EFFECTOR T LYMPHOCYTES IN DEFENCE AGAINST MURINE INFLUENZA VIRUS INFECTION

by

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獻於父母

TO MY PARENTS

. . . a scientific life is . . .

in reality exciting, rather passionate and - in terms of hours of work - a very 'demanding' and and sometimes 'exhausting' occupation. It is also likely to be tough on a wife or husband and children who have to live with an obsession without the compensation of being possessed by it themselves.

Advice to a young scientist

Sir Peter Medawar.

CONTENTS

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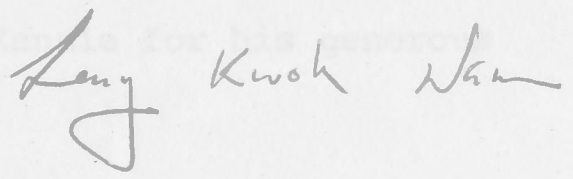
S T A T E M E N T

The experiment described in Table 8 of Chapter 7 was performed by Dr. R.B. Ashman. All other experiments described in this thesis constitute my own original work and were carried out by myself under the supervision of Professor G.L. Ada.

Part of the concluding discussion (Chapter 9) has been published in a review article which was written jointly with Professor G.L. Ada and Dr. H. Ertl.



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A C K N O W L E D G E M E N T S

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ABBREVIATIONS

ADCC	- Antibody-dependent cell-mediated cytotoxicity
ATS	- Anti-thymocyte serum
B cell	- Bursa of Fabricius (or mammalian equivalent) - derived lymphocyte
BCG	- Bacilli-Calmette-Guerin
CBH	- Cutaneous basophil hypersensitivity
CMI	- Cell-mediated immunity
CML	- Cell-mediated lympholysis
Con A	- Concanavalin A
CRBC	- Chicken red blood cells
CS	- Concanavalin A-activated spleen cell supernatant
Cy	- Cyclophosphamide
DNFB	- Dinitrofluorobenzene
DNP	- Dinitrophenyl
DTH	- Delayed-type hypersensitivity
EID_{50}	- Median egg infectious dose
E:T	- Effector to target cell ratio
FCA	- Freund's complete adjuvant
FCS	- Foetal calf serum
h	- hour(s)
H-2	- MHC in mouse
HA	- Haemagglutinin
HAU	- Haemagglutination units
HI	- Haemagglutination inhibition
HLA	- MHC in man
HSV	- Herpes simplex virus
H-Y	- Male specific antigen

Ia	- I region associated
Ig	- Immunoglobulin
i.n.	- Intranasal
i.p.	- Intraperitoneal
^{125}I-UdR	- ^{125}I-5-iodo-2'-deoxyuridine
i.v.	- Intravenous
LCM	- Lymphocytic choriomeningitis virus
LPS	- Bacterial lipopolysaccharides
Lyt	- Lymphocyte differentiation antigen
M protein	- Matrix or membrane protein
MCMV	- Murine cytomegalovirus
2-ME	- 2-Mercaptoethanol
MEM	- Eagle's minimum essential medium
MHC	- Major histocompatibility complex
MHV	- Mouse hepatitis virus
MIF	- Migration inhibitory factor
MLR	- Mixed lymphocyte reaction
MLV	- Murine leukemia virus
NA	- Neuraminidase
NDV	- Newcastle disease virus
NIP	- (4-hydroxyl-5-iodo-3-nitrophenyl) acetyl hapten
NK	- Natural killer
NP	- (4-hydroxyl-3-nitrophenyl) acetyl hapten
PBL	- Peripheral blood leukocytes
PBS	- Phosphate-buffered-saline
PEC	- Peritoneal exudate cells
PHA	- Phytohemagglutinin

PMN	- Polymorphonuclear leukocytes
s.c.	- Subcutaneous
S.E.	- Standard error
SFV	- Semliki forest virus
SRBC	- Sheep red blood cells
T cell	- Thymus-derived lymphocyte
Tc	- Cytotoxic T cells
Td	- T cells mediating DTH reactions
Th	- Helper T cells
Ts	- Suppressor T cells
(T,G)-A,L	- Poly (L-Tyr, L-Glu)-poly (D,L-Ala)-poly (L-Lys)
Thy 1	- Theta antigen
TNP	- Trinitrophenyl
TNP-KLH	- Trinitrophenyl-keyhole limpet hemocyanin
TNP-SAV	- Trinitrophenyl-Streptococcus A vaccine
UV	- Ultra-violet
VSV	- Vesicular stomatitis virus

A B S T R A C T

Increasing evidence has suggested that cell-mediated immunity (CMI) is an important component of the host defence mechanisms against influenza virus infection. The work presented in this thesis clearly demonstrated that there are many components in the CMI responses to murine influenza virus infection and these involve at least four functionally distinct T cell subsets. Some act as effector cells {e.g. cytotoxic T cells (Tc) and T cells that mediate delayed-type hypersensitivity reactions (Td)} whereas others have regulatory functions {e.g. helper (Th) and suppressor (Ts) T cells}.

The kinetics of sensitization and elicitation of DTH in mice to both infectious and non-infectious preparations of influenza virus were found to be similar to that of some protein antigens and to other viruses. Sensitization was achieved without added adjuvant. Maximum DTH was elicited in the mouse footpad 6 days after primary in vivo sensitization. Adoptive transfer experiments showed that the DTH responses were mediated by T cells. DTH in mice sensitized with non-infectious virus was elicited only by virus which shared haemagglutinin specificity with the sensitizing virus whereas those sensitized with infectious influenza A virus could be elicited by any A strain viruses, including some not sharing haemagglutinin or neuraminidase specificity.

Cells recovered from infected mouse lungs and secondary effector cells generated in vitro transferred DTH if injected directly into the footpads with the eliciting virus. It was found that there are two classes of T lymphocytes which can mediate DTH to influenza virus. Thus, if non-infectious virus was used to sensitize for or to elicit a DTH response, the effector cells were found to be Lyt $1^{+}2^{-}3^{-}$ and I region restricted. If infectious

virus was used to sensitize for and to elicit the reaction, a second set of DTH effector cells was also detected which were Lyt $1^-2^+3^+$ and K,D region restricted. This class of DTH effector cells also possessed cytotoxic activity and attempts to distinguish them from the cytotoxic T cells with respect to inductive requirements, specificity and surface markers failed.

A DTH response to influenza virus could also be generated in athymic BALB/c nude (nu/nu) mice if Concanavalin A-activated spleen cell supernatant was injected into the mice in addition to the sensitizing virus. The response showed similar kinetics and specificity as seen in normal mice. The DTH effector cells were found to be Thy 1^+ and showed H-2 restriction, suggesting that nude mice do contain precursor Td cells that can be activated under appropriate conditions.

Effector T cells which mediate DTH reactions to influenza virus could be generated in tissue cultures using normal mouse spleen cells as the responder population. Both Lyt 1^+, I region restricted and Lyt $2,3^+$ K,D region restricted Td cells were generated. Negative selection experiments with anti-Lyt antibodies and complement suggested that the precursor Td cells were mostly likely to be Lyt $1^+2^+3^+$. Depleting the responder and stimulator cell populations of phagocytic and plastic adherent cells resulted in a failure to produce Td cells, which showed a requirement for macrophage-like cells as accessory cells in the primary in vitro generation of Td cells.

Injection of mice with infectious or non-infectious preparations of influenza virus induced the early formation of T cells which, when added to primary cultures of normal spleen cells exposed to influenza virus, enhanced the generation of primary effector T cells which mediate DTH reactions. The enhancing cells (Th) were found to possess Thy 1 and Lyt 1 surface antigens, were radioresistant and antigen-specific. The activity

of these Th cells was found to be IA subregion restricted and this was shown to operate at the level of stimulator cells so that the delivery of help to the responder cells was not H-2 restricted.

Cell preparations that contain only I region restricted DTH activity but with negligible K,D region restricted cytotoxic and DTH activities, when adoptively transferred to influenza virus-infected syngeneic recipients, failed to protect the mice but mice so treated had a higher mortality rate. This increase in mortality was not observed if the infected recipients and the donors of the transferred cells were K,D region compatible and I region incompatible. The cellular infiltration which occurred in the lungs of virus-infected mice, as estimated by a radioisotopic procedure, was increased by transferring cells with only I region restricted DTH activity and was decreased by transferring cells with only K,D region restricted cytotoxic and DTH activities.

Mice injected with UV-inactivated influenza virus generated suppressor cells in the spleens 5 days after virus injection which, upon adoptive transfer, could inhibit Tc cell formation if transferred within 24 h of injection of infectious virus. The specificity pattern of suppression observed was cross-reactive within the A strains of influenza virus. The activity of the cells mediating suppression was destroyed by monospecific anti-Thy 1.2 antibody and complement.

Natural killer (NK) activity in normal mice could be greatly augmented by injection of either infectious or non-infectious preparations of influenza virus. After intravenous injection of infectious virus, peak NK activity was found in the spleens 2 days after virus injection and the activity rapidly dropped to low levels by day 4-5. In contrast, intranasal inoculation of mice with a lethal dose of virus resulted in the early appearance (2-3 days) of NK activity in the lungs and the level remained high until the death of the mice ensued.

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CHAPTER 1

GENERAL INTRODUCTION:
A LITERATURE REVIEW
AND
AIMS AND SCOPES OF THIS THESIS

PREFACE

Despite several decades of extensive research, influenza still remains one of the major infectious diseases in man. It is well-known that influenza is basically an unvarying disease caused by a varying virus. Recent research has shown that cell-mediated immunity is an important component of the host defence mechanisms against influenza virus infection. The work presented in this thesis was an attempt to study the properties and roles of different T cell subsets in response to murine influenza virus infection. This introductory chapter is a literature review which aims at providing a background knowledge for the work done in this thesis. It has been broadly divided into 4 main sections. Section 1 presents a current view about the host defence mechanisms, both immunologically specific and nonspecific, against viral infections in man and animals. Section 2 specifically looks at the immune responses to influenza virus, mainly in man and the mouse model, with particular emphasis on the cell-mediated immune responses. Since a large proportion of the work reported in this thesis has been devoted to the understanding of the delayed-type hypersensitivity (DTH) responses to influenza virus in mice, therefore, a brief review of DTH reactions in general is given in Section 3. The last section primarily deals with the heterogeneity of effector T lymphocytes and finally the influence of the major histocompatibility complex on T cell activation and functions is presented. Because of the vast literature in these areas, this introductory chapter is neither comphrensive nor referenced in details. Only those areas that are most relevant to the work done in this thesis will be highlighted.

1. DEFENCE MECHANISMS AGAINST VIRAL INFECTIONS

Viruses are a large group of intracellular parasites which cause a number of acute and chronic infectious diseases in plants, animals and man. Viral infections may be classified as either acute, chronic, congenital, latent or persistent. It is therefore not surprising that vertebrates have evolved a multitude of defence mechanisms, operative at many levels, to cope with invading viruses. The body's defence mechanisms may be immunologically specific or nonspecific for the invading organisms. They range from simple physical barriers to sophisticated, complex interactions among different cell types of the immune system. Disease manifestation may result not only from a failure of primary or secondary defence mechanisms but also in some cases from the immune reactions of the host to cope with the viruses.

Extensive studies have revealed a great complexity of host defences against viral infections. This complexity arises from the multiplicity of the host defence mechanisms and their interactions with one another. Multiplicity of the host defence mechanisms become necessary in view of the great diversity of viruses, hosts, routes of infection, body compartments or cell types that viruses may infect as well as different mechanisms of viral multiplication and mode of spread of infections. The presence of multiple defences helps to explain why impairment of one or a few mechanisms does not entirely abrogate host's resistance to certain viral infections. Most of the present knowledge concerning defence mechanisms of the host against viral infections has been derived from studies in tissue cultures and experimental animal models, although some valuable information has come from studies in man.

In many experimental models of viral diseases, it is quite clear that two or more host defence mechanisms cooperate to terminate the infection whereas in some other models, one component of the immune responses appears

to be more important. In the following sections, individual components of the host defence system will be discussed separately. However, it should be remembered that in most natural infections, there are interactions among different mechanisms which resulted in the overall resistance of the host to the viral infections. Moreover, it is well-known that many types of physiological parameters such as hormonal levels, body temperature, nutritional status, concurrent infections and age etc. will undoubtedly affect the susceptibility of the host to and/or recovery from viral infections. They do so by influencing viral multiplication, the immune responses of the host or the production of interferon. Furthermore, there is a good deal of evidence which suggests that genetic factors in the host may influence the susceptibility or resistance to viral infections. All these factors have been described in detail elsewhere (Fenner et al., 1974; Mims, 1976; Bang, 1978).

1.1 Nonspecific mechanisms

1.1.1 Defence of the mucosal surface

The physical barrier presented by the intact skin or mucous membrane is the first barrier and is often the critical determinant in viral infections. Mucus or other secretions from mucous surfaces can have antiviral activity. The inhibitory activity may be merely an effect of pH. Viruses such as rhinoviruses and influenza virus are rapidly inactivated at low pH levels, e.g. in the stomach. Consequently these acid-labile viruses cannot infect the small intestine as do the more acid-stable enteroviruses, adenoviruses and reoviruses (Fenner et al., 1974). Apart from acids, the secretions may contain other materials inhibitory to viruses. For example, viral inhibitors have been detected in homogenates of lungs and intestinal mucosa (Wasserman, 1968) and the bile (Lee & Hanson, 1975).

1.1.2 Macrophages in resistance to viral infections

Another major component of the host nonspecific defence mechanisms against viral infections is the mononuclear phagocytic system. This comprises circulating blood monocytes, macrophages in various tissues, macrophages lining the sinusoids in the liver and spleen as well as those monitoring serous spaces such as pleural and peritoneal cavities. Macrophages with their nonspecific phagocytic activity, their strategic location at the portal entry of many viruses, their wide distribution in most organs of the body, can play an important role in viral infections and represent an important barrier to the establishment and dissemination of many viral infections (reviewed by Allison, 1974a; Mogensen, 1979). After inoculation by various routes into mice, viruses are generally taken up by macrophages in local tissues or in the liver (Mims, 1964; Allison, 1974b). In many cases, permissive replication of viruses does not take place in macrophages of the adult host (Allison, 1974b) so these cells may prevent spread of viruses from the primary sites of infection to more susceptible cells, such as those of the liver parenchyma or brain. However, viruses such as ectromelia virus (Roberts, 1964), yellow fever virus (Tiggert et al., 1960), infectious canine hepatitis virus (Fenner et al., 1974), vaccinia virus (Koszinowski et al., 1975) and human vesicular stomatitis virus (VSV) (Edelman & Wheelock, 1967) can replicate in normal macrophages and the viral progeny escapes to infect nearby susceptible tissues. In these cases, phagocytosis and subsequent ingestion of viral particles by macrophages not only fail to prevent the spread of virus but facilitate the establishment of the infection. A number of other viruses such as lymphocytic choriomeningitis virus(LCM) (Mims & Subrahmanyan, 1966), lactic dehydrogenase-elevating virus (Evans, 1970) and Aleutian mink disease virus (Porter et al., 1969) which can cause persistent viral infections are also capable of replicating in the host macrophages. Although permissive replication of murine cytomegalovirus

(MCMV) has not been observed in infected macrophages, however, the infected cells can aid the transmission of infectious virus to other susceptible cells and thus persistent infection can be achieved by presenting the virus with the macrophages (Booss, 1980). The importance of macrophages in limiting viral infections is highlighted by the following observations. Firstly, treatment of mice with agents that impair macrophage functions (e.g. anti-macrophage serum or certain chemical agents such as silica and carrageenan) prior to infection with Coxsackie B3 virus, yellow fever virus or herpes simplex virus (HSV) (Burns & Allison, 1975; Mogensen, 1979) resulted in an increase in virus titres in target organs and enhanced the mortality of the infected adult mice. Similarly, mice treated with silica had greatly reduced resistance to subsequent infection with ectromelia (i.c.) or Kunjin virus (s.c.) and death may result within 5 days after infection (Hapel, 1975). Secondly, the ability of a virus strain or virus type to replicate in macrophages from an animal correlates with the virulence of that particular virus strain or type for the host. This has been demonstrated with different strains of Newcastle disease virus (NDV), ectromelia virus, mouse hepatitis virus (MHV), LCM and HSV viruses (Mogensen, 1979). Thirdly, genetically determined macrophage-dependent resistance to viral infections in mice is known to occur for a large number of viruses such as flaviviruses, MHV type 2 & 3, HSV type 2, MCMV and influenza A virus (Bang, 1978; Mogensen, 1979). Fourthly, age-related resistance of mice to viral infections has been attributed to the maturation of macrophages during the first few weeks of life. The nature of the maturation process that enables macrophages from adult mice to resist virus infections is not well understood. For example, in herpes simplex virus infection of mice, neonatal but not adult animals were susceptible to infection (Johnson, 1964). This is because HSV undergoes an incomplete replication cycle in adult macrophages and fails to produce

infectious progeny (Stevens & Cook, 1971). Hirsch et al.(1970) showed that resistance to HSV infection could be transferred from resistant adults to suckling animals with syngeneic peritoneal macrophages. A similar age-determined increase in resistance of macrophages to viral replication was also demonstrated in mice infected with CMV (Selgrade & Osborn, 1974) or yellow fever virus (Zisman et al., 1971).

There are many ways in which macrophages can contribute to the resistance of the host to viral infections. It has been shown that macrophages which become activated in vivo during the course of a viral infection can gain an enhanced capacity to control a viral infection in vitro, in comparison with unstimulated macrophages (Koszinowski et al., 1975; Schleupner et al., 1979). Macrophages are also reported to act as effector cells in antibody-dependent cytolysis (ADCC) of virus-infected cells (MacFarlan et al., 1977; Kohl et al. 1979). Experiments with Sindbis (McFarland et al., 1972) and ectromelia (Blanden, 1974) viruses suggest the recruitment of mononuclear cells, including macrophages, at the sites of viral infection is an important component of the host defence system and may help in the recovery of the host from the infections. Furthermore, macrophages can produce interferons both systemically and at the foci of infections (De Maeyer & De Maeyer-Guignard, 1979), thus limiting virus replication. It is also well documented that macrophages are one of the major cell types that participate in the inflammatory response. Although the specific contribution of macrophages is difficult to identify in the inflammatory response, the general environment at the site of inflammation (including a low pH level, a decrease in oxygen tension and the production of proteolytic enzymes) tends to adversely affect virus replication (Baron, 1973).

Unlike mononuclear phagocytes, the role of other phagocytic cells such as the polymorphonuclear leukocytes (PMN) in the resistance to viral infections in the host has not yet been investigated thoroughly (Notkins et al.,

1970). These cells do not continuously monitor the tissue and body fluids but they may accumulate in large numbers at the foci of inflammation. In vitro studies have shown that PMN can ingest viral particles and either destroy them or inhibit virus replication (Mims, 1972). However, there is as yet no evidence that these cells play a prominant role in the antiviral defence mechanisms of the body.

1.1.3 Interferon and natural killer cells

Interferon, a product of mammalian cells, was first discovered by Isaacs & Lindenmann in 1957 (Isaacs & Lindenmann, 1957). Interferons are a heterogenous, complex group of glycoproteins (molecular weight: 15,000-100,000 daltons) that have several antiviral and non-antiviral properties (Gresser, 1977). The induction and properties of interferons and their mechanisms of action have been a subject of many recent reviews (Ho & Armstrong, 1975; Clemens & Tyrrell, 1977; De Maeyer & De Maeyer-Guignard, 1979) and will not be deal with here. Only the possible role of interferons in resistance to viral infections and their influence on natural killer (NK) activity during viral infections will be discussed.

Early studies in vitro have established that interferons can inhibit the replication of many viruses including cytopathic and tumorigenic RNA and DNA viruses (DeClercq & Stewart, 1973; Friedman, 1977). Interferon causes the induction of a second antiviral protein which inhibits the transcription and/or translation of viral RNA (Clemens & Tyrrell, 1977; De Maeyer & De Maeyer-Guignard, 1979). The early production of interferons during viral infections together with their broad spectrum of antiviral activity would suggest that these substances may represent an important nonspecific first line defence mechanism during viral infections. Evidence has recently been obtained which shows that interferons can play a role in some natural viral infections in vivo. Indirect evidence which suggests

an effect of interferons on *in vivo* viral infections was obtained from studies which showed that (1) a temporal relationship existed between the appearance of interferons in viral infections and the progress of the diseases (Sonnenfeld & Merigan, 1979). It was reported that a decrease in the production of interferons was associated with an increase in the severity of a viral infection in experimental animals and led to impaired recovery (Friedman & Rabson, 1964; Chang & Rasmussen, 1965), and (2) treatment of animals or human volunteers infected with different viruses with exogenous interferons or interferon inducers often resulted in less severe infections. This is particularly so in the case of infections by togavirus, picornavirus, herpesvirus and hepatitis virus (Sonnenfeld & Merigan, 1979). However, the most direct evidence for a role of interferons in natural viral infections has come from murine studies involving the use of an anti-interferon immunoglobulin preparation. It has been shown that application of anti-interferon globulin to mice infected with different viruses such as Semliki forest virus, encephalomyocarditis virus, HSV type 1, VSV, NDV, MHV type 3 or Moloney sarcoma virus increased the severity of the disease and indicates that endogenous interferons are important for controlling the early spread and replication of the virus (Sonnenfeld & Merigan, 1979). The action of interferon may simply be due to its antiviral effect on virus replication or due to its non-antiviral effects such as inhibition of cell growth and differentiation, expression of cell surface antigens or the interaction with the immune system which indirectly affect the host resistance to viral infections (Gresser, 1977; Sonnenfeld & Merigan, 1979; De Maeyer & De Maeyer-Guignard, 1979). It is well-known that interferon can activate macrophages both *in vivo* and *in vitro* and this may indirectly limit viral infections by causing an enhancement of the phagocytosis of both virus and virus-infected cells (Donahoe & Huang, 1976; Schultz *et al.*, 1978;

Degré & Rollag, 1979; Hamburg et al., 1980). Moreover, pretreatment of L cells with interferons enhanced their cytolysis by virus-specific cytotoxic T cells (Sethi & Brandis, 1978).

Treatment of human lymphocytes with interferons or interferon inducers greatly increases nonspecific natural killer activity (Santoli et al., 1978; Trinchieri & Santoli, 1978). Similarly, a significant enhancement of of NK cell activity has also been observed in mice injected with different types of viruses or interferon inducers (Herberman et al., 1977; Macfarlan et al.,1977)and it seems likely that this effect is mediated through the production of interferon. Very little is known about the role of NK cells in host resistance to viral infections. It has been proposed that interferon could limit viral infection indirectly by activating nonspecific NK activity which would kill the virus-infected cells and at the same time interferon could also protect other uninfected cells from nonspecific lysis (Santoli & Koprowski, 1979; Trinchieri et al., 1981).

1.1.4 The complement system

Complement is a complex group of proteins in the blood which form an important part of an animal's defence mechanisms against pathogenic microorganisms, notably bacteria and viruses. In association with antibodies, it destroys foreign cells, stimulates opsonization and generates local inflammation. Complement activation can be achieved by two independent mechanisms, namely, the classical and the alternative pathways (reviewed by Fothergill & Anderson, 1978). Complement can assist antibodies in the neutralization of viruses by coating, aggregating or lysis of viral particles (Daniels, 1975). Complement deposition on antibody-aggregated viral particles may facilitate opsonization of the particles by phagocytic cells bearing C3b receptors. Moreover, complement in the presence of antibodies may also directly lyse a variety of viruses that have lipid envelopes

(Sissons & Oldstone, 1980). In addition, some viruses such as VSV and retroviruses are neutralized by complement alone in the complete absence of antibodies (Mills & Cooper, 1978; Cooper, 1979). The participation of complement in resistance to viral infections *in vivo* is still unclear. Decomplementation of mice has been shown to increase the severity of influenzal pneumonia (Hicks *et al.*, 1978) and of Sindbis virus encephalitis (Hirsch *et al.*, 1980)but such treatment protected mice from encephalitis due to LCM virus infection (Oldstone & Dixon, 1975). More recent work in human system shows that virus-infected cells may be lysed by antibody and complement *in vitro* and that this lysis is dependent on the alternative complement pathway (Sissons & Oldstone, 1980). It was suggested that in the measles system and possibly for other viruses, virus-infected cells themselves actually activate the alternative pathway independent of antibody, but the presence of divalent IgG antibodies is necessary for lysis to occur (Sissons & Oldstone, 1980).

1.2 Specific mechanisms

1.2.1 Humoral immunity

Most viral infections induce humoral antibody production. The sequence of appearance of circulating antibodies are IgM, followed by IgG and IgA antibodies (Ogra *et al.*, 1975; Burns & Allison, 1975). IgM production usually occurs for a short period of time whereas IgG production persists for much longer period of time. If viral infection is initiated at the mucosal surface, secretory IgA antibodies are also produced, which can be quite independent of the systemic antibody response. The production of specific antibodies to many viruses is known to be T cell dependent (Burns *et al.*, 1975).

The role of antibodies in mediating host defence against viral infections aims at the prevention of initial infection as well as termination of a primary infection, thereby limiting the spread of virus and prevention of

reinfection. These can be achieved either by neutralization of virus or lysis of virus-infected cells (Sissons & Oldstone, 1980). The extent to which antibodies may contribute to the recovery from primary infection varies considerably for different viruses. In many cases, the antibodies are produced too late to be effective and other host defence mechanisms such as cell-mediated immunity or interferon can be shown to be more important. Also the method of virus spread is important. If the virus spread by extracellular routes (e.g. Togaviruses, Polioviruses and Coxsackieviruses) , then further infection can be minimized by the neutralizing properties of the antiviral antibodies. Other viruses, such as herpes simplex virus and rabies virus , which often pass directly from one cell to another, are much less vulnerable to neutralization by antibodies and very high levels of antibodies may be required to minimize the further spread of the viruses (Kaplan et al., 1975; Baron et al., 1976).

The initial step in the neutralization of virus by antibodies is the attachment of antibodies to the virion. If the amount of antibody bound to the virion is small or if the antibody is not attached to the 'critical' sites, the result may not be neutralization but the formation of infectious virus-antibody complexes (Daniels, 1975). Antibodies may limit virus infection by aggregating viral particles and thereby reducing the number of infectious particles or by covering the surface of the virion thereby interfering with the normal process of attachment, penetration or uncoating of the virus so that potentially susceptible cells are not infected. Furthermore, antibodies may attach to viral antigens on the surface of infected cells and thereby allow complement-mediated cytolysis.

Apart from neutralization of virus, antibodies can act in a number of other ways which indirectly help to protect the host from infection. Firstly, antibodies may act to trigger the recruitment of inflammatory cells to virus-infected loci in solid tissues; these cells can mediate

phagocytosis and subsequently cause effective destruction of virus (Berger,1980). Secondly, antibodies can 'arm' macrophages which may then specifically attack virus-infected cells (Zisman et al., 1971; Rager-Zisman & Bloom, 1974; MacFarlan et al., 1977). Thirdly, mononuclear cells known as 'K' cells have an Fc receptor for antibody that allows them to bind antibodies directed against infected cells (Shore et al., 1976; Rager-Zisman & Allison, 1976; Greenberg et al., 1977). It has been reported that this mechanism of cell lysis is many times more efficient than complement-mediated antibody-dependent cell lysis (Shore et al., 1976). However, antibody may not always have a protective effect. For example, by covering antigens on the cell surface, antibody can impair sensitized T cells from destruction of cells infected with VSV, Sindbis virus or Bebaru virus (Hale et al., 1978; McFarland, 1974;Mullbacher et al., 1979) Finally, the antibody response may cause disease manifestation by formation of antibody-virus immune complex, whose deposition can cause complications such as vascular damage and nephritis (Oldstone & Dixon, 1975).

1.2.2 Cell-mediated immunity

The available evidence strongly suggests that cell-mediated immunity (CMI) plays a central role in host defence against many viral infections (Woodruff & Woodruff, 1975; Bloom & Rager-Zisman, 1975). The CMI response is a complex phenomenon involving many specific and nonspecific components. Although it is generally recognized that sensitized T cells are the predominant effector cells of the CMI response, other cell types such as K cells and macrophages may also play a part. Specifically sensitized T cells are often the early manifestation of the specific host defence mechanism against viral infections. They can damage and lyse virus-infected cells directly or secrete soluble mediators of cellular immunity called lymphokines. Sensitized T cells have been detected as early as 2-3 days

after antigen stimulation, generally reach peak activity between 5-7 days and then decline markedly at about 2 weeks after infection. Sometimes this type of activity can still be detected several months after the initial infection. Sensitized T cells may be detected in the circulation, in lymphoid tissues and at the local site of viral infection (Gadol et al., 1974; Blanden, 1977). They can differentiate into memory cells which under certain conditions can be restimulated both in vivo and in vitro (Burns & Allison, 1975; Bloom & Rager-Zisman, 1975; Blanden, 1977). The mechanisms by which CMI responses control viral infections are not fully understood and at least four mechanisms are thought to operate in vivo. These are (1) direct killing of virus-infected cells; (2) activation of macrophages; (3) recruitment of mononuclear cells into the sites of infection and (4) production of immune interferon. Each of these mechanisms will be briefly described as follows.

Specifically sensitized T cells can directly lyse virus-infected cells in vitro, provided viral antigens are expressed on the surface of the infected cells. It is thought that the virus-infected cells are recognized as non-self and thus destroyed in a manner analogous to the current ideas about allograft rejection. The time of appearance of viral antigens on the surface of infected cells is sufficiently early in the growth cycle of many viruses to permit immune destruction before viral progeny can be produced and liberated (Jackson et al., 1976; Zinkernagel & Althage, 1977). Generation of virus-specific cytotoxic T cells has been demonstrated in a large number of viral infections (Zinkernagel & Doherty, 1979). The lysis of virus-infected cells by cytotoxic T cells requires sharing of the K,D region of the H-2 gene complex between donors of effector cells and the target cells (Zinkernagel & Doherty, 1974a). Adoptive transfer of cell preparations with cytotoxic T cell activity has shown to protect mice in a number of viral infections such as LCM (Zinkernagel & Welsh, 1976), ectromelia (Kees & Blanden, 1977), influenza (Yap et al., 1978), HSV (Howes et al., 1979) and MCMV (Ho, 1980), thus suggesting that

the cytotoxic T cells may play a significant role *in vivo* in the recovery of host from primary infections.

An alternative mechanism for the lysis of virus-infected cells which has been shown to occur *in vitro* is demonstrated by cells which bear Fc receptors and which are able to lyse antibody-coated virus-infected target cells in the absence of complement. This antibody-dependent cell-mediated cytotoxicity (ADCC) was shown to be prominent in the human system but is more difficult to detect in mouse (Sissons & Oldstone, 1980). The effector cells which could mediate ADCC include monocytes-macrophages, 'K' cells and polymorphonuclear leukocytes (Kohl *et al*., 1979). ADCC was shown to operate against mumps (Härfast *et al*., 1975), HSV type 1 & 2 (Ramshaw, 1975; Shore *et al*., 1976; Kohl *et al*., 1979), vaccinia (Perrin *et al*., 1977b) and measles (Perrin *et al*., 1977a; Kreth & ter Meulen, 1977) virus-infected cells. Unlike T cell mediated cytolysis, ADCC is not H-2 restricted, i.e., histocompatibility between killer cells and target cells is not required for lysis to occur. Recent *in vivo* work suggests an important role for macrophage-mediated ADCC in the prevention of HSV dissemination in the mouse (Rager-Zisman & Allison, 1976; Oakes & Rosemond- Hornbeak, 1978), but the *in vivo* significance of ADCC in control of human viral infections has not been elucidated.

Macrophages may be activated to attack virus and virus-infected cells not only by antibody but also by sensitized T cells (Bloom & Rager-Zisman, 1975; Allison, 1974a). It has been demonstrated that *in vitro*, normal macrophages can be activated by sensitized T cells with the result that the yield of Venezuelan equine encephalitis virus in cultured mouse L cells was reduced (Rabinowitz & Procter, 1974). Similarly, although Sindbis virus-infected cells were destroyed by sensitized T cells alone, the virus yield was not decreased unless macrophages were added (McFarland, 1974). Although the mechanism of activation of macrophages by T cells is not clear, it is possible that the activation can be mediated by immune interferon or by a

macrophage activation factor, products that are known to be secreted by sensitized T cells (Wallen et al., 1973; Schultz et al., 1978; Degre & Rollag, 1979).

Another CMI mechanism to control viral infections is the recruitment of blood monocytes to the foci of infection following specific antigen stimulation of the sensitized T cells. On contact with the viral antigens or virus-infected cells, the sensitized T cells produce a number of soluble mediators (lymphokines) (Dumonde et al., 1969). These mediators may function by recruiting mononuclear phagocytic cells for the participation in the immune and inflammatory responses (e.g. transfer factor, chemotactic factors and blastogenic factors), by retaining inflammatory cells within infected tissues (migration inhibitory factor) and by activating macrophages (macrophage activation factor and immune interferon). Once activated, macrophages may take part in the control of and clearance of the virus from the foci of infection. The ability of sensitized T cells to attract and recruit blood monocytes to the foci of viral infection has been well documented in vivo using the ectromelia system (Blanden, 1974) and such T cell-mediated recruitment of blood monocytes to foci of infection is clearly important in the recovery of mice from ectromelia infection.

Finally, a more efficient and less damaging CMI mechanism of defence would be the release by sensitized cells of factors capable of inhibiting viral replication without destroying the infected cells or tissues. Immune interferons appear to play such a role. Their secretion by virus-immune lymphocytes is immunologically specific, although their action is nonspecific. Secretion of immune interferon by sensitized lymphocytes is known to occur in response to many viral infections. Examples are infection with vaccinia virus (Epstein et al, 1972), HSV (Rasmussen et al., 1974), Rubella virus (Buimorici-Klein et al., 1977) or MCMV (Rytel & Hooks, 1977).

In conclusion, although cellular immunity is clearly a major host defence mechanism against many viral infections, the precise manner by which it operates in vivo is far from being fully understood. In any experimental model, it seems

TABLE 1

Relative Importance of Humoral Versus CMI Responses in Recovery from Various Viral Infections in Man and Mouse[a]

System	Treatment / Immunodeficiency	Impairment of immune response		Higher susceptibility to the viral diseases (or infection aggravated)
		Humoral	CMI	
Mouse	Anti-thymocyte serum	-	+	Vaccinia
Mouse	Anti-thymocyte serum	-	+	Ectromelia
Mouse	Anti-thymocyte serum	N.D.[b]	+	Herpes Simplex
Mouse	Neonatal thymectomy	N.D.	+	Herpes Simplex
Mouse	Cyclophosphamide	+	N.D.	Coxsackie B3
Man	Bruton-type agammaglobulinemia (congenital)	+	-	Poliomyelitis; Coxsackie B3
Man	Nezelof's syndrome (congenital)	-	+	Vaccinia
Man	Cartilage hair hypoplasia (congenital)	-	+	Varicella-Zoster
Man	Wiscott-Aldrich syndrome (congenital)	Normal IgG, IgA lowered IgM	+	Measles; Varicella-Zoster; Herpes Simplex; Cytomegalovirus
Man	Hodgkin's disease	-	+	Herpes Simplex; Cytomegalovirus; Varicella-Zoster
Man	Leukemia	-	+	Measles; Hepatitis; Cytomegalovirus

a Modified from Bloom & Rager-Zisman, 1975; Fenner et al., 1974.

b N.D.: Not determined.

that multiple factors may operate together or in parallel or as complementary components in the ultimate host defence against the infections.

1.2.3 Evaluation of the relative importance of humoral immunity versus cell-mediated immunity in viral infections

The relative importance of specific cellular and humoral immune responses in the host defence against viral infections is determined by the modes of replication and spread of virus. Generally speaking, antibody is effective in controlling viral infections in which the virus spreads by extracellular routes so that viraemia occurs. In contrast, CMI is more likely to play an important role in the recovery from viral infections in which the virus passes from cell to cell so that viraemia is limited or does not occur and in those situations where infection causes antigenic modifications of the surface of infected cells (Burns & Allison, 1977; Blanden et al., 1977b). Studies of viral infections in experimental animals and patients with congenital or acquired immunodeficiency and those undergoing immunosuppressive therapy have yielded valuable information on the relative role of CMI versus humoral immunity in controlling viral infections.

Humans with impaired T cell functions frequently have an increased susceptibility to certain viral infections such as vaccinia, measles, cytomegalovirus, herpes simplex and varicella-zoster. Patients with hypogammaglobulinemia recover normally from these infections but are more prone to poliovirus infection (Table 1). Studies in experimental animals have shown that impairment of CMI functions by treatment of mice with anti-lymphocyte or anti-thymocyte serum or by neonatal thymectomy can markedly increase the susceptibility of mice to infections with viruses such as ectromelia, vaccinia or herpes simplex whereas similar treatments have no obvious effect on the host susceptibility to enteroviruses such as yellow fever or Coxsackie viruses (Table 1).

TABLE 2

Protective effect of T cells in Viral Infections

Virus	Group	Immune cell transfer which conferred protection[a]	Reference
Ectromelia (Mousepox)	Poxvirus	Day 6 ISC[b]	Blanden, 1971
Venezuelan equine encephalomyelitis (VEE)	Togavirus (Alphavirus)	Day 7-13 ISC	Rabinowitz & Adler, 1973
Lymphocytic choriomeningitis (LCM)	Arenavirus	Day 6 or 8 ISC	Johnson & Cole, 1975
Bebaru	Togavirus (Alphavirus)	Day 5 or 6 ISC	Hapel, 1975
Herpes simplex (HSV)	Herpesvirus	Day 6 ISC	Rager-Zisman & Allison, 1976
Murine cytomegalovirus (MCMV)	Herpesvirus	Day 6 ISC	Starr & Allison, 1977
Influenza	Orthomyxovirus	Day 6 ISC	Yap & Ada, 1978b
Banzi virus	Togavirus (Flavivirus)	Day 5 ISC	Jacoby et al.,1980

a The immune cells which conferred protection were shown to be T cell dependent.

b ISC: immune spleen cells.

More direct evidence which suggests that T or B cells may play a more decisive role in recovery from viral infections has been derived from studies using adoptive or passive transfer of immune cells or sera. The protective effect of passively transferred virus-specific antibodies in immunosuppressed mice has conclusively shown that an adequate humoral response is critical for recovery during infections with yellow fever, West-nile and Coxsackie viruses (Zisman et al., 1971; Rager-Zisman & Allison, 1973). Thus, it is clear that humoral antibodies are important in protection of host against infections with certain groups of viruses such as enteroviruses and arboviruses. On the other hand, adoptive transfer of immune T cells but not immune sera has been shown to protect mice in a large number of viral infections (Table 2), thus strongly suggests that cellular immunity can play an important role in defence of the host against many viral infections.

In summary, by interfering with the early stages of virus invasion, multiplication and spread to susceptible organs, the nonspecific host defence mechanisms may be important in determining the outcome of a viral infection. Final recovery from a fully established infection, however, is probably determined by the specific immune responses, involving cell-mediated and humoral immunity, which appear some days after the initiation of infection. Complex interactions between the various specific and nonspecific host defence mechanisms undoubtedly occur in vivo and an imbalance in these systems can lead to defective elimination of virus which leads to disease manifestation and/or subsequent persistence of a viral infection.

2. INFLUENZA VIRUS AS A MODEL FOR THE STUDY OF IMMUNE RESPONSES TO ACUTE VIRUS INFECTIONS

2.1 The viruses

Influenza viruses belong to the group Orthomyxovirus. They can be classified into types A, B and C on the basis of serological relatedness of their internal proteins. Influenza A virus has been studied in great detail as it infects many species of birds, domestic animals and man.

Influenza A and B viruses contain eight RNA segments which code for eight virus-specific proteins (seven are structural components of the virus and the other non-structural protein is demonstrable in the nucleolus of infected cells) and that Influenza C virus contains at least 4 RNA segments (Palese et al., 1978; Scholtissek, 1978; Barry & Mahy, 1979). The structure of influenza A virus is characterized by spikes of glycoproteins that project from a lipid membrane and consist of the rod-shaped haemagglutinin (HA) which is present as trimers and the mushroom-shaped neuraminidase (NA) which is present as tetramers. The haemagglutinin is responsible for the adsorption of the virus to the host cell surface, via neuraminic acid receptors. The neuraminidase, on the other hand, is an enzyme that cleaves neuraminic acids from the cell surface, thereby permitting the release of virus particles from the surface of infected cells and enables subsequent spread of infectious virus particles. Recent evidence suggests that penetration of the cell by some strains of influenza virus involves a fusion mechanism and neuraminidase seems to play an essential role in such a process (Huang et al., 1980). Under the lipid membrane of the virus is a layer of matrix (M) protein that surrounds the core of the virus particle. The nonglycosylated M protein is the most abundant protein of the virus (about 40% of total virus proteins) and is thought to play a role in virus assembly by attaching to plasma membrane bearing viral glycoproteins, thereby providing a binding site for the ribonucleoprotein during viral assembly. In addition, the M protein was suggested to be responsible for stabilizing the virion

(Robertson et al., 1979). The core of the virion is made up of 8 RNA segments, each of which is coated with nonglycosylated nucleoprotein (NP) to form helical ribonucleoprotein (RNP) complex. Three other large internal proteins, called polymerase-associated (P1, P2, P3) proteins, are believed to be associated with the RNP complex. It was suggested that the P proteins and the nucleoproteins are involved in the transcription and replication of viral RNAs (Barry & Mahy, 1979).

The internal antigens of the virion (NP, M & P proteins) appear to be antigenically stable and are common to all influenza virus subtypes, although minor differences in antigenicity and in their amino acid sequences have been identified by peptide mapping (Kilbourne, 1978). In contrast, the viral surface glycoproteins, HA and NA, are subjected to antigenic variations. Two types of changes have been recognized. Minor changes in the amino acid sequence of the surface glycoproteins, known as 'antigenic drifts', are thought to result from spontaneous point mutations during virus replication followed by selection of mutants in a partially immune host population. Major changes called 'antigenic shifts', are due to radical changes of the antigenic properties of the surface glycoproteins, leading to emergence of new pandemic strains in which the population has no or little specific immunity against them. Whether these viruses arise by genetic reassortments between human and animal strains of influenza virus, or by re-emergence of a previous prevalent influenza virus into human population, or by some other mechanisms is not known (Laver & Webster, 1979). Both kinds of variation occur with influenza A virus, whereas with influenza B virus, only antigenic drift has been observed. Two antigenic shifts have occurred to the HA and one to the NA of human influenza A virus. The HA are classified as H0 (H1), H2 and H3 and the NA as N1, N2 so that an A strain may be designated as, for example, H0N1, H2N2 and H3N2 etc..

2.2 The animal models

The immune response to influenza A virus infection is more easily analyzed in experimental animal models than in human infections. Several animal species, including mouse (Fazekas de St. Groth, 1950), ferret (Potter et al., 1972), guinea pig (Wetherbee, 1973) and hamster (Jennings et al., 1976) have been commonly used as experimental animal models for studying human influenza virus infection and the mechanisms of immunity against the disease. Probably the experimental animal most suited for the study of human influenza is the ferret, since this animal suffers an infection which is clinically similar to that seen in man (Potter et al., 1972) and develops demonstrable humoral and cellular immune responses following infection or immunization with influenza virus. However, ferrets are expensive to maintain and have a cellular immune response which is distinct from that of man (Potter et al., 1974). In addition, the lack of inbred strains and well-characterized reagents for lymphocyte surface markers together with the problems of handling large numbers of ferrets make these animals impractical for extensive studies. Guinea pigs, on the other hand, provide a good model for the study of localized upper respiratory tract infection (Phair et al., 1979). The skin test reaction against influenza virus is histologically similar to those seen in man and distinct from those of ferrets. The cellular immune response persists as in man (Habershon et al., 1973) whereas those of hamsters and ferrets are relatively short-lived. However, guinea pigs require a much larger dose of virus to establish infection than the ferrets or hamsters (Phair et al., 1979). Moreover, infection does not induce a febrile reaction in guinea pigs and no local antibody could be found in nasal washings. Therefore, guinea pig is not a good model for the study of the humoral response and pathogenicity of human influenza virus infection. The mouse is the most widely used animal for experimental studies of influenza virus infection. It has certain advantages over other animal models.

The ease of breeding, the availability of large numbers of well-defined inbred strains, the well-established procedures for cell separation and characterization, the precise information about genetic control of immune responses and detailed knowledge about immunoglobulin classes and subclasses together with extensive knowledge of mouse biology make this animal well-suited for the study of the host immune response to influenza virus. It is known that influenza virus infection in man is normally confined to the upper respiratory tract whereas in mice the lungs are more involved. Fazekas de St. Groth (1950) pointed out that the difference in the disease patterns between man and mouse was quantitative rather than qualitative. Although viral pneumonia is the most common result of murine influenza virus infection, the work of Iida & Bang (1963) showed that an influenza virus infection could also be restricted to the nasal epithelium if small volume of inoculum was given to mice while they were awake, thereby more closely resembling the upper respiratory infection as seen in man. Moreover, influenzal pneumonia does occur in man and it shares many similarities with the disease observed in mice (Mulder & Hers, 1972).

2.3 Nonspecific factors in resistance to influenza virus infection

A number of nonspecific factors are involved in the resistance of the host to influenza virus infection. These include the action of a mucociliary blanket, the possible inhibitory action of surface commensals in the respiratory tract, the presence of nonspecific virus inhibitors such as α, β and γ mucoproteins in mucus, serum and tissue fluids, genetic factors which affect the resistance, the actions of macrophages and interferons etc. (Sweet & Smith, 1980). Lindenmann *et al.* (1978) have shown that some strains of mice (e.g. A2G) which are homozygous or heterozygous for the dominant gene Mx, are resistant to influenza A virus administered by any route. Resistance was found to be independent of T and B cells but expressed at the level of

macrophages. *In vitro* studies have shown that the genetic basis of resistance is due to the selective resistance of macrophages but not of other cell types to infection with influenza virus (Lindenmann *et al.*, 1978). The injection of resistant mice with a small amount of anti-interferon globulin rendered the mice more susceptible to influenza virus infection so that death could occur (Haller *et al.*, 1979a). This indicates that interferons are important in the resistance to influenza virus which is controlled by the gene Mx. How interferons cooperate with the gene Mx to create a resistant state that is highly specific for a group of closely related viruses is far from clear. More recently, work done with radiation chimeras indicated that *in vivo* resistance followed the genotype of the recipient host and was independent of the Mx gene expression in cells of the haemopoietic system (Haller *et al.*,1979b). Since mice are a very unlikely host for influenza virus in nature, the evolutionary meaning of genetic resistance to the virus is unknown. On the other hand, evidence for an innate resistance to influenza virus infection in man has been conflicting. One unresolved issue is whether susceptibility to influenza virus infection is linked to blood groups or HLA types (Potter & Oxford, 1979).

The contribution of macrophages to the host defence against influenza virus infection is not well-defined. Activated macrophages have been recovered from lungs of influenza virus-infected mice (Wyde & Cate, 1979). *In vitro* studies have demonstrated that active replication of influenza virus does not take place in macrophages, yet they are capable of spreading the infection by having virus attaching onto their surfaces (Wells *et al.*, 1978). The mechanism by which influenza virus attaches to and its release from macrophage resulting in infection of respiratory epithelial tissues is unknown. Moreover, immunization of mice with Bacillus-Calmette-Guerin (BCG) or *Staphylococcus aureus* not only rendered their macrophages more resistant to influenza virus infection *in vitro* but also conferred protection of the animals against subsequent infection with influenza virus (Shayegani *et al.*, 1974; Spencer *et al.*, 1977).

How this nonspecific protection was achieved is unknown but the nonspecifically activated macrophages may play a potential role.

A decisive role for interferon in primary murine influenza virus infection has not yet been established. Gresser et al.(1976) have shown that administration of a potent anti-interferon serum did not alter the course of influenza virus infection in mice whereas Suzuki et al.(1975, 1977) found that injection of dextran phosphate (DP40) or 9-methylstreptrimide (9-MS) which are potent interferon inducers , can protect mice from subsequent challenge with influenza virus. In the former case, the absence of a detectable effect of anti-interferon antibody may be related to the failure of the antibody molecules to reach the critical site of infection in effective concentration, and does not necessarily imply that interferon is unimportant in the recovery of mice from influenza virus infection. In the latter case, the protective effect of DP40 or 9-MS against influenza virus infection in mice might be due to its enhancement of interferon production in the lungs of mice , the nonspecific activation of natural killer cells or might be due to some other unknown effect of the drugs on the host function.

Finally, it was shown that an intact complement pathway is important in recovery of mice from influenza virus infection (Hicks et al., 1978). Though decomplemented mice or congenitally C5 deficient mice have similar antibody and cytotoxic T cell responses, they demonstrated more severe pulmonary damages and have a higher morbidity and mortality rate than the control mice. It was suggested that complement may play an accessory role in the host recovery from primary influenza virus infection, perhaps by chemotaxis of mononuclear cells into the site of infection or by complement-mediated lysis of infected cells coated with antiviral antibodies (Hicks et al, 1978; Verbonitz et al., 1978).

2.4 Role of antibody

It is generally believed that humoral immunity is a major defence mechanism of the host against influenza virus infection. Natural infection or vaccination with influenza virus results in the formation of serum haemagglutination-inhibition (HI) antibodies. The HI antibodies can neutralize virus *in vitro* and inhibit virus release from infected cells (Dowdle *et al.*, 1974). Serum anti-haemagglutinin (anti-HA) antibody has clearly been shown to protect mice from death due to influenza (Virelizier, 1975; Yap & Ada, 1979). Immunization of mice with purified neuraminidase or passive transfer of anti-neuraminidase (anti-NA) antibody resulted in reduction of virus titres in lungs and also reduced the size of lung lesions (Schulman *et al.*, 1968). Unlike anti-HA antibodies, anti-NA antibodies generally do not prevent infection but do modify the disease when given in sufficiently large amounts. Thus, resistance to infection is more closely associated with anti-HA than anti-NA antibody levels. Although the transfer of antibodies to the external antigens provided some protection, the transfer of large amounts of either anti-matrix (anti-M) or anti-nucleoprotein (anti-NP) antibodies did not protect mice against influenza virus infection (Virelizier *et al.*, 1976). Webster & Hinshaw (1977) have reported that mice immunized with purified M protein cleared virus more rapidly from the lungs than did the controls, though they were not protected against live virus challenge. In man, it was reported that the level of anti-M antibody reflected the clinical severity of infection in volunteers inoculated with live H3N2 influenza virus (Creteson *et al.*, 1978). The significance of anti-M antibody in influenza virus infection in human remains unclear, however.

The relative role of secretory IgA antibody and serum IgG antibody in protective immunity against influenza virus infection has not yet been resolved and is controversial. It has been argued that serum antibody is of primarily importance in protection against influenza virus infection in man

and animals (Schulman et al., 1968; Hobson et al., 1972; Virelizier, 1975). Moreover, the observation by Aho et al.(1976) that a correlation between resistance to influenza virus and pre-epidemic HI antibody titre also existed in IgA deficient subjects strongly favours a role for serum HI antibody as an important determinant against influenza virus infection. In contrast, other studies have shown that in mice, ferrets and man, protection against influenza appears to be more closely related to secretory than serum antibodies (Fazekas de St. Groth & Donnelley, 1950; Shore et al., 1972; Freestone et al., 1972). Similarly, no pronounced correlation between the level of circulating antibody and immunity to infection with influenza virus was found in many other studies (Shvartsman & Zykov, 1976). Studies in ferrets by Small et al.(1976) also indicated that serum antibody did not prevent infection, although it minimized the severity of the disease. The work of Ramphal et al.(1979) suggested that the relative role of serum or secretory antibody depends on the primary site of infection. In mice it was found that serum antibody was protective against severe pulmonary parenchymal disease but not for disease of the ciliated epithelium in the upper respiratory tract.

In summary, the current evidence suggests that the antibody response is the major determinant in providing protection of the host from reinfection with the homologous virus. The extent to which antibody may contribute to the host's recovery from a primary infection is unclear. A recent report suggests that a major role of humoral antibody is to limit infection by the virus, and in this aspect it complements the action of cytotoxic T cells (Yap & Ada, 1979).

2.5 Cell-mediated immunity

2.5.1 Evidence for CMI response to influenza virus infection

The CMI response to influenza virus infection has been demonstrated in man and experimental animals using a variety of assays such as the delayed cutaneous response and the macrophage migration inhibition test (Habershon, 1973; Feinstone *et al.*, 1969), *in vitro* lymphocyte transformation assays (Ruben *et al.*, 1973; Hellman *et al.*, 1972) and lymphocyte-mediated cytotoxicity assays (Greenberg *et al.*, 1977; Cambridge *et al.*, 1976). Until early 1978, the time when the work reported in this thesis began, there were only few reports on the DTH response to influenza virus. The nature of the effector cells involved, the role of these cells in the recovery process and the pathogenesis of the disease had not been investigated. Habershon *et al.* (1973) have studied the DTH response to influenza virus in man using the skin test. They found that the DTH response did not correlate with the presence of serum HI antibody level. Lymphocyte transformation *in vitro* has been used frequently as a measure of the CMI response to influenza virus. However, the assay itself does not provide a measure of the effector cell function nor indicates any antiviral mechanism and hence is of doubtful value in elucidating the role of the CMI response to influenza virus infection. ADCC to influenza virus-infected cells has been demonstrated by Greenberg *et al.*(1977). They found that peripheral blood lymphocytes (PBL) obtained from volunteers who had no known exposure to either inactivated or live influenza virus for at least two months had cytotoxic activity to xenogeneic or allogeneic influenza virus-infected target cells. The PBL were shown to have small amounts of anti-HA antibodies associated on their surface which can only be detected by radioimmunoassay. The effector cells involved were shown to be non-adherent, non-phagocytic, Fc-receptor bearing cells which are characteristics of 'K' cells. Such ADCC for influenza virus-infected cells has not been reported in the mouse system and there is a lack

of experimental evidence on the protective effect of ADCC *in vivo*.

One aspect of the CMI response to influenza virus infection which has studied extensively is the induction of cytotoxic T cell response. Mice infected intranasally or injected parenterally with infectious influenza virus generate virus-specific cytotoxic T cells (Tc) which kill infected, H-2 compatible target cells *in vitro* (Doherty *et al*., 1977; Ennis *et al*., 1977a; Yap & Ada, 1977). Memory Tc cells were found following primary infection and may persist for at least one year after initial infection (Webster & Askonas, 1980). Potent secondary Tc cell preparations were obtained if the memory Tc cells were restimulated *in vitro* or in irradiated mice (Yap & Ada, 1977; 1979). Injection of anti-HA antibody prior to virus infection abolished the ability of the mice to generate a primary or a secondary Tc cell response whereas injection of anti-NA antibody had only a minor suppressive effect (Yap & Ada, 1979). Tc cells can be recovered from various organs of mice such as spleens, lungs,draining lymph nodes or from the peripheral blood (Yap & Ada, 1977; 1978a; Ennis *et al*., 1978; Bennink *et al*. 1978) during influenza virus infection. There is disagreement about the specificity of the cytotoxic T cell killing of influenza virus-infected cells. Most investigators found that the lysis of target cells is virus type-specific, but cross-reactivity in the killing of target cells infected with any type A influenza viruses has also been observed (Braciale, 1977a; Effros *et al*., 1977; Zweerink *et al*., 1977a). In contrast, there are two reports that the killing is haemagglutinin-specific (i.e. subtype-specific) (Cambridge *et al*., 1976; Ennis *et al*., 1977a). The more recent demonstration of a clone of T cells which kills target cells infected with different A strains of influenza virus (Lin & Askonas, 1980; Braciale *et al*., 1981) and parallel findings of cross-reactivity of influenza virus-specific Tc cells in humans (McMichael & Askonas, 1978; Biddison *et al*., 1979) show beyond any doubt that there are at least two populations of Tc cells to

influenza virus, a major subpopulation which is highly cross-reactive within the A strains and a minor subpopulation which is subtype-specific. The precise nature of the antigen that is recognized by the cross-reactive Tc cells in the surface of virus-infected cells is not completely resolved. The type-specific matrix protein was at first thought to be a likely candidate as the target antigen that is recognized by the cross-reactive Tc cells (Ada & Yap, 1977; Braciale, 1977b; Biddison et al., 1977; Reiss & Schulman, 1980). However, this possibility now appears much less likely as more recent work using monoclonal antibodies against matrix protein (Hackett et al., 1980; Askonas & Webster, 1980) or reconstitution of influenza virus surface antigens into liposomes (Koszinowski et al., 1980) argues against a role for matrix protein but suggests that cross-reactive Tc cells recognize a common antigenic determinant(s) on the haemagglutinin molecule of influenza virus. The question of Tc cell induction by inactivated influenza virus has also been controversial. Braciale & Yap (1978) did not observe any significant cytotoxicity in the spleens of mice after injection of UV-inactivated (non-infectious) influenza virus. Furthermore, the UV-inactivated virus elicited only a subtype-specific Tc response in a secondary in vitro restimulation of memory spleen cells from mice primed with infectious virus. In contrast, subtype specific Tc cells were described by Ennis et al.(1977b) after injection of inactivated influenza virus vaccine into mice. Most recent work in this laboratory has shown that the difference observed between various groups may be quantitative rather than qualitative (see Chapter 9).

Cytotoxic T cells specific for influenza A virus can also be generated from human peripheral blood lymphocytes in vitro (McMichael & Askonas, 1978). After an induction period of 4-14 days, cytotoxic cells which lyse autologous influenza virus-infected lymphoid cells could be demonstrated. This is probably a secondary immune response as all volunteers tested had been exposed to a natural infection(s) with influenza A virus. Memory for

influenza virus-specific Tc cells was boosted by immunization with killed whole virus vaccines but not with subunit vaccines (McMichael et al., personal communication). Attempts to demonstrate T cell-mediated cytotoxicity in freshly prepared peripheral blood of vaccine recipients without in vitro culture were largely unsuccessful (McMichael et al., personal communication) Parallel to observations in mice, the human influenza virus-induced Tc cells are type-specific but not subtype specific (McMichael & Askonas, 1978; Biddison et al., 1979). Cross-reactivity at the level of priming has also been observed (McMichael, 1980). The killing process is HLA restricted, normally sharing of either HLA-A or HLA-B locus is sufficient for lysis to occur, HLA-Dr or HLA-C coded antigens are not involved (McMichael, 1978). It was also found that the antigen recognized by virus-immune T cells can be distinguished from the serologically defined HLA-A and B antigenic determinants and there may be multiple self determinants on individual HLA-A molecules that T cells can recognize in conjunction with virus (Biddison et al., 1980).

2.5.2 Role of the CMI response to influenza virus infection

Early attempts to demonstrate a role for the CMI response in recovery of mice from primary influenza virus infection gave conflicting results. Reports from several laboratories suggested that the CMI response to murine influenza virus infection may be either unimportant, beneficial, detrimental or a combination of both, to the host. Hirsch & Murphy (1968) showed that pretreatment of mice with ATS had no effect on the progress of the disease or the mortality rate so that the CMI response seemed to be unimportant under those conditions. Similarly, Virelizier et al. (1976) suggested that the humoral immunity rather than cellular immunity was important in protection against influenza A virus infection in mice, as adoptive transfer of immune spleen cells (taken from mice primed 2 months before with influenza virus) was poorly protective against a lethal challenge

with A/PR8 virus. The immune spleen cells were only fully protective when they were secreting antibody. A protective role for the CMI response was suggested by Hurd & Heath (1975) who treated mice with cyclophosphamide (Cy) and found that this converted a nonfatal influenza virus infection into a fatal one. Based on the observation that Cy treatment prevented a cellular infiltrate in the lungs of influenza virus-infected mice so that a fatal infection ensued, these authors concluded that CMI may be important in the recovery of mice from influenza virus infection. In contrast, other studies suggested that the CMI response may contribute significantly to the pathogenesis of influenza disease. For example, transfer of immune spleen cells can increase the mortality of mice subsequently challenged with influenza virus (Kurimura et al., 1973; Cate & Mold, 1975). Moreover, using a highly mouse-adapted influenza virus strain to infect mice, Wyde et al. (1977) found that nude mice exhibited an increase in survival time and manifested minimal cellular infiltration and no tissue damage in the lungs when compared to normal littermates. They concluded that T cells play a significant role in the inflammatory response to influenza virus infection. In contrast to the finding of Hurd & Heath (1975), an earlier report by Singer et al.(1972) showed that Cy treatment decreased the mortality of treated mice during the first week of infection compared to control mice. The Cy treated mice also had higher lung virus titres which persisted longer with lower antibody and interferon levels and a decreased cellular infiltrate in the lungs. The finding that a decrease in cellular infiltration in the lungs was associated with a decrease in mortality and an increase in lung virus titre suggested that a CMI response had both an antiviral and an immunopathological effect. Consistent with such an interpretation was the work of Suzuki et al.(1974) who found that the administration of ALS to mice infected with an A2 strain of influenza virus not only increased the survival rate but also reduced the development

of lung consolidation without any apparent effect on lung virus titre or the humoral antibody response. In fact, the lung virus titre was higher later in the infection. A report by Sullivan et al.(1976) also suggested that T cells may have both beneficial and harmful effects on the host. These investigators showed that nude mice did not eliminate virus as did the controls and virus could persist up to 2-3 weeks in the lungs after infection, yet the nude mice died somewhat later than the normal littermates. Thus, T cells may be responsible for limiting the dissemination of influenza virus in the lungs but at the same time they may also contribute to the pathology of the disease. These conflicting reports did not establish whether or not the CMI response had a definitive role during murine influenza virus infection.

More clear-cut and convincing evidence for a role of T cells in the recovery of mice from influenza virus infection came from the experiments of Yap & Ada (1978b) who showed that adoptive transfer of specific immune T cells not only protected mice from death following inoculation of mice with a lethal dose of virus but also significantly reduced the virus titres in the lungs. Additional experiments indicated that the responsible T cell subset in the transferred cell population had the characteristics of cytotoxic T cells, i.e. they were Lyt $2,3^{+}$ and K,D region restricted (Yap et al., 1978). Further studies with athymic nude mice indicated that if a low dose (e.g. $10^{3}EID_{50}$) of influenza virus was administered, T cells were not necessary for survival although their presence was an advantage. However, in more severe infections, T cells were essential for survival and they facilitated the clearance of virus from the infected lungs (Yap et al., 1979). More recently, Lin & Askonas (1980) have demonstrated that a cloned T killer cell line can lyse target cells infected with different A strains of influenza virus. Adoptive transfer of these cloned cells into irradiated mice 24 h after intranasal infection

with influenza A virus was found to protect the recipient mice from an otherwise lethal infection.

Thus the results so far suggest that Tc cells are important in the recovery of mice from primary influenza virus infection. The possibility that B cells or other T cell subsets (e.g. Th, Td, Ts) might also be involved in the recovery process has not been ruled out. This was because conditions were deliberately chosen which optimized the production of Tc activity. The same conditions might not be necessarily optimal for the generation of other cell types. Therefore, the relative importance of other T cell subsets in the defence of the host against influenza virus infection has yet to be determined.

2.6 Heterotypic immunity

Earlier work by Schulman et al.(1965) had shown that previous infection of mice with an A strain influenza virus could protect them from subsequent infection with another A strain virus differing in both HA and NA serological specificity. This phenomenon, known as 'heterotypic immunity', was not observed when the primary immunization was done with inactivated virus and even with live virus, heterotypic immunity did not take place between type A and B influenza virus. The mechanisms involved was not completely resolved but recent studies (Floc'h & Werner, 1978; Yap & Ada, 1978c; Webster & Askonas, 1980) have indicated that heterotypic immunity is more related to T cell function rather than to antibody response. Floc'h & Werner (1978) showed that the heterotypic immunity observed in mice was abolished by ATS but not by Cy treatment and they suggested that T cells rather than B cells were involved in this phenomenon. Yap & Ada (1978c), on the other hand, showed that Tc cells raised by a type A influenza virus was able to express significant antiviral activity against recipient mice infected with another type A influenza virus sharing neither HA nor NA

serologically specificities with the first virus. They also found that mice preprimed with one subtype of influenza A virus and rechallenged with a different subtype which shared neither HA nor NA antigenic specifities with the first virus had enhanced cross-reactive Tc activity in the lungs compared with the unprimed mice. These results altogether strongly suggested that cross-reactive Tc cells are responsible for the cross-protection observed. Similarly, Webster & Askonas (1980) also found a correlation between cross-protection from infection with different type A influenza virus with the production of high levels of memory Tc cells, again indicating that Tc cells may be involved in heterotypic immunity.

Heterotypic immunity has also been described in ferrets (Yetter et al., 1980) but convincing evidence in man is still lacking. A report by Hoskins et al.(1976) showed that children that were naturally infected with influenza A/England were better protected against subsequent infection with antigenically distant strain A/Port Chalmers 18 months later compared to those who were vaccinated just before the first outbreak and who had similar antibody titres at the time of the second epidemic. The significance of heterotypic immunity in human influenza virus infection is unclear. It appears to play a role in the recovery from but not in the prevention of reinfection and perhaps can account for the progressively lower clinical attack cases observed with increasing age (Kilbourne, 1978).

3. DELAYED-TYPE HYPERSENSITIVITY

3.1 Historical development

Probably the first description of a delayed-type hypersensitivity (DTH) reaction was reported by Edward Jenner in 1798. He noticed that vaccines injected into the skin of people with prior smallpox infection or vaccination caused an inflammatory reaction. Robert Koch in 1890 described certain hypersensitivity phenomena following the subcutaneous injection of tuberculin. The concept of hypersensitivity, in which an immunological response can lead to tissue damage, was introduced by Portier & Richet (1902). In 1906, von Pirquet coined the term 'allergy' which was used to describe the state of altered reactivity that occurs after exposure of human beings or animals to antigenic stimuli (von Pirquet, 1906). 'Delayed-type hypersensitivity' is a name originally applied by Zinsser (1921) to describe the erythematous, indurated reactions elicited in specifically sensitized subjects by intracutaneous challenge with bacterial antigens. The allergic reactions of the host to simple chemical compounds, known as 'contact sensitivity', was studied by Landsteiner & Chase in early 1940s. They showed that passive transfer of contact sensitivity to picryl chloride in naive guinea pigs could be achieved using peritoneal exudate cells obtained from sensitized donors (Landsteiner & Chase, 1942). Their experiments were considered as a landmark in the development of the understanding of DTH reactions. Chase (1945) further showed that passive transfer of tuberculin reaction by lymphoid cells and this suggested that bacterial allergy and contact sensitivity might involve the same mechanism. The relationship between DTH reactions and skin allograft rejection was studied extensively by Medawar and his colleagues in the late 1950s. Following the demonstration by Miller (1961) that skin allograft rejection failed to take place in neonatally thymectomized animals, it became clear that DTH and related phenomena depend on the integrity of the thymus in

neonatal life. By the 1970s it had become well established that DTH reaction is a T cell dependent immune phenomenon which is manifestated as an inflammatory response at the site of antigen deposition. When sensitized T cells are stimulated by a second contact with the antigen, soluble substances called lymphokines are released from the sensitized cells and these cause a cellular infiltration to the site of antigen injection. The DTH reaction usually reaches its peak activity 24-48 h after antigen challenge.

3.2 Classification of DTH responses

In general, three types of DTH reactions can be distinguished in man and animals. They are the classical (tuberculin-type) delayed hypersensitivity, the contact sensitivity and the Jones-Mote type hypersensitivity. The induction of these three types of DTH responses depends on multiple factors such as the dose and route of immunization, the immunogenicity of the sensitizing antigen and the use of adjuvants etc. (Godfrey & Gell, 1978).

3.2.1 Classical (tuberculin-type) delayed hypersensitivity

The classical type of DTH is readily induced by immunization with foreign protein antigens incorporated into Freund's complete adjuvant (FCA) or by priming with microorganisms such as BCG (Dvorak et al., 1980). This type of response can be conveniently demonstrated by the use of the 'skin reaction test'. The skin reaction first appears 4-6 h after antigen injection as local areas of erythema which become progressively larger and the reaction is also characterized by induration. There is a parallel increase in microvascular permeability and a mononuclear cell infiltration with the development of erythema which reaches maximum intensity at 24 h. The mononuclear cellular infiltrate consists of mainly lymphocytes and monocytes in guinea pigs whereas in man there

are only few monocytes participate in the reaction (Dvorak et al., 1980). Basophil infiltration is uncommon in the classical DTH reactions. Other important features of the classical DTH reactions are the thickening of the basement membrane, variable amount of endothelial cell injury and substantial deposition of fibrin in the intervascular reticular dermis. It has been shown that fibrin deposits are a common feature of the classical DTH reactions seen in man and guinea pigs and they contribute to the induration of the skin (Dvorak et al., 1980). The ability of eliciting a classical DTH response in the host persists for a long time after sensitization. Moreover, these type of reactions can be transferred by immune cells but not by immune serum.

3.2.2 Contact sensitivity

A second form of DTH called contact sensitivity is seen after sensitization, usually by skin painting, with small highly reactive substances such as picryl chloride, 2-ethoxymethylene-5-oxazolone, dinitrofluorobenzene (DNFB) and dinitrochlorobenzene (DNCB) etc.. These low molecular weight (<1000) chemicals are strongly electrophilic and thus able to form covalent bonds with the sulfhydryl groups or amino groups of proteins present in the skin. These substances can sensitize man and animals in such a way as to produce a DTH response after a second contact. Contact sensitivity is also characterized by an intensive mononuclear cell infiltration. Induration is seen in man but rather seldom in guinea pigs (Dvorak et al., 1980). Like classical DTH, it also requires T cells for its induction and transfer. The main differences between contact sensitivity and classical DTH are the antigens used and the sites of sensitization. Moreover, the cellular infiltrate seen in contact sensitivity consists of numerous basophils and few monocytes.

3.2.3 Jones-Mote hypersensitivity

The Jones-Mote reaction was initially described by Jones & Mote (1934) in humans sensitized to foreign proteins in saline. The reaction is an evanescent form of DTH, resulting in an erythmatous lesion but no induration. A similar response can also be elicited in guinea pigs immunized with protein antigens administered in saline or in Freund's incomplete adjuvant (Nelson, 1964). A number of features of Jones-Mote hypersensitivity suggest that it is a delayed allergic reaction. The reaction can be evoked prior to the detection of circulating antibodies. It can be passively transferred with lymphoid cells but not with serum from sensitized donor. The skin reaction is similar to tuberculin reaction -- primarily erythema with little or no edema or necrosis. The major difference between the Jones-Mote and tuberculin reactions is the evanescent character of the former. Moreover, the cellular infiltrate seen in Jones-Mote hypersensitivity usually contains many basophils. Therefore this type of response has sometimes been called cutaneous basophil hypersensitivity (CBH).

Since the discovery of basophils in DTH reactions (Richerson et al., 1970; Dvorak et al., 1970), a reclassification of DTH reactions into two separate subtypes known as the 'classical DTH' and 'CBH' was proposed by Dvorak and his co-workers. Thus, immunization with antigens in FCA results in tuberculin-type DTH reactions that are strongly indurated and lacking basophils whereas animals immunized with antigens in the absence of FCA also have lymphocyte-mediated delayed reactions which are less indurated, more transient and contain large amounts of basophils in the cellular infiltrates and are therefore called CBH. Basophil-containing delayed-onset reactions have been most extensively studied in guinea pigs in response to a variety of immunogens including contact allergens, vaccinia virus, allogeneic tumor cells, insect bites and skin allografts

(Dvorak et al., 1980). In humans, basophils have also been demonstrated to be present in Jones-Mote type skin reactions and in contact sensitivity reactions (Askenase, 1977). Basophils have not been noted in DTH responses in the ears, the flanks or the footpads of mice as these cells are exceedingly rare in mice and none has been detected in the blood or bone marrow of mice (Dvorak & Dvorak, 1974). In recent years, numerous findings have rendered the distinctions between classical DTH and CBH less precise (Askenase, 1977). For example, many delayed basophil-containing reactions are not evanescent but long-lasting (Askenase, 1977; Dvorak et al., 1980) and some are hapten specific and can be transferred by serum antibodies (Askenase, 1973). Thus, it was recommended that the term 'CBH' should be used to denote the occurrence of basophils in delayed reactions bearing in mind that the presence of these cells may be due to antibodies, T cells and various regulatory factors (Askenase, 1977).

3.3 Measurement of DTH reactions in the mouse

3.3.1 In vivo methods

Many in vivo manifestations have been associated with the DTH responses, such as the disappearance of macrophages from the peritoneal cavity after antigen challenge, skin thickening, tissue damage and cell proliferation at the skin test site etc. (Ruddle, 1972). However, none of these can be measured in a precise and quantitative manner as has been done with antibody response. A measure of the intensity of DTH reaction in comparative studies must allow for the quantification of the inflammatory response at the site of antigen injection. Quantitative or semi-quantitative measurements of the DTH reactions in vivo can be readily achieved in guinea pigs and man by measuring the diameter of the skin test site or the amount of induration and these can be related with reasonable accuracy to the level of sensitization. In mice this approach

is unsatisfactory as mice usually do not produce a strong skin reaction and the results sometimes may be quite variable. There are two approaches widely used for the in vivo measurement of DTH response in mice. One measures the relative increase in footpad or ear thickness and the other measures the amount of radioactivity in the cellular infiltrate at the site of antigen injection.

The skin of many animal species such as mice and rats is not suitable for intradermal skin testing for DTH reaction. The skin in mice is very thin and is almost laminated in structure. Intradermal injections are usually unsatisfactory as the injected antigen may not persist long enough at the injection site to allow the reaction to occur. Antigens injected into the footpad or the ear remain localized in these vascular tissues which can be reached by lymphoid cells and immunoglobulins. The increase in thickness resulting from antigen injection in either the ear pinna or the footpad of sensitized mice can be measured conveniently by either a pair of calipers (Crowle, 1975), by the amount of a fluid that the swollen foot will displace in a plethysmograph (Uyeki et al., 1969) or by an electronic device (van Dijk et al., 1976). The test is simple to perform, usually does not require sophisticated equipments, and with sufficient practice and experience, the test can be made reasonably accurate and objective. This method has the additional advantages that the DTH reaction can be read at various intervals after antigen challenge and local adoptive transfer of cells is possible in the case of footpad swelling test. Therefore the footpad test is the most widely used method for measurement of DTH responses in mice (Crowle, 1975). However, the footpad test also has many disadvantages: (1) it does not indicate whether the swelling results from cellular infiltration or oedema and a histological examination of the reaction site is often desirable; (2) quantification is more difficult when one had to deal with minute increase in thickness of the footpad; and (3) large amounts of sensitized cells are required to demonstrate a significant activity in

adoptive transfer studies.

