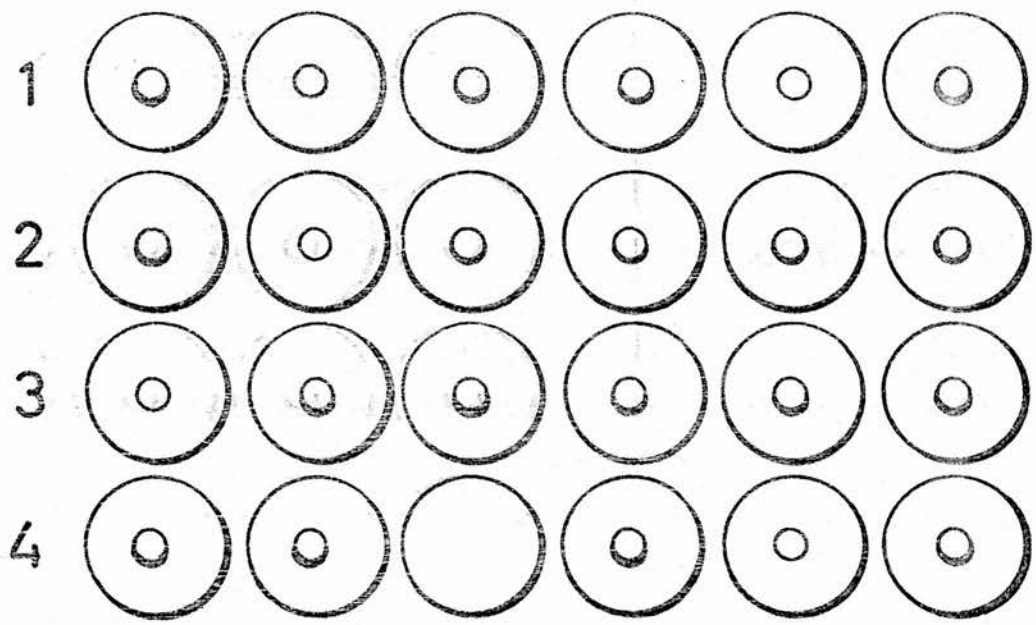

FIG 2. Appearance of positive and negative haemagglutination patterns in mini-microtitre system, including artist's impression.

FIG 3. Partially purified Au-Ag prior to sensitization (E.M. mag. = 50 000).

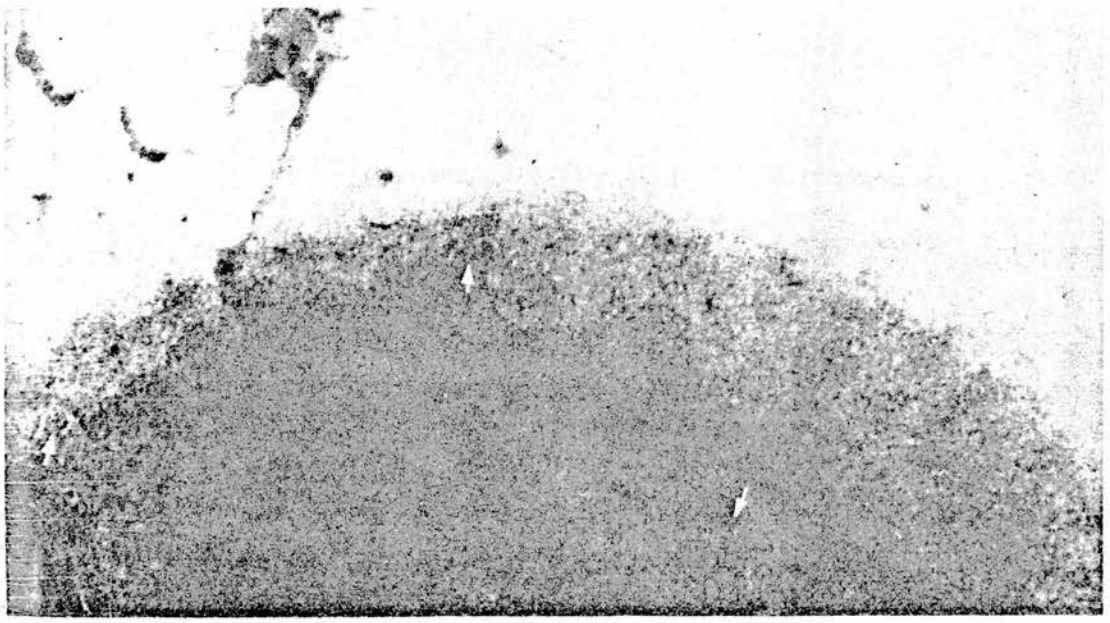

FIG 4. Sensitized cell with Au-Ag bound to surface. No free Au-Ag visible in supernatant (E.M. mag. = 28 000).

monitoring the levels of passively administered hyperimmune anti-Au (details of dose and inoculation schedule to be published elsewhere) in a number of cases where CIEOP has consistently failed to detect any antibody. Fig 7 shows the levels of anti-Au detected in one such case. No antibody could be detected prior to administration of the gamma-globulin, while circulating anti-Au could be detected within 48 hr after injection and persisted up to 15 weeks later.

Fig 8 shows the results obtained when Au-Ag positive and negative sera were coded and tested by a variety of techniques. The haemagglutination-inhibition test detected all positive samples as did CIEOP and RIA, and gave no false positive reaction, while one batch of latex, ID and IEM failed to detect several of the positive (Au-Ag) sera, and latex agglutination from both commercial sources gave false positive reactions.

TABLE I. Results obtained when sera from selected donors and dialysis patients were screened for Au-Ag and anti-Au by haemagglutination-inhibition and CIEOP

		CIEOP		*Tanned-cell haemagglutination*		
	No. tested	*Anti-HAA positive*	*HAA positive*	*Anti-HAA positive*	*HAA positive*	*'Plasma-cell' agglutination*
Donors	76	4	7	4	7	0
Dialysis patients	14	2	0	2	0	2*

* Different samples from those possessing anti-HAA.

The results shown in Table I confirm the specificity of the haemagglutination method, showing complete agreement with CIEOP when sera from selected donors and dialysis patients were tested for Au-Ag and anti-Au. In two of these patients low levels of antibody capable of agglutinating antigen sensitized cells were, in fact, due to reactions against serum protein(s) as demonstrated by agglutination of plasma sensitized cells. This antibody did not inhibit the reactivity of purified ad^+ or ay^+ antigens whereas a known anti-Au (diluted to a similarly low level) did have an inhibitory effect under the same circumstances.

DISCUSSION

The technique described in this report provides a simple, rapid, economical and highly sensitive test within the scope of most routine laboratories. Preparation of reagents is straightforward, and the use of inactivated antigen reduces the risk from accidental exposure to personnel involved in routine screening. The use of a 0.75% cell suspension (microtitre system) allows a reasonable incubation time, removes the need to centrifuge plates and facilitates interpretation of results by personnel receiving only a short period of instruction, while retaining a sensitivity of the same order as that of the radio-immuno techniques. The mini-microtitre system, on the other hand, reduces the volume of reagents considerably although it then becomes advisable to centrifuge the trays to obtain the best definition between positive and negative results. The apparent absence of variation amongst different batches of cells eliminates any dependence upon a select group of red cell donors and reflects

FIG 9. Situation which may arise in a multitransfused patient possessing antibodies to both Au-Ag and certain plasma proteins.

the stability of the technique. The extreme sensitivity suggests that antisera may be used at least 100–1000 times more dilute than for immunodiffusion or counter-electrophoresis, thereby alleviating problems arising from expense or shortage of reagents. A shelf life of 3 weeks is acceptable if routine sensitization is performed fortnightly. Storage of fresh cells in liquid nitrogen and the use of glutaraldehyde fixed cells are at present under investigation in this laboratory with a view to increasing the shelf life of the reagents. It remains to be seen whether or not tanned cells sensitized with Au-Ag will prove suitable for application to an autoanalyser.

Experience in a variety of other biological systems, including hormone estimation, where the tanned cell haemagglutination system has been used extensively, has shown that non-specific inhibition can occur in the assay system despite the use of specific antibody (Stavisky & Ingraham, 1964). However, the specificity of the present technique for detection of Au-Ag seems good as no false positive inhibition reactions have occurred amongst the number of samples so far tested. A prozone effect was noted in the direct haemagglutination test when testing hyperimmune sera containing high titre antibody; this was eliminated by using a starting dilution of test sera of 1/2.

