B CELL RESPONSES

IN

MURINE INFLUENZA

BY

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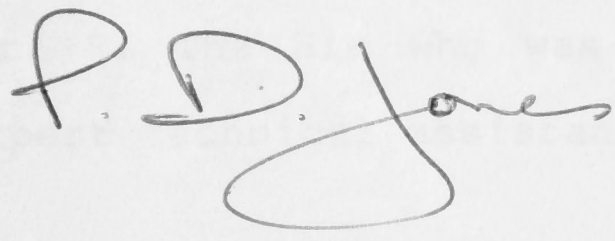
STATEMENT

The work described in this thesis represents my own original work with the exception of the cytotoxicity assays and limiting dilution assays described in Chapter 6 which were performed by Mr. R. Tha Hla.



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CONTENTS

ABSTRACT

The B cell response to influenza virus infection has been characterized largely on the basis of measurements of serum antibody and antibody in respiratory secretions. However, the cellular component of this response has received little attention principally because a reliable method for quantitation has been lacking. An ELISA-plaque assay has been developed to enumerate influenza-specific antibody-secreting cells (ASCs) of different antibody isotypes. This assay can accurately and reproducibly detect cells secreting antibodies of IgM, IgG and IgA isotypes to influenza virus type specific antigens. Antibodies of different subtype specificity could be distinguished by modifying the assay so that only the surface glycoproteins are recognized. This assay has been used to study the B cell response in the murine lung, spleen and circulation to influenza infection and immunization.

During primary influenza infection, ASCs of each isotype were detected in the lungs, demonstrating that antibody in respiratory secretions of each type, not only IgA, was locally produced. Furthermore, the pulmonary response was of greater magnitude than the splenic response for each isotype, and markedly so for the IgA component. In vitro stimulation of lungs from unprimed mice could also generate ASCs, suggesting that resident B cell precursors in

the lungs could contribute to the local response during primary infection.

Influenza A virus haemagglutinin-specific antibodies, capable of preventing re-infection, may persist for decades in humans after the original influenza A virus subtype is no longer circulating in the population. To study the possible mechanisms involved, the local and systemic components of the cellular events underlying the duration of the antibody response in murine influenza were analyzed. ASCs, predominantly IgG-secreting, were found in the lung and spleen up to 18 months after primary murine influenza infection. B cell memory was determined by enumerating ASCs generated in stimulated lung and spleen cell cultures obtained from primed mice. There was a 50-200 fold increase in ASCs generated in vitro after influenza infection. Whereas there was no change in the level of B cell memory in the spleen up to 18 months after infection, the number of B memory cells in the lung progressively declined 6 months after infection. By 18 months the number of ASCs generated in lung cell cultures was comparable to that in unprimed mice, although the predominant isotype, being IgG, differed. The effect of subsequent viral challenge on ASCs and B cell memory was also studied. Homotypic challenge increased ASCs in the lung only, whereas B cell memory increased in both the lung and spleen. Although ASCs, and to a lesser extent B cell memory, increased in both the lung

and spleen after heterotypic challenge, ASCs and B cell memory specific for the original subtype were not increased.

The development of regional B cell responses was also studied in mice immunized intranasally with a cold-adapted (ca) variant of influenza A virus. The ca-variant virus was 100 fold less efficient than the parental virus in the induction of influenza-specific ASCs in the lung and failed to induce ASCs in the spleen. The ca-variant virus was less efficient in priming for secondary IgG and IgA responses generated in vitro in both lung and spleen cell cultures. Protection against homotypic challenge in mice immunized by different priming strategies was correlated with the development of pulmonary B cell responses rather than splenic responses. In particular, protection was correlated with the presence of ASCs and IgG and IgA memory in the lung at the time of challenge.

Local cellular immune responses were studied in mice immunized intranasally with the surface glycoproteins of influenza A virus presented in either micelles or immuno-stimulatory complexes (iscoms). Iscoms were more immunogenic that micelles in unprimed mice. Iscoms (given in two doses) elicited a primary ASC response in the lung but failed to elicit a primary cytotoxic (Tc) cell response. However, both local B and Tc cell memory was established after immunization with iscoms. In mice primed by infectious virus, both iscoms and micelles elicited

secondary ASC and Tc cell responses in vivo. The ability of iscoms to prime for secondary Tc cell responses and to elicit secondary Tc cell responses in vivo contrasts with the failure of previous preparations of inactivated influenza vaccines to elicit Tc cell responses.

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ABBREVIATIONS

ABC	Avidin-biotin-perixodase complex
ADCC	Antibody-dependent cell-mediated cytotoxicity
ALM	Alveolar macrophage
APC	Antigen-presenting-cell
ARI	Acute respiratory infections
ASCs	Antibody-secreting cells
BALT	Bronchus-associated-lymphoid-tissue
BSA	Bovine serum albumin
CRBCs	Chicken red blood cells
CsA	Cyclosporin A
DTH	Delayed-type hypersensitivity
EID_{50}	Median egg infective dose
ELISA	Enzyme-linked immunosorbent assay
GALT	Gut-associated-lymphoid-tissue
HA	Haemagglutinin
HAU	Haemagglutination units
HI	Haemagglutination inhibition
IFN	Interferon
Iscoms	Immunostimulatory complexes
M protein	Matrix protein
MHC	Major histocompatibility complex
NA	Neuraminidase
NK	Natural killer
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
RSV	Respiratory syncytial virus

SC	Secretory component
sIgA	Secretory IgA
SRBCs	Sheep red blood cells
TBW	Tracheobronchial washings
Tc	Cytotoxic T cells
Td	Cells mediating DTH reactions
Th	T helper cells
Ts	T suppressor cells

CHAPTER ONE

INTRODUCTION

CONTENTS

1.1 Preface

Acute respiratory infections (ARI) are the single largest cause of use of health services in both developed and developing countries (Douglas & Kerby-Eaton, 1985). In both settings, children experience approximately seven episodes of ARI each year for the first five years of life; viruses being the major primary cause of these episodes. However children in developing countries are much more likely, principally as a result of malnutrition, to develop secondary bacterial infections leading to a higher mortality rate for ARI in developing countries (Pio et al., 1985). ARI, diarrhoeal diseases and malnutrition are the three major causes of approximately 15 million deaths in children under the age of five each year in developing countries (Grant, 1985).

Although absolute mortality from ARI has fallen drastically in developed countries, mortality from ARI in Australia is still nearly four times higher than all other infectious diseases combined (Douglas, 1985). In addition, the morbidity from ARI in this country is estimated to account for a minimum of 50 million dollars in medical and drug costs per year in young children alone (Douglas, 1985). Furthermore, the impact of ARI in developed countries also includes the increased risk of chronic lung diseases following ARI (Woolcock & Peat, 1985).

The nature of the association between viral respiratory pathogens and ARI is remarkably similar in developed and developing countries. The major viral pathogens are influenza virus types A and B, respiratory syncytial virus (RSV), the parainfluenza viruses, rhinoviruses, adenoviruses and coronaviruses. The viruses associated with exanthematous illnesses may also cause ARI, particularly in developing countries.

Except for influenza virus vaccine, no vaccines are available for the control of other major viral respiratory pathogens. Problems encountered in the development of effective vaccines have included the wide antigenic diversity of some viruses e.g. rhinovirus, antigenic variation e.g. influenza and the development of pathological immune responses to some inactivated vaccines e.g. RSV. In view of the short incubation period between infection and the development of disease in viral ARI, usually less than 4-5 days, a vaccine, to be successful, must elicit a durable immune response capable of preventing infection at the respiratory epithelial surface. It is generally accepted that neutralizing antibody in respiratory secretions is the principal mediator of prevention against viral ARI. The development of local immune responses to viral ARI will be reviewed in the first part of this introduction.

Influenza A virus infection was chosen as the model system to study the development of local B cell responses in

this thesis. Influenza A virus has caused recurrent epidemics of febrile respiratory disease every 1 to 3 years for at least the past 400 years. Epidemics of world wide scope (pandemics) have occurred less often. The greatest pandemic occurred in 1918/1919 when 21 million deaths were recorded. Even between epidemics, influenza is an important cause of mortality responsible for 10,000 deaths per annum in the U.S.A. (Mostow, 1986) and considerably greater morbidity. The immune response to influenza virus and vaccines is reviewed in the second part of this introduction.

1.2 Respiratory Tract Immunity

Before reviewing the development of local immunity to viral infections of the respiratory tract, the structural basis of immune responses in the respiratory tract, at both the tissue and cellular level, and general concepts of respiratory tract immunity will be briefly described.

1.2.1 Organization of Lymphoid Tissues in the Lung

The respiratory tract may be structurally and functionally divided at the broncho-alveolar junction. The upper division includes the mucosal surfaces of the trachea, bronchi and terminal bronchioles. The lower division, the lung parenchyma, includes the respiratory bronchioles and alveoli, which are lined by alveolar epithelium only and the supporting interstitium. Within the mucosal division of the respiratory tract, there are several compartments of organized lymphoid tissue (Bienenstock, 1984a). In contrast, the normal lung parenchyma contains only a small number of lymphocytes and macrophages scattered diffusely throughout the interstitium.

The most organized compartment of mucosal lymphoid tissue is the submucosal lymphoid follicle referred to as bronchus-associated-lymphoid-tissue (BALT) (Bienenstock, 1984b). These follicles are morphologically and probably functionally analogous to the gut-associated-lymphoid-tissue

(GALT). BALT has been identified, in varying degrees, in humans and many animals (McDermott et al., 1982).

Morphologically, BALT consists of well-organized lymphoid aggregates with an overlying specialized lymphoepithelium containing lymphocytes and non-ciliated epithelial cells possessing microvilli (M cells). The lymphoepithelium appears to be important in the uptake of soluble and particulate antigens from the lumen of the respiratory tract. The lymhoid tissue of BALT, divisible into dome, follicular and parafollicular areas, contains predominantly B cells, most of which express IgA and is considered to be the principal source of precursors of IgA-antibody secreting cells (ASCs).

A second compartment of lymphoid tissue consists of intra-epithelial lymphocytes. The majority of these cells have not been identified, though 30% express Thy 1 and 55% express Lyt 2, but surprisingly not Thy 1. Whilst the function of the majority of these cells is unknown, small subsets have been identified as natural killer cells, cytotoxic T cell precursors and mast cell precursors (Bienenstock, ASI Proceedings, 1986).

A third compartment of lymphoid tissue consists of loosely organised lymphoid aggregates scattered diffusely throughout the submucosa and lamina propria. These

aggregates contain T and B cells, of which the major class is IgA.

The cellular constituents of the air spaces of peripheral bronchioles and alveoli have been sampled by broncho-alveolar lavage (Young & Reynolds, 1984). In most species, approximately 90% of broncho-alveolar cells are macrophages and the remainder are mostly lymphocytes.

1.2.2 Immunocompetent Cells in the Lung

1.2.2.1 Alveolar Macrophages

The alveolar macrophage (ALM) is a mononuclear phagocytic cell present in broncho-alveolar air spaces and in the lung interstitium and mucosa. These cells are originally derived from the bone marrow and have the capacity to proliferate locally in the lung. The principal functions of the ALM appear to be the nonspecific phagocytosis of inhaled substances and the regulation of inflammation through the release of soluble mediators including leukotrienes, prostaglandins and complement components. However, it is the interaction of the ALM with specific components of the immune response which will be considered further.

There are several lines of evidence supporting an important role for the ALM in antigen presentation:

(i) ALMs possess the characteristics essential for an antigen-presenting-cell (APC) i.e. they express Ia antigens (Lipscomb et al., 1981; Mason et al., 1982), are able to process antigens (Schuyler & Todd, 1981) and can synthesize and release interleukin-1 (McDermott et al. 1982)

(ii) ALMs can support mitogen and antigen induced lymphoproliferation in vitro (Lipscomb et al., 1981);

(iii) antigen-pulsed ALMs can induce specific T cell proliferative responses in vivo (Lyons & Lipscomb, 1983).

Although the site of antigen presentation has not been determined, ALMs may migrate within the lung to enter the interstitium or mucosal lymphoid tissue, including BALT, or migrate outside the lung to extrapulmonary lymphoid tissue. ALMs may also selectively recruit sensitized T cells into the lung (Lyons & Lipscomb, 1983).

ALMs also participate as effector cells in the immune response by phagocytosing opsonized organisms and in antibody-dependent cell-mediated cytotoxicity (Whitcomb, 1979).

The role of ALMs in the regulation of immune responses is variable (McDermott et al., 1982; Holt, 1986). Although ALMs have been shown to support lymphoproliferation, ALMs may also suppress lymphoproliferation and ASC responses by the release of soluble products, including prostaglandin (Ansfield et al., 1979; Demenkoff et al., 1980). This functional heterogeneity of ALMs may be explained by different stages in maturation or activation (Shellito & Kaltreider, 1984 & 1985).

1.2.2.2 Antigen-presenting Cells other than ALMs

Cells other than ALMs have been identified within the respiratory tract which, by virtue of their ability to express Ia antigens or their dendritic morphology, may act as APCs, although there is no direct evidence to support this.

Cells morphologically resembling Langerhan's cells and expressing the F4/80 antigen of the murine mononuclear phagocyte system have been found spread along the basement membrane of the bronchial and bronchiolar epithelium (Hume et al., 1984).

Epithelial cells of the trachea and bronchi may express Ia antigens (Wiman et al, 1978; Natali et al., 1981). Whether the epithelial cells (M cells) of the lymphoepithelium overlying BALT are capable of

antigen-presentation is unknown. In addition, there are cells within BALT (Racz, 1977) resembling the interdigitating cells of T cell areas which are thought to be the in vivo counterpart of the isolated Steinman lymphoid dendritic cells.

1.2.2.3 Natural Killer (NK) cells

Natural killer activity has been found in normal murine (Puccetti, 1980) and human (Bordignon et al., 1982) lung cell suspensions, though in varying degrees. Although the precise location of NK cells in the lung has not been determined, intra-epithelial lymphocytes in the gut possess NK cell activity (Tagliabue et al., 1982).

In addition, approximately 20-30% of lymphocytes in broncho-alveolar cell preparations are "null" cells which may include NK cells. However NK cell activity has not been detected in broncho-alveolar cell preparations (Puccetti, 1980; Bordignon et al., 1982).

1.2.2.4 B Lymphocytes

The distribution of B cells throughout the lung has been largely characterized by immunofluorescence studies. The principal Ig-bearing cells found throughout the respiratory mucosa express IgA. Although over 50% of the lymphocytes in BALT are surface IgA positive (Rudzik et al.,

1975), mature plasma cells have rarely been found within the lymphoid follicles of the BALT (Chamberlain et al., 1973). BALT, like GALT, is thought to be a source of precursors of IgA plasma cells which are already committed to specific antigens (Gearhart & Cebra, 1979). By analogy with the findings in GALT, antigen-specific precursor IgA B cells in BALT might locally proliferate upon exposure to antigen in the presence of IgA-specific T helper cells. IgA ASCs would then migrate within the respiratory mucosa or enter the lumen via the lymphoepithelium. Cells with surface IgG, IgE and to a lesser extent IgM, have also been found throughout the respiratory mucosa. The source of precursor cells for these different isotypes is unknown, although based on the finding of percursor IgE cells in GALT (Ngan & Kind, 1978) it is likely that the BALT is also a source of precursor IgE cells.

B cells constitute 10-20% of the lymphocytes found in broncho-alveolar cell preparations. The proportion of isotype-specific ASCs in human broncho-alveolar cell preparations quantitated by a reverse haemolytic-plaque assay differs from that in peripheral blood lymphocytes by virtue of the higher proportion of IgA ASCs in broncho-alveolar cells (Lawrence et al., 1978). Overall, IgA- and IgG-secreting cells are more numerous than IgM-secreting cells. B cells in broncho-alveolar cell preparations may be either derived from the respiratory mucosa and/or the blood (Danielle et al., 1977).

1.2.2.5 T Lymphocytes

T cells have been identified within the respiratory epithelium, the lamina propria, the lymphoid follicles of BALT and in broncho-alveolar cell preparations. However, there is little data available on the precise identification of these cells according to phenotypic and functional characteristics.

Whilst approximately 20% of BALT lymphocytes are T cells (Rudzik et al., 1975) there is no evidence that there is a fixed T cell area within BALT analogous to other lymphoid follicles. IgA specific T helper (Th) cells (Elson et al., 1979) and IgE specific T suppressor (Ts) cells (Ngan & Kind, 1978) have been found in GALT and probably exist in BALT as well.

T cells constitute approximately 50% to 70% of lymphocytes in broncho-alveolar cell preparations. In human studies, approximately 45% of broncho-alveolar T cells were identified as helper/inducer cells ($OKT4^+$) and approximately 25% were identified as T suppressor/cytotoxic cells ($OKT8^+$) (Hunninghake & Crystal, 1981).

1.2.3 Immune Responses in the Lung

In this section the induction and expression of specific immune responses in the lung, including the

generation of local memory, will be considered. The biological activities of the various responses with particular regard to their role in viral immunity will be considered later (Section 1.2.4).

1.2.3.1 Induction of Local Immune Responses

Within the upper division of the respiratory tract, antigen uptake may occur across the mucosa via the lymphoepithelium of BALT and where the epithelial integrity of the mucosa has been disrupted as in viral infections. Antigen presentation may subsequently be mediated by submucosal macrophages, including those in BALT and by other putative APCs. In the parenchymal division, local immune responses may be induced by antigen presented by ALMs either within the alveoli or the interstitium.

There are three basic mechanisms by which specific immune responses expressed in the lung may arise (Kaltreider, 1984):

(i) local antigen may stimulate specific T and B cell precursors already present in the lung;

(ii) local antigen may stimulate specific T and B cell precursors which have been recruited into the lung;

(iii) circulating antigen may stimulate specific T and B cell precursors in extrapulmonary lymphoid tissue; effector cells being subsequently recruited into the lung by local antigen.

The relative roles of these mechanisms in the expression of T and B cell responses in the mucosal and parenchymal divisions in the lung have not been adequately defined.

1.2.3.2 B Cell Responses

IgA

Secretory IgA (sIgA) constitutes the major Ig found in bronchial secretions under normal conditions and following antigenic stimulation. Although specific precursor IgA B cells are found in BALT, the local cellular interactions responsible for B cell activation have not been determined. However, by analogy with events within the gut, antigen in the presence of sensitized Th cells initially activates precursor IgA cells in BALT (Cebra et al., 1983) which then migrate to other mucosal sites where final differentiation into ASCs occurs. Dimeric IgA with J-chain is secreted into the interstitium and binds to the secretory components (SC) expressed in epithelial cells. The sIgA molecule is transported across the cell and secreted into the lumen. Whereas the bulk of sIgA found in respiratory secretions is

derived from local synthesis, additionally serum dimeric IgA may be selectively transported into the lumen via SC receptors on epithelial cells (McDermott et al., 1982).

IgM

Under normal conditions, the relative concentration of IgM in respiratory secretions is very low. This is presumably derived from local synthesis as transudation from serum would be limited by the high molecular weight of IgM. In the presence of inflammation, IgM levels may increase as a result of exudation. In addition, specific IgM ASCs may appear in the lung parenchyma following intrapulmonary immunization.

Using a haemolytic-plaque assay, direct plaque-forming cells were detected in broncho-alveolar cell preparations of dogs immunized with sheep red blood cells (SRBCs) (Kaltreider et al, 1974; Kaltreider & Turner, 1976; Bice et al., 1980). In contrast, primary intrapulmonary immunization of mice with SRBCs failed to induce the appearance of ASCs in the lung (McLeod et al., 1978). However, both direct and indirect (IgG) plaque-forming cells were detected in the lungs of mice after repeated immunization. In both the canine and murine models, it was concluded that pulmonary ASCs were derived from extrapulmonary lymphoid tissue according to the third

mechanism described above. This conclusion was based on the following observations:

(i) ASCs appeared first in hilar lymph nodes and subsequently in the blood and lung (Kaltreider & Turner, 1976);

(ii) ASCs appeared in the hilar lymph node only, after low immunizing doses (Kaltreider & Turner, 1976);

(iii) intratracheal challenge resulted in the local recruitment of adoptively transfered sensitized splenic lymphocytes (Kaltreider et al., 1983).

Whilst these observations support the conclusion that ASCs may be derived from extrapulmonary sources, the alternate possibility that ASCs may also originate from resident precursors has not been excluded.

IgG

The relative concentration of IgG in respiratory secretions progressively increases further down the respiratory tract to become the principal isotype in the lung parenchyma (Kaltreider, 1984). IgG in bronchial secretions is thought to be principally derived from the serum by transudation, although some local synthesis by submucosal plasma cells does occur. In the lung

parenchyma, transudation is also important as indicated by the similar IgG to albumin ratios in broncho-alveolar lavage fluid and in serum under both normal conditions and in response to antigenic stimulation (Young & Reynolds, 1984). As indicated above though, specific IgG ASCs presumably derived from extra-pulmonary sources do appear in the lung parenchyma after immunization with foreign erythrocytes.