More recently, a second approach has been commonly used for the *in vivo* measurement of DTH response in the mouse. This method depends on the measurement of the infiltration of radioisotope-labelled cells to the site of antigen injection. It is based on the observation that macrophages or monocytes, one of the predominant cell types found in DTH lesions, are derived from a pool of rapidly replicating precursor cells in the bone marrow (Volkman & Gowans, 1965; Vadas *et al.*, 1975). These rapidly dividing cells can be labelled *in vivo* a few hours after antigen challenge by injection of I^{125}-5-iodo-2'-deoxyuridine (Vadas *et al.*, 1975) or tritiated thymidine (Sabolovic *et al.*, 1972). The amount of radioactivity localized in the site of antigen injection, usually the ear, is proportional to the intensity of the DTH reaction. Although this method is objective, sensitive and accurate, it also suffered from a number of drawbacks. Firstly, since the animals have to be killed to remove their ears for counting of the radioactivity, only single measurements are possible. Secondly, local transfer of cells into the ear cannot be achieved. Thirdly, with certain antigens such as viruses, the response may be so weak that borderline values may be obtained. Finally, the test is often associated with radiation hazards especially when large number of animals are used.

3.3.2 *In vitro* method

It is well established that sensitized lymphocytes react with specific antigens *in vitro* with the liberation of a large number of soluble mediators called lymphokines. The measurement of the *in vitro* activities of these mediators form the basis of a large number of *in vitro* methods for detecting DTH in the mouse. Perhaps the most widely used method is the migration inhibition test (George & Vaughan, 1962; Feinstone *et al.*, 1969;

Friedman et al., 1969). The capillary tube method for studying inhibition of macrophage migration in vitro has been shown to be a useful correlate of the in vivo DTH response in experimental animals and man (Bloom & Bennett, 1971). Sensitized lymphoid cells challenged with antigen in vitro produce a soluble factor, called migration inhibitory factor (MIF), and the effect of this lymphokine is measured by its capacity to inhibit migration of macrophages in vitro (Bloom & Bennett, 1971). In mice , the MIF test for DTH activity may or may not correlate with the in vivo test, depending partly on the mode of sensitization or the source of the sensitized cells (Crowle, 1975). In certain case, a dissociated development of the delayed footpad reactivity and the MIF activity was observed (Mitsuyama et al., 1980). Moreover, the MIF test usually does not give a quantitative dose response relationship.

Lymphocytes are transformed when they are restimulated with an antigen to which they have been sensitized. The lymphocyte transformation test is sometimes used as an in vitro correlation of DTH reactions in mice (Ruddle, 1972; Miller & Jones, 1973). The blastogenic response, usually measured by the tritiated thymidine incorporation, is best detected in mice by means of the mixed leucocyte culture (Crowle, 1975). It may represent an in vitro model for the initial events in the early phase of induction of DTH response. However, recent work by Moorhead (1978) has showed that in a single pool of DNFB immunized cells, Ia^- T cells are responsible for the transfer of DTH whereas Ia^+ T cells are involved in the in vitro proliferative response. Thus different cells may be involved in DTH reaction in vivo and proliferative response in vitro and also they may be regulated in different ways.

Other in vitro methods which have been reported to correlate with the in vivo DTH reaction include macrophage spreading inhibition (Fauve & Dekaris,

1968), macrophage aggregation test (Phillips et al., 1972), random monocyte migration test (Hawes et al., 1979) and cytotoxicity against innocent bystander cells (Ruddle, 1979). These methods have not been widely used for measurement of DTH response in mice.

3.4 DTH response to viral antigens

DTH reactions in man and animals have been known to occur in response to a large number of antigens, such as bacteria, viruses, fungi, protozoa, insect saliva antigens, foreign proteins and synthetic polypeptides (Turk, 1975). DTH response to a virus was first described by Jenner in 1798 as the 'reaction of immunity' in revaccinated subjects who had previous exposure to vaccinia virus. Since then, similar skin reactions have been reported in man in reponse to measles virus, mumps virus and herpes simplex virus (Turk, 1975). The first systematic study of the DTH response to a virus in mice was Fenner's work on the pathogenesis of mousepox infection (Fenner, 1948). Subsequently,the DTH response to a number of other viruses such as influenza and mumps (Feinstone et al., 1969), vaccinia (Ueda & Nozima, 1969), LCM (Tosolini & Mims, 1971) and murine leukemia virus (MLV) (Peters et al., 1975) have been reported in mice using either the in vivo footpad swelling test or the in vitro macrophage migration inhibition test. The maximum response which can be elicited usually occurs at day 5-10 after virus injection and declines thereafter. In most cases, cell transfer studies have not been carried out and the characteristics of the cells involved have not been elucidated. Little is known about the role of DTH in acute and chronic viral infections. In case of ectromelia virus infection, it was suggested that the skin hypersensitivity was not responsible for localizing virus in the skin and/or development of the rash, as the former appeared usually 10 days after virus infection whereas the latter developed on the sixth day after infection (Fenner, 1948). In contrast, Lawrence & Valentine (1970)

suggested that DTH may play a protective role in vaccinia virus infection in humans, as treatment of patients who had depressed cellular immunity with transfer factor had been shown to eradicate disseminated vaccinia. Peters et al.(1975), on the other hand, have found that MLV-immunized mice giving a DTH response also resisted the challenge with live leukemia virus. However, definitive evidence that DTH response may play a direct role in protection against leukemia virus infection in mice is still lacking. Part of the work reported in this thesis was an attempt to elucidate the characteristics and the role of the cells that take part in a DTH reaction during murine influenza virus infection. Since the work reported in this thesis was undertaken, a number of reports dealing with the DTH responses to many other viruses have been published. The significance of the findings in this thesis and of others' work in contributing to the understanding of DTH responses to viral antigens will be discussed in the final concluding chapter (Chapter 9).

3.5 Biological significance of DTH reactions

Despite the fact that DTH responses have been known to occur in immune responses to infectious agents, tumors, allografts and foreign proteins etc., the functional significance of the DTH reactions remains obscure. Whether they play a beneficial or destructive role to the host has not yet been completely resolved. It is well established that sensitized T cells can secrete a variety of lymphokines during a DTH reaction. Lymphokines such as macrophage activation factor and chemotactic factor can cause substantial activation and mobilization of macrophages into the target areas, such as infectious foci in liver or lungs, an allograft or a tumor mass. Thus, the DTH response may play a potential role as part of the host's defence mechanisms against foreign invasion.

The role of DTH response in bacterial infections has been studied for a long time. T cells are known to play a central role in immunity to facultative intracellular bacterial infections (North, 1973) and that there is a good correlation between the DTH response and the acquired cellular resistance to infections (Mackaness, 1971; Salvin & Neta, 1975). However, whether DTH contributes directly to the acquired cellular resistance is unclear (Salvin & Neta, 1975). It has been suggested that the inflammatory reactions of DTH play little or no role in the cellular immune reactions against organisms such as the tubercule bacilli (Turk, 1975; Youmans, 1975). In contrast, recent work of Kaufmann et al.(1979) have found that specific peritoneal exudate T cells with the Lyt 123^{+} phenotype are involved both in protection against Listeria monocytogenes and in DTH response to the listerial antigens. The protective role of DTH response in Listerial infection in mice was supported by the work of Mitsuyama et al.(1980) who found that there was an enhanced elimination of the bacteria at the site of specific delayed footpad reaction. Hsu et al.(1980) also suggested that DTH reaction can has a beneficial role in enhancing the host resistance to infection. They observed that DTH induced by tuberculin in BCG-vaccinated mice can provide, to a limited extent, an increase in resistance of the mice to a subsequent challenge with virulent Salmonella typhimurium. Such an increase in resistance was thought to be due to an accelerated influx of phagocytic cells into the site of infection (Hsu et al., 1980). On the other hand, Easmon & Glynn (1975) have found that DTH reactions did not protect mice against Staphylococcus aureus infections but could enhance the severity of subsequent staphylococcal lesions. Similarly, DTH also appears to play a role in the pathogenesis of certain granulomatous disease such as Schistosomiasis (Warren et al., 1967).

Although there are many studies concerning the possible role of the DTH response in the rejection of foreign or neoplastic cells, yet the results

so far obtained do not allow a definitive conclusion to be drawn and hence they are not discussed in here. Finally, DTH reactions have also thought to be involved in the manifestation of many experimental and clinical autoimmune diseases, although circulating antibodies could play a role in some cases (Turk, 1975).

CHROMOSOME 17 OF THE MOUSE

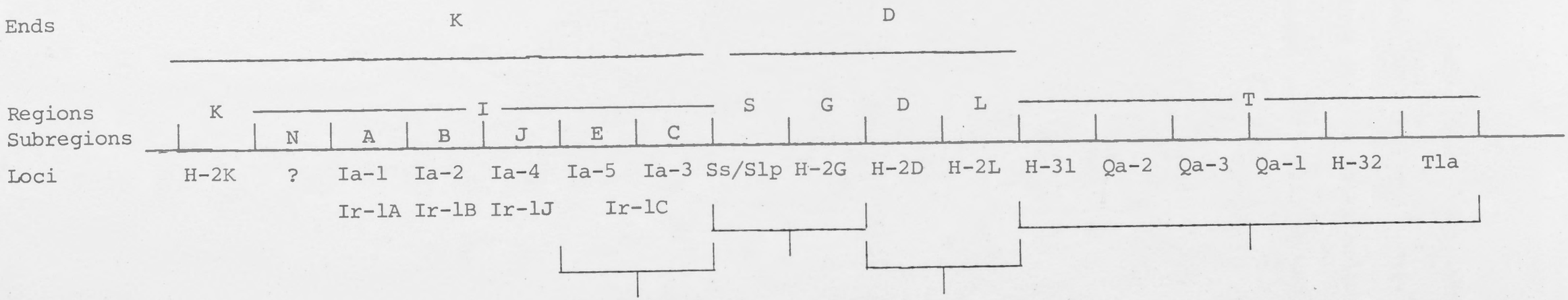


Fig. 1: A genetic map of the MHC complex in mouse (H-2 gene complex). (Modified from Klein, 1979).

The gene products of the loci are as follows:

H: Histocompatibility antigens; Ia: I-region-associated antigens; Ss: Serum serological;

Slp: Sex-limited protein; Qa: Q-region antigen; Tla: Thymus-leukemia antigen.

Brackets indicate that the order of loci within the bracket is uncertain, the arrangement shown here is arbitrary.

4. THE ROLE OF THE MAJOR HISTOCOMPATIBILITY COMPLEX ON T CELL ACTIVATION AND FUNCTIONS

4.1 The major histocompatibility complex of the mouse

The major histocompatibility complex (MHC), known as H-2 in the mouse or HLA in man, consists of a cluster of genetic loci that represent the strongest transplantation barrier and also are involved in the defence of the body against infections and tumors. To date, the MHC has been clearly defined in at least ten different animal species (Meruelo & Edidin, 1980). With the exception of the chicken, all are found in mammalian species. The occurrence of similar systems in amphibians suggests that MHC had its origin at a rather early stage in vertebrate evolutionary history (Frelinger & Shreffler, 1975). The H-2 gene complex of the mouse is located in the middle portion of chromosome 17. A current genetic map of the H-2 gene complex is shown in Fig. 1. It can be divided into six regions (K, I, S, G, D and T) with the I region further divided into six subregions (N, A, B, J, E and C). With the exception of the S region, the gene products of all other regions are expressed at the cell surface.

The K and D regions code for the major histocompatibility or transplantation antigens which are found on almost all cells with the highest density on lymphoid tissues such as the spleen and lymph nodes. These antigens are responsible for rapid allograft rejection (Meruelo & Edidin, 1980). The H-2K and H-2D antigens are serologically complex and genetically extremely polymorphic. They are membrane bound glycoproteins with molecular weight of about 45,000 daltons. Each H-2K or H-2D molecule is a single polypeptide chain with two carbohydrate side chains. The K and D molecules show a strong homology in their primary structures. They are associated noncovalently with a short polypeptide (m.w., 12,000 daltons) called β2-microglobulin (Klein, 1979). Recent work has demonstrated that the D region in fact codes for at least two glycoproteins, the H-2D and H-2L

molecules (Neauport-Sautes et al., 1977). The H-2L locus is also polymorphic and codes for products that are structurally and functionally very similar to those of H-2K and H-2D loci, therefore it was suggested that L, D and K molecules are three structurally homologous cell surface glycoproteins that might have evolved from a common primordial gene (Hansen et al., 1979).

The I region encodes for another set of cell surface antigens called I-region-associated (Ia) antigens. It is generally accepted that Ia antigens represent a family of glycoprotein molecules of molecular weight of about 58,000 daltons and that each Ia molecule consists of two subunits called α (m.w., 35,000-38,000 daltons) and β (m.w., 25,000-28,000 daltons) chains (Cullen et al., 1976). The antigenic determinants of these molecules appear to reside in their protein portion. Ia antigens have been demonstrated on a high proportion of B cells, certain subsets of T cells, sperms, epidermal cells, some macrophages and tumor cells but absence on fibroblasts, erythrocytes, brain, liver or kidneys (Hämmerling, 1976). Recent work in two laboratories has demonstrated the presence of Ia antigens in serum. Thus, Callahan et al.(1976) found Ia antigens associated with the high density serum lipoproteins. In contrast, Parish et al.(1976) reported that substantial amounts of glycolipid Ia antigens are present in normal mouse serum. These glycolipid Ia antigens appear to be secreted by T cells and their level in serum can be greatly increased after activation of T cells by antigens, mitogens or in graft-versus-host reactions (Parish & McKenzie, 1977). The work of Parish and his colleagues has suggested that a family of carbohydrate-defined Ia antigens exists both in man and mouse. These carbohydrate-defined antigens probably exist as glycolipids in serum and on cell surfaces and are distinct from the conventional protein-defined Ia antigens (Parish et al., 1978). The Ia antigens are thought to play a role in T cell activation, T cell-macrophage and

T cell-B cell interactions and antigen presentation. They are also found to be associated with several immunoregulatory molecules such as helper and suppressor factors. All these aspects have been reviewed elsewhere (Shreffler & David, 1975; Thomas et al., 1977; Benacerraf & Germain, 1978).

The I region genes are also involved in the regulation of immune responses (Benacerraf & Germain, 1978). At least four immune response (Ir) genes have been described. Three enhancing and one suppressing immune responses. The enhancing genes map in the IA, IB, IC/IE subregions and the suppressor gene maps in the IJ subregion (Klein, 1979). The relationship between the Ir gene products and Ia antigens is not fully understood and all attempts to find an Ir gene product distinct from the Ia antigens have been unsuccessful.

The S region gene codes for serum proteins Ss(serum serological) and Slp (sex-limited protein). It was thought that Slp protein may represent an allotype variant of the Ss protein. Both Ss and Slp proteins consist of three covalently linked polypeptides, called α, β and γ (Klein, 1979). These molecules represent the C4 component of the classical complement pathway. Very little is known about the gene products of other H-2 regions. It was suggested that the G region codes for genetic markers on erythrocytes whereas the Tla locus of the T region codes for antigens expressed in leukemia and some normal thymocytes.

Undoubtedly the MHC serves a diversity of biological functions. It has been shown to play a key role in a variety of immunological and physiological phenomena, including allograft rejection, genetic control of immune responses and complement activity, genetic association with susceptibility and/or resistance to diseases and genetic control of a large number of traits which are classified as non-immunological (reviewed by Klein, 1978 and Meruelo & Edidin, 1980).

4.2 Heterogeneity of T lymphocytes

Thymus-derived (T) lymphocytes play a central role in a wide range of immunological phenomena. It is well established that T cells are heterogeneous with regard to their physical properties, immune reactivities and expression of cell surface markers. Thus T cell subsets can be defined by their differential sensitivities to various chemical and physical manipulations such as treatment of their host with corticosteriods, cyclophosphamide, X-irradiation, anti-lymphocyte serum or adult thymectomy etc. (Katz, 1977). Moreover, physical procedures such as electrophoretic separation, buoyant density separation, sedimentation separation and fluorescence-activated cell sorting have also revealed a great deal of T cell heterogeneity, permitting the isolation and characterization of many discrete T cell subpopulations. Perhaps the most widely adopted criteria for classification of T cell subsets are based on their functional activities as well as the differential expression of surface antigenic markers, such as Lyt and Ia alloantigens.

Functionally T cells can be broadly divided into two main categories: those which act as effector cells and those which have regulatory functions. Effector T cells include those cells that participate in cytotoxicity reactions (Tc) as well as those mediating DTH responses (Td). T cells which can either enhance or suppress the effector cell functions are known as helper T cells (Th) and suppressor T cells (Ts) respectively. Lymphocyte differentiation (Lyt) antigens are useful markers of T cell subpopulations. Although many Lyt antigens are known to exist (McKenzie & Potter, 1979), the Lyt 1,2,3 antigens are the most useful. They are expressed almost exclusively on T cells and there are two alleles for each Lyt locus and in heterozygous animals these are expressed codominantly (Swain, 1980). Lyt 1 locus is situated on chromosome 19 whereas Lyt 2 and Lyt 3 loci are located on chromosome 6. The Lyt 2 and Lyt 3 loci are closely linked and have not

yet been separated by recombination. Studies with antisera specific for the Lyt 1,2,3 alloantigens have led to the identification of at least three classes of T cells, i.e., cells bearing the phenotype Lyt $1^+2^-3^-$, Lyt $1^-2^+3^+$ or Lyt $1^+2^+3^+$. In the thymus Lyt $1^+2^+3^+$ cells predominant whereas in the peripheral T cells there are 50-55% Lyt $1^+2^+3^+$ cells, 30-35% Lyt $1^+2^-3^-$ cells and 5-10% Lyt $1^-2^+3^+$ cells (McKenzie & Potter, 1979).

The Lyt antigens have been extremely useful in defining functionally distinct subpopulations of T cells. The current view is that helper T cells, proliferating T cells in MLC reactions and T cells mediating DTH reactions are Lyt $1^+2^-3^-$ whereas suppressor T cells and cytotoxic T cells are Lyt $1^-2^+3^+$ (Cantor & Boyse, 1977). The functions of Lyt $1^+2^+3^+$ are less well-defined and it was thought that they are the precursors for the Lyt 1^+ and Lyt $2,3^+$ cells. In addition, Lyt 1^+ cells seem to recognize primarily I region coded determinants, either as alloantigens or as self antigens in combination with foreign antigens. Lyt $2,3^+$ cells, on the other hand, recognize K or D region products in alloreactive response or foreign antigens in combination with K or D region products in syngeneic response (Nagy et al., 1976). However, more recent findings have shown that the original functional classification of T cell subsets based on the Lyt phenotypes is not entirely correct and needs modifications. Firstly, the use of more sensitive techniques such as monoclonal antibodies and quantitative immunofluorescence with a fluorescence-activated cell sorter have shown that virtually all T cells carry the Lyt 1 antigens, though quantitative differences do exist among T cell subsets (Hogarth et al., 1980; Ledbetter et al., 1980). Secondly, it was found that nonimmune Lyt 1^+ spleen cells can nonspecifically suppress IgE antibody responses in irradiated recipients (Watanabe et al., 1977). Moreover, suppressor cells for the induction of DTH responses to SRBC (Ramshaw et al., 1977) and influenza virus (Liew & Russell, 1980) were found to be Lyt 1^+. Thirdly, it has been established in several systems

that Lyt $1^+2^+3^+$ cells may be directly involved in either cytotoxicity, helper activity, suppression, DTH reactions or *in vitro* proliferative responses (Kaufmann *et al.*, 1979; McKenzie & Potter, 1979; Swain, 1980). Thus the evidence so far suggests that the Lyt phenotypes correlate better with the MHC regions which the T cells recognize,rather than the T cell functions, and no example has been found of an I region recognizing cell without Lyt 1 or of a K,D responsive cell without Lyt 2,3 (Swain, 1980).

Apart from the Lyt 1,2,3 antigens, the presence of other cell surface markers is also useful in delineating T cell subsets. For instance, cytotoxic T cells and DTH T cells are generally found to be Ia^- whereas suppressor T cells are Ia^+ (McKenzie & Potter, 1979). The presence or absence of Ia or Qa-1 antigenic determinants has provided a useful tool for dissecting heterogeneity within helper T cell subpopulations (Tada *et al.*, 1978; Cantor *et al.*, 1978). Furthermore, the Ly 7.2 alloantigen may also be useful as a marker of T cell subsets as it was found to be selectively expressed on Th cells but not on Tc or Ts cells in an *in vitro* allogeneic response (Pilarski *et al.*, 1980).

4.3 Influence of the MHC on T cell activation and functions

It is well documented that the MHC exerts a profound influence on the T cell reactivities. Thus, the gene products of MHC play a key role in controlling a variety of different T cell functions, including cell-mediated lympholysis (CML), DTH reactions, proliferative responses and T-B collaboration (Sprent *et al.*, 1980). Current evidence suggests that the activation of most T cells requires that antigens be presented on the cell surface in association with the MHC gene products. Furthermore, the immune functions of the sensitized T cells are restricted by the MHC gene products which they have encountered during the induction phase, a phenomenon called 'H-2 restriction'. At least two models have been proposed

TABLE 3

Influence of MHC on Effector T Cell Functions in Mice

Experimental system	H-2 regions involved	References
Cytotoxicity of T cells to		
(a) virus-infected cells	K,D	Zinkernagel & Doherty, 1974a; 1979
(b) minor histocompatibility antigen	K,D	Bevan, 1975
(c) chemically modified syngeneic cells	K,D	Shearer et al., 1975
(d) male-specific (H-Y) antigen	K,D	Gordon et al., 1975
(e) tumor antigens	K,D	Germain et al., 1975; Schrader et al., 1975
Adoptive transfer of DTH to[a]		
(a) proteins & polypeptides	IA	Miller, 1978a; 1978b
(b) contact chemicals (DNFB)	K,D or I	Vadas et al., 1977
(c) LCM virus	K,D	Zinkernagel, 1976
T cell-B cell cooperation for antibody responses	IA	Katz, 1976; Sprent, 1978; Sprent et al., 1980
Proliferative response of T cells to		
(a) allogeneic cells	I	Alter et al., 1973
(b) antigen-pulsed macrophages	IA	Schwartz & Paul, 1976; Paul et al., 1977
(c) Mls locus-coded antigen	IA	Janeway et al., 1980b
Suppressor T cells for		
(a) cytotoxic T cell response to ectromelia virus	D	Pang & Blanden, 1978
(b) contact sensitivity to DNFB	D	Miller, Sy & Claman, 1978

a See also Table 1, Chapter 9.

to explain the phenomenon of H-2 restriction. The altered self model, as originally proposed by Zinkernagel & Doherty (1974b), postulated that each T cell has one receptor specific for the neoantigenic determinant(s) formed by the H-2 gene product and the foreign antigen. This model can be interpretated in two different versions: the modified-altered self model and the complexed-altered self model (Langman, 1978). In the modified-altered self model it is conceivable that self antigens can be modified by the foreign antigens or vice versa. In the complexed-altered self model, foreign antigen becomes closely associated with self H-2 antigen to form a new antigenic complex which is recognized by the T cell. The two receptors model (or dual-recognition model) was proposed independently by several investigators (Doherty et al., 1976b; Janeway et al., 1976). The essence of this model is that each T cell has two distinct receptors, one directed to foreign antigen (X) and the other to self H-2 antigen and that both anti-H-2 and anti-X receptors are clonally distributed. At present both models fail to accomodate all the available experimental data. Until more is known about the nature of the T cell receptor, the mechanism of T cell recognition remains entirely speculative.

Most immune phenomena associated with T cells are subjected to H-2 restriction. A summary of these results is given in Table 3. Thus, Tc cells generated during a viral infection will only lyse virus-infected target cells that share the K or D region with the donor of the Tc cells (Zinkernagel & Doherty, 1979). Similarly, Tc cells specific for the minor histocompatibility antigen (Bevan, 1975), chemically modified self (Shearer et al., 1975), H-Y antigen (Gordon et al., 1975) and tumor antigens (Germain et al., 1975; Schrader et al., 1975) are also known to be K,D region restricted. Nevertheless, there are several reports that CML to non-K,D antigens do not show H-2 restriction. For examples, lysis directed

to Qa-1 determinants (Wernet & Klein, 1979; Fischer-Lindahl, 1979), I region determinants (Klein et al., 1977), mammary tumors (Stutman & Shen, 1978) and plasmacytoma antigens (Burton et al., 1977) appeared to be H-2 unrestricted. Furthermore, Tc cells specific for H-2 negative F9 teratocarcinoma cells have been described (Wagner et al., 1978).

It was found that for successful adoptive transfer of DTH responses, there existed a requirement for H-2 compatibility between donors of sensitized T cells and naive recipients. In case of proteins and polypeptides, successful transfer of DTH was IA subregion restricted (Miller et al., 1975) whereas for contact chemicals such as DNFB, successful transfer was K,D and I region restricted (Vadas et al., 1977). In contrast, Zinkernagel (1976) demonstrated that adoptive transfer of DTH to LCM virus was K,D but not I region restricted.

Although it is generally agreed that helper T cells show H-2 restriction in the induction phase, whether there exists a similar H-2 restriction pattern in the delivery of help from T cells to B cells is still controversial. Many investigators argue that the delivery of helper signal from T cells to B cells depends upon cell-to-cell contact and is H-2 restricted (Sprent, 1978; Yamashita & Shevach, 1978) whereas others have reported that the delivery of T cell help is H-2 unrestricted (McDougal & Cort, 1978; Singer et al., 1979).

The specific antigen-induced proliferation of sensitized T cells in vitro has been shown to be controlled by specific immune response (Ir) genes encoded within the H-2 complex of the mouse. Thus, for two antigens, poly (L-Tyr, L-Glu) - poly (D,L-Ala) - poly (L-Lys) and the BALB/c IgA myeloma protein (TEPC-15), the Ir genes controlling the T lymphocyte proliferative responses were mapped to the K region or IA subregion of the MHC (Schwartz & Paul, 1976). Similarly, the proliferative response of T cells to the Mls-locus antigen was found to be IA subregion

restricted (Janeway et al., 1980b). Furthermore, the I region was also associated with the proliferative response of T cells to allogeneic cells (Alter et al., 1973) or to antigen-pulsed macrophages (Paul et al., 1977).

The influence of the MHC on suppressor cells activity is less well-defined. It is generally believed that the function of the 'classical' Ts cells is not H-2 restricted (Vadas & Greene, personal communication). However, there are a few reports which showed an apparent H-2 restriction in the action of the suppressor T cells. For example, Kumar & Bennett (1977) have shown that the interactions between suppressor T cells and mitogen-responsive cells in cultures infected with Friend leukemia virus required identity at the H-2D region. Further work has demonstrated that such a H-2 restriction was mediated by a third cell type, called the interfering cells,that were present in the suppressor cell population. It was found that sharing of the H-2D region between the interfering cells and mitogen-responsive cells was required to demonstrate suppression and that the action of Ts on the mitogen-responsive cells was not H-2 restricted (Kumar & Bennett, 1979). A requirement for H-2D compatibility between donor and recipient mice was also reported for suppressor T cells active in suppression of cytotoxic T cell response to ectromelia virus infection (Pang & Blanden, 1978). However, in this case, the observed suppression was most likely due to feedback inhibition by the cytotoxic T cells which effectively removed the antigenic stimulus and that the classical Ts cells did not seem to be involved. On the other hand, Miller, Sy & Claman (1978) have shown that intravenous injection of DNP-coupled allogeneic lymphoid cells (spleen or lymph node cells) generated Ts (alloinduced Ts) cells that could suppress subsequent induction of contact sensitivity to DNFB in the immune mice. Such alloinduced Ts cells were only suppressive when transferred to recipient mice which shared the D region with the donors of the DNP-coupled lymphoid cells.

However, the alloinduced Ts cells have not been shown to belong to the classical Ts cells and that the possibility remains that these cells may be the highly active Tc cells which inhibit antigen presentation rather than the immune response which ensues thereafter.

AIMS AND SCOPES OF THIS THESIS

Influenza virus, together with polioviruses and reoviruses, were among the first viruses to be studied both antigenically and biochemically. Despite the fact that the influenza virus has been studied extensively for almost half a century, influenza is still a major infectious disease of man and the virus often causes epidemics of varying severity which cannot be effectively prevented by the vaccines currently available. The mechanisms underlying the host defence against influenza virus infection are far from being fully understood. Indeed, influenza virus and the disease it causes are still of great interest to both virologists and immunologists, with respect to its antigenic variation and specificity, humoral and cellular immunity as well as immunological memory and heterotypic immunity.

A study of the literature has shown that a considerable understanding of the immune responses to influenza virus infection has been acquired in recent years, particularly in the mouse model. However, up to early 1978, the time when the work reported in this thesis began, there were many aspects of the CMI response to influenza virus which remained unclear. It has been well documented that the humoral immunity to influenza virus is of great importance in protection of the host from subsequent reinfection. The possibility that the CMI response was also important as a host defence mechanism against influenza virus infection has been seriously considered only in the past few years. There are two major arms of the CMI response, namely, the cytotoxic T cell response and the delayed-type hypersensitivity response. The former has been shown to play a potentially important role in the recovery of mice from primary influenza virus infection. In contrast, little was known about the role of DTH response and the properties of the cells involved in the DTH reaction had not been studied in detail. Therefore,

the main purpose of the work presented in this thesis is to elucidate the inductive requirements and the properties of the effector cells involved in the DTH responses to influenza virus in the mouse model. Attempts are also made to evaluate the role these cells play during influenza virus infection. In view of the potential importance of the cytotoxic T cell response in the recovery of mice from influenza virus infection, factors governing their generation are studied. Furthermore, the differences in the immune reactivities of the host towards infectious and non-infectious viruses have been analyzed as this may throw some light on the strategy to be followed for the production of the most effective vaccines. Finally, resistance to influenza virus infection is a result of complex interactions among the specific and nonspecific defence mechanisms of the host. One aspect of the nonspecific mechanisms----the induction and properties of natural killer cells, is also examined.

CHAPTER 2

MATERIALS AND METHODS

TABLE 1

H-2 Haplotypes of Mice Used in the Experiments

Mouse strain	H-2 haplotype	H-2 regions K ABJEC I SGD
CBA	k	k kkkkk kkk
BALB/c	d	d ddddd ddd
DBA/2	d	d ddddd ddd
SJL	s	s sssss sss
DBA/1	q	q qqqqq qqq
C57BL/6J	b	b bbbbb bbb
A.TL	t1	s kkkkk kkd
A.TH	t2	s sssss ssd
C3H.OL	o1	d ddddd kkk
C3H.OH	o2	d ddddd ddk
B10.A(2R)	h2	k kkkkd ddb
B10.A(3R)	i3	b bbbkd ddd
B10.A(4R)	h4	k kbbbb bbb
B10.A(5R)	i5	b bbkkd ddd
B10.AQR	y1	q kkkkd ddd

MICE

The various strains of inbred specific pathogen-free mice used in this work were bred at the John Curtin School of Medical Research. Mice of the same sex and age (usually 6-10 weeks old) were used for each experiment. The H-2 haplotypes of the mouse strains are given in Table 1.

Congenitally athymic BALB/c nude (nu/nu) mice were obtained either from the John Curtin School or occasionally from the Walter and Eliza Hall Institute of Medical Research, Melbourne. Nude mice that were used for experiments were always supplied with antibiotics {kanamycin sulfate and tetracycline hydrochloride (Bristol-Myers Co., N.S.W)} in their drinking water.

The beige mutant mice C57BL/6J bg^J/bg^J (bg/bg) and their heterozygous (bg/+) littermates were originally obtained from Dr. G.R. Shellam, University of Western Australia. They were bred in the John Curtin School under conventional conditions.

Outbred mice were multicolored Walter and Eliza Hall Institute strain (WEHI). They were used for production of ascites using Sarcoma 180/TG cells and for production of Con A-activated supernatant (Interleukin-2).

VIRUSES

Influenza virus strains A/WSN/33 (H0N1), A/JAPAN/305/57 (H2N2), A/RHODE ISLAND/5/57 (H2N2), A/PORT CHALMERS/1/73 (H3N2), A/JAP-BEL (A/JAPAN/305/57 x A/BELLAMY/42) (H2N1), B/LEE/40 virus and Sendai virus were grown in the allantoic cavity of 10-day-old embryonated eggs for 40-48 h. The infectious allantoic fluid was harvested, distributed into small ampoules and stored at -70^0C until use. The infectivity of the virus was determined in eggs and expressed as the median egg infectious dose (EID_{50}) per millilitve according to the modified Spearman & Karber method

(Irwin & Cheeseman, 1939). Virus titres are usually expressed as EID_{50} or as haemagglutination units (HAU).

Concentrated preparations of infectious virus were obtained by light centrifugation (2000xg, 30 min) of freshly harvested infectious allantoic fluid to remove red cells and debris followed by sedimenting (85,000xg, 35 min) the virus from the supernatant by ultracentrifugation. The virus pellet was resuspended in one-tenth the original volume of allantoic fluid and this was distributed into small aliquots and immediately frozen and kept at -70^0C until use.

Purification of viruses

Influenza virus was purified from infectious allantoic fluid according to the method of Laver (1969) with minor modifications. Briefly, the virus was partially purified and concentrated 10-fold by one cycle of adsorption and elution from fowl erythrocytes followed by ultracentrifugation (85,000xg, 35 min) to pellet the virus particles. These were resuspended in a small volume ($\sim$2-3 ml) of Ca^{++}-Mg^{++}-saline and then further purified by sedimentation through a continuous 10-50% sucrose gradient (SW 27 rotor, 17,000 RPM, 40 min). The virus band was collected, dialyzed in cold phosphate-buffered-saline (PBS, pH 7.3) overnight and then diluted to a concentration of $2x10^5$ HAU/ml in PBS. Purified virus was usually stored at 4^0C with 0.1% sodium azide.

Egg-grown Sendai virus was purified from allantoic fluid by pelleting it in a Beckman 21 Spinco rotor at 18,000 RPM for 110 min. The allantoic fluid had previously been clarified by centrifugating at 4,000xg for 10 min. The virus pellet was resuspended in PBS and then layered onto a 20-60% sucrose gradient and centrifuged at 24,000 RPM for 1 h in a SW 27 rotor. The resulting virus band was collected, dialyzed in cold PBS and stored at 4^0C with 0.1% sodium azide.

Ultraviolet-light inactivation of viruses

For inactivation of virus, purified virus contained in a glass petri dish (diameter:3 inches) was exposed to a 30-Watt Phillips Germicidal ultraviolet (UV) lamp at a distance of 15 cm for 7 min (intensity:320 $\mu W/cm^2$ as measured by a Blak-Ray Ultraviolet meter, UV products, Inc. San Gabriel, California). Such a procedure rendered the virus non-infectious (judged from egg infectivity test) without any change in haemagglutinating activity.

CELL LINES

The various murine tumor cell lines such as P815-X2 cells ($H\text{-}2^d$) (methylcholantrene-induced mastocytoma of DBA/2 mice), EL-4 cells ($H\text{-}2^b$) (benzopyrene-induced T cell lymphoma of C57BL/6J mice), RBL-5 cells ($H\text{-}2^b$) (Rauscher leukemia virus-induced T cell lymphoma of C57BL/6J) were maintained in Dulbecco's modified Eagle's medium (Grand Island Biological Co., N.Y., Cat. no. 430-1600 or H-16) supplemented with 10% heat-inactivated (56^0C, 30 min)-foetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Cat. no. 5023) and containing 100 units/ml of penicillin G, 100 µg/ml of streptomycin sulfate and 100 µg/ml of neomycin sulfate (usually referred as PSN). YAC-1 cells ($H\text{-}2^a$) (Moloney leukemia virus-induced T cell lymphoma of A/Sn mice) were grown in RPMI 1640 medium (Grand Island Biological Co., N.Y., Cat. no. 430-1800 or H-18) supplemented with 10% heat-inactivated (HI)-FCS and antibiotics. The BALB/c mice derived myeloma line P3-NSI/1-Ag 4-1 (designated NS-1, $H\text{-}2^d$) were cultured in RPMI 1640 medium supplemented with 10% horse serum (Commonwealth Serum Laboratories, Melbourne, Cat. no. 5031) and 10% HI-FCS and antibiotics.

HSB2 cells and CEM cells are human T cell lymphomas which were a gift from Dr. E. Peterhans. These cell lines were maintained in vitro in RPMI medium supplemented with 10% HI-FCS and antibiotics.

All cultures were kept at 37^{0}C under a gas phase of 10% CO_2 in air in a CO_2 incubator (Kevatron model 102). The cells were seeded at an initial concentration of 10^5 cells/ml in Falcon tissue culture flasks (25 or 75 cm^2, Falcon Plastics, Oxnard, California) and subcultured at about 2-day interval when the cell concentration was about 10^6 cells/ml.

The C3H mouse derived L929 continuous fibroblasts were cultured in 75 cm^2 Falcon tissue culture flasks containing 30 ml of modified Minimum Essential Medium (Auto-Pow, Flow Laboratories, Australasia Pty. Ltd., Cat. no. 11-110-24) supplemented with 10% bovine serum and antibiotics. Each flask was seeded with 10^6 cells and incubated at 37^{0}C for 5-7 days until the cells formed an almost confluent monolayer. The cell monolayer was then trypsinized (0.05% trypsin) and washed three times with the culture medium, counted and usually used on the same day for the experiments.

ANTISERA

Anti-viral antiserum

Anti-A/WSN virus antiserum was prepared by immunization of mice with 10^3 HAU (or 10^7 EID_{50}) A/WSN virus intravenously. Mice were boosted at 1-2 months interval with the same dose of virus. Blood was collected 9 days after the second boost from anesthetized mice by cardiac puncture under sterile conditions. The blood was allowed to clot at 37^{0}C for 10 min, and then kept at 4^{0}C for 4-6 h. The serum was obtained by centrifugation (3,000 RPM, 15 min) of the clotted blood at 4^{0}C and the supernatant was harvested, distributed in small aliquots and immediately frozen at -70^{0}C.

Anti-Thy 1 ascitic fluid

Anti-Thy 1.2 ascitic fluid was prepared in AKR mice which were given up to 10 intraperitoneal (i.p.) injections of 5-10x10^7 CBA thymocytes at weekly intervals. The first injection was given with 10^9 killed B. pertussis

(phase 1) organisms (Commonwealth Serum Laboratories, Melbourne). Three days before the final dose of thymocytes, the mice were injected i.p. with 0.2 ml of a 10% suspension of Sarcoma 180/TG cells in saline. The ascitic fluid was harvested 7 days after the final thymocyte injection. Unimmunized AKR mice were used as donors of control (normal) ascitic fluid. The batch of anti-Thy 1.2 ascitic fluid used in this work lysed about 95% of thymocytes and 25-30% spleen cells when used in conjunction with rabbit complement.

Monoclonal anti-Thy 1.2 IgM antibody (F7D5) was a gift from Dr. P. Lake, Clinical Research Centre, Harrow, U.K.. It was produced by somatic cell hybridization of NS-1 cells with spleen cells of AKR mice responding to immunization with CBA thymocytes. The specificity of the antibody is directed to the Thy 1.2 alloantigenic determinant. This was determined by tests with Thy 1 congenic mice and in studies of tissue distribution.

Anti-Lyt and anti-Ia antisera

Monoclonal anti-Lyt 1.1 (1147A) and anti-Lyt 2.1 (49-31.1) antibodies , anti-Lyt 2.1 (As 145) and anti-Ia^k antisera were generous gifts from Professor I.F.C. McKenzie, Melbourne. Anti-Lyt 1.1 or anti-Lyt 2.1 antibodies were obtained from tissue culture supernatants produced by hybrid cells resulting from fusion of NS-1 cells with spleen cells from 129/ReJ mice immunized with B6.Ly-1^a(Cy) or CBA thymus, lymph node and spleen suspensions respectively. Anti-Lyt 2.1 antiserum was obtained from B10.Br mice hyperimmunized with thymus cells from CE mice. Anti-Ia^k antiserum was produced by immunizing recipient mice (A.TH) with 6 weekly injections of lymph node and spleen cells from A.TL mice.

Anti-Thy 1.2 antibody and complement treatment

$5x10^7$ cells were incubated at room temperature for 30 min with 1 ml anti-Thy 1.2 antibody (for ascitic fluid, 1:8 dilution; for monoclonal antibody, 1:1,000 dilution in Eagle's minimum essential medium (F-15 or MEM)

plus 10% HI-FCS). The cells were then washed once, resuspended in 1 ml normal rabbit serum (1:5 dilution, preadsorbed with allogeneic and syngeneic spleen cells at 4^0C for 30 min each to remove any nonspecific cytotoxic activity) and incubated at 37^0C for 30 min. The cells were washed twice and cell viability was determined by the trypan blue dye exclusion method. Such a treatment killed ∿95% thymocytes and ∿30% spleen cells. Furthermore, similar treatment had shown to abrogate completely both the cytotoxicity of 6 days immune spleen cells generated *in vivo* by i.v. injection of 10^7 EID_{50} A/WSN virus into mice and of the cytotoxic activity of secondary effector cells to influenza virus generated *in vitro*.

Anti-Lyt antibody and complement treatment

The use of monoclonal anti-Lyt 1.1 antibody and anti-Lyt 2.1 antiserum have been described previously (Hogarth *et al.*, 1980; Pang *et al.*, 1976; Beverley *et al.*, 1976). Briefly, $5x10^7$ cells were incubated with 1 ml of anti-Lyt antibody (anti-Lyt 1.1, 1:32 dilution; anti-Lyt 2.1 , 1:5 dilution) for 30 min at room temperature, the cells were washed once, resuspended in 1 ml of 1:5 dilution of preadsorbed normal rabbit serum and incubated at 37^0C for 30 min. The cells were then washed twice and the viability determined. I am aware of the fact that Lyt 1 antigen was demonstrated to be present in all T cells using very sensitive assay systems (Ledbetter *et al.*, 1980; Hogarth *et al.*, 1980), though quantitative difference may exist in various T cell subpopulations. However, in this thesis (and also a number of other workers in this area, e.g. Swain, 1980; Shen *et al.*, 1980; Simon & Abenhardt, 1980; Leclerc & Cantor, 1980a; Smith & Miller, 1979a etc.) the notations Lyt 1^+ and Lyt $2,3^+$ are still used to denote cell populations that are sensitive to anti-Lyt 1.1 and anti-Lyt 2.1 antibodies and complement treatments respectively, under the prescribed experimental conditions.

Anti-Ia antiserum and complement treatment

Cells from CBA mice were suspended at a concentration of $5x10^7$ cells/ml and treated with anti-Ia^k antiserum at a final dilution of 1:6 for 30 min at room temperature. After washing once, the cells were incubated with guinea pig complement at a final dilution of 1:2 for 40 min at 37^0C. The viability was determined by a trypan blue dye exclusion method.

PREPARATION OF SPLEEN CELL SUSPENSION

Spleens were aseptically removed, minced with scissors and then gently pressed through a stainless steel sieve into 10 ml MEM medium. Cell clumps were dissociated by pipetting and fibrous material was removed by a quick centrifugation for 30 sec. The cells were washed 3 times with medium and viability determined by trypan blue dye exclusion method. Spleen cell suspension free of erythrocytes can be obtained either by exposure to a lysing solution for 5 min or by centrifugation in a Ficoll-Isopaque gradient, according to the procedures of Davidson & Parish (1975).

ISOLATION OF LYMPHOCYTES FROM PERIPHERAL BLOOD, LUNGS AND DRAINING LYMPH NODES

Blood was collected into Alsever's solution (0.8% sodium citrate, 0.42% sodium chloride and 2.1% glucose) and viable lymphocytes were obtained by centrifugation in a Ficoll-Isopaque gradient at 20^0C for 15-20 min at 2,000xg. The lungs were removed aseptically and washed with MEM medium to remove as much adherent blood as possible. Draining lymph nodes were obtained by removing the superficial and deep cervicals, the mediastinal, the axillary and brachial lymph nodes from the mice. Cells from the lungs and lymph nodes were prepared by cutting the tissues

into small pieces and pressing through a stainless steel sieve. Cell debris was first removed by a quick centrifugation and viable cells were obtained by sedimentation through a Ficoll-Isopaque gradient.

LYSIS OF ERYTHROCYTES

Removal of red blood cells from mouse lymphoid cells was achieved by exposure of the cells to a 10 ml lysing solution for 4-5 min at room temperature. The lysing solution is a 0.184 M NH_4Cl solution in 0.19 M Tris buffer, pH 7.2. It is usually stored at 4^0C as a 10x concentrate.

Ig-POSITIVE AND Ig-NEGATIVE CELL SEPARATION

The method for separation of mouse lymphoid cells into Ig-positive and Ig-negative populations as described by Parish et al.(1974) was adopted with slight modifications. Briefly, sheep IgG specific for mouse Ig was coupled to sheep red blood cells (SRBC) by $CrCl_3$ treatment. These SRBC were then used to form rosettes with mouse lymphoid cells. Rosettes were separated from non-rosetting cells (Ig-negative cells) by centrifugation through a gradient of Ficoll-Isopaque solution. The Ig-negative cells were collected from the Ficoll-Isopaque/medium interface whereas Ig-positive cells were collected at the bottom of the tubes. The Ig-positive cells were freed of SRBC by lysis with lysing solution as described above.

REMOVAL OF ADHERENT/PHAGOCYTIC CELLS

Several procedures were used to remove adherent/phagocytic cells from mouse lymphoid cell populations. They are briefly described as follows:

(1) Uptake of carbonyl iron powder

$2x10^8$ cells in 10 ml culture medium (MEM + 10% HI-FCS) were incubated with 80 mg carbonyl iron powder (Atomergic Chemetals Co., N.Y.) for 45 min

at 37^{0}C and the carbonyl iron powder was removed with an electromagnet.

(2) Adherence to plastic surface

$5x10^7$ cells were suspended in 5 ml warm and slightly acidic MEM medium supplemented with 10% HI-FCS. The cell suspension was placed in a Falcon tissue culture flask (75 cm^2 growth area) and incubated for 3 h at 37^{0}C. Nonadherent cells were then collected and the flask rinsed gently twice, each time with 10 ml warm MEM medium to remove any remaining nonadherent cells.

When dealing with large amounts of cells (e.g. 10^9 cells), a two step procedure which includes first the carbonyl powder treatment followed by adherence onto plastic surface was used for more effective removal of adherent/phagocytic cells from the cell population.

(3) Treatment with λ-carrageenan

Lambda-carrageenan (λ-Car, from aciculaire and pistillata of the genus Gigartina) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A) and was dissolved in warm normal saline at a concentration of 2.4 mg/ml and sterilized by boiling for about 10 min. Cells at a concentration of 10^7 cells/ml in MEM medium plus 10% HI-FCS were exposed to λ-carrageenan at a final concentration of 300 or 600 μg/ml. The mixture was incubated at 37^{0}C for 4 h before the activity of the residual cells was tested.

(4) Treatment with silica

Silica Min-U-Sil (5 microns in diameter) was a gift from the Whittaker, Clark & Daniels, Inc. N.J.. Cells at a concentration of 10^7 cells/ml in MEM medium plus 10% HI-FCS were exposed to silica at a final concentration of 125 or 250 μg/ml. The silica particles were suspended in MEM medium and sonicated immediately before use. The mixture of cells and silica was incubated at 37^{0}C for 4 h before the activity of the residual cells was tested.

FRACTIONATION OF ANTIBODIES IN MOUSE SERUM

(1) Reverse chromatography using Sephadex G-200 column

2 ml of 6 days immune serum from BALB/c mice primed with 10^3 HAU UV-inactivated A/WSN virus was applied to a Sephadex G-200 column and the sample was eluted with cold PBS at a flow rate of 10 ml per hour. 5 ml fractions were collected at 4^0C and the optical density at 280 nm was determined using a UV-spectrophotometer. The first 19S peak and the second 7S peak were each concentrated to 1 ml by vacuum ultrafiltration.

(2) Affinity chromatography using Protein A Sepharose column

IgM and IgG antibodies were separated from mouse immune serum by affinity chromatography using Staphylococcal Protein A covalently linked to Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described by Ey et al.(1978). 2 ml of 6 days immune serum was dialyzed against 100 ml 0.1 M phosphate buffer, pH 8.0 for 4 h. The protein A-Sepharose column was pre-equilibrated with 0.1 M phosphate buffer, pH 8.0 before application of the dialyzed serum sample. The unbound proteins (containing IgM) were removed by washing the column with 25-30 ml of the phosphate buffer and then reconcentrated to 1 ml volume by vacuum dialysis against cold PBS. IgG was eluted from the column using 0.1 M sodium citrate/ citric acid (pH 4.0) at a flow rate of 0.4 ml per min. The eluted fractions were immediately neutralized with 1 M Tris-HCl buffer, pH 9.0. They were pooled and reconcentrated to 1 ml volume by vacuum dialysis against cold PBS. The entire fractionation procedure was done at 4^0C.

PREPARATION OF CONCANAVALIN A-ACTIVATED CELL SUPERNATANTS

The method used was essentially the same as that described by Lafferty et al.(1980). Briefly, $3x10^8$ normal spleen cells from either inbred or outbred mice were resuspended in 15 ml serum-free MEM medium in a 75 cm^2 Falcon tissue culture flask. These were incubated for 2 h at 37^0C with Con A (Pharmacia Fine Chemicals, Uppsala, Sweden) at a final concentration

of 5 µg/ml. Under these conditions most cells stick firmly to the plastic surface of the flask. The monolayer so obtained was washed gently 3 times with warm (37^{0}C) Hank's balanced salt solution and incubated for a further period of 17 h in 30 ml serum-free fresh MEM medium supplemented with $2x10^{-4}$ M 2-mercaptoethanol in a gas phase of 10% CO_2, 7% O_2 and 83% N_2. The supernatant was then harvested and concentrated 8-fold through a diaflow Amicon PM10 membrane system and then sterilized by filtration through a millipore membrane filter. The Con A-activated supernatant was distributed into small aliquots and kept at -20^{0}C until use. Samples were taken for assay of costimulator and maintenance activities using standard procedures as described elsewhere (Lafferty et al., 1980).

CYCLOPHOSPHAMIDE PRETREATMENT

Cyclophosphamide (Cy) (Endoxan-Asta, Bristol-Myers Co., N.S.W.) was dissolved in sterile normal saline immediately before use. A volume of 0.2 ml was given intraperitoneally 2 days before immunization. Dosages used are recorded in the result sections.

γ-IRRADIATION

Radiation was provided by a 100 Ci ^{60}Co (Cobalt) source at the C.S.I.R.O., Canberra. Lymphoid cells received a dose of 2000 rads whereas tumor cells (P815) received 5000 rads at a dose rate of ∿660 rads/min.

MITOMYCIN C TREATMENT

Mitomycin C (from Streptomyces caespitosus, Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in sterile PBS in a final concentration of 200 µg/ml. 10^7 cells were incubated with 20 µg mitomycin C in a final volume of 1 ml at 37^{0}C for 30 min. The treated cells were washed 3 times before use.

HAEMAGGLUTINATION

Serial 2-fold dilutions of the virus suspension was carried out in PBS in 96-wells round-bottomed tissue culture plate (Linbro, Flow Laboratories, Inc., Conn.) finishing with a final volume of 50 µl in each well. An equal volume of 0.5% chicken red blood cells (CRBC) in PBS was added to each well. The plate was shaken gently and stood on ice for about 45 min before the patterns were read. The virus dilution in the final well which gave a positive reaction (haemagglutination) was considered as having a titre of one haemagglutination unit (HAU).

HAEMADSORPTION TEST

Influenza virus-infected cells were washed twice at the end of the incubation period and cell concentration was adjusted to $2x10^6$ cells per ml. 0.1 ml cell suspension was taken and 0.3 ml of 0.5% CRBC was added. The cell mixture was centrifuged at 400xg for 5 min at 4^0C. The cell pellet was resuspended very gently with a Pasteur pipette and a fraction of the cell suspension was examined for the percentage of rosettes in a haemacytometer (AO Spencer Bright-line haemacytometer, American Optical, N.Y.).

HAEMAGGLUTINATION INHIBITION TEST

All sera to be tested were first heat-inactivated (56^0 C, 30 min) to remove nonspecific inhibitors. Four HAU of virus in 25 µl volume was added to serial 2-fold dilutions of serum in a final volume of 25 µl in PBS. After about 30 min at room temperature, 50 µl of a 0.5% CRBC in PBS was added to each well and the haemagglutination-inhibition endpoint was determined 30 min later. The titre of the serum was expressed as the reciprocal of the highest dilution of the serum which still inhibited the haemagglutination.

HISTOLOGY

Mouse footpads were fixed for at least 48 h in 10% formol saline. The tissues were first decalcified, then embedded in paraffin wax. Sections were cut and stained with haematoxylin-eosin by the Histology Section of the John Curtin School. Slides were examined and then photographed using an Orthomat-W (Leitz) fully automatic microscope camera attached to an Orthoplan microscope (Leitz).

MEASUREMENT OF PULMONARY PATHOLOGY

Estimation of pulmonary pathology was carried out as described previously (Wagner, 1956; Yap & Ada, 1978a). Briefly, lungs were removed from infected mice and the surface of all lobes were inspected for areas of dark purple 'consolidation'. The total 'purple' area of each pair of lungs was scored (based on visual inspection) from 0-100% with respect to total lung surface.

INTRANASAL INOCULATION OF VIRUS INTO MICE

Mice, under light ether anaesthesia, were inoculated intranasally (i.n.) by placing 50 µl of virus suspension diluted in cold PBS into both nostrils with a 50 µl micropipette, thus facilitating inhalation.

SENSITIZATION OF MICE FOR DTH

Normal mice or mice injected 48 h previously with cyclophosphamide were injected subcutaneously (s.c.) with normal allantoic fluid, infectious allantoic fluid or UV-inactivated virus. Optimal doses for sensitization with infectious & inactivated virus were 10^3 HAU and 5×10^3 HAU respectively.

ADOPTIVE TRANSFER AND MEASUREMENT OF DTH REACTIONS

(1) Cell transfer studies

Cell preparations were tested for their ability to transfer DTH as follows: (a) In systemic cell transfer, 5-10x10^7 primary immune spleen cells were injected i.v. and 2-8 h later, the footpads of the recipients were challenged with virus (see below). (b) In local syngeneic cell transfer, cultured cells (usually 5-10x10^6), lung cells (1-2x10^6), lymph node cells (1x10^7), peripheral blood leukocytes (2x10^6) or Ig-negative immune spleen cells (5x10^6) were mixed with virus and the mixtures(40-50 µl volume) were injected directly into the footpads of naive recipients. In semi-syngeneic or allogeneic cell transfers, the effector cells were either injected 6 h after injection of virus into the same footpad or alternatively,they were first depleted of plastic-adherent cells and then injected at the same time with virus into the mouse footpads. In all cases it was found that injection of cells alone seldom gave an increase of footpad swelling at 24 h of more than 8% and averaged about 5%.

(2) Measurement of DTH reactions

(a) Footpad swelling test

The method is essentially the same as described previously (Cooper, 1972). Briefly, sensitized mice or naive mice adoptively transferred with sensitized cells were injected into the right hind footpad with an eliciting dose of either purified, UV-inactivated virus (6x10^3 HAU) or concentrated preparation of infectious virus (2.5x10^3 HAU) in a volume of 30 µl using a 50 µl syringe (Hamilton Company, Reno, Nevada) fitted with a 30-gauge needle (Yale, Becton, Dickinson & Company, Rutherford, N.J.). The same volume of PBS was injected into the left hind footpad as a control. The dorso-ventral thickness of each hind footpad was measured 24-72 h after virus challenge with a dial-gauge caliper (H.C. Kröplin, Schlüchtern,

Hessen, Germany) calibrated to 0.05 mm. Each footpad was measured three times and the mean reading was taken. Results were expressed as the mean percentage increase in footpad thickness, which is calculated as follow :

$$\frac{\text{Thickness of right hind footpad - thickness of left hind footpad}}{\text{Mean thickness of the normal feet}} \times 100\%$$

In all experiments described in chapters 3, 4.1 and 4.2, unless otherwise mentioned, purified, UV-inactivated virus was used to elicit the DTH response, as it was found that injection of infectious allantoic fluid per se gave higher control readings. In some experiments described in chapters 4.3 and 5, mice were challenged with concentrated, infectious preparations of virus (which is essential for detection of the K,D region restricted Td population). However, it was found that injection of concentrated, infectious virus alone into naive mice gave a significant background increase in footpad swelling (12-15%) whereas that given by UV-inactivated virus was low (<5%) and often negligible. To allow for this effect when infectious virus was used, the mean increase in footpad thickness was calculated by substracting the background swelling (injection of infectious virus alone into naive mice) from the swelling obtained when cells were adoptively transferred with infectious virus into naive recipients.

(b) The localization of I^{125}-labelled cells in the ear

The procedure for the ear test was described by Vadas et al.(1975). Briefly, mice were injected with 15 µl purified virus ($3x10^3$ HAU) in the left ear lobe. Same volume of PBS was injected into the right ear as a control. 5-Fluoro-deoxyuridine (Sigma Chemical Co., U.S.A) (0.2 ml, $5x10^{-4}$ M) was injected i.p. 9½ h after virus challenge. Thirty minutes later

2 µCi I^{125}-5-iodo-2-deoxyuridine (I^{125}-UdR) (The Radiochemical Centre, Amersham, U.K., specific activity: 5 Ci/mg) in 0.2 ml volume was injected i.v. into each mouse, and 16 h later the ear lobes were cut off at the hairline and their radioactivity measured. Results were expressed as the ratio of radioactivity in the left ear to that in the right ear.

A RADIOISOTOPIC METHOD FOR MEASURING THE MONONUCLEAR CELLULAR INFILTRATION IN LUNGS OF MICE INFECTED WITH INFLUENZA VIRUS

The procedures used were adopted from Anders et al.(1979) with minor modifications. Briefly, mice were infected i.n. with a mouse-adapted batch of A/WSN virus, the dose administered was specified in the text. Six days later, all mice were given 0.2 ml of $5x10^{-4}$ M 5-fluoro-deoxyuridine i.p. followed 30 min later by 1 µCi I^{125}-UdR injected i.p.. Twenty-four hours after the injection of I^{125}-UdR, mice were killed by exsanguination under ether anaesthesia to reduce excessive bleeding around the lungs. The lungs from each mouse were removed and single cell suspension was made. Contaminating red cells were removed by exposure of the cells to 10 ml lysing solution for 4 min at room temperature. Radioactivity associated with the remaining lung cells was measured in a gamma counter (Packard Auto-gamma counter or Beckman gamma 9000 counter).

MEASUREMENT OF T-CELL-MEDIATED CYTOTOXICITY

$5\text{-}10x10^6$ target cells (tumor cells or fibroblasts) were labelled with ~400 µCi ^{51}Cr (sodium chromate, 200 mCi/mg, CEA commissariat A L'energie Atomique, Siren) for 1 h at 37^0C in a total volume not greater than 0.5 ml. After labelling, the cells were washed once with MEM medium and then divided into two equal lots. One lot was infected with virus (15-20 EID_{50} per cell) for 1 h at 37^0C and the other lot left uninfected and used as a

control. The target cells were then washed twice and the cell concentration adjusted to $1x10^5$ cells/ml. The target cells in 0.1 ml aliquots were distributed into each well of the 96-wells flat-bottomed tissue culture trays (Linbro Scientific CO. , Hamden, Conn.). Effector cells (spleen cells, lung cells, peritoneal cells or in vitro cultured cells) at various effector to target cell ratios (25:1, 50:1, 100:1) were added in 0.1 ml portions to the wells containing the labelled targets. Each test sample was plated in triplicate or quadruplicate. The trays were gassed with 10% CO_2, 7% O_2 and 83% N_2 in a gas box and incubated at 37^0C for 6 h, after which 0.1 ml of the supernatant from each well was carefully removed and the radioactivity measured in a gamma counter. For spontaneous lysis (or medium release), 0.1 ml medium was added to each well containing only 0.1 ml labelled target cells. For total releasable ^{51}Cr (water lysis), 2 ml distilled water was added to 0.1 ml labelled target cells and then allowed to stand on the bench for 6 h. This was centrifuged and the supernatant removed and radioactivity measured. The specific release of ^{51}Cr was determined, using the formula

$$\frac{\text{Mean counts from immune effector cells} - \text{mean counts from normal effector cells}}{\text{Water lysis supernatant count}} \times 100\%$$

The data were processed with a digital pdp 11/34 computer which calculated the specific ^{51}Cr release and the standard errors for each set of data.

NATURAL-KILLER-MEDIATED CYTOTOXICITY ASSAY

The assay for natural killer (NK) activity was similar to that described for the assay of T-cell-mediated cytotoxicity except that (1) in most cases, NK-sensitive targets such as YAC-1 or EL-4 cells (Roder et al., 1979) were used and the target cells were usually uninfected; and (2) a short term 5 h ^{51}Cr release assay was used unless otherwise mentioned. The specific ^{51}Cr release was calculated as follow using the formula:

$$\frac{\text{Mean counts in the presence of effector cells} - \text{mean spontaneous release counts}}{\text{Mean water lysis counts} - \text{mean spontaneous release counts}} \times 100\%$$

GENERATION OF INFLUENZA OR SENDAI VIRUS-SPECIFIC CYTOTOXIC T CELL ACTIVITY

(1) Primary in vivo

Mice were injected i.v. with 10^7 EID_{50} (or 10^3 HAU) infectious virus. Optimal cytotoxic activity was generated in spleens 6 days after virus injection. Cytotoxic activity can also be recovered from the lungs of mice 6 days after i.n. inoculation with infectious influenza virus.

(2) Secondary in vitro

10^8 spleen cells from mice primed (i.v. or i.p.) with 10^3 HAU (or 10^7 EID_{50}) infectious virus 3 weeks or more previously were cultured with 10^7 virus-infected (3 EID_{50}/cell) syngeneic spleen cells in a 75 cm^2 Falcon tissue culture flask containing 40 ml MEM medium supplemented with 10% FCS, 10^{-4} M 2-mercaptoethanol (2-ME) and antibiotics. The responders (the primed spleen cells) to stimulators (virus-infected syngeneic spleen cells) ratio was always 10:1. The flask was incubated at 37^{0}C for 5 days in a gas phase of 10% CO_2 in air. Viable cells were obtained from the culture after centrifugation on a Ficoll-Isopaque gradient and they were tested for cytotoxic activity in a 5-6 h ^{51}Cr release assay.

A LIMITING DILUTION ASSAY FOR IN VITRO GENERATION AND ASSAY OF VIRUS-SPECIFIC CYTOTOXIC T CELLS

The method has been described in detail elsewhere (Ashman & Müllbacher, 1979). Briefly, appropriate numbers of the Ig-negative responder cells (normal BALB/c spleen cells, $5x10^4$), virus-infected, γ-irradiated stimulator cells ($5x10^4$) and Ig-negative, γ-irradiated helper cells (spleen cells from BALB/c mice primed with virus 3 weeks or more, $2x10^5$) were suspended in MEM medium supplemented with 10% FCS, 10^{-4}M 2-ME and antibiotics and then dispensed in a total volume of 0.1 ml into 96-wells, round-bottomed Linbro tissue culture trays. After incubation at 37°C for 5 days in a gas phase of 10% CO_2, 7% O_2 and 83% N_2, the trays were centrifuged at 300xg for 5 min, the supernatants removed and each well was replaced by 0.1 ml fresh MEM medium supplemented with 10% HI-FCS and antibiotics. P815 cells labelled with ^{51}Cr and then infected with virus (15-20 EID_{50}/cell) were added to each well (10^3 cells/well) and the mixture incubated at 37°C for 6 h. 0.1 ml supernatant was removed from each well and the radioactivity measured. Wells showing ^{51}Cr release greater than three standard deviations above the mean of the spontaneous release were scored as positive.

IN VITRO GENERATION OF SECONDARY EFFECTOR T CELLS WITH I REGION RESTRICTED DTH ACTIVITY BUT NEGLIGIBLE CYTOTOXIC T CELL ACTIVITY TO INFLUENZA VIRUS

The procedure is essentially the same as that described for the *in vitro* generation of secondary cytotoxic T cells (see above) except that the initial sensitization of the donor mice was done with UV-inactivated influenza virus ($5x10^3$ HAU injected s.c.). After 3 weeks or more, the memory spleen cells were restimulated *in vitro* with syngeneic spleen cells exposed to either infectious or non-infectious homologous virus (1 h, 37°C) and the cultures kept at 37°C for 5 days. Viable cells were tested for

DTH activity by adoptive transfer with antigen (purified, UV-inactivated virus) into the right hind footpads of naive recipients and for cytotoxic activity using the conventional ^{51}Cr release assay.

IN VITRO GENERATION OF SECONDARY EFFECTOR T CELLS WITH K,D REGION RESTRICTED CYTOTOXIC AND DTH ACTIVITIES BUT NEGLIGIBLE I REGION RESTRICTED DTH ACTIVITY TO INFLUENZA VIRUS

The procedure is similar to that described for the *in vitro* generation of secondary influenza virus-specific cytotoxic T cells except that the stimulator cells used do not have Ia antigenic determinants on their surface (e.g. fibroblasts, P815 cells etc.).

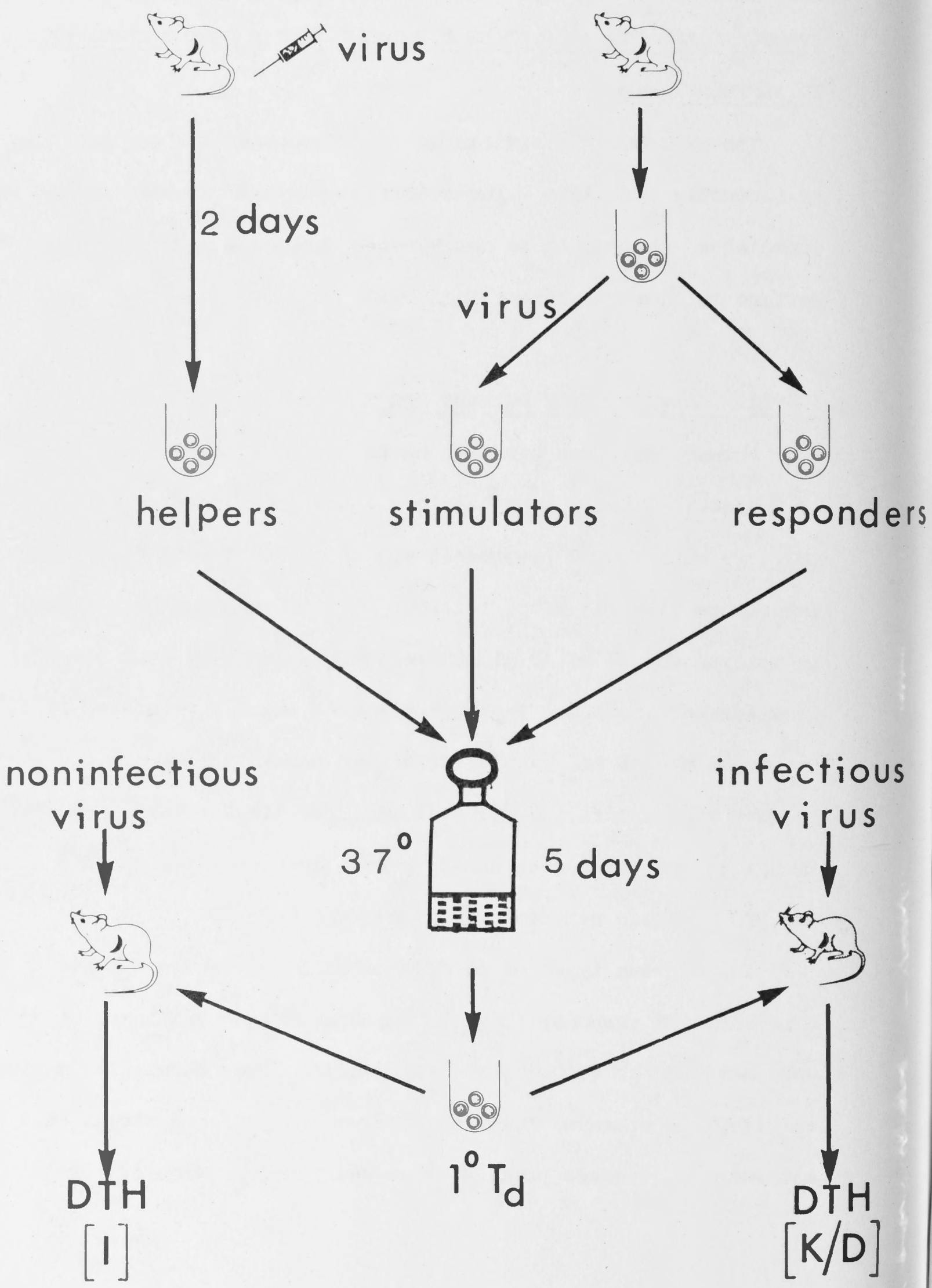
IN VITRO GENERATION OF PRIMARY DTH EFFECTOR CELLS

Primary cultures were set up in 75 cm^2 Falcon tissue culture flasks as follows: $7.5x10^7$ normal spleen cells (responder cells) were stimulated *in vitro* with $1.5x10^7$ syngeneic spleen cells exposed (1 h, 37^0C) to either infectious virus (3 EID_{50}/cell) or UV-inactivated virus ($1.5x10^3$ HAU/10^7 cells) in a total volume of 40 ml MEM medium supplemented with 10% FCS, 10^{-4} M 2-ME and antibiotics. The cultures were usually incubated at 37^0C in a gas phase of 10% CO_2 in air for 5 days before harvesting the viable cells. In most experiments, $7.5x10^7$ γ-irradiated (2000 rads) 2 days *in vivo* primed immune spleen cells were added to each culture flask as this would augment the DTH response generated *in vitro* (see below).

The various types of stimulator cells which can be used to generate a primary DTH response *in vitro* were prepared as follows: Lymph node cells were obtained by removing the superficial, deep cervicals, mediastinal, axillary and brachial lymph nodes from the mice and single cell suspension was made by standard procedures as described previously. Peritoneal exudate

Figure 1

ASSAY OF HELPER ACTIVITY



cells were induced by injection of 3 ml of 3% thioglycollate solution (Difco Bacto Fluid Thioglycollate Medium) into the peritoneal cavity of mice. The cells were harvested 3 days later by peritoneal lavage with 5 ml cold Puck's saline (0.8% NaCl, 0.06% KCl, 0.08% $CaCl_2$, 0.005% $MgCl_2$, 0.03% glucose, 0.00025% phenol red, 100 µg/ml chloramphenicol and pH adjusted to 7.0 with 1 M NaOH).

Con A blasts and LPS blasts were produced by culturing 10^8 normal spleen cells with 125 µg Concanavalin A (2.5 µg/ml) or 500 µg Lipopolysaccharide (LPS) (from E. coli 0128:B12, Sigma Chemical Co., U.S.A., 10 µg/ml) in 50 ml culture medium for 2 days at 37^0C.

L929 continuous fibroblasts were grown as monolayers in Auto-Pow medium, supplemented with 10% bovine serum and antibiotics. They were mitomycin C treated before use as stimulator cells in primary cultures.

GENERATION OF HELPER T CELLS IN VIVO AND ASSAY OF HELPER ACTIVITY

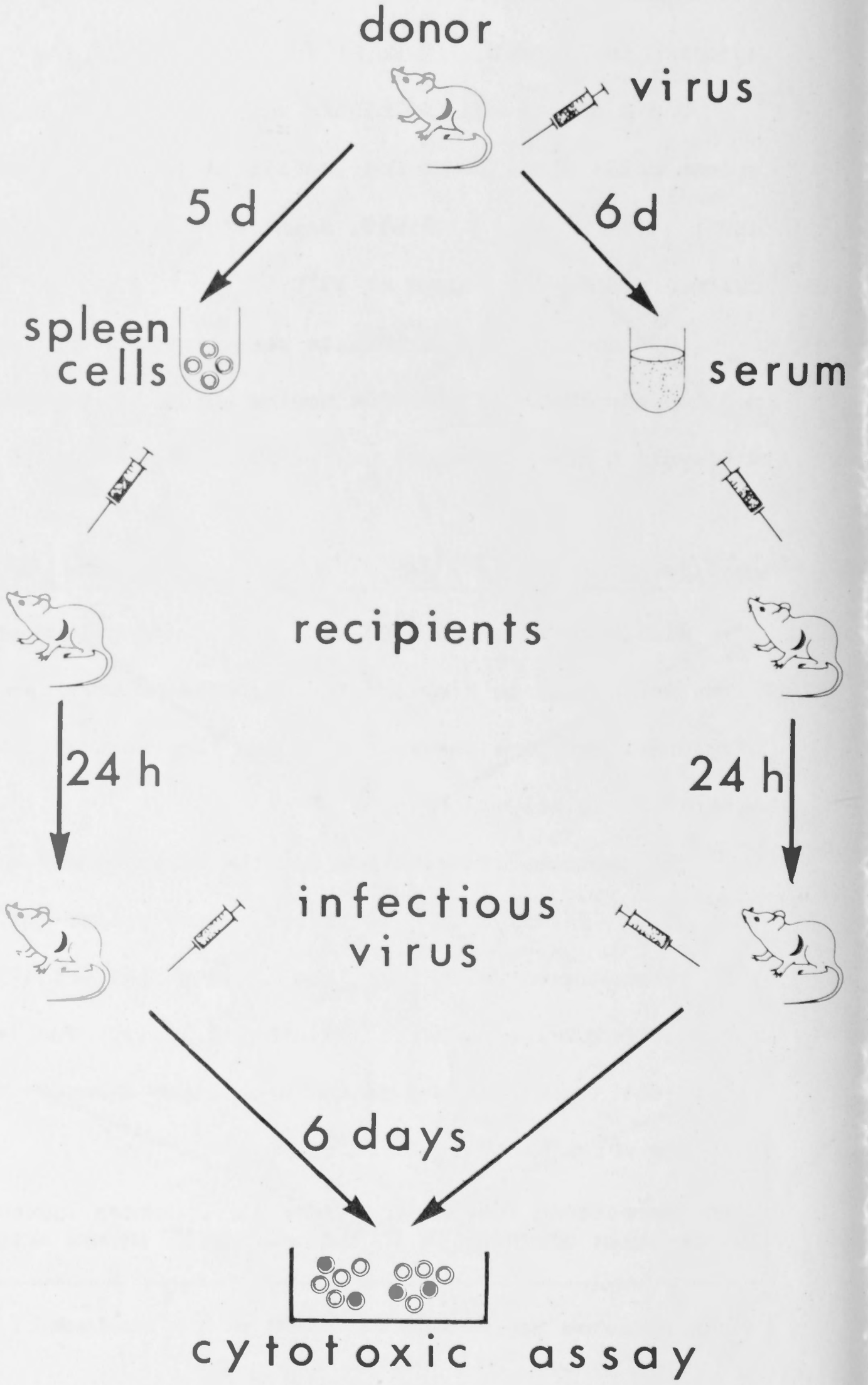
Mice were injected i.v. with 10 HAU infectious or UV-inactivated virus and except in kinetic studies, spleens were removed 2 days later, single cell suspensions were made and they were γ-irradiated (2000 rads) before use as helper cells.