It is probable that a proportion of the normal, apparently healthy, population may show positive reactions due to low levels of antibody against plasma proteins when screened by this sensitive method. Brumelhuis *et al* (1971) found 13 such reactions in 30 000 samples tested by the Netherland Red Cross Transfusion Service using the less sensitive technique of immunodiffusion. Patients, particularly multitransfused patients, are likely to produce such reactions due to the development of iso-precipitins (Blumberg, 1964; Langenhuysen, 1971). One possible way of circumventing this problem is to use highly purified Au-Ag for coating the cells. However, when 'purified' Au-Ag was used in the haemagglutination method of Vyas & Shulman (1970), as modified by Prince *et al* (1971), the sensitized cells were agglutinated by commercial anti-whole human sera and an anti-human IgG (Prince, 1972). This finding may indicate the presence of anti-Au in the commercial antisera, but is more likely to represent contaminating serum protein(s) in the 'purified' antigen. Whether the contaminant(s) is a part of the Au-Ag moiety (Millman *et al*, 1971), or plasma protein, is not known. However, the practical conclusions from these observations are that any serum giving a positive reaction, particularly for antibody, must be retested using plasma sensitized cells, and further confirmed by the means outlined in this paper. Fig 9 represents the situation which may arise in a multitransfused patient possessing antibodies to both HAA and certain plasma proteins (Vierucci *et al*, 1970). Further investigation of this phenomenon is required, but such results are of interest in the light of the high incidence of apparently low levels of anti-Au in multi-transfused patients reported by Lander *et al* (1971) and Levy & Hawrisiak (1972).

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Results and Comments

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	C.E.P.	Hepanosticon	Turkey Cells	Auscell	E.H.A.I.*	E.H.A.I.†	Ausria I	Ausria II
ad	2	400	1000	64 000	2000	32 000	16 000	8000
ay	16	800	4000	32 000	8000	64 000	2000	4000

* With four units of antibody for inhibition.
† With three units of immune absorbed high affinity antibody for inhibition.

TABLE III—*Results of Antigen Panel Studies*

	Public Health Laboratory Panel		Canadian Red Cross Panel*		American Red Cross Panel				
					(1)			(2)	
	Positive	Negative	Positive	Negative	True Positive	False Positive	False Negative	Positive	Negative
C.E.P.	20	5	3	6	6	0	4	23	3
Turkey cells ..	25	0			9	0	1		
Ausria I	25	0							
U.S.A.					10	9	0		
U.K.					10	0	0		
Hepanosticon ..	23	2							
Auscell					10	3	0		
E.H.A.I.	25	0	9	0	10	0	0	26†	0

* This panel also contained another specimen with antibody (anti-HBsAg).
† 13 *ad*, 13 *ay*.

TABLE IV—*Results of Reinvestigation of Donors implicated in 10 Cases of Post-transfusion Hepatitis. All Donations were Originally Found to be Negative by C.E.P.*

Case no.:	1	2	3	4	5	6	7	8	9	10
HBsAg	+	+	−	+	+	+	−	−	+	N.T.
No. of donations involved	8	14	18	18	32	14	3	10	4	17
No. of donations found to be positive and method..			1 by A.I., E.H.A.I., and T.C.			1 by A.I., E.H.A.I., and T.C.			1 by A.I., and E.H.A.I.	1 by E.H.A.I. and T.C.

A.I. = Ausria I. E.H.A.I. = Economical haemagglutination inhibition. T.C. = Turkey cells. N.T. = Not tested.

Detection of Hepatitis B Surface Antigen among Scottish Blood Donors: Evaluation of Sensitive Tanned-cell Haemagglutination-inhibition Test

R. HOPKINS, M. ROBERTSON, D. ROSS, W. M. TURNBULL, P. C. DAS

Summary

A total of 70 224 blood donations were tested at three Scottish blood transfusion centres for hepatitis B surface antigen (HBsAg) by an economical haemagglutination-inhibition method (E.H.A.I.) and the results compared with those of counterelectrophoresis (C.E.P.). A further 4086 donations were tested using the Wellcome turkey cell haemagglutination test, C.E.P., and E.H.A.I. E.H.A.I. was also compared with commercial haemagglutination and radioimmunoassay reagents for sensitivity and specificity against several established antigen panels and used to reinvestigate counterelectrophoresis-negative blood donations implicated in post-transfusion hepatitis.

E.H.A.I. combines the inherent specificity of an inhibition reaction with a sensitivity equal to that of commercial radioimmunoassay and haemagglutination kits but at a fraction of the cost. The assessment of 70 224 blood donations in three regions showed that E.H.A.I. detected more antigen-positive blood donations than C.E.P. Results of retesting more than 100 blood donors implicated in 10 cases of post-transfusion hepatitis suggested that the use of E.H.A.I. or a test of similar sensitivity in place of C.E.P. may significantly reduce the incidence of this complication.

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Introduction

Despite the identification of the causal agent of type B post-transfusion (serum) hepatitis,[1] and the subsequent removal of blood identified as positive for HBsAg by counterelectrophoresis (C.E.P.) hepatitis continues to complicate the therapeutic use of blood and blood products.[2] More sensitive serological tests for surface antigen might lead to a reduction in the number of cases of post-transfusion hepatitis but are unlikely to eliminate it completely because of the potential infectivity of blood containing antibody to the Dane particle core[3] and the possible existence of a reputed hepatitis C virus[4] —quite apart from other causes such as hepatitis virus A (infectious hepatitis) and other microbial agents.[5]

The relatively insensitive technique of C.E.P.[6] is most commonly used in blood banks. Much more sensitive assays, using principles of haemagglutination or radioimmunoassay, are now commercially available.[7-10] Some reports suggest that these kits can detect carriers of HBsAg too weak to be positive on C.E.P.[7-9] Unfortunately, their effective use in the blood transfusion service will prove expensive and only time will tell whether such expense is justified.

We report here the experience of three Scottish blood transfusion centres in evaluating a sensitive and economical tanned-cell haemagglutination-inhibition test (E.H.A.I.).[11] In addition to a field assessment involving 70 224 blood donations the test was compared with commercial haemagglutination and radioimmunoassay kits for specificity and sensitivity. We also present data on the retesting of 138 blood donors' sera which were HBsAg negative on initial screening by C.E.P. but were later implicated in post-transfusion hepatitis in 10 patients.

Methods

Haemagglutination Inhibition.—The test used was the "mini-micro" version of that described by Hopkins and Das[11] in which washed, β-propiolactone/ultra-violet light-treated antigen was used to sensitize human O Rh-negative erythrocytes. Test serum was diluted 1/10 or 1/5 in phosphate-buffered saline and the test conducted in Terasaki trays using only 5 μl of reagent. Antibody was detected by

direct haemagglutination. Sera positive for HBsAg were confirmed by subtyping (*ad*/*ay*) using a modification of the test assay.[12]

Other Assays.—C.E.P. was performed as previously described,[6] and the plates were washed with 1% tannic acid before reading. Radioimmunoassay (Ausria, Abbott Laboratories) and haemagglutination (Auscel, Abbott Laboratories; Hepanosticon, Organon; and turkey cells; Wellcome Reagents) were performed as recommended by the manufacturers.

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patients were antigen positive) showed that in two cases an antigen-positive unit of blood had been transfused which was undetectable by the electrophoresis method during the initial screening as well as on reinvestigation. In both cases antigen was detected only by the more sensitive tests (table IV). On reinvestigation of the two cases from Manchester (cases 9 and 10) a positive unit was detected in both cases, though one was missed by turkey cells and the other by Ausria I. The antigen-positive unit transfused in case 9 was subtyped as *ad*, while that transfused in case 10 was found to be *ay*, confirming the finding of the titration study (table II) in which Ausria I showed a bias towards detection of the *ad* subtype, despite that all other tests except Auscel found *ad* to be weaker. A similar bias in favour of the *ad* subtype was recorded by Vanderweld *et al.*,[14] but this fault seems to have been corrected to some extent in the newer Ausria II test (table II).