IgE

IgE is present in minute quantities in respiratory secretions under normal conditions. Precursor IgE cells are present in BALT and presumably most of the IgE in bronchial secretions is locally synthesized whereas IgE in broncho-alveolar lavage fluid is thought to be largely derived from serum. Following local antigenic stimulation IgE ASCs increase in the respiratory tract (Sedgwick & Holt, 1983b).

1.2.3.3 T Cell Responses

Apart from the role of T cells in BALT regulating IgA and IgE responses, there are no data conclusively demonstrating the occurrence of regulatory T cells, determined by either phenotypic or functional characteristics, in the lung during immune responses. However, antigen-reactive cells in lymphoproliferative assays, which have been reasonably assumed to represent Th

cells, do appear in the lung after antigenic stimulation (Lipscomb et al. 1981).

Specific cytotoxic T (Tc) cells have been detected in lung cell suspensions during viral infections (Section 1.2.4) and after local stimulation with allo-antigens (Emeson et al., 1982; Liu et al., 1982). Evidence for the appearance of T cells (Td) mediating delayed type hypersensitivity (DTH) reactions was initially based on the demonstration of macrophage inhibition factor (MIF) in broncho-alveolar cell preparations following local antigenic stimulation (Galindo & Myrvik, 1970; Waldman et al., 1972). However, as the secretion of MIF is not exclusively restricted to Td cells, more convincing evidence was provided by demonstrating that cells mediating DTH reaction could be recovered from the lungs of influenza infected mice (Leung & Ada, 1980). These cells were identified as Class II restricted Lyt 2^- T lymphocytes.

The source of T cells in immune responses in the lung is largely unknown. Following adoptive transfer of sensitized T cells, Th and Tc cells can be recovered in the lung, although the effect of local antigen on recruitment is variable. Using influenza-specific T cell clones, Bienenstock et al. (1983) showed striking increases in numbers of cells in the lung parenchyma of infected mice compared with non-infected mice but only with the Lyt 2^+ (Tc) clone and not with an Lyt 2^- (Th) clone. The alternate

source of T cells involving stimulation of resident T cell precursors in the lung is suggested by the finding of influenza-specific Tc cell precursors in the normal murine lung at a frequency comparable to that in the spleen (Mak et al., 1984).

1.2.3.4 Interaction of Pulmonary and Extrapulmonary Immune Responses

The concept of a common mucosal immune system describes the integration of mucosal immune responses through the selective migration of immunocompetent cells and antibodies originating at one mucosal site to other mucosal sites. There is considerable evidence that oral presentation of antigen will result in the appearance of antigen-specific precursor IgA cells in the mucosa of the gut and lung as well as in other mucosal sites (Weisz-Carrington et al., 1979; Pierce & Koster, 1980). Furthermore, T-lymphoblasts from mesenteric lymph nodes may localize in the epithelium and lamina propria of the bronchial mucosa (McDermott & Bienenstock, 1979). These findings provide a rationale for the successful immunization against adenoviruses (Smith et al., 1970) and influenza virus (Waldman et al., 1981) using oral vaccines.

The corollary that cells from the respiratory tract may migrate to other mucosal surfaces is also true. However, B and T lymphocytes obtained from bronchial lymph

nodes localize preferentially, though not exclusively, to the lung (McDermott & Bienenstock, 1979).

Systemic immune responses may also contribute to immune responses in the lung, especially within the parenchymal division. Although there is considerable evidence that antibody in respiratory secretions may be derived from extrapulmonary sources, the migration of immunocompetent cells into the lung parenchyma has not been studied in any great detail.

1.2.3.5 Immunological Memory in the Lung

Immunological memory in the respiratory tract has been demonstrated by either increases in antigen-specific cells or by the kinetics of secondary responses. Antigen-specific IgA containing cells have been demonstrated in the bronchial mucosa of primed animals (Pierce & Koster, 1980; Joel et al., 1980). However, anamnestic IgA responses have not been consistently observed following secondary stimulation (Clancy & Bienenstock, 1984). This may reflect the effect of local regulatory influences, including Ts cells, and the nature and dose of the boosting antigen. In particular, secondary IgA responses may not develop after challenge with homologous virus if infection is prevented by pre-existing neutralizing antibody.

Immunological memory for T cell responses has also been demonstrated by increases in antigen-reactive cells in lymphoproliferative assays (Clancy & Pucci, 1978) and Tc cell precursor frequency after priming (Mak et al., 1984). In addition, local T cell responses develop earlier in previously sensitized animals (Yap et al., 1979; Ganguly & Waldman, 1983).

1.2.4 Viral Immunity in the Lung

Immune responses to viral infections of the respiratory tract may lead to recovery from infection and protection against re-infection or may contribute to the pathogenesis of disease. These roles will be considered in the broader context of viral immunity initially and then specifically for the major respiratory viral pathogens. Data on local immune responses to viruses are largely limited to the development of secretory antibody responses and to a lesser extent cell mediated responses. There is little information on non-specific immune responses, although the components of this system have been identified in the respiratory tract.

1.2.4.1 Protective Role of Local Immune Responses

Non-Specific Responses

The components of the non-specific immune system, including macrophages, NK cells, interferon (IFN) and

complement appear to be important in limiting viral spread and initiating recovery.

Macrophages may limit viral spread by several mechanisms (reviewed by Morahan et al., 1985):

(i) virus phagocytosis leading to inactivation of virus or abortive replication;

(ii) lysis of virus-infected cells by either antibody-dependent or independent mechanisms;

(iii) release of alpha-IFN;

(iv) presenting viral antigens to T cells and activating T cell responses by the release of interleukin-1.

All of these functions have been ascribed to ALMs.

The direct antiviral effects of IFN relate to inhibition of viral replication. In addition, IFN, either alpha or gamma, may modulate other immune responses (Rager-Zisman & Bloom, 1985) by:

(i) augmenting the expression of MHC antigen on cells;

(ii) activating macrophages and increasing the expression of Fc receptors for IgG;

(iii) augmenting NK cell activity.

In addition, antibody synthesis may be either enhanced or suppressed by IFN depending on the dose and the time of exposure relative to antigen stimulation.

NK cells may non-specifically lyse virus-infected cells in vitro. However, there is little evidence documenting the role of NK cells in resistance to viral infections in vivo (Rager-Zisman & Bloom, 1985).

The complement system either independently of, or in the presence of specific antiviral antibody, plays a role in host defence against viral infection (Lachman, 1985). Complement can interact with viruses in the absence of detectable antiviral antibody resulting in either lysis of enveloped viruses (virolysis) or neutralization of viruses. The interaction of complement with antiviral antibody enhances antibody-mediated virus neutralization, virolysis and lysis of virus-infected cells.

B Cell Responses

Virus-specific sIgA constitutes the principal antibody response to viral respiratory pathogens. Responses involving other isotypes also occur, although whether responses originate locally or systemically is largely unknown. The degree of local antibody response is

influenced by the local availability of antigen and the ability of the virus to replicate in the respiratory tract.

Anti-viral activities of antibodies include:

(i) neutralization of viral infectivity by inhibition of virus attachment, penetration, uncoating or transcription of viral nucleic acid;

(ii) enhancement of virus phagocytosis by opsonization;

(iii) lysis of virus-infected cells by antibody-dependent cell-mediated cytotoxicity;

(iv) virolysis and lysis of virus-infected cells by complement-mediated antibody-dependent cytotoxicity.

sIgA has been shown to be capable of virus neutralization (Ogra et al., 1968; Taylor & Dimmock, 1985a). In contrast to IgM and IgG, sIgA is relatively ineffective in opsonization and in activation of the complement cascade. Although ALMs were intially thought not to possess Fc receptors for IgA, more recently IgA receptors have been detected on activated ALMs (Gauldie et al., 1983). IgA does not activate complement by the classical pathway and it is uncertain whether non-aggregated IgA can activate the alternate pathway (McDermott et al., 1982).

The principal role of local antibody is the prevention of re-infection. However, protection may not necessarily correlate with sIgA and may be mediated by other isotypes.

T Cell Responses

T cells interact with virus-infected cells, rather than cell-free virus, in an MHC restricted manner, either by direct lysis of infected cells or by the release of lymphokines (Doherty, 1985). The generation of virus-specific Tc cells and their ability to clear virus in vivo in mice has been shown for many viral infections. Mononuclear phagocytes are recruited and activated by the release of chemotactic factors, gamma-IFN, macrophage activating factor and migration inhibition factor.

T cell responses appear to be principally involved in recovery from viral infections. Patients with selective deficits of T cell immunity may continue to shed RSV, parainfluenza virus and other respiratory viruses from the respiratory tract for prolonged periods, often in association with extrapulmonary spread of the virus (Fishaut et al., 1980).

1.2.4.2 Immunopathology of Viral Respiratory Infections

The only available data on the role of immune responses in the pathogenesis of viral respiratory infections pertain

to antibody and T cell responses. Pathological consequences may arise from antibody responses due to the formation of immune complexes and development of homocytotropic IgE responses. Whereas there is no evidence to suggest that sIgA contributes to immunopathology, other isotypes may do so. Immune complex formation has been demonstrated during RSV infection and may contribute to the development of bronchospasm through the release of arachidonic acid metabolites (Faden et al., 1983). The development of a virus-specific homocytotropic IgE response, associated with increased histamine levels in naso-pharyngeal secretions, has been implicated in the development of bronchial hyperactivity during viral respiratory infections (Welliver & Ogra, 1983).

The role of T cell responses in the pathogenesis of viral respiratory infections has only been studied with influenza. Although the development of a Tc cell response is essential for complete recovery, evidence for a pathological role of Tc cells is suggested in murine studies using live attenuated influenza viruses (Section 1.3.7.4). In addition, influenza-specific Td cells mediating Class II restricted DTH activity are known to have a detrimental effect on recovery, although the induction of these cells may be regulated by Ts cells during infection (Section 1.3.7.3).

1.2.5 Local Immune Responses to Specific Viral Respiratory Pathogens (excluding influenza)

1.2.5.1 Respiratory Syncytial Virus (RSV)

RSV is the major cause of lower respiratory tract infections (bronchiolitis and pneumonia) in infants and small children and is also frequently responsible for croup and minor upper respiratory infections. Re-infection occurs frequently, although attack rates are lower in older children and adults and the clinical course is usually milder.

Local antibody responses, involving IgA, IgM, IgG and IgE develop in the respiratory tract after natural RSV infection (Scott & Gardner, 1970). However, the development and persistence of RSV specific sIgA in infants correlates poorly with protection against re-infection and development of bronchiolitis in subsequent infections (McIntosh et al., 1978). The development of bronchiolitis and bronchospasm during subsequent infections appears to relate to the development of homocytotropic IgE responses. IgE bound to respiratory epithelial cells develops following infection and usually disappears in 10 to 14 days. However, patients with persistent IgE responses are significantly more likely to have bronchiolitis and bronchospasm. The presence of RSV IgE is associated with increased histamine levels in nasopharyngeal secretions and the development of

bronchospasm during subsequent infections (Welliver et al., 1981). The persistence of IgE responses in these patients is associated with a defect in RSV specific Ts cell function and recovery of Ts function correlated with protection against RSV-induced bronchospasm (Welliver et al., 1983).

Local cellular responses, including the development of NK cell activity and RSV specific Tc cell responses, have been described in the lungs of cotton rats infected with RSV (Taylor et al., 1985; Kumagai et al., 1985). In children infected with RSV there is an increase in lymphoproliferative responses of peripheral blood lymphocytes which correlates with the severity of illness (Welliver et al., 1979). The detrimental effect of inactivated RSV vaccine is thought to have resulted from the induction of Td cells mediating DTH reactions (Sissons & Borysiewicz, 1985).

1.2.5.2 Parainfluenza Viruses

Parainfluenza viruses (Types 1-3) are important causes of respiratory illnesses, especially croup, in young children. Specific antibody is detectable in serum and respiratory secretions after natural parainfluenza infection. Clinical studies with parainfluenza virus, Types 1 and 2, have suggested a closer correlation between protection against re-infection and nasal antibody levels rather than serum antibody levels (Smith et al., 1966; Tremonti et al., 1968). However, neutralizing activity in

nasal secretions has not always correlated with IgA levels (Yanagihara et al., 1978). Protection mediated by antibody is type specific (Welliver et al., 1982a). Virus specific IgE responses occur regularly, though are of greater magnitude in patients with croup and are associated with increased histamine levels in nasopharyngeal secretions (Welliver et al., 1982b). The role of these responses in the pathogenesis of croup is unknown.

There is no data available on cell-mediated responses to these viruses in humans. Considerable data, however, exist on Sendai virus, a murine paramyxovirus closely related to parainfluenza Type 1 virus. Specific Class I restricted Tc and Td cells are induced by Sendai virus, provided the virus retains its fusion capability (F^+). F^- Sendai virus preparations (inactivated by ultraviolet irradiation) fail to elicit Tc cell responses but induce Class II restricted Td cells (Ada et al., 1981).

1.2.5.3 Rhinovirus

Rhinovirus, of which there is over 100 serotypes, is the major known cause of the common cold. Unlike RSV and parainfluenza virus infections, rhinovirus infection does not commonly involve the lower respiratory tract. Specific secretory antibody of the IgA, IgM and IgG classes appear in nasal secretions and may protect against homotypic re-infection. It is not known whether antibody contributes

to immunopathology in rhinovirus infections, although bronchospasm may develop in patients with bronchial reactivity. There are no data on cell-mediated responses (Ogra et al., 1984).

1.2.5.4 Adenovirus and Coronavirus

The data on the immune responses to adenovirus and coronavirus are limited to studies correlating protection with serum antibody responses.

1.3 Immune Responses to Influenza Infection

The purpose of this section is to review the nature of the immune response to influenza A virus, both in hosts which experience natural infection, particularly man, and in hosts which are experimentally infected, particularly mice.

1.3.1 Properties of the Components of the Influenza Virion

Influenza is a myxovirus which can be highly pleomorphic but, where well adapted, most particles are spheres with a diameter between 80 and 120nm. When examined by negative staining, the particles show the presence of "spikes" which represent the two surface glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA).

1.3.1.1 The Haemagglutinin

The HA molecule accounts for about 25% of the viral protein. It mediates the initial attachment of the virion to a cellular receptor and possesses a fusion capability that enables the virus envelope to integrate with an intracellular membrane, so allowing the internal components access to the cell cytoplasm. The HA molecule is present in the virion as a trimer (= "spike"). Each monomer exists as two chains, HAI and HA2, linked by a disulfide bond. The precursor single polypeptide must be cleared to produce HAI and HA2 for the virus particle to be infectious. HA2 is

anchored in the membrane by a sequence of 25-32 hydrophobic amino acids at the C terminus. Each HA molecule contains two main regions, a triple-strand coil of alpha helices and a globular region of anti-parallel beta-sheets (Wiley et al., 1981; Wilson et al., 1981). The cell-receptor binding site and the variable antigenic determinants are located on the globular domain. Complete amino acid sequences of the HA for two H3 strains and partial sequences, deduced from cDNA copies, for 32 A strains are available. The highest homology (80%) is between H3 and H5, and the lowest (25%) between H1 and H3. There is generally > 90% homology between strains of a given subtype.

1.3.1.2 The Neuraminidase

The NA is an enzyme which cleaves sialic acid residues from any oligosaccharide chain possessing that terminal sugar with the correct glycosidic linkage, including residues on both the HA and NA. Before replication, the NA may act on non-specific inhibitors in the extracellular fluids and possibly release virus from mucous. After assembly of the virus, the NA acts not only on substrates in or on the infected cell, but also on the virion itself. The NA exists as a spike on the virion with a "head" containing four coplanar and approximately spherical subunits, a centrally attached "stalk" with a transmembrane hyrophobic region (Laver & Valentine, 1969). Complete amino acid sequences of five N2 neuraminidases and partial sequences of

others are known. The overall homology between N1 and N2 proteins is 39%. The three-dimensional structure of pronase-isolated NA heads has been determined by X-ray crystallography to a 2.9Å resolution (Varghese et al., 1983; Colman et al., 1983). The polypeptide chain is arranged in six topologically identical four-stranded anti-parallel beta-sheets, giving an overall appearance of propeller blades. The catalytic site appears to be a large pocket on the distal surface which is formed by some 18 residues which are conserved between subtypes.

1.3.1.3 The Matrix Protein

The virion contains a third envelope associated protein - the matrix (M) protein, which is the major component in the virion. The complete amino acid sequence of the protein has been deduced from base sequences and indicates that the sequences are very highly conserved.

1.3.1.4 Other Virion Proteins

Inside the viral envelope there are eight RNA segments and products of five of these. The influenza virus genome consists of eight single-stranded (negative sense) RNA molecules ranging in size from 890 to 2,341 nucleotides. All RNA segments encode a single polypeptide, except the two smallest, each of which encodes two polypeptides.

The three polymerase (P) proteins - PA, PB1 and PB2 - transcribe the viral RNA. The nucleoprotein (NP) probably constitutes the backbone of the helical complex that is sometimes displayed by disrupted virions. The virus also encodes two non-structural proteins - NS1 and NS2 - neither of which are found in the virion, and an M2 protein which is not found in the virion but is expressed on the surface of infected cells.

An aspect of increasing interest is the expression of viral proteins other than HA and NA at the surface of the infected cell. It has been shown (Virelizier et al., 1977) and confirmed (Yewdell et al., 1981) that NP is expressed at the infected cell surface and this may also occur in cells transfected with DNA encoding NP (Townsend et al., 1984). Different results have been found with matrix protein. Investigators using polyclonal antisera suggested that M was expressed at the cell surface (Ada & Yap, 1977; Biddison et al., 1977; Reiss & Schulman, 1980a); in contrast, workers using monoclonal antibody preparations have found only very small amounts expressed at the cell surface (Hackett et al., 1980; Yewdell et al., 1981). The different results may either be explained by contamination of the polyclonal antisera with anti-HA antibodies, though this has not been demonstrated and steps were taken to eliminate such a possibility (Reiss & Schulman, 1980a) or possibly by the anti-matrix sera recognizing those amino acid sequences shared between M1 and M2. M2 protein has been found to be

expressed in considerable amounts at the infected cell surface (Lamb & Choppin, 1983), as has NS1, a nonstructural protein (Shaw et al., 1981).

The extent to which other internal virion proteins may be expressed at the infected cell surface is not clear. It is assumed that a similar surface expression pattern occurs in infected cells in vivo.

Recent data on the susceptibility of virus infected target cells to Tc activity suggests that antigenic fragments of some viral proteins are expressed at the cell surface and are recognized in an MHC restricted fashion. This has been demonstrated for NP and HA and may be the case for other influenza viral proteins (Germain, 1986).

1.3.2 Antigenic Properties of the Viral Proteins

1.3.2.1 B Cell Recognition

It has been known for a long time that antibodies which neutralize the infectivity of influenza virus bind predominantly to the HA. There is general agreement that there are four major antigenic sites which have been variously designated as: Site A (loop), a.a. 140-146; Site B (tip), 187-196; Site C (hinge), a bulge in the tertiary structure at the disulfide bond between Cys 52 and Cys 277;

and Site D (interface), which is the interface between subunits in the HA trimer (Webster & Laver, 1980; Wiley et al., 1981; Gerhardt et al., 1981). Recently a panel of monoclonal antibodies were tested for their binding to different physical forms of the HA molecule and their ability to neutralize virus infectivity (Nestorowicz et al., 1985). Antibodies, including neutralizing antibodies to important antigenic sites of a viral surface antigen, reacted with conformational determinants, some of which may be quaternary structures and therefore are nonlinear (discontinuous) amino acid sequences.

Variation in amino acid sequence of the NA has been observed to occur in regions which form a nearly continuous surface at the top of a subunit. The catalytic site, which is a large pocket of the distal surface, thus appears to be surrounded by variable regions. Studies with monoclonal antibodies to laboratory-derived mutants indicate that these variable sequences are also antigenic (Colman et al., 1983).

The internal viral proteins of the virus have not been examined to the same extent as the two surface glycoproteins. Antigenic variability in the NP and M protein have been detected by the binding of monoclonal antibodies; these changes occurred independently of those in the surface glycoproteins (Van Wyke et al., 1980 & 1984).