The experimental protocol for the assay of helper activity is depicted in Fig. 1. Briefly, in vivo primed immune spleen cells (helper cells) were γ-irradiated which were then added to the primary cultures at a fixed ratio of helper:responder:stimulator of 5:5:1. The helper activity of the primed cells was assessed as the percentage enhancement of the primary DTH response which was calculated as follow :

$$\frac{\text{Mean increase in footpad thickness in the presence of Th cells in the culture} - \text{mean increase in footpad thickness in the absence of Th cells}}{\text{Mean increase in footpad thickness in the absence of Th cells in the culture}} \times 100\%$$

Figure 2

ASSAY OF SUPPRESSOR ACTIVITY



ASSAY OF SUPPRESSOR ACTIVITY FOR THE CYTOTOXIC T CELL RESPONSE TO INFLUENZA VIRUS

The experimental protocol is depicted in Fig. 2. Briefly, donor mice were injected i.v. with either infectious or UV-inactivated influenza virus (10^3 HAU). Five or six days later, spleen cells or sera from a group of 3-4 mice were pooled. The 5 days immune spleen cells ($5\text{-}10\text{x}10^7$) or 6 days immune serum (0.3 ml) were transferred into naive recipients which were challenged i.v. 24 h later with 10^7 EID_{50} infectious virus. Normal spleen cells or normal mouse serum were transferred as controls. Six days after virus injection, the spleen cells were harvested from the recipients and tested for cytotoxicity in a 6 h ^{51}Cr release assay. The degree of suppression was calculated using the following formula:

$$\frac{\text{Specific } ^{51}Cr \text{ release with cells from mice transferred with normal cells or serum} - \text{Specific } ^{51}Cr \text{ release with cells from mice transferred with immune cells or serum}}{\text{Specific } ^{51}Cr \text{ release with cells from mice transferred with normal cells or serum}} \times 100\%$$

TITRATION OF INFLUENZA VIRUS IN MOUSE LUNGS OR FOOTPADS

Mouse lungs or footpads were removed as aseptically as possible and stored at -70^0C until ready for titration. All subsequent procedures until the harvesting of the allantoic fluids were carried out aseptically. The organs were allowed to thaw at 4^0C, each footpad or each pair of mouse lungs was homogenized in 3 ml standard medium (or known as gelatin saline, which is a solution made up of 0.85% NaCl. 0.03% $CaCl_2$, 0.08% $MgCl_2$, 0.5% gelatine and buffered to pH 7.2 with 0.2 M borate buffer) with a motorized teflon pestle. The homogenized suspension was centrifuged at 1500xg for

20 min at 4^0C. Serial 10-fold dilutions of the supernatant were made in standard medium and 0.1 ml volumes were inoculated into the allantoic cavity of 10-day-old embryonated eggs. Usually 3-5 eggs were used for each dilution point. After 40-48 h of incubation, 50 µl of allantoic fluid from each egg was tested for the presence of virus by mixing with an equal volume of 0.5% CRBC and haemagglutination was noted. The virus titre was expressed as the log EID_{50} per organ extract which was determined by the modified Spearman & Karber method (Irwin & Cheeseman, 1939).

STATISTICAL ANALYSIS

All results were expressed as the arithmetic mean ± standard error. Student's *t* test was used to determine the confidence limits in group comparisons.

CHAPTER 3

PRODUCTION AND PROPERTIES OF T CELLS MEDIATING DELAYED-TYPE HYPERSENSITIVITY (DTH) RESPONSES TO INFLUENZA VIRUS

CHAPTER 3.1

PRODUCTION OF DTH IN THE MOUSE TO INFLUENZA VIRUS: A COMPARISON WITH CONDITIONS FOR STIMULATION OF CYTOTOXIC T CELLS

INTRODUCTION

Very little is known about the role which delayed-type hypersensitivity (DTH) reactions play in acute and chronic viral infections (Allison, 1967). Using murine models and the traditional footpad swelling test after challenge with antigen, DTH responses have been shown to occur following inoculation of a variety of viruses, including LCM virus (Tosolini & Mims, 1971), ectromelia virus (Fenner, 1948), Semliki Forest virus (SFV) (Kraaijeveld et al., 1979), St Louis encephalitis virus (Hudon et al., 1979), rabies virus (Lagrange et al., 1978), murine leukaemia virus (Peters et al., 1975) and influenza virus (Webster & Hinshaw, 1977; Floc'h & Werner, 1978). DTH responses to influenza virus have also been reported in humans, using skin test reactions (Habershon et al., 1973; Cole & Molyneux, 1975). Despite these observations, many characteristics of the DTH response have been inadequately studied, such as the conditions of induction and elicitation (including genetic requirements), the Lyt phenotype, the influence of extrinsic reagents and so on. This is in contrast to our knowledge about the properties of cytotoxic T lymphocytes (Tc) generated during influenza virus infection in man (McMichael et al., 1977; Biddison et al., 1979) and mice (Doherty et al., 1977; Effros et al., 1977; Ennis et al., 1977a; Yap & Ada, 1977). Furthermore, adoptive transfer experiments suggest that cell preparations enriched for Tc activity reduce lung virus titres in mice given a lethal dose of virus and protect the mice from death (Yap & Ada, 1978b; Yap et al., 1978). It has been suggested that DTH reactions, on the other hand, may contribute to the pathology of the infectious process. Definitive experiments to establish this have yet to be carried out, however, and this cannot be done until more is known about the effector cells which

take part in DTH reactions (Td) and the conditions for their generation. This chapter describes the production and some of the properties of Td cells generated in mice following injection of infectious and non-infectious influenza virus. Some of these findings are compared with conditions necessary to induce Tc cells production and substantial differences shown to occur. Succeeding chapters will deal with experiments designed to study their role during recovery from infection by the virus.

RESULTS

Comparison of two methods - footpad swelling and ear localization - for measurement of DTH

There are several methods available for the measurement of DTH responses. The traditional procedure is measurement of the increase in footpad thickness 24 h after injection of the challenge antigen into the footpad. Another involves the localization of ^{125}I-UdR labelled cells into the site of antigen injection, usually the ear. The latter procedure, introduced by Miller et al. (1975) was claimed to be a more sensitive and objective procedure for the quantitative in vivo measurement of DTH. Though these and other methods have recently been compared (Robinson & Naysmith, 1976), measurement of footpad swelling seems still to be the most commonly used (Floc'h & Werner, 1978; Lagrange et al., 1978; Hudson et al., 1979; Kraaijeveld et al., 1979); however, before deciding on this procedure, tests were carried out comparing this with the ear-localization method.

Mice were injected subcutaneously (s.c.) with 10^3HAU infectious A/WSN virus. Two days later they were injected into the right hind footpad with 6×10^3HAU purified UV-inactivated A/WSN virus and into the left ear lobes with 3×10^3HAU of the same virus. The opposite footpads and ear lobes were injected with the same volume of phosphate-

buffered saline (PBS). Ten h after the injection of the challenge antigen, the mice were injected intravenously (i.v.) with 2μCi ^{125}I-UdR. The increase in footpad thickness and the radioactivity in the ear lobes were measured 24 h after antigen injection, with the results shown in Table 1. In the three experiments quoted, there is a reasonable correlation between the two procedures. For the bulk of the work to be reported, however, the footpad technique has been used because it has been found technically simple and in some circumstances, antigen and cell can be injected into the footpad with successful results, whereas this has not been possible with the ear procedure.

Sensitization of mice with infectious or non-infectious virus

A DTH response to many antigens can only be achieved if an adjuvant is used as well (Katsura et al., 1977). Infectious and non-infectious (UV-inactivated) influenza virus preparations were compared for their ability to sensitize mice for a DTH response in the absence of added adjuvant. The results of a dose-response study are shown in Fig. 1. In this experiment, an increase in the amount of UV-inactivated virus (from 10^1-$5x10^3$HAU) used to sensitize the mice (s.c. injection) resulted in some increase in the DTH response when challenged with a standard dose ($6x10^3$HAU) of purified, UV-inactivated virus. The maximum effective dose for infectious virus was 10^3HAU. There was a reduction in the response if a higher dose ($5x10^3$HAU) of infectious virus was used. However, if infectious virus was given i.v., a dose of only 1-10 HAU gave the optimal sensitization, higher doses giving a progressively less effect (Fig. 1).

Kinetics of induction of the DTH response

Normal mice and mice which had been injected with cyclophosphamide (Cy; 200 mg/kg) 2 days previously, were immunized with $5x10^3$HAU UV-inactivated A/WSN virus. The mice were challenged in the footpad at

various intervals thereafter with $6x10^3$HAU purified UV-inactivated virus. The kinetics of the DTH response to the virus (Fig. 2) is similar to that of some protein antigen (Cooper, 1972). A response can be elicited as early as 2 days after sensitization and the peak occurred at day 6 and declined thereafter so that a small response could still be elicited at 16 days, but not later. Cy-pretreated mice showed a similar pattern of response, the major difference being that there was an increase in the magnitude of the response at days 6-8 (Fig. 2). Immunization with infectious virus (10^3HAU, s.c.) gave similar kinetics of induction of a DTH response (Fig. 3).

Development of the footpad response after injection of antigen

The kinetics of response in the footpad after challenge with antigen were examined in three groups of mice. 1. unprimed mice; 2. mice sensitized 6 days earlier with $5x10^3$HAU UV-inactivated virus; 3. mice injected 8 days earlier with Cy (200 mg/kg) and sensitized with virus, as above, 2 days later. In each case, the challenge was into the right hind footpad ($6x10^3$HAU purified UV-inactivated virus), PBS being injected into the left hind footpad. The results (Fig. 4) show that all groups gave a rapid but transient response peaking at 4 h after injection, and reducing by 12 h to background levels. No further swelling occurred in the unprimed mice. Primed mice (no Cy) gave a second, delayed response which peaked at 24 h, but had reduced to a low level by 72 h. The effect of Cy pretreatment was to enhance the magnitude of the response over the period 24-72 h.

The 4 h reaction cannot be attributed to the presence of antibody, as it occurred in unprimed mice. A similar effect has been previously observed for Semliki Forest virus (Kraaijeveld et al., 1979) and the most likely explanation is that it represents a non-specific inflammatory response due to an innate toxic effect of the virus

particles (Kato & Okada, 1961). The size of this reaction depended on the amount of virus injected, lesser amounts giving a smaller reaction.

Effect of dose of eliciting antigen in the DTH response

It was seen earlier that variation in the amount of virus, either infectious or non-infectious, used to sensitize mice for DTH affected the size of the subsequent response. In the reverse experiment, using a standard dose ($5x10^3$HAU) of non-infectious virus to sensitize the mice, the amount of antigen used to elicit the response also affected the extent of the observed reaction (Fig. 5). A significant reaction was not observed unless a substantial amount of the virus ($3-6x10^3$HAU) was used as the challenge.

Specificity of the DTH response

Mice were sensitized with infectious A/WSN virus, B/LEE virus, Sendai virus or normal allantoic fluid. Six days later they were challenged with $6x10^3$HAU purified, UV-inactivated A/WSN virus and the footpads measured 24 h later with the results shown in Table 2. A positive DTH response was only given if A/WSN virus was used to elicit the reaction.

Adoptive transfer of cells mediating a DTH response

Adoptive transfer experiments have shown that the DTH response to influenza virus can be transferred to naive recipients by 6 days immune spleen cells but not by 6 days immune serum from sensitized donor mice. (Data not shown.) The nature of the cells involved in the transfer of DTH was then investigated. The cells could be transferred systemically or directly into the footpad with the challenge antigen. The results of several experiments are given in Table 3. The first experiment shows that the Ig-negative fraction of 6 days immune spleen cells was capable of transferring DTH. It was found that the response obtained was dose-dependent; local injection of less than $5x10^5$ Ig-negative cells with

antigen failed to give a significant increase in footpad thickness (data not shown).More cells needed to be injected i.v. if a substantial DTH response was to be transferred (Expt. 2, Table 3). Treatment of the unfractionated immune spleen cells with anti-Thy 1.2 ascitic fluid and complement destroyed the activity of the transferred cells (Expt. 3, Table 3), indicating that the activity was a property of T cells.

Influence of the route of injection on sensitization for DTH and generation of Tc

One major aim of this work is to elucidate the role of different classes of T cells in the recovery from viral infection and to determine the factors important in the presentation of antigen, ways of obtaining cell preparations enriched for one or the other activity were sought. Table 4, Expt. 1, indicates that the route of inoculation is important. Using the same dose of infectious virus (10^3HAU), s.c. injection sensitized efficiently for DTH, but induced Tc least efficiently. In contrast, i.v. injection of virus gave the opposite effect. To make a more direct comparison, mice were injected either i.v. or s.c. with 10^3HAU infectious virus and the spleens removed 6 days later. Ig-negative cell preparations were made and these tested directly for Tc activity or injected with or without virus into the footpads. The results are shown in Expt. 2, Table 4. They show a similar pattern to the results in Expt. 1.

Differential effect of cyclophosphamide pretreatment on the generation of Tc and Td activity

The second approach was to study the effect of pre-treatment of mice with cyclophosphamide (Cy) before sensitization with antigen. Two groups of mice were injected intraperitoneally (i.p.) with different doses of Cy 2 days prior to i.v. injection (group 1) or s.c. injection (group 2) of 10^3HAU of infectious A/WSN virus. After 6 days, the mice

in group 1 were killed and the Tc activity of the spleen cells measured and those in group 2 injected with virus into the footpads and the increase in thickness measured 24 h later (Table 5). High doses of Cy inhibit the production of Tc but enhance the DTH response. The enhancement of DTH response by Cy treatment was supported by histological examination of the footpads of mice. As shown in Fig. 6, an increase of mononuclear cell infiltration into the footpad was accompanied by an increase in footpad swelling in Cy pretreated mice.

There are three possible explanations for the enhancement of the DTH response by Cy pretreatment. Firstly, this could be due to reduction in antibody levels at the time when the challenge virus was administered. In the mice receiving 200 mg/kg Cy and s.c. injection of virus, a decrease of 71% in HI antibody level as compared to controls (no Cy pretreatment) was found at day 6 after sensitization. The role of antibody was examined further. Two of three groups of mice were preinjected with Cy 200 mg/kg 2 days before all groups were sensitized s.c. with infectious A/WSN virus (10^3HAU). Five days after sensitization, one Cy-pretreated groups was injected i.v. with 0.3 ml syngeneic anti-A/WSN serum (HI titre = 1280) and serum from all mice collected 20 h later. All mice were then challenged with virus into the footpads and the increase in swelling measured as usual. The results, antibody levels (a) and percent increase in footpad thickness (b) were as follows. Group 1 (no Cy) a = 93 ± 13, b = 32.3 ± 1.1; Group 2 (Cy), a = 27 ± 7, b = 51.1 ± 5.2; Group 3 (Cy + antibody), a = 320 ± 80, b = 14.6 ± 2.1.

Secondly, Cy may have had an effect on the direct generation of Td cells. To check this, day 6 immune spleen cells from a control group of mice (no Cy and virus primed) and from mice preinjected with the high dose of Cy and primed with virus 2 days later were transferred i.v. ($8x10^7$ cells/mouse) to naive recipients which were then challenged

with virus in the footpads 6 h later. The DTH response(% mean increase in footpad thickness) of the two groups of recipients 24 h after virus injection were similar (no Cy, 32.8 ± 1.6; Cy pretreatment, 27.9 ± 1.6).

A third possiblity is that Cy may have an effect on the circulating monocytes, cells that are known to participate in the sites of DTH reactions. To test for this possibility, mice were divided into three groups A, B and C. Groups A and B were treated with Cy (200 mg/kg) and group C was untreated. Seven days later, mice in group A were injected i.v. with 0.3 ml syngeneic anti-A/WSN serum (HI titre = 1280). All three groups were injected with $8x10^7$ day 6 immune spleen cells (from mice primed with 10^3HAU infectious A/WSN virus) 8 days after Cy treatment. Six h after cell transfer, mice were challenged with virus and the DTH responses measured 24 h later for the three groups were as follows:

Group A (Cy + antibody)	:	10.4 ± 1.1
Group B (Cy)	:	30.2 ± 1.1
Group C (No Cy)	:	32.3 ± 1.1

Thus, pretreatment of <u>recipients</u> with Cy did not affect their ability to mount a DTH response to injected immune cells and virus (compare groups B and C). However, transfer of virus-specific antibodies caused a suppression of the DTH response in Cy pretreated recipients (compare groups A and B) and these results further support the first possibility that Cy acts primarily on antibody response with the resultant enhancement of DTH response to influenza virus.

DISCUSSION

The work reported in this chapter is part of a programme to assess the role of the CMI response during murine influenza infection. Earlier work from this and other laboratories (Doherty et al., 1977; Effros et al., 1977; Yap & Ada, 1977; Zweerink et al., 1977a;Braciale & Yap, 1978; Wells et al., 1979) have described the properties of cytotoxic T cells (Tc) produced in the mouse during infection. By contrast, little is known about another arm of the CMI response - those effector cells that take part in a DTH response (Td). This chapter describes (a). some of the characterisitcs of the Td cells generated in mice in response to an injection of infectious or non-infectious virus; (b). conditions which influence the generation of Tc and Td cells. In estimating and comparing these effector cell activities, it would be preferable to describe each activity in terms of the number of each cell type present in different preparations, but this is not yet possible. Nevertheless, the estimates of activities made, using admittedly cruder procedures, do allow some conclusions to be drawn.

The kinetics of sensitization and elicitation of DTH to both preparations of influenza A virus was found to be similar to that of some protein antigens (Cooper, 1972) and to other viruses (Peters et al., 1975; Lagrange et al., 1978; Hudson et al., 1978; Kraaijeveld et al., 1979). Adequate sensitization could be achieved by the injection of the virus without any added adjuvant, and under these conditions maximum DTH was elicited in the footpad 6 days after sensitization. Adoptive cell transfer showed that the cells that mediated DTH were in the Ig-negative fraction of the spleen and were sensitive to the action of anti-Thy 1 and complement. They were virus-specific, as indicated in the experiments with either B/LEE virus or Sendai virus (Table 1).

A major purpose of this study was to compare the role of various subsets of T cells during the infection, and this required cell preparations enriched for one or the other subset. In this chapter, different routes of inoculation and the effect of cyclophosphamide were examined.

The route of inoculation of virus clearly influences the response obtained. Subcutaneous inoculation of antigen is frequently used for sensitizing for a DTH response, and in the experiments reported here, an increase in response occurred over the range 10^1 to 10^3HAU. As was found originally for sheep erythrocytes (Lagrange et al., 1974a), intravenous injection could also give good sensitization when low doses of virus were injected (1-10 HAU), whereas higher doses gave substantially reduced responses. In contrast, a linear response in the formation of Tc cells after intravenous injection of infectious virus (10^{-3} to 10^3HAU) has been obtained, whereas little or no Tc activity was recovered from the spleens when a wide dose range of UV-inactivated virus (10^{-2} to 10^4HAU) was injected intravenously (Braciale & Yap, 1978).

The reasons for these differences are not well understood. One recent hypothesis (Greene & Bach, 1979) proposes that the immune response comprises predominantly two compartments: one central, consisting of the bone marrow thymus and spleen, and the other peripheral, consisting of lymph nodes arranged in proximity to all other organs. Experimentally, it was found that injection of some antigens subcutaneously preferentially leads to a cell-mediated immune response, whereas the injection of large amounts of antigen intravenously leads to a preponderance of regulatory or suppressor cells (Miller & Claman, 1976) or enhanced skin graft survival when allogeneic cells are used (Greene & Bach, 1979). These authors mainly considered DTH responses, and in this respect the data in this chapter would be consistent with their proposal. However, the

conditions reported in this chapter and other work (Yap & Ada, 1977; Braciale & Yap, 1978) for the production of Tc cells in the spleen are not consistent with this proposal in that there was a linear response when infectious virus was injected intravenously. Furthermore, when mice were inoculated intranasally with infectious virus, Tc and Td cells were found both in the lungs and in the draining lymph nodes (Cambridge et al., 1976; Yap & Ada, 1978a; Chapter 4.1). Perhaps replicating antigens such as viruses behave differently from other antigens in this respect.

An alternative way of influencing the production of various T cell subsets is to pre-inject mice with Cy. Table 5 shows that with increasing amounts of Cy, the level of DTH which could be elicited with antigen was increased, whereas the activity of Tc cells present in the spleen decreased. A similar finding concerning Tc cells production was also reported for LCM virus (Doherty & Zinkernagel, 1974). The enhanced reaction was most noticeable between 6 and 8 days after sensitization (Fig. 2). The reaction elicited by antigen injection into Cy-treated mice was not only enhanced at the 24 h time point but persisted until 72 h (Fig. 4). Cy acts by inhibition of rapidly dividing cells, and these could be B or T cells (Stockman et al., 1973; Turk & Poulter, 1975). The protocol used in these experiments was such that either B cells (Katz et al., 1974; Lagrange et al., 1974b) or suppressor T cells (Askenase et al., 1975; Mitsuoka et al., 1976; Röllinghoff et al., 1977) might be affected. In the former case, antibody levels in the Cy-injected mice would be expected to be reduced, and this was found to be true in our case. Furthermore, injection of virus-specific antibody into Cy-treated mice before challenge with antigen greatly decreased the subsequent DTH response. In contrast, comparison of the spleens of Cy-treated and control sensitized mice showed that the levels of DTH activity were similar.

Thus, the major reason for the enhancement of the DTH response after Cy injections was probably the decreased antibody production.

The reason why Tc levels in the spleen of Cy-treated mice are depressed is less clear. It has recently been shown that Cy pre-treatment depresses two H-2-restricted Tc responses, namely to TNP-coupled syngeneic cells and the in vitro response to H-Y antigen, but does not depress the allogeneic response (Hurme, 1979). It was suggested that precursor cells for the former (Lyt $1^+2^+3^+$) and for the latter (Lyt $1^-2^+3^+$) differed in their susceptibility to Cy, since the former are immature cells and might be rapidly dividing (Hurme, 1979). The Lyt phenotypes of primary and secondary anti-ectromelia Tc cells have been studied, but no conclusion was reached about the phenotypes of the precursor cells (Pang, et al., 1976). It has also recently been shown that the generation of anti-influenza virus primary Tc cells in tissue culture is enhanced by the addition of T helper cells (Th) (Ashman & Müllbacher, 1979). Whether the lack of production of anti-influenza Tc cells in Cy-treated mice in the present experiments was due to an effect of Cy on precursor Tc or Th cells, or both, remains to be established.

Nevertheless, pretreatment of mice with Cy before sensitization is a useful method for obtaining cell preparations with high Td but low Tc activity. Other methods for achieving cells enriched for either activity depend upon determining the Lyt phenotype of the Td cells and the histocompatibility requirement for activation to occur. These studies will be reported in Chapter 3.2.

SUMMARY

The kinetics of sensitization and elicitation of DTH in mice to both infectious and non-infectious preparations of influenza virus were found to be similar to that of some protein antigens and to other viruses. Sensitization was achieved without added adjuvant. Maximum DTH was elicited in the footpad 6 days after sensitization. Adoptive transfer experiments showed that the effector cells were in the Ig-negative fraction of the spleen and were sensitive to anti-Thy 1 ascitic fluid and complement. A comparison was made of conditions for the generation of DTH activity and cytotoxic T cells. The route of inoculation was important. With a high dose (10^3HAU) of virus, subcutaneous inoculation was the most efficient and intravenous injection the least efficient for sensitizing for DTH, whereas the reverse was found for cytotoxic T cell generation. Secondly, treatment of mice with cyclophosphamide (Cy) had differential effects. Preinjection of a large dose (200 mg/kg) into mice 2 days before sensitization with virus resulted in an increase in the DTH response and a 90% reduction in cytotoxic T cell activity in the spleens of the treated mice. The Cy injected mice had reduced (70%) anti-haemagglutinin levels compared to the controls. This may be the explanation for the enhanced DTH response as transfer of specific antibody to sensitized mice prior to injection of the eliciting virus substantially reduced the DTH response. Pretreatment with Cy did not affect the generation of DTH effector cells as spleen cells from these and control mice had similar levels of activity.

TABLE 1

Comparison of DTH Response to Influenza A Virus as Measured by the Footpad Swelling and ^{125}I-UdR Localization Test

Experiment No.	Sensitization[a]	DTH measurement[b]	
		Mean increase in footpad thickness at 24 h (%)	$\frac{L}{R}$ ^{125}I-UdR uptake
1	Normal allantoic fluid	6.3 ± 1.3[c]	1.10 ± 0.17[c]
	Infectious A/WSN	32.8 ± 2.0[d]	1.78 ± 0.19[e]
2	Normal allantoic fluid	7.1 ± 0.8	0.95 ± 0.08
	Infectious A/WSN	32.1 ± 1.6[d]	1.54 ± 0.06[e]
3	Normal allantoic fluid	2.4 ± 0.8	0.92 ± 0.02
	Infectious A/WSN	27.4 ± 2.4[d]	1.59 ± 0.03[e]

a Groups of 4-5 CBA mice were sensitized subcutaneously with normal allantoic fluid or 10^3HAU infectious A/WSN in 0.2 ml volume.

b Mice were challenged with 6×10^3HAU purified, UV-inactivated A/WSN virus 6 days after sensitization. Methods of DTH measurement were given in Materials and Methods section (Chapter 2).

c Mean ± standard error.

d Significantly greater than control (mice sensitized with normal allantoic fluid); $p < 0.001$.

e Significantly greater than control (mice sensitized with normal allantoic fluid); $p < 0.01$.

TABLE 2

Specificity of DTH Response

Sensitization[a]	Elicitation[b]	Mean increase in footpad thickness at 24 h (%)
Normal allantoic fluid	A/WSN	6.3 ± 1.3[c]
A/WSN	A/WSN	32.8 ± 2.0
B/LEE	A/WSN	3.1 ± 1.3
Sendai	A/WSN	4.3 ± 0.8

a Mice were sensitized s.c. with either normal allantoic fluid or infectious virus (10^3 HAU).

b Sensitized mice were elicited with purified, UV-inactivated A/WSN virus ($6x10^3$ HAU).

c Mean ± standard error for groups of 4 mice.

TABLE 3

Adoptive Transfer of Influenza Virus Specific DTH Response is T Cell Dependent

Experiment No.	Sensitization of donor[a]	Cell preparation transferred	Route of cell transfer[b]	Injection into the footpad of recipients	Mean increase in footpad thickness at 24 h (%)
1	Nil	-	-	Virus alone	7.1 ± 1.5[c]
	Nil	Ig^- fraction of normal spleen cells (NSC) ($5x10^6$)	f.p.	Cells + virus	9.4 ± 1.3
	A/WSN	Ig^- fraction of 6-day immune spleen cells (ISC) $5x10^6$)	f.p.	Cells alone	2.4 ± 0.8
	A/WSN	Ig^- fraction of 6-day ISC ($5x10^6$)	f.p.	Cells + virus	38.3 ± 0.8
2	Nil	NSC (10^8)	i.v.	Virus alone	5.5 ± 0.8
	A/WSN	D_6 ISC (10^8)	i.v.	Virus alone	32.8 ± 1.6
	A/WSN	Ig^+ fraction of D_6 ISC ($7x10^7$)	i.v.	Virus alone	6.3 ± 1.3
	A/WSN	Ig^- fraction of D_6 ISC ($3x10^7$)	i.v.	Virus alone	25.8 ± 0.8
3	Nil	NSC (10^8)	i.v.	Virus alone	3.2 ± 0.0
	A/WSN	D_6 ISC (10^8)	i.v.	Virus alone	28.2 ± 1.3
	A/WSN	Normal ascitic fluid + C' treated D_6 ISC (10^8)	i.v.	Virus alone	27.4 ± 1.3
	A/WSN	Anti-Thy 1.2 ascitic fluid + C' treated D_6 ISC_6 (10^8)	i.v.	Virus alone	4.2 ± 0.9

a CBA mice were sensitized s.c. with 10^3 HAU infectious A/WSN virus.

b f.p.: footpad injection; i.v.: intravenous injection.

c Mean ± standard error.

TABLE 4

Influence of Routes of Virus Administration on the Induction of Cytotoxic and DTH Responses

Route of virus inoculation[a]	Effector cells	Specific ^{51}Cr release on infected L929 cells (effector:target = 50:1) (%)	Cell transfer for DTH	Injection into footpad[e]	Mean increase in footpad thickness at 24 h (%)
Experiment 1					
Nil	NSC	3.5 ± 0.2[c]	No[d]	Virus	3.1 ± 1.8[c]
i.v.	D_6 ISC	35.8 ± 0.7	No	Virus	19.8 ± 1.1
i.p.	D_6 ISC	22.5 ± 1.0	No	Virus	21.9 ± 2.2
s.c.	D_6 ISC	12.6 ± 0.1	No	Virus	30.5 ± 1.5
Experiment 2					
Nil	NSC, Ig^- fraction[b]	6.9 ± 0.7	Yes	Cells + virus	5.2 ± 1.1
i.v.	D_6 ISC, Ig^- fraction[b]	34.0 ± 0.7	Yes	Cells alone	1.9 ± 1.9
				Cells + virus	14.9 ± 1.5
s.c.	D_6 ISC Ig^- fraction[b]	15.0 ± 0.6	Yes	Cells alone	1.6 ± 1.6
				Cells + virus	31.3 ± 1.3

Legends for Table 4

a CBA mice were sensitized in various routes with 10^3 HAU infectious A/WSN virus.

b The Ig^- fraction of the spleen cells was prepared, part of them were measured for cytotoxicity in a 6 h ^{51}Cr release assay while others were tested for their capacity to mount a DTH response on adoptive transfer into syngeneic recipients.

c Mean ± standard error.

d Sensitization and elicitation of DTH response were done on the same animals.

e Either virus alone (purified UV-inactivated A/WSN, $6x10^3$ HAU), cells alone (Ig^- fraction of spleen cells, $5x10^6$), or both were injected into the right hind footpad.

TABLE 5

Differential Effects of Cyclophosphamide Pretreatment on the Generation of the Cytotoxic T cell Response and the DTH Response to Influenza A Virus in Mice[a]

Cyclophosphamide pretreatment	Virus injection	Specific ^{51}Cr release by infected targets (E:T ratio 50:1) (%)	Reduction in cytotoxicity[b] (%)	Mean increase in footpad thickness at 24 h (%)
Nil	Nil	5.0 ± 0.75		7.1 ± 1.5
Nil	Infectious A/WSN	40.4 ± 1.69		32.1 ± 2.7
50 mg/kg	Infectious A/WSN	40.0 ± 0.69	1.0	34.4 ± 2.6
100 mg/kg	Infectious A/WSN	29.3 ± 1.45	27.5	46.6 ± 2.7
200 mg/kg	Infectious A/WSN	3.7 ± 0.41	90.8	51.6 ± 5.2

a CBA/H mice were pretreated with different doses of cyclophosphamide. Two days later, they were injected with $1x10^3$ HAU infectious A/WSN virus (i.v. for optimal cytotoxic T cell response and s.c. for optimal DTH response). Six days after virus injection, cytotoxic T cell response was tested by the ^{51}Cr release assay and the DTH response was measured by the 24 h footpad swelling method.

b % reduction was calculated relative to the control group of mice which was injected with virus only.

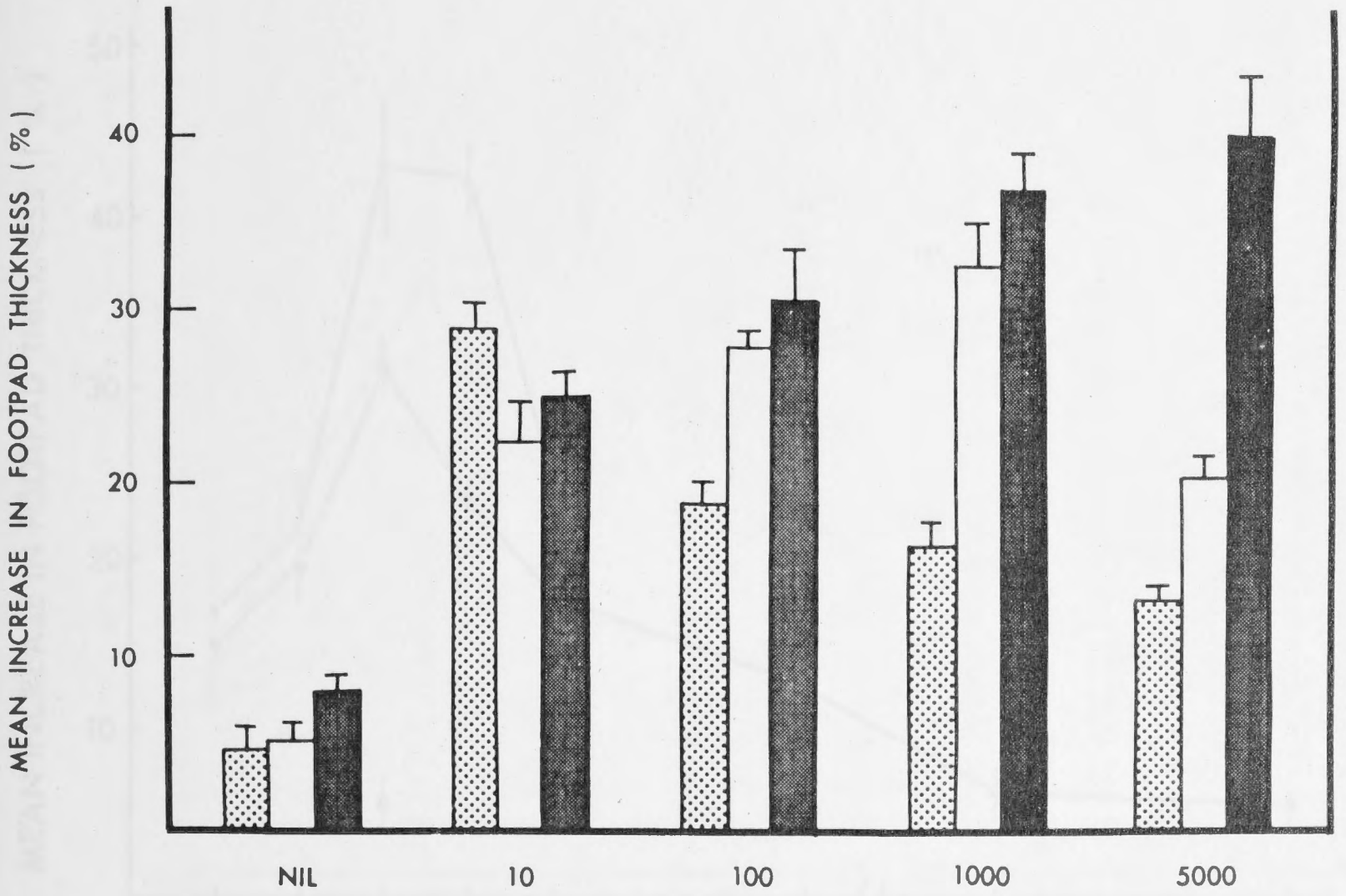


Fig. 1: Influence of viral infectivity, routes of administration and sensitizing dose in the induction of a DTH response to influenza A virus. CBA mice were sensitized with A/WSN virus and purified, UV-inactivated A/WSN virus (6×10^3 HAU) was used for elicitation of the DTH response 6 days later.
(▩) infectious A/WSN injected i.v.;
(▩) UV-inactivated A/WSN injected s.c.;
(□) infectious A/WSN injected s.c..
Vertical bars represent one standard error.

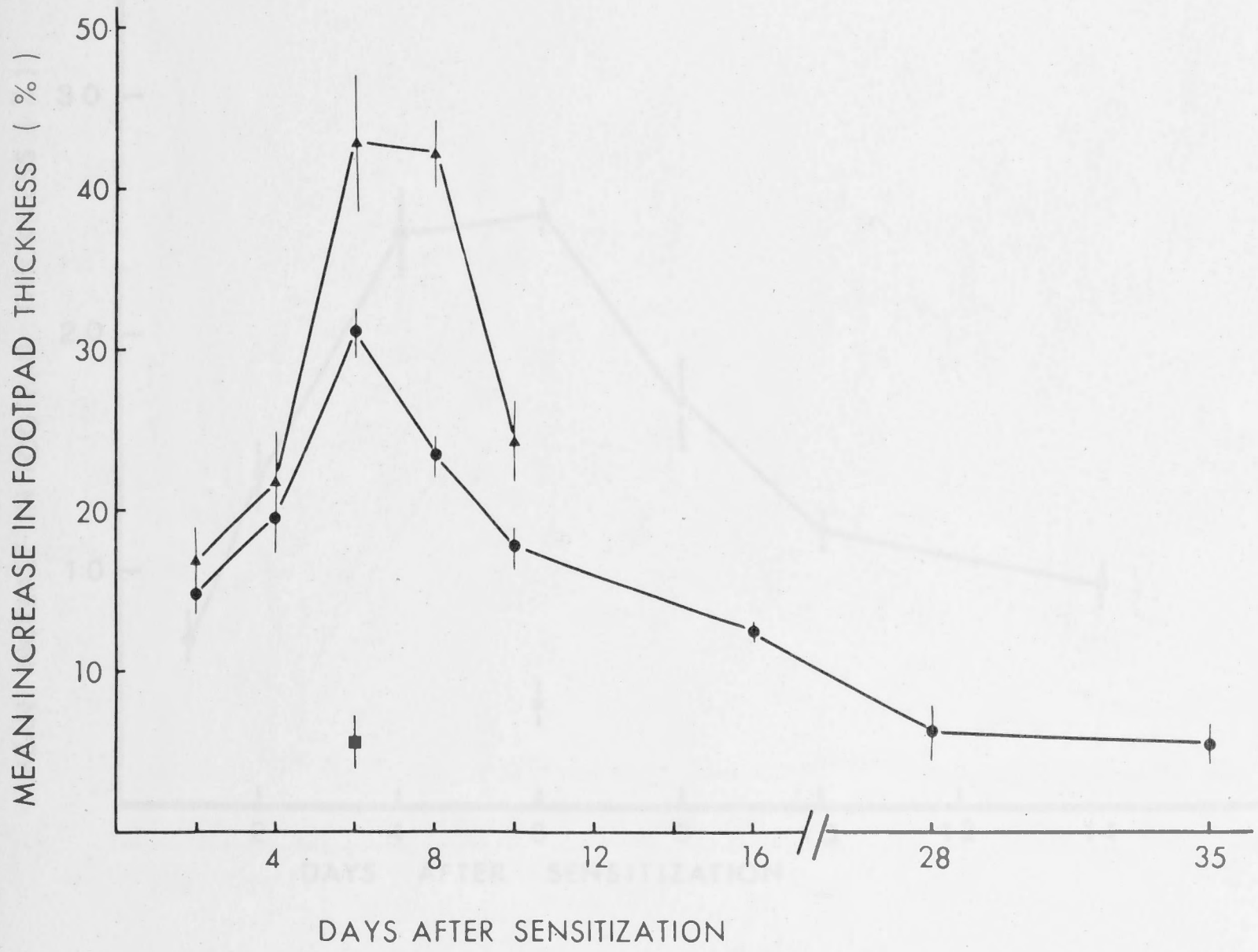


Fig. 2: Kinetics of induction of a DTH response to noninfectious influenza A virus. CBA mice were either unprimed (■), primed s.c. with $5x10^3$ HAU purified UV-inactivated A/WSN virus (●), or Cy pretreated (▲) (200 mg/kg) 2 days before priming (UV-inactivated A/WSN, $5x10^3$ HAU). They were elicited at different intervals after sensitization with UV-inactivated A/WSN virus ($6x10^3$ HAU) and footpad swelling was measured 24 h after virus challenge. Vertical bars represent ± one standard error.

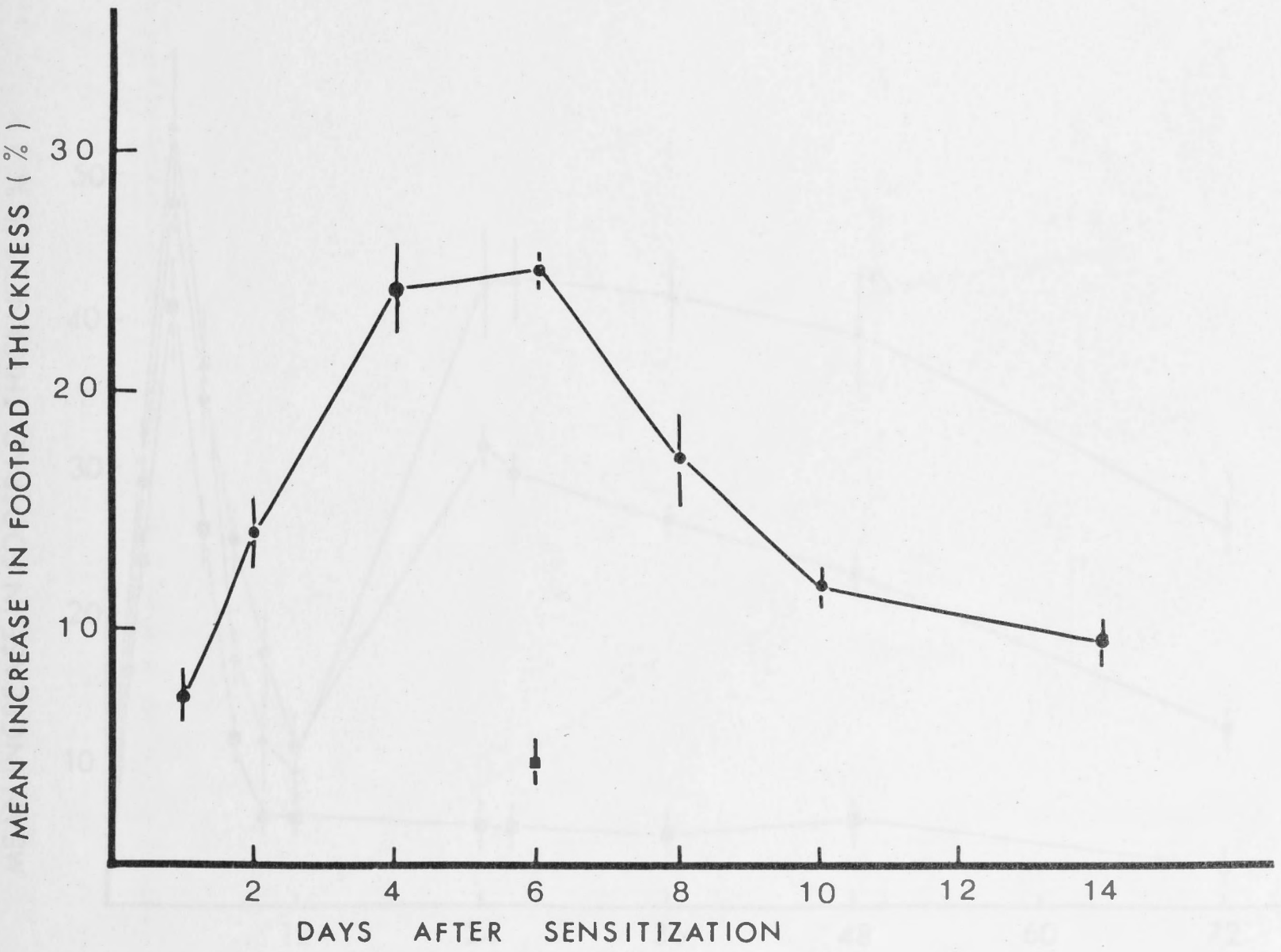


Fig. 3: Kinetics of induction of a DTH response to infectious influenza A virus. CBA mice were either unprimed (■), or primed s.c. with 10^3 HAU infectious A/WSN virus (●). They were elicited at different intervals after sensitization with UV-inactivated A/WSN virus (6×10^3 HAU) and footpad swelling was measured 24 h after virus challenge. Vertical bars represent ± one standard error.

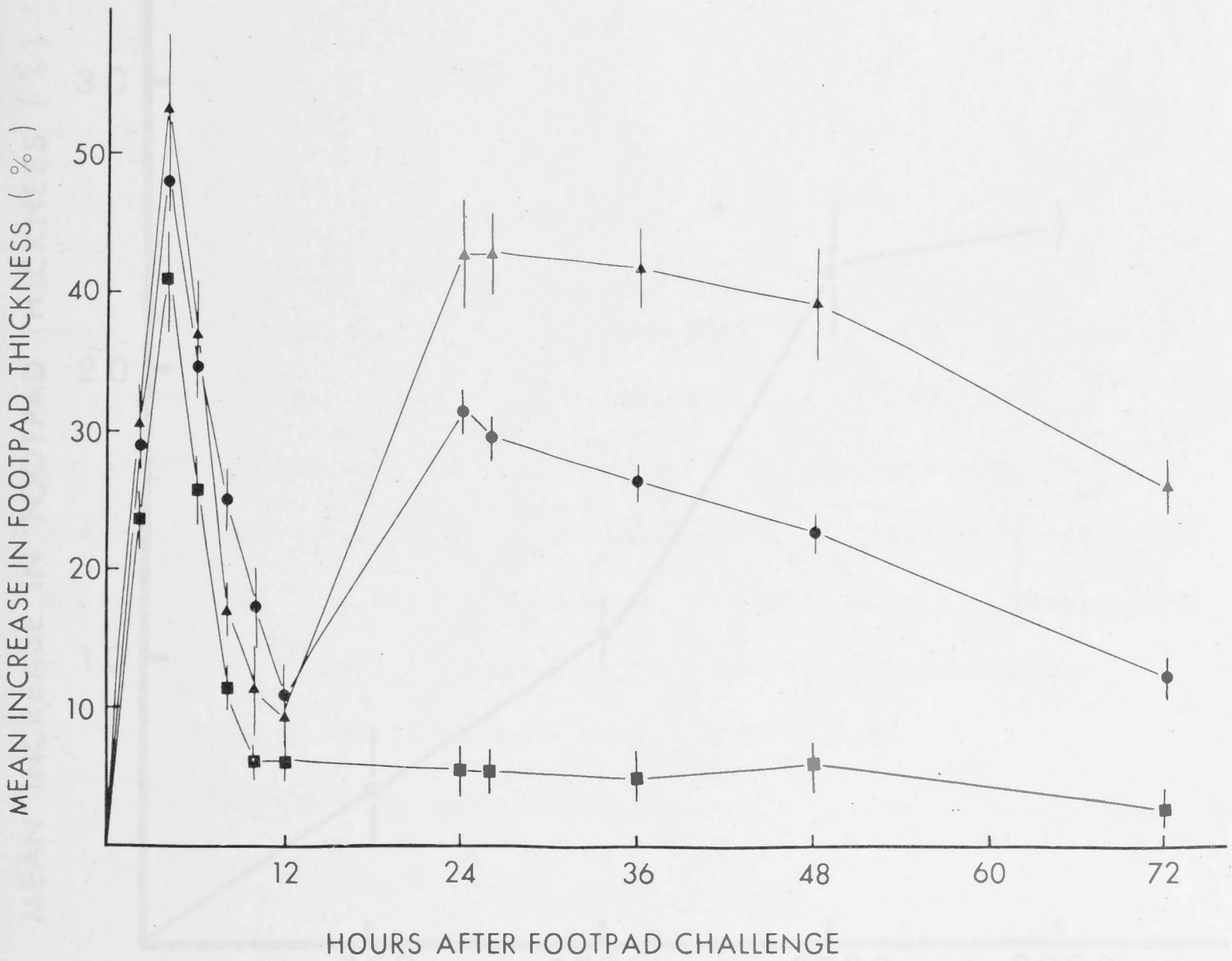


Fig. 4: Development of footpad swelling. Unprimed CBA mice (■), A/WSN primed (UV-inactivated, $5x10^3$HAU) mice (●) and mice that were Cy pretreated (200 mg/kg) 2 days before priming with A/WSN virus (UV-inactivated, $5x10^3$HAU) (▲) were all injected with $6x10^3$HAU purified, UV-inactivated A/WSN virus 6 days after primary sensitization. Footpad swelling was measured over a period of 72 h and vertical bars represent ± one standard error.

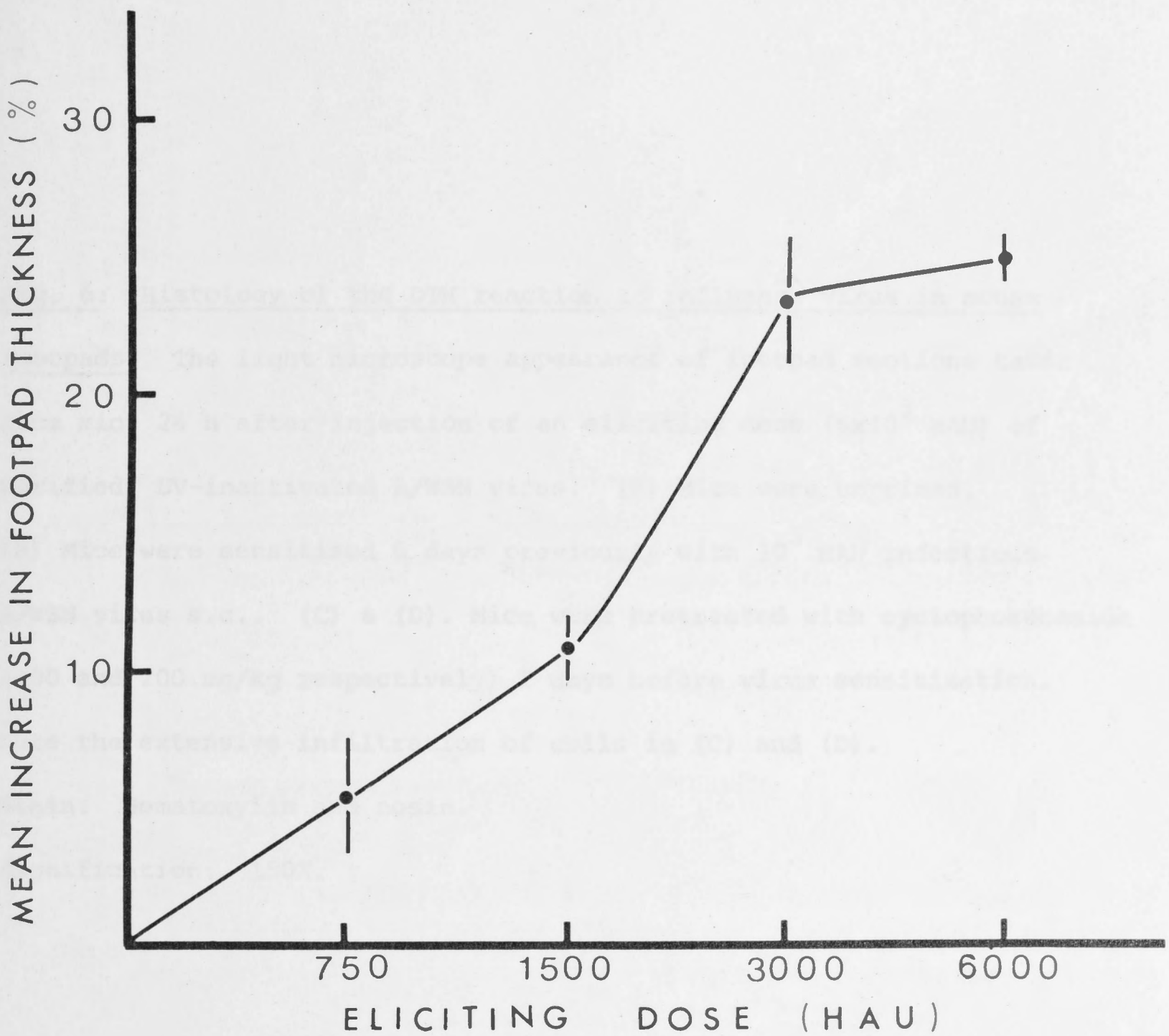
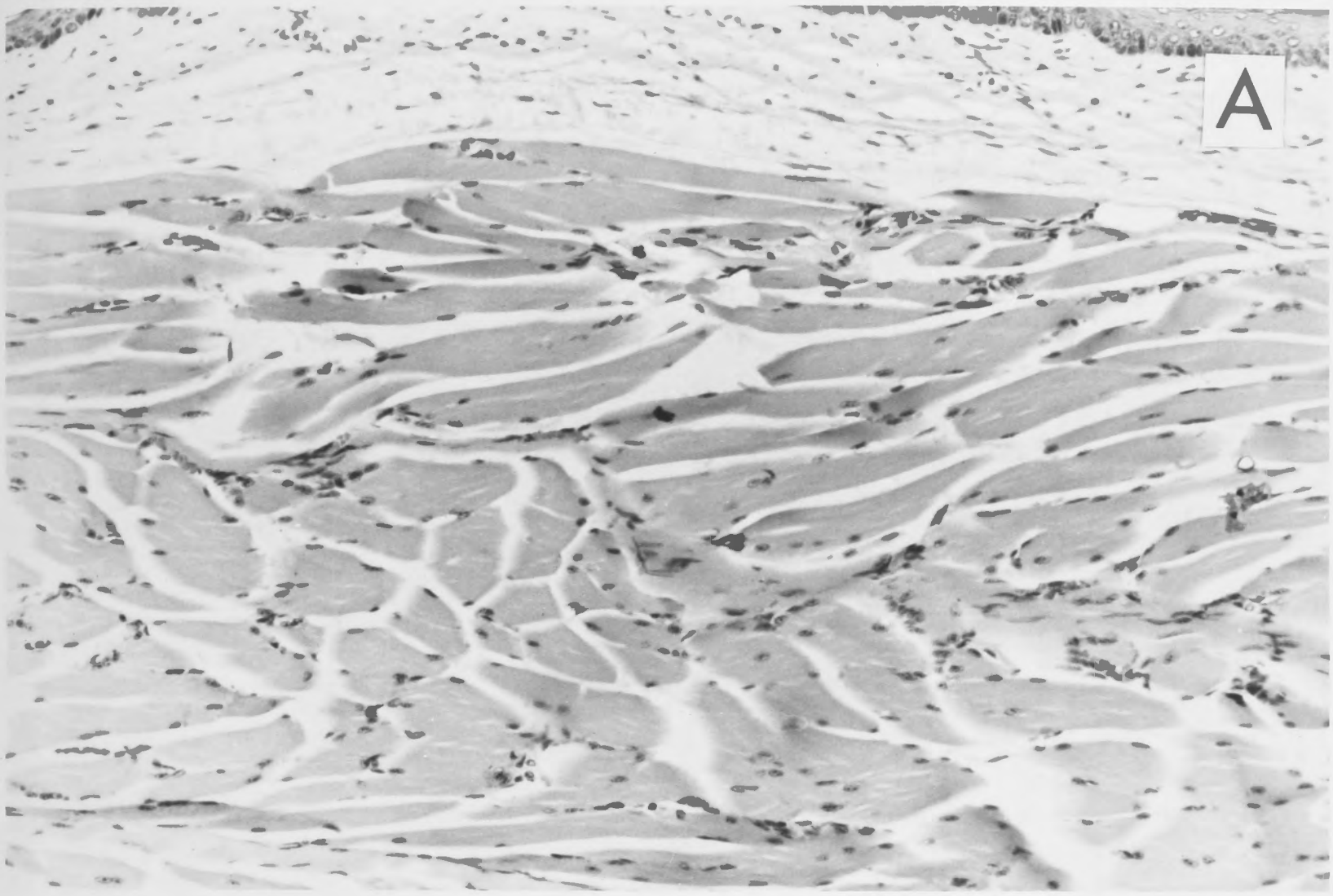


Fig. 5: Virus dose dependency for elicitation of DTH. CBA mice sensitized s.c. with 10^3 HAU infectious A/WSN virus 6 days previously were elicited with different doses of purified, UV-inactivated A/WSN virus. Footpad swelling was measured 24 h after elicitation. Vertical bars represent ± one standard error.

Fig. 6: Histology of the DTH reaction to influenza virus in mouse footpads. The light microscope appearance of footpad sections taken from mice 24 h after injection of an eliciting dose ($6x10^3$ HAU) of purified, UV-inactivated A/WSN virus. (A) Mice were unprimed. (B) Mice were sensitized 6 days previously with 10^3 HAU infectious A/WSN virus s.c.. (C) & (D). Mice were pretreated with cyclophosphamide (100 and 200 mg/kg respectively) 2 days before virus sensitization. Note the extensive infiltration of cells in (C) and (D).

Stain: Hematoxylin and eosin.

Magnification: 150X.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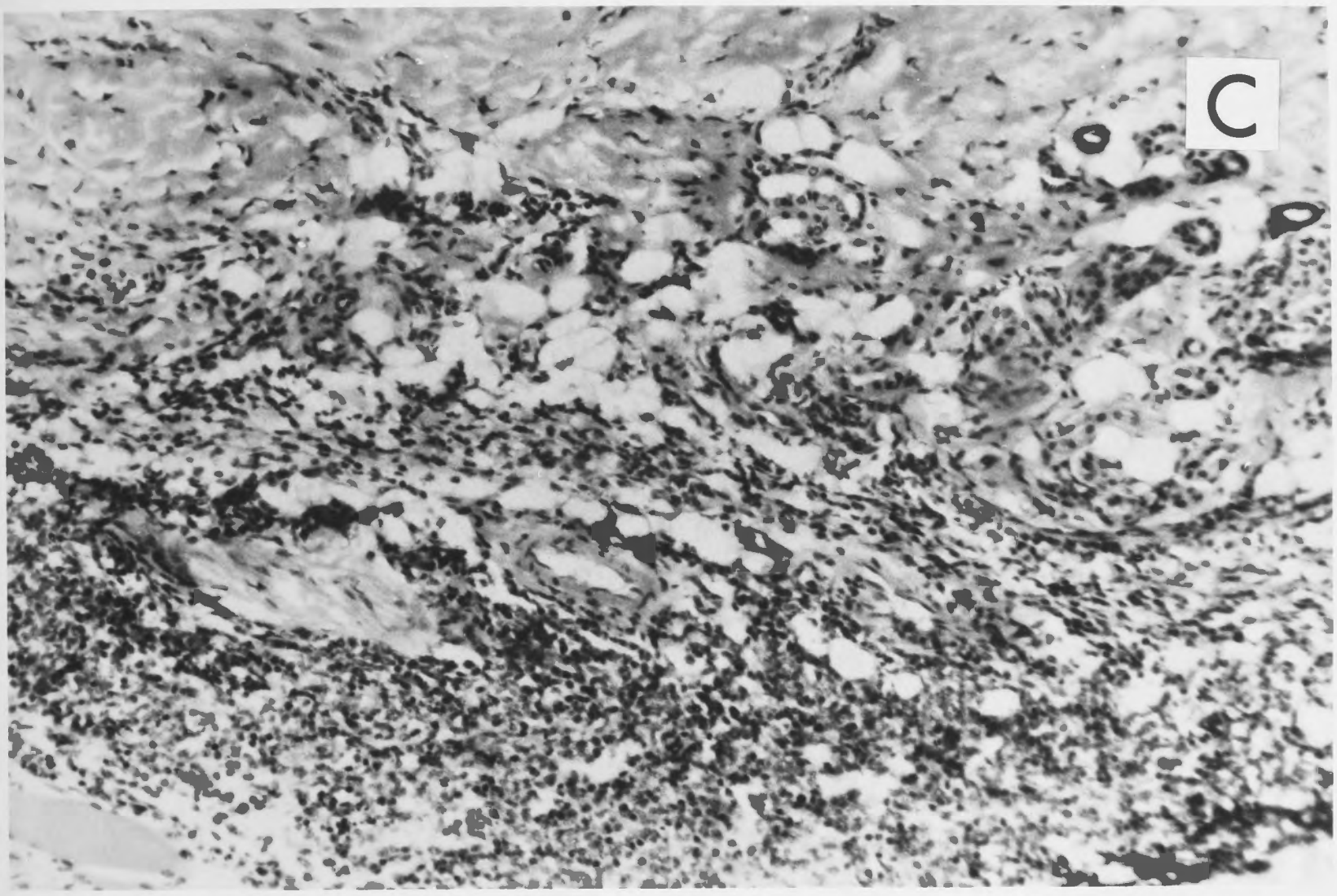
B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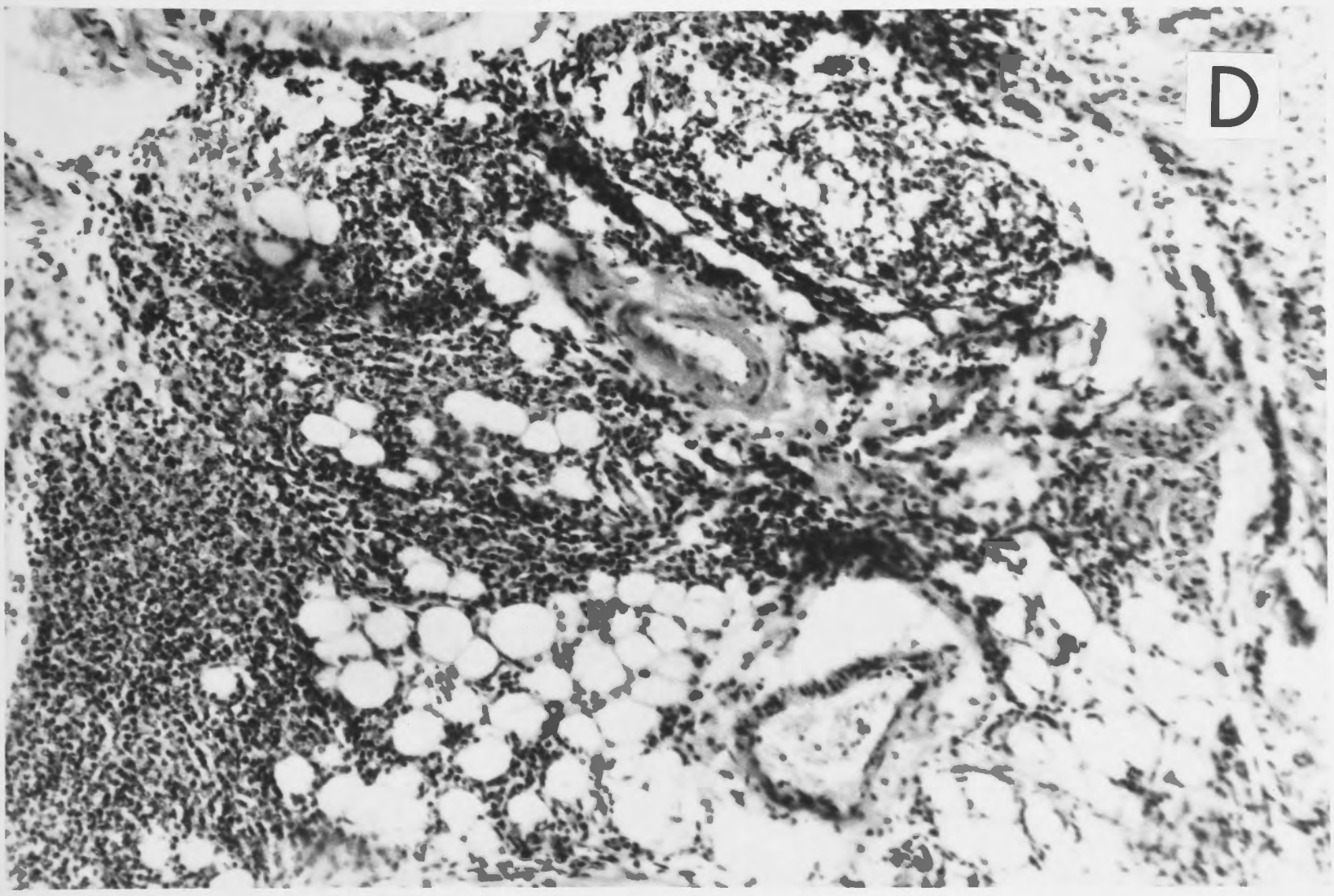

D

CHAPTER 3.2

SPECIFICITY, LYT PHENOTYPE AND H-2 COMPATIBILITY REQUIREMENTS OF EFFECTOR CELLS IN DTH RESPONSES TO MURINE INFLUENZA VIRUS INFECTION

INTRODUCTION

There is currently much interest in the cell-mediated immune response to viral infection which largely derives from the initial finding of Zinkernagel and Doherty (1974a) that cytotoxic T cells (Tc) generated in mice infected with LCM virus would lyse LCM infected target cells only if effector and target cells were compatible at the K,D region in the H-2 gene complex. The findings have been extended to many other systems (Doherty et al., 1976a; Blanden, 1977). Another component of the CMI response to viral infection is the DTH response. Though there are a number of recent reports of DTH responses to viral infections (Tosolini & Mims, 1971; Peters et al., 1975; Lagrange et al., 1978; Kraaijeveld et al., 1979; Hudson et al., 1979), the genetic requirements and antigenic properties of the effector cells (Td) have not been studied in the same detail as have Tc cells. It is generally thought that Td cells require I region compatibility in cell transfer systems (Miller et al., 1975), but in the one example where a viral system was examined (Zinkernagel, 1976), K,D compatibility was required for a successful transfer of DTH. The Lyt antigenic specificity of the cells was not examined.

It has been shown in Chapter 3.1 that the DTH response to influenza virus in mice can be measured reliably by measuring footpad swelling or by localization in the ear of labeled mononuclear cells. The kinetics of development and factors affecting their production were also described. The fine specificity of the effector cells, their Lyt phenotype and requirement for H-2 compatibility are reported in this chapter. The results obtained with influenza virus are different, at least in respect to the last parameter, to the earlier result with LCM virus.

RESULTS

Specificity of the DTH response in mice sensitized with infectious or non-infectious virus

Previous work using mice infected with influenza virus had shown that the population of cytotoxic T cells (Tc) generated to this antigen in spleen would lyse target cells infected with either the homologous virus or any other A strain virus (Braciale, 1977a; Effros et al., 1977; Zweerink et al., 1977a). In contrast, secondary effector Tc cells generated in tissue culture after exposure to UV-inactivated virus yielded potent effector cells which were specific for the homologous virus (Braciale & Yap, 1978). It was previously shown that the DTH response to influenza A virus in mice was specific in that the response in mice sensitized with an A strain virus could be elicited with the homologous virus but not with a B strain virus or Sendai virus (Chapter 3.1). Table 1 compares the fine specificity of the DTH response of mice when infectious or non-infectious virus was used for sensitization and shows that (1). Mice sensitized with infectious virus generate Td cell populations which are cross-reactive within the A strains. (2). Mice sensitized with non-infectious virus generate a Td cell population which is specific for the homologous virus and probably for the haemagglutinin.

The Lyt phenotype of Td cells

The results in Table 2 demonstrate that the cells which, on adoptive transfer to naive mice, confer DTH activity are sensitive to anti-Lyt 1.1 antibody and complement and are not lysed by anti-Lyt 2.1 serum and complement. This is the case for cells from donor mice which were sensitized with either infectious or non-infectious virus.

Requirement for H-2 sharing between donor and recipient strains of mice for expression of DTH

(A) Sensitization of donor mice with UV-inactivated virus

In preliminary experiments, CBA($H\text{-}2^k$), C57BL/6($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice were used as donor and recipient mice of immune cells. Transfer of DTH occurred between syngeneic but not allogeneic mice (Table 3, Expt. 1). However, successful transfer occurs if the donor and recipient mice shared H-2 genes but otherwise differed in their genetic background (Table 3, Expt. 2). Moreover, in studies with (BALB/c x CBA)F_1 mice, transfer of DTH occurred when primed cells from the F_1 were transferred into either parent, but not into another strain (Table 3, Expt. 3). Groups of experiments were then carried out to determine the region or subregion of the H-2 gene complex required for successful cell transfer. The results are shown in Table 4. In all experiments, A/WSN was used to both sensitize and elicit the reaction. Group 1 experiments show that differences in K,D region do not affect the transfer of DTH. Group 2 experiments show that difference at S,G or D region is unimportant but sharing at I region is required for DTH transfer. Group 3 experiments show that difference at IJ subregion is unimportant and that difference at K,D and IA regions prevents transfer of DTH. The experiments in group 4 indicate that sharing at the IA subregion alone is sufficient for DTH transfer to be successful.

Taking all these results together, it is concluded that IA subregion sharing is both necessary and sufficient for DTH to be demonstrated.

(B) Sensitization of donor mice with infectious virus

A similar but more limited series of experiments to those described in the previous section were carried out with the results shown in Table 5. In the first three groups of experiments, the same virus (A/WSN) was used to sensitize for and to elicit the DTH reaction.

Again sharing of the IA subregion is necessary and sufficient for successful transfer of DTH. These results suggest that the T cell subset which was responsible for cross-reactivity between different A strains of virus was also I region restricted, and an extra group of experiments (Expt. 4, Table 5) confirmed this.

DTH activity of secondary effector cell populations with enhanced Tc activity

In view of the earlier demonstration (Zinkernagel, 1976) that DTH in the LCM system was transferred by cells which required K,D sharing between donor and recipient, cell populations were tested which were known to contain influenza virus-specific effector T cells requiring K,D compatibility. Memory cells from mice primed with infectious virus were restimulated in vitro with infectious homologous virus. Such populations have high Tc activity (Yap & Ada, 1977). A/WSN immune cells (1.5×10^7) prepared in this way were injected i.v. into syngeneic (CBA/H) mice and the eliciting virus then injected into the footpad. Measurement of footpad swelling 24-72 h after antigen injection showed a minor, non-significant effect compared to the control. One explanation of this result might be that cultured cells circulate relatively poorly to distant areas, such as feet. To circumvent this, cells (5×10^6) with virus or virus alone were injected directly into the footpad. Feet injected with cells plus virus gave a significant swelling (Table 6) at 24 h and remained swollen until 72 h after injection. However, the effector cells in this preparation which transferred DTH were sensitive to anti-Lyt 1.1 and complement and not to anti-Lyt 2.1 sera and complement (Table 6) and were I region restricted (see Chapter 4.2).

The properties of Td cells from lungs of infected mice

Mice were inoculated i.n. with a lethal dose (5×10^4 EID_{50}) of A/WSN virus. Previous work (Yap & Ada, 1978a) showed that after such

an inoculation, very high levels of infectious virus were present in the lungs 24 h later and these increased until death ensued 7-9 d after infection. The lungs of the infected mice were removed at 6 d, a cell suspension made (Yap & Ada, 1978a) and 1-2x10^6 of these injected with virus into the hind footpads of mice with the same or different H-2 haplotypes as the donor mice. It was found that (1). that I region sharing was necessary for successful transfer of DTH; (2). that the Lyt phenotype of the Td cells from the lungs was Lyt 1. (For data see Chapter 4.1).

DISCUSSION

Earlier work of Yap & Ada (1977, 1978a,b) has described the production of cytotoxic T cells (Tc) in mice infected with influenza virus and produced evidence suggesting that these cells were important in the recovery of mice during infection with these viruses. Part of the evidence that Tc cells were the responsible effector cells was the abrogation of the protective effect when the cell preparation was treated with anti-Lyt 2,3 sera and complement before transfer to syngeneic hosts and the requirement for K,D sharing between the donor of the transferred immune cells and the recipient mouse (Yap *et al.*, 1978). In contrast, the effector cells which effect DTH are considered to be Lyt 1 positive and insensitive to anti-Lyt 2,3 sera and complement (Vadas *et al.*, 1976). Miller and his colleagues (1975) who used the 'ear localization' of labeled cells procedure, showed that DTH to fowl γ-globulin was I region restricted. Weinberger *et al.* (1979) showed that the DTH to the haptens NIP and NP in mice required at least IA compatibility and in a preliminary study, Asherson *et al.* (1979) reported that in the transfer of contact sensitivity in mice to the agent oxazalone, 'the left hand side of the MHC was more important than

the right hand side'. There are two reports that DTH may be K,D restricted. Vadas et al. (1977) found that DTH to the hapten dinitrofluorobenzene (DNFB) was both I and K,D regions restricted, and Zinkernagel (1976) found that cells expressing DTH in the mouse to the virus, LCM, required K,D region compatibility. Zinkernagel suggested that antigen parameters determine whether T cells are specific for 'altered' I or 'altered' K,D coded structures, postulating that multiplying infectious agents like viruses which actively invade cells and interfere in the genetic and metabolic pathways of the cells would alter K and D rather than I coded structures.

Because of the result with LCM and previous transfer experiments which suggested that Tc cells were important in the recovery of mice from influenza virus infection, it was necessary to check the properties of influenza virus specific Td cells. In this chapter, I have (1). characterized cells transferring DTH activity both by their sensitivity to anti-Lyt sera and complement and their H-2 compatibility requirements, and (2). compared the specificity patterns seen with infectious and non-infectious preparations of influenza virus. That infectivity does have an effect is shown clearly by the specificity of the Td cells generated. Injection of UV-inactivated virus yields Td cells which are specific for the homologous virus, and most likely for the haemagglutinin (Table 1). Injection of infectious virus yields Td cells which are cross-reactive within the A strains of influenza virus (Table 1). A similar cross-reactivity has been observed previously (Floc'h & Werner, 1978) although the results quoted were very variable. Irrespective of this difference, all cell preparations showing DTH were sensitive to the action of anti-Lyt 1 and complement and not to anti-Lyt 2 and complement (Table 2). The genetic requirements for successful transfer of DTH are summarized in Fig. 1. In all situations tested, I region and not

K,D region compatibility was necessary; for infectious and non-infectious virus, the requirment was for IA subregion compatibility (Tables 4 and 5).

Despite this clear pattern, the question arose whether cells expressing Lyt 2,3 antigens and a known requirement for K,D compatibility can transfer DTH in this system. In the experiments recorded in Table 4, the cells transferring DTH were generated following sensitization of mice with UV-inactivated virus and such are known to contain negligible Tc activity (Braciale & Yap, 1978). In the experiments recorded in Table 5, the cells transferred would have possessed Tc activity. At this stage, there is no available method for the production of Tc and not Td cells. One of the last two experiments was designed to test the DTH potential of cell populations possessing high Tc activity, and therefore containing K,D restricted and Lyt 2,3 positive cells (Yap et al., 1978). These cells were produced in tissue culture but did not transfer DTH activity when injected i.v. into the recipient mice. However, they caused prolonged footpad swelling when injected directly into the footpad with virus. The responsible cells were Lyt 1 positive and I region restricted. The other experiment was to test cell populations from infected lungs which, in contrast to spleen, are known to contain high levels of infectious virus (Yap & Ada, 1978a). Such cell populations also contain Tc activity (Yap & Ada, 1978a), yet upon transfer into footpads with virus the observed DTH was again due to Lyt 1 positive cells which required I region compatibility. It seems therefore that the property of infectivity per se does not determine the genetic requirements which the cells transferring DTH will display.

It should be emphasized that these results do not unequivocally show that Lyt 2,3 positive influenza immune cells which recognize K,D gene products cannot transfer DTH; they show that the major activity is

carried by Lyt 1 positive cells which require IA compatibility. What then is the explanation for the difference between LCM and influenza virus infections? One obvious difference is the kinetics of the DTH reaction. The footpad swelling observed both in the non-transfer experiments and in those experiments involving transfer of influenza virus specific immune spleen cells reached peak values at 24 h, the value at 72 h being much reduced. In contrast, the reaction observed after transfer of LCM immune spleen cells usually peaked at 48-72 h and on occasions there was minimal swelling at 24 h (Zinkernagel, 1976). Though it could be postulated that migration properties of the two cell populations differed, the possibility remains that the effector cells differed in other respects apart from surface antigen and H-2 antigen recognition, such as lymphokine production.

