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THE ROLE OF MACROPHAGES IN INFLUENZA VIRUS INFECTION IN MICE

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THE ROLE OF MACROPHAGES IN INFLUENZA VIRUS INFECTION IN MICE

by

JONATHAN S. ROSENBERG

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

1979

"He had been eight years upon a project for extracting sunbeams out of cucumbers to be put in phials hermetically sealed and let out to warm the air in raw inclement summers."

JONATHAN SWIFT: GULLIVER'S TRAVELS

To the memory of my father, Arthur Rosenberg, whose love and understanding carried me into the future.

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

7/27/79 Jerome L. Schulman

date Chairman of Examining Committee

8/1/79

date Executive Officer

James G Wetmur

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The City University of New York

I. ABSTRACT

THE ROLE OF MACROPHAGES IN INFLUENZA VIRUS INFECTION IN MICE

by

Jonathan S. Rosenberg

Advisor: Jerome L. Schulman, M.D.

The roles of macrophages in influenza virus infection were characterized *in vitro* and *in vivo*. The effects of macrophage depletion on the course of infection in mice defined both accessory and effector functions of macrophages.

Virus antigens were detected on the surface of macrophages infected *in vitro*. Hemadsorption of erythrocytes to infected cells verified the presence of hemagglutinin. The lysis of infected macrophages by antineuraminidase antibody and complement provided evidence of neuraminidase on the cell surface. Infection of macrophages was abortive since no evidence of newly synthesized virus was obtained. Failure to detect a greater frequency of recombinant progeny in supernatants of doubly infected macrophage cultures than in control cultures furnished proof that influenza viruses go through abortive replication in macrophages. Further evidence of an incomplete replicative cycle was demonstrated by an absence of an increase in virus titers detected by direct assay of virus in infected macrophage supernatants, or by indirect assay by enumeration of infectious foci on permissive monolayers to which treated or untreated infected macrophages were added.

A murine model provided evidence of the participation of macrophages in the

recovery from influenza virus infection. Depletion of macrophages by i.p. administration of silica on the day of infection depressed the generation of humoral, cellular, and interferon responses to infection illustrating the necessity of functional monocytes for the induction of immune responses. The clearance of virus and the development of lung lesions were delayed in macrophage depleted mice. Despite the presence of normal serum hemagglutinating-inhibiting (HI) antibody responses in mice treated with silica 3 days or later after infection, virus titers remained elevated and lung consolidation development was still less extensive than in normal mice.

Pulmonary interferon titers measured in a plaque inhibition assay with VSV, were no different in macrophage depleted mice than in control animals at the time when virus titers in the lungs of control animals had declined while virus titers remained elevated in silica treated mice. A cytotoxicity assay was developed to determine the development of cell mediated responses to infection.

Unlike antibody or interferon responses, cytotoxic responses were generated in the lung at the time when virus titers were declining. The predominant cytotoxic effector cells in the lung mononuclear cell populations during the recovery period were characterized as cytotoxic T cells. However, adherent cells with the characteristics of macrophages made a minor contribution to the cytotoxic response.

Cytotoxic activity was temporally associated with virus clearance but evidence was obtained which indicated that macrophages may also directly participate in the elimination of virus. Adoptive transfer of primary or secondary immune spleen cells with demonstrable cytotoxic potential, accelerated the rate of virus clearance in control mice. However, antiviral effects of primary spleen cells were not detected in macrophage depleted animals. Although secondary immune spleen cells could partially compensate for the defect in virus clearance observed in silica treated mice, virus persisted in the lungs for an extended period of time after infection when no detectable virus was observed in the lungs of control animals.

The accumulation of lymphocytes and monocytes in the lungs of infected mice coincided with the time at which cytotoxic activity appeared and virus clearance as well as the development of lung lesions became apparent. In contrast, antibody forming cells in the lung were detected well after recovery was underway.

The data support the hypothesis that both T cells and macrophages serve as effector cells facilitating clearance of virus from the lung and at the same time contribute to the characteristic lesions which develop during the recovery from influenza virus infection.

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There are no words which adequately express how fortunate I have been to have had the love and support of Monica. I hope someday I can show her how I feel for all the ways she has enriched my life by sharing it with me.

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VI. INTRODUCTION

A. INFLUENZA VIRUS

The influenza viruses are enveloped, negative stranded RNA viruses containing segmented genomes. Three types of influenza viruses have been identified, types A, B, and C. All the members within a subtype possess an immunologically related nucleoprotein and matrix protein but there is no cross reactivity between different types of influenza viruses (Bull. W.H.O., 1972).

Influenza A viruses have 8 RNA segments (reviewed in Palese, 1977; Pons, 1976; Bean and Simpson, 1976) each of which codes for a single protein after transcription and synthesis of mRNA. Like influenza A viruses, the genomes of influenza B and C viruses are also segmented containing 8 and 7 distinct RNA segments respectively (Racaniello and Palese, 1978; Racaniello, 1979, personal communication). In addition, influenza C viruses are unique by virtue of the absence of a virus coded neuraminidase.

A complete genetic map for influenza A viruses was constructed by the analysis of the migration of RNAs and proteins of parental viruses parallel with recombinant viruses on polyacrylamide gels which is reviewed in detail (Palese, 1977). Similar results have been obtained by using different techniques. Inglis et al. (1977) used cytoplasmic mRNA extracts of influenza A virus-infected cells to direct virus-specific protein synthesis in a cell free translation system. When individual vRNA segments were hybridized to the mRNA extract, translation of the resulting double stranded form was blocked; however, translation of the remaining single stranded RNAs continued. Thus, it was possible to correlate specific genes with viral polypeptides for which they coded. A different approach to establishing genetic maps of influenza viruses involved the formation of ts^+ recombinants derived from crosses of ts mutants and ts^+ virus of a different subtype. Analysis of the inheritance of genes in ts^+ recombinants by hybridization permitted identification of the genes derived from the ts$^+$ parent (for review see Scholtissek, 1978).

There are two distinctive features of the influenza virus replication cycle. The first is the nuclear phase of replication evidenced by inhibition of macromolecular synthesis by actinomycin D (Barry et al., 1962), and lack of replication in enucleated cells (reviewed in Scholtissek and Klenk, 1975). The other interesting feature of influenza viruses is their ability to undergo genetic recombination after co-infection of cells with two different viruses. This is due to the reassortment of gene segments from each parental virus into new combinations resulting in recombinant progeny. In fact, these observations led to the proposal that influenza viruses possessed segmented genomes (reviewed in Palese, 1977).

As the influenza virion matures, it inserts two glycoproteins, hemagglutinin and neuraminidase, into the cell membrane which later serves as the viral envelope. Each HA and NA molecule contains several virus specific antigenic determinants (Laver et al., 1974) as well as host determined carbohydrate antigens. The component sugars of the oligosaccharide chain may vary with the strain of virus (Choppin, 1975) or the cell in which the virus is grown (Harboe, 1963; Laver and Webster, 1966; Nakamura and Compans, 1978).

The hemagglutinin subunit is composed of one polypeptide cleaved into two peptides HA1 and HA2 which have molecular weights of 55000 and 25000 daltons, respectively (Compans et al., 1970; Skehel and Schild, 1971; reviewed in Schulze, 1975). In cell systems where host proteases fail to cleave the HA polypeptide, noninfectious progeny virus is produced (Lazarowitz et al., 1973; Schulman and Palese, 1977). The hemagglutinin binds to the sialic acid residue of cell membrane glycoproteins, thus initiating the critical first step in the virus replicative cycle (reviewed in Hughes, 1973).

The enzymatically active form of neuraminidase is a tetramer (Kendal and Eckert, 1972; Bucher and Kilbourne, 1972) in which each subunit is bound to the other by disulfide bonds. The subunit is composed of a stalk which is embedded in the viral envelope and a bulb-like knob exposed to the exterior (Wrigley et al., 1973; Laver and Kilbourne, 1966). Various functions which have been attributed to neuraminidase in the replication cycle have included the prevention of aggregation of progeny after exit from the cell (Palese et al., 1974), release of virus from the cell by cleavage of terminal sialic acid residues on cell membrane glycoproteins (Compans et al., 1969; Webster, 1970; reviewed in Bucher and Palese, 1975), facilitation of cleavage of hemagglutinin into the HA1 and HA2 peptides, and the removal of neuraminic acid from mucins which bind nonspecifically to hemagglutinin (Schulman and Palese, 1977).

It has been speculated that the matrix and nucleoproteins interact at the cell membrane and play a role in virus maturation (reviewed in Compans and Choppin, 1975). The matrix protein located underneath the lipid envelope might serve as an organizer of the segmented RNA complexes or as a support structure for virion physical integrity. The transcriptase complex in the interior of the virion is composed of RNA, nucleoprotein and the three P or polymerase proteins (reviewed in Palese, 1977). Nucleoprotein and P2 are associated with RNA replicase activity (Krug et al., 1975; Ritchey and Palese, 1977), P1 and P3 proteins are necessary for complementary RNA synthesis (Krug et al., 1975; Palese et al., 1977).

Epidemiology of influenza virus.

Descriptions of epidemics similar to influenza can be traced back as far as the 12th century and the records since the 15th century contained numerous reports of influenza-like epidemics. Analyses of sera from individuals alive in 1899 suggested a relationship in neutralization tests of the virus which caused the epidemic of 1899 with viruses prevalent in 1958 (reviewed in Pereira, 1969). The most severe recorded pandemic in history was caused by the "swine flu" virus in 1918-1919 which caused the death of approximately 20 million people around the world. Antigenic shifts occurred in 1929 when the H0N1 strain appeared, in 1947 when the H1N1 virus was isolated, in 1957 when both surface antigens changed and the H2N2 virus first surfaced, and in 1968 with the emergence of the Hong Kong H3N2 virus (reviewed in Laver and Webster, 1975). Each antigenic shift was accompanied by a pandemic disease, presumably due to the transmission of the new virus in the immunologically virgin population.

After the abortive appearance of the swine influenza virus in Fort Dix, New Jersey, in 1976, the H1N1 serotype reappeared in 1977. Comparison of oligonucleotide maps of these current H1N1 isolates with H1N1 strains isolated in 1950 showed similarities which could not be explained by a series of step-wise mutations over a 27 year period. Furthermore, comparison of oligonucleotide maps of H1N1 isolates from 1947, 1950, and 1956 revealed more differences in the maps of 1950 and current H1N1 isolates despite the 28 year period which had elapsed (Nakajima et al., 1978). The authors speculated that perhaps the virus remained in an animal reservoir in which little genetic change occurred, or that by some mechanism, influenza viruses are capable of latent infections in humans in which genetic information is conserved. The possibility that these viruses were introduced into man after having been in the frozen state for an extended period was also raised.

Influenza A viruses isolated from birds have been shown to be serologically related to human strains (Pereira et al., 1967). Similarly, antigenic relatedness has been observed with equine and human strains of virus (Kasel et al., 1965). Hemagglutinin subunits of A/Equine/Miami/1/63 (Heq2Neq2) and A/Duck/Ukraine/1/63 (Hav7Neq2) cross reacted in hemaggluination-inhibition and immuno-diffusion tests with those of the human influenza A/Hong Kong/1/68 (H3N2) although five years time passed between the isolation of the animal viruses and the human virus. HA2 peptide maps of the duck, equine, and human viruses were almost identical suggesting that equine, duck, and human Hong Kong viruses arose from a common ancestor (Laver and Webster, 1973). These observations were consistent with the hypothesis that animals may serve as reservoirs for new influenza viruses (reviewed in Andrewes, 1959). It has been demonstrated that recombinant viruses could be detected *in vivo* in swine (Webster et al., 1971). The analysis of changes in nucleotide maps of some of the genes of avian influenza A viruses Hav6N2 and Hav6Nav4 and the identical maps of the M and HA genes suggested that at least one of these two avian viruses arose from a recombinational event in nature (Desselberger et al., 1978). In another study, Scholtissek et al., (1978) compared base sequence homologies between RNAs of H2N2 influenza viruses and other human influenza subtypes by hybridization techniques. Although 7 of 8 RNA segments of the H2N2 virus and the H3N2-Hong Kong strain were almost identical, but the HA sequence homology was different between H2N2 and H3N2 viruses. There was 90% homology, however, between the Hong Kong HA gene and the HA gene of the A/Duck/Ukraine (Hav7Neq2) although other RNA segments showed less homology. These studies furnished substantial evidence that pandemic strains of virus may result from recombination between human and animal influenza A viruses (Kilbourne, 1968; Tumova and Pereira, 1965; Andrewes, 1959).

Host range.

Influenza A viruses which naturally infect man, also infect swine, birds, horses and a variety of other mammalian species. In fact, of the influenza viruses type A alone may persist in subhuman mammals and birds. Although occasional influenza B virus isolates have been reported in animals, there is no evidence of transmission in species other than man. Influenza C viruses have not been found in animals and are only an infrequent cause of epidemic disease in man (reviewed in Kilbourne, 1975).

Virtually all influenza A viruses replicate in most strains of mice. Although mice are not naturally infected with influenza virus some of the pathological and biological features of experimental infection of mice is similar to that in man and murine models have been used extensively to study the pathogenesis and immune responses to influenza virus infection. In mice, disease is characterized by a significant degree of pulmonary inflammation and a predominance of mononuclear cell infiltration into the lungs (Loosli, 1949; Hers, 1955; Mulder and Hers, 1972). The lungs of infected animals appear purplish in appearance, the visual manifestation of the consolidation and marked inflammatory exudative lesion formation. After repeated passage of A/Turkey/England/63 (Hav1Nav3) in mice, a hepatotropic strain was isolated capable of replicating in the liver and causing extensive liver destruction and death within 4 or 5 days after intraperitoneal administration in mice (Haller and Lindenmann, 1974). Two strains derived from A/NWS which have neurovirulent properties were also isolated after serial intracerebral passage in mice.

Antigenic variation.

There are two types of antigenic variation characteristic of influenza A viruses. Major changes, antigenic shift, may result from recombination of human and/or animal strains as was discussed in the preceding section. Minor variations are thought to be due to stepwise mutations involving the antigenic determinants of the surface glycoproteins.

The use of monoclonal antibody to influenza virus hemagglutinin has furnished useful information regarding the molecular aspect of antigenic drift. Gerhard and his associates have utilized the monoclonal antibody to select variant viruses with altered antigenic structure. The inability of monoclonal antibody directed to strain-specific determinants of the parental virus to restrict the growth of variants in eggs provided information that any new epidemic strain probably evolved from the change of several amino acid residues in the HA of the parental strain (Gerhard and Webster, 1978) and not a change in a single amino acid residue (Fazekas de St. Groth, 1969).

Analysis of antigenic variants selected from a cloned preparation of PR8 virus by means of monoclonal antibody has revealed that as many as 50 antigenic determinants exist on the HA molecule just below the hydrophilic tip (Laver et al., 1979). These determinants may be either strain specific or cross-reactive (Laver and Webster, 1968; Virelizier et al., 1974a, 1974b). Polypeptide maps of antigenic variants selected by monoclonal antibody have revealed that the changes in amino acid residues of HA polypeptides may be related to antigenic differences, but that a change does not necessarily affect the antigenicity of the HA molecule (Gerhard, 1976; Laver et al., 1979). Determination of the primary structure of HA subunits which have previously shown stepwise changes in the amino acid backbone of the HA glycoprotein during the interpandemic period (Skehel and Waterfield, 1975) in concert with the use of monoclonal antibodies has deepened our understanding of the ability of the influenza virus to avoid neutralization by antibody in the immune population. The use of monoclonal antibodies has also provided information regarding the nature of strain specific and cross-reactive determinants and their location on the hemagglutinin molecule.

B. IMMUNOLOGY

Humoral immunity to influenza viruses.

A few years after influenza was isolated from swine it was isolated from man (Smith et al., 1933). A filtrate of a human throat washing were used to infect ferrets which exhibited respiratory complications. Shortly afterward, intranasal installation of swine virus into mice was shown to cause a respiratory disease, similar to the illness observed in ferrets (Andrewes et al., 1934; Francis, 1934; Shope, 1935).

In these experiments both ferrets or mice infected with either the human or swine influenza viruses were more resistant to reinfection with the homologous virus (Smith et al., 1933; Shope, 1935). It was also demonstrated that ferrets inoculated with mixtures of convalescent sera and heterologous virus were protected, but in the light of more recent evidence of the inhibitory effects of anti NA antibody on virus replication, the results of these studies are now interpreted differently. These earlier studies in animal models also demonstrated that reinfection with homologous virus was prevented by the presence of circulating antibody. An impressive amount of data in human populations also supports the contention that individuals with serum hemagglutinating-inhibiting antibody are more resistant to infection (reviewed in Schulman, 1975).

Evidence of the mechanism by which antibody to hemagglutinin or neuraminidase modifies influenza virus infection has accumulated over the years.

Antibody directed to hemagglutinin prevents virus attachment to cells in cell culture and in the intact host, antibody to HA reduces the susceptibility to acquisition of infection. Mice immunized with a recombinant virus containing the same hemagglutinin but a different neuraminidase, upon subsequent challenge, were as resistant to infection as mice immunized with a virus containing the same HA and NA as the challenge virus (Schulman et al., 1968).

When infection does occur in the presence of HA antibody, virus titers are lower and pulmonary consolidation is less severe. The reduced likelihood of becoming infected suggests that antibody to hemagglutinin may also prevent the transmission of disease. However, mice immunized with inactivated virus or passively given antibody to HA transmit infection as readily as unimmunized control mice (Schulman, 1967).

Antibody to neuraminidase does not block attachment or penetration of virus into cells. In one study the release of virus from the cell surface was not affected by Fab fragments which were capable of inhibiting neuraminidase activity *in vitro* (Brecht et al., 1971). In another study, univalent Fab' fragments of anti-neuraminidase antibody inhibited the neuraminidase activity and the release of virus from the infected cell membrane. In addition, plaque size was reduced when anti-neuraminidase antibody was incorporated into agar overlays. Parallel results were obtained when the neuraminidase inhibitor FANA was used in the agar overlay demonstrating that infection can be modified in part by direct enzyme inhibition (Kilbourne et al., 1975).

In contrast to the reduced capacity to acquire infection in the presence of anti-hemagglutinin antibody, antibody to neuraminidase does not prevent infection. Mice with antibody to neuraminidase at the time of challenge are no more resistant to the acquisition of infection than control animals (Schulman, 1970). However, antibody to neuraminidase modifies infections in mice by reducing virus titers and the extent of lung lesions (Schulman et al., 1968). Unlike mice with hemagglutinin antibody, mice with anti-neuraminidase antibody have a reduced ability to transmit infection. Similar effects have been shown in humans (Murphy et al., 1972).

There is really no clear evidence whether humoral immune responses to internal proteins have any bearing on the course of the disease. Antibody to the three P proteins located on the interior of the virion is not detected after infection. Although antibody to nucleoprotein and matrix proteins have been detected after natural infection or artificial immunization of humans with inactivated virus (Virelizier et al., 1979) there is no evidence that subsequent infection is modified. In one study, injection of M protein (Webster and Hinshaw, 1977) resulted in a low incidence of anti-M antibody production (4 of 9 mice had detectable anti-M antibody one week after infection). These results suggested that virus clearance and lung lesions in mice immunized with M protein may be due to a cellular immune response to this antigen which may be responsible for the cross-reactive lysis of target cells infected with influenza A subtypes *in vitro* (Effros et al., 1977) and cross protection observed between different influenza

A subtypes after infection with one subtype and challenge with another (Schulman and Kilbourne, 1965).

Heterotypic immunity and original antigenic sin.

Earlier experiments and more recent experiments have demonstrated that infection with one subtype of virus increases the resistance to subsequent challenge with another subtype in the absence of detectable antibody to the challenge virus (Francis and Shope, 1936; Francis and Magill, 1936; Henle and Lief, 1963; McClaren and Potter, 1974). Furthermore, sequential infection with serologically unrelated viruses resulted in the development of enhanced antibody titers to the hemagglutinin of the virus used in the second challenge (Schulman and Kilbourne, 1965). The possible relationship of heterotypic immunity and cross-reactive T cells is discussed in another section of this manuscript. However, humoral immune responses during sequential infection or immunization are addressed here.

Francis described a different type of humoral response in humans infected sequentially with two heterotypic influenza viruses. The kinetics and nature of the response are as follows. Antibody produced in response to the second virus is characterized by greater reactivity with the primary virus (Davenport et al., 1953; Fazekas de St. Groth and Webster, 1966a, 1966b). This type of immunological recall called “original antigenic sin,” has also been demonstrated at the level of purified hemagglutinin antigens. H0 antigen primed mice boosted with the cross-reacting H1 antigen demonstrated a secondary anti-H0 response (Virelizier et al., 1974b). This secondary response to the H0 hemagglutinin was directed to both common antigenic determinants and strain specific determinants found on H0 alone. Antibody reactive with H1 hemagglutinin was directed to cross-reactive determinants. The antibody raised in response to the second virus could be almost totally absorbed by the first virus, whereas the second H1 virus, could absorb only antibody directed to H1 determinants. The T cell dependence of anti-hemagglutinin antibody response was also demonstrated in these experiments (Virelizier et al., 1974a, 1974b). Transfer of lymphocytes from H0 hemagglutinin primed mice into an irradiated thymectomized host boosted with H0 hemagglutinin resulted in greater antibody production when both T and B lymphocytes were transferred than when the donor cell population was depleted of T cells by treatment with anti-theta serum and complement. Interestingly the cross-reactive antibody response appeared to be less T cell dependent than the strain specific response. Thus, thymectomized irradiated bone marrow reconstituted TxBM, mice which were hyperimmunized with hemagglutinin produced antibody to cross-reactive determinants. The response to cross-reactive determinants in nonreconstituted animals was delayed in appearance but more vigorous than in animals reconstituted with thymocytes. The authors concluded that the injected thymocytes suppressed the cross-reactive response suggesting the presence of suppressor cells (Virelizier et al., 1974a).

The phenomenon of original antigenic sin has been explained by stimulation of cross-reactive memory B cell clones and cross stimulation of strain specific memory B cell clones in response to the heterotypic virus. The antigenic relatedness of influenza virus subtypes may cause broadening of the repertoire of memory B cells. Phenomena similar to original antigenic sin have been observed in systems using closely related chemically defined antigens (Klinman et al., 1973; Deutsch et al., 1973).

Cross-reactivity between different subtypes of influenza A viruses have been detected in other assays besides the single radial immuno-diffusion tests employed by Virelizier and his colleagues (1974a, 1974b). Cross-reactivity among influenza A subtype virus have been detected by HI tests (Sugiura and Kilbourne, 1966), immunodiffusion assays (Schild et al., 1972), and modified Jerne plaque assays (Reiss, 1978).

Local immunity.

It was first suggested by Francis (1941) that resistance to influenza did not appear to be directly related to serum antibody but was more closely associated with antibody in nasal secretions. This led to investigations of the possible role of local immunity in influenza virus infections.

An "agent" isolated from the nasal washings of infected individuals had the capacity to inactivate virus. This substance had properties very much like antibodies (Francis, 1941; Burnet et al., 1939) including virus specificity. It was postulated that the presence of antibody in nasal washings could explain the resistance of mice and ferrets (Francis and Shope, 1936) to reinfection with homotypic virus by intranasal installation (Francis, 1941).

In 1950, Fazekas de St. Groth and Donelley demonstrated that immunization of mice with inactivated virus by intranasal inoculation conferred greater resistance to reinfection than did parenteral immunization (Fazekas de St. Groth and Donnelley, 1950). Later experiments conducted by Schulman and Kilbourne (1967) demonstrated that previously infected mice were much more resistant to reinfection than animals previously immunized by injection of inactivated virus. Despite the presence of comparable levels of serum antibody, previously infected mice were not reinfected even by large doses of virus while mice which had been immunized with inactivated virus by the parenteral route were infected when exposed to lower doses of virus. A similar finding was reported by Potter and his associates using ferrets as the experimental animal (Potter et al., 1973).

More recently, Iwasaki and Nozima (1977) observed that increasing titers of IgA antibody in the tracheobronchial washings of PR8 virus infected mice coincided with the decline in virus titers. IgA antibody was not produced in T cell depleted mice who succumbed to the lethal infection. On the basis of these observations, IgA was thought to play an important role during the recovery process.

In human subjects, evidence suggests that infection induced immunity against influenza is of longer duration and more protective than immunity evoked by vaccine administration (reviewed in Schulman, 1975). Parenteral immunization of primed individuals with inactivated virus increased serum antibody titers relative to antibody titers in the bronchial secretions. Aerosol infection elicited higher antibody titers in bronchial secretions than in the serum (Waldman, 1973).

The available evidence suggests that infection induced immunity confers greater protection to reinfection than does immunity from parenterally administered influenza vaccines (reviewed in Schulman, 1975). Perhaps, this is due to the specific stimulation of secretory antibody, IgA, in the respiratory tract. Upon subsequent virus challenge it is possible that secondary IgA responses may protect against the intranasally installed virus. In an analogous study, Smith et al., (1966) demonstrated that injection of parainfluenza type I vaccine was less effective in stimulating neutralizing antibody production in nasal secretions than was infection with parainfluenza virus. These inactivated vaccine recipients were quite susceptible to reinfection. Titers of neutralizing antibody in nasal secretions was associated with protection against infection to a greater degree than the correlation of serum antibody titers.

Similar observations have been made with respiratory syncytial virus. The level of virus replication in adult volunteers was inversely correlated with the titer of neutralizing antibody in nasal secretions (Mills et al., 1971). No such correlation was evident with serum antibody levels. Thus it appears that local antibody produced in response to respiratory viruses may be more protective than serum antibody. In addition, the intranasal route of administration of live, attenuated virus vaccines which induces secretory antibody production appears to provide better protection against reinfection than does parenterally administered vaccines which elicits better serum antibody responses. This compartmentalization of humoral immune responses has a bearing on the route of administration, dose, and antigenic composition of vaccines which will stimulate protective antibody titers in respiratory secretions.

Cell mediated immunity.

The literature reviewed in the previous section clearly demonstrates that antibody present at the time of challenge protects against reinfection with a homotypic or heterotypic virus. However, the kinetics of antibody production in the serum and respiratory tract during virus infection suggest that antibody may not have important impact on the recovery process during primary infection. In the last decade the development of techniques for the measurement of cell mediated responses provided new avenues of research on the cellular immune response to influenza virus antigens. Among these techniques are cytotoxicity assays which measure lysis of virus infected and labeled target cells. Migration-inhibition assays have been used to demonstrate that sensitized T cells elaborated soluble factors when exposed to antigens. Blastogenesis assays which measure the rate of cell proliferation in response to antigens proved to be invaluable in MLR, mixed lymphocyte reactions, with its clinical application, as well as in assays of T cell responses to viral antigens *in vitro.*

The discovery of other effector cell populations besides T cells has added extra levels of complexity to the problem of determining which cells contribute to recovery from influenza virus infection – T cells, K cells, NK cells, and cytotoxic macrophages have all been postulated as potentially important effector cells during recovery processes from viral infections.

T cells.

Thymus derived lymphocytes of mice are grouped into subpopulations on the basis of the functions they perform or the surface Ly alloantigens or I region determinants they bear. These T lymphocyte subpopulations include helper T cells, amplifier T cells, suppressor T cells, effectors of delayed type hypersensitivity reactions and cytotoxic T cells.

Helper T cells. Helper T cells along with histocompatible macrophages and B cells are necessary for the production of antibody by plasma cells in response to most antigens (Claman et al., 1966; Katz and Benacerraf, 1972; Mitchison, 1969). Helper T cells bear the Ly 1 alloantigen on their cell membrane and have been shown to produce antigenic specific helper factors (reviewed in Golub, 1977).

The necessity for helper T cells in the production of antibody to hemagglutinin has been observed in a number of systems (Virelizier et al., 1974a, 1974b; Iwasaki and Nozima, 1977; Sullivan et al., 1976; Schulman et al., 1977).

Virelizier demonstrated that when spleen cells obtained from mice sensitized with virus a month before were transferred to thymus deprived animals along with a non-immunogenic dose of purified hemagglutinin the day after infection, mice survived the infection. However, without the dose of purified HA, transfer of immune spleen cells alone, had no protective effect. From these results it was concluded that T cells served as helper cells in antibody production and not as effector cells (Virelizier, 1975). Non-immune T lymphocytes have also been shown to produce interferon in response to influenza virus *in vitro* (Tsukui, 1977). Interferon production was abolished in thymus deprived mice (Iwasaki and Nozima, 1977). However, the subpopulation of T cells involved in interferon production was not determined in these experiments.

Amplifier T cells. Amplifier T cells are to cell mediated responses as helper T cells are to antibody responses. This subpopulation of T cells also have Ly 1 antigen in the cell membrane. T:T cell cooperation in cell mediated responses was first shown by Cantor and Asofksy in 1970 when they demonstrated synergy between parental thymus cells and peripheral blood cells in the production of a graft-versus-host response in F_1 hosts (Asofsky et al., 1971). Other experiments demonstrated that treatment with anti Ly 1 or anti Ly 2, 3 and complement inhibited cytotoxic reactions (Cantor and Boyse, 1975a, 1975b). T amplifier cells may also produce a soluble factor which aids in the generation of cytotoxic T cells (Plate, 1971).

Suppressor T cells. Both suppressor T cells and cytotoxic T cells of mice bear Ly 2, 3 alloantigens on their cell membrane. The two subpopulations are distinguished by the I-J region product on the suppressor cell membrane (Golub, 1977) and the ability to induce suppressor cells by treatment with Con A (Rich and Pierce, 1974). Suppressor cells may also produce a suppressive factor (Roman and Golub, 1976) analogous to the helper factor produced by helper T cells (Taussig, 1974). Suppressive effects mediated by T cells were first shown by Gershon in the early 1970s (reviewed in Gershon, 1974).

There is little evidence of the effects of suppressor T cells in the influenza virus system. Indirect evidence for the generation of suppressor T cells during influenza virus infections has been provided by the observation that infected recipients of influenza immune cells from syngeneic mice have lower HI antibody titers than recipients of normal spleen cells (Schulman et al., 1977; Schulman, personal communication). The suppression of antibody responses in TxBM mice observed by Virelizier et al. (1974a) also provided evidence that cells capable of suppressor activity were present in the transferred cell population.

Delayed type hypersensitivity (DTH). Delayed type hypersensitivity reactions are measured *in vivo* by the injection of antigen into the skin or footpad of a primed animal. Two days later the amount of swelling at the injection site is measured. An *in vitro* correlate of DTH is the mixed lymphocyte reaction (MLR) (Bach and Hirschhorn, 1963).

DTH reactions to influenza virus antigens as well as mumps virus were detected by an *in vitro* macrophage migration inhibition assay (George and Vaughan, 1962). Peritoneal cells from A/WSN/33 infected mice were used in this assay. In the presence of sensitized lymphocytes and influenza antigen, migration of peritoneal macrophages was inhibited (Cambridge et al., 1976). Migration of peritoneal cells from sensitized mice was inhibited when exposed to a homologous antigen but only slightly inhibited in the presence of heterologous influenza virus antigens (Feinstone et al., 1969). DTH reactions in man have also been shown to be type specific (Beveridge and Burnet, 1944). DTH reactions measured by skin tests have also been demonstrated in man (Habershon et al., 1973) and guinea pigs (Cole and Molyneux, 1975). These studies demonstrated that T cells recognized and responded to influenza antigens by secreting soluble factors which recruited inflammatory cells. The role of DTH in influenza virus pathogenesis is discussed elsewhere in this section.