1.3.2.2. T Cell Recognition

Regulatory T Cells

Studies on the ability of human and murine HA-specific T cell clones to respond in proliferation assays to either synthetic peptides of the HA molecule or virus mutants with known amino acid substitutions have yielded comparable results (Lamb et al., 1982; Hackett et al., 1983; Hurwitz et al., 1984). Three immunological regions were identified - a 25 amino acid segment at the carboxyterminus of HAI, a nonapeptide (residues 111-119) located at the globular head region and a third sequence including residue 136. These sites are distinct from the four proposed antibody-binding sites involved in virus neutralization. Interestingly, T cell antigenic sites tend to have amphipathic structures, that is structures with separated hydrophobic and hydrophilic surfaces, with the hydrophobic residues displaying periodicity (Allen et al., 1985; Streicher et al., 1984). Amphipathic periodicity has also been demonstrated for the two well-defined determinants of HAI (De Lisi & Berzofsky, 1985).

There have been several reports of T cell clones which recognize viral proteins other than HA, including NA, M and NP and which exhibit helper activity (Lamb et al., 1982; Sterkers et al., 1985). An early report that Th cells recognizing an internal antigen (M) could provide help for a

subsequent anti-HA antibody response (Russell & Liew, 1979) has recently been confirmed but only for an IgM response (Scherle & Gerhard, 1986). In contrast, Th cells recognizing the HA are cross-reactive for an IgG response.

There is little information available about antigen recognition by Ts cells. High concentrations of a synthetic peptide of HA rendered cloned Th cells unresponsive to subsequent re-stimulation, however the dose of the peptide required was sufficiently high to doubt whether such a mechanism could operate in vivo (Lamb et al., 1983).

Effector T Cells

Our knowledge about antigen recognition by effector T cells comes almost entirely from recent studies of one subset of T cells - cytotoxic cells. Several groups have demonstrated that Tc cells raised to one A strain virus could lyze target cells infected with any A strain, but not a B strain virus (Doherty et al., 1977; Zweerink et al., 1977; Braciale, 1977). Limiting dilution analysis subsequently showed that the majority of Tc cells were "cross-reactive" (Owen et al., 1982) and work with murine T cell clones showed that they could be divided into three groups: those that were specific for the HA of the stimulating virus; those which recognized HA of all viruses of a given subtype; and those which recognize target cells infected with any A strain virus (Braciale et al., 1981).

The pattern of association required to render a target cell susceptible to lysis is generally considered to reflect a similar pattern on the presenting cell responsible for the induction or effector stage, but has been mainly done at the latter.

A variety of methods has been applied. The most direct involve the treatment of inducer or target cells with one of three techniques: (1) transfection with known DNA sequences coding for particular proteins; (2) infection with recombinant (hybrid) viruses which express the protein coded for by the inserted DNA; and (3) fusion with preparations, usually liposomes, containing defined proteins. Another approach has been to compare parental viruses with reassortant or mutant strains and this may implicate a specific viral protein. In Table 1.1 results are assembled which indicate the viral proteins recognized by Tc cells. There can be little doubt that in both murine and human studies HA is recognized by Tc cells, but the evidence now suggests that (i) about 10-15% of Tc precursor cells recognize HA, and (ii) there is some cross-recognition between H1 and H2, but very little, if any, between H2 and H3. There is little known about the epitopes recognized by Tc cells (Section 1.3.1.4). There are no reports unequivocally demonstrating that NA is recognized by Tc cells, though this is likely to be the case (Sterkers et al., 1985). Kees and Krammer (1984) using limiting dilution analysis of early responses to reassortant viruses found

that up to 90% of Tc cells in C57Bl/6 mice recognized internal proteins. Yewdell et al. (1985) claim a "significant" proportion of cross-reactive Tc cells in BALB/c mice recognize NP. Andrew et al. (1986) using limiting dilution to determine precursor frequency find about 30% recognizing NP.

Pala et al. (1986), using L cells transfected with NP and Db genes found a great variation in the frequency of NP-specific Tc between individuals in an inbred mouse strain, with some showing no recognition of NP, though having a strong A virus-specific cross-reactive Tc response. It has also been pointd out that there are at least two classes of NP-specific Tc, one which is fully cross-reactive between all A strains and another which distinguishes NP in two groups of type A viruses, isolated between 1934-1943 and 1943-1979 (Townsend & Skehel, 1984). There may be three determinants on the NP molecule recognized by Tc (Townsend et al., 1985). Bennink and colleagues (J. Bennink, personal communication) have constructed vaccinia virus recombinants containing the DNA coding for other viral antigens and have found that all three polymerases are recognised by murine Tc cells.

Are nonstructural viral antigens - M2, NS1, and NS2 - recognized by Tc cells because it is clear that M2 and NS1 are well expressed at the infected cell surface, a finding which per se would suggest that these antigens are

candidates for recognition? Bennink and colleagues (J. Bennink, personal communication), using recombinant vaccinia preparation, find that NS1 but not M2 is recognized by Tc cells.

In contrast to the extensive work with effector cells expressing Tc-cell activity, little work has been done on effector cells expressing DTH, except to show that populations which are specific to one subtype or are cross-reactive may be produced (e.g. Ada et al., 1981). Preparations may be class I or II MHC antigen-restricted. In view of the fact that cloned T cells may show both cytotoxic and DTH activities (e.g. Lin and Askonas, 1981) and cells expressing cytotoxic activity may be class I or II MHC antigen-restricted (Lukacher et al., 1985), the antigenic recognition patterns seen with Tc cells probably apply also to cells expressing only (detectable) DTH activity.

1.3.3 Antigenic Drift and Shift

The two surface antigens of influenza A viruses undergo two types of antigenic change. Antigenic drift is the term describing minor changes in the HA and NA; antigenic shift involves major changes in these two surface glycoproteins. At present, there is no method available for predicting the precise changes which occur in either of these two processes.

Antigenic drift in both the HA and NA occurs by point mutation in the genes, resulting in an accumulation of amino acid sequence changes. Three mechanisms have been proposed to explain antigenic shift. The change which occurred in 1968 when H3 replaced H2 is so large that it has been proposed that the H3 human virus could only have been derived by genetic reassortment between human and animal or human viruses. Viral genetic reassortment between humans and between humans and lower animals have been observed and several studies involving genetic and biochemical analysis support this mechanism for the appearance of H2 and H3 strains. In contrast, the strain of H1N1 that appeared in northern China in 1977 and spread worldwide is so similar in all genes to the virus which caused an epidemic in 1950 that it is reasonable to propose that the earlier virus had remained dormant for the 27-year interval, e.g. in a frozen state. The third possibility is that an animal or bird virus changed and became infectious for humans. An example is the isolation of identical influenza viruses from pigs and humans on the Wisconsin farm in the mid-1970s (Hinshaw et al., 1978).

1.3.4 Patterns of Infection

1.3.4.1 Influenza in Humans

Human influenza is primarily an infection of the upper respiratory tract and major central airways. The pathology,

characterized by desquamation of the epithelium, involves the nasal mucosa, larynx and tracheobronchial tree. Influenza pneumonia occurs rarely and is characterized histologically by a desquamative interstitial pneumonitis. Although physiological abnormalities of small airways function may be demonstrated in uncomplicated influenza (Hall & Douglas, 1980), these abnormalities relate to airway hyperreactivity and do not necessarily reflect local infection.

The major method of transmission of influenza virus is airborne; direct spread via infectious droplets is less common (Murphy & Webster, 1985). Inhalation of infected aerosols may result in the deposition of virus throughout the respiratory tract, including the alveoli. It is unclear why there is subsequently less pathological involvement at the alveolar level. Influenza virus may be more efficiently cleared by local defense mechanisms, especially the alveolar macrophage, preventing alveolar infection. Alternatively, the alveolar epithelium may be less susceptible to infection as suggested by in vitro studies (Rosztoczy et al., 1975).

1.3.4.2 Influenza in Animals and Birds

Influenza infection occurs naturally in many avian species and in some mammals including pigs, horses, seals and mink. The study of infection in these hosts has

provided valuable data on the epidemiological links between avian, mammalian, and human influenza viruses, particularly on the basis of antigenic shift of human influenza viruses. However, studies on the immune response have been largely restricted to experimental infections in mice and ferrets.

The mouse has been the most widely used animal for experimental studies of influenza virus infection. Its advantages over other animal models, aside from the similarities between murine and human influenza pneumonia, include the ease of breeding and availability of inbred strains, the extensive background knowledge of the murine immune system and the MHC, and the availability of well-defined reagents for characterizing the components of the immune response. The pattern of infection in mice depends on the degree of host adaptation of the virus. Infection with unadapted strains generally does not produce overt disease, unless very high inocula are used, although virus may replicate in the lungs, bronchi, and nasal mucosa. Infection with mouse-adapted strains, either intranasally or by aerosol, results in severe disease with alveolar involvement as the predominant finding. The pulmonary pathology in mice is similar to that seen in influenza pneumonia in humans. Lung viral titres peak within 3-4 days and decline to undetectable levels by 10 days, although viral antigen may be detected, by ELISA, up to 2 months after infection (Astry et al., 1984).

Intranasal inoculation of ferrets with influenza virus results in a similar disease to uncomplicated human influenza, with a similar distribution of viral antigen in the respiratory tract. There is little evidence for alveolar involvement even during infection with virulent clones (Sweet et al., 1981). These observations are consistent with the demonstration that ciliated epithelial cells from ferrets are better able to support virus replication and to release virus than are alveolar cells (Cavanagh et al., 1979). However, the ferret is limited as a model for studying the immune response, primarily by lacking the advantages of the murine model as well as by the difficulty and expense in maintaining sufficient numbers of ferrets for detailed experiments.

1.3.5 Nonspecific Responses

The nonspecific immune system in influenza infection has been evaluated either by delineating the components of the system separately - namely fever, macrophages, NK cells, IFN and complement - or by examining the system collectively and independently of the specific arm of the immune response. The complex interactions within this system and with the specific arm of the immune response, however, make it difficult to dissect the roles of the respective components in recovery from influenza infection.

1.3.5.1 In Experimental Influenza

In experimental influenza the nonspecific immune response is implicated in limiting viral spread and initiating recovery. Several observations have been made which tend to substantiate this role. Firstly, pretreatment of mice with P. acnes (C. parvum) before a lethal virus challenge results in lower lung viral titres and lower mortality (Mak et al., 1983; Gangemi et al., 1983). This protective effect correlates with increased lung IFN levels, macrophage content, and NK cell activity in the absence of any demonstrable change in either T or B cell function. Secondly the survival of athymic mice following a sublethal infection with influenza virus is dependent on either the nonspecific immune response or IgM antibodies (Yap & Ada, 1979). The persistence of virus in the lungs of surviving mice for prolonged periods suggests, however, that neither mechanism is sufficient for complete recovery. Finally, the early decline, during the 2nd and 3rd day, of nasal virus titres in infected ferrets precedes the development of a measurable specific immune response and correlates with the degree of preceding pyrexia and inflammatory cellular infiltrate (Toms et al., 1977).

The role of fever as a host defense mechanism is suggested by the correlation between the height of fever and subsequent rate of decline of nasal virus titres in ferrets. Furthermore viral replication in nasal turbinate

organ cultures is inhibited at pyrexial temperatures (Sweet et al., 1978) and suppression of fever in ferrets by nonpharmacological means results in delayed clearance of virus (Husseini et al., 1982).

ALMs from normal murine lungs are susceptible to infection by influenza virus in vitro (Rodgers & Mims, 1981; Wells et al., 1978). Although the virus undergoes abortive replication only, infectious virus, possibly on the surface of macrophages, may form infectious foci when cocultured with susceptible cells. Alveolar macrophages recovered from influenza-infected mice acquire resistance to in vitro infection which is induced by IFN (Rodgers & Mims, 1982b). Macrophages recovered from influenza-infected lungs may also mediate lysis of infected cells independent of antibody (Mak et al., 1982a). Influenza-infected macrophages also produce IFN and may act as APCs (Wyde et al., 1982; Lyons & Lipscomb, 1983). The effect of influenza virus infection on release of interleukin-1 has not been studied.

NK cells may play a role in limiting viral spread by lysis of virus-infected cells and production of IFN (Welsh, 1981). Enhanced levels of NK cells can be detected in pulmonary lymphocytes 48h after influenza infection in mice (Leung & Ada, 1981; Stein-Streilein et. al., 1983).

Pulmonary levels of type 1 (alpha-, beta-) IFN rise rapidly during murine influenza infection and correlate directly with the degree of viral replication (Wyde et al., 1982). Alveolar macrophages and lymphocytes recovered early from infected lungs are the major sources of IFN released in vitro. Intranasal instillation of anti-IFN serum results in an increase in viral titres and an increase in host mortality (Hoshino et al., 1983). In mice bearing the Mx gene an increased resistance to influenza virus, attributable to enhanced sensitivity to alpha/beta IFN can be abolished by pretreatment with anti-IFN serum (Haller, 1981). These observations indicate a definite role for IFN in limiting viral spread.

Virus particles themselves, virus-infected cells, and virus-induced desialation of cells may activate complement by either the classical or alternate pathways (Lambre et al., 1983). Subsequently binding of complement in the absence of specific antibody may neutralize virus or result in lysis of virus particles. The importance of complement in influenza infection is shown by the increased mortality in mice depleted of complement and in C5-deficient mice, although the major protective effect of complement was mediated late in infection and probably through antibody-dependent lysis of infected cells (Hicks et al., 1978).

1.3.5.2 In Human Influenza

While there are no data in humans comparable to that in experimental influenza which support a role for the nonspecific immune system in recovery from influenza, descriptions of the responses in humans are consistent with those in animal models.

ALMs recovered from human bronchoalveolar cell preparations are susceptible to influenza in vitro, though replication is abortive (Rodgers & Mims, 1982a). ALMs infected in vitro produce alpha-IFN and retain the ability to act as accessory cells (Ettensohn & Roberts, 1984).

Large granular lymphocytes (LGLs) from peripheral blood lymphocytes may be stimulated in vitro by influenza virus (Djeu et al., 1982), resulting in enhanced NK activity and production of alpha- and gamma-IFN. Furthermore, LGLs provide an accessory function in the generation of influenza-specific Tc cells through unidentified soluble factor(s) (Burlington et al., 1984). Enhanced NK activity may also be detected in circulating lymphocytes early after influenza infection in volunteers (Ennis et al., 1981a).

Alpha-IFN is detected in acute-phase sera during natural infection (Ennis et al., 1981a; Green et al., 1982). Local nasopharyngeal IFN levels peak early after artificial challenge and correlate directly with nasal virus

titres (Murphy et al., 1973). In vitro stimulation of peripheral blood lymphocytes results in the production of both alpha- and gamma-IFN, higher levels of gamma-IFN being produced in recently vaccinated volunteers (Ennis & Meager, 1981).

1.3.6 The Antibody Response

1.3.6.1 Dynamics of the Response

The class-specific antibody response to the haemagglutinin subunit has been studied in persons challenged with live attenuated vaccines, using the ELISA technique. A characteristic primary serological response was observed in children experiencing a primary infection (Murphy et al., 1982). Serum IgM and IgG responses occurred in all cases whereas IgA responses occurred less frequently and were less marked. IgA formed the major response in nasal secretions and occurred in most cases. A secondary antibody response was detected in young adults primed by natural infection and challenged subsequently with a live attenuated subtype variant (Burlington et al., 1983). Serum IgG and IgA responses occurred in most cases; there was a correlation between serum and secretory IgA responses but not IgG responses. IgM responses were detected infrequently in serum and not in nasal secretions.

Serum HI titres gradually decrease over the first 6 months after infection and may then persist for several

years as subsequent infections by related virus strains boost titres ("original antigenic sin"). The finding of antibody to H3 in 1968 in persons born before 1892, and to H1 in 1976 in persons born before the early 1950's, illustrates this (Couch & Kasel, 1983). In contrast, only about 30% of adults will have detectable neutralizing antibody in nasal secretions 1 year following intranasal immunization with an inactivated vaccine (Waldman et al., 1973). Nasal wash IgA was detectable, by ELISA, in children 3-6 months after natural infection but not 10-18 months after immunization with a live attenuated virus vaccine (Wright et al., 1983).

Data on the antibody response in the lower respiratory tract of humans are limited. Waldman has demonstrated a threefold increase in HI antibody in bronchoalveolar lavage fluid following aerosol administration of an inactivated vaccine (Waldman et al., 1973). Following intranasal challenge with a low dose of live attenuated vaccine, an antibody response in the lower respiratory tract was observed in primed individuals and was predominantly IgG in nature (Zahradnik et al., 1983). Antibody responses in all classes develop in mice within the 1st week, though titres in bronchial washings were markedly lower than those in serum (Zee et al., 1979).

ASCs have been detected by measuring antibody production from cultured human peripheral blood

lymphocytes. Immunization with either live or inactivated vaccines has been shown to result in the appearance of circulating ASCs (Yarchoan et al., 1981; Mitchell et al., 1982). In experimental infection in ferrets and mice, ASCs detected by the haemolytic-plaque technique have been found in mediastinal lymph nodes and spleen (McLaren & Butchko, 1978, Reiss & Schulman, 1980b).

Responses to Other Viral Proteins

Using sensitive assays, antibody to the NA subunit can be detected in serum during both primary and secondary infections and may subsequently persist for many years. The dynamics of anti-NA antibody formation and duration in nasal secretions in primed individuals after natural infection are similar to those for the anti-HA response, with IgA the major isotype (Hruskova et al., 1976). Antibody responses to the M protein and NP also develop frequently after infection. There are no reports of the antibody response in man to minor viral proteins though polyclonal antisera and monoclonal antibodies to the polymerase and NS1 protein have been prepared in animals.

1.3.6.2 Specificity of the Response

The specificity of antibody to the HA subunit in the context of the broad antibody response following infection has been characterized using virus-adsorbed sera. The

antibody population consists of antibodies to strain-specific determinants of the HA of the infecting strain and of previous infecting subtype variants and cross-reacting antibodies to shared determinants of different subtype variants. The relative composition of the antibody response is largely dependent on the host's prior antigenic experience. After natural infection, the majority of unprimed children produce predominantly strain-specific antibodies to the infecting virus; cross-reacting antibodies constitute only a small component of the response (Oxford et al., 1981a). In contrast, the majority of adults, previously exposed to an earlier subtype variant, produce predominantly cross-reacting antibodies as well as strain-specific antibodies to the previously encountered strain (Oxford et al., 1979).

These observations have been subsequently confirmed at the clonal level by analysis of the specificity of antibodies produced by stimulation of individual B cell precursors in limiting dilution cultures (Yarchoan & Nelson, 1984). It was further shown that viruses of one subtype may stimulate the production of antibodies specifically directed to the HA of a different subtype or cross-reactive antibodies to shared determinants on either the HA or other proteins of different subtype viruses. The availability of monoclonal antibodies to known epitopes on the HA molecule now allows an examination of the response of individuals to particular epitopes, by testing the ability of their sera to

compete with the monoclonal preparations for binding to the HA.

The specificity of the secretory antibody response is less defined although similar patterns of a predominantly homotypic response with reports of a broadened heterotypic response dependent on previous antigenic exposure have been described (Waldman et al., 1970; Shvartsman et al., 1977). In some instances the specificity of the secretory response has been broader than that of the corresponding serum response.

1.3.6.3 Antiviral Effects of Antibody

Neutralization of Infectivity

Dimmock and colleagues (Possee et al., 1982; Taylor & Dimmock, 1985a,b) have recently studied this aspect using different forms of antibody - IgG, IgM and monomeric and sIgA. Profound differences were found between the monomeric (IgG, IgA) and oligomeric (IgM, sIgA) forms. The surprising initial finding was that type A influenza viruses, neutralized by polyclonal or monoclonal anti-HA IgG, attached to a variety of susceptible cell types, at temperatures between 4° and 37°C, with kinetics indistinguishable from those of nonneutralized virus. Furthermore, the kinetics of internalization of neutralized virus, its subsequent uncoating, and the transport of virion

RNA to the cell nuclei was unchanged compared with infectious virus. Loss of infectivity seemed to result from the inhibition of a later stage in the replication cycle, thought to be inactivation of the viral transcriptase. Virus neutralized by monomeric IgA did not prevent attachment of the virus to susceptible cells, but its action was not investigated further.

In contrast, neutralizing IgM and sIgA prevented the attachment of up to half the virus to the cells and the portion which did attach was not internalized. These authors point out that virus-IgM complexes on the cell surface could well render that cell susceptible to complement-mediated lysis.

Lysis of Virus-Infected Cells

Antibody may modify infection by restricting viral spread by lysis of virus-infected cells. This effect is mediated either by the lytic action of complement or by antibody-dependent cell-mediated cytotoxicity (ADCC).