Discussion

Recent reports have highlighted the inadequacies of C.E.P. in detecting HBsAg in blood donations.[2 7-10] The American Red Cross seem to have opted for R.I.A., while regional transfusion centres in the U.K. seem to favour haemagglutination. Three commercial haemagglutination assays, all using agglutination of antibody-coated erythrocytes, are currently available. The cheapest of these (about 10p per test if ordered in bulk) is marketed by Wellcome Reagents, and consists of fixed turkey erythrocytes coated with affinity column purified antibody raised in horses. In terms of speed and simplicity it is well suited to the large-scale testing of blood donations,[10 15] and its sensitivity is much greater than that of C.E.P. but less than that of R.I.A. The other two haemagglutination tests—Hepanosticon, which consists of fixed sheep erythrocytes coated with antibody raised in sheep, and Auscel, which consists of fixed human erythrocytes coated with antibody raised in guinea pigs—have been found to be more sensitive than C.E.P.[8 9] All three tests are susceptible to a few false-positive reactions, the problem being solved by absorption-titration experiments.

Our E.H.A.I. test is about as sensitive as R.I.A. and, being an inhibition reaction, has an inherent specificity not found in the haemagglutination tests. The use of monospecific antibody (prepared by absorption or affinity chromatography) allows E.H.A.I. to be used to subtype antigen-positive sera, which provides an essential confirmatory step.[12]

The cost of E.H.A.I. is negligible owing to the availability of raw material, the considerable expertise in haemagglutination technology within the Blood Transfusion Service, and the simplicity of reagent preparation combined with the small quantities (5 μl) needed for testing. In one week 500 ml of high-titre HBsAg-positive serum was processed to provide enough antigen for the sensitization of sufficient cells to test 720 000 donations for both antigen and antibody. We estimate that about 40 tests can be done for 1p.

The prime criterion in evaluating a new screening test must be the number of additional antigen carriers detected in comparison with current techniques. Our results indicate that E.H.A.I. detects several donations possessing HBsAg in concentrations below the sensitivity of C.E.P. The importance of these findings is emphasized by the results obtained from the retesting of blood donations implicated in post-transfusion hepatitis, which serves as a measure of the efficiency of HBsAg testing within the Blood Transfusion Service. On the three occasions in which a C.E.P. false-negative donation was detected by R.I.A. it was also detected by E.H.A.I.; furthermore, E.H.A.I. detected one out of 17 units transfused which remained R.I.A. negative (table IV). This tends to confirm the findings of Koretz *et al.*,[16] who reported that a positive haemagglutination reaction was more closely related with hepatitis or seroconversion than was a positive R.I.A. reaction.

E.H.A.I. may prove acceptable in large-scale blood donor testing. The incidence of antibody, detected by direct haemagglutination, is about 10-20 times greater than that detected by C.E.P. (252/31 999 *v.* 13/31 999), thus not only providing valuable materials for laboratory reagents but also increasing the quantity of antibody for subsequent fractionation of hepatitis B immunoglobulin for therapeutic use. It seems that with appropriate training the technique may be readily introduced into any regional transfusion centre, thereby keeping reagent costs to a minimum. The basic technology has been adapted to a rapid microcapillary test for use in emergencies[17] and is being automated in Inverness and modified for the detection of antibody to the Dane particle core in Edinburgh.

We thank Dr. J. D. Cash, Dr. I. A. Cook, and Dr. C. Cameron, regional directors of the South-East, North, and East regions respectively, for continued support and encouragement during this project. We also thank Mr. R. Y. Dodd and Dr. T. J. Greenwalt (American National Red Cross), Dr. B. P. L. Moore (Canadian Red Cross Society), and Dr. P. Bradstreet (Public Health Laboratory Service) for kindly providing the antigen panels. We are grateful to Dr. L. D. Wadsworth (Manchester Blood Transfusion Service) for allowing us to report on two cases of post-transfusion hepatitis. Finally, we thank Abbott Laboratories for the timely arrival of an Ausria II evaluation kit.

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Bulk preparation of reagents for hepatitis B testing

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Technical method

Bulk preparation of reagents for hepatitis B testing

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Hepatitis B surface antigen (HB_sAg) is a marker of one of the causative agents of post-transfusion (serum) hepatitis (Giles *et al*, 1969) and as such must be identified in donor blood before transfusion. Over the past few years, a number of techniques have been applied to the detection of HB_sAg, ranging in sensitivity and sophistication from agar-gel diffusion (AGD, first generation test) to radioimmunoassay (RIA, third generation test). The experience of many laboratories clearly indicates that third generation methodology must be adopted if maximum efficiency of blood donor testing is to be achieved (Ling and Overby, 1972; Cayzer *et al*, 1974; Hopkins *et al*, 1975). Unfortunately, third generation reagents are available to the majority of blood transfusion centres only on a commercial basis, the cost ranging from 10 pence per test (Hepatest—Reverse Passive Haemagglutination (RPHA) Wellcome Reagents) to 50 pence per test or more, apparently depending on geographical location (AUSRIA-II, Radioimmunoassay (RIA), Abbott Laboratories).

In 1973 details were published of a tanned-cell haemagglutination inhibition (HAI) technique, utilizing Terasaki tissue culture trays, which achieved a sensitivity similar to RIA but at a fraction of the cost (Hopkins and Das, 1973). This technique has recently been evaluated by three Scottish regional transfusion centres testing over 70 000 blood donations together with various well documented HB_sAg panels (Hopkins *et al*, 1975). The results indicated that, with appropriate training, the technique could be introduced into most regional transfusion centres, thereby keeping reagent costs to a minimum. A valid comparison between centres was possible only because reagents were prepared in bulk at one centre and were thus completely standardized.

This communication describes the bulk preparation of HB_sAg coated glutaraldehyde fixed spherocytes in a quantity sufficient for approximately one million tests.

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Preparation of Fixed Spherocytes

PREPARATION OF SPHEROCYTES

Reagent standardization should be enhanced if a uniform cell population is used for HB_sAg coating. Uniformity is, in this case, achieved by selection of the most robust cells using a controlled hypotonic medium.

One unit (approximately 450 ml) of human group O rhesus negative blood collected in anticoagulant (citrate phosphate dextrose), and not more than three weeks old, was obtained from the blood bank. It was first necessary to determine the molarity of phosphate buffer pH 7·2 required for the selection process. Isotonic (0·2 M) phosphate buffer containing 0·1 % sodium azide was serially diluted in deionized water, and 0·1 ml of fresh, well mixed blood was added to 2 ml of each dilution of phosphate buffer in a test tube, mixed thoroughly, and allowed to stand for 60 minutes. That solution showing at least 50% haemolysis was arbitrarily taken as optimum. The molarity at this point was usually 0·045 M to 0·030 M. The remainder of the unit of blood was then mixed with phosphate buffer (adjusted to optimum molarity) in the ratio 1 volume of cells to 4 volumes of buffer, and the bulk volume was split into 200 ml aliquots for ease of subsequent washing. After 60 minutes at room temperature the aliquots were spun at 1000 rev/min for 15 minutes at 20°C or room temperature using an MSE 6L centrifuge, the supernatant was removed, and fresh hypotonic buffer was added. This procedure was repeated until only a minimum of haemolysis was observed in the supernatant, and the remaining cells accounted for approximately 50% of those originally present. Wet film microscopy showed that the cells had lost their conventional biconcave morphology and become rounded. They were subsequently referred to as 'spherocytes'.

FIXATION OF SPHEROCYTES

In a pilot experiment some spherocytes were divided into aliquots to which glutaraldehyde (Koch Lights, 25%) was added to give the following concentrations: 1 in 100, 1 in 200, 1 in 400, 1 in 800, 1 in 1600, 1 in 3200, and 1 in 6400. After thorough mixing, the cell aliquots containing fixative were left at room temperature overnight. The following morning cells from each aliquot were examined microscopically and subjected to a 'water-resistance' test, which

involved adding 25 μl of 'settled' spherocytes to 2 ml of deionized water, when properly fixed cells resisted haemolysis. Dilutions of glutaraldehyde up to 1 in 1600 (0·06%) produced acceptable fixation, although dilutions up to 1 in 400 (0·25%) tended to distort the shape of the spherocytes, causing them to revert to biconcave discs. The remaining spherocytes (from the unit pack) were fixed in the appropriate concentration of glutaraldehyde (approximately 0·06%) as above and stored at 4°C in phosphate buffer containing 0·1% sodium azide. All solutions contained 0·1% sodium azide.