Cytotoxic T cells. Cytotoxic T cells lyse target cells most efficiently when the targets and effector cells share H-2K or H-2D histocompatibility genes (Doherty and Zinkernagel, 1974). Similar histocompatibility requirements have been demonstrated in AG-B genes in rats (Marshak et al., 1977) or HL-A-B or HL-A-A genes in man (McMichael et al., 1977). However, murine alloreactive cells have been demonstrated with influenza virus infected targets (S. Finberg, personal communication). Alloaggressive cytotoxic T cells have been generated by *in vitro* stimulation of primed spleen cells with reconstituted membranes of histocompatible cells in the presence of soluble virus proteins. Secondary alloreactive effector cells have also been generated *in vitro* by exposure to liposomes into which partially purified H-2 antigens and virus proteins have been incorporated. Mice which are irradiated and reconstituted with H-2 incompatible lymphoid cells are radiation chimeric mice. Lymphocytes sensitized in radiation chimeric mice will lyse target cells which share the H-2K or H-2D products of the chimeric environment. In this case, the cytotoxic T cells apparently recognize alloantigens in association with H-2 products (Doherty et al., 1976a; Zinkernagel, 1976a, 1976b). However, it has been demonstrated that in semiallogeneic chimeras produced by injection of (AxB) F_1 fetal liver cells or bone marrow cells into lethally irradiated type A or type B hosts, cytotoxic T cells generated during infection of the A or B host with ectromelia virus exhibit a bias but not an absolute restriction to host H-2 antigens. Approximately 75% of the (AxB) $F_1 \rightarrow$ A chimeras recognized and lysed virus infected B macrophages (Blanden and Andrew, 1978). Immune cells recognize both self, H-2, and nonself, viral antigens, or a neoantigen determined by both host and viral genomes (Doherty et al., 1976a, 1976b; Blanden et al., 1976). The nature of the T cell receptor unit is still unidentified.

Lysis of target cells by cytotoxic T lymphocytes is energy dependent and requires contact between target and effector cells (Cerottini and Brunner, 1973). Divalent cations are necessary in the early and intermediate (attachment) phases of the cytotoxic process (Henney and Bubbers, 1973) but not later in the lytic stages (Martz and Benacerraf, 1973). Killing is also inhibited when the assay is conducted at 4^{o} (Berke et al., 1972).

Cytotoxic T lymphocytes generated in mice in response to viral infection was initially reported with lymphocytic choriomeningitis virus (Zinkernagel and Doherty, 1973). Since then, cytotoxic T cell generation has been induced by infections with vaccinia (Koszinowski and Thomssen, 1975), ectromelia virus (Gardner et al., 1974), Sendai virus (Doherty and Zinkernagel, 1976), parainfluenza virus (Lewandowski et al., 1975) and Friend leukemia virus (Blank et al., 1976). These are all enveloped viruses which make it more obvious that viral antigens in infected cell membranes are recognized by T cells. The mechanism by which cytotoxic T cells recognize nonenveloped viruses like adenovirus (Inada and Uetake, 1978) and Coxsackie virus (Wong et al.,

1977) is not understood at this time.

Both infection and sensitization with influenza virus result in the generation of cytotoxic T cells in mice (Cambridge et al., 1976; Doherty et al., 1977; Braciale, 1977a; Yap and Ada, 1977; Ennis et al., 1977; Zweerink et al., 1977b; Schulman and Reiss, 1977, personal communication).

After infection or immunization, cytotoxic T cells are generated in the spleen (Braciale, 1977a; Doherty et al., 1977) in which peak activity occurs about 6 days after primary immunization, in the lymph nodes (Ennis et al., 1977) and in the lungs (Yap and Ada, 1978c; Bennink et al., 1978; Rosenberg and Schulman, 1979).

Type A influenza virus infections generate cytotoxic T cells which have the capacity to lyse target cells infected with type A viruses which possess different hemagglutinin and neuraminidase surface glycoproteins (Effros et al., 1978; Doherty et al., 1977; Braciale, 1977a). For instance, effector cells generated by infection with PR8 (H0N1) virus lyse H2N2 and H0N1 virus infected targets. However, one laboratory determined that cytotoxic T cells in influenza-infected mice were hemagglutinin specific (Ennis et al., 1977) when tested against syngeneic primary kidney cells but cross-reactive lysis was still observed with L929 target cells. The authors concluded that the specific cytotoxic response in the kidney fibroblasts reflected the importance of the response to the hemagglutinin, the major surface antigen during influenza infection.

Cytotoxic T cells can be generated *in vitro* by exposure of spleen cells from influenza virus primed mice to influenza virus infected lymphoblasts or syngeneic macrophages or inactivated virus (Zweerink et al., 1977a) for five days. Heterotypic viruses were as effective in generating cytotoxicity during secondary stimulation *in vitro* regardless of the strain of virus used for the *in vivo* immunization 5-12 weeks previously. The cytotoxic cells so generated, efficiently lysed target cells infected with viruses possessing different surface antigens (Zweerink et al., 1977a). However, the cytotoxic T cells generated in response to *in vitro* exposure to purified HA antigen from the same strain originally used for priming were highly specific for the targets infected with viruses bearing the same hemagglutinin.

Cross-reactive lysis is thought to be mediated by the recognition of the common matrix protein of influenza A viruses (Braciale, 1977b; Biddison et al., 1977; Reiss and Schulman, 1978). Both hemagglutinin and neuraminidase are glycoproteins and their formation is susceptible to the effects of 2-deoxy-D-glucose (2-DG), an inhibitor of glycoprotein synthesis (Klenk et al., 1972).

Influenza virus infected cells treated with 2-DG were lysed by cytotoxic T cells. Lysis of these cells was also effected by antibody to matrix protein and complement, but no lysis occurred with anti-HA or anti-NA antibody in the presence of complement (Braciale et al., 1977b) confirming the hypothesis that cross-reactive lysis by T cells might be due to the recognition of matrix protein. Antibody to matrix protein has been used to block the lysis of cells infected with a virus different than the virus used for sensitization and generation of cytotoxic T cells (Reiss, 1978). This furnished additional evidence for the importance of the matrix protein in cross-reactive lysis.

It has been postulated that the expression of matrix protein on the target cells used in these studies, L929 fibroblasts and P815 mastocytoma cells, may be a reflection of abortive infection in these cell lines associated with higher concentrations of M protein at the cell surface. However immunofluorescent studies with specific anti-M antibody revealed the presence of M protein on the membranes of abortively and productively infected cell lines (Reiss and Schulman, 1978, submitted for publication).

Antibody dependent cell mediated cytotoxicity (ADCC).

The effector cells of ADCC are thymus independent lymphoid cells called K cells

(Harding et al., 1971). Association of antibodies of the IgG class with Fc receptors on the surface of noncommitted K cells brings about lysis of xenogeneic, autologous, or tumor cells (reviewed in Perlmann et al., 1972). K lymphocytes in human peripheral blood mononuclear cells have been shown to lyse measles virus infected targets (Kreth and ter Meulen, 1974; Perrin et al., 1977a; Galama et al., 1978), vaccinia virus infected targets (Perrin et al., 1977b) and in mumps virus (Andersson et al., 1975) and herpes simplex virus (Shore et al., 1976) systems. K cells have not been demonstrated in mice.

K cell activity in peripheral blood lymphocytes (PBL) was demonstrated against influenza virus infected hamster kidney cells. The elution of antibody to influenza virus antigens by treatment of PBL for 30 minutes at 37°C provided evidence that cells which were present in the PBL population had specific antibody bound to their membranes (Greenberg et al., 1975, 1977).

Natural killer cells.

The possibility that natural killer cells may be important in the surveillance of tumors has led many researchers to examine these cells in detail. NK activity in man is reviewed elsewhere (Pross and Baines, 1977) but murine NK cells will be discussed here (reviewed in Kiessling and Wigzell, 1979).

Natural killer cells are x-ray resistant. They may be prethymic T cells since small amounts of theta antigen and low concentrations of Fc receptors have been detected on their surface (Herberman et al., 1978; Herberman et al., 1977a). However, athymic nude mice, which do not generate T cell responses or T cell dependent B cell or macrophage responses, are capable of rejecting virus infected allogeneic or xenogeneic tumor cells (Welsh, 1978a).

Many viruses induce NK cell activity in mice. Among these are C-type viruses, lactic dehydrogenase virus, minute virus of mice, polyoma virus, mouse adenovirus, Sendai virus, mouse hepatitis virus (Herberman et al., 1977b), Moloney leukemia virus (Kiessling et al., 1975), Coxsackie virus (Wong et al., 1977), Newcastle disease virus (Gidlund et al., 1978), LCM virus (Welsh, 1978b), and influenza virus (Wyde et al., 1978; Anderson and Heath, 1977).

Nonspecific immune mechanisms.

Interferon.

In the 20 years since interferon was first discovered by Isaacs and Lindenmann, research has shown that it may be an important host defense against many viral infections.

Interferon is a term used to describe a number of glycoproteins separated into different classes based on physiochemical and antigenic properties. Type I standard interferon is produced by almost all nucleated cells. Type II immune interferon is pH 2.0 labile (Youngner and Salvin, 1973).

Mechanism of action.

The evidence of interferon induced enzymatic activities in cells grown in tissue culture has increased our understanding of its mechanism of action.

Double stranded RNA, which may represent a replicative intermediate formed during RNA virus replication induces interferon synthesis (reviewed in Baglioni, 1979) in animal cells and inhibits protein synthesis in extracts of interferon treated cells (Kerr et al., 1974). Numerous experiments have demonstrated that two interferon induced dsRNA dependent enzymatic activities are present in the extracts of interferon treated cells.

The first enzyme is an oligonucleotide polymerase which synthesizes a series of oligonucleotides from ATP which contain 2'5' phosphodiester bonds. The product of the oligonucleotide polymerase (designated 2,5A polymerase) is pppA(2'5'A)n (Kerr and Brown, 1978) which then activates an endoribonuclease which subsequently degrades both cellular and viral mRNA. Translation of message into proteins inhibits the formation of progeny virus preventing viral assembly (reviewed in Baglioni, 1979).

The second enzyme is a protein kinase whose activation requires both dsRNA and ATP (Farrell et al., 1977). The protein kinase phosphorylates the alpha subunit of initiation factor eIF-2 which inhibits binding of initiator tRNA to 40s ribosomal subunits. Phosphorylation of another P1 may also occur with the protein kinase (reviewed in Baglioni, 1979). The protein kinase activity increases with the log of the interferon concentration used to pretreat the cells (Samuel, 1979). This activity correlates with the induction of a ribosome-associated inhibitor of translation perhaps by inhibiting the formation of the Met-$tRNA_f$/40s ribosomal subunit complex which initiates the translation of proteins (Farrell et al., 1978).

The combined action of 2,5A induced endoribonuclease which blocks accumulation of viral mRNA and the protein kinase which blocks protein synthesis may prevent viral replication. Finally, the inhibition of methylation of the 5' terminal guanosine of mRNA in extracts of interferon treated cells (Sen et al., 1975) may represent another mechanism which impairs translation of capped viral mRNA of both RNA and DNA viruses but this effect of interferon has not yet been studied in great detail.

Although these studies have shown interferon induces enzymatic activities which are effective in the maintenance of the antiviral state in tissue culture, enzymatic activities induced by interferon remain to be examined using animal models. However, the advances made in our understanding of the molecular basis of interferon action *in vitro* have shed some light on how to design experiments focusing on the effects of interferon *in vivo* during viral infections.

Antiviral effects in vitro.

In vitro experiments have established that interferon alone reduces virus yields in the absence of other immune mechanisms. Antibody to interferon enhances virus replication in cell cultures (Fauconnier, 1969). Pretreatment of cells in culture with interferon prior to infection decreased the size of plaques caused by Western equine encephalitis virus (Stanton, 1976). Poly I:C, a potent interferon inducer, inhibited the growth of influenza A2, respiratory syncytial virus, parainfluenza virus and rhinovirus 13 (Hill et al., 1969). Type II interferon produced by mixed lymphocyte cultures protected murine macrophages against the cytopathic effects of the hepatotropic influenza A/Turkey/England/63 (Virelizier et al., 1977).

Antiviral effects in vivo.

Extracts of infected organs have been used as interferon sources. Lung extracts from influenza virus infected mice enhanced the resistance to infection with Bunyawera virus (Hitchcock and Isaacs, 1960) and supernatants from infected cell cultures increased resistance to vaccinia virus infection in rabbits (Isaacs and Westwood, 1959) and man (Isaacs, 1962).

Pretreatment of young mice with anti-interferon type I antibody exacerbated infection with Semliki Forest virus, encephalomyocarditis virus, herpes simplex virus, vesicular stomatitis virus, and Moloney sarcoma viruses but no effect was observed in influenza virus infected mice (Gresser et al., 1976).

Pretreatment of mice with NDV administered intravenously three hours before exposure to littermates which had been infected with Japan 305 virus a day previously, partially protected these mice against the acquisition of infection. The NDV treated mice had significant interferon titers in their serum and lungs as opposed to saline treated animals in which interferon was undetected. Two days after exposure to infected mice (three days after NDV or saline treatment) pulmonary virus titers in infected contact mice were identical in NDV and saline treated contact mice which had become infected by exposure to infected cage-mates.

In a separate series of experiments NDV pretreatment afforded protection against infection at the same dose of virus which infected all control mice. These experiments indicated that interferon induced by NDV increased resistance of mice to transmitted infection or direct aerosol infection. However, once infected, the interferon induced by NDV did not appear to affect the rate of virus clearance (Rytel and Schulman, 1969).

In another experiment, however, interferon production was associated with recovery from infection (Iwasaki and Nozima, 1977). T cell depleted, B cell depleted, and normal mice were infected with influenza A/PR/8/34 virus. T cell depleted mice were unable to produce IgA or interferon and virus multiplied exponentially in the lungs of these mice until death. B cell depleted mice produced normal levels of interferon but were unable to clear virus later in infection. In normal mice pulmonary interferon titers reached maximum levels on day 7, and immediately after, both interferon titers and virus titers in the lung declined slowly. IgA in the respiratory tract reached maximal levels when virus titers sharply declined. The authors concluded that interferon was important in the stage of the disease when virus titers began to decline slowly, whereas IgA mediated the rapid decline in virus titers observed late after infection. Furthermore, interferon and IgA produced in response to influenza virus infection was T cell dependent.

Interferon production in influenza infections of mice is associated with T cells. T cell depleted mice did not produce either serum interferon or pulmonary interferon (Iwasaki and Nozima, 1977) after infection with PR8 virus. It had been demonstrated earlier by De Maeyer-Guignard and De Maeyer (1971) that anti lymphocyte serum treatment on three consecutive days prior to intravenous inoculation of influenza virus led to a depression of interferon produced in response to the virus. The depression in interferon produced ranged from a reduction of 64-89% of the interferon titers in influenza infected mice pretreated with normal serum.

These observations regarding the source of interferon in response to myxovirus infection were confirmed in tissue culture experiments by Tsukui (1977). Exposure of spleen cells from noninfected nonimmune mice to influenza virus resulted in the production of interferon. Depletion of T lymphocytes by treatment with anti-theta serum and complement abolished most of the interferon responses to the virus. In this study, B cells and macrophages were also shown to be capable of producing interferon under the proper conditions. Unfortunately, the interferons obtained in this study were not characterized with respect to pH, heat stability or other properties.

These reports established that in mice, interferon production after intravenous inoculation of a myxovirus or exposure of spleen cells to influenza virus *in vitro* requires intact T cell function.

In studies with human subjects, interferon has been detected in the serum and nasal washings early after artificial infection (Jao et al., 1965; Gresser and Dull, 1964) but interferon was absent in convalescent sera. In another report, intranasal inoculation of human interferon the day before challenge with influenza A or B viruses did not alter the frequency or severity of infection (Merigan et al., 1973). Interferon has an immunoregulatory effect in addition to its well studied antiviral properties. Injection of interferon preparations has been shown to suppress humoral responses (Brodeur and Merigan, 1975) and cell mediated responses (De Maeyer et al., 1975) to SRBC in mice. Addition of interferon to human peripheral blood lymphocytes inhibited the mitogenic response but enhanced the lytic capacity of the cells *in vitro* (Heron et al., 1976). Depending on the time and dose of interferon administered, immune responses may be enhanced or depressed (reviewed in Allison, 1978).

Macrophages.

Although phagocytes were the first cells found to mediate host defenses against infection (Metchnikoff, 1884), throughout the early part of this century many immunologists disregarded these classic experiments. In the 1920s and 1930s it was determined that the macrophage was responsible for clearing antigen although it was also thought that macrophages synthesized antibody (Sabin, 1923). Once again interest in macrophages waned. In the early sixties Fishman and Adler obtained results which indicated that macrophages were required for the stimulation of lymphocytes by presentation of antigen-RNA complexes (Fishman and Adler, 1963). No supporting evidence was found and interest once again turned to the lymphocytes.

In our current understanding of the immune system, the macrophage has crucial accessory functions in immune responses. Immune responses to T dependent antigens require three classes of immunocompetent cells – macrophages, T cells, and B cells (Claman and Mosier, 1972; Katz and Benacerraf, 1972; Unanue, 1972; Pierce and Kapp, 1976).

According to modern schema, macrophages take up antigens, process them and present them in association with Ia antigens to lymphocytes. This subpopulation of macrophages which express Ia membrane antigens are required for an *in vitro* antibody response. The specific I region determinants required for the response and for the macrophage T lymphocyte interaction may be restricted to the I-J subregion (reviewed in Niederhuber, 1978). T cells respond to antigen only after the antigen-Ia complex is recognized. Antisera to the antigen on the macrophage surface does not block T cell proliferation (Ellner et al., 1977), but alloantisera to the Ia on the macrophage does block proliferation of T cells (Greineder et al., 1976).

T cell activation depends on the repertoire of major histocompatibility complex (Ir) immune response genes. Ability to respond to chemically defined antigens has been determined to be under the control of the macrophage (Rosenthal and Shevach, 1973). Using inbred strains of guinea pigs these investigators showed that only responder parental macrophages were effective in evoking T cell activation in progeny of responder-nonresponder crosses (Shevach and Rosenthal, 1973).

Effective macrophage lymphocyte interaction requires histocompatibility between macrophages and lymphocytes and is enhanced by contact between the two cell types (Rosenthal and Shevach, 1973; Lipsky and Rosenthal, 1973). Cell-to-cell contact may be antigen independent (Lipsky and Rosenthal, 1973) or antigen dependent in which case proliferation occurs (Werdlin et al., 1974; Lipsky and Rosenthal, 1975) in response to antigen.

Apart from its role in the induction of immunity, a great deal of information is available on the metabolism, ultrastructure, biochemistry, and phagocytosis by macrophages (reviewed in Cohn, 1968; Unanue, 1972; and Immunological Reviews, Volume 40, 1978).

The environment from which the macrophage is obtained (age and immune status of the animal, metabolic state of the cell) is also important when certain activities of the macrophage are measured (reviewed in North, 1978; Karnovsky and Lazdins, 1978; Cohn, 1978).

Macrophages play an essential role in immune surveillance against neoplasia (Fink, 1977) and in resistance to bacterial infection (Mackaness and Blanden, 1967). These will only be briefly mentioned. The focus of this section is macrophage mediated resistance to viral infections.

In vitro studies on virus-macrophage interactions.

The ability of viruses to replicate in macrophages and produce infectious progeny can be an important factor in the pathogenesis of the ensuing disease since macrophages are strategically located at portals of entry or points of access to susceptible target organs or tissues (Mogenson, 1979). Various *in vitro* methods have been employed to determine the outcome of virus-macrophage interactions.

Direct assay of virus growth.

Titration of extracellular virus in the culture medium or release of intracellular virus by freeze thaw cycles or sonication of infected cells is a direct means of determining whether or not virus replication occurs.

Type 2 mouse hepatitis virus replicates to high titers in cultures of peritoneal macrophages from susceptible Princeton (PRI) mice (Shif and Bang, 1970). Lactic dehydrogenase virus replicates almost exclusively in macrophages (du Buy and Johnson, 1966). Ectromelia virus is capable of undergoing several replicative cycles but fewer progeny are produced with each round of replication (Roberts, 1964) and infection is relatively non-productive.

The outcome of infection of macrophages may also depend on the metabolic state of the cell, the immune state, strain, or age of the animal and the strain of the particular infecting virus.

Type or strain related resistance.

A virulent strain of Newcastle disease virus which rapidly kills chickens also destroys chicken macrophages in culture. An avirulent strain which causes only mild respiratory infection replicates poorly in chicken macrophages (Bang and Warwick, 1957). Two strains of ectromelia virus, the Hampstead mouse strain and the Hampstead egg adapted strain, differed in their pathogenicity in mice and their growth in murine peritoneal macrophages (Roberts, 1963). The mouse adapted strain replicated well in these cells and the egg passaged strain replicated poorly, as determined by fluorescent antibody techniques. Mouse Kupffer cells were used to ascertain whether differences in infectivity in mice of two vaccinia virus strains was correlated with the ability to replicate in macrophages. Although the CL-R strain and the CL strain both grew in liver parenchymal cells, only the CL-R strain grew in Kupffer cells (Mims, 1960). Immunofluorescent examination of peritoneal macrophages harvested 2 days after i.p. infection with the virulent WE-3 strain of LCM and the less virulent Armstrong strain of LCM showed that 70% and 4% of the cells were infected respectively. Infection of macrophage cultures resulted in 40% and 3% of the WE-3 and Armstrong strain infected cells exhibiting fluorescence (Tosolini and Mims, 1971).

As stated previously MHV-2 replicates efficiently in macrophage cultures as does mouse hepatitis virus type 3 (Malluci, 1965; Bang and Warwick, 1960). Both these viruses produce fatal hepatitis in mice after i.p. administration. In contrast, MHV-1 is nonpathogenic in mice and does not replicate in macrophages.

Utilizing infectious center assays, evidence was obtained that herpes simplex virus type 1 infection in macrophages was much more restricted than herpes simplex virus type 2 infection when numbers of plaques were enumerated (Mogenson, 1977a). Similarly, HSV-1 produced little liver disease while HSV-2 caused progressive focal necrotizing hepatitis. That this restriction *in vivo* and *in vitro* was macrophage specific was supported by evidence that HSV-1 replicated to higher titers in fibroblast cultures than did HSV-2. Intravenous administration of silica, a specific macrophage toxin, 2 hours before infection eliminated the differences in size and number of liver lesions after i.p. infection (Mogenson and Andersen, 1977).

Genetically determined resistance.

In certain virus macrophage systems macrophages express at the cellular level the resistance observed *in vivo*. Murine resistance to myxoviruses is controlled by an autosomal dominant Mx gene (Haller and Lindenmann, 1974; Lindenmann and Klein, 1966). A2G mice which carry this allele are resistant to infection with neurovirulent and hepatovirulent strains of influenza virus. Progeny of the cross between A2G mice and another strain of susceptible mice resist infection with influenza viruses (Lindenmann et al., 1978). Following infection by intraperitoneal administration, mice of the susceptible strains succumb to the fulminant hepatic infection within 4-6 days. Macrophages from these mice do not curtail replication of this virus (Lindenmann et al., 1978).

In contrast, macrophages from A2G mice are resistant to infection *in vitro* with A/Turkey/England/63 (Hav1Nav3). After repeated passage of peritoneal fluid of infected mice, a hepatotropic strain was isolated from liver homogenates obtained from these infected mice (Haller et al., 1976).

As an additional note, no evidence of productive infection of peritoneal or alveolar macrophages was found with other influenza viruses (Wells et al., 1978; Rosenberg and Schulman, unpublished observations; Nugent and Pesanti, 1978). This will be discussed in great detail elsewhere in this manuscript.

The genetically determined resistance of PRI mice to yellow fever virus and the susceptibility of the C3H strain was shown to be due to an autosomal dominant resistance allele (Sabin, 1952). Although initial rates of virus clearance after i.p. administration of the flavivirus West Nile virus were similar in the susceptible C3H and resistant PRI mice a period of viremia occurred in the susceptible C3H but not in the resistant PRI mice (Goodman and Koprowski, 1962). Interferon titers in the C3H mice were higher than in the PRI mice, however macrophages from the PRI mice were more readily protected by interferon treatment than cells from the susceptible strain (Hanson et al., 1969). Interferon sensitivity of the host cells may be a factor governing resistance. Resistance has been shown to be governed by an autosomal dominant trait in murine cytomegalovirus infection (Selgrade and Osborn, 1974) as well as in influenza A virus infection (Lindenmann et al., 1978). Resistance to HSV-2 has been shown to be an X-linked dominant trait (Mogenson, 1977b).

Susceptibility to infection with MHV-2 and MHV-3 is an autosomal dominant trait (Bang and Warwick, 1960; Virelizier and Allison, 1976). It is interesting that C3H macrophages do not support replication of MHV while PRI macrophages are susceptible to infection. In these two mouse strains the resistance to MHV-2 and West Nile viruses are reversed.

Age related resistance.

Explanation for the age dependent increase in resistance have included maturation of the immune system, increased interferon production, and changes in virus receptors (Fenner et al., 1974). Recent studies have shown that rough endoplasmic reticulum is much more developed in adult macrophages and almost nonexistent in macrophages from newborn mice (Mogenson, 1979). Numerous examples provide evidence that macrophage maturation may be important in resistance.

Peritoneal macrophages from weanling and adult mice were equally susceptible to infection with HSV-1 *in vivo* and *in vitro*, but adult macrophages did not spread infection to other cells (Johnson, 1964) or release as much virus *in vitro* as did weanling macrophages (Hirsch et al., 1970). Blockade of macrophage function by silica or anti-macrophage serum transformed a mild HSV-1 infection of weanling mice into a fatal encephalitic infection. Transfer of 6 x 10^6 peritoneal macrophages from adult mice also protected weanling mice infected with a lethal dose of virus (Zisman et al., 1970). Similar experiments have shown that RES blockade abolishes resistance of adult mice infected with rabies virus (Turner and Ballard, 1976), cytomegalovirus

(Selgrade and Osborn, 1974), and yellow fever virus (Zisman et al., 1971).

Resistance and the immune or metabolic state of the macrophage.

In the course of an infection macrophages become metabolically active and have increased microbicidal capacity. This process may be immunologically mediated by the elaboration and secretion of soluble lymphokines by sensitized T cells. The activated macrophage may exhibit increased phagocytic capacity, enhanced spreading on glass surfaces, and increased lysosomal enzyme contents (Cohn and Benson, 1965). These properties can also be observed in macrophages which have been stimulated by irritants such as mineral oil, proteose peptone, etc. There are examples in which immunologically activated macrophages and stimulated macrophages have augmented antiviral activity.

In an infectious center assay proteose-peptone stimulated adult macrophages restricted replication of HSV-1 to a much greater degree than did normal adult macrophages or peptone stimulated weanling macrophages (Hirsch et al., 1970). Stimulated macrophages have also inhibited plaque formation in infectious center assays, or virus replication when HSV-1, HSV-2 (Lodmell et al., 1973), encephalomyocarditis virus (Morahan et al., 1977), and vaccina viruses have been employed as the infecting agent. Inoculation of *S. aureus* before infection with influenza virus *in vitro* increased the restriction of influenza replication measured by the development of hemadsorption and formation of S and V antigens (Shayegani et al., 1974).

Treatment of mice with immunomodulators like BCG or the putative macrophage stimulators *C. parvum* or *C. acnes* does not always increase resistance to infection. BCG inoculation only partially protected adult rabbits from the development of encephalitis after corneal infection with HSV-2 (Larson et al., 1972). BCG treatment did not increase resistance after vaginal infection with HSV-2 in either rabbits (Larson et al., 1972) or adult mice (Baker et al., 1974) but live BCG administration 6 days before viral challenge reduced mortality among i.p. inoculated suckling mice while other stimulators did not. However, Spencer and his associates have demonstrated that BCG administered intranasally or systemically confers protection against subsequent challenge with influenza virus (Spencer et al., 1977).

Models using infected immunosuppressed, athymic nude mice, or mice with a GVH reaction have also demonstrated the potential of macrophage mediated resistance against viruses.

Inoculation of sensitized parental lymphoid cells into F_1 adult hybrid mice renders these recipients immunoincompetent. Although humoral (Blanden 1969, 1971b) and cell mediated immune responses (Blaese et al., 1964; Blanden, 1969) to viral and bacterial agents are depressed, the phagocytic capacity of the RES is increased. In mice with a GVH reaction clearance of intravenously inoculated ectromelia virus was more rapid (Blanden, 1971b) and resistance to *L. monocytogenes* was increased as well (Blanden, 1969). Despite the increased rate of clearance and initial protection of liver and spleen, in mice with the GVH reaction, necrotic liver foci were larger than in normal mice, perhaps due to decreased migration of phagocytes to the liver.

Nude mice have an enhanced primary resistance to such bacterial pathogens as *Listeria monocytogenes* (Cheers and Wallar, 1975), *Brucella abortus, Salmonella typhimurium* (reviewed in Mogenson, 1979) and *Candida albicans* (Cutler, 1976).

However, nude mice were more susceptible than normal mice when infected with HSV-2 (Mogenson and Andersen, 1978) and murine cytomegalovirus (Starr and Allison, 1977) although the development of hepatitis was observed in immunocompetent mice and not in nude mice. *In vitro,* macrophages from nude mice were more resistant to infection with HSV-2 (Mogenson and Andersen, 1978) and vaccinia virus (Rao et al., 1977) than normal macrophages. Macrophages from germ free nude mice were not tumoricidal whereas macrophages from conventionally housed nude mice maintained tumoricidal activity (Meltzer, 1973). The mechanism for the increased resistance displayed by macrophages from nude mice is thought to be due to increased stimulation of macrophages by the products of the bacterial flora harbored in these animals. This concept is supported by the observation that antibiotic treatment eliminates the elevated resistance of nude mice to *L. monocytogenes* challenge (Nickol and Bonventre, 1977).

Macrophages also mediate resistance through their own production of interferon or by the stimulation of other cells to produce interferon.

Spread of HSV in rabbit kidney cells was curtailed by leukocytes from immune rabbits, but leukocytes from nonimmune rabbits and antibody to HSV were ineffective in preventing the spread of the virus. High levels of interferon were measured in the immune leukocyte cultures and no interferon was detected in the cultures to which nonimmune cells and antibody had been added. Since HSV spreads via intercellular bridges, it was proposed that interferon may be an essential defense against viruses which spread cell to cell without exposure to the extracellular environment (Lodmell et al., 1973; Lodmell and Notkins, 1974).

Interferon production by macrophages also enhanced the elimination of vaccinia. Rabbits were sensitized with CFA 3-6 months before intradermal inoculation of vaccinia virus along the paraspinal region. One day later PPD or phosphate buffered saline were injected into the vaccinia virus sites. A DTH reaction was observed in sensitized animals which also were shown to have 1000-fold lower virus titers compared to controls. High concentrations of pH 2.0 stable interferon appeared in PPD-vaccinia injection sites. It is believed that viral induced interferon produced by inflammatory cells was responsible for the rapid elimination of virus in this system.

Although there is a wealth of information regarding macrophage mediated resistance to other viruses, little information was available regarding the role of the macrophage in influenza virus infections when this research project was undertaken.

Throughout these experiments BALB/c mice of 6-8 weeks of age were used, exclusively, to measure immune responses to infection. It is recognized that these responses and the pathogenesis of infection may be quite different in other strains of mice. In part, these differences could be due to genetically determined differences in immune responses under the control of the H-2 locus. For example, mice which possess a specific H-2 haplotype combination do not generate cytotoxic T cells to vaccinia or influenza viruses (Doherty et al., 1978). Other genetically determined immune responses may not be linked to the major histocompatibility locus. An example is the autosomal dominant Mx allele which confers resistance to influenza viruses (Lindenmann and Klein, 1966).

C. MECHANISMS OF RECOVERY FROM INFLUENZA VIRUS INFECTION

The availability of assay systems to measure humoral and cell mediated immunity *in vitro* are useful in kinetic studies of the responses of animals infected *in vivo.* The demonstration that particular immune responses are elicited, does not provide definitive information that these immune mechanisms exert biologic effects during infection which can alter the course of the disease. Thus, to study the role of antibody or cellular immune mechanisms in the recovery from infection requires either the passive transfer of immune serum or cells, or the use of reagents which abolish specific cells which generate the responses. In this section, a review of the experiments which helped to define the role of each of the immune responses in the resistance or recovery from infection is discussed.

Dysgammaglobulinemias in man have provided strong evidence that humoral antibodies may not be critical in recovery from many viral diseases. The patient who lacks IgG but has intact DTH responses recovers normally from most viral infections.