The results in this chapter suggest that the influenza virus specific Td cells, like Tc cells (Braciale, 1977a, 1979; Effros et al., 1977; Zweerink et al., 1977a,b) recognize HA and possibly another viral antigen. In fact, the two T cell subsets show some similarities in this respect. If non-infectious virus was used for stimulation, both primary Td cells (in vivo) and secondary Tc cells (in vitro) were specific for the homologous virus. If infectious virus was used for stimulation, both primary Td and Tc cells generated in vivo showed cross reactivity with the A strains of virus. It seems as though the influenza viral antigens which are recognized by these effector cells may be associated on stimulator cells with both K,D and I region products. The identity of the viral antigen(s) recognized by LCM effector cells is unknown but it can be speculated that it might associate more effectively with K,D than with I region products.

SUMMARY

Delayed-type hypersensitivity (DTH) to infectious and to non-infectious (UV-inactivated) influenza A viral preparations was measured in mice by the increase in footpad swelling 24 h after injection of the eliciting virus. Mice sensitized with non-infectious virus were elicited only by virus which shared haemagglutinin specificity with the sensitizing virus, whereas footpad injection of a given A strain virus (A/WSN) could elicit DTH in mice sensitized with a variety of infectious A strain viruses, including some not sharing haemagglutinin or neuraminidase specificities. The effector T cells generated in mice sensitized with either form of virus were sensitive to anti-Lyt 1 antibody and complement, but not to anti-Lyt 2 antiserum and complement. Adoptive transfer of DTH was H-2 restricted. Sharing of the IA sub-region was both necessary and sufficient for successful transfer to occur, using spleen cells from mice sensitized s.c. with either infectious or non-infectious virus. Cells recovered from infected mouse lungs and secondary effector cells generated *in vitro* transferred DTH if injected into the footpad with the eliciting virus. The effector cells had the Lyt 1 phenotype and, in both cases, the cells were I region restricted. These results contrast with earlier findings that transfer of DTH to LCM virus infection required K,D sharing between donor and recipient. Thus, the earlier hypothesis that multiplying infectious agents like viruses would 'alter' K and D rather than I region coded structures is not generally correct.

TABLE 1

A Comparison of the Specificity of the DTH Response in Mice Sensitized with Infectious or Non-Infectious Influenza Virus[a]

Strain	Sensitizing virus infectivity[b]	Challenge virus	Mean increase in footpad thickness at 24 h (%)
Normal Allantoic Fluid	N.I.	A/WSN (NON1)	6.3 ± 1.3
A/WSN	I.	A/WSN	32.8 ± 2.0[c]
	N.I.	A/WSN	33.6 ± 2.0[c]
A/RI (H2N2)	I.	A/WSN	23.4 ± 1.6[c]
	N.I.	A/WSN	4.4 ± 0.8
A/PC (H3N2)	I.	A/WSN	26.6 ± 3.3[c]
	N.I.	A/WSN	7.1 ± 0.8
B/LEE	I.	A/WSN	3.1 ± 1.3
	N.I.	A/WSN	6.3 ± 1.0
A/WSN	N.I.	A/RI (H2N2)	2.4 ± 0.8
A/RI	N.I.	A/RI	29.7 ± 2.0[c]
A/PC	N.I.	A/RI	6.9 ± 1.2
A/JAP-BEL (H2N1)	N.I.	A/RI	28.1 ± 1.3[c]

a Groups of 4-5 CBA mice were injected s.c. with infectious or non-infectious virus; 6 d later they were challenged into the footpad with purified, UV-inactivated A/WSN virus ($6x10^3$ HAU), footpad swelling was measured 24 h after virus challenge. Details are given in Materials and Methods (Chapter 2).

b I. = infectious; N.I. = non-infectious.

c Significantly greater than control group of mice (sensitized with normal allantoic fluid only). $p < 0.001$.

TABLE 2

The Effect of Anti-Lyt Sera on the Ability of Immune Cells, Taken from Mice Sensitized with Infectious or Non-Infectious Virus to Transfer DTH[a]

Sensitizing virus		Cell transfer	Treatment of cells[d]	Injection into footpad	Mean increase in footpad thickness at 24 h (%)
Strain	Infectivity[b]				
A/WSN (HON1)	I.	-[c]	-	Virus[g]	31.3 ± 1.3
A/WSN	I.	+	Nil	Cells	2.1 ± 1.1
A/WSN	I.	+	Nil	Cells + virus	25.0 ± 1.0
A/WSN	I.	+	Anti-Lyt 1.1 + C'	Cells + virus	6.3 ± 1.3[e]
A/WSN	I.	+	Anti-Lyt 2.1 + C'	Cells + virus	25.6 ± 1.6[f]
A/WSN	N.I.	-[c]	-	Virus	33.6 ± 2.0
A/WSN	N.I.	+	Nil	Cells + virus	25.0 ± 1.3
A/WSN	N.I.	+	Anti-Lyt 1.1 + C'	Cells + virus	5.5 ± 0.8[e]
A/WSN	N.I.	+	Anti-Lyt 2.1 + C'	Cells + virus	25.0 ± 2.2[f]
Nil	-	-[c]	-	Virus	3.1 ± 1.8
A/PC (H3N2)	I.	-[c]	-	Virus	33.3 ± 2.8
A/PC	I.	+	Nil	Cells + virus	26.6 ± 2.0
A/PC	I.	+	Anti-Lyt 1.1 + C'	Cells + virus	4.2 ± 1.1[e]
A/PC	I.	+	Anti-Lyt 2.1 + C'	Cells + virus	28.9 ± 1.5[f]

Legends for Table 2

a Local adoptive transfer of virus-specific DTH response was performed 6 d after sensitization of CBA mice. Details were described in Materials and Methods (Chapter 2).

b I. = infectious; N.I. = non-infectious.

c No cell transfer means sensitization and elicitation of DTH reaction occurred in the same animal.

d The Ig^- fraction of day 6 immune cells was either untreated or treated with Anti-Lyt + C' and same number of viable cells were injected into the footpad in each case.

e Significantly lower than the control (no treatment of cells) ($p < 0.01$).

f Not significantly different from the control (no treatment of cells).

g Purified A/WSN virus (6×10^3 HAU) was used for elicitation.

TABLE 3

MHC Control of Successful Transfer of DTH Response

Donor strains	Sensitization[a]	Recipient strains	Mean increase in footpad thickness at 24 h (%)
Experiment 1			
None	-	CBA	5.5 ± 0.8
CBA ($H\text{-}2^k$)	-[b]	CBA	5.5 ± 2.0
CBA	+	CBA	29.7 ± 1.6
BALB/c ($H\text{-}2^d$)	+	CBA	7.1 ± 0.8
C57BL/6J	-[b]	CBA	2.4 ± 0.8
C57BL/6J	+	CBA	5.5 ± 0.8
C57BL/6J	-[b]	C57BL/6J	3.9 ± 0.8
C57BL/6J	+	C57BL/6J	21.9 ± 1.8
CBA	+	C57BL/6J	4.3 ± 0.7
BALB/c	+	C57BL/6J	3.1 ± 0.0
Experiment 2			
DBA/2 ($H\text{-}2^d$)	+	DBA/2	29.7 ± 0.8
BALB/c	+	DBA/2	28.1 ± 1.8
DBA/2	+	BALB/c	30.5 ± 0.8
Experiment 3			
(BALB/c x CBA)F_1	+	(BALB/c x CBA)F_1	34.4 ± 3.4
(BALB/c x CBA)F_1	+	C57BL/6J	6.3 ± 3.1
(BALB/c x CBA)F_1	+	BALB/c	27.1 ± 1.1
(BALB/c x CBA)F_1	+	CBA	21.1 ± 0.8

a Donor mice were sensitized with $5x10^3$ HAU purified, UV-inactivated A/WSN virus s.c. Six days later, $8x10^7$ immune spleen cells were transferred i.v. to each naive recipient which was challenged with $6x10^3$ HAU purified, UV-inactivated A/WSN 6 h after cell transfer. Footpad swelling was measured 24 h after challenge injection.

b Transfer of normal spleen cells ($8x10^7$) to recipients.

TABLE 4

The Adoptive Transfer, Using Various Strain Combinations, of DTH Using Cells from Donor Mice Sensitized with Non-Infectious Influenza Virus

Mouse strains		H-2 Regions shared	Mean increase in footpad thickness at 24 h (%)
Donor[a]	Recipient		
Experiment 1			
A.TL	A.TL	All	31.3 ± 0.0
CBA	A.TL	I	30.1 ± 1.3
CBA	CBA	All	29.7 ± 1.6
A.TL	CBA	I	33.3 ± 3.9
Experiment 2			
A.TH	A.TH	All	24.3 ± 1.5
A.TL	A.TH	K,D	7.1 ± 0.8[b]
SJL	A.TH	K,I	23.9 ± 1.1
A.TL	A.TL	All	26.3 ± 0.8
A.TH	A.TL	K,D	7.1 ± 2.0[b]
SJL	A.TL	K	2.1 ± 1.1[b]
C3H.OH	C3H.OH	All	30.5 ± 3.1
C3H.OL	C3H.OH	All except S,G	27.4 ± 2.4
C3H.OL	C3H.OL	All	31.3 ± 1.3
C3H.OH	C3H.OL	All except S,G	30.5 ± 2.0
Experiment 3			
B10.A(5R)	B10.A(5R)	All	27.1 ± 1.1
B10.A(3R)	B10.A(5R)	All except IJ	26.1 ± 1.1
B10.A(3R)	B10.A(3R)	All	25.0 ± 0.9
B10.A(5R)	B10.A(3R)	All except IJ	31.3 ± 0.0
B10.A(2R)	B10.A(2R)	All	27.1 ± 3.8
B10.A(4R)	B10.A(2R)	K, IA, D	28.1 ± 2.2
B10.A(4R)	B10.A(4R)	All	26.1 ± 2.8
B10.A(2R)	B10.A(4R)	K, IA, D	25.0 ± 1.3
Experiment 4			
B10.A(4R)	B10.A(4R)	All	34.3 ± 2.6
B10.A(4R)	B10.AQR	IA	33.8 ± 0.8
B10.A(4R)	CBA	K, IA	29.7 ± 1.6
B10.A(4R)	C57BL/6J	All except K,IA	4.7 ± 1.6[b]

a Donor spleen cells ($8x10^7$) were transferred i.v. to recipient mice 6 d after sensitization. A/WSN virus was used to sensitize mice and to elicit DTH.

b These values are significantly lower than control values of mice which received syngeneic immune cells ($p < 0.01$).

TABLE 5

The Adoptive Transfer, Using Various Strain Combinations, of DTH Using Cells from Donor Mice Sensitized with Infectious Influenza Virus

Mouse strains		H-2 Regions shared	Mean increase in footpad thickness at 24 h (%)
Donor[a]	Recipient		
Experiment 1			
CBA	CBA	All	23.4 ± 0.9
A.TL	CBA	I	23.4 ± 1.6
A.TL	A.TL	All	22.7 ± 1.5
CBA	A.TL	I	23.9 ± 2.1
A.TH	A.TL	K,D	1.1 ± 1.1[b]
A.TH	A.TH	All	22.7 ± 2.0
A.TL	A.TH	K,D	3.1 ± 1.8[b]
Experiment 2			
B10.A(2R)	B10.A(2R)	All	24.3 ± 2.0
B10.A(4R)	B10.A(2R)	K,IA,D	24.4 ± 1.2
B10.A(4R)	B10.A(4R)	All	25.0 ± 2.0
B10.A(2R)	B10.A(4R)	K,IA,D	24.3 ± 1.5
Experiment 3			
B10.AQR	B10.AQR	All	37.2 ± 1.9
B10.AQR	B10.A(4R)	IA	40.5 ± 0.8
Experiment 4			
A.TL	A.TL	All	26.6 ± 4.7
A.TH	A.TL	K,D	3.9 ± 0.8[b]
CBA	A.TL	I	25.8 ± 0.8
CBA	CBA	All	27.4 ± 1.5
A.TL	CBA	I	25.8 ± 1.5
A.TH	A.TH	All	26.6 ± 1.6
A.TL	A.TH	K,D	2.4 ± 0.8[b]

Legends for Table 5

a Donor spleen cells ($8x10^7$) were transferred i.v. to recipient mice 6 d after sensitization. In experiments 1, 2 and 3, A/WSN virus was used to sensitize mice and to elicit DTH. In experiment 4, mice were sensitized with A/PC virus and DTH elicited with A/WSN virus.

b These values are significantly lower than the control values of mice which received syngeneic immune cells ($p < 0.01$).

TABLE 6

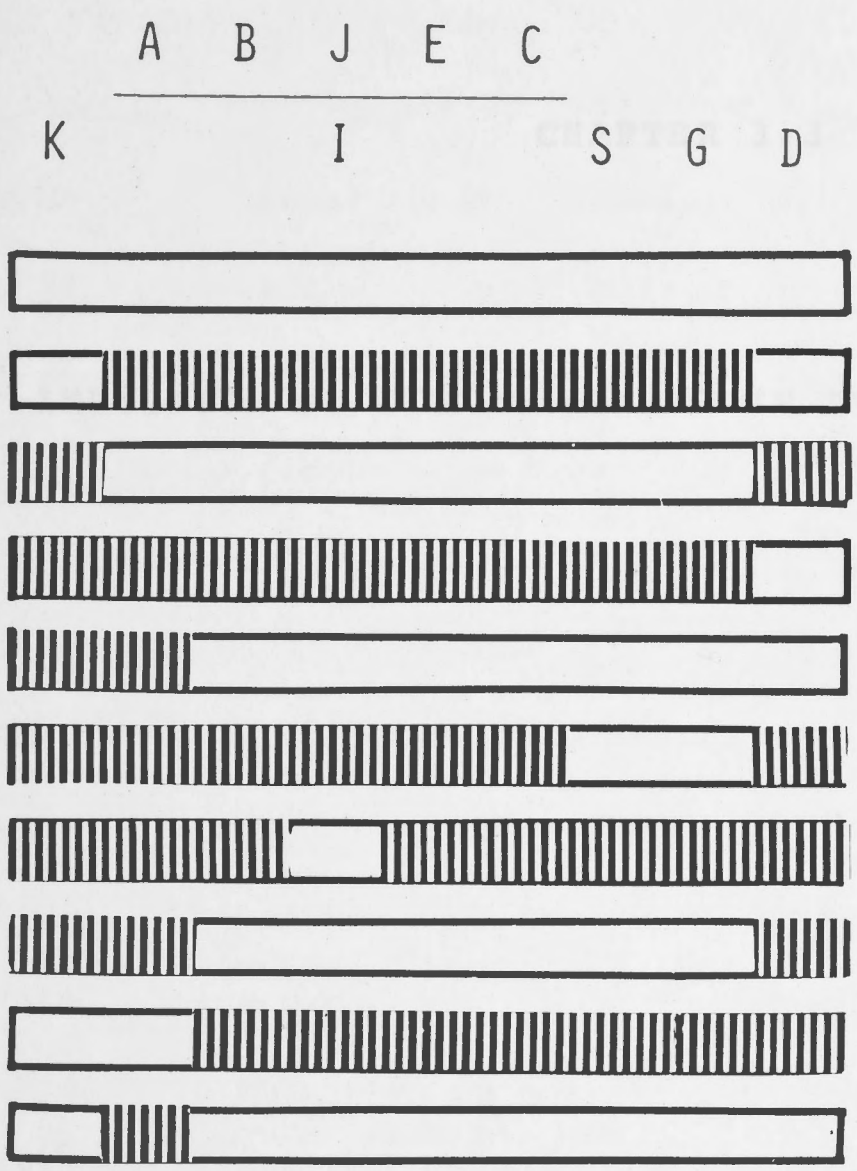
The Effect of Anti-Lyt Sera on the Ability of Secondary Immune Cells Generated in Tissue Culture to Transfer DTH

Tissue culture preparation of cells[a]	Treatment of cells	Injected into footpad	Mean increase in footpad thickness at 24 h (%)
No cells	-	Virus	3.1 ± 0.0
Infected stimulator plus memory spleen cells	-	Cells	2.4 ± 0.8
Infected stimulator plus memory spleen cells	-	Cells + virus	35.2 ± 2.0
Infected stimulator plus memory spleen cells	Anti-Lyt 1.1 + C'	Cells + virus	7.3 ± 1.1[b]
Infected stimulator plus memory spleen cells	Anti-Lyt 2.1 + C'	Cells + virus	39.1 ± 3.0[c]
Uninfected stimulator plus memory spleen cells	-	Cells + virus	9.4 ± 1.3

a Preparation described under Materials and Methods (Chapter 2). A/WSN virus was used to sensitize mice and to elicit DTH.

b Significantly lower than the control (no treatment of cells) ($p < 0.01$).

c Not significantly different from the control (no treatment of cells).



	INFECTIOUS VIRUS	
NON-INFECTIOUS VIRUS	TOTAL	CROSS REACTIVE
-	-	N.D.
+	+	+
-	-	-
+	N.D.	N.D.
+	N.D.	N.D.
+	N.D.	N.D.
+	N.D.	N.D.
-	N.D.	N.D.
+	+	N.D.

Fig. 1: MHC control of successful transfer of DTH to influenza virus. Summary of the genetic requirements for transfer of DTH, as described in Tables 3-5. Shared (▥) and unshared (□) regions or subregions of the H-2 gene complex between donor and recipient mice. Successful (+) and unsuccessful (-) transfer. N.D.: not done.

CHAPTER 3.3

INDUCTION OF DTH RESPONSES IN ATHYMIC (NUDE) MICE

INTRODUCTION

It is generally thought that T cell differentiation and maturation take place in the thymus which then give rise to immunocompetence and diversity of T cell repertoire (Stutman, 1978). Nude (nu/nu) mice which lack a thymus gland are known to be generally devoid of T cell functions (Pantelouris, 1968; Kindred, 1979). However,asmall number of Thy 1 positive cells have been detected in spleens of nude mice born to either homozygous or heterozygous mothers (Raff, 1973; Loor et al., 1976), although their functional significance is far from understood. More recently, Kirchner et al. (1980) have shown that Thy 1 positive cells in nude mouse spleen cells were required for interferon production when the nude spleen cells were stimulated by PHA. Gillis et al.(1979), on the other hand, have reported that Thy 1 positive cytotoxic effector cells against alloantigens can be generated foom nude lymphoid cells in vitro in the presence of allogeneic stimulator cells and supernatants from Concanavalin A (Con A)-activated rat spleen cells. Subsequently, a number of laboratories have confirmed that nude mice do contain cytotoxic T cell precursors. Thus, alloreactive cytotoxic T cells (Tc) and H-2 restricted Tc cells against TNP-modified syngeneic cells have been shown to be generated in nude mice in vivo (Wagner et al., 1980b,Hale, 1980) or from nude lymphoid cells in vitro (Hünig & Bevan, 1980; Ando & Hurme, 1981). However, the development of a T cell response to viral antigens has not yet been reported in nude mice. In this chapter, the in vivo induction of a DTH response to influenza virus in nude mice was investigated. It was found that a DTH response to influenza virus can be generated in nude mice if Con A-activated spleen cells supernatant (CS) was also injected into the mice. The characteristics

of the DTH effector cells in nude mice are also described.

RESULTS

In vivo primary induction of a DTH response to influenza virus in nude mice

BALB/c nude (nu/nu) mice were divided into four groups. Group A mice were unprimed, group B mice were primed with 10^3HAU infectious A/WSN virus subcutaneously (s.c.) and group C mice were primed s.c. with 10^3HAU infectious A/WSN virus and then injected intravenously (i.v.) with 0.1 ml CS for 3 consecutive days. Group D mice were the controls which received CS only. All mice were challenged with UV-inactivated A/WSN virus ($6x10^3$HAU) into the right hind footpads 6 days after virus sensitization and footpad swelling was measured 24 h later. The results in Table 1 show that unprimed mice or mice injected with virus or CS only did not induce any significant DTH response. In contrast, injection of CS into virus-sensitized nude mice induced a very significant DTH response, with a magnitude comparable to that obtained with normal BALB/c mice (group E). Furthermore, the DTH response was found to be virus-specific as sensitization of nude mice with A/WSN virus can only be elicited with A/WSN virus but not with Sendai virus (data not shown).

The next few experiments were performed to determine the optimum conditions for the generation of a DTH response in nude mice. Firstly, the batch of CS used for injection of nude mice was characterized by testing for its lymphokine activity. It was found that it possesses both costimulator activity as well as maintenance activity (data not shown)as determined by the standard assays (Lafferty et al., 1980). Secondly, the dependence of nude mice on CS on the induction of a DTH response was studied. Results

in Table 2 show that a single injection of CS into nude mice at the same time of virus sensitization induced a low but significant level of DTH response. However, consecutive injection of CS for 2 or 3 days gave better responses. Finally, the kinetics of induction of a DTH response in nude mice were also investigated. The results obtained are very similar to that seen in normal mice (Chapter 3.1). No significant DTH activity can be detected in nude mice 2 days after virus sensitization. The % mean increase in footpad thickness at day 4 and day 6 after virus sensitization were 20.3±1.6 and 31.6±3.1 respectively, values that are significantly higher than that of the controls (mice injected with CS only: 1.5±1.5).

Characterization of DTH effector cells in nude mice

The effector cells mediating DTH response in nude mice were characterized by systemic (i.v.) transfer of spleen cells from nude mice primed with virus and CS into normal recipient mice which were then challenged with UV-inactivated virus. It was found that the DTH response could be transferred by i.v. injection of $8x10^7$ 6 days immune nude spleen cells into normal BALB/c recipients. The ability of the cells to transfer DTH activity was abolished by anti-Thy 1.2 ascitic fluid and complement treatment (Table 3, Expt. 1). Successful transfer of DTH activity occurred when donors and recipients shared K,D and I regions or K and I regions but not D region alone (Table 3, Expt.2).

DISCUSSION

This chapter demonstrates that a DTH response to influenza virus can be generated in nude mice if Con A-activated supernatant is injected into nude mice in addition to the sensitizing virus. The induced DTH response shows virus specificity as sensitization with A/WSN virus can only be elicited with homologous A/WSN virus but not with heterologous Sendai virus.

The effector cells are found to be Thy 1 positive and show H-2 restriction. Thus, it is quite clear that the DTH response induced in nude mice has the same basic characteristics as that induced in normal mice (Chapters 3.1, 3.2). Although nude mice are known to have small number of Thy 1 positive cells and possibly ectopic thymic tissues (Kindred, 1979), the T cell functions in nude mice are usually severely depressed or completely absent. However, there is one report which claimed that 'normal' nude mice could give a good T cell response to monomeric flagellin, as measured in an *in vitro* test for helper activity and in an *in vivo* test for DTH activity (Kirov, 1974). The author suggested that the mice may have been primed by flagellar or cross-reacting antigens due to previous exposure to bacterial antigens. In contrast, this chapter shows that injection of influenza virus alone did not induce any significant DTH response in nude mice. However, the present results show that nude mice do contain precursor DTH T cells that can be activated *in vivo* by a viral stimulus and Con A-activated supernatant with the resulting generation of Thy 1 positive DTH effector cells. Therefore, these results are parallel to those of Wagner *et al.* (1980 b) who found that injection of T cell-derived helper factors (interleukin 2) allowed the *in vivo* induction of cytotoxic T cells against alloantigens in nude mice. Similarly, the presence of cytotoxic T cell precursors in nude mice was also reported by a number of laboratories (Hale, 1980; Hünig & Bevan, 1980 ; Ando & Hurme, 1981). On the other hand, nude mice derived helper T cells for antibody response have been described (Ishikawa & Saito, 1980; Stötter *et al.*, 1980). Interesting enough, Kirchner *et al.* (1980) have reported that nude mice do contain a subset of T cells that respond to PHA but not Con A with interferon production. Taking these altogether it is clear that nude mice do have T cells and precursor T cells for a variety of T cell functions which can be activated under appropriate

conditions both *in vivo* and *in vitro*.

It has been well established that Con A-activated supernatants contain lymphokines that are essential for the activation of T cell response to alloantigens or mitogens (costimulator activity) and also for the continuous proliferation of activated T cells (maintenance activity) (Woolnough & Lafferty, 1979). The present data shows that Con A-activated supernatant with both costimulator and maintenance activities *in vitro* could also have activity *in vivo* by restoring a T cell function in nude mice. The mechanism of induction of a DTH response in nude mice is unknown. It has been suggested that the production of a T cell helper factor in GvH reaction *in vivo* (Hale, 1980) or the injection of a T helper factor into the animal (Wagner *et al.*, 1980b;Stötter *et al.*,1980) may circumvent a defect within the nude mice and thus lead to the generation of a T cell response. The possibility that the injection of Con A-activated supernatant either provides a function which is totally absent from the nude mice or simply enhances a functional activity which is present in low level in nude mice have not yet been distinguished.

SUMMARY

A DTH response to influenza virus can be generated in athymic BALB/c nude (nu/nu) mice if Concanavalin A-activated spleen cell supernatant is injected into the mice in addition to the sensitizing virus. The response shows similar kinetics and specificity as seen in normal mice. The DTH effector cells are found to be Thy 1 positive and show H-2 restriction. The possibility that nude mice do have T cells and precursor T cells for a variety of T cell functions is discussed.

TABLE 1

In Vivo Induction of a DTH Response to Influenza Virus in Nude Mice[a]

Group	Mice	Injection of mice with		Mean increase in footpad thickness at 24 h (%)
		Virus	CS	
A	Nude	-	-	4.7 ± 1.6
B	Nude	+	-	7.3 ± 1.1
C	Nude	+	+	35.4 ± 2.1[b]
D	Nude	-	+	4.7 ± 1.6
E	Normal	+	-	38.6 ± 2.8

a Normal BALB/c or BALB/c nude (nu/nu) mice in group of 3-4 were injected with infectious A/WSN virus (10^3 HAU, s.c.) and/or CS (Con A-activated cell supernatant, i.v. injection of 0.1 ml for the first 3 consecutive days). All mice were challenged with UV-inactivated A/WSN virus ($6x10^3$ HAU) into the right hind footpads 6 days after virus sensitization and footpad swelling was measured 24 h later.

b Significantly higher than control group A, $p<0.001$

TABLE 2

Dose Dependency of Con A-Activated Supernatant (CS) on the Induction of a DTH Response in Nude Mice

Virus sensitization [a]	Number of days of injection of CS into nude mice [b]	Mean increase in footpad thickness at 24 h [c] (%)
-	3	4.7 ± 1.6
+	-	4.2 ± 2.8
+	1	14.6 ± 2.1
+	2	25.0 ± 0.0
+	3	28.1 ± 1.8

a BALB/c nude mice in groups of 3 were primed s.c. with 10^3 HAU infectious A/WSN virus.

b CS was injected i.v. in 0.1 ml volume for the first 1-3 days. The first injection was always given at the same time as the sensitizing virus.

c All mice were elicited with UV-inactivated A/WSN virus (6×10^3 HAU) 6 days after virus sensitization and footpad swelling was measured 24 h later.

TABLE 3

Characterization of DTH Effector Cells in Nude Mice[a]

Experiment no.	Sensitization with		Mouse strain			Treatment of effector cells	Mean increase in footpad thickness at 24 h (%)
	Virus	CS	Donor	Recipient	H-2 region shared		
1	+	-	BALB/c nude	BALB/c	All	Nil	6.3 ± 1.8
	+	+	BALB/c nude	BALB/c	All	Nil	34.4 ± 3.1
	+	+	BALB/c nude	BALB/c	All	Anti-Thy 1.2 + C'[b]	4.2 ± 2.1
2	+	+	BALB/c nude	BALB/c	All	Nil	28.1 ± 3.1
	+	+	BALB/c nude	C3HOH	All except D	Nil	25.0 ± 1.8
	+	+	BALB/c nude	C57BL/6J	Nil	Nil	1.6 ± 1.6
	+	+	BALB/c nude	ATH	D	Nil	1.9 ± 1.0

a BALB/c nude donor mice were injected with 10^3 HAU infectious A/WSN virus and Con A-activated cell supernatant (0.1 ml each time for the first 3 consecutive days). Six days later, spleen cells were prepared and 8×10^7 cell were transferred i.v. to recipient mice which were challenged 4 h later with UV-inactivated A/WSN virus (6×10^3 HAU) into the right hind footpads and footpad swelling was measured 24 h afterwards.

b Anti-Thy 1.2 + C' killed ∿11% of 6 days immune spleen cells from nude mice.

CHAPTER 4

IN VIVO AND IN VITRO GENERATION OF TWO DISTINCT POPULATIONS OF EFFECTOR T CELLS MEDIATING DTH REACTIONS

CHAPTER 4.1

CELLS MEDIATING DTH IN LUNGS OF MICE INFECTED WITH AN INFLUENZA A VIRUS

INTRODUCTION

There is a great deal of information on the role of specific antibodies, both in the serum and in respiratory secretion, in the protection of human beings from influenza virus infection (Potter & Oxford, 1979). However, little is known about the T lymphocyte response that occurs in such infections and the role such responses might play. Delayed-type hypersensitivity (DTH) to influenza virus has been demonstrated in natural human infections (Haberson *et al.*, 1973; Cole & Molyneux, 1975), and peripheral blood lymphocytes from people who have experienced the infection can be stimulated *in vitro* to produce cytotoxic T lymphocytes (Tc) that are HLA-restricted (McMichael *et al.*, 1977). Although influenza virus is not a natural pathogen of mice, influenzal pneumonia occurs, with substantial cell damage, if infectious virus is instilled into the nose, and in this respect it is similar to human influenzal pneumonia. Thus the mouse is a suitable model for some aspects of the human disease and is much more suitable for defining the relative roles of different classes of lymphocytes. Thus, T helper cells are important in the production of IgG antibodies to the virus (Virelizier, 1975) and injection of such antibodies, either before or after virus infection, will prevent or limit the infectious process (Yap & Ada, 1979). Tc cells can be recovered from both the lung washings and lung tissues, and high activities can be recovered by 6-7 days after intranasal inoculation of a lethal dose of virus (Yap & Ada, 1978a). Furthermore, primary or secondary cultures of immune cells rich in Tc activity could prevent mice from dying if they were transferred to the animals 24 h before or after administration of a lethal dose of virus (Yap & Ada, 1978b). The active cells were H-2 restricted - sharing of K or D region was

necessary - and were Lyt 2,3 positive, as shown by complement-mediated lysis with appropriate specific antisera (Yap et al., 1978). In contrast, little is known about the occurrence of cells with DTH activity (Td) in the lungs of infected mice. A recent paper (Liew et al., 1979) shows that mice injected with a large dose (200 mg/kg) of cyclophosphamide (Cy) and then injected intranasally with virus aerosol show a marked DTH reaction when challenged 7 days later with inactivated virus in one hind footpad. Presumably, effector cells that took part in the DTH footpad reactions had previously been sensitized in the infected lungs. A much smaller reaction was elicited if Cy pretreatment was omitted. In this chapter, the kinetics of appearance and the properties of Td cells in the lungs of mice infected with A strains of influenza virus were studied.

RESULTS

Generation of DTH effector cells (Td) after intranasal inoculation of virus

Mice were inoculated intranasally with a lethal dose ($5x10^4 EID_{50}$) of A/WSN virus. Under these conditions, high titres of infectious virus (c.$10^6 EID_{50}$) are found in the lungs 24 h after inoculation, and the mice die between days 7 and 10 (Yap & Ada, 1978a). Cells were harvested from the lungs, blood, spleen, and the lymph nodes draining the lungs 6 days after infection and injected either intravenously or directly into the feet of normal recipient mice and tested for DTH activity. Cells from uninfected lungs and spleen were used as controls. Relatively small numbers of cells from lungs, lymph nodes and blood showed high activity. Spleen cells from such immunized mice were also active (Table 1).

The kinetics of generation of Td cells in the lungs of mice receiving a lethal dose of virus intranasally are shown in Table 2. Again because of the small numbers available, all were injected with antigen directly into the footpad of recipient mice. DTH activity was just detectable on day 4, when traces of lung consolidation were also noticed, but both gross pathological lesions and DTH activity increased markedly in the next 48 h (Table 2). As also noticed previously (Bennink et al., 1978; Yap & Ada, 1978a) increasing numbers of cells could be recovered from the lungs as consolidation increased. The effect of the virus dose on the generation of Td cells is shown in Table 3. This experiment was done in two parts - one to test the survival rate of mice (observed over a period of 21 days), and the other to test lung consolidation and DTH effector cell activity. Between doses of 10-10^4 EID_{50}, there is a positive correlation between the degrees of lung consolidation observed and the levels of DTH activity detected.

Properties of the DTH effector cells

The Td cells present in the lung cells from 6 days virus-infected CBA mice were examined for their surface antigen characteristics. Their activity was found to be destroyed by anti-Thy 1.2 ascitic fluid and complement and by anti-Lyt 1.1 antibody and complement but not by anti-Lyt 2.1 antiserum and complement (Table 4). They are thus clearly T lymphocytes, and, as would be expected from this finding, removal of adherent cells from the cell preparation did not diminish the DTH activity.

The H-2 requirements for expression of DTH activity of lung cells was tested by using donor and recipient strains of mice which shared different regions of the H-2 gene complex. The experiment shown in Table 5 shows, first, that I-region and not K,D-region homology is

required for successful transfer and, second, that homology at the IA subregion is sufficient for this to occur.

Finally, the specificity of the Td cells present both in the lungs and in the draining lymph node 6 days after intranasal inoculation of virus was examined. The results in Table 6 indicate that the Td cells found in both the infected lung and the draining lymph nodes were completely cross-reactive, using A strains of virus of different haemagglutinin and neuraminidase specificities. The cross-reactivity did not extend to Sendai virus (Chapters 3.1, 3.2).

DISCUSSION

In this chapter attempts were made to answer two questions directly: (1). Can Td cells be detected in the lungs of mice that have been inoculated intranasally with infectious virus? (2). If so, what are the characteristics of the cells so produced?

It was found that Td cells could be recovered from infected lungs, and that 6 days after inoculation of mice the activity recovered was reasonably proportional to the dose of virus instilled and to the extent of lung consolidation observed (Table 3). Furthermore, Td activity could be recovered from the draining lymph nodes and from the peripheral blood. The lung cells with DTH activity were Lyt 1 positive and required I region sharing (Tables 4 and 5). If a particular A-strain of virus was used to infect the mice, then DTH activity could be elicited with another A-strain virus. Thus, the cells in the lungs were similar in these three properties to the cells recovered from the spleens of mice previously immunized subcutaneously with an infectious A-strain virus. Apart from demonstrating the presence of Td cells in infected lungs, these results are important in another respect. Even though large doses of infectious virus ($10^7 EID_{50}$) are

injected intravenously or subcutaneously to generate Tc cells or to sensitize mice for a DTH response, no infectious virus is recovered from the spleen. Zinkernagel (1976) earlier found that K,D and not I region compatibility was required for the successful transfer of DTH to LCM virus infection. The Lyt phenotype was not reported. LCM virus is a natural mouse pathogen and can replicate in many tissues (Lehmann-Grube, 1971), so it is possible that cells with these characteristics were generated in response to an active, on-going infection. Such a process occurs in the mouse lung with influenza virus. After intranasal inoculation of about $10^3 EID_{50}$ of influenza virus, high titres (about $10^6 EID_{50}$) can be recovered from the lungs 24 h later, and in the succeeding 5-7 days these rise to titres of 10^7-$10^8 EID_{50}$ (Yap & Ada, 1978a). Yet the cells recovered from the lungs, where active replication takes place, and from spleen, where abortive replication takes place, had similar properties.

There are, however, two caveats to this finding. The first is that the Td cells recovered from the lung may not have been generated there. The kinetics of appearance of Td cells (this chapter) and of Tc cells (Yap & Ada, 1978a) are similar. Since a previous attempt to identify the site of production of Tc cells recovered from infected lungs gave equivocal results (Yap & Ada, 1978a), it is possible that at least some of the Td cells in the lung may have been generated in other organs. The second consideration is that elicitation of all the DTH reactions in the present work and also those reported in Chapter 3 was done with purified, non-infectious virus. Attempts to elicit the reaction with preparations of virus with a high ratio of infectious to non-infectious virus (e.g. freshly harvested infectious allantoic fluid) gave poor responses, possibly because the injected virus did not replicate significantly in the footpad. Thus it is conceivable that as well as Lyt 1^+, IA subregion restricted Td cells being present in the infected

lungs, there may also be Lyt 2^+, K,D-region restricted Td cells, but their presence, if that is the case, has not been detected by the present experimental protocols (see Chapter 4.3).

SUMMARY

Effector cells that demonstrate delayed-type hypersensitivity (DTH) on transfer with antigen to naive mice can be recovered from the lungs of mice inoculated intranasally 6 days earlier with a lethal dose (usually $5x10^4 EID_{50}$) of influenza A virus. The activity recovered was proportional to the dose of virus instilled intranasally and the extent of lung consolidation observed. Active cells could also be recovered from the draining lymph nodes and from the peripheral blood. The effector cells were identified as T lymphocytes of Lyt 1 phenotype and required I region sharing between donor and recipient for activity to be elicited. They were cross-reactive within the A group of influenza viruses.

TABLE 1

Generation of Delayed-Type Hypersensitivity in CBA Mice after Intranasal Instillation of a Lethal Dose (5×10^4 EID_{50}) of A/WSN Virus

Cells transferred[a]	Route of cell transfer[b]	Injection into footpad of recipients	Mean increase in footpad thickness at 24 h (%)
Normal lung cells (2×10^6)	f.p.	Cells + virus[c]	4.7 ± 1.4
6-Day immune lung cells (2×10^6)	f.p.	Cells	3.9 ± 0.7
	f.p.	Cells + virus	33.6 ± 2.1[d]
6-Day immune peripheral blood lymphocytes (2×10^6)	f.p.	Cells + virus	32.8 ± 2.0[d]
6-Day immune draining lymph node cells (1×10^7)	f.p.	Cells	2.1 ± 2.1
	f.p.	Cells + virus	32.3 ± 2.1[d]
Normal spleen cells (10^8)	i.v.	Virus	3.1 ± 1.3
6-Day immune spleen cells (10^8)	i.v.	Virus	18.8 ± 2.2[d]

Legends for Table 1

a The number of cells recovered from one pair of uninfected lungs or spleen was about $3x10^5$ and $7x10^7$, respectively. Cells recovered from various organs of mice 6 days after virus inoculation were as follows: lungs, $2\text{-}3x10^6$; spleen, $1x10^8$; draining lymph nodes, about $1x10^7$; and blood, about $4x10^6$/ml.

b f.p.: footpad injection; i.v.: intravenous injection.

c The eliciting dose of purified, untraviolet light (UV)-inactivated A/WSN virus was $6x10^3$ haemagglutination units (HAU).

d Significantly greater than control mice (injected with non-immune cells and virus); $p < 0.001$.

TABLE 2

Kinetics of Generation of Delayed-Type Hypersensitivity in Mouse Lungs 6 Days after Intranasal Inoculation of Mice with a Lethal Dose (5×10^4 EID_{50}) of A/WSN Virus

Days after inoculation[a]	Degree of lung consolidation	Lung cells recovered per mouse ($\times 10^5$)	Mean increase in footpad thickness at 24 h (%)
0	0	3	4.7 ± 1.4
2	0	8	4.7 ± 0.9
4	4-10	16	9.4 ± 1.8[b]
6	55-60	30	33.6 ± 2.1[c]

a Groups of 5-8 CBA mice were killed at various time intervals, lung suspensions made and 2×10^6 cells injected together with 6×10^3 HAU purified, UV-inactivated A/WSN virus into the hind footpad of each recipient mouse and the degree of swelling measured 24 h later.

b,c Significantly greater than controls (lung cells from uninfected mice). b, $p < 0.05$; c, $p < 0.001$.

TABLE 3

Effect of Virus Dose on the Appearance in Lungs of Immune Cells with Delayed-Type Hypersensitivity Activity

Dose of virus[a] inoculated intranasally (EID_{50})	Lung consolidation[b] (%)	Mice died[c]	Mean increase in footpad thickness at 24 h (%)
Nil	0	-	4.7 ± 1.4
10	0-5	-	10.4 ± 2.1
10^2	10-15	-	13.6 ± 2.8
10^3	30-35	-	18.8 ± 0.0
10^4	55-60	+	36.8 ± 1.5
10^5	75-80	+	37.5 ± 1.3

a Mouse-adapted A/WSN virus.

b Mean of estimated values from gross observation of groups of 6-8 CBA mice, 6 days after virus inoculation.

c Deaths usually occurred 7-11 days after virus inoculation, and the remaining mice were observed for 21 days.

TABLE 4

Surface Antigen Characterization of Delayed-Type Hypersensitivity Effector Cells from Lungs of 6-Day Infected Mice

Treatment of immune cells[a]	Injection into recipient footpad[b]	Mean increase in footpad thickness at 24 h (%)
Nil	Cells	3.9 ± 0.7
Nil	Cells + virus	33.6 ± 2.1
Normal ascitic fluid + C'	Cells + virus	32.1 ± 0.7[c]
Anti-Thy 1.2 ascitic fluid + C'	Cells + virus	5.2 ± 0.9[d]
Removal of adherent cells	Cells + virus	32.8 ± 1.4[c]
Anti-Lyt 1.1 + C'	Cells + virus	6.3 ± 1.3[d]
Anti-Lyt 2.1 + C'	Cells + virus	34.4 ± 1.8[c]

a These cells were recovered from the lungs of CBA mice 6 days after inoculation of 5×10^4 EID_{50} of A/WSN virus.

b 2×10^6 cells and/or 6×10^3 HAU purified, UV-irradiated A/WSN virus were injected.

c Not significantly different from control values (untreated immune cells).

d Significantly lower than control values (untreated immune cells), $p < 0.01$.

TABLE 5

H-2 Restriction of Delayed-Type Hypersensitivity Activity of Cells from Infected Lungs[a]

Mouse strains		H-2 regions shared	Mean increase in footpad thickness at 24 h (%)
Donors	Recipients		
Experiment 1			
CBA	CBA	All	27.4 ± 1.5
CBA	A.TL	I	29.2 ± 1.1
A.TL	A.TL	All	30.5 ± 1.3
A.TL	CBA	I	34.4 ± 1.5
A.TL	A.TH	K,D	4.7 ± 1.4[b]
Experiment 2			
B10.A(4R)	B10.A(4R)	All	33.3 ± 1.1
B10.A(4R)	C57BL/6J	All except K, IA	4.2 ± 2.1[b]
B10.A(4R)	B10.AQR	IA	33.3 ± 4.2

a Donor mice were infected intranasally with 5×10^4 EID_{50} A/WSN virus and the lung cells harvested 6 days later. 2×10^6 cells with virus (6×10^3 HAU) were injected into the hind footpad of recipients and swelling measured 24 h later.

b Values are significantly lower ($p < 0.001$) than the control value of mice receiving syngeneic immune cells.

TABLE 6

Specificity of Delayed-Type Hypersensitivity Effector Cells from Infected Lungs and Draining Lymph Nodes

Virus strains		Cells transferred[c]	Mean increase in footpad thickness at 24 h (%)
Donor inoculation[a]	Recipient challenged[b]		
Nil	A/WSN	Lung	3.1 ± 2.2
A/WSN (HONl)	A/WSN	Lung	32.1 ± 0.7[d]
A/WSN	A/RI	Lung	27.4 ± 1.3[d]
A/WSN	Sendai	Lung	1.1 ± 0.9
A/RI (H2N2)	A/RI	Lung	34.4 ± 1.5[d]
A/RI	A/WSN	Lung	37.5 ± 1.8[d]
A/RI	A/RI	Lymph nodes	34.4 ± 1.3[d]
A/RI	A/WSN	Lymph nodes	32.8 ± 0.9[d]
A/RI	A/PC	Lung	33.3 ± 1.1[d]
A/RI	A/PC	Lymph nodes	31.3 ± 1.3[d]
A/PC (H3N2)	A/PC	Lung	30.2 ± 0.9[d]
A/PC	A/RI	Lung	32.3 ± 0.9[d]
A/PC	A/WSN	Lung	31.3 ± 1.3[d]
A/PC	A/PC	Lymph nodes	32.3 ± 2.1[d]
A/PC	A/RI	Lymph nodes	32.3 ± 1.1[d]
A/PC	A/WSN	Lymph nodes	31.3 ± 1.8[d]

a Donor CBA mice were injected intranasally either with A/WSN ($5x10^4$ EID_{50}), A/RI ($1x10^5$ EID_{50}) or A/PC ($1x10^5$ EID_{50}).

b All recipient CBA mice were challenged with $6x10^3$ HAU purified, UV-inactivated virus.

c Number of lung or lymph node cells transferred were $2.5x10^6$ or $1x10^7$, respectively.

d Not significantly different from each other.

CHAPTER 4.2

GENERATION OF INFLUENZA VIRUS-SPECIFIC DTH T CELLS IN VITRO: SECONDARY EFFECTOR CELLS

INTRODUCTION

The ability to generate populations of cycotoxic T lymphocytes (Tc) in tissue culture has been of great importance in defining some of the necessary events, such as the nature of the stimulator cells, and the requirement for helper T cells (Th), during their generation. Studies have been made with allogeneic systems (Davidson, 1977; Pilarski, 1979; Röllinghoff & Wagner, 1975; Schilling et al., 1976) and with virus-infected syngeneic cells (Blanden et al., 1977a; Pang & Blanden, 1977; Plata et al., 1975). Recently, primary induction of delayed type hypersensitivity (DTH) responses in vitro to non-replicating, particulate antigens such as heterologous red blood cells have been described (Bretscher, 1979; Ramshaw & Eidinger, 1979), but the conditions for generation of such a response using virus (a replicating antigen) have not been examined. Previous chapters have described the DTH response in mice to influenza virus infection (Chapters 3.1, 3.2, 4.1) and this chapter examines more closely factors such as antigenic and genetic requirements as well as cellular collaborations in the induction of a DTH response which sometimes cannot be so readily determined in in vivo experiments. The conditions for the preparation in tissue culture of secondary effector cells which can mediate DTH reaction (Td) were described. Two of the results described in detail in this chapter have been briefly referred to in Chapter 3.2.

RESULTS

The in vitro generation of secondary effector cells with DTH activity

It has been shown (Yap & Ada, 1977) that potent secondary influenza virus-specific cytotoxic T cells can be generated *in vitro* by restimulation of spleen cells from mice primed with *infectious* virus (10^3HAU) 3 weeks or more previously. Maximal cytotoxicity was detected after 5 days culture. In view of similar conditions for the generation of Tc and Td cells *in vivo* (Chapter 3.1), it was of interest to see whether Td cells were also generated *in vitro* under the same conditions which resulted in high Tc activity. Normal spleen cells (10^7) were exposed to infectious ($3x10^7 EID_{50}$) or non-infectious A/WSN virus ($1.5x10^3$HAU) for 1 h, washed, and 10^8 spleen cells from mice primed 3 weeks or more earlier with infectious A/WSN virus ($1x10^7 EID_{50}$) i.p. were added. After incubation for 5 days at 37°C, the cells were harvested, dead cells removed and the viable cells tested for Tc activity and for DTH activity by adoptive transfer with antigen into syngeneic naive recipients. As shown in Table 1, unstimulated memory cells did not yield detectable levels of Tc or Td activity. In contrast, stimulated memory cells demonstrated high levels of cytotoxic activity on virus-infected, K,D compatible target cells but not with uninfected target cells. If the cells were injected directly into the footpad with antigen, a DTH reaction was observed 24 h later. It should be noted however, that injection of the cells i.v., followed by antigen into the footpad did not result in a DTH reaction in the footpad, even though this procedure is successful when Td cells generated *in vivo* are tested.

Kinetics of induction of Td in vitro

Spleen cells from mice primed 21 days or more previously were cultured for different times with virus-infected syngeneic stimulator

cells before harvesting and DTH activities were measured. Fig. 1 shows that cells in day 1 or day 3 cultures were inactive, but that considerable activity was present in day 5 and 7 cultures.

Development of the footpad response

The response in the mouse footpad to antigen injection following s.c. immunization with infectious or UV-inactivated virus has been described in Chapter 3.1. In such a primary *in vivo* response, there is an initial (4 h) brief inflammatory reaction due to the injection of virus, and this is followed by a delayed reaction which reaches a peak at 24 h and then declines. However, after injection of virus and cultured cells into the footpad, the brief swelling at 4 h occurred but the delayed reaction extended from 24-72 h before there was a significant decrease in the swelling (Fig. 2).

The dose response with cultured cells injected into the mouse footpad

The level of footpad swelling (24 h) observed after injection of different quantities of cultured cells is shown in Fig. 3. DTH activity was detected following injection of as few as $5x10^5$ cells. Increasing the number of cells injected resulted in a substantial increase in footpad swelling but (as expected) a linear response was not seen.

Influence of viral infectivity on the generation of Tc and Td effector cells in vitro

The influence of viral infectivity was tested at two points in the generation of Tc and Td cells: (1). at the priming phase when mice were immunized to produce memory cells which were subsequently used as responder cells; (2). at the restimulation phase, when the responder cells were restimulated *in vitro*. Experiments in which either infectious or UV-inactivated virus were used at these points in the experimental protocol are reported in Table 2. It can be seen

that: (1). only mice primed with infectious virus produced immune cells which could be restimulated *in vitro* to demonstrate high cytotoxic activity; (2). in contrast, the infectivity of the virus used at either point had little influence on the level of DTH activity generated. The properties of the effector cells generated under these different conditions differed, however, as will be shown in the next section.

Specificity of the DTH response generated in vitro

It was previously shown (Floc'h & Werner, 1978; Chapter 3.2) in *in vivo* experiments that injection of infectious influenza A virus into mice produced a cross-reactive (within A strain) DTH response, whereas injection of UV-inactivated virus induced a specific DTH reaction which was probably directed towards the haemagglutinin molecule of the virus. Similar specificity studies were carried out on Td cells produced in tissue culture (Table 3). Three main conclusions can be drawn from these results: (1). The DTH activity generated *in vitro* was specific. If influenza virus was used to prime the donor mice and to restimulate the memory cells *in vitro*, a strong DTH response in the footpad was obtained if influenza virus was used to elicit the response but not if Sendai virus was used. (2). Cross-reactive (within influenza A strains) Td cells were generated only if infectious virus was used both for priming the donor mice and infecting the stimulator cells *in vitro*. If UV-inactivated virus was used either for priming the donor mice and/or treating the stimulator cells *in vitro*, the Td cells so generated were specific for the virus which had been used to prime the donor mice. (3). Priming mice with an infectious A strain yielded cells which could be restimulated *in vitro* by a heterologous infectious A strain virus to yield Td cells which were cross-reactive within the A strain viruses.

Characterization of DTH activity generated in tissue culture

The surface antigen phenotype of the virus-specific DTH effector cells from CBA mice generated *in vitro* was established using appropriate antiserum. The DTH activity was found to be abolished by treatment with anti-Thy 1.2 ascitic fluid and complement (Table 5). They were also found to be of Lyt $1^{+}2^{-}$ phenotype (Chapter 3.2).

A requirement for H-2 sharing in the adoptive transfer of DTH activity with secondary effector T cells generated in vitro

If secondary effector cells generated *in vitro* were injected into the footpad at the same time as antigen, there was no apparent requirement for H-2 antigen sharing between donor and recipient mice. However, if the immune cells were depleted of plastic surface adherent cells before injection with antigen, H-2 restriction was observed (Table 5). Sharing of I region but not K,D region between the donor and recipient mice was sufficient for the transfer of activity. A similar effect was observed if the immune cells were injected into the footpad 6-8 h after the injection of virus and this procedure was followed in the characterization of the H-2 sharing requirement. It was found that sharing of the IA subregion alone was both necessary and sufficient for successful adoptive transfer of DTH activity generated *in vitro* (Table 6).

DISCUSSION

It is perhaps not surprising that conditions which were previously found suitable for the generation in tissue culture of cytotoxic T cells should also allow the production of effector cells which mediate DTH reactions. Previous work in both allogeneic and viral systems had shown that these conditions of tissue culture satisfied the requirements for antigenic stimulation, generation of Th

cells and production of Tc cells. The two points from this present work which need to be discussed are: (1). What is the relationship between Td and Tc cells generated in this way? (2). Are there any differences in the properties of Td cells generated *in vivo* and *in vitro*?

Several findings indicated that the secondary effector Td and Tc cells generated in tissue cultures represent different lymphocyte subpopulations. One is the effect of viral infectivity on the production of each subpopulation. Use of either infectious or non-infectious virus to produce 'memory' responder cells *in vivo* and to restimulate them *in vitro* results in Td cell generation, as elicited using purified, UV-inactivated virus. However, unless infectious virus is used for the initial priming event *in vivo*, Tc cells are not generated from responder cells in tissue culture irrespective of the use of infectious or non-infectious virus as the antigenic stimulus at this stage. This finding extends an earlier observation that injection of UV-inactivated virus does not result in Tc cell production (Braciale & Yap, 1978), even though Tc precursor cells can be recovered from their spleen (Chapter 7). It is now seen that 'memory' Tc cells may also not be generated under these conditions. Another finding is the requirement for H-2 sharing for successful demonstration of cellular activity. Tc cells generated either *in vivo* or *in vitro* require K,D compatibility between effector cells and target cells for killing to be observed (Yap & Ada, 1977). However, the Td cells generated *in vitro*, like their counterparts generated *in vivo*, require IA compatibility between donor and recipient for successful transfer of DTH activity. It is of interest that the cultured cells had to be injected directly into the footpad in order to demonstrate their DTH activity (see below); and that H-2 restriction was only demonstrated if cells which could adhere to

plastic were removed from the injected cell population or viral antigen was injected into the footpad some time (6-8 h) before injection of the cells. A similar finding that DTH to sheep red blood cells could be transferred locally but not systemically irrespective of H-2 compatibility between donor and recipient has been recently reported (Lubet & Kettman, 1979). The removal of potential accessory cells from the cell population to be injected or the prior injection of antigen into the footpad - procedures which might minimize the involvement of one class of accessory cells in the injected cell population - allowed H-2 restriction to be demonstrated.

Td cells generated in vivo or in vitro have many properties in common, such as antigenic phenotype, specificity of action and H-2 sharing requirements. A major difference is the requirement for the local injection of cultured cells to demonstrate their activity, whereas cells generated in vivo can be successfully transferred systemically. A likely explanation for this is the different migratory properties of the cultured cells compared to those produced in vivo. Kees & Blanden (1977) produced some evidence which suggests that, compared to primary immune cells produced in vivo, parenterally injected cultured cells are detained in lung capillaries. It is known that i.v. injection of cultured cells which possess both Tc and Td activities can reduce virus titres in the lungs after intranasal inoculation of infectious influenza virus (Yap & Ada, 1978b), so it is presumed that these cells reach the lungs in adequate numbers to achieve this result. However, if they are detained in lung (and other) capillaries early after their injection, they may not reach the hind footpads in sufficient quantity to effect a DTH reaction. When injected directly into the hind footpads however, as few as 5×10^5 cells can initiate a significant reaction (Fig. 3). The observation that the delayed swelling of the footpad persists for up to 72 h after

injection of cultured cells is consistent with the interpretation that the injected cells remain at the site of injection because of their different migratory properties.

This chapter and earlier finding (Chapter 3.1) show clearly that there are different requirements, both *in vivo* and *in vitro*, for the generation of Tc and Td effector cells to influenza virus in mice. Yet with respect to specificity patterns, these two different effector cell populations have at least one characteristic in common, i.e. both subtype-specific and cross-reactive subpopulations can be distinguished in each preparation (Yap & Ada, 1977; Table 3). It is possible that the same virus-coded antigenic structure(s) can be recognized by these different effector cell populations but this still has to be determined.

The ability to generate Td effector cells *in vitro* allows the study of a number of factors which cannot be so readily investigated *in vivo*. Experiments in subsequent chapters will examine some of these as are also attempts to initiate a primary response *in vitro*.

SUMMARY

When spleen cells from mice injected 3 weeks or more previously with influenza A virus (responder cells) are mixed with normal spleen cells exposed 1 h previously to influenza A virus (stimulator cells) and the mixture cultivated at 37°C for 5-6 days, the surviving cell population contains effector T cells (Td) which can mediate delayed type hypersensitivity reactions. If infectious virus is used to prime both the donor mice and to infect the stimulator cells, the cell population also contains cytotoxic T cells (Tc). In this case, both Tc and Td cells have similar specificity patterns, as cells raised to one subtype A virus are cross-reactive to other A strain viruses but

not to Sendai virus. If non-infectious virus is used to immunize the donor mice, Td but not Tc cells are generated and these cells are specific for the subtype A virus used in the original immunization. Both preparations of Td cells are Lyt $1^{+}23^{-}$ and require IA sharing of donor and recipient mice for transfer of DTH activity to be successful. Td cells produced this way are similar to those produced <u>in vivo</u> except they may have different migratory properties and must be injected directly into the footpad for DTH activity to be elicited. In such transfers, H-2 restriction can be clearly demonstrated if the challenging antigen is injected into the footpad some hours before the injection of cells.

TABLE 1

Generation of Secondary Cytotoxic and DTH Activities *in Vitro*

Secondary cultures[a]	E:T ratio	% Specific ^{51}Cr release from L929 targets		Route[c] and dose of cultured cells transferred for DTH activity	Injection into footpads of recipients	Mean increase in footpad thickness at 24 h (%)
		Uninfected	A/WSN infected			
A/WSN primed spleen cells (10^8) + normal spleen cells (10^7)	2:1	1.0 ± 0.4[b]	4.7 ± 1.0[b]	f.p. ($5x10^6$)	Cells + virus	9.4 ± 1.3[b]
	8:1	1.2 ± 0.6	9.4 ± 0.9			
A/WSN primed spleen cells (10^8) + A/WSN infected spleen cells (10^7)	2:1	1.9 ± 0.6	44.5 ± 0.6	f.p. ($5x10^6$)	Cells alone	2.4 ± 0.8
	8:1	6.3 ± 0.2	68.5 ± 4.6			
				f.p. ($5x10^6$)	Cells + virus	38.1 ± 2.1
				i.v. ($1.5x10^7$)	Virus[d]	4.2 ± 2.1

a Spleen cells from CBA mice primed with infectious A/WSN virus (10^7 EID_{50}) 3 wk or more were used as a source of responder cells; responder:stimulator ratio was always 10:1. Secondary cells were harvested after 5 days culture.

b Mean ± standard error,

c f.p.: footpad; i.v.: intravenous.

d A standard dose ($6x10^3$ HAU) of UV-irradiated A/WSN virus was used for elicitation of the DTH reaction.

TABLE 2

Influence of Viral Infectivity on the *in Vitro* Generation of Cytotoxic and DTH Activities

Secondary Cultures[a]		% ^{51}Cr release on A/WSN-infected L929 target cells (E:T = 4:1)	Mean increase in footpad thickness[c] at 24 h (%)
Stimulators	Responders[b]		
Normal spleen cells (NSC)	Infectious A/WSN primed spleen cells	1.6 ± 0.2	9.4 ± 2.8
A/WSN-infected NSC	Infectious A/WSN primed spleen cells	40.5 ± 1.2	35.2 ± 2.0
UV-inactivated A/WSN treated NSC	Infectious A/WSN primed spleen cells	37.3 ± 2.5	38.4 ± 1.7
Normal spleen cells	UV-inactivated A/WSN primed spleen cells	1.3 ± 0.5	8.6 ± 1.5
A/WSN-infected NSC	UV-inactivated A/WSN primed spleen cells	6.1 ± 1.1	46.9 ± 1.8
UV-inactivated A/WSN treated NSC	UV-inactivated A/WSN primed spleen cells	7.3 ± 0.9	39.1 ± 1.6

a Secondary cells were harvested after 5 days culture.

b Spleen cells from CBA mice primed with infectious or UV-inactivated A/WSN virus (10^3 HAU) 3 wk or more previously were used as a source of responder cells.

c Secondary cells (5×10^6) were injected with purified, UV-inactivated A/WSN virus (6×10^3 HAU) and footpad swelling measured 24 h later.

TABLE 3

Specificity of Secondary Td Generated *in Vitro*

Virus strains used					
Treatment of normal spleen cells (stimulator cells)		Priming of donor mice (responder cells)		Elicitation of DTH reaction in recipient mice	Mean increase in footpad thickness at 24 h (%)
Virus	Infectivity[a]	Virus	Infectivity[a]	Virus[b]	
Experiment 1					
Nil		A/WSN	+	A/WSN (HONl)	9.4 ± 2.8[c]
A/WSN	+	A/WSN	+	A/WSN	35.2 ± 2.0
A/WSN	+	A/WSN	+	Sendai	5.2 ± 1.1[c]
A/PC	+	A/PC	+	A/PC (H3N2)	36.4 ± 1.3
A/PC	+	A/PC	+	A/WSN	31.3 ± 2.9
A/PC	+	A/PC	+	A/RI (H3N2)	32.8 ± 2.0
A/RI	+	A/RI	+	A/RI	31.3 ± 1.3
A/RI	+	A/RI	+	A/WSN	33.6 ± 1.5
Experiment 2					
A/WSN	-	A/WSN	-	A/WSN	39.6 ± 1.1
A/WSN	-	A/WSN	-	A/PC	6.3 ± 0.5[c]
A/WSN	-	A/WSN	-	A/RI	6.3 ± 1.8[c]
A/WSN	-	A/WSN	-	Sendai	4.2 ± 2.8[c]
Experiment 3					
A/WSN	-	A/WSN	+	A/WSN	40.6 ± 4.8
A/WSN	-	A/WSN	+	A/PC	6.3 ± 1.3[c]
A/WSN	-	A/WSN	+	A/RI	5.5 ± 2.0[c]
A/WSN	-	A/WSN	+	Sendai	3.9 ± 1.5[c]
A/WSN	+	A/WSN	-	A/WSN	38.6 ± 1.1
A/WSN	+	A/WSN	-	A/PC	5.2 ± 2.1[c]
A/WSN	+	A/WSN	-	A/RI	6.3 ± 1.0[c]
A/WSN	+	A/WSN	-	Sendai	5.2 ± 1.1[c]
Experiment 4					
A/WSN	+	A/PC	+	A/JAP	33.3 ± 1.1
A/WSN	+	A/PC	+	A/PC	32.3 ± 1.1
A/WSN	+	A/PC	+	A/WSN	39.6 ± 1.1
A/WSN	+	A/JAP	+	A/JAP	35.9 ± 0.9
A/WSN	+	A/JAP	+	A/WSN	39.6 ± 3.8
A/WSN	+	A/JAP	+	A/PC	36.4 ± 1.1

Legends for Table 3

a +: infectious; -: UV-inactivated.

b Purified and UV-inactivated viruses ($6x10^3$ HAU) were used.

c Significantly lower than controls (in which the same virus was used for both priming, restimulation and elicitation); $p < 0.01$.

TABLE 4

Effect of Treatment with Anti-Thy 1.2 Ascitic Fluid and Complement on the DTH Activity Generated *in Vitro*

Treatment of secondary cultured cells	Mean increase in footpad thickness at 24 h (%)
Nil	42.7 ± 3.8
Normal ascitic fluid + C'	39.9 ± 0.8
Anti-Thy 1.2 ascitic fluid + C'	7.3 ± 1.1[a]

a Significantly lower than control (untreated cells) ($p < 0.01$).

TABLE 5

Influence of Adherent Cells on the H-2 Requirement for Successful Transfer of DTH Activity with Secondary Effector Cells Generated *in Vitro*

Mouse strains		H-2 region shared	Removal of adherent cells before injection into footpad together with virus	Mean increase in footpad thickness at 24 h (%)
Donors	Recipients			
CBA	CBA	All	-	37.5 ± 3.6
			+	32.8 ± 0.9[a]
CBA	BALB/c	Nil	-	30.5 ± 3.3
			+	6.3 ± 1.3[b]
A.TL	CBA	I	-	34.4 ± 1.8
			+	32.3 ± 1.1[a]
A.TL	A.TH	K,D	-	29.7 ± 0.9
			+	5.2 ± 1.1[b]
A.TL	A.TL	All	-	38.6 ± 3.8
			+	34.4 ± 3.1[a]

a Not significantly different from controls (no removal of plastic adherent cells).

b Significantly lower than controls ($p < 0.01$).

TABLE 6

Adoptive Transfer of DTH Activity with Secondary Effector Cells Generated *in Vitro* is H-2 Restricted[a]

Mouse strains		H-2 region shared	Mean increase in footpad thickness at 24 h (%)
Donors	Recipients		
CBA	BALB/c	None	4.2 ± 2.1[b]
CBA	CBA	All	35.4 ± 2.1
CBA	A.TL	I	31.3 ± 1.8
CBA	C3H.OH	D	6.3 ± 1.3[b]
CBA	B10.A(4R)	K,IA	36.4 ± 2.8
CBA	B10.A(5R)	IJ, IE	8.3 ± 1.1[b]
CBA	B10.AQR	IA, IB , IJ, IE	39.1 ± 1.6
A.TL	A.TL	All	34.4 ± 3.1
A.TL	A.TH	K,D	5.2 ± 1.1[b]
A.TL	CBA	I	32.3 ± 1.1
A.TL	BALB/c	D	6.3 ± 0.0[b]
B10.A(4R)	B10.A(4R)	All	39.6 ± 2.1
B10.A(4R)	C57BL/6J	All except K,IA	3.9 ± 1.5[b]
B10.A(4R)	CBA	K,IA	34.4 ± 1.8
B10.A(4R)	B10.AQR	IA	35.6 ± 0.8

a $5x10^6$ secondary effector cells were injected into the right hind footpad 6-8 h after injection of viris ($6x10^3$ HAU).

b These values are significantly lower than control values of mice which received syngeneic immune cells ($p < 0.01$).

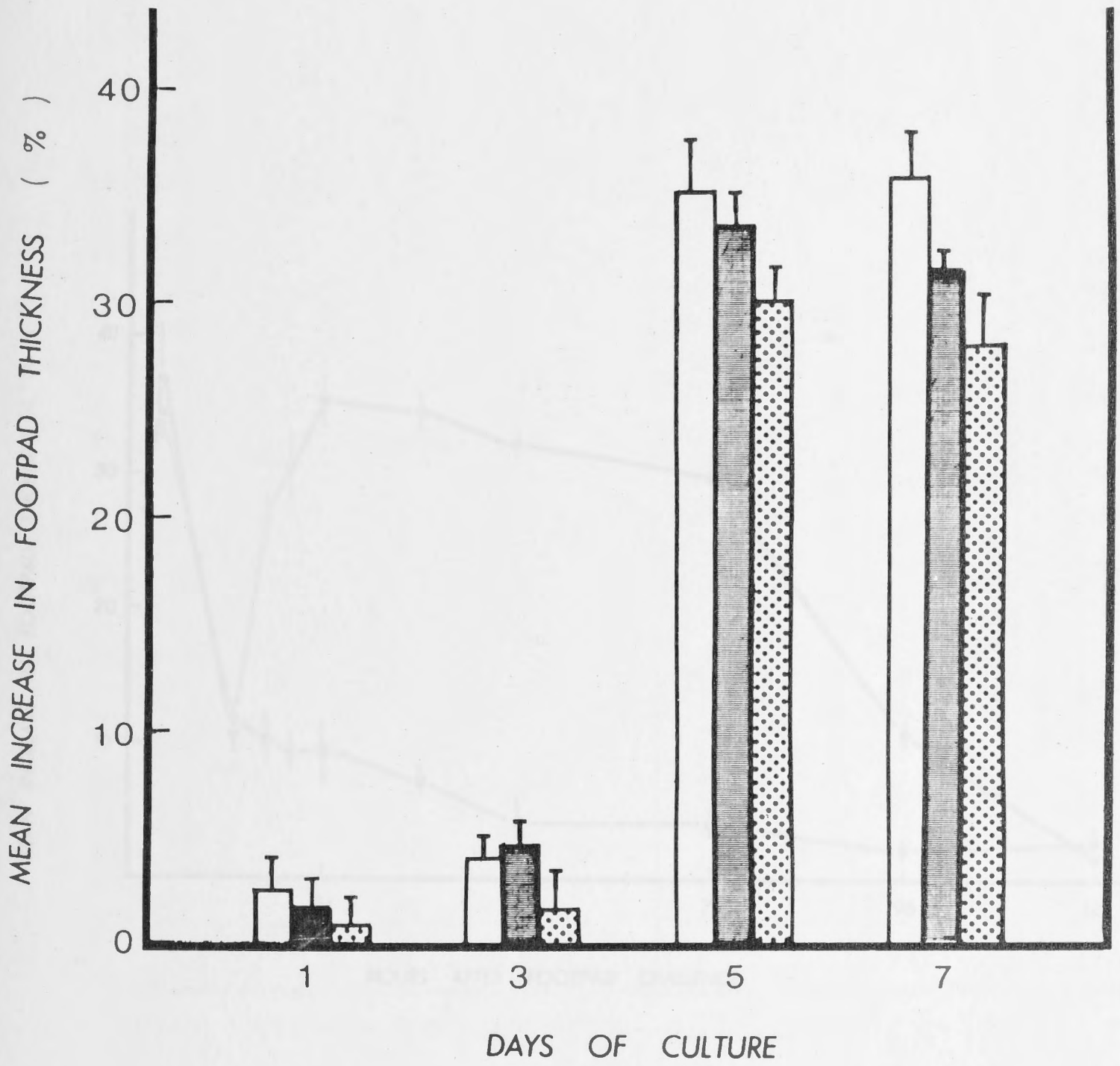


Fig. 1: Kinetics of generation of secondary DTH activity in vitro. 'Memory' CBA spleen cells were cultured for different times with virus-infected syngeneic stimulator cells before harvesting. $5x10^6$ viable secondary effector cells were injected with virus into the hind footpad of each normal CBA mouse and footpad swelling was measured at (□) 24 h; (▩) 48 h; and (⊡) 72 h after challenge. Vertical bars represent one standard error.

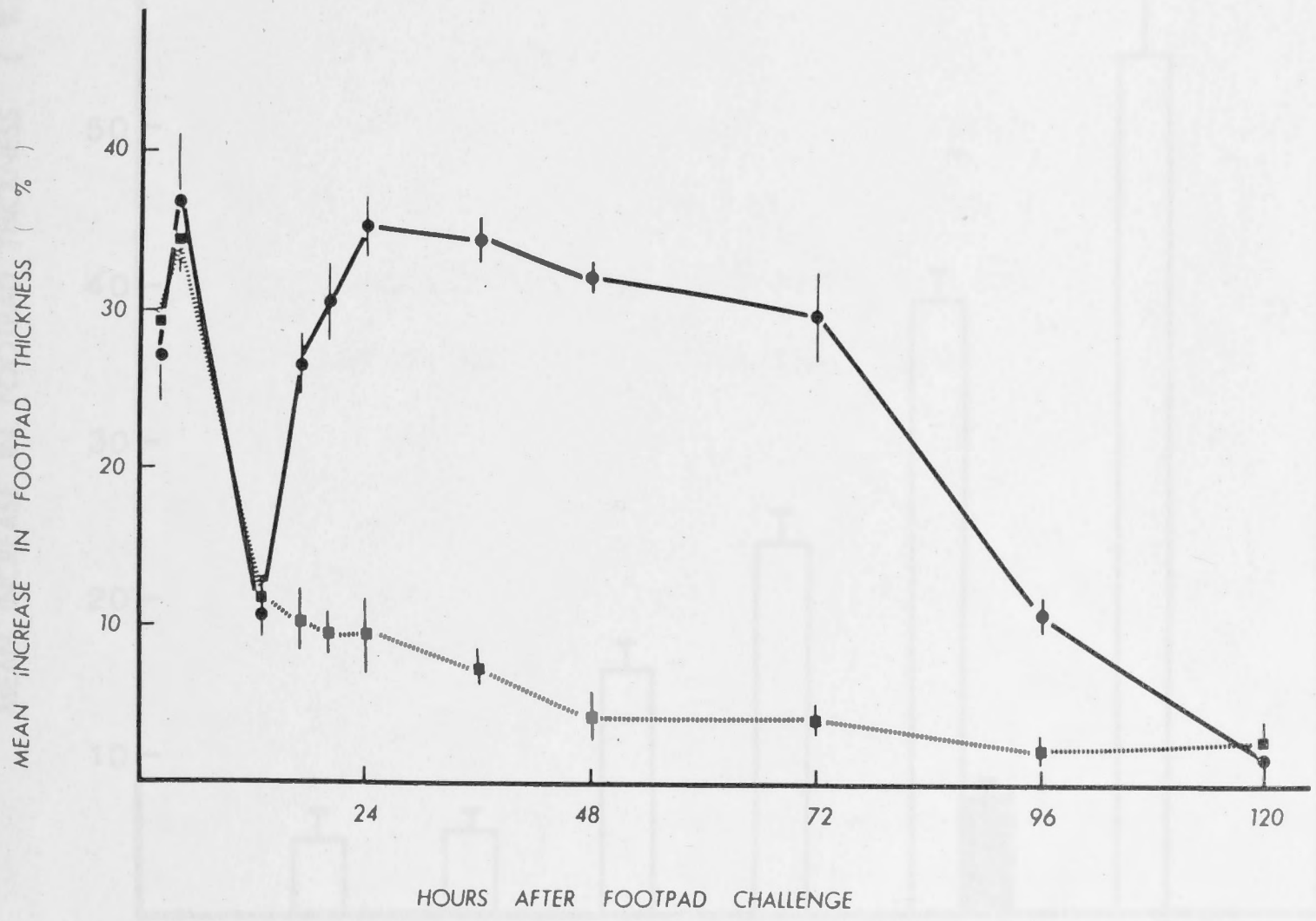


Fig. 2: Development of footpad swelling. Normal CBA mice were either injected with virus only (UV-inactivated A/WSN, $6x10^3$ HAU) or secondary effector cells ($5x10^6$) plus virus into the right hind footpads and equal amount of PBS was injected into each left hind footpad as a control. Footpad swelling was measured over a period of 120 h and vertical bars represent ± one standard error.
(▪▪▪▪▪▪▪) virus only; (▬▬) cells + virus.