TANNING, SENSITIZATION, AND STABILIZATION OF FIXED SPHEROCYTES
(for one million HAI tests)

Sixteen ml of packed glutaraldehyde-fixed spherocytes were washed twice with 500 ml volumes of physiological saline (MRC bottle spun at 1000 rev/min for 5 minutes in an MSE 6L centrifuge at room temperature). The deposited cells were resuspended in 500 ml of tannic acid (M & B at a 1 in 60 000 dilution in phosphate buffer 0·15 M pH 7·2 containing 0·1% sodium azide and previously heated to 37°C) and left in a 37°C waterbath for 15 minutes with occasional mixing. The tanned cells were spun down as before, washed twice with 500 ml of phosphate buffered saline (PBS) pH 6·4 containing 0·1% sodium azide, and finally resuspended in 400 ml PBS pH 6·4.

Twenty millilitres HB_sAg, prepared as previously described (Hopkins and Das, 1973) and containing both 'd' and 'y' antigenic determinants, was added, and the spherocyte-antigen suspension was mixed continuously at room temperature (see addendum). After 20 hours a 1 ml aliquot was removed, stabilized as previously described (Hopkins and Das, 1973), and used to titrate a known standard HB_sAb containing serum. Sampling was repeated at approximately 20-hour intervals until a satisfactory sensitivity was achieved. The bulk reagent was removed from the mixer and the cells were recovered by centrifugation as before. The sensitized cells were then stablilized by resuspension and mixing for 4 hours at room temperature in 500 ml PBS pH 7·2 containing 2% normal human serum to give a cell suspension of approximately 3·2% (or 10 times working concentrations).

Storage of these cells overnight at 4°C was followed by a repetition of the stabilizing procedure. Thereafter, the 3·2% suspension of cells was dispersed into 50 ml aliquots and stored at 4°C ready for pasteurization.

PASTEURIZATION AND STORAGE OF HB_sAg COATED SPHEROCYTES

Immediately before pasteurization the coated spherocytes were pelleted and resuspended to the same volume with PBS pH 7·2 containing 0·05% phenol. They were then left for 12-16 hours in a 60°C waterbath, allowed to cool, divided into smaller aliquots (for convenience), and stored at 4°C until required when each aliquot would be diluted 10-fold with PBS pH 7·2 to achieve working strength.

The coated cells could also be stored at −20°C or in the lyophilized state without appreciable loss of sensitivity.

Comment

Recent reports have highlighted the inadequacies of first and second generation methods of HB_sAg testing (Ling and Overby, 1972; Cayzer *et al*, 1974; Hopkins *et al*, 1975). The American Red Cross appear to have opted for RIA as a means of blood donor screening, while transfusion centres in the United Kingdom seem to favour haemagglutination.

It is the purpose of this communication to emphasize that third generation testing efficiency is available at a fraction of the cost of purchasing commercial reagents. Preparation of sufficient HAI reagents for approximately one million tests is described in detail using a process which is readily reproducible (we have recently prepared a second batch of reagents sufficient for 3·5 million HAI tests) and requires relatively unsophisticated laboratory equipment. The cost of one million RPHA tests is £80 000, while a similar number of RIA tests bought commercially could cost £500 000.

Experience has shown that HAI reagents are extremely stable in that they will survive 4°C for over one year, repeated freezing and thawing, lyophilization and exposure to 2·5 mega rads of gamma irradiation from a cobalt source (Courtesy of Ethicon Ltd, Edinburgh), neither does transport over thousands of miles via air freight appear to have an adverse effect, even when cells are maintained in the liquid state.

The safety of laboratory staff handling hepatitis B testing reagents is of paramount importance. Until it is possible to evaluate the biological activity of HBAg it is essential that every reasonable precaution should be taken to ensure the safety of such reagents. For this reason we have attempted to incorporate into reagent preparation accepted methods of virus inactivation, combining the principles of chemical and physical inactivation, namely, (*a*) betapropiolactone treatment and ultraviolet irradiation of raw serum (LoGrippo *et al*, 1971), (*b*) pasteurization of HB_sAg coated glutaraldehyde fixed spherocytes, and, if required, (*c*) gamma irradiation of the lyophilized reagents.

We wish to express our sincere thanks to Drs J. D. Cash and P. C. Das, of the South East of Scotland

Regional Blood Transfusion Centre, for their support and encouragement in the course of this project.

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Addendum

We observed that cell sensitization was successful when prepared by mixing in an MRC 500 ml bottle by mechanical turntable but unsuccessful when performed in the same type of bottle using a magnetic mixer with plastic-coated follower. Adsorption of HB_sAg onto the plastic follower has been eliminated but the electrochemical or electrophysical possibilities for the failure have yet to be investigated.

British Journal of Haematology, 1971, **21**, 673.

Rapid Identification of Hepatitis Associated Antigen and Antibody by Counter-Immunoelectroosmophoresis

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Summary. A modification of the counter-immunoelectroosmophoresis method is described which will allow rapid identification of hepatitis associated antigen and antibody within 2 hr by producing a reaction of identity with known reference materials. The specificity of the reaction is established by the demonstration of non-identity and partial identity by this technique.

The exact relationship between Australia/SH antigen, presently known as hepatitis associated antigen (HAA), and A or B hepatitis virus remains to be clearly defined. However, there is little doubt that products containing HAA are capable of transmitting the infection to susceptible recipients. Amongst the various immunological techniques used for the detection of HAA, the counter-immunoelectroosmophoresis (CIEOP) (Passendorfer *et al*, 1970) has recently gained popularity for its sensitivity and the rapid formation of precipitin lines due to an enhanced reaction between the antigen and antibody. Combining the principle of electrophoresis and immunoprecipitation simultaneously, the basic technique involves two wells per sample between which a precipitin line is observed when the antibody immunoglobulin present in the anode well reacts with the material in the cathode well containing HAA. When a precipitin line does occur, it is necessary to confirm its specificity by demonstrating its identity with known reference material. Occasionally, erroneous results may be obtained because the antisera of human origin which are presently used for detection of HAA, are mostly derived from multitransfused patients and may contain, besides anti-HAA, other antibodies to plasma proteins (Blumberg, 1964). Zuckerman & Taylor (1970) and Passendorfer *et al* (1970) suggested that CIEOP is unable to produce reaction of identity readily and recommended the Ouchterlony double diffusion method for this purpose. This has been a major drawback to the adoption of CIEOP for identification of HAA and its antibody.

It was suggested (F. Reicht, personal communication 1970) that CIEOP could be used for identification purposes if the precipitin bands are allowed to form sufficiently close between the wells containing reference material such as known HAA and the test substance reacting simultaneously against the complementary antibody present in a third well. This paper describes such a modification and allows the identification of antigen and/or antibody within an hour by showing reaction of identity, partial and non-identity between the reactants.

MATERIAL AND METHOD

Materials

HAA. Plasma and serum containing HAA were obtained from a hepatitis patient; the reference antigen was received from Dr Okochi, Tokyo.

Fibrinogen. Normal plasma obtained from a healthy blood donor was used as a source of human fibrinogen and hooded rat plasma for rat fibrinogen.

Anti-human fibrinogen (Behringwerke AG, Germany) produced in the rabbit was used. This antiserum reacts only with fibrinogen but not with other plasma proteins.

Anti-HAA was obtained from a multiply transfused haemophiliac and its reactivity had been tested and compared with other reference antisera (Das *et al*, 1971).

Preparation of Plates

A 0.9% agarose solution in 0.025 M veronal buffer (pH 8.6) was used. Aliquots of 25 ml molten agarose were poured on to a 10 cm square plastic petri dish (Sterilin) on a levelled table and allowed to solidify. Circular wells 2 mm in diameter were cut in the agarose 3 mm apart along the electrophoresis axis (Fig 1). If HAA was suspected, the pair of wells 1 mm apart were placed towards the cathode and vice versa.

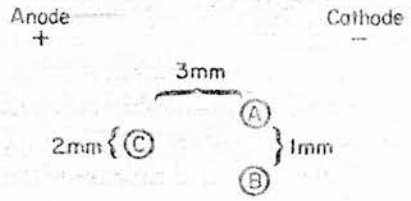

FIG 1. Position of wells in the agarose gel for the CIEOP technique. A: HAA control; B: suspected HAA; C: anti-HAA serum.

Method

The anode well(s) were filled with antiserum by means of a capillary tube. The petri dish was then placed in the electrophoresis chamber (Shandon, Model U.177) and maintained at 30°C by means of a water jacket (Shandon) through which water was circulated at the rate of 1 litre/2.5 min from a water bath using a peristaltic pump. Glass fibre paper (Whatman) was used as connecting wicks with veronal buffer (pH 8.6, 0.05 M) in the electrophoresis chamber and a current of 40 mA (about 200 volts) was passed for 5 min. The current was then switched off and the antiserum well(s) 'topped' up. Antigen was added to the cathode well(s) and and the current re-started. Under these conditions, the precipitin lines appear in 1 hr. If the reactants in the two vertical wells both contain HAA (or antibody) a line of identity will be obtained. The specificity of the technique is further demonstrated by the production of reactions of non and partial identity.