However, in persons with immunodeficiency states affecting cellular responses, infections by a number of viruses can become lethal. Examples of these are the severe generalized vaccinia which follows smallpox immunization and dissemination of herpes zoster virus in immunocompromised individuals. Measles infections are also severe in individuals with cell mediated deficiency states (Bloom and Rager-Zisman, 1975).

Antibody.

In the case of influenza virus infections there is a large body of evidence which illustrates that actively or passively acquired antibody protects against exposure to homotypic viruses (reviewed in Schulman, 1975).

Loosli (1953) and his associates demonstrated that transfer of immune serum prior to infection of nonimmune mice had a protective effect. This approach of immune serum transfer was employed by Schulman and his associates who demonstrated the protective effects against infection by transfer of anti-neuraminidase antibody (Schulman et al., 1968).

Evidence has accumulated in the recent past which has shown that antibody may not be directly associated with the mechanisms of recovery from influenza virus infection. In the first place, both serum antibody titers and numbers of antibody forming cells in the lymph nodes and lungs of mice do not reach maximal levels until well after virus clearance has begun (Reiss, 1978). Influenza virus infected mice pretreated with antithymocyte serum are incapable of mounting a serum antibody response and display an inability to clear virus from the lungs. Passive administration of immune serum to ATS treated mice one week after infection did not enhance the rate of virus clearance (Schulman et al., 1977), although antibody titers were equivalent to untreated mice. Thus, serum antibody may not be responsible for virus clearance.

Interferon.

In experiments described previously contradictory results have been obtained regarding the participation of interferon in the recovery process. Iwasaki and Nozima (1977) maintained that the interferon present in respiratory secretions was responsible for the initial clearance of virus before IgA antibody titers reached maximal levels. However, pretreatment of influenza virus infected mice with antibody to type I interferon did not have any effect on the severity of infection (Gresser et al., 1976). The role of type II (immune) interferon has not been characterized in any studies to date.

T Cells.

The requirement for helper functions of T cells in antibody production was demonstrated by Virelizier (1975). The first part of the experiment reiterated the protective role of antibody. Virelizier demonstrated that TxBM or cyclophosphamide treated mice could not produce antibody to PR8 virus hemagglutinin. Passive administration of specific IgG antibody had a protective effect when administered to TxBM or cyclophosphamide treated mice one day after infection with PR8 virus. The specificity of the transferred antibody was critical. Transfer of anti HA antibody to PR8 virus had a protective effect whereas antibody to HA molecules of a different subtype did not afford protection.

Spleen cells from mice sensitized a month before with PR8 virus did not have a protective effect unless recipients were treated with a dose of purified hemagglutinin protein at the time of adoptive transfer. *In vitro,* the spleen cells did not produce antibody unless purified hemagglutinin was included in the culture system. These results were interpreted to mean that T cells functioned as helper cells and not effector cells during infection.

In another murine model, the transfer of H0N1 immune spleen cells 2 days after infection with the recombinant H0N2 virus or H3N2 virus did not result in the acceleration of virus clearance or the production of serum antibody, unless inactivated HA antigens and cells were administered simultaneously. These antiviral effects were observed exclusively in the H0N2 infected recipients of both cells and antigen and not in the H3N2 infected mice. Passive administration of H0N1 antiserum one day after H0N2 infection had the same effects as simultaneous administration of immune cells and antigen. As in the previously cited experiment, it was concluded that transferred cells were precursor cells. However, the author did not eliminate the possibility that mechanisms other than antibody were important in the recovery process (Russell, 1977).

Effector T cells.

As discussed previously, treatment of mice with ATS before infection with influenza virus was characterized by elevated virus titers in the lungs and depressed serum HI antibody titers. However when ATS treatment was delayed until six days after infection a normal serum HI response was observed but virus titers remained elevated in the ATS treated mice (Schulman et al., 1977).

Immune spleen cells obtained from Japan 305 sensitized mice 6 days after immunization were transferred to syngeneic recipients one day after infection with Japan 305 virus. Infection in recipients of immune cells was characterized by lower virus titers, lower serum HI titers, and less extensive lung consolidation than in recipients of normal spleen cells. This experiment (Schulman et al., 1977) established an effector function for T cells in the immune spleen cell population. It also showed that the presence of normal titers of antibody did not accelerate the delayed rate of virus clearance in T cell depleted animals.

Similar results have been obtained by Yap et al., (1978) demonstrating that treatment of secondary immune spleen cells with anti Ly 2 sera and complement before transfer to syngeneic recipients the day after infection with A/WSN/33 (H0N1) abolished the antiviral effects. However, treatment of the cells with Ly 1 and complement did not reduce the ability of the immune cells to eliminate pulmonary virus. Transfer of these cells into recipients which were syngeneic or histocompatible at the K or D end of the H-2 locus resulted in an augmented rate of virus clearance. Transfer into a host with homology in the I region had no effect on virus titers (Yap et al., 1978). These investigators also demonstrated that treatment with antiserum to the theta alloantigen, a T cell surface marker (Reif and Allen, 1964) plus complement abrogated the antiviral effects of transferred immune cells (Yap and Ada, 1978a). These experiments demonstrated that the effector cells had the characteristics attributed to cytotoxic T cells. Ly 1 positive cells, helper T cells and effector cells of DTH, which require homology at the I region to exert effects, did not appear to be important in the recovery process.

In a series of adoptive transfer experiments Yap and Ada (1978a, 1978b) demonstrated that the transfer of primary or secondary immune spleen cells caused a reduction in virus titers in recipient mice when transferred immediately before or after infection. Transfer of immune cells protected from death as well. Significant reductions in virus titers was observed in the lungs of immune cell recipients infected with either a homologous (Yap and Ada, 1978a) or a heterologous virus (Yap and Ada, 1978b). This observation provides a possible explanation for the previously observed heterotypic immunity. Perhaps, cross-reactive cytotoxic T cells or other mechanisms of cellular immune reactions may be important in the protection against or recovery from heterotypic influenza virus infections.

In summary, these adoptive transfer experiments have demonstrated that the presence of antibody at the time of challenge protects against exposure to subsequent infection. However antibody production in the course of infection does not appear to be closely related to virus clearance. In contrast, these experiments provide evidence that virus clearance is partially mediated by a cell population with the characteristics of cytotoxic T cells and that cell mediated immune mechanisms are more closely associated with the recovery from infection.

D. IMMUNOPATHOLOGY

T cell mediated injury occurs in response to a number of viral infections. Experiments in which the depletion of T cells decrease the extent of disease caused by members of the arenavirus group (Cole et al., 1972) is the best example of cell mediated immunopathology (Gledhill, 1967; Hirsch et al., 1967). Cytotoxic T cells have been shown to localize in the cerebrospinal fluid of mice with meningitis caused by lymphocytic choriomeningitis virus (Zinkernagel and Doherty, 1973) or poxviruses (Hapel and Gardner, 1974).

Infiltration of the lungs of influenza virus infected mice by sensitized T cells and mononuclear phagocytes may both aid in elimination of virus, and contribute to the lung pathology which is characteristic of influenza virus infection in immunocompetent mice. Evidence that cell mediated immune mechanisms may be central to the immunopathological process occurring in the lungs of influenza virus infected mice is the theme of this section of the manuscript.

Treatment of PR8 virus infected mice with cyclophosphamide (CY), a potent immunosuppressive drug, results in less extensive lung consolidation, lower antibody titers and interferon titers, elevated virus titers, and in extended survival rates compared to untreated infected mice (Singer et al., 1972).

Hirsch and Murphy (1968) could not demonstrate an effect on immune responses or the course of disease in influenza virus infected mice following pretreatment with anti-lymphocyte serum. They concluded that cell mediated immune responses did not appear to be important against influenza virus infection. This is the singular study in which an immunosuppressive drug was employed which failed to demonstrate an effect on infection of mice with influenza virus.

Injection of rabbit anti-mouse lymphocyte serum beginning one day before infection of mice with the Kumamoto strain of H2N2 virus and four consecutive days after infection, resulted in delayed mortality rates. Similar treatment with purified immunoglobulin obtained from the same rabbit antiserum (ALG) resulted in enhanced survival rates, higher virus titers on day 7 after infection, and lower serum HI antibody titers on day 5. Both ALS and ALG treated mice exhibited less extensive lung pathology. These investigators concluded that lymphocytes, particularly T cells, contributed to the pulmonary pathology in influenza disease. They did not comment on their observations of the delayed clearance of virus in the T cell depleted mice (Suzuki et al., 1974).

Virulent and avirulent strains of influenza virus evoked different responses in immunodepressed mice. Infection with the normally avirulent Kunz strain of H1N1 virus was converted into a lethal infection in mice treated with cyclophosphamide on the day of infection and the first five days afterward. Similarly, following infection with a low dose of a virulent strain of H1N1 virus the survival time of CY treated mice was decreased compared to the mean survival time of control mice. In contrast, cyclophosphamide treated mice infected with a higher dose of the virulent virus had a longer survival time than control mice (Hurd and Heath, 1975). The authors concluded that these results agreed with the earlier cyclophosphamide experiment conducted by Singer et al. (1972).

A comparison of the pathogenicity of virulent and avirulent strains of the same virus was studied in congenitally athymic nude mice. An avirulent low passage strain of Hong Kong virus (H3N2) caused minimal lung pathology in immunocompetent

BALB/c mice. In contrast, infection with a high passage virulent isolate of the same virus caused extensive pneumonia. Infection of nude mice with the virulent virus was characterized by minimal lung pathology as well as prolonged survival, and dissemination of virus to the brain in association with histologic evidence of encephalitis (Wyde et al., 1977). This study in nude mice confirmed previous observations that suppression of functions in normally immunocompetent mice was associated with retarded pulmonary inflammatory responses to influenza virus (Suzuki et al., 1974; Singer et al., 1972). In another study, infection of nude mice with PR8 virus was marked by longer survival rates, persistence of virus in the lung, and relatively low seroconversion rates and HI antibody titers (Sullivan et al., 1976).

In summary, these experiments have demonstrated that cell-mediated immune responses following influenza virus infection of mice participate in the evolution of the pulmonary lesions which develop. Conversely, intact T-cell responses may play a role in the elimination of influenza virus.

VII. AIMS OF RESEARCH

Much information is available regarding the interaction of macrophages with other viruses. Relatively little information exists about the role played by macrophages in resistance to or recovery from primary influenza virus infections. The aims of the project were to establish the role of macrophages during *in vitro* and *in vivo* infection with influenza virus.

The specific aims were:

1) to determine whether macrophages could support productive infection of influenza viruses.

2) to elucidate the accessory functions of macrophages in cellular and humoral immune responses of mice infected with influenza viruses.

3) to determine whether or not macrophages provided important effector functions during the recovery process from influenza virus infection in mice.

VIII. MATERIALS AND METHODS

Mice. Male and female Swiss albino mice obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts, were used in the *in vitro* studies of macrophage-influenza virus interaction. BALB/c mice were obtained from Jackson Laboratories, Bar Harbor, Maine, or West Seneca Laboratories, Buffalo, New York. C3H mice were obtained from West Seneca Laboratories. The age of mice varied between 6-15 weeks in any experiment.

Viruses. Influenza A viruses A/PR/8/34 (H0N1), A/Hong Kong/8/68 (H3N2), A/NWS/33 (H0N1), and A/Japan/305/57 (H2N2) were obtained from stocks of viruses maintained in the Department of Microbiology, Mount Sinai School of Medicine. Stocks of seed viruses were grown in 10-11 day old embryonated eggs and stored at -70°C. Samples of influenza B/Lee/40 were generously provided by Vincent Racaniello of the Department of Microbiology at Mount Sinai. Vesicular stomatitis virus, New Jersey serotype was a gift from Dr. Colin Brand during his sabbatical in our department.

Influenza Virus Titrations. Influenza seed virus titers were determined by a standard microtiter hemagglutination assay (Hierholzer and Sugg, 1969) or an egg infectious titer (EID_{50}) after 40+ hours growth in 10-11 day old embryonated hens eggs (Reed and Muench, 1938).

Formalin inactivation of virus. Formalin was added to allantoic fluids of stock PR8 virus at a final concentration of 1:400. After 72 hours at room temperature, the treated virus was dialyzed in 500 volumes of saline for 48 hours to remove formalin.

Serologic Assays of Influenza Viruses. The surface antigens of influenza viruses were determined by microtiter hemagglutination-inhibition assays (Hierholzer and Sugg, 1969) and neuraminidase-inhibition assays (Aymard-Henry et al., 1973).

Infection of Mice. Mice were infected with 50-100 50% mouse infectious doses, MID_{50}, by exposure to a small particle aerosol suspension of influenza virus according to established procedures (Schulman and Kilbourne, 1963).

Immunization of Mice. Mice were immunized by intraperitoneal injection of 0.2 ml of virus diluted in phosphate buffered saline supplemented with antibiotics (ABS). The inoculum used for immunization was diluted to an HA titer of 1:1024–1:2048.

Cell Lines. P815 murine mastocytoma cells were generously provided by Dr. Walter Gerhard of the Wistar Institute, Philadelphia, Pennsylvania. The cell line was maintained in suspension cultures in RPMI 1640 medium (Microbiological Associates) with 10% FCS and antibiotics and passaged every 2-3 days in 25 cm^2 tissue culture flasks (Falcon Plastics, Oxnard, California). Two lines of L929 cells were used. One L cell line was a gift from Dr. Peter C. Doherty from the Wistar Institute. The other L cell line was obtained from the American Type Culture Collection. L cells were maintained in Dulbecco's modified Eagle's medium with 5% FCS and antibiotics and passaged every 3-4 days. Madin-Darby canine kidney fibroblast cells were passaged in Reinforced Eagle's Medium (Microbiological Associates) with 10% FCS and antibiotics. This cell line was provided by the Department of Microbiology at Mount Sinai.

Macrophage Culture Conditions. Peritoneal exudate cells (PEC's) were obtained by peritoneal lavage with 5.0 ml of Hank's balanced salt solution without Ca^{++} or Mg^{++} (Flow Laboratories, McLean, Virginia) supplemented with antibiotics (HBSS). PEC's were washed twice in 10 ml of HBSS then resuspended in Eagle's minimal essential medium with 10% FCS and antibiotics (Microbiological Associates). After counting, the cells were adjusted to a concentration which varied according to experimental needs. Macrophage monolayers were prepared by plating the cell suspensions onto tissue culture dishes. After overnight incubation at 37°C, the monolayers were washed extensively with antibiotic saline, ABS, before they were considered to be macrophage monolayers. Suspension cultures of macrophages were established by incubation of PEC's containing 1-2 x 10^6 monocytes/5 ml in 50 ml conical centrifuge tubes (Falcon) which were gassed with CO_2 and tightly capped before incubation in a 37°C shaker water bath (New Brunswick Scientific Co., New Brunswick, New Jersey).

Infection of Macrophage cultures. Adherent cultures of macrophages were infected with PR8 virus, NWS virus or HK virus at a multiplicity of infection (M.O.I.) of 10 plaque forming units/cell. After one hour incubation, monolayers were washed thoroughly with ABS. Residual virus was removed by incubation with 0.2 ml of virus specific rabbit antiserum. After 30' incubation at 37°C the monolayers were washed and medium was readded to the cultures. Macrophages in prepared suspension cultures were infected at a M.O.I. of 10. Aliquots were collected at various intervals after infection, washed and treated with antiserum for 30' before resuspending in MEM and returned to the shaker bath in tightly capped conical tubes.

Detection of influenza virus proteins in infected macrophages by hemadsorption.

The expression of hemagglutinin on infected macrophages was determined by hemadsorption specific adsorption of erythrocytes bound to hemagglutinin expressed on the infected cell membrane (White et al., 1965). A 0.1% suspension of type "O" human red blood cells in PBS was added to infected macrophage cultures for 30'. After washing extensively with PBS, (macrophages in suspension cultures were plated onto culture dishes and allowed to adhere for 30' before washing free of erythrocytes) the proportion of cells with 3 or more erythrocytes adsorbed to the cell membrane was determined.

Antibody and complement mediated lysis. The presence of virus neuraminidase on infected macrophage membranes was determined by lysis of cells by antiserum specific to PR8 virus neuraminidase in the presence of complement. Macrophage cultures were established in Lab-Tek tissue culture slides (Lab-Tek Products, Naperville, Illinois) infected with PR8 virus and treated with antisera as described previously. Serial dilutions of rabbit antiserum specific for PR8 virus neuraminidase were added in 0.1 ml aliquots to the infected cultures along with 0.85 ml of medium for 30'. Aliquots of 0.1 ml of a 1:10 final concentration of guinea pig complement was added to individual slide chambers for an additional 45'. The percentage of cells lysed by anti-neuraminidase antibody and complement was determined by failure to exclude trypan blue.

Indirect immunofluorescence. In addition, presence of virus proteins on infected macrophage membranes was assessed by indirect immunofluorescence. Macrophage cultures established in tissue culture slides were infected as described above with PR8 virus or B/Lee virus at a M.O.I. of 10. The monolayers were washed and incubated for 30' at 4°C with either normal rabbit serum or PR8 or B/Lee specific antiserum. After washing, fluorescein isothiocyanate conjugated goat anti-rabbit IgG (Cappel Laboratories) was added for one hour at 4°C. The slides were washed and allowed to dry. After 10' fixation with 50% ethanol followed by a 10' period of fixation in 95% ethanol, the slides were mounted in a 9:1 glycerol:PBS solution for observation by phase contrast microscopy. A Zeiss microscope with a mercury arc lamp was used with Zeiss excitation and barrier filters to observe the immunofluorescent intensity of the infected macrophage membranes.

Virus titers in supernatants of infected macrophage cultures.

Aliquots of supernatants from infected macrophage monolayers were collected at intervals after infection and inoculated into 10 or 11 day old embryonated eggs to determine virus titers. In some experiments 5.0 ml of pooled supernatants from infected cultures were treated with 1.0 ml of a trypsin-versene solution for 15 or 30' before inoculation into eggs. In other experiments 1.0 ml of actinomycin D at a concentration of 2ug/ml were added to macrophage cultures for one hour before infection. Actinomycin D is an inhibitor of influenza virus RNA and polypeptide synthesis (Gregoriades, 1970; Barry et al., 1962; Scholtissek and Rott, 1979; Pons, 1975). Receptor destroying enzyme RDE (Behring) was used to treat cells after infection in an attempt to eliminate residual input virus from the system. A 0.1 ml aliquot containing 50 units of RDE was incubated with infected macrophages commencing for 30' after infection.

Infectious Center Assay. Madin-Darby canine kidney (MDCK) cells were utilized as indicator cells to determine whether PR8 or HK influenza viruses replicate in macrophages. Infected macrophages were treated immediately after infection with antiserum, RDE, actinomycin D, or trypsin as described previously. Macrophages were gently scraped from the dishes with a sterile rubber policeman, counted and plated onto MDCK monolayers for 1.5-2.0 hours. The dishes were overlaid with an agar overlay containing 50% medium 199 (Gibco, Catalog No. 118E) antibiotics, 2mM glutamine, vitamins (Microbiological Associates) 0.005% $NaHCO_3$, 0.01% DEAE, and 0.6% agar (Oxoid Ltd., London, England, Lot L28) and 0.5 ug/ml trypsin. Dishes were incubated for 72 hours to allow plaques to develop. Dishes were stained with crystal violet for 30'. The number of plaques were counted, each plaque theoretically representing an infectious center caused by the spread of newly synthesized virus from the macrophage to adjacent MDCK cells in which influenza viruses replicate productively (Gaush and Smith, 1968).

Recombination assay. Adherent cultures of peritoneal macrophages were established in 60 mm tissue culture dishes (Lux Scientific Corp., Newbury Park, California). The macrophages were doubly infected with PR8 and HK viruses at a M.O.I. of 10 for each virus. Control cultures were infected separately with either PR8 or HK virus. Ten hours after infection 0.1 ml aliquots were collected and immediately frozen at -70°C. MDCK monolayers were infected with 0.1 ml of supernatants from the doubly infected cultures or combined supernatants from separately infected cultures. After 60' incubation an agar overlay was added to the dishes. The overlay contained a 1:800 final concentration of antisera to recombinant HK-PR8 (H3N1) or PR8-HK (H0N2) viruses. After 72 hours incubation at 37°C, a equal number of plaques were picked from each group of dishes. The antigenic composition of virus isolated from each plaque was determined by standard hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests. The frequency of recombinant progeny found in the supernatants of doubly infected macrophage cultures was compared to the frequency of recombinants observed in the combined supernatants from singly infected cultures.

Anti-macrophage reagents. Silica Dorentrüp Quartz $<$ 5 mu diameter was generously provided by Dr. I. M. Reisner, Hauptstelle für Staub - und Silikosebekämpfung, Essen-Kray, West Germany. The preparation of silica for *in vivo* use was similar to that described by Levy and Wheelock (1975). Silica dust was autoclaved and suspended in antibiotic saline. The silica suspension was briefly sonicated (Ultra-Sonic Cleaner, Cole-Parmer, Chicago, Illinois) just prior to i.p. injection of 50 mg. (0.5 ml) per mouse.

Anti-macrophage serum. Rabbit anti-mouse macrophage serum was prepared in rabbits with mouse peritoneal macrophage in a manner described by Gallily (1971). Peritoneal exudate cells (PEC) were obtained 3 days after stimulation with 10% proteose-peptone (Difco, Detroit, Michigan) by peritoneal lavage with 3.0 ml Hank's balanced salt solution (Flow Laboratories) containing antibiotics. The peritoneal cell population was comprised of 75-80% monocytes. These PEC were washed three times Rabbits were immunized initially with 5 x 10^7 PEC in complete Freund's adjuvant. After that rabbits were immunized by injections of 10^7-10^8 cells every 2-3 weeks over a three month period. Rabbits were bled one week after the last of 5 injections. Sera were inactivated by heating for 30' at 56°C and then were adsorbed with non-adherent splenic lymphocytes at a concentration of 10^7 cells/ml of serum for 1 hour at room temperature. Adsorption was repeated a total of 5 times. Samples of sera then were adsorbed with equal volumes of packed sheep erythrocytes to remove Forssman antibody. The sera then were filtered and stored at -20°C.

***In vitro* cytotoxicity assay for macrophages.** The cytotoxic activity of the anti-macrophage serum was tested with PEC obtained by peritoneal lavage as described above. After suspension in Eagle's minimal essential medium (EMEM) containing 10% FCS (Microbiological Associates, Walkerville, Maryland) cells were plated in 35 mm plastic petri dishes (Lux Scientific Co., Newbury Park, California) in 2.0 ml aliquots containing 2.5 x 10^5 monocytes. After 2-3 hours incubation at 37°C, monolayers were washed with saline and fresh medium was added. After overnight incubation monolayers were washed 5 times and considered to be macrophage monolayers, serial dilutions of 0.1 ml of anti-macrophage serum and EMEM were added to the adherent cell monolayers for 60' at 4°C. A commercial source of guinea pig complement (Flow Laboratories) was added to a final concentration of 5% in veronal buffer (Microbiological Associates) for 60' at 35°C. Cell viability was determined by exclusion of 0.4% trypan blue (Flow Laboratories). The cytotoxicity of AMS was expressed as the dilution which caused death of 50% of the cells (failure to exclude trypan blue). Duplicate samples of 200 cells were enumerated. The assay was conducted twice. The cytotoxicity titer was 1:250 for macrophages and 1:50 for thymocytes.

Phagocytic activity of macrophages *in vitro*. PEC or lung mononuclear cells from 3-4 mice infected with PR8 virus were collected at intervals after infection. After resuspension in medium at a concentration of 10^6 PEC/ml or 2 x 10^5 alveolar macrophages/ml a phagocytic assay was performed similar to that described by Tolnai

(1975). Aliquots of 0.1-0.3 ml of the cell suspensions were diluted 10-fold in medium containing a 10^3 dilution of washed polystyrene latex beads (Dow Chemical Company, Indianapolis, Indiana, Lot No. 763L) for PEC and a 10^2 dilution for alveolar macrophages. The suspensions were gassed with CO_2, tightly capped and placed into a 37°C shaker bath for 15'. Cell suspensions then were plated into 35 mm plastic dishes and incubated for 20'. Adherent monolayers were washed 5 times and stained. The number of latex beads phagocytosed/cell was determined under oil immersion. At least 100 cells/dish were counted. Duplicate or triplicate samples were used for each experimental group.

Interferon Assay. L929 cells plated in 35 mm plastic dishes (Lux Scientific Corporation) were grown in DMEM containing 5% FCS and antibiotics. Pools of lung suspensions separated into experimental groups were thawed one day before assay. An equal volume of packed human erythrocytes was added to the lung suspensions to remove influenza virus. After overnight adsorption at 4°, the supernatants obtained after centrifugation were diluted in DMEM supplemented with 2% FCS and antibiotics. Aliquots of 0.5 ml of the lung supernatant dilutions were added to each L cell dish. After incubation at 37°C for 18 hrs., the monolayers were washed twice with saline and infected with vesicular stomatitis virus (VSV) 30-50 PFU/dish. After 1 hr. incubation at 37°C, 2.5 ml agar overlay was added to each dish. The overlay consisted of 50% medium 199 (Gibsco Catalog No. 118E) antibiotics, 2 mm glutamine, vitamins (Microbiological Associates) 0.005% $NaHCO_3$, 0.01% DEAE and 0.6% agar (Oxoid Ltd., London, England, Lot L28). After 48 hours incubation at 37°C interferon titers were determined in units/ml, the endpoint being the dilution which caused a 50% reduction in plaque number. Standard mouse interferon (Lot No. 6-002-904-511) obtained from the Research Reference Branch of the National Institute for Allergy and Infectious Diseases (NIAID) served as an internal control for each assay.

Pulmonary Virus Titrations. Mice were killed with phenobarbital at various intervals after infection. Lungs were removed aseptically and placed in saline containing antibiotics and 0.1% gelatin. Individual lungs were ground with a homogenizer (Lourdes Instrument Company, Brooklyn, New York) centrifuged at room temperature and then stored at -70°C. Serial 10-fold dilutions of lung supernatants were assayed in 10-11 day old eggs to determine the titers of egg infectious virus, EID_{50}, using the standard Reed-Muench method.

Antibody Titrations. Individual sera were obtained by cardiac puncture. After overnight storage at 4°C, the serum was drawn off, treated with RDE and stored at -20°C until hemagglutinating-inhibiting antibody titers were individually measured by standard microtiter assay.

Antibody Forming Cell Assay. Sheep red blood cells in Alsever's solution were provided by Pocono Rabbit Farm, Canadensis, Pennsylvania. Erythrocytes less than two weeks old were coated with allantoic fluid seed PR8 virus according to the method of Russell (1977) and modified by Reiss and Schulman (submitted for publication), so that KIO_4 was used at a concentration 2.5×10^{-4} M in PBS. Virus coated SRBC were washed, suspended to a final concentration of 20% in HBSS and stored at 4°C until used.

Direct AFC Assay. Mononuclear cells were obtained from the lungs of infected mice as described in the section on preparation of effector cells for cytotoxicity assays. The number of IgM secreting cells in the lung cell population was determined according to the method of Reiss and Schulman (submitted for publication). The following reagents were added to glass culture tubes in a 45°C water bath. 0.5% indubiose A-37 (Accurate Chemical and Scientific Company, Hicksville, New York) virus coated SRBC to a final concentration of 1% V/total volume, and 0.1 ml of undiluted or ten-fold dilutions of the lung mononuclear cell suspension in HBSS. Immediately following the addition of the cells to the mixture, the tubes were mixed vigorously and the contents were poured onto 0.1% indubiose primed microscope slides. After allowing the agarose to set at room temperature, the slides were incubated on slide trays bathed in HBSS supplemented with 15mM HEPES buffer and antibiotics (Microbiological Associates). After 5 hours incubation, the slides were incubated with guinea pig complement (Flow Laboratories) diluted 1:20 in veronal buffer (Microbiological Associates). The number of plaques were enumerated.

Indirect AFC Assay. The number of IgG and IgA secreting cells in the lung cell populations was determined by the procedures of Pierce et al. (1971) and Reiss and Schulman (submitted for publication). Slides were prepared in the same manner as in the direct AFC assay described above except that 25 ul of goat anti-mouse IgM (blocking antibody) was incorporated into the mixture of agarose, virus coated SRBC and lung cells before pouring onto slides. After an hour incubation at 35°C, a 1:20 dilution of rabbit anti-mouse IgA (Miles Research Products) or rabbit-anti-mouse IgG (Bionetics) were added to the slide trays. After 4 hours incubation these facilitating antibody solutions were discarded, the slides were washed, and complement was added as in the direct AFC assay. The number of plaques in duplicate slides were enumerated.

Cell-Mediated Cytotoxicity Assays.

Target cell preparation. P815 target cells were labeled in suspension in serum free medium RPMI 1640 (Microbiological Associates) for one hour with $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Massachusetts) at a concentration of 100-200 uCi $^{51}Cr/10^6$ cells. L929 cells were labeled in adherent cultures in 25 cm^2 tissue culture flasks at the same concentration of ^{51}Cr used to label P815 cells. P815 cells were washed twice, resuspended in serum free medium and divided into two equal aliquots. Similarly, separate flasks of L cells were washed. Virus was added to half the P815 cells or separate flasks of L cells at a ratio of 10-20 EID_{50} virus/cell. The cells were incubated for one hour at 37°C. After washing three times, the P815 cells were adjusted to a concentration of 10^4 viable cells/ml in lung cell cytotoxicity assays and 10^5 cells/ml in spleen cell cytotoxicity assays unless indicated otherwise. L cell targets were trypsinized from the flasks after infection and adjusted to the desired concentration after washing three times similar to the treatment of P815 cells.

Effector Cell Preparation.

Lung cells. Mononuclear cells from the lungs of infected mice were prepared by teasing small pieces of lung tissue through stainless steel grids into Hank's balanced salt solution supplemented with 10% FCS, 15mM HEPES buffer solution, and antibiotics. These cells were washed once and resuspended in fresh saline. Debris and dead cells were removed by centrifugation for 30 minutes at room temperature in a ficoll-isopaque gradient (Lymphoprep, Accurate Chemical and Scientific Company, Hicksville, New York). The density of this solution is 1.077 g/ml which is optimal for the recovery of human leukocytes (Boyum, 1968). Although the recovery of murine lymphocytes is maximum when a gradient with a density of 1.09 g/ml is used (Parish, et al., 1974), sufficient number of mononuclear cells were recovered from the interface between the HBSS and gradient to conduct cytotoxicity assays.

Spleen cells. Primary immune spleen cells were obtained from mice 6 days after parenteral sensitization by injection of 0.2 ml of virus. Secondary immune spleen cells were obtained 5 days after the last dose of virus. Single cell suspensions were made by gently teasing the tissue through stainless steel grids into HBSS with 10% FCS, 15mM HEPES buffer and antibiotics. After one wash in this solution, erythrocytes were removed by treatment with 5.0 ml of 0.184 M NH_4Cl for 5' at room temperature (Zweerink et al., 1977b). After washing in PRMI medium the cells were resuspended to an appropriate dilution of viable cells for use as effector cells in a ^{51}Cr release assay.

Cytotoxicity Assays.

Lung cells. In a 0.1 ml volume 10^3 target cells and 0.1 ml effector cells at 100:1

or 50:1 effector:target cell ratio were added to individual wells of microtiter test plates (Linbro Chemical Company, New Haven, Connecticut). All cell counts were adjusted on the bases of cell viability as assessed by trypan blue exclusion. Samples were incubated for 8-8.5 hours at 37°C in a 5% CO_2 atmosphere. The assay plates were centrifuged at 1000 rpm for 10 minutes before 0.1 ml of supernatant was removed for counting in a Beckman gamma counter. Background release was determined by incubation of target cells with 0.1 ml medium and maximum release by incubation with 0.1 ml 5% Triton X-100. Specific immune release was calculated by the following formula:

$$\text{Specific } {}^{51}\text{Cr release} = 100 \text{ X } \frac{\text{Experimental (cpm)} - \text{Spontaneous (cpm) release}}{\text{Maximum release (cpm)} - \text{Spontaneous (cpm) release}}$$

All results are expressed as the mean percentage of ${}^{51}Cr$ release from 3-4 replicates. Standard errors were between ± 5-10%.

