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RECOGNITION OF AND RESPONSE BY MICE TO INFLUENZA VIRUS ANTIGENS

by

CAROL S. REISS

A dissertation submitted to the Graduate Faculty in Microbiology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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I. ABSTRACT

RECOGNITION OF AND RESPONSE BY MICE
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by
Carol S. Reiss

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In this project, recognition by mice of influenza virus glycoprotein antigens presented in different forms was investigated. The specificities of the T and B cell responses were compared after infection and after immunization with untreated or vaccine forms of influenza viruses.

A modified Jerne plaque technique was developed utilizing sheep erythrocytes coated with influenza viruses. The use of recombinant viruses or heterotypic viruses to coat red cells facilitated distinction between the reactivities with hemagglutinin, neuraminidase, host-coded oligosaccharide, and type A cross-reactive specificities among the antibody forming cells (AFC). The AFC response to influenza virus sensitization was found to be virus-specific and primarily directed at the hemagglutinin. AFC responses were compared with the development of serum antibody.

A cell mediated cytotoxicity assay using influenza virus infected cell lines was developed. It was characterized as histocompatibility restricted, influenza A virus specific,

but cross-reactive among the subtypes of Type A viruses. The heterotypic recognition could be blocked by antiserum to M protein.

Mice were immunized with intact untreated virus, intact inactivated viruses (UV, formalin) or with Triton X-100 extracted glycoprotein heteropolymers of the same virus. Immunization with intact untreated virus produced the maximum AFC and cytotoxic responses. Sensitization with UV inactivated virus was found to be as effective as untreated virus in stimulating an AFC response, but consistantly elicited a cytotoxic response of somewhat lower magnitude. Formalin inactivated virus induced an equivalent AFC response, but did not elicit a primary cytotoxic response or induce memory for a secondary cytotoxic response. However, formalin inactivated virus did evoke a secondary cytotoxic response in mice previously primed with whole virus. Aggregated isolated glycoproteins were much less effective in inducing an antibody response, and did not evoke either a primary or a secondary cytotoxic response.

Influenza virus infected mice developed cytotoxic T cells and AFC specific for viral antigens both in mediastinal lymph nodes and in the spleens. Cytotoxic T cell activity appeared prior to the development of AFC, and at a time when a precipitous decline in pulmonary virus titer was observed.

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The questions addressed in this project concern the recognition by mice of influenza virus glycoprotein antigens presented in different forms. The influence of the different forms of antigen presentation on the kinetics of the responses of thymus derived cytotoxic effector cells and of antibody forming cells was assessed. Experiments designed to determine whether all forms of viral antigens stimulated qualitatively and quantitatively equivalent responses at a cellular level, or if different forms of the glycoproteins could distinguish structures which were capable of eliciting B cell responses in the absence of T cell responses. The specificities of the cytotoxic T cell responses and the antibody forming cellular responses were compared using appropriate recombinant or heterotypic viruses to ascertain whether the determinants recognized by T and B cells comprised one set or rather were two partially overlapping repertoires.

VI. INTRODUCTION

A. IMMUNOLOGY

The Cells of the Immune System, Cellular Interaction

The discussions which follow require a brief introductory review of the cell types of the immune system and their interrelated functions. This description is not intended to be an exhaustive survey of the literature, but rather, serves to introduce ideas necessary to interpret the results of the experiments presented.

Four basic cell types have been identified in the immune systems of higher animals: macrophages, B cells, T cells, and null cells.

1) Macrophages can be divided into two subsets, those which bear a surface glycoprotein antigen (Ia), and those that do not. Among the peritoneal macrophages in mice and guinea pigs, a small proportion (10 - 30%) are capable of taking up antigen, "processing" it and expressing antigenic determinants on their surface membrane in association with Ia. It is this configuration which is recognized by T and B cells (reviewed in Rosenthal, 1978). The remainder of the macrophages remove debris, antigen-antibody complexes, etc., by phagocytosis and subsequent degradation (Cohn, 1968).

2) B cells. Upon appropriate stimulation, B cells differentiate to plasma cells and secrete antibody. These cells, like macrophages, and T cells, are of bone marrow cell origin. In the fetus, precursor B cells are matured in the liver, the bursal equivalent (Owen et al., 1975; Miller and Philips,

1975). While undergoing maturation in the fetal liver, there is sequential expression of immunoglobulin classes inside the cell and on the membrane (Abney et al., 1978). This antibody serves as the antigen receptor and determines the specificity to which the cell is committed (Julius et al., 1973). Early in the maturation of B cells, before surface IgD is expressed in concert with IgM on the cell membrane, antigen contact produces tolerance; subsequent to the expression of IgD outside cells, however, antigen contact triggers B cells (reviewed by Vitetta and Uhr, 1978). Once triggered by antigen, these cells differentiate along two lines to produce memory cells and antibody forming cells. The former are long-lived whereas the latter are not.

3) T cells are so called because of the requirement for thymic influence in their maturation (Miller, 1962). There are numerous subsets, some of which may represent the same subpopulation defined by different assay systems. There are: a) helper cells which collaborate with macrophages and B cells in the antibody response to most antigens (Miller et al., 1968; Miller et al., 1971); b) suppressor cells which inhibit a required step during B cell differentiation (Tada and Takemori, 1974; Eardley and Gershon, 1975); c) cells which give rise to delayed type hypersensitive (DTH) reactions in the primary host or to recipients when adoptively transferred (Chase, 1945); d) cells which are capable of rejecting transplanted tissues (Govaerts, 1960) or mounting a graft versus host response (Mitchison, 1971); e) cells which suppress DTH

responses or graft versus host responses (Greene et al., 1978; Gershon et al., 1976); f) cells which participate in mixed lymphocyte culture responses upon recognition of foreign antigenic determinants in vitro (Bach and Hirschhorn, 1964); g) cytotoxic T cells which can inhibit the growth of tumor cells (Brunner et al., 1966) or lyse target cells to which the effector cells have been sensitized in vivo (Cerottini and Brunner, 1974) or in vitro (Hayry and Defendi, 1970). The role of cytotoxic T cells in vivo is not as clear as it appeared to be only a year ago since it has been shown that grafts can be rejected in the absence of demonstrable cytotoxic cell activity (Hurme et al., 1978).

The purpose of the preceeding catalogue is to emphasize the presence of positive and negative controls for almost all of the functions described. The interactions of those cell populations are in dynamic equilibrium.

4) Null cells neither bear surface immunoglobulin nor T cell surface antigens. At least two functions have been ascribed to this population: "natural killer cell" activity (Kiessling et al., 1975) and antibody dependent cell mediated cytotoxicity (Greenberg, A.H. et al., 1973). The latter mechanism required a surface receptor for the Fc portion of IgG.

With the exception of a few highly repetitive antigens, generally polysaccharides, most soluble and particulate antigens encountered by the immune system in vivo or under laboratory manipulations require the interaction of macrophages, T cells, and B cells to elicit an antibody response

(Janeway et al., 1975; reviewed in Katz, 1977). For the optimal expression of this response in vitro, all of the cells must share surface antigens which are encoded by the major histocompatibility complex. In the intact animal host, of course, all of these cells share these antigens. The idealized soluble protein antigen is phagocytosed by macrophages and processed. Antigenic determinants are expressed in association with Ia. T cells committed to that antigen recognize the Ia-Antigen complex and are triggered (extensively reviewed in volume 40, Immunological Reviews, 1978). In some species, soluble factor(s) including Ia-Ag are released from the macrophage and can effect T cell activation (Taussig and Munro, 1975). However, in other species, intimate contact of T cells and macrophages is necessary (Rosenwasser and Rosenthal, 1978). T cells subsequently secrete a number of factors, some of which are antigen-specific (Harwell et al., 1976). B cells recognize antigen on the surface of macrophages and receive an obligatory "second signal" from T cells. T cell factors also activate macrophages, cause accumulation of cells in the locale of antigen deposits, induce proliferation of both B and T cells, and amplify the immune response (reviewed in Cohen et al., 1977). T cells may also differentiate to effectors of DTH or differentiate to cells which suppress either antibody production or DTH. Memory cells of both B and T cell origin store expanded populations of antigen-specific reactivity.

Humoral Antibody Response to the Idealized T cell Dependent Antigen

It has long been appreciated that in the primary response to the idealized antigen, IgM appeared first in serum, followed by IgG antibody. After secondary immunization with the same antigen, the response was characterized by a more rapid appearance of IgG antibody, and some, but a small amount of IgM antibody was produced (Dean and Webb, 1928). In the primary response, the relative affinity of antibody generally was lower initially with the appearance of higher avidity antibody later; the affinity observed after restimulation was generally not different from that observed in sera after the primary immunization (Eisen and Siskind, 1964). Different classes of antibody have been discriminated on the basis of size (Kabat, 1961), electrophoretic mobility (Poulik, 1968), or sensitivity to mercaptoethanol (Deutsch and Morton, 1957). More recently, much more sensitive techniques have been developed for distinguishing antibody class and subclass such as radioimmune assay (Klinman and Taylor, 1969) and the facilitated antibody forming cell assay (Pierce *et al.*, 1971). Both detect the products of individual cells and clones *in vitro*. Determinations of the avidity of antibody in serum and the product of cells in culture have been described (reviewed in DeLisi *et al.*, 1977).

Modulation of the Immune Response by Antigen

The responses discussed above may be influenced by a variety of factors including the form of the antigen, dose,

route of immunization, and by host factors.

The immune response to comparatively simple protein antigens such as bovine serum albumin is complex. In general, both antibody and DTH responses are elicited when animals are inoculated with soluble albumin (Gell and Benacerraf, 1961). If the same protein is polymerized, DTH is preferentially stimulated. However, in the virtual absence of antibody secretion, B cell memory is generated (Kostiala, 1978). In animals given a large dose of albumin, high dose tolerance can be induced (reviewed in Weigel, 1973). The affinity of the antibody response can be controlled by the dose of the antigen administered to elicit the response. High affinity antibody forming cells are preferentially stimulated with low doses of immunogen (reviewed by Siskind and Benacerraf, 1969). In addition, T cells with high affinity receptors for antigen are also triggered by small quantities of antigen (Paul et al., 1968).

There are sets of immune response genes which control the responses to a variety of antigens from simple synthetic polymers composed of only three amino acids (reviewed in Benacerraf and McDevitt, 1972) to the male-specific cell surface antigen H-Y (Hurme et al., 1978) or to a T cell surface antigen, Thy 1 (Zaleski, 1975). Recent experiments suggest that non-responder mice generate suppressor regulatory T cells which are responsible for the lack of responsiveness (reviewed in Benacerraf and Germaine, 1978). When appropriate alloantisera are used to remove the suppressor

population, a response can be elicited (Pierres et al., 1978; Cantor et al., 1978).

Modification of Antigen can Effect the Relative Responses of T and B Cells

Almost 20 years ago it was observed that denatured protein antigens could stimulate DTH responses to the native molecule, whereas antibody induced by the native molecule bound poorly to the denatured protein (reviewed in Gell and Benacerraf, 1961). There are mutants of the H-2 coded "serologically determined" proteins (the structural proteins, H-2D and H-2K) which induce strong cytotoxic responses but do not elicit antibody responses (Klein, 1978). In addition, fragments of staphylococcal nuclease which retain neither enzymatic activity nor native conformation, maintain the ability to stimulate T cells in vitro (Schwartz et al., 1978).

The route of administration of antigen is critical to the modulation of the response. Trinitrophenyl derivitized syngeneic cells administered subcutaneously are good inducers of contact sensitivity responses to picryl chloride, but inoculation of the same cells intravenously causes a suppression of the generation of the contact sensitivity response (Greene et al., 1978). Administration of diphtheria toxoid subcutaneously, but not intramuscularly, was shown to result in the induction of DTH responses (Uhr et al., 1957). Complete Freund's adjuvant, when administered with allogeneic cells, specifically suppresses the development of cytotoxic

T cells, and at the same time, amplifies the specific antibody response (Reinisch et al., 1976). Subcutaneous administration of antigen-antibody complexes in concentrations of antibody excess specifically stimulates the development of DTH responses in the absence of an antibody response, whereas in concentrations of antigen excess or at equivalence, the DTH response is suppressed (Uhr et al., 1957). However, in concentrations of antigen excess, the effector T cell response to tumor cells is augmented (Faanes et al., 1976). Silver and Benacerraf (1974) demonstrated that helper T cell activity is much more sensitive to tolerization by deaggregated bovine gamma globulin than DTH responses.

Mechanisms of Cytotoxicity

There are four basic mechanisms by which tumor cells, allogeneic cells, transplanted organs, or virus infected syngeneic cells can be killed. These include natural killer cells, cytotoxic antibody, antibody dependent cell mediated lysis, and cell mediated cytotoxicity. A fifth mechanism had been described, but the physiological role of mitogen induced cytotoxicity is uncertain (Kirchner and Blaese, 1973). 1) Natural killer cell activity has been found to be greatest in young mice. Kiessling and his co-workers (1975) identified the cell type responsible for the killing of Maloney leukemia cells as the null cell. Rodda and White (1976) observed that early in Semliki Forest virus infection there was a transient appearance of a macrophage-like cell which killed cells infected with another, non-cross-reactive virus. Zinkernagel

and Welsh (1977) described a cell population obtained from mice infected with lymphocytic choriomeningitis virus (LCM virus) which was capable of lysing uninfected syngeneic cells. A similar observation was made in mice following coxsackie B3 virus infection (Wong et al., 1977).

2) Cytotoxic Antibody recognizing transplantation antigens (Wigzell, 1965) or viral antigens expressed on infected cells (Measles virus: Kibler and terMeulen, 1975; Perrin et al., 1977; Semliki Forest virus: Rodda and White, 1976; influenza virus: Ennis et al., 1977a) have been well documented. It has been estimated that at least 10^6 complement fixing antibody molecules must coat a measles virus infected cell in order to irreversibly damage the cell membrane and cause cell death (Joseph et al., 1976).

3) Antibody Dependent Cell Mediated Cytotoxicity has been described using a variety of targets including heterologous erythrocytes (von Boxel et al., 1974; Greenberg, AH, et al., 1973), Schistasoma mansoni (Butterworth et al., 1974), cells infected with mumps virus (Horfast et al., 1975) or herpes simplex virus (Gershon et al., 1976; Romano and Shore, 1977), or measles virus (Perrin et al., 1977) or influenza virus (Greenberg, SB, et al., 1975; Greenberg, SB, et al., 1978). The effector cell bears a receptor for the Fc region of IgG antibody and may also have a complement receptor. This activity has been found to be present in populations of activated T cells (Evans et al., 1978) as well as in null cells (Greenberg, AH, et al., 1975). Specificity is con-

ferred by the antibody. This mechanism does not require histocompatibility antigens in common.

4) Cell mediated cytotoxicity is effected by sensitized T cells recognizing syngeneic cells modified by tumor antigens, chemical treatment or virus infection, allogeneic cells, or xenogeneic cells. In man, mouse and rat, the target of sensitized lymphocytes must share a portion of the major histocompatibility complex in common with the stimulating cell (Doherty and Zinhernagel, 1974; McMichael et al., 1977; Marshak et al., 1977). This requirement for shared histocompatibility antigens between stimulator and target cell has also been found in chimeric mice sensitized to minor histocompatibility antigens or to viral antigens (Bevan, 1977; Zinkernagel et al., 1977b). Killing requires intimate cell contact between effector and target; supernatant fluids are not cytotoxic (Klein, 1978). In addition, cytotoxicity is temperature and energy-sensitive (Martz and Benacerraf, 1975). The effector cells express Ly 2 antigens (Pang et al., 1976).

The Role of Cell Mediated Immunity in Infection

Whether cell mediated immune responses are beneficial or detrimental to the infected host seems to be dependent not only on the nature of the agent, but also upon the virulence of the agent, the locus of infection, and a variety of host factors. Several different models of the effects of cell mediated immunity during infection have been studied, leading to three general categories of effects: advantageous, dis-

advantageous, no effect. The two archetypal models of the extremes are lymphocytic choriomeningitis (LCM) and ectromelia virus/Listeria monocytogenes infections.

Intracerebral injection of LCM virus into adult mice results in infection, cellular infiltration of the meninges and the cerebral spinal fluid, and a breakdown of the blood-brain barrier. Within 7-10 days after infection, mice usually die. Manipulation of the immune system by T cell depletion (neonatal thymectomy, adult thymectomy with irradiation and bone marrow reconstitution, or by administration of rabbit anti-thymocyte serum) before infection prevents overt disease and its rapidly fatal outcome. However, even in the presence of passively transferred antibody, reconstitution of infected T cell deprived mice with immunocompetent thymus derived cells invariably results in immunopathological disease (reviewed in Doherty and Zinkernagel, 1974).

In contrast to the destruction mediated by T cells in LCM virus infection of adult mice, an intact immune system is ultimately responsible for recovery in either ectromelia virus infection (mouse pox) or Listeria monocytogenes infection. Mackaness demonstrated 16 years ago that the bacteria were not cleared from the liver and spleen of infected animals until DTH to listeria antigens could be detected. The essential role of T cells in controlling listeria infection was demonstrated by treatment of mice with ATS or by thymectomy prior to infection. These manipulations combined with immunological reconstitution provided insights into the

effector mechanisms. In the control of listeria infection, activation of macrophages by immune T cells is required. In ectromelia virus infection, the development of cytotoxic T cells and the production of immune interferon is associated with recovery. In each of these infections serum neutralizing antibody develops later and does not appear to influence the cell-associated infection (reviewed in Blanden, 1974). It has recently been demonstrated that cytotoxic T cells can prevent the release of infectious vaccinia virus from infected cells *in vitro* (Zinkernagel and Althage, 1978), thus the spread of infection might be limited, *in vivo*. In addition, it has also been observed that cytotoxic T cells, generated during listeria infection can kill cells infected with the intracellular bacteria (Zinkernagel, *et al.*, 1977a).

In addition to ectromelia virus infection (Gardner, *et al.*, 1974), the development of cytotoxic T cells has been described in many other viral infections of mice including, vaccinia virus (Koszinowski and Thomssen, 1974), LCM virus (Zinkernagel and Doherty, 1974), sendai virus, (Shrader and Edelman, 1977; Anderson *et al.*, 1977; Sugamura *et al.*, 1978), coxsackie virus (Wong *et al.*, 1977), parainfluenza virus (Lewandowski *et al.*, 1975), reovirus (Burakoff *et al.*, personal communication, 1978), adenovirus (Inada and Uetake, 1978) and influenza virus (Cambridge *et al.*, 1976; Doherty *et al.*, 1977; Braciale, 1977; Yap and Ada, 1977; Ennis *et al.*, 1977; Zweerink *et al.*, 1977).

Virelizier and his co-workers (1974) observed that stimulation of an antibody response to influenza virus antigens in mice requires functional T cells. Burns and his co-workers (1975) found that T cell help was required for antibody synthesis in response to all of the viruses tested. T cell function other than helper function is required for the limitation of virus spread and recovery from herpes virus infection (Oakes, 1975). Although the mechanism has yet to be defined, the effector function(s) involved may possibly be activation of macrophages or the induction of immune interferon responses (Morahan and McCord, 1975).

Another form of immunopathology which indirectly requires T cell function is immune complex disease. Neonatal mice infected with LCM virus do not develop the central nervous system disease characteristic of the adult infection. In contrast, infection of neonatal mice results in chronic virus replication and formation of complexes between viral antigens and antibody which are deposited in the kidneys (Oldstone and Dixon, 1971). In this disease, the T cell dependent antibody which is made is neither neutralizing nor cytotoxic. As a result, the infection persists in spite of a vigorous antibody response, and death results from renal damage secondary to the deposition of immune complexes. A similar disease has been reported in mink infected with Aleutian disease virus (Porter et al., 1969).

There is evidence that T cell function is required in recovery from murine hepatitis virus infection (Allison, 1974),

and influenza virus infection (Sullivan, et al., 1975). Like LCM infection, in other infections of the central nervous system, T cell function contributes to immunopathology as in arbovirus infection (Hapel, 1975) and sindbis virus infection (McFarland et al., 1972).

B. INFLUENZA VIRUS

Biology of the virus

Influenza virus, classified as an orthomyxovirus, is an enveloped virus, containing a segmented single stranded genome of negative polarity. The eight segments of RNA code for seven structural proteins and one non-structural protein (reviewed in Palese, 1977). Two of the structural proteins are glycoproteins: the hemagglutinin (HA) and the neuraminidase (NA), both of which recognize sialic acid on cell surfaces. HA mediates viral attachment and penetration (reviewed in Schulze, 1975) whereas NA functions in release of virus from cells (reviewed in Bucher and Palese, 1975). Although the genetic information of influenza virus is in the form of RNA, there is an actinomycin D sensitive phase in its replicative cycle (Barry, 1964) and influenza virus will not undergo productive infection in enucleated cells (Kelly et al., 1974).

Type specificity of influenza virus is determined by antigenic determinants present on internal proteins. Type specificity is usually assessed by complement fixation tests using solubilized internal antigens (Lennette and Horsfall, 1941). Three types of influenza virus have been described:

A,B,C. Subtypes of influenza A viruses which infect man, classified by antigenic differences in the surface antigens, include: HswlNl, HONl, HlNl, H2N2, and H3N2 (W.H.O., 1971). Influenza B viruses have not been divided into subtypes (Chakraverty, 1971). The appearance of a new subtype of influenza A viruses ("antigenic shift") is associated with the absence of immunity in the population, and results in pandemic disease. During the years intervening between pandemics, minor antigenic changes can be detected in the NA and HA ("antigenic drift"). The latter changes are associated with epidemic disease (reviewed in Kilbourne, 1975).

Early structural studies on the influenza virion suggested that M protein (also known as matrix protein or membrane protein) is located inside the lipid bilayer (Choppin and Compans, 1975). However, more recent studies, undertaken to answer questions arising from cross-reactive recognition of infected cells by cytotoxic T cells, have suggested that in infected cells, M protein may be exposed to extracellular antibody (Braciale, 1977b; Ada, and Yap, 1977; Biddison et al., 1977). In addition, one research group has reported that in infected cells, nucleoprotein is accessible to extracellular antibody early in infection (Virelizier et al., 1977).

Glycoproteins of influenza virus are synthesized on the rough endoplasmic reticulum of the host cell. In analogy to other well studied translation systems, it is thought that a "signal sequence", that is a short series of amino acids at

the N-terminus of the nascent polypeptide chain directs the sequestration of the protein into the lumen of the endoplasmic reticulum. Once within this organelle, the signal sequence is proteolytically removed and glycosylation takes place (Blobel and Dobberstein, 1975). Recent studies suggest that the pattern of glycosylation of influenza virus glycoproteins is like the glycosylation of other glycoproteins: a branched oligosaccharide comprised mostly of mannose and glucosamine is transferred en block from an isoprene carrier to asparagine residues along the nascent chain (Klenk et al., 1978). Once the polypeptide has been synthesized, it travels along the membrane to the smooth endoplasmic reticulum and to the Golgi region where galactosamine, fucose, and N-acetyl neuraminic acid are added to the oligosaccharide backbone (Compans, 1973). NANA is soon removed by the viral neuraminidase. There is some evidence that not all influenza virus hemagglutinins are glycosylated in the same manner in the same host cell. Choppin and his colleagues (1975) observed that HA_2 (the lipophilic portion of the protease-cleaved hemagglutinin) of influenza B/GL/1760 virus lacked fucose when the virus was cultivated in a hamster kidney cell line (HKCC); in contrast, A/WSN/33 virus propagated in the same cell line was found to possess fucose in the oligosaccharides associated with the HA_2 fragment. This difference may be accounted for by differences in tertiary structure of the nascent polypeptides and subsequent folding back of the protein after the initial branched structure is added. Those differences may determine

whether glycosyl transferases can gain access to the carbohydrate.

In addition, different host cell systems determine the nature of the structure of the oligosaccharide side chains (Choppin et al., 1974; Schwarz et al., 1977; Nakamura and Choppin, 1973; Laver and Webster, 1966; Harboe, 1963; Lindenmann, 1971). The nature of the added oligosaccharide influences antigenic determinants of the virus and contributes to what has been termed "host antigen", (Harboe, 1963; Lindenmann, 1971; Laver and Webster, 1966). Harboe has reported that antibody to the host antigen is capable of inhibiting hemagglutination. Gerhard (1976) has reported that a substantial portion of the monoclonal antibody response of mouse spleen cell fragments is directed at the host antigen(s) of PR8 virus.

Early studies of inhibition of glycosylation employed the analogue 2-deoxy-glucose to analyze the effects of inhibition of glycosylation of virus production. When 25 mg of the antimetabolite were injected into the allantoic cavity of embryonated chicken eggs, there was a reduction in the titers of hemagglutinating virus, especially early in virus replication (Kilbourne, 1959). Klenk and his co-workers (1978) reported that in the presence of tunicamycin, an inhibitor of the transfer of oligosaccharide from the isoprene carrier, viral HA was synthesized in reduced amounts. The radiolabled unglycosylated protein which was synthesized migrated more rapidly on polyacrylamide gels.

Influenza virus is capable of replicating in several species and many cell culture systems. In some hosts, no special treatment is required for the production of infectious virus progeny (i.e. embryonated chicken eggs, allantois on shell, lungs of mice or ferrets). In contrast, multicycle growth in other culture systems required either a particular gene constellation (McCahon and Schild, 1971; Schulman and Palese, 1977) or the presence of exogenous proteases such as plasminogen or trypsin to cleave the hemagglutinin molecule, (Lazarowitz et al., 1973; Lazarowitz and Choppin, 1975; Klenk et al., 1975). In other cell culture systems no progeny virus is produced following infection with influenza virus (Franklin and Breitenfeld,1959; Rott and Scholtissek, 1963; Avery, 1976). The latter category includes the target cells most frequently used in assays of cell mediated cytotoxicity, L929 and P815 cells.

Isolation and initial studies of immunity

Influenza virus was initially isolated not from man, but from fowl in 1901 (Pereira et al., 1965) then from swine in 1930 (Shope, 1931). Some three years later Smith, Andrewes and Laidlaw obtained a filterable virus from throat washings of clinically symptomatic people which, when instilled into ferrets, produced respiratory symptoms. Subsequently, the virus was propagated by serial animal passage. In 1935 it was determined that mice could be infected experimentally, when anesthetized and their nares immersed in fluid containing infectious virus of ferret origin (Shope, 1955). Swine were

found to be susceptible to infection with influenza viruses of human origin, and if co-infected with Hemophillus suis, developed influenza pneumonia (Francis and Shope, 1936). Rabbits, however, were found to be resistant to infection by either viruses of human or porcine origin (Francis and Magill, 1935).

Infection of swine, ferrets, or mice results in the production of neutralizing antibody and in immunity to reinfection with the same virus (Smith et al., 1933; Shope, 1935; Shope, 1937; Shope and Francis, 1936). Mice were afforded protection from infection if immune serum (to homotypic virus) was passively administered prior to infection (Francis and Magill, 1935). Shope reported that mice immune to either human or swine influenza viruses were resistant to later infection with heterologous virus (1935) although these mice did not possess serum antibody which was cross-reactive in neutralization tests (Francis, 1935).

Surveys of human sera for the presence of neutralizing antibody demonstrated that newborn infants had measurable levels, and that active immunity was acquired later in life by most individuals. Convalescent sera possessed the highest titers of neutralizing antibody (Francis and Magill, 1936). Most older individuals were found to have substantial titers of antibody to swine virus (Shope, 1936). These observations lent credence to the hypothesis that swine virus represented a human influenza virus which had infected swine sometime after the 1918-1919 pandemic (Laidlaw, 1935).

Cross-neutralization studies run with immune sera demonstrated the relatedness of various human influenza virus isolates made in the 1930's, and their distinction from the porcine isolates (Francis and Shope, 1936; Smith and Andrewes, 1938). It is on the basis of such distinctions in the hemagglutinin antigen that influenza A viruses are classified.

In 1936 a widespread epidemic of respiratory illness similar to that engendered by influenza A virus infection was observed. The virus isolated from acutely ill patients was propagated in ferrets. However, attempts to identify the virus with known immune sera to human or swine influenza A viruses were not successful. The new virus was designated influenza B. Cross-immunization of ferrets with influenza A and B viruses failed to produce any evidence of cross-protection, in contrast to the responses of ferrets, mice and swine to influenza A viruses (Francis, 1940; Francis, 1941).

In addition to the observations of neutralizing antibody in serum, experiments indicated a role for local immunity. Francis noted that "immunity resulting from infection was more effective than that acquired under other conditions" (1941). Passive immunization with convalescent serum resulted in transient immunity, but mice and ferrets could be readily infected after titers of circulating antibody had dropped (due to catabolism of the antibody). This observation was in contrast to the virtually permanent immunity to reinfection afforded experimental animals by "natural infection" (Francis and Magill, 1935; Francis and

Shope, 1936).

Infection of mice with influenza viruses is characterized by destruction of the mucous membrane of the pulmonary parenchyma followed by leukocyte infiltration and edema. In some infections, the epithelial cells desquamate (Loosli, 1949). The bronchial epithelium of infected animals has been shown by immunofluorescence to contain viral antigens in both the cytoplasm and the nuclei (Hers et al., 1962). On a macroscopic level first focal, then larger areas of purple lesions develop (Horsfall, 1939).

Rickard and Francis (1938) observed that intraperitoneal inoculation of 100,000 to 1,000,000 minimal lethal doses of mouse-lung adapted influenza A/PR/8/34 virus (PR8 virus) resulted in the appearance of the gross pulmonary lesions observed in mice inoculated by intranasal instillation of virus. However, injection of smaller quantities of virus did not produce influenza pneumonia.

Analysis of the Antibody Response

As described above, high titers of serum neutralizing antibody to homotypic virus are protective. Immunity following passive transfer of antibody was shown to be of short duration (Loosli et al., 1953). Experimental studies involving the infection of mice with recombinant influenza viruses were undertaken by Schulman, Kilbourne and co-workers. They observed that immunization with a virus which possessed the same HA but a different NA as the challenge virus provided complete protection from subsequent infection. In contrast,

mice immunized with a virus which had the same NA but a different HA as the challenge virus were as readily infected as unimmunized controls, but these mice experienced a modified disease and had lower peak titers of virus (Schulman et al., 1968). Homotypic immunity on mice resulting from previous infection was compared to immunity resulting from inoculation with inactivated virus. Although the two groups of mice had comparable serum antibody titers, previously vaccinated infected mice could not be reinfected for periods lasting as long as a year (Schulman, 1973).

A consequence of the antigenic relatedness of influenza viruses is "original antigenic sin" (Francis et al., 1953). Following initial exposure to one influenza A virus, exposure to an influenza A virus of a different subtype results in the production of serum HI antibody which not only reacts with the second antigen, but also with the first virus encountered. In addition, all of the newly synthesized antibody can be adsorbed by the initial virus, whereas only the antibody reacting with the second antigen can be adsorbed by the latest virus. Similar observations have been made with other cross-reactive antigens including 2,4-dinitrophenyl and 2,4,6-trinitrophenyl haptenic groups both in vivo: (Russell and Eisen, 1969) and in vitro: (Klinman et al., 1973). Other antigens with which the same phenomenon has been observed are streptococcal group A and A-variant antigens (Cramer and Braun, 1973), meta-aminobenzoic acid and meta-sulfanilic acid haptenic groups (Deutsch et al., 1973), bovine serum albumin

and human serum albumin (Gilden, 1963), and histocompatibility antigens (Dorf and Eguro, 1973). Virelizier et al. (1974a) determined that mice normally respond to two categories of antigens, termed "specific" and "cross-reactive" on influenza virus hemagglutinin molecules. In sequential immunization with heterotypic viruses, it is the latter category of clones which are restimulated.

Schulman (1965) observed that in the absence of serum antibody to the challenge virus, mice previously infected with influenza A viruses were protected against challenge with heterotypic virus, whereas mice which had been immunized with inactivated virus or infected with B/Lee virus were not protected. Similar observations were made in ferrets (McLaren and Potter, 1974) and in hamsters (Potter et al., 1973).

Experimental vaccination with formalin inactivated virus has been shown to be as effective as untreated virus in stimulating a serum antibody response (reviewed in Salk et al., 1945; Fazekas de St. Groth and Donnely, 1950). This form of immunization, which has been applied routinely in man, has been shown to be effective in producing an increase in serum antibody titers, but has been shown to be associated with toxicity (Parkman et al., 1977). Consequently, in recent years other methods of immunization have been studied, principally subviral forms of the surface antigens (chemically disrupted virus or isolated glycoprotein preparations) and attenuated virus vaccines. Detergent disrupted virus, while relatively free of toxicity, is poorly immunogenic in unprimed

mice (Barry et al., 1974; McLaren et al., 1977), in ferrets (McLaren et al., 1974) and in children (Wright et al., 1977). Vaccines comprised of isolated glycoproteins have also been shown to have reduced immunogenicity in unprimed hamsters (Potter and Jennings, 1976; Laver and Webster, 1976) and in man (Webster et al., 1977). However, in rabbits (Laver and Webster, 1976) or in primed animals of other species, these forms of the viral antigens have been found to be effective in recalling immunological memory.