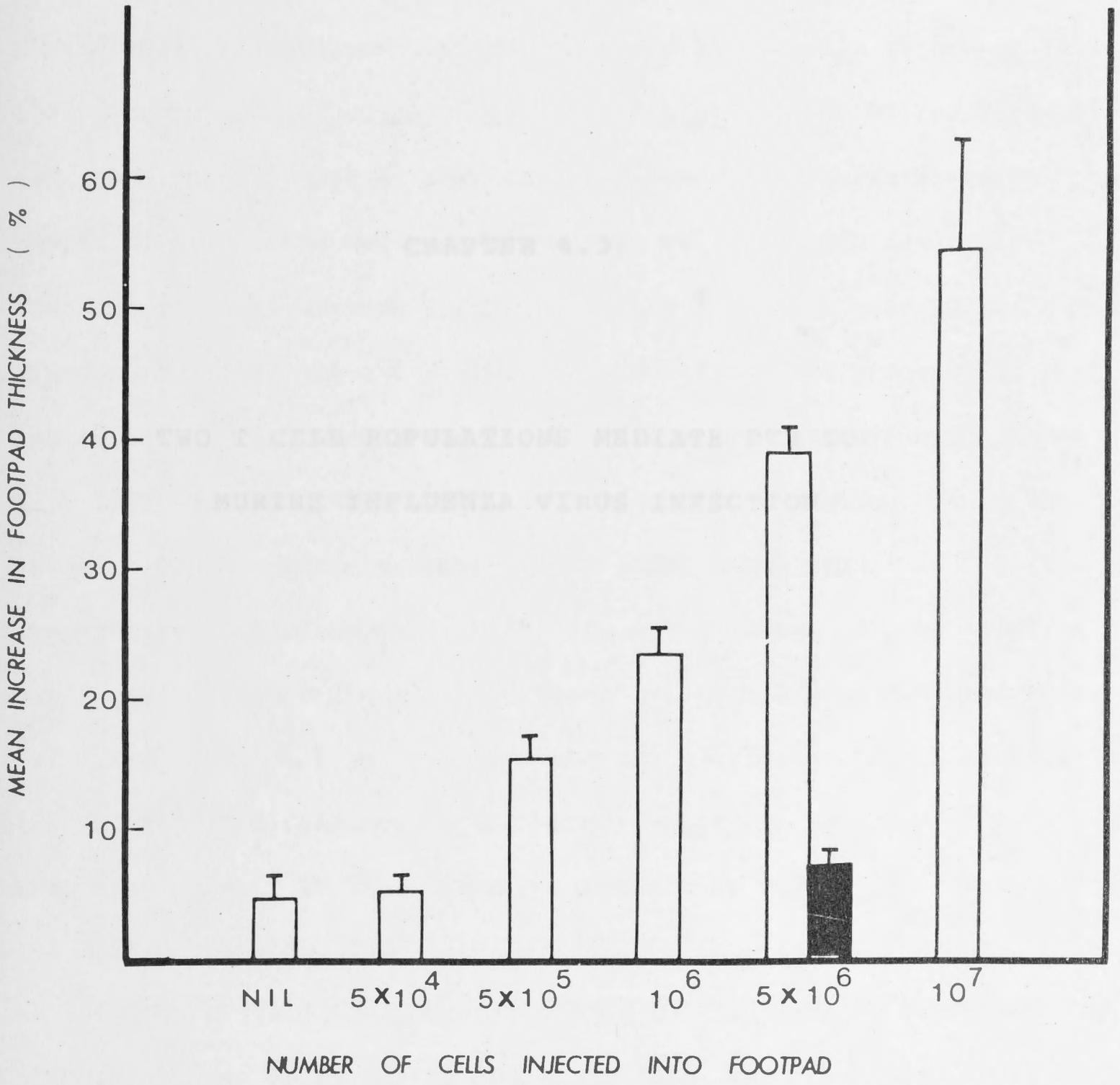


Fig. 3: Dose dependency of DTH activity with secondary effector cells generated in vitro. Different amounts of cells were injected with a standard dose of purified, UV-inactivated virus ($6x10^3$HAU) into the right hind footpad of each CBA mouse and footpad swelling was measured 24 h later (□). On one occasion $5x10^6$ secondary effector cells were injected alone without virus as a control (■). Vertical bars represent one standard error.

CHAPTER 4.3

TWO T CELL POPULATIONS MEDIATE DTH TO MURINE INFLUENZA VIRUS INFECTION

INTRODUCTION

Effector T cells mediating delayed-type hypersensitivity (Td) to non-replicating antigens such as sheep red blood cells (Huber et al., 1976), proteins and polypeptides (Vadas et al., 1977; Miller 1978a,b) are phenotypically Lyt 1^+ and have been found to require I region compatibility at the H-2 gene complex in cell transfer systems, although the delayed-type hypersensitivity (DTH) response to the hapten 2,4-dinitro-1-fluorobenzene (DNFB) was found to be a property of both Lyt 1^+ and Lyt $2,3^+$ cells and are I and K,D region restricted (Vadas et al., 1977; Miller, 1978b). In contrast, cells mediating DTH in the mouse to lymphocytic choriomeningitis (LCM) virus require K,D region compatibility (Zinkernagel, 1976) (the Lyt phenotype of the effector cells was not determined). More recently, it had been reported that Lyt $1,2,3^+$ and Lyt 1^+ T cells are the major effector cells involved in the specific DTH response to the bacteria Listeria monocytogenes (Kaufmann et al., 1979) and the murine sarcoma virus (Leclerc & Cantor, 1980b) respectively. Thus, it seems likely that DTH response can be mediated by different subpopulations of T cells bearing different Lyt phenotypes, depending on the characteristics and the nature of the antigens used in the induction of the response. Earlier chapters in this thesis showed that murine cells which mediate DTH to influenza virus were I region restricted and were Lyt 1^+, irrespective of whether infectious or non-infectious preparations of virus were used to sensitize the mice. However, in these experiments, non-infectious (ultraviolet light (UV)-irradiated) virus was used to elicit the DTH reaction as it was technically difficult to obtain preparations which contained only infectious virus. The possibility remained therefore that infectious virus would sensitize a second population of DTH

effector cells to influenza virus, which would be K,D region restricted but not detected in the previous assay system because the eliciting antigen was non-infectious (Chapters 3.1, 3.2, 4.1, 4.2). This chapter shows that if concentrated preparations of virus which contain infectious virions were used to elicit the DTH reactions, it was found that injection of mice with infectious virus or restimulation of immune spleen cells in vitro generates two distinct populations of effector T cells which mediate DTH reactions.

RESULTS

Three groups of experiments were carried out to determine the nature of effector T cells which take part in DTH reactions to influenza virus. Two examined the conditions of their generation in tissue culture and their properties. The third group examines the properties of the cells present in infected tissues.

Generation and detection of cells mediating DTH that are K,D-restricted depend upon the use of infectious virus

Donor A.TH mice were injected with either infectious or with ultraviolet light (UV)-irradiated (non-infectious) A/WSN virus. After three weeks or more, their spleen cells were restimulated in vitro with syngeneic A/WSN virus-infected spleen cells. After 5 days in culture, the viable cells were first depleted of plastic adherent cells and then injected with infectious A/WSN virus into the footpads of recipient mice which were either H-2 incompatible or shared various regions of the H-2 gene complex with the donor mice. The 24 h footpad swellings are reported in Table 1 and show that using infectious preparations of virus to challenge the recipient mice, DTH activity which is D or K,D-restricted can be detected only if infectious virus was used to sensitize the donor mice.

Effector T cells which mediate DTH can be distinguished by both Lyt phenotype and H-2 requirements

Experiments were carried out to determine the Lyt phenotype of cells mediating DTH that are K,D-restricted. Secondary effector cells were generated as in Table 1 but before injection into the footpads of recipient mice, they were treated with the appropriate antibodies and complement with the results shown in Table 2. The main findings are as follows: If the initial stimulus is non-infectious virus, then the DTH reaction elicited using infectious virus preparation is I-region restricted and has the Lyt 1 phenotype. In contrast, if infectious virus is used both to sensitize mice and to elicit the footpad response, two populations of effector T cells are generated. One is I region restricted and of Lyt 1 phenotype (as seen above); the other is D region restricted and of Lyt 2,3 phenotype.

Mice infected with influenza virus generate two populations of T cells mediating DTH

Following the demonstration that two populations of T cells could be generated in tissue culture, it was important to see if these were also produced *in vivo*. In the first set of experiments (Table 3), mice were inoculated intranasally with a lethal dose of A/WSN virus ($1x10^4 EID_{50}$) and the lungs harvested 6 days later (before death) when there were high titres of infectious virus (Yap & Ada, 1978a). Cell suspensions were made and viable cells injected with infectious virus into the footpads. Clearly again two populations of effector cells were generated, one I region and the other K,D region restricted. A similar set of experiments was performed (Table 3, group 2) in which infectious virus was injected subcutaneously into the donor mice, conditions which result in abortive infections in the spleen (Toms *et al.*, 1974). The immune spleen cells, when tested, again showed the

presence of the two populations of effector cells. Finally (Table 3, group 3), the donor mice were injected with cyclophosphamide (Cy) 2 days prior to subcutaneous injection of infectious virus, conditions which are known to greatly decrease the production of cytotoxic T cells which are K,D region restricted in their action (Doherty & Zinkernagel, 1974; Chapter 3.1). The results of this experiment show quite clearly that whereas the production of I region restricted cells mediating DTH are unaffected, as had been seen previously (Chapter 3.1), the generation of K,D-restricted effector DTH cells is abolished. In each of the above experiments, the tissue cell suspensions were also tested for cytotoxic T cell activity. Both lung and spleen (experiments 1 and 2) cells were quite active, whereas pre-injection of Cy decreased markedly the cytotoxic activity recovered from the spleen, as found previously (Chapter 3.1).

The specificity of D region restricted secondary effector cells mediating DTH

Memory spleen cells from CBA mice primed 3 weeks previously with A/WSN, A/JAP or A/PC virus were restimulated in vitro for 5 days with syngeneic spleen cells infected with homologous virus. These cells were injected ($5x10^6$ per mouse) into the footpads of either H-2 incompatible (C57BL/6J) or D-region compatible (C3H.OH) naive recipient mice that had received infectious A/WSN virus ($2x10^3$ HAU) 6 h previously. The 24 h footpad swellings were shown in Table 4. It was clearly demonstrated that the D-region restricted DTH effector cells are totally cross-reactive within the A strains of influenza virus.

The specificity of I region restricted DTH response in mice sensitized with non-infectious virus

In Chapter 3.2, it has been shown that if inactivated virus was used for elicitation, mice sensitized with UV-inactivated virus

generated I region restricted DTH response that is specific for the homologous virus whereas mice sensitized with infectious virus generated I region restricted DTH response that is cross-reactive within the A strains of influenza virus. In the following experiment, mice were sensitized with UV-inactivated A/WSN or A/PC virus and 6 days later, the mice were challenged into the footpads with infectious preparations of either A/WSN, A/PC, A/JAP or Sendai viruses and footpad swelling measured 24 h later. As shown in Table 5, mice sensitized with UV-inactivated virus only generate DTH response that is specific for the homologous virus, irrespective whether infectious or non-infectious preparation of virus was used for elicitation (compare results in Table 1, Chapter 3.2). Thus the specificity of the DTH response to influenza virus seems to be determined by the infectivity of the virus used for sensitization rather than that used for elicitation.

Failure to recover infectious virus from the footpads of mice

Infectious A/WSN virus (30µl, $1.8 \times 10^7 EID_{50}$) was injected into the hind foodpads of two mice and 24 h later, the feet removed aseptically, cut into small pieces and homogenised in standard medium. After low speed centrifugation, aliquots of the supernatant were injected into 11-day-old embryonated eggs. No infectious virus was recovered.

DISCUSSION

This chapter demonstrates that at least two subpopulations of T lymphocytes can mediate delayed-type hypersnesitivity to influenza virus in mice. The evidence for this was provided by two sets of experiments in which conditions for the generation of these cells in tissue culture and their antigenic characteristics were investigated and from a third experiment in which effector lymphocytes present in virus-infected mouse lungs were analysed.

It was previously found (Chapter 3.2) that, irrespective of whether infectious or non-infectious preparations of influenza virus were used to sensitize cells for a DTH reaction, the use of non-infectious preparations of virus to elicit the reaction activated effector cells which were Lyt 1 positive and I region restricted. The present experiments indicated that a similar result was obtained if non-infectious virus was used as the initial stimulus and an infectious virus preparation was used to elicit the reaction (Tables 1 and 2). However, if infectious preparations of virus were used both to sensitize for and to elicit the reaction, two cell populations with DTH activities can be demonstrated - one that had the Lyt 1 phenotype and was I region restricted and the other that was Lyt 2,3 positive and was D or K,D region restricted (Tables 1 and 2). When cells from the lungs of mice infected intranasally with a lethal dose of virus were transferred to naive recipients, challenge of the recipients with infectious virus also demonstrated that these two cell populations were present in the infected lungs (Table 3), thus showing that the previous finding was unlikely to be an artefact of in vitro culture. The simplest interpretation of these results is that (1). K,D region restricted, Lyt 2,3 positive DTH T cells are only generated if infectious viruses are used both to sensitize the mice and to elicit the response; (2). I region restricted, Lyt 1 positive DTH T cells are generated if non-infectious viruses are used for one or both purposes. There are two possible explanations why infectious influenza virus preparations generate both populations of DTH T cells. The first is that even freshly harvested virus preparations contain an excess of non-infectious virions and these may persist in the mouse because permissive replication does not take place in the spleen (Toms et al., 1974) or footpad (this chapter). Even after the initial rapid replication of influenza virus in the lungs, the virus titre levels off

(Larson et al., 1976; Yap & Ada, 1978a) and this may reflect an accumulation of non-infectious virions. Thus, the mouse would be sensitized to produce both classes of DTH effector cells when infectious influenza virus preparations were injected. A second possibility is that infectious virions can also sensitize mice to give the I-region restricted Lyt 1 positive population of DTH T cells and previous work (Chapter 3.2) on the antigen-receptor specificity of T cells generated when infectious virus was used to sensitize mice and non-infectious virus was used to elicit the response, supports this alternative. However, both situations may occur.

These results are consistent with the earlier finding with LCM virus (Zinkernagel, 1976) that the use of infectious virus can generate a K,D region restricted population of DTH T cells. The reason why LCM infection does not also generate an I region restricted population of DTH T cells is not clear. It may be related to the different abilities of the viral antigens to associate preferentially with the K,D or with the I region gene products or due to the fact that, in contrast to influenza virus, LCM virus replicates permissively in a variety of mouse tissues (Lehmann-Grube, 1971). If other viruses that are known to replicate permissively in mouse tissues, such as ectromelia, Sendai or reoviruses, are found to preferentially produce K,D region restricted, Lyt 2,3 positive DTH T cells, this would favour the second proposal. However, this still remains to be determined.

These findings have several implications. It is now clear that lymphocyte populations activated during a viral infection and which are Lyt 2,3 positive and D or K,D-region restricted can demonstrate two types of activities, namely, cytotoxicity and delayed-type hypersensitivity. Can both of these activities be mediated by the same cells or are they functions of different cells with similar surface antigenic characteristics and specificities? The answer may not be known with

certainty until a clone of cells can be demonstrated to possess or not to possess both properties. However, two additional observations reported here are consistent with the first notion. Firstly, pre-treatment of mice with a high dose of cyclophosphamide before sensitization with infectious virus decreases the generation of cytotoxic T cells in the spleen (Chapter 3.1; Table 3, this chapter) without affecting the production of Lyt 1 positive, I region restricted DTH effector cells (Chapter 3.1). The results in Table 3 show that production of Lyt 2,3 positive, D region restricted DTH effector cells is also diminished if mice were pretreated with cyclophosphamide. Secondly, it was found that Lyt 2,3 positive, K,D region restricted DTH effector cells are cross-reactive within the A strains of influenza virus as was previously found for cytotoxic T cells (Braciale, 1977a; Effros et al., 1977; Zweerink et al., 1977a) whereas DTH effector cells generated after sensitization and elicitation with non-infectious virus that are I region restricted and Lyt 1 positive are specific for the viral haemagglutinin (Chapter 3.2).

It was earlier reported that DTH response to the hapten DNFB was K,D or I region restricted and was the property of two distinct subsets of T cells of different Lyt phenotypes (Vadas et al., 1977; Miller, 1978b). The demonstration of a similar situation with viruses allows the opportunity of studying differences in the presentation of viral antigens to the host when infectious or non-infectious virions are used as the antigens. It is important to study this, as earlier report (Yap et al., 1978) and work in progress (see Chapter 6) have shown that these two different subsets of T cells might have different roles in the recovery of the host from the viral infection.

SUMMARY

Two classes of T lymphocytes can mediate delayed-type hypersensitivity (DTH) to influenza virus in the mouse. If non-infectious virus preparations are used to sensitize for or to elicit a DTH response, the effector cells are found to be Lyt 1 positive and are I region restricted. If infectious virus is used both to sensitize for and to elicit the reaction, a second set of effector cells is also detected which are Lyt 2,3 positive and are D or K,D region restricted. The latter cells are cross-reactive within the A strains of influenza viruses and pretreatment of the mice with high doses of cyclophosphamide markedly decreases their generation in the spleens of sensitized mice, suggesting that the cells which demonstrate DTH activity *in vivo* may also have cytotoxic activity *in vitro*.

TABLE 1

K,D Restriction of DTH Activity is Dependent upon Initial Stimulation and Elicitation with Infectious Virus[a]

Experiment	Donor mice	Initial virus stimulus	Recipient mice	H-2 region shared	Injection into recipient footpad	Mean increase in footpad thickness at 24 h (%)
1	-	-	A.TH	-	Virus	16.7 ± 1.1[b]
	A.TH	Infectious	A.TH	All	Cells + virus	43.8 ± 2.6[c]
	A.TH	Infectious	CBA	Nil	Cells + virus	17.7 ± 1.1[d]
	A.TH	Infectious	BALB/c	D	Cells + virus	42.2 ± 3.0[c]
	A.TH	Infectious	A.TL	K,D	Cells + virus	41.7 ± 2.1[c]
2	-	-	A.TH	-	Virus	11.4 ± 1.1
	A.TH	Non-infectious	A.TH	All	Cells + virus	39.6 ± 2.1
	A.TH	Non-infectious	A.TL	K,D	Cells + virus	12.5 ± 1.8[d]
	A.TH	Non-infectious	CBA	Nil	Cells + virus	11.8 ± 0.8[d]
	A.TH	Non-infectious	BALB/c	D	Cells + virus	13.3 ± 0.8[d]

Legends for Table 1

a A.TH mice were primed with either infectious ($1x10^7$ EID_{50}) or ultraviolet light (UV)-inactivated (10^3 HAU) A/WSN virus 3 weeks or more previously. Spleen cells were harvested and restimulated *in vitro* with A/WSN infected syngeneic spleen cells for 5 days. Viable cells remaining after 5 days culture were depleted of plastic adherent cells and tested for DTH activity by adoptive transfer into footpads of normal recipients ($5x10^6$ cells/mouse footpad) together with infectious virus ($2x10^3$ HAU/mouse). Footpad swelling was measured 24 h after footpad challenge.

b Mean ± standard error.

c Not significantly different from each other but significantly different from controls in which mice were challenged with infectious virus only ($p < 0.05$).

d Not significantly different from controls in which mice were challenged with infectious virus only.

TABLE 2

DTH Effector T Cells[a] Can be Distinguished by Both Lyt Phenotype and H-2 Antigen Requirements

Donor mice	Initial viral stimulus	Recipient mice	H-2 region shared	Treatment of cells before transfer	Injection into recipient footpad	Mean increase in footpad thickness at 24 h (%)
-	Infectious	CBA	-	-	Virus	10.9 ± 1.6
CBA	Infectious	CBA	All	-	Cells + virus[d]	41.7 ± 1.1[e]
CBA	Infectious	BALB/c	Nil	-	Cells + virus	13.6 ± 1.1[f]
CBA	Infectious	C3H.OH	D	-	Cells + virus	36.4 ± 2.1[e]
CBA	Infectious	C3H.OH	D	Anti-Lyt 1.1 + C'[b]	Cells + virus	38.6 ± 2.1[e]
CBA	Infectious	C3H.OH	D	Anti-Lyt 2.1 + C'[c]	Cells + virus	11.4 ± 2.1[f]
CBA	Infectious	A.TL	I	-	Cells + virus	43.8 ± 1.8[e]
CBA	Infectious	A.TL	I	Anti-Lyt 1.1 + C'	Cells + virus	11.4 ± 1.1[f]
CBA	Infectious	A.TL	I	Anti-Lyt 2.1 + C'	Cells + virus	42.7 ± 1.1[e]
CBA	Non-infectious	CBA	All	-	Cells + virus	42.2 ± 1.6[e]
CBA	Non-infectious	BALB/c	Nil	-	Cells + virus	13.6 ± 1.1[f]
CBA	Non-infectious	A.TL	I	-	Cells + virus	43.8 ± 0.0[e]
CBA	Non-infectious	A.TL	I	Anti-Lyt 1.1 + C'	Cells + virus	11.4 ± 1.1[f]
CBA	Non-infectious	A.TL	I	Anti-Lyt 2.1 + C'	Cells + virus	42.7 ± 2.8[e]

Legends for Table 2

a Normal CBA spleen cells were infected with A/WSN virus (2-3 EID_{50}/cell, 1 h, 37°C) and then mixed with syngeneic spleen (memory) cells from mice primed earlier with either infectious (10^7 EID_{50}) or UV-inactivated (10^3 HAU) A/WSN virus. After 5 days *in vitro* culture, the viable cells were harvested and tested for their DTH activities by adoptive transfer, as described in Table 1.

b Anti-Lyt 1.1 + C' treatment killed about 60% of the cells so about $2x10^6$ cells were injected per footpad.

c Anti-Lyt 2.1 + C' treatment killed about 33% of the cells so about $3.5x10^6$ cells were injected.

d $5x10^6$ secondary effector cells (depleted of plastic adherent cells) plus $2x10^3$ HAU infectious A/WSN virus.

e Significantly different from controls (mice challenged with infectious virus only), $p < 0.05$, but not significantly different from each other.

f Not significantly different from controls (mice challenged with virus only).

TABLE 3

Detection of Both K,D and I region Restricted DTH Activities in Lungs or Spleens of Mice Given Intranasal or Subcutaneous Inoculation of Infectious Virus

Exp. No.	Source of effector cells	Donor mice	% specific ^{51}Cr release[d] (E:T ratio 20:1)	Recipient mice	H-2 region shared	Cell transfer (i.v.)	Injection into recipient footpad	Mean increase in footpad thickness at 24 h (%)
1	Lungs[a]	A.TL		A.TL	All	-	Cells + virus	37.5 ± 3.1
		A.TL		CBA	I	-	Cells + virus	35.9 ± 4.7
		A.TL	54.3 ± 4.1	A.TH	K,D	-	Cells + virus	32.8 ± 1.6
		A.TL		C57BL/6J	Nil	-	Cells + virus	10.9 ± 1.6[e]
		-		A.TL	-	-	Virus	10.2 ± 0.8
2	Spleen[b]	A.TL	21.8 ± 0.8	A.TL	All	+	Virus alone	32.3 ± 1.1
		A.TL		CBA	I	+	Virus alone	34.4 ± 1.8
		A.TL		A.TH	K,D	+	Virus alone	25.0 ± 1.8
		A.TL		C57BL/6J	Nil	+	Virus alone	8.3 ± 1.1[e]
3	Spleens[c] from Cy-treated mice	A.TL	7.5 ± 1.3	A.TL	All	+	Virus alone	33.3 ± 1.1
		A.TL		CBA	I	+	Virus alone	34.4 ± 3.1
		A.TL		A.TH	K,D	+	Virus alone	9.4 ± 3.1[e]
		A.TL		C57BL/6J	Nil	+	Virus alone	10.9 ± 1.6[e]

Legends for Table 3

a A.TL mice were inoculated intranasally with a lethal dose (10^4 EID_{50}) of A/WSN virus and lungs harvested 6 days later. Lung cells were injected into the footpads of normal recipients (10^6/mouse) 6 h after injection of infectious virus ($2x10^3$ HAU/mouse) into the same footpad.

b A.TL mice were injected subcutaneously with 10^3 HAU infectious A/WSN virus, the spleens harvested 6 days later and $7x10^7$ immune cells injected intravenously into each recipient mouse, followed 4 h later by injection of infectious virus ($2x10^3$ HAU/mouse) into the footpad.

c As in experiment 2, except the donor mice were injected with cyclophosphamide (150 mg/kg) 2 days before sensitization with virus and $4x10^7$ day 6 immune cells were injected into recipient mice.

d Donor spleen cells in each experiment were tested for cytotoxicity on A/WSN-infected P815 (H-2 compatible) target cells in a ^{51}Cr release assay. Results are expressed as mean ± standard error.

e Not significantly different from controls in which recipient mice were challenged with virus only.

TABLE 4

Specificity of D Region Restricted DTH Activity Generated Secondary *in Vitro*[a]

Donor mouse strain	Virus strain used for: Priming *in vivo*	Restimulation *in vitro*	Elicitation in recipient mice	Recipient mouse strain	H-2 region shared	Injection into footpad of recipients	Mean increase in footpad thickness at 24 h (%)
-	-	-	-	C3H.OH	-	Virus alone	14.1 ± 1.6
CBA	A/WSN (H0N1)	A/WSN	A/WSN	C3H.OH	D	Cells + virus	39.9 ± 2.4[b]
				C57BL/6J	Nil	Cells + virus	15.6 ± 3.1[c]
CBA	A/JAP (H2N2)	A/JAP	A/WSN	C3H.OH	D	Cells + virus	41.4 ± 1.5[b]
				C57BL/6J	Nil	Cells + virus	14.1 ± 1.6[c]
CBA	A/PC (H3N2)	A/PC	A/WSN	C3H.OH	D	Cells + virus	44.6 ± 0.8[b]
				C57BL/6J	Nil	Cells + virus	15.6 ± 3.1[c]

a CBA mice were primed with infectious A/WSN, A/JAP or A/PC (10^7 EID_{50}/mouse i.p.). After 3 wk or more, memory spleen cells (responder cells) were restimulated with syngeneic spleen cells infected with homologous virus ($3EID_{50}$/cell, 1 h, 37°C). Effector cells harvested after 5 days culture were injected ($5x10^6$/mouse) into footpads of recipient mice that had received infectious A/WSN virus ($2x10^3$ HAU) 6 h previously. Footpad swelling was measured 24 h after injection of cells.

b Significantly different from controls (mice challenged with infectious virus only), $p < 0.05$ but not significantly different from each other.

c Not significantly different from controls.

TABLE 5

Specificity of I Region Restricted DTH Response in Mice Sensitized with Non-Infectious Influenza Virus

Virus used for		Mean increase in footpad thickness at 24 h (%)
Sensitization[a]	Elicitation[b]	
Nil	A/WSN (H0N1)	15.6 ± 1.8[c]
Nil	A/JAP (H2N2)	12.5 ± 1.8[c]
Nil	A/PC (H3N2)	15.6 ± 1.8[c]
Nil	Sendai	12.5 ± 2.1[c]
A/WSN	A/WSN	47.9 ± 1.1[d]
A/WSN	A/JAP	13.6 ± 1.1
A/WSN	A/PC	18.8 ± 1.8
A/WSN	Sendai	13.6 ± 2.8
A/PC	A/WSN	19.8 ± 1.1
A/PC	A/JAP	17.7 ± 1.1
A/PC	A/PC	47.9 ± 2.1[d]
A/PC	Sendai	10.9 ± 1.6

a CBA mice were sensitized with 5×10^3 HAU UV-inactivated virus s.c.

b Mice were elicited with infectious virus (2.5×10^3 HAU) 6 days after sensitization.

c Background nonspecific swelling due to injection of infectious virus alone.

d Significantly higher than background swelling ($p < 0.001$).

CHAPTER 5

INDUCTION OF A PRIMARY DTH RESPONSE TO INFLUENZA VIRUS IN VITRO

CHAPTER 5.1

EFFECTS OF HELPER T CELLS ON THE PRIMARY IN VITRO PRODUCTION OF DTH RESPONSES TO INFLUENZA VIRUS

INTRODUCTION

A requirement for helper T cells in the production of IgG antibody by B cells has been known for a long time and has been extensively studied (Katz & Benacerraf, 1972; Katz, 1976; Sprent et al., 1980). More recently, evidence has accumulated that there may be two classes of helper T cells for antibody production (Tada et al., 1978; Keller et al., 1980; Janeway, 1980a)and that T cell-T cell collaboration may be involved in the in vitro generation of cytotoxic T lymphocytes (Tc) (Wagner, 1973; Cantor & Boyse, 1975; Wagner & Röllinghoff, 1978; Corley et al., 1980). Moreover, Pilarski and her colleagues have shown that there is an absolute requirement for antigen-specific helper T cells in the generation of Tc cells from thymocyte precursors (Pilarski, 1977; Baum & Pilarski, 1978) as well as for Tc responses to metabolically inactivated stimulator cells (Pilarski, 1979). The possible participation of helper T cells in anti-viral Tc responses is suggested from recent work both in vivo (Zinkernagel et al., 1978) and in vitro (Pang et al., 1976; Ashman & Müllbacher, 1979; Kreeb & Zinkernagel, 1980). A similar requirement for helper cells in the generation of H-Y-specific CTL has also been suggested by von Boehmer & Haas (1979). Finberg et al. (1979), on the other hand, have shown that radioresistant helper T cells generated in vivo can augment TNP-specific Tc activity generated in vitro and Fujiwara et al. (1980) have found a similar situation with respect to cell-mediated immunity (CMI) against syngeneic tumors in vitro. Another major arm of the CMI response is the delayed-type hypersensitivity (DTH) response and there are now at least three reports that such activity can be generated in vitro from normal spleen cells against heterologous erythrocytes (Bretscher, 1979; Ramshaw & Eidinger,

1979) or to a soluble protein and a synthetic antigen (Eshhar et al., 1979). A recent paper has suggested that there is a requirement for T-T cooperation for the manifestation of a DTH response to the synthetic polypeptide (T,G)-A-L (Strassmann et al., 1980). Earlier chapters in this thesis have studied the DTH response to influenza virus as an antigen, both in vivo (Chapters 3.1; 3.2; 4.1) and in vitro (Chapter 4.2). It has been found that a weak primary DTH response to influenza virus can also be obtained in vitro (Chapter 5.2). This chapter demonstrates that this response can be greatly augmented by radioresistant helper T cells and the antigenic specificity as well as H-2 requirement of the helper cells will be described.

RESULTS

Evidence for radioresistant helper cells for primary DTH response generated to influenza virus in vitro

Normal CBA spleen cells were incubated with A/WSN virus-infected syngeneic spleen cells ($3EID_{50}$/cell) at a responder to stimulator cell ratio of 5:1. After 5 days of culture at 37°C, viable cells were tested for DTH activity by adoptive transfer with antigen (purified, UV-inactivated A/WSN virus) into syngeneic naive recipients. As shown in Table 1, a low but significant level of DTH activity was generated without addition of other cells (group A). However, when spleen cells from mice primed in vivo 2 d previously with a low dose of infectious A/WSN virus (10 HAU/mouse injected i.v.) were used as the responder cells, considerable enhancement of the DTH response was observed (Table 1, group B). If without a further period of culture in vitro, these 2 d immune spleen cells were adoptively transferred i.v. to naive recipients or the Ig-negative

fraction prepared from them injected locally into footpads of naive recipients and the mice subsequently challenged with virus, little or no DTH activity can be detected (mean increase in footpad swellings of i.v. injection of $9x10^7$ unfractionated 2 d immune spleen cells and footpad injection of $1x10^7$ Ig-negative fraction of 2 d immune spleen cells were 3.1 ± 1.8% and 4.2 ± 2.1% respectively). On the other hand, if these 2 d immune spleen cells were cultured alone for 5 d *in vitro* and then injected into footpads of mice with virus, a low but significant level of DTH activity was observed (Table 1, group G). This suggests that sufficient antigen was carried over to allow the continuation of the *in vivo* primed response *in vitro*. One possible explanation for the enhancement of the DTH response by *in vivo* priming was that the 2 d *in vivo* primed cell population contained helper cells. If this was so, then this preparation would still be active after γ-irradiation. This was found to be the case. Two day primed cells were γ-irradiated and added to infected stimulators and normal spleen cells (responders). An enhanced DTH response was shown when the cultured cells were adoptively transferred into naive recipients (Table 1, group C). If these γ-irradiated 2 d immune cells were cultured only with infected stimulator cells, no DTH activity was recovered (Table 1, group E). This would argue against the possibility that the observed increase in DTH activity in group C was due to an additional DTH activity contributed by the primed cell population.

The next few experiments were carried out to determine optimum conditions for the production of this helper activity and in all cases the helper cells were γ-irradiated before use.

Effect of in vivo priming dose on the generation of helper activity

CBA mice were primed i.v. with various doses (1-1000 HAU) of infectious or UV-inactivated A/WSN virus and 2 d later, spleen cells were harvested and used as the source of helper cells. Primary DTH activity was generated by co-culturing of γ-irradiated helper cells with A/WSN virus-infected spleen cells (stimulator cells) and normal spleen cells (responder cells) at a fixed ratio of 5:1:5. Viable cells were tested for their DTH activity 5 d later. As seen in Table 2, significant levels of helper activity were generated by priming with low doses (1-10 HAU) of infectious virus; priming with higher doses of virus was less efficient. In contrast, if UV-inactivated virus was used for priming, doses of 10 HAU or higher were required to demonstrate significant levels of helper activity, and the helper activity was slightly increased at higher doses (e.g. up to 10^3HAU).

Factors affecting the helper activity for the DTH response generated in vitro

In the first group of experiments, the kinetics of generation of helper activity were studied with the results shown in Fig. 1. Day 1 immune spleen cells from mice primed with 10 HAU infectious virus were inactive, whereas optimal helper activity was obtained with 2 d immune spleen cells. Thereafter the helper activity declined gradually. About 40% enhancement of the DTH response as compared to control (normal spleen cells were used as helper) was still observed when 21 d immune cells were tested for helper activity but 35 d cells were no longer active. In the second set of experiments, the dose-dependency of helper cells in augmenting primary DTH response generated in vitro to influenza virus was examined. The results in Fig. 2A clearly showed that the helper activity was dose-dependent. Of the ratios of the responders to helper cells tested, equal numbers of helper and

normal responder cells gave maximal DTH response. No helper activity could be observed at a ratio of responder to helper cells of 8:1 or above. Finally, the influence of the time of addition of helper cells to the culture in the enhancement of DTH response generated in vitro was investigated. Fig. 2B shows that significant helper activity was observed if the cells were added within the first 24 h but not 48 h or later after the initiation of the culture.

Characterization of the helper cells

Day 2 immune spleen cells from CBA mice primed with a low dose of infectious A/WSN virus (10 HAU) were treated with anti-Thy 1.2 ascitic fluid plus complement or with complement alone before their addition to cultures of stimulator cells and normal responder cells. Helper activity was completely abrogated by anti-Thy 1.2 ascitic fluid plus complement but not by complement treatment alone (Table 3, Expt. 1). Removal of plastic adherent cells from the primed cell population did not affect their helper activity and fractionation of the resultant non-adherent cells into Ig-positive and Ig-negative fractions showed that only the Ig-negative cells contained helper activity (Table 3, Expt. 2), suggesting that the helper activity was a property of the T cells. Further confirmation of this was obtained by showing that nude mice failed to generate helper activity (Table 3, Expt. 3). The helper T cells were further characterized by anti-Lyt antibodies and complement treatment. It was found that the helper activity was sensitive to anti-Lyt 1.1 antibodies plus complement, but not to anti-Lyt 2.1 antibodies plus complement treatment (Table 3, Expt. 4). An experiment also showed that only viable cells could deliver help, as heat-killed cells or supernatant from sonicated cells were unable to demonstrate any helper activity (Table 3, Expt. 4). Therefore, the data clearly show that radioresistant, viable plastic

non-adherent, Lyt 1^+ helper T cells can augment the primary DTH response generated to influenza virus *in vitro*.

The effects of viral infectivity and antigen specificity on T cell help

Four sets of experiments were carried out in which the infectivity of the virus used to generate the stimulator cells and helper T cells were varied. In addition, the effect of homologous or heterologous virus used to generate helper T cells was also examined. Normal CBA spleen cells (responders) were stimulated *in vitro* with syngeneic spleen cells exposed (1 h, 37°C) to either infectious ($3EID_{50}$/cell) or purified, UV-inactivated virus ($1.5x10^3$HAU/10^7 cells) and the cultures also contained syngeneic γ-irradiated helper cells which were obtained from spleens of mice primed 2 d earlier with either homologous or heterologous and with infectious or UV-inactivated virus (10 HAU). Primary effector cells were harvested after 5 d and tested for their specificities of DTH activity by eliciting the reaction with different strains of purified, UV-inactivated virus ($6x10^3$HAU). The results of several experiments are shown in Tables 4 and 5. As far as infectivity of the virus is concerned, the following two conclusions can be drawn: (1). If the virus used to generate stimulator cells *in vitro* is non-infectious, then the DTH effector cells (Td) so generated will be specific for the homologous virus used for stimulation irrespective of whether infectious or non-infectious virus is used to generate helper T cells (Table 4, Expt. 1) and (2). If the virus used to generate stimulator cells is infectious, then the final DTH effector cells will be cross-reactive within the A strains of influenza virus, again irrespective of whether infectious or non-infectious virus is used to generate helper cells (Table 4, Expt. 2). That is, the specificity of the effector cells is predominantly a function of the infectivity of the virus used for their *in vitro* stimulation, and is independent of the infectivity of the virus used

for *in vivo* priming for the helper activity. When the antigen specificity of the helper T cells was examined, the results showed that (1). Helper activity is antigen-specific as helper cells generated from *in vivo* priming with Type B influenza virus cannot help the primary response generated to Type A virus (Table 5) and (2). *In vitro* stimulation of DTH response by infectious virus can be helped by helper cells generated from *in vivo* priming with either homologous or heterologous A strain viruses, whereas stimulation by non-infectious virus can only be helped by homologous but not heterologous A strain viruses (Table 5).

H-2 restriction of helper activity

γ-irradiated helper cells were obtained from mouse strains which differed from the donors of the normal responder cells and of the virus-infected stimulator cells at various regions or subregions of the major histocompatibility complex (MHC). After culturing for 5 d, the effector cells were adoptively transferred to naive recipients which were either syngeneic to the donors of the responder cells, when the challenge antigen used was UV-inactivated virus; or were K,D compatible but I region incompatible to the donors of the responder cells, when the challenge antigen was infectious virus. The results in Table 6 show clearly that the helper cell activity was IA subregion restricted, irrespective of whether the DTH effector cells generated were I or K,D region restricted.

In the above experiments, the stimulator and responder cells were syngeneic so it was not clear whether the restriction occurred at the stimulator or responder cell level. So further experiments were carried out in which the helper cells were syngeneic at the I region either with the stimulator cells or with the responder cells. The results in Table 7 show that the I region restriction of helper cell

activity was at the level of stimulator cells and not with the responder cells.

DISCUSSION

This chapter shows that two days after i.v. injection of influenza virus into mice, the spleen contains T cells which can function as helper T cells (Th) in the *in vitro* generation of effector T cells which mediate DTH reactions. The cells are radioresistant, are sensitive to anti-Thy 1.2 ascitic fluid and complement and to anti-Lyt 1.1 antibodies and complement treatment. Both inactivated and infectious virus induce Th cell formation but the dose response curves are different. With infectious virus, low doses were more efficient; with inactivated virus, higher doses were better. The reason for the different responses is not clear. It may in part be a question of antigen load. Although infectious virus has not been recovered from the spleen after i.v. injection of infectious virus (Braciale & Yap, 1978), non-permissive replication is thought to take place and this could result in a substantial increase in the amounts of antigen expressed at the cell surface. Although low levels of DTH activity can sometimes be detected in spleens 2 or 3 days after sensitization with virus, peak activity occurs at about day 6 (Chapter 3.1). In contrast, peak Th activity occurs 2-3 days after sensitization and then slowly decreases and is no longer detected 5 weeks later. In tissue culture, the Th cells are only effective if added to the culture within the first day but not two days after the initiation of culture. This is unlikely to be the fact that enough helper activity has been generated in the culture within the first two days as the lack of addition of Th cells usually gives only a low DTH response (Table 1). It may well be that the responder cells can no longer respond to the Th cells after 2 days in culture.

There are several aspects which warrant discussion. Though the Th cells show antigen specificity with respect to Type A and B influenza viruses, the specificity patterns of T cell help and of the effector DTH cells (Td) depend both on the infectivity of the virus used for in vitro stimulation and on the specificity (i.e., homologous or heterologous virus within the A strains) of the virus used to generate the Th cells in vivo. The data, as shown in Tables 4 and 5, are summarized in Table 8. Briefly, two conclusions can be drawn: (1). If infectious virus is used for in vitro stimulation, help is delivered whether homologous or heterologous virus is used to generate Th cells in vivo. (2). If non-infectious virus is used for in vitro stimulation, help is delivered only when homologous virus is used to generate Th cells in vivo. Infection of cells by virus may allow expression at the cell surface of a crossreactive determinant(s) of a viral antigen, presumably the haemagglutinin (Hackett et al., 1980; Koszinowski et al., 1980), and this may be recognized by the Th cells primed with a heterologous virus. It has been shown that IgG response to influenza virus is T cell-dependent (Virelizier et al., 1974), and both infectious virus and non-infectious virus are effective at inducing an antibody response after i.v. injection. A recent report (Anders et al., 1980) indicates that the Th cells present in the spleens of mice previously infected with an influenza A strain virus could deliver cross-reactive help (within the A strains) to B cells. It may be that the same Th cells can serve both roles, that is, to co-operate with both T and B cells.

Another interesting aspect is the H-2 restriction pattern of the Th cells and the main data for this, as recorded in this chapter, are summarized in Fig. 3. The activity of these cells is IA subregion-restricted and by using stimulator and responder cells which varied in specificity at the K,D or I region with the helper cells, it was clearly

shown that the restriction operates at the level of the stimulator cells and that the delivery of help to the responder cells is not H-2 restricted. There are many examples where it has been shown either *in vivo* or *in vitro* that Th cell activity is H-2 restricted (Kindred & Shreffler, 1972; Katz *et al.*, 1973; Sprent, 1978; Waldmann, 1978; Singer *et al.*, 1979; Andersson *et al.*,1980; Martinez-Alonso *et al.*, 1980). There are few *in vitro* experiments where the H-2 requirements between Th cells and stimulator cells on one hand and Th cells and effector lymphocytes on the other hand have been studied. Singer *et al.* (1979) showed that in a T-B collaboration system, the Th cells recognize H-2 determinants on accessory cells but not on B cells so that the delivery of help to the B cells is not H-2 restricted. Ashman & Müllbacher (1979) have also shown that the delivery of help by the radioresistant Th cells to the Tc precursors is H-2 unrestricted; the H-2 requirements between Th cells and stimulator cells was not analysed.

It was earlier found that DTH activity to influenza virus can be mediated by two distinct subpopulations of T cells (Chapter 4.3). One is Lyt 23^+ and is K,D region restricted; the other is Lyt 1^+ and is IA subregion-restricted. The former is only detected if infectious virus is used both to sensitize the host and to elicit the response and in this situation, both T cell subpopulations are demonstrated. If non-infectious virus is used at either step, only IA subregion restricted DTH is observed. It was found that both the K,D and IA region restricted DTH reactions could be elicited from cell populations generated in the primary *in vitro* culture and the Th cells were active in augmenting the DTH activity mediated by both cell types. The question arises - what is the relationship between the precursor DTH cells, the effector DTH cells and the helper cells? Recently, an interaction between two distinct T cell subpopulations, with the

phenotypes Lyt 123^+ and Lyt 1^+ was shown to be necessary for the manifestation of the DTH response to a synthetic polypeptide (T,G)--A--L (Strassmann et al., 1980). It has shown elsewhere (Chapter 5.2) that the precursors for primary DTH responses to influenza virus are most likely to be Lyt 123^+ cells so such precursors can give rise to two subpopulations of effector T cells, one is Lyt 1^+, I region restricted, and the other Lyt 23^+ and K,D region restricted. Presumably the helper T cells described in this work also arise from Lyt 123^+ precursor cells. Do they represent an intermediate stage between the precursor cells and the Lyt 1^+, IA subregion restricted DTH effector cells? The answer to this question is not known at present and I know of no finding which eliminates this possibility. If this is so, then in the differentiation process, the ability to act as a Th cell precedes the ability to mediate a DTH response as the 2 days immune γ-irradiated cells are active as helpers but not as mediators of DTH reaction. Whatever the answer is, the present work adds to the growing evidence that provision of help via T cells is a common immunological phenomenon. The extent to which helper activity in the generation of DTH response may occur in vivo has yet to be determined.

At present, little is known about the action of Th cells in enhancing DTH response in vitro. A tentative mechanism is shown in Fig. 4. Whether the delivery of help requires cell-to-cell contact between Th cells and precursor Td cells or via soluble factor(s) has not yet been established.

SUMMARY

Injection of mice with infectious or non-infectious preparations of influenza virus induces the formation of T cells which, when added to primary tissue cultures of normal spleen cells exposed to influenza virus, enhance the generation of effector T cells which mediate DTH reaction. The enhancing cells possess Thy 1 and Lyt 1 surface antigens, are radioresistant and antigen-specific. If infectious virus was used to stimulate the DTH response *in vitro*, help was delivered whether homologous or heterologous A strain influenza virus was used to generate the helper cells (Th) *in vivo*. In contrast, only Th cells generated using homologous virus were effective if non-infectious virus was used to stimulate the DTH response *in vitro*. Peak helper activity occurred two days after virus injection and the Th cells were only effective if added to the primary cultures within 24 h after addition of the stimulating antigen. The Th cells enhanced the generation of both classes of DTH effector cells, i.e., those which are Lyt 1^+ and IA subregion restricted and those which are Lyt 23^+ and K,D region restricted. The activity of the Th cells was found to be IA subregion restricted and this was shown to operate at the level of the stimulator cells so that the delivery of help to the responder cells was not H-2 restricted. The possibility that the Th cells might be a precursor to the Lyt 1^+, IA subregion restricted DTH effector cells is discussed.

TABLE 1

Evidence for Radioresistant Helper Cells for Primary DTH Response Generated to Influenza Virus *in Vitro*

Group No.	Primary *in vitro* culture[a]				Mean increase in footpad thickness at 24 h (%)[c]	Enhancement of DTH response[d] (%)
	A/WSN virus-infected spleen cells (1.5×10^7)	Day 2 immune spleen cells[b] (7.5×10^7)	Day 2 immune spleen cells[b] (γ-irradiated, 2000 rads) (7.5×10^7)	Normal spleen cells (7.5×10^7)		
A	+	-	-	+	15.6 ± 1.8	
B	+	+	-	-	34.4 ± 1.8	121
C	+	-	+	+	32.3 ± 2.8	107
D	+	+	-	+	32.3 ± 1.1	107
E	+	-	+	-	3.1 ± 0.0	-
F	-	-	+	+	10.2 ± 1.5	-
G	-	+	-	-	14.6 ± 1.1	-

a CBA mice were used as donors of spleen cells. Viable cells were harvested after 5 days culture. 1×10^7 cells were then injected together with purified, UV-inactivated A/WSN virus (6×10^3 HAU) into syngeneic mouse footpad. Footpad swelling was measured 24 h after challenge.

b Mice primed with 10 HAU infectious A/WSN virus.

c Mean ± standard error for groups of 3-4 mice.

d % enhancement was calculated as described in Materials and Methods (Chapter 2).

TABLE 2

The Effect of *in Vivo* Priming Dose Using Infectious or Non-infectious Virus on the Generation of Helper Activity[a]

Experiment No.	Virus dose used for priming	Infectivity of the virus used	Mean increase in footpad thickness at 24 h[b] (%)	Enhancement of DTH response (%)
1	Nil	-	14.9 ± 1.5	-
	1	Infectious	35.2 ± 0.8	136
	10	Infectious	35.2 ± 1.5	136
	100	Infectious	26.6 ± 2.0	79
	1000	Infectious	22.7 ± 2.7	52
2	Nil	-	13.6 ± 1.1	-
	1	Non-infectious	17.7 ± 1.1	30
	10	Non-infectious	32.3 ± 1.1	138
	100	Non-infectious	34.4 ± 1.3	153
	1000	Non-infectious	39.9 ± 2.0	193

a CBA mice were primed i.v. with various doses of A/WSN virus. 2 d immune spleen cells were used as the source of helper cells. Primary DTH activity was generated by co-culturing of γ-irradiated (2000 rads) helper cells (7.5×10^7) with A/WSN virus-infected spleen cells (1.5×10^7) and normal spleen cells (7.5×10^7) for 5 d at 37°C.

b Primary effector cells (1×10^7) were injected into the footpad of each syngeneic recipient together with purified, UV-inactivated A/WSN virus (6×10^3 HAU) and footpad swelling measured 24 h later.

TABLE 3

Helper Cell Characterization

Exp. No.	Source of helper cells[b]	Treatment of helper cells before culture[c]	Mean increase in footpad thickness at 24 h (%)	Enhancement of DTH response (%)
1[a]	Normal spleen cells	Nil	17.7 ± 1.1	-
	Day 2 immune spleen cells	Nil	44.8 ± 2.8[d]	153
	Day 2 immune spleen cells	C' alone	45.8 ± 2.1[d]	159
	Day 2 immune spleen cells	Anti-Thy 1.2 ascitic fluid + C'	16.7 ± 1.1	-6
2[a]	Normal spleen cells	Nil	14.1 ± 1.6	-
	Day 2 immune spleen cells	Nil	30.2 ± 1.1[d]	114
	Day 2 immune spleen cells	Ig-positive fraction	12.5 ± 1.3	-11
	Day 2 immune spleen cells	Ig-negative fraction	34.4 ± 1.8[d]	144
	Day 2 immune spleen cells	Removal of adherent cells	31.3 ± 0.0[d]	122
3	BALB/c normal spleen cells	Nil	16.4 ± 2.4	-
	BALB/c day 2 immune spleen cells	Nil	39.6 ± 3.8[d]	142
	BALB/c nude (nu^+/nu^+) day 2 immune spleen cells	Nil	15.6 ± 3.1	-5
4[a]	Normal spleen cells	Nil	12.5 ± 0.0	-
	Day 2 immune spleen cells	Nil	42.7 ± 1.1[d]	242
	Day 2 immune spleen cells	Anti-Lyt 1.1 + C'	10.9 ± 0.9	-13
	Day 2 immune spleen cells	Anti-Lyt 2.1 + C'	47.7 ± 1.9[d]	282
	Day 2 immune spleen cells	Heat killed (56°C, 30 min)	14.6 ± 1.1	17
	Day 2 immune spleen cells	Sonicated and supernatant used for culture	12.5 ± 1.8	0

Legends for Table 3

a CBA mice were used.

b Normal spleen cells or cells from mice primed 2 days before with a low dose of infectious A/WSN virus (10 HAU, i.v.) were used as a source of helper cells.

c Helper cells were γ-irradiated in all the cases (untreated or treated). 7.5×10^7 treated or untreated helper cells were co-cultured with virus-infected stimulator cells (1.5×10^7) and normal responder cells (7.5×10^7) for 5 d at 37°C before harvesting for effector cells.

d Significantly higher than controls (normal spleen cells were used as a source of helper cells); ($p < 0.01$) but not significantly different from each other for each experiment.

Influence of Viral Infectivity on DTH Response Generated to Influenza Virus *in Vitro*

Exp. No.	Virus used: Priming mice for help[a] Strain	Infectivity[b]	Stimulating cells in vitro[c] Strain	Infectivity	Elicitation in recipients[d] (UV-inactivated)	Mean increase in footpad thickness at 24 h (%)	Enhancement of DTH response[e] (%)
1	Nil	-	A/WSN	NI	A/WSN (HON1)	17.7 ± 1.1	-
	A/WSN	I	A/WSN	NI	A/WSN	42.2 ± 1.6[f]	138
	A/WSN	NI	A/WSN	NI	A/WSN	40.6 ± 3.1[f]	129
	Nil	-	A/WSN	NI	Sendai	6.3 ± 1.3	-
	A/WSN	I	A/WSN	NI	Sendai	7.8 ± 1.6	24
	A/WSN	NI	A/WSN	NI	Sendai	3.1 ± 1.8	-50
	Nil	-	A/WSN	NI	A/RI (H2N2)	6.3 ± 1.8	-
	A/WSN	I	A/WSN	NI	A/RI	4.7 ± 1.6	-25
	A/WSN	NI	A/WSN	NI	A/RI	5.2 ± 1.1	-18
2	Nil	-	A/WSN	I	A/WSN	19.8 ± 2.1	-
	A/WSN	I	A/WSN	I	A/WSN	43.8 ± 3.6[f]	121
	A/WSN	NI	A/WSN	I	A/WSN	41.7 ± 2.1[f]	111
	Nil	-	A/WSN	I	Sendai	6.3 ± 1.8	-
	A/WSN	I	A/WSN	I	Sendai	6.3 ± 2.1	0
	A/WSN	NI	A/WSN	I	Sendai	3.1 ± 0.0	-50
	Nil	-	A/WSN	I	A/RI	18.0 ± 1.5	-
	A/WSN	I	A/WSN	I	A/RI	45.8 ± 1.1[f]	154
	A/WSN	NI	A/WSN	I	A/RI	39.1 ± 1.6[f]	117
	Nil	-	A/WSN	I	A/PC (H3N2)	17.7 ± 1.1	-
	A/WSN	I	A/WSN	I	A/PC	43.8 ± 1.8[f]	148
	A/WSN	NI	A/WSN	I	A/PC	40.6 ± 1.8[f]	129

Legends for Table 4

a CBA mice were primed with 10 HAU virus 2 days before harvest spleens for helper cells.

b I: infectious; NI: non-infectious.

c Normal spleen cells exposed to either infectious (3 EID_{50}/cell) or purified, UV-inactivated (1.5×10^3 HAU/10^7 cells) A/WSN virus for 1 h at 37°C.

d Purified, UV-inactivated virus (6×10^3 HAU/mouse) was injected together with primary effector cells (1×10^7/mouse) into footpads of syngeneic recipient mice.

e Relative increase in footpad thickness compared to controls (mice received effector cells generated in the absence of helper cells).

f Significantly higher than controls (no helper cells added); $p < 0.01$ but not significantly different from each other.

TABLE 5

Specificity of Helper T Cells

Exp. No.	Virus used: Priming mice for help[a] Strain	Infectivity[b]	Stimulating cells in vitro[c] Strain	Infectivity	Elicitation in recipients[d] (UV-inactivated)	Mean increase in footpad thickness at 24 h (%)	Enhancement of DTH response[e] (%)
1	Nil	-	A/WSN	NI	A/WSN	13.6 ± 1.1	-
	A/WSN (HON1)	I	A/WSN	NI	A/WSN	32.8 ± 1.8[f]	141
	A/WSN	NI	A/WSN	NI	A/WSN	32.3 ± 1.1[f]	138
	B/LEE	I	A/WSN	NI	A/WSN	14.1 ± 1.6	4
	A/PC (H3N2)	I	A/WSN	NI	A/WSN	15.6 ± 1.8	15
	A/PC	NI	A/WSN	NI	A/WSN	14.6 ± 2.1	7
	A/JAP (H2N2)	I	A/WSN	NI	A/WSN	16.7 ± 3.8	23
	A/JAP	NI	A/WSN	NI	A/WSN	15.6 ± 1.8	15
2	Nil	-	A/WSN	I	A/WSN	14.1 ± 1.6	-
	A/WSN	I	A/WSN	I	A/WSN	41.7 ± 2.1[f]	196
	A/WSN	NI	A/WSN	I	A/WSN	39.6 ± 2.1[f]	181
	B/LEE	I	A/WSN	I	A/WSN	16.7 ± 2.1	18
	A/PC	I	A/WSN	I	A/WSN	39.6 ± 2.8[f]	181
	A/PC	NI	A/WSN	I	A/WSN	35.4 ± 2.8[f]	151
	Nil	-	A/WSN	I	A/PC	13.6 ± 2.8	-
	A/PC	I	A/WSN	I	A/PC	33.3 ± 1.1[f]	145
	A/PC	NI	A/WSN	I	A/PC	33.3 ± 2.1[f]	145

Legends: Refer to Table 4.

TABLE 6

H-2 Restriction of Helper T Cells in Primary DTH Response to Influenza Virus Generated *in Vitro*

	Donor mouse strains		H-2 region shared between helper and responder cells	Injection into footpads of recipients[a]	Mean increase in footpad thickness at 24 h (%)	
					Elicitation in	
	Helper cells (Day 2 Immune spleen cells, γ-irradiated)	Normal responder cells and virus-infected stimulator cells			Recipients syngeneic to donors of responder cells using UV-irradiated virus[b]	Recipients K,D compatible to donors of responder cells using infectious virus[c]
(a)	-	-	-	Virus only	3.1 ± 3.1[d]	14.1 ± 1.6[d]
(b)	Nil	A.TL	-	Cells + virus	14.1 ± 1.6	23.4 ± 1.6
(c)	A.TL (s kkkkk kkd)	A.TL	All	Cells + virus	34.4 ± 0.0[e] (144)[f]	45.3 ± 4.7[e] (94)[f]
(d)	C57BL/6J (b bbbbb bbb)	A.TL	Nil	Cells + virus	12.5 ± 1.8 (-11)	23.9 ± 1.1 (2)
(e)	CBA (k kkkkk kkk)	A.TL	I	Cells + virus	32.3 ± 1.1[e] (129)	41.7 ± 1.1[e] (78)
(f)	B10.A (4R) (k kbbbb bbb)	A.TL	IA	Cells + virus	32.8 ± 1.6[e] (133)	39.6 ± 1.1[e] (69)
(g)	A.TH (s sssss ssd)	A.TL	K,D	Cells + virus	13.6 ± 1.1 (-4)	23.9 ± 2.1 (2)
(h)	BALB/c (d ddddd ddd)	A.TL	D	Cells + virus	13.6 ± 1.1 (-4)	22.9 ± 1.1 (-2)

a Primary effector cells (5×10^6) were injected at the same time as UV-inactivated virus (6×10^3 HAU) or 6 h after injection of infectious virus (2.5×10^3 HAU).

b Only I-region restricted DTH activity could be elicited using UV-inactivated virus.

c Only K,D-region restricted DTH activity could be demonstrated by elicitation with infectious virus in K/D-compatible recipients. In all cases, A.TH mice were used as recipients except in group (g) where BALB/c mice were used to mimic any possible allogeneic effect of the cultured cells.

d Nonspecific swelling due to injection of virus only.

e Not significantly different from each other but significantly higher than control (group (b), no helper cells were added), $p < 0.05$.

f Values in parentheses represent percentage enhancement of DTH response as compared to those controls without addition of helper cells.

TABLE 7

H-2 Requirement for Delivery of Helper Activity

Donor mouse strains			H-2 region shared		Recipient mice strains (D-region compatible)	Injection into footpad of recipients[b]	Mean increase in footpad thickness at 24 h (%)
Helper cells[a] (γ-irradiated)	Stimulator cells (γ-irradiated)	Responder cells	Helper vs Stimulator	Helper vs Responder			
Experiment 1							
-	-	-	-	-	BALB/c (ddd)	Virus only	14.1 ± 0.9
Nil	A.TH	A.TH	-	-	BALB/c	Cells + virus	25.0 ± 0.0
A.TH (ssd)	A.TH	A.TH	KID	KID	BALB/c	Cells + virus	39.9 ± 2.4[c]
CBA (kkk)	A.TH	A.TH	Nil	Nil	BALB/c	Cells + virus	23.9 ± 1.1
A.TH	A.TL (skd)	A.TH	K,D	KID	BALB/c	Cells + virus	23.4 ± 1.6
A.TH	A.TH	A.TL	KID	K,D	BALB/c	Cells + virus	44.8 ± 2.8[c]
Nil	CBA	CBA	-	-	C3HOH	Cells + virus	23.9 ± 1.1
CBA	CBA	CBA	KID	KID	C3HOH	Cells + virus	42.2 ± 1.6[c]
Experiment 2							
-	-	-	-	-	BALB/c	Virus only	12.5 ± 0.0
Nil	A.TL	A.TH	-	-	BALB/c	Cells + virus	22.7 ± 1.5
CBA	A.TL	A.TH	I	Nil	BALB/c	Cells + virus	38.3 ± 2.0[c]

a Both helper and responder cells were depleted of plastic adherent cells to minimize the possibility of cross-infection from stimulator cells, even though the latter were always extensively washed to remove any residual virus.

b 5×10^6 primary effector cells were injected 6 h after injection of infectious A/WSN virus (2.5×10^3 HAU) into the same footpads.

c Not significantly different from each other but significantly higher than controls (no helper cells were added), $p < 0.05$.

TABLE 8

A Summary of Specificity of Helper T Cells

Virus used for priming		Help for DTH virus used for elicitation	
Stimulator cells	Helper cells	Homologous	Heterologous
Infectious	Homologous, infectious	+	+
	Homologous, non-infectious	+	+
	Heterologous, infectious	+	+
	Heterologous, non-infectious	+	+
Non-infectious	Homologous, infectious	+	-
	Homologous, non-infectious	+	-
	Heterologous, infectious	-	-
	Heterologous, non-infectious	-	-

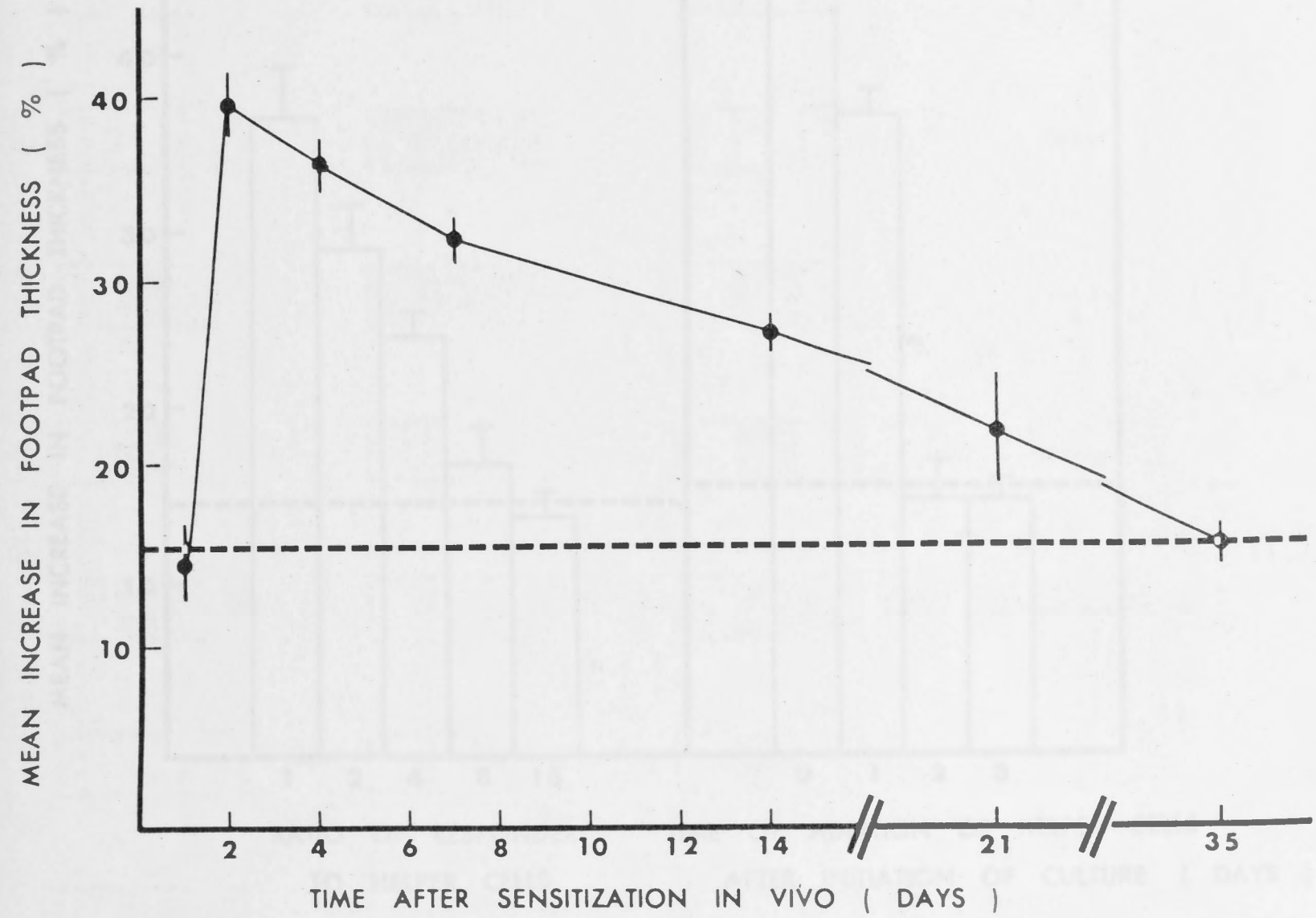


Fig. 1: Kinetics of generation of helper activity. CBA mice were primed at different intervals with 10 HAU infectious A/WSN virus i.v.. Normal or primed spleen cells were γ-irradiated, incubated with A/WSN virus-infected syngeneic spleen cells and normal syngeneic spleen cells at a ratio of 5:1:5. Cells were harvested at day 5 and tested for DTH activity by adoptive transfer ($1x10^7$ per mouse) with antigen ($6x10^3$ HAU purified, UV-inactivated A/WSN virus) into footpads of naive recipients. Vertical bars represent ± one standard error.

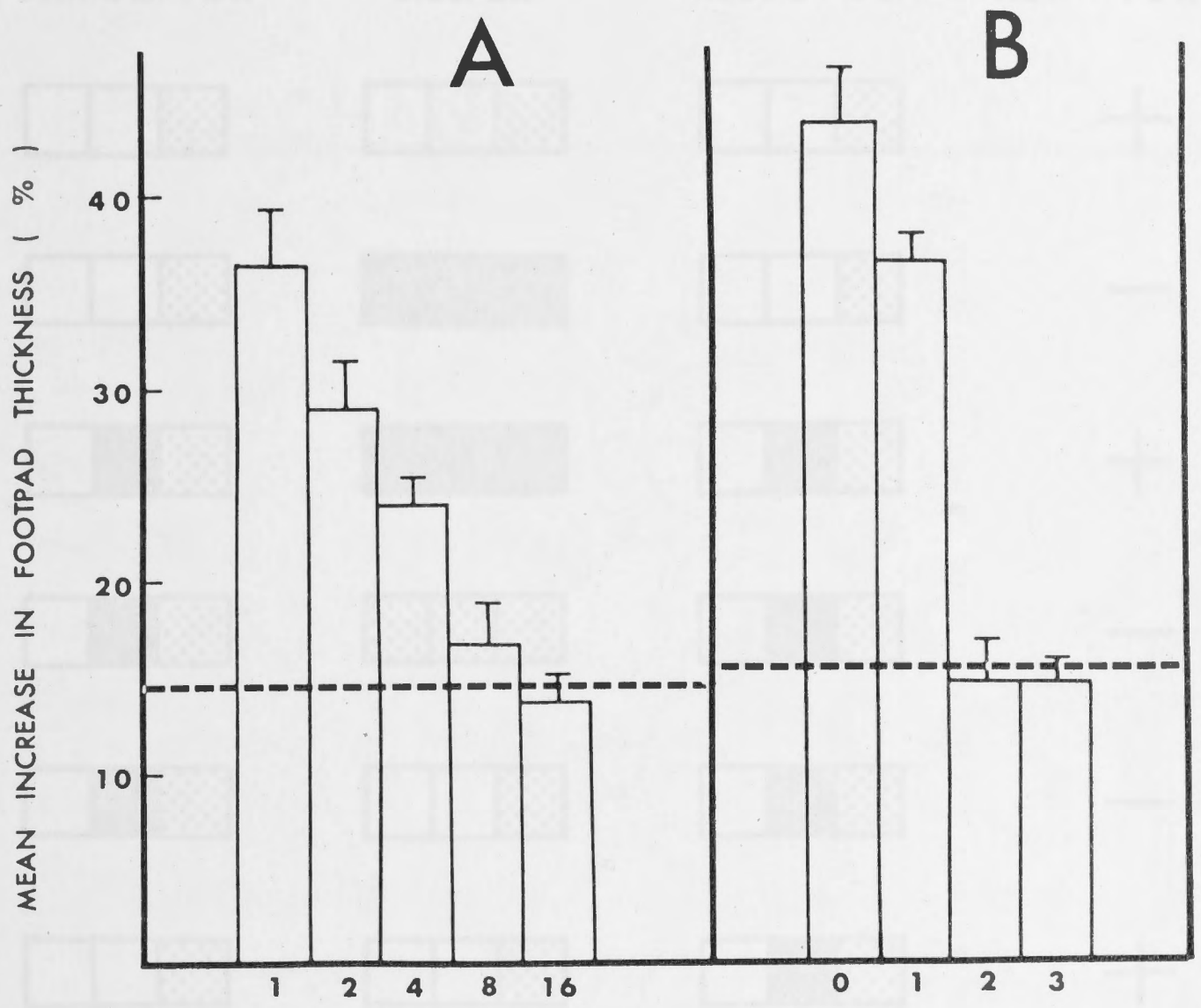


Fig. 2: (A) Dose dependency of helper cells in augmenting primary DTH response generated to influenza virus in vitro. Day 2 immune spleen cells from CBA mice primed with 10 HAU infectious A/WSN virus were γ-irradiated and added at different ratios with respect to normal responder cells.

(B) Influence of the time of addition of helper cells in the enhancement of DTH response generated in vitro. Day 2 immune spleen cells from CBA mice primed with 10 HAU infectious A/WSN virus were γ-irradiated and added at various times after initiation of primary culture. Ratio of helper, stimulator and responder was always 5:1:5.

Effector cells in (A) and (B) were harvested at day 5, and tested for DTH activity as described in Fig. 1. Horizontal lines indicate values of DTH activity from controls (no helper cells were added to cultures). Vertical bars represent one standard error.

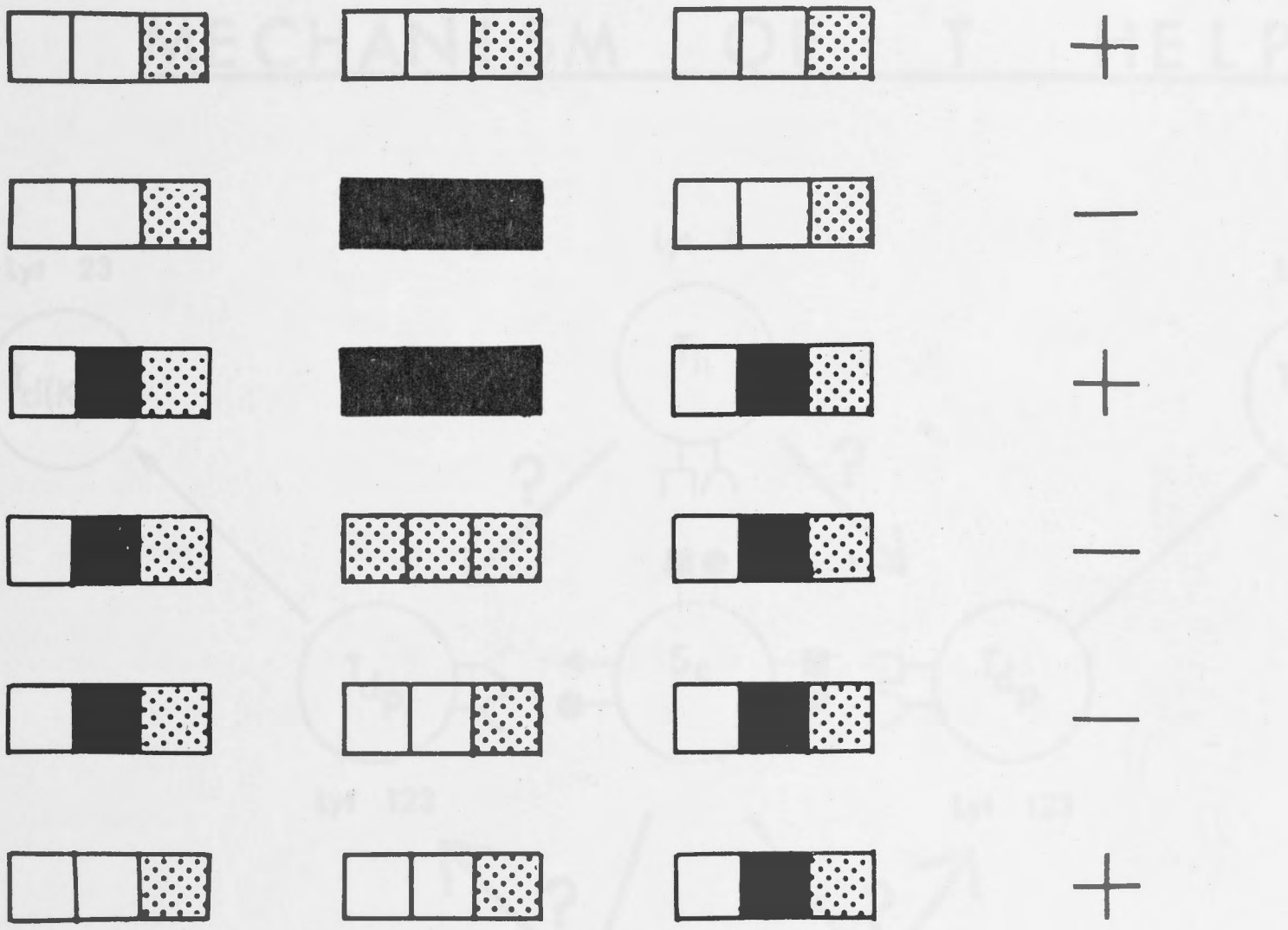


Fig. 3: Summary of H-2 restriction data on helper T cells. First panel, K region; Second panel, I region; Third panel, D region. (□) s specificity; (■) k specificity; (⊡) d specificity. The data show that the helper activity is I region restricted and that this is directed to the stimulator and not to the responder cells.

MECHANISM OF T HELP

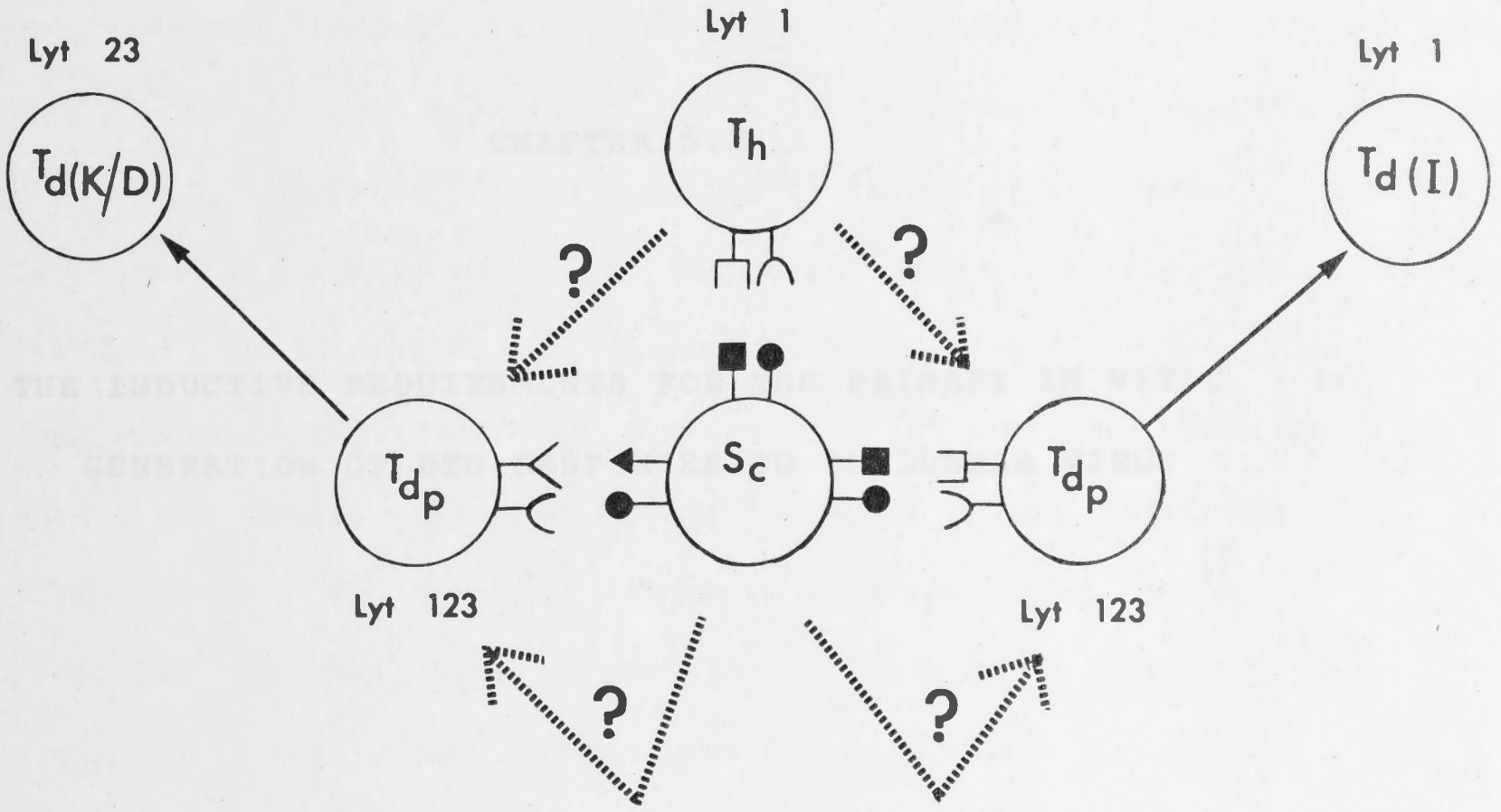


Fig. 4: A hypothetical model for the action of helper T cells in enhancing influenza virus-specific DTH response in vitro. Precursor DTH T cells (Td_p), presumably bearing Lyt 123 phenotype, recognize both viral antigens (●) and MHC coded determinants (■ , I region gene product; ▲ , K,D region gene products) on the surfaces of stimulator cells (Sc) which then give rise to either Lyt 1, I region restricted effector Td cells or Lyt 23, K,D region restricted effector Td cells. Sharing of I region between helper T cells (Th) and Sc cells is both necessary and sufficient for expression of T cell help. Whether the delivery of helper signal (⇢) requires cell-to-cell contact or via soluble factor(s) has not yet been established.