Adherent lung cells. Lung mononuclear cells were prepared as effector cells as in previous cytotoxicity assays. In preliminary experiments no effort was made to quantitate the number of monocytes or lymphocytes. In these experiments cytotoxic activity in adherent cell populations, nonadherent cell populations or whole lung cell populations was determined. In later experiments the number of monocytes and lymphocytes was determined and the same ratio of lymphocytes to target cells and monocytes (adherent cells):target cells were used. Cells were dispensed in 0.1 ml aliquots to individual wells of the assay plate along with 0.1 ml of medium. After 1.5-2.0 hours incubation the plates were inverted and immersed in a sterile saline solution and shaken vigorously. After repeating this process 3 more times, 0.1 ml of target cells and 0.1 ml of medium was added to each well. The plates were centrifuged at 1000 rpm for 10' and incubated for 8-8.5 hours. After centrifugation at 1000 rpm for 10', 0.1 ml supernatant from each well was collected for counting. Specific immune release was calculated as described above.

Spleen cell assays. Primary or secondary immune spleen cells were prepared as described above. These assays were identical to the lung cell assays except that 10^4 target cells were added to individual wells in 0.1 ml aliquots along with 0.1 ml of the spleen cells in ratios of 25:1, 50:1, or 100:1 effector:target cells. Specific immune release was calculated by the formula described previously.

Nylon wool columns. Nylon wool columns were prepared according to the method of Julius et al. (1973). Approximately 0.6 gram aliquots of washed and dried nylon wool (LP-1 Leuko-Pak Leukocyte Filter, Fenwal Laboratories, Morton Grove, Illinois) were packed into 12 ml plastic syringes (Monoject, Sherwood Medical Industries, Inc., St. Louis, Missouri) up to the 6.0 ml mark. The syringe barrels were autoclaved in the plastic syringe covers. The lids were sealed until used for experiments.

The columns were incubated with MEM +5% FCS for at least one hour at 37°C prior to the loading of lung cells collected from infected animals. The ficoll gradient enriched lung cells were loaded onto the column. A total of 3.6 x 10^6 lymphocytes in a volume of 2.5 ml were washed into the column with 1.5 ml of warm medium. The columns were resealed and returned to the incubator for 45'. At that time, prewarmed medium was added slowly to the column. The effluent flow rate was adjusted to 1 ml/min. and the first 25 ml of effluent was collected in 50 ml comical tubes (Falcon Plastics). These cells were washed in cold medium and used as effector cells in appropriate ratios in cytotoxicity assays.

Anti-theta ascitic fluid and complement. 3 x 10^6 ficoll gradient enriched lymphocytes obtained from BALB/c mice 10 days after infection were suspended for 45' at 37°C in 1.0 ml of a 1:4 dilution of anti-theta ascitic fluid raised in AKR mice sensitized with C3H thymocytes by standard procedure (Reif and Allen, 1964). To obtain non-adherent cells, lung cells were incubated for 1.0 hr in a 35 mm tissue culture dish.

After retaining the wash medium, 3 x 10^6 nonadherent cells were also treated with anti-theta serum identically to the whole lung cell population. Both the non-adherent and whole lung cell populations were washed and resuspended in 1.0 ml of a 1:10 dilution of guinea pig complement (Flow Laboratories) in veronal buffer for 45' at 37°C. The cells were washed and resuspended to the desired number of viable cells for use as effector cells in a cytotoxicity assay.

Adoptive Transfer Experiments.

Transfer of primary immune spleen cells. BALB/c mice were immunized parenterally with 0.2 ml of PR8 or Japan 305 viruses. Spleens were obtained 6 days later from both sensitized and normal mice. Single cell suspensions were prepared by teasing the organ through stainless steel grids into MEM and 5% FCS. Cells were washed 3 times in cold medium and counted. Each recipient mouse received 10^8 normal or immune lymphocytes by i.p. injection of 0.2 ml of the cell suspension. Cell transfers were conducted one day after infection of the recipient mice. Groups of 5 mice were sacrificed at various intervals after infection. The effects of cell transfer on pulmonary virus titers, lung lesion formation, and serum HI antibody were analyzed.

Transfer of secondary immune spleen cells. BALB/c mice were sensitized with either PR8 or Japan viruses. Five weeks later mice were inoculated with a second injection of the heterotypic virus to prevent neutralization by antibody elicited by the first virus injection. Single cell suspensions were prepared five days after the last virus dose. Mice which were infected with the same virus used in the last dose to immunize donor mice, received 10^8 cells one day after infection. The effects of immune cell transfer on pulmonary virus titers, pulmonary lesions and antibody responses was determined.

Statistics. Statistical analyses were conducted by employing Student's t test to determine levels of significance indicated in the text.

IX. RESULTS

A. INFLUENZA VIRUS – MACROPHAGE INTERACTION

In preliminary experiments the interaction of influenza virus with murine peritoneal macrophages was studied. The aim of these experiments was to determine whether infection of macrophages with a number of influenza A viruses was abortive or productive.

Expression of influenza virus envelope proteins.

Hemagglutinin.

Infection of adherent cells or suspension cultures of peritoneal exudate cells from the peritoneum of mice stimulated with proteose-peptone 3 days previously was accomplished as described in the Materials and Methods section of the manuscript. After one hour infection with A/PR/8/34 (H0N1) virus, the infected cultures were washed extensively with saline and treated with PR8 specific antiserum for 30 minutes at 37°C to remove input virus. A 0.1% suspension of human type "O" erythrocytes was added to the infected cultures for 30 minutes.

Figure 1 demonstrates that in both adherent and suspension cultures of macrophages no hemadsorption was observed in the first few hours after infection. By 10-12 hours after infection, in this experiment, 80-100% of the cells exhibited hemadsorption. In different experiments the percentage of cells which were positive for hemadsorption varied but the kinetics were always similar, no hemadsorption within the first few hours, and an increase at 8-12 hours after infection. The kinetics of hemadsorption observed in adherent cultures infected with either NWS or PR8 viruses are also illustrated in Figure 1. Although the initial kinetics of hemadsorption were different for the NWS virus infected macrophages than for the PR8 virus infected macrophages, 24 hours after infection 25-35% of the cells infected with either virus continued to express hemagglutinin.

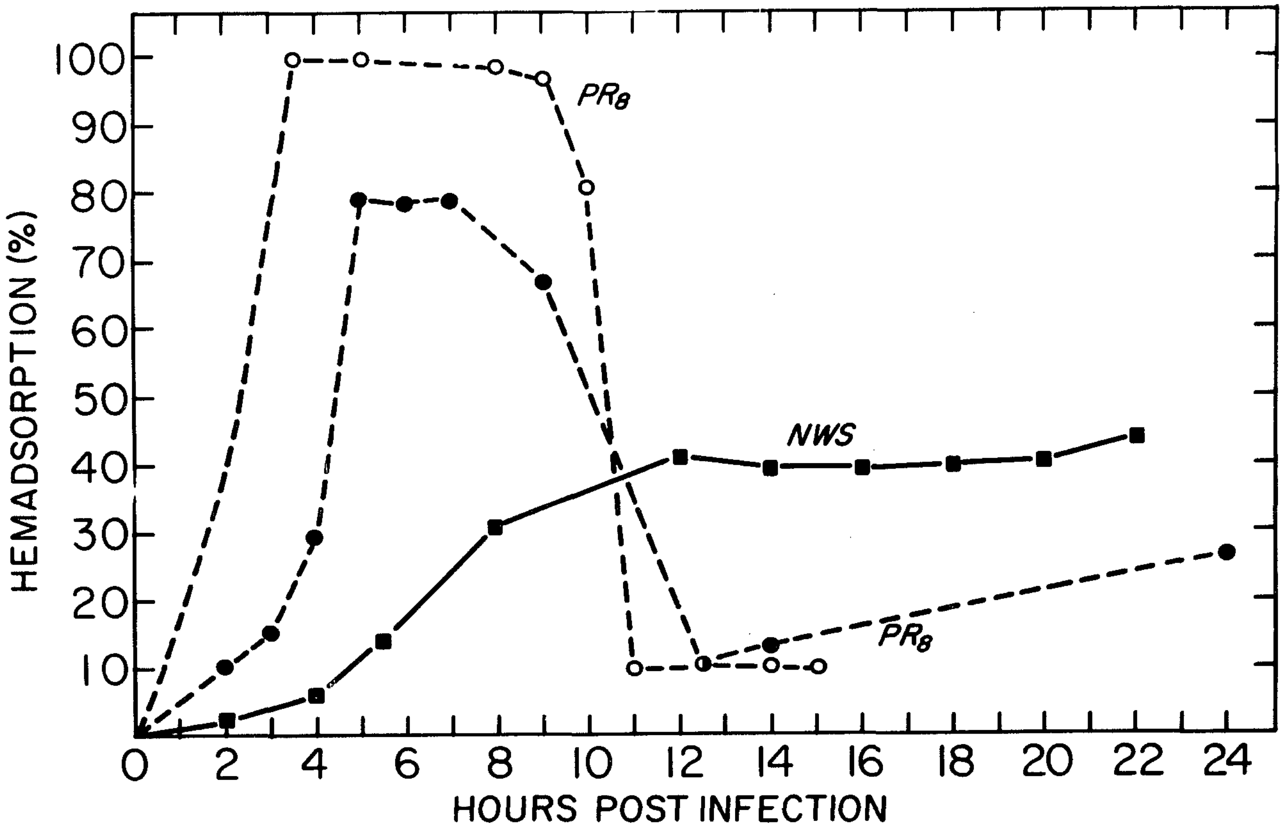

FIGURE 1. Kinetics of hemadsorption of erythrocytes to infected macrophages. HRBC type "O" were added to A/PR/8/34 (H0N1) virus infected macrophages in adherent (•—•) or suspension (o—o) cultures and A/NWS/33 (H0N1) virus in adherent cultures (■—■). Cells were considered to be positive for hemadsorption if 3 or more erythrocytes adhered to the membrane.

Neuraminidase.

Neuraminidase is a distinct glycoprotein present in the virus envelope (Laver and Valentine, 1969) which has also been found to be present on the surface of infected cells in which virus protein synthesis occurs. The percentage of macrophages expressing PR8 virus neuraminidase was analyzed by the proportion of cells lysed by specific anti NA antibody and complement. Eighteen hours after infection 0.1 ml of various dilutions of antiserum specific for PR8 neuraminidase was added to macrophage cultures along with 0.85 ml of medium. After 30 minutes incubation 0.05 ml of guinea pig complement was added to the culture system. After an additional 45' incubation, cell lysis was determined by the failure to exclude uptake of trypan blue dye. Approximately 30% of the macrophages were lysed, the same proportion of cells which expressed hemagglutinin on the surface 24 hours after infection. The results shown in Figure 2 represent the data obtained from two separate experiments in which duplicate macrophage cultures were used for each dilution of antiserum. It should be noted that normal rabbit serum or Heq1Neq1 (EqEq) rabbit antiserum did not cause lysis of cells in the presence of complement. In the absence of complement, cell lysis was not observed even in the presence of the highest concentration of antiserum specific for PR8 virus neuraminidase.

Indirect immunofluorescent techniques were used to confirm the increased expression of virus hemagglutinin and neuraminidase over time. The intensity of fluorescence increased to a maximum at 8-12 hours after infection coinciding with the time that the greatest number of macrophages expressed hemagglutinin by hemadsorption assays. The specificity of the fluorescence was confirmed by the absence of fluorescence on the surface of macrophages when antiserum to B/Lee virus instead of antiserum to PR8 virus was employed in the assay. An early study using fluorescent antibody added to influenza virus infected macrophages demonstrated that macro-

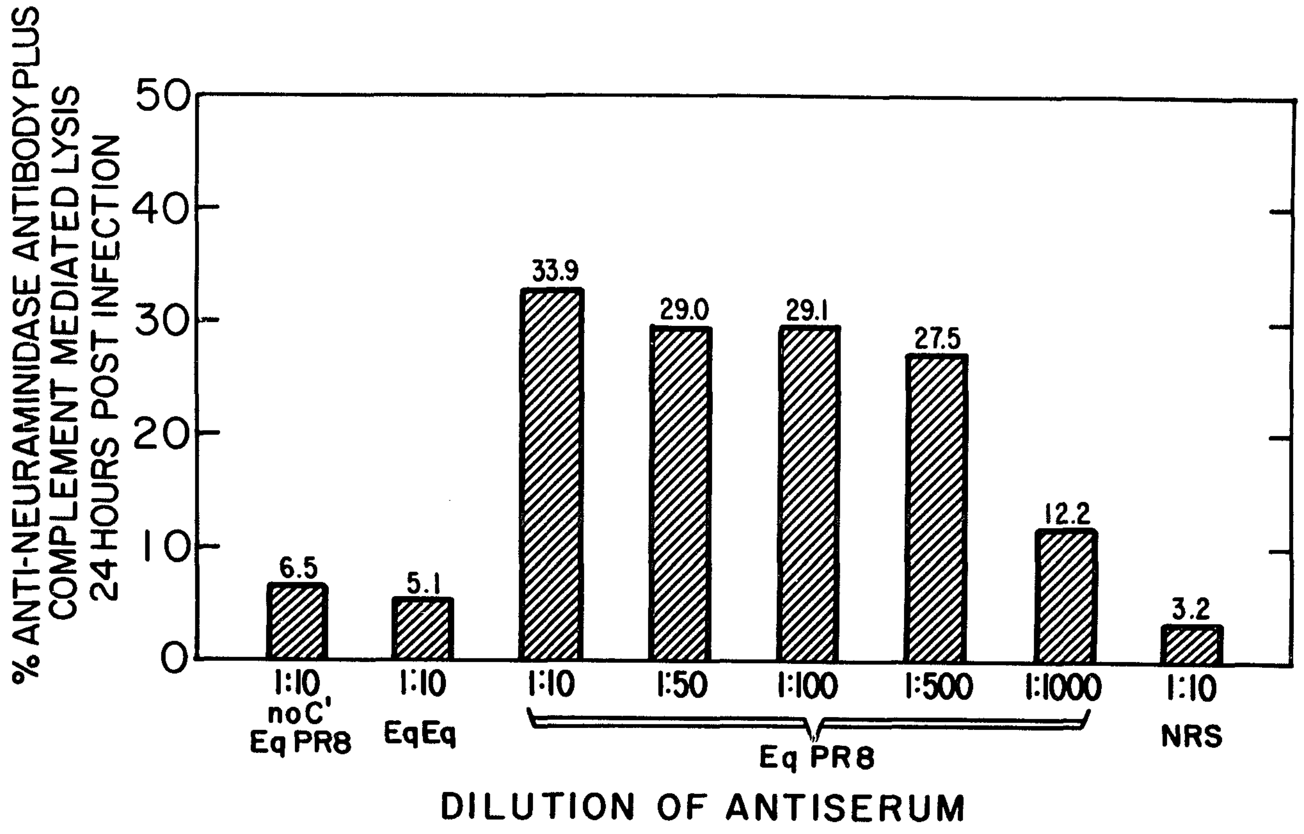

FIGURE 2. Antineuraminidase antibody and complement mediated lysis of PR8 infected macrophages one day after infection. Macrophages were infected and treated with antiserum as described in MATERIALS AND METHODS before addition of antiserum to EqPR8 virus, EqEq virus, or normal rabbit serum for 30' followed by the addition of complement for 45'. Cell lysis was determined by the inability to exclude trypan blue dye. Values represent average from two experiments in which duplicate cultures were used for each dilution of antiserum.

phages expressed virus antigens (Boand et al., 1957; Hanson et al., 1957).

These techniques provided evidence that following infection of macrophages virus antigens were present on the cell surface. Exposure of adherent cultures of macrophages to formalin inactivated virus did not result in the expression of virus hemagglutinin on the cell membrane, indicating that hemagglutinin present on the macrophage membrane after infection with untreated virus represented newly synthesized virus protein and not processed antigen (see Figure 3).

Antigen expression of macrophages infected in vivo.

Mice were infected by i.p. injection of an inoculum of 0.2 ml of a suspension containing PR8 virus on three consecutive days. On the next day peritoneal exudate cells were obtained by lavage and 5 x 10^5 mononuclear cells were plated onto tissue culture dishes. After 90 minutes incubation, nonadherent cells were removed by washing with PBS. Rabbit antiserum to PR8 virus was added for 30 minutes to remove residual input virus. The percentage of cells expressing virus hemagglutinin was assayed by hemadsorption. The percentage of cells expressing virus hemagglutinin from mice infected 1, 2, or 3 days previously was assayed. One day after infection 40% of the cells were positive for hemadsorption, approximately the same proportion which expressed hemagglutinin or neuraminidase one day after *in vitro* infection. The proportion of peritoneal cells which were positive for hemadsorption two days and three days after infection was 15% and 8%, respectively. In this preliminary experiment virus antigens continued to be expressed on macrophage membranes in decreased proportions after *in vivo* infection. These observations provided evidence which suggested that macrophages were capable of supporting at least an abortive cycle of replication following infection with influenza virus. However, these findings did not distinguish between abortive and productive infection and additional experiments were

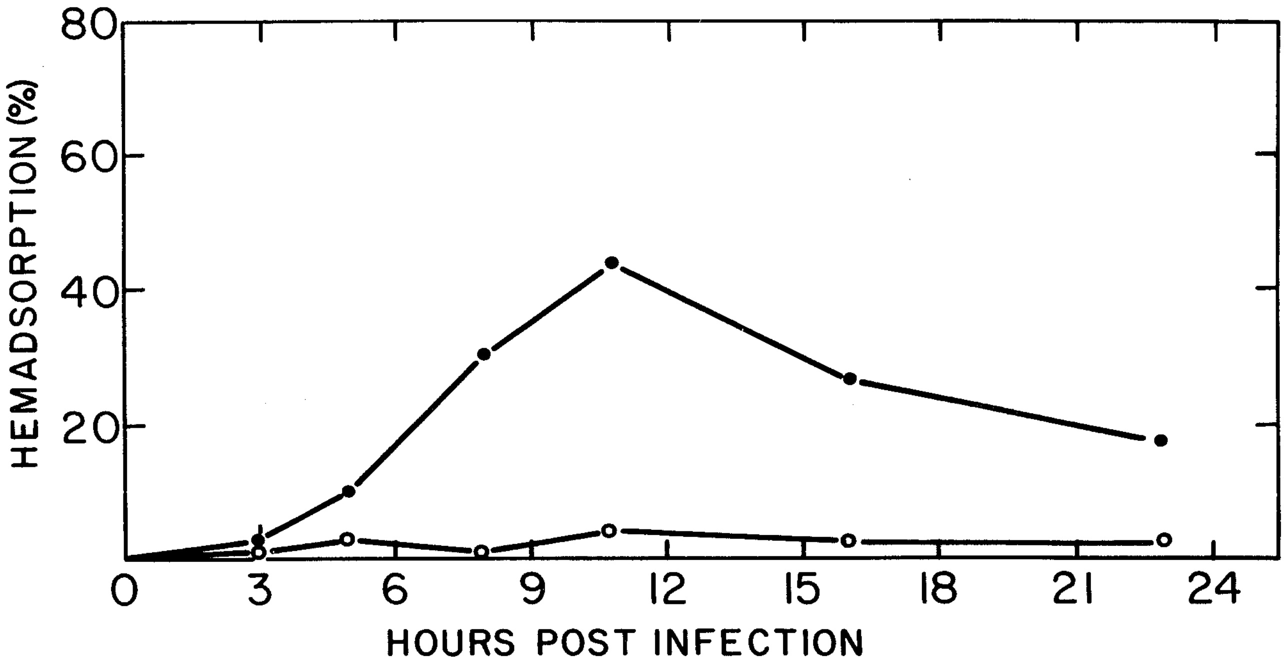

FIGURE 3. Kinetics of hemadsorption by macrophages infected with untreated (●—●) or formalin inactivated (o—o) A/PR/8/34 (H0N1) virus. Virus was inactivated by treatment described in MATERIALS AND METHODS section.

conducted to discriminate between these possibilities.

Infectious center assays.

Adherent cultures of macrophages were infected with PR8 virus at an M.O.I. of 10 PFU/cell. Two hours later after treatment of half the cultures with antiserum and the other half with saline, the cells were gently scraped from the monolayers with a sterile rubber policeman. The macrophages were counted, assayed for viability by trypan blue exclusion and plated onto MDCK monolayers in serial dilutions containing 2×10^1, 2×10^2, 2×10^3 monocytes after which agar was added to the cells. Three days later plaques were observed on the dishes onto which saline treated macrophages had been added. However, no plaques were observed on any of the dishes in which antiserum treated macrophages had been added to the indicator monolayers. These results suggested that the infectious centers observed with the saline treated macrophages were due to residual input virus on the surface of macrophages. However, it was possible that antibody bound to Fc receptors on macrophages neutralized low levels of infectious virus produced in macrophages. Hence, reagents other than antibody were used to eliminate residual input virus.

Actinomycin D treatment of infected macrophages.

The addition of actinomycin D early after infection inhibits influenza virus RNA synthesis (Barry et al., 1962; Pons, 1973; Scholtissek and Rott, 1970). Although actinomycin D interacts with DNA, the inhibition of influenza virus replication by the drug has been attributed to the intervention of the nuclear phase of the influenza virus replicative cycle (Barry et al., 1962).

Treatment of macrophages with actinomycin D at a concentration of 2ug/ml one hour before infection, did not affect the formation of plaques observed in MDCK

TABLE 1

EFFECTS OF ACTINOMYCIN D AND TRYPSIN TREATMENT OF PR8 VIRUS INFECTED MACROPHAGES ON NUMBER OF INFECTIOUS FOCI IN MDCK MONOLAYERS

Treatment		# of Infectious Foci / # of Monocytes			
Actinomycin D[a]	Trypsin[b]	2×10^3 [c]	2×10^2	2×10^1	4×10^0
+	+	TNTC[d]	172	26	3
+	+	TNTC	205	27	6
+	+	TNTC	241	57	5
–	+	TNTC	252	70	10
–	–	TNTC	194	20	7

[a] macrophage monolayers were treated with 1.0 ml of actimonycin D at a concentration of 2 ug/ml 1 hour before infection.

[b] macrophages were treated for 15' with EDTA-Trypsin 4 hours after infection.

[c] number of viable monocytes plated onto indicator cells.

[d] too numerous to count.

monolayers. Pretreatment of macrophages for one hour before infection with actinomycin D and treatment with trypsin for 30 minutes after infection had no effect on the number of infectious foci observed in MDCK monolayers. Table 1 illustrates that in dishes to which actinomycin D treated macrophages had been added, the same number of plaques were observed as in dishes to which trypsin treated macrophages or actinomycin D and trypsin treated macrophages had been added. At the highest and lowest serial dilutions, similar numbers of plaques were enumerated on dishes to which treated or untreated macrophages had been added. The failure to remove infectious centers with actinomycin D suggested that these infectious foci were caused by residual input virus.

Other reagents were used in an attempt to remove residual input virus from the membrane of macrophages before transfer to indicator MDCK cells. Infected macrophages were treated with trypsin for 15-30' before washing and transfer to MDCK monolayers. Trypsin was used in an attempt to remove virus receptors in the cell membrane. Partial digestion or alteration of the influenza virus receptor could prevent adherence of virus to the membrane, much like trypsin treatment destroyed reovirus receptors on type "O" erythrocytes (Lerner et al., 1963). However, treatment with trypsin did not effect the number of plaques on the monolayers compared to untreated cultures (Table 2).

Another approach to remove input virus by removing receptors was the use of receptor destroying enzyme, RDE (Hoyle, 1968) which could mimic the effects of the viral neuraminidase in cleaving sialic acid containing receptors on the cell membrane. It was assumed that treatment with RDE for 30 minutes, would cause elution of the virus from the membrane. However, when macrophages were treated with 0.1 ml of RDE (50 units) and used in an infectious center assay, no difference in the number of plaques was observed between RDE treated or untreated macrophages. It is not clear

TABLE 2

EFFECTS OF TRYPSIN TREATMENT OF PR8 VIRUS INFECTED MACROPHAGES ON NUMBER OF INFECTIOUS FOCI IN MDCK MONOLAYERS

Trypsin Treatment[a]		# Infectious Foci / # of Monocytes			
10 minutes	30 minutes	2×10^3 [b]	2×10^2	2×10^1	2×10^0
+		150	29	0	0
+		162	45	4	0
+		134	35	0	0
	+	172	42	2	0
–	–	132	18	0	0

[a] Infected macrophages were treated with EDTA-Trypsin solution containing 0.25% trypsin and 0.02% EDTA.

[b] Number of viable monocytes plated onto indicator cells after trypsin treatment and washing.

why RDE or trypsin did not remove the input virus from the cell membranes. It is possible that attachment of the virus to receptors on the macrophage cell membrane somehow protects against the enzymatic action of the reagents employed. The potency of the RDE preparation used was confirmed by the inhibition of infection produced by treatment of macrophages with RDE **before** infection (data not shown).

Recombination assays.

As stated above, it was possible that antiserum treatment may have neutralized virus which was newly synthesized in macrophages. Attempts to remove input virus with other reagents, RDE or trypsin, were not effective in removing input virus. The possibility remained that small quantities of newly synthesized virus were not detectable in the presence of residual input virus. To increase the sensitivity of the assay a different approach involving recombination was employed which would circumvent the problem of residual input virus masking a possible low level of infection in macrophages.

Macrophage cultures were doubly infected with Hong Kong (H3N2) and PR8 (H0N1) viruses at a M.O.I. of 10 PFU/cell each. Control cultures consisted of those fluids collected from macrophage cultures infected separately with PR8 or HK virus. These supernatants were combined just prior to plating onto monolayers. Any recombinant progeny found on these monolayers would be due to recombination in MDCK cells from residual virus in macrophage culture supernatants. Supernatants from doubly infected macrophage cultures and combined supernatants from singly infected macrophage cultures were collected at 10 hours after infection and added to MDCK monolayers. After one hour incubation, agar was added to the dishes. A 1:800 final dilution of either HK-PR8 (H3N1) or PR8-HK (H0N2) antisera was incorporated in the agar overlay. After 72 hours incubation equal numbers of plaques were observed on monolayers to which supernatants from doubly infected macrophage cultures as were observed on monolayers to which combined supernatants had been added. Plaques which were thought to be due to recombinant viruses were picked and the surface antigens of the plaque picked viruses were determined by hemagglutination-inhibition and neuraminidase-inhibition tests. The numbers of recombinant viruses from doubly infected macrophage cultures were no different from the numbers of control culture supernatants, and it was concluded that no detectable productive infection occurs in influenza infected macrophages.

Virus titers in supernatants from infected macrophage cultures.

A direct assessment of the amount of virus present in the supernatants of infected macrophage cultures was accomplished by collecting aliquots at intervals after infection.

Effects of Actinomycin D. Supernatants from infected macrophages pretreated for one hour before infection with actinomycin D were collected and inoculated into eggs. No difference in virus titers were observed in the supernatants immediately after infection or at 8, 12, or 24 hours after infection in untreated and actinomycin D treated macrophage culture supernatants.

Effects of RDE. Treatment of macrophages after infection for 30 minutes with 50 units of RDE did not prevent input virus from adhering to infected membranes. When aliquots of RDE treated cell supernatants were titered in eggs, no difference in virus titers were detected between RDE treated and untreated supernatants.

On the basis of the failure to find a greater frequency of recombinants in doubly infected macrophage cultures than in control cultures and the failure to detect an increase in virus titers in supernatants of infected macrophages it was concluded that PR8 influenza virus undergoes an abortive, nonproductive cycle of replication in macrophages.

Summary of influenza virus macrophage interaction in vitro.

The experimental results described up to this point lead to the following conclusions regarding influenza virus infection of macrophages.

1. Virus protein synthesis occurs in peritoneal macrophages from young adult Swiss Albino mice as evidenced by:
    a) detection of hemagglutinin on the surface of infected cells by hemadsorption;
    b) detection of neuraminidase in infected macrophage membranes by antibody and complement mediated lysis;
    c) increased intensity of fluorescence on macrophages over time;
    d) absence of the expression of hemagglutinin on macrophages infected with non-replicating formalin inactivated virus.
2. PR8 influenza virus undergoes an abortive cycle of replication in macrophages as evidenced by:
    a) a failure to detect an increase in virus titers in supernatants from infected macrophage cultures;
    b) an inability to detect a greater frequency of recombinant progeny in supernatants from doubly infected macrophage cultures than in control culture supernatants.
3. Residual input influenza virus remains on the surface of macrophages for extended periods of time after infection and is not removed by RDE or trypsin as evidenced by:
    a) the plaques observed on MDCK monolayers in infectious center assays which are absent if macrophages are treated immediately after infection with antiserum.

b) the absence of any effect with actinomycin D on titers of supernatants from infected macrophages or the numbers of plaques produced in infectious center assays.

B. MACROPHAGE FUNCTIONS DURING INFLUENZA VIRUS INFECTION OF BALB/c MICE

Reticuloendothelial system (RES).

The fixed macrophages and circulating monocyte populations of an organism comprise its RES. Macrophages are neither clonally restricted nor antigen specific but function as nonspecific accessory cells (Unanue, 1972). Macrophages also act as effector cells by the phagocytosis or pinocytosis of microbes and antigens. The participation of macrophages in inflammatory responses and delayed type hypersensitivity reactions can also lead to tissue destruction. The relationship of these complex macrophage functions to the resistance to and recovery from influenza virus infections in mice was the subject of this research project.

Effects of anti-macrophage reagents on phagocytic activity.

In initial experiments, efforts were made to demonstrate that the 2 methods for macrophage depletion, silica and anti-macrophage serum, were capable of depressing macrophage function *in vivo.* For this purpose measurements of phagocytic activity with latex beads by macrophages obtained from control mice and macrophage depleted mice were conducted.

Peritoneal exudate cells obtained from PR8 virus infected mice treated with silica on the day of infection or 3 days after infection, as well as PEC from mice treated with AMS on day 0 and day 3 of infection or untreated mice were tested for phagocytic activity with latex beads 5 days after infection. These results are summarized in Table 3. A marked reduction in phagocytosis of latex beads was observed with macrophages from silica treated mice. A significant, but less dramatic depression of phagocytosis was observed with macrophages obtained from AMS treated mice.

TABLE 3

IN VITRO PHAGOCYTOSIS OF LATEX BEADS BY PERITONEAL MACROPHAGES FROM NORMAL OR MACROPHAGE DEPLETED MICE FIVE DAYS AFTER INFECTION WITH A/PR/8/34 (H0N1) VIRUS

	Number of Beads Phagocytosed				
Treatment	0–4	5–10	11–20	21–30	31–40
Silica-Day 0*	96$^+$	4			
Silica-Day 3	56	35	9		
AMS**-Days 0, 3	26	41	25	8	
Saline-Days 0, 3	8	36	34	14	8

* Day 0 = day of aerosol infection with PR8 virus.

** Antimacrophage serum, 0.2 ml intraperitoneally.

$^+$ % of macrophages.

Consistent with the depression of phagocytic activity induced by silica or AMS treatment on peritoneal macrophages is the demonstration of reduced phagocytosis with alveolar macrophages from silica treated mice. As summarized in Table 4, on day 4 after infection, phagocytosis of latex beads by alveolar macrophages from silica treated mice was depressed when compared to phagocytosis by alveolar macrophages from control animals. A less impressive effect was observed on day 6 after infection when slight decreases in phagocytic activity were observed in alveolar macrophages from silica treated animals. The numbers in parentheses which represent the median number of beads phagocytosed, were essentially unchanged in the control mice but slightly increased in the silica treated mice from day 4 to day 6. These results furnished evidence that i.p. injection of silica impaired the phagocytic function of alveolar macrophages for at least a few days after influenza virus infection. These results lent support to the hypothesis that the effects of silica administration observed in the lungs of silica treated mice during infection related to macrophage depletion.

Effects of macrophage depletion on influenza virus infection.