The route of vaccine administration is also a critical consideration. Webster (1968) observed that rabbits responded with the highest HI antibody titers after intravenous administration of vaccine and less efficiently to intraperitoneal immunization. Subcutaneous administration of formalin inactivated virus was the poorest of the three routes studied. Although parenteral administration of formalin inactivated virus to mice is effective, in unprimed animals, intranasal inoculation failed to elicit either an antibody response or protection against challenge with homotypic virus. In contrast, parenteral immunization followed by intranasal immunization with inactivated homotypic virus resulted in a boost in antibody levels and in enhanced immunity (Schulman, in Kilbourne et al., 1973).

Immunosuppression

With the advent of modern immunological techniques such as thymectomy, employment of antibody to specific lymphocyte populations, and pharmacologic manipulation of experimental

animals, understanding of the cells and factors important in the immune response to influenza virus has grown. Nevertheless, our knowledge is still incomplete with respect to genetic differences in the immune response to influenza virus antigens, effector cell pupulations important in recovery and the relative efficacy of different forms of antigen presentation in eliciting immune responses.

The antibody response to the hemagglutinin antigen has been shown to be T cell dependent (Virelizier et al., 1974; Sullivan et al., 1975; Burns et al., 1975; Wyde et al., 1977; Iwasaki and Nozima, 1977; Schulman et al., 1977). Immune interferon production in response to influenza virus had been found to be T cell dependent (deMaeyer-Guignard, and deMaeyer, 1971; Iwasaki and Nozima, 1977).

Antithymocyte serum treatment did not alter the course of a lethal infection with influenza virus, but mice treated with ATS had increased mortality in a less virulent infection (Hirsch and Murphy, 1968). In other studies, anti-lymphcyte serum treated mice showed increased survival rates and reduced development of lung consolidation when infected with an influenza H2N2 virus. The mice also showed increased duration of high virus titers in lung and developed lower titers of serum antibody (Suzuki et al., 1974). However, other investigators, using ATS observed that treated mice developed more severe lung lesions, had higher virus titers in lungs and showed increased mortality due to H2N2 virus infection. ATS treated mice produced less HI antibody than

NRS treated controls animals, and the immune deficit could not be overcome with passive transfer of physiological quantities of immune serum (Schulman et al., 1977).

Cyclophosphamide-treated lethally infected mice died later than untreated mice, and were found to have impaired virus clearance, decreased levels of interferon, and lower titers of HI antibody (Singer et al., 1972). On the other hand, in a non-lethal infection, cyclophosphamide treatment was associated with mortality. In an infection with a more virulent H2N2 virus, treated mice died later than untreated controls (Hurd and Heath, 1975). In another series of experiments, cyclophosphamide treatment increased susceptibility to fatal PR8 virus infection. This defect, however, could be overcome with passive transfer of immune serum (Virelizier, 1975).

Athymic nude mice have been used in several studies. Although death was delayed, in infected nude mice virus clearance from lungs was impaired. Moreover, increased mortality and spread of virus to spleens was observed in infected nude mice (Sullivan et al., 1975). Priming of nude mice with influenza virus vaccine did not alter the course of fatal infection (Lucas et al., 1978). In other studies, nude mice showed increased survival times and little cellular infiltration or lung lesion development when infected with a H3N2 virus. Virus was, however, isolated from the brains of infected nude mice (Wyde et al., 1977).

In experiments employing adult thymectomy, irradiation and bone marrow reconstitution, infection of mice with PR8 virus was found to be characterized by diminished serum antibody titers, isolation of virus from brain tissue and increased mortality. Administration of immune serum prevented the spread of virus to the brain and death (Virelizier, 1975). Another group found that this treatment diminished the immune interferon response and antibody response to PR8 virus infection. Similar to the observations of Sullivan, the virus replication was not limited, and isolation was made of virus in brain tissues. However, in contrast to the observations in infected nude mice, lung lesions were observed (Iwasaki and Nozima, 1977).

These experiments have shown uniform depression of serum antibody responses in immunosuppressed infected mice. In contrast, in some studies, lung consolidation was more or less severe in control animals. Immunosuppressed animals almost uniformly succumb to infection. However, when especially virulent infections were employed, immunosuppressed animals died a few days after intact controls. In some experiments, passive administration of hyperimmune serum prevented death, but in these experiments large quantities of antibody were administered within a day of infection, long before an intact host would mount a response. It is probable that the transferred serum neutralized virus, preventing spread and modulating infection.

One strain of mice, A2G, has been described which is genetically resistant to neurotropic influenza A/WSN/33 virus infection and relatively resistant to respiratory infection with the same virus (Lindenmann and Klein, 1966). When A2G mice were immunosuppressed with cyclophosphamide or by X-irradiation, the resistant state was unaltered, even though mice were incapable of generating either a detectable DTH or antibody response. In addition, interferon levels were not different in the infected immunosuppressed mice and in untreated A2G mice (Fiske and Klein, 1975). However, unpublished studies by Meyer, Schulman and Kilbourne demonstrated a decrease in the resistance of A2G mice following cyclophosphamide treatment. In other studies, the autosomal dominant mx gene of A2G mice was introduced into athymic BALB/c nude mice. In the F_2 generation, nu/nu,mice which were mx/+ were found to be resistant to infection with influenza viruses, while in contrast, their nu/nu mx+/+ and nu/+, mx+/+ littermates were susceptible to infection (Haller and Lindenmann, 1974). These studies indicate that the resistance of AG2 mice is not based on immune responsiveness as it is currently understood. Surveys of inbred and randombred strain of mice have shown no association between the mx trait and the major histocompatibility complex (Lindenmann and Klein, 1966).

Effector T Cell Functions

Several investigators have assessed effector T cell functions in lymphocytes obtained from animals which were not immunosuppressed. Rat lymphocytes (Woodruff and Woodruff, 1974),

peripheral blood leukocytes of rat and human origin (Boand et al., 1957) and mouse peritoneal macrophages (Shayegani et al., 1974) have been found to bear receptors for influenza viruses. In an in vitro proliferation assay, Butchko and his co-workers (1977) have demonstrated that influenza viruses with the H2 hemagglutinin are mitogenic for murine lymphocytes, and Dolin et al. (1978) observed antigen-specific blastogenic responses in human peripheral blood lymphocytes taken from donors immunized with influenza virus vaccines. Hanson and colleagues (1957) observed that peritoneal exudate cells from influenza virus immune mice phagocytosed PR8 virus more rapidly than peritoneal cells from naive mice. In other studies, peritoneal exudate cells from immune mice were shown to exhibit antigen specific DTH sensitivity in a migration inhibition assay (Feinstone et al., 1968). At a time when virus shedding was curtailed and when serum antibody began to appear, migration inhibition responses could be elicited in peritoneal exudate cells from guinea pigs which had been infected with HK virus (Wetherbee, 1973).

Another T cell function, the development of cytotoxic T cells, has been detected in the spleens of mice after peritoneal injection of intact influenza A viruses or after infection of mice (Cambridge et al., 1976; Doherty et al., 1977; Ennis et al., 1977d; Braciale, 1977a; Yap and Ada, 1977, Zweerink et al., 1977a). As in other examples of the development of cytotoxic T cells after stimulation with viruses, the effector cells are H-2 restricted. AG-B restricted cyto-

toxic T cells have also been described in rats after inoculation of influenza viruses (Marshak et al., 1977); and as mentioned above, McMichael and his colleagues (1977) described a HL-A restricted human peripheral blood T cell mediated killing of infected mitogen-stimulated target cells.

There is some disagreement in the published literature about the nature of the specificity in the response of murine effector cells to infected target cells. Two groups have described a system in which the specificity of the cytotoxicity is restricted to infected cells bearing a hemagglutinin antigen shared by the immunogen (Cambridge et al., 1976; Ennis et al., 1977d). The remaining investigators have found that cytotoxicity is type specific (not reactive with target cells infected with other types of influenza virus) but is cross-reactive among the subtypes of influenza A virus. It has been suggested that the cross-reactive recognition might be due to the expression of M protein on the surface of infected cells in vivo and on the target cells used in the assay in vitro. (Braciale, 1977b; Ada and Yap, 1977; Biddison et al., 1977). The expression of M protein on some of the target cells might be related to the abortive nature of influenza virus infection in those cells (Ennis et al., 1977b). There is also disagreement whether inactivated viruses can stimulate a cytotoxic T cell response in vivo (Ennis et al., 1977c; Braciale, 1978). Furthermore, much

larger quantities of isolated hemagglutinin were required to stimulate an *in vitro* secondary response in primed murine spleen cells than intact virus (Zweerink, *et al.*, 1977b).

Adoptive Transfer Studies

Experiments have been designed utilizing adoptive transfer of sensitized lymphocytes to study the effects of these cells on the course of influenza virus infection in recipients. Several laboratories have undertaken experiments utilizing this technique, but the data reported and the interpretations of the results are conflicting. Cate and Mold (1973; 1975) hyperimmunized donor mice by repeated subcutaneous injection of either formalin inactivated or untreated virus. Eight days after the last immunization, spleen cells and lymph node cells were collected for transfer. Recipients were infected on the day of transfer. They observed that infected mice which received washed lymph node cells from mice sensitized with formalin inactivated PR8 virus had increased mortality. In contrast, if the same donor cells were not washed before transfer, no increase in mortality was observed in infected recipients. When washed cells derived from mice sensitized with formalin inactivated virus were treated with ATS and complement, the response of the infected mice was similar to that of control mice to the 50% lethal infection. If donor cells were obtained from mice which had been previously infected or from mice which had been hyperimmunized with untreated PR8 virus, the course of infection was not altered, whether or not the cells were washed, prior to transfer. The

authors concluded that immunization with formalin inactivated virus induced a cell mediated immunopathology which was absent in mice which had been primed with live virus. However, the immunological specificity of these observations is unclear, since B/Lee virus infected recipients which had been given washed lymphocytes from donors sensitized with formalin inactivated virus (PR8) were found to have increased mortality, as described above.

Russell (1977) primed donor mice with UV inactivated PR8 virus (H0N1) 10 days prior to transfer of spleen cells. At the time of cell transfer, some recipient mice were also sensitized with inactivated PR8 virus. Challenge 3 days later was with A/PR/8/34(HO)-Eng/939/69(N2) virus infection. Infected mice which received sensitized cells produced more antibody and had less virus in lungs two days after infection than did mice which received inactivated PR8 virus but no spleen cells. Infected recipients which received immune spleen cells but no inactivated virus boost prior to infection made significant quantities of antibody, but had the same virus titers in lungs as did control infected mice. Russell concluded that antibody is an important factor soon after infection, but that other mechanisms might also be involved. It is interesting that despite the presence of high titers of serum antibody to the PR8 virus hemagglutinin, mice which had received immune spleen cells but no antigen boost, still had high titers of H0N2 virus in their lungs.

Virelizier and his co-workers (1975, 1976) primed donors by sublethal infection 2 months prior to spleen cell transfer. Immune spleen cells were inoculated 8 days prior to infection. Some mice also received a large dose of bromelain isolated PR8 virus hemagglutinin 7 days prior to infection. Mice which received only the glycoprotein before infection did not have measurable serum HI antibody 7 days after immunization, and all succumbed to infection. Mice which received spleen cells from donors which had been primed by infection, transferred one week before challenge infection did not have measurable antibody on the day of infection. Of these mice, 10/13 died. In contrast, recipients of the same cells and of isolated glycoprotein one week before infection had high antibody titers at the time of infection and all the mice survived. The investigators found that they could duplicate the protection afforded by passive administration of "physiologic quantities" of immune serum (rabbit antibody to either whole virus or to isolated hemagglutinin) at the time of infection. They also observed that mice immunosuppressed with cyclophosphamide or by thymectomy, irradiation and reconstitution of bone marrow cells, could be protected by passive transfer of antibody prior to infection. Therefore they concluded that the specific immune defects in these mice were related to the inability to make antibody, and that antibody has an important role in protection against infection. They stated, "transferred immune spleen cells were shown to provide full protection only when they were actually secreting antibody". However, it is generally accepted that B cell function can be

transferred poorly, at best, to adult recipient mice, unless the recipients have been lethally irradiated (Celada, 1966). It is very likely that among the cells which were transferred were memory T cells of both effector (DTH, cytotoxic) and helper subsets. Helper cells of donor origin probably collaborated with recipient macrophages and B cells to elaborate the high level of HI antibody observed. The antibody alone probably contributed to the protection in these mice, particularly as it was present before challenge infection and undoubtedly neutralized some, if not all, of the virus. Moreover, the donor lymphocytes were not fractionated prior to transfer and it is possible that the same effect would have been observed using B cell depleted populations of immune spleen cells. The experiments were not designed to ascertain the role of DTH activity in the recipients. The fact that death was delayed in the 10/13 mice which received immune spleen cells but no antigen prior to infection, suggests that an immune response was mounted even in the absence of measurable serum HI antibody.

Schulman and his associates (1977) used adoptive transfer to study the effect of immune spleen cells on the course of sublethal influenza virus infection. Donor mice were sentitized with untreated A/Japan/305/57 virus 6 days before spleen cell transfer. Recipient mice were infected with the same virus one day before cell transfer. Recipients of immune spleen cells had less virus in lungs, less extensive lung lesions and lower antibody titers than recipients of naive

spleen cells. Cell fractionation studies were done on the transferred cell populations. The same protective effect was demonstrated in T cell enriched lymphocytes obtained from a nylon wool column (Julius et al., 1973).

The comparative analysis of these reports using different experimental procedures, immunization protocols and intervals between transfer of cells from sensitized donors and infection, is difficult. In two of these studies, high titers of serum antibody was found to be present before infection. Since serum antibody is protective, cell mediated effector responses could not be segregated. It would have been interesting if Virelizier had used a less severe infection, to determine whether protection was correlated with cell transfer. Twenty-three percent of adoptively immunized mice survived an infection which was 100% fatal in untreated mice, and the deaths which occurred in the adoptively immunized group occurred two days after the deaths in control animals. Cate and Mold's studies are not easily interpretable in light of the absence of immunological specificity (sensitization of donors with formalin inactivated PR8 virus provided cells which resulted in increased mortality in B/Lee virus infected recipients). In addition, transfer of cells from mice immunized with formalin inactivated virus had no effect on mice undergoing more moderate infections with either PR8 or B/Lee virus.

Schulman, in contrast, observed decreased virus titers, less extensive lung lesions, and lower serum HI antibody titers in recipients of spleen cells from mice sensitized 6

days previously. Since this transferred activity could be effected with column purified T cells, it is strongly suggestive of a T cell effector function(s). Two explanations of the lower serum HI antibody titers are possible. First, that with less virus in lungs, the antigenic stimulus might have been diminished. Second, it is possible that the T cell subsets transferred were enriched for suppressor cells. In Russell's experiments, donor cells were taken 10 days after sensitization, and administered to recipients 3 days before infection. It was possible that the antibody responses in recipient mice, even those not given antigen boost, was due to the predominance of helper cells, not suppressor T cells in the transferred population. Sercarz and his co-workers (1978) sensitized mice with β-galactosidase and took spleen cells at intervals. They assessed the ability of sensitized T cells to collaborate with B cells in an in vitro response to the same antigen. They found that helper cells predominated in lymphocytes harvested within the first 4-5 days after sensitization, followed by a period of net suppressor cell activity. From 9-10 days after immunization onward, helper cell function again was dominant. These observations support the findings of Schulman and of Russell, and suggest a mechanism for the serum antibody titers detected.

Kinetics of T Cell Responses During Infection

In other experiments designed to study the kinetics of effector cell responses in infected mice, varying results have been obtained. In part this might be due to infection by viruses

of varying virulence and infections which differed in severity. In mice undergoing an infection with influenza A/WSN/33 virus, an *in vitro* correlate of DTH, migration inhibition was detected in lymph node cells as early as 5 days after infection. In contrast, activity in splenic lymphocytes was not demomstrated until 6-7 days after infection (Cambridge *et al.*, 1976). In these same mice, cytotoxic T cells were detected at peak levels 7 days after infection in lymph node lymphocytes, and 9 days after infection in splenic lymphocytes. In a brief report, Doherty described peak levels of cytotoxic activity in lymph nodes 7 days after infection; no measurement of splenic cell cytotoxic activity was reported (Doherty *et al.*, 1978). Ennis and his co-workers (1977a) described cytotoxic T cell activity peaking 4 days after A/Pt.Chalmers/1/73 virus infection in cells obtained from local lymph nodes, and at moderate levels of cytotoxic activity in splenic lymphocytes, between days 6 and 12 after infection (Ennis *et al.*, 1977a). Yap and Ada (1978) described the simultaneous appearance of cytotoxic T cell activity in lymph node cells, spleen cells, and in Ficoll-Hypaque gradient isolated cells from lungs of WSN virus infected mice. They noted a temporal association between the appearance of cytotoxic T cells, the development of lung lesions, and the clearance of virus. They concluded that the spleen does not contribute substantially to the local cytotoxic T cell response since splenectomized mice respond with high levels of cytotoxic activity in the lymphocytes obtained from lungs. However, because ATS

treated mice had diminished levels of cytotoxic activity in the lung-associated lymphocytes, they suggested that the local response in part derived from recirculating T cells.

The rapid progress being made in studying the immune response to influenza virus antigens, can be attested to by the fact that while these studies were in progress, several other laboratories developed cell mediated lympholysis assays similar to the one described in this report. In addition, another laboratory has developed an antibody forming cell assay, similar in many respects to the one described in these studies. During the same period of time several laboratories developed radioimmuno assays, and rocket immunoelectrophoresis has also been applied to influenza virus research. These events suggest that this active field will continue to provide new insights, increasing our understanding of the immune responses to influenza virus antigens.

VII. MATERIALS AND METHODS

Mice. Female BALB/c, special pathogen free, mice (Charles River, Inc.) and female C3H/StHa mice (West Seneca Labs., Buffalo) 6-20 weeks of age were studied in groups of 3-7.

Viruses. A catalogue of the viruses used in these studies is found in Table 1. All influenza viruses were obtained from laboratory stocks of the Microbiology Department, Mount Sinai School of Medicine. Influenza viruses were cultivated in the allantoic cavity of 10 or 11 day embryonated chicken eggs (Shamrock Poultry Farm) for 40 hours at 37°. Viruses were stored as allantoic fluids at -70°. Vesicular stomatitis virus was provided by Jonathan Rosenberg, Microbiology Department, Mount Sinai School of Medicine. Cultivation conditions of VSV can be found in the section entitled "Host Antigen Specificity".

Cell Lines. The tissue culture cell lines used in these studies are listed in Table 2.

Immunization of Mice. In most experiments mice were immunized with untreated allantoic fluid PR8 virus. Stock virus having an HA titer of 1:4096 was diluted 1:4 in phosphate buffered saline (PBS; supplemented with penicillin and streptomycin) and 0.2 ml was injected intraperitoneally.

Infection of Mice. Mice were infected with 30-100 50% mouse infectious doses (MID_{50}) in the form of a small particle aerosol of either PR8 virus or Japan virus (Schulman and Kilbourne, 1963a).

Table 1

VIRUSES USED IN THESE STUDIES

Wild Type Influenza Viruses	Reference	Antigens	Abbreviation
A/PR/8/34		HON1	PR8 virus
A/Japan/305/57		H2N2	Japan virus
B/Lee/40			B/Lee virus
Recombinant Infuenza Viruses	a		
A/Japan/305/57 x PR/8/34		H2N2	Jap-Jap virus
A/Japan/305/57 (H2)-PR/8/34 (N1)		H2N1	Japan-PR8 virus
A/PR/8/34 (HO)-Japan/ 305/57 (N2)		HON2	PR8-Japan virus
A/PR/8/34 (HO)-Hong Kong/8/68 (N2)	b	HON2	PR8-HK virus
A/Hong Kong/8/68 (H3)-PR/8/34 (N1)	b	H3N1	HK-PR8 virus
A/Hong Kong/8/68 x PR/8/34	b	H3N2	X-31 virus
A/Pt.Chalmers/1/73 x PR/8/34		H3N2	MRC-11 virus
A/NWS/33 (HO)-RI/5+/ 57 (N2)	c	HON2	X-7 virus
A/PR/8/34 (HO)-Equine/ 1/56 (Neq1)		HONeq1	PR8-Equi virus
A/Japan/305/57 (H2)-Equine/1/56 (Neq1)		H2Neq1	Japan-Equi virus
Other Virus			
Vesicular Stomatitis Virus		Indiana	VSV

a Bulletin W.H.O., 1971

b Palese and Schulman, 1976

c Laver and Kilbourne, 1966

Table 2

CELL LINES USED IN THESE EXPERIMENTS

Line, Reference	Origin	Where Obtained	Cultivation Conditions
L929 cells (Sanford, et al., 1958)	C3H mouse ($H\text{-}2^k$) Connective tissue	Two lines were used: 1-Microbiology Dept.- Mount Sinai School of Medicine; 2-Dr. Peter Doherty, Wistar Institute	Dulbecco's Modified Eagle's medium with 5% calf serum.
P815 cells (Dunn and Potter, 1957)	DBA/2 mouse ($H\text{-}2^d$) Mastocytoma	Dr. G. Dennert, Salk Institute	Same as L929
BALB/c3T3 (Aaronson and Todaro, 1968)	BALB/c mice ($H\text{-}2^d$) Embryo Fibroblasts	Dr. S. Rohrlich, Rockefeller Institute	Same as L929
MDCK cells (Krug, 1971)	Madin-Darby canine kidney, fibroblasts	Mount Sinai School of Medicine	Reinforced Eagle's Medium
MDBK cells (Choppin, 1969)	Madin-Darby bovine kidney, fibroblasts	Microbiology Department, Mount Sinai School of Medicine	Reinforced Eagle's Medium

Inoculation of Rabbits. Groups of 3 adult albino rabbits (Pocono Rabbit Farm) were inoculated subcutaneously with purified whole PR8 virus or the isolated glycoprotein preparation of PR8 virus as described in the text, and bled at 10,20, and 42 days after inoculation.

Preparation of Rabbit Antisera. Antisera to sucrose gradient purified PR8 virus was obtained from laboratory stocks. Antisera to internal influenza virus proteins were generously donated by Dr. Doris Bucher. M protein and nucleoprotein (NP) were isolated from X-7 virus (H0N2), by SDS disruption and electrophoresis on polyacrylamide gels, first under non-reducing then under reducing conditions (Bucher, 1975; Bucher et al., 1976). The isolated proteins were judged chromatographically pure by the presence of only one Coomassie blue-staining band on analytical SDS-PAGE gels. Rabbits were immunized by foot-pad inoculation of 50 ug protein dissolved in PBS with complete Freund's adjuvant. Six weeks later the rabbits were restimulated with a second injection of 50 ug of either M protein or NP. Sera were collected one week later and stored at -20°. Antiserum to M protein and to NP did not inhibit hemagglutination or neuraminidase activity of PR8 or HK viruses.

Adsorption of anti-M protein antiserum with X-7 virus. Preliminary experiments showed significant immunofluorescence in the presence of anti-M protein. In order to determine whether this antiserum made to M protein isolated from X-7 virus contained antibody recognizing other determinants ex-

pressed on the surface of the virus, X-7 virus was purified by sucrose gradient centrifugation. One half ml of the serum was adsorbed with 2 mg of purified virus at 4° for 3 hours. The virus was removed by ultracentrifugation and then by adsorption of the serum with 0.5 ml packed human erythrocytes.

Preparation of Inactivated Viruses. One large batch of 175 ml allantoic fluid PR8 virus (HA titer 1:8196, $EID_{50}10^{8.4}/0.1$ ml) was concentrated on a continuous 30-60% sucrose gradient. The virus was pelleted at 25,000 rpm for 30 min., and resuspended in phosphate buffered saline (PBS) containing Ca++ and Mg++ (0.01% each as Cl salt), and aliquots were treated with UV light or formaldehyde or extracted with Triton X-100.

UV Inactivation: A 5 ml aliquot of the gradient purified resuspended virus was irradiated in a 60 mm tissue culture dish at a distance of 15 cm with a GE G8T5 bulb for 15 seconds under conditions of continuous mixing. This dose of irradiation was chosen to avoid excessive genetic damage to the virus which would preclude its capacity to undergo even an abortive infection of cells. The inactivated virus preparation was divided into several portions and frozen for storage. This preparation was capable of abortive infection of L929 cells as determined by the development of hemadsorption in 80% of the cells 8 hours after infection. The preparation retained some egg infectivity but this was reduced ten-thousand fold from the infectivity of the initially purified virus. Initial experiments in C3H mice utilizing

untreated virus diluted to contain an equivalent amount of infectious virus as the UV inactivated preparation ($10^{4.4}EID_{50}$/ 0.1 ml) failed to stimulate an antibody response or to evoke either an AFC or cytotoxic response.

Formalin Inactivation: Another aliquot of the gradient purified virus was treated overnight at 4° with formalin (0.25%) and then dialyzed overnight against 500 volumes of PBS to remove unreacted formalin. No egg infectious virus was detected in this preparation, but its HA titer was unaffected. The formalin inactivated virus was unable to undergo abortive infection in L929 cells, as demonstrated by the absence of hemadsorption.

Triton X-100 Extraction: The procedure of Scheid and Choppin (1973) was used. Following treatment with triton, the glycoprotein fraction was extracted with cold butanol and dried with ether, resuspended in PBS, dialyzed against water, brought up to isotonicity with 10X PBS. The preparation retained both neuraminidase activity and hemagglutinating activity with human and sheep erythrocytes, but had no residual infectivity.

Inoculation of Mice with Different Forms of PR8 Virus Antigens. Allantoic fluid virus, gradient purified virus, UV inactivated virus, or formalin inactivated virus preparations were inoculated intraperitoneally into mice in a volume of 0.2 ml containing an HA titer of 1:1024. In order to administer an equivalent quantity of viral antigenic mass of the isolated glycoprotein heteropolymer, this suspension was diluted in

PBS to contain the same neuraminidase activity present in the other preparations (5.5 I.U., nmoles N-acetyl neuraminic acid/min/ml).

Commercial vaccines In some experiments, commercial vaccines containing influenza A/Pt. Chalmers/1/73 (H3N2) virus antigens were used to extend our observations. These were Merrell National produced MRC-11 formalin inactivated whole virus vaccine (Lot 26537A) and Wyeth Laboratories N-butyl phosphate disrupted MRC-11 (Lot 1607#5). These vaccines, generously provided by Dr. F.A. Ennis of the Bureau of Biologics, had been used for related studies in that laboratory (1977c). As an infectious virus control, we used MRC-11 virus propagated in our laboratory. Both vaccines contained 1000 CCA units/ml. In some experiments groups of mice received 0.5 ml (500 CCA units) of either vaccine. Other groups of mice received a dilution of either vaccine, calibrated to contain the same neuraminidase content as the standard PR8 virus vaccine described above. For the Merrell National vaccine, the adjusted dose was 0.2 ml of 1:4 dilution of the vaccine; for the Wyeth preparation, the standard dose was 0.2 ml of a 1:8 dilution of the vaccine.

Preparation of lymphocytes, lungs, and serum samples

In most experiments, mice were sacrificed at intervals after sensitization by injection of sodium pentobarbital. Mice were exsanguinated and individual serum samples were stored at -20°.

Spleens of mice were removed immediately upon sacrifice and were placed in 5 ml cold Hanks Balanced Salt Solution (HBSS) buffered with 15 mM HEPES. Single cell suspensions were made by forcing spleens through 100 mesh stainless steel screens. Clumps of cells were removed by settling at 4°. Lymphocytes were washed, resuspended, and serially diluted in HBSS. Lymphocytes were counted in hypotonic medium (99.4 ml distilled water, 0.6 ml glacial acetic acid, tetrachrome dye). Viability was determined by trypan blue exclusion, and routinely was greater than 90%.

After infection, lungs were removed, scored for macroscopic lesion, (measured as the percent of the lung surface involved)(Horsfall, 1939). Individual lungs were homogenized and stored at -70° for determinations of virus titers. Mediastinal lymph nodes within a group were pooled as were spleen cells from the same animals.

Serologic Assays. Serum hemagglutination inhibiting (HI) antibody was measured by standard microtiter assay using human type 0 RBC (Hierholzer and Sugg, 1969). Neuraminidase inhibiting (NI) antibody was measured by using fetuin as substrate during an 18-hour incubation (Aymard-Henry et al., 1973). Studies were done on individual serum samples using appropriate recombinant viruses. To determine mercaptoethanol sensitivity, serum specimens from groups of mice were pooled and an aliquot was treated with mercaptoethanol by the method of Yasuda et al., (1977). It was not possible to asses the mercaptoethanol resistant fraction of NI antibody

because of the sensitivity of enzyme activity to reduction (Bucher and Palese, 1975).

Determinations of Infectious Virus in Lungs. Lungs were individually homogenized, serially diluted, and inoculated into embryonated chicken eggs (3 eggs/dilution). After 40 hours of incubation at 37°, allantoic fluids were harvested and tested for the presence of virus by hemagglutination (Schulman and Kilbourne, 1963b).

Antibody Forming Cell Assay:

Preparation of Target erythrocytes. Sheep red blood cells (SRBC) in Alsever's solution were obtained fresh weekly (Pocono Rabbit Farm). For most experiments the SRBC were coated with different unpurified allantoic fluid seed viruses in accordance with the method of Russell et al., 1975, except that KIO_4 was used at a concentration of 2.5×10^{-4}M in PBS. The virus-coated SRBC were washed and resuspended to a final concentration of 20% in HBSS and stored at 4°. In some experiments SRBC were coated with isolated glycoprotein subunits (HA-NA) obtained after Triton X-100 extraction according to the method of Scheid and Choppin (1973).

Table 3 demonstrates how AFC responses to HA and NA antigens were compared using different virus-coated SRBC targets. SRBC were coated with virus antigenically identical to the immunizing strain (HON1); HON2 virus containing the same HA and a different NA; H3N1 containing the same NA and a different HA; or with a completely heterotypic virus H3N2.

Table 3

ANALYSIS OF AFC RESPONSE BY THE USE OF RECOMBINANT VIRUSES TO COAT TARGET ERYTHROCYTES

Mice Sensitized With	SRBC Coated With	Measured AFC To
A/PR/8/34 virus (H0N1)	PR8 virus (HON1)	Both Surface Antigens
A/PR/8/34/ virus	PR8-HK virus (HON2)	Hemagglutinin
A/PR/8/34 virus	HK-PR8 virus (H3N1)	Neuraminidase
A/PR/8/34/ virus	X-31 virus (H3N2)	Heterotypic Antigens
A/PR/8/34 virus	B/Lee/40 virus	Host Specific Antigens

Direct AFC Assay. (IgM secreting cells): The following were combined in 10 x 75 mm glass culture tubes immersed in a 45° water bath to give a total volume of 0.45 ml: 0.5% Indubiose A-37 (Accurate Chemical and Scientific Co., Hicksville, N.Y.); virus coated SRBC, 1%; and 0.1 ml of ten-fold dilutions of spleen cell suspensions in HBSS. Immediately after the addition of the spleen cells, the duplicate or triplicate tubes were mixed and the contents poured onto 0.1% Indubiose-primed microscope slides. The agarose was allowed to gel at room temperature before incubation on trays at 35° in a humidified, 5% CO_2 gassed incubator for 5 hours. During the last hour each slide was incubated with 2 ml guinea pig complement (lyophilized, virus and virus antibody free; Flow Laboratories) diluted 1:20 in veronal buffer.

Indirect AFC Assay. (IgG and IgA secreting cells): IgG and IgA secreting cells were determined by the method of Pierce et al., (1971). Slides were set up in the same manner as those above except that 25 µl goat anti-mouse IgM (heavy chain specific, Cappell Labs.) was added to the agarose mixture before pouring onto the slides. During the third and fourth hours of incubation 2 ml rabbit anti-mouse IgG (Bionetics) or rabbit anti-mouse IgA (Miles Research Products) diluted 1:15 in HBSS was added to each slide. During the final hour of incubation this fluid was removed and replaced with guinea pig complement as above.