Complement-mediated lysis of virus-infected cells proceeds through the alternate pathway of complement (Perrin et al., 1976). Although the alternate pathway may be activated by virus-infected cells independent of antibody, lysis only occurs in the presence of specific antibody. Antibody mediating this effect is subtype specific and

develops after influenza infection and vaccination (Verbonitz et al., 1978).

Similarly, antibody responsible for ADCC appears after natural infection and vaccination (Hashimoto et al., 1983). Effector cells in human blood mediating ADCC include NK cells, neutrophils, and monocytes. The NK cells are the most efficient effector cells and require less antibody to produce cytolysis than the complement-mediated mechanism. As the responsible antibody primarily recognizes the HA molecule on the infected cell surface, ADCC is also subtype specific.

1.3.6.4 Protective Role of Antibody

Anti-HA Antibody

Evidence for the protective role of antibody is either based on demonstrating a correlation between antibody level and resistance to infection or, more directly, prevention or modification of infection mediated by passive transfer of antibody. Field and volunteer studies have shown that resistance to infection is correlated with serum anti-HA antibody levels. However, direct evidence that antibody is the mediator of protection has largely come from animal studies. Passive transfer of immune serum will protect recipients against homotypic challenge (Virelizier 1975). This is more clearly seen in immunosuppressed recipient mice

unable to mount an active host response. That antibody is essential for protection is demonstrated by the finding that mice selectively suppressed for antibody production can recover from infection, yet are susceptible to reinfection in the absence of RIA-detectable antibody (Kris et al., 1985). A beneficial effect of seroprophylaxis in human influenza has also been claimed in studies cited by Shvartsman & Zykov (1976).

Infection also confers a significant but lesser degree of immunity to subtype variants, although the extent to which this is due to antibody is unclear. The observations that heterotypic immune serum passively transferred in mice is less protective than homotypic serum (Virelizier 1975), and that cross-reacting antibody to shared determinants on subtype variants is less effective in neutralizing virus in vitro (Haaheim & Schild, 1980), are not inconsistent with a role of antibody in modifying infection.

Epidemiological observations in humans also indicate that infection with one subtype does not confer immunity to other subtypes and mice are not protected by the passive transfer of immune serum prior to challenge with a different subtype virus (Virelizier, 1975).

Relative Importance of Serum and Secretory Antibody

To be protective, anti-HA antibody must be present at

the mucosal surface, having been either produced locally or derived from serum. Resistance to infection in humans has been correlated with anti-HA in nasal washings of either the IgG or IgA isotypes (Couch et al., 1981; Clements et al., 1983). Further evidence that IgG is protective has been inferred from the inverse relationship between transplacentally acquired antibody in infants and the frequency of influenza infection (Puck et al., 1980). Maternally derived antibody in animal studies has also been shown to be protective though protection is mainly acquired during suckling (Reuman et al., 1983; Husseini et al., 1984). However, the protective effect of passively acquired antibody from immune mothers or in transfer experiments is limited to the lower respiratory tract, primarily the lung, in ferrets and mice - infection in the upper respiratory tract and trachea is not prevented. The protection afforded by serum-derived IgG to the lower respiratory tract is consistent with the greater proportions of IgG relative to IgA in lower respiratory tract secretions compared with the lower proportion of IgG in nasal washings.

Immunoglobulin isotypes are distributed in the human respiratory tract in a similar pattern with the relative proportion of IgG to IgA increasing in the lower part. The degree to which serum-derived IgG contributes to prevention of infection may accordingly vary, depending on the site of virus deposition. IgA in nasal washings may assume an apparent greater importance in protection against virus challenge in human volunteer studies when virus is usually

administered by intranasal droplets. When virus is transmitted naturally by aerosol, it is deposited throughout the respiratory tract and locally produced antibody, IgA and possibly IgG, with an additional effect from serum-derived IgG, primarily to the lower respiratory tract, prevent infection.

Role of Antibody in Recovery

Since patients with agammaglobulinemia recover from influenza infection, it is evident that antibody is not essential for recovery. However, the beneficial effect of hyperimmune serum in modifying disease in humans with influenza suggests an additive role for antibody in recovery (Shvartsman & Zykov, 1976), and this is supported by animal studies. Passive transfer of immune serum to normal mice early after infection had an additive effect in reducing lung virus titres (Yap & Ada, 1979). However, passive transfer of antibody to infected nude mice, though it initially lowers lung virus titres and prevents dissemination, has a transient effect as virus shedding reappears in 1-2 weeks (Askonas et al., 1982a). By selectively suppressing the antibody response with anti-IgM, Kris et al. have shown that mice can recover in the absence of antibody (Kris et al., 1985).

Antibody to Other Viral Proteins

Antibody to the NA can neutralize viral infectivity in vitro or in vivo only in extremely high concentrations, probably due to steric hindrance of HA-binding sites. However, smaller amounts of anti-NA antibody inhibit the release of virus from infected cells, possibly by cross-linking budding virus particles. The protective effect of anti-NA antibody in human influenza has been evaluated in field studies following the introduction of the H3N2 subtype and in challenge studies, with volunteers primed naturally or artificially by NA-specific vaccines, allowing an assessment of protection independent of an anti-HA antibody response. The presence of anti-NA antibody has been inversely correlated with infection rates and more consistently with a reduction in clinical illness (Schulman, 1975). Similarly, studies in animals have shown a protective effect of actively acquired anti-NA antibody, primarily in modifying infection rather than prevention. This effect can be reproduced by passive transfer of anti-NA serum though the effect is less pronounced than that of anti-HA antibody (Virelizier, 1975).

There is no evidence that antibodies to the M protein or NP have a significant role in immunity to influenza. Mice passively immunized against these components are not protected and antisera to either component has no neutralizing activity in vitro (Virelizier et al., 1976).

1.3.7 The Cell-Mediated Immune (CMI) Response

1.3.7.1 Cell Characteristics

T-lymphocytes have in the past been classified according to their activities as follows: regulatory T cells, Th and Ts, with helper-inducer and suppressor activity respectively; and effector T cells, Tc and Td, mediating cytotoxic and DTH activities. Table 1.2 lists the characteristics of T cells from human and murine sources with respect to their MHC requirements for activation, some surface markers commonly used to distinguish them, and the biological properties which cell preparations from primary responses (in vivo responses in naive hosts, e.g. mice) or secondary responses (usually in tissue culture with responder cells derived from primed hosts) or which cloned cells display. It is apparent that T cells occur in two major cell lineages according to (1) the MHC activation profile and (2) the presence of specific surface markers, Lyt 2 and L3T4 in the mouse (Lyt 1 differs only quantitatively) and T4 and T8 in humans. In contrast, work on influenza virus-specific T cells has been largely responsible for showing that a particular cloned cell line may mediate a number of activities, e.g. in the case of class I MHC restricted cells, Tc and Td activities. Though it has not been formally shown yet, it may well be found that one clone of class I or II MHC restricted T cells may mediate cytotoxicity, DTH, and helper-induced activity. (An

alloreactive, class II MHC-restricted T-cell clone has shown all these activities - Dennert et al., 1981).

Until recently, all Th cells tested were shown to be class II MHC restricted. Recently, Sinickas et al. (1985) have found that class I MHC-restricted Th cells could help Tc cell responses to murine cytomegalovirus infections (Sinickas et al., 1985). Both classes of Th cells produced IL-2.

1.3.7.2 Kinetics of T Cell Response

In a primary response, effector T cells expressing Tc activity are first detected in the lung 4 to 5 days after virus inoculation and depending on the dose of virus inoculated, reach a maximum 6 to 8 days later and decline thereafter to be no longer detectable after about 14 days. In mice primed to a serologically different A strain virus, Tc cells were detected in the lungs of challenged mice between day 2 to 3 and were usually absent by day 14. The kinetics of generation of Td cells after intranasal inoculation of virus was similar to that seen for Tc cells. There is no data on the kinetics of regulatory T cells in the infected lung. By analogy with findings in the spleen after intravenous injection of virus, Th cells would be found in the lung within 48 hours after virus inoculation.

1.3.7.3 Regulation of the Response

There are few studies on the role of Th or Ts cells in regulating the cell-mediated response. Although there is a basic requirement for T help in effector T cell generation, there is little evidence that in normal mice the availability of Th cells is a limiting factor in the generation of a cellular response to influenza virus (e.g. Leung et al., 1982).

Liew & Russell (1983) showed that Lyt 2^- cells which suppressed DTH activity could be detected in the murine spleen between 2 and 7 weeks after intranasal inoculation of infectious virus.

1.3.7.4 Roles for Effector T Cells

Effector T cells or their precursors per se cannot prevent infection of susceptible cells by virus. The induction of effector T cells requires that viral antigen be presented by appropriate cells, such as macrophages or dendritic cells, or possibly by B cells.

Effector T cells may contribute to two processes - to recovery from infection and/or to immunopathology which is part of the infectious process in normal hosts. Yap et al. (1978) showed that secondary effector T cells, obtained from the spleens of mice immunized with infectious virus and

restimulated in vitro with infectious virus, were able to reduce lung virus titres when transferred to infected syngeneic mice. The cells responsible were Lyt $1^{-}2^{+}$, were class I MHC antigen restricted and possessed Tc activity. Transfer of the same cell preparation to infected recipients which were class II MHC antigen compatible did not cause a reduction in lung virus titres. Similar cell preparations upon transfer to syngeneic recipients were found to cross-protect against different A strain viruses (Yap & Ada, 1978b). These findings were later confirmed (Wells et al., 1983). Lin & Askonas (1981) and Braciale and colleagues (e.g. Lukacher et al., 1984) subsequently showed that, when adoptively transferred, cloned T cells would reduce lung virus titres and protect mice from death. These findings agree with other related work. Thus, when nu^{+}/nu^{+} mice are infected with virus, the virus persists to high titre in the lungs for prolonged periods; transfer of immune spleen cells or secondary effector T cells to such mice rapidly reduces the level of virus in the lungs.

Experiments with human volunteers also support the contention that Tc cells are important in the recovery from influenza virus infection. In studies at the Common Cold Centre in England, Tc cell memory (in PBLs) correlated with rapid clearing of administered virus in individuals, some of whom lacked specific antibody to HA or NA (McMichael et al., 1983).

Following the early work of Cate and Mold (1975), there is considerable evidence showing that the action of effector T cells induces pathological damage in the lungs, but it is not the only cause of such damage. Thus, Yap et al. (1979) showed that nu^+/nu^+ mice inoculated with about one LD_{50} dose of infectious virus displayed marked pathological lesions. On the other hand, a vigorous CMI response to an influenza virus infection can be mounted in the mouse lung with minimal pathology occurring, a deciding factor probably being the level of virus replication in the lung (Mak et al., 1982b). A limited amount of replication, occurring with a cold-adapted mutant virus, gave rise to a low level only of lung consolidation. If mice are treated with Cyclosporin A (CsA) and infected with virus, the virus replicates to high titre but little immunopathology occurs because, although effector T cells develop, their activity is inhibited (Schiltknecht & Ada, 1985a).

Adoptive transfer of class II MHC-restricted effector T cells with DTH but undetectable Tc activity has been shown by two groups (Leung & Ada, 1982; Liew & Russell, 1980) not to reduce lung virus titres but to reduce the survival of infected mice given a high dose of virus. Some evidence was obtained to show a linkage between this effect and the DTH activity of the transferred cells.

1.3.7.5 Mode of Action of Effector T Cells

Effector T cells mediate DTH activity by secreting lymphokines; in contrast, the in vitro expression of cytotoxic activity by Tc cells does not require lymphokine secretion, as witnessed by the failure of Cyclosporin A to affect their activity (Borel, 1981; Andrus & Lafferty, 1982; Schiltknecht & Ada, 1985b). As expected from the in vitro results, effector Td cells, upon transfer to CsA-treated mice, do not mediate a DTH reaction, but a similar finding with transferred Tc cells was unexpected, even though it was shown that CsA did not affect the traffic of the transferred cells to the mouse lung. This finding provided, however, a possible key to understanding the in vivo findings. The first suggestion of a requirement for lymphokine release by Tc cells was the observation (Taylor & Askonas, 1983) on the activity of virus-specific Tc cell clones. Only the clone which secreted gamma-IFN on contact with a target cell in vitro could upon transfer reduce lung virus titres. The release of gamma-IFN by the Tc cell was dependent upon antigen recognition (Askonas & Pala, 1985). In apparent contrast to this finding, Lukacher et al. (1984) using two Tc cell clones in transfer experiments, one subtype-specific and the other cross-reactive, showed the induction and expression of antiviral activity (reduction of lung viral titres) by both clones was highly specific. This result did not support the earlier finding that lymphokine secretion by these cells was a critical factor in the reduction of lung

virus titres. Yap and Ada (1978b) had earlier observed that upon transfer of Tc cells to mice either 24h before or after infection of the mice, lung viral titres did not decrease until about day 4-5 after initiation of viral infection. This is in contrast to findings in the ectromelia virus (Kees & Blanden, 1977) and LCM virus (Dunlop, 1978) systems where transfer of specific Tc cells results in a rapid drop (within 24h) of viral titres in target organs. A possible explanation of these apparently conflicting findings is to postulate that the function of the lymphokine(s) secreted by the effector Tc cells was to enhance the expression of MHC antigens on the infected lung cells so that they became more susceptible to Tc cell lysis.

1.3.8 The Generation of Memory

1.3.8.1 B Cells

The persistence of serum antibody over decades and the occurrence of secondary antibody responses during successive influenza infections dictate that B cell memory is long lasting. Specific B "memory" cells have been detected, following in vitro stimulation of peripheral blood lymphocytes (PBLs), in the majority of primed adults (Callard, 1979). IgG-producing cells have been the predominant cell type detected. The duration of B "memory" cells in the circulation after natural infection is unknown. However, B memory cells have been detected in the

spleen, lymph nodes, and tonsils of primed individuals in the absence of circulating B memory cells (Callard et al., 1982). In mice, HA-specific B cell precursor frequency in the spleen increased 10- to 50-fold after primary infection (Cancro et al., 1978). In addition, specific ASCs generated in spleen cell cultures and detected by the haemolytic-plaque assay increased tenfold after priming (McLaren & Pope, 1980).

Evidence for B cell memory in the respiratory tract is suggested by the brisk IgA response in nasal secretions in primed children, after intranasal challenge with inactivated virus vaccine. Nasal wash IgA was not detectable by ELISA prior to viral challenge (Wright et al., 1983). Furthermore, in vitro stimulation of human tonsillar tissue results in the production of specific IgA as well as IgG and IgM (McGaughan et al., 1984).

1.3.8.2 T Cells

Memory for Tc cell responses is the only aspect which has been examined in detail. One to 6 months after i.v. injection of virus, the frequency of Tc cell precursors found in mouse spleens was at least tenfold higher than in spleens from immunized mice (Askonas et al., 1982b). The level of Tc precursor cells in mouse spleens 2 years after i.v. injection of the virus was shown to be about half the level observed 3 weeks after i.v. injection of virus

(Ashman, 1982). Cells taken from the immunized mice at 2 years also showed Th cell activity but this was not quantitated.

Possibly more relevant were studies on Tc cell precursor frequency in mouse lung. Mice were inoculated intranasally with moderate doses of egg-grown influenza A virus, either of a parental strain or a cold-adapted strain, with a mouse-adapted strain or with an UV-inactivated virus preparation. Infection with either of the egg-grown virus preparations caused a 20-fold increase in Tc cell precursor frequency in the lungs of mice 2-6 weeks after virus administration, despite the fact that the parental strain replicated to much higher titres. With the mouse-adapted strain, precursor cell frequency was about 100-fold higher, whereas administration of inactivated virus had very little effect on precursor cell frequency (Mak et al., 1984). Though the possibility was not eliminated, little evidence was obtained for major traffic of Tc cells to or from the lungs during infection so the findings suggest that the precursor cells found in the lungs some time after viral replication are to a large extent progeny of the resident cells in normal lung.

Experiments in humans have been confined to PBL stimulation. Using PBLs from 198 human volunteers, Mitchell et al. (1985) measured Tc cell levels following stimulation with virus and culture for 6 days. Natural infection with

influenza virus was shown to stimulate Tc cell "memory" two- to fourfold. McMichael et al. (1983) reported that there had been a low prevalence of influenza A infection in the Oxford and surrounding "catchment" areas since 1978, and they noticed that over a 5-year period there had been a sharp decline in the proportion of subjects examined who gave positive Tc cell responses following in vitro stimulation of PBLs. They were led to conclude from this that the half-life of Tc memory cells in the circulation was 2-3 years. This is surprising in view of (1) the prolonged production of antibody to influenza virus and (2) the prolonged memory, sometimes said to be lifelong and in many cases 10 or more years, to a number of diseases such as smallpox and measles in which T cell memory is probably an important component. It needs to be demonstrated in some way that sampling of PBLs is a reliable indication of the T cell immune status of an individual at different times.

1.3.9 Vaccination Against Influenza

1.3.9.1 Inactivated Virus Vaccines

Inactivated virus vaccines are prepared from the allantoic fluid from virus-infected eggs which is purified and concentrated by zonal centrifugation and inactivated. The main procedures used for inactivation of virus are treatment with formalin or β-propionolactone or UV light

irradiation. Differences in the immunogenicity of inactivated vaccines, primarily the ability to prime the host for a Tc response, have been related to different methods of activation (Ada et al., 1981).

Whole-virus vaccines contain intact inactivated virus. Split-product vaccines are prepared from purified formalin-treated virus disrupted with chemicals to solubilize the viral envelope. The virus HA and NA may be isolated and purified to produce subunit vaccines.

Immunogenicity

Sufficient data are now available to make an assessment of the immunogenicity of inactivated virus vaccines (Potter, 1982). In primed individuals, parenteral vaccination with either whole-virus or split-product vaccines results in a protective level of serum HI antibody in over 85% of recipients shortly after vaccination. In contrast, protective levels of serum HI antibody develop in approximately only 60% of unprimed recipients of whole-virus vaccines and to an even lesser extent after the split-product and subunit vaccines, requiring the administration of two doses of these vaccines to achieve an adequate response.

The induction of a secretory antibody response to inactivated virus vaccines is dependent both on the route of

administration and on the recipient's prior antigenic experience. In unprimed subjects, local antibody responses (i.e. in nasal washes) are of low magnitude and occur infrequently after both parenteral and intranasal administration of vaccine. Parenteral administration produced a local IgG response, detected by ELISA, in 94% of primed recipients, whereas local IgA responses developed in only 38% (Clements et al., 1985). In contrast, local IgA responses develop in the majority of primed recipients after intranasal administration (Wright et al., 1983). Although intranasal immunization with inactivated vaccine can induce protection against challenge virus infection, there are no comparative studies with parenteral administration. The oral administration of inactivated virus vaccine also induces a nasal wash antibody response in primed individuals, though data on efficacy are lacking (Waldman et al., 1981).

The duration of serum HI antibody after vaccination also varies according to the recipient's prior antigenic experience. Primed subjects retain protective levels of antibody for at least 1 year, whereas antibody levels decline rapidly in unprimed subjects.

The specificity of the antibody response after vaccination with inactivated virus in primed individuals is similar to that following natural infection (Oxford et al., 1979), although a lesser degree of cross-reactivity to

subtype variants is induced in persons initially immunized to a new subtype virus by inactivated vaccine rather than by natural infection (Masurel et al., 1981).

The T cell responses to inactivated virus vaccine differ in several respects to those to infectious virus. Several groups (e.g. Braciale & Yap, 1978; Webster & Askonas, 1980) have shown that in mice, infectious virus was far more effective at inducing a primary class I restricted Tc cell response than was inactivated virus. Furthermore, inactivated virus was relatively ineffective in priming mice for a secondary Tc response. A secondary in vitro Tc cell response can be obtained from human PBLs by exposure to different virus preparations (McMichael et al., 1981; Ennis et al., 1981b). There is general agreement that whole inactivated virus adequately stimulates the response though the duration of the response has not been determined.

Efficacy

The protective efficacy of the different inactivated vaccines, including whole-virus and split-products, after parenteral administration are comparable, though the latter are less reactogenic, ranging from 60% to 80%.

In primed people (i.e. adults who have experienced one or more infections by influenza virus) immunized parenterally with inactivated vaccines, either whole- or

split-virus preparations, and challenged with infectious homologous virus, vaccination may afford protection for several years. Though it is known that cross-reactive Tc cells are generated under these conditions, the extent to which they contribute to this result is not clear. In unprimed people, usually young children, this immunization is less protective, probably because of the poor ability of parenterally administered inactivated virus to prime for a local (respiratory tract) humoral or Tc cell response. Even if given intranasally, inactivated virus does not induce a primary local humoral response in unprimed people. In contrast, a primary infection in children induces a local antibody response (Murphy et al., 1982) and primes for a secondary IgA response (Wright et al., 1983).