In a preliminary experiment, the effects of silica injected intraperitoneally on the day of infection on virus clearance, lung lesions, and serum HI antibody responses were examined in mice infected with PR8 virus. As demonstrated in Figure 4 (upper panel) virus clearance was markedly delayed in silica treated mice. Ten days after infection mean virus titers were 100-fold higher in silica treated animals than in control mice, and 14 days after infection, virus was no longer detectable in any of the lungs of control mice whereas the lungs of all silica treated mice still contained high titers of virus. Similarly, as shown in the middle panel of Figure 4, lung lesions developed later and were less extensive in silica treated animals than in control mice. The bottom panel of Figure 4 demonstrates that serum hemagglutinating-inhibiting antibody

TABLE 4

IN VITRO PHAGOCYTOSIS OF LATEX BEADS BY ALVEOLAR MACROPHAGES FROM NORMAL OR MACROPHAGE DEPLETED MICE FOUR AND SIX DAYS AFTER INFECTION WITH A/PR/8/34 (H0N1) VIRUS

Treatment	Days after Infection	Number of Beads Phagocytosed					Median
		0–4	5–10	11–20	21–30	>30	
Silica-Day 0*	4	74+	20.5	5.5			3.9
Control		35	41.5	17.5	5.5	0.5	8.1
Silica-Day 0	6	57	27	13	1.5	0.5	5.7
Control		29.5	43	17	3.5	2	8.0

* Day 0 = day of aerosol infection with PR8 virus.

\+ % of macrophages.

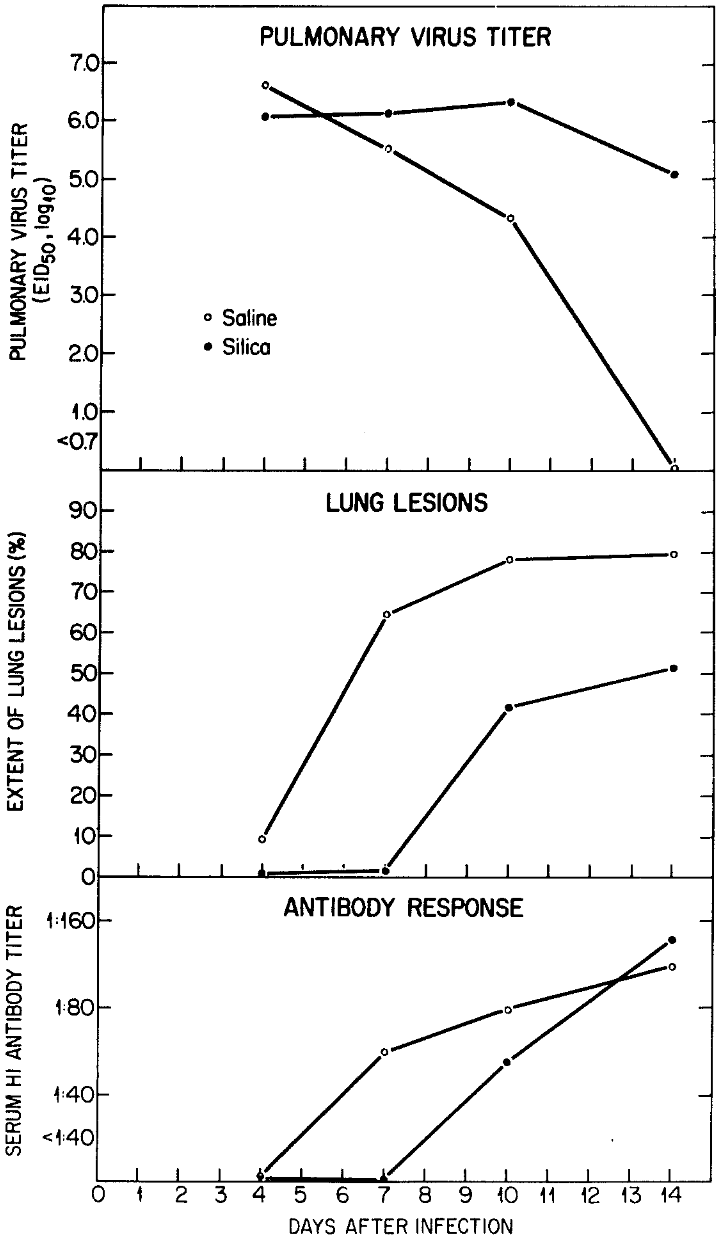

FIGURE 4. Effects of macrophage depletion on pulmonary virus titers (upper panel), the development of lung lesions (middle panel), and serum HI antibody responses (lower panel). Animals were infected with influenza A/PR/8/34 (H0N1) as described in MATERIALS AND METHODS. On the day of infection mice were inoculated i.p. with saline (o—o) or 50 mg. silica (●—●). Values represent the mean of five mice/group. Each point represents the mean percent gross pulmonary consolidation.

appeared late in silica treated mice. Thus, 7 days after infection all control mice had low levels of serum HI antibody whereas none of the silica treated mice had detectable serum antibody.

Effects of silica administration on the day of infection on cytotoxic activity of lung mononuclear cells from infected mice.

To measure cytotoxic activity in lung lymphocytes at different times after infection in a single assay, it was necessary to infect control and silica treated mice at staggered intervals. Lung mononuclear cells obtained from PR8 virus infected mice at various intervals after infection were isolated from a Ficoll-isopaque gradient and tested for cytotoxicity against PR8 virus infected P815 target cells. These P815 cells are of DBA/2 origin, grow in suspension culture, and have been used extensively as target cells in ^{51}Cr release assays as histocompatible cell lines for both DBA/2 and BALB/c mice which share the H-2^{d} haplotype. The results summarized in Tables 5 and 6 demonstrate that significant levels of cytotoxic activity were not demonstrable in lung mononuclear cells until 8 days after infection in either group. In both experiments, control mice developed appreciably more cytotoxic activity than silica treated animals 8 days after infection. In the first of these experiments (Table 5) no cytotoxic response was detected in silica treated mice 8 days after infection whereas in the second experiment (Table 6), reduced levels of cytotoxicity were observed using lymphocytes from silica treated mice. The data in this table also demonstrates that by 11 days after infection the level of cytotoxic activity in mononuclear cells obtained from the lungs of silica treated mice are equivalent to that observed with immune cells from control mice. Tables 5 and 6 also show that lung lesions were delayed in appearance and reduced in intensity in silica treated mice confirming the results shown previously in Figure 4.

TABLE 5

PULMONARY LESIONS AND CYTOTOXIC ACTIVITY OF LUNG MONONUCLEAR CELLS IN SILICA AND CONTROL MICE INFECTED WITH PR8 VIRUS

Treatment group	Days after infection	Lesion[a] score (%)	Effector: target ratio	% ^{51}Cr release[b]	
				PR8 infected targets	Uninfected targets
Silica[c]	5	0	100:1	–3.6 (±3.7)	–2.3 (±1.0)
			50:1	–2.0 (±1.4)	–2.3 (±0.6)
Control	5	9	100:1	4.2 (±3.0)	–4.2 (±2.4)
			50:1	4.4 (±3.0)	–3.4 (±0.9)
Silica	8	16	100:1	1.6 (±2.4)	–5.2 (±0.4)
			50:1	2.1 (±2.6)	–3.9 (±2.9)
Control	8	70	100:1	28.3 (±5.6)[d]	–1.6 (±3.2)
			50:1	18.3 (±2.2)[d]	–1.8 (±1.1)
Control	uninfected	–	100:1	3.5 (±3.7)	1.5 (±2.1)

[a] mean extent of lung consolidation (%) 5 animals/group.

[b] $$\frac{\#\text{ counts released} - \text{spontaneous release}}{\text{total releasable counts} - \text{spontaneous release}} \times 100$$

values represent mean of four replicate wells (±SD). Spontaneous release from uninfected and infected P815 target cells was 25.2% and 26.7% respectively.

[c] 50 mg i.p. on the day of infection.

[d] significantly greater release than with normal lymphocytes or with lymphocytes from silica treated mice $P < 0.001$

TABLE 6

PULMONARY LESIONS AND CYTOTOXIC ACTIVITY OF LUNG MONONUCLEAR CELLS IN SILICA TREATED AND CONTROL MICE INFECTED WITH PR8 VIRUS

Treatment group	Days after infection	Lesion[a] score (%)	Effector: target ratio	% ^{51}Cr release[b]	
				PR8 infected targets	Uninfected targets
Silica[c]	8	13	50:1	16.3(±2.3)	−1.7(±2.0)
Control	8	49	50:1	40.3(±4.8)[d]	−4.2(±2.0)
Silica	11	27	50:1	64.5(±4.9)	−1.8(±1.8)
Control	11	85	50:1	57.8(±2.0)[e]	−0.8(±0.7)
Control	uninfected	––	50:1	−1.4(±1.9)	2.8(±2.7)

[a] mean extent of lung consolidation (%) 4 animals/group.

[b] $$\frac{\#\text{ counts released} - \text{spontaneous release}}{\text{total releasable counts} - \text{spontaneous release}} \times 100$$

values represent mean of four replicate wells (±SD). Spontaneous release from uninfected and infected P815 target cells was 24.0% and 20.5% respectively.

[c] 50 mg i.p. on the day of infection.

[d] significantly greater release than with normal lymphocytes or with lymphocytes from silica treated mice $P < 0.001$

[e] not significantly different release than with lymphocytes from silica treated mice.

Effects of silica treatment on the day of infection on pulmonary interferon titers.

Lung suspensions treated as described in the Materials and Methods section, were assayed for interferon titers on L cells employing plaque inhibition with VSV as the assay system. As shown in Figure 5, early after infection pulmonary interferon titers were lower in silica treated mice than in control mice. On day 5 after infection, interferon titers were still slightly lower in silica treated mice but by day 7 interferon titers in the two groups were equivalent. In addition Figure 5 demonstrates that 10 days after infection, virus titers in the lungs of macrophage depleted animals were 1000-fold higher than titers in the lungs of control mice despite the presence of equivalent interferon titers in the two groups. Comparison of serum interferon titers in mice from similar experiments demonstrated equivalent responses in silica treated and control mice (data not shown). It should be noted that lung suspensions used in the interferon assays were not treated at pH 2.0 to avoid any assumptions regarding the type of interferon present in the lungs after influenza virus infection.

In summary, silica treatment on the day of infection caused delays in virus clearance, serum antibody responses, cytotoxic responses in the lung, interferon responses in the lung, and the development of lung consolidation. To determine which of these immune responses was associated with recovery from infection, the time at which silica was administered was varied in an attempt to produce differential effects on different components of the immune response and to relate these effects to virus clearance and lung consolidation in silica treated animals.

Effects of delayed silica treatment or anti-macrophage serum treatment on infection.

The effects of silica administered parenterally on days 3 and 6 after infection or AMS injected i.p. on days 0 and 3 of infection were analyzed. In the upper panel of Figure 6 it can be seen that virus clearance was delayed in all macrophage depleted

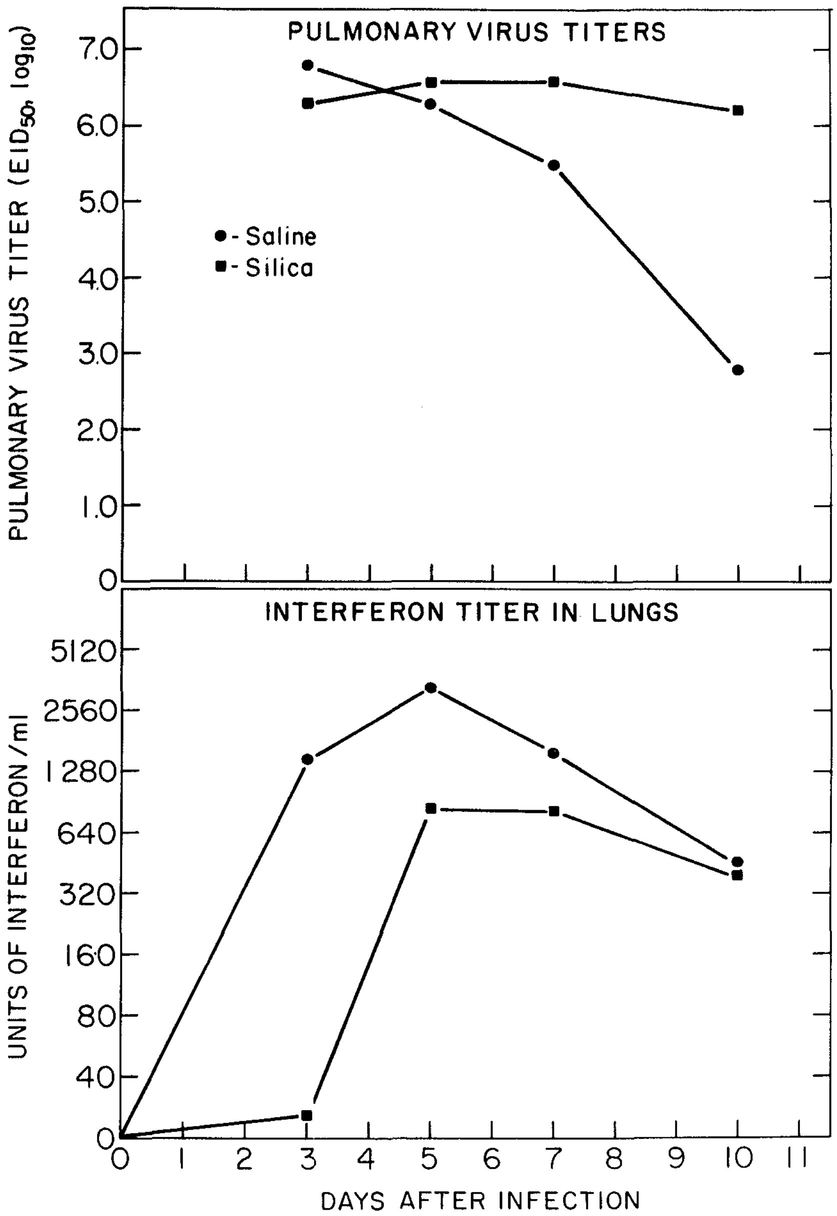

FIGURE 5. Interferon titers in the lungs of PR8 virus infected mice treated with saline (●—●) or silica (■—■).

mice regardless of the time of silica administration. Virus titers in both of the silica treated groups and the AMS treated group were much higher than control animals on day 10 of infection. Although virus titers declined by day 12 in the AMS treated group and in mice which were silica treated on day 3, clearance of virus was still depressed compared to control animals in which virus was no longer detectable. Virus titers remained at higher levels for an extended period of time in the lungs of mice treated with silica on the day of infection. Pulmonary consolidation was less extensive and slower in development in all groups of macrophage depleted mice. Despite the delayed clearance and less extensive lung consolidation observed in the delayed silica treated mice, normal serum antibody responses were noted. In contrast, antibody responses of mice treated with silica on the day of infection and in AMS treated mice were depressed throughout the infection. Thus, these experiments provided evidence that the effects of silica treatment on antibody response could be dissociated from the clearance of virus and the development of lung lesions. Delayed clearance of virus and decreased lung lesions were still evident in mice in which silica treatment was delayed despite a normal HI response.

Effects of delayed silica treatment on cytotoxic activity of lung mononuclear cells from infected mice on the course of disease.

In a preliminary experiment lung mononuclear cells were obtained on day 8 after infection from mice treated with silica on day 0 or day 3 of infection. The cytotoxic activity of these cells were much lower than the activity observed in the lung cells of the control animals. These results, illustrated in Table 7, compelled us to conduct a more elaborate experiment in which cytotoxic activity of murine lung mononuclear cells could be measured along with virus titers, lung lesions, and antibody responses in delayed silica treated mice.

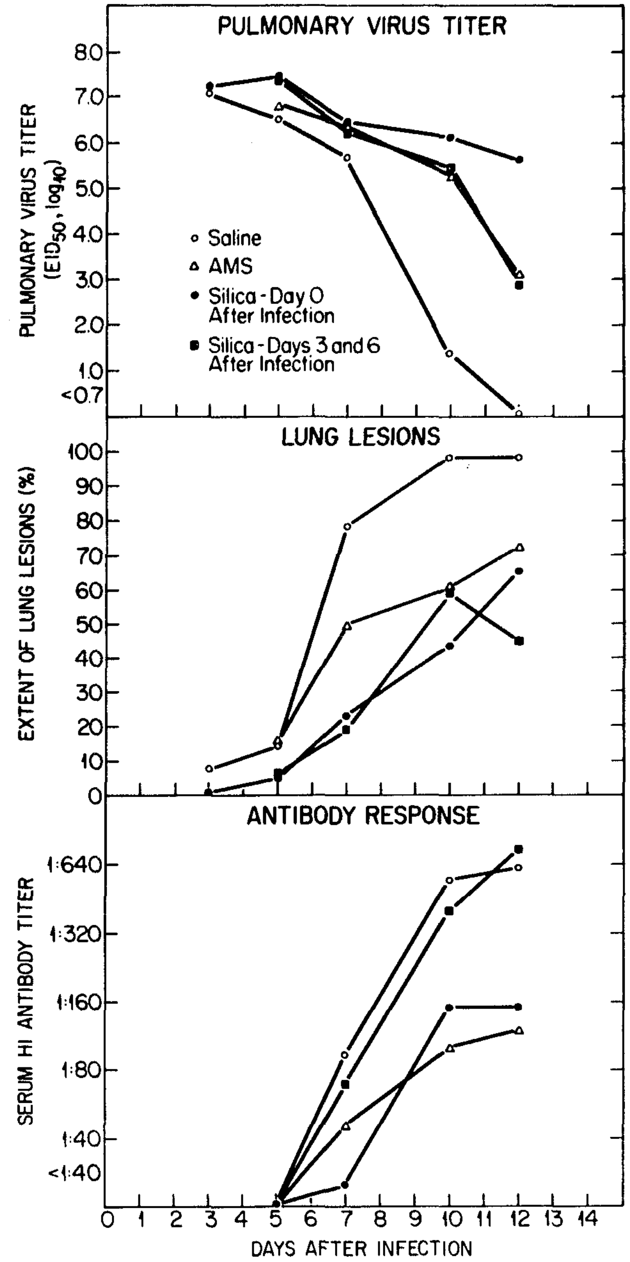

FIGURE 6. Effects of different regimens of macrophage depletion on pulmonary virus titers (upper panel), the development of lung lesions (middle panel), and serum HI antibody responses (lower panel). Animals were infected with influenza A/PR/8/34 (H0N1) as described in MATERIALS AND METHODS. Control mice were injected with saline on the day of infection and three days later (o—o). One group of mice received 50 mg. of silica i.p. on the day of infection (•—•). Another group received 50 mg. silica i.p. 3 days after infection and 25 mg. silica 6 days after infection (▪—▪). A fourth group received antimacrophage serum on days 0 and 3 of infection (△—△). Values represent the mean of five mice/group.

TABLE 7

EFFECTS OF SILICA TREATMENT ON DAY 0 OR DAY 3 AFTER PR8 VIRUS INFECTION ON CYTOTOXIC ACTIVITY IN LUNG MONONUCLEAR CELLS 8 DAYS AFTER INFECTION

Treatment Group	Effector: Target Ratio	% Specific ^{51}Cr release PR8 infected targets	 Uninfected targets
Silica – Day 0[a]	100:1	18.0[b]	–1.0
	50:1	11.2[b]	0
Silica – Day 3 after infection	100:1	17.4[b]	3.5
	50:1	8.4	2.0
Untreated	100:1	41.0[c]	3.1
	50:1	21.9[b]	0
Uninfected	100:1	–1.0	0

[a] 50 mg. i.p. on the day indicated.

[b] Significantly greater release than with normal lymphocytes or uninfected targets ($P < 0.01$).

[c] Significantly greater release than any other group ($P < 0.001$).

Separate groups of mice were treated with a single dose of silica on day 3, day 5, or day 7 after infection. Cytotoxic activity of lung mononuclear cells from each of the delayed silica groups of mice and untreated infected mice were measured on day 8 after PR8 virus infection. The results are summarized in Table 8. No detectable cytotoxic activity was observed in lung cells from mice injected with silica on day 3 of infection. However, the level of cytotoxic activity of lymphocytes from the lungs of mice treated with silica on day 5 was only slightly depressed compared to lymphocytes from the lungs of control mice and the cytotoxic activity of the cells from mice treated with silica on day 7 was comparable to the activity observed in lung cells of control animals.

The effects of this delayed silica treatment on virus clearance, lung lesions, and antibody responses are illustrated in Figure 7. Clearance of virus was delayed in all silica treated mice regardless of the time of silica administration. Late in infection, on days 10 and 12, when virus titers in the lungs of control mice had declined appreciably, virus titers in the macrophage depleted mice were significantly higher. The extent of lung consolidation in mice treated with silica on day 7 was comparable to that observed in control mice on day 8. Lung lesions were less extensive in animals treated with silica earlier than day 7 and cytotoxic activity was lower in these groups as well. In this experiment, therefore, a correlation between cytotoxic responses and the extent of pulmonary pathology was observed. Serum HI antibody responses in all silica treated mice were not significantly different from those of control mice on days 8, 10, or 12 after infection. These results confirmed the earlier observation that when macrophage depletion is delayed until 3 days or later after infection a normal antibody response is mounted while virus clearance is still delayed. Therefore, it was concluded that serum antibody responses are not directly associated with either virus clearance or the lung lesions.

TABLE 8

EFFECTS OF DELAYED SILICA TREATMENT ON CYTOTOXIC ACTIVITY IN LUNG MONONUCLEAR CELLS 8 DAYS AFTER PR8 VIRUS INFECTION

Treatment Group	Effector:Target Ratio	% Specific ^{51}Cr release[a]	
		PR8 infected targets	Uninfected targets
Silica[b] Day 3 after infection	100:1	6.1±1.7[c]	1.4±2.2
	50:1	3.8±1.1[c]	4.1±2.2
Silica[b] Day 5 after infection	100:1	48.9±4.5[d]	6.0±0.7
	50:1	30.3±3.9[d]	3.4±3.8
Silica[b] Day 7 after infection	100:1	61.3±4.0[d]	1.9±1.8
	50:1	31.4±1.7[d]	−1.4±0.7
Control Untreated	100:1	68.6±3.6[d]	3.2±2.5
	50:1	43.1±2.4[d]	4.4±2.8
Normal	100:1	2.3±2.7	−2.3±3.4
	50:1	3.6±3.0	2.7±3.0

[a] $\frac{\text{\#counts released} - \text{spontaneous release}}{\text{total releasable counts} - \text{spontaneous release}} \times 100$

[b] 50 mg. i.p. on the day indicated after infection.

[c] not significant.

[d] significantly greater release than with normal lymphocytes $P < 0.001$

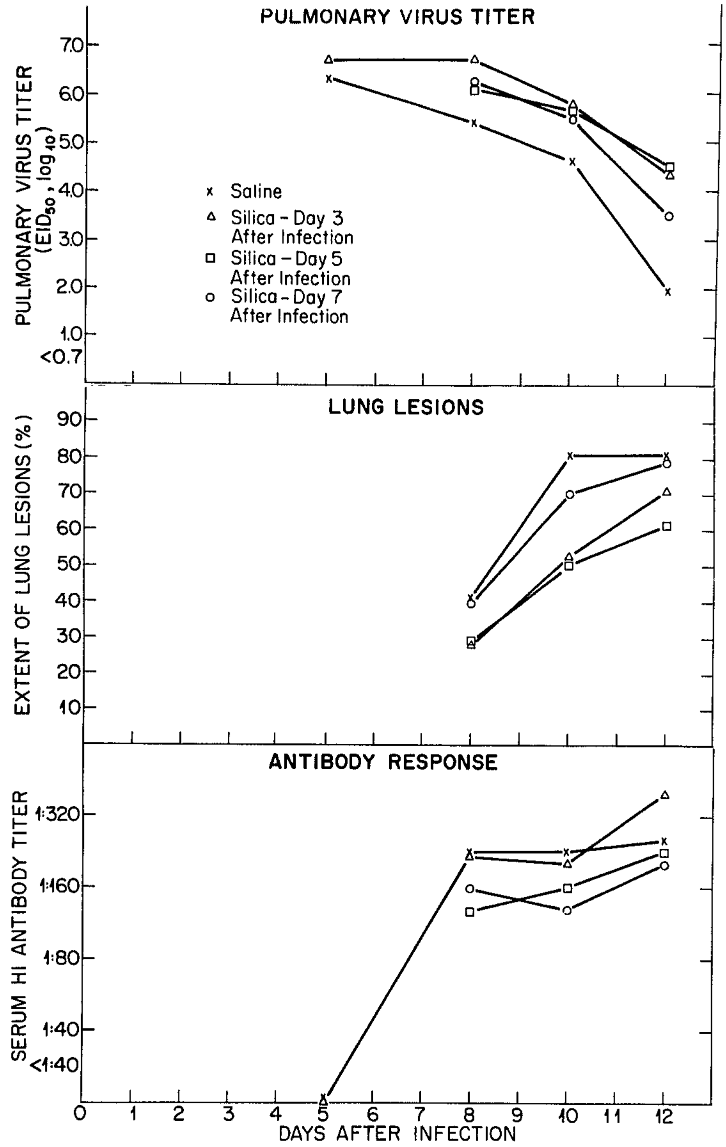

FIGURE 7. Effects of delayed silica treatment on pulmonary virus titers (upper panel), development of lung lesions (middle panel), and serum HI antibody responses (lower panel). Mice were inoculated i.p. with saline on day 3 (x—x) or 50 mg. of silica on day 3 (△—△), day 5 (□—□), or day 7 (o—o) after infection. Values represent the mean of five mice/group.

In this same experiment the lung suspensions of mice treated with silica on day 3 and the lung suspensions of control animals were assayed for interferon titers. These results are shown in Figure 8. The virus titers are the same as those in Figure 7 (upper panel) and are included for ease of comparison with interferon responses. Interferon titers in the lungs of mice treated with silica on day 3 of infection were similar to those measured in the lungs of control animals on day 5 and on day 8, although interferon titers in control mice had declined while the titers remained unchanged in the silica treated mice. Virus titers in macrophage depleted mice remained elevated throughout the remainder of the experiment, but interferon titers in both groups of mice were similar on day 10, and on day 12 no interferon was detected in the lungs of either group. Once again, despite the presence of equivalent interferon titers late in infection, a more rapid clearance of virus was observed in the untreated mice. These observations confirmed earlier results which suggested that virus clearance may not be directly related to the inhibitory effects of interferon.

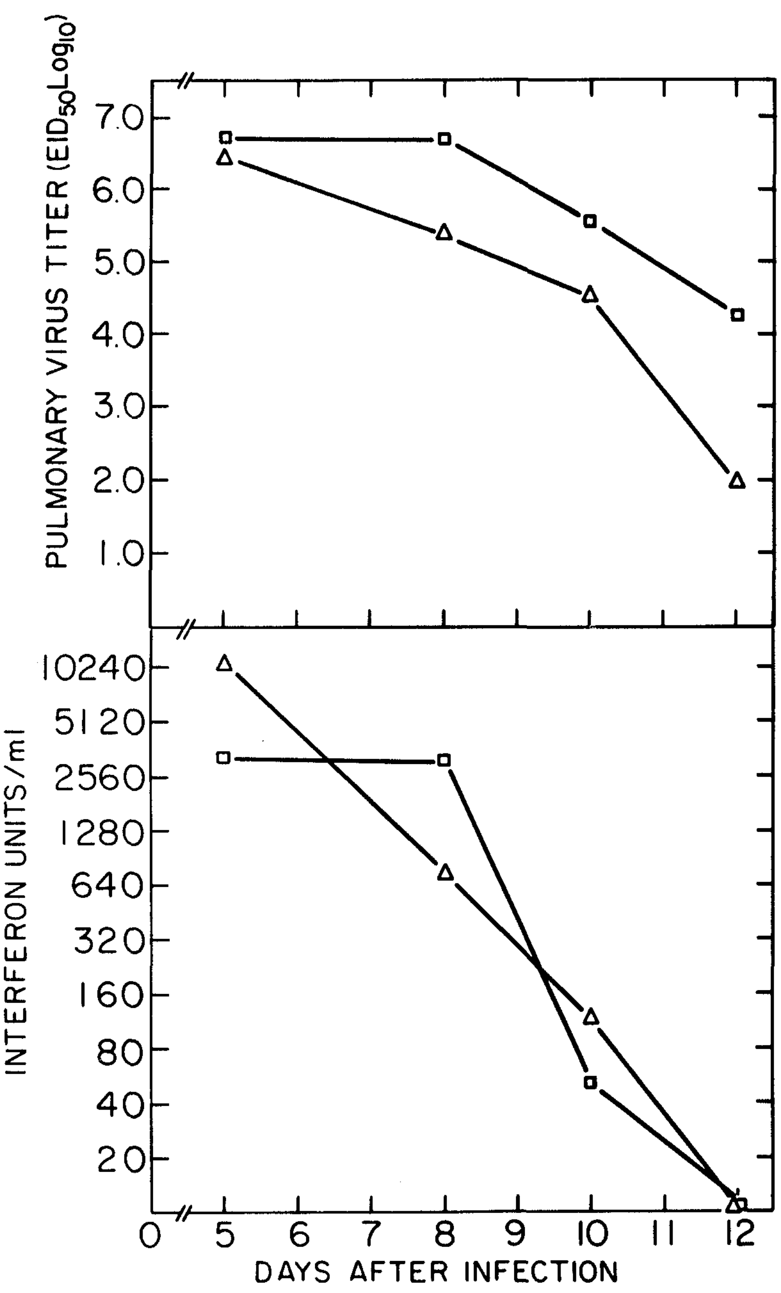

FIGURE 8. Interferon titers in the lungs of PR8 virus infected mice treated with saline (△—△), or silica on day 3 after infection (□—□). Data shown is that obtained from the same animals used in Figure 7. (Virus titers are identical to those shown in Figure 7.)

C. CHARACTERISTICS OF CYTOTOXIC EFFECTOR CELLS IN LUNG MONONUCLEAR CELL POPULATIONS

The possible association of cytotoxic activity with the clearance of virus and pulmonary consolidation necessitated a more thorough characterization of the effector cells recovered from the lungs of infected mice. A number of experiments were conducted to determine the salient features of the cytotoxic effector cells. Most cytotoxic assays were conducted on day 10 of infection, at which time cytotoxic activity was maximum and virus titers were declining most rapidly.

H-2 restriction of cytotoxic activity.

BALB/c and C3H mice which were 6-8 weeks old were infected with PR8 virus. Ten days after infection lung mononuclear cells were obtained from infected and uninfected mice. Lung mononuclear cells from C3H mice which have the H-2^k haplotype were tested for cytotoxic activity against both histocompatible L cells and incompatible P815 cells. Similarly, BALB/c cells were tested for cytotoxic activity against histocompatible H-2^d P815 cells and L cells. The results are shown in Table 9. Lysis effected by BALB/c lung lymphocytes was observed exclusively with histocompatible infected P815 mastocytoma cells, and C3H lung cells were capable of lysing the infected histocompatible L cells but not the P815 histoincompatible cells. It should be noted that no cell lysis was mediated by cells from uninfected mice and cells from infected animals did not cause lysis of uninfected histocompatible or incompatible cells indicating that in these assays NK activity was not detected. Similar experiments conducted in our laboratory have demonstrated that like pulmonary effector cells, splenic effector cells were also restricted to killing H-2 compatible cells (Reiss, 1978).

TABLE 9

CHARACTERIZATION OF EFFECTOR CELLS IN LUNGS OF INFLUENZA VIRUS INFECTED MICE: H-2 RESTRICTION

	% Specific ^{51}Cr release[b]	
	P815 (H-2^d)	L929 (H-2^k)
BALB/c[a] infected	18.4 ± 0.9[c]	5.3 ± 1.0
C3H infected	4.2 ± 0.6	40.2 ± 0.9[c]

[a] lung cells were obtained 10 days after infection with PR8 virus, cytotoxic response at a 50:1 effector:target cell ratio is shown.