Host Antigen Specificity. In some experiments SRBC were coated with viruses grown in tissue culture. In these ex-

periments confluent cultures of canine kidney (MDCK) cells were infected with 10^4 to 10^5 EID_{50} of influenza PR8, Japan, X-31, or B/Lee viruses or with VSV. The infected monolayers were incubated for 40 hours under liquid medium (Reinforced Eagles medium, Microbiological Assoc.; supplemented with bovine albumin, Miles; and 1 ug TPCK trypsin/ml, Worthington). Culture fluids were harvested, clarified by centrifugation and adjusted to HA titers comparable to those of egg grown virus. The MDCK grown viruses were coated onto SRBC at the same time and under the same conditions as egg grown virus.

VSV was also cultivated in the 7 day embryonated chicken egg, in the allantoic cavity for 24 hours. Both the MDCK grown and the egg grown VSV preparations were treated in vitro with 66 TBA units/ml neuraminidase from V. cholerae (Behring lot #475), for 45 minutes at 37° in a water bath. The viruses were pelleted by ultracentrifugation and resuspended in 3 ml PBS. Both treated VSV preparations were used to coat SRBC.

Cytotoxicity Assays. L929 cells, the generous gift of Dr. Peter Doherty (Wistar Institute), were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% fetal calf serum. Initial studies indicated that a ratio of 100 effector cells to one target cell was optimal. Preliminary observations confirmed that the assay system showed an H-2 restriction, that the cytotoxicity was specific for influenza A virus infected cells in influenza A virus sensitized mice, or specific for influenza B virus infected targets in influenza

B immunized mice. Initial experiments also demonstrated equivalent levels of cytotoxic activity when the means of spleen cells from individual animals and pooled populations of spleen cells were compared.

In most experiments 0.5-1.0 x 10^5 L929 cells were seeded into 35 mm tissue culture dishes. The following day all dishes were washed with serum free medium and half were infected with influenza viruses (approximately 1000 EID_{50} per cell). After adsorption, the dishes were washed to remove any unadsorbed virus, and 25 uCi $Na_2{}^{51}CrO_4$ (New England Nuclear) in one ml of serum free medium was added per dish. After a 5 hour pulse, the dishes were washed three times before effector cells were added in 5% serum containing DMEM. This was followed by an additional 12 hour incubation at 35°. In general, four replicates were used and the standard deviations were approximately 2-5%. Total releasable counts were determined after the addition of 1 ml 5% Triton X-100 in distilled water to the washed labeled dishes. Aliquots of supernatants were counted in a Beckman Biogamma machine. Specific Immune Release (SIR) was determined by the formula:

$$SIR = 100 \times \frac{\text{(counts released by sensitized lymphocytes} - \text{counts released by control lymphocytes)}}{\text{total releasable counts} - \text{counts released by control lymphocytes}}$$

Statistical significance was determined by the Student's t test.

Histocompatibility Restriction. In early experiments both BALB/c 3T3 cells and L cells were used in cytotoxicity experiments. BALB/c 3T3 cells were the gift of Dr. Susan Rohrlich of the Rockefeller University. BALB/c 3T3 were cultivated in the same manner as L929 cells.

In the H-2 restriction assays, both BALB/c and C3H mice were sensitized 6 days prior to the determination with 0.2 ml PR8 virus, HA 1:1024. Unimmunized controls were also used. The cytotoxicity assay was done under the conditions described above.

Indirect Immunofluorescence. Three tissue culture cell lines were used in these studies: murine fibroblasts (L929 cells) which are abortively infected with most influenza A viruses (Franklin and Breitenfeld, 1959; Rott and Scholtissek, 1963; Avery, 1975); bovine kidney cells, MDBK cells, which are productively infected by most influenza viruses but produce non-infectious particles, in the absence of exogenous proteases (Schulman and Palese, 1977); and canine kidney cells, MDCK cells, which are productively infected with influenza viruses and in the presence of trypsin, produce infectious particles. Cells were seeded at 10^5 cells/2 ml culture medium supplemented with serum into Lab Tex culture slides; 24 hours later the cells were washed with serum-free medium and infected with a multiplicity of infection of 50-100 EID_{50} PR8 virus. After 60' adsorption, cells were washed extensively and incubated for 6 hours with serum-free medium at 35°. Cells were washed, incubated for 60' at 4° with normal

rabbit serum (NRS) or with one of the rabbit antisera (anti-M protein, anti-NP, anti-PR8), washed and incubated at 4° for 60'. with FITC-conjugated goat anti-rabbit IgG (Cappel Labs). Slides were washed, air dried, fixed with ethanol, dried, and mounted in 9:1, glycerol:PBS. Fluorescence was observed with a Zeiss research microscope, using a mercury arc lamp and dark-field condenser, FITC excitation and barrier filters (Zeiss). Cells were examined both under dark-field conditions and with the filters in place. Fluorescence was scored 0 (None), ± (dull) to ++++ on the basis of brightness.

In one experiment with anti-NP antiserum, cells were fixed with acetone for 10' before incubation with anti-NP, to assess the ability of anti-NP to detect internal antigens.

VIII. RESULTS

A. Preliminary Results: Testing the Accuracy of the Systems Employed

AFC Assay

Preliminary experiments attempted to use human type "o" red blood cells coated with PR8 virus as targets for antibody and complement mediated lysis, with rabbit antibody to whole PR8 virus and guinea pig complement diluted in veronal buffer. Although the virus coated erythrocytes were agglutinated by specific antibody, lysis was not observed. However, when sheep erythrocytes were substituted for human red cells, lysis was evident. Virus coated red cells immobilized in agarose were exposed to specific antiserum in a radial diffusion assay, for 18 hours at 4^{o} (Russell et al., 1974); subsequently, when incubated at 37^{o} for 1 hour, complement mediated hemolysis was observed in discrete zones surrounding wells containing antiserum, but was absent with normal rabbit serum (Fig. 1) or with antiserum to heterologous viruses (data now shown). A very small zone of hemolysis was observed with antiserum to M protein (data not shown).

Jerne Plaque Determination

In initial experiments BALB/c mice were immunized with 0.2 ml of a 10% SRBC suspension. Five days later, immunized mice and unimmunized control BALB/c mice were sacrificed. Serial 10-fold dilutions of spleen cell suspensions were combined with 25 ul 10% SRBC and 0.3 ml .6% agarose in HBSS in 10 x 75 mm glass tubes in a 45^{o} water bath. Tubes were

FIGURE 1

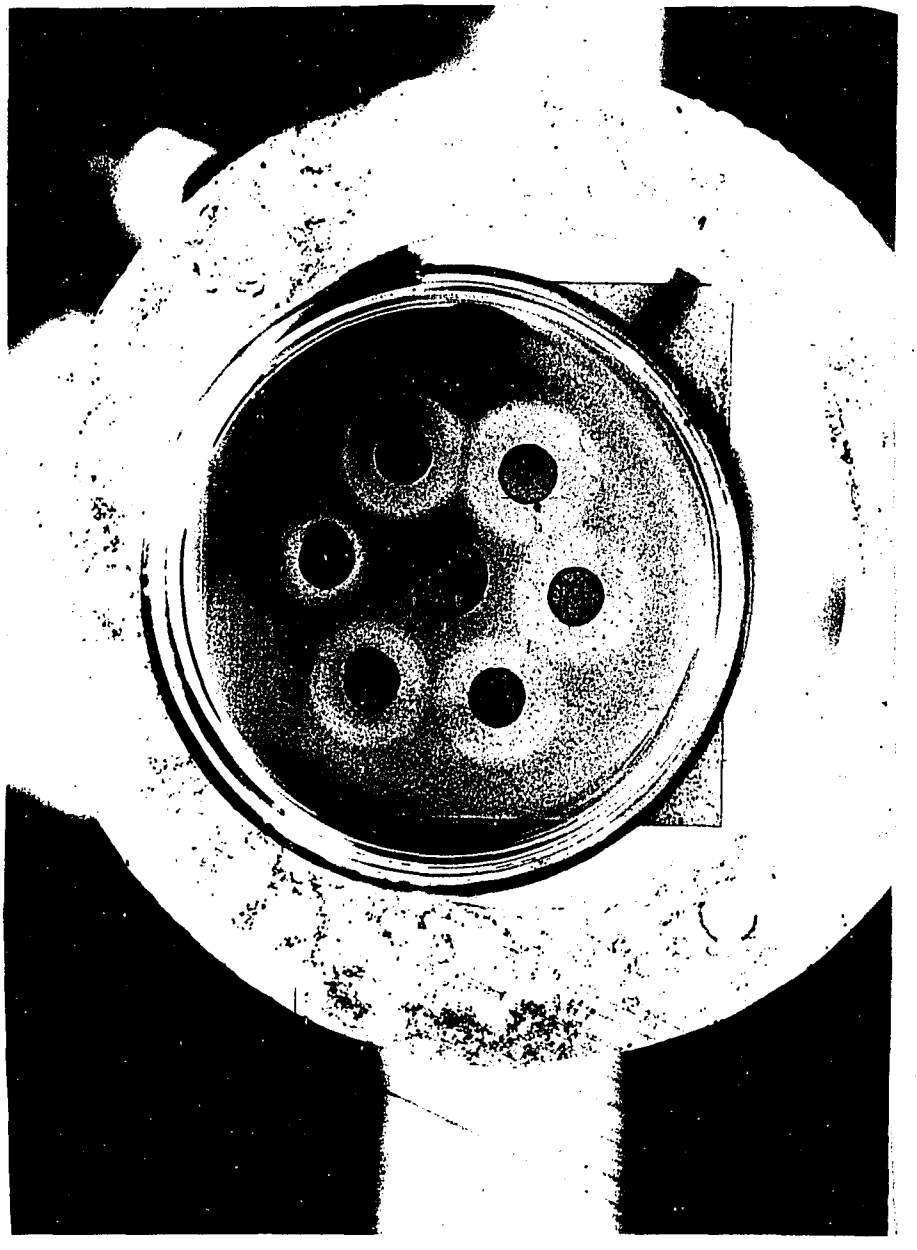

Figure 1. Radial immune hemolysis of PR8 virus coated sheep erythrocytes with antiserum to PR8 virus, method of Russell, et al., (1975). Center well, normal rabbit serum; top well, undiluted antiserum; clock-wise, serial two-fold dilutions of antiserum.

mixed and poured onto microscope slides. Representative slides were incubated with goat anti-mouse IgM to block IgM secreting AFC and facilitated with rabbit anti-mouse IgG. All slides were developed with guinea pig complement during the third hour of incubation at 37^{o} in a 5% CO_2, humidified incubator. Both IgM and IgG secreting AFC were counted. The results demonstrated 85,000 IgM secreting AFC/spleen and 32,000 IgG secreting AFC/spleen. These observations are in agreement with previously published values (Hege and Cole, 1966). The preliminary assays indicated that the conditions for measurement of AFC were not inefficient or toxic and were suitable for assay of AFC responses to influenza virus.

AFC Determinations with Influenza Virus Coated Targets

Preliminary studies were done to determine the optimum ratio of virus and periodate-treated SRBC for good quality hemolytic plaques. It was found that equal volumes of packed SRBC and viruses like PR8 virus with an HA titer of 1:4096, gave useful targets. Therefore, recombinant viruses which grew to high titers in eggs were used rather than the wild type virus. A sample plaque is shown in Fig. 2.

Initial Studies for the Cell Mediated Cytotoxicity Assay

Preliminary experiments were carried out to determine the multiplicities of infection required for influenza A and B viruses, and the time of expression of influenza virus antigens on the surface of infected L929 cells, P815 cells, and BALB/c 3T3 cells. The results were assayed by measurement of

FIGURE 2

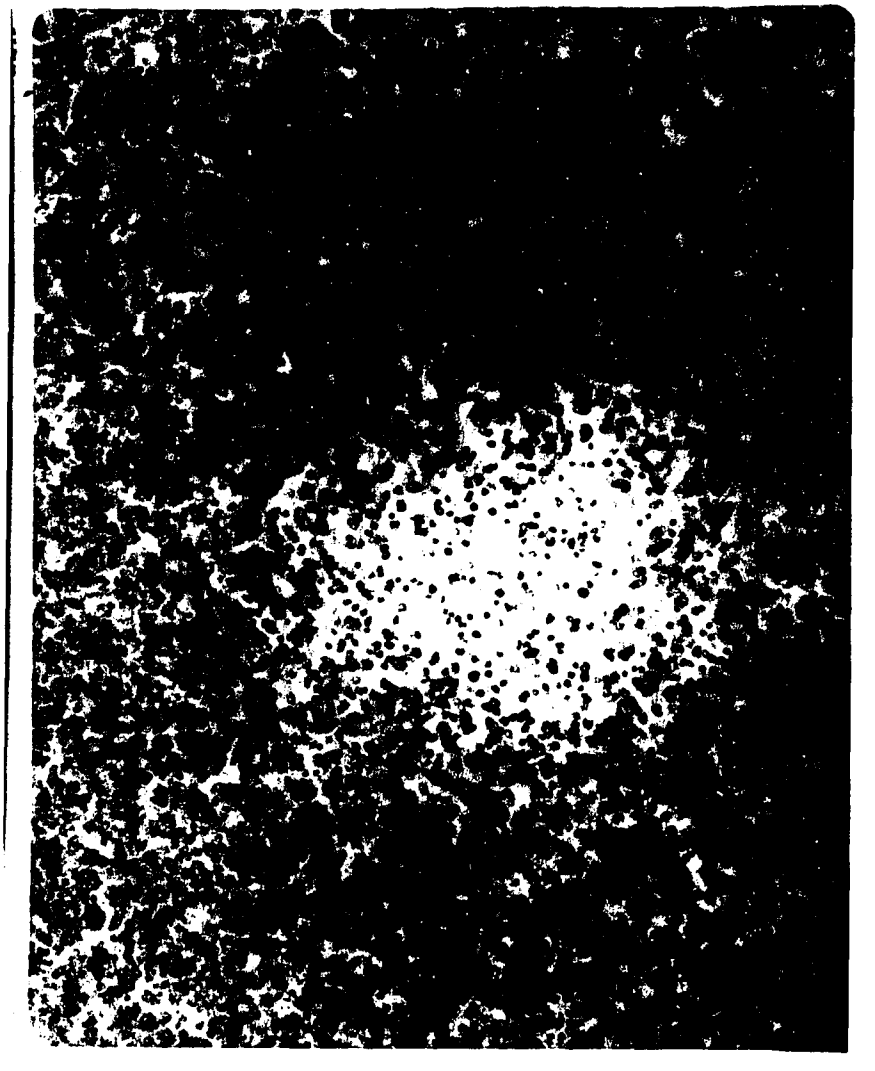

Figure 2. Representative hemolytic plaque formed by a splenic lymphocyte secreting antibody to PR8 virus. Erythrocytes coated with PR8 virus.

the development of hemadsorption (Fig. 3) and by immunofluorescence (Figs. 4 and 5). Using dilutions of allantoic fluid viruses (1:2 for Japan virus and B/Lee virus, to 1:5 for PR8 virus) to infect the cell culture lines, hemadsorption and immunofluorescence were detected as early as four hours after infection, and increased in intensity over the next 8-12 hours. Studies were carried out to determine if the infections of these cell lines were productive. Measurements were made of supernatant culture fluids to assess the presence of hemagglutinating virus particles and the titer of infectious virus. The three cell lines were found to be abortively infected, without particle production.

Preliminary experiments were run to assess the optimal means of lysing cells to determine the total amount of ^{51}Cr present in pulsed cells. Various detergents (Triton X-100 and sodium laurel sulfate) and caustic methods (1N and 2N NaOH) were tried. It was found that 5% Triton X-100 in distilled water was effective. Initial studies were done to assess the optimal ratio of effector lymphocytes to target cells. Ratios of 1:1, 3:1, 10:1, 30:1, and 100:1 were tested. A ratio of 100:1 produced the maximum cytotoxicity. In early studies the standard deviations of replicate dishes were too high, therefore a means of making results more uniform were studied. It was found that using 2 ml pipets rather than portions of 10 ml pipets eliminated most of the variability. Assay length was tested by varying the time of preinfection before addition of lymphocytes and the length of incubation

FIGURE 3

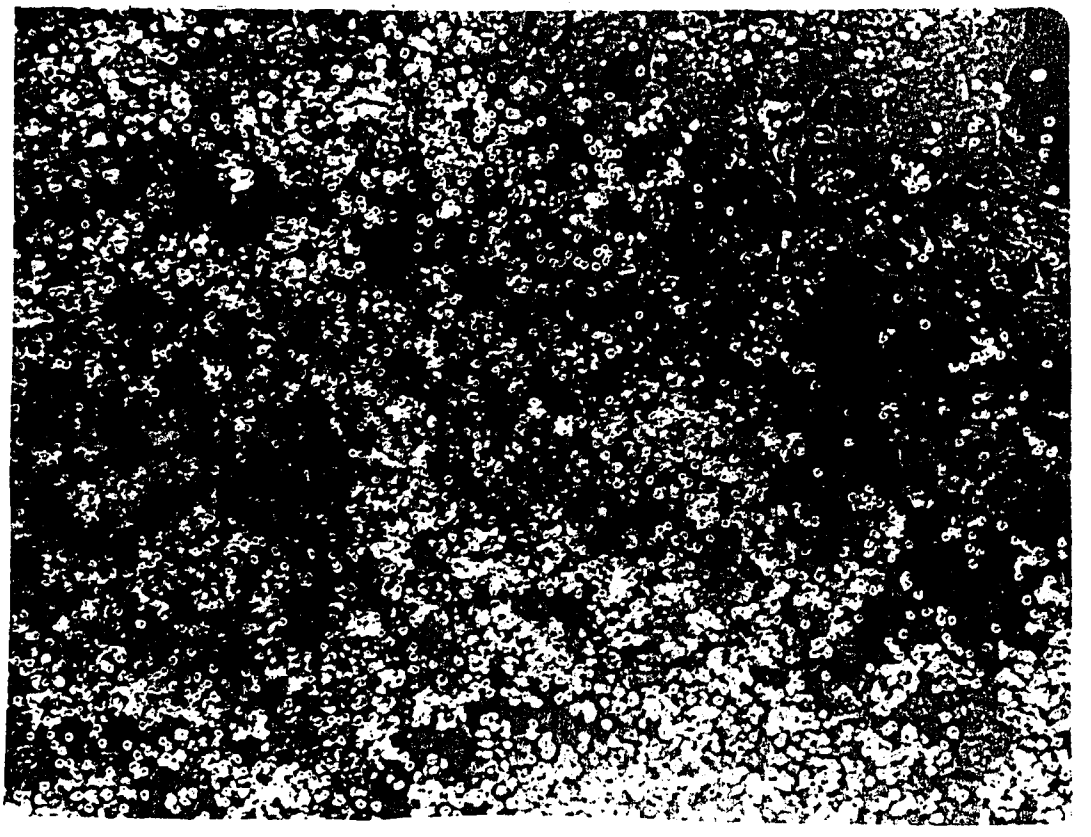

Figure 3. Representative hemadsorption: NWS virus infected BALB/c3T3 cell monolayer and human type "O" erythrocytes. BALB/c3T3 cells were grown to confluency, washed and infected with NWS virus (multiplicity of infection, 10 EID_{50}/cell). After 30' adsorption, cell sheets were washed and incubated for 1 hr in serum-free medium. Antiserum to NWS virus was used to remove extracellular virus. The dish was incubated an additional 5.5 hours at 37° prior to extensive washing. Human type "O" erythrocytes (0.1%) were added for a 15' incubation period at 25° prior to extensive washing with PBS. Mag. = 63x.

FIGURE 4

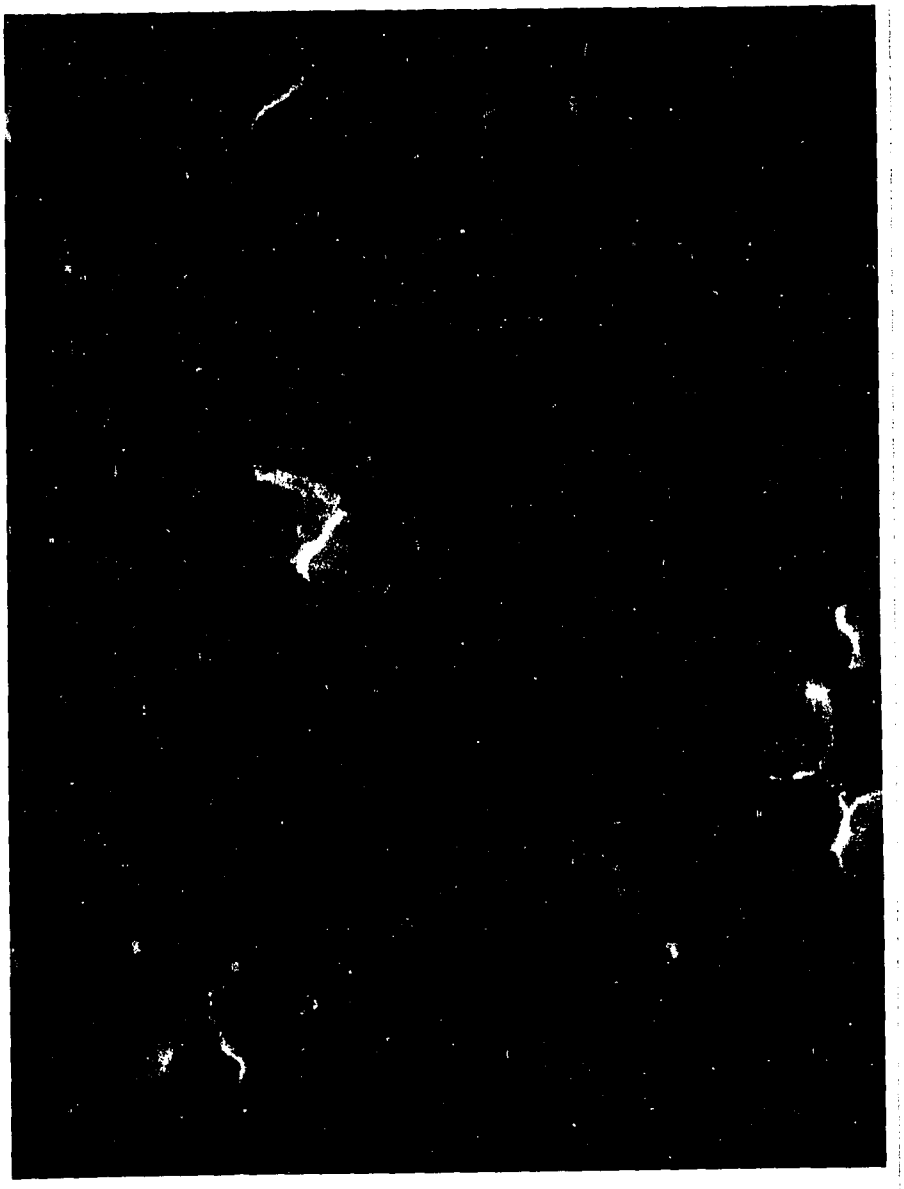

Figure 4. Indirect immunofluorescence of PR8 virus infected L929 cells. See Materials and Methods.

FIGURE 5

Figure 5. Indirect immunofluorescence of PR8 virus infected P815 cells. See Materials and Methods.

of effectors and target cells. It was determined that prior to addition of lymphocytes, 5-8 hours of infection, and a 12 hour incubation period of the complete system before taking aliquots of supernatant for gamma counting were optimal. Generally for assays, background ^{51}Cr release was 20-30% of the total counts incorporated.

Variation Between Mice

The variation between mice in a group was studied to determine if pooled specimens of splenic lymphocytes could be used for either the cytotoxicity assay or for the AFC determinations. The use of pooled samples would allow more flexibility in experimental design. The results are shown in Table 4. It was determined that the variation among mice within a group was small and that therefore pooled samples could be used.

B. Experimental Results

Primary AFC Response in BALB/c Mice

Female BALB/c mice were sensitized with untreated PR8 virus as described above. As shown in Figure 6, IgM secreting AFC recognizing PR8 virus coated SRBC are detectable above background levels by four days after sensitization. (In several other experiments IgM AFC recognizing PR8 virus coated SRBC targets have been seen as early as 2 days after inoculation). The peak of the IgM response is 11 days after sensitization following which there is a rapid decline in the number of IgM secreting AFC. IgG secreting AFC are initially

Table 4

VARIABILITY OF INDIVIDUAL MICE

AFC Assay:

Control Mice	1) 60 AFC/spleen[a]	Sensitized Mice[b]	1) 139,300	
	2) 60		2) 158,700	
	3) 40		3) 137,000	
	4) 30		4) 121,700	

CML Assay:

Total releasable counts[c]:	34,014 ± 1185		
Control mice:	1) 15,258 ± 390		
	2) 16,534 ± 317		
	3) 15,119 ± 882		
	pool 15,318 ± 1172		
Sensitized mice[d]:	1) 29,612 ± 1630	76.4%[e]	
	2) 27,841 ± 1144	66.9	
	3) 27,298 ± 770	64.1	
	4) 28,909 ± 285	72.7	
	pool 27,683 ± 549	66.1	

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[a] direct AFC with PR8 virus coated SRBC

[b] 5 days after sensitization with the standard dose of PR8 virus

[c] quadruplicate cultures, PR8 infected cells; no difference was observed on uninfected L929 cells between naive and sensitized mice. Mean ± SEM.

[d] six days after sensitization with the standard dose of PR8 virus

[e] specific immune release

FIGURE 6

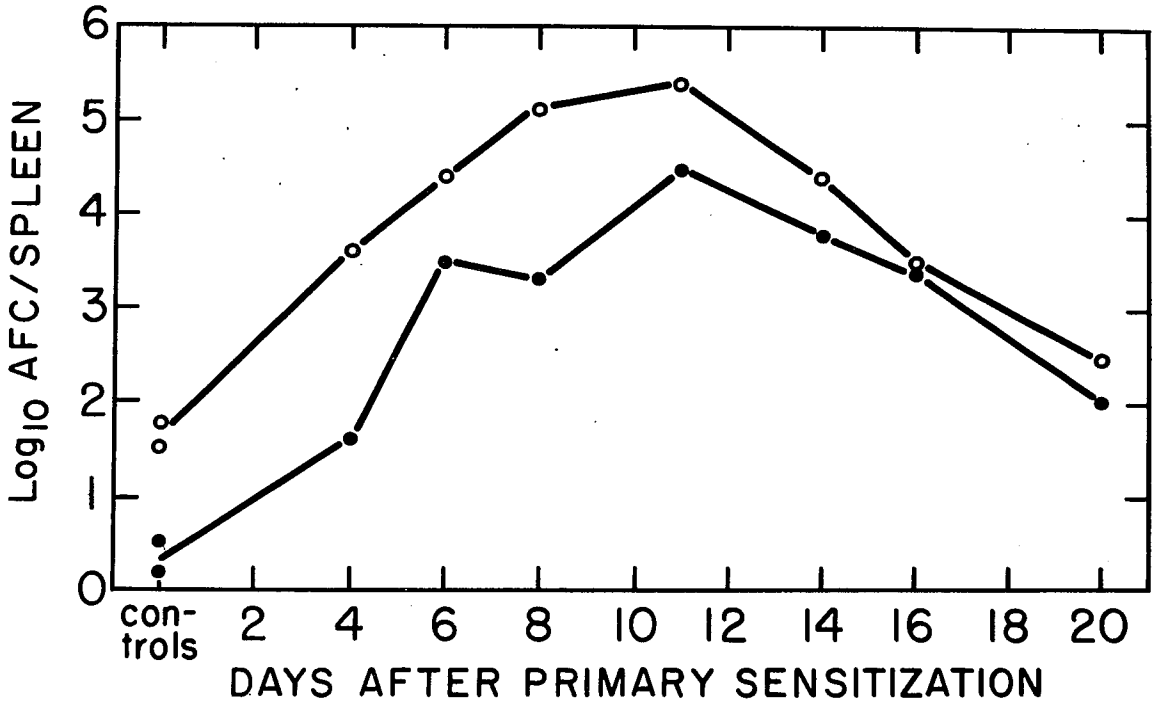


Figure 6. Kinetics of the primary splenic AFC response of BALB/c mice sensitized parenterally with PR8 virus. Mice were immunized with 0.2 ml of egg grown PR8 virus which had an HA titer of 1:1024 (standard dose). Lymphocytes were assayed against PR8 virus coated SRBC targets. IgM secreting cells (0); IgG secreting cells (●).

detectable on day 4 and are also at peak numbers on day 11, but never reach the magnitude of the IgM response. By three weeks after immunization, the primary cellular proliferative response has declined to background levels.

Kinetics of Specific AFC Responses to Hemagglutinin (HA) and Neuraminidase (NA) Antigens

Using spleen cells from BALB/c mice immunized in the same manner and SRBC coated with the appropriate hybrid viruses (Table 1) IgM and IgG AFC responses to HA and NA antigens were compared. As shown in Figure 7, IgM secreting AFC are primarily directed to the HA antigen, outnumbering IgM AFC recognizing NA by approximately 10:1. In contrast, the kinetics of the IgG AFC responses to HA and NA are identical. Measurements of serum HI and NI antibody in these mice (Fig. 8) demonstrated that antibody is detectable by day 4, that peak titers are reached by day 6 and that the titers remain elevated for at least three months after AFC responses have returned to background levels. Assay of the same sera for HI activity after reduction with mercaptoethanol revealed that IgM antibody predominated until after day 10. Serum antibody obtained later was resistant to mercaptoethanol treatment, presumably reflecting the predominance of IgG Ab, Figure 9.

The Effects of Antigen Dose on the Kinetics of the Response

To determine whether the antigen dose influences the kinetics of the response, BALB/c mice were immunized with one tenth the dose of PR8 virus employed in the previous experi-

FIGURE 7

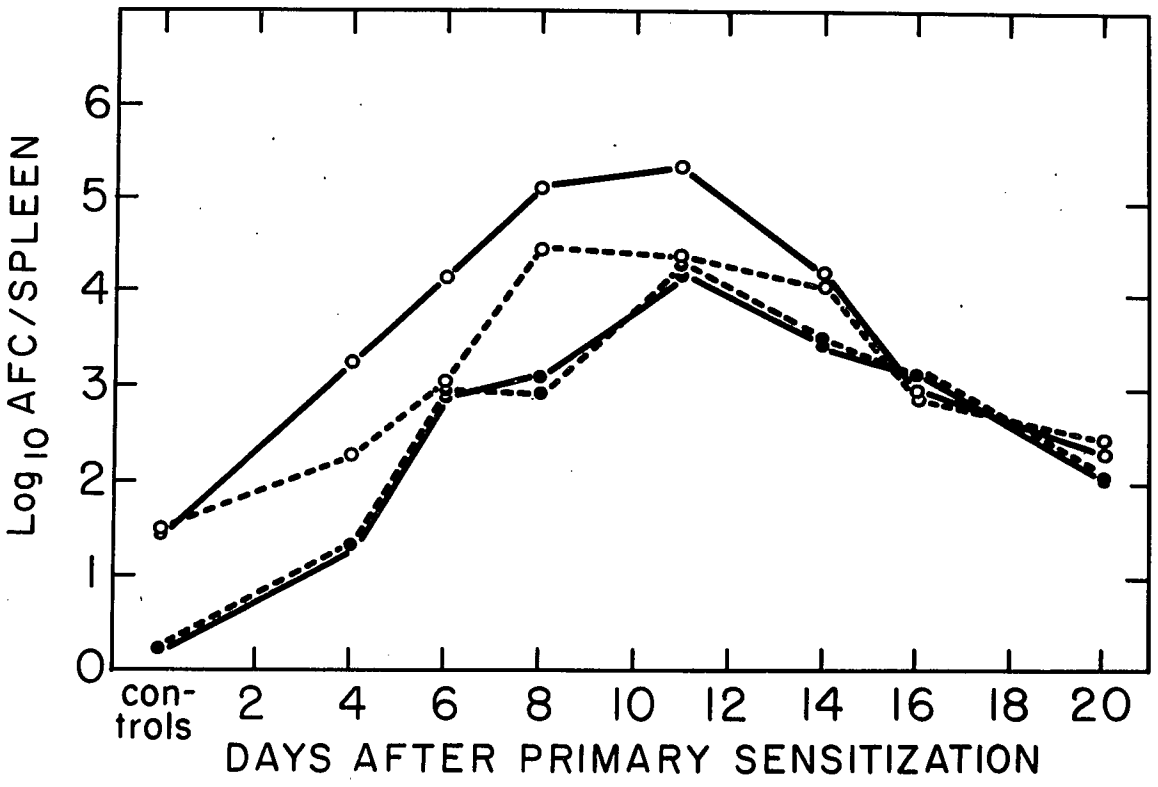


Figure 7. Comparison of the hemagglutinin specific and neuraminidase specific AFC responses following primary immunization. BALB/c mice were immunized with the standard dose of PR8 virus. Lymphocytes were assayed against PR8-HK virus coated SRBC targets (HA-specific)(————) or HK-PR8 virus coated targets (NA-specific)(– – – –); IgM secreting cells (0); IgG secreting cells (●).