RESULTS

Fig 2(a) shows a complete reaction of identity by CIEOP using serum and plasma from the same patient. Complete identity was also obtained when the test serum was compared with the reference antigen (Fig 2b). This basic observation was not exclusive to the HAA and anti-HAA system and could be extended to plasma fibrinogen as shown in Fig 2(c). However,

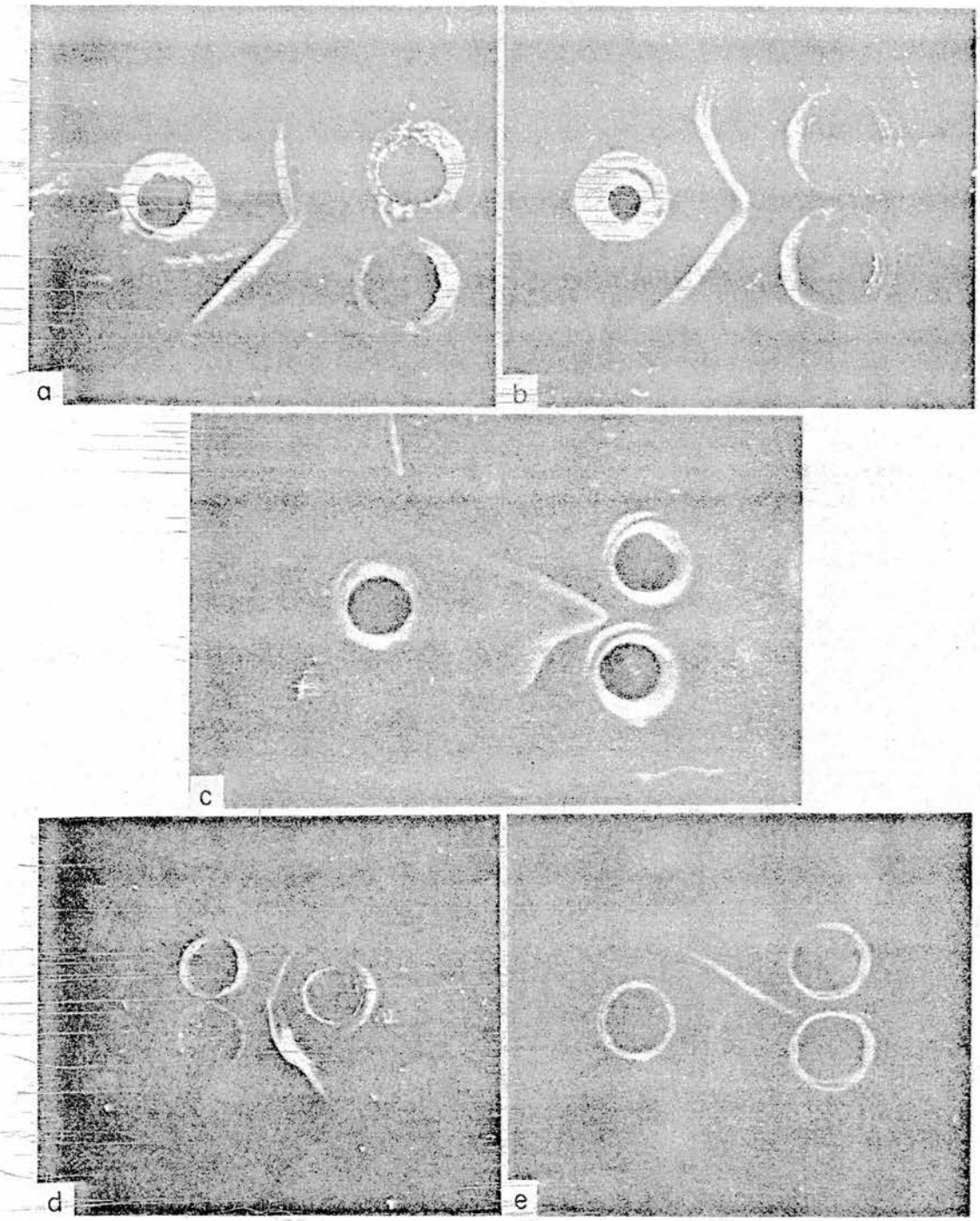

FIG 2. Different types of immunological reaction obtained by the CIEOP. (a), (b) and (c) Reaction of identity; (d) non-identity reaction; (e) reaction of partial identity.

(*Facing p* 674)

when the patient's plasma was allowed to react simultaneously against the anti-HAA and anti-fibrinogen serum, a reaction of non-identity showing the characteristic intersected precipitin lines was obtained (Fig 2d). When human and rat plasma were allowed to react against an anti-human fibrinogen by this method, the resultant precipitin lines showed partial-identity due to the known serological differences in rat and human fibrinogen. The size and direction of the 'spur' indicate the degree of dissimilarities between the antigens (Fig 2e).

DISCUSSION

In examining 158 transfused thalassaemic patients, Vierucci *et al* (1970) reported that 20% of those showing anti-Ag (low density lipoprotein) also contained anti-HAA and suggested that use of such antisera in HAA detection might give rise to false positives. Establishment of the nature of reaction by identifying the precipitin bands is therefore of major importance since much of the anti-HAA presently available is derived from multiply transfused patients in whom iso-precipitins, not related to HAA may be present (Blumberg, 1964). During the last year at least one such reaction has been demonstrated in this laboratory in a para-proteinaemic patient's serum which produced a precipitation line against anti-HAA, but failed to demonstrate virus like particles on electronmicroscopy.

In using the CIEOP technique, the size of the wells does not appear to be critical and some workers preferred to alter the well-diameter according to the concentration of the reagents used. The optimal distance between the anode and cathode wells seems to be 3 mm (Alter *et al*, 1971). For accurate identification one must ensure that the margins of the wells are intact (Ouchterlony, 1964). We have found that layering the gel with 1% freshly made tannic acid solution at room temperature for 10–15 min significantly increases the sensitivity of the system by sharpening the weak precipitation bands (Alpert *et al*, 1970), and has the added advantages of time and simplicity over the conventional staining methods.

Despite the increasing use of CIEOP for the detection of HAA many laboratories still base their final conclusion on a reaction of identity produced by the technique of Ouchterlony. A major disadvantage of this method is that it is relatively time consuming requiring 1–7 days (Prince & Burke, 1970) before a definite answer is available. It is therefore ill suited for blood transfusion purposes, particularly in making available fresh whole-blood and platelet concentrates which may be required for transfusion within a few hours of withdrawal. The technique we describe seeks to reduce substantially this delay, so that in less than 2 hr initial screening and identification is possible. Besides the HAA system, this method could also be applied in other antigen–antibody systems such as plasma fibrinogen and alpha-feto-protein (Kohn, 1970).

ACKNOWLEDGMENTS

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Improved sensitivity of the electrophoresis method by tannic acid for detection of Australia antigen

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Improved sensitivity of the electrophoresis method by tannic acid for detection of Australia antigen

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From the Regional Transfusion Service, Royal Infirmary, Edinburgh

For detection of Australia antigen (Au-Ag) by counter-immunoelectroosmophoresis (CIEOP) staining the agarose gel plates with certain dyes has been claimed to improve the sensitivity (Combridge and Shaw, 1971). In our experience, however, a simpler and less time-consuming procedure is that of layering the gel plates with 1% freshly made tannic for 10 minutes (Alpert, Munroe, and Schur, 1970) after the routine CIEOP procedure (Das, Hopkins, Cash, and Cumming, 1971). This has resulted in a significantly increased sensitivity by improving visualization of precipitin lines.