[b] $$\frac{\text{\# counts released} - \text{spontaneous release}}{\text{total releasable counts} - \text{spontaneous release}} \times 100$$

[c] significantly greater release than with H-2 incompatible target cells $P < 0.001$

Cytotoxic activity is anti-theta and complement sensitive and cross-reactive among different influenza A virus infected targets.

Lung mononuclear cells recovered from BALB/c mice 10 days after PR8 virus infection were treated with anti-theta serum and complement. These cells then were tested for cytotoxicity against PR8 virus infected targets and Japan 305 virus infected cells. As illustrated in Table 10 it was observed that anti-theta and complement treatment reduced cytotoxic activity by 75-80%. This experiment also demonstrated that the cytotoxic effector cell population was capable of lysing both PR8 (H0N1) and Japan 305 (H2N2) virus infected targets with equal efficiency. This cross-reactive lysis was also eliminated by treatment with anti-theta serum and complement. From these observations it became apparent that the predominant cell which was the cytotoxic effector had the characteristics of cytotoxic T cells.

Absence of cross-reactive lysis between influenza A and influenza B virus infected targets.

BALB/c mice were infected with B/Lee/40 virus. Ten days after infection lung mononuclear cells were recovered and tested for cytotoxic activity against B/Lee or PR8 virus infected P815 target cells. No cross-reactive lysis between PR8 and B/Lee virus infected target cells was observed in either the whole cell population or the adherent cell population from the B/Lee infected mice (Table 11A). B/Lee immune cells lysed only B/Lee virus infected targets.

Similarly, in a separate experiment, summarized in Table 11B, it was observed that PR8 virus infection did not generate cells which could lyse B/Lee virus infected target cells. PR8 virus infected target cells were lysed by both the whole cell population and the population of nonadherent cells recovered after 90 minutes incubation at 37°C in plastic dishes. In these experiments the cytotoxic activity in both adherent

TABLE 10

CHARACTERIZATION OF EFFECTOR CELLS IN LUNGS OF INFLUENZA VIRUS INFECTED MICE: CROSS REACTIVITY BETWEEN INFLUENZA A VIRUSES AND EFFECTS OF ANTI-THETA TREATMENT

	% ^{51}Cr Release[a]		
Treatment group*	PR8 infected targets	JAP infected targets	Uninfected targets
Untreated infected	46.9 ± 5.4[b]	43.4 ± 2.7[b]	3.2 ± 2.4
ATS + C^1	12.0 ± 1.1[c]	14.6 ± 4.3[c]	0.8 ± 0.8
ATS + C^1 nonadherent	13.0 ± 2.4[c]	13.8 ± 4.6[c]	4.5 ± 4.0
ATS	44.5 ± 6.7[b]	39.9 ± 6.3[b]	4.4 ± 4.3
Normal (uninfected)	1.7 ± 2.9	2.4 ± 0.7	1.2 ± 1.1

[a] $$\frac{\text{\# counts released} - \text{spontaneous release}}{\text{total releasable counts} - \text{spontaneous release}} \times 100$$

[b] significantly greater release than with anti-theta serum and complement treated cells or normal lymphocytes $P < 0.001$

[c] significantly greater release than with normal lymphocytes $P < 0.01$

* a 50:1 effector:target ratio was used; lymphocytes were obtained from infected mice 10 days after infection with A/PR/8/34 (H0N1) virus.

TABLE 11

CHARACTERIZATION OF EFFECTOR CELLS IN LUNGS OF INFLUENZA VIRUS INFECTED MICE: ABSENCE OF CROSS REACTIVITY BETWEEN INFLUENZA A AND B VIRUSES; CYTOTOXIC ACTIVITY IN ADHERENT AND NON-ADHERENT CELL POPULATIONS

	% Specific ^{51}Cr release*	
A. Effector cells[a]	PR8 infected targets	B/Lee infected targets
PR8 infected	59.7 ± 4.9[b]	7.9 ± 1.9
PR8 infected - nonadherent cells	44.3 ± 4.9[c]	5.6 ± 4.4
Normal	2.7 ± 2.3	4.5 ± 3.0
B. B/Lee infected	−1.9 ± 2.7	39.4 ± 7.7[d]
B/Lee infected adherent cells	2.1 ± 2.3	12.2 ± 2.8[e]
Normal	0.0 ± 0.6	4.5 ± 4.2

[a] mice were infected with B/Lee/40 or A/PR/8/34 (H0N1) viruses lung lymphocytes were assayed 10 days after infection in different experiments, a 50:1 effector:target ratio was employed.

[b] significantly greater release than with B/Lee targets or normal lymphocytes $P < 0.001$ and non-adherent cells $P < 0.05$

[c] significantly greater release than with normal lymphocytes or B/Lee targets, $P < 0.001$

[d] significantly greater release than all other groups in Experiment B, $P < 0.001$

[e] significantly greater release than with uninfected targets $P < 0.001$ or PR8 infected targets $P < 0.01$

* release from uninfected targets did not exceed ± 5.0 in any group

and nonadherent cell populations were measured to determine whether an adherent cell population contributed to the cytotoxic activity in these assays. The observation of significant levels of activity in the adherent cell population compelled us to conduct additional assays (Table 13).

Taken together these two experiments confirm earlier reports that cytotoxic activity of sensitized lymphocytes is not observed with influenza A and B virus infected cells (Braciale, 1977a; Doherty et al., 1977). When the lung cell populations were separated into adherent and nonadherent populations cross-reactivity was not observed in either population.

Nylon wool passage diminishes cytotoxic activity.

Nylon wool columns are used to enrich for T lymphocytes and deplete B cells and macrophages. Ten days after infection with PR8 virus, gradient purified lung mononuclear cells were passed through a Julius column according to established procedures (Julius et al., 1969). In two separate experiments, comparison of cytotoxic activity of nylon wool passaged cells with the activity of untreated lung cells revealed that approximately one-third of the activity was removed by nylon wool passage (illustrated in Table 12). The results of these experiments provided evidence that cytotoxic activity was mediated in part by nylon wool and plastic adherent cells, which were resistant to treatment with anti-theta serum and complement suggesting that a cell population with the characteristics of macrophages contributed to the cytotoxic activity which was measured.

Cytotoxic activity in whole lung cell populations and adherent cell populations.

Lung mononuclear cells recovered from BALB/c mice 10 days after PR8 virus infection were enumerated by morphological criteria as lymphocytes or monocytes.

TABLE 12

CHARACTERIZATION OF EFFECTOR CELLS IN LUNGS OF INFLUENZA VIRUS INFECTED MICE: EFFECTS OF NYLON WOOL PASSAGE

Treatment group	Effector:Target Ratio	% Specific ^{51}Cr release: PR8 infected targets	% Specific ^{51}Cr release: Uninfected targets
Experiment 1			
Nylon wool	50:1	46.3 ± 6.4[a]	1.4 ± 0.7
Control	50:1	66.5 ± 11.4[b]	0.5 ± 0.1
Normal	50:1	−0.8 ± 0.5	−2.8 ± 0.0
Experiment 2			
Nylon wool	50:1	42.1 ± 3.2[a]	−3.7 ± 3.1
Control	50:1	65.3 ± 4.0[c]	2.2 ± 2.0
Normal	50:1	3.6 ± 2.1	−1.4 ± 2.7

[a] significantly greater release than with normal lymphocytes $P < 0.001$

[b] significantly greater release than with normal lymphocytes $P < 0.001$ or with nylon wool passaged cells $P < 0.05$

[c] significantly greater release than with all other groups in the same assay $P < 0.001$

Equal numbers of unseparated, adherent cells, and nonadherent cells were compared for cytotoxic activity. When standardized to the same effector:target cell ratio, a low level of cytotoxic activity was again observed in the adherent cell population (Table 13). In this experiment cytotoxic activity was not diminished in the cell population after removal of plastic adherent cells by incubation for 90 minutes at 37°C. This might be due to the enrichment of a cytotoxic T cell population which are able to make contact with target cells more often in the absence of the larger, adherent cells.

TABLE 13

CHARACTERIZATION OF EFFECTOR CELLS IN LUNGS OF INFLUENZA VIRUS INFECTED MICE: CYTOTOXIC ACTIVITY IN WHOLE LUNG CELL AND ADHERENT CELL POPULATIONS

		% Specific ^{51}Cr release[b]	
Group[a]	Effector:Target Ratio	PR8 infected targets	Uninfected targets
Infected	50:1	63.5 ± 4.1[c]	−2.2 ± 0.5
Infected nonadherent	50:1	68.0 ± 5.0[c]	−2.7 ± 0.6
Normal	50:1	−1.9 ± 2.8	−3.0 ± 0.8
Infected adherent	50:1	12.5 ± 4.0[d]	−3.8 ± 0.8
Normal adherent	50:1	2.5 ± 2.8	1.3 ± 2.5

[a] mice were infected with A/PR/8/34 (H0N1) 10 days before lung lymphocytes were assayed for cytotoxic activity. Nonadherent cells were obtained from lung cells after depletion of adherent cells by incubation for 90 min at 37°C.

[b] $$\frac{\text{\# counts released} - \text{spontaneous release}}{\text{total releasable counts} - \text{spontaneous release}} \times 100$$

Spontaneous release for PR8 virus infected target cells and uninfected target cells in whole cell populations and adherent cell assays was 11.2%, 10.4%, and 10.5% and 12.4%, respectively.

[c] significantly greater than all other groups $P < 0.001$

[d] significantly greater than normal adherent cells $P < 0.01$

D. ADOPTIVE TRANSFER EXPERIMENTS

A series of experiments were conducted to determine whether immune cells transferred to intact or macrophage depleted animals facilitated recovery from influenza virus infection. In each of the immune cell transfer experiments the cytotoxic activity in the donor cells transferred was measured in a ^{51}Cr release assay employing spleen cells from littermates of immune cell donors. Preliminary experiments were conducted to determine the kinetics of the development of cytotoxic activity in the spleens of sensitized mice. Table 14 demonstrates that the maximal amount of cytotoxic activity in spleen cells occurred on day 6 after sensitization with PR8 virus. This observation concurred with the earlier reports by Braciale (1977a), Doherty and his associates (1977) and Schulman and his co-worker (Schulman and Reiss, personal communication).

After establishing the time at which splenic effector cell activity was maximum, cells were transferred 6 days after sensitization in all primary immune cell experiments. Specific immune release mediated by primary immune spleen cells was usually between 25 and 35% for BALB/c mice. The results of a typical ^{51}Cr release assay using spleen cells from BALB/c mice 6 days after sensitization is shown in Table 15.

Antiviral effects of primary immune cell transfer.

Adoptive transfer of 10^8 immune spleen cells obtained 6 days after sensitization of donors accelerated the clearance of virus from the lungs of normal immune cell recipients late in infection as illustrated in Table 16. The antiviral effect of immune cells first became evident on day 10 after infection when the difference in virus titers between control recipients of immune cells and control recipients of normal cells became statistically significant. These effects mediated by immune cells were not

TABLE 14

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

Day after[a] infection	Effector:Target cell ratio	% Specific ^{51}Cr release: PR8 infected targets	% Specific ^{51}Cr release: Uninfected targets
3	100:1	3.6	0.5
	50:1	2.4	0.0
6	100:1	28.5[b]	0.9
	50:1	17.2[c]	0.0
9	100:1	11.8[d]	0.0
	50:1	8.3	0.9
Normal	100:1	3.7	1.0
	50:1	2.0	0.6

[a] BALB/c mice sensitized i.p. with 0.2 cc PR8 virus having HA 1:1024

[b] significantly greater release than with uninfected targets or normal lymphocytes $P < 0.001$

[c] significantly greater release than with uninfected targets or normal lymphocytes $P < 0.01$

[d] significantly greater release than with uninfected targets or normal lymphocytes $P < 0.05$

TABLE 15

CYTOTOXIC ACTIVITY IN SPLEEN CELLS FROM LITTERMATES OF DONORS IN ADOPTIVE TRANSFER OF CELLS 6 DAYS AFTER SENSITIZATION

		% Specific ^{51}Cr release[c]	
Spleen cell source[a]	Effector:Target ratio	PR8 infected targets	Uninfected targets
A/PR/8 immune donors	100:1	27.6[d]	5.7
	75:1	21.6[d]	5.1
	50:1	17.7[d]	4.5
	25:1	8.3	1.4
Normal donors	100:1	4.0	4.8
	75:1	4.2	4.5
	50:1	2.8	3.8
	25:1	2.2	3.2

[a] BALB/c mice were sensitized with PR8 virus 6 days before cell transfer. Littermates were used as spleen cell sources to assess the cytotoxic activity in cells transferred.

[b] 2×10^4 target cells/well.

[c] mean from four wells.

[d] significantly greater release than uninfected targets or normal cells $P < 0.005$

TABLE 16

EFFECTS OF PRIMARY IMMUNE SPLEEN CELL TRANSFER ONE DAY AFTER PR8 VIRUS INFECTION ON PULMONARY VIRUS TITERS

Days after infection	Treatment of recipient	Donor cells transferred	Pulmonary virus titer EID_{50} Log_{10}
4	– –	– –	6.7 ± 0.2
	Silica	– –	6.1 ± 0.4
7	– –	– –	5.6 ± 0.4
	– –	Normal	5.6 ± 0.6
	– –	Immune	5.1 ± 0.6[a]
7	Silica	– –	6.2 ± 0.4
	Silica	Normal	5.7 ± 0.2
	Silica	Immune	6.3 ± 0.8
10	– –	– –	4.5 ± 0.5
	– –	Normal	4.0 ± 1.0
	– –	Immune	$<$ 1.7[b]
10	Silica	– –	6.4 ± 0.3
	Silica	Normal	5.8 ± 0.1[c]
	Silica	Immune	5.4 ± 0.5[d]

[a] Not significantly lower than silica treated normal cell recipients 7 days after infection ($P < 0.01$). Significantly lower than silica treated immune cell recipients 7 days after infection.

[b] Significantly lower than all other experimental groups ($P < 0.001$).

[c] Significantly higher than all control groups 10 days after infection ($P < 0.01$).

[d] Not significantly lower than other silica treated groups 10 days after infection.

observed in silica treated mice. In contrast to the antiviral effects of immune cells in control animals, control or silica treated recipients of normal cells did not clear virus any more rapidly than animals which had not received any cells.

In Figure 9 it can be seen that in this same experiment, transfer of sensitized cells retarded the development of lung lesions in normal animals 7 days after infection but by 10 days after infection the lung pathology in the control recipients of immune cells was not significantly reduced from that observed in the other control groups of mice. In this same experiment control recipients of normal cells appeared to have slightly higher HI titers but these results were not observed in other experiments. Characteristic delays in the development of lung lesions and antibody responses were observed in all silica treated mice regardless of the source of the cells they received.

Table 17 and Figure 10 depict the results of an experiment similar to the one described above. In this experiment an acceleration of virus clearance was observed on day 11 after infection in normal recipients of immune cells (Table 17). Silica treated recipients of immune cells did not clear virus more rapidly than control recipients of normal cells. Indeed, both groups of macrophage depleted mice maintained higher levels of virus compared to virus titers in the lungs of control animals. Figure 10 also demonstrates that both the development of lung lesions and serum antibody responses were delayed in silica treated mice regardless of whether they received immune or normal spleen cells. On day 11, the extent of pulmonary pathology and serum HI antibody titers were nearly equivalent in all groups of mice.

In another experiment, when immune cells were transferred four days after infection instead of one day after infection, no acceleration of virus clearance was observed in the lungs of control immune cell recipients (data not shown). Thus, it appeared that immune cells must be administered to recipients immediately before (Yap and Ada, 1978a) or one day after infection to produce the desired effects.

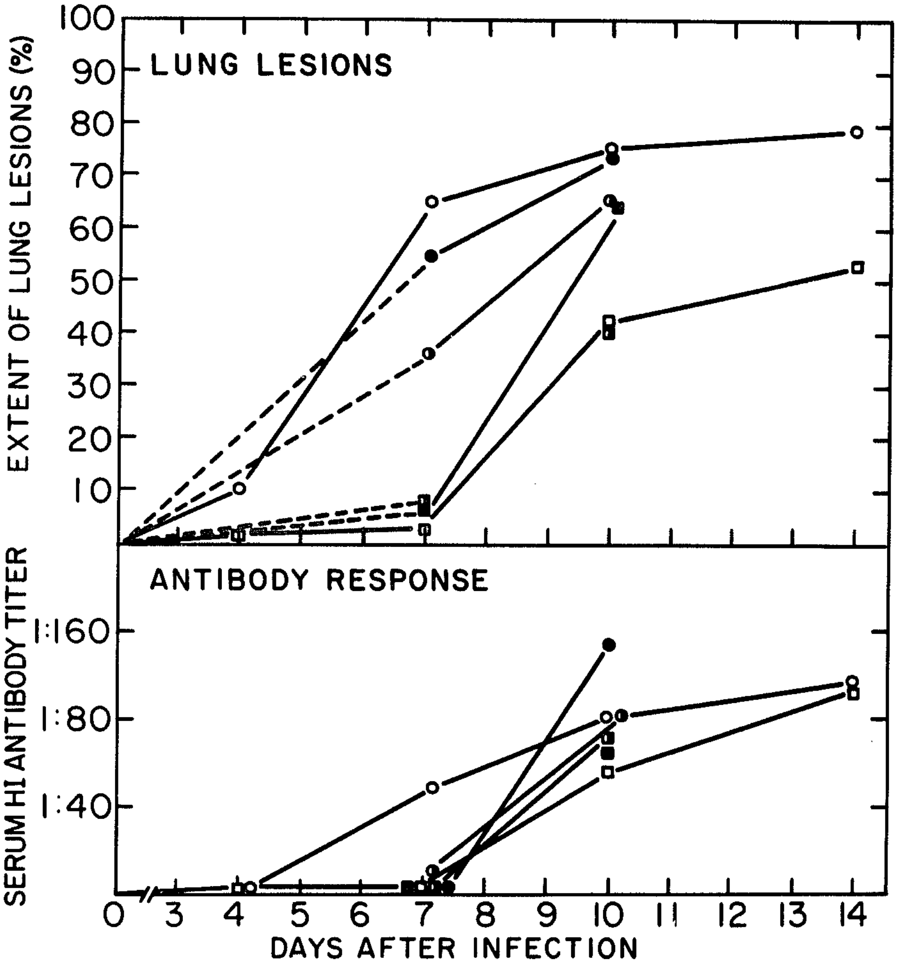

FIGURE 9. Effects of adoptive transfer of primary immune spleen cells on the development of lung lesions (upper panel) and serum HI antibody responses (lower panel). Animals received 10^8 spleen cells one day after infection with PR8 virus. Control *no* cells (o—o); control immune cell recipients (◐—◐); control normal cell recipients (•—•). Macrophage depleted animals inoculated i.p. with 50 mg. silica on the day of infection received *no* cells (□—□), immune (◧—◧) or normal spleen cells (▪—▪). Spleen cells obtained 6 days after sensitization with PR8 virus. Values represent the mean of five mice/group.

TABLE 17

EFFECTS OF PRIMARY IMMUNE SPLEEN CELL TRANSFER
ONE DAY AFTER PR8 VIRUS INFECTION
ON PULMONARY VIRUS TITERS

Days after infection	Treatment of recipient	Donor cells[a] transferred	Pulmonary virus titer EID_{50} Log_{10}
3	– –	Normal	7.1 ± 0.4
	– –	Immune	7.1 ± 0.4
3	Silica	Normal	7.0 ± 0.4
	Silica	Immune	6.6 ± 0.5
7	– –	Normal	5.8 ± 0.4[b]
	– –	Immune	5.9 ± 0.4[b]
7	Silica	Normal	7.3 ± 0.3
	Silica	Immune	6.9 ± 0.4
11	– –	Normal	2.5 ± 1.5[c]
	– –	Immune	< 0.7[d]
11	Silica	Normal	5.1 ± 1.2
	Silica	Immune	5.0 ± 0.8

[a] 1.5×10^8 cells transferred.

[b] Significantly lower than silica treated groups 7 days after infection ($P < 0.001$).

[c] Significantly lower than silica treated groups 11 days after infection ($P < 0.01$).

[d] Significantly lower than all other groups. Compared to control recipients of normal cells 11 days after infection ($P < 0.025$). Compared to silica treated groups ($P < 0.001$).

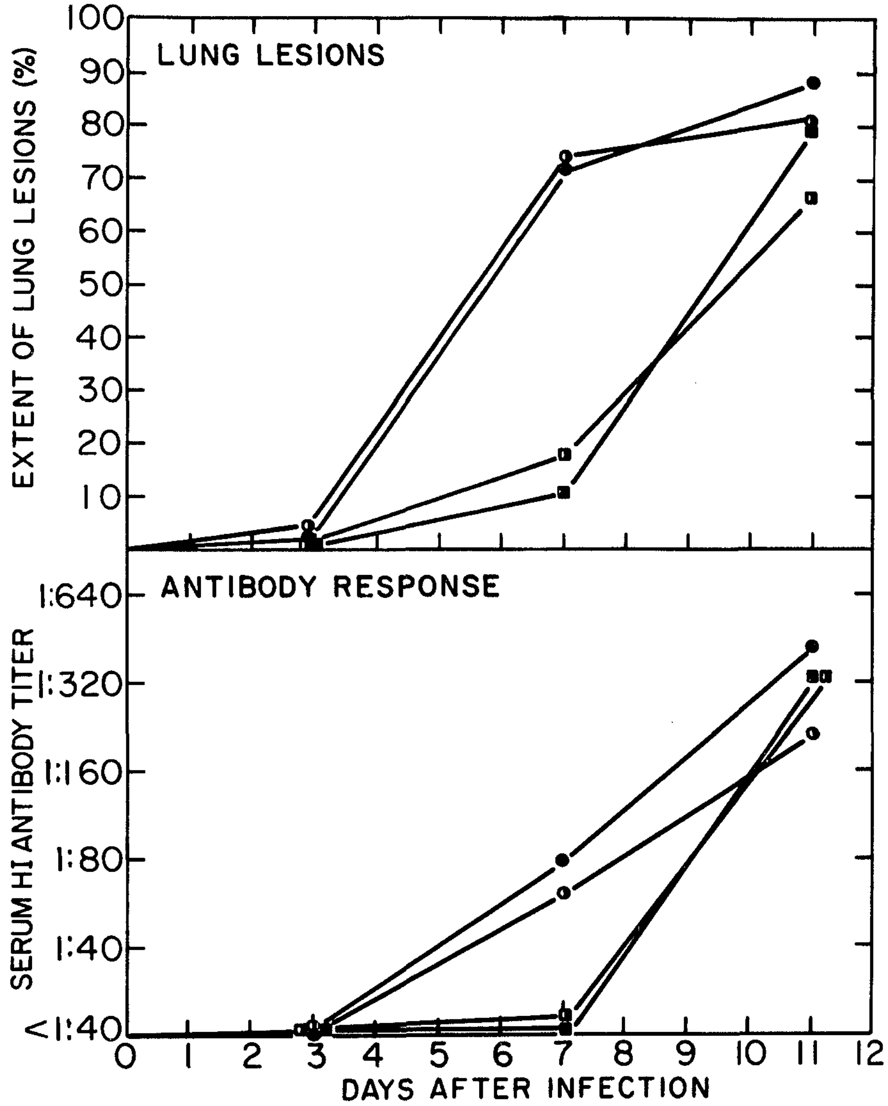

FIGURE 10. Effects of adoptive transfer of primary immune spleen cells on the development of lung lesions (upper panel) and serum HI antibody responses (lower panel). Animals received 1.5 x 10^8 spleen cells one day after infection with PR8 virus. Control immune cell recipients (◐—◐); control normal cell recipients (•—•). Macrophage depleted mice inoculated i.p. with 50 mg. silica on the day of infection, immune cell recipients (◧—◧), normal cell recipients (▪—▪). Spleen cells were obtained 6 days after sensitization with PR8 virus. Values represent the mean of five mice/group.

In all these adoptive transfer experiments in which primary immune cells were employed an effect was observed in control recipients of immune cells as evidenced by a more rapid rate of clearance of virus than that observed in control recipients of normal cells. The transfer of primary immune spleen cells did not have similar effects on the rate of virus clearance in macrophage depleted mice. The possibility was raised that transfer of immune cells with greater cytotoxic activity might enhance the rate of virus clearance in macrophage depleted mice. Yap and Ada (1978a) had demonstrated that secondary immune spleen cells obtained after secondary stimulation *in vitro* had greater cytotoxic activity and were more effective in all transfer experiments. Hence it was decided that secondary immune spleen cells should be used in adoptive transfer experiments to determine whether their antiviral effects could compensate for the defect in virus clearance in macrophage depleted mice.

Transfer of secondary immune spleen cells.

BALB/c mice were sensitized with PR8 virus by i.p. inoculation of 0.2 ml of a virus suspension in saline with an HA titer of 1:1024. Five weeks later mice were immunized with an equivalent dose of a second virus, Japan 305. Two antigenically different viruses were used to eliminate the possibility of virus-antibody complexes and inhibition of evoking T cell responses. Five days after the second virus administration, 10^8 immune cells were transferred to Japan 305 virus infected mice one day after infection. Table 18 shows the cytotoxic activity in spleen cells from littermates of mice used as immune cell donors in this experiment. Secondary immune spleen cells were more potent cytotoxic effectors than primary immune spleen cells. In this experiment the specific immune release effected by secondary immune cells was 45%, which was higher than the cytotoxic activity in the spleen cells used in primary immune cell transfer experiments which was 28% (Table 15). Again, in this experiment consider-

TABLE 18

CYTOTOXIC ACTIVITY IN SECONDARY IMMUNE SPLEEN CELLS FROM LITTERMATES OF DONORS OF SPLEEN CELLS 5 DAYS AFTER SECONDARY SENSITIZATION

		Specific ^{51}Cr release from P815 targets (%)		
Spleen cell source[a]	Effector:Target ratio[b]	A/Japan/305 (H2N2)	A/PR/8 (H0N1)	Uninfected
Secondary immune	100:1	45.5[c]	24.1[c]	−1.1
	50:1	40.2[c]	14.5[d]	0.1
	25:1	19.8[c]	9.7	−0.6
Normal	100:1	9.8	2.0	0.0
	50:1	5.5	1.8	0.0
	25:1	3.0	−2.0	0.0

[a] Littermates of mice used for adoptive transfer in Table 19 were primed with PR8 virus, 5 weeks later mice received a dose of A/Japan/305 virus and spleens were removed 5 days later.

[b] 1×10^4 target cells/well.

[c] Significantly greater release than from uninfected targets or normal cells ($P < 0.001$).

[d] Significantly greater release than from uninfected targets or normal cells ($P < 0.01$).

Spontaneous release from A/Japan/305 infected cells, A/PR/8 infected cells and uninfected cells was 15.9%, 14.1%, and 14.8%, respectively.

able cross-reactive lysis of different influenza A virus infected targets was observed. This infection proved to be lethal in control mice. Mice began to die a few days after infection, preventing analysis of virus titers and the development of lung lesions late in infection. The virulence of this infection is demonstrated by the results shown in Table 19. In other experiments, virus titers had declined appreciably by 8 days after infection, but in the present experiment no evidence of virus clearance was observed even in control recipients of immune cells. Despite the absence of any differences in virus titers 8 days after infection the transfer of immune cells protected normal recipients from subsequently succumbing to the lethal infection. Conversely, silica treated recipients of immune cells began to die sooner than either control or silica treated recipients of normal cells. Perhaps this was due to the suppressive effects of immune cell transfer which compounded the immunodepression brought on by silica treatment. The results shown in Figure 11 illustrate that mice which were silica treated and received normal or immune cells, as well as control recipients of non-immune cells did not survive beyond day 9 after infection. In contrast, all normal mice which received immune spleen cells survived until day 12 after infection when the experiment was terminated. The results summarized in Figure 11 were derived from tabulating the mortality rate of 15-18 mice per experimental group.

The results of assays of cytotoxic activity of mononuclear cells 8 days after infection with Japan 305 virus are shown in Table 20. The highest level of cytotoxic activity was observed in the lungs of normal mice which were the recipients of immune cells. As noted above this was the only group which subsequently survived the infection. Although cytotoxic activity in the lungs of silica treated recipients of immune cells was slightly higher than that observed in the lungs of normal or silica treated recipients of non immune cells, no protective effect was demonstrable.

In this experiment, a correlation between numbers of cells recovered from the

TABLE 19

EFFECTS OF SECONDARY IMMUNE SPLEEN CELL TRANSFER ONE DAY AFTER JAPAN 305 VIRUS INFECTION ON PULMONARY VIRUS TITERS

Days after infection	Treatment of recipient	Donor cells[a] transferred	Pulmonary virus titers EID_{50} Log_{10}
5	Silica	– –	7.6 ± 0.4
	Control	– –	7.7 ± 0.4
7	Silica	Immune	7.1 ± 0.5
		Normal	6.9 ± 0.3
	Control	Immune	6.2 ± 0.4
		Normal	6.7 ± 0.2
8	Silica	Immune	6.6 ± 0.6
		Normal	7.6 ± 0.5
	Control	Immune	5.6 ± 0.6[b]
		Normal	7.1 ± 0.3

[a] Secondary immune spleen cells obtained from mice 5 days after i.p. sensitization with Japan 305 virus; an initial dose of PR8 virus was administered 5 weeks before the second booster dose.

[b] Significantly lower than silica and control normal cell recipients ($P < 0.01$).

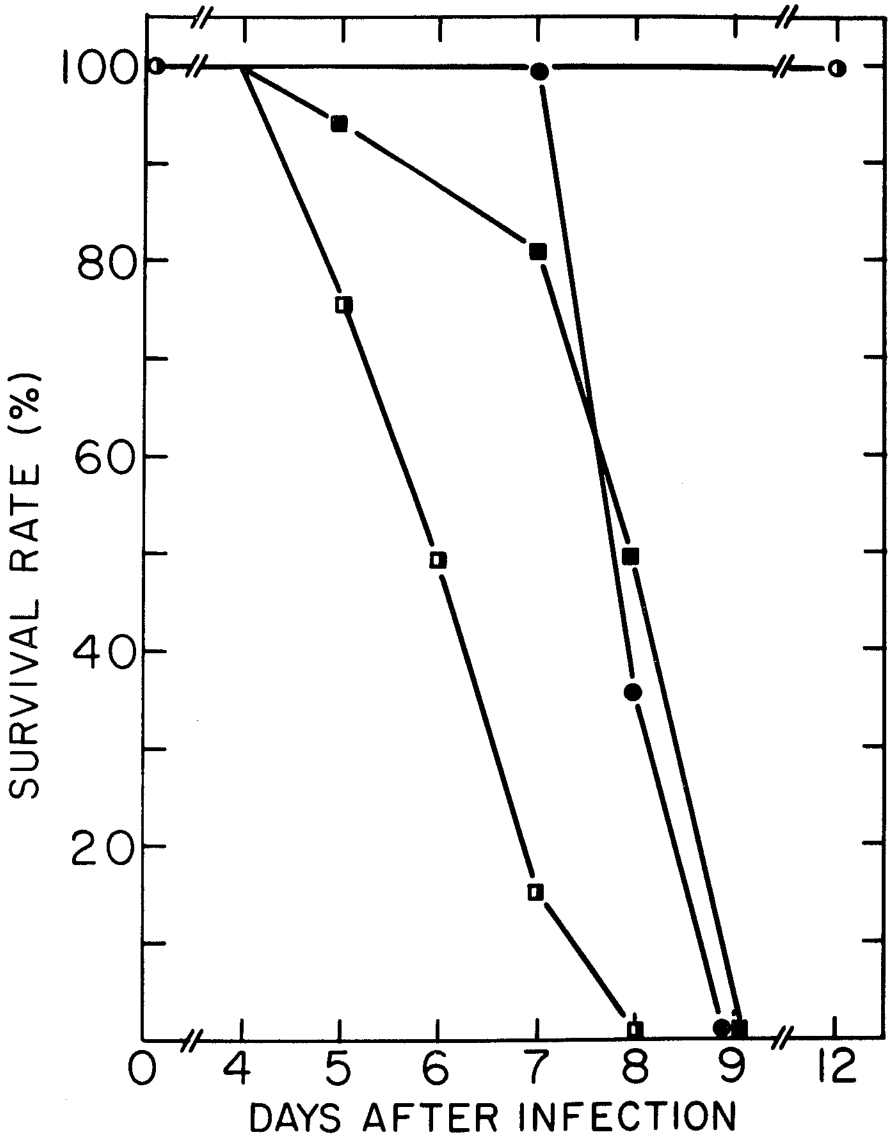

FIGURE 11. Survival of intact or macrophage depleted recipients of secondary immune spleen cells one day after infection with A/Japan/305/57 (H2N2) virus. Control immune cell recipients (◐—◐), control normal cell recipients (●—●). Macrophage depleted immune cell recipients (◧—◧), and normal cell recipients (■—■). Animals received 10^8 secondary immune spleen cells obtained from mice restimulated with Japan 305 virus 5 days previously. Primary parenteral sensitization with PR8 virus was conducted 5 weeks earlier. Values represent survival rate of 15-18 mice/group.