FIGURE 8

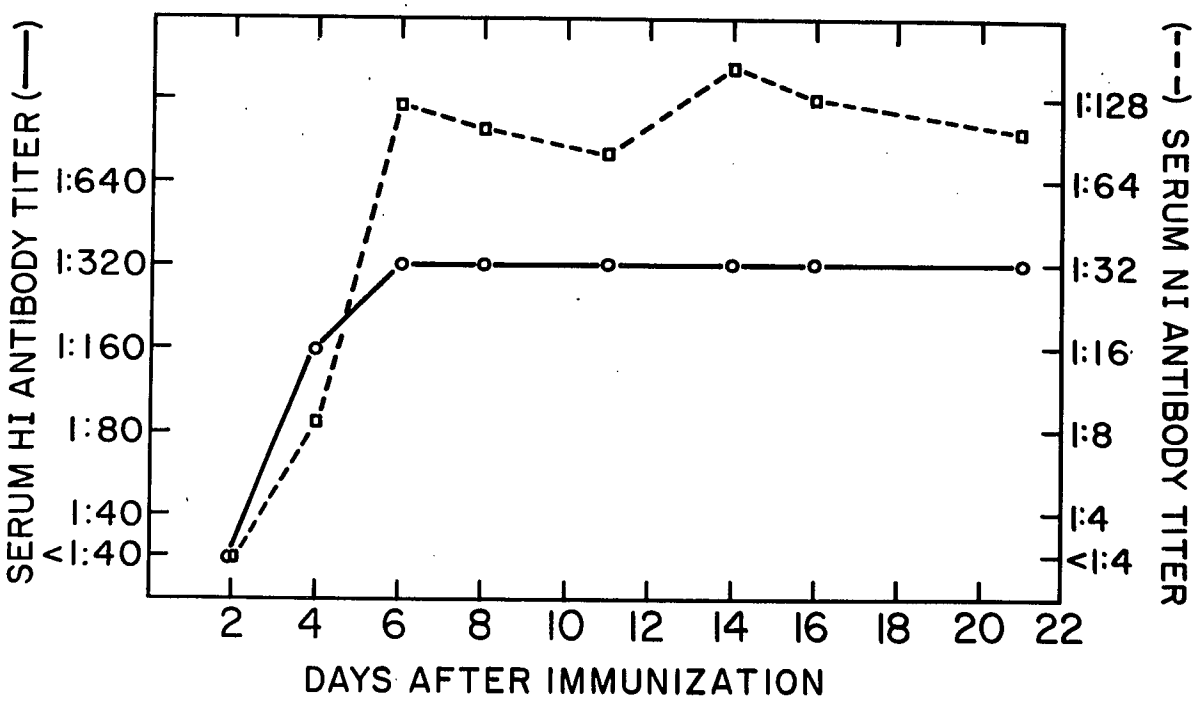


Figure 8. Serum hemagglutination inhibiting and neuraminidase inhibiting titers of BALB/c mice after primary immunization with PR8 virus. Sera were obtained from mice whose AFC responses are shown in Fig. 2. HI titer (log_2) (———); NI titer (log_2) (------).

FIGURE 9

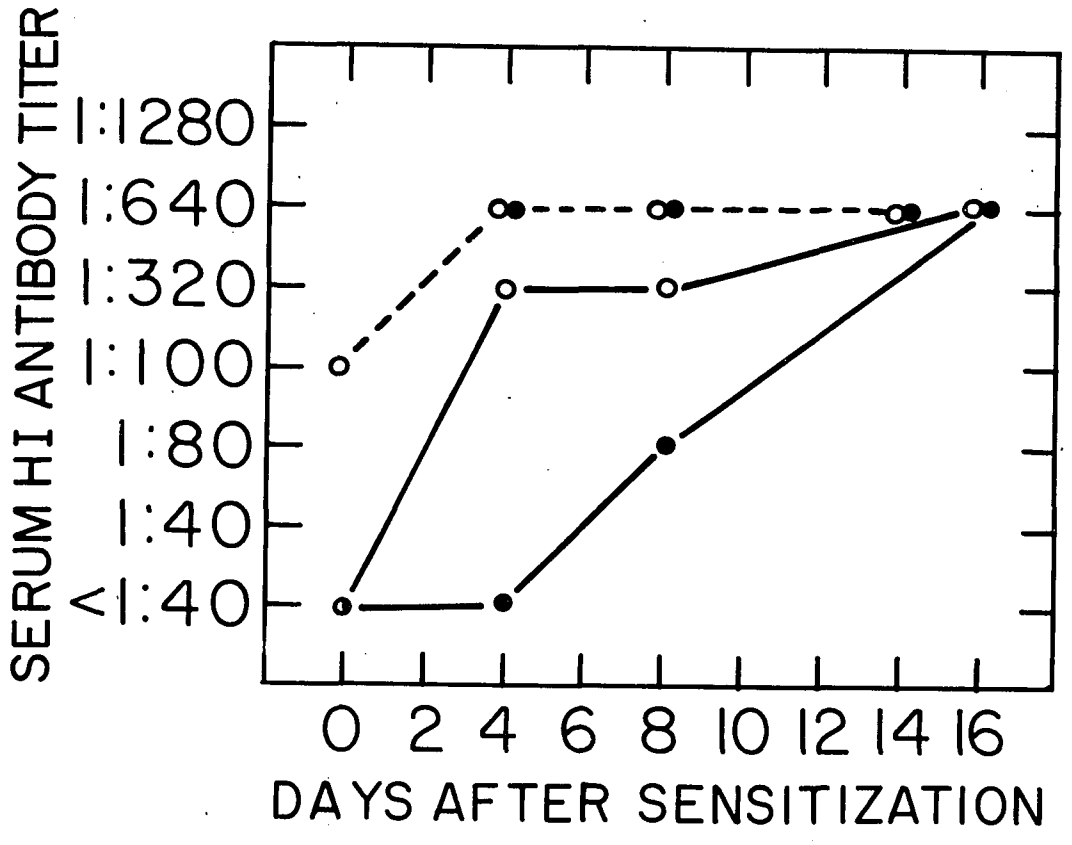


Figure 9. Effect of mercaptoethanol treatment on serum HI antibody titers of BALB/c mice. Sera were derived from mice undergoing a primary (———) or a secondary (------) response to PR8 virus. Untreated (0); mercaptoethanol resistant (●). Antigen: PR8-Equi virus, 32 HA units.

ments. The kinetics of the splenic AFC repsonses are shown in Figure 10 along with the responses obtained with the standard dose for comparison. For the first 6 days the kinetics of the responses to the two doses of antigen are identical. However, following immunization with the lower dose of virus, the number of IgM and IgG secreting AFC do not continue to increase after day 6, and from day 8 onward, the AFC response in these mice declines more rapidly than does the response of mice immunized with the standard dose. Comparisons of the serum HI antibody responses at intervals after immunization revealed that mean serum HI antibody titers in the two groups were similar initially, but that after day 7, titers in mice immunized with the smaller dose were consistently slightly lower than those of mice immunized with the larger dose of antigen (Fig. 11).

Secondary AFC Responses in BALB/c Mice

BALB/c mice were immunized with the standard dose of PR8 virus described above, and 4 weeks later were given a booster injection of the lower dose of antigen; in addition, previously unimmunized mice were injected with the lower dose of virus. Comparison of the AFC responses shown in Fig. 12 demonstrates that both IgM and IgG AFC responses develop more rapidly in previously primed animals. In addition, it can be seen that the secondary response is characterized by higher peak numbers of AFC, the predominance of IgG secreting cells, and a more prolonged period in which AFC responses above background are evident. In contrast to the primary response, AFC specific

FIGURE 10

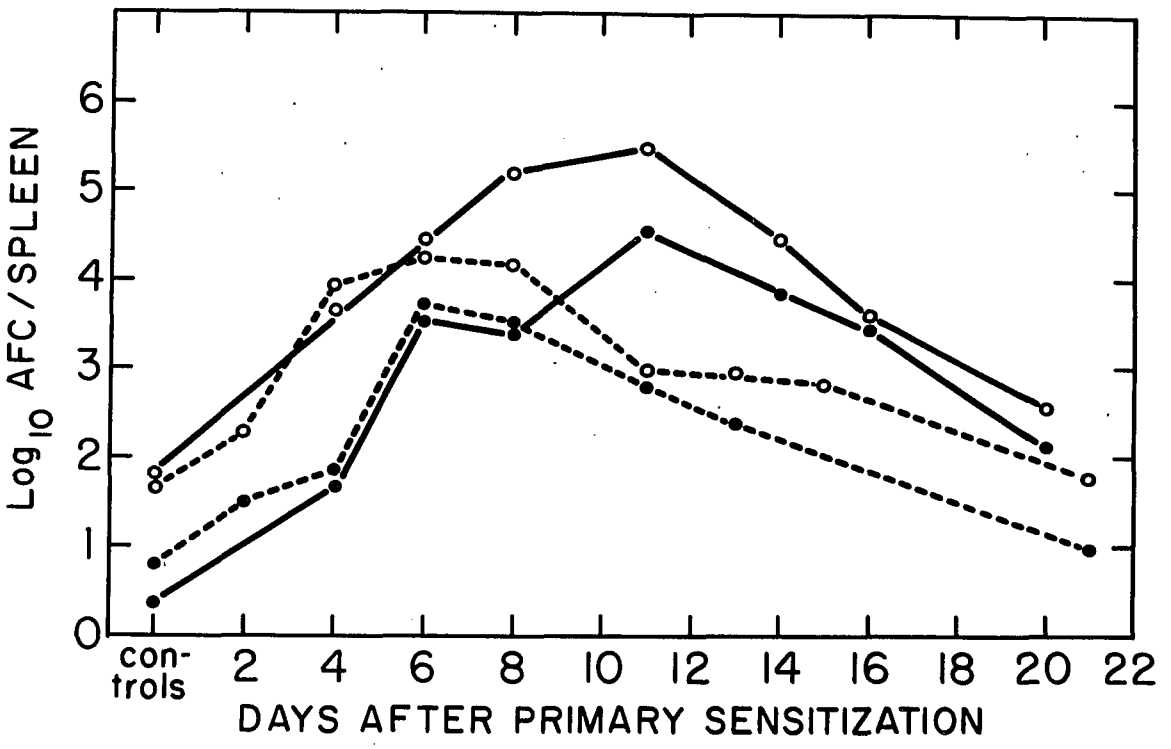


Figure 10. Kinetics of the primary AFC response of BALB/c mice immunized with 2 doses of PR8 virus. Mice immunized with the standard dose (———); mice immunized with the 10-fold lower dose (------); IgM secreting cells (0); IgG secreting cells (●). Target erythrocytes were coated with PR8 virus.

FIGURE 11

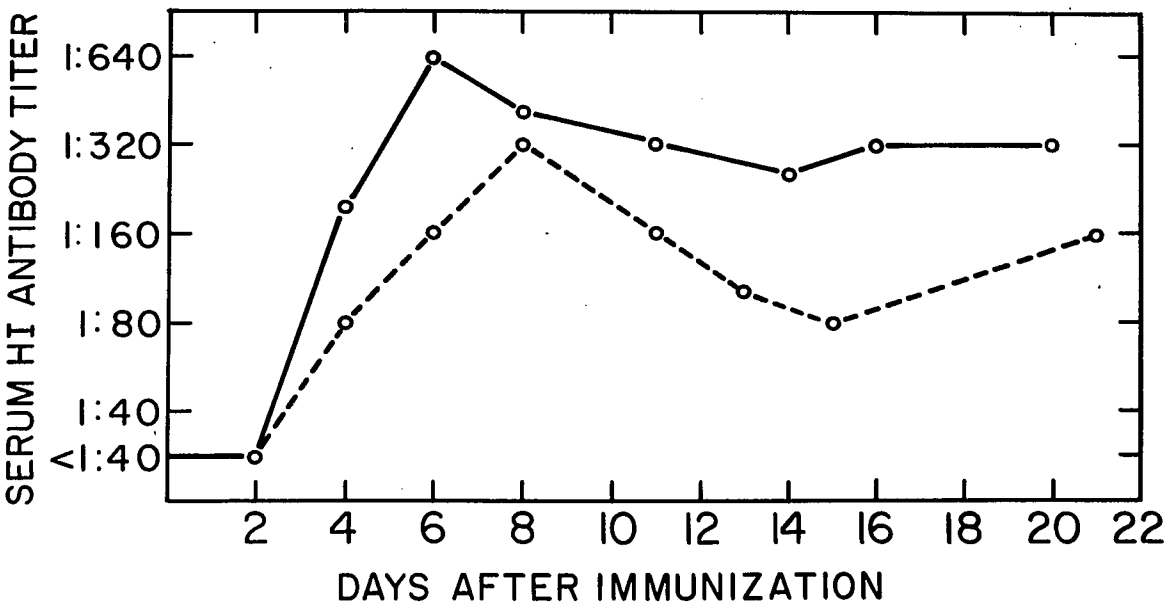


Figure 11. Serum HI antibody titers of mice immunized with two doses of PR8 virus. Sera were obtained from the mice whose AFC response is shown in Fig. 10. Standard dose (———); one-tenth standard (------).

FIGURE 12

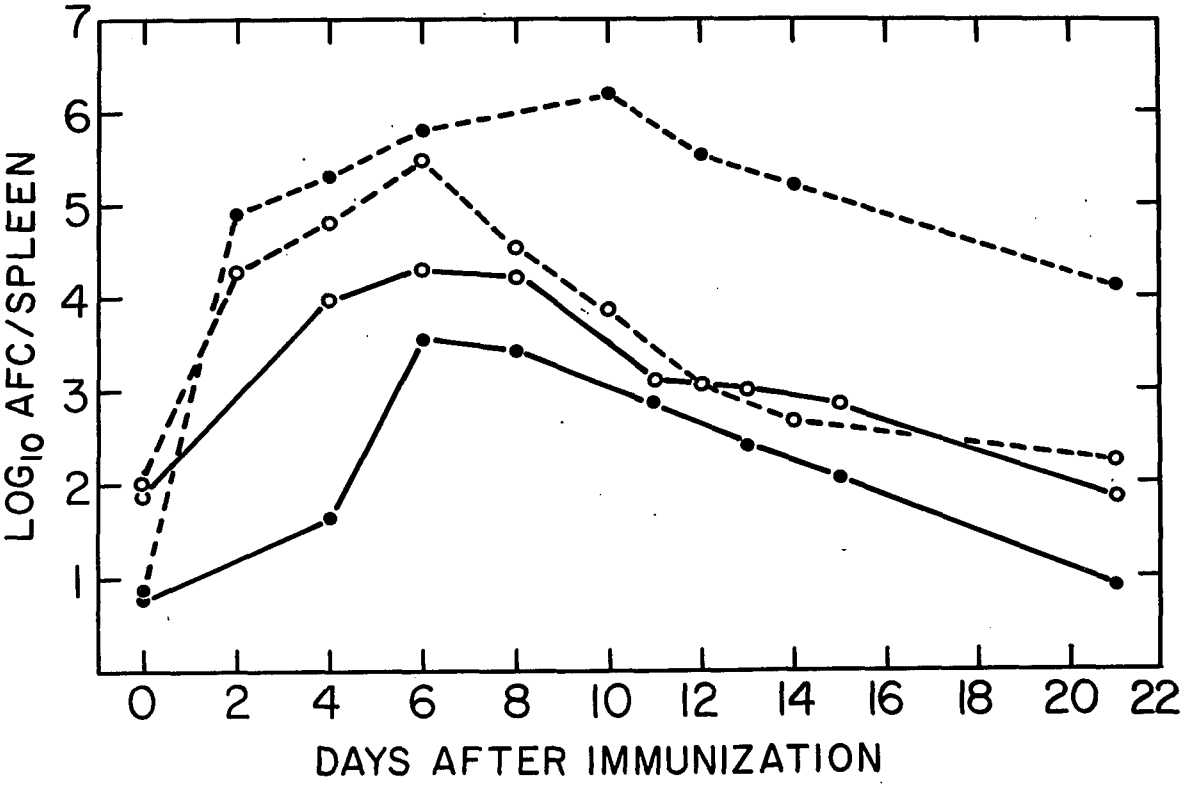


Figure 12. Comparison of the primary and secondary AFC responses of BALB/c mice to PR8 virus. Mice were immunized with the standard dose of PR8 virus and restimulated one month later with the one-tenth standard dose (------); the primary response to the one-tenth standard dose is shown for comparison (———); IgM secreting cells (O); IgG secreting cells (●). Target SRBC were coated with PR8 virus.

for HA predominate in both IgM and IgG secreting cells. Analysis of antibody responses following booster immunization revealed a more rapid increase of anti-NA and anti-HA antibody than is observed following primary immunization (increases in titer are seen as early as 2 days after secondary sensitization). In addition there is a 2-4 fold increase in both HI and NI Ab over preexisting elevated levels. Treatment with mercaptoethanol did not effect titers of HI antibody following secondary immunization (Fig. 9).

Initially, I planned to compare AFC and cytotoxic responses in BALB/c mice. However, it became evident that cytotoxic assays could be performed more reproducibly (in my hands) using a cell line compatible with C3H mice, rather than BALB/c mice. Consequently, AFC assays were also performed in C3H mice. The results provided an opportunity to compare the kinetics of AFC responses in the two strains.

AFC Responses in C3H Mice

Comparisons then were made of the AFC responses and antibody synthesis in another strain of mice following primary immunization with two different doses of PR8 virus antigen and following secondary immunization as described for BALB/c mice. Figure 13 demonstrates that following primary immunization of C3H mice with the standard dose of PR8 virus the numbers of AFC responding to hemagglutinin exceeds the numbers of AFC recognizing NA among both IgM and IgG secreting cells. This observation is in contrast to the data shown in Figure 7 which demonstrates that in the primary response of BALB/c mice, HA-specific AFC predominate only

FIGURE 13

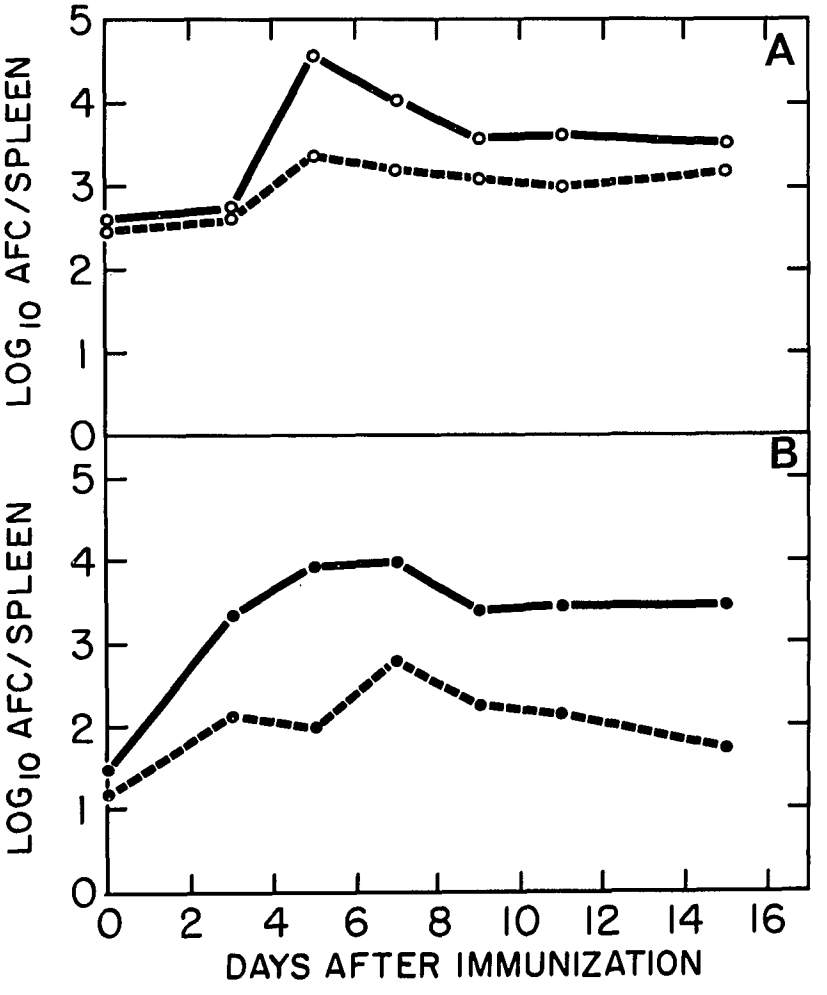


Figure 13. Comparison of the primary hemagglutinin-specific and neuraminidase-specific AFC responses following primary immunization. C3H mice were sensitized with the standard dose of PR8 virus. Lymphocytes were assayed against SRBC targets coated with PR8-HK virus (HA-specific (_____) or HK-PR8 virus (NA-specific (------); IgM secreting cells, panel A; IgG secreting cells, panel B.

among IgM secreting AFC. Moreover, examination of the kinetics of the response in C3H mice demonstrates that for the first 5 days they are similar to those observed in BALB/c mice. However, whereas the peak AFC response in BALB/c mice is not obtained until 11 days after immunization (Fig. 6), in C3H mice the numbers of AFC do not continue to increase after day 5. Another difference can be seen in the ratio of IgM and IgG secreting cells; in C3H mice the numbers of IgM and IgG secreting cells are approximately equal, whereas at the peak of the response in BALB/c mice, IgM secreting cells outnumber IgG secreting cells by 10:1. Serum antibody titers of specimens from the mice whose AFC responses are shown in Figure 13 are found in Figure 14.

Effect of Antigen Dose on the Kinetics of the Response

Comparison of the AFC responses in C3H mice following immunization with the standard dose and a 10-fold lower dose of virus (Figure 15) reveals that initially the IgM secreting AFC response develops slightly more slowly in mice which had received the smaller inoculum. The delay in the IgG response is even more striking: 5 days after sensitization there is more than a 10-fold difference in the numbers of IgG secreting AFC between the two groups. After this initial delay, both the IgM and the IgG AFC response in mice immunized with the smaller inoculum reached the same magnitude as seen with the standard dose. Consistent with the kinetics of the AFC response, serum HI antibody was delayed in appearance in sera obtained from C3H mice sensitized with the lower dose (Fig.

FIGURE 14

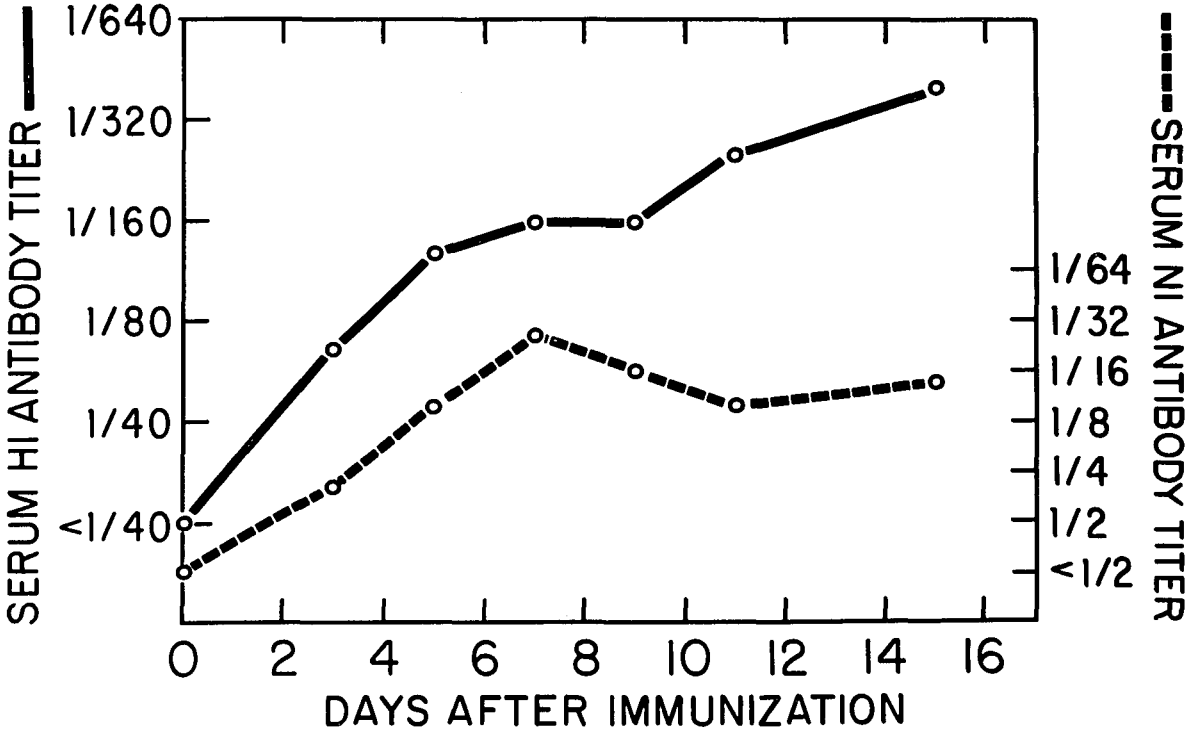


Figure 14. Serum hemagglutination inhibiting and neuraminidase inhibiting titers of C3H mice after primary immunization with PR8 virus. Sera were obtained from mice whose AFC responses are shown in Fig. 13. HI titer (log_2) (———); NI titer (log_2) (------).

FIGURE 15

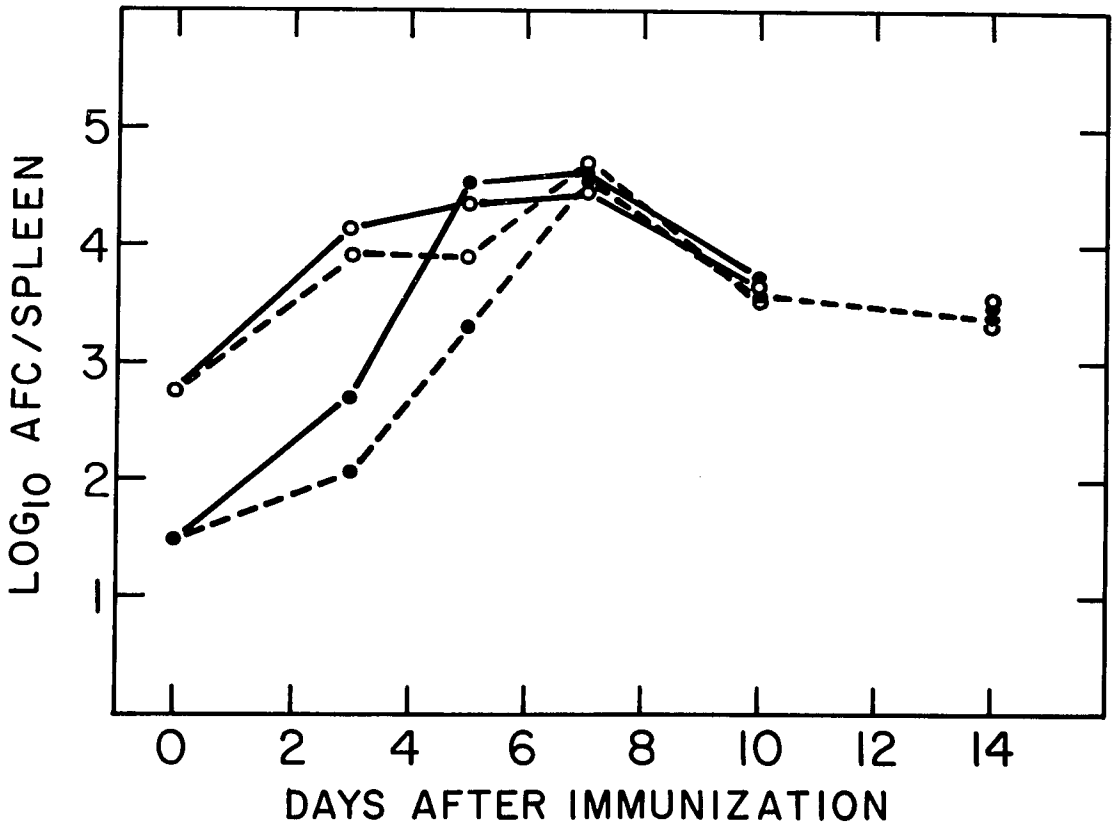


Figure 15. Kinetics of the AFC responses of C3H mice to 2 doses of PR8 virus. Standard dose (———); one-tenth standard dose (------); IgM secreting cells (0); IgG secreting cells (●). Targets erythrocytes were coated with PR8 virus.

16). However, peak HI antibody titers were equivalent to those observed in mice immunized with the standard dose. Mercaptoethanol treatment of serum indicated that in both groups, resistant antibody appeared on day 7, and that by 2 weeks after immunization, all measurable HI antibody was resistant to reduction. The differences in kinetics of the AFC responses of C3H mice immunized with 2 different doses of antigen were not comparable to those observed with BALB/c mice sensitized with the standard and lower antigen doses. In BALB/c mice the kinetics of response following the lower dose were initially identical to those seen following the standard dose. After day 6, the numbers of AFC did not continue to increase in mice sensitized with the lower dose.

Secondary AFC Responses of C3H Mice

C3H mice were primed with the high dose of PR8 virus and restimulated with the low dose 4 weeks later. Like the secondary response of BALB/c mice, the secondary AFC response in C3H mice is characterized by a predominance of IgG secreting cells (Fig. 17), but the peak numbers of AFC are much lower than that observed with BALB/c mice (5×10^4 compared to 10^6 AFC/spleen). As was seen in the primary response, in the secondary AFC response of C3H mice, HA-specific AFC predominate in both classes. The serum antibody titers of BALB/c and C3H mice during the secondary response are shown in Figure 18 for comparison. It is apparent that the serum HI antibody responses are virtually identical in contrast to the very different AFC profiles in the two strains of mice. No re-

Figure 16

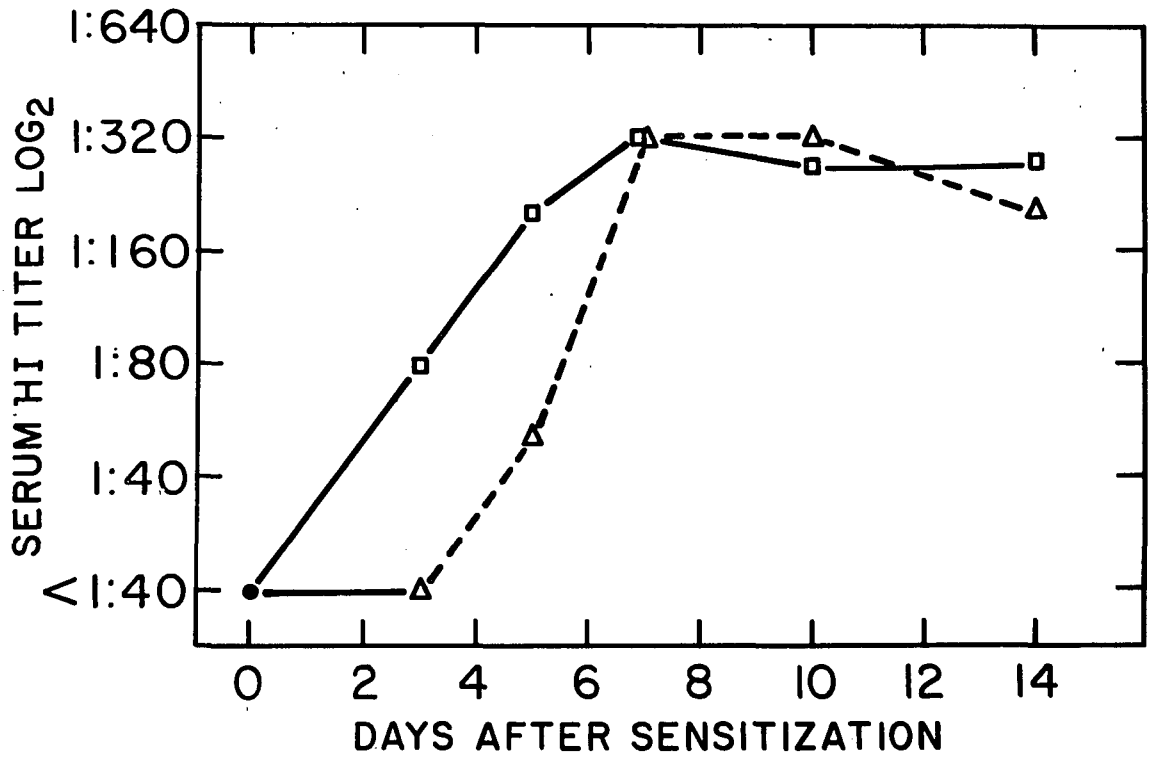


Figure 16. Serum HI responses of C3H mice sensitized with 2 doses of PR8 virus. Sera were obtained from the mice whose AFC responses are shown in Fig. 8. Standard dose (———); ten-fold lower dose (------).