In view of antigenic drift, how effective is inactivated vaccine in protection against a challenge with a subtype variant virus? The study which best addresses this question is by Hoskins et al. (1979), who showed that the protective effect of inactivated virus vaccine was limited to nonimmune schoolchildren who were vaccinated for the first time with the prevailing strain. Revaccination with later prevailing strains (inactivated) did not provide protection against subsequent challenge, whereas natural infection afforded almost complete protection during successive outbreaks involving drift viruses for more than 4 years. As the specificity of the antibody response after vaccination and infection is similar, differences in the

extent of cross-reactive Tc responses to vaccination and infection may account for the lesser degree of heterotypic protection seen after vaccination.

1.3.9.2 Attenuated Virus Vaccines

Influenza viruses have been effectively attenuated and the genes determining attenuation, which are located on the RNA segments coding for nonsurface antigens, transferred into reassortant viruses bearing the desired surface glycoproteins of current human influenza viruses.

Host Range Mutants

Attenuation of human influenza virus can be affected by the transfer of genes from host range mutants which are selected during repeated passage of human virus in eggs (e.g. A/PR/8/34(H1N1)). However, attenuation is not achieved if all of the six transferable PR8 genes are transferred, as unexpectedly a mixture of the PR8 and wild-type human influenza polymerases is required to restrict replication of the reassortant virus. Subsequently attenuation of reassortant viruses cannot be checked by simple in vitro methods, imposing a significant restriction in their use.

Temperature-Sensitive (ts) Mutants

ts mutants, produced by exposing virus-infected cells to a chemical mutagen (5-fluorouracil), are identified by being restricted in their ability to replicate in vitro at 37°-38°C. Although influenza A reassortant viruses bearing ts genes were satisfactorily attenuated, their genetic instability, leading to reversion to virulence, has precluded further use.

Cold-Adapted (ca) Mutants

ca mutants are produced by serial passage at successively lower temperatures (33°-25°C) in primary chick kidney cell culture. This causes mutations in each of the six transferable genes. The resulting ca mutant is also temperature sensitive and reassortant viruses receiving all six of the nonsurface antigen genes exhibit the ca and ts phenotypes. These reassortants are genetically stable (Cox & Kendall, 1984) and retain their ca phenotype even during infection in unprimed children who continue to shed virus for up to 12 days (Belshe & Van Voris, 1984). Furthermore, ca reassortant viruses were not transmitted to fully susceptible children who were exposed to vaccinated children (Wright et al., 1982).

The median human infective dose (HID_{50}), determined by antibody response and virus shedding, in seronegative adult

volunteers (though presumably primed), is approximately $10^{5.5}$ to $10^{6.1}$ $TCID_{50}$ for H1N1 and H3N2 reassortant viruses (Murphy & Webster, 1985). At a dose of $10^{7.5}$ $TCID_{50}$ or greater in adults, mild reactogenicity is observed. The HID_{50} for a H1N1 reassortant in unprimed children is 100-fold lower (Belshe & Van Voris, 1984). In children immunized with a comparable dose ($10^{6.3}TCID_{50}$) of either an H1N1 or H3N2 reassortant, the H1N1 reassortant was less immunogenic than the H3N2 reassortant (Wright et al., 1982). The dynamics of the serum and secretory antibody responses in primed and unprimed individuals have been previously described (Section 1.3.6.1).

Data on the T-cell response to live attenuated vaccines in humans are limited to the development of secondary Tc responses in primed individuals vaccinated with a PR8 reassortant virus (Ennis et al., 198b1); there are no data on the Tc responses in humans to ca reassortants. In mice, ca reassortants can induce a primary Tc response and can sensitize the lungs for a secondary Tc response (Mak et al., 1982b; Mak et al., 1984). H3N2 reassortants are more effective than H1N1 reassortants at inducing a Tc response (Tao et al., 1985). The dose of a ca mutant of A/AA/6/60(H2N2) required to induce the same level of Tc response was 100-1000 times greater than that of the parental strain (Mak et al., 1982b). A similar difference in dosage was required to prime mice to resist subsequent challenge with a mouse-adapted strain, A/WSN(H1N1). The

difference in dosage required for priming could be overcome by giving two small doses 3 weeks apart (Tannock et al., 1984). Using this approach, ca reassortants may induce cross-protection against different subtype viruses, although the duration of cross-protection in mice is short (Tannock & Paul, 1985).

The protection afforded by ca reassortants has not been extensively evaluated. Seronegative adult volunteers were challenged with the homologous wild-type virus 1-2 months after vaccination with either an H3N2 ca reassortant ($10^{7.5}TCID_{50}$) or inactivated vaccine (Clements et al., 1984). Ca recipients were completely protected against illness compared with a 72% efficacy in the inactivated virus vaccinees. Infection occurred in 19% of ca vaccinees and in 63% of inactivated virus vaccinees. Furthermore, nasal wash viral titres were 1000-fold lower in ca vaccinees shedding virus. Adult recipients of H1N1 ca reassortant ($10^{7.6}TCID_{50}$) were similarly protected against illness on challenge 1-3 months later (82% efficacy) and against infection - 18% of vaccinees were infected with 1000-fold lower viral titres than nonvaccinated controls (Betts et al., 1985). At 6-7 months after vaccination, protective efficacy remained at 91% (Clements et al., 1985). Field trials on the protection afforded by ca reassortants against natural infection have not demonstrated superiority over inactivated virus vaccines in adults within the first year after vaccination, though ca reasssortants were claimed to

be more efficacious 2 years after vaccination against natural infection with heterotypic H1N1 virus (Couch et al, 1985).

The efficacy of ca reassortants has also been studied in unprimed children. Children receiving an H3N2 ca reassortant vaccine appeared to be protected against subsequent natural infection with related H3N2 strains (Wright et al., 1982; Belshe et al., 1984).

Avian Influenza Viruses

The genetic determinants of attenuation of avian viruses in primate cells reside on one or more of the genes coding for nonsurface antigens. These attenuating genes have been transferred into an avian-human reassortant which is restricted in its replication in primate cells. Infection with avian-human reassortant virus in monkeys induces significant resistance to subsequent challenge with wild-type human influenza virus. In humans, the reassortant was satisfactorily attenuated and immunogenic (Murphy & Webster, 1985). However, the difficulty in confirming attenuation in vitro and the possible interaction with wild avian viruses may restrict this approach.

Deletion Mutants

Deletion mutants may be produced by treatment of DNA with restriction endonucleases. These mutants should be

stable because of their inability to revert and low likelihood of suppression by a new mutation at another site on the viral genome. The potential for producing deletion mutants of influenza virus exists, as cloned DNA of the influenza genome has been inserted into SV40 vectors and the HA expressed in eukaryotic cells. However, the transfer of genetic information in cloned DNA into a reassortant influenza virus has not been reported.

1.4 Aims of Thesis

Local humoral immunity of the respiratory tract plays an important role in the prevention of viral respiratory tract infections, including influenza. However, the B cell response to viral infections in the respiratory tract has been largely characterized by measurements of antibody in serum and respiratory secretions. Essential data on local B cell responses pertaining to the origin of antibody in respiratory secretions and the generation of B cell memory in the respiratory tract are limited. The aim of this thesis has been to study the generation of influenza virus-specific ASCs and B cell memory in the murine lung. The characterization of these responses following influenza infection and following immunization with existing and potential influenza vaccines will provide original data relevant to the development of an effective influenza vaccine as well as broadening our knowledge of viral respiratory tract immunity.

Specific areas relating to influenza infection which have been addressed in this thesis aim to provide data on the following questions:

(i) Do influenza-virus specific ASCs appear in influenza-infected lungs?

(ii) What is the origin of influenza-virus specific ASCs in influenza-infected lungs?

(iii) Do influenza virus-specific ASCs persist in the lung after influenza infection?

(iv) Is there any evidence for influenza virus-specific B cell memory in the lung?

These questions are also addressed in mice immunized with different vaccination strategies comparing the ability of different vaccines to induce local B cell responses. Finally, the development of local cellular immune responses, including NK cells, ASCs and Tc cells, to a novel influenza vaccine preparation as described.

TABLE 1.1 Viral Proteins Recognized by Influenza Virus-specific Tc Cells

Proteins	Cellular Expression		Species[a]	Method[b]
	Stimulator	Target		
Structural:				
HA	+	+	H,M	T,RV,F,Ab
NA		+	H	RM
M	+	+	H,M	Ab, RM
	-		M	RV
NP		+	H,M	T,RV,RM
Polymerases				
PA		+	M	RV
PB1		+	M	RV
PB2		+	M	RM,RV
Non-Structrual				
M2		-	M	RV
NS1		+	M	RV
NS2		-	M	RV

a. H, Human; M, mouse.

b. T, transfection; RV, recombinant virus; RM, reassortant or mutant virus; F, cell fusion; Ab, inhibition by antibody.

TABLE 1.2 Characteristics of Human and Murine Influenza-immune T Cells

MHC antigen requirement for activation	Lymphocyte antigen expressed		Biological Activities	Cell Preparation
	Human	Mouse		
Class I	T8		Cytotoxic ?Suppressor ?DTH	Secondary
		Lyt 2^+ L3T4^- Lyt 1^-	Cytotoxic DTH Helper	Primary, Secondary, Clones Primary, Secondary, Clones Primary
Class II	T4		Helper Cytotoxic ?DTH	Secondary, Clones Clones
		L3T4+ Lyt 2^- Lyt 1^+	Helper DTH Cytotoxic	Primary, Secondary, Clones Primary, Secondary, Clones Secondary, Clones

CHAPTER TWO

MATERIALS AND METHODS

CONTENTS

CONTENTS

MATERIALS AND METHODS

2.1 Mice

BALB/c mice were bred at the John Curtin School of Medical Research. All primary inoculations were performed in mice 8 to 12 weeks old and mice of the same sex were used for each experiment.

2.2 Viruses

2.2.1 Normal Virus Preparations

Influenza virus strains used in this work included:

A/PR8	A/Puerto Rico/8/34 (H1N1)
A/Jap	A/Japan/305/57 (H2N2)
A/AA	A/Ann Arbor/6/60 (H2N2)
A/PC	A/Port Chalmers/1/73 (H3N2)
A/Jap-Bel	[A/Japan/305/57 (H2N2)xA/Bellamy/42 (H1N1)] (H2N1)
B/Sing	B/Singapore/79

Influenza viruses and Sendai virus were grown in the allantoic cavity of 10 day old embryonated eggs for 40 to

48h at 35°C. The infectious allantoic fluid was harvested, centrifuged at 2000g for 30 min. to remove cell debris and then aliquoted and stored at -70°C. The virus titre was expressed as the median egg infective dose (EID_{50}) or as haemagglutination units (HAU).

2.2.2 Cold-adapted Virus Preparations

The cold-adapted influenza virus A/Ann Arbor/6/60-ca (H2N2) was kindly supplied by Dr. G. Tannock, University of Newcastle (Mak et al., 1984).

2.2.3 Purification of Viruses

Influenza virus was purified from infectious allantoic fluid by sucrose gradient centrifugation (Laver, 1969). Initially the virus was partially purified and concentrated by one cycle of adsorption and elution from chicken red blood cells (CRBC) followed by ultracentrifugation (85,000g, 90 min.). Virus particles were resuspended in a small volume (approximately 2-3ml) of Ca-Mg saline and then further purified by sedimentation through a continuous 10-55% sucrose gradient (SW 28 motor, 20,000 r.p.m., 60 min.). The virus band was collected, dialyzed against Ca-Mg saline for 48h at 4°C and stored at 4°C in Ca-Mg saline with 0.1% sodium azide.

2.2.4 Disruption of Virus

Purified A/Jap virus was disrupted by sodium deoxycholate as previously described (Webster & Laver, 1966). Virus was diluted to a concentration of $4x10^5$ HAU/ml and dialyzed against 0.15M NaCl containing 0.01M Tris-HCl pH7.5. 2ml of virus suspension was mixed with 0.5ml 5% (w/v) sodium deoxycholate and allowed to stand for 5 min. at 35°C leading to a reduction in opalescence of the virus suspension. Intact virus particles were removed by ultra-centrifugation (32,000g, 30 min.) and the supernatant dialyzed against water.

2.2.5 Ultraviolet Inactivation of Virus

Infectious allantoic fluid was dialyzed against PBS for 2-3h at 4°C. The virus suspension was then exposed to a 30 watt Philips germicidal ultraviolet lamp at a distance of 15cm for 7 min. (intensity: $320uW/cm^2$ as measured by a Blak-Ray ultraviolet meter, U.V. Products Inc.). This procedure rendered the virus non-infectious, as judged by egg infectivity, with minimal change in the haemagglutinating activity.

2.3 Influenza-specific Micelles and Iscoms

Micelles and iscoms ('immuno-stimulatory complexes') containing the HA and NA of A/PR8 virus were kindly prepared

by B. Morein, Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden. Briefly, 10mg of purified A/PR8 virus (10mg/ml) were solubilized in 2% of the detergent MEGA-10 (Hildreth, 1982) for 1h at room temperature. The solubilized virus was applied on a cushion of 20% (w/w) sucrose containing 0.5% MEGA-10 and centrifuged for 1h at 200,000g, 20°C. Fractions containing the solubilized envelope proteins were traced by their absorbance at 280nm and pooled.

Micelles were prepared by dialysing 1mg of solubilized proteins (0.5-1.0mg/ml) extensively against PBS. The micelles were further purified by pelleting through a cushion of 5% and 10% sucrose, the upper layer containing 0.1% Triton X-100. The pelletted micelles were resuspended in PBS, monitored by electron microscopy and filtered through a 0.45um sterile filter and stored at 4°C (Fig. 2.1a).

Iscoms were prepared in a similar manner except that the solubilized envelope proteins were mixed with Quil A (Dalsgaard, 1978) to a final concentration of 0.1% (w/v) before dialysing against PBS. Free Quil A was removed by pelleting the iscoms through a cushion of 5% and 10% sucrose, the upper laying containing 0.1% Triton X-100 (Fig. 2.1b).

2.4 Virus Titrations

2.4.1 Haemagglutination Test

Two-fold serial dilutions of virus suspension in PBS were prepared in a final volume of 50ul in round bottomed 96 well microtitre trays (Cooke, Microtitration). An equal volume of 0.5% CRBCs in PBS was added to each well. Control wells contained CRBCs only. The trays were shaken gently and kept at 4°C for 30 to 45 min. before reading. The virus titre was the reciprocal value of the highest dilution of virus which gave a positive (haemagglutination) reaction, and was expressed as HAU/ml.

2.4.2 Egg Infectivity

Serial 10-fold dilutions of virus suspension were made in standard medium (0.85% NaCl, 0.03% $CaCl_2$, 0.8% $MgCl_2$ and 0.5% gelatine, pH adjusted to 7.2 with 0.2M borate buffer) containing antibiotics. 100ul volumes were inoculated into the allantoic cavity of 10 day old embryonated eggs, 4 to 5 eggs per dilution. After 40 to 48h incubation at 35°C, 50ul of allantoic fluid was removed from each egg and tested for haemagglutination. The virus titre was expressed as the median egg infective dose (EID_{50}) per ml which was determined by the modified Spearman and Karber method (Irwin & Cheeseman, 1939).

2.5 Experimental Procedures in Mice

2.5.1 Intranasal Inoculation

Mice were inoculated intranasally with 50ul of saline, containing virus or viral preparations, under light ether anaesthesia.

2.5.2 Lung Consolidation

Lungs were removed from infected mice and the surface of all lobes inspected for areas of dark purple discoloration due to consolidation. This area of discoloration was scored from 0 to 100% with respect to total lung surface. Lung consolidation was also assessed by dry lung weight.

2.5.3 Lung Virus Titration

Infected mouse lungs were removed aseptically and stored at -70°C until titrated. The lungs were thawed and homogenized in 3ml standard medium using a chilled glass homogenizer. The homogenized suspension was centrifuged at 1500g for 20 min. at 4°C. The supernatant was collected and virus titre determined by egg infectivity and expressed as the EID_{50} per organ extract.

2.5.4 Cell Preparations

2.5.4.1 Spleen

Spleen cell suspensions were prepared by finely mincing spleens and gently pressing them through a stainless steel sieve. Erythrocytes and dead cells were removed by centrifugation on a Ficoll-Isopaque gradient. Viability counts were performed by trypan blue exclusion method. The cells were suspended to the desired concentration in Eagle's minimal essential medium supplemented with 10% foetal calf serum.

2.5.4.2 Lung

Lung cell suspensions were prepared similarly except that the finely minced lungs were digested with collagenase (Boehringer). Each pair of minced lungs was suspended in minimal essential medium (2ml per pair) containing 2mg collagenase per ml and incubated at 37°C for 30 min. The cell suspensions were washed 3x to remove the collagenase and then pressed through a stainless steel sieve and prepared as for spleen cell suspensions.

2.5.4.3 Blood

Blood was collected by cardiac puncture into 0.1ml sodium citrate (3.8%) in a 1ml syringe. The citrated blood

was cenrifuged at 10,000g, 4°C for 15 min. The plasma was removed and stored at -20°C. The cells were resuspended in minimal essential medium, washed twice and separated on a Ficoll-Isopaque gradient.

2.5.5 Tracheobronchial Washings

The trachea was exposed in the neck and cannulated with a blunt 20G needle attached to a 1ml syringe containing 0.5ml PBS. The lungs were gently lavaged 2 to 3x recovering 0.3 to 0.4ml of the original volume. The washings were centrifuged at 10,000g, 4°C for 15 min. and the supernatant stored at -20°C.

2.6 Antibody Titrations

2.6.1 Haemagglutination Inhibition (HI) Assay

All sera and tracheobronchial washings (TBW) were first heat-inactivated (56°C, 30 min.) to remove nonspecific inhibitors of haemagglutination. Serial two-fold dilutions of serum or TBW in PBS were prepared in a final volume of 25ul. 4 HAU of virus in 25ul was added to each well. After 30 min. at room temperature, 50ul of 0.5% CRBC in PBS was added to each well and the haemagglutination pattern read 30 min. later. The titre was expressed as the reciprocal of the highest dilution which still inhibited haemagglutination.

2.5.2 Indirect Enzyme-linked Immunosorbent Assay (ELISA)

An indirect ELISA was used to measure isotype-specific anti-influenza antibodies. The method is shown schematically in Fig. 2.2 and described as follows:

(i) 100ul of 1000 HAU of purified virus per ml in carbonate-bicarbonate buffer, pH 9.6, was added per well to 96 well polyvinylchloride microplates (Titertek ImmunoAssay-Plate) and left overnight at 4°C;

(ii) virus suspension was removed and the plates washed 3x with PBS-0.05% Tween 20 (PBS-Tw20);

(iii) 125ul of PBS-Tw20-1%bovine serum albumin (PBS-Tw20-BSA) was added to each well and incubated (37°C, 1h) to block nonspecific binding sites on the plate;

(iv) after removing the BSA solution, 50ul of two-fold serial dilutions of serum or TBW in PBS-Tw20-BSA were added to duplicate wells and incubated (37°C, 1h);

(v) the plates were washed 3x with PBS-Tw20 and 100ul of goat anti-mouse antiserum of appropriate heavy chain specificity (Cooper Biomedical Inc., Philadelphia;

diluted 1:2000 in PBS-Tw20-BSA) was added and incubated (37°C, 1h);

(vi) after washing, 100ul of rabbit anti-goat IgG alkaline phosphatase conjugate (Cooper; 1:3000 dilution) was added and incubated (37°C, 1h);

(vii) the plates were then washed 3x with PBS-Tw20 and once with diethanolamine buffer, pH 9.8. The substrate p-nitrophenyl phosphate (Sigma Chemical Co; U.S.A.) was dissolved in diethanolamine buffer at a concentration of 1mg/ml and 100ul added to each well and incubated (37°C, 1h);

(viii) after 1h, 50ul of 3M NaOH was added to each well to stop the enzyme reaction and optical density (O.D.) measured in an Auto ELISA Reader (Dynatech) at 405nm.

Two negative controls were included in each plate: (a) wells to which no serum or TBW was added, and (b) wells containing normal mouse serum and TBW in dilutions corresponding to the test sample. The endpoint was defined as the highest dilution with a mean O.D. greater than twice the mean O.D. of the negative control with the higher O.D. The titre was expressed as $\log_2$ of the reciprocal of the endpoint dilution. All titrations presented in individual experiments were performed in parallel.