Serial dilutions of Au-Ag containing serum and anti-Au (human origin) were set up in the test system using a 'chessboard' design. After the electrophoresis 'run' the gel plates were observed at an angle under direct light over a dark background. The results were scored as + for sharp precipitin line, ± for weak precipitation, and − for no reaction. Table I shows that the titre of Au-Ag against the neat antiserum was 1/4, and no significant improvement was noticed when the same plate was reviewed after overnight incubation. Tannic acid was now added and the plate read after 10 minutes: the titre was now 1/16. This improvement reflects an increased sensitivity of the system as a whole; thus, before tannic acid treatment, the total number of

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positives in the 'chessboard' was 14, and after tannic acid treatment they were 21. This was further confirmed over a period of two weeks during which 212 selected specimens from patients, including drug addicts, with clotting disorders and hepatitis, as well as blood donors, some of them already known to be carriers of Au-Ag, were subjected to the procedure described above. Results show (Table II) that the number of positive samples were eight before and 12 after tannic acid treatment; the additional

	No. of Samples Tested			
	Blood Donor	*Patient*	*Laboratory Staff*	*Total Positive*
	178	27	7	
Before tannic acid	3	5	0	8
After tannic acid	4	8	0	12

Table II *Number of positive specimens amongst 212 selected samples before and after tannic acid treatment of CIEOP plates*

positive results consisted of three from the patient group and one from the blood donors. In view of these results the above procedure was extended to the 'routine' laboratory where every unit of blood donated was screened for the presence of Au-Ag. During a period of five months a total of 19 423 donors were tested, 10 were found to be positive by routine procedure and no additional positive appeared, however, after tannic acid treatment of the CIEOP plates.

The mechanism by which tannic acid increases the sensitivity of the system is not clear, but from a practical point of view there is no doubt that it is capable of bringing out Au-Ag-antibody precipitin lines, especially amongst the patient's sera. The present procedure in this laboratory is to score the results immediately after the routine CIEOP 'run', the plates are washed, then treated with tannic acid, re-read and photographed immediately. All positive samples are re-investigated for identity reaction, if necessary, after concentration.

We are grateful to Dr R. A. Cumming and Dr J. D. Cash of this Department for constant encouragement, support, and reading the typescript.

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Latex agglutination test for detection of Australia antigen (HB-Ag) among blood donors and patients

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SUMMARY The application of the latex test for the detection of Australia antigen (Au-Ag) was investigated. Reagents from two commercial sources were compared with the electrophoresis method with regard to sensitivity and specificity using samples from blood donors, hospital patients, and plasma fractions. Discordant results were further investigated by electron microscopy and radioimmunoassay.

Differences were noted in the results between these reagents and the significance of the findings together with suggestions for minimizing false positive results are discussed.

The introduction of a latex agglutination test for the detection of Au-Ag (HAA/HB-Ag) by Leach and Ruck (1971), which can be read in minutes and is claimed to be as sensitive as the generally recognized counter immunoelectroosmophoresis technique, may be regarded as an important advance. This report describes our experience with this technique applied to blood donors and patients and compares it with the counter immunoelectroosmophoresis method. Electron microscopy and radioimmunoassay were performed on those specimens showing consistent discrepancy between the results by counter immunoelectroosmophoresis and latex agglutination.

Materials and Methods

STANDARD SERA

Known Au-Ag positive serum and plasma samples from our reference panel were collected either locally or from abroad including the reference panel no. 2 of the Department of Biologics Standard, NIH (Bethesda, Maryland), and the American Red Cross (Washington). Serum and plasma taken into different anticoagulants (citrate, oxalate, heparin, and EDTA) from nine healthy males (39-64 years) were tested both serologically including radioimmunoassay, and by electron microscopy for use as negative controls. Further, a group of sera selected from the panel that had been tested by different laboratories (by multiple methods) and giving concordant results, were used in this study as positive and negative controls (table I).

TEST SAMPLES

Test samples were obtained from several sources: from plasmaphoresis donors, from known contacts to Au-Ag, from miscellaneous patients including some with rheumatoid arthritis, and from coagulation factor concentrates prepared by the Scottish National Plasma Fractionation Centre. Also, 10 known Au-Ag positive sera were derived from the screening, by counter immunoelectroosmophoresis, of 15 000 donors. Of these 10 Au-Ag positive sera, four were subtyped as ad+ and a further four as ay+ by Le Bouvier (1971) at Yale University; all 10 were coded and randomly dispersed among the other test samples.

LATEX AGGLUTINATION TEST

Two separate batches (PF1, PF2) of latex particles coated with anti-Au-Ag, prepared in guinea-pigs and supplied by Pfizer Ltd,[1] were tested according to the method of Leach and Ruck (1971). A further set of reagents (anti-Au-Ag prepared in rabbits) was also supplied by Hoechst Pharmaceuticals Ltd,[2] and the tests were performed as recommended by the manufacturers. By the same procedure further tests were carried out using latex tagged with normal rabbit IgG and with guinea-pig immunoglobulin (obtained commercially from Wellcome Reagents[3]

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[1]Kent, UK. [2]Brentford, UK. [3]Beckenham, UK.

Samples with (*) or Without (°) Au-Ag	*Latex Pfizer (2)*	*Latex Hoechst*	*Counter Immuno-electroosmophoresis*	*Electron Microscopy*	*Radio-immunoassay*
°A	+	±	—	—	—
*B	+	—	+	—	+
°C	—	—	—	—	—
*D	+	+	+	+	+
*E	+	+	+	+	+
°F	—	—	—	—	—
*G	+	+	+	+	+
°H	—	—	—	—	—
°I	—	—	—	—	—
°J	—	—	—	—	—
*K	+	+	+	+	+
*L	+	+	+	—	+
°M	—	—	—	—	—
°N	—	—	—	—	—
*O	+	+	+	—	+
°P	—	—	—	—	—
*Q	+	+	+	+	+
°R	—	—	—	—	—

Table I *Coded reference panel samples tested by radioimmunoassay, two different preparations of latex, and other methods*

and Pfizer Ltd). The results were scored as + for strong, ± for weak, and − for no agglutination.

OTHER METHODS

Counter immunoelectroosmophoresis was performed by the modification of Das, Hopkins, Cash, and Cumming (1971) and read after tannic acid treatment (Hopkins and Das, 1972).

Basic electron microscopy and immune electron microscopy were performed as described by Kelen, Hathaway, and McLeod (1971). Solid phase radioimmunoassay using radioiodinated marker, anti-Australia antigen 1251(Austria-125), obtained from the Abbott Laboratories[1], was used in accordance with the manufacturer's directions.

Results

Preliminary studies on the various latex preparations set up against known standard Au-Ag positive sera demonstrated that although all apparently reacted satisfactorily, differences in sensitivity could be shown between the reagents (table II). Further, the 10 positive sera derived from routine screening of 15 000 blood donors, when tested with one batch of latex (PF1), all reacted strongly; four of these which were subtyped ad+ had a mean reaction time of 1·9 minutes (range 0·8-2·5 minutes) and four which were subtyped ay+ had a mean reaction time of 2·8 minutes (range 1·5-4·5 minutes).

Based on these findings 100 selected test samples were set up against the PF2 and Hoechst latex preparations. The results are summarized in table III; all samples shown to be positive by counter immuno-electrophoresis were also strongly positive with the PF2 latex preparation. However, the Hoechst material failed to detect one out of six of the Au-Ag-positive samples, and both the latex preparations produced five false positives.

As the one false negative reaction occurred in a serum from a patient, a further 10 patients' sera were tested. These sera were kindly supplied by the Edinburgh University Department of Bacteriology's diagnostic service and were tested against the PF2 and Hoechst reagents. The results are summarized in table IV which shows that the PF2 reagent detected all six Au-Ag positive sera. However, the Hoechst latex preparation reacted to only two of these sera. Both reagents gave false positive reactions all of which were also negative to electron microscopic and immune electron microscopic examination. Prior heat treatment of these sera (56°C for 30 minutes) did not influence the non-specific latex agglutinin titre, except in one sample out of the six so treated, and the addition of normal guinea-pig serum to the test as recommended by one manufacturer produced false negative result in a haemophiliac (table IV), probably due to physical dilution. However, in all of these sera the presence of non-

[1]Queenborough, Kent, UK.

Counter-Immunoelectro-osmophoresis	*Latex*		
	Pfizer Batch 1	*Pfizer Batch 2*	*Hoechst*
1:16	1:4	1:256 (1:512)[1]	1:4 (½)

Table II *Comparative antigen titration with different batches of latex and by counter immuno-electroosmophoresis*

[1]Figures in parenthesis are before addition of normal animal serum.