FIGURE 17

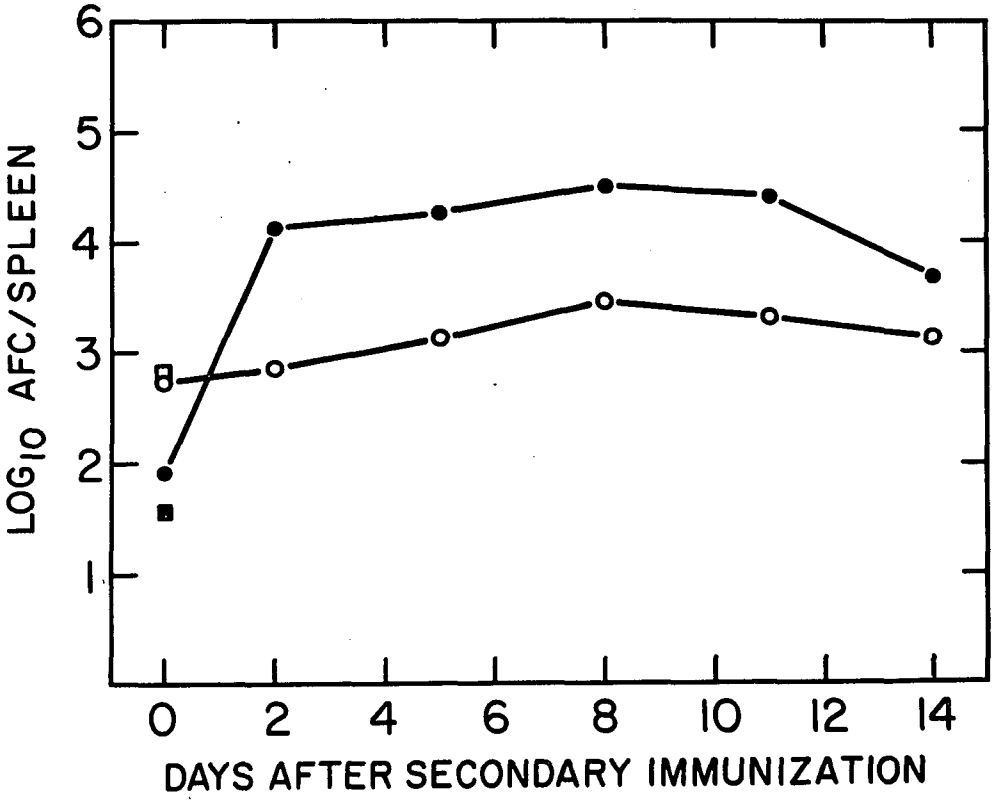


Figure 17. Kinetics of the secondary AFC response of C3H mice immunized with PR8 virus. Mice were primed with the standard dose; four weeks later they were restimulated with the ten-fold lower dose. IgM secreting cells (0); IgG secreting cells (●). Target SRBC were coated with PR8 virus.

FIGURE 18

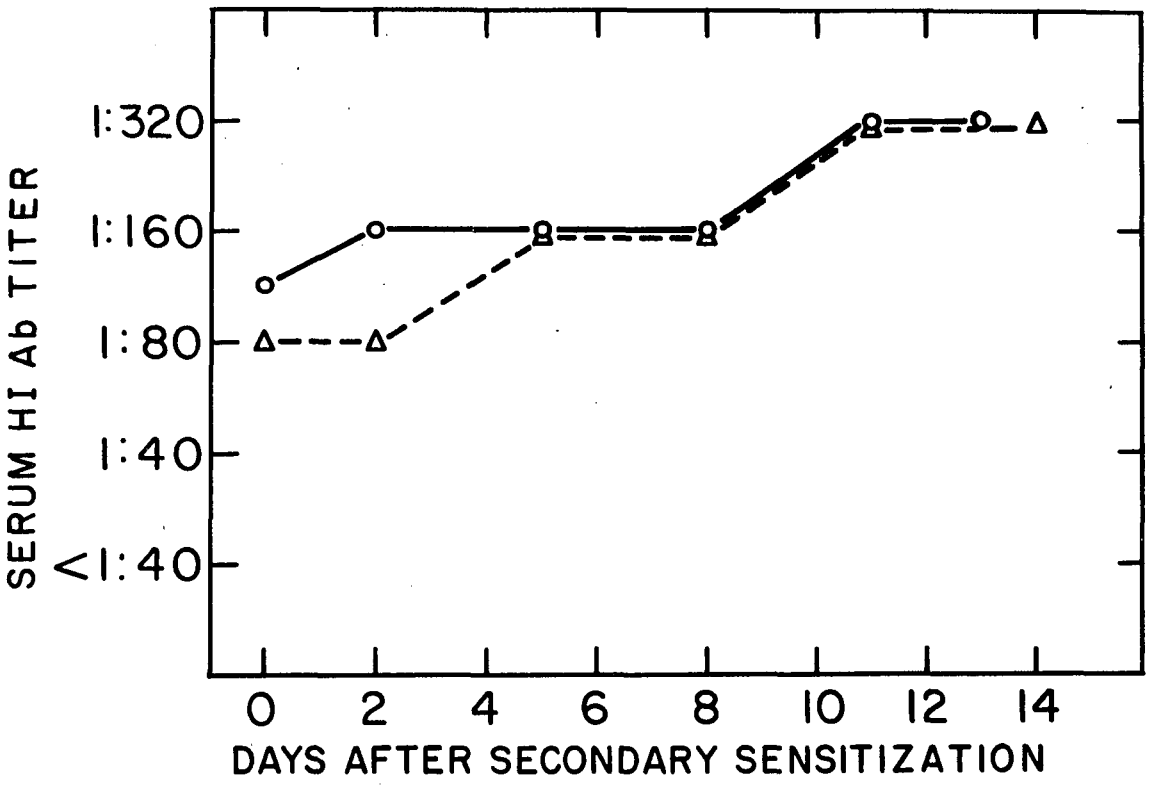


Figure 18. Serum HI responses of BALB/c and C3H mice during the secondary response to PR8 virus. Mice were primed with the standard dose and boosted with the one-tenth standard dose one month later. C3H mice (------); BALB/c mice (———).

duction of serum HI antibody titers was observed after mercaptoethanol treatment of sera obtained after secondary immunization of either strain of mice (Fig. 19). This observation is consistent with the predominance of IgG secreting cells which was seen in both groups.

AFC Responses to Heterotypic Determinants and Host Cell Coded Antigen(s)

In all of these studies a standard target cell control of SRBC coated with allantoic fluid X-31 (H3N2) virus was included to measure AFC responses to heterotypic viral antigenic determinants. Following primary immunization of mice, a 2-4 fold increase above background levels of IgM secreting AFC recognizing H3N2 targets was noted (Fig. 20). However, following secondary immunization, a marked increase (100-400 fold in different experiments) principally among IgG secreting cells was detected with X-31 virus coated targets. Sera from these mice did not contain antibody which was detectable in either HI or NI assays with X-31 antigens. Following secondary stimulation with egg grown PR8 virus, spleen cells of BALB/c and C3H mice were assayed in an AFC determination with SRBC coated with three subtypes of influenza A and influenza B/Lee viruses. Figure 21 demonstrates that in both strains of mice the secondary IgG response is primarily directed against homologous viral antigens (HON1). A marked cross-reactive response was detected using SRBC coated with influenza A viruses of two other subtypes: Japan virus (H2N2) and X-31 virus (H3N2); and a lesser degree of cross-reactivity was

FIGURE 19

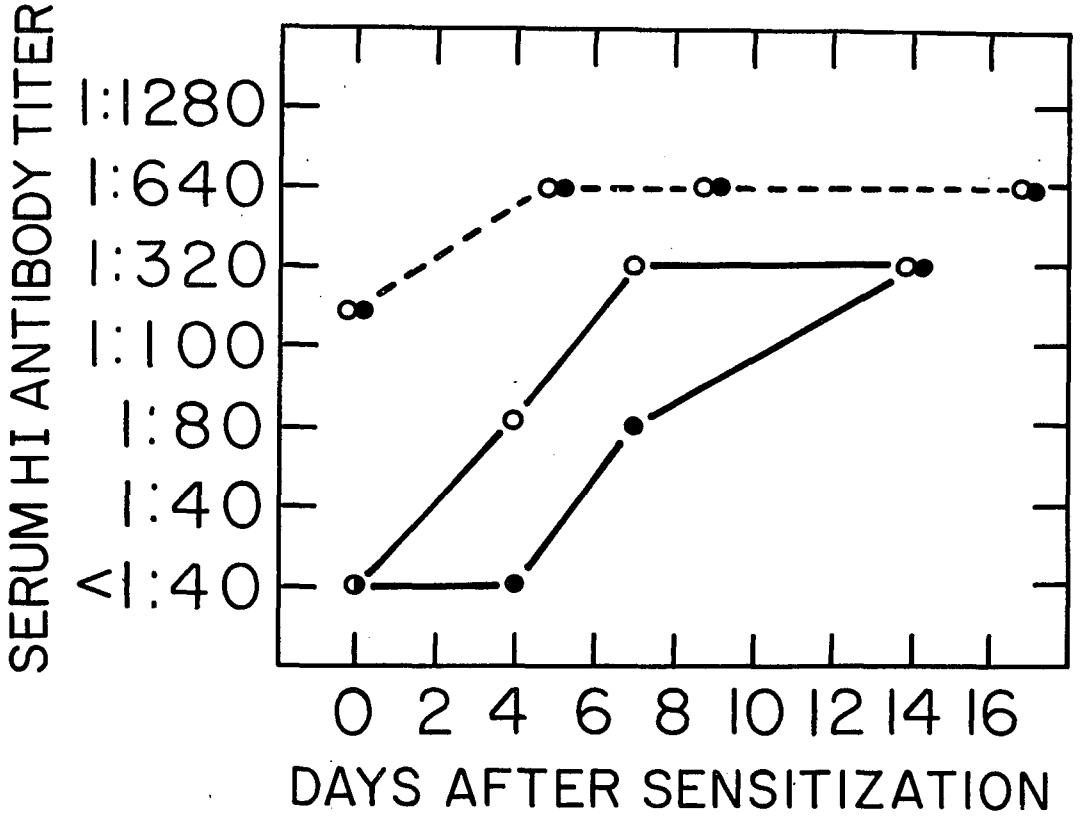


Figure 19. Effect of mercaptoethanol treatment on serum HI antibody titers of C3H mice. Sera were derived from mice undergoing a primary (———) or a secondary (- - - - - -) responses to PR8 virus. Untreated (0); mercaptoethanol resistant (●). Antigen: PR8-Equi virus, 32 HA units.

FIGURE 20

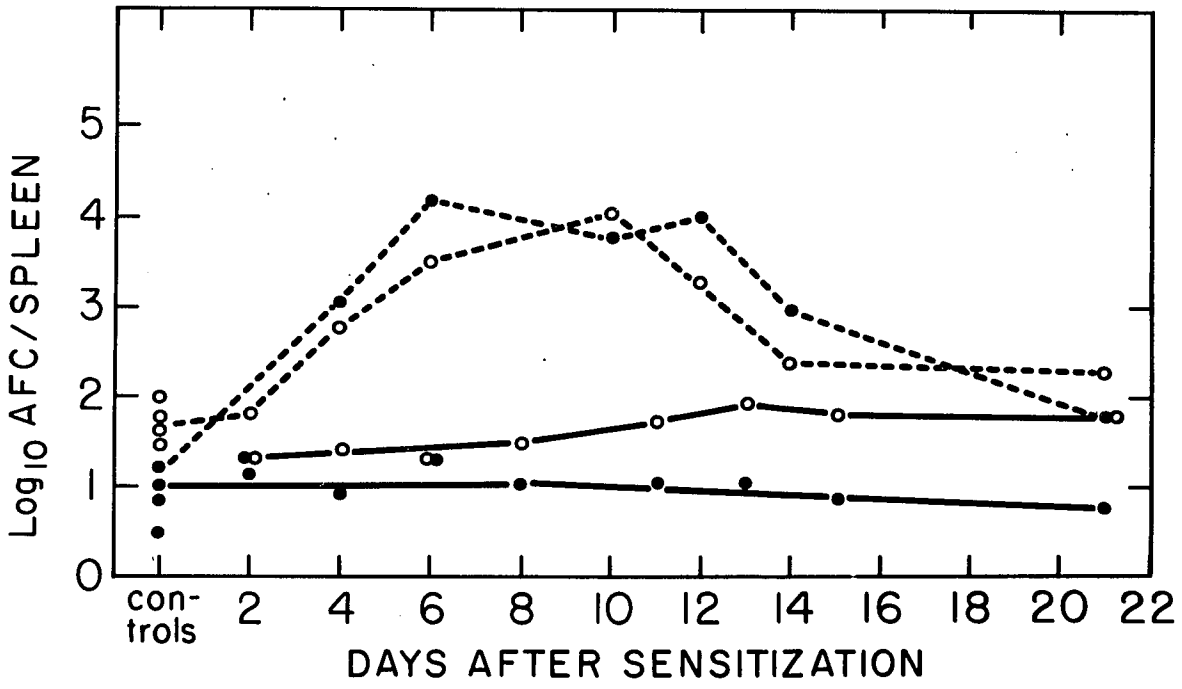


Figure 20. Kinetics of the heterotypic AFC responses of BALB/c mice after PR8 virus immunization. Primary response (———); secondary response (------); IgM secreting AFC (0); IgG secreting AFC (●). Target erythrocytes coated with X-31 virus.

FIGURE 21

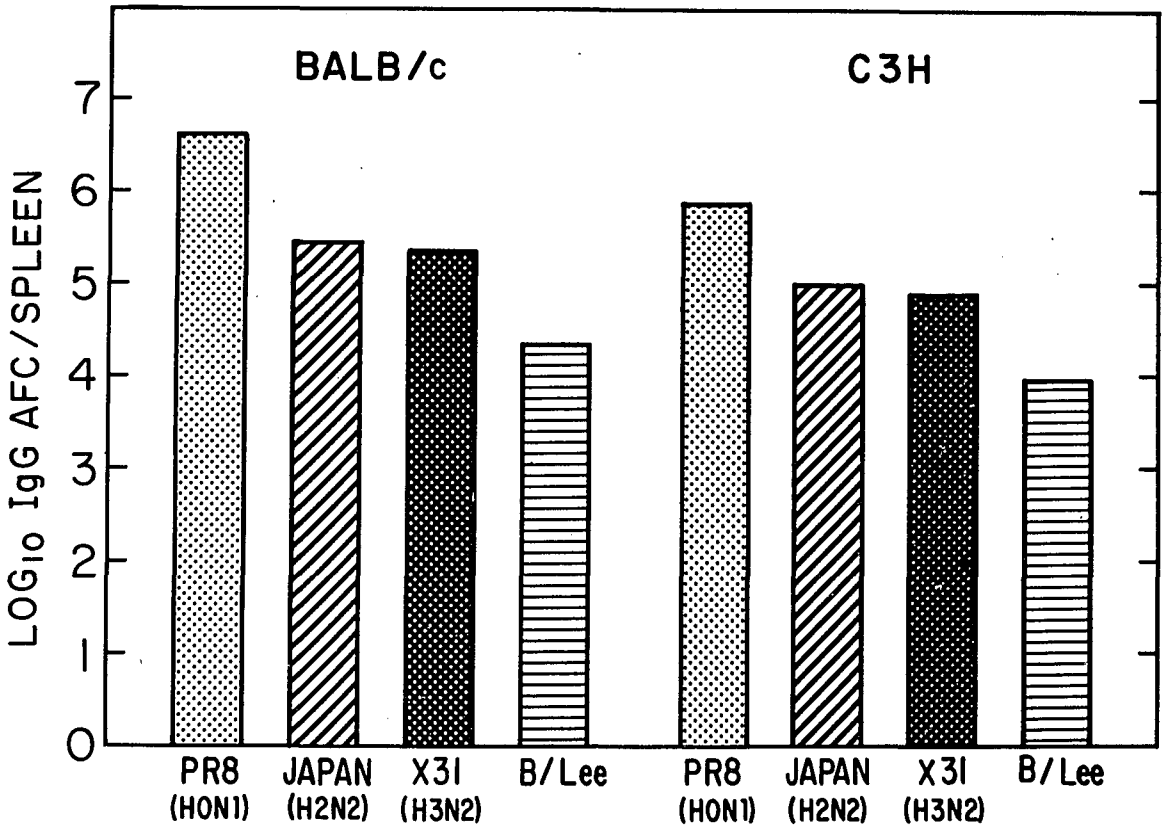


Figure 21. Analysis of the secondary heterotypic response after PR8 virus immunization of C3H and BALB/c mice. Mice were primed with the standard dose and restimulated a month later with the ten-fold lower dose of egg grown PR8 virus. Spleen cells were assayed 7 days later against erythrocytes coated with egg grown PR8 virus (HON1), Japan virus (H2N2), X-31 virus (H3N2), or B/Lee/40 virus. Control mice had fewer than 10 IgG AFC/spleen which reacted with any of the targets.

observed with influenza B/Lee virus coated SRBC targets. To determine if the antigens recognized were solely related to the propogation of the virus in the chicken embryo host, viruses used to coat SRBC were cultivated in canine kidney (MDCK) cells. About 90% of the reactivity observed with egg grown type A viruses was lost when MDCK grown Japan virus or X-31 virus coated SRBC targets were used. However, there was a 1000-fold increase above background levels in the secondary response to heterotypic influenza A viruses (H2N2 and H3N2) in mice immunized twice with egg grown PR8 virus.

Fewer AFC recognized B/Lee virus coated SRBC targets than recognized either H2N2 or H3N2 virus coated targets. However, equivalent numbers of AFC recognizing B/Lee virus coated SRBC and VSV coated erythrocyte targets were observed. When SRBC were coated with viruses grown in MDCK cells, a substantial portion of the reactivity was lost. These observations are consistent with the proposal that reactivity with B/Lee virus constitutes mostly recognition of host (chicken) antigen. The remaining level of AFC detected with MDCK grown B/Lee and VSV- viruses cannot be accounted for by this explanation. However, this amounts to a small increment above the background level of AFC detected in mice which have been primed but not restimulated.

To determine if the recognition of heterotypic influenza A virus antigen(s) was due to clones responding to internal antigens or to glycoprotein antigens, SRBC targets were coated with Triton X-100 extracted glycoproteins of PR8 or X-31 viru-

ses, or isolated M protein derived from PR8 virus. No difference in the numbers of AFC was observed when targets coated with whole virus or isolated glycoproteins of the same subtype were compared: i.e., responses to X-31 virus and to isolated X-31 virus glycoproteins were equivalent as were those to PR8 virus and to isolated PR8 virus glycoproteins (Table 5). No increase above background was observed when targets coated with M protein were used (data not shown). An additional control was to employ periodate treated SRBC targets which has been incubated with normal allantoic fluid. Using these cells as targets, no increase in AFC above background was evident (data not shown). Taken together, these results suggest that the heterotypic response to B/Lee virus coated targets seen during the secondary response to egg grown PR8 virus reflects recognition of host cell coded carbohydrate antigen(s) associated with the viral glycoproteins. However, all of the reactivity with other subtypes of influenza A viruses cannot be explained in the same manner. We interpret these results to suggest that in the secondary response there are specificities which are cross-reactive among subtypes of influenza A viruses which are not recognized by murine antibody in HI determinations. The data also suggests that this cross-reactivity among A strain viruses is probably not due to reaction with M protein in the AFC assay.

Sequential Immunization with Influenza Viruses of Different Subtypes

In other experiments C3H mice were primed with PR8 virus

Table 5

AFC RESPONSE OF BALB/c MICE SENSITIZED WITH PR8 VIRUS, DETECTED BY ERYTHROCYTE TARGETS COATED WITH ALLANTOIC FLUID VIRUSES OR TRITON X-100 EXTRACTED GLYCOPROTEINS

Source of Spleen Cells	Class AFC	Erythrocyte Targets: PR8 Virus AFV[a]	PR8 Virus GP[b]	X-31 Virus AFV	X-31 Virus GP	B/Lee Virus AFV
Naive	IgM	450[c]	415	335	405	500
	IgG	5	5	<5	5	5
Primary Response[d]	IgM	109,000	91,000	605	515	535
	IgG	4,150	2,700	5	10	5
Primed, not re-stimulated	IgM	242	292	245	229	250
	IgG	4	<4	<4	4	<4
Secondary response[e]	IgM	14,500	18,000	900	750	950
	IgG	205,000	155,000	1,650	1,350	600

[a] allantoic fluid virus

[b] isolated glycoproteins

[c] AFC/spleen

[d] five days after immunization with the standard dose PR8 virus

[e] mice were primed with the standard dose and one month later immunized with a 1:10 dilution of the standard dose.

and one month later inoculated with Japan virus. AFC studies were performed. Control groups of mice included mice undergoing primary responses to PR8 virus or to Japan virus. Figure 22 illustrates the results. Mice immunized first with PR8 virus and then with Japan virus initially responded more quickly to Japan virus coated SRBC targets. The amplified AFC response was detected as increases in both IgM and IgG secreting cells. After 6 days, however, the response was not different from that observed in mice which had not been primed but were immunized at the same time with Japan virus. A portion of the expanded population of cells recognizing Japan virus coated SRBC were also recognizing PR8 virus coated targets (Fig. 22 middle panel). An even smaller number of AFC recognized B/Lee virus coated targets indicating that some of these cells were specific for host antigen(s). This observation is consistent with the data presented which suggests that cross-reactive determinants are present on the glycoproteins. Serum HI responses to Japan virus HA were not different in mice which had been primed with PR8 virus and those which had not (Fig. 23). No boost in the serum HI antibody to PR8 virus HA was detected in mice which were primed then boosted with Japan virus. These observations are consistent with the hypothesis that the common determinants are not hemagglutination inhibiting.

Cell Mediated Cytotoxicity Responses:

Kinetic Studies of the Cytotoxicity Response

The kinetics of the primary cell mediated cytotoxicity response of mice immunized in the same manner as the mice

Figure 22. Kinetics of AFC responses of C3H mice primed with PR8 virus and immunized one month later with Japan virus (——▲——). Primary response to PR8 virus sensitization (——●——). Primary response to Japan virus sensitization (——■——). Left panels, IgM secreting cells. Right panels, IgG secreting cells. Targets: Jap-Jap virus coated SRBC (top); PR8 virus coated SRBC (middle); B/Lee virus coated SRBC (bottom).

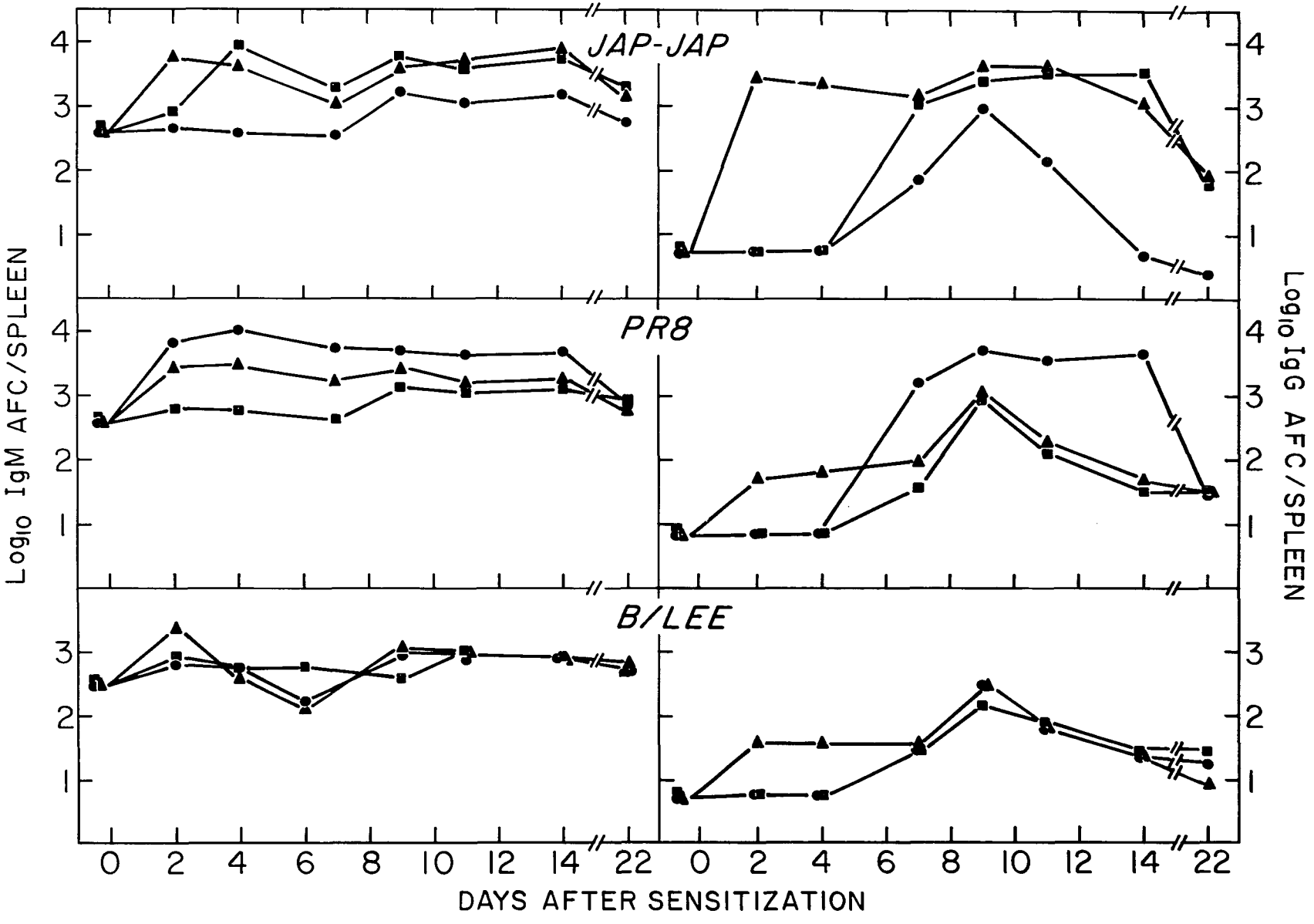

JAP-JAP
PR8
B/LEE
Log10 IgM AFC/SPLEEN
Log10 IgG AFC/SPLEEN
DAYS AFTER SENSITIZATION
0 2 4 6 8 10 12 14 22
1 2 3 4

FIGURE 23

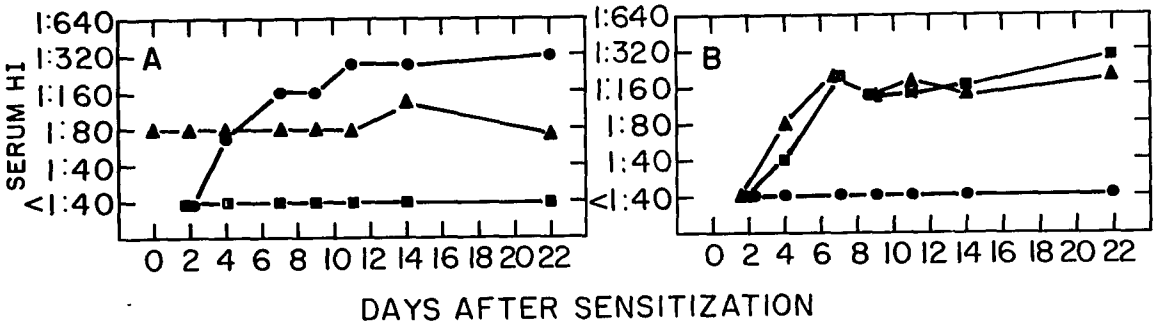


Figure 23. Serum hemagglutination inhibiting antibody titers of C3H mice primed with PR8 virus and immunized one month later with Japan virus (——▲——). Primary response to PR8 virus sensitization (——●——). Primary response to Japan virus sensitization (——■——). Antigens: PR8-Equi virus (left); Japan-Equi virus (right), 32 HA units.

studied for their AFC responses to PR8 virus described above, was examined. The variation between assays of this sort is large (it is common to find levels of specific immune release ranging from 20-60% in assays of sensitized spleen cells taken from mice immunized in exactly the same manner). Therefore, in order to make comparisons between groups of mice at different intervals after sensitization, it was necessary to stagger the immunizations, and sacrifice all of the mice on the same day. Results of 2 such assays are shown in Table 6. Subsequently, most assays were done at the peak of cytotoxic activity, between days 5 and 7 after immunization with PR8 virus. The kinetics of cytotoxic response were identical in both BALB/c and C3H mice, in contrast to the differences of their AFC responses to the same antigen.

The secondary in vivo response was studied. Restimulation with the same virus failed to elicit a measurable response. Subsequent immunization with a heterotypic influenza virus resulted in cytotoxic activity which was detectable at significant levels 3 days after inoculation. Peak activity levels were observed 5 days after restimulation. For discrimination between primary and secondary responses, the earlier time was chosen, because, at that time, there is very little activity in spleen cells taken from mice undergoing a primary response.

Histocompatibility Restriction of Cytotoxicity

Zinkernagel and Doherty (1974) had observed that recognition and killing of lymphocytic choriomeningitis virus infected target cells by lymphocytes from mice sensitized by LCM virus required identity at the major histocompatibility complex

Table 6

KINETICS OF DEVELOPMENT OF CYTOTOXIC T CELL ACTIVITY IN SPLENIC LYMPHOCYTES DERIVED FROM C3H MICE SENSITIZED PARENTERALLY WITH PR8 VIRUS

Experiment	Days After Sensitization	% S.I.R.	Student's t Test
I	2	20.5	$p < .05$
	5	68.9	$p < .005$
	7	70.5	$p < .005$
	9	37.4	$p < .02$
II	3	5.7	$p > .05$
	6	31.2	$p < .005$
	10	10.2	$p < .05$

Target L929 cells infected with PR8 virus;

Ratio: effectors to targets was 100:1.

between the lymphocytes and target cells. Subsequently this was demonstrated in other virus infections of mice (reviewed in the introduction). Consequently, this analysis of the response of mice to influenza virus was undertaken, in March of 1976. BALB/c ($H\text{-}2^d$) mice and C3H mice ($H\text{-}2^k$) were sensitized with PR8 virus six days prior to the assay. L929 cells ($H\text{-}2^k$) and BALB/c 3T3 cells ($H\text{-}2^d$) were used as targets. The results of the cytotoxic determination are shown in Table 7. The data confirmed the fact that cytotoxicity in the influenza virus system is H-2 restricted, as is the cytotoxicity in the other viral systems which have been studied.

Specificity of the Cytotoxic T Cell Response

The specificity of the cytotoxic recognition was studied. In Table 8 are results from a typical assay. Mice sensitized with PR8 virus (HON1) recognized both PR8 virus and X-31 virus (H3N2) infected L929 cells. Conversely, mice sensitized with X-31 virus lysed L929 cells infected with either X-31 virus or PR8 virus. Similar observations were made with Japan virus (H2N2) infected target cells in other experiments (data not shown). Therefore, in December of 1976, it was concluded that recognition of influenza virus infected cells was cross-reactive within influenza A viruses.

Later experiments were undertaken using lymphocytes from mice sensitized with either influenza A or influenza B/Lee viruses against L929 cells infected with those viruses. The results are shown in Table 9. Mice sensitized with influenza A viruses did not recongize influenza B virus infected cells,

Table 7

HISTOCOMPATIBILITY RESTRICTION OF CELL MEDIATED CYTOTOXICITY OF PR8 VIRUS SENSITIZED BALB/c AND C3H MICE

	PR8 Virus Infected Targets (% S.I.R.)	
	L929 Cells	BALB/c 3T3 Cells
BALB/c lymphocytes	5.3 ^{0}	17.8 *
C3H lymphocytes	19.1 *	-1.5 ^{0}

Effector to target cell ratio 30:1.

No significant lysis was observed by any lymphocyte population with uninfected target cells.