TABLE 20

CYTOTOXIC ACTIVITY IN LUNG MONONUCLEAR CELLS FROM RECIPIENTS OF NORMAL OR SECONDARY IMMUNE SPLEEN CELLS 8 DAYS AFTER INFECTION WITH A/JAPAN/305 (H2N2) VIRUS

Treatment group	Cells transferred[b]	% Specific ^{51}Cr release: Jap/305 infected targets	% Specific ^{51}Cr release: Uninfected targets
Silica[a]	Immune	14.4[c]	0.0
	Normal	4.9	0.0
Control	Immune	77.5[d]	1.2
	Normal	4.2	0.0
Uninfected	– –	3.5	0.8

[a] 50 mg. i.p. on the day of infection.

[b] 1 x 10^8 cells transferred 5 days after the second dose of virus (Japan/305) administered 5 weeks after initial sensitization with PR8 virus.

[c] Significantly greater release than all groups except control-immune cell recipients ($P < 0.01$).

[d] Significantly greater release than all other groups ($P < 0.001$).

TABLE 21

AVERAGE NUMBER OF LYMPHOCYTES RECOVERED FROM THE LUNGS OF SECONDARY IMMUNE SPLEEN CELL OR NORMAL CELL RECIPIENTS 8 DAYS AFTER JAPAN/305 VIRUS INFECTION

Group	Cells received	# mice	Average number of lymphocytes recovered/ mouse lung X 10^5
Silica	Normal	4	1.8
	Immune	4	1.7
Control	Normal	4	6.0
	Immune	4	8.4

lungs and cytotoxic activity was observed. As demonstrated in Table 21, control immune cell recipients had the highest level of cytotoxic activity and the highest numbers of lymphocytes recoverable from their lungs 8 days after infection. Normal recipients of non-immune cells did not have as many lymphocytes present in their lungs nor was any cytotoxic activity detectable on day 8. Despite the lower number of lymphocytes in the lungs of silica treated immune cell recipients, slightly higher levels of cytotoxic activity were present in the lungs of this group than in normal recipients of non-immune cells. Silica treated recipients of non-immune cells had slightly lower numbers of lymphocytes than silica recipients of immune cells and no cytotoxic activity was detectable in their lungs at the time of assay.

Another experiment was conducted with secondary immune spleen cells to obtain data on virus titers later in infection with PR8 virus instead of Japan 305 virus as the challenge virus.

Mice were sensitized with 0.2 ml of a Japan 305 virus suspension with an HA titer of 1:1024. Five weeks later an equivalent dose of PR8 virus was administered. Spleen cells were recovered five days later and inoculated i.p. into control and silica treated mice the day after infection with PR8 virus.

As shown in Table 22, transfer of secondary immune spleen cells one day after infection accelerated the rate of virus clearance as early as day 7 after infection in macrophage depleted recipients of immune cells but only slight differences were observed in normal recipients of immune and non-immune cells. On day 10 after infection neither group of normal mice had any detectable virus in the lungs. Hence in this experiment it was not possible to measure the effects of immune cells in normal mice. Silica treated immune cell recipients cleared virus more rapidly than silica treated recipients of normal cells. However, they did not clear virus as rapidly as either group of mice with intact macrophage function. Therefore, in this experiment, transfer of

TABLE 22

EFFECTS OF SECONDARY IMMUNE SPLEEN CELL TRANSFER ONE DAY AFTER PR8 VIRUS INFECTION ON PULMONARY VIRUS TITERS

Days after infection	Treatment group	Donor cells[a] transferred	Pulmonary virus titer EID_{50} Log_{10}
4	Silica	Immune	6.7 ± 0.2
		Normal	6.8 ± 0.5
	Control	Immune	6.3 ± 0.6
		Normal	7.0 ± 0.4
7	Silica	Immune	6.2 ± 0.5
		Normal	7.3 ± 0.4
	Control	Immune	5.5 ± 0.7
		Normal	6.0 ± 0.5
10	Silica	Immune	4.5 ± 1.1[b]
		Normal	6.6 ± 0.2
	Control	Immune	0[c]
		Normal	0[c]

[a] Secondary immune cells obtained 5 days after i.p. inoculation of PR8 virus; mice were initially sensitized with Japan 305 virus five weeks before the second dose.

[b] Significantly lower than virus titers in silica recipients of normal cells on day 10 ($P < 0.01$).

[c] Significantly lower than both silica groups on day 10 ($P < 0.001$).

TABLE 23

CYTOTOXIC ACTIVITY IN SECONDARY IMMUNE SPLEEN CELLS 5 DAYS AFTER SECONDARY SENSITIZATION

Spleen cells source[a]	Effector:Target ratio[b]	% Specific ^{51}Cr release PR8 infected targets	Japan 305 infected targets	Uninfected targets
Secondary immune	100:1	64.0[c]	48.4[c]	4.0
	50:1	65.0[c]	42.6[c]	4.2
	25:1	25.2[c]	15.2[d]	2.8
Normal	100:1	11.5[d]	0.4	–4.3
	50:1	7.2	0.3	1.4
	25:1	–0.9	–0.5	0.5

[a] BALB/c mice were primed with Japan/305 (H2N2) virus; 5 weeks later mice received a dose of PR8 (HON1) virus, spleen cells were obtained 5 days after the last dose of virus.

[b] 2 x 10^4 target cells/well.

[c] Significantly greater release than uninfected targets or release by normal lymphocytes ($P < 0.001$).

[d] Significantly greater release than uninfected targets.

secondary immune spleen cells partially compensated for the defect in virus clearance in macrophage depleted animals. As shown in Table 23, cytotoxic activity in secondary immune spleen cells used in a similar experiment was much higher than primary immune spleen cells. Once again, a cross-reactive lysis is observed between heterologous influenza A virus infected targets.

In this same adoptive transfer experiment, cytotoxic activity of lung mononuclear cells was measured in recipients 9 days after PR8 virus infection. The highest level of activity was detected in lung cells from normal recipients of immune cells although significant levels of cytotoxic activity was observed with lung cells from all groups of mice as illustrated in Table 24. Although virus clearance was more rapid in silica treated immune cell recipients, there was no difference in the level of activity in the lungs of these animals when compared with the activity in silica treated recipients of normal cells.

In comparison to the first adoptive transfer experiment, cytotoxic activity in this experiment appeared to be even more closely associated with the numbers of lymphocytes accumulating in the lungs on day 9 after infection as shown in Table 25. The greater number of lymphocytes observed in the lungs of control mice was associated with higher levels of cytotoxic activity in the lungs of these animals. Similar results are obtained when the comparison between the two silica treated groups were obtained. It should be emphasized that in silica treated animals, regardless of the source of cells received, the cytotoxic activity and numbers of lymphocytes in the lungs were lower than normal mice.

Figure 12 demonstrates the development of lung lesions in these mice during the experiment. The characteristic delays in development of lesions were observed in macrophage depleted mice. Once again, the transfer of immune cells appeared to suppress the development of lung lesions early after infection in control animals.

TABLE 24

CYTOTOXIC ACTIVITY IN LUNG MONONUCLEAR CELLS FROM RECIPIENTS OF NORMAL OR SECONDARY IMMUNE SPLEEN CELLS 9 DAYS AFTER INFECTION WITH PR8 VIRUS

Group[a]	% Specific ^{51}Cr release[b] PR8 infected targets	% Specific ^{51}Cr release[b] Uninfected targets
Control – sensitized	59.5 ± 3.0[c]	1.8 ± 1.4
Control – normal	32.4 ± 3.5[d]	2.3 ± 2.3
Silica – sensitized	20.8 ± 1.4[e]	1.1 ± 1.3
Silica – normal	16.5 ± 1.4[e]	1.8 ± 1.3
Normal	3.2 ± 1.3	2.0 ± 0.1

[a] Animals were infected with A/PR/8/34 (H0N1) virus, one day after infection mice received 10^8 secondary immune or normal syngeneic splenic lymphocytes. Lung lymphocytes were assayed for cytotoxic activity 9 days after infection at a 50:1 effector:target ratio.

[b] $$\frac{\#\text{counts released} - \text{spontaneous release}}{\text{total releasable} - \text{spontaneous release}} \times 100$$

[c] Significantly greater release than all other groups ($P < 0.001$).

[d] Significantly greater release than silica-sensitized ($P < 0.02$) or silica-normal ($P < 0.01$) groups.

[e] Significantly greater release than uninfected cells or release mediated by normal lymphocytes ($P < 0.01$).

TABLE 25

AVERAGE NUMBER OF LYMPHOCYTES RECOVERED FROM THE LUNGS OF SECONDARY IMMUNE SPLEEN CELL OR NORMAL CELL RECIPIENTS 9 DAYS AFTER INFECTION WITH PR8 VIRUS

Group	Cells received	# mice	Average number of lymphocytes recovered/ mouse lung X 10^5
Silica	Normal	4	2.6
	Immune	4	3.0
Control	Normal	4	6.4
	Immune	4	12.0

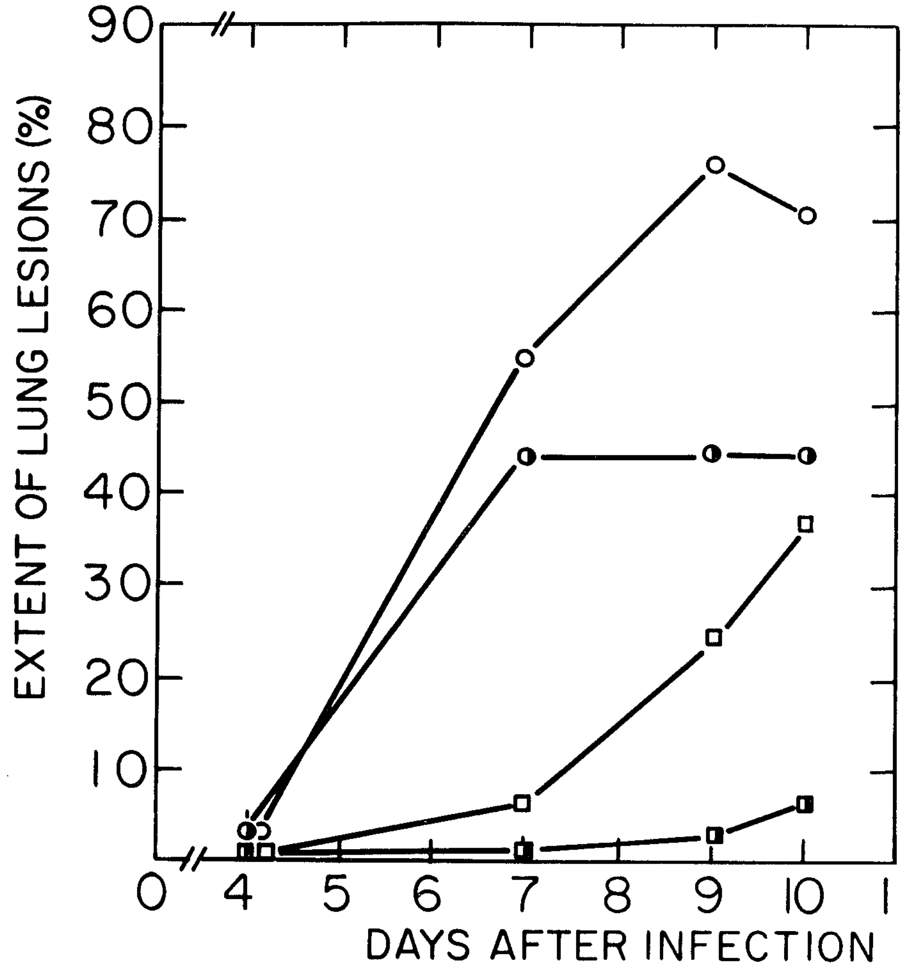

FIGURE 12. Effects of adoptive transfer of secondary immune spleen cells on the development of lung lesions. Animals received 10^8 spleen cells one day after infection with PR8 virus. Control immune cell recipients (◐—◐), control normal cell recipients (•—•). Macrophage depleted immune cell recipients (◧—◧), and normal cell recipients (▪—▪). Spleen cells were obtained 5 days after restimulation with PR8, primary sensitization was conducted 5 weeks earlier with A/Japan/305/57 (H2N2) virus. Values represent the mean of five mice/group.

These results confirmed the phenomenon of immune cell suppression of lung lesions or antibody responses described in earlier experiments and may explain the decreased survival rate of silica treated immune cell recipients (Figure 11) whose immune responses are already suppressed by silica treatment.

E. ANTIBODY FORMING CELLS IN THE LUNGS

In previous experiments it had been demonstrated that serum antibody responses were not closely associated with either the clearance of virus or the development of pulmonary consolidation (Rosenberg and Schulman, 1978; Schulman et al., 1977). However, it was not known whether antibody forming cells accumulated in the lungs during the period when virus clearance and lung lesion development were apparent. An antibody forming cell assay (AFC) performed in this laboratory using mediastinal lymph node cells demonstrated that IgM, IgG and IgA secreting cells appeared as early as day 3 after infection. After a decline, IgM forming cells appeared to reach maximal levels on day 7 after infection when approximately 100 cells/10^6 lymph node cells secreted this class of immunoglobulin. IgG and IgA secreting cells increased to a maximum on day 11 after infection when approximately 100 AFC/10^6 lymph node cells appeared. At this time IgM, IgG, and IgA antibody forming cells appeared to be present in equivalent numbers. The number of antibody forming cells which secreted IgM, IgG, or IgA varied between 75-100 AFC/10^6 lymph node cells until day 18 when the experiment was terminated (Reiss, 1978).

Lung mononuclear cells were collected from both control mice and mice treated with silica on the day of infection and days 5, 7, 9, and 14 after infection and used in an AFC assay with SRBC coated with PR8 virus. In this experiment no AFC were detected in the lung cells of either silica or control mice earlier than day 14 of infection. On day 14 small numbers of cells secreting IgM, IgA, or IgG were detected in approximately equal numbers in both control and macrophage depleted mice as shown in Table 26. The absolute number of AFC was very low compared to the number of AFC in mediastinal lymph nodes (about 100-fold less). Nevertheless, these results provided evidence that AFC were not detected in the lungs of either intact or macrophage

TABLE 26

NUMBER OF IgM, IgG AND IgA ANTIBODY FORMING CELLS IN THE LUNG MONONUCLEAR CELL POPULATION 14 DAYS AFTER INFECTION WITH PR8 VIRUS

Group	Class of antibody secreted	Number of AFC X 10^2 per mouse lung
Control	IgM	1.5
	IgG	1.5
	IgA	2.4
Silica	IgM	1.7
	IgG	1.5
	IgA	2.0

depleted mice until after virus had been cleared from the lungs and lung pathology had developed. Therefore, it was concluded that cells capable of producing antibody in the lung did not participate significantly in the recovery from infection with influenza viruses.

F. KINETICS OF CELLULAR INFILTRATION IN THE LUNGS OF PR8 VIRUS INFECTED MICE

The number of cells recovered from the lungs of PR8 virus infected BALB/c mice were enumerated in all assays in which lung mononuclear cells were used as effector cells in either a ^{51}Cr release assay or an AFC assay. Although the absolute numbers of cells varied from one experiment, to another, an influx of both lymphocytes and monocytes into the lung was consistently observed one week after infection in normal infected mice. This sudden increase in the numbers of cells recovered from the lungs of normal infected mice was not observed in silica treated mice. Figure 13 illustrates the mean number of lymphocytes recovered from the lungs of PR8 virus infected mice from several similar experiments. It can be seen that the increased number of lymphocytes present in the lungs of normal mice coincided with the appearance of cytotoxic responses in these animals. Hence, on day 8 when the number of lymphocytes was highest, significant levels of cytotoxic activity were observed in control mice. However, fewer lymphocytes and lower levels of cytotoxic activity were detected in the lungs of silica treated mice than in the lungs of control animals. Although the numbers of cells recovered from the lung was maximum on day 8, cytotoxic activity was not maximum until day 11 by which time the numbers of lymphocytes in the lung were decreasing.

Figure 14 illustrates that lymphocytes and monocytes accumulated in the lungs in approximately equal numbers and that the infiltration to the lung coincided with the appearance of gross lung lesions. Similarly, the decreased numbers of mononuclear cells infiltrating the lungs of silica treated mice correlated with less extensive lung consolidation observed in these animals.

Infiltration of mononuclear cells in the lungs of mice infected with influenza

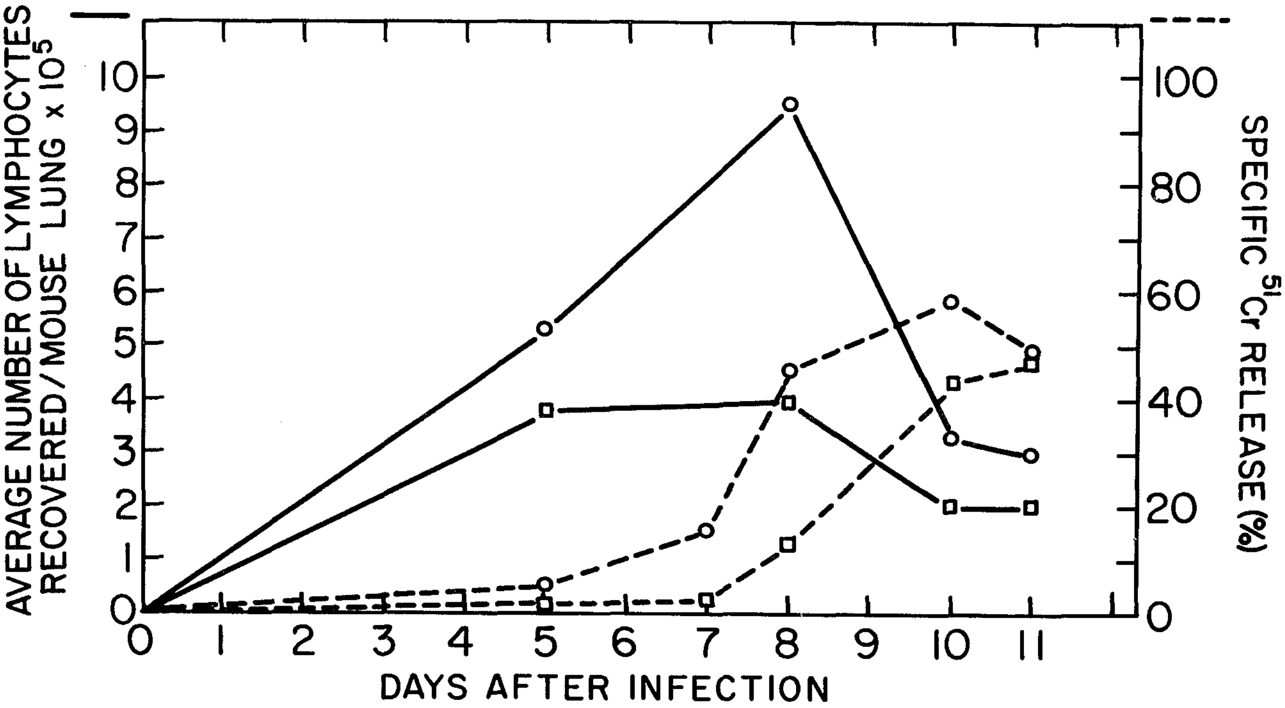

FIGURE 13. Comparison of the accumulation of lymphocytes and cytotoxic activity in the lungs of intact (o—o) and macrophage depleted mice (□—□) treated with 50 mg of silica on the day of PR8 infection. Values represent average number of lymphocytes recovered from the lungs on a given day after infection in several experiments. Cytotoxic activity represents the average response in lung mononuclear cells in control and macrophage depleted animals.

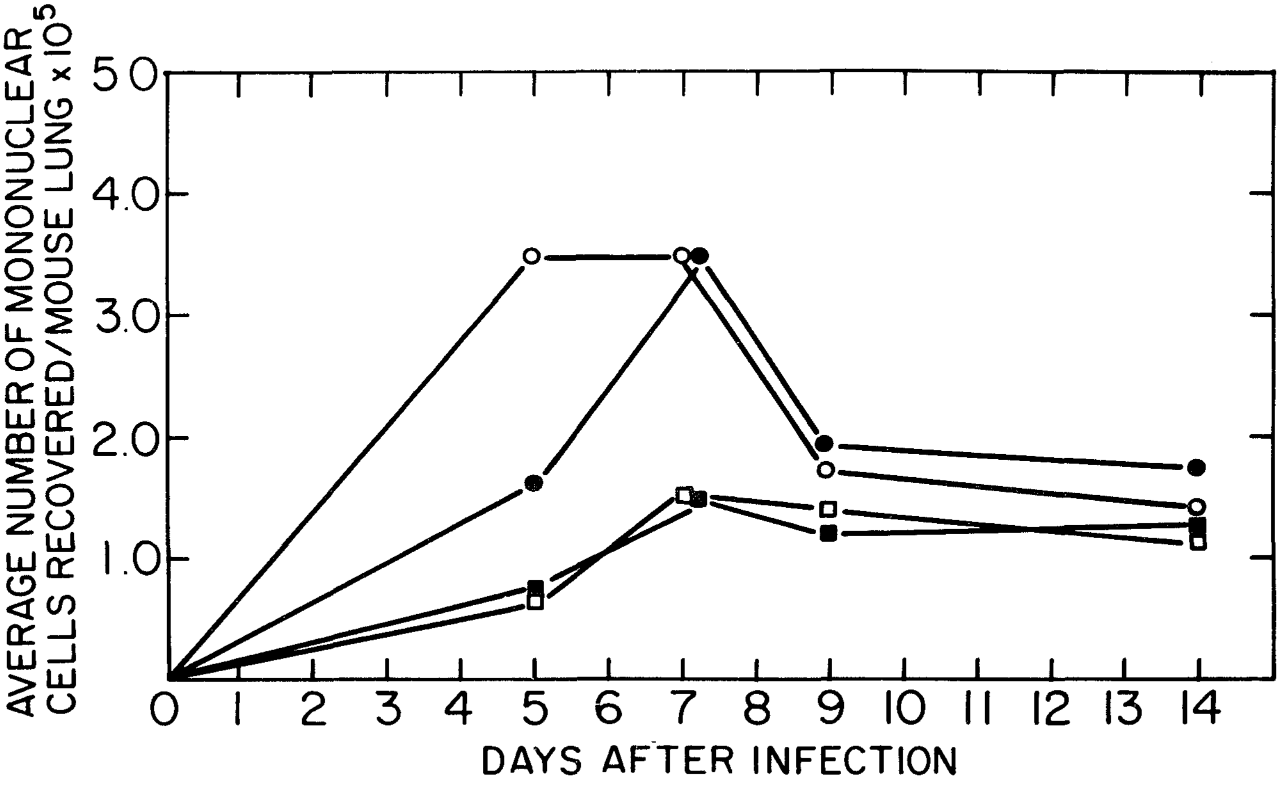

FIGURE 14. Comparison of the kinetics of infiltration of lymphocytes and monocytes in the lungs of intact and macrophage depleted mice. Average number of lymphocytes (o—o) or monocytes (•—•) from control mice. Average number of lymphocytes (□—□) or monocytes (▪—▪) from mice treated with silica on the day of PR8 virus infection. All values represent the cells obtained at intervals after infection during the course of one experiment.

virus was also examined after ATS treatment. Mice were treated with 0.5 ml of a commercial source of rabbit antiserum to murine thymocytes or with normal rabbit serum on days 1 and 3 after infection and with 0.5 ml of a 1:2 dilution of ATS or NRS on day 6 after infection. Cytotoxic activity in lung mononuclear cells was measured on day 9 after infection. ATS treatment abolished cytotoxic activity in both unseparated cell populations and adherent cell populations when tested against PR8 or Japan 305 virus infected target cells (Table 27). The effects of ATS treatment on the activity of adherent cells suggest that a T cell functional activity is required for the generation of adherent cells with cytotoxic activity, however the mechanism by which ATS inhibited adherent cell activity is unclear.

Other parameters were also measured in this experiment. The number of lymphocytes recovered from the lungs of ATS treated mice was 1.2×10^5 per mouse as opposed to 9×10^5 per mouse in the NRS treated group. Lung pathology in the ATS treated mice averaged 45% and in the NRS group the extent of pathology was 65%. Thus, the correlation between numbers of cells, cytotoxic activity and pulmonary pathology was evident in this experiment and others described in this manuscript.

TABLE 27

EFFECTS OF ANTI-THYMOCYTE SERUM ON CYTOTOXIC ACTIVITY IN LUNG MONONUCLEAR CELLS

	% ^{51}Cr release[b]		
Treatment group[a]	PR8 infected targets	Jap infected targets	Uninfected targets
Normal rabbit serum	31.7 ± 4.1[c]	25.0 ± 3.6[c]	0.4 ± 0.4
Anti-thymocyte serum	−1.1 ± 3.3	−2.0 ± 3.3	−2.0 ± 2.9
Normal rabbit serum adherent cells	14.2 ± 3.9[d]	6.1 ± 2.1	0.0 ± 0.6
Anti-thymocyte serum adherent cells	−2.0 ± 2.8	−2.3 ± 2.2	n.d.
Normal uninfected	2.0 ± 2.8	3.0 ± 3.6	−2.6 ± 0.2

[a] Animals were treated with 0.5 ml normal rabbit serum or rabbit anti-mouse thymocyte serum on days 1 and 3 after infection and 0.5 ml of a 1:2 dilution on day 6 after infection. Lung lymphocytes were obtained on day 9 after infection with A/PR/8/34 (H0N1) virus. A 50:1 effector:target ratio was used in the assay.

[b] $$\frac{\text{\#counts released} - \text{spontaneous release}}{\text{total releasable counts} - \text{spontaneous release}} \times 100$$

[c] Significantly greater release than all other groups ($P < 0.001$).

[d] Significantly greater release than with normal lymphocytes ($P < 0.001$) or with Jap infected targets ($P < 0.05$).

[e] Spontaneous release with PR8 infected, Jap infected, or uninfected targets was 10.8%, 11.1%, and 10.2%, respectively. Release from uninfected targets did not exceed ±2.6% in any case.

G. SUMMARY OF MACROPHAGE DEPLETION EXPERIMENTS: IN VIVO OBSERVATIONS

Collectively, the major observations made in the experiments described in sections B through F can be summarized as follows:

I. Virus clearance and pulmonary consolidation can be dissociated from antibody responses in silica treated influenza virus infected mice as illustrated by delays in the development of lung lesions and virus clearance in mice treated with silica 3 days or later after infection although serum antibody responses measured in HI tests were normal.

II. The kinetics of interferon responses in silica treated and normal mice do not correlate with virus clearance in the two groups. Equivalent interferon titers later in infection during the recovery period are not associated with virus clearance in silica treated mice in which higher levels of virus are detectable than in control animals at the same time.

III. Cell mediated immune responses in which cytotoxic T cells may be an important component contribute significantly to the recovery process shown by:

    A. temporal association between virus clearance and the development of cytotoxic responses in the lung mediated, for the most part, by cells with characteristic of cytotoxic T cells, and the accumulation of lymphocytes in the lungs.

    B.. the protective effects of adoptive transfer of immune spleen cells:

        1. Adoptive transfer of primary immune cells accelerated virus clearance in normal but not macrophage depleted mice.

        2. Secondary immune spleen cells had greater cytotoxic activity than primary immune spleen cells and accelerated virus clearance in macro-

phage depleted mice. However, these mice still did not clear virus as rapidly as normal animals.

C. The inhibition of cytotoxic activity in lung mononuclear cell populations by treatment with anti-thymocyte serum.

IV. Functional macrophages are essential during influenza virus infection:

A. as accessory cells the absence of which in silica treated mice is associated with a delay in the appearance of antibody and cytotoxic responses.

B. as cells which may participate in the development of lung lesions since the accumulation of monocytes in the lung associated with the development of pulmonary consolidation are inhibited in macrophage depleted mice.

C. possibly as an effector cell which contributes directly to virus clearance:

1. since cytotoxic activity in the lung and virus titers in delayed silica treated mice can be dissociated 8 and 11 days after infection.
2. because immune cell transfer, at best, partially compensates for the defect in virus clearance in macrophage depleted mice.
3. because of the simultaneous accumulation of monocytes in the lungs and the decline in virus titers in control mice.
4. because cytotoxic effector cells with the characteristics of macrophages make a contribution to cytotoxic activity although the activity is not observed in adherent cells obtained from ATS treated mice.

X. DISCUSSION

The results presented here clearly demonstrate that influenza virus infection of macrophages is abortive. However, macrophages continue to express virus antigens for a period of time after infection *in vitro*. Presentation of viral antigens by macrophages are required for the development of immune responses to infection. This critical role of macrophages is more readily demonstrated in the *in vivo* infection of macrophage depleted mice. In addition to this accessory function, macrophages may be important mediators of virus clearance and may also contribute to lung lesions. Collaboration between macrophages and immune T cells appears necessary for the recovery from influenza virus infection. The implication of these studies are discussed fully in the following pages.

Interactions in vitro.

Infection of macrophages with influenza viruses A/PR/8 and A/NWS viruses resulted in the expression of both virus glycoproteins, hemagglutinin and neuraminidase, on the macrophage membrane as shown in Figures 1-3. Earlier observations by Boand (1957) and Hanson (1957) and a later report by Shayegani (1974) verified the presence of viral antigens on the surface of macrophages by hemadsorption of erythrocytes and immunofluorescent techniques. On the basis of their results, these investigators concluded that influenza virus replicated in macrophages. Yet, no attempt was made to determine whether infectious virus progeny was produced.

In a series of experiments it was determined that the HA present in the infected macrophage membranes represented newly synthesized antigen (Figure 3). Despite the *de novo* production of viral antigens the replication of PR8 virus in macrophages is abortive. This conclusion is based on a variety of experimental results. The failure to detect recombinant progeny above background levels, the failure to detect a greater number of infectious foci above background levels in infectious center assays, and the inability to show an increase in virus titers in supernatants of infected macrophage cultures are consistent with the conclusion that an incomplete cycle of replication occurs. The one exception to the interaction is the ability of the hepatotropic M-TUR (A/Turkey/England/63) virus to replicate productively in murine macrophages (Virelizier et al., 1979; Lindenmann et al., 1978; Haller and Lindenmann, 1974). Even with this virus, sequential passage in peritoneal fluid was necessary before it could replicate in macrophages. Replication of all other influenza A virus strains tested so far is nonproductive in both peritoneal and alveolar macrophages (Wells et al., 1978). In fact, Nugent and Pesanti (1978) demonstrated that although PR8 virus hemagglutinin is present on the surface of murine alveolar macrophages, infection is abortive, as is PR8 infection of peritoneal macrophages.

There is insufficient evidence as to where the defect in replication occurs. But the synthesis of HA and NA proteins suggests that the block in replication occurs late in the replicative cycle at the stage of maturation or release of the virus.

It should be noted that not all macrophages infected *in vitro* gave evidence of infection when analyzed by hemadsorption assays. Even after high M.O.I. a maximum of 80% of adherent cells were positive for hemadsorption. Whether or not this reflects heterogeneity of subpopulations of macrophages is not clear. Not all macrophages are adherent and different cells may be in different stages of activation when levels of enzyme released are measured. The ability to support abortive infection may be related to any of these functional properties.