Source	No. of Samples Tested	Number of Positives		
		Counter Immuno-electroosmophoresis	Latex Pfizer II	Latex Hoechst
Plasmaphoresis subjects	65	0	0	3
Coagulation disorders	9 (1)	1	1	0
	9 (8)	0	1	1
Plasma fraction (DEF-IX)	4	0	2	1
Carrier (Au-Ag) blood donor	5	5	5	5
Contacts and staff	11	0	0	0
Transplant donors	2	0	1	0
Alcoholic liver	1	0	0	0
Myeloma	1	0	1	0
Normal sera	2	0	0	0
Total	100	6	11	10

Table III *Tests by two different latex reagents on 100 selected specimens and comparison with counter-immunoelectroosmophoresis*

Clinical Diagnosis		Counterimmuno-electroosmophoresis (×5 Conc. Sera)	Without	With	Hoechst Latex
			Pfizer Latex (2)		
			Normal Guinea-pig Sera		
J6703	Diabetis	—	+	+	—
J5821	Serum jaundice	+	+	+	—
J6566	Chronic myeloid leukaemia	—	—	—	—
J6477	Haemophilia	+	+	—	+
J6508	Haemophilia	+	+	+	—
J6655	Aplastic anaemia	+	+	+	—
J6046	Obstructive jaundice	+	+	+	+
J6018	After bypass	—	—	—	—
J5979	Haemophilia	+	+	+	—
J5983	Obstructive jaundice	—	—	—	+

Table IV *Results obtained on patients' sera using Pfizer and Hoechst latex agglutination test*

specific latex agglutinins could be readily demonstrated by latex-coated normal rabbit or guinea-pig immunoglobulins.

In order to ascertain whether this latter observation could be used in monitoring false positive results, sera from 19 patients with rheumatoid arthritis (seven of whom were positive for rheumatoid factor) were tested with rabbit anti-Au-Ag coated latex (Hoechst) and with normal rabbit immunoglobulin sensitized latex (Wellcome Reagents). Table V shows that nine samples were negative to both preparations, one serum showed a weak positive reaction to anti-Au-Ag coated latex only, but a further nine samples reacted to both specific and non-specific latex reagents; the agglutination titres against the specific anti-Au-Ag coated latex (mean 24 ± 23) and non-specific gamma globulin-coated latex (13 ± 11) showed highly significant correlation ($r = 0{\cdot}91$, $P < 0{\cdot}001$) in the later group of nine sera. All 19 samples were negative by counter immunoelectrophoresis.

Attempts to reduce further the time required for the latex technique by using plasma instead of serum proved disappointing. Fresh normal plasma, irrespective of the anticoagulant used, produced weak but definite latex agglutination and heat treatment (56°C for 30 minutes) did not modify this reaction. Sera from the same donors (tested after two hours at 37°C) were negative.

Number of Samples	Anti-Au-coated Latex	Normal Rabbit Gamma Globulin-coated Latex	Counter-immuno-electro-osmophoresis
9	—	—	—
9	+	+	—
1	±	—	—

Table V *Sera from rheumatoid arthritis patients tested by normal rabbit gamma globulin-coated latex and anti-Au-sensitized latex (Hoechst)*

In a further investigation, radioimmunoassay was used in parallel with both preparations of anti-Au-Ag-coated latex (Hoechst and Pfizer). Positive and negative specimens from the Reference Panel were coded and tested by a variety of other methods.

Table I shows that all the eight known positive samples were correctly identified by radioimmunoassay, counter immunoelectroosmophoresis, and Pfizer latex (PF2). However, one remained negative with the Hoechst reagent and three with electron microscopy. No false positive results were obtained by radioimmunoassay, counter immunoelectroosmophoresis, and electron microscopy, but one occurred with both specific latex reagents. This was confirmed by negative results obtained by these three techniques.

Discussion

The surprising feature of this study was the good agreement between both the latex preparations and the counter immunoelectroosmophoresis technique when applied to sera obtained from known Au-Ag-positive blood donors. On the other hand undoubted false negatives occurred in sera obtained from Au-Ag-positive patients with the Hoechst and PF1 preparations. This may have been due to a limited specificity of the coating antibody, for preliminary studies in this laboratory have suggested that the antigen typed as ay+ may not react as effectively as those designated ad+ with the latex reagents we used. It seems more likely, however, that the problem, which is of some clinical importance, may be more closely related to the low sensitivity of these reagents. This conclusion is supported by the absence of false negatives with the PF2 preparation.

False positive reactions to both the latex reagents used in this study have been reported previously (Banatvala, Best, Almeida, and Dane, 1971; Cossart, Field, March, and Porter, 1972; Burrell, Dickson, Gerber, McCormick, and Marmion, 1972) and our results confirm these observations. In investigating the proficiency of anti-Au-Ag coated latex, Perkins, Perkins, Chen, and Vyas (1972) noted about 2·5 times higher false positivity amongst hospital samples compared with those from normal volunteer donors, and while using basically a similar latex agglutination principle for the detection of rheumatoid factor, Caplan (1963) noted a very high degree of reactivity amongst the non-rheumatoid patients compared with blood donors. The increased reactivity in patients' sera may reflect the presence of substances such as rheumatoid factor, heterophil antibody, or species specific substances, whose diverse properties may affect comparisons between normal individuals and patients (Hoq, Cash, Das, and Cumming, 1971). In addition Langenhuysen (1971) demonstrated the presence of antibodies against gamma globulin causing agglutination in the latex fixation test in patients following transfusions and cytomegalovirus infection. One factor responsible for these non-specific reactions in some sera seems to be associated with the IgM fraction (Zalan, Wilson, and Labzoffsky, 1972), for three out of five rheumatoid sera which agglutinated both specific and non-specific latex reagents became negative following mercaptoethanol treatment (table V).

With regard to the false positive results, none of the preparations tested appeared to have any advantage over the others. The fact that different latex preparations react falsely with different Au-Ag-negative sera may reflect differences in animal species from which the immunoglobulins were derived, and in the methods of coating the latex, all of which may influence the primary structure of the immunoglobulin molecule (Stanworth and Pardoe, 1967).

To eliminate this problem heating at 56°C for 30 minutes has been claimed to be effective (Ziegenfuss, 1972), but this was not confirmed by our experience. However, our findings do suggest that latex coated with appropriate normal immunoglobulin introduced as 'control' would help to identify spurious positive results, although simultaneous presence of Au-Ag and non-specific agglutinin(s) could cause agglutination of both 'test' and 'control' reagents. Since the positive Au-Ag sera gave 85% agreement between the two manufacturers' reagents tested, this approach might be useful for emergency screening, provided that the reagents are of high quality and sensitivity; the result, however, is available in about five minutes and repeating the test after an absorption procedure with appropriate normal gamma globulin-coated latex could augment the specificity of this reaction. The simplicity and short length of time involved in the latex test for detecting Au-Ag appear to make it an ideal reagent for rapid monitoring of purification stages of Au-Ag containing materials, and this approach has been successfully employed as one of the methods for standardization of the antigen for sensitizing human red cells employed in a passive haemagglutination test (Hopkins and Das, 1973) for detection of Au-Ag and its antibody.

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A rapid micro-capillary haemagglutination method for detection of Australia antigen (HB-Ag) and its antibody

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A rapid and a simple micro-capillary haemagglutination test is described for screening and identification of HB-Ag and its antibody in sera. While retaining the specificity and very small volume of reagents required it has a similar degree of sensitivity to the microtitre plate method. The reagents are glutaraldehyde pre-fixed human RBCs coupled to beta propiolactone-inactivated HB-Ag, which retain their reactivity for at least 6 months after storage.

Introduction

With the demonstration of the association between Australia antigen (HB-Ag) and Hepatitis B, blood has been screened for its presence by a variety of immunologic methods, including haemagglutination (HA) which requires 1–2 hr for completion of the test and has a similar degree of sensitivity to radioimmune assay (Hopkins & Das, 1973). This communication describes a modification of the conventional HA plate procedure to a micro-capillary system which, while retaining the sensitivity and specificity of the former, has the primary advantage that the tests can be read within 20 min, and also requires smaller amounts of the reagents. Other advantages are that the antigen-coated human erythrocytes may be stored for at least 6 months, and the presence of both HB-Ag and HB-Ab can be detected and confirmed by the same technique. A similar technique has been described for the detection of serum fibrin degradation products by Israels, Rayner, Israels & Zipursky (1968).