* $p < .01$

^{0} $p > .05$

Table 8

SPECIFICITY STUDIES OF THE CYTOTOXIC T CELL RESPONSE: HETEROTYPIC RECOGNITION OF INFLUENZA A VIRUS INFECTED L929 CELL TARGETS

	Target L929 Cells		
	Uninfected	PR8 Virus Infected	X-31 Virus Infected
Total releasable counts	70,628 ± 3252	78,111 ± 1820	77,143 ± 825
Spontaneous release	23,399 ± 116	31,411 ± 1208	36,802 ± 266
Control spleen cells	28,301 ± 1111	47,625 ± 817	47,998 ± 527
PR8 virus sensitized spleen cells	27,747 ± 126	59,785 ± 198 (39.9%)	59,240 ± 1628 (38.6%)
X-31 virus sensitized spleen cells	27,614 ± 324	60,616 ± 1855 (42.6%)	63,849 ± 2041 (54.4%)

Six days after sensitization; ratio was 15 effectors to 1 target.

Table 9

SPECIFICITY STUDIES OF THE CYTOTOXIC T CELL RESPONSE: INFLUENZA TYPE A AND TYPE B VIRUSES ARE NOT RECOGNIZED AS CROSS-REACTIVE

	L929 Targets (% S.I.R.)	
Effector Cells	PR8 Virus Infected	B/Lee Virus Infected
PR8 virus sensitized	39.4 ± 1.5 *	-1.2 ± 0.8 ^{0}
B/Lee virus sensitized	0.9 ± 1.3 ^{0}	27.5 ± 2.0 *

Ratio 100 effectors to 1 target cell

* $p < .005$

^{0} $p > .05$

and conversely lymphocytes from mice sensitized with influenza B virus did not kill L929 cells infected with an influenza A virus.

On rare and unreproducible occasions, spontaneous cytotoxic activity was observed with lymphocyte populations. When a rigorous attempt to detect natural killer cells of the sort described by Rodda and White (1976) or by Zinkernagel and Welsh (1977) was made, no non-specific activity was detected (data not shown).

Experiments with Antiserum to M Protein

The cross-reactivity at the level of the cytotoxic T cell was further investigated. Dr. Bucher generously provided antisera to M protein and to nucleoprotein; these antisera are described in detail in the Materials and Methods section. Antibody plus complement mediated lysis of infected target cells, immunofluorescence studies and experiments to determine the effect of anti-M protein antiserum on cell mediated cytotoxicity were begun.

Antibody Plus Complement Mediated Lysis

The cell damage produced by antibody plus complement mediated lysis was assessed in 2 ways, the release of ^{51}Cr from damaged cells or the failure of these cells to exclude trypan blue dye. In data not shown, it was determined that ^{51}Cr release was a more sensitive tool to determine cytolysis. Results of antibody plus complement mediated killing of MDCK, MDBK, and L929 cells 7 hours after infection with PR8 virus are shown in Table 10. These cell lines were selected because

Table 10

ANTIBODY PLUS COMPLEMENT MEDIATED LYSIS OF PR8 VIRUS INFECTED CELL LINES

Cell Line [0]	Antiserum to PR8 Virus	Antiserum to M Protein
L929	39.8 *	20.4
MDEK	78.4	24.7
MDCK	65.9	44.8

* Specific Release = $\frac{\text{(Antiserum - Normal Rabbit Serum)}}{\text{Total - Normal Rabbit Serum}}$ X 100

[0] Assay was performed 7 hours after infection. No significant release was detected using uninfected cells.

of different patterns of virus replication. L929 cells are nonproductively infected with PR8 virus. MDBC cells are productively infected, but noninfectious particles are made. MDCK cells are productively infected, releasing infectious particles. From the ^{51}Cr release studies it was concluded that M protein is expressed on the surface of all three cell lines. No lysis was observed with antiserum to nucleoprotein and complement, therefore, it was concluded that nucleoprotein was not expressed outside infected cells.

Immunofluorescence Studies

Indirect immunofluorescence studies were also undertaken. Data is presented in Table 11, and a typical series of micrographs are shown in Figure 24. No fluorescence was detected on uninfected cells exposed to antiserum to influenza viruses or proteins. No fluorescence was detected on infected cells treated with normal rabbit serum. Bright fluorescence was observed using antiserum to PR8 virus on all three cell lines. Dull fluorescence was observed on L929 and MDCK cells infected with PR8 virus after exposure to antiserum to nucleoprotein. In contrast, if the cells were fixed prior to antiserum treatment, bright, perinuclear fluorescence was observed. Infected cells treated with antiserum to M protein fluoresced brightly. Although adsorption with X-7 virus reduced the intensity of the fluorescence, antiserum to M protein still reacted with infected L929 and MDCK cells. It was concluded that nucleoprotein is probably not expressed on the surface of infected cells, but is detected within the same cells. M protein was

Table 11

SURFACE AND INTERNAL IMMUNOFLUORESCENCE OF PR8 VIRUS INFECTED CELL LINES

Treatment	L929[a]	MDCK[b]	MDBK[c]
Uninfected Cells:			
NRS[d]	0	0	0
Anti-PR8 virus[e]	0	0	0
PR8 Virus Infected Cells:			
NRS	0	0	0
Anti-PR8 virus	++++	++++	++++
Anti-NP[f]	±	±	ND[g]
Fixed cells[h] + anti-NP	++	++	++
Anti-M protein[i]	++	++	+
Anti-M protein adsorbed with X-7[j]	+	+	ND

[a] L929 cells, non-permissive infection

[b] Canine kidney cells, permissive infection, infectious particles

[c] bovine kidney cells, permissive infection, non-infectious particles

[d] normal rabbit serum

[e] rabbit anti-PR8 virus

[f] rabbit anti-nucleoprotein (X-7 virus)

[g] not done

[h] cells were acetone-fixed and then incubated with antiserum

[i] rabbit anti-M protein (X-7 virus)

[j] see Materials and Methods

FIGURE 24

A

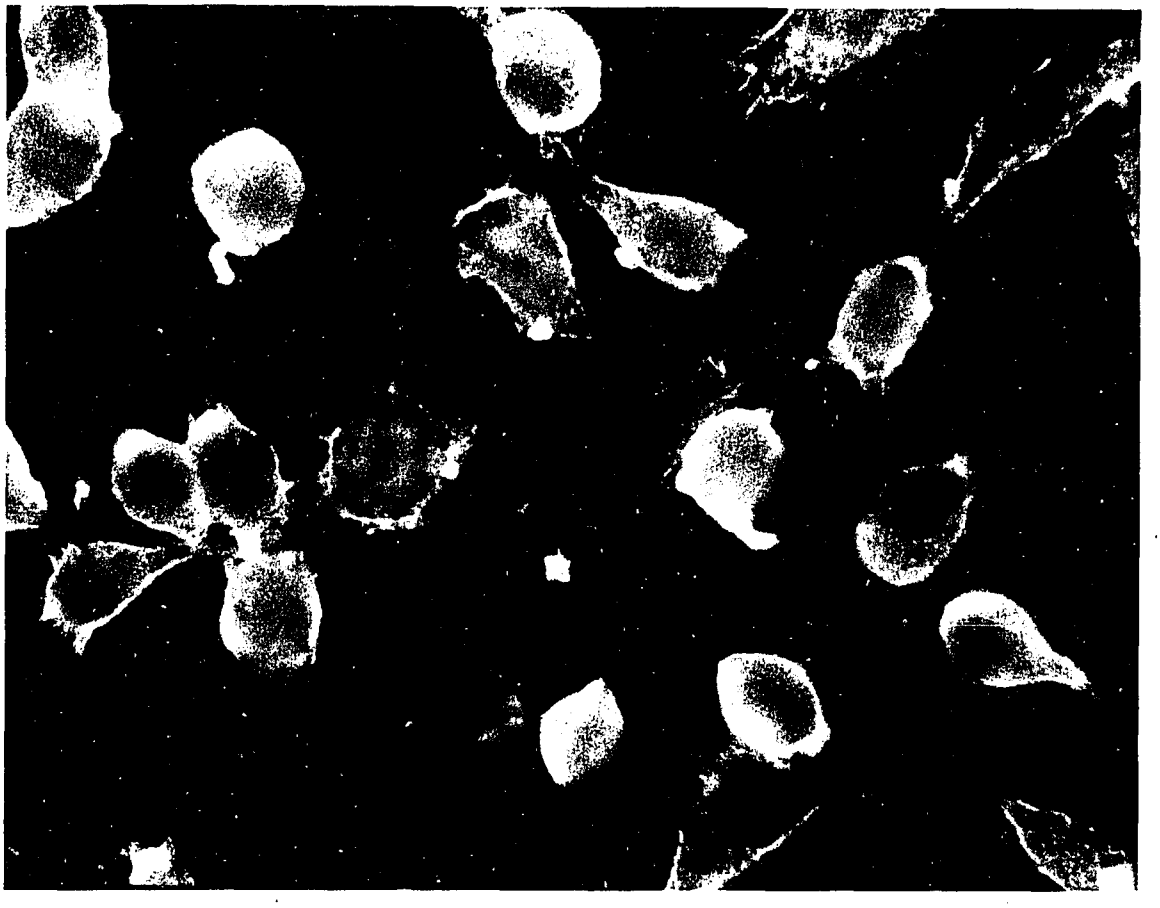


Figure 24. Immunofluorescence studies of PR8 virus infected L929 cells.

A. antiserum to whole PR8 virus, unfixed cells;

B. antiserum to nucleoprotein (X-7), unfixed cells;

C. antiserum to nucleoprotein, fixed cells;

D. antiserum to M protein (X-7), unfixed cells;

E. antiserum to M protein adsorbed with X-7 virus, unfixed cells.

FIGURE 24

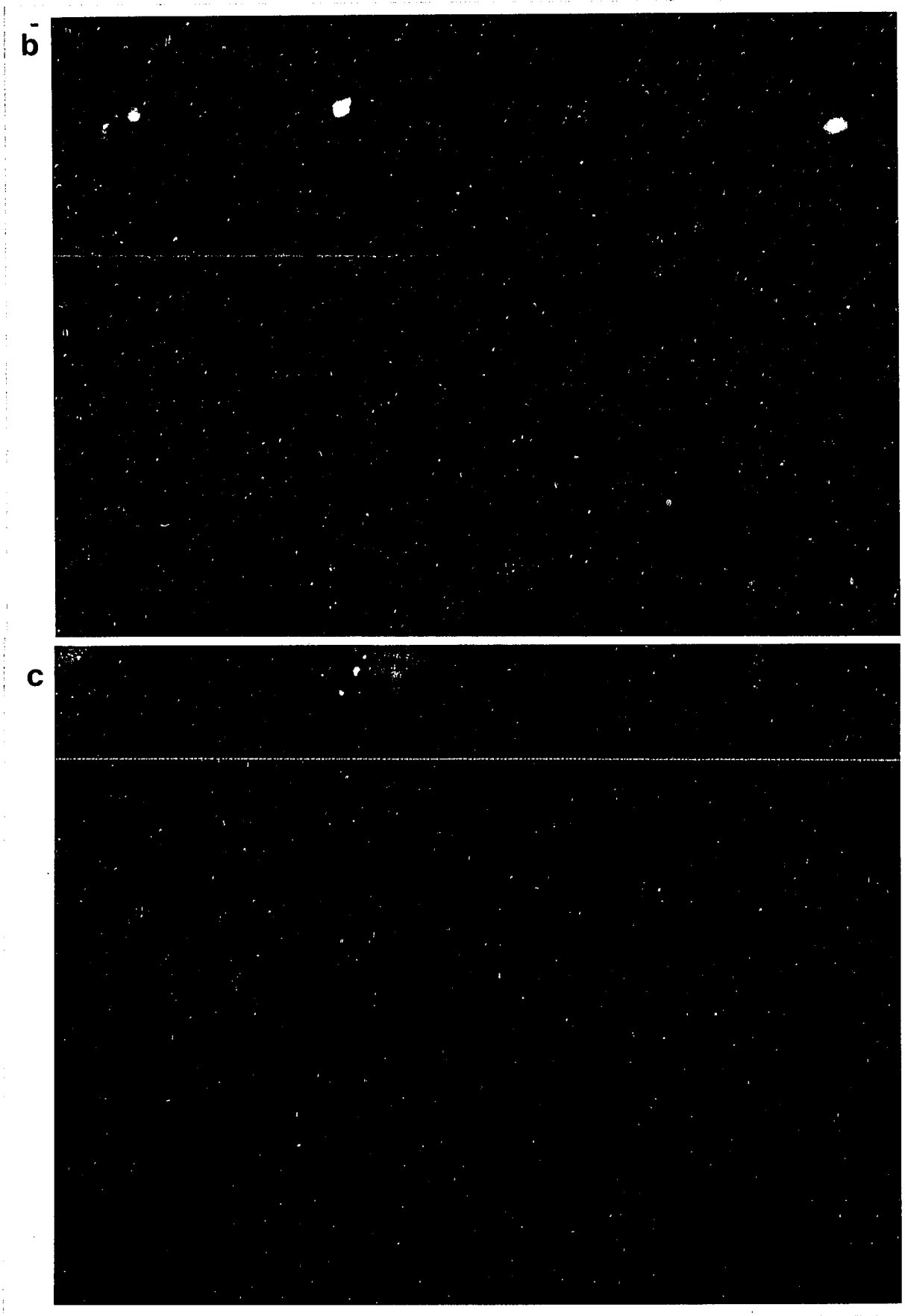
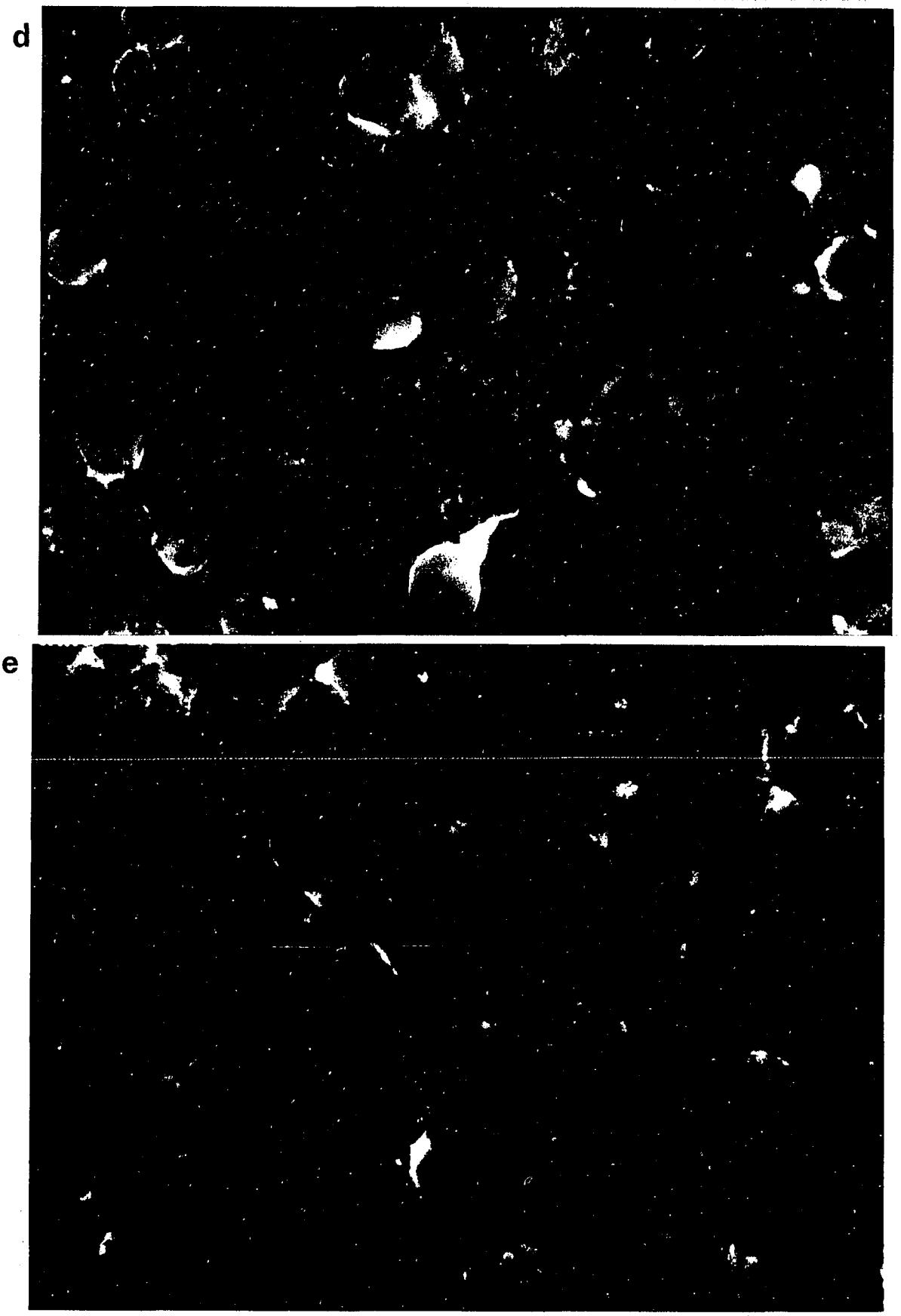


FIGURE 24



found to be present on the outside of infected cells.

Effect of Antiserum to M Protein on Cytotoxic Recognition

Additional experiments were conducted to determine whether antibody to M protein could block the cross-reactive cytotoxic T cell response. Spleen cells from PR8 virus sensitized mice were tested with target cells infected with homotypic PR8 virus and with cells infected with heterotypic Japan virus. As is shown in Table 12, simultaneous addition of anti-M protein antibody and sensitized lymphocytes resulted in a significant reduction in the cytotoxic activity with cell targets infected with heterotypic virus, but caused only a slight decrease in the activity against cells infected with homotypic virus. These results suggest that the cross-reactive killing could be due to the presence of a common M protein on the surface of infected cells. These observations are in accord with the findings of other investigators (Ada and Yap, 1977; Braciale, 1977b; Biddison et al., 1977) made while this work was in progress.

Effects of Different Forms of PR8 Virus Antigens on the Immune Response of C3H Mice:

Antibody Forming Cell Responses to Different Forms of PR8 Virus Antigens

The splenic lymphocyte AFC response and serum antibody titers were compared following immunization of mice with untreated allantoic fluid virus, gradient purified virus, virus inactivated with UV or formalin, or isolated glycoproteins derived from PR8 virus. The IgM secreting AFC responses to

Table 12

BLOCKING EFFECT OF ANTISERUM TO M PROTEIN ON CELL MEDIATED CYTOTOXICITY

	Target L929 Cells (% SIR)	
	PR8 Virus Infected	Japan Virus Infected
PR8 virus sensitized lymphocytes[a] + NRS[b]	24.3	36.0
PR8 virus sensitized lymphocytes + anti-M Protein[c]	22.4	10.1
% Reduction in Cytotoxicity[d]	7.7	71.9
	$p > .05$	$p < .01$

[a] Mice were sensitized 6 days previously with 0.2 ml PR8 virus, HA titer 1:1024. Ratio used was 100 effectors: 1 target cell.

[b] 10% normal rabbit serum in Dulbecco's medium during the 12 hour incubation period with lymphocytes and ^{51}Cr pulsed target cells.

[c] 10% rabbit anti serum to M protein.

[d] $$\frac{100 \times [SIR\ (+NRS) - SIR(+Anti\text{-}M)]}{SIR\ (+NRS)}$$

PR8 virus, shown in Figure 25a, demonstrate that the AFC responses to untreated allantoic fluid virus, purified untreated virus, and to UV inactivated virus were very similar. Although the IgM secreting AFC response to formalin inactivated virus followed similar kinetics to those seen after immunization with untreated or UV inactivated virus, the magnitude of the AFC response was consistently 3-5 fold lower. Following immunization with the isolated glycoprotein vaccine, only a minimal increase above the background level of IgM secreting AFC was observed. Figure 25B demonstrates the IgG secreting AFC response in the same groups of animals. Again, the responses to untreated and UV inactivated virus antigens were very similar. Formalin inactivated virus and the glycoprotein vaccine generated a small but measurable IgG AFC response. Results using target cells coated with appropriate recombinant viruses confirmed earlier observations that over 90% of the AFC response of both classes was directed at the hemagglutinin antigen (data not shown).

Comparison of the serum antibody titers in the same groups of mice correlated with the AFC responses. Serum HI antibody titers were equivalent in mice immunized with untreated and UV inactivated virus (Fig. 26A). The serum antibody titers after immunization with formalin inactivated virus were initially equivalent to those elicited by untreated virus, but HI titers did not continue to increase after day 5 in mice immunized with the formalin inactivated preparation. The glycoprotein vaccine evoked a small but measurable HI antibody

FIGURE 25

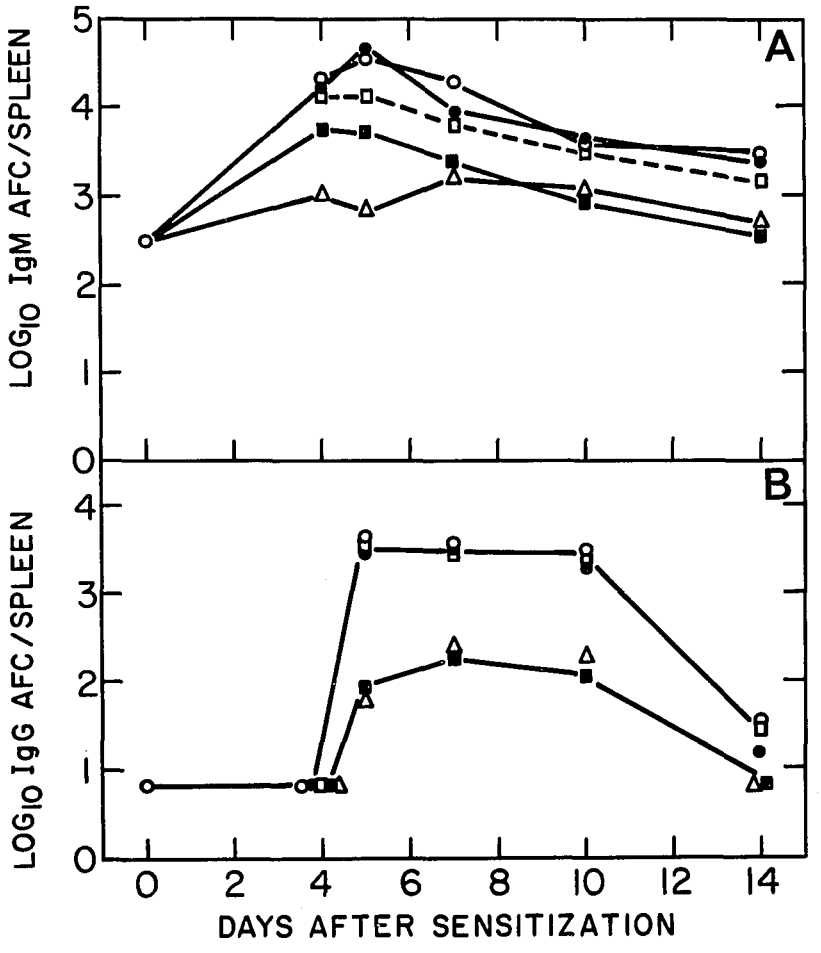


Figure 25. A. IgM secreting specific AFC in spleens of mice immunized as described in the Materials and Methods with untreated and inactivated forms of Influenza A/PR/8/34 virus. Target SRBC were coated with PR8 virus, B. IgG secreting specific AFC. Untreated allantoic fluid virus (—0—); Gradient purified PR8 virus (—●—); UV inactivated PR8 virus (—□—); Formalin inactivated PR8 virus (—■—); Glycoprotein heteropolymer of PR8 antigens (—△—).

FIGURE 26

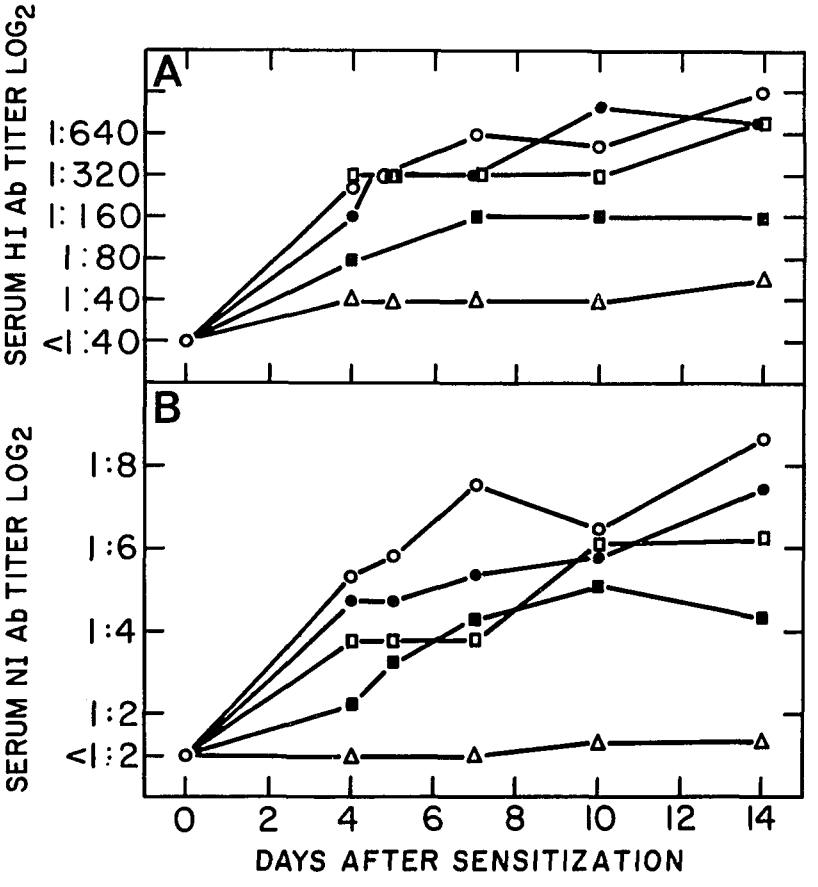


Figure 26. Serologic studies on specimens derived from the mice whose AFC are shown in Fig. 1. A. Hemagglutination inhibiting antibody titers. B. Neuraminidase inhibiting antibody titers. Untreated allantoic fluid PR8 (-○-); Gradient Purified PR8 virus (-●-); UV inactivated PR8 virus (-□-); Formalin inactivated PR8 virus (-■-); Isolated glycoproteins derived from PR8 virus (-△-).

response. Comparisons of NI antibody titers (Fig. 26b) gave similar results to those seen with the HI antibody responses.

Immunogenicity of the Extracted Glycoprotein in Rabbits

The virtual lack of AFC or serum antibody responses to the isolated glycoprotein preparation raised the possibility that denaturation might have occurred during the isolation procedure. To examine this possibility different doses of untreated intact virus and isolated glycoprotein vaccine were diluted to contain the same NA activity and were administered to groups of three rabbits each. Serum HI antibody was measured at intervals. Although the antibody response in rabbits which received the glycoprotein vaccine was slower to appear, the final serum antibody titers were similar to those observed in rabbits which received equivalent doses of whole virus (Table 13). The "antigenic extinction" (the minimum dose capable of evoking a measurable response) was identical for the two forms of antigen. These results indicated that the glycoprotein preparation retained immunogenicity in the rabbit, and that the absence of AFC and serum antibody responses in mice was not attributable to denaturation of the antigen.

AFC Responses to Commercial Vaccine

In another experiment, AFC and serum antibody responses to intact untreated virus and to two commercial vaccines were compared. One vaccine was a formalin inactivated intact MRC-11 (H3N2) virus preparation and the other was an N-butylphosphate disrupted preparation of the same virus. The vaccines were administered to mice in two different dosages, 500 CCA

Table 13

SERUM HEMAGGLUINATION INHIBITION ANTIBODY TITER OF RABBITS IMMUNIZED WITH PURIFIED PR8 VIRUS OR ISOLATED GLYCOPROTEINS OF PR8 VIRUS

Group		Prebleed	10 Days	20 Days	40 Days
Purified PR8 virus	1	<1:40*	1:160	1:320	1:40
Standard dose	2	<1:40	1:160	1:320	<1:40
	3	<1:40	1:320	1:320	1:80
Purified PR8 Virus	1	<1:40	<1:40	<1:40	1:160
1:20 dilution of	2	<1:40	1:80	1:80	1:80
standard dose	3	<1:40	<1:40	<1:40	<1:40
Isolated glyco-	1	<1:40	1:40	1:160	1:160
proteins	2	<1:40	<1:40	<1:40	1:80
Standard dose	3	<1:40	<1:40	<1:40	1:80
Isolated glycopro-	1	<1:40	<1:40	<1:40	<1:40
teins, 1:20 dilu-	2	<1:40	<1:40	<1:40	1:80
tion of standard	3	<1:40	<1:40	<1:40	1:80
dose					

* Antigen: A/PR/8/34/ (HO)-Equine/1/56 (Neql), 32 HA units.

units/mouse or a dose standardized by neuraminidase activity to contain the same quantity of antigen as the PR8 virus preparations described above. An additional group of mice received untreated intact MRC-11 virus at the standard dose. The results are summarized in Table 14. Mice immunized with the standard dose of the formalin inactivated vaccine attained the same titers of serum antibody as observed in mice immunized with untreated virus, although five days after immunization somewhat fewer AFC were detected in spleen cell suspensions of the group sensitized with the formalin inactivated virus preparation. Mice inoculated with the larger (500 CCA unit) dose of inactivated virus had higher titers of serum antibody initially, but did not attain higher peak titers than mice immunized with the smaller dose of formalin inactivated virus. Mice sensitized with disrupted virus vaccine had barely demonstrable serum antibody responses. Antibody was detectable in small quantities only 11 days after immunization with the 500 CCA unit dose. Similarly, AFC responses in mice immunized with the standard dose of disrupted virus preparation were limited to a 5-fold increase above background level. These results are consistent with those obtained using PR8 virus vaccines, except that mice immunized with the commerically prepared formalin inactivated virus responded with AFC and antibody titers even closer to those observed in mice sensitized with untreated virus.

Cell Mediated Cytotoxicity Studies

Cytotoxic responses were compared in mice immunized with the same PR8 virus preparations. Six days after immunization,

Table 14

SERUM HI ANTIBODY AND AFC RESPONSES OF MICE IMMUNIZED BY COMMERCIAL INFLUENZA VIRUS VACCINES AND UNTREATED VIRUS

Immunogen	Dose	Serum HI Antibody[a]			AFC[b]	
		Day 5	Day 8	Day 11	Day 5	Day 8
Whole virus, untreated[c]	standard[d]	40	160	640	11,400	2,000
Whole virus vaccine, formalin inactivated[e]	standard	80	160	640	5,300	4,100
	500 CCA	320	ND[f]	640	ND	ND
Disrupted virus vaccine[g]	standard	< 40	< 40	< 40	1,300	1,700
	500 CCA	< 40	ND	80	ND	ND

[a] reciprocal dilution of serum heamgglutination inhibiting antibody titer; 16 HA units of A/Pt. Chalmers/1/73 (H3)-Equine/1/56 (Neq1) virus was used as antigen.

[b] IgM secreting AFC/spleen; target SRBC were coated with A/MRC-11 a recombinant virus with A/Pt. Chalmers/1/73 (H3N2) surface antigens; background level was 240 AFC/spleen.

[c] untreated A/MRC-11 virus.

[d] see Materials and Methods.

[e] Merrell National vaccine (MRC-11)

[f] not determined

[g] Wyeth Laboratories vaccine (MRC-11)

mice were sacrificed and their splenic lymphocytes were assayed using PR8 virus infected L929 cell targets. The results are summarized in Table 15. UV inactivated virus was almost as effective as untreated virus in stimulating a primary cytotoxic response. However, in repeated experiments, the level of cytotoxic activity in mice immunized with UV inactivated virus was always significantly lower than that in mice immunized with untreated virus. Table 15 also demonstrates that no primary cytotoxic response was elicited by either formalin inactivated virus or by the isolated glycoprotein vaccine. Similar results were obtained in two additional experiments (data not shown).

Because Ennis and his co-workers (1977c) had reported a delayed appearance of the cytotoxic response in mice inoculated with commerical formalin inactivated virus vaccine, the observation period was extended. The results are summarized in Table 16. In mice immunized with untreated or UV inactivated virus, the peak cytotoxic response was observed on day 5 after immunization, and declined by day 10. Although a slight increase in the immune release was observed 10 days after administration of formalin inactivated virus vaccine, this increase was not statistically significant ($p > .05$). With the mice immunized with the isolated glycoprotein preparation, no significant cytotoxic activity was detected 6 or 10 days after sensitization.