CHAPTER 5.2

THE INDUCTIVE REQUIREMENTS FOR THE PRIMARY IN VITRO GENERATION OF DTH RESPONSES TO INFLUENZA VIRUS

INTRODUCTION

Delayed-type hypersensitivity (DTH) reactions in the mouse to a wide variety of antigens have been studied (Crowle, 1975). With most antigens, it has been demonstrated that successful transfer of DTH responses are I region restricted (Vadas *et al.*, 1977; Miller, 1978a; Chapter 3.2) but DTH reaction to virus has sometimes been found to be K,D region restricted (Zinkernagel, 1976; Weiner *et al.*, 1980). Recently, it has been found that if non-infectious influenza A virus was used both to sensitize the host and to elicit the reaction, the reaction was I region restricted. However, if infectious virus was used at both steps, the reaction was K,D and I region restricted (Chapter 4.3). The mechanisms which lead to activation of DTH precursor cells are not well understood and cannot be defined by *in vivo* studies. It was necessary therefore to develop an *in vitro* system.

Primary induction of DTH responses *in vitro* to non-replicating antigens such as heterologous erythrocytes (Bretscher, 1979; Ramshaw & Eidinger, 1979) protein and synthetic polypeptides (Eshhar *et al.*, 1979) have been described but there has been little study of the role of different cell types in the process. To my knowledge, a primary *in vitro* response to a replicating antigen has not been previously described. In Chapter 5.1, it has been shown that a good primary DTH response can be induced *in vitro* if helper T cells are added to the culture and the properties, and mode of action of these helper cells are also described. The availability of such a model has now enabled the study of the several aspects of the process in more detail. This chapter describes some of the characteristics of the precursor and DTH effector T cells (Td), the response to different stimulator cells and

the genetic requirements for induction to occur.

RESULTS

Three main aspects of the reaction were examined. They were the kinetics of the induction process, the properties of the precursor and effector T lymphocytes and the inductive requirements for a primary DTH response to occur.

Kinetics of induction

Spleen cells from normal BALB/c mice were stimulated *in vitro* with A/WSN virus-infected syngeneic spleen cells ($3EID_{50}$/cell) at a responder cell to stimulator cell ratio of 5:1. After 5 days culture at 37°C, the viable cells were injected with antigen (either infectious or UV-inactivated A/WSN virus) into the footpads of naive recipients. As shown in Chapter 5.1, low but significant levels of DTH activity could be generated in this way but considerable enhancement of the DTH response was achieved if γ-irradiated, 2 days *in vivo* primed (10 HAU infectious A/WSN virus) immune spleen cells were added as a source of helper T cells. Figure 1 shows the DTH response of a constant number of cells taken at different times from cultures which were incubated with or without helper T cells. Peak activity occurred at day 5 and this was also the time when the greatest enhancement due to the prior addition of helper T cells was observed. As a result, cultures were incubated for 5 days as a routine procedure.

The kinetics of the footpad swelling after injection of antigen and cells was also examined and the results found were similar to those reported previously (Chapter 3.1). There was an initial peak of swelling at 4-6 h after the injection but this rapidly subsided and a second peak of swelling occurred at 24 h (Fig. 2). Therefore, footpad swelling measurements were routinely carried out at 24 h.

Nature of the precursor cells

Normal spleen cells from CBA mice were pretreated with anti-Lyt 1.1 or anti-Lyt 2.1 antibodies and complement. The surviving cells were cultured either separately or after mixing in equal proportions. The different preparations were tested in mice which shared the I or D regions with the donors. Untreated or complement treated responder cells were included as controls. The results in Table 1 show that (1). unselected responder cells generated D or I region restricted Td cells; (2). treatment of responder cells with either anti-Lyt 1.1 or anti-Lyt 2.1 antibodies and complement abrogated their ability to generate the D or I region restricted Td cells; and (3). mixtures of cells selected for Lyt $1^+2\ 3^-$ and Lyt $1^-2\ 3^+$ cells were unable to generate primary DTH effector cells. Thus, the results indicated that the presence of Lyt $1^+2\ 3^+$ cells in the responder population was necessary for the induction of a primary DTH response to influenza virus *in vitro*.

Properties of the effector cells

The surface antigen characteristics and properties of the primary DTH effector cells generated *in vitro* were established (Table 2). The effector cells were sensitive to anti-Thy 1.2 ascitic fluid and complement treatment but insensitive to anti-Ia^k serum and complement or to complement alone. Separation into Ig^- and Ig^+ fractions and removal of plastic adherent cells showed that only the Ig^- fraction was active. Thus the effector cells were clearly T lymphocytes.

The H-2 restriction pattern of adoptive transfer of virus-specific Td cells was also examined. Effector cells generated *in vitro* were transferred into the footpads of mice which were syngeneic, allogeneic or shared I, IA, K,D or D regions of the H-2 gene complex with the donor mice. The recipient mice were challenged with UV-inactivated virus or with infectious virus as the former has been

shown to elicit only a IA subregion restricted DTH response whereas the latter can stimulate in addition a K,D restricted DTH response (Chapter 4.3). The results in Table 3 indicate that two Td cell populations with different H-2 requirements for adoptive transfer were generated in primary *in vitro* culture. The Lyt phenotypes of these effector cells were established using appropriate antibodies and complement treatments. The results shown in Table 4 confirmed earlier work (Chapter 4.3) which also showed that the IA restricted population was Lyt 1^+ and the K,D restricted population was Lyt 23^+.

The specificity of the Td generated primary *in vitro* was also examined. The results shown in Table 5 were very similar to those obtained in *in vivo* experiments (Chapter 3.2). Briefly, if the responder cells were stimulated *in vitro* with UV-inactivated virus, a DTH response was only obtained if the same A strain virus was used to elicit the response. In contrast, stimulation with infectious virus *in vitro* generated effector cells that were cross-reactive within the A strains of influenza virus.

Induction of DTH responses

(A) Requirement for accessory cells

Experiments were carried out to see whether plastic-adherent and phagocytic cells acted as accessory cells in the induction of a DTH response *in vitro*. Stimulator and responder cell preparations were depleted of phagocytic and adherent cells by treatment with carbonyl iron powder followed by adsorption onto plastic surface. The results in Table 6 show that whereas treatment of the responder population alone did not affect the generation of a DTH response, removal of phagocytic and plastic-adherent cells from *both* stimulator and responder cells abolished the ability of the cultured cells to generate DTH effector cells. This was so whether infectious or non-infectious virus was used for the *in vitro* stimulation.

(B) Nature of stimulator cells

In order to test different cell types for their ability to act as stimulator cells, responder and helper cell populations were depleted of plastic-adherent and phagocytic cells as described previously. Cells to be tested as stimulators were infected with virus and then washed four times before adding to the culture containing responder and helper cells. The results are shown in Table 7. Spleen cells, lymph node cells, peritoneal exudate cells, Con A and LPS blasts were all able to function as stimulator cells to induce the generation of Td cells which could mediate D or I region restricted DTH responses. Peritoneal exudate cells were slightly superior to other cell preparations. L929 fibroblasts stimulated only the D region restricted DTH response.

(C) H-2 requirements for induction of Td cells in vitro

In order to study the genetic requirements for the induction of Td cells *in vitro*, stimulator cells (γ-irradiated virus-infected spleen cells) were used which differed from the responder cells at various regions or subregions of the H-2 gene complex. The helper cells added were syngeneic to the stimulator cells. Both the responder and helper populations were depleted of adherent and phagocytic cells. I region restriction was studied by cell transfer to syngeneic recipients and challenge with UV-inactivated virus. K,D region restriction was studied by cell transfer to recipients which shared K,D or D regions with the donors which were challenged with infectious virus. As shown in Table 8, sharing of the K and/or D region between stimulator and responder cells only generated Td cells which are K,D or D region restricted. Similarly sharing of I region or IA subregion between stimulator and responder cells only gave rise to I region restricted responses. Incompatibility at the K,I and D region failed to yield DTH effector cells.

DISCUSSION

This chapter shows that DTH effector T cells (Td) specific for influenza A virus can be generated in tissue culture using normal spleen cells as the source of precursor cells. Addition of helper T cells enhanced the production of Td cells as described in detail in Chapter 5.1. An enhancement of the *in vitro* DTH response to heterologous erythrocytes has been reported in which spleen cells taken from mice a few days after injection of antigen were used instead of naive spleen cells as responder cells (Bretscher, 1979; Ramshaw & Eidinger, 1979). Such preparations probably contained helper cells. Two results presented here are consistent with previous findings (Chapters 3.2; 4.3) in which studies of the DTH response to influenza virus were carried out *in vivo*. Firstly, in both cases, two types of Td cells are produced, i.e., those that are I region restricted and Lyt 1^+ and those that are K,D region restricted and Lyt 23^+. Secondly, analysis of specificity showed that like Td cells generated *in vivo*, *in vitro* generated effector cells are specific for the homologous virus when inactivated virus was used as the stimulating antigen. A cross-reactive (within the A strains of influenza virus) Td cell population could also be detected if infectious virus was used for *in vitro* stimulation. These results strongly suggested that the *in vitro* system can be regarded as a valid model of the *in vivo* response so that analysis of the *in vitro* response should give relevant answers to questions that cannot be approached in *in vivo* experiments.

One aspect which could be resolved by *in vitro* studies is the nature of the precursor DTH T cells. The availability of monoclonal preparations of anti-Lyt 1 and anti-Lyt 2 antibodies enabled experiments to be done which showed that Lyt 123^+ cells were necessary for

the generation of primary DTH effector cells *in vitro*. The procedure used was essentially negative selection. Treatment of the responder cell population with either anti-Lyt 1 or anti-Lyt 2 antibodies and complement prevented the generation of both Lyt 1^+ and Lyt 23^+ effector Td cells. Furthermore, when responder cells pretreated with anti-Lyt 1 and complement were mixed with anti-Lyt 2 and complement treated responder cells, the generation of DTH activity *in vitro* was not restored. The simplest explanation of such a result is that Lyt 123^+ cells are essential for the generation of both types (Lyt 1^+, I-region restricted and Lyt 23^+, K,D-region restricted) of effector Td cells. However the present results do not distinguish between two possibilities - that Lyt 123^+ cells are the direct precursors of Td cells or simply that their presence in the culture is necessary. The first possibility could be established by using cells with distinguishable markers as precursors which permit one to follow the development of Lyt 123^+ T cells in unselected T cell populations after sensitization. This has been achieved in several systems (Burakoff, *et al.*, 1978; Simon & Abenhardt, 1980; Leclerc & Cantor, 1980a;Shen *et al.*, 1980) using appropriate congeneic mice, but these were not available to us. Nonetheless, the present finding which indicates an absolute requirement for Lyt 123^+ cells agrees with several other groups who found that Lyt 123^+ T cells contain the precursors for both alloreactive and H-2 restricted primary Tc responses (Simon & Abenhardt, 1980; Wagner *et al.*, 1980a). For example, Lyt 123^+ cells are found to be precursors of cytotoxic T lymphocytes against Moloney leukemia virus (Leclerc & Cantor, 1980a),TNP-modified syngeneic cells and Sendai virus-infected syngeneic cells (Burakoff *et al.*, 1978), syngeneic mammary tumor cells (Stutman *et al.*, 1977) and alloantigens (Simon & Abenhardt, 1980; Teh & Teh, 1980). Similar requirements for Lyt 123^+ cells as precursors in the generation of helper T cells for TNP-SAV

has also been reported (Shen et al., 1980). In contrast, Feldmann et al. (1977) using another system, have found that helper and suppressor precursors to TNP-KLH are Lyt 1^+ and Lyt 23^+ respectively. Therefore, the properties of the precursor cells seem to differ according to the system studied.

A second aspect which can be examined is the cellular requirement for induction of a primary DTH response. In studies on the generation of Tc, a variety of cell types such as macrophages, T and B cells as well as mitogen-activated spleen cells were found to be effective stimulators of the cytotoxic T cell responses (Pang & Blanden, 1977; Davidson, 1977). Koszinowski & Simon (1979) have shown that in the generation of primary Tc cells to Sendai virus, there is no requirement for a particular type of stimulator cell provided that the viral antigens are able to associate with the membrane of the stimulator cells, which can be achieved either by fusion of the virus with the cell membrane or by the process of infection. Experiments shown in Table 7 have used the infectivity of the virus in order to achieve 'integration' of the viral antigens into the cell plasma membrane. In this case, a variety of cell types such as spleen cells, lymph node cells, mitogen activated blasts, were able to function as stimulator cells for the generation of both K,D or I region restricted DTH responses. In contrast, the use of L929 fibroblasts generated only the D (and presumably K) region restricted DTH response; this is probably due to the fact that L929 cells lack Ia antigens on their surface (Hämmerling, 1976). On the other hand, stimulator cells exposed to non-infectious virus generate exclusively the I region restricted response (Table 6). Thus, this is the first time that the generation of DTH effector cells which are only K,D region restricted has been achieved. This will be particularly useful in studying the role of these cells in recovery from infection.

The importance of macrophage-like accessory cells in the generation of Tc cells is well documented in several systems (Glaser, 1980; Gorczynski, 1976; Pang & Blanden, 1976; Wagner et al., 1972; Lutz & Fitch, 1979; Rouse & Lawman, 1980). A requirement for macrophages in the induction of helper cells has also been reported (Erb & Feldmann, 1975). Our present study also suggests that there is a requirement for phagocytic and adherent cells in the generation of primary influenza virus specific Td cells in vitro, since the depletion of these cells from the cultures abrogated the ability of the remainder cells to generate a DTH response. The need for macrophage-like accessory cells in the in vitro induction of DTH response may be due to the fact that they are required to improve culture conditions (Pang & Blanden, 1976). Alternatively, they might act as antigen presenting cells in the activation of DTH response, as thioglycollate elicited peritoneal adherent cells, which consist mainly of macrophages, were found to be efficient stimulator cells. Moreover, others (Bach et al., 1978; Vadas et al., 1977; Mottram & Miller, 1980) have found that antigens and haptens bound to macrophages are efficient immunogens for the in vivo sensitization of DTH response and Eshhar et al. (1979) have demonstrated that DTH response to a soluble protein (fowl-γ-globulin) and to a synthetic polypeptide (T,G)-A-L could only be generated in vitro if these antigens were presented on plastic-adherent cells.

Finally, the in vitro system has allowed a closer examination of the H-2 requirements in the inductive phase of a DTH response. If the stimulator and responder cells were allogeneic, the DTH response could not be induced. If they shared the K or D region, then K,D region but not I region restricted Td cells were generated and vice versa. Thus, the H-2 restriction imposed at the level of elicitation in the adoptive transfer of DTH was also reflected at the level of

induction. Similar H-2 requirements have been seen in the *in vitro* induction of Tc to viral antigens as well as in the stimulation of responder cells to undergo virus-specific proliferative responses (Hapel, *et al*., 1978).

The findings reported in this work open the way to a closer examination of the interactions between the stimulator and responder cells in the activation of a DTH response. Is the method of stimulation of responder cells by viral antigen-I-region product the same as that of viral antigen-K,D-region product? Is one a soluble complex, the other cell bound? Studies to answer these questions are under way.

SUMMARY

Effector T cells (Td) which mediate delayed-type hypersensitivity reactions to influenza A virus can be generated in tissue culture using normal mouse spleen cells as the responder population. Addition of helper T cells enhance but is not essential for the production of Td cells. Both Lyt 1^+, I-region restricted and Lyt 23^+, K,D-region restricted effector cells are generated. Treating the responder cell population with anti-Lyt 1 or anti-Lyt 2 antibodies and complement prevented the generation of both classes of effector T cell, suggesting that the precursor Td cells are Lyt 123^+. Effector cells which are specific for the homologous virus or cross-reactive within the A strains of influenza virus are produced, as has been found previously in *in vivo* experiments. Depleting the cell population of phagocytic and plastic-adherent cells resulted in a failure to produce Td cells, which showed a requirement for macrophage-like cells as accessory cells in the primary *in vitro* generation of Td cells. A variety of cells, such as peritoneal exudate cells, mitogen stimulated blasts or L929 fibroblast cells could serve as stimulator

cells. Only Lyt 23^{+}, K,D region restricted Td cells were produced when L929 cells were used as they lack I region-coded surface antigens. The I region restricted DTH response was mapped to the IA subregion of the H-2 gene complex.

TABLE 1

Lyt Phenotype of Primary Precursor Td Cells[a]

Group No.	Responder cell population		Recipient mice	H-2 region shared between donors of primary effector cells and recipients	Mean increase in footpad thickness at 24 h[b] (%)
	Treatment	Selected for			
1	Nil	Unselected	CBA	All	24.7 ± 2.1
	Nil	Unselected	BALB/c	Nil	0
	Nil	Unselected	C3H.OH	D	24.7 ± 2.1
	Nil	Unselected	A.TL	I	23.7 ± 1.1
2	C' alone	Unselected	CBA	All	24.2 ± 1.6
	C' alone	Unselected	BALB/c	Nil	0
	C' alone	Unselected	C3H.OH	D	27.3 ± 1.6
	C' alone	Unselected	A.TL	I	24.7 ± 2.8
3	Lyt 1.1 + C'[c]	Lyt $2,3^+$	C3H.OH	D	0[d]
	Lyt 1.1 + C'	Lyt $2,3^+$	A.TL	I	0[d]
4	Lyt 2.1 + C'[c]	Lyt 1^+	C3H.OH	D	0.7 ± 0.5[d]
	Lyt 2.1 + C'	Lyt 1^+	A.TL	I	0[d]
5	Lyt 1.1 + C' &	Lyt 1^+ &	C3H.OH	D	0[d]
	Lyt 2.1 + C'	Lyt $2,3^+$ (1:1 ratio)	A.TL	I	0[d]

Legends to Table 1

a Primary effector cells were generated by incubating 7.5×10^7 normal CBA spleen cells (unselected or selected with anti-Lyt antibodies and C') with 1.5×10^7 virus-infected, γ-irradiated syngeneic stimulator cells. 7.5×10^7 γ-irradiated day 2 immune syngeneic spleen cells were added to amplify the DTH response generated *in vitro*.

b 5×10^6 primary effector cells were injected into each mouse footpad 6 h after injection of infectious virus (2.5×10^3 HAU) into the same footpad.

c Anti-Lyt 1.1 + C' killed ~ 19% of total spleen cells.
Anti-Lyt 2.1 + C' killed ~ 15% of total spleen cells.

d Significantly lower than the corresponding unselected responder cells (underlined values); $p < 0.001$.

TABLE 2

Characterization of Influenza Virus-Specific Primary DTH Effector Cells Generated *in Vitro*[a]

Treatment of primary CBA effector cells[b]	Mean increase in footpad thickness at 24 h (%)
Nil	30.2 ± 1.1
Complement alone	31.3 ± 0.0
Anti-Thy 1.2 ascitic fluid + complement	4.7 ± 2.0[c]
Anti-Ia^k antiserum + complement	32.1 ± 1.9
Removal of plastic adherent cells	28.9 ± 1.5
Ig^+ and Ig^- cell fractination:	
Plastic non-adherent Ig^+ cells	3.9 ± 1.5[c]
Plastic non-adherent Ig^- cells	34.4 ± 1.8
Exposure to 2000 rads γ-irradiation	30.5 ± 1.5

a Primary CBA effector cells were generated *in vitro* as described in Materials and Methods (Chapter 2).

b $5x10^6$ untreated or treated cells were injected with $6x10^3$ HAU purified, UV-inactivated A/WSN virus into footpads of syngeneic recipient mice.

c Significantly lower than control (untreated effector cells); $p < 0.01$.

TABLE 3

H-2 Restriction for Adoptive Transfer of Influenza Virus-Specific DTH T Cells Generated Primary *in Vitro*

Mouse strains		H-2 region shared	% Mean increase in footpad thickness at 24 h elicited with[a]	
Donor	Recipient		UV-inactivated virus	Infectious virus
A.TL	A.TL	All	27.1 ± 1.1	24.1 ± 1.6
A.TL	C57BL/6J	Nil	4.2 ± 2.1[b]	0[b]
A.TL	CBA	I	29.7 ± 1.6	20.4 ± 1.1
A.TL	B10.A(4R)	IA	30.2 ± 2.8	20.4 ± 1.1
A.TL	A.TH	K,D	3.1 ± 1.8[b]	22.5 ± 3.1
A.TL	SJL	K	3.1 ± 3.1[b]	26.7 ± 3.8
A.TL	BALB/c	D	6.3 ± 0[b]	20.9 ± 4.7

a 5×10^6 primary effector cells were injected into each mouse footpad 6 h after injection of either infectious (2.5×10^3 HAU) or UV-inactivated (6×10^3 HAU) virus into the same mouse footpad and footpad swelling measured 24 h later.

b These values all significantly lower than the control values of mice that received syngeneic effector cells ($p < 0.01$).

TABLE 4

Lyt Phenotype of I-Region Restricted and D-Region Restricted Primary DTH Effector Cells Generated to Influenza Virus *in Vitro*

Treatment of primary effector cells[a]	Mouse strains used		H-2 region shared	Mean increase in footpad thickenss at 24 h (%)
	Donors	Recipients		
Nil	CBA	CBA	All	27.6 ± 2.1
Nil	CBA	BALB/c	Nil	1.5 ± 1.1
Nil	CBA	A.TL	I	25.5 ± 2.1[b]
Nil	CBA	C3H.OH	D	30.5 ± 1.5[c]
C' alone	CBA	A.TL	I	25.5 ± 1.1[b]
C' alone	CBA	C3H.OH	D	26.5 ± 0.0[c]
Anti-Lyt 1.1 + C'	CBA	A.TL	I	0.0 ± 0.0[d]
Anti-Lyt 1.1 + C'	CBA	C3H.OH	D	30.5 ± 1.9[c]
Anti-Lyt 2.1 + C'	CBA	A.TL	I	27.3 ± 2.9[b]
Anti-Lyt 2.1 + C'	CBA	C3H.OH	D	3.1 ± 0.9[e]

a CBA primary effector cells generated *in vitro* were either untreated or treated with C' alone (~3% cells were killed); anti-Lyt 1.1 + C' (~33% cells were killed) or anti-Lyt 2.1 + C' (~27% cells were killed). 5×10^6 viable cells (untreated or treated) were injected into mice footpads 6 h after injection of infectious virus (2.5×10^3 HAU) into the same footpads.

b,c Not significantly different from each other.

d Significantly lower than control (untreated cells injected into I region compatible recipients); $p < 0.001$.

e Significantly lower than control (untreated cells injected into D region compatible recipients); $p < 0.001$.

TABLE 5

Specificity of Primary DTH Effector Cells Generated *in Vitro*[a]

Virus strains used		Mean increase in footpad thickness at 24 h (%)
Stimulation *in vitro*	Elicitation in mouse footpad[b]	
Infectious A/WSN	A/WSN (H0N1)	43.8 ± 3.6
	A/PC (H3N2)	35.4 ± 1.1
	A/RI (H2N2)	37.5 ± 1.8
	Sendai	5.2 ± 2.8[c]
Non-infectious A/WSN	A/WSN	42.2 ± 1.6
	A/PC	1.6 ± 1.6[c]
	A/RI	5.2 ± 1.1[c]
	Sendai	3.1 ± 1.8[c]

a Primary CBA effector cells were generated *in vitro* as described in Materials and Methods (Chapter 2).

b Purified, UV-inactivated virus ($6x10^3$ HAU) was injected together with primary effector cells ($5x10^6$) into footpads of syngeneic recipient mice.

c Significantly lower than control (homologous virus was used for elicitation), $p<0.001$.

TABLE 6

Requirement for Accessory Cells in the Induction of Primary Td Cells *in Vitro*

Infectivity of virus used for generating stimulator cells[a]	Treatment of stimulator cells[b]	Treatment of responder cells[b]	% Mean increase in footpad thickness at 24 h elicited by	
			UV-inactivated virus in syngeneic recipients (BALB/c)	Infectious virus in D-region compatible recipients (A.TH)
Infectious	Nil	Nil	31.3 ± 1.8	28.6 ± 2.8
Infectious	Nil	Removal of phagocytic and adherent cells	29.7 ± 1.6	28.1 ± 1.6
Infectious	Removal of phagocytic and adherent cells	Removal of phagocytic and adherent cells	6.3 ± 1.8[c]	3.6 ± 1.1[c]
Non-infectious	Nil	Nil	28.1 ± 1.1	0.8 ± 0.8
Non-infectious	Removal of phagocytic and adherent cells	Removal of phagocytic and adherent cells	3.1 ± 1.8[c]	0.5 ± 0

a Normal BALB/c spleen cells were exposed to either infectious A/WSN virus ($3EID_{50}$/cell) or UV-inactivated A/WSN virus ($1.5x10^3$ HAU/10^7 cells) for 1 h at 37°C, stimulator cells were washed 4 times after exposure to virus before addition to primary cultures.

b Stimulator, responder and helper cells were depleted of phagocytic and adherent cells by carbonyl iron powder treatment (45 min, 37°C), followed by adherence onto plastic surface (2 h, 37°C).

c Significantly lower than controls (both stimulator and responder cells were untreated); $p < 0.01$.

TABLE 7

Capacity of Various Cell Types to Stimulate a Primary DTH Response *in Vitro*[a]

Stimulator cell types[b]	% Mean increase in footpad thickness at 24 h elicited by	
	UV-inactivated virus in syngeneic recipients (CBA)	Infectious virus in D-region compatible recipients (C3H.OH)
Spleen cells[c]	29.2 ± 1.1	25.5 ± 1.1
Lymph node cells[c]	26.6 ± 1.6	26.2 ± 3.2
Thyioglycollate-elicited peritoneal cells[d]	35.9 ± 0.9	32.3 ± 2.1
Con A blasts[c]	28.1 ± 1.8	25.0 ± 4.8
LPS blasts[c]	27.4 ± 1.5	27.1 ± 2.1
L929 fibroblasts[e]	4.2 ± 1.1	24.2 ± 0.8

a 7.5×10^6 virus-infected ($3EID_{50}$/cell) stimulator cells were added to cultures containing 7.5×10^7 normal CBA spleen cells and 7.5×10^7 γ-irradiated day 2 immune CBA spleen cells which have previously been depleted of plastic-adherent and phagocytic cells. The mixtures were incubated at 37°C for 5 days before the effector cells were harvested and tested for DTH activity.

b CBA mice were used as donors of stimulator cells.

c γ-irradiated (2000 rads) after exposure to infectious virus for 1 h.

d Cells harvested from peritoneal cavity were adhered onto plastic tissue flasks for 3 h, nonadherent cells were removed, and the adherent cells were then infected with virus.

e Mitomycin C treated after exposure to infectious virus for 1 h.

TABLE 8

H-2 Requirement for Induction of Primary Td Cells *in Vitro*[a]

Mouse strains used		H-2 region shared	% Mean increase in footpad thickness at 24 h elicited by[b]	
Stimulator cells	Responder cells		UV-inactivated virus in syngeneic recipients	Infectious virus in K,D or D region compatible recipients
CBA	CBA	All	32.1 ± 2.0 (CBA)[d]	30.2 ± 1.1 (C3H.OH)[d]
C57BL/6J	CBA	Nil	4.7 ± 0.9[c] (CBA)	4.2 ± 1.1[c] (C3H.OH)
A.TL	CBA	I	31.3 ± 1.3 (CBA)	5.0 ± 1.5[c] (C3H.OH)
A.TH	A.TH	All	30.5 ± 1.5 (A.TH)	32.3 ± 3.1 (A.TL)
BALB/c	A.TH	D	4.7 ± 2.0[c] (A.TH)	31.2 ± 1.1 (A.TL)
A.TL	A.TH	K,D	5.5 ± 1.5[c] (A.TH)	30.0 ± 2.1 (BALB/c)
B10.AQR	B10.AQR	All	31.3 ± 1.8 (B10.AQR)	29.2 ± 3.6 (A.TH)
DBA/1	B10.AQR	K	7.8 ± 1.6[c] (B10.AQR)	26.0 ± 3.1 (A.TH)
B10.A(4R)	B10.AQR	IA	27.1 ± 2.1 (B10.AQR)	1.0 ± 0.5[c] (A.TH)

a Primary Td cells were generated *in vitro* by co-culturing of virus-infected ($3EID_{50}$/cell), γ-irradiated spleen cells (stimulator cells) with normal spleen cells (responder cells) and helper cells (syngeneic to stimulator cells) for 5 d at 37°C. The responder cells and the helper cells were depleted of phagocytic and plastic adherent cells before use.

b $5x10^6$ primary effector cells were injected at the same time with UV-inactivated virus ($6x10^3$ HAU) into each mouse footpad or injected 6 h after injection of infectious virus ($2.5x10^3$ HAU) into the same footpad.

c Significantly lower than controls in which stimulator cells were syngeneic to responder cells, $p < 0.01$.

d Recipient mouse strains are indicated in parenthesis.

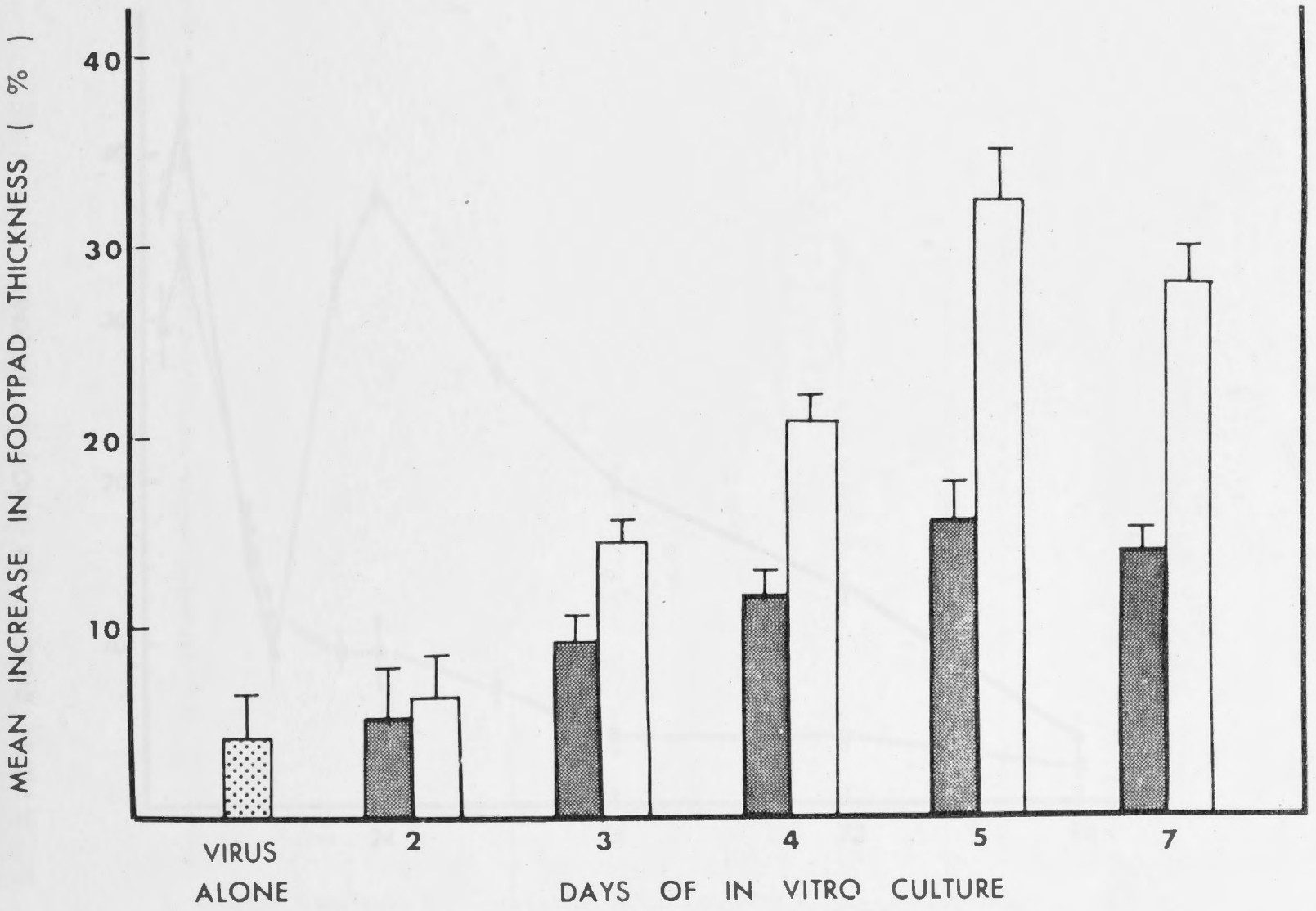


Fig. 1: Kinetics of generation of primary DTH response in vitro. Normal BALB/c spleen cells were cultured for different times with A/WSN virus-infected syngeneic stimulator cells in the absence (▩) or presence (□) of syngeneic γ-irradiated 'helper' cells. Primary effector cells were harvested and $5x10^6$ viable cells were injected with $6x10^3$ HAU purified, UV-inactivated A/WSN virus into the footpad of each mouse and footpad swelling was measured 24 h later. (⊡) injection of virus alone. Vertical bars represent one standard error.

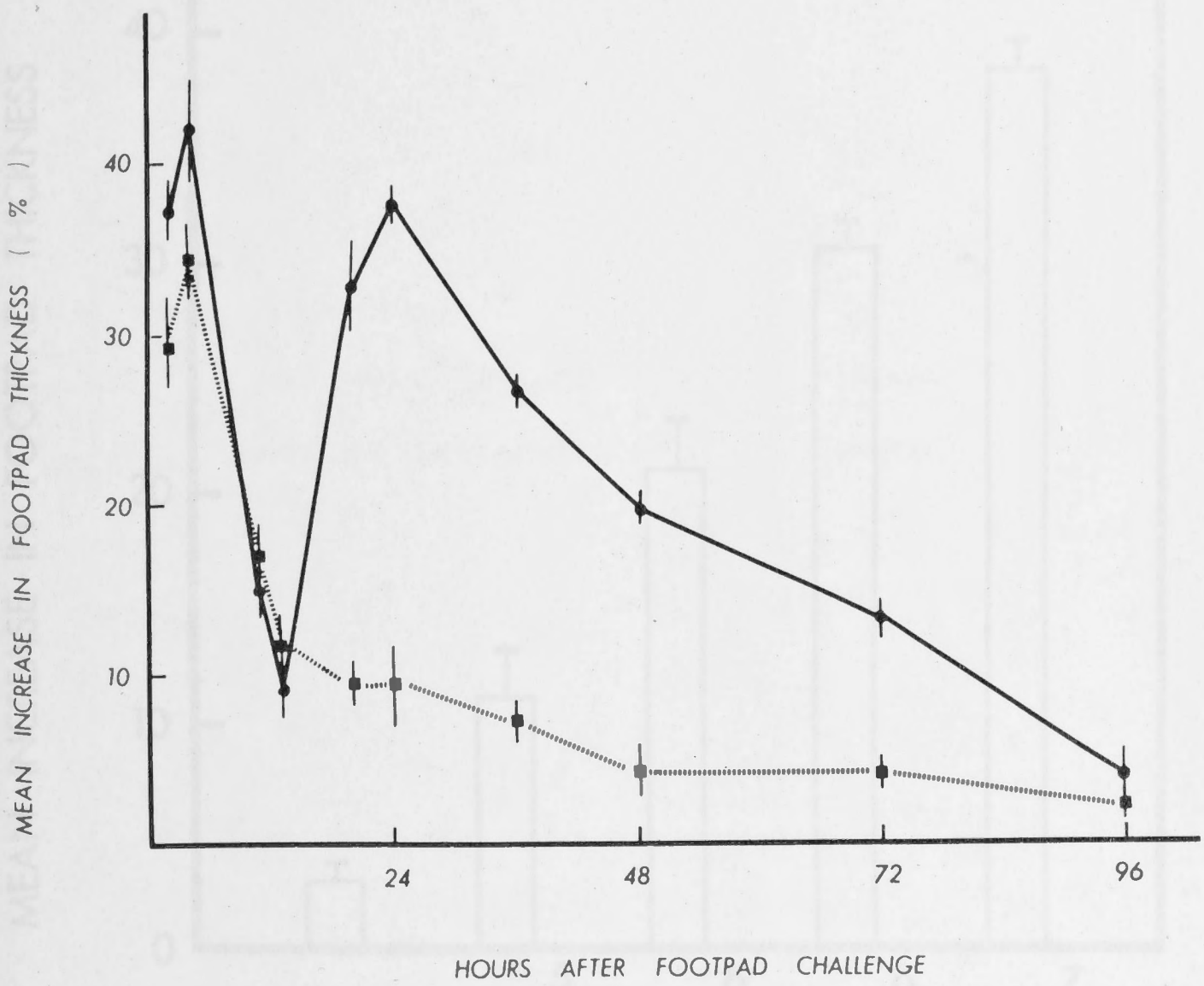


Fig. 2: Development of footpad swelling. Normal CBA mice were either injected with virus only (UV-inactivated A/WSN, $6x10^3$ HAU) or $1x10^7$ in vitro generated primary effector cells plus virus into the right hind footpads and equal amount of PBS was injected into each left hind footpad as a control. Footpad swelling was measured over a period of 96 h and vertical bars represent ± one standard error. (■) virus only; (●) cells + virus.

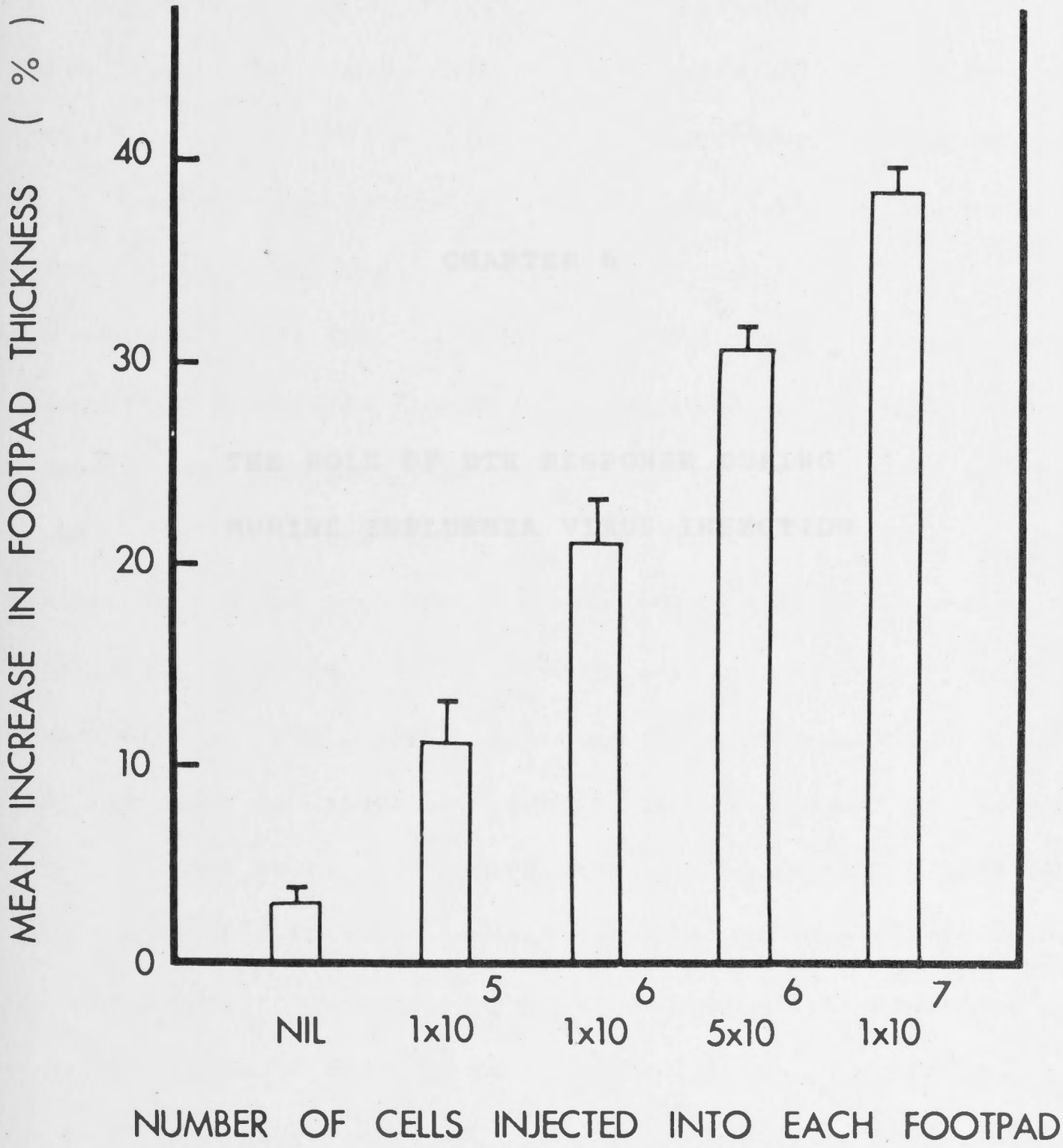


Fig. 3: Dose dependency of DTH activity with primary effector cells generated in vitro. Different amounts of cells were injected with a standard dose of purified, UV-inactivated virus ($6x10^3$ HAU) into the right hind footpad of each CBA mouse and footpad swelling was measured 24 h later. Vertical bars represent one standard error.

CHAPTER 6

THE ROLE OF DTH RESPONSE DURING MURINE INFLUENZA VIRUS INFECTION

INTRODUCTION

Earlier chapters in this thesis (Chapters 3 & 4) have shown that T cells with cytotoxic and DTH activities can be recovered from the lungs or the spleens of mice 6 days after intranasal or intravenous inoculation of mice with infectious influenza virus. The results in chapter 4.3 further demonstrated that at least two populations of DTH effector T cells (Td) can be activated in response to infectious influenza virus: one is Lyt 1^+ and I region restricted whereas the other is Lyt $2,3^+$ and K,D region restricted. The latter is not distinguishable from the K,D region restricted cytotoxic T cells (Tc), as far as the inductive requirements, antigen specificity and surface phenotypes are concerned. Previous work from this (Yap & Ada, 1978b; Yap et al., 1978) and other (Lin & Askonas, 1980) laboratories have unequivocally demonstrated that the K,D region restricted Tc cells play a protective role in the recovery of mice from primary influenza virus infection. In contrast, little if any, information is available concerning the role of the I region restricted Td cells in host resistance against the viral infection. Studies on the conditions for the in vitro generation of Tc and Td activities have enabled experimental protocols to be designed for the selective generation of K,D region restricted Tc (and Td) cells but not I region restricted Td cells and vice versa (Chapters 4.2, 5.2). The availability of these cell preparations provides an excellent tool for the investigation of the role played by each T cell subset during murine influenza virus infection. The results in this chapter show that in contrast to the K,D region restricted Tc cells, adoptive transfer of I region restricted cells with DTH activity failed to protect infected mice from death but appeared to increase the severity of the disease.

RESULTS

Two approaches have been used to investigate the role of DTH response during murine influenza virus infection. One approach is to study the effect of adoptive transfer of effector cells with DTH activity on the susceptibility of mice to influenza virus infection, which can be determined by observing the mortality pattern and measuring the virus titres in the lungs of the infected mice. A second approach is to compare the effect of transferring cell preparations with I region restricted DTH activity and/or K,D region restricted cytotoxic and DTH activities on the inflammatory response occurring in the virus-infected lungs, which can be measured by a sensitive radioisotopic method.

Failure to protect influenza virus-infected mice from death with cell preparations enriched for I region restricted DTH activity

Previous work (Yap & Ada, 1978b) has shown that adoptive transfer of secondary effector cells with cytotoxic activity had a protective effect in mice infected with a lethal dose of influenza virus. Results in Chapter 4.3 showed that such cell preparations also contained K,D and I regions restricted DTH activity. More detail analysis has shown that the antiviral effect of the secondary effector cells was attributable to the K,D region restricted Lyt $2,3^{+}$ cells (Yap *et al.*, 1978). These results implied that K,D region restricted Tc (and Td) cells have a protective effect whereas the I region restricted Lyt 1^{+} cells do not. This could be directly checked by adoptive transfer of cell preparations with *only* I region restricted DTH activity to infected mice and then examines the effect of these cells on the severity of the disease.

Cells taken from CBA mice 3 weeks or more after injection of UV-inactivated virus can be stimulated *in vitro* to give cell preparations with high levels of I region restricted DTH activity but negligible, if any, K,D region

restricted Tc and Td activities (Chapters 4.2, 4.3). Two such preparations were made. Upon testing for DTH activity by adoptive transfer ($5x10^6$ cells per mouse footpad) with antigen (UV-inactivated virus, $6x10^3$ HAU) into the hind footpads of syngeneic recipients, the % increase in footpad thickness were 32.3±1.1 and 38.3±2.7 respectively, as compared to a control value of less than 8% (mice challenged with virus only). The same cell preparations, when tested for cytotoxic activity on H-2 compatible target cells (L929 fibroblasts) at an effector/target cell ratio of 4:1, gave specific ^{51}Cr release values of 2.5±.8% and 1.7±.4% respectively,and these are less than 5% of the usual level of cytotoxic activity obtained if infectious virus is used to prime the donor mice. In two experiments, these cell preparations were transferred to mice 1 or 2 days after receiving an intranasal inoculation of $5x10^3 EID_{50}$ of infectious A/WSN virus, and the results are shown in Fig. 1. In each experiment, the control mice received no cells, since past experience had clearly shown that transfer of normal cells to mice made no difference to the mortality rate (Yap & Ada, 1978b). The results of the two experiments are similar and show that mice receiving these immune secondary effector cells died more rapidly than the controls. In addition, in one of the two experiments, the virus titres in the lungs of mice 5 days after infection were also examined. It was found that mice receiving $2x10^7$ secondary cells 2 days after virus infection had similar levels of virus in the lungs as compared to control mice infected with virus only (Virus titres expressed in $\log_{10}$ EID_{50}/lung extract: cells + virus, 6.63±0.26; virus alone, 6.88±0.19).

It was previously shown (Chapters 3 & 4) that mice injected s.c. with UV-inactivated virus generate maximal I region restricted DTH activity but negligible cytotoxic activity in the spleens 6 days after immunization. The effect of adoptive transfer of these <u>in vivo</u> generated primary effector cells with only I region restricted DTH activity on the severity of the influenza disease in recipient mice was examined. Similar to results obtained

with secondary effector cells, adoptive transfer of $3.3x10^7$ Ig-negative 6 days immune spleen cells from mice primed with UV-inactivated virus increased the mortality of mice to influenza virus infection (Fig. 2).

The increase in mortality of mice adoptively transferred with cells with I region restricted DTH activity requires I region sharing between donors of the effector cells and the infected recipients

If the observed increase in mortality is due to the I region restricted Td cells in the transferred cell population, it would be expected that such phenomenon only occurs if recipients are I region compatible with the donors of the effector cells. The results of two experiments shown in Table 1 indicate that this is indeed the case. Enhanced mortality was seen in recipient mice adoptively transferred with syngeneic secondary effector cells but no significant increase in mortality was observed if the recipients and donors are K,D but not I region compatible.

Measurement of the inflammatory response in the lungs of mice after intranasal inoculation with infectious influenza virus

Groups of CBA mice were either uninfected or infected i.n. with increasing doses of A/WSN virus ($5x10^2$-$5x10^4$ EID_{50}). Six days later, all mice were given 0.2 ml of $5x10^{-4}$ M 5-fluorodeoxyuridine i.p. followed 30 min later by 1 uCi I^{125}-5-iodo-2-deoxyuridine (I^{125}-UdR) injected i.p.. Twenty-four hours after the injection of I^{125}-UdR, mice were killed, the lungs were removed and radioactivity in the whole lungs was counted. Preliminary experiments showed that the total lung radioactivity was always much higher in infected than uninfected mice. However, a substantial amount of radioactivity was not cell-associated and this varied with the extent of lung damage. In order to estimate the cell-associated radioactivity in the lungs, single cell suspensions were made, contaminating erythrocytes were lysed and the radioactivity in the residual cells was counted. The results in Table 2 showed that there was a positive correlation in the

degree of lung consolidation, the level of cell-associated radioactivity in the lungs and the dose of virus used to infect the mice.

The kinetics of cellular infiltration into the lungs as a function of time after intranasal inoculation of mice with a lethal dose of influenza virus was investigated. As shown in Fig. 3, the extent of cellular infiltration increased fairly linearly with the time after virus infection, until death ensued. At day 7 after infection, lung cells from infected mice were about 20 fold more radioactive than lung cells from uninfected mice.

Effect of adoptive transfer of cell preparations with cytotoxic and/or DTH activity on the extent of cellular infiltration in mouse lungs during influenza virus infection

Four secondary cell preparations were made (Table 3a). The first contained only the influenza virus-specific K,D region restricted Tc (and Td) activity whereas the second one contained only the I region restricted DTH activity. The third one contained both types of activities and the fourth one was a control which contained Sendai virus-specific Tc and Td activities. The first cell preparation was produced by restimulation of memory spleen cells from mice primed with infectious influenza virus 3 weeks or more using stimulators that lack Ia antigenic determinants such as fibroblasts (Hämmerling, 1976). The second and the third preparations were generated by in vitro restimulation of memory spleen cells from mice primed with either UV-inactivated or infectious influenza virus respectively. These 4 preparations were transferred i.v. into groups of syngeneic recipient mice that were infected 24 h previously with 5×10^3 EID_{50} A/WSN virus. Five days after cell transfer, the degree of cellular infiltration and virus titres in the mouse lungs in the various groups were measured. The results in Table 3b showed that(1). transfer of cells with cytotoxic

activity lowered the virus titres in the lungs of infected recipients whereas transfer of cells with only I region restricted DTH activity had no antiviral effect and (2). a significant decrease in the cellular infiltration was found when cells with only K,D region restricted cytotoxic (and DTH) activity but negligible I region restricted DTH activity were transferred. In contrast, transfer of cells with only I region restricted DTH activity enhanced the degree of cellular infiltration in the mouse lungs. The transfer of Sendai virus-specific secondary cells or influenza virus-specific secondary effector cells possessing both K,D region restricted cytotoxic and DTH activities and I region restricted DTH activity had no significant effect on the extent of cellular infiltration as compared to the control mice receiving no cells.

DISCUSSION

Previous work (Chapter 4.1) has shown that T cells mediating a DTH response to influenza virus (as tested by adoptive transfer with antigen into the footpads of naive recipients) can be recovered from infected mouse lungs 6 days after intranasal inoculation of mice with a lethal dose of virus. Two questions immediately arise: (1) To what extent do DTH responses occur in the target organ of influenza virus infection, i.e., the infected mouse lungs? and (2) What is the role of the DTH response in recovery from and in the pathogenesis of the influenza disease? Attempts to answer these questions have taken advantage of two recent findings. Firstly, Anders et al.(1979) have reported that an estimate of the inflammatory response occurring in the brains of Sindbis virus-infected mice with encephalitis can be made by measuring the influx of rapidly dividing mononuclear cells into the organ by means of a radioisotopic technique. Whether the same approach could be used to

estimate the extent of any inflammatory response occurring in mouse lungs during influenzal pneumonia was investigated in this chapter. Secondly, studies of the conditions for the in vitro generation of Tc and Td activities have enabled experimental protocols to be designed for selective generation of K,D region restricted Tc and Td cells but not I region restricted Td cells and vice versa (Chapters 4.2, 5.2). Adoptive transfer of these cells to infected mice allowed a closer examination of the role of the DTH response during murine influenza virus infection.

One method to estimate the inflammatory response occurring in virus-infected lungs is to measure the infiltration of I^{125}-labelled cells into lungs during infection. The response was virus-dose dependent and increased almost linearly as a function of time during a lethal infection. Adoptive transfer of different preparations of cells showed that (1) only preparations containing cytotoxic activity could lower virus titres in the mouse lungs, results that are entirely consistent with the previous observation of others that such preparations are protective against influenza virus infection in mice (Yap & Ada, 1978b; Lin & Askonas, 1980). In contrast, the transfer of secondary cells with I region restricted DTH activity had no antiviral effect and (2) the cellular infiltration in the lungs was increased by transferring cells with only I region restricted DTH activity and was decreased by transferring cells with only K,D region restricted cytotoxic (and DTH) activity. Transfer of Sendai virus-specific secondary cells or influenza virus-specific secondary cells possessing both K,D region restricted cytotoxic and DTH activities and I region restricted DTH activity did not result in any significant change in the extent of cellular infiltration in the lungs as compared to control mice receiving no cells. One possible explanation for the latter result is that the enhancing action of the

I region restricted cells on the degree of cellular infiltration was offset by the opposing effect of the K,D region restricted cells. Furthermore, immune cells generated either primary *in vivo* or secondary *in vitro* enriched for I region restricted DTH activity but with negligible K,D region restricted Tc (and Td) activity did not protect infected mice from death but increased the mortality rate of the mice. Such an increase in mortality was not seen if the donors and the recipients were K,D region compatible but I region incompatible, suggesting that I region compatibility is required for these immune cells to demonstrate an immunopathological effect.

A similar observation of an increase in mortality following the adoptive transfer of immune cells was described previously by Cate & Mold (1975) who found that adoptive transfer of immune cells from mice primed with formalized influenza virus but not *live* virus increased the mortality of mice after challenge with either homologous or heterologous virus. The increase in mortality was prevented by treatment of the immune cells with anti-thymocyte serum. It was suggested that immunization with inactivated virus but not live virus created a state of cell-mediated immune reactivity which could result in greater severity of influenza virus infection. The mechanism for the enhancement of mortality of mice by transferring cells with I region restricted DTH activity in the present investigation is unclear. The observation that recipient mice with greater mortality rate was always associated with an increase in lung cellular infiltration suggests that the I region restricted Td cells may contribute, at least in part, to the enhanced pathological effect. The involvement of T cells in the pathogenesis of influenza disease is further supported by the observation that nude mice have decreased lung cellular infiltrates and that lung pathology progressed more slowly than in the normal littermates (Wyde *et al.*, 1977; Wells *et al.*, 1981). Indeed, T cell mediated pathogenesis has been

frequently observed in a number of viral infections, such as LCM virus (Gilden et al., 1972), parainfluenza type 1 virus (Gilden et al., 1978), Theiler's murine encephalomyelitis virus (Lipton & Dal Canto, 1976) or Coxsackie B3 virus (Woodruff & Woodruff, 1974) infection of mice. A more recent report also indicates that immune T cells can trigger pathological lesions in the brains of mice infected with an avirulent form of Semliki forest virus (Berger, 1980).

The findings reported in this chapter support the concept that T cells activated during influenza virus infection may have both beneficial and detrimental effects on the host (Singer et al., 1972; Suzuki et al., 1974; Sullivan et al., 1976). Thus, transfer of K,D region restricted Tc cells has a protective effect by lowering virus titres in the infected lungs whereas transfer of I region restricted cells with DTH activity increased the extent of cellular infiltration into the lungs and resulting in higher mortality of the recipients. As both Tc and Td cells are found in the infected mouse lungs to a considerable extent after intranasal infection, it is inevitable that these cell types must contribute to a greater or lesser extent to the pathology observed. Effector T cells that are I region restricted and mediate DTH activity may secrete lymphokines which can result in an inflammatory response in the virus-infected lungs. On the other hand, effector T cells that are K,D region restricted and which mediate cytotoxic activity must also contribute to the lung pathology but this is in part offset by their effective 'removal' of the infected cells before viral progeny is assembled (Jackson et al., 1976; Zinkernagel & Althage, 1977).

Earlier work (Chapters 3.1, 3.2; Yap et al., 1978) has shown that influenza virus-specific K,D region restricted Tc cells and I region restricted Td cells differed in a number of aspects such as inductive

requirements, cell surface phenotypes, H-2 requirements for adoptive transfer of activity and sensitivity to cyclophosphamide pretreatment etc.. The present chapter further suggests that these T cell subsets may play a different role during murine influenza virus infection. There are clearly two other aspects which require further studies. The first one is -- what is the relative contribution of the Tc and Td cells to the in vivo inflammatory response occurring in the mouse lungs during influenza virus infection? Adoptive transfer of these cell preparations to infected recipients does not give a direct answer to this question as 'endogenous' Tc and Td activities are always found in the mouse lungs as a result of influenza virus infection. Perhaps adoptive transfer studies using athymic nude mice may allow a more definitive conclusion to be drawn. The second aspect is that in view of the potential importance of these T cell subsets in the recovery from and pathogenesis of the influenza virus infection. Studies on the factors governing their production such as induction of suppressor cells are considered to be important as these may be of relevance in the strategy to be followed for the production of more effective vaccines. This aspect will be examined in the following chapter (Chapter 7).

SUMMARY

Effector T cells containing only I region restricted DTH activity but with negligible K,D region restricted cytotoxic and DTH activities can be generated either primary in vivo or secondary in vitro by immunization of donor mice with UV-inactivated virus. Such cell preparations, when adoptively transferred to influenza virus-infected syngeneic recipients, did not protect the mice from death but increased their mortality rate. This increase in mortality was not observed if the infected recipients and the donors of the effector cells were K,D region compatible and I region incompatible. The inflammatory response occurring in the virus-infected

lungs can be estimated by measuring the infiltration of I^{125}-labelled mononuclear cells into the lungs during infection. The response was virus-dose dependent and increased fairly linearly as a function of time when a lethal dose of virus was administered. Adoptive transfer of different preparations of secondary effector cells showed that the cellular infiltration in the lungs was increased by transferring cells with only I region restricted DTH activity and was decreased by transferring cells with only K,D region restricted cytotoxic (and DTH) activity. Transfer of Sendai virus-specific secondary cells or influenza virus-specific secondary cells possessing both K,D region restricted cytotoxic and DTH activities and I region restricted DTH activity did not result in any significant change in the extent of cellular infiltration in the lungs as compared to control mice receiving no cells.

TABLE 1

A Requirement of I Region Compatibility for the Increase in Mortality of Virus-Infected Mice Adoptively transferred with Secondary Effector cells with I Region Restricted DTH Activity but Negligible Cytotoxic Activity[a]

Experiment no.	Mouse Strain			Fraction of virus-infected recipient mice dying within 21 days	
	Donors of effector cells	Recipients	H-2 region shared	No cell transfer	Cell transfer
1	A.TH	A.TL	K,D	3/8	4/8[b]
2	A.TL	A.TL	K,I,D	3/9	7/9
	A.TL	A.TH	K,D	8/10	7/9[b]

a Secondary effector cells were raised *in vitro* by restimulation of memory spleen cells from mice primed with UV-inactivated A/WSN virus ($5x10^3$ HAU injected s.c.) 3 weeks or more previously. Recipient mice were adoptively transferred with $2x10^7$ secondary effector cells 24 h after i.n. inoculation of A/WSN virus ($5x10^3$ EID_{50} per mouse). Mortality was observed for 21 days after virus infection.

b Not significantly different from the control group (no cell transfer).

TABLE 2

Virus Dose Dependency on the Infiltration of I^{125}- Labelled Cells into the Lung of Mice Infected Intranasally with Influenza Virus[a]

Dose of virus inoculated intranasally (EID_{50})	Lung consolidation[b] (%)	Mice died[c]	Radioactivity in the lung cells (cpm)
Nil	Nil	-	27 ± 6
$5x10^2$	20-30	-	431 ± 57
$1x10^3$	30-45	-	602 ± 63
$5x10^3$	60-80	+	1030 ± 108
$1x10^4$	75-80	+	1233 ± 229

a CBA mice in groups of 4-5 were either uninfected or infected i.n. with increasing doses of A/WSN virus (a mouse-adapted batch). Six days later, all mice were given 0.2 ml of $5x10^{-4}$M 5-fluorodeoxyuridine injected i.p. followed 30 min later by 1 µCi I^{125}-UdR injected i.p. Twenty-four hours after the injection of I^{125}-UdR, mice were killed and the cell-associated radioactivity in each pair of lungs was measured.

b Estimated values from gross observation of groups of 4-5 mice, 7 days after virus inoculation.

c. Mortality was observed for 21 days after virus inoculation and death usually occurred at day 7-12.

TABLE 3a

Properties of Different Cell Preparations for Adoptive Transfer Studies

Preparation no.	Generation of effector cells in vitro		Properties of cells generated in vitro[b]		
	Responder cells[a]	Stimulator cells	% specific ^{51}Cr release on A/WSN-infected L929 cells (E/T = 8:1)	Mean increase in footpad thickness at 24 h (%) Recipients[c] C3HOH[d]	ATL[e]
1	Infectious A/WSN-primed spleen cells	A/WSN-infected L929 cells	40.9 ± 0.3	25.0 ± 1.6	1.6 ± 0.0
2	UV-inactivated A/WSN primed spleen cells	Spleen cells exposed to UV-inactivated A/WSN	6.7 ± 0.5	4.7 ± 3.1	26.6 ± 1.8
3	Infectious A/WSN-primed spleen cells	A/WSN-infected spleen cells	38.3 ± 0.4	23.5 ± 1.8	26.6 ± 3.1
4	Infectious Sendai-primed spleen cells	Sendai-infected spleen cells	7.6 ± 0.4	N.D.[f]	N.D.[f]

a Donor CBA mice were primed either with infectious (10^3 HAU, i.p.) or UV-inactivated ($5x10^3$ HAU, s.c.) virus 3 weeks or more previously.

b Effector cells were obtained after 5 days in culture.

c Recipients were injected with $5x10^6$ effector cells into the footpads 6 h after injection of infectious A/WSN virus ($2.5x10^3$ HAU) into the same footpad. Footpad swelling was measured 24 h after injection of cells.

d D region compatible with donors of effector cells.

e I region compatible with donors of effector cells.

f N.D. : Not determined.

TABLE 3b

Effect of Adoptive Transfer of Different Cell Preparations on Virus Titres and Cellular Infiltrations in the Lungs of Influenza Virus-Infected Mice[a]

Virus inoculated i.n.	Cell preparation transferred to virus-infected CBA mice			Lung virus titres (log EID_{50}/lung extract)	P value[c]	Radioactivity in lung cells (cpm)	P value[c]
	Cell preparation no.	Activity[b] I region restricted Td activity	Activity[b] K,D region restricted Tc and Td activities				
Nil						39±6	
+				6.37 ± 0.07		1209±129	
+	1	-	+	3.84 ± 0.51	P<0.01	599±155	P<0.02
+	2	+	-	6.19 ± 0.79	N.S.[d]	1830±39	P<0.01
+	3	+	+	4.44 ± 0.21	P<0.01	1036±148	N.S.[d]
+	4	-	-	N.D.[d]		1317±189	N.S.[d]

a $2x10^7$ secondary effector cells were transferred i.v. into groups of syngeneic recipient mice that were infected i.n. 24 h previously with $5x10^3$ EID_{50} A/WSN virus. Five days after cell transfer, the degree of cellular infiltration and virus titres in the mouse lungs in the various groups were measured (see Chapter 2: Materials and Methods).

b Influenza virus-specific T cell-mediated activity (see Table 3a).

c Comparison between the group of mice transferred with secondary effector cells and the control group that received no cells.

d N.D. : Not determined; N.S. : Not significantly different from control.

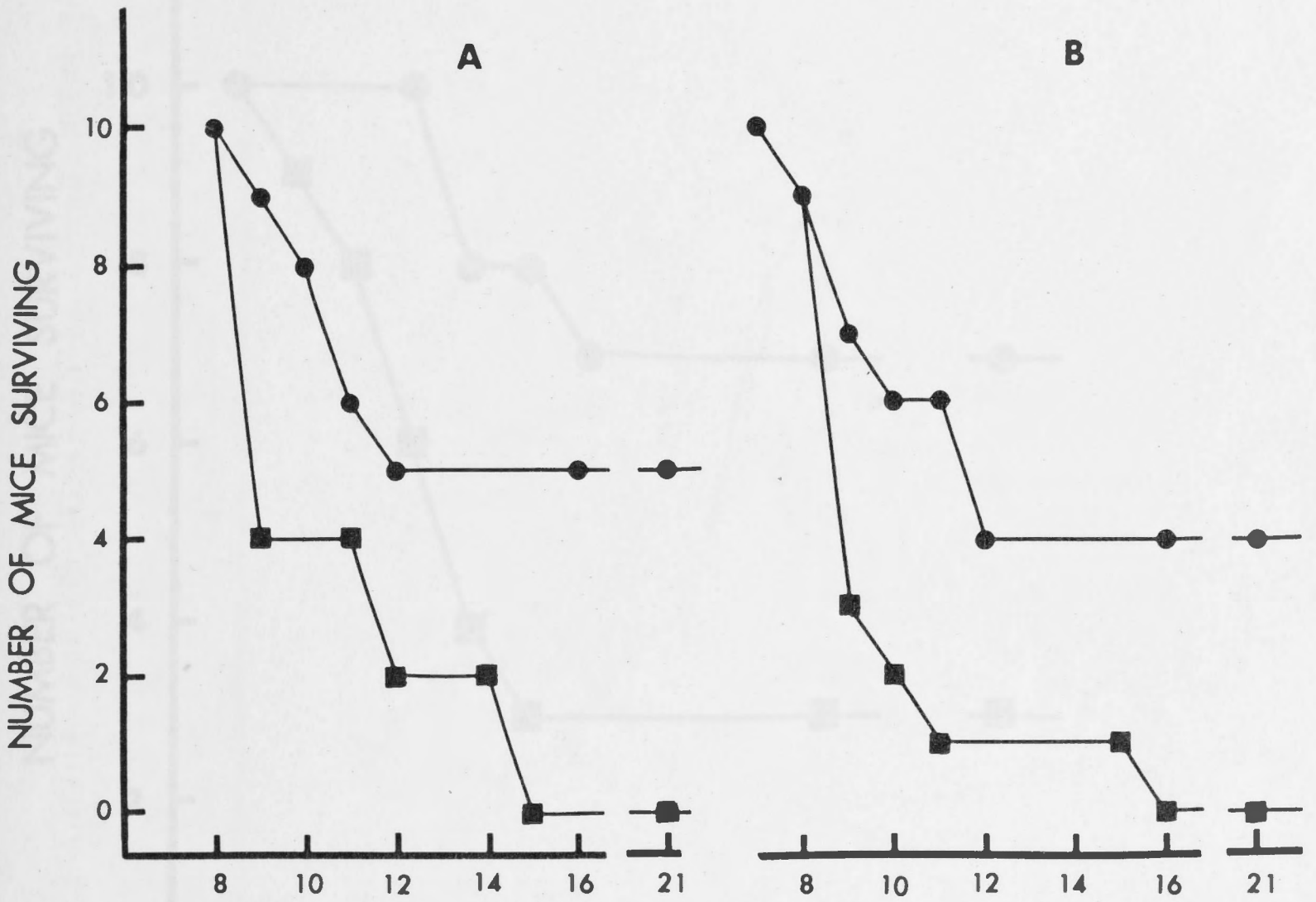


Fig. 1: Increase in mortality of A/WSN virus-infected CBA mice ($5x10^3$ EID_{50} given i.n.) after adoptive transfer of 5 days secondary effector cells raised in vitro. Secondary effector cells were generated in vitro by culturing of A/WSN virus-infected, syngeneic spleen cells with memory spleen cells from mice primed i.p. with UV-inactivated A/WSN virus (10^3 HAU) 3 weeks or more previously. Cells prepared in this way have potent I region restricted DTH activity but little or low levels of cytotoxic activity (data see text). (●) No cell transfer; (■) mice were given intravenously $1.3x10^7$ cells 24 h after virus inoculation (A) or $2x10^7$ cells 48 h after virus inoculation (B).

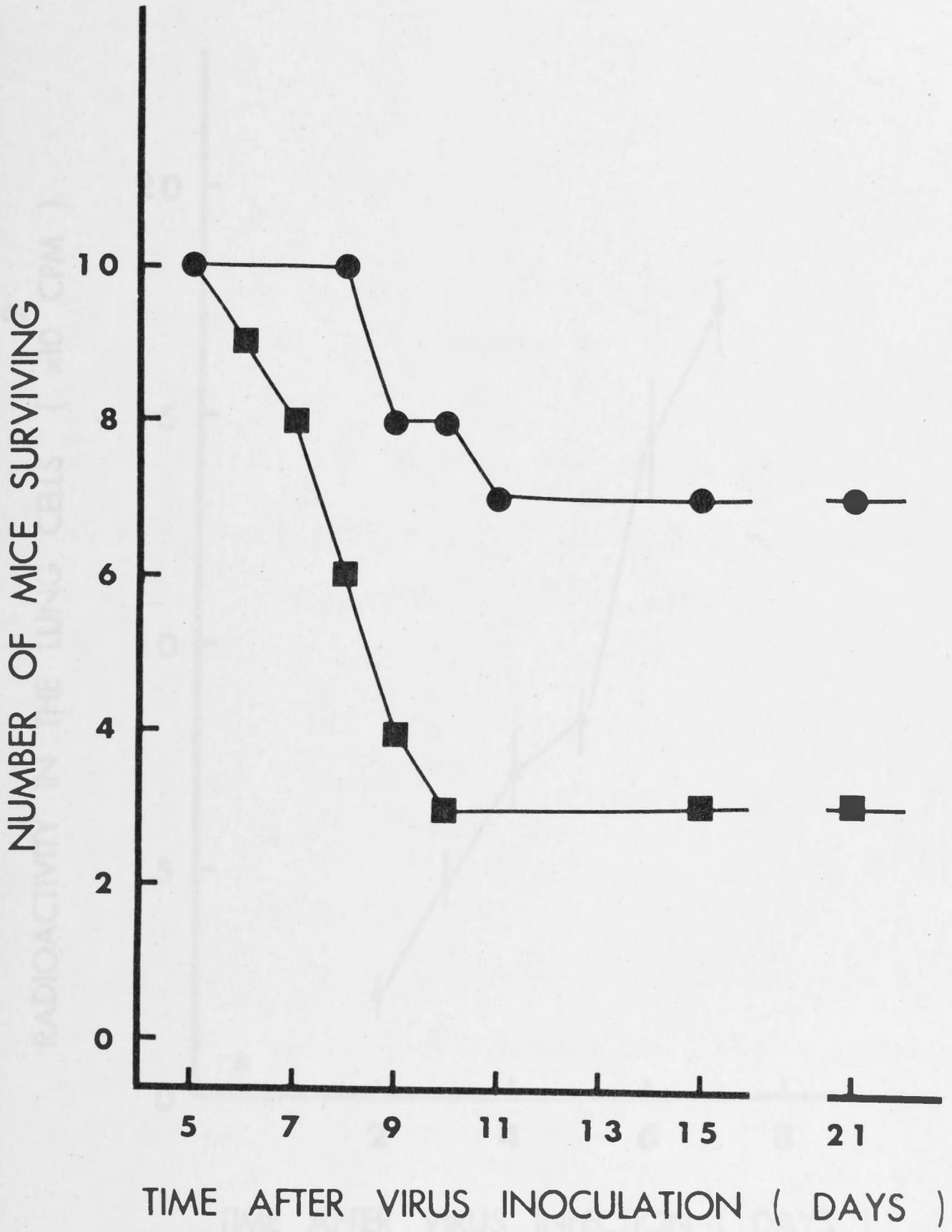


Fig. 2: Increase in mortality of A/WSN virus-infected CBA mice ($5x10^3$ EID_{50} given i.n.) after adoptive transfer of 6 days immune spleen cells generated in vivo. CBA mice were primed s.c. with $5x10^3$ HAU UV-inactivated A/WSN virus. Six days later, spleen cells harvested were separated into Ig^+ and Ig^- populations by a rosetting technique. $3.3x10^7$ Ig^- cells were transferred i.v. to syngeneic recipients 24 h after virus infection (■). Control mice received no cells (●). Mortality in both groups were observed for at least 21 days after virus inoculation.