It should be emphasized that presentation of viral antigens on the surface of macrophages following infection involves events which are different from those which take place following ingestion and processing of nonreplicating antigens by macrophages and that these differences may have an important bearing on subsequent immune responses. Formalin inactivated virus has been shown repeatedly to elicit antibody responses as well as untreated virus and has been used routinely in influenza virus vaccines.

However, Reiss (1978) demonstrated that although formalin inactivated virus induced antibody responses equivalent to those produced by untreated virus, formalin inactivated virus did not elicit primary cytotoxic T cell responses in unprimed mice or secondary cytotoxic responses in primed mice. In contrast, UV inactivated virus evoked both antibody and cytotoxic responses (Reiss, 1978) equivalent to those produced by untreated virus. These results suggested that infection and antigen presentation by appropriate stimulator cells are required for immune responses by some T cell populations.

Perhaps the processing of UV inactivated virus by macrophages results in the presentation of viral antigens in an immunogenic form which stimulate helper T cells. The absence of a cytotoxic response to the formalin treated virus suggests that antigen presentation by macrophages following this form of immunization is poorly immunogenic. In contrast, UV inactivated virus is capable of abortive infection as evidenced by expression of viral glycoproteins on infected cell surfaces *in vitro* (Reiss, 1978). The property of the UV inactivated virus may also be expressed *in vivo* leading to the expression of viral antigens and H-2 antigens on appropriate macrophages. The inability of formalin-inactivated virus to undergo any replication may result in inefficient or inappropriate presentation of antigen which fails to stimulate cytotoxic responses. The absence of any increase in hemadsorption by macrophages after exposure to formalin treated virus (Figure 3) may also indicate that in this form, virus antigens may undergo more rapid clearance and intracellular degradation.

The detection of infectious foci on susceptible MDCK monolayers demonstrated that virus remained attached to macrophages without being internalized for a period of time after infection. The use of trypsin (Table 2) or receptor destroying enzyme to alter virus receptors on the cell surface proved to be ineffective in the removal of virus. Yet the addition of antibody to infected macrophages before transfer to monolayers neutralized input virus and prevented formation of infectious foci. In these experiments, infected macrophages were added to MDCK monolayers immediately after antiserum treatment. It is possible that the prevention of plaque formation was effected by phagocytosis and intracellular degradation of virus as well as virus neutralized by antibody. Phagocytosis has been shown to be an important means of virus clearance with a number of other viruses (reviewed in Silverstein, 1970). Phagocytosis of viruses like herpes (Johnson, 1964; Stevens and Cook, 1971; Hirsch et al., 1970; Mogenson and Andersen, 1978) and ectromelia (Blanden, 1971b) appears to be a major factor in limiting infection and allowing recovery of the host. Antibody coating of of vaccinia and other viruses, which are normally taken into the cytoplasm of the cell where they may replicate, are diverted instead into phagolysosomes where they are degraded (Silverstein, 1970). Opsonization of influenza virus may also be an important defense during *in vivo* influenza virus infection. Perhaps antibody bound to Fc receptors of macrophages facilitated phagocytosis and prevented virus from eluting from the macrophage surface and initiating plaques on the indicator MDCK cells. Opsonization will be addressed later in the discussion.

Role of macrophages in recovery from infection.

The extensive use of silica in other murine models has shown its potency and selectivity in the impairment of macrophage function (Allison et al., 1966). Intraperitoneal doses of 50 mg and intravenous doses of 3 mg have been used to study macrophage functional activities in infections with HSV (Zisman et al., 1970), yellow fever virus (Zisman et al., 1971), coxsackie B-3 virus (Rager-Zisman and Allison, 1973), murine cytomegalovirus (Selgrade and Osborn, 1974), Friend leukemia virus (Larson et al., 1972b; Wirth et al., 1976), rabies virus (Turner and Ballard, 1976), lactic dehydrogenase virus (du Buy, 1975), and M-TUR influenza virus (Haller et al., 1976) as well as influenza A/PR/8/34 (H0N1) (Rosenberg and Schulman, 1978). Although some unresolved questions were raised regarding the effects of silica, in recognition of its repeated use in other models it was utilized in these studies to delineate the role of macrophages in resistance to influenza virus infections. A second anti-macrophage reagent, antiserum raised in rabbits by immunization with peritoneal macrophages, anti-macrophage serum (AMS), was characterized as described in earlier studies (Unanue, 1968; Gallily, 1971).

Both silica and AMS depressed phagocytic uptake of latex beads by peritoneal macrophages, and silica impaired phagocytic uptake by alveolar macrophages (Tables 3 and 4). It is possible that the cells in the peritoneal cavity may be different in phagocytic activity in silica or AMS treated mice from the cells in control animals. Alternatively, the cells in the peritoneum of macrophage depleted and infected mice may not be the cells which affect the course of the disease. However, the two reagents produced similar effects in influenza virus infected mice as evidenced by depressed immene responses and failure to clear virus from the lungs (Figures 4, 6, 7, 9, 10). The fact that 2 different reagents acting on macrophages had similar effects on at least one parameter of macrophage function and identical effects on virus infection in mice, provided reassuring evidence that the *in vivo* effects were related specifically to macrophage depression. Whether the altered infection observed in macrophage depleted mice was a consequence of depressed phagocytic activity of the macrophages and/or defects in specific immune responses, became the focus of much of this work.

Correlations of virus titers and lung lesions, with induction of various components of the immune response in macrophage depleted mice, permitted the analysis of defense mechanisms which participate significantly in the recovery process.

PR8 virus infection in mice treated with silica on the day of infection or treatment with AMS on day 0 and day 3 of infection was marked by lower serum HI antibody titers (Figures 4 and 6), less extensive lung consolidation (Figures 4 and 6; Tables 5 and 8) delays in cytotoxic responses (Figures 5 and 6) and interferon responses in the lung (Figure 5) and elevated virus titers (Figures 4, 5, 6, and 9) compared to control animals. With all these effects, it became necessary to manipulate the basic model to determine the associations of these responses with virus clearance.

Schulman and his colleagues had demonstrated that depletion of T cells by ATS treatment beginning four days after infection permitted mice to mount an antibody response equivalent to that observed in normal animals. However, virus clearance was still delayed (Schulman et al., 1977). In an analogous treatment regimen, silica treatment was postponed until day 3 after infection. In these delayed silica treated mice serum HI antibody titers were equivalent to those of control mice throughout the infection (Figure 6) but the delays in virus clearance and the development of lung lesions was as marked as in animals in which macrophages were depleted on the day of infection (Figure 6).

In a separate experiment the effects of silica treatment on the third day of infection on serum antibody responses and cytotoxic responses were compared and it was noted that whereas antibody responses were normal (Figures 6 and 7), cytotoxic responses in lung lymphocytes were still delayed in appearance (Tables 7 and 8). These observations were pursued by experiments in which silica treatment was delayed until 5 or 7 days after infection. The results indicated that the cytotoxic responses on day 8 were only slightly reduced in animals injected with silica on day 5 and were equivalent to those of control animals or animals treated on day 7 after infection (Table 8).

Nevertheless, 10 and 12 days after infection pulmonary virus titers were higher in the silica treated groups. Thus functional macrophages are required for both humoral and cellular responses to influenza virus antigens *in vivo.* Silica treatment on the third day after infection provided enough opportunity for induction of a normal antibody response, but functionally intact macrophages were required for a longer period during infection for the development of cytotoxic immune responses. These results are in accord with numerous experiments which have demonstrated the requirement of macrophages for the induction of immune responses *in vitro* and also provide one of the few demonstrations of the requirement of functional macrophages for immune responses to virus infection *in vivo.*

Besides presentation of antigen to lymphocytes, the interactions between macrophages and lymphocytes in the induction and expression of immune responses also involve lymphoregulatory soluble factors which modulate responses in a positive manner by activation of T cells or in a negative mode by suppression of immune responses in an antigen specific or nonspecific way (reviewed in Persson et al., 1978; Unanue, 1978; reviewed in Allison, 1978). These factors are poorly defined as to biochemical and functional properties, but they may be involved in the manifestations which were observed in macrophage depleted mice.

Besides secretion of regulatory factors, it has been shown that macrophage depletion *in vitro* may also lead to induction of suppressor activity. Responses to the chemically defined synthetic antigen GAT, composed of L-glutamine, L-alanine, and L-tyrosine were dependent on the strain of mouse or guinea pig used in the study. F_1 hybrids produced by crosses between responders to GAT, Black-6 mice, and nonresponders to GAT, DBA/1 mice, make antibody responses to GAT. Spleen cells from the GAT sensitized F_1 mice cultured with antigen pulsed macrophages from Black-6 mice produce antibody. In contrast, exposure of the spleen cells to nonresponder macrophages bearing GAT determinants do not produce antibody (Benacerraf, 1978). Perhaps the absence of immune responses in silica treated animals is not only due to the inhibition of appropriate antigen bearing macrophage interaction with T cells, but also due to a suppressed state produced in the absence of macrophages and the virus antigen excess which macrophage depletion brings on.

Returning to the results of these experiments, it was apparent that in the presence of normal levels of antibody in delayed silica treated mice, virus titers remained at elevated levels (Figures 6 and 7) and pulmonary inflammation did not develop. Antibody responses do not appear to participate directly in either the elimination of virus from the lung or in the development of lung lesions. It is possible, however, that cytophilic antibody bound to Fc receptors on macrophage membranes may promote the uptake and eventual degradation of virus thereby eliminating it from the lungs. It is also possible that antibody on other cells with Fc receptors such as K cells may play a role by other mechanisms such as ADCC.

The kinetics of the development of serum HI antibody (Figures 4 and 6), and the absence of antibody producing cells in the lungs (Table 26) until after virus clearance occurs, and the dissociation of antibody response and virus clearance in mice in which silica treatment was delayed, reduced the likelihood of antibody playing an important role in the recovery process in this model.

Interferon titers in lung suspensions of mice treated with silica on the day of infection or 3 days after infection, was delayed in appearance when compared to interferon responses of control mice (Figures 5 and 8). However, later in infection when virus titers were declining in control mice, interferon titers in the lungs of control and macrophage depleted mice were similar. This separation of interferon responses from virus titers served as proof that interferon present later in infection may not exert antiviral effects by eliminating virus from the lungs. Other previously cited reports support our findings. Antibody to interferon type I did not increase severity of the disease in influenza infected mice (Gresser, 1976).

Virelizier and his co-workers (1977) demonstrated that type II interferon produced by mixed lymphocyte cultures protected murine macrophages from the cytocidal effects of A/Turkey/England/63 replication. Since specific antibody to type II interferon is not available, the question as to whether type II interferon is important is still open.

The delay in interferon production in macrophage depleted animals (Figures 5 and 8) may reflect the ability of macrophages to produce interferon themselves (Tsukui, 1977; reviewed in Notkins, 1975) in response to influenza antigens. It has been known for over ten years that alveolar macrophages produce interferon in response to virus infections (Acton and Myrvik, 1966). Macrophages are also necessary for the production of interferon by T cells (Milstone and Waksman, 1970). Although macrophages are also capable of synthesizing immune interferon (Neumann and Sorg, 1977) it is unclear whether the interferon detected in these assays or the other previously cited reports is type I or type II interferon. More studies are necessary before the role of interferon is established in influenza virus infections, since it is possible that macrophages and T cells collaborate to remove virus by interferon type II production as proposed by Virelizier et al. (1979).

Cellular immune responses to influenza virus infection appeared to be most closely associated with recovery from infection. Significant levels in cytotoxic activity in the lungs of immunocompetent mice were demonstrable 8 days after infection (Tables 5 and 8). This time coincided with the clearance of virus, increased accumulation of mononuclear cells into the lungs, and development of lung lesions. In certain experiments, cytotoxic activity in the lung did not appear to correlate with virus clearance. Indeed, mice treated with silica on day 5 or day 7 after infection had levels of cytotoxic activity similar to those observed in normal infected mice (Table 8, Figure 7) when lung mononuclear cells were assayed for activity 8 days after infection, but still demonstrated delays in virus clearance. These results suggest that cell mediated immune mechanisms other than cytotoxic cells participate in the clearance of virus.

The ability of transferred primary immune spleen cells to facilitate virus clearance in intact mice but not macrophage depleted mice (Tables 16–19) further served as evidence that macrophages as well as cytotoxic T cells act as effector cells which participate in virus clearance. Transfer of secondary immune cells also exerted antiviral effects in control mice by accelerating virus clearance in control mice (Tables 19 and 20) and in one experiment protecting against death (Figure 11). Transfer of secondary immune cells to macrophage depeleted mice one day after PR8 virus infection increased the rate of virus clearance in these animals (Table 22) but virus titers remained significantly higher than both control groups on day 10 after infection.

The ability to demonstrate increased virus clearance in macrophage depleted recipients of secondary immune cells (Tables 18 and 23) and not primary immune cells may be related to the cytotoxic potency of the cells (Table 15). Cytotoxic activity in the lungs of control recipients of secondary immune cells (Table 20) during a lethal infection probably protected them against death. Similarly, cytotoxic activity in the lungs of 2^o immune cell recipients were higher than other groups in the experiment (Table 24). Thus cytotoxic activity was related to virus clearance and number of lymphocytes accumulating in the lungs of control animals (Table 21 and 25). These associations were not as apparent in macrophage depleted mice.

Transfer of primary or secondary immune spleen cells to immunocompetent mice appeared to reduce the extent of lung consolidation in these animals (Figures 9, 10, and 12). In the primary immune cell control recipients HI antibody titers were lower than those of normal cell recipients (Figures 9 and 10). Thus, in contrast to the additive effects of immune cells of host and donor origin in facilitating virus clearance, these inhibitory effects of immune cells on antibody responses and the formation of lung lesions may be attributed to suppressor cells in the transferred cell population. Similar observations were made by Schulman et al. (1977) in other adoptive transfer experiments. However, it is possible that the more rapid clearance of virus from the lungs of control immune cell recipients prevent the normal development of lung lesions which accompany recovery from infection in untreated influenza virus infected mice. Prevention of virus replication and spread to other cells may inhibit virus induced cytopathology.

Taken together, the transfer of immune cells accelerated virus clearance in control animals. At best, secondary immune cells partially compensated for defects in virus clearance observed in macrophage depleted mice. The transfer of primary immune cells had no effect on virus clearance in macrophage depleted mice. The work of Yap and Ada (1978a, 1978b) suggests that cells with the characteristics of cytotoxic T cells participate in virus clearance. However, the results in the present experiment demonstrating that the defect in virus clearance in macrophage depleted mice cannot be compensated by secondary immune cell transfer suggest that other cells, particularly macrophages, may participate in virus clearance.

It is also possible that adoptively transferred cells are more effective in control animals because the effects of these cells are additive to those produced as a result of the host's own immune response which are absent or delayed in macrophage depleted animals. In this connection it is important to note that adoptive transfer, in this model, has no demonstrable effect until 6 days after transfer (seven days after infection of the recipients) an interval in which the normal host is mounting its own immune responses. However, the delays in virus clearance in macrophage depleted mice which display normal cytotoxic responses (Table 8) lends support to the hypothesis that macrophages may have an effector function during the recovery process.

Consistent with the scheme is the increased presence of both T cells and macrophages in the lung at the time of virus clearance and the development of lung lesions (Table 14, Figures 4 and 6). In another study, characterization of the cells populating the lung at the time of lesion development and virus clearance verified that macrophages and theta positive lymphocytes comprised the major proportion of mononuclear cells in the lungs (Wyde et al., 1978). Macrophage depletion by silica administration reduced both the accumulation of lymphocytes in the lung and cytotoxic activity in lung mononuclear cells. Thus influenza virus infection in macrophage depleted mice is similar to infection in mice treated with ATS (Schulman et al., 1977), ALS, ALG (Suzuki et al., 1974), cyclophosphamide (Singer et al., 1972), or in nude mice, in that infection with influenza virus is characterized by higher virus titers and less lung consolidation than are observed in immunocompetent animals. These effects are associated with lower numbers of immunoreactive cells during the period of recovery from infection.

The delay in lesion formation caused by macrophage depletion may not be immunologically specific. The accumulation of macrophages at the site of inflammation leads to the possibility that degradative enzymes released by activated macrophages may contribute to the lung pathology (Davies and Allison, 1976). Macrophages normally secrete lysozyme. However, activated macrophages also secrete collagenase and elastase (Werb and Gordon, 1975a, 1975b) which may contribute to tissue destruction.

Virus infection of macrophages may also cause the release of degradative enzymes which cause injury. Pulmonary cytomegalic inclusion disease induced in mice after treatment with antilymphocyte serum by subcutaneous administration of cytomegalovirus, resulted in the accumulation of virus containing monocytes in the pulmonary capillaries and interstitium. Although no evidence for virus replication in endothetial cells could be found, these cells were damaged during the infection. The cellular destruction was attributed to the release of lysosomal enzymes by macrophages lysed by the virus (Brody and Craighead, 1974). Virus infection of macrophages may also cause the release of lysosomal enzymes without killing the macrophage (Allison and Malluci, 1965).

In the present experiments the evidence which was obtained indicated that cytotoxic effector cells in the lungs were important in the recovery process. There was a temporal relationship between virus clearance and the accumulation of mononuclear cells in the lungs. In order to characterize the cell types involved in the cytotoxicity, a series of experiments were conducted in which the biologic properties of these cytotoxic cells obtained 10 days after infection were examined. It should be noted that in all of the experiments there was no evidence of a significant level of NK cell activity. The predominant effector cell capable of cytotoxic activity was H-2 restricted, anti-theta and complement sensitive (Table 10), specific for type of influenza virus (Tables 11A, 11B) but non-specific for subtypes of influenza A virus (Tables 10 and 18), nonadherent to plastic (Tables 10, 11A, 11B, and 13) or nylon wool (Table 12). These characteristics are compatible with those ascribed to cytotoxic T cells. This analysis agrees with the earlier report of Yap and Ada (1978c) and Doherty's group (Bennink et al., 1978).

In addition, a population of effector cells which were resistant to anti-theta and complement (Table 10) and were adherent to plastic (Tables 11A, 11B, and 13) or nylon wool (Table 12) consistently accounted for approximately 25% of the total cytotoxic activity. These cells had the characteristics of macrophages. Cytotoxic macrophages have been detected in another virus system, as well, Semliki Forest virus infection in mice (Rodda and White, 1976).

Intraperitoneal or subcutaneous infection with Semliki Forest virus results in the appearance of splenic mononuclear cells 2 or 3 days after infection which were anti-theta and complement resistant. These cells were plastic and nylon wool adherent and capable of the lysis of target cells infected with SFV or the immunologically unrelated kunjin virus (Rodda and White, 1976). Six days after infection the predominant effector cells were T cells. The authors concluded that perhaps virus-infected cells were unusually susceptible to lysis by activated macrophages. The characteristics of these adherent cytotoxic cells in our studies and those of Rodda and White appear to be quite similar and characteristic of macrophages except that in the present studies virus specific activity was observed and activity was not observed in adherent cells from ATS treated mice (Table 27). More studies are necessary to further characterize the adherent cell population and to determine whether lysis by these cells requires antibody, uncharacterized soluble products of other immunoreactive cells, and whether the lysis is H-2 restricted.

Influenza virus infection in man is often accompanied by secondary bacterial pneumonia (Finland et al., 1942). The use of murine models of virus infection have shown repeatedly that influenza virus impairs the ability to clear intranasally inoculated bacteria from the infected lung (Finland et al., 1942; Harford et al., 1942; Francis and de Torregrosa, 1945; Sellers, et al., 1961). Recent studies in man and mice suggest that influenza virus infection causes a depression of monocyte activities. In humans, it has been shown that infection impairs the chemotactic responsiveness of monocytes (Kleinerman et al., 1975). Murine alveolar macrophages obtained from influenza virus infected lungs displayed a decreased ability to ingest and degrade inhaled bacteria (Warshauer et al., 1977). Similar defects in anti-bacterial activity of murine alveolar macrophages has been observed in Sendai virus infections (Jakab and Warr, 1976). In fact the viral induced suppression of intracellular bacterial killing was related to the inhibition of phagosome-lysosome fusion following *in vitro* phagocytosis of *Candida krusei* by lavaged phagocytes (Jakab and Warr, 1978). Maximal phagocyte dysfunction occurred one week after Sendai virus infection, the time when maximal suppression of pulmonary bactericidal activity was demonstrated. Similarly, temporary depression of pulmonary bactericidal activity observed in influenza virus infected mice intranasally inoculated with *S. aureus* (Sellers et al., 1961) was most apparent early after virus infection.

Thus it is clear that secondary bacterial pneumonia which occurs during influenza virus infection is probably caused by temporary macrophage dysfunction. In retrospect, it was apparent that macrophage functions were important in the recovery process. However, until recently, the technological sophistication did not allow a more detailed analysis of mononuclear phagocyte activities in respiratory diseases.

Thus, these experiments have demonstrated that macrophage depletion profoundly alters the pathogenesis of influenza virus infection in mice as well as the immune responses to infection. The normal antibody responses of mice treated with silica on day 3 or later after infection did not affect the rate of virus clearance or the formation of lung lesions which are delayed in macrophage depleted mice. Thus, humoral immune responses can be dissociated from these indicators of recovery from infection. In contrast, cytotoxic responses appear to be an important component of the immune responses which participate in the recovery process. In this connection the evidence also suggests that besides functioning as accessory cells which elicit immune responses, macrophages may also function as effector cells which aid in the elimination of pulmonary virus. Furthermore, macrophages appear to participate in the development of lung lesions which accompany the recovery from influenza virus infection in mice. Although these studies have provided evidence that macrophages are important for recovery from influenza virus infection, there is much more to be learned about this cell type and its role during influenza virus infection. It should be emphasized that a deeper understanding of the immune mechanisms which effect the recovery from influenza virus infection is required for the future application of immunotherapy and the development of influenza virus vaccines.

The underlying mechanisms which are involved in recovery from influenza virus infection appear to be associated with the cellular immune responses to the virus. The generation of specific cytotoxic T cells in the lungs of influenza virus infected mice (Yap and Ada, 1978a; Rosenberg and Schulman, 1979) coincides with the time pulmonary virus titers begin to decline and lung lesions develop. Transfer of cells with the characteristics of cytotoxic T cells to recipient mice infected with homotypic or heterotypic virus accelerates the rate of virus clearance in immunocompetent mice (Schulman et al., 1977; Yap and Ada, 1978a, 1978b) demonstrating that these cells participate in recovery from infection. However, adoptive transfer of immune cells to macrophage depleted recipients partially compensates for defects in virus clearance, suggesting that macrophages may function as an important effector cell which eliminates virus from the lungs of infected mice.

The mechanism by which macrophages aid in recovery by eliminating virus may be by phagocytosis of virus or by destruction of virus infected cells. Although cytotoxic activity was detected in an adherent cell population the means by which lysis is effected is unclear. Among other things, it is not known whether lysis is H-2 restricted. Since target cells infected with serologically different influenza A viruses are lysed by adherent cells, it is unlikely that the lysis is antibody dependent unless previously unrecognized antibody to cross-reactive determinants is present in the system. It is unknown whether cross-reactive lysis by these cells is due to the recognition of a common M protein as may be the case with cytotoxic T cells (Braciale, 1977a; Biddison et al., 1977; Reiss, 1978).

The ability of cells with the characteristics of cytotoxic T cells to reduce virus titers in syngeneic mice infected with a heterotypic virus (Yap and Ada, 1978b), perhaps by recognition of M protein, may be one of the mechanisms underlying heterotypic immunity (Webster and Hinshaw, 1977) first described by Schulman and Kilbourne (1965).

Although natural killer cells are elicited in mice by infection with influenza virus (Anderson and Heath, 1977), more studies are necessary before any role can be assigned to these cells. Similarly, no evidence is available regarding the roles of other effector cell populations in recovery from influenza virus infection in mice. Due to the contradictory results of experiments designed to determine whether interferon functions in recovery (Gresser et al., 1966; Iwasaki and Nozima, 1977) its role remains unclear. In this manuscript the virus inhibitory effects of interferon could not be clearly distinguished from other immune mechanisms operating at the same time.

The results of the experiments presented in this thesis also support the hypothesis that the lung lesions which develop during influenza virus infection in mice are predominantly due to the host's immune response to the virus. Within hours after infection, virus antigens are present on the cell membranes of epithelial cells lining the respiratory tract (Hers et al., 1962). Although polymorphonuclear leukocytes and neutrophils accumulate in the lung one or two days after infection (Loosli et al., 1949) an increased number of macrophages are present in the lung at the same time. Thus, of the phagocytic cells present in the early inflammatory response to infection, only macrophages recognize viral antigens present on the apical membrane of bronchial cells but the form of these antigens and how the macrophages recognize viral antigens remains unclear. Perhaps macrophages recognize antigens composed of viral and H-2 determinants on alveolar cell membrane. Alternatively, macrophages in the lymph nodes draining the lungs of infected mice trap virus. In particular, dendritic cells may serve to focus antigen in the lymph nodes. However, it should be noted that little is known about these cells, except that they are a novel cell type present in peripheral lymphoid organs (Steinman and Cohn, 1973), are glass adherent (Steinman and Cohn, 1974), bear Ia antigens (Steinman et al., 1979), and are potent stimulators of the mixed leukocyte reaction (Steinman and Witmer, 1978). It is also possible that circulating monocytes endocytose virus particles in the lung and present the virus antigens in association with Ia antigens to T and B cells in the lympho nodes, draining the lungs in a form similar to that first recognized by monocytes on infected epithelial cells of the lung.

After immunologic induction, cytotoxic activity becomes detectable in the lungs a few days later (7 days after infection). The generation of cytotoxic effector cells accompanies the appearance of lung lesions. Silica treatment delays the development of both the cytotoxic response and the concomitant pathology. A cytotoxic T cell recognizes the viral antigens present on the inner surface of the infected cells and not on the basal membrane, since the antigens recognized are not exposed to the circulation. This may explain why the cytotoxic response in lung mononuclear cells are not detected sooner and why the antiviral effects of immune cell transfer are not observed until 10 days after infection–it takes time for sufficient numbers of immunoreactive cells to traverse the membrane and reach the cell surface bearing the viral antigen.

Once there are enough effector cells present in the lungs, virus replication is inhibited by the destruction of the infected cells. Macrophages and theta-bearing lymphocytes are present in increased numbers (Wyde et al., 1978) during the period in which cytotoxic responses are generated, lung lesions are becoming more extensive, and rapidly declining virus titers are observed. Silica treatment delays this series of events. In macrophage depleted animals virus titers remain elevated and lung pathology is minimal. This suggests that virus induced cytopathic effects may not contribute significantly to the development of lung lesions as had been implied in earlier studies (Loosli et al., 1949; Hers et al., 1962).

The influx of both T cells and macrophages into the lungs of influenza infected mice during the recovery period suggests that these two cell types collaborate in the clearance of virus and participate in the development of lung lesions. As stated previously, cytotoxic T cells may destroy virus infected cells, eliminating virus but at the same time cause widespread necrosis which constitutes the lesions which are observed. The two cell types may also collaborate in an entirely different series of events. T cells activated by macrophages may elaborate soluble factors, among which is migration inhibition factor. Macrophages and T cells may interact to produce a DTH reaction in the lung which contributes both to the visible lung lesions and participates in the clearance of virus from the lung.

The results of these experiments are in accord with the results of histological studies (Hers et al., 1962; Loosli et al., 1949) and the studies of inflammatory cell responses in the lungs of influenza virus infected mice (Wyde et al., 1978). Therefore, the pneumonia which develops during influenza virus infection in mice may be an immunopathological process in which T cells (Wyde et al., 1978; Sullivan et al., 1976; Singer et al., 1972; Suzuki et al., 1974) and macrophages participate (Rosenberg and Schulman, 1979, submitted for publication).

In conclusion it is clear that intact T cell and macrophage function contribute significantly both to recovery and to the development of lung lesions in mice infected with influenza virus. Although from the results presented in this thesis antibody alone does not appear to be a significant factor in either event in conjunction with other immune mechanisms antibody responses may play a role. Further definition of the precise importance of each of these immune mechanisms requires appropriate model systems which can be studied separately.

XI. SIGNIFICANCE OF THE WORK

1. The definition of the roles of macrophages during influenza virus infection *in vivo* provides insights into the complex interactions between macrophages and lymphocytes which result in the generation of immune responses. Humoral immune responses do not appear to be crucial in the recovery process. An association between T cell and macrophage effector activities and the elimination of pulmonary virus during influenza virus infection in mice establish the importance of cell mediated immunity in the recovery from infection.

2. These studies provide a broad view of the immune responses which allow recovery from infection and at the same time contribute to the pathogenesis of the disease in mice. This knowledge may facilitate the development of prophylactic and therapeutic approaches to influenza disease in man.

XII. APPENDIX – ABBREVIATIONS

ABS	antibiotic saline
ADCC	antibody dependent cell mediated cytotoxicity
AFC	antibody forming cells
AG-B	major histocompatiblity complex of the rat
ALG	anti-lymphocyte globulin
ALS	anti-lymphocyte serum
AMS	anti macrophage serum
ATS	anti thymocyte serum
AVP	antiviral protein
BALB/c	inbred strain of mice
BCG	Bacille-Calmette-Guerin
B/Lee virus	influenza B/Lee/40 virus
C3H	inbred mouse strain
^{51}Cr	radioactive chromium
CY	cyclophosphamide
DMEM	Dulbecco's modified Eagle's medium
DTH	delayed type hypersensitivity
EID_{50}	50% egg infectious dose
EMEM	Earle based minimal essential medium
EqEq antiserum	influenza Eq/1/56 (Heq1Neq1) antiserum
EqPR8 antiserum	influenza Eq/1/56 (Heq1)–A/PR/8/34 (N1) antiserum
FCS	fetal calf serum
GAT	synthetic antigen L-glutamine, L-alanine, L-tyrosine
HA molecule	hemagglutinin glycoprotein

HA titer	hemagglutination titer
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxy-piperazine-N'-2.ethane-sulfonic acid
HI	hemagglutinating inhibiting
HK virus	influenza A/Hong Kong/8/68 (H3N2) virus
HK-PR8 virus	influenza A/Hong Kong/8/68 (H3)–PR/8/34 (N1) virus
HL-A	major histocompatibility complex of man
HSV	herpes simplex virus
H-2	major histocompatibility locus of mice
Ia	cell surface antigen associated with the I region of the H-2 locus
Japan 305 virus	A/Japan/305/57 (H2N2) virus
L929	connective tissue fibroblast cell-line of C3H mice
LCM	lymphocytic choriomeningitis virus
Ly 1, Ly 2	cell surface antigens of T cells
M protein	matrix or membrane protein
MDCK cells	Madin-Darby canine kidney cells
MHV	mouse hepatitis virus
MID_{50}	50% mouse infectious doses
MLR	mixed lymphocyte reaction
M.O.I.	multiplicity of infection
M-TUR virus	influenza A/Turkey/England/63 (Hav1Nav3)
Mx	autosomal dominant trait of the A2G strain; phenotype is myxovirus resistant
NA molecule	neuraminidase glycoprotein
NDV	Newcastle disease virus
NI	neuraminidase-inhibiting

NP	nucleoprotein
NRS	normal rabbit serum
NWS	influenza A/NWS/33 (H0N1)
P815	mastocytoma cell line of DBA/2 mice
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PEC	peritoneal exudate cells
PFU	plaque forming unit
PR8 virus	influenza A/PR/8/34 (H0N1) virus
PR8-HK virus	influenza A/PR/8/34 (H0)–Hong Kong/8/68 (H2) virus
PRI	Princeton-Rockefeller Institute strain of mice
RDE	receptor destroying enzyme
RES	reticuloendothelial system
SFV	Semliki forest virus
S.I.R.	specific immune release
SRBC	sheep erythrocytes
TxBM	thymectomized–X irradiated–bone marrow–reconstituted
2-DG	2-deoxy-D-glucose
UV	ultraviolet light
VSV	vesicular stomatitis virus

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