Secondary Cytotoxic Responses

Although formalin inactivated virus and isolated glyco-

Table 15

CYTOTOXIC ACTIVITY OF SPLENIC LYMPHOCYTES OF C3H MICE SENSITIZED PARENTERALLY SIX DAYS PREVIOUSLY WITH WHOLE OR INACTIVATED FORMS OF INFLUENZA A/PR/8/34 VIRUS (HON1).

Sensitization	% Specific Immune Release ± SEM
Untreated PR8 virus	61.7 ± 3.9 *
Gradient Purified PR8 virus	66.0 ± 3.7 *
UV inactivated PR8 virus	41.7 ± 6.3 *
Formalin inactivated PR8 virus	2.9 ± 2.2 +
Isolated glycoproteins of PR8 Virus	0.1 ± 1.9 +

Ratio used was 100 effectors to 1 PR8 virus infected L929 cell target.

* $p < .005$

\+ not significant

Table 16

CYTOTOXIC ACTIVITY OF SPLENIC LYMPHOCYTES OF C3H MICE SENSITIZED PARENTERALLY SIX OR TEN DAYS PREVIOUSLY WITH WHOLE OR INACTIVATED FORMS OF INFLUENZA A/PR/8/34 (HON1) VIRUS.

Sensitization	% Specific Immune Release ± SEM	
	6 Days	10 Days
Untreated PR8 virus	54.1 ± 1.0 *	20.5 ± 2.4 *
Gradient purified PR8 virus	55.3 ± 1.2 *	23.8 ± 0.7 *
UV inactivated PR8 virus	31.9 ± 0.3 *	13.0 ± 2.5 ^{0}
Formalin inactivated PR8 virus	4.3 ± 3.3 +	8.6 ± 1.8 +
Isolated glycoproteins of PR8 virus	0.4 ± 3.3 +	4.4 ± 0.9 +

Ratio used was 100 effectors to 1 PR8 virus infected L929 cell target

* p <.005

^{0} p <.01

\+ not significant

protein vaccines were unable to engender a primary cytotoxic response, it was possible that these forms of the antigens might be able to induce memory. In subsequent experiments, the capacity to stimulate memory populations for a secondary cytotoxic response was examined. Mice were primed with each of the antigens as in Table 15, and one month later were challenged with heterotypic untreated whole Japan virus (H2N2). Mice were sacrified three days later and their spleen cells were assayed for cytotoxicity against Japan virus infected L929 cells. The results are summarized in Table 17. Three days after heterotypic challenge, the cytotoxic responses of mice sensitized initially with whole or UV inactivated PR8 virus were significantly greater than the primary response of previously unimmunized mice. In contrast, the cytotoxic responses to the same dose of Japan virus in mice previously primed with formalin inactivated virus or the isolated glycoprotein heteropolymer vaccine were somewhat lower than those seen with mice undergoing a primary response to Japan virus. These results indicate that formalin inactivated virus and the glycoprotein vaccine were not only unable to stimulate a primary cytotoxic response, but were also incapable of priming for memory which could be recalled on secondary challenge with heterotypic whole virus.

Next we attempted to determine whether formalin inactivated virus or the glycoprotein vaccine could elicit a secondary cytotoxic response in mice which had been previously immunized with untreated heterotypic virus. Mice were primed

Table 17

SECONDARY CYTOTOXIC RESPONSES OF SPLENIC LYMPHOCYTES FROM C3H MICE PRIMED WITH UNTREATED OR INACTIVATED FORMS OF INFLUENZA A/PR/8/34 (HON1) VIRUS

Sensitization	Challenge[a]	% Specific Immune Release ± SEM[b]
None	Japan virus[c]	13.2 ± 0.6 *
Untreated PR8 virus	Japan virus	31.2 ± 2.2 *
UV inactivated PR8 virus	Japan virus	24.0 ± 2.6 *
Formalin inactivated PR8 virus	Japan virus	7.9 ± 2.5 ^{0}
Isolated glycoproteins of PR8 virus	Japan virus	6.9 ± 1.2 +

[a] mice were primed with the standard dose of PR8 virus and one month later challenged with Japan virus, 3 days before assay.

[b] ratio was 100 effectors to one Japan virus infected L929 cell target

[c] influenza A/Japan/305/57 virus (H2N2), untreated whole virus

* $p < .005$

\+ $p < .01$

^{0} $p < .05$

with intraperitoneal injection of intact Japan virus and one month later were stimulated with untreated, UV inactivated, or formalin inactivated PR8 virus, or with the glycoprotein vaccine. Secondary responses were evident in mice previously primed with Japan virus then boosted with untreated intact virus, UV inactivated, intact virus or formalin inactivated intact virus. However, even in previously primed mice, no cytotoxic response was observed following stimulation with the isolated glycoprotein vaccine (Table 18).

Employing the MRC-11 vaccines used by Ennis (1977c) to induce a primary cytotoxic response, we were unable to find any activity with either the formalin inactivated virus or with the disrupted virus vaccine at any dose in several experiments. In contrast, untreated MRC-11 virus was an effective stimulator of a primary cytotoxic response. The response was cross-reactive as demonstrated by cytotoxicity with target cells infected by different subtypes of influenza A virus. However, no cytotoxicity for targets infected with influenza B/Lee/40 virus was observed using effector cells from mice which had been sensitized with intact influenza A virus. Data from 2 separate experiments is summarized in Table 19.

Immunization of Mice with B/Lee Virus and Isolated PR8 Virus Glycoproteins

Laver and his associates (1977) had observed that immunization of hamsters with both isolated glycoproteins of an influenza A virus and whole B/Lee virus could induce a serum antibody response to the antigens of the isolated glycopro-

Table 18

SECONDARY CYTOTOXIC RESPONSES OF SPLENIC LYMPHOCYTES OF PRIMED C3H MICE WITH WHOLE OR INACTIVATED FORMS OF INFLUENZA A A/PR/8/34 (HON1) VIRUS

Secondary Sensitization[a]	% Specific Immune Release ± SEM[b]
Untreated PR8 virus	29.8 ± 4.4 *
UV inactivated PR8 virus	10.7 + 1.0 *
Formalin inactivated PR8 virus	11.4 ± 1.8 ^{0}
Isolated glycoproteins of PR8 virus	2.1 + 1.2 +

[a] mice were primed with untreated intact A/Japan/305/57 virus (H2N2) and immunized one month later with PR8 virus antigens, three days before assay.

[b] ratio used was 100 effectors to 1 PR8 virus infected L929 target cell

* $p < .005$

^{0} $p < .01$

+ not significant

Table 19

CYTOTOXIC ACTIVITY OF SPLENIC LYMPHOCYTES OF C3H MICE SENSITIZED PARENTERALLY WITH WHOLE OR DISRUPTED COMMERCIAL VACCINES OF INFLUENZA A/MRC-11 (H3N2) VIRUS OR UNTREATED MRC-11 VIRUS

	Target Cells (% Specific Immune Release)[a]		
Sensitization	PR8 virus infected	MRC-11 virus infected	B/Lee virus infected
PR8 virus, untreated	73.6 ± 3.5 *	36.1 ± 2.3 *	ND[b]
MRC-11 virus, untreated	48.5 ± 6.1 *	21.8 ± 1.7 *	ND
Formalin inactivated MRC-11 virus vaccine[c]	0.0 ± 2.7 +	-0.1 ± 2.5 +	ND
Disrupted MRC-11 vaccine[d]	-0.9 ± 5.2 +	3.1 ± 6.5 +	ND
PR8 virus, untreated	20.5 ± 0.7 *	ND	0.2 ± 0.8 +
MRC-11 virus, untreated	13.0 ± 1.0 *	ND	-0.7 ± 0.6 +
Formalin inactivated MRC-11 vaccine	-0.5 ± 0.2 +	ND	-2.1 ± 0.5 +
Disrupted MRC-11 vaccine	-0.8 ± 0.4 +	ND	-2.0 ± 0.5 +
B/Lee virus, untreated	1.3 ± 1.1 +	ND	19.4 ± 0.5 *

[a] ratio used was 100 effectors to 1 target L929 cell

[b] not done

[c] Merrell National vaccine

[d] Wyeth Laboratories vaccine

* $p < .005$

\+ not significant

tein preparation. A similar series of experiments was undertaken using the same PR8 virus glycoprotein preparation which had previously been shown to be poorly immunogenic for both antibody forming cellular and cell mediated cytotoxicity responses in C3H mice. Mice were immunized with the standard dose of PR8 virus glycoproteins and 0.2 ml of a B/Lee virus preparation which had an HA titer of 1:256. There was no adjuvant effect observed in either the generation of an AFC response to PR8 virus antigens 6 or 10 days after immunization. In addition, no cytotoxic T cell response to PR8 virus infected L929 cells was detected. No change in the number of anti-B/Lee virus AFC or of lysis of B/Lee virus infected L929 cells was observed (Table 20). Therefore, it was concluded that intact B/Lee virus did not act as an adjuvant for the isolated glycoprotein either in the generation of an AFC response or of a cytotoxic T cell response in mice.

Immune Responses During Influenza Virus Infection

Fifty mice were infected with approximately 500 MlD_{50} of PR8 virus by exposure to a small particle aerosol. At intervals after infection, 5 mice were sacrificed, individual serum specimens obtained, lungs scored for gross lesions and ground suspensions prepared for infectious virus titration. Separate pools of lymph node cells and spleen cells from the same animals were prepared, and assayed for AFC using PR8 virus coated targets. Figure 27a demonstrates the response of C3H mice to infection as measured by virus titers, lung lesions, serum antibody, and deaths. Peak virus titers were observed

Table 20

AFC AND CYTOTOXIC T CELL RESPONSES IN C3H MICE AFTER IMMUNIZATION WITH BOTH B/LEE VIRUS AND ISOLATED PR8 VIRUS GLYCOPROTEINS

Immunization	AFC 6 Days After Inoculation		% SIR 6 days PI	
	PR8-SRBC	B/Lee-SRBC	PR8-L929	B/Lee-L929
Naive Mice	275	310	--	--
PR8 virus	30,080	360	35.8	1.1
PR8-GP*	305	295	1.3	-0.9
PR8-GP + B/Lee	295	10,550	0.7	29.8
B/Lee	280	11,000	2.0	31.2

* isolated glycoprotein

FIGURE 27

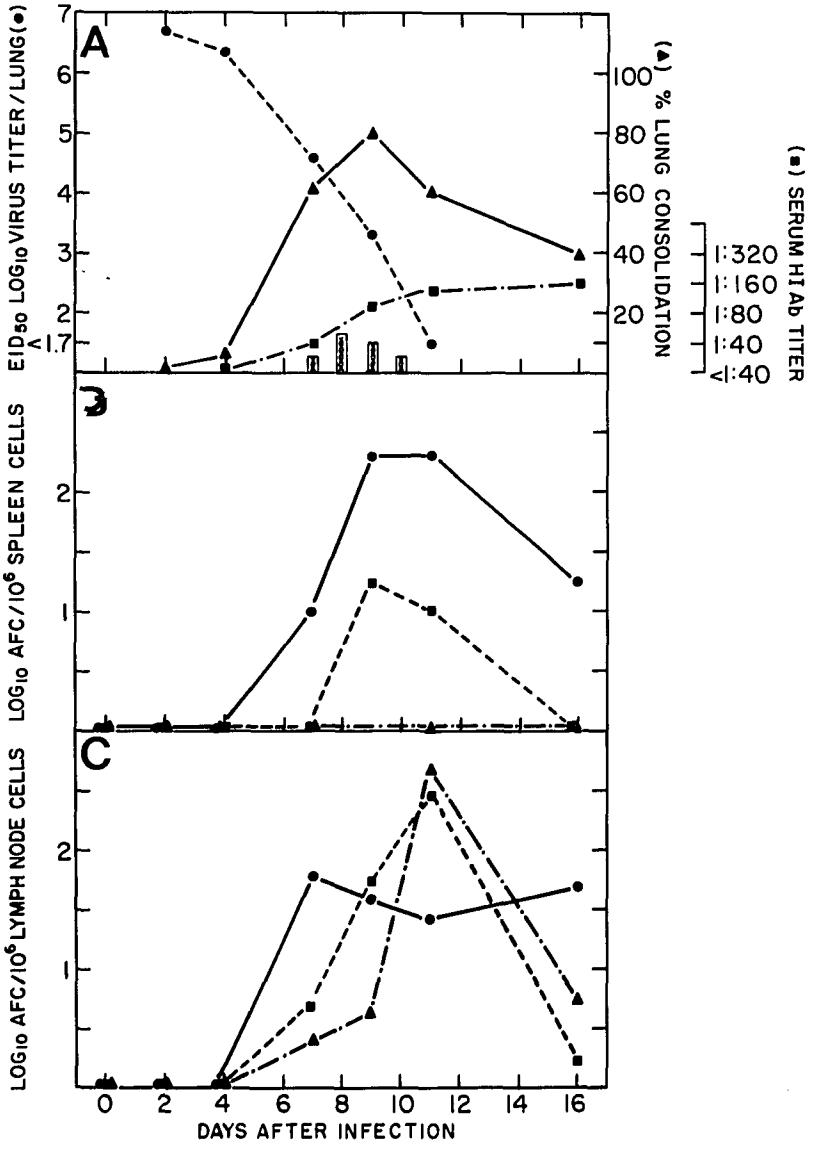


Figure 27. Kinetics of the response of C3H mice to infection with a small particle aerosol of influenza A/PR/8/34 virus (50 MID_{50}). Panel A: titer of infectious virus present in lung homogenates ($log_{10}EID_{50}$); ●— – —●. Lung lesions expressed as % consolidation; ▲———▲. Serum

hemagglutination inhibiting antibody titer; ■— — —■. The number of mice which died is represented by an X within a box. Panel B: Splenic AFC: IgM secreting cells ●———●; IgG secreting cells ■— — —■ ; IgA secreting cells ▲— — —▲ . Target SRBC were coated with PR8 virus. Panel C: AFC present in the mediastinal lymph nodes: IgM secreting cells ●———●; IgG secreting cells ■— — —■ . IgA secreting cells ▲— — —▲ . Target SRBC were coated with PR8 virus.

within the first 2-3 days following infection and decreased rapidly thereafter. Gross pulmonary lesions initially appeared on the 4th day after infection and reached a maximum between the 7th and 9th days, and then declined in severity. A total of 13 mice died during the course of this infection. Most deaths occurred between the 7th and 10th days after infection. Serum HI antibody was demonstrable 7 days after infection and reached peak titers 4 days later.

Figure 27b shows the kinetics of the AFC response in the spleens of the same mice. No specific AFC of any class were detected until 7 days after infection, when IgM secreting cells were observed. IgM secreting cells reached a peak between days 7 and 9 and then declined. Two days after the appearance of IgM secreting cells, IgG secreting cells were detected in the spleens; only one in 10 AFC was secreting IgG antibody to PR8 virus. During the observation period, IgA secreting cells were not detected in the spleens of mice infected with influenza virus.

AFC responses in lymphocytes obtained from mediastinal lymph nodes also were not observed until 7 days after infection (Fig. 27c) when there was a predominance of IgM secreting cells, but IgG and IgA secreting cells were also present. The numbers of IgM secreting cells rapidly reached a plateau, which was maintained throughout the observation period. IgG and IgA secreting cells reached a peak of about 500/million lymphocytes harvested, 11 days after infection.

Using SRBC targets with recombinant viruses appropriate

to measure hemagglutinin- or neuraminidase-specific responses, hemagglutinin-specific AFC responses outnumbered NA-specific responses by approximately 10 to 1. These ratios are similar to those obtained following parenteral immunization.

In another experiment, 60 C3H mice were infected with approximately 100 MID_{50} of Japan virus. As illustrated in Figure 28a, peak pulmonary virus titers were not as high as with PR8 virus infection, and virus persisted in the lungs of mice for a longer period of time. Thus, on day 7, the geometric mean titers of virus were as high as those found two days following infection. (In several infections, PR8 virus has been seen to reach a higher peak titer and be cleared more rapidly than Japan virus- data not shown). In addition, the peak lesion score and the appearance of serum antibody occurred later following infection with Japan virus. Death also tended to occur one day later with Japan virus infection.

Figure 28b illustrates the AFC responses observed in the spleens of mice infected with Japan virus. As with PR8 virus infection, IgM secreting cells dominate the splenic response and no IgA secreting cells above background were detected. In general, the kinetics of the AFC response is similar to that observed following PR8 virus infection, although the predominance of IgM secreting cells is not as great and the rate of decline in the numbers of AFC in both classes is somewhat slower. Similar kinetics of the AFC response were observed in several other experiments (data not shown) except for the appearance of a small increase over background levels on day

FIGURE 28

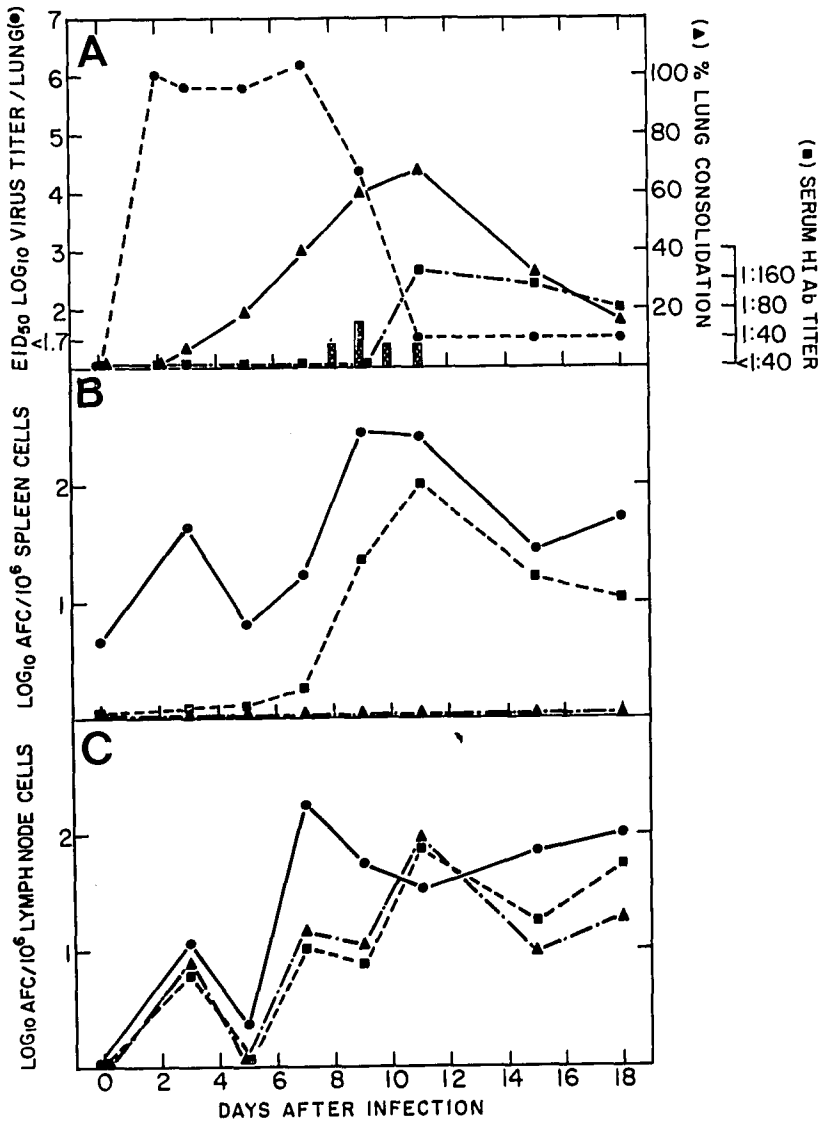


Figure 28. Kinetics of the response of C3H mice to infection with A/Japan/305/57 virus (10 MID_{50}). Panel A: titer of infectious virus present in lung homogenates ($log_{10}EID_{50}$); ●— — —●. Lung lesions (% consolidation); ▲————▲. Serum hemagglutination inhibiting antibody titer; ■— — —■. The number of mice

which died due to infection is illustrated by an X within a box. Panel B: Splenic AFC: IgM secreting cells ●———●. IgG secreting cells ■— — —■. IgA secreting cells ▲— —▲. Target SRBC were coated with Japan virus. Panel C: AFC present in the mediastinal lymph nodes: IgM secreting cells ●———●. IgG secreting cells ■— — —■ . IgA secreting cells ▲— — —▲. Target SRBC were coated with Japan virus.

3 after infection which was not consistent in all experiments.

The kinetics of the AFC response in the mediastinal lymph nodes (Fig. 28c) also was similar to that observed after PR8 virus infection. The peak IgM AFC response was on day 7, whereas the peak IgG and IgA secreting cellular response did not occur until day 11. Following Japan virus infection a predominance of IgG and IgA secreting cells over IgM secreting cells in mediastinal lymph node cells was not clearcut as that observed following PR8 virus infection.

The effect of mercaptoethanol treatment of hemagglutination inhibiting antibody titers of sera from the mice whose AFC studies are depicted in Figures 27 and 28 are shown in Figure 29. Resistant hemagglutination inhibiting antibody appeared at detectable levels 4 days after the initial appearance of serum HI antibody following either PR8 virus infection (Fig. 29a) or Japan virus infection (Fig. 29b). By 17 or 18 days after infection, most of the serum antibody was resistant to reduction. This indicated that serum antibody is of the IgM class during recovery and that IgG serum antibody is a late component of the recovery process.

To make meaningful comparisons of cytotoxic responses in lymph node and spleen at different times after infection, using the same infected targets on the same assay day, it was necessary to stagger infections of mice. Previous experiments had shown that if similar groups of mice were infected with the same stock virus on different days under the same conditions, the kinetics of the response to infection were similar. Groups of mice were infected at intervals with about

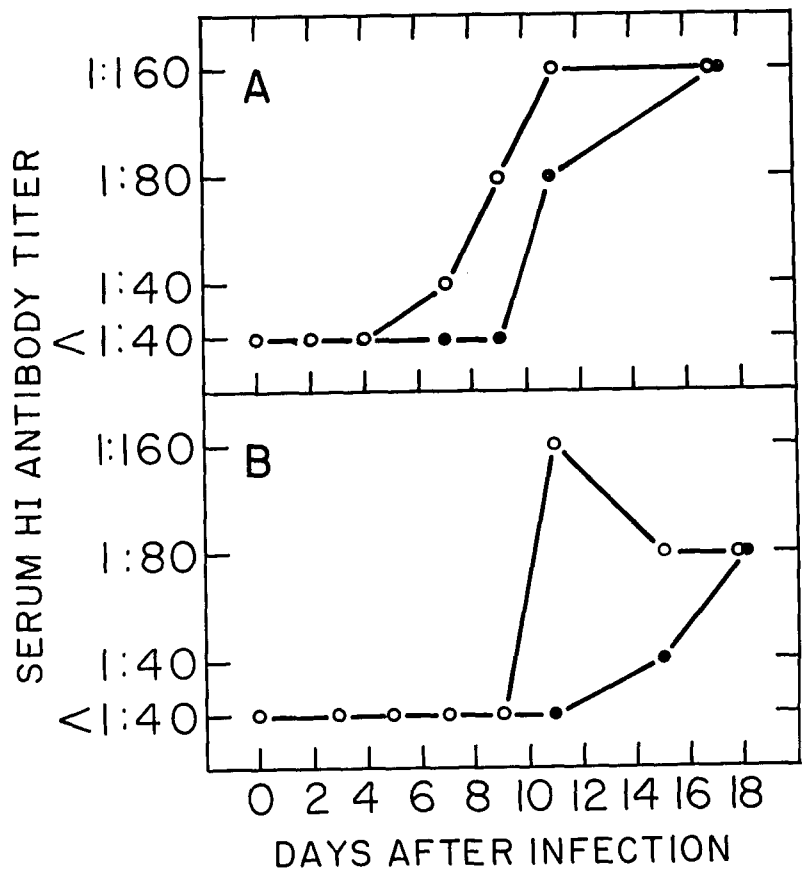


Figure 29. Effect of mercaptoethanol treatment on serum HI antibody titers of C3H mice during influenza virus infection. A. PR8 virus infection. B. Japan virus infection. Untreated serum (0); mercaptoethanol resistant (●). Antigens: A. PR8-Equi virus; B. Japan-Equi virus, 32 HA units.

500 MID_{50} PR8 virus in a small particle aerosol. All mice were sacrificed on a single day, pulmonary lesions were scored, individual specimens, lung homogenates were obtained and separate pools of lymph nodes and spleen cells were made using 7 mice per group. Figure 30a demonstrates that the infection followed a similar course in these mice as was shown in Figure 27a, although pulmonary virus titers declined somewhat more slowly. Figure 3b illustrates the specific cytotoxic activity of lymph node lymphocytes and splenic lymphocytes (at a ratio of 50:1 each) from mice infected at varying times earlier. On a per cell basis, splenic lymphocytes exhibited consistently higher activity than lymphoctyes from mediastinal lymph nodes. Peak effector activity was detected in both lung and spleen 7 days after infection. Equivalent cytotoxicity was observed using L cell targets infected with heterotypic influenza A viruses, but no activity was observed with influenza B/Lee/40 virus infected L cells targets (data not shown). This is consistent with the cross-reactivity of cytotoxic responses following intraperitoneal immunization (Doherty et al., 1977; Braciale, 1977a; Yap and Ada, 1977; Zweerink et al., 1977a).

Table 21 is a composite of three experiments, two using different doses of PR8 virus and a third using Japan virus. In Experiment I (also shown in Fig. 30), cytotoxic activity was observed in the spleen but not in the lymph nodes 4 days after infection. Lung lesions were most extensive 10 days after infection, whereas cytotoxic activity was at its peak 7 days after infection in both mediastinal lymph nodes and spleens. In infection II, mice were infected with twice as

FIGURE 30

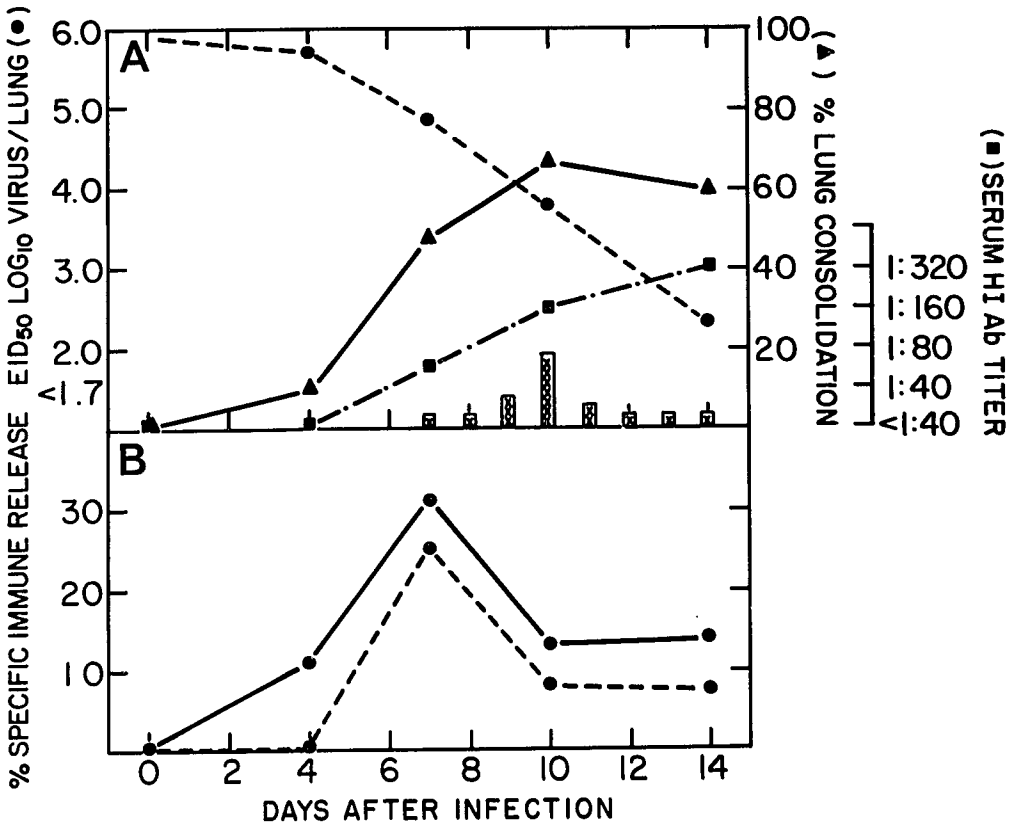


Figure 30. Kinetics of the response of C3H mice to infection with A/PR/8/34 virus (50 MID_{50}). Panel A; titer of infection virus present in lung homogenates ($log_{10}EID_{50}$); 0----0. Lung lesions (% consolidation) ▲——▲. Serum hemagglutination inhibiting antibody titer; ■——■. The number of mice which died is represented by an X within a box. Panel B; Specific immune release of mediastinal lymph node lymphocytes; ●----●. Spleen lymphocytes ●——●. Target L929 cells were infected with PR8 virus and assay was run at an effector to target cell ratio of 50:1.

Table 21

	Experimental Infection[a]	Days after Infection or Sensitization	Specific Cytotoxic Activity: Lymph Node	Specific Cytotoxic Activity: Spleen	Lung Lesions[a]
I	PR8 virus (500 MID_{50})	4	none	10.9 ± 1.11*	10
		7	25.8 ± 0.50*	31.4 ± 1.73*	48
		10	8.2 ± 1.95[0]	13.3 ± 1.68*	66
		14	8.6 ± 0.86[0]	14.9 ± 1.31*	59
	PR8 virus sensitized[d]	6		24.8 ± 2.80*	
II	PR8 virus (1000 MID_{50})	4	none	37.9 ± 6.97+	9
		7	none	27.6 ± 7.28+	41
	PR8 virus sensitized	6		26.5 ± 9.50+	
III	Japan virus (30 MID_{50})	4	none	none	5
		7	none	20.3 ± 1.39*	30
		10	none	43.3 ± 2.59*	45
		15	24.8 ± 2.66*	32.4 ± 2.66*	15
	PR8 virus sensitized	6		16.2 ± 1.36*	

[a] experimental infection I target L929 were infected with PR8 virus; effector to target ratio 50:1 for both mediastinal lymph node cells and for spleen cells.

experimental infection II target L929 were infected with PR8 virus; effector to target ratios were 37:1 for lymph node cells and 100:1 for splenic lymphocytes.

experimental infection III target L929 cells were infection with Japan virus; effector to target cell ratios were 50:1 for mediastinal lymph node cells and 100:1 for splenic lymphocytes.

Legend to Table 21 - Continued....

[b] specific immune release ± standard error of mean

[c] % lung consolidation

[d] mice were immunized parenterally with 0.2 ml containing 1000HA units of egg grown PR8 virus six days before the assay.

* p <.001 student's t test

+ p <.01

[0] p <.05

much initial virus as in infection I (1000 MID_{50} PR8 virus); all mice which had been reserved for the later time points died between days 8 and 10 of infection. No cytotoxic activity was detected in lymph nodes at either 4 or 7 days after infection. The spleen effector population developed more rapidly in this infection than in infection I; maximum cytotoxic activity was evident on day 4 in this experiment, but the extent of macroscopic lesions did not reach maximal levels until three days later. In infection III, in which mice were infected with Japan virus (30 MID_{50}) only 7% of the mice expired. Lesion development followed the same kinetics as in the first two infections. No splenic cytotoxic activity was detected until a week after initiation of infection and the peak of cytotoxic activity was not reached until day 10. In contrast to experiment I, cytotoxic effector cells in mediastinal lymph nodes were not demonstrable until day 15.

IX. DISCUSSION

The series of experiments, comprising this work have compared, for the first time, the development of cytotoxic thymus derived lymphocytes and the development of antibody forming cells following infection or immunization with influenza virus antigens in different forms. The specificities of the response have also been examined.

A. Kinetics and Specificity of the Antibody Forming Cellular Response

The antibody forming cellular responses were detected using a modified Jerne plaque assay system with wild type or recombinant influenza virus coated sheep erythrocyte targets. The kinetics and specificity of the AFC detected in lymphocytes obtained from immunized mice were compared to the appearance of serum antibody (hemagglutination inhibiting and neuraminidase inhibiting). Thus it was possible to make several observations which would not have been apparent from antibody determinations alone.

Although the serum antibody responses of C3H mice to primary immunization with two different antigen doses, and to secondary sensitization are equivalent to those of BALB/c mice, the kinetics of the cellular responses in the 2 strains of mice are different in several respects. The primary AFC response in BALB/c mice to a "standard" dose of PR8 virus is characterized by a clearcut predominance of IgM secreting cells throughout the response (Fig. 6), and peak numbers of

AFC of both classes 11 days after sensitization. In contrast, C3H mice respond with an earlier attainment of a lower maximum number of AFC/spleen. In addition, there is only a transient early predominance of IgM secreting cells in C3H mice (Fig. 14).