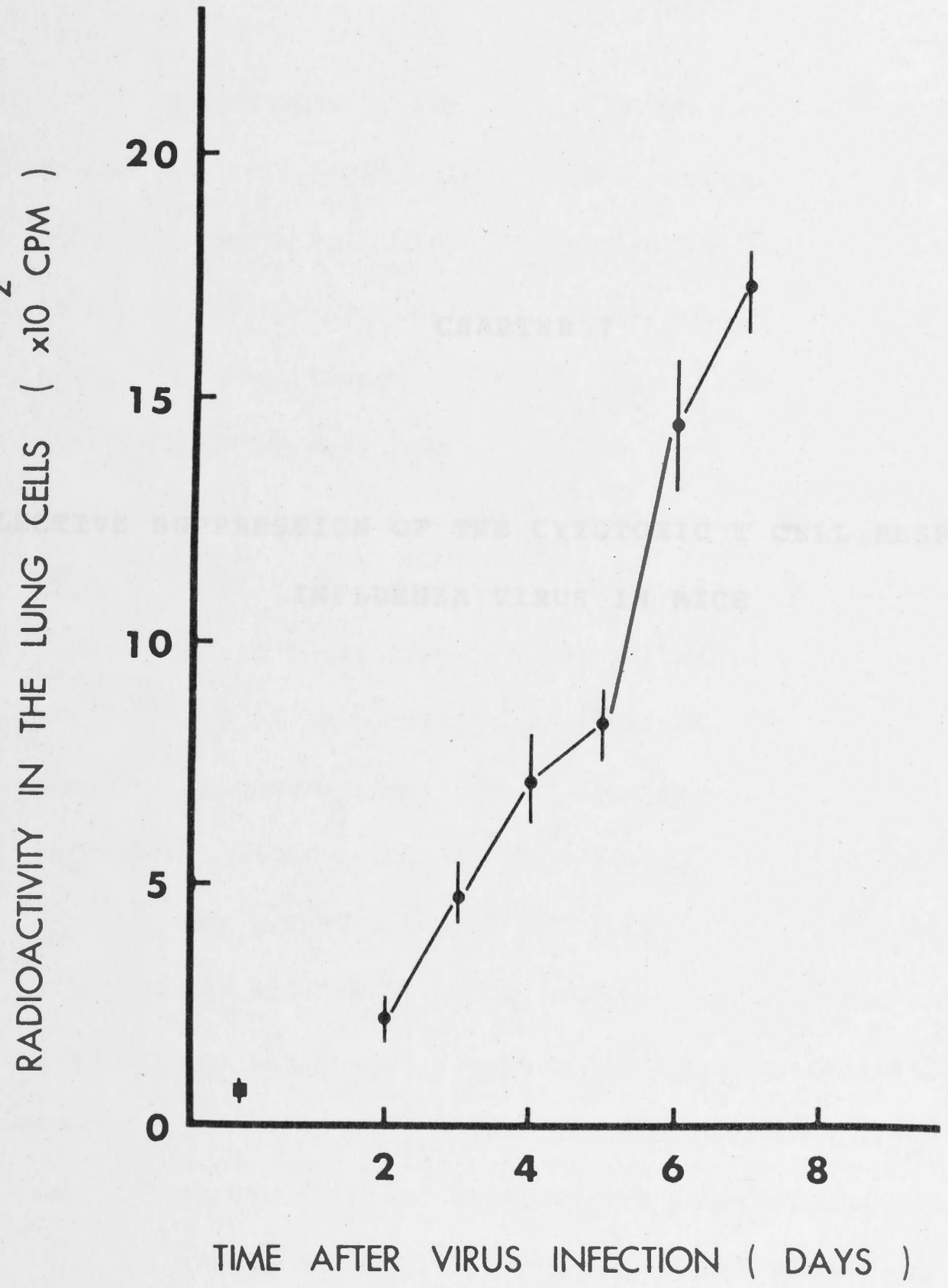


Fig. 3: Appearance of cell-associated radioactivity in the lungs of mice as a function of time after influenza virus infection. CBA mice were inoculated i.n. with the same dose ($5x10^4 EID_{50}$) of A/WSN virus and at different times after virus infection 3-5 mice were injected i.p. with 1 uCi I^{125}-UdR. Lungs were removed 24 h after pulse labelling and single cell suspensions were made. Contaminating erythrocytes were lysed and the radioactivity associated with the remaining cells was measured.

(●) Cells from infected mice;

(■) Cells from normal mice.

CHAPTER 7

SELECTIVE SUPPRESSION OF THE CYTOTOXIC T CELL RESPONSE TO INFLUENZA VIRUS IN MICE

INTRODUCTION

Previous work of Yap & Ada (1978b) has shown that adoptive transfer of virus-specific immune T cells were able not only to protect mice from death following injection of a lethal dose of virus but also to significantly reduce the titre of infectious virus in the lungs. Additional experiments indicated that the responsible T cell subset in the transferred cell population had the characteristics of cytotoxic T cells (Tc), i.e., were Lyt 23^+ and were K,D region restricted (Yap et al., 1978). This result did not exclude the possibility that other T cell subsets could also play a similar role but this would await the availability of appropriate preparations. In view of this finding, however, it became important to examine conditions which influenced the generation of Tc cells following injection or inoculation of virus.

Primary preparations of Tc cells are usually formed by intravenous injection of infectious virus into mice and maximum activity is recovered in the spleen six days later. Despite another report to the contrary (Ennis et al., 1977b),it was found that injection of UV-inactivated virus failed to cause the generation of specific Tc cells in mouse spleen although as much specific antibody was formed as if live virus had been injected (Braciale & Yap, 1978). This finding was confirmed in this chapter and in Chapter 3.1 it has been shown that injection of UV-inactivated virus also induces the formation of effector T cells involved in delayed type hypersensitivity reactions. As the antibody response to influenza virus is T cell dependent (Virelizier et al., 1974), the lack of a Tc response is selective and warrants further investigation. Two possible explanations are:
(1). injection of inactivated virus simply failed to induce the formation of Tc cells, possibly because antigen was not presented in

the appropriate way; (2). UV-light inactivated virus preferentially induced the formation of inhibitory cells. Evidence is presented in this chapter which supports the latter proposition.

RESULTS

Inhibition of the in vivo generation of Tc cells by prior injection of inactivated virus

Mice injected i.v. with $10^7 EID_{50}$ ($10^3 HAU$) A/WSN virus generate specific Tc cells and the maximum recovery from the spleen is 6 days after injection of virus (Yap & Ada, 1977). If mice were injected with the same amount ($10^3 HAU$) of UV-inactivated (non-infectious) virus and then challenged i.v. 4 days later with infectious virus, only about 10% of the usual Tc activity was recovered from the spleen at day 10 (6 days after the final virus injection) (Table 1). Similar low levels of activity were observed if the spleen were removed 2 or 4 days after injection of infectious virus (Fig. 1). Inhibition was observed if the dose of non-infectious virus was varied over the range 10^4-$10^1 HAU$ but the effect was negligible if the dose was 1 HAU or less (Fig. 2). Inhibition of the formation of Tc cells also occurred if other strains of influenza virus were used. Thus, for A/PC the inhibition found varied from 91-95% (mean = 92%) and for B/LEE virus, from 77-79%.

A smaller but substantial effect was seen if infectious A/WSN rather than non-infectious virus was first injected (Table 1). This suggested that the effects seen with pre-injection of inactivated or infectious virus differed only quantitatively. An additional experiment suggested a qualitative difference. It was previously shown (Braciale & Yap, 1978) that injection of live virus, 10^{-3} to $10^2 HAU$, gave a linear response with respect to Tc cells formation. If however, UV-inactivated virus was injected ($10^3 HAU$) at the same time as

infectious virus, the kinetics of production of Tc cells in the spleen were similar but the highest level reached was 40% lower than the control (injection of infectious virus only) (Fig. 3).

In contrast to the inhibition of Tc activity observed in these experiments, there was no depression in the antibody (anti-haemagglutinin) titres which occurred in mice pre-injected with the non-infectious virus (Table 2), thus confirming the original finding of Braciale & Yap (1978).

Specificity of inhibition of Tc response

The specificity of the suppression of Tc cells formation was examined with the results shown in Table 3. As well as suppression occurring with the homologous influenza A strain virus, substantial suppression also occurred with A strains which shared neither haemagglutinin nor neuraminidase serological specificity. Suppression however was not seen if the challenge virus was B/LEE or Sendai. As a control, it was shown that mice injected first with UV-inactivated B/LEE showed a decreased Tc response to subsequent challenge with infectious B/LEE virus, but not to a subsequent challenge with A/PC virus. Thus, the suppression found showed specificity between major types of influenza virus but little specificity within a subtype.

In considering the possible mechanism of suppression of Tc cells formation, possible candidates are: (1). a humoral factor; (2). a cell subpopulation.

Inhibition of the Tc response by transfer of immune serum

It was recently demonstrated that anti-viral antibody injected i.v. either previously or with infectious virus inhibited the formation of specific Tc cells in the spleen (Yap & Ada, 1979) and that neutralizing antibody (anti-haemagglutinin) was by far the most effective. Furthermore, injection of antibody at as short a time as 6 h after virus did not inhibit the generation of Tc cells.

Injection of serum (0.3 ml), taken from mice 6 days after i.v. injection of 10^3HAU of infectious or non-infectious virus, into recipient mice 24 h before injection of infectious virus inhibited the subsequent generation of Tc cells in the recipient mice by 66-70%. The active factor(s) in the serum was first detected at day 3-4 after virus injection and reached peak levels at day 6 and remained high until at least day 10 (Fig.4). The kinetics of production of serum suppressor factor(s) correlated well with the formation of serum HI antibodies (Fig. 4). Samples of 6 days serum were fractionated on G-200 Sephadex columns (Fig. 5) and the 19S and 7S peaks collected and tested. Both showed low levels of anti-haemagglutinin activity and both upon injection into mice (as above) inhibited Tc cells formation to approximately equal extents (19S peak, 68% inhibition; 7S peak, 79% inhibition; original serum, 71% inhibition). Samples of serum were also chromatographed on Protein A-Sepharose column. Both the effluent (62% inhibition) and the eluate (62% inhibition) were active (original serum, 76% inhibition).

The specificity of the serum inhibitory effect was next examined. Normal mouse serum or serum taken from mice 6 days after injection of UV-inactivated or infectious A/WSN virus was injected into recipient mice 24 h before injection of infectious A/WSN virus, A/PC virus or Sendai virus. Similar results were obtained whether infectious or UV-inactivated virus was used initially; Table 4 reports the result obtained when infectious virus was used and shows that the inhibition was specific for the homologous virus. This is in contrast to the specificity pattern in Table 3.

Inhibition of the Tc response by transfer of immune cells

Spleens were removed from mice 5 days after i.v. injection of 10^3HAU of UV-inactivated or infectious A/WSN virus. Immune or normal spleen cell suspensions ($7x10^7$ cells) were injected into syngeneic recipients (3 mice/group) which were challenged 24 h later with 10^7EID_{50}

of A/WSN virus (i.v.) and Tc activity in the spleen determined 6 days after virus injection. Representative results of two types of experiments are shown in Table 5. In experiment 1, injection of immune spleen cells from mice injected with non-infectious virus, inhibited the level of splenic cytotoxic activity by greater than 50%; the mean reduction from 10 such experiments was 61.2 ± 2.3%. In most experiments, injection of normal spleen cells slightly increased Tc activities rather than decreased them, as seen in Table 5. In contrast to these results, the injection of immune cells from mice pre-injected with infectious virus (Expt. 2) resulted in a substantial (about 50%) enhancement of the Tc response in the recipient mice and preliminary results showed that both donor and recipient cells contributed to the total cytoxicity.

The suppressive effect was further examined, with the following results: (1). injection of cells i.v. rather than i.p. gave slightly higher inhibition; (2). day 3 immune spleen cells were also active in suppression, but maximum suppression was obtained with 5 days immune spleen cells and suppressor activity dropped thereafter (Fig. 6); (3). dose response study has shown that at least $2x10^7$ 5 days immune spleen cells were required for transfer of significant suppression, although high dose such as 10^8 cells gave slightly higher suppression (Fig. 7); (4). the maximum suppression achieved was 70% and this occurred when 10^8 5 days immune cells were transferred to recipient mice 48 h before the injection of the infectious virus. Inhibition of the Tc activity was reduced to 40% if immune cells were transferred 24 h after challenge of the recipient mice with infectious virus. No suppression was observed if cells were transferred 48 h or later after challenge. (5). viable cells are required for transfer of suppression, as heat-killed (56°C, 30 min) immune cells or supernatant from sonicated immune cells failed to transfer suppression (Table 6).

The specificity of the inhibition by transferred immune cells was examined and is reported in Table 7. The results are similar to those in Table 3 and show cross-reactivity within the influenza A strains with no significant effect of A/WSN immune cells on Tc cells generation to Sendai virus.

Mechanism of action of suppressing cells

Virus-specific, radioresistant Th cells can augment the clonal expansion of H-2-restricted anti-viral Tc cells *in vitro* (Ashman & Müllbacher, 1979) and hence it may be inferred that collaboration between these two types of cells may occur *in vivo*. The action of suppressing cells might be to interfere with the generation of Th cells or the expansion of the Tc clones. Two types of experiments were carried out to elucidate the mechanism of action of the transferred immune cells. A dilution assay was used to examine the status of both cell classes in the spleens of normal mice and of mice injected with live or UV-inactivated virus. Two experiments with A/JAP and A/WSN virus are reported in Table 8. As expected, full clonal expression of Tc cells in normal (unprimed) spleens only occurred if helper T cell preparations were added. In contrast, there was little or no requirement for help when the spleens from mice primed with infectious or UV-inactivated virus were tested, showing clearly that the spleens of mice receiving UV-inactivated virus contained Tc precursor cells which could undergo clonal expansion and that the Th cells present were primed.

The second experimental approach was to see if the injection of radioresistant Th cells at the same time as injection of infectious or UV-inactivated virus influenced the level of Tc activity subsequently recovered in the spleens. The results of several experiments showed that injection of 10^8 γ-irradiated memory spleen cells (containing Th cells) to mice which also received infectious ($10^7 EID_{50}$) or

UV-inactivated virus (10^3HAU) made little difference to the level of cytotoxicity generated by the virus itself (data not shown). This suggested either that such Th cells, when injected, were incapable of delivering help or that such help upon delivery was ineffective, for unknown reasons. The first possibility was tested by showing that such helper T cell preparations, when injected with sub-optimal amount of infectious virus (10^5EID_{50} instead of 10^7EID_{50}, as in the above experiments), increased the level of cytotoxic T cell activity recovered in the spleens at day 6 (specific ^{51}Cr release 50% ± 1.0 compared to 36% ± 1.1 which is a significant increase $p < 0.01$). Thus the result favoured the second interpretation.

Experiments concerning the nature of the inhibitory cells

(A) Failure of nude mice to generate splenic suppressor cells

Nude mice were injected with a standard dose of UV-inactivated A/WSN virus and 5 days later the spleens removed and $1x10^8$ cells transferred to normal littermates which were challenged 24 h later with infectious A/WSN virus. Tc levels were measured in the recipient mouse spleens 6 days later and indicated that transfer of immune compared to normal nude mouse spleen cells did not decrease Tc levels in the recipient mice. There was in fact a slight enhancement (Table 9). These experiments indicated that formation of the inhibitory cells was T cell-dependent.

(B) Requirement for H-2 compatibility between donor and recipient mice

If one active component of the inhibiting cell population was T cells, a direct way to establish this would be to see if there was a requirement for H-2 compatibility between donor cells and recipient mice. In the first experiment, transferred cells were found to inhibit Tc cells formation if the donor and recipient mice shared H-2 genes

but otherwise differed in their genetic background (Table 10). Experiments demonstrating the importance of shared regions of the H-2 gene complex are reported in Table 11. The three panels of the table demonstrate: (1). ablation (<90%) of the suppressor effect if there was complete H-2 incompatibility (top panel), or incompatibility at the I region between donor and recipient (middle panel); and (2). a highly significant reduction (77-85%) in suppression if donor and recipient were incompatible at the K and/or D region (bottom panel).

These results suggested that one (or more) class of T lymphocytes was an active component in the suppressing cell population. Additional approaches were followed to obtain further information on the nature of the active cells.

(C) Cell fractionation studies

Spleen cell preparations were treated with carbonyl iron powder to remove phagocytic cells or depleted of adherent cells by adsorption onto plastic. The resulting cell populations had undiminished inhibitory activity on adoptive transfer to recipients, compared to the unfractionated control preparations (data not shown).

(D) Susceptibility to anti-Thy 1.2 antibody and complement

Five days immune spleen cells from mice previously injected with UV-inactivated A/WSN virus (10^3HAU) were either treated with mono-specific anti-Thy 1.2 antibody and complement, with complement alone or untreated. The cell preparations were adoptively transferred to syngeneic recipient mice which were injected with infectious A/PC virus. (A different A strain was used to eliminate any suppressive effect of antibody which is specific for the homologous virus - see Tables 4 and 7). The result in Table 12 indicates that the cells mediating the suppression were susceptible to anti-Thy 1.2 and complement treatment.

DISCUSSION

This chapter describes experiments which confirm and extend the original finding of Braciale & Yap (1978) that intravenous injection of UV-inactivated influenza virus, in contrast to infectious virus, fails to generate cytotoxic T cells though normal amounts of antibody are formed and T cells mediating delayed type hypersensitivity are generated (Chapters 3.1, 3.2). Furthermore, the work reported in this chapter also established that inactivated virus induced specific suppression which results in a state of inactivity in one arm of the immune system.

Tolerance to virus infections is known in two other systems, lymphocytic choriomeningitis virus, LCM (Traub, 1960) and avian leukosis virus (Rubin et al., 1962). In both cases, the hosts are exposed to the virus in utero or as neonates and as far as has been examined, the tolerance involves both humoral and cell-mediated arms of the immune system. It has been proposed in LCM (Dunlop & Blanden, 1977) that Tc cells are generated but self destruct as they become infected by the virus. The experiments in this chapter suggest a different cellular mechanism operating in the influenza virus system.

The basic observation is that mice injected with UV-inactivated virus followed some days later with infectious virus almost completely fail to generate Tc cells in their spleens. Transfer experiments indicate that the suppression is due to humoral and cellular factors. Pre-injection of inactivated virus caused the production of (both IgM and IgG) antibodies which were specific for the A strain virus used, and most likely were haemagglutinin specific. The antibody may simply neutralize some of the injected antigen, thus giving a lower Tc response which is known to be dose-responsive (Braciale & Yap, 1978). Suppression also occurs but to a lesser extent if infectious rather than inactivated virus is first injected (Table 1), but this is due

solely to antibody (Table 4) so that suppression was not observed when cells were transferred (Table 5).

The cellular component which inhibits Tc cells generation has concerned me more. Evidence that this is a cellular phenomenon and not due to antibody is shown by: (1). Simultaneous injection of infectious and inactivated virus resulted in a 60% drop in Tc activity in the spleen. This decrease was similar in magnitude to that seen when cells from mice immunized with inactivated virus were transferred to recipients that were then challenged with infectious virus; (2). cells from mice immunized with inactivated virus were still suppressive if transferred to recipients 24 h after the latter were injected with infectious virus. It has been shown by Yap & Ada (1979) that antibody given with or before infectious virus abolished the generation of Tc cells but that their generation was unaffected if antibody was injected only 6 h after the virus; (3). the specificity patterns of the inhibition were different. Antibody inhibited generation of Tc cells only to the homologous virus. The cellular component showed cross-reactivity within the A strain viruses when the inactivated virus was an A strain but this did not extend to Sendai virus or to a B strain virus; (4). the transfer of inhibitory effect was H-2 restricted. The requirement for I region sharing was almost complete (Table 11); there was also a partial requirement for K,D sharing. As suppression was only observed if the cells were transferred to the recipient within 24 h after injection of infectious virus, the possibility that the apparent partial requirement was due to rejection of the transferred cells was unlikely. But this possibility has <u>not</u> been formally excluded and experiments are under way to test this point; (5). the suppressor activity of the cellular fraction was abolished by treatment with monospecific anti-Thy 1.2 antibody and complement. These five considerations argue against B lymphocytes or their products being candidates for the major effect observed.

Experiments which were carried out to elucidate the mechanism of action of the inhibitory cells took advantage of two recent findings: (1). that spleen cells from mice injected three weeks earlier with infectious virus after γ-irradiation, can function as Th cells in tissue culture. Under appropriate conditions, injection of sub-optimal amounts of virus, they may also have this activity *in vivo* (this chapter); and (2). that clonal expansion of Tc cells in dilution assays can be used to estimate Tc cell precursor frequency (Ashman & Müllbacher, 1979). Application of this technique indicated that spleen cells from mice pre-injected with inactivated virus contained Tc precursors and primed cells which upon stimulation *in vitro* were effective helper cells. (It is assumed that this *in vitro* assay system, the suppressor activity was either diluted out or did not function.) The failure of injected Th cells to significantly increase Tc cells generation in mice given inactivated virus (control group had injection of virus only) is consistent with the belief that the spleens of these mice already contained primed cells which could act as Th cells after stimulation *in vitro* or that receipt of help was blocked.

Results in this chapter therefore indicate that injection of UV-inactivated virus may activate both Th and Tc precursor cells but that the further development of the latter is inhibited by suppressor cells. Infectious or inactivated virus may differ only quantitatively (or not at all) in their ability to induce Tc and Th cells but inactivated virus is more efficient at inducing suppressor activity (see Table 5). Nevertheless, as Tc cells are important in the recovery of mice from influenza virus infection (Yap & Ada, 1978b; Yap *et al.*, 1978) it is necessary to establish why inactivated virus is more efficient at inducing a mechanism which prevents Tc cells generation, as this has implications for the development of viral vaccines.

There are several aspects which clearly need further investigation. One is the H-2 sharing requirements for the transferred cells to function. Experiments designed to define more closely the genetic requirements for successful transfer are in progress. A second aspect is the elucidation of the different methods of antigenic stimulation which occur when inactivated or infectious virus is administered to the mice, and why inactivated virus is so selective in its effect. Thus, antibody production (and presumably Th cells) and Td cells sensitization are unaffected and yet production of active Tc cells is suppressed. It seems that the immune system has different mechanisms to deal with a replicative and a non-replicative form of a virus (Zinkernagel, 1976).

SUMMARY

Mice injected with inactivated (UV light-irradiated) influenza virus produce specific antibody, become sensitized for a DTH reaction, but do not generate specific cytotoxic T cells (Tc). If injected 4-5 days later with infectious virus, the formation of Tc cells is suppressed by >90%. If A strain viruses are used, the suppression observed is cross-reactive within A strain viruses but does not extend to B/LEE or to Sendai virus. Serum from the mice injected with UV-inactivated virus contains antibodies, which on adoptive transfer can inhibit Tc cells formation when infectious homologous virus is used to challenge the recipients. Spleen cells from the same mice, upon adoptive transfer, also inhibit (50-70%) Tc cells formation if transferred within 24 h of injection of infectious virus and the specificity pattern observed is cross-reactive within A strains. The activity of the cells mediating suppression was destroyed by monospecific anti-Thy 1.2 antibody and complement. The immune cells

required I region sharing between donor and recipient mice for their suppressor activity to be effective. There was also a partial requirement for K,D region sharing but the possible rejection of transferred cells was not excluded. Dilution assays in which clonal expansion of Tc precursors is used to estimate their frequency and the presence of T helper cells (Th) indicate that suppressed mice possess Tc precursors and primed cells which, upon restimulation, acted as Th cells. Furthermore, injection of irradiated Th cells with inactivated virus did not significantly reduce the ensuing suppression.

TABLE 1

Effect of Pre-immunization of Mice with UV-Inactivated (Non-infectious) or Infectious Virus Upon the Subsequent Generation of Cytotoxic Activity by a Standard Dose of Infectious Virus[a]

Pre-immunization of mice	% Specific ^{51}Cr release at effector:target cell ratios of		% Suppression at E:T ratios of[c]	
	25:1	50:1	25:1	50:1
Nil	27.7 ± 0.7[b]	46.4 ± 3.6	-	-
UV-inactivated A/WSN	2.4 ± 0.8	4.7 ± 2.1	91	90[d]
Infectious A/WSN	7.0 ± 0.3	11.2 ± 1.9	75	76

a Groups of 3 BALB/c mice were either untreated or pre-injected i.v. with infectious or UV-inactivated A/WSN virus (10^3HAU/mouse). Four days later, all mice were challenged i.v. with a standard dose of infectious A/WSN virus ($10^7 EID_{50}$/mouse). Spleens were removed and pooled in each group and assayed for cytotoxic activity 6 days after the second injection.

b Specific lysis from infected, H-2 compatible P815 target cells. (Specific lysis from uninfected targets was always <5%.)

c % suppression =

$$\frac{\text{^{51}Cr release with cells from mice not pre-injected with virus} - \text{^{51}Cr release with cells from mice pre-injected with virus}}{\text{^{51}Cr release with cells from mice not pre-injected with virus}} \times 100$$

d The mean value of 10 experiments was 94.7 ± 1.2% suppression.

TABLE 2

Effect of Pre-immunization of Mice with UV-Inactivated Influenza Virus Upon the Subsequent Production of Antibodies Against a Second Challenge with Infectious Virus

Pre-immunization of mice[a]	Haemagglutination-inhibition antibody titer[b]			
	Days after i.v. injection of infectious A/WSN virus (10^3HAU)			
	2	7	11	15
Nil	<20	50 ± 6	70 ± 10	80 ± 0
UV-inactivated A/WSN virus	40 ± 0	130 ± 19	110 ± 19	140 ± 20

a Groups of 3 BALB/c mice were either untreated or pre-injected i.v. with 10^3HAU UV-inactivated A/WSN virus. Five days later, all mice were challenged i.v. with 10^3HAU infectious A/WSN virus. Blood was collected from the tail veins of mice at different times after the second challenge with infectious virus.

b Determined by standard procedure as described in Materials and Methods (Chapter 2).

TABLE 3

Specificity of Suppression of Tc Responses Following the First Injection of UV-Inactivated Virus

Inoculation of mice with virus (i.v.)		% Specific ^{51}Cr release at effector:target cell ratios of		% Suppression at effector: target ratios of	
First injection of UV-treated virus[a]	Challenge injection of infectious virus[a]	50:1	100:1	50:1	100:1
Nil	A/WSN (HONl)	38.1 ± 1.9[b]	46.0 ± 1.1[b]		
A/WSN	A/WSN	3.0 ± 1.6	3.2 ± 0.2	92	93
Nil	A/RI (H2N2)	32.9 ± 1.7[b]	47.3 ± 1.2[b]		
A/WSN	A/RI	9.6 ± 1.1	19.9 ± 0.4	71	58
Nil	A/JAP (H2N2)	10.8 ± 0.7[b]	21.8 ± 0.4[b]		
A/WSN	A/JAP	4.8 ± 0.8	5.4 ± 0.2	63	75
Nil	A/PC (H3N2)	42.4 ± 1.6[b]	57.5 ± 2.2[b]		
A/WSN	A/PC	11.4 ± 0.9	19.5 ± 2.6	73	66
Nil	Sendai	63.8 ± 2.3[c]	73.7 ± 4.1[c]		
A/WSN	Sendai	69.1 ± 1.9	75.4 ± 2.9	Nil	Nil
Nil	B/LEE	18.5 ± 1.5[b]	31.6 ± 1.2[b]		
B/LEE	B/LEE	3.9 ± 0.5	7.4 ± 1.3	79	77
Nil	A/PC	N.D.[d]	61.9 ± 2.7[c]		
B/LEE	A/PC	N.D.	58.5 ± 1.7	-	5

Legends for Table 3

a Dose of inactivated virus was 10^3HAU/mouse and of infectious virus was $10^7 EID_{50}$/mouse. The challenge virus was given 4-5 days after the first injection and cytotoxic activity in the spleen determined 6 days after the challenge injection.

b The difference in ^{51}Cr release is significant ($p < 0.01$).

c The difference in ^{51}Cr release is not significant.

d N.D.: not determined.

TABLE 4

Specificity of Immune Sera from Mice Injected with Infectious Virus on the Inhibition of Cytotoxic T Cell Response

Serum transfer[a]	Challenge virus	% Specific ^{51}Cr release[b] at E:T ratios of		% Suppression at E:T ratios of	
		50:1	100:1	50:1	100:1
Normal serum	A/WSN (HON1)	37.6 ± 0.5	40.8 ± 1.1		
A/WSN immune serum	A/WSN	8.1 ± 1.0	13.4 ± 1.3	79	67
Normal serum	A/PC (H3N2)	34.4 ± 0.2	49.4 ± 0.9		
A/WSN immune serum	A/PC	32.6 ± 2.5	45.8 ± 0.6	5	7
Normal serum	Sendai	47.8 ± 2.0	58.1 ± 0.6		
A/WSN immune serum	Sendai	44.5 ± 2.8	62.5 ± 0.3	7	Nil

a 0.3 ml sera were injected into BALB/c mice which were challenged 24 h later with $1x10^7 EID_{50}$ infectious virus. 6 days after virus injection, spleens were removed and tested for cytotoxic activity.

b Specific ^{51}Cr release from P815 target cells infected with viruses which are homologous with those used for challenging the mice.

TABLE 5

Suppression or Enhancement of Tc Activity in Mice Adoptively Transferred with 5-day Immune Spleen Cells 24 h Before Challenge with the Homologous Infectious Virus

Experiment	Group	Pre-injection (i.v.) of mice with virus (10^3 HAU A/WSN virus)	Cell transfer[a]	% Specific ^{51}Cr release at E:T ratios of		% Suppression at E:T ratios of[b]		% Enhancement at E:T ratios of[b]	
				25:1	50:1	25:1	50:1	25:1	50:1
1	a	Nil	Nil	30.1 ± 0.8	41.8 ± 2.5				
	b	UV-inactivated virus	Nil	2.4 ± 0.5	5.5 ± 1.4	92	87		
	c	Nil	Normal	27.0 ± 2.0	38.7 ± 1.7	10	7		
	d	UV-inactivated virus	Immune	13.7 ± 0.8	18.3 ± 1.1	55	56		
2	a	Nil	Nil	21.9 ± 0.5	29.6 ± 0.6				
	b	Infectious virus	Nil	7.1 ± 0.3	7.8 ± 0.2	68	74		
	c	Nil	Normal	21.4 ± 0.7	31.0 ± 0.1	2			5
	d	Infectious virus	Immune	33.6 ± 0.7	45.6 ± 0.4		53		54

a 7×10^7 normal or 5 days immune spleen cells were adoptively transferred i.v. into syngeneic recipients 24 h before challenge with infectious A/WSN virus ($10^7 EID_{50}$/mouse).

b % suppression or enhancement were calculated by comparing the specific lysis of group b-d relative to specific lysis of group a only.

TABLE 6

Requirement of Viable Cells for Transfer of Suppression

Cell transferred[a]	Treatment before transfer	% Specific ^{51}Cr release at E:T ratio of 25:1	% Suppression[c]
Normal spleen cells	Nil	26.5 ± 1.0	
5 Days immune spleen cells	Nil	14.1 ± 0.2	47
5 Days immune spleen cells	Heat-killed (56°C, 30 min)	27.4 ± 0.9	-3
5 Days immune spleen cells[b]	Sonicated	24.3 ± 0.6	8

a $7x10^7$ syngeneic spleen cells were transferred to naive BALB/c recipients which were then challenged with infectious A/WSN virus ($1x10^7 EID_{50}$) 24 h later.

b Supernatant from sonicated cells was transferred.

c % Suppression was calculated by comparing specific lysis with the control group (transfer of normal spleen cells).

TABLE 7

Specificity of the Splenic Suppressor Cells

Spleen cells injected[a]	Challenging virus	% Specific ^{51}Cr release[b] at E:T ratios of		% Suppression at E:T ratios of	
		25:1	50:1	25:1	50:1
Normal cells	A/WSN (H0N1)	27.9 ± 0.8	47.4 ± 2.5	63	69
A/WSN immune cells	A/WSN	10.2 ± 0.5	14.7 ± 0.6		
Normal cells	A/PC (H3N2)	35.1 ± 1.0	47.2 ± 0.9		
A/WSN immune cells	A/PC	8.5 ± 0.6	15.3 ± 0.5	76	68
Normal cells	A/JAP (H2N2)	27.0 ± 1.3	36.6 ± 1.2		
A/WSN immune cells	A/JAP	9.3 ± 0.5	15.7 ± 1.3	66	57
Normal cells	A/RI (H2N2)	21.7 ± 0.5	27.4 ± 1.0		
A/WSN immune cells	A/RI	5.9 ± 0.9	8.1 ± 0.1	73	70
Normal cells	Sendai	47.3 ± 1.5	68.6 ± 1.5		
A/WSN immune cells	Sendai	47.5 ± 1.0	64.8 ± 0.3	Nil	6

a $1x10^8$ normal spleen cells or 5 days immune spleen cells from donor mice pre-injected with 10^3HAU UV-inactivated virus were injected i.v. into syngeneic recipients and these mice were challenged with $1x10^7 EID_{50}$ virus 24 h later. Cytotoxic activity of the spleens were determined 6 days after virus infection.

b Specific ^{51}Cr release from P815 target cells infected with viruses which are homologous to those used for challenging the mice.

TABLE 8

Secondary *in Vitro* Stimulation, with or without Added T Helper Cells, of Cytotoxic T Cells in the Spleens of Mice Injected Earlier with Infectious or UV-Inactivated Virus[a]

Virus used	Responder cells	Number positive wells/number tested	
		Th cells not added	Th cells added
A/WSN	Unprimed	7/31	30/31
	Primed with infectious virus	31/31	31/31
	Primed with UV-inactivated virus	22/31	31/33
A/JAP	Unprimed	4/31	28/31
	Primed with infectious virus	31/31	30/31
	Primed with UV-inactivated virus	31/31	31/31

a Mice were primed by i.p. injection of $10^7 EID_{50}$ infectious virus or 10^3HAU of UV-inactivated virus, and spleens removed and used as a source of responder cells in an *in vitro* assay 6 days later. Wells contained either $7x10^4$ cells/well (A/WSN) or $5x10^4$ cells/well (A/JAP). $2x10^5$ Th cells were added to each well.

TABLE 9

Failure of Nude Mice to Generate Splenic Suppressor Cells

Donor mice[a]	Spleen cells injected[b]	Recipient mice	% Specific ^{51}Cr release at E:T ratios of		% Suppression[c] at E:T ratios of	
			25:1	50:1	25:1	50:1
BALB/c	Normal	BALB/c	16.9 ± 0.9	29.5 ± 1.0		
BALB/c	Immune	BALB/c	9.0 ± 0.1	15.9 ± 1.0	47	46
BALB/c (nu^+/nu^+)	Normal	BALB/c	20.6 ± 0.9	34.1 ± 1.4	-22	-16
BALB/c (nu^+/nu^+)	Immune	BALB/c	23.7 ± 0.6	36.5 ± 0.8	-40	-24

a Donor mice were either untreated or primed with 10^3HAU UV-inactivated A/WSN virus i.v. and spleens removed 5 days later.

b $1x10^8$ normal or 5 days immune spleen cells were injected i.v. into recipient mice which were challenged i.v. with 10^7EID_{50} infectious A/WSN virus and cytotoxic activity of the spleen cells were determined 6 days after virus infection.

c % Suppression was calculated by comparing the specific ^{51}Cr release in each case with the specific ^{51}Cr release from cells of mice which were adoptively transferred with syngeneic, normal, spleen cells.

TABLE 10

MHC Control Successful Transfer of Suppression

Donor mice (H-2 haplotype)[a]	Spleen cells injected[b]	Recipient mice	% Specific ^{51}Cr release at E:T ratios of		% Suppression[c] at E:T ratios of	
			25:1	50:1	25:1	50:1
BALB/c (ddd)	Normal	BALB/c	24.4 ± 0.6	38.4 ± 0.2		
BALB/c	Immune	BALB/c	7.3 ± 0.2	13.7 ± 0.7	70	64
DBA/2 (ddd)	Normal	BALB/c	22.8 ± 0.9	45.5 ± 0.8	7	-19
DBA/2	Immune	BALB/c	10.7 ± 0.2	19.7 ± 0.6	56	49

Legends: Refer to Table 9.

TABLE 11

Requirement for H-2 Compatibility Between Donor and Recipient Mice

Donor mice (H-2 haplotype)[a]	Spleen cells injected[b]	Recipient mice	% Specific ^{51}Cr release at E:T ratios of		% Suppression[c] at E:T ratios of	
			25:1	50:1	25:1	50:1
BALB/c (ddd)	Normal	BALB/c	25.4 ± 0.8	40.4 ± 0.1	-	-
BALB/c	Immune	BALB/c	7.5 ± 0.3	13.1 ± 0.4	71	68
CBA/H (kkk)	Normal	BALB/c	23.8 ± 2.3	36.8 ± 1.5	6	9
CBA/H	Immune	BALB/c	24.8 ± 1.5	38.7 ± 0.9	2	4
A.TH (ssd)	Normal	A.TH	20.5 ± 0.6	45.0 ± 2.5	-	-
A.TH	Immune	A.TH	9.0 ± 0.8	25.7 ± 1.3	56	43
A.TL (skd)	Normal	A.TH	20.7 ± 0.7	46.6 ± 1.5	-	-
A.TL	Immune	A.TH	19.4 ± 1.6	43.4 ± 1.4	5	4
A.TL	Normal	A.TL	20.6 ± 0.2	27.4 ± 1.2	-	-
A.TL	Immune	A.TL	4.3 ± 0.8	7.9 ± 2.2	79	71
CBA	Normal	A.TL	17.5 ± 0.8	26.2 ± 0.3	15	4
CBA	Immune	A.TL	16.9 ± 0.1	24.4 ± 0.2	18	11
CBA	Normal	CBA	19.1 ± 1.0	30.8 ± 0.6	-	-
CBA	Immune	CBA	4.9 ± 0.3	9.8 ± 0.2	74	68

Legends: Refer to Table 9.

TABLE 12

Effect of Anti-Thy 1.2 Antibody and Complement Treatment on Splenic Suppressor Activity[a]

Cell transferred	Treatment before cell transfer	% Specific ^{51}Cr release[b] at E:T ratios of		% Suppression at E:T ratios of	
		25:1	50:1	25:1	50:1
Normal spleen cells	Nil	48.1 ± 1.9	67.5 ± 2.1		
5 days immune spleen cells	Nil	18.3 ± 0.9	25.3 ± 1.5	62	63
5 days immune spleen cells	C' alone	21.8 ± 1.1	34.7 ± 0.3	55	49
5 days immune spleen cells	Anti-Thy 1.2 + C'	44.8 ± 1.2	63.9 ± 2.7	7	5

a Splenic suppressor cells were generated by immunization of donor mice with 10^3HAU UV-inactivated A/WSN virus. The spleen cells were then harvested 5 days later, $9x10^7$ cells were injected i.v. into the syngeneic recipients which were then challenged 24 h later with another A strain virus (A/PC, $10^7 EID_{50}$). The cytotoxic activity of the spleen cells was determined 6 days after virus injection.

b Specific ^{51}Cr release from A/PC-infected P815 cells.

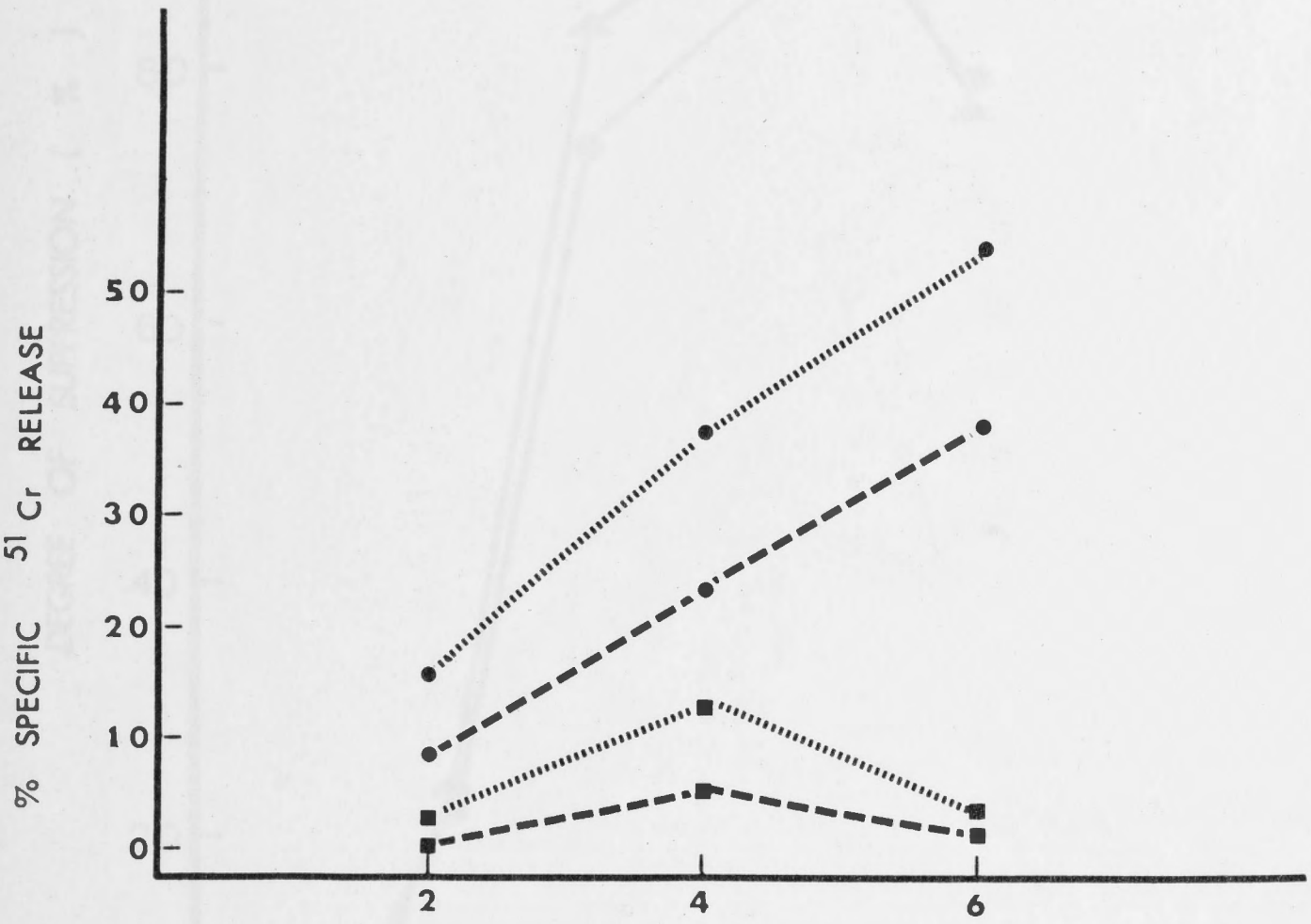


Fig. 1: Effect of preimmunization of mice with noninfectious virus on the kinetics of the subsequent generation of cytotoxic T cell response to a standard dose of infectious virus. BALB/c mice were either unprimed (●) or primed (■) i.v. with 10^3 HAU UV-inactivated A/WSN virus. Four days later, the mice were injected with 10^7 EID_{50} infectious A/WSN virus i.v.. Cytotoxic activity of the spleen cells was determined at different times after injection of infectious virus. Specific lysis on A/WSN-infected P815 targets are presented as means of 4 wells. Specific lysis on uninfected targets were often less than 10% and hence are omitted from the figure. Results for two effector/target ratios are shown here. (■▬▬■) 25:1; (\\\\\\\\) 50:1.

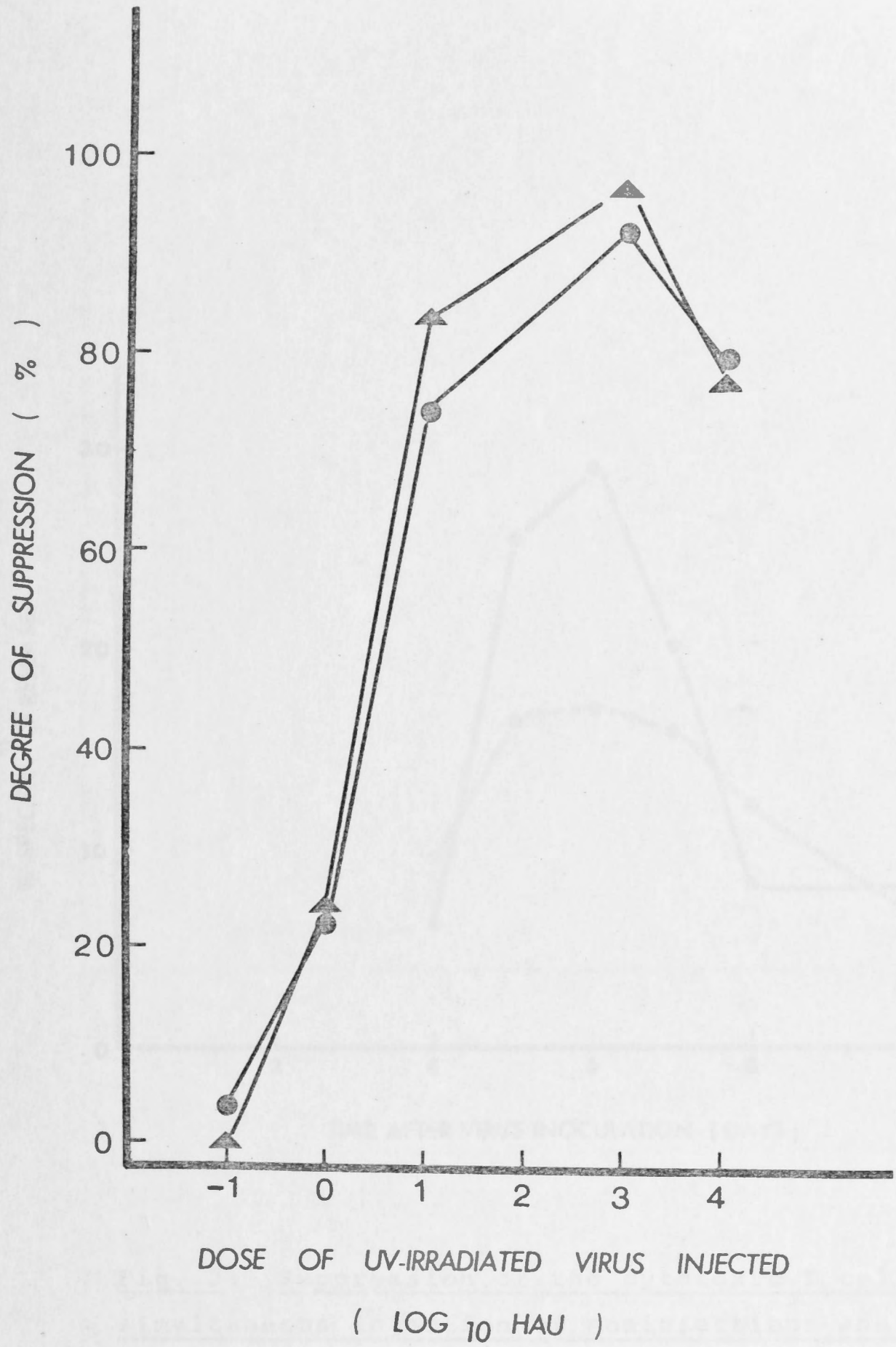


Fig. 2: Virus dose dependency on the suppression of cytotoxic T cell response to influenza virus. BALB/c mice were pre-injected i.v. with various doses of UV-inactivated A/WSN virus. Four days later all mice were given 10^7 EID_{50} infectious A/WSN virus i.v. and cytotoxic activity in their spleens were determined 6 days after the final injection. The degree of suppression was obtained by comparing the cytotoxic activity in spleens of mice which were pre-injected with UV-inactivated virus to mice which were given infectious virus only. Results for two effector/target ratios are shown here. (▲) 50:1; (●) 100:1.

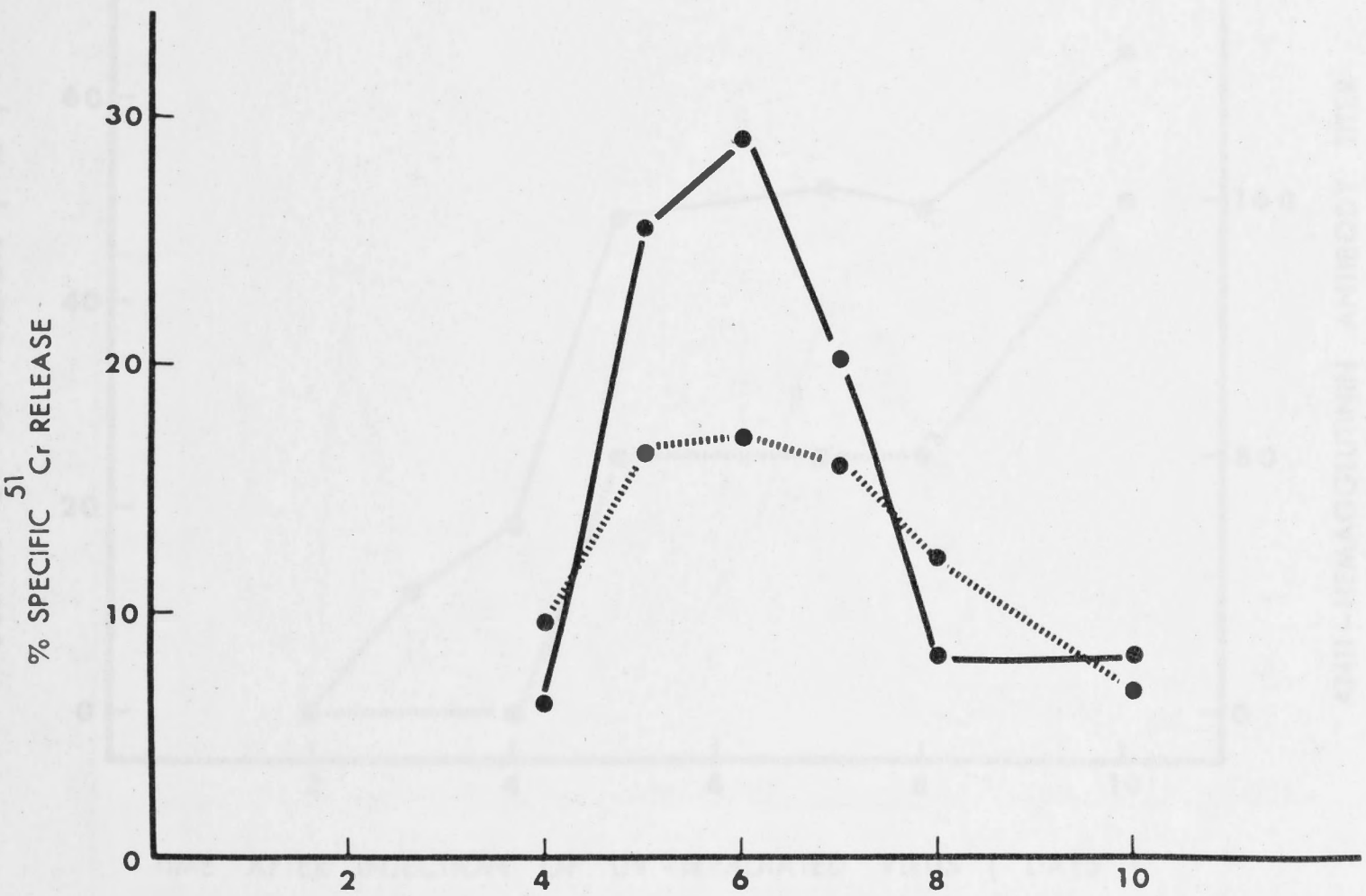


Fig. 3: Suppression of the cytotoxic T cell response by simultaneous injection of noninfectious and infectious influenza virus. BALB/c mice in groups of 3 were injected i.v. at different times either with 10^3 HAU infectious A/WSN virus only (▬▬) or with 10^3 HAU UV-inactivated A/WSN virus followed by 10^3 HAU infectious A/WSN virus (▮▮▮▮▮▮▮). Spleens from each group of mice were pooled and their cytotoxicity was determined in the same cytotoxic assay. Only results for effector/target ratio of 25:1 are shown here. Specific lysis on uninfected targets were negligible and are omitted from the figure.

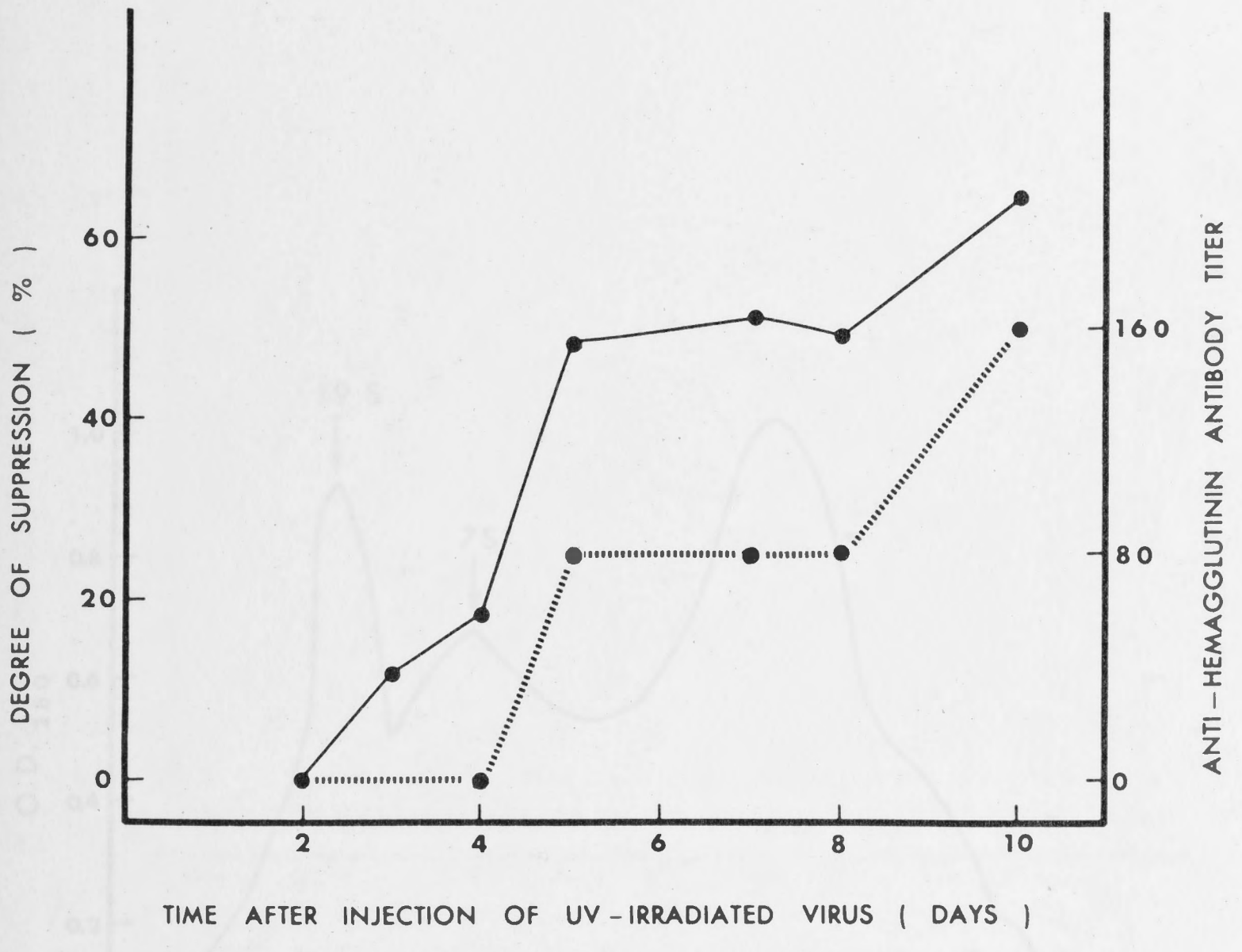


Fig. 4: Kinetics of induction of humoral suppressor factor(s) and production of anti-haemagglutinin antibodies in mice immunized with UV-inactivated influenza virus. BALB/c mice were primed with 10^3 HAU UV-inactivated A/WSN virus i.v. and sera were collected at different intervals after immunization. The anti-HA antibody titers (▮▮▮▮▮▮) were determined using standard procedures. 0.3 ml immune serum was also adoptively transferred into each naive syngeneic recipient which was then infected 24 h later with 10^7 EID_{50} A/WSN virus. Cytotoxic activity of the spleen cells was determined 6 days after virus injection and the degree of suppression (▬▬) was calculated by comparing the cytotoxic activity with that of the control mice which received normal mouse serum.

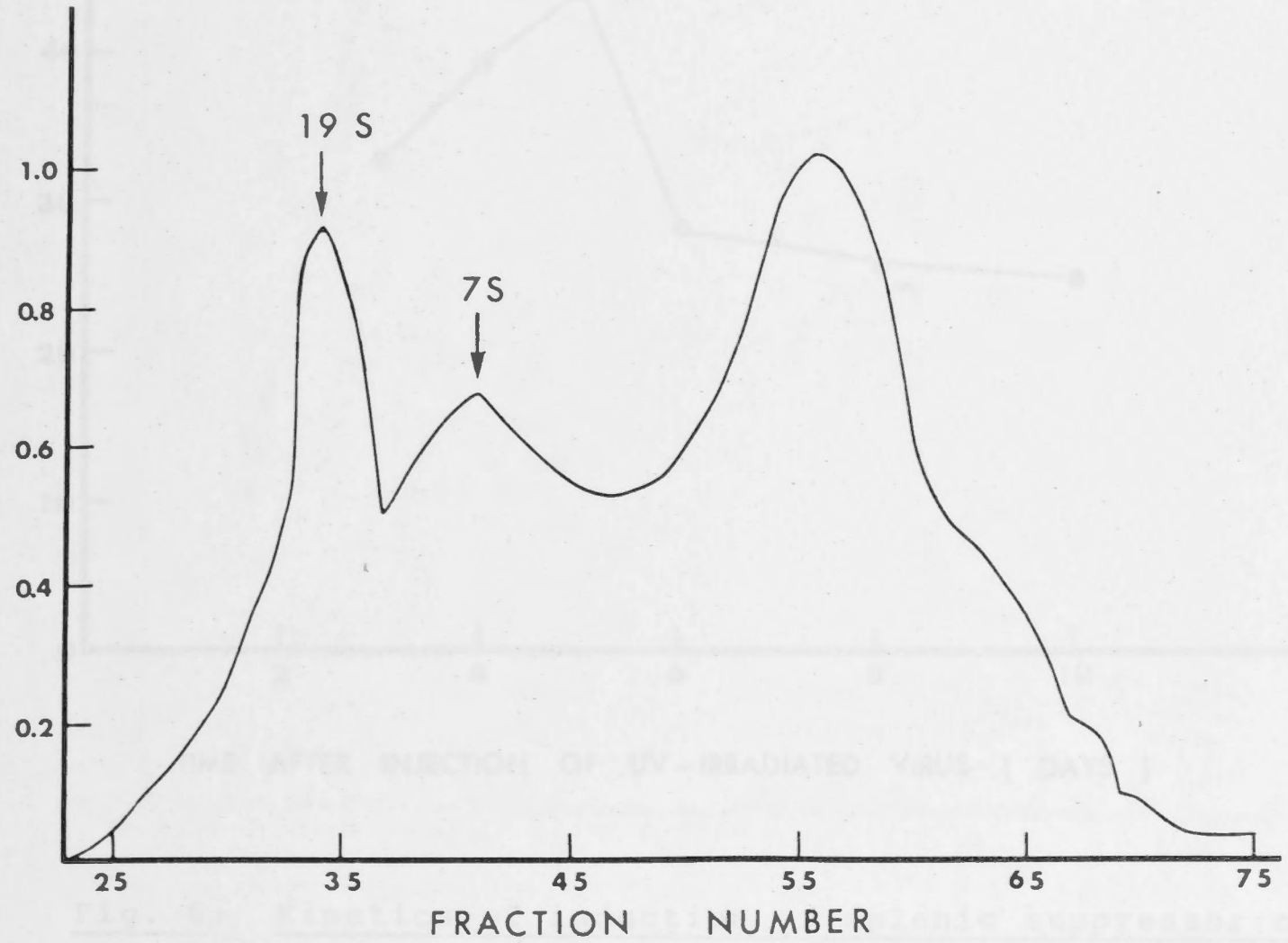


Fig. 5: Fractionation of immune mouse serum using Sephadex G-200 column by reverse chromatography. 2 ml of 6 days immune serum from BALB/c mice primed with 10^3 HAU UV-inactivated A/WSN virus was applied to a Sephadex G-200 column and the sample was eluted out with cold PBS at a flow rate of 10 ml per hour. Fractions of 5 ml were collected at 4^{o}C and optical density at 280 nm was determined by a UV spectrophotometer.

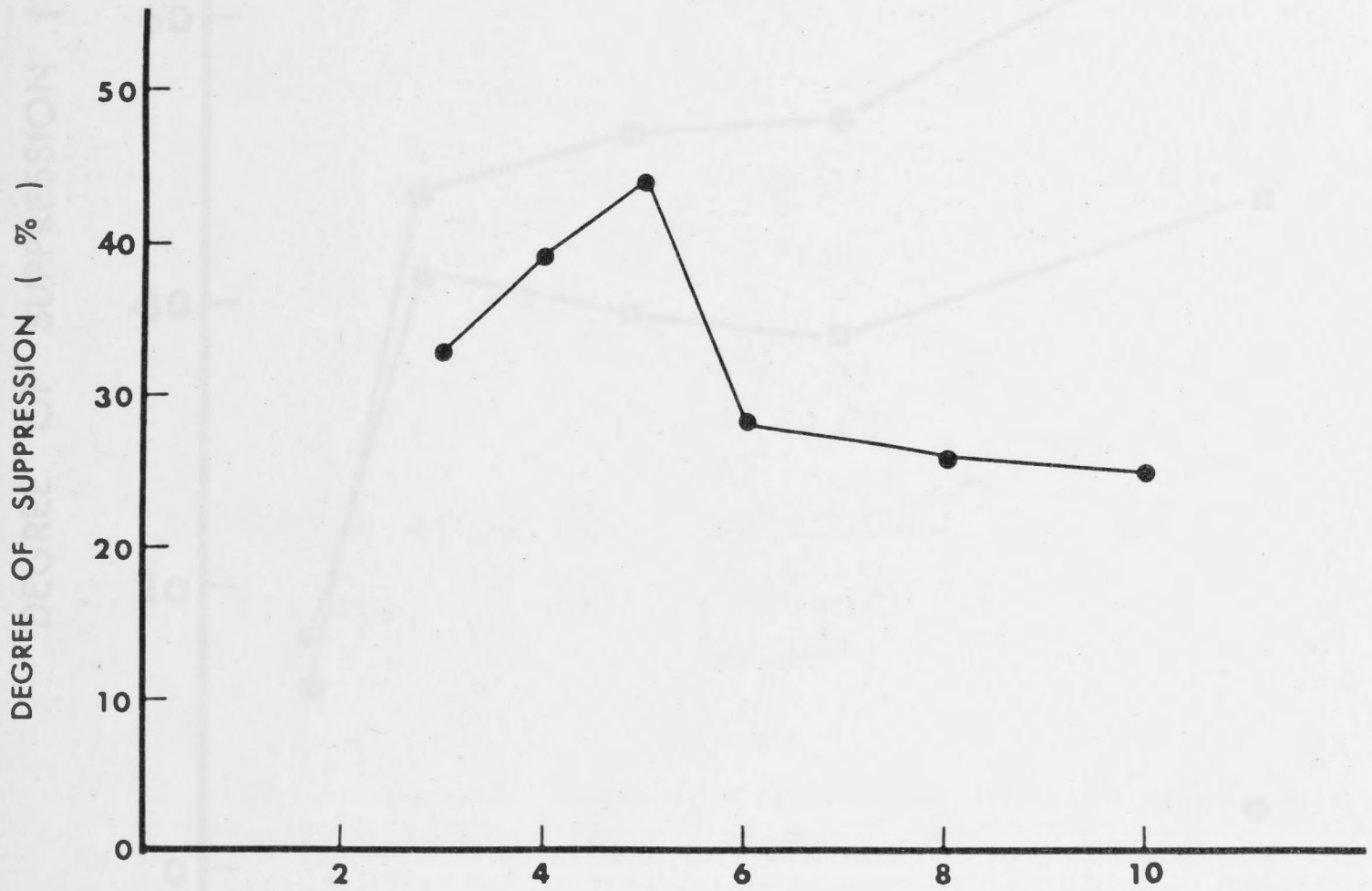


Fig. 6: Kinetics of induction of splenic suppressor cells. BALB/c mice were primed at different times with 10^3 HAU UV-inactivated A/WSN virus. Spleen cells were prepared and $1x10^8$ cells were adoptively transferred into each naive syngeneic recipient mouse which was then infected with $10^7 EID_{50}$ infectious A/WSN virus 24 h later. Cytotoxic activity of all groups was determined 6 days after virus injection. The degree of suppression was obtained by comparing the cytotoxic activity in spleens of mice which received immune spleen cells to mice which received same amount of normal spleen cells. Only results at the effector to target ratio of 25:1 are shown here.

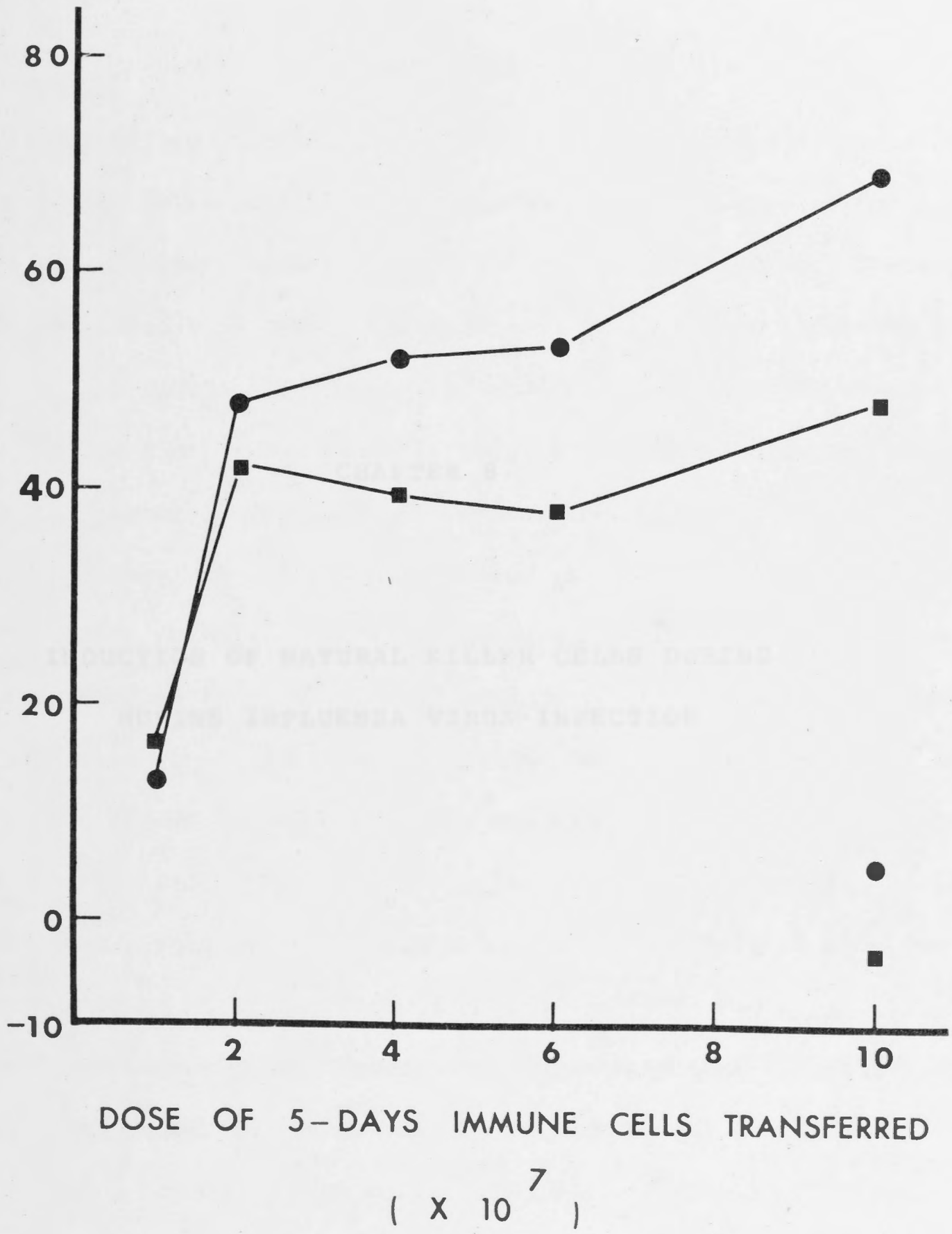


Fig. 7: Dose response curve of immune spleen cells on the suppression of cytotoxic T cell response to influenza virus. BALB/c mice were primed 5 days previously with 10^3 HAU UV-inactivated A/WSN virus and different doses of immune spleen cells were adoptively transferred into naive syngeneic recipients which were then infected with 10^7 EID_{50} A/WSN virus 24 h later. Cytotoxic activity of all groups was determined 6 days after virus injection. The degree of suppression was obtained by comparing the cytotoxic activity in spleens of mice which received immune cells to mice received no cells. Only results at two effector/target ratios are shown here. (●—●) 25:1; (■—■) 50:1. (● ■) mice which received $1x10^8$ normal spleen cells.

CHAPTER 8

INDUCTION OF NATURAL KILLER CELLS DURING MURINE INFLUENZA VIRUS INFECTION

INTRODUCTION

Natural killer (NK) cells cytotoxic for a wide range of tumor cells or transformed cells have been found in many species, such as the mouse, rat and chicken (Herberman & Holden, 1978; Fleischer, 1980). There is increasing interest in the possibility that NK cells are important in the defence of the host against virus-infected and malignant transformed cells (Welsh, 1978b; Kiessling & Wigzell, 1979; Santoli & Koprowski, 1979). Treatment of human lymphocytes with interferon or interferon inducers greatly increases nonspecific NK activity (Santoli et al.,1978; Trinchieri & Santoli, 1978). Similarly, a significant enhancement of NK activity has also been observed in mice infected with different viruses (Herberman et al., 1977; Macfarlan et al.,1977; Welsh, 1978a; Quinnan & Manischewitz, 1979). However, the extent to which NK cells are important in the resistance of the host to a viral infection is not clear. Previous work in this laboratory has studied the role of effector T cells in the recovery of mice from primary influenza virus infection (Yap & Ada, 1978b; Yap et al.,1978). However, experiments with athymic nude mice showed that effector T cell formation and action is not an obligatory requirement for the recovery of host following infection with a low dose (e.g. $10^3 EID_{50}$) of infectious virus, as both nude mice and their normal littermates survived this challenge equally well (Yap et al., 1979). Thus, other defence mechanisms such as the early production of interferons or IgM antibodies (Iwasaki & Nozima, 1977), the nonspecific activation of macrophages (Wyde & Cate, 1979; Mak, Leung & Ada, submitted for publication) and induction of NK cells may well be important in controlling virus replication and dissemination and therefore aid in the host's recovery whether functional T cells are present or not. This chapter describes the induction and properties of NK cells

following injection of mice with influenza virus. The results show that 1. after parenteral injection of mice with influenza virus, cytotoxic cells can be recovered from the spleens in the first few days after virus injection and these cells possess many basic characteristics of NK cells as have been described by others (Herberman & Holden, 1978; Kiessling & Wigzell, 1979) and 2. NK-like cells can be directly demonstrated in the target organ of virus replication, i.e., the infected mouse lungs.

RESULTS

Induction of cytotoxic cells after intravenous injection of influenza virus

CBA mice ($H\text{-}2^k$) were injected intravenously with infectious A/WSN virus (10^3 HAU) and spleen cells were harvested 2 days later. They were then tested for cytotoxicity in a short term (5 h) ^{51}Cr release assay using two histoincompatible targets, one known to be resistant to NK-mediated killing (P815, $H\text{-}2^d$), the other being susceptible (YAC-1, $H\text{-}2^a$) (Roder et al., 1979). As shown in Table 1, normal CBA spleen cells had little or no cytotoxicity on both infected and uninfected P815 targets but had significant killing on YAC-1 targets. The killing of both P815 and YAC-1 targets was greatly enhanced by preinjection of mice with influenza virus, and both infected and uninfected targets were killed equally well. Preinjection of normal allantoic fluid had no such effect.

Target cell sensitivity to influenza virus-induced early cytotoxic cells

A large number of cultured cell lines were tested for their sensitivity to lysis by influenza virus-induced 2 days cytotoxic cells. With the exception of YAC-1 cells, all the target cells tested were rather resistant to killing by normal CBA spleen cells (Table 2). However, injection of mice with influenza virus induced the formation of cells which were cytotoxic

for a wide range of target cells. Lysis was most efficient on T cell lymphomas and least efficient on fibroblasts (Table 2). Furthermore, the virus-induced cytotoxic cells killed both syngeneic (L929), allogeneic (P815, NS-1, EL-4, RBL-5 & YAC-1) and xenogeneic (HSB, CEM) targets. In most subsequent experiments, the more sensitive targets such as EL-4 and YAC-1 cells were used to study the properties and characteristics of the influenza virus-induced early cytotoxic cells.

Kinetics of induction of cytotoxic cells and lysis of target cells

CBA mice were injected i.v. at different time intervals with 10^3 HAU infectious A/WSN virus and their spleen cells were assayed for cytotoxicity using EL-4 target cells in a 5 h ^{51}Cr release assay. It was found that peak cytotoxic activity occurred 1-2 days after virus injection which dropped rapidly to low levels by day 4-5 (Fig. 1). The kinetics of target cell lysis were determined on two targets, EL-4 and YAC-1 cells. As seen in Fig. 2, specific ^{51}Cr release from EL-4 target cells was only detected after 5 h incubation with normal spleen cells but after 12 h incubation the specific ^{51}Cr release reached 35%. In contrast, rapid killing of EL-4 targets was observed in the first few hours of incubation using 2 days virus-induced effector cells so that a 40% specific ^{51}Cr release occurred after 5 h incubation. Once lysis was detected, the rate of release of ^{51}Cr using normal cells was similar to that using 2 days virus-induced immune cells as effector cells. The apparent difference in the action of immune and normal cells on EL-4 targets was much less obvious when YAC-1 cells were used as targets. In the first 4 h incubation, the release of ^{51}Cr using normal spleen cells as effector cells was ∿50% or less than that using immune spleen cells. After this period, the rates of release of ^{51}Cr using either source of effector cells were similar.

Characterization of splenic cytotoxic cells

The properties of virus-induced 2 days splenic cytotoxic cells were

established using standard procedures. Cytotoxicity was not diminished by anti-Thy 1.2 and complement treatment (Table 3, Expt. 1). Cytotoxic activity was enriched in the Ig-negative fraction of the spleen cells with little activity in the Ig-positive cell population (Table 3, Expt. 1). Treatment of effector cells with carbonyl iron powder or removal of plastic adherent cells only slightly diminished the cytotoxic activity (Table 3, Expt. 1). Similarly, in vitro preincubation of effector cells with silica or λ-carrageenan, agents that are known to be selectively cytotoxic for macrophages (Allison et al., 1966; Catanzaro et al., 1971), did not affect the level of cytotoxic activity (Table 3, Expt. 2 & 3). Therefore, the virus-induced cytotoxicity was mediated by cells which were neither T , B, phagocytic nor plastic adherent. These results indicate that cells displaying cytotoxic activity fall into the same cell population as has been described by many others for NK cells (Herberman & Holden, 1978; Kiessling & Wigzell, 1979; Welsh, 1978a; Macfarlan et al., 1977).

Influence of viral infectivity on the generation of NK activity

Viral infectivity is important in the activation of cytotoxic T cell response to influenza virus. Non-infectious virus generates cells with either no or negligible Tc activity (Braciale & Yap, 1978; Hosaka et al., 1978). The influence of viral infectivity on the activation of NK cells by influenza virus was examined. It was found that both infectious and UV-inactivated influenza virus were capable of inducing NK activity in spleen of mice after i.v. injection with a wide range of virus doses (100-5000 HAU) (Table 4). However, it should be noted that infectious virus was a better inducer of NK activity than non-infectious virus, especially when lower doses were used for immunization.