Materials and Methods

Human erythrocytes (group O) were fixed with glutaraldehyde and treated with tannic acid according to the procedure of Hoq & Das (1971). Partially purified HB-Ag, treated with beta-propiolactone was coupled to the tanned cells by following the method of Hopkins & Das (1973). A 4% (v/v) suspension of these cells in phosphate buffered saline (PBS) at pH 7.2, containing 1% normal absorbed rabbit serum and Na-azide (1 mg/cm^3, PBS) was prepared and stored at 4° C in aliquots sufficient for one day's expected requirements. PBS was used as diluent in all experiments and the supernatant of the stored cells replaced with fresh PBS at monthly intervals. Under these conditions the cells retain their reactivity for at least six months for both the capillary and microtitre plate tests. Fixed cells similarly coated with normal pooled plasma, and uncoated cells, were used as controls. In the preliminary experiments 38 samples containing either HB-Ag or HB-Ab were obtained from previously screened blood donors. Of these, 19 were coded and dispersed at random among 18 normal sera (Table 1).

The tests were performed with appropriate laboratory precautionary measures as described in a report by a scientific group of the WHO (1973). Capillary tubes with blunt

TABLE I

Micro-capillary test for detection of HB-Ag and anti-HB-Ag

Nature of sample	Test Number	Results HB-Ag	HB-Ab	Negative
HB-Ab	25	0	25	0
HB-Ag	13	13	0	0
Normal sera	18	0	0	18

ends, 90 mm in length and 0.5 mm internal diameter were selected. The details of the methodological procedures are shown in Fig. 1. During the entire performance of the test the capillary tube was held (while wearing surgical gloves) only at the upper end (U) which remains uncontaminated throughout the procedure. By capillary action test sera, reagents,

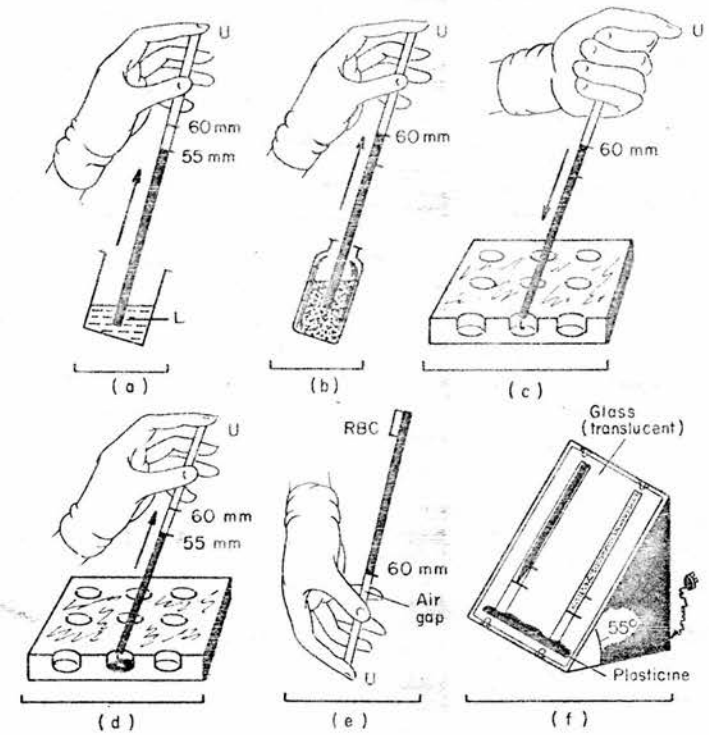

Fig. 1. Stages of microcapillary method. Capillary was held only at the upper end (U) throughout the test procedure. Reagents and tests enter by capillary action through the lower end (L). (a) Sera containing HB-Ab. (b) HB-Ag coated RBC. (c)–(d) Known antibody plus test expelled out of capillary, mixed and refilled via L. (e)–(f) Inversion and embedding of capillary (note RBC now positioned at the top). (f) RBC gravitating down to form negative or positive reaction pattern.

and control sera enter the capillary tube through the lower end (L). The index finger of the performer rests gently on the upper end of the tube, releasing the capillary lumen at appropriate times, thereby allowing the reactants to reach the desired levels inside the capillary.

The capillaries were marked at intervals of 55 and 60 mm from the lower end. For HB-Ab detection serial dilutions of the test serum were drawn by capillary action to the 55 mm mark [Fig. 1(a)] followed by sensitized cells to the 60 mm mark [Fig. 1(b)]. For the

detection of HB-Ag, the antiserum was selected by determining the titration end point of a given serum and using a ten-fold concentration for the test (e.g. the working dilution of an antibody with a titre of 1 in 10 000 would be 1 in 1000). By capillary action the antiserum was drawn to the 55 mm mark [Fig. 1(a)] followed by test serum to the 60 mm mark, then a rubber teat was fitted at the upper end of the capillary and the contents gently expelled to the bottom of a well of a microtitre (Flow Laboratory, Irvine) plastic plate [Fig. 1(c)]. The teat was removed and with the capillary held at the upper end, the index finger resting over the capillary lumen (U), the contents were mixed inside the well with the tip of the lower end of the capillary (L) for 10 sec. The finger was removed and the mixture was re-drawn by capillary action to the 55 mm mark [Fig. 1(d)], followed by coated cells to the 60 mm mark [Fig. 1(b)]. After filling the tubes an air gap (30 mm) remained between the column of reagent (inside the capillary tube) and the upper end of the capillary [Fig. 1(b)]. The capillaries were inverted thus the red cells were positioned at the top of the liquid [Fig. 1(e)], and the ends (U) embedded in plasticine fixed to a sheet of translucent glass, tilted to produce an angle of 55° and mounted in a metal viewing box illuminated by a fluorescent lamp [Fig. 1(f)]. Observations were made at intervals of 2–3 min over a period of 10–20 min, after which the results were recorded. As the red cells gravitate down from the top and through the liquid in the capillary, they form their characteristic pattern in approximately 5 min. In the absence of agglutination this takes the form of a well-defined line tapering to a pencil point [Fig. 1(f)]. In the presence of haemagglutination the characteristic 'fir tree' is first formed followed eventually by frank agglutinates [Fig. 1(f)]. Reactions were recorded as + (agglutination), − (no agglutination) and ± (intermediate). For antibody titrations reactions were designated +++, ++ and +.

The presence of HB-Ag was confirmed by further tests using two specific antibodies. Samples showing direct agglutination (antibody) were retested using red cells coated with normal plasma and the specificity confirmed by utilizing the test serum as anti-HB-Ag in an inhibition reaction with HB-Ag positive controls and normal serum. The following controls were incorporated in each batch of tests:

Positive:	Anti-HB-Ag with PBS and normal serum
Negative:	Anti-HB-Ag with known HB-Ag positive serum
Test control:	PBS with test samples
Cell control:	PBS with sensitized cells

Results and Discussion

Table 1 shows that all 38 samples in the preliminary experiments were correctly identified. Titration of Hb-Ab material showed that, when undiluted, some immune sera of animal origin (chimpanzee and rabbit) produced an unusual reaction pattern suggestive of a prozone effect. The cells remained on the surface of the column even after standing for 3 hr, in contrast to the expected negative (non-agglutination) pattern. Human serum containing HB-Ab has not so far shown this phenomenon (Table 2). Comparative titrations of HB-Ab

TABLE 2

Capillary HB-Ab titration showing prozone effect in some immune animal sera but not in human sera*

HB-Ab	Neat	1/10	1/100	1/1000	1/10 000
Chimpanzee	−	+	++	+++	++
Rabbit	±	++	++	+	+
Human (1)	+	+	+	±	−
Human (2)	++	++	+	+	−

* Not all animal antisera show this effect.

containing materials so far suggest that the capillary method as described has 'one tube' of advantage over the plate procedure, but by comparison with doubling dilutions of known HB-Ag the capillary method is shown to be slightly less sensitive (1 tube difference). This is consistent with our experience in the determination of serum fibrin degradation products (Hoq & Das, 1971) and is probably related to the larger volumes and to the higher concentrations of antiserum relative to the test sample (55 mm/5 mm), used in the capillary method. The sensitivity of the micro-capillary test for the detection of antigen could be increased by pre-incubation of equal volumes of test serum and antibody, drawing the mixture to the 55 mm mark followed by cells to the 60 mm mark, or by reducing the concentration of antibody. For antigen screening however, larger volumes of antisera were used to improve definition. The test does not require costly equipment and even some of the items described above could be simplified. Its simplicity, rapidity, and sensitivity render it superior to the rapid, but problematic latex test (Hopkins & Das, 1974*b*) for emergency requirements involving a limited number of samples. The red cells are capable of HB-Ag subtyping into *ad* and *ay* varieties and can be used for this purpose in the analysis of the specific antibody content of HB-IgG preparations (Hopkins & Das, 1974*a*).

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