The kinetics of the secondary AFC responses are also distinguishable. Both strains responded with comparable rapid sustained increases in serum antibody resistant to mercaptoethanol and with rapid increases in splenic AFC dominated by IgG secreting cells (Figs. 9,18,19). However, the magnitude of the secondary AFC responses in BALB/c mice was approximately 10-fold greater (range 6-20 fold in different experiments) than that of C3H mice (Figs. 12,17).

Furthermore, a 10-fold reduction in the immunizing dose of virus influenced the kinetics of primary AFC responses in the two strains differently. In BALB/c mice the AFC response to the standard dose and to the 10-fold lower dose were indistinguishable for the first 6 days, after which, in mice immunized with the larger dose, the numbers of AFC continued to increase, whereas, the numbers of AFC began to decline in mice which had received the smaller inoculum (Fig. 10). In contrast, in C3H mice, the AFC response to the lower dose was initially delayed, but realized the same peak level as observed following the standard dose (Fig. 15).

These differences in the kinetics of the AFC responses in BALB/c and C3H mice contrasted with the similarities in serum antibody responses cannot be explained fully by the techniques employed in these studies. It is possible that

C3H mice produce antibody which is more avid, so that fewer molecules secreted by fewer cells can achieve the same result in inhibition assays. Minga and his co-workers (1975) have described 60-fold differences in the avidity of anti-DNP hapten antibody in BALB/c and C3H mice. In the present experiments, such differences in antibody avidity might also explain the more rapid shut-off of the C3H proliferative response by a mechanism of high avidity negative feedback as has been described in the immune response to staphylococcal nuclease (Pisetsky et al., 1978). It is possible that AFC vary in the quantity of antibody secreted. Such differences could not be discriminated by the techniques described in this report, but the monoclonal splenic focus technique used by Gerhard (Braciale et al., 1976) could measure the relative amounts of antibody produced by individual clones in vitro. It is improbable that plaques from one strain of mice were recognized unevenly by the Fc-specific facilitating antiserum used in these studies, as BALB/c and C3H mice are congenic at the IgCH a haplotype (Herzenberg et al., 1965).

The differences in the kinetics of the AFC and antibody responses in the two strains of mice are also consistent with the hypothesis that the continued increase in the numbers of AFC in BALB/c mice after primary immunization and the more pronounced AFC response after secondary immunization reflects a preferential response to determinants which are not detected in antibody inhibition assays.

With the use of target cells coated with appropriate

recombinant viruses, it was observed that at least 90% of the AFC response was directed to determinants on the viral hemagglutinin. However, not all HA-specific antibody secreting cells may produce antibody which is reactive in the HI assay. This implies that the HA glycoprotein possesses a complex of antigenic determinants.

Clones producing antibody detectable in HI determinations may be preferentially stimulated early in the primary response of BALB/c mice to the standard dose of PR8 virus, and may dominate the AFC response after low dose immunization. Response to another set of HA-specific determinants, not detected in HI tests, may characterize the later (after day 6 or 7) proliferative response and most of the secondary response in BALB/c mice. Such an explanation would imply a hierarchy in the responses to HA-specific antigenic determinants in BALB/c mice. At high antigen concentration, the subsets of determinants are recognized sequentially. However, at low levels of antigen, the subset detected in HI assays dominates the response. Precedent for such a hierarch is provided by the evidence that in the immune response of BALB/c mice to phosphorylcholine, antibody of multiple idiotypic specificities is secreted early, and is displaced by the TEPC-15 idiotype later in the response (Ruppert and Claflin, 1978). Although the TEPC-15 idiotype proceeds to dominate the response in BALB/c mice, this idiotype represents a minority of the response in other strains of mice (Cancro et al., 1978b). Compared to BALB/c mice, C3H mice

may produce a diminished response to HA-specific determinants not detected in the HI test while undergoing a primary response to PR8 virus.

The AFC assay enabled us to examine the specificities of the immune response to PR8 virus by means of target erythrocytes coated with a variety of antigens. In the primary response of BALB/c and C3H mice to PR8 virus, there is a barely detectable increase over background levels of cells recognizing target SRBC coated with viruses of different subtypes. However, in the secondary response of both strains, a substantial cross-reactive component is evident (Fig. 21). To characterize which determinants are involved in this secondary heterotypic response, we used a variety of SRBC coated with different antigens. The numbers of AFC observed using targets coated with the isolated glycoproteins of X-31 virus (H3N2) were equivalent to those observed using whole X-31 virus coated red cell targets. Only negligible numbers of AFC above background were noted when SRBC coated with M protein or normal allantoic fluid were used as targets. These observations eliminate the possibility that the secondary heterotypic AFC responses were due to recognition of egg proteins which had been adsorbed to the targets and reduced the likelihood that recognition of internal viral antigens is responsible.

Sera were not examined for complement fixing antibody to soluble influenza virus antigens. In the AFC assay using M protein coated SRBC targets, no increase in the background

level of AFC was observed in spleen cells from mice undergoing either a primary or a secondary response to PR8 virus. However, it must be noted that no direct measurement of the quantity of M protein coupled to periodate treated erythrocytes was made. Moreover, no information is available from these studies about the humoral immune response to M protein in mice immunized with PR8 virus. Previous observations of human serologic response to influenza virus infection or to immunization with vaccines have indicated that infection generally induces an increase in complement fixing antibody to the soluble virus antigens, whereas intact virus vaccine generally does not. In contrast, immunization with disrupted virus vaccine stimulates an antibody response to internal virus antigens (Tauraso in Kilbourne et al., 1972). Hence, the absence of a significant response to internal proteins observed in these studies may either reflect a bias of the AFC assay or it may be possible that immunization with intact PR8 virus is a poor inducer of antibody to internal soluble virus proteins.

Further studies using B/Lee virus grown in 2 host cell systems to coat erythrocyte targets were consistent with the hypothesis that virtually all of the heterotypic cross-reactive response to B/Lee virus was due to recognition of host coded oligosaccharide sidechains on the glycoproteins. However, the cross-reactivity observed among type A influenza viruses was not due exclusively to recognition of host-antigen. This was evidenced by the fact that there were consistently 10-fold

or more AFC recognized by targets coated with H2N2 virus than were observed with B/Lee virus coated targets; and the demonstration that more than 60,000 AFC/spleen were detected with canine kidney cell propogated H2N2 and H3N2 virus coated targets. It is possible that there are antigenic determinants on the glycoproteins of X-31 (H3N2) and Japan (H2N2) viruses which are recognized in the secondary response of mice immunized with PR8 virus (HON1).

Additional evidence of cross-reactive determinants shared by PR8 virus and Japan virus comes from the response to sequential immunization of C3H mice with the two viruses. In comparison to unprimed mice, mice previously primed with PR8 virus responded during the first 6 days after challenge with Japan virus with increased numbers of AFC of both classes which reacted with PR8 virus and Japan virus coated SRBC targets (Fig. 22). This response was not due to recognition of common host antigen because only small increments over the background response to B/Lee virus coated SRBC targets were detected. However, the increased AFC responses to Japan virus coated targets in PR8 virus primed mice was not reflected in a boosted serum HI antibody response to Japan virus (Fig. 23). These findings are consistent with the hypothesis that there are common cross-reactive determinants on the glycoproteins of viruses of different influenza A virus subtypes, and that these determinants stimulate an antibody response which does not inhibit hemagglutination.

The cross-reactive clones reactive with H2N2 and H3N2 detected in the secondary response to PR8 immunization might recognize determinants which elicit antibody not active in HI determinations; this could include antibody to determinants on the neuraminidase. Furthermore, it cannot be excluded completely that these clones are recognizing determinants on internal viral proteins.

In both BALB/c and C3H mice, the primary AFC response to PR8 virus sensitization is associated with a barely detectable increment in the background number of AFC which react with B/Lee virus coated SRBC targets. In the secondary AFC responses of both strains of mice to PR8 virus, the numbers of AFC recognizing B/Lee virus coated targets is appreciably increased. However, in the secondary response, the host antigen specific response comprises 10% or less of the total AFC response. Gerhard and his co-workers have attributed a much greater proportion of the primary response to recognition of host determined oligosaccharide antigen(s) (Cancro et al., 1978a). The differences in the results may be related to differences in immunization and assay protocols. In most of the studies by Gerhard and his colleagues, spleen cell fragments derived from primed BALB/c mice are cultured with large quantities of whole virus (either homotypic or heterotypic) and the antibody secreted in vitro is characterized by radioimmunoassay (Braciale et al., 1976; Gerhard et al., 1975; Gerhard, 1976). The studies presented in this report employed sensitization in vivo with smaller doses of

virus. Among the findings included in this communication is the modulating effect of antigen dose in BALB/c mice. In the primary response of BALB/c mice, a small dose of PR8 virus preferentially stimulates HI reactive clones, whereas immunization with larger doses of virus is associated with stimulation by determinants on the glycoproteins which evoke antibody that does not inhibit hemagglutination. It is possible that the very large dose of virus used in culture by Gerhard and his co-workers to evoke a secondary response, altered the spectrum of the response, stimulating populations of B cells of lower affinity (reviewed in Siskind and Benacerraf, 1969). In their assay, the method used to define clones responding to host antigen was any clone of antibody secreting cells which reacted with heterotypic H2N2 or H3N2 viruses. In contrast, observations described in this report included discrimination of clones responding to common determinants on the glycoproteins of heterotypic influenza A viruses but not with B/Lee virus. Even taking into account, the broader definition of host antigen used by Gerhard, all of the differences between the findings on the proportion of the response associated with oligosaccharide antigen(s) cannot be completely explained. There are other differences in the studies, as Gerhard and his colleagues can only survey a finite number of clones for the reactivity patterns. However, using the AFC assay, as many as 10^7 splenic lymphocytes can be sampled with one virus coated SRBC target on a slide. In addition, in the radioimmuno assay, a positive response is defined by 10% or greater

binding to the immunoadsorbant. It is possible that clones producing antibody of lower affinity might register in the AFC assay and not in the radioimmunoassay.

Evidence of the relatedness of the glycoprotein antigens of influenza viruses of different subtypes has been shown in several different ways. The phenomenon of "Original Antigenic Sin" was discussed in the Introduction. Other experiments employing repeated infections of guinea pigs with the same influenza virus resulted in the appearance of serum antibody which reacted in a virus specific complement fixation test with viruses of different subtypes (Henle and Lief, 1963). In double immunodiffusion assays, HswlNl, HONl, and HlNl viruses have been found to cross-react at the level of the hemagglutinin when antiserum made to isolated hemagglutinin is used (Schild, 1970; Schild et al., 1972). Radioimmunoassay using antibody produced in vitro has confirmed the cross-reactivity among those subtypes (Gerhard et al., 1975; Gerhard, 1976). In hemagglutination inhibition tests, Dowdle and his co-workers (1972) have detected antigenic relatedness among the hemagglutinins of the H2N2 and H3N2 subtypes. Radioimmunoassay has also been used to find evidence of relatedness between HlNl and H2N2 viruses (Kurimura et al., 1973). Anker and his co-workers (1978) observed cross-protection between influenza H2N2, H3N2, and Heq2Neq2 viruses in mice hyperimmunized with incomplete Freund's adjuvant. They observed protection from death following lethal injection with heterotypic virus in the absence of serum HI or NI antibody to the challenge virus prior to infection.

Thus, in summary, these studies have detected at least 4 levels of recognition in the AFC response of mice to PR8 virus. The principal feature of the response is that it is virus specific, and primarily directed at the hemagglutinin. Among the clones recognizing the HA, many secrete antibody which inhibits hemagglutination. Other clones recognize structural determinants on the glycoprotein and secrete antibody which is not detected in HI tests. Some of the latter clones recognize determinants present on the glycoproteins of other subtypes of influenza A viruses, but not on influenza B/Lee virus. In addition, there are clones which recognize host oligosaccharide antigen(s) present on all influenza viruses grown in that host cell system. The latter population accounts for all of the cross-reactivity observed with egg grown B/Lee virus coated SRBC targets.

These observations suggest that the AFC assay may prove to be a useful tool in the analysis of the cellular responses to influenza virus antigens and in defining the specificities of those responses beyond what is possible with antibody analysis alone.

B. Kinetics and Specificity of the Cytotoxic T Cell Response

After parenteral immunization of mice with untreated PR8 virus, a cytotoxic response develops, peaking in activity 5-7 days after immunization (Table 6). The response is characterized by cross-reactivity among influenza A virus infected targets, but no activity with influenza B virus infected targets (Tables 8 and 9). Target recognition requires not only

the presence of influenza A virus antigens, but also antigens of the major histocompatibility complex, H-2 (Table 7). Secondary responses cannot be elicited by the virus used to prime mice, but can be stimulated by heterotypic viruses, in vivo. All of these observations are consistent with the findings published by Doherty et al. (1977), Braciale (1977a), Yap and Ada (1977), and by Zweerink et al. (1977a). However, Cambridge and her colleagues (1976) and Ennis and his co-workers (1977b; 1977d) have described hemagglutinin specific recognition of infected cells. When Ennis and his colleagues compared (1977a) L929 cells and a "primary" mouse kidney cell line with respect to cytotoxic target cell specificity, they found considerable cross-reactivity with L929 targets in contrast to hemagglutinin specific recognition of the kidney cell targets.

To determine whether common nucleoprotein or M protein antigens are expressed on the surface of infected cells, immunofluorescent techniques and studies employing antibody plus complement mediated lysis were undertaken. With either assay system, nucleoprotein was not detectable on the surface of infected cells. In contrast, M protein, accessible to external antibody, was found in both assays. In addition, M protein could be demonstrated at the external cell surface of abortively infected L929 cells, and at the surface of infected MDCK and MDBK cells, both of which are productively infected with influenza viruses. Therefore, the expression of M protein at the surface of cells does not require abortive infection.

Other investigators have demonstrated the blocking of cytotoxic T cell activity by antiserum to antigenic determinants which are recognized, but these observations have been made only in alloreactive systems, not for virus infected target cells (Cerottini and Brunner, 1974; Nabholz et al., 1974; Germain et al., 1975; Burakoff et al., 1977). In the present experimental studies, most of the heterotypic cytotoxic T cell recognition could be blocked by antiserum to M protein (Table 12). This observation suggests that a portion of the heterotypic cytotoxic recognition was specific for M protein expressed in conjunction with H-2 antigens on the surface of infected cells. It does not, however, rule out the possibility that a component of the cytotoxic T cell population recognizes determinants present on the glycoprotein antigens, similar to the cross-reactivity detected in the AFC response, described above.

Ada and Yap (1977), Braciale (1977b), and Biddison and his co-workers (1977) have described the presence of M protein on the surface of infected cells. They also suggest that this protein may be recognized by cytotoxic T cells and may be responsible for the cross-reactive component of cytotoxic assays. All of these studies and those presented in the present report require certain assumptions concerning the specificity of the antiserum used. If the antiserum purported to be specific for isolated M protein is contaminated with antibodies which react with glycoprotein antigens, then the conclusions drawn are open to question.

Braciale (1977b) treated infected cells with deoxyglucose and used the infected cells in competition assays with sensitized cytotoxic T cells. He observed that the antigen specific competition was lost when cells treated with deoxyglucose were used, but the heterotypic component of the cytotoxic response was retained. He therefore concluded that the expression of viral hemagglutinin on the surface of infected cells is recognized by one portion of the cytotoxic T cell pool. In infected cells treated with the antimetabolite, hemagglutinin was not detectable, but heterotypic cytotoxic recognition, probably mediated by recognition of M protein was unaffected.

In contrast to the recognition of AFC, it is probable that a large segment of the cytotoxic T cell population recognizes M protein on infected cells. Both cytotoxic T cells and AFC recognize virus specific determinants. In addition, AFC and probably cytotoxic T cells also recognize cross-reactive influenza A specific determinants associated with the glycoproteins. AFC, but not cytotoxic T cells respond to chicken host antigen(s) on the glycoproteins.

C. The Responses of C3H Mice to Different Forms of Influenza Virus Antigens

Previously, comparisons by different forms of presentation of influenza virus antigens have emphasized antibody responses, in recognition of the primary role of antibody in protection against infection with homotypic virus (Salk et

al., 1945; Fazekas de St. Groth and Donnely, 1950). More recently, with the development of techniques to measure T cell responses to immunization with influenza virus antigens, some attention has been given to the relative efficiency with which different forms of antigen generate such responses. For example, it has been reported that formalin inactivated virus is capable of eliciting a primary cytotoxic response in unprimed mice (Ennis et al., 1977c) and that high concentrations of isolated glycoproteins in vitro can stimulate a secondary cytotoxic response, but not a primary cytotoxic response (Zweerink et al., 1977b).

In the present studies for the first time, the antibody forming cellular responses and cell mediated cytotoxic responses of mice immunized with equivalent doses of different forms of influenza virus antigens were compared. The results are consistent with previous observations demonstrating that formalin inactivated virus induces antibody production at levels comparable to those induced by untreated virus (Fazekas de St. Groth and Donnely, 1950). However, in the present studies, formalin inactivated virus was found to be ineffective in inducing a primary cytotoxic T cell response or in eliciting memory for a secondary cytotoxic response. In contrast, mice immunized with UV inactivated virus responded by producing high titers of antibody and generating cytotoxic T cell responses (Fig. 25 and Table 15). The striking differences in the antibody and cytotoxic respones of mice immunized with UV or formalin inactivated PR8 virus are not likely

to be due to the threshold of sensitivity or our cytotoxic assay. The absence of priming following immunization with formalin inactivated virus for a secondary cytotoxic response (Table 4) argues that formalin inactivated virus is far less efficient than untreated virus in inducing proliferation and maturation of the appropriate subsets of T cells involved in the cytotoxic response.

It is possible that the cellular processing and recognition of antigen for helper T cell and for B cell responses are different from the recognition structure(s) which stimulate cytotoxic T cell differentiation. It has been demonstrated that replicating agents are more effective in generating another T cell effector function, delayed hypersensitivity responses (Gell and Benacerraf, 1961). In several other systems, the development of delayed hypersensitivity (DTH) and resistance to infection has been linked to immunization with infectious and not inactivated agents. Francisella tularensis infected animals develop DTH and cellular resistance to infection; whereas killed organisms, although effective in stimulating an antibody response, did not either elicit a measurable DTH response or confer cellular resistance to challenge in adoptive transfer experiments (Kostiala et al., 1975). Mice sensitized with formalin inactivated Venezuelan equine encephalomyelitis virus developed high titers of specific antibody. However, adoptive immunity could be conferred by spleen cells from mice immunized with live virus, but not by spleen cells from mice sensitized with formalin inactivated

virus (Rabinowitz, 1976). Although lacking antibody to the challenge virus, mice which had been primed with infectious virus were partially protected when challenged with a heterotypic influenza A virus; in contrast, mice immunized with inactivated viruses were not protected (Schulman and Kilbourne, 1965). In the light of more recent experiments, it is not unlikely that the cross-protection observed may have been due to cross-reactive recognition by T cells of influenza virus infected targets. Whereas infectious virus is capable of stimulating Ly 2 positive T cell effector responses as well as Ly 1 positive helper T cell functions (Cantor and Boyse, 1977), formalin inactivated virus stimulates only the latter response.

In contrast to formalin inactivated virus, UV inactivated PR8 virus was capable of abortive infection as evidenced by expression of viral glycoproteins on infected cell surfaces in vitro. It is likely that the UV inactivated PR8 virus retained the same capacity in vivo, leading to the expression of viral antigens in association with H-2 antigens on appropriate stimulator cells, and consequently was able to stimulate a primary cytotoxic T cell response.

Macrophage processing of non-replicating antigen may be adequate in producing the appropriate macrophage-T cell-B cell interaction requisite for antibody production. Even when live virus is inoculated intraperitoneally, it is unlikely that newly synthesized antigens contribute significantly to the antibody response as evidenced by the observation that a

large proportion of the antibody response to egg grown virus is specific for chicken host carbohydrate antigen(s) (Harboe, 1963; Gerhard, 1976). Thus, processed antigen not newly synthesized antigen, provides the major stimulus for antibody production. Conversely, newly synthesized antigen appears to be far more effective in generating a cytotoxic response.

The present studies of AFC and antibody responses of mice immunized with purified glycoproteins or with disrupted virus have demonstrated that these subviral forms of antigen are poorly immunogenic. Even with a 20-fold higher dose of antigen, antibody responses were significantly lower than those produced with whole virus, and no cytotoxic activity was evident. Similar observations with respect to the poor immunogenicity of subviral forms of viral antigen in stimulating an antibody response have been made previously in unprimed mice (Barry et al., 1974; McLaren et al., 1977), in unprimed ferrets (McLaren et al., 1974), in unprimed hamsters (Laver and Webster, 1976) and in young children (Wright et al., 1977). Paradoxically, as has been previously reported, rabbits generate effective antibody responses to the isolated glycoprotein antigens (Laver and Webster, 1976). The basis for this species difference is unexplained.

The fact that mice mount a low level primary antibody response to higher doses of the isolated glycoprotein antigens suggests that there is no barrier to a primary response to antigens in this form, rather, that the isolated glycoproteins are far less efficient in stimulating an antibody response. It is possible that the processing of virus-sized

particles (even inactivated virus) and the processing of disrupted virus or isolated glycoprotein heteropolymers are different. It is also possible that the clearance of virus particles occurs more slowly than with heteropolymers, producing a relative increase in the period of high antigen concentration. The explanation for the absence of a primary cytotoxic T cell response with the isolated glycoprotein or disrupted virus immunization is probably similar to that for the failure of formalin inactivated virus to stimulate a primary cytotoxic response. However, whereas formalin inactivated virus was capable of stimulating a secondary cytotoxic response, equivalent concentrations of antigen in the form of isolated glycoprotein or disrupted virus, failed to achieve the same result. Again, it is likely that this is due to different handling of intact virus and antigens in the form of disrupted particles. The fact that with high concentrations of isolated hemagglutinin, a secondary cytotoxic response was elicited *in vitro*, (Zweerink *et al.*, 1977b) argues that there is no absolute block to stimulation of a cytotoxic response with this form of antigen, but that the efficiency is markedly reduced.

The possible implications of the results of the present experiments for influenza virus immunization should not be overlooked. At the present time it has not been resolved whether T cell effector functions other than helper activity (Virelizier *et al.*, 1974; Sullivan *et al.*, 1975; Burns *et al.*, 1975; Iwasaki and Nozima, 1977) are beneficial to the host.

On the one hand, effector T cells may be involved in virus clearance (Fig. 29; Yap and Ada, 1978). Evidence for such a mechanism is suggested by the observation that subtype specific protection can be adoptively transferred to infected unirradiated hosts (Schulman et al., 1977). However, T cells also may contribute to immunopathological consequences. The production of cytotoxic T cells and the development of lesions have been reported to follow similar time courses (Yap and Ada, 1978). In addition, athymic nude mice have been reported to have less severe lung lesions following infection than heterozygous littermates (Sullivan et al., 1975; Wyde et al., 1977).

Evidence obtained in man demonstrates that T cell functions of blastogenesis, development of delayed hypersensitivity and cytotoxic effector responses are stimulated following exposure to influenza virus antigens (Dolin et al., 1978; Kantzler et al., 1974; McMichael et al., 1977), but no basis exists to assess the role of these responses to recovery or to pathogenesis. Neither have any comparisons been made of the relative efficiency with which different forms of vaccines stimulate these responses in vivo. If the cytotoxic responses of humans are comparable to those of mice, immunization of primed subjects with formalin inactivated virus vaccine may be effective in stimulating a secondary but not a primary cytotoxic T cell response. Consideration should also be given to the possibility that the more prolonged immunity reported to follow natural infection compared to the immunity seen following parenteral immunization with formalin inactivated virus

(Kilbourne et al., 1973) might be due, in part to, stimulation of a cytotoxic response and not exclusively to the production of secretory antibody. In any case, it seems likely that knowledge of the capacity of different forms of antigen presentation to stimulate T and B cell responses will be helpful in designing better vaccines.

D. The Responses of C3H Mice to Influenza Virus Infection

In these studies we have directly examined the kinetics of two cellular responses of mice to influenza A virus infection by comparing the development of AFC and cytotoxic effector cells. The present studies provide evidence that cytotoxic T cells appear earlier and attain peak activity sooner than antibody forming cellular responses (Figs. 27 and 29). The data also demonstrate a temporal relationship between the development of cytotoxic effector T cells and the disappearance of virus, both of which preceed the appearance of AFC. Hence, on the basis of the kinetics of the responses the clearance of virus was related to the development of cytotoxic T cells rather than to antibody or to antibody forming cellular responses, which develop after significant virus clearance has already occurred. Moreover, the fact that antibody as well as AFC appear relatively late disputes the suggestion that antibody is present early in infection, but is inaccessible to measurement due to the formation of immune complexes. It should be noted that the threshold sensitivity of the AFC and CMC assays might be very different. It is well within the

sensitivity of the AFC assay to detect a 10-fold increase in the background level of antibody secreting cells which react with virus coated SRBC targets, an increase of less than 10% of the total AFC response. In contrast, an increase of 10% of the cytotoxic activity would have been undetectable over the background release of ^{51}Cr in the presence of naive lymphocytes. However, it is possible that a portion of the AFC response in the local lymph nodes was not detectable because of migration of a portion of the cell population into the lungs. Such an interpretation seems unlikely considering the homing patterns of most B cells, and would require that influenza-specific plasma cells behave in a different manner than other B cell populations (Mitchell, 1972).

In contrast to the lack of correlation of antibody or antibody forming cellular responses and virus clearance, antibody responses appeared at a time when pulmonary lesions were developing. We do not intend to suggest that there is a causal relationship between antibody responses and the development of immunopathology. In other experiments carried out in this laboratory, it has been demonstrated by means of functional impairment of macrophages, that serum antibody production and lesion formation can be dissociated (Rosenberg and Schulman, 1978). Similarly, the temporal association of the development of cytotoxic T cells and virus clearance does not necessarily imply a causal relationship. It has been demonstrated that cytotoxic T cells can prevent the release of infectious vaccinia virus from infected cell targets in vitro (Zinkernagel and Althage, 1977), providing a mechanism by which effector

T cells can contribute to virus clearance. Furthermore, in adoptive transfer experiments, influenza virus infected recipients of T cell enriched populations of immune spleen cells cleared virus more rapidly than recipients of T cell populations from naive mice (Schulman et al., 1977). However, in vivo, it is probable that other mechanisms such as the generation of an immune interferon response, activation of macrophages, and other manifestations of delayed hypersensitivity reactions contribute to interrupting virus replication and also to the development of lesions.

In some experiments, cytotoxic T cells were not detected in mediastinal lymph nodes early in the response. It is possible that the cytotoxic effectors had migrated to infected tissues, and that immature developing effector cells and/or subthreshold levels of cytotoxic T cells remained in the lymph nodes. It should be noted that other investigators have described different kinetics in lymph node and spleen cell activity following infection (Cambridge et al., 1976; Ennis et al., 1977a; Yap and Ada, 1978; Doherty et al., 1978). Yap and Ada (1978) reported that concurrent development of cytotoxic activity occurred in local lymphoid tissue and spleen. They observed a correlation between the appearance of cytotoxic activity and the development of lung lesions. On the other hand, Cambridge and her co-workers, and Ennis and his associates, observed that cytotoxic activity in the lymph node preceeded cytotoxic activity in the spleen. The lack of complete correlation between our results and those of others might be due to the use of different strains of mice at

different ages (3-10 weeks), different target cell systems, a variety of influenza A viruses, inoculated by different routes (intravenous, intranasal instillation, aerosol), and at different doses.

Other experiments have shown that thymus derived cells are required not only for helper activity (Virelizer et al., 1974; Burns et al., 1975; Sullivan et al., 1975; Iwasaki and Nozima, 1977) but also for the containment of virus in the lungs of infected mice (Schulman et al., 1977; Sullivan et al., 1975; Wyde et al., 1977; Virelizier, 1976). Whether T cells contribute to recovery from infection or lead to the development of immunopathological disease may depend upon the interplay of many factors including the virulence of the strain of virus, the immune response genotype of the host, how fast the immune response is mobilized, and previous exposure to related antigens, and non-specific factors.

X. SIGNIFICANCE OF THIS WORK

1. By the simultaneous measurement of antibody forming cellular and cytotoxic T lymphocyte responses to different forms of influenza virus antigens, it was possible to discriminate forms of antigen presentation which were capable of stimulating antibody forming cellular but not cell mediated immune responses. Whether or not such differences may be significant in immunity of man is not clear. However, an appropriate understanding of whether or not a particular antigen stimulates cell mediated immune responses may enable us to design more effective vaccines.

2. These studies have elucidated antigenic relatedness among type A influenza viruses which is not apparent from standard serological testing. The specificities of the antibody forming cellular and cytotoxic T cell responses indicate cross-reactivity which may contribute to our understanding of partial protection or "Original Antigenic Sin".

3. During infection of mice by influenza viruses, the kinetics of the cellular responses suggest that T cells may contribute substantially to virus clearance and recovery. Antibody and AFC responses appear too late to effect virus clearance, but are undoubtedly important in protection.

XI. APPENDIX: ABBREVIATIONS

A2G	inbred mouse strain resistant to myxovirus infection
AFC	antibody forming cells
AG-B	major histocompatibility complex of the rat
ATS	anti-thymocyte serum
BALB/c	inbred mouse strain
BALB/c3T3	embryonic fibroblast cell line of BALB/c mice
B/Lee virus	influenza B/Lee/40 virus
C3H	C3H/StHa, inbred mouse strain
CCA unit	chick cell agglutination unit
^{51}Cr	radioactive chromium
DTH	delayed type hypersensitivity
EID_{50}	50% egg infectious doses
GP	glycoprotein
H-2	major histocompatibility complex of mice
HA	hemagglutinin glycoprotein
HA titer	hemagglutination titer
HBSS	Hank's balanced salt solution
HI	hemagglutination inhibiting antibody
HL-A	major histocompatibility complex of man
HK virus	influenza A/HongKong/8/68 virus (H3N2)
HK-PR8 virus	influenza A/HK/8/68(H3)-PR/8/34(N1) virus
Ia	cell surface antigen present on B cells and macrophages
Japan virus	influenza A/Japan/305/57 virus (H2N2)

Japan-Equi virus	influenza A/Japan/305/57(H2)-Equine/1/56(Neq1) virus
Japan-PR8 virus	influenza A/Japan/305/57(H2)-PR/8/34(N1) virus
Jap-Jap virus	influenza A/Japan/305/57(H2N2) x PR8 virus
L929	connective tissue fibroblast cell line of C3H mice
LCM virus	lymphocytic choriomeningitis virus
Ly 1, Ly 2	cell surface antigens of T cells
M protein	also called matrix or membrane protein
MDBK cells	Madin-Darby bovine kidney cell line
MDCK cells	Madin-Darby canine kidney cell line
MID_{50}	50% mouse infectious doses
MRC-11	influenza A/Pt. Chalmers/1/73 (H3N2) x PR8 virus
Mx	autosomal dominant trait of the A2G strain, phenotype is myxovirus resistant
NA	neuraminidase glycoprotein
NANA	N-acetyl neuraminic acid, sialic acid
ND	not done
NI	neuraminidase inhibiting antibody
NP	nucleoprotein, also known as ribonucleoprotein
NRS	normal rabbit serum
Nu	autosomal recessive trait of nude mice, phenotype is thymic atrophy
P815	mastocytoma cell line of DBA/2 mice
PBS	phosphate buffered saline
PR8 virus	influenza A/PR/8/34 virus (H0N1)
PR8-Equi virus	influenza A/PR/8/34(H0)-Equine/1/56(Neq1) virus

PR8-HK virus	influenza A/PR/8/34(HO)-Hong Kong/8/68(N2) virus
PR8-Japan virus	influenza A/PR/8/34(HO)-Japan/305/57(N2) virus
RIA	radioimmunoassay
RI/5+ virus	influenza A/RI/5+/57 virus (H2N2)
SEM	standard error of mean
SIR	specific immune release
SRBC	sheep erythrocytes
UV	ultraviolet light
VSV	vesicular stomatitis virus
WSN virus	influenza A/WSN/33 virus (H0N1)
X-7 virus	influenza A/NWS/33(HO)-RI/5+/47(N2) virus
X-31 virus	influenza A/Hong Kong/8/68(H3N2) x PR/8/34 virus

XII. BIBLIOGRAPHY

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