2.7 ELISA-Plaque Assay

The ELISA-plaque assay for enumerating ASCs is similar in principle to the indirect ELISA (Fig. 2.2). The steps involved include:

(i) 4ml of 1000 HAU of purified virus per ml in carbonate-bicarbonate buffer was added per well to Linbro six-well plates (3.5 x 1.0cm) and left overnight at 4°C;

(ii) the wells were washed with PBS-Tw20 3x. PBS-Tw20-BSA was added (2ml per well) and incubated (37°C, 1h);

(iii) the BSA solution was removed and 1.5ml of cell suspension containing Cytodex microcarriers (1mg/ml) was added to the plates on a levelled surface in a gas chamber. The plates were incubated for 3-4h at 37°C in a humid atmosphere containing 10% CO_2.

(iv) after washing 3x with PBS-Tw20, 1ml of goat anti-mouse antiserum of appropriate heavy chain specificity (Cooper; 1:2000) was added and incubated (37°C, 90 min.);

(v) after washing, 1ml of rabbit anti-goat IgG alkaline phosphatase conjugate (Cooper; 1:1000) was added and left overnight at 4°C;

(vi) the substrate solution used was 5-bromo-4-chloro-3-indolyl phosphate dissolved in 2-amino-2-methyl-1-proponal buffer as described previously (Sedgwick & Holt, 1983a) in a final concentration of 0.05%. The substrate solution was warmed to 40°C and 3% agarose solution (Sigma Type 1) was added to a final concentration of 0.6% agarose;

(vii) after the plates were washed, emptied and placed on a level surface, 1ml of warmed agarose substrate mixture was added to each well. After the mixture gelled, the plates were incubated (37°C, 2h);

(viii) plaques appearing as blue spots were enumerated using a dissecting microscope (x12) (Fig. 2.3).

The ELISA-plaque assay was modified in some experiments by initially coating the plates with a monoclonal antibody to the HA of A/Jap virus (supplied by Mr. R. Tha Hla). The optimal concentration of the anti-A/Jap MAb in the bicarbonate buffer was coated onto plates overnight at 4°C. After blocking with PBS-Tw20-BSA, 4ml of purified A/Jap virus (1000 HAU/ml) in PBS-BSA was added to each well and

incubated for 1h at 37°C. After washing, cell suspensions were added and the assay completed as above.

2.8 Cytotoxicity Assays

2.8.1 Tc Cell Mediated Cytotoxicity

P815 cells ($H\text{-}2^d$, methylcholanthrene-induced mastocytoma of DBA/2 mice) were used as target cells. The cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated foetal calf serum, 100 units/ml of penicillin and 100ug/ml of streptomycin sulphate. The cell cultures were kept at 37°C under a gas phase of 10% CO_2 and subcultured every 2 to 3 days.

4 to $10x10^6$ target cells were labelled with 300 to 400uCi ^{51}Cr (sodium chromate, Amersham, UK) for 1h at 37°C in a total volume of 0.5ml. During labelling the cells were shaken occasionally to prevent settling. After labelling the cells were washed once and resuspended in medium and divided into two equal lots. One lot was infected with virus (15-30 EID_{50}/cell) for 1h at 37°C and the other lot left uninfected. After infection the cells were washed twice and diluted to $1x10^5$/ml and 0.1ml was added to each well of a 96 well flat bottomed tissue culture plate containing 0.1ml of appropriate dilutions of effector cells.

Spontaneous ^{51}Cr release (medium release) was determined by adding 0.1ml of target cells to wells containing 0.1ml of medium. For total releasable ^{51}Cr release, 0.1ml of target cells were added to 0.1ml of 1% Triton X-100. All samples were tested in quadruplicate wells.

Effector and target cell contact was initiated by centrifugation at 100rpm (300g) for 1 min. The plates were incubated for 6h at 37°C in a gas chamber (10% CO_2, 7% O_2, 83% N_2). After incubation 0.1ml of supernatant was removed from each well and the radioactivity measured in a gamma-counter.

The specific ^{51}Cr release was determined by the following formula

$$\% \text{ Specific lysis} = \frac{\text{mean counts from effector cells} - \text{mean counts from medium release}}{\text{mean counts from total release} - \text{mean counts from medium release}}$$

The data was processed with a digital PDP 11/34 computer which calculated % specific lysis and standard errors for each set of data.

2.8.2 NK Cell Mediated Cytotoxicity

YAC-1 cells (H-2^a Moloney leukaemia virus-induced T cell lymphoma of A/Sn mice) were used as target cells and maintained as for P815 cells.

The assay for NK cell activity was similar to that described for Tc cell cytotoxicity assays except that target cells were uninfected.

2.9 Depletion of Ig-bearing Cells

The method for depleting cell suspensions of Ig-bearing cells was adopted, with slight modifications, from Parish et al. (1974). Briefly SRBCs were coupled with sheep anti-mouse Ig by $CrCl_3$ treatment. Rosettes formed between SRBC and Ig-bearing cells were removed from the cell suspensions by centrifugation through a Ficoll-Isopaque gradient. The Ig-negative cells were collected from the Ficoll-Isopaque/medium interface.

2.10 Depletion of L3T4$^+$ Cells

Spleen and lung cell suspensions, at a dilution of 10^7 cells/ml, were incubated with a 1:10 dilution of rat monoclonal antibody to the L3T4 lymphocyte marker (supplied by Dr. R. Ceredig) at 37°C. After 20 min. a 1:10 dilution of low-toxicity rabbit complement (Cedarlane Laboratories)

was added and incubated for a further hour. After washing twice dead cells were removed on a Ficoll-Isopaque gradient.

2.11 Generation of ASCs in vitro

Spleen and lung cell suspensions were cultured in minimal essential medium supplemented with 10% foetal calf serum, 10^{-4}M 2-mercaptoethanol and antibiotics (1 to 2 x 10^6 cells/ml). The flasks were incubated at 37°C in a gas phase of 10% CO_2 in air. Viable cells were obtained from the culture after centrifugation on a Ficoll-Isopaque gradient.

Cultures were stimulated by the addition of purified influenza virus at the beginning of the culture period. Cultures from normal mice were stimulated with 20 HAU/ml and incubated for 4 days. Cultures from primed mice were stimulated with 10 HAU/ml and incubated for 5 days.

2.12 Limiting Dilution Analysis

Lung cell suspensions from normal and primed mice were used as responder cell populations. Syngeneic spleen cells, infected with 10 to 20 EID_{50}/cell of A/PR8 virus for 60 min at 37°C, were irradiated at 2000 rads from a ^{60}Co source and used as stimulator cells. Dead cells and erythrocytes were removed from both responder and stimulator cell populations by centrifugation on Ficoll-Isopaque gradients.

Graded numbers of responder living cells, 24 replicates for each cell dose, were cultured with $2x10^6$ stimulator cells in a final volume of 200ul in 96 well Linbro round-bottom tissue culture plates for 6 days at 37°C in a gas phase of 10% CO_2. The culture medium was supplemented with T cell growth factor (Mak et al., 1984).

After culture, the plates containing effector cells were centrifuged at 300g for 1 min., the medium removed and replaced with 0.1ml of fresh medium supplemented with 10% foetal calf serum. ^{51}Cr labelled P815 cells, either infected or uninfected with A/PR8 virus, were used as target cells. $1x10^4$ target cells in 0.1ml medium was added to each well. Effector and target cell contact was initiated by centrifugation of the plates at 300g for 1 min. and plates were then incubated for 6h at 37°C in a gassed incubator. 0.1ml of medium was removed from each well and counted in a gamma-counter. Spontaneous release from control cultures consisting of virus-infected irradiated stimulator cells alone was never significantly higher than spontaneous medium release. Cultures which caused ^{51}Cr release greater than 3 standard deviations above the spontaneous medium release of cultures without responder cells were scored positive for cytotoxic activity.

The logarithm of the percentage of negative cells was plotted against the number of responder cells per well. Tc cell precursor frequency was determined as the reciprocal of

the number of responder cells that gave 36.8% negative cultures.

2.13 Immunohistochemistry

2.13.1 Fixation

1mm strips of lung tissue were fixed by immersion in periodate-lysine-paraformaldehyde (McLean & Nakane, 1974) for 3 to 4h at 4°C. After fixation the strips were washed in several changes of 7% sucrose in 0.05M phosphate buffer, pH 7.4, and stored in this buffer for less than 24h.

2.13.2 Embedding

Excess buffer was removed by blotting and the strips of lung tissue snap frozen in liquid monochloro-difluoromethane (freon 22). The frozen tissue was placed into 15mm diameter alfoil cups and covered with cold (4°C) OCT embedding compound (Ames Tissue Tek). The cups were suspended over liquid nitrogen until the OCT compound was frozen. The frozen blocks were removed from the alfoil cups and stored at -20°C until use.

2.13.3 Sectioning

The blocks were sectioned using a cryostat at -20°C. Frozen sections (5-10um) were allowed to adhere to poly-L-

lysine coated slides and air dried.

2.13.4 Immunoperoxidase Technique

Sections were stained using the indirect avidin-biotin-complex (ABC) immunoperoxidase technique (Hsu et al., 1981) as previously modified (Hume & Gordon, 1983):

(i) the OCT compound was removed by washing in PBS:

(ii) the sections were dehydrated through graded alcohol baths (50-100%), and placed in 0.1% H_2O_2 in methanol for 30 min. to eliminate endogenous peroxidase activity;

(iii) after passing the sections back down the graded alcohol baths (100-50%) the sections were covered with PBS containing 2% normal horse serum and rabbit anti-mouse IgG (Litton Bionetics, 1:100 dilution) for 30 min. to block non-specific antibody-binding sites. The anti-mouse IgG was omitted when staining for Ig-bearing cells.

(iv) the blocking solution was removed and the primary antibody applied to the sections for 2h. Primary rat monoclonal antibodies to the following murine antigens were used - F4/80 and PGP, markers of murine phagocytes (supplied by Dr. D. Hume), 1a

antigens (ATCC TIB120), L3T4 antigen (supplied by Dr. R. Ceredig). In addition, Ig-bearing cells were stained using affinity purified anti-mouse Ig antiserum (Cooper Biomedical);

(v) sections were washed 3x in PBS over 10 min. and biotinylated sheep anti-rat Ig (Amersham U.K.; 1:200 dilution) applied to the sections for 1h;

(vi) after washing, the sections were covered with a preformed avidin-biotin-peroxidase complex (Vectastain ABC System, Vector Labs) for 1h;

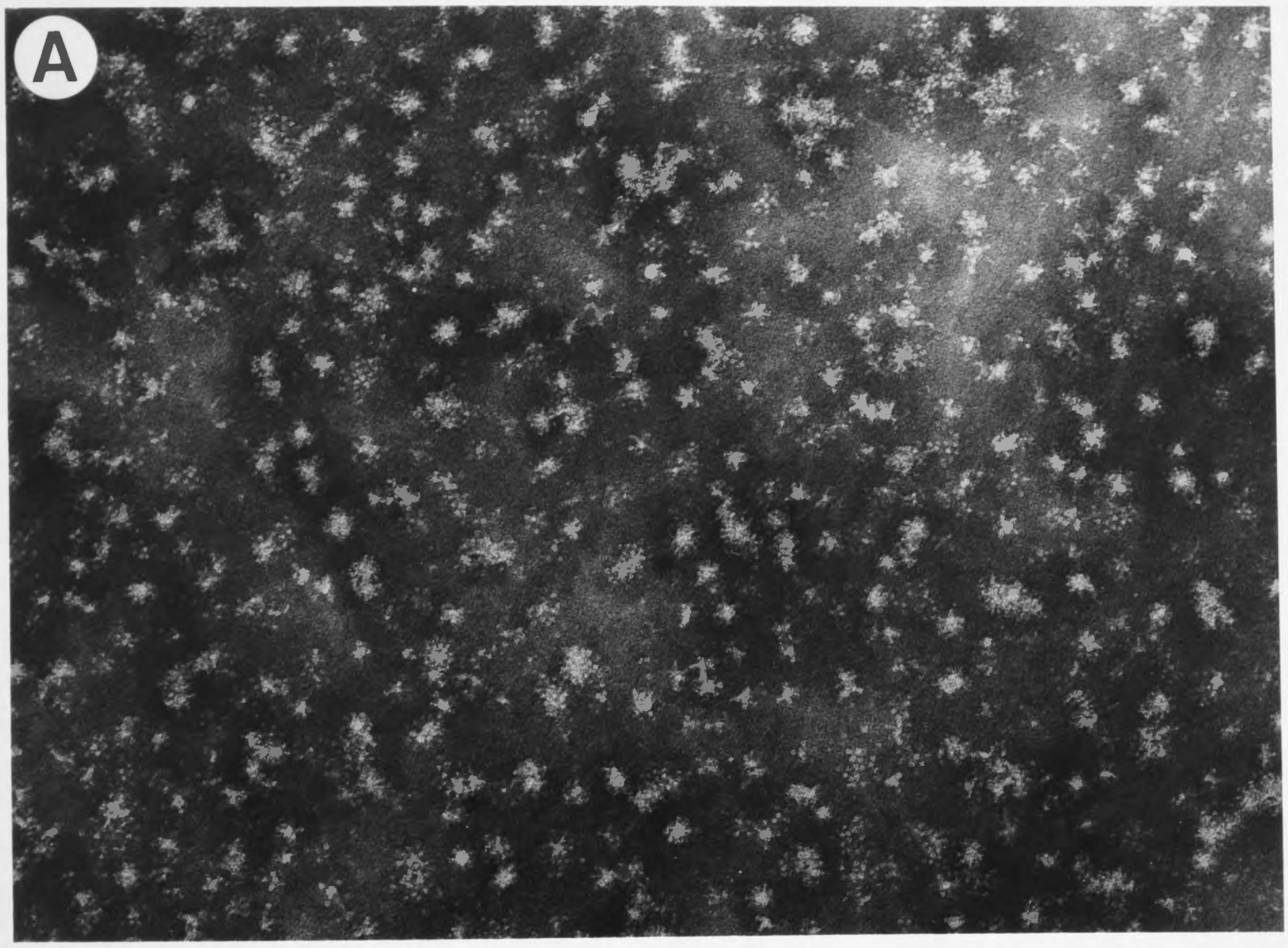
(vii) after washing in PBS, sections were stained with the peroxidase substrate, diaminobenzidine and 0.02% H_2O_2, made up in PBS plus 10mM imidazole, pH 7.4, for 5 min.;

(viii) the sections were washed in water and lightly counterstained with Mayers haematoxylin, dehydrated through graded alcohol baths, cleared in xylene and mounted under coverslips using Depex.

2.14 Statistical Methods

Student's t test was used to compare continuous variables and linear correlation coefficients determined for bivariate groups.

FIG. 2.1 Electron micrographs of HANA micelles (a) and iscoms (b).


A

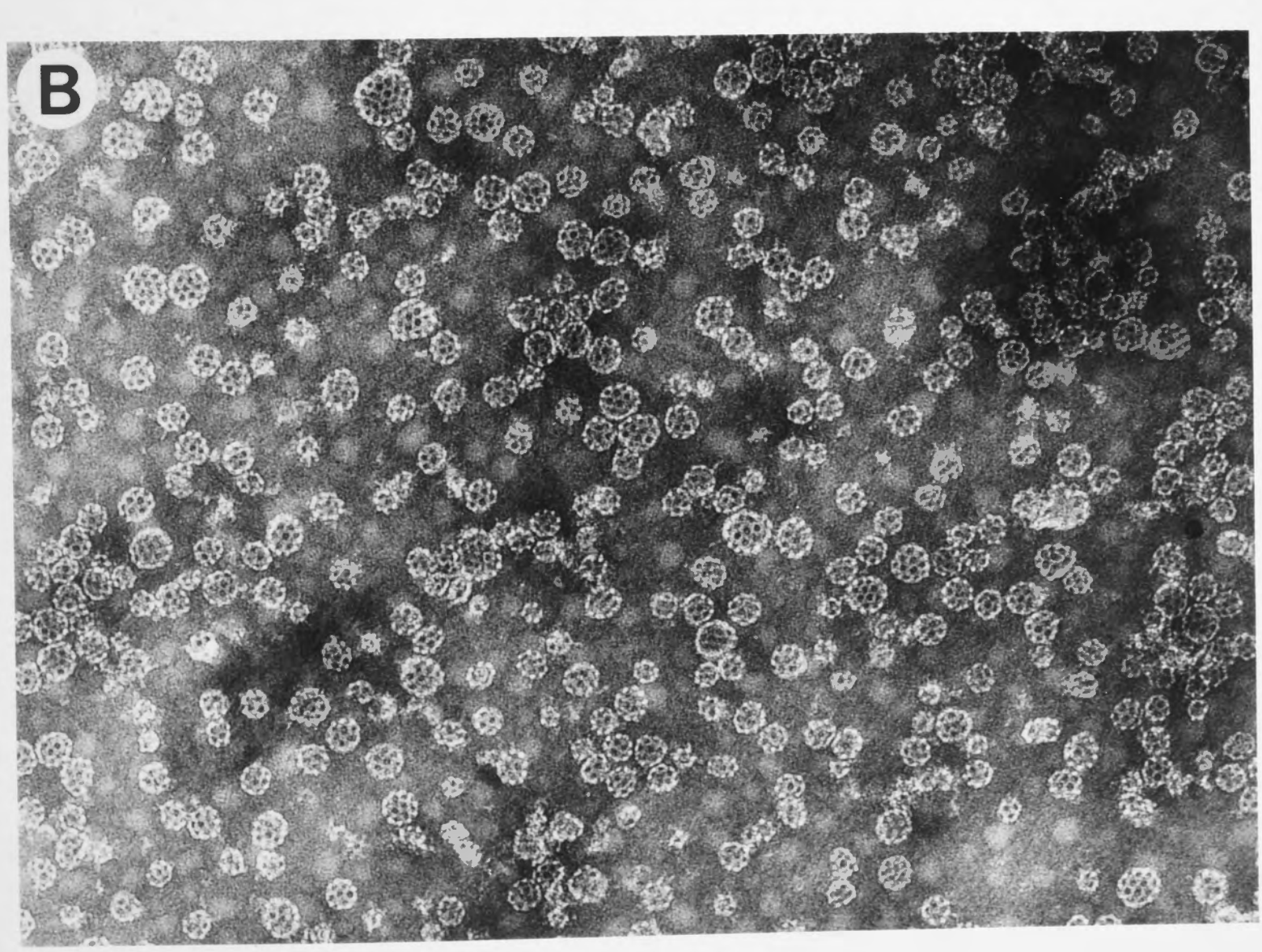

B

FIG. 2.2 Schematic outline of indirect ELISA and ELISA-plaque assay.

Indirect ELISA

1 Antigen adsorbed to plate

Wash

2 Add serum: specific antibody attaches to antigen.

Wash

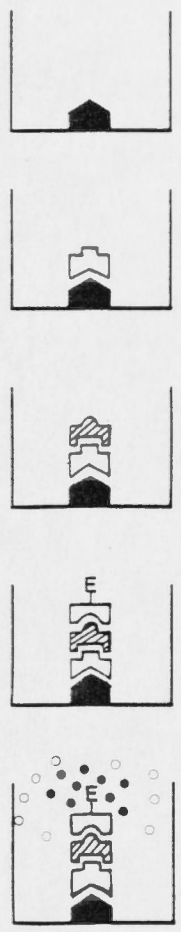
3 Add heavy chain specific antiglobulin(secondary antibody).