Preferential lysis of virus-infected but not uninfected target cells by NK cells on prolonged incubation

Normal CBA spleen cells or 2 days immune spleen cells from mice primed

i.v. with 10^3 HAU infectious A/WSN virus were assayed for cytotoxicity on either A/WSN virus-infected or uninfected target cells (P815, L929) for incubation periods of 5 h and 16 h. NK-sensitive targets such as YAC-1 cells were not used as they were poorly infected by influenza virus, as determined by the haemadsorption test (unpublished observation). The results in Table 5 showed that 1. both infected and uninfected target cells were not lysed by normal spleen cells in a 5 h assay whereas significant lysis of infected but not uninfected targets occurred after 16 h incubation; and 2, using day 2 immune spleen cells as effector cells, a significant difference between lysis of infected and uninfected targets occurred during the 16 h assay but not in a 5 h assay. These results suggest that either infected cells were more fragile than uninfected cells (and hence more easily lysed) and/or virus-infected cells somehow activate NK activity in vitro during prolonged incubation.

Induction of NK cells in different mouse strains

The ability of influenza virus to induce NK activity in various mouse strains was investigated. Firstly, normal BALB/c nude (nu/nu) have a higher level of NK activity than their normal littermates, a finding that is consistent with others (Herberman & Holden, 1978; Welsh, 1978b). Intravenous injection with influenza virus also caused an enhancement of splenic NK activity in nude mice (Table 6, Expt. 1). Secondly, C57BL/6 mice with the beige mutation which are known to be defective in NK functions while retaining normal macrophage and T cell functions (Roder & Duwe, 1979) did generate significant NK activity after i.v. injection with influenza virus, although lower levels were obtained when compared to their corresponding heterozygous littermates and their parental strain C57BL/6 (Table 6, Expt. 2). Finally, C3H/HeJ mice which have a known genetic defect in macrophage activation (Ruco et al., 1978) could also be induced to generate high level of NK activity (Table 6, Expt. 2).

Induction and characterization of cytotoxic cells in the lungs of mice infected with an influenza A virus

Following the demonstration of the induction of NK activity in the spleens of mice after i.v. injection with influenza virus, it is equally important to show whether such cells can be recovered from the target organ of virus replication, i.e., the mouse lungs. CBA mice were inoculated i.n. with a lethal dose ($5x10^4 EID_{50}$) of A/WSN virus and 2 days later, the lung cells and the peripheral blood lymphocytes were harvested and tested for NK activity on EL-4 targets. As shown in Table 7, high levels of cytotoxic activity can be detected in both lung cells and peripheral blood lymphocytes of immune but not in normal mice. The kinetics of induction of cytotoxic cells in the lungs were studied. CBA mice in groups of 4-6 were infected i.n. at different intervals with a lethal dose ($5x10^4 EID_{50}$) of A/WSN virus and lungs from each group were pooled and viable lung cells were tested for cytotoxicity on EL-4 targets in a 5 h ^{51}Cr release assay. It can be seen that low level of cytotoxic activity was detected in lungs of mice 24 h after virus inoculation, reaching peak activity at day 2 and then persisted until death (Fig. 3).

The effector cells in the lungs were found to be insensitive to anti-Thy 1.2 ascitic fluid and complement, and removal of adherent cells or treatment with silica or λ-carrageenan did not diminish their activity (Table 8). Therefore the cytotoxic cells recovered from the infected-lungs have the same characteristics of NK cells found in the spleens. As seen in Table 4, the dose of virus used to immunize mice i.v. has little influence on the level of NK activity recovered in the spleens 2 days after virus injection. In contrast, induction of NK cells in the lungs was virus-dose dependent, only higher doses that are lethal for mice could induce high levels of NK activity in the lungs 2 days after virus infection (Table 9).

DISCUSSION

This chapter shows that after adminstration of influenza virus to mice by various routes (intravenous or intranasal), cytotoxic cells are recovered from the target 'organs' (spleen or lungs) which lyse both uninfected and virus-infected target cells. The following reasons suggest that the effector cells belong to the class of cells termed natural killer (NK) cells as described by others (Herberman & Holden, 1978; Kiessling & Wigzell, 1979; Welsh, 1978b). 1. A similar but lower activity is present in uninfected animals (Table 1); 2. The cytotoxic activity is H-2 unrestricted, as both syngeneic, allogeneic and xenogeneic target cells are killed (Table 2); 3. The cells are Ig-negative, they are resistant to anti-Thy 1 antibody and complement treatment under conditions which are known to destroy effector T cells, such as cytotoxic T cells. They do not adhere to plastic surface and agents such as silica and λ-carrageenan which are selectively cytotoxic to phagocytic cells leave the cytotoxic activity unimpaired (Table 3). After i.v. injection of virus, the level of cytotoxic activity reaches a peak in the spleen at 1-2 days and then drops to a low level by day 4-5. These results obtained with influenza virus are similar in many aspects to other recently described NK activity in mice infected with Kunjin virus (Macfarlan et al., 1977), LCM virus (Welsh, 1978a) and cytomegalovirus (Quinnan & Manischewitz, 1979; Bancroft et al., 1981). Thus, the induction of NK activity appears to be a general phenomenon in the early cellular response to viral infections. In contrast to the requirement for infectious virus for the generation of cytotoxic T cells (Braciale & Yap, 1978; Hosaka et al., 1978), both infectious and non-infectious virus preparations induce the generation of NK activity. The apparent greater efficiency in induction of NK activity by low doses of infectious virus compared to non-infectious virus may simply reflect

a difference in the amount of viral antigens present at the site of induction in this circumstance.

In contrast to the kinetics of NK cell induction after i.v. injection of infectious virus, intranasal inoculation of mice with a lethal dose of virus results in the early appearance (1-2 days) of NK activity in the lungs and the level remains high until death of the mouse ensues (Fig. 3). This is presumably due to the continuous high level of virus present in the lungs up until the death of the mice (Yap & Ada, 1978a). As seen in Table 4, the induction of splenic NK activity is not virus dose dependent after i.v. adminstration over a wide range of infectious virus doses. By contrast, induction of NK activity in the lungs is dose dependent. Intranasal inoculation of a sublethal dose resulting in only a minimal increase in the level of NK activity in the lungs of the mice 2 days after virus infection. Substantially enhanced levels of NK activity occurred only when high doses of virus inoculum was used. Such large inocula resulted in the death of the animals.

The results in this chapter provide the background information on the activation of NK cells during murine influenza virus infection. There are many questions which must be answered in order to evaluate the role of such cells in the host resistance to a viral infection. The first is -- what is the relationship between the NK cells in the normal host compared to those seen in virus-infected animals? Are the former precursors of the latter? It has been reported that virus-induced NK cells may have different characteristics compared to 'endogenous' NK cells. Kiessling et al, (1980) found that LCMV-induced NK cells are more adherent than 'endogenous' NK cells to nylon wool columns and they also expressed more Fc receptors on their surface. Macfarlan et al.(1979), on the other hand, found that Kunjin virus-induced NK cells differed from 'endogenous' NK cells in target

cell specificity. In this chapter it was found that influenza virus-induced NK cells but not normal cells are cytotoxic for a wide range of continuous cell lines, results that are entirely consistent with Macfarlan et al.(1979). However, one of the puzzling features of the present findings is the kinetics of lysis of EL-4 and YAC-1 cells by normal versus 'immune' spleen cells. In both cases, the initial rates of target cell destruction are much higher using 'immune' cells than normal cells. Particularly with the EL-4 targets, 'immune' cells cause the immediate lysis of the target cells whereas there is a significant delay (∿5 h) before the target cells exposed to normal cells show specific release of ^{51}Cr. After this period, however, the rate of release of ^{51}Cr from both target cells appears to be similar. Clearly, further work is required to establish the nature of the killing process by the normal and the virus-induced cytotoxic cells.

The second question is -- do activated NK cells discriminate between infected and uninfected targets? A selective killing of virus-infected but not uninfected cells by 'NK-like' cells has been reported in many systems, both in man (Härfast et al., 1978; Ault & Weiner, 1979; Weston et al., 1980) and mouse (Minato et al., 1979). On the other hand, in this chapter it was found that influenza virus-induced NK cells, as with all other virus-induced NK cells (Macfarlan et al., 1977; Welsh, 1978a; Bancroft et al., 1981), can kill both infected and uninfected target cells. However, it was also shown that in longer period of assay (e.g. 16 h), there is greater lysis of infected versus uninfected targets, and this is particularly pronounced when normal effector cells are used. At present, it is not clear whether the apparent increase in killing of infected over uninfected cells is due to higher fragility of the infected cells or alternatively it is due to the in vitro activation of NK cells by the virus-infected cells during prolonged incubation, perhaps because of

interferon production. Whether the lysis of both infected and uninfected targets by virus-induced NK cells as observed *in vitro* has any relevance in the *in vivo* situation is unknown but it has been suggested that uninfected cells can be protected from NK-mediated lysis by interferon treatment *in vitro* (Trinchieri *et al.*, 1981). Perhaps interferons produced *in vivo* during viral infections may have a similar effect but this remains to be determined.

Finally, do the NK cells have a protective role during a viral infection? There are suggestions that they may do so (Santoli & Koprowski, 1979; Bloom *et al.*, 1980) and there is no evidence presented in this chapter which argues against this. However, the results in Table 9 suggest that when a lethal dose of virus is administered, the action of the NK cells might contribute to the pathological lesions. Substantial evidence for the *in vivo* role of NK cells against tumor growth has been accumulated in recent years (Welsh, 1978b; Kasai *et al.*, 1979; Kiessling & Wigzell, 1979; Ojo, 1979; Kärre *et al.*, 1980), yet direct evidence that NK cells do play a protective role in viral infections is still lacking. Beige mice which are known to be defective in NK cell functions (Roder & Duwe, 1979), do have low but still significant levels of NK activity when infected with viruses (Table 6; Welsh & Kiessling, 1980; Bancroft *et al.*, 1981). Nevertheless, on the basis of the observation that beige mice with low NK activity and control mice with high NK activity produce similar levels of virus and interferon in the spleen, Welsh & Kiessling (1980) suggested that NK cells may not play a significant role in control of early viral synthesis during early stage of acute LCM virus infection. In contrast, Bancroft *et al.* (1981) found that beige mice with low level of NK activity during MCMV infection often have greater susceptibility to MCMV infection than their normal littermates. So far the question whether NK cells play a definitive role in host resistance to viral

infections has not been resolved. Perhaps this question can be examined more closely by using adoptive transfer of a cloned line of NK cells to see what effect this has on the outcome of a viral infection. Experiments are in progress to determine this using the influenza virus model.

SUMMARY

Cytotoxic cells can be recovered in the spleens or lungs of mice 2 days after intravenous or intranasal inoculation with infectious influenza A virus. Both uninfected or virus-infected target cells are lysed equally well in a 5 h short term ^{51}Cr release assay. The action of the cytotoxic cells is found to be H-2 unrestricted, as a variety of syngeneic, allogeneic or xenogeneic tumor cells or transformed cell lines are lysed. The virus-induced cytotoxicity is mediated by cells which are neither T, B, phagocytic nor plastic adherent, indicating that the cells displaying cytotoxic activity fall into the same cell population as has been described by many others for natural killer (NK) cells. Both infectious and UV-inactivated virus preparations are capable of inducing NK activity in the spleens of mice although the former is generally found to be very effective, even at low doses, whereas the latter requires higher doses in order to induce significant levels of splenic NK activity. After intravenous injection of influenza virus, peak NK activity is found in the spleens 2 days after virus injection and the activity rapidly drops to low levels by day 4-5. In contrast, intranasal inoculation of mice with a lethal dose of virus results in the early appearance (2-3 days) of NK activity in the lungs and the level remains high until the death of the mice ensues.

TABLE 1

Induction of Early Cytotoxic Cells during Murine Influenza Virus Infection[a]

Immunization of CBA mice	Effector cells	% Specific ^{51}Cr release (E/T=50:1)			
		P815		YAC-1	
		Uninfected	Infected	Uninfected	Infected
Nil	Normal spleen cells	1.1 ± 0.7[b]	0.1 ± 0.6	24.6 ± 1.2	23.9 ± 2.7
Normal allantoic fluid	Day 2 spleen cells	-0.2 ± 0.3	0.2 ± 0.2	15.0 ± 0.0	20.8 ± 2.1
Infectious allantoic fluid	Day 2 immune spleen cells	12.6 ± 1.5	11.8 ± 0.6	68.5 ± 1.9	68.3 ± 0.4

a Groups of 3 CBA mice were either unprimed or injected i.v. with 0.3 ml normal or infectious (10^3 HAU A/WSN virus) allantoic fluid. Two days later, spleen from each group were pooled and tested for cytotoxicity on both uninfected and A/WSN virus-infected P815 and YAC-1 targets in a short term (5h) ^{51}Cr release assay.

b Mean ± standard error for replicates of four wells.

TABLE 2

A Comparison of Target Cell Sensitivity to Early Cytotoxic Cells Induced by Influenza Virus Infection

Experiment No.	Target cell used	Strain of origin	Characteristics	% Specific ^{51}Cr release[a]			
				Normal spleen cells		Day 2 immune spleen cells	
				Effector to target ratios			
				50:1	100:1	50:1	100:1
1	L929	C3H (H-2^k)	Continuous fibroblasts	-1.6 ± o.1	-0.8 ± 0.3	10.6 ± 0.8	19.3 ± 0.5
	P815	DBA/2 (H-2^d)	Mastocytoma	0.3 ± 0.6	0.7 ± 0.7	16.5 ± 1.2	29.5 ± 0.9
	NS-1	BALB/c (H-2^d)	Myeloma	1.8 ± 1.0	2.6 ± 0.5	26.7 ± 0.7	44.0 ± 0.4
	EL-4	C57BL/6J (H-2^b)	T lymphoma	0.9 ± 0.4	1.4 ± 0.4	29.0 ± 0.3	48.9 ± 0.8
	RBL-5	C57BL/6J (H-2^b)	T lymphoma	-2.7 ± 0.4	-3.9 ± 1.0	30.4 ± 1.0	53.4 ± 1.2
	YAC-1	A/Sn (H-2^a)	T lymphoma	15.0 ± 1.1	21.8 ± 1.0	55.4 ± 0.7	71.0 ± 0.4
2	EL-4	C57BL/6J	T lymphoma	4.2 ± 0.6	8.8 ± 0.8	30.2 ± 1.4	45.4 ± 1.5
	HSB2	Human	T lymphoma	6.4 ± 1.1	8.8 ± 1.4	20.5 ± 2.6	34.5 ± 2.7
	CEM	Human	T lymphoma	1.3 ± 0.5	6.0 ± 0.5	19.4 ± 1.4	28.6 ± 1.2

a Groups of 3 CBA mice were either unprimed or injected i.v. with 10^3 HAU infectious A/WSN virus. Two days later, spleen from each group were pooled and tested for cytotoxicity on various uninfected targets in a 5 h ^{51}Cr release assay.

TABLE 3

Characterization of Cytotoxic Cells in Spleens of Mice 2 Days after Intravenous Injection of Influenza Virus

Experiment No.	Effector cells[a]	% Specific ^{51}Cr release[b] (E/T=50:1) EL-4	YAC-1
1	Untreated cells	25.3 ± 0.1	52.5 ± 1.3
	Ig-positive cells	6.3 ± 0.6	9.7 ± 0.0
	Ig-negative cells	52.5 ± 2.5	67.7 ± 2.5
	Carbonyl-powder treated cells	18.1 ± 1.2	48.5 ± 2.2
	Plastic nonadherent cells	14.5 ± 0.5	46.1 ± 1.3
	Complement treated cells	22.3 ± 0.5	49.8 ± 0.1
	Anti-Thy1.2 + C' treated cells	31.1 ± 1.5	56.2 ± 1.8
2	Untreated cells	23.0 ± 0.4	45.4 ± 2.6
	Silica (125 ug/ml) treated cells[c]	21.4 ± 0.4	46.4 ± 2.2
	Silica (250 ug/ml) treated cells[c]	23.9 ± 0.1	43.8 ± 1.2
3	Untreated cells	27.9 ± 0.5	45.3 ± 4.0
	λ-carrageenan (300 ug/ml) treated cells[c]	27.9 ± 0.9	43.8 ± 1.0
	λ-carrageenan (600 ug/ml) treated cells[c]	31.8 ± 4.0	48.3 ± 1.4

Legends for Table 3

a Effector cells were 2 days immune spleen cells from CBA mice primed with 10^3 HAU A/WSN virus i.v. The procedures for the various treatments of the effector cells were described in details in Materials and Methods (Chapter 2).

b A 5 h ^{51}Cr release assay on unifected target cells was used.

c Effector cells (in 0.1 ml volume) were preincubated for 4 h at 37°C in the absence or presence of silica or λ-carrageenan before addition of target cells (0.1 ml). The mixture was further incubated for another 5 h before harvesting supernatant for assay of released ^{51}Cr. The presence of silica or λ-carrageenan in the assay wells had no significant effect on the spontaneous lysis of the target cells.

TABLE 4

Influence of Viral Infectivity in the Induction of NK Cells

Virus dose used for immunization (HAU)[a]	Infectivity[b]	% Specific ^{51}Cr release[c] (E:T=50:1)	
		EL-4	YAC-1
Nil	-	5.1 ± 0.3	37.4 ± 0.2
10^2	I	41.9 ± 1.0	65.5 ± 2.2
	NI	13.6 ± 1.3	46.4 ± 0.6
10^3	I	40.6 ± 3.0	66.3 ± 1.9
	NI	22.1 ± 0.3	58.2 ± 0.3
$5x10^3$	I	50.9 ± 1.7	73.0 ± 0.9
	NI	30.1 ± 1.1	61.3 ± 1.9

a A/WSN virus was injected i.v. into CBA mice. 2 days immune spleen cells were used as effector cells.

b I : Infectious NI : Non-infectious

c A 5 h ^{51}Cr release assay on uninfected targets.

TABLE 5

Preferential Lysis of Virus-Infected Cells by NK Cells on Prolonged Incubation

Effector cells[a]	Time of incubation[b]	% Specific ^{51}Cr release							
		P815				L929			
		Uninfected		Infected[c]		Uninfected		Infected[c]	
		50:1	100:1	50:1	100:1	50:1	100:1	50:1	100:1
NSC	5	1.7 ± 0.5	2.5 ± 0.6	3.4 ± 0.7	6.0 ± 0.3	-0.1 ± 0.4	1.0 ± 0.3	3.8 ± 0.8	7.1 ± 2.0
ISC	5	21.7 ± 0.4	27.1 ± 0.2	25.9 ± 2.2	32.0 ± 1.2	15.8 ± 0.4	19.5 ± 0.5	17.3 ± 0.7	25.0 ± 1.9
NSC	16	6.2 ± 1.2	5.1 ± 0.9	48.1 ± 1.5	49.3 ± 0.6	5.9 ± 0.1	4.7 ± 0.5	37.5 ± 1.7	43.3 ± 1.0
ISC	16	32.5 ± 0.8	33.9 ± 1.0	38.8 ± 1.0	51.1 ± 2.2	22.3 ± 0.5	30.3 ± 0.9	39.9 ± 0.5	47.9 ± 1.0

a NSC : Normal CBA spleen cells; ISC : 2 days immune spleen cells from CBA mice primed with 10^3 HAU infectious influenza A/WSN virus.

b Effector and target cells were incubated together for 5 or 16 h before harvesting supernatants for assay of released ^{51}Cr.

c A/WSN virus-infected targets (15-20 EID_{50}/cell, 1 h at 37°C).

TABLE 6

Induction of NK Cells in Different Mouse Strains during Murine Influenza Virus Infection

Mouse strain used	Effector cells[a]	% Specific ^{51}Cr release[b] (E/T=50:1) EL-4	YAC-1
Experiment 1			
BALB/c	NSC	2.5 ± 0.9	16.2 ± 0.4
	D_2ISC	26.2 ± 0.9	50.5 ± 0.5
BALB/c nude (nu/nu)	NSC	24.7 ± 0.3	44.9 ± 3.3
	D_2ISC	33.9 ± 0.6	59.0 ± 1.0
Experiment 2			
C57BL/6J	NSC	0.4 ± 0.3	27.1 ± 1.3
	D_2ISC	10.4 ± 0.6	38.5 ± 2.5
Beige heterozygous (bg/+)	NSC	-0.3 ± 0.5	12.3 ± 0.8
	D_2ISC	9.1 ± 1.3	34.3 ± 0.8
Beige homozygous (bg/bg)	NSC	0.5 ± 0.9	2.2 ± 1.4
	D_2ISC	4.6 ± 1.5	20.5 ± 0.7
C3H/HeJ	NSC	1.8 ± 0.3	25.1 ± 0.3
	D_2ISC	42.9 ± 2.2	71.7 ± 1.9

a Different mouse strains were unprimed or injected i.v. with 10^3HAU infectious A/WSN virus. 2 days later, the spleen cells were used as a source of effector cells.

b Cytotoxicity in a 5 h ^{51}Cr release assay on uninfected targets.

TABLE 7

Induction of Cytotoxic Cells in Mouse Lungs and Peripheral Blood

Effector cells[a]	Effector/target ratios[b]	% Specific ^{51}Cr release from EL-4 target
Normal lung cells	50:1	5.7 ± 0.9
	100:1	11.0 ± 0.8
Immune lung cells	50:1	40.0 ± 1.7
	100:1	54.9 ± 2.1
Normal peripheral blood leucocytes	50:1	5.7 ± 1.5
	100:1	15.4 ± 0.4
Immune peripheral blood leucocytes	50:1	48.7 ± 2.0
	100:1	66.5 ± 3.5

a Immune cells were obtained from CBA mice 2 days after inoculation of $5x10^4$ EID_{50} A/WSN virus.

b $5x10^3$ target cells were added to each well.

TABLE 8

Characterization of Cytotoxic Cells in the Lungs of Mice 2 Days after Intranasal Inoculation of Influenza Virus[a]

Experiment No.	Treatment of effector cells	% Specific ^{51}Cr release			
		EL-4		YAC-1	
		25:1	100:1	25:1	100:1
1	Nil	22.8 ± 1.0	N.D.	47.4 ± 0.2	N.D.
	C' alone	23.4 ± 0.8	N.D.	47.9 ± 3.3	N.D.
	Anti-Thy 1.2 + C'	24.7 ± 2.5	N.D.	57.7 ± 3.5	N.D
	Removal of plastic adherent cells (37°C, 2 h)	26.9 ± 0.7	N.D.	55.0 ± 2.0	N.D.
2	Nil	N.D.	33.4 ± 0.6	N.D.	56.9 ± 1.2
	λ-carrageenan (600 ug/ml)	N.D.	30.1 ± 1.3	N.D.	57.9 ± 0.7
	Silica (125 ug/ml)	N.D.	32.4 ± 1.1	N.D.	60.8 ± 0.5
	Silica (250 ug/ml)	N.D.	29.4 ± 0.4	N.D.	55.7 ± 1.3

a CBA mice were infected i.n. with $5x10^4$ EID_{50} A/WSN virus and 2 days later, lung cells were used as effector cells in a 5 h ^{51}Cr release assay on uninfected targets ($5x10^3$/well).

TABLE 9

Virus Dose Dependence on the Induction of NK Cells in the Lungs of Mice 2 Days after Intranasal Inoculation of Influenza Virus[a]

Dose of A/WSN virus inoculated i.n. (EID_{50})	Lethal for mice	% Specific ^{51}Cr release EL-4	
		45:1	90:1
Nil	No	5.9 ± 0.1	7.3 ± 0.6
50	No	7.4 ± 0.6	8.2 ± 0.4
500	No	8.3 ± 0.1	13.0 ± 1.8
5000	Yes	22.1 ± 0.2	29.3 ± 0.1
50000	Yes	32.1 ± 1.1	39.1 ± 0.7

a Groups of 4-8 CBA mice were infected i.n. with various doses of influenza virus and 2 days later, the lungs from each group were pooled and used as a source of effector cells. Cytotoxicity was determined in a 5 h ^{51}Cr release assay using uninfected EL-4 targets ($5x10^3$ cells/well).

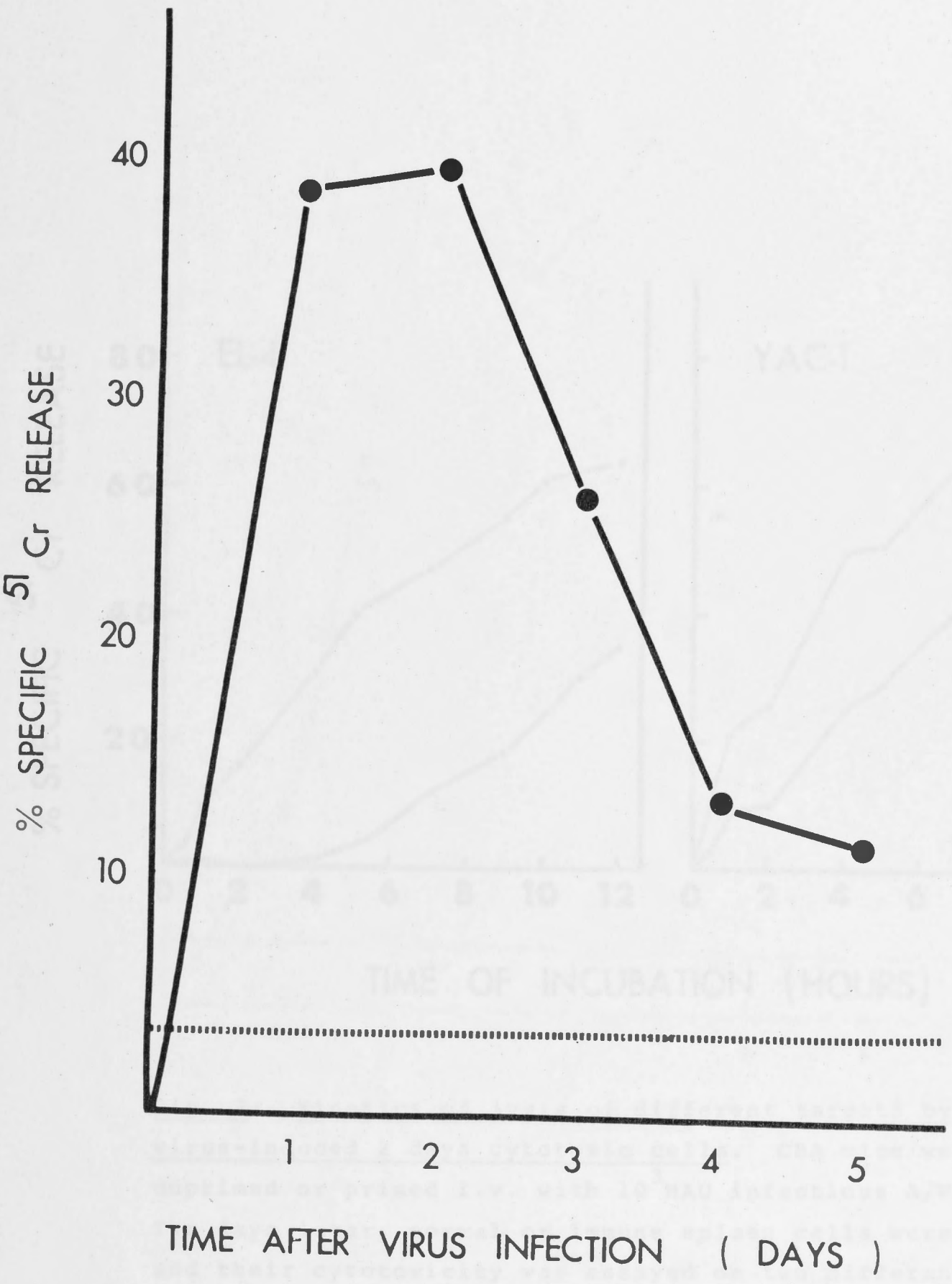


Fig. 1: Kinetics of induction of splenic cytotoxic cells after intravenous injection of mice with influenza virus. CBA mice in groups of 3 were injected i.v. at different days with the same dose (10^3HAU) of A/WSN virus. Spleens from each group of mice were pooled and tested for cytotoxicity on the same day using uninfected EL-4 cells as targets in a 5 h ^{51}Cr release assay. (▪▪▪▪▪▪▪▪▪) Cytotoxic activity of normal spleen cells.

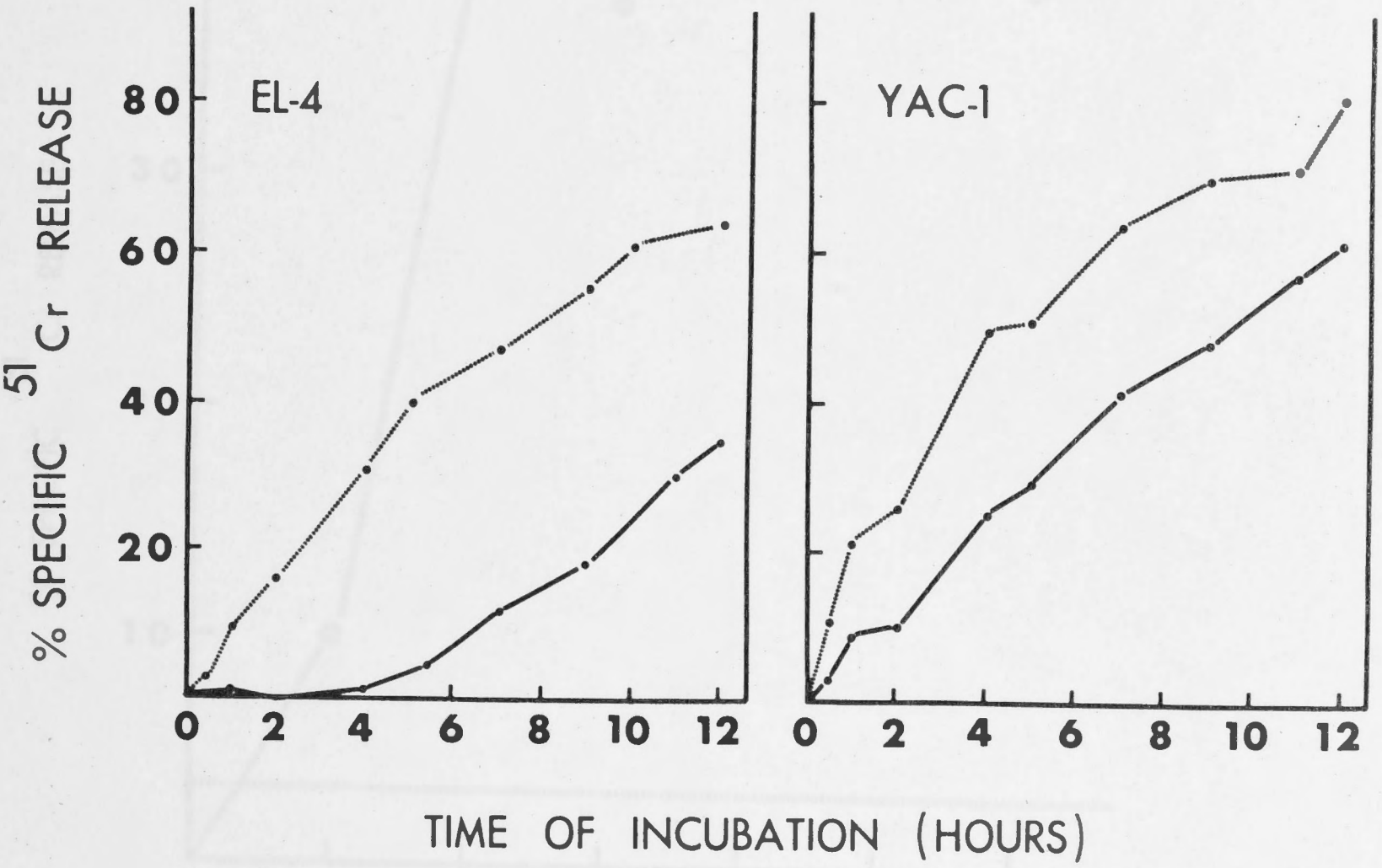


Fig. 2: Kinetics of lysis of different targets by influenza virus-induced 2 days cytotoxic cells. CBA mice were either unprimed or primed i.v. with 10^3 HAU infectious A/WSN virus. Two days later, normal or immune spleen cells were prepared and their cytotoxicity was assayed on two different targets, EL-4 and YAC-1 cells. The kinetics of lysis of each target were determined for the first 12 h after addition of target cells to normal spleen cells (▬▬) or immune spleen cells (▪▪▪▪▪▪▪▪).

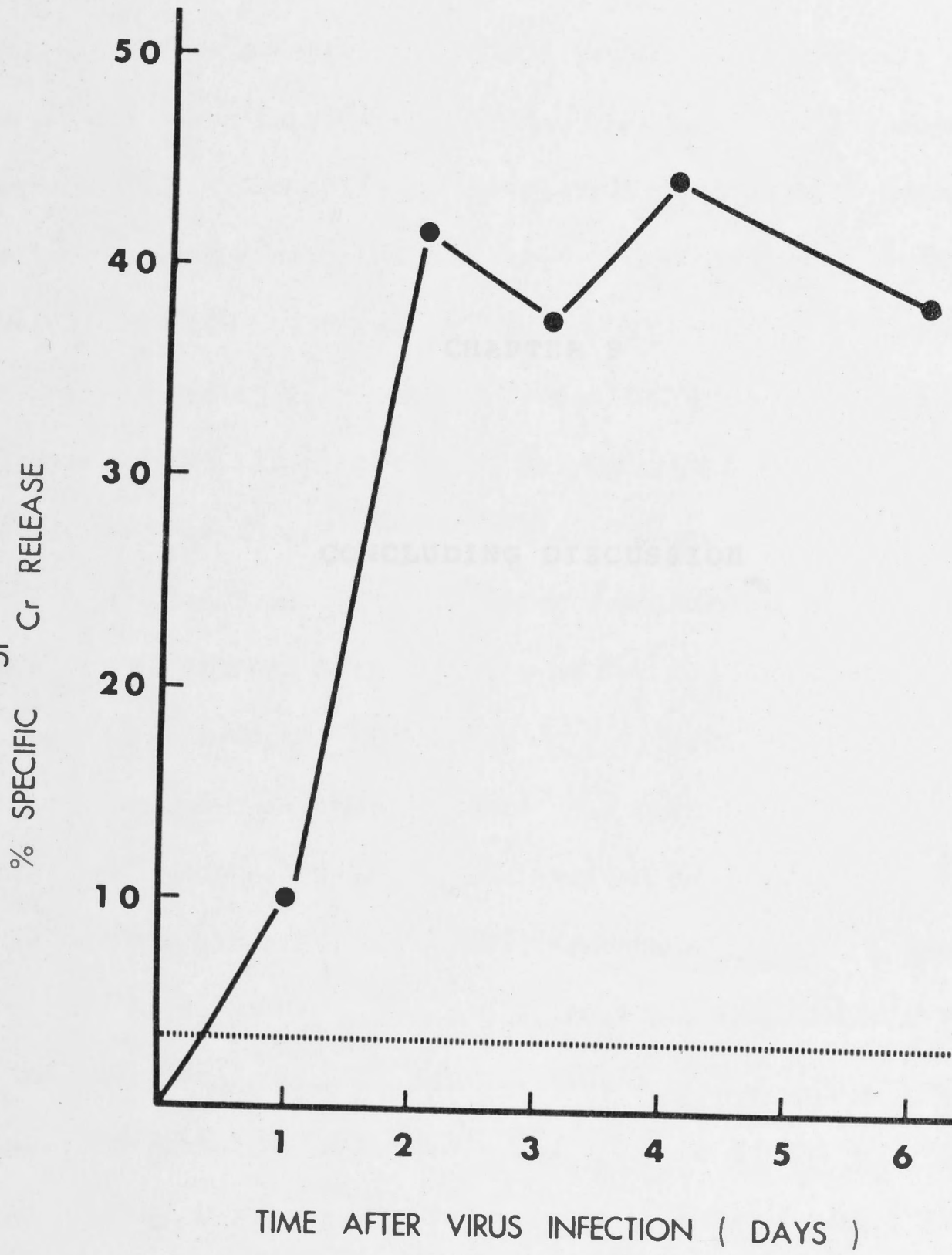


Fig. 3: Kinetics of induction of cytotoxic cells in the lungs of mice after intranasal inoculation with a lethal dose of influenza virus. CBA mice in groups of 4-6 were inoculated i.n. at different days with a lethal dose ($5x10^4 EID_{50}$) of A/WSN virus. Lung cells from each group of mice were pooled and their cytotoxicity was determined on the same day using uninfected EL-4 cells as targets in a 5 h ^{51}Cr release assay. (▪▪▪▪▪▪▪▪▪) Cytotoxic activity of normal lung cells.

CHAPTER 9

CONCLUDING DISCUSSION

The activation and effector function of T cells are governed by a dual specificity of recognition system involving on one hand, a foreign antigen such as a hapten, a soluble protein molecule or a viral antigen; and on the other hand, a self antigen coded for by the major histocompatibility complex (MHC). According to the H-2 region involved, mouse T lymphocytes can be subdivided into two subsets - T cells which recognize antigen in association with the I region MHC products and T cells which recognize antigen associated with the K,D region MHC products. The H-2 restriction pattern and the Lyt phenotype of T lymphocytes are closely linked: I region restricted T cells are Lyt $1^{+}2^{-}3^{-}$; K,D region restricted T cells have the Lyt $1^{-}2^{+}3^{+}$ phenotype. In the latter case, it may be that the observed correlation reflects participation of Lyt 2,3 antigens in the structure of the T cell receptor (Hollander et al., 1980; Sarmiento et al., 1980).

T cells of both these subsets take part in a variety of functions. It has been proposed that T cells evolved as part of the defence mechanisms to infectious diseases, and viral infections may have played a particularly important role. Thus, infection of cells by many different viruses results in the early expression of viral antigens at the surface of infected cells. These cells could be destroyed by an effector T cell long before infectious viral progeny is produced which could overwhelm the host (Jackson et al., 1976; Koszinowski & Ertl, 1976; Zinkernagel & Althage, 1977). Thus for these reasons, viruses are a very suitable probe to investigate T cell activation and effector function, to analyze the important role of viral infectivity in these events, and to determine the function of different T cell subsets during a viral infection. Influenza virus was chosen as a model for the present study as the molecular biology of the virus is well understood. The virus has been characterized both biochemically and antigenically and fewer viral antigens are known to be expressed in the plasma membrane of the infected cells than many other viruses. The availability of many different strains of the virus with serologically

defined antigenic relationship enables one to examine more closely the fine specificity of the immune responses of the host directed against the virus.

This concluding chapter will first discuss the recent advances (within the past three years) in the understanding of the DTH response in viral infections as well as the influence of the MHC on the adoptive transfer of DTH. Subsequently the use of influenza virus as a tool for analysis of T cell activation and function based on the contributions from the work reported in this thesis together with earlier results from this and other laboratories are discussed. Finally, the relevance of this work to prophylaxis against human influenza will be highlighted.

Is DTH response a general phenomenon in viral infections?

As mentioned previously in the introductory chapter, when the work on this thesis started, there were few systematic studies on the induction of a DTH response to viruses in mice. Viruses previously examined include ectromelia (Poxvirus) (Fenner, 1948), influenza and mumps (Myxoviruses) (Feinstone et al., 1969), vaccinia (Poxvirus) (Ueda & Nozima, 1969), LCM (Arenavirus) (Tosolini & Mims, 1971) and MLV (Oncornavirus) (Peters et al., 1975). In most cases, the nature and characteristics of the effector cells involved in the DTH reaction and their role in host resistance against viral infections have not been established. Since the work reported in this thesis was undertaken, and within a short period of time, a number of reports on the induction of a DTH response using many other viruses have been published. Thus, DTH response was found to occur in mice in response to rabies virus (Rhabdovirus) (Lagrange et al., 1978), St. Louis Encephalitis virus (Togavirus) (Hudson et al., 1979), Semliki forest virus (Togavirus) (Kraaijeveld et al., 1979), reovirus (Weiner et al., 1980), herpes simplex type 1 (Herpesvirus) (Nash et al., 1980),

adenovirus (Inada & Uetake, 1978) and Sendai virus (Paramyxovirus) (Ertl,1981).

Although the phenomenon of DTH reaction to a virus had been known since Jenner's discovery of the 'reaction of immunity' to vaccinia virus in revaccinated subjects in 1798, the progress in this field is relatively slow, in contrast to the rapid development in our knowledge of the cytotoxic T cell response in viral infections. The presence of virus-specific Tc cells in mice was first demonstrated with LCM virus (Cole et al., 1973; Doherty et al., 1974), ectromelia virus (Gardner et al., 1974) and Sindbis virus (Mcfarland, 1974). Within the next few years, at least 20 different viruses were reported to induce cytotoxic T cell response in mice (Zinkernagel & Doherty, 1979). The nature of the cytotoxic response and the characteristics of the effector cells involved have been established by extensive studies both in vivo and in vitro. Moreover, substantial evidence has accumulated which suggests that Tc cells have a protective role in a number of viral infections (see Chapter 1). In contrast, little is known about the characteristics of the cells mediating the DTH response and the role these cells may play in host defence against viral infections. One of the reasons which may account for the relative paucity of our knowledge about Td cells compared to Tc cells is the difference in the sensitivity and accuracy of the methods employed to study these responses. Cytotoxic T cell activity is usually measured by the amount of radioisotope ^{51}Cr released from the infected target cells after interaction with the effector cells. The ^{51}Cr release assay is simple to perform, accurate, objective, reliable and requires only a small number of effector cells. By contrast, the study of DTH response is often hampered by the insensitivity of the assay systems used. Currently the most popular method for measuring DTH response in mice is the relative increase in thickness of the footpad or the ear after antigen challenge. These methods are less sensitive and often require

large number of cells in order to demonstrate significant activity. Moreover, a linear quantitative response is difficult to obtain (Chapters 3, 4 & 5). Although radioisotopic methods based on the localization of H^3- or I^{125}-labelled cells into the sites of antigen injection have been adopted for measurement of the DTH response to viruses in mice, the values obtained are often low and to some extent are unsatisfactory (Chapter 3.1; Hudson et al., 1979). Also these methods suffered from a number of drawbacks which have been described in details in chapter 1. Nevertheless, using admittedly cruder procedures, DTH responses have been demonstrated in mice infected or immunized by a diverse group of viruses. These results strongly suggest that, like the cytotoxic T cell response, induction of DTH response in mice is a general phenomenon during viral infections. In many cases, however, the kinetics of induction of Tc and Td cells are very much similar so that analysis of the role played by these T cell subsets during a viral infection is difficult. One obvious approach is to deplete a subset of cells expressing a particular activity. This can be achieved by selective lysis of cells using anti-Lyt antibodies and complement. (Yap et al., 1978). Another approach is to make use of the differences in the inductive requirements of these T cell subsets. Thus, in the influenza virus model, injection of UV-inactivated virus generated I region restricted Td activity but failed to generate significant levels of cytotoxic activity (Chapter 3.1; Braciale & Yap, 1978). On the other hand, restimulation of memory spleen cells from mice primed with infectious virus with virus-infected stimulators that lack Ia antigenic determinants (e.g. fibroblasts or P815 mastocytoma cells) preferentially led to the generation of K,D region restricted Tc (and Td) activity but little, if any, I region restricted Td activity (Chapters 5.2, 6). In this way, the role played by each T cell subset can be analyzed in a more precise way. This subject will be explored in greater detail in the subsequent sections.

What is the influence of the MHC on the adoptive transfer of DTH in mice?

The discovery of the 'H-2 restriction' phenomenon for cytotoxic T cell response (Zinkernagel & Doherty, 1974a) had led Miller and his colleagues (Miller et al., 1975) to investigate whether similar restriction would be imposed on the DTH response in mice. It was found that in order to achieve a successful transfer of DTH, there is always a requirement for H-2 compatibility between donors of sensitized T cells and naive recipients. In case of soluble proteins (e.g. fowl gammaglobulin, keyhole limpet haemocyanin) and synthetic polypeptides (GAT), adoptive transfer of DTH was IA subregion restricted whereas with contact chemicals such as dinitrofluorobenzene (DNFB) identity at the K,D or I region was sufficient (Vadas et al., 1977, Miller, 1978a, 1978b). Such a restriction was thought to reflect the requirement for H-2 sharing between sensitized Td cells and antigen presenting cells, such as macrophages and epidermal cells. Direct evidence for an in vivo antigen presenting function of macrophages in DTH response was provided by Miller et al.(1979) who showed that antigen-pulsed macrophages can sensitize and elicit a DTH reaction. It was also found that sensitization of a DTH response is directed not to the antigen as such but to a complex structure on the macrophage surface determined partly by the 'processed' antigen and partly by the MHC coded gene product. In other words, induction of a DTH response is also H-2 restricted (Chapter 5.2). Experiments with thymus-grafted chimeras showed that I region compatibility between thymus graft donor and the naive recipients was essential for successful transfer of DTH response to a protein antigen (KLH), indicating the importance of the thymus genotype in influencing DTH responsiveness (Smith et al., 1979).

Recently, there was an increasing interest in the study of the influence of the MHC on the DTH response in mice. A large number of reports which have a direct bearing on this topic has been published in the past three years. They are summarized in Table 1. After the earlier work of Miller

TABLE 1

H-2 Restriction on Adoptive Transfer of DTH

Antigen	H-2 region restricted	Lyt phenotype of effector cells	Reference
Soluble proteins and polypeptides			
Fowl gamma globulin	IA	Lyt 1^+	Vadas et al, 1976, 1977
Keyhole limpet haemocyanin (KLH)	IA	N.D.	Miller, 1978a
GAT	IA	N.D.	
Contact chemicals			
Dinitrofluorobenzene (DNFB)	K,D or I	Lyt 1^+ & Lyt $2,3^+$	Vadas et al, 1977, Miller, 1978b
Oxazolone	K, IA, IB	N.D.	Asherson et al, 1979
Haptens			
4-hydroxy-3-nitrophenyl acetyl (NP)	IA	N.D.	Weinberger et al, 1979
Azobenzenearsonate	IA	N.D.	Bach et al, 1978
H-Y	IB	Lyt 1^+	Liew & Simpson, 1980
Viruses			
LCM	K,D	N.D.	Zinkernagel, 1976
Influenza: non-infectious	IA	Lyt 1^+	This thesis
infectious	K,D or I	Lyt $2,3^+$ & Lyt 1^+	
Sendai : fusion-negative	IA	Lyt 1^+	Ertl, 1981
fusion-positive	K,D or I	Lyt $2,3^+$ & Lyt 1^+	
Reovirus	K,IA or D	N.D.	Weiner et al, 1980
Herpes simplex type 1	IA	N.D.	Nash et al, 1981b
Allogenic cells			
Non-H-2 antigens	'K' end	Lyt 1^+ & Lyt $2,3^+$	Smith & Miller, 1979a,b
H-2 antigens	unrestricted		

N.D. Not determined

and his colleagues, it came as a surprise that when a viral system (LCM) was examined, K,D but not I region compatibility between donors and recipients was found to be necessary for adoptive transfer of DTH (Zinkernagel, 1976). The difference between these results was unknown but it was suggested that 'antigen parameters' determined whether T cells were specific for 'altered' I or 'altered' K and D region coded products, and that multiplying infectious agents like viruses that actively invade cells and interfere with the genetic and metabolic pathways of the cell would alter K,D rather than I region coded gene products. These observations prompted an investigation into the adoptive transfer of DTH using the influenza virus model. The results from this research allowed a mechanism to be proposed which might explain the requirements for different regions of the H-2 gene complex on the adoptive transfer of DTH. It was found that at least two T cell populations can mediate DTH responses to influenza virus in mice. If UV-irradiated (non-infectious) virus was used for sensitizing and/or eliciting the reaction, only I region restricted DTH response was detected, which is analogous to the DTH response to a protein antigen. However, if infectious virus preparations were used for both sensitization and elicitation, two populations of Td cells were generated and detected; one was Lyt $2,3^+$ and K,D region restricted and the other Lyt 1^+ and I region restricted (Chapter 4.3). Subsequent work with Sendai virus gave similar results (Ertl, 1981). Fusion-positive (infectious or UV-inactivated) Sendai virus generates Td cells that are K,D or I region restricted whereas fusion-negative (cell-cultured virus) Sendai virus stimulates a Td population that is I region restricted. There are two other reports which studied the H-2 restriction on adoptive transfer of DTH to viruses. Weiner et al.(1980) showed that cells mediating DTH to infectious reovirus (a mouse pathogen) were D or K/IA region restricted. The combination of congenic mouse strains used in their study did not

allow a determination of whether a separate I region restricted subset of T cells was stimulated. Nash et al.(1981b), on the other hand, found that adoptive transfer of DTH to herpes simplex virus was IA subregion restricted. The significance of these findings with the viruses is not entirely understood. It appears that at least in the case of influenza virus and Sendai virus, induction of a DTH response to non-infectious or fusion-negative virus may involve processing of the viral antigens by the macrophages so that a I region restricted DTH response is activated, in a manner that is analogous to the DTH response generated to soluble proteins or polypeptides. In contrast, integration of the viral antigens into the cell membrane by the process of infection or fusion may enable the association of the viral antigens with the K,D region coded gene products in such a way so as to generate a K,D region restricted DTH response.

The current results strongly suggest that the nature of the antigen may govern the MHC region that is responsible for restriction imposed on DTH responses. Thus, Td cells generated to a variety of non-replicating antigens such as proteins and polypeptides (Miller, 1978a), haptens such as azobenzenearsonate (Bach et al., 1978) and 4-hydroxyl-3-nitrophenyl acetyl (Weinberger et al., 1979) and H-Y antigen (Liew & Simpson, 1980) are found to be I region restricted. In contrast, with replicating antigens such as viruses, it appears that the ability to integrate into the cell membrane may determine whether the DTH response activated is K,D or I region restricted. Obviously, further studies on viruses other than influenza and Sendai viruses may help to clarify these points.

The subsequent sections of this chapter will discuss why influenza virus is particularly useful in the study of T cell activation and functions. Several aspects such as the role of viral infectivity, the manner of antigen presentation and the participation of different T cell subsets in the immune responses of the host to the virus will be examined.

The role of viral infectivity in the induction of immune responses to influenza virus in mice

1. The antibody response

If the antigen mass is sufficiently large, the amount of antibody formed by the host is similar whether infectious or non-infectious virus is injected. Thus, Braciale & Yap (1978) injected different doses of infectious or UV-inactivated influenza A virus i.v. into mice and measured the anti-HA antibody ten days later. Although the antibody response to infectious virus was greater at lower immunizing doses, similar antibody levels were achieved upon immunization with higher doses of UV-inactivated virus (e.g. 10^3-10^4 HAU). Reiss & Schulman (1980b)have reported similar results using infectious, UV-irradiated or formalin-treated influenza virus.

2. The cytotoxic T cell response

In contrast to the above results, viral infectivity is very important for the generation of cytotoxic T cells (Tc) to influenza virus. Earlier work from this laboratory is summarized in Table 2. The conclusions are (1) All preparations of cytotoxic T cells were K,D region restricted and had the Lyt 2,3 phenotype. (2) If infectious virus was used both to sensitize the host and the target cells, specific and cross-reactive (within the A strains) populations of Tc cells were generated. These two findings have been previously reported independently by several other groups (Doherty et al., 1977; Zweerink et al., 1977a; Braciale, 1977a). (3) If non-infectious virus (UV-irradiated virus, 7 min exposure) was used to immunize the mice (i.v. injection), Tc cell generation was suppressed (see below). (4) If non-infectious virus was used as a secondary stimulus for memory cells obtained from donors primed with infectious virus, effector cells specific for the homologous virus were generated. Furthermore, the work in this laboratory has shown that most inactivated (UV-irradiated)

TABLE 2

Viral Infectivity and the Generation and Properties of Cytotoxic T Cells to A Strain Influenza Viruses

Stimulation		Sensitization of target cell	Specificity of effector cells	H-2 sharing required	Lyt phenotype
Primary[a]	Secondary[b]				
Infectious	-	Infectious	S[c] + C.R.[d]	K,D	2,3
Infectious	Infectious	Non-infectious	S (1)[e]	K,D	2,3
Infectious	Infectious	Infectious	S + C.R.	K,D	2,3
Infectious	Non-infectious	Infectious	S	K,D	2,3
Non-infectious			Suppression C.R.		

Legends for Table 2

a All primary stimulations were carried out in vivo. The effector cells were either spleen cells or lung cells obtained from mice 6 days after i.v or i.n. inoculation of virus.

b All secondary stimulations were carried out in vitro. The responder cells were obtained from the spleens of mice injected i.v. or i.p. three weeks or more previously with infectious virus.

c Effector cells only lyse target cells which are exposed to the homologous virus, i.e., a virus-strain specific response (S).

d Effector cells lyse target cells which are exposed to any A strain influenza virus. It was initially shown by cold target competition experiments that such preparations of cytotoxic cells contained at least two populations of effector T cells, one subtype-specific and the other is type-specific but cross-reactive within the A strains (C.R.).

e One recombinant strain was found to be effective (Ertl & Ada, 1981).

influenza A viruses could not sensitize a target cell for T cell mediated lympholysis (CML) but for some unknown reasons, one recombinant A strain virus (A/JAP-BEL) was found to be quite effective (Ertl & Ada, 1981). In this case HA-specific Tc cells were generated. There are apparent contradictions in the literature about the minimum requirements for influenza virus to stimulate Tc cell production or to sensitize target cells for CML. These will be discussed later.

3. The delayed-type hypersensitivity response

The infectivity of the influenza A virus preparation used to sensitize for and to elicit a DTH response is important in determining the nature of the DTH effector cells (Td) being predominantly activated. The results reported in previous chapters of this thesis are summarized in Table 3. Briefly, the following conclusions can be drawn: (1) Two subsets of T cells which mediate a DTH response can be generated. One is Lyt $1^-2^+3^+$ and K,D region restricted; the other is Lyt $1^+2^-3^-$ and I region restricted. (2) Infectious virus must be used both to stimulate and to elicit the response in order to generate and to detect the K,D region restricted Td cells; the cells so generated are cross-reactive within the A strains of the influenza virus. (3) If non-infectious virus is used as the stimulating antigen, only Lyt $1^+2^-3^-$ and I region restricted cells are generated and they are specific for the HA of the stimulating virus. Liew (personal communication) has also shown that the DTH reaction to non-infectious influenza virus is mediated by cells that are Lyt 1^+ and I region restricted. (4) In contrast, if infectious virus is used to initiate the primary (either in vivo or in vitro) or both primary (in vivo) and secondary (in vitro) responses, and non-infectious virus used to elicit the reaction, the I region restricted DTH responses detected are also cross-reactive within the A strains of the influenza virus. (5) If infectious virus is used to initiate a primary response in vivo or in vitro

TABLE 3

Viral Infectivity and the Generation and Properties of DTH T Cells (Td) to A Strain Influenza Viruses

Stimulation		Elicitation	Specificity of effector cells	H-2 sharing required	Lyt phenotype
Primary[a]	Secondary[b]				
Infectious	-	Infectious	C.R.[c]	K,D[e]	2,3
Infectious	-	Infectious	C.R.[c]	I[e]	1
Infectious	-	Non-infectious	C.R.[c]	I	1
Non-infectious	-	Infectious	S[d]	I	1
Non-infectious	-	Non-infectious	S[d]	I	1
Infectious	Infectious	Non-infectious	C.R.[c]	I	1
Non-infectious	Infectious	Non-infectious	S[d]	I	1
Infectious	Non-infectious	Non-infectious	S[d]	I	1

Legends for Table 3

a Primary stimulation was usually carried out in vivo. Effector cells were either spleen cells obtained from mice 6 days after i.v. injection of virus or lung cells obtained from mice 6 days after i.n. inoculation of infectious virus. Primary stimulation could also be carried out in vitro. The responder cells were normal spleen cells and γ-irradiated helper T cells (2 days immune spleen cells from mice primed with a low dose of virus) were added to enhance the response. Cells were harvested from the cultures 5 days after in vitro stimulation with virus (Chapter 5).

b All secondary stimulations were carried out in vitro. The responder cells were taken from the spleens of mice three weeks or more after injection of infectious virus.

c DTH can be elicited by any A strain influenza virus. There is no formal proof that such preparations contain two populations of cells, one strain-specific (S) and the other cross-reactive within the A strains of influenza virus (C.R.), but this seems a reasonable possibility, by analogy with the Tc findings (see Table 2).

d DTH can only be elicited by the homologous virus.

e It should be noted that DTH reactions, compared to some other tests such as CML, are poorly defined. A detailed analysis of the reactions mediated by these two cell classes has yet to be made.

and non-infectious virus used both to stimulate the memory cells *in vitro* and to elicit the reaction *in vivo*, cells specific for the homologous virus are detected. The last two points illustrate an important phenomenon. Though non-infectious virus does not induce the generation of the cross-reactive Td cells from naive or memory precursor cells, once generated these cells can be recognized by non-infectious virus. It is another example of conditions of stimulation being more demanding than recognition by effector T cells. The results further indicate that each population of Td cells contains two subsets of effector cells, one of which is HA-specific and the other cross-reactive.

4. *The helper T cell response*

Infection of mice with influenza virus induces formation of helper T cells (Th) which enhance the generation of effector Td cells *in vitro* (Chapter 5.1). The Th cells are Lyt 1^+ and IA subregion restricted at the level of induction comparable to Th cells for B cell response. The delivery of help to both subsets of Td cells is H-2 unrestricted. If UV-inactivated influenza virus was used to stimulate Td precursor cells, only Th cells induced with the homologous virus could increase the response. If infectious virus was used for induction of Td cells, Th cells induced by the homologous or a heterologous A strain influenza virus could increase the response. Th cells in both cases could be induced by infectious or UV-inactivated virus. Thus the requirements for induction of Th cells for Td cell generation were similar to the requirements for the induction of helper cells enhancing the immune response of other effector lymphocytes (T as well as B cells). Pfizenmaien *et al.* (1980) have shown that antigen-specific Th cells for H-2 restricted cytotoxic T cell response followed similar rules.

5. *The suppressor T cell response*

A state of tolerance to an antigen may be due to the generation of a

fourth type of T effector cells, called suppressor T cells (Ts). The induction and properties of these cells to influenza virus in mice have also been studied (Chapter 7; Liew & Russell, 1980).

Two subsets of suppressor T cells, directed against the generation of distinct T cell populations, can be induced either by infectious or by UV-inactivated influenza virus. Liew & Russell (1980) showed that mice inoculated intranasally with infectious virus subsequently gave a reduced DTH response if sensitized and then challenged with inactivated virus. Suppression could not be induced with non-infectious virus. The suppression was due to the generation of Thy 1^+, Lyt 1^+ cells, which expressed specificity for the HA. They were found in the spleen two weeks after the initial stimulus and suppressed the induction but not the effector function of the I region restricted Td cells. I have confirmed this observation and also shown that the generation of the K,D region restricted T cells was not inhibited (unpublished results).

A converse situation was found if mice were immunized with UV-inactivated influenza virus. Intravenous injection of UV-inactivated virus into mice caused the generation of suppressor T cells which inhibited the generation of Lyt $1^-2^+3^+$ K,D region restricted Tc and Td cells (Chapter 7 and unpublished observations) without influencing the induction of Lyt $1^+2^-3^-$, I region restricted Td cells. The specificity pattern of these suppressor cells showed cross-reactivity within the influenza A strains. The suppressed mice were found to possess both normal levels of Tc precursor cells and primed cells which could act as Th cells in the in vitro generation of Tc cells. This is consistent with results reported previously for the generation of Th cells.

The most intriguing aspect of these findings on suppressor T cell formation is the apparent symmetry. Inoculation of infectious influenza virus intranasally induces the formation of antigen-specific suppressor

cells which inhibit the induction of I region restricted effector T cells. In contrast, intravenous injection of non-infectious influenza virus induces Ts cells which inhibit the generation of K,D region restricted effector T cells. Such a differential suppression of distinct T effector cells may well be very important in maintaining the balance between protective and/or immunopathological effects of the T cell mediated immune reactions *in vivo*. It has also recently been found that i.v. injection of infectious herpes simplex virus induces tolerance to DTH reactivity (Nash *et al.*, 1981a).

There are four aspects which warrant further discussion. The first concerns the difference in antigen presentation which results in a K,D region or an I region restricted response to influenza virus. The second point involved the questions: How many T cell subsets are there? Is the ability to lyse a target cell and to mediate a DTH reaction a property of the same cell? The third aspect concerns the role played by each T cell subset during murine influenza virus infection. Do the T cells protect mice or to what extent they may contibute to the pathology? A final point is -- what is the relevance of the study of CMI response to influenza virus to the prophylaxis against human influenza?

Antigen presentation and the generation of a K,D region restricted T cell response

All the available evidence supports the belief that the main if not the only factor in determining which class of T cell will be generated is the manner of antigen presentation. Kurrle *et al.*(1979) found that target cells could be sensitized for CML provided that the HA of the influenza virus used was cleaved, irrespective of whether the virus preparation was infectious or not. Rott (1980) later showed that if cleaved or uncleaved HA was incorporated into liposomes, only liposomes containing cleaved HA fused with the target cell membrane. Liposomes containing uncleaved HA would fuse only if they were treated with trypsin to cleave the HA. The

molecular mechanism of fusion was not clear as it only occurs if neuraminidase as well as HA were present in the liposome.

There is some uncertainty in the literature whether inactivated influenza virus can sensitize or prime the host for a Tc response or sensitize a target cell for lysis by Tc cells. The main procedures used for inactivation of virus are UV-light irradiation or treatment of the virus with β-propionolactone or formaldehyde. The former two procedures preferentially damage the viral nucleic acids whereas formaldehyde reacts with both proteins and nucleic acids (Skinner & Hugo, 1976). It should be noted that inactivation by either UV-light or β-propionolactone shows a 'tailing' effect so that complete loss of infectivity is hard to achieve and that the two procedures act synergistically (Logrippo, 1960). Thus, Reiss & Schulman (1980b)have shown that formaldehyde-treated influenza virus could not sensitize mice for a measurable primary Tc response and also it did not prime mice for a secondary response, yet the treated virus could evoke a secondary Tc response in mice primed with infectious virus. The formaldehyde-treated virus had no detectable infectivity, could not induce an abortive infection of cells (as shown by lack of haemadsorption) but could induce antibody formation in mice in amounts similar to infectious virus. The same authors further showed that influenza virus exposed to UV-light irradiation for 15 sec (at a 15 cm distance)could undergo abortive infection and induce a strong Tc response even though infectivity was reduced to approximately 10,000 fold . Virus exposed for 1 min (15-Watt, at a 11 cm distance)was able to stimulate the generation of secondary Tc activity *in vitro* from spleen cells of mice previously primed with infectious virus (Zweerink *et al.*, 1977b). Similarly, virus (A/Hav2 Neq1) exposed to UV-light for 5 min (40-Watt, at a 8 cm distance)could sensitize target cells for lysis by Tc cells (Kurrle *et al.*, 1979). Ertl & Ada (1981) also demonstrated that one recombinant A strain (A/JAP-BEL) was quite effective

in sensitizing target cells for lysis by Tc cells, even though after UV-irradiation (40-Watt, at a 10 cm distance for 7 min). On the other hand, Hosaka et al.(1978) reported that infectious A/PR8 virus was about 10^5 times more efficient than UV-irradiated virus (1 min at a distance of 20 cm) at inducing a Tc response. Virus exposed for 7 min (25-Watt, at a distance of 20 cm) was non-infectious, did not sensitize target cells nor mice for CML but could elicit a subtype specific secondary Tc response in vitro (Braciale & Yap, 1978), a result reminiscent of that obtained with virus treated with formaldehyde (see above). Furthermore, virus rendered non-infectious in a similar way was able to induce a virus-specific suppressor T cell response (Chapter 7).

The above conflicting results concerning the ability of UV-irradiated virus at inducing a K,D region restricted Tc response may reflect the differences in the fusion capacity of the virus strain used and/or the degree of UV-inactivation of the virus. The latter possibility was tested by studying the time of exposure to UV-light relative to the loss of infectivity, and to the ability to induce both haemadsorption in target cells and a specific Tc response in mice. It was found (data not shown in this thesis) that a 10^4-10^5-fold reduction in infectivity resulted in a great decrease in the haemadsorption of exposed target cells but the virus was still able to induce very significant Tc formation. It is only when no haemadsorption was detected that the ability to induce a Tc response became very low. Thus, continuing exposure of the virus to UV-light clearly results in a progressive effect in which egg infectivity (permissive replication) is lost at a very much faster rate than are other properties -- defective replication(as indicated by haemadsorption) as well as the ability to induce a Tc response. It seems likely that this progressive effect may explain the different findings on UV-light inactivation on the ability of influenza virus to induce a Tc response. A similar type of phenomenon may underlie the report that rabies virus

exposed to β-propionolactone can still stimulate mice to give a Tc response *in vivo* (Wiktor *et al.*, 1977).

How many T cell subsets are there?

Another interesting aspect is the degree of functional heterogeneity of the different T cell subsets generated in response to influenza virus. Are there two subsets of H-2 restricted cells, one of which is I region restricted and Lyt 1^+ and the other K,D region restricted and Lyt $2,3^+$? Or are there further divisions within each of these two subsets as shown by distinct functional activities? An immediate question raised by the work presented in this thesis is whether a K,D region restricted , Lyt $2,3^+$ cell can mediate two functions, CML and DTH? Some information indirectly suggests that the answer to this question may be yes. Thus, pretreatment of mice with a high dose of cyclophosphamide before sensitization with infectious virus decreases the generation of K,D region restricted T cell population(s) mediating both CML and DTH activities without affecting the production of I region restricted cells mediating DTH activity (Chapter 4.3). Similarly, the antigenic specificities of the K,D region restricted cells are similar for both CML and DTH activities, as far as can be judged.

The most unequivocal answer would be to test whether clones of cells can exhibit multiple functional activities. Lin & Askonas (1980) have described some properties of a cloned Tc cell line active against influenza virus-infected target cells and they have tested these cells for their ability to mediate a DTH reaction. When infectious influenza virus and $3x10^5$ or $6x10^5$ cloned cells were injected directly into the footpads of naive mice, the 24 h specific increases in footpad thickness were 16% & 23% respectively, values significantly different from the controls. These compare with a 25-30% specific footpad swelling found when infectious virus and $5x10^6$ cultured, secondary effector cells were injected (Chapter 4.3).

The finding of Lin & Askonas (manuscript in preparation) suggests that a single T cell can mediate two functions -- CML and DTH. Using a different system, Dennert and his colleagues (personal communication) have also shown that a T cell clone directed against the private specificity of IA subregion can deliver help, mediate a DTH reaction and lyse target cells, again showing multifunctional activities of individual T cells.

The role of the different T cell subsets during influenza virus infection

Most recent work to define T cell effector function has been done in the mouse and in asking what is the function of T cell subsets during influenza virus infection, it should also be asked whether the murine disease is a suitable model for the human disease. Mild infection in man is thought to be limited to the upper respiratory tract whereas in the mouse, the lungs are more involved. Fazekas de St. Groth (1950) pointed out that the difference in the disease pattern between the two hosts was quantitative rather than qualitative. Thus, just as extensive lung involvement in man may lead to severe and possibly fatal pneumonitis, so Taylor (1941) showed that a nonlethal infection in the mouse can be converted into a lethal infection by simply intranasal inoculation of saline, thus carrying viral particles deeper into the lungs. A crucial test is to show whether important findings made in the mouse are relevant to the human disease.

It has been known for some time that virus specific T cell reaction (Feinstone et al., 1969) and T cell mediated cellular damage (Suzuki et al., 1975) occurred in influenza virus-infected mice. These two aspects will be considered separately.

(A) Do T cells help in the recovery from influenza virus infection?

Early attempts to protect mice from a lethal viral infection by transfer of immune spleen cells were largely unsuccessful and it was concluded that

any effect of T cells was an indirect effect of Th cells augmenting antibody production (Virelizier, 1975). The first evidence that one subset of T cells might play a protective role during influenza virus infection in mice was the demonstration that a secondary culture of T cells transferred to mice 24 h before or after inoculation of a lethal dose of virus would protect the mice from death. The protective cells were K,D region restricted and of Lyt $1^{-}2^{+}3^{+}$ phenotype so the effect was attributed to the cytotoxic T cells known to be present in the preparation (Yap et al., 1978). The transfer of these immune cells also resulted in a significant reduction of infectious virus in the lungs. This finding was extended by Lin & Askonas (1980) who showed that a cloned line of killer T cells transferred to mice 24 h after virus infection had a similar effect. McMichael (Fourth International Congress of Immunology, Paris, 1980; personal communication) has compared the severity of disease in patients infected with influenza virus with the ability of their peripheral blood lymphocytes to mount a cytotoxic T cell response. The more severe infections occurred in those patients who gave the weakest cytotoxic T cell response, which is the first indication that observations made about the importance of the effector T cell response in murine influenza could also apply to the human disease. It should also be remembered that cytotoxic T cells to influenza A virus lyse target cells infected with any A strain virus (Doherty et al., 1977; Zweerink et al., 1977a; Braciale, 1977a; Table 2, this chapter) and that transfer of cytotoxic cells raised to one A strain lowered lung virus titres in mice infected with a different A strain (Yap & Ada, 1978c). Furthermore, mice primed with one influenza A strain virus were more resistant to infection by a different A strain compared to unprimed mice (known as heterotypic immunity) and this correlated with the level of cross-reactive cytotoxic T cells which developed in the primed mice. Thus the evidence at present available shows convincingly for mice and suggests for man that cytotoxic T cells help to control influenza virus infection.

In contrast to the above results, experiments involving the transfer of secondary IA subregion restricted, Lyt $1^{+}2^{-}3^{-}$ effector T cells and containing DTH activity, to mice infected 24 h earlier with a lethal dose of virus resulted not only in a lack of protection and when a critical dose of mouse-adapted virus was inoculated, the group of mice receiving the immune cells had higher mortality than the control mice (Chapter 6). The first finding is perhaps not surprising. I region restricted T cells are generated to inactivated influenza virus so that immune cells which react against host cells presenting viral antigens associated with I region gene products would not be expected to affect the infected cells that produce viable viral progeny.

Howes et al.(1979) found that immune protection against HSV-1 could be conferred by immune spleen cells possessing either I or K,D compatibility with the recipients and that long lasting protection was conferred only by cells requiring I region compatibility. They speculated that help for B cells in the production of antiviral antibody was the most likely explanation of the latter finding. The protocols of their experiments were very different from those employed in the work reported in this thesis. The more recent results of Nash et al.(1981b) are perhaps more relevant to the findings in this thesis. Successful transfer of DTH to HSV-1 was achieved when IA subregion sharing was present but additional sharing of K (and presumably D) region was necessary for the rapid clearance of infectious virus. Possible reasons why the mice receiving influenza virus-specific IA subregion restricted Td had greater mortality than control mice are discussed in the next section.

(B) Do T cells contribute to the pathology of influenza virus infection?

Sweet & Smith (1980) have recently discussed the pathogenesis of influenza virus infection. It is in part due to the toxic effects of the virus and these may be related to virulence. They stated that immunopathology appears to be minimal in humans but more evident in murine infections. Both Wells et al. (1979) and Wyde & Cate (1978) suggested that cellular components of the host

may be important in clearing virus from the lungs and contribute to the observed pathology, the latter in particular suggesting that local T cell response was a major contributor to the pneumonia in murine influenza virus infection. Their evidence was indirect although they and others (Yap & Ada, 1978a; Ennis et al., 1978) noticed that the rise in effector T cell activity in the infected lungs slightly preceded the development of lung consolidation observed during influenza virus infection.

Direct lysis of infected cells by cytotoxic T cells (if it occurs in vivo) contributes to organ damage not only by target cell death but may also by release of chemotactic substances which may lead to cellular infiltration. DTH reactions are inflammatory responses which are characterized by mononuclear cell infiltration. As both cytotoxic and DTH T cell activities are found in infected mouse lungs to a considerable extent (Yap & Ada, 1978a; Chapter 4.1), it is inevitable that these cell types must contribute to a greater or lesser extent to the pathology. It is therefore to be expected that mice transferred with specific I region restricted Td cells would show increased lung immunopathology which could result in the increased mortality rate that was observed (Chapter 6). Effector T cells which mediate cytotoxic activity must also contribute to the pathology but this is in part offset by their effective 'removal' of the infected cells which are the source of viral progeny. Experiments which examined the degree of cellular infiltration which occurs in the lungs of infected mice to which different effector T cell preparations have been transferred showed that transfer of cells with only I region restricted DTH activity not only failed to lower lung virus titres but instead enhanced the cellular infiltration in the lungs of mice. In contrast, transfer of cells with K,D region restricted Tc (and Td) activity but negligible I region restricted DTH activity lowered the lung virus titres with a concomitant decrease in the cellular infiltration in the virus-infected lungs (Chapter 6). These results are consistent with the interpretation that

cells with K,D region restricted Tc (and Td) activity are protective whereas cells with I region restricted DTH activity may have a detrimental effect by increasing the severity of the influenza disease.

The extent to which findings with murine influenza virus infection of differential protective/immunopathological effects by different T cell subsets is relevant to infections by other viruses may depend upon whether the virus concerned is a natural pathogen for the host and the organ which is infected and being studied. Thus, it has been shown (Berger, M.L., manuscripts submitted for publication) that immunopathological effects which occur in the brains of mice infected with ectromelia virus can be transferred by K region restricted but not I region restricted T cells. The probable reason for this is the absence in the brain of macrophages which may be the only or at least the major stimulator cells in different organs for I region restricted specific responses.

It should be stressed that interpretation of the biological effects observed (protection from death, lowering of virus titres, increased immunopathological effects) is helped considerably if there is knowledge of the particular cellular activities present in the cell preparations at the time of transfer.

The relevance of this work to the prophylaxis against human influenza

It has been the hope of many who study the murine system that significant findings would be found to apply to the human disease. Provided future work continues to demonstrate the importance of the cytotoxic T cell response during virus infections in humans, there are five aspects discussed in this chapter which have significant implications in determining the criteria to be used in evaluating the effectiveness of prospective human influenza vaccines.

(1) Infectious virus efficiently generates cytotoxic T cell activity and all current work suggests that these cells effectively limit viral replication. Inactivated virus induces suppressor T cells which selectively inhibit the generation of cytotoxic T cells. (2) Cytotoxic T cells show cross-reactive specificity within the A strain viruses, thus may help to overcome the continuing difficulty of antigenic drift and shift. (3) Inactivated virus induces I region restricted DTH T cells which, in the short term, are not protective and contribute significantly to the immunopathology. In the longer term, helper T cells production which is also I region restricted will be important in antibody production. (4) Intranasal inoculation of mice with infectious virus induces suppressor T cells which inhibit the generation of I region restricted DTH T cells. and (5) To boost Tc cell generation, strains of virus should be chosen which, even when non-infectious, have the ability to fuse with the cell membrane.

These considerations added to other accumulating evidence that infectious but not non-infectious virus activates nonspecific defence mechanisms such as induction of cytotoxic macrophages (Mak, Leung & Ada, submitted for publication) indicates that more attention should be directed to developing suitable attenuated live virus vaccines (Tannock, 1980) for use against influenza virus infection in humans in preference to the continued use of existing killed whole virus or subunit vaccines.

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