Wash

4 Add enzyme labelled antiglobulin to secondary antibody.

Wash

5 Add soluble substrate (p-nitrophenyl phosphate): measure O.D. of solution.



ELISA Plaque Assay

1 Antigen adsorbed to plate.

Wash

2 Add cell suspension: secreted antibody attaches to antigen.

Wash

3 Add heavy chain specific antiglobulin(secondary antibody).

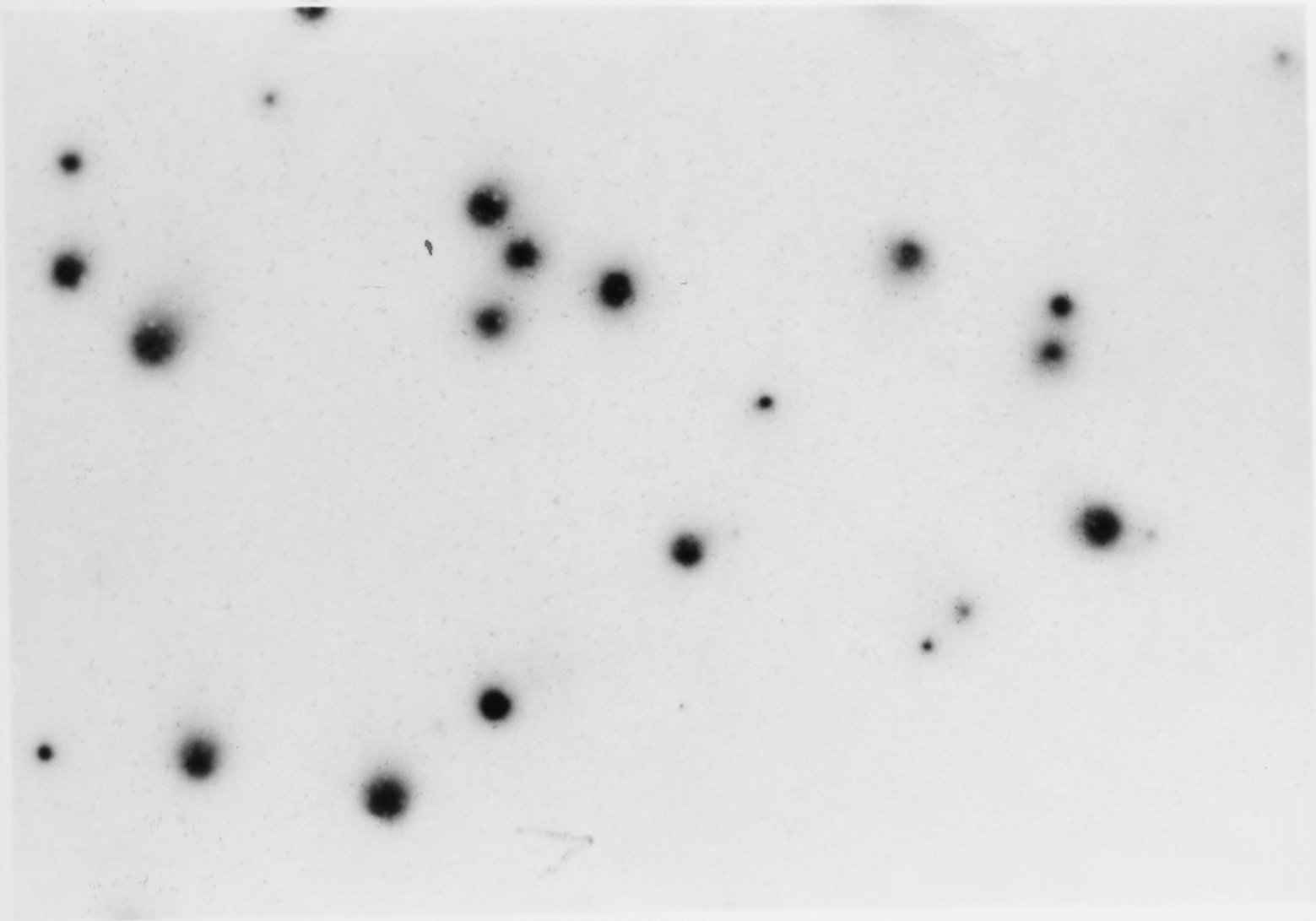
Wash

4 Add enzyme labelled antiglobulin to secondary antibody.

Wash

5 Add soluble substrate (5-bromo-chloro-indolyl phosphate): count plaques formed by insoluble color product.

FIG. 2.3 Appearance of plaques in ELISA-plaque assay.



CHAPTER THREE

THE ENUMERATION OF INFLUENZA VIRUS-SPECIFIC ASCs

INTRODUCTION

In order to address the aims of the thesis, it was essential to be able to accurately enumerate cells secreting influenza-specific antibodies. Since its discovery, the Jerne haemolytic-plaque assay, and its modifications, has been the most commonly used technique for enumerating ASCs. This technique relies upon the complement-mediated antibody-dependent lysis of untreated SRBCs or SRBCs coupled with either antigen, protein A or antiglobulin for the detection of either antigen specific or non-specific ASCs. The limitations of the haemolytic-plaque assay include its relative insensitivity and the need to enumerate IgG and IgA secreting cells by indirect plaque formation.

Whilst the haemolytic-plaque assay has been extensively utilized in studies on immune response to non-replicating antigens, it has found only limited application in the study of B cell responses to replicating antigens. Influenza-specific ASCs have been previously detected using the haemolytic-plaque technique by coupling the virus to periodate treated SRBCs (McLaren et al., 1978; Reiss & Schulman, 1980b). However this method has not been universally reproducible.

A technique employing ELISA methodology for enumerating specific ASCs to soluble antigens was recently described by two separate groups employing either the alkaline

phosphatase or horseradish-peroxidase enzyme-substrate systems (Sedgwick & Holt, 1983a; Czerkinsky et al., 1983). This technique referred to as either the ELISA-plaque assay or ELISPOT assay, is more sensitive than the haemolytic-plaque assay and can be readily adapted to accurately distinguish antibody isotypes. Previous applications of this method at the time work on this thesis commenced included the detection of cells secreting antibodies specific for ovalbumin (Sedgwick & Holt, 1983b) rheumatoid factor (Tarkowski et al., 1984) and tetanus toxoid (Czerkinsky et al., 1984).

The ELISA-plaque assay employing the alkaline phosphatase enzyme-substrate system was adapted to enumerate influenza-specific ASCs. The technique is described in detail in Section 2.7. In this chapter, the experiments designed to standardize the assay and demonstrate its specificity are described.

RESULTS

3.1 Standardization of the ELISA-plaque assay

3.1.1 Antigen Titration

The optimal amount of viral antigen needed for coating plates was determined by incubating spleen cell suspensions from mice immunized i.v. with 1000 HAU A/Jap virus on plates coated with variable concentrations of purified intact A/Jap virus. There was a significant increase in the IgM secreting cells detected with increasing concentrations of purified virus up to 1000 HAU/ml (Fig. 3.1). Plaques could also be reliably discerned at this concentration whereas at lower concentrations plaques progressively became fainter. Although the sensitivity of the assay is slightly sub-optimal at 1000 HAU/ml, this concentration was selected for subsequent experiments to achieve efficient use of virus stocks.

3.1.2 Culture Conditions

The volume of medium used during incubation of cell suspensions has been previously reported to be an important parameter affecting the accuracy of the assay (Czerkinsky et al., 1983). This was examined by enumerating IgM secreting cells in spleen cell suspensions from immunized mice using constant cell density ($2x10^5$ cells/ml) in

variable volumes of medium - 1.0ml, 1.2ml, 1.5ml and 2.0ml. There was no significant difference in the number of ASCs/10^6 cells detected under these conditions. 1.5ml was chosen for subsequent experiments as smaller volumes did not cover the surface of the wells completely.

In order to determine whether the cell density affected the accuracy of the assay, variable numbers of immunized spleen cells were incubated in a constant volume of medium. There was a significant correlation ($r = 0.998$, $p<0.01$) between the number of cells incubated and the number of ASCs detected (Fig. 3.2). Thus in subsequent experiments the number of ASCs detected could be accurately adjusted to be expressed as ASCs/10^6 cells depending on the number of cells incubated.

The culture medium used for incubating cell suspensions was supplemented with microcarrier beads (Cytodex 1) as previously described (Czerkinsky et al., 1983). This resulted in improved definition of plaques without affecting the sensitivity of the assay. The effect of microcarrier beads on plaque formation is supposedly due to accelerated sedimentation of the cells onto the solid phase. In addition, the beads may also absorb free immunoglobulins secreted into the medium either directly or via Fc receptors on macrophages which selectively bind to the beads (Ren, 1982).

3.1.3 Reagents

Chequerboard titrations were performed to determine the optimal dilutions of the primary (goat anti-mouse Ig) and secondary (rabbit anti-goat Ig) antibodies. The substrate solution was used at a concentration of 0.05% as lower concentrations reduced the accuracy of the assay and higher concentrations increased the background staining. The incubation time for the substrate solution was, however, varied with the concentrations of the primary and secondary antibodies (Table 3.1). There was no significant difference in the number of ASCs detected within the range of concentrations tested for each of the primary antibodies. Subsequent experiments were performed using a 1:2000 dilution. There was both a reduction in the number of plaques and in plaque definition with concentrations of the secondary antibody below 1:1000; a 1:1000 dilution was used subsequently. There were no plaques formed if either the primary or secondary antibody was omitted. Plaque numbers and definition were increased by 2h incubation of the substrate; longer incubation periods resulted in greater background staining.

3.2 Precision of the ELISA-plaque Assay

The precision of the ELISA-plaque assay was estimated by determining the intra-plate and inter-plate variation in plaque counts using the same cell suspensions so as to avoid that source of biological variation. The coefficient of

variation for intra-plate counts, determined in over fifty experiments, was 9%. Inter-plate variation, determined in five experiments, was 12%. The total assay variation calculated from these results was 15%.

3.3 Effect of Enzyme Digestion of Cell Suspensions

The effect of digesting lung and spleen cell suspensions with collagenase prior to assaying for ASCs was evaluated to determine if the yield of viable cells and in particular ASCs could be increased.

Lung and spleen cell suspensions were prepared as described in Section 2.5.4. Preliminary experiments showed that the maximal yield of viable cells from normal lungs was achieved by incubating lung cell suspensions with 2ml of collagenase (2mg per ml of medium) per lung. This resulted in a 10 to 20 fold increase in the yield of total viable cells from each lung. The effect of collagenase digestion on the yield of ASCs from immunized mice is shown in Table 3.2. Collagenase digestion had no significant effect on the yield of viable cells or ASCs from spleen cell suspensions. In contrast, the yield of viable cells and ASC's from lung cell suspensions was increased by 20 to 30 fold.

3.4 Dependence of Plaque Formation on Antibody Production and Secretion

3.4.1 Effect of cycloheximide on plaque formation

To demonstrate that plaque formation was dependent on antibody production, variable concentrations of cycloheximide were incubated with spleen cell suspensions for 1h at 37°C. The cell suspensions were washed to remove secreted pre-formed antibodies and then assayed for ASCs after re-adding the corresponding cncentration of cycloheximide (Table 3.3). Under these conditions, incubation with cycloheximide shortly before and during the assay reduced plaque counts but did not completely ablate plaque formation probably as a result of the continued secretion of preformed antibodies. In order to inhibit antibody synthesis completely, cycloheximide was added to primary spleen cell cultures during the final 18 hours of culture. Prior to assaying for ASCs, the cell suspensions were washed and dead cells removed by centrifugation on Ficoll. The corresponding concentration of cycloheximide was added to the cell suspensions at the time of the plaque assay. Although increasing concentrations of cycloheximide reduced the yield of viable cells after culture, greater that 10^6 cells were recovered for each assay. Plaque formation was completely ablated by lug cyloheximide/ml.

3.4.2 Effect of depleting cell suspensions of Ig-bearing cells

To demonstrate that plaque formation was dependent on the presence of ASCs, spleen cell suspensions were depleted of Ig-bearing cells by separating out cells forming rosettes with SRBCs coupled with anti-mouse Ig. IgM and IgG plaque counts in the non-rosetting fraction were reduced to less than 5% of the unseparated cell suspensions (Fig. 3.3).

3.5 Specificity of the ELISA-plaque Assay

3.5.1 Detection of influenza-specific ASCs by the ELISA-plaque assay

Several experiments demonstrated the specificity of the ELISA-plaque assay to detect influenza-specific ASCs (Table 3.4). Plaques were infrequently detected in spleen and lung cell suspensions from normal unimmunized mice when assayed on influenza-coated plates. These were mostly IgM plaques (< 5 per 10^6 cells), and only occasionally IgG and IgA plaques (<1 per 10^6 cells). Plaques were rarely detected when cell suspensions from influenza infected mice were assayed on plates coated with either uninfected allantoic fluid or bicarbonate buffer only. In addition, influenza-specific ASCs were not detected in significant numbers in cell suspensions from mice immunized with Sendai virus.

The number of plaques formed by influenza-specific ASCs was also shown to be dependent on the dose of virus used for immunization (Fig. 3.4).

3.5.2 Effect of influenza virus type and subtype on the specificity of the ELISA-plaque assay

The specificity of the assay was further evaluated by immunizing mice with influenza viruses of different type and subtype specificities. Groups of mice were immunized with either A/Jap, A/Jap-Bel, A/PC, A/PR8 or B/Sing viruses. The ELISA-plaque assay was performed on spleen and lung cell suspensions using plates coated with the above viruses (Table 3.5). The greatest number of plaques for all isotypes was seen with the homologous combinations. However, significant cross reaction in each isotype was seen within the Type A influenza viruses. A very small number of cross reactive plaques, of the IgM isotype only, was seen with the Type B virus.

The observation that some cross reaction was seen between viruses with different surface antigens suggested that this was due to the recognition of non-surface antigens. This possibility was supported by the demonstration that plaque formation was completely inhibited by adding virus disrupted by detergent to the cell suspension during the assay, but was still observed in the presence of intact virus (Fig. 3.5).

3.5.3 Detection of subtype specific ASCs by a modified ELISA-plaque assay

The recognition of non-surface antigens occurs as a result of disruption of the whole virus during adsorption to the plate. To avoid disruption of the virus, A/Jap virus was bound to plates initially coated with a monoclonal antibody to the HA of the A/Jap virus. The effect of this modification on the specificity of the ELISA-plaque assay is shown in Table 3.6. Whereas approximately 40% of the IgM ASCs induced by immunization with A/PR8 formed plaques on plates coated with A/Jap virus alone, less than 2% formed plaques on plates initially coated with antibody. Approximately 55% of the IgM ASCs induced by immunization with A/Jap virus formed plaques on the antibody coated plates.

FIG. 3.1 Antigen Titration. IgM ASCs in day 4 spleens after primary i.v. immunization (1,000 HAU of A/Jap virus). Plates were coated with various concentrations of A/Jap virus.

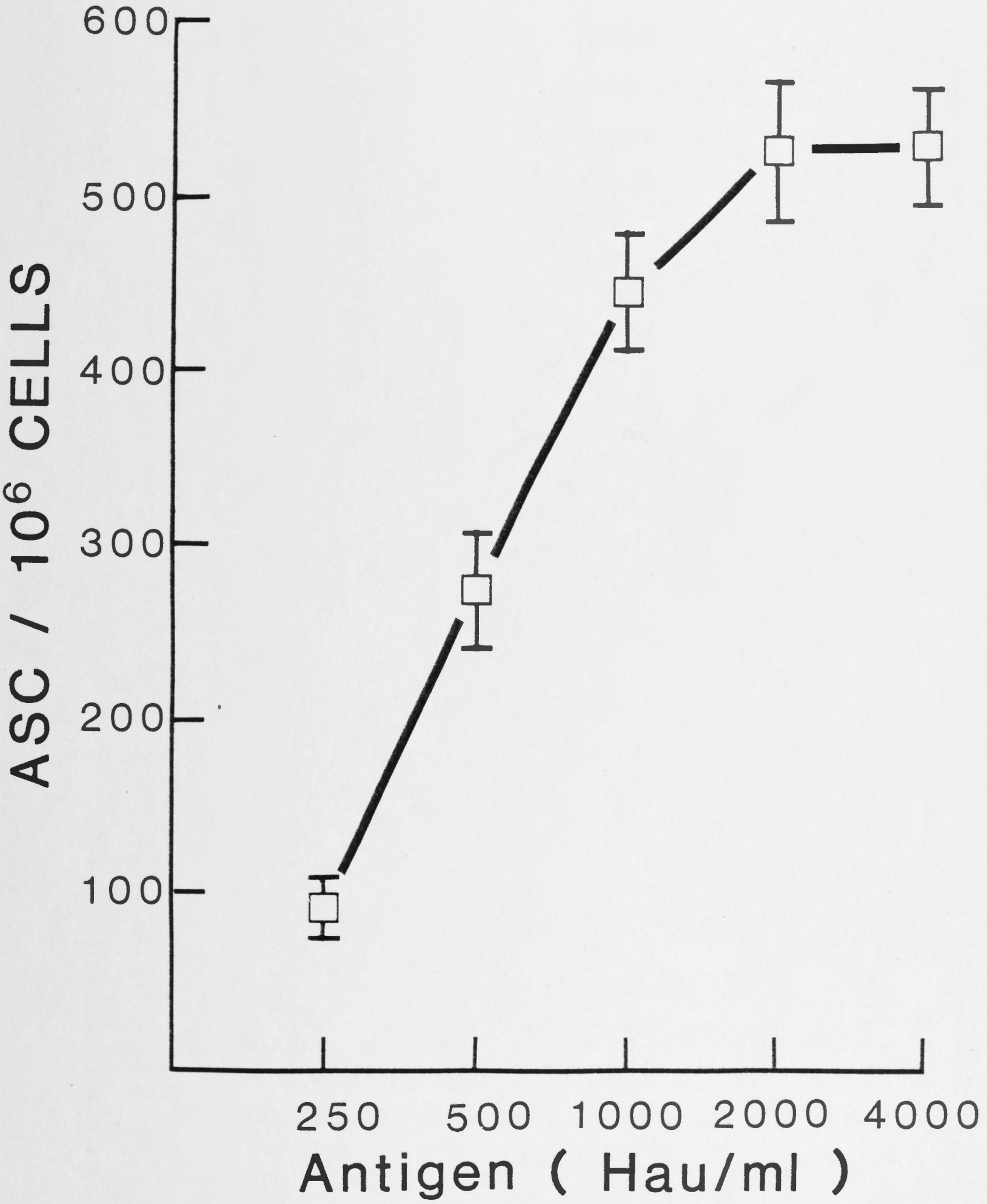

600
500
400
300
200
100
ASC / 10^6 CELLS
250
500
1000
2000
4000
Antigen (Hau/ml)

FIG. 3.2 IgM ASCs in day 6 spleens after primary i.v. immunization (1,000 HAU of À/Jap virus). Variable number of cells incubated in constant volume of medium.

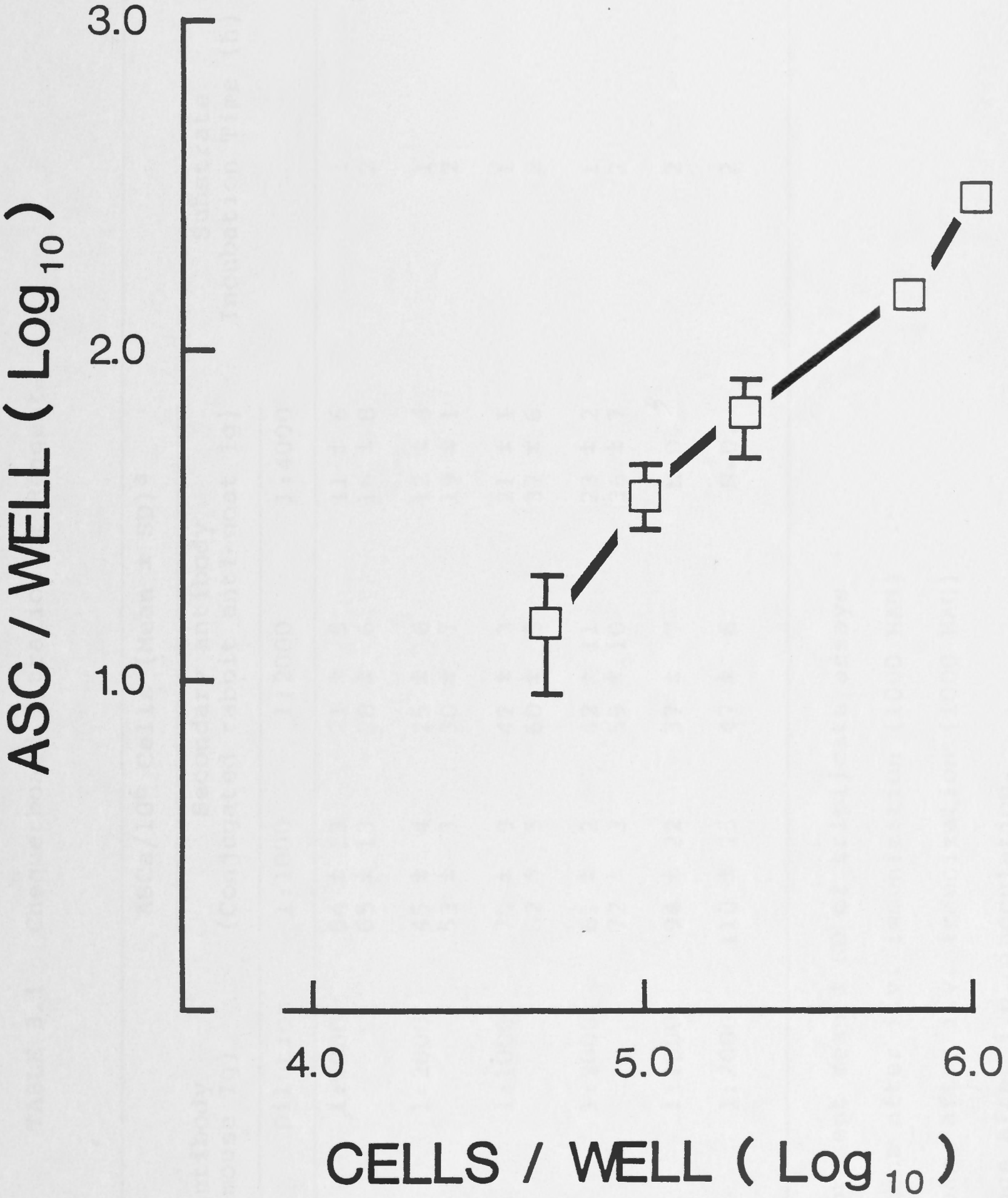

3.0
2.0
1.0
ASC / WELL (Log 10)
4.0
5.0
6.0
CELLS / WELL (Log 10)

TABLE 3.1 Chequerboard Titration of Reagents

Primary antibody (goat anti-mouse Ig)		ASCs/10^6 Cells (Mean ± SD)[a] Secondary antibody (Conjugated rabbit anti-goat Ig)			Substrate Incubation Time (h)
Specificity	Dilution	1:1000	1:2000	1:4000	
IgM^b	1:1000	54 ± 13	21 ± 5	11 ± 6	1
		65 ± 13	28 ± 6	16 ± 8	2
	1:2000	45 ± 4	25 ± 6	12 ± 4	1
		53 ± 3	30 ± 7	19 ± 1	2
IgG^c	1:1000	70 ± 9	42 ± 3	21 ± 1	1
		82 ± 5	60 ± 3	37 ± 6	2
	1:2000	61 ± 2	42 ± 11	23 ± 2	1
		72 ± 3	59 ± 10	36 ± 7	2
IgA^d	1:1000	94 ± 22	37 ± 7	N.D.	2
	1:2000	110 ± 25	47 ± 6	N.D.	2

a. Values represent mean ± SD of triplicate assays

b. Day 4 spleens after i.v. immunization (1000 HAU)

c. Day 6 spleens after i.v. immunization (1000 HAU)

d. Day 14 lungs after i.n. inoculation.

TABLE 3.2 Effect of Collagenase on Cell Yields and ASCs

Collagenase Treatment	Viable cells per mouse		ASCs/mouse (mean ± SD)[a]				
			Spleen[b]		Lung[c]		
	Spleen[b]	Lung[c]	IgM	IgG	IgM	IgG	IgA
-	1.7×10^8	9.6×10^5	32,640 ±2,040	18,190 ±2,210	208 ± 10	86 ± 15	119 ± 12
+	1.24×10^8	1.63×10^7	27,652 ±1,612	15,872 ±1,612	3,896 ±244	3,683 ±342	3,081 ±326

a. Values represent mean ± SD of 5 replicate assays.

b. Day 6 spleens after i.v. immunization (1,000 HAU).

c. Day 12 lungs after i.n. inoculation ($10^3 EID_{50}$).

TABLE 3.3 Effect of Cycloheximide on Plaque Formation

Cycloheximide Concentration (ug/ml)	IgM ASCs/10^6 cells (mean ± SD)[a]	
	Number of Hours Cycloheximide added to Cell Suspensions before Assay	
	1h[b]	18h[c]
Nil	176 ± 11	381 ± 37
0.5	119 ± 5	12 ± 5
1.0	91 ± 5	1 ± 1
2.0	56 ± 8	0
4.0	57 ± 8	0

a. Values represent mean ± SD of triplicate assays

b. Day 7 spleens after primary i.v. immunization (1000 HAU)

c. Suspensions of pooled spleen cells obtained from 2 normal mice stimulated in vitro for 4 days. Cell suspensions from unstimulated cultures produced < 5 IgM ASCs/10^6 cells.

FIG. 3.3 (A) IgM ASCs in day 4 spleens after primary i.v. immunization (1,000 HAU of A/Jap virus). (B) IgG ASCs in day 4 spleens after secondary i.v. immunization (two injections of 1,000 HAU of A/Jap each). 1, normal spleens; 2, suspensions of unseparated cells from immunized mice; 3, suspensions depleted of Ig-bearing cells from immunized mice.

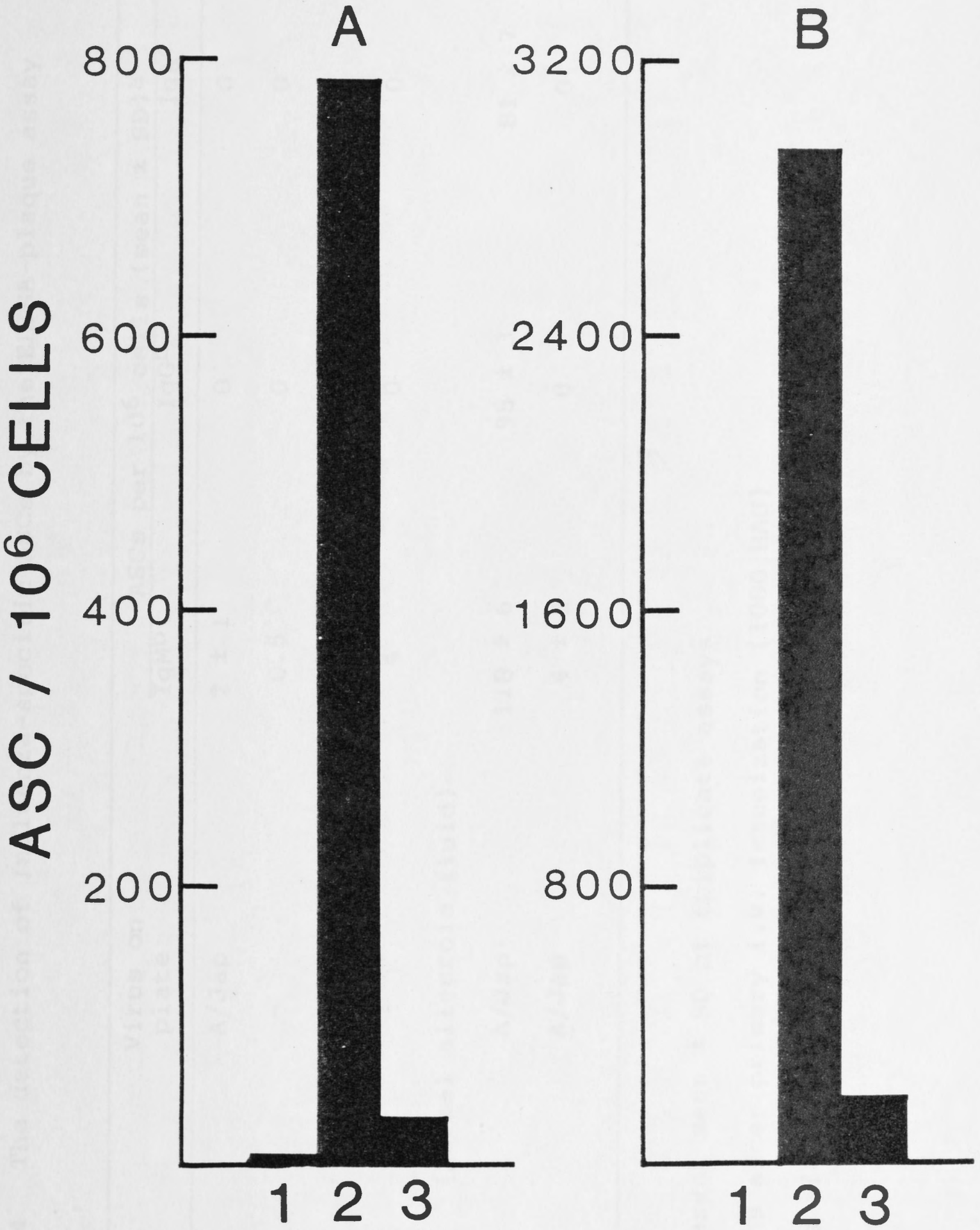

A
B
800
600
400
200
3200
2400
1600
800
ASC / 10^6 CELLS
1 2 3
1 2 3

TABLE 3.4 The detection of Influenza-specific ASCs by the ELISA-plaque assay

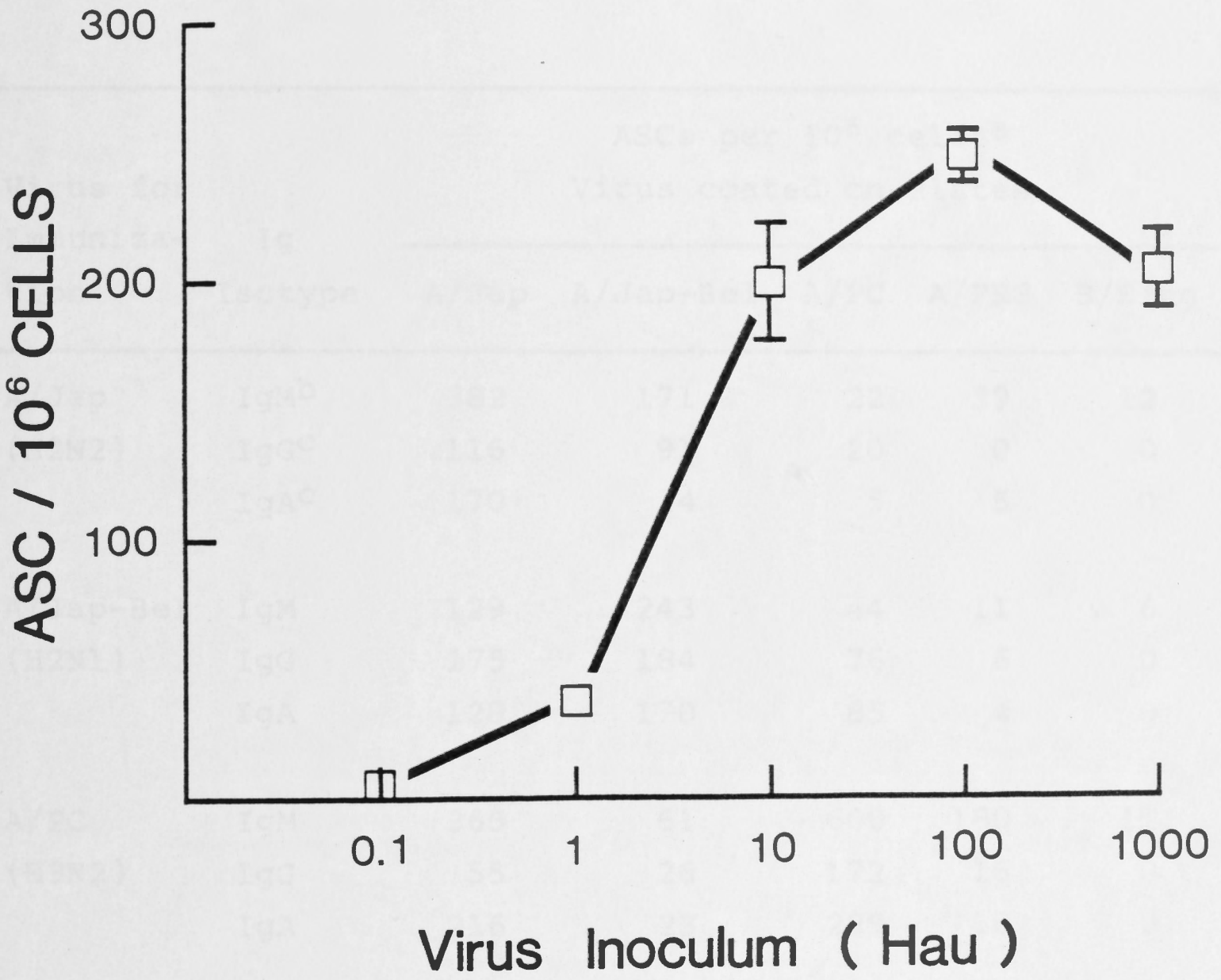
Virus for Immunization	Virus on plate	ASCs per 10^6 cells (mean ± SD)[a]		
		IgM[b]	IgG[b]	IgA[c]
-	A/Jap	2 ± 1	0	0
A/Jap	- (Buffer only)	0.5	0	0
A/Jap	- (Normal allantoic fluid)	4	0	0
A/Jap	A/Jap	118 ± 6	98 ± 3	81 ± 7
Sendai	A/Jap	4 ± 2	0	0

a. Values represent mean ± SD of triplicate assays

b. Day 6 spleens after primary i.v. immunization (1000 HAU)

c. Day 12 lungs after primary i.n. inoculation

FIG. 3.4 IgM ASCs in day 4 spleens after primary i.v. immunization with graded doses of A/Jap virus.


300
200
100
ASC / 10^6 CELLS
0.1
1
10
100
1000
Virus Inoculum (Hau)

TALE 3.5 Effect of influenza virus type and subtype on the specificity of the ELISA-plaque assay

Virus for Immunization	Ig Isotype	ASCs per 10^6 cells[a] Virus coated on Plates				
		A/Jap	A/Jap-Bel	A/PC	A/PR8	B/Sing
A/Jap (H2N2)	IgM[b]	382	171	22	39	12
	IgG[c]	116	91	20	0	0
	IgA[c]	170	14	5	5	0
A/Jap-Bel (H2N1)	IgM	129	243	44	11	6
	IgG	175	184	76	6	0
	IgA	128	170	85	4	0
A/PC (H3N2)	IgM	365	61	600	180	11
	IgG	55	26	172	16	0
	IgA	216	23	299	10	0
A/PR8 (H1N1)	IgM	53	23	25	422	13
	IgG	31	20	12	88	0
	IgA	102	8	0	158	0
B/Sing	IgM	9	4	6	13	110
	IgG	0	0	0	0	40
	IgA	0	0	0	0	70

a. Values represent mean of duplicate assays.

b. Day 5 spleens after primary i.v. immunizations (1000 HAU).

c. Day 14 lungs after primary i.n. inoculations.

FIG. 3.5 IgM ASCs in day 4 spleens after primary i.v. immunization (1,000 HAU of A/Jap virus). Various concentrations of either purified intact A/Jap virus (solid line) or detergent-disrupted virus (broken line) were added to cell suspensions at the time of assay.

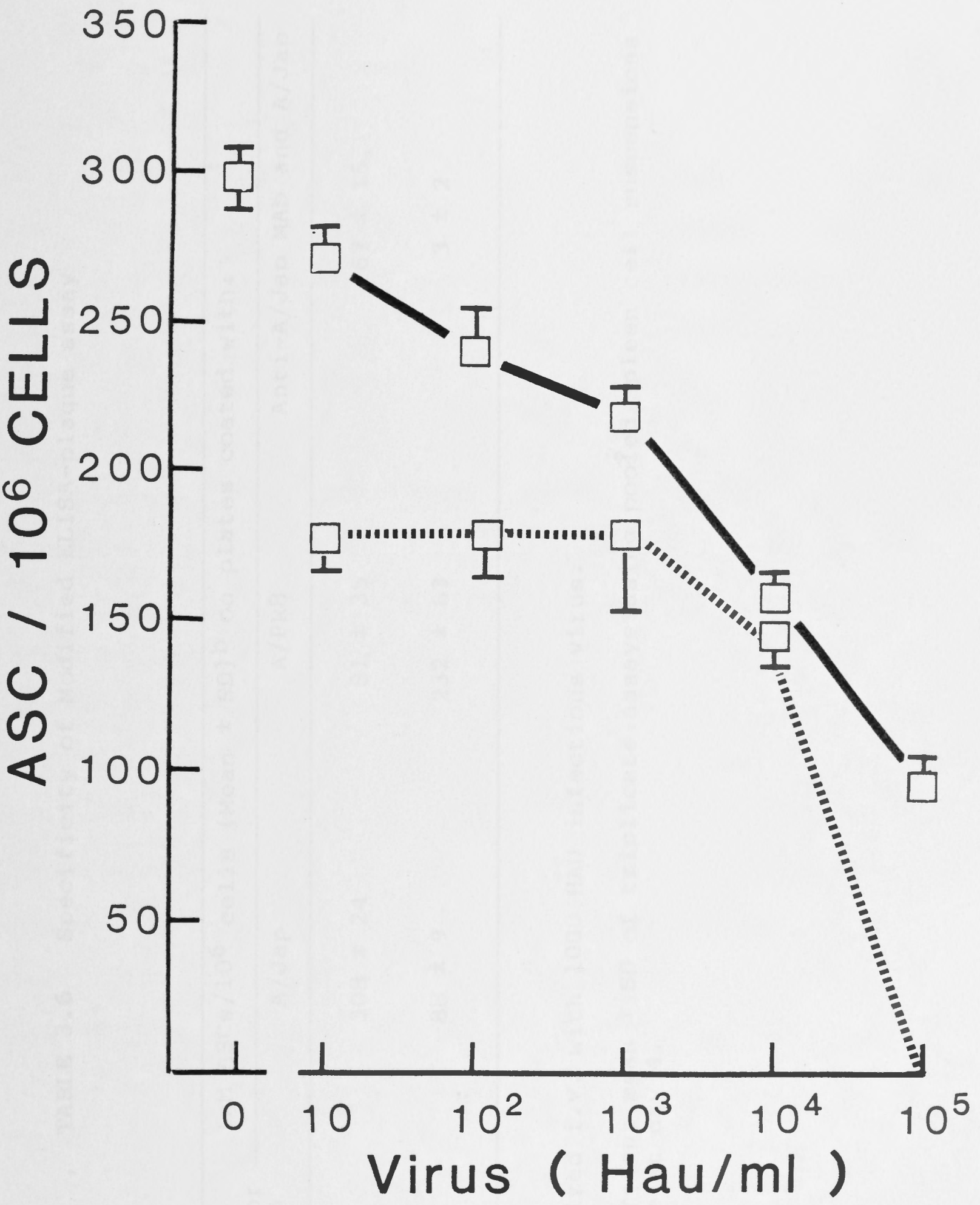

ASC / 10^6 CELLS
350
300
250
200
150
100
50
0
10
10^2
10^3
10^4
10^5
Virus (Hau/ml)

TABLE 3.6 Specificity of Modified ELISA-plaque assay

Virus used for immunization[a]	IgM ASCs/10^6 cells (Mean ± SD)[b] on plates coated with:		
	A/Jap	A/PR8	Anti-A/Jap MAb and A/Jap
A/Jap	308 ± 24	81 ± 35	167 ± 15
A/PR8	88 ± 9	232 ± 63	3 ± 2

a. Mice immunized i.v. with 1000 HAU infectious virus.

b. Values represent mean ± SD of triplicate assays using pooled spleen cell suspensions from 3 mice at day 4.

DISCUSSION

An ELISA-plaque assay is described which can reliably enumerate influenza-specific ASCs. The ELISA-plaque assay was initially chosen in preference to the haemolytic-plaque assay because of the limitations of the haemolytic-plaque assay. Subsequently however, in experiments designed to compare the sensitivity of the ELISA-plaque assay with the haemolytic-plaque assay, the previously reported successful use of the haemolytic-plaque assay to detect influenza-specific ASCs could not be reproduced. At the time work on this thesis was initiated, the ELISA-plaque assay had not been applied to enumerate ASCs specific for infecting agents. Subsequently, a report describing splenic ASCs following intravenous immunization with lymphocytic choriomeningitis virus and vesicular stomatitis virus employed the ELISA-plaque assay (Moskophidis & Lehmann-Grube, 1984). Just as the ELISA technique for antibody detection can be used to measure antibodies of any specificity, the ELISA-plaque technique could most likely be adapted to estimate the numbers of cells secreting antibodies to any infectious agent and probably in any host organ.

A variety of experiments attest to the reliability and reproducibility of the ELISA-plaque assay for enumerating cells secreting influenza-specific antibodies of different isotypes. Plaque formation was shown to be dependent on

antibody production and on the presence of cells secreting antibody. Although plastic adherent cells are not removed by washing after incubation of cell suspensions, the possibility that macrophages to which specific cytophilic antibody was bound formed plaques is unlikely, as plaques do not develop when cell suspensions from immunized mice are incubated on antigen-free plates, despite the presence of adherent cells.

The assay specifically detected influenza-specific ASCs essentially only in mice immunized with influenza virus. Cells producing type specific antibodies but not cells producing antibodies of different subtype specificities could clearly be distinguished. This latter result is similar to previous experiences with the indirect ELISA where whole virus was used as the antigen for measuring anti-influenza antibody (Hammond et al., 1980; Al-Kaissi & Mostratos, 1982) and is due to the recognition of common antigenic determinants, principally on the internal proteins. These determinants are exposed in the well probably as a result of physical alteration to the virus structure during adsorption to the solid phase, an interpretation supported by the complete inhibition of plaque formation only by detergent-disrupted virus.

In view of the lack of subtype specificity of the ELISA-plaque assay using whole virus to coat the plates, it was necessary to modify the assay so as subtype specific

ASCs could be detected. This was achieved by firstly coating the plates with a monoclonal antibody to the HA molecule to avoid disruption of the virus. This approach was preferred to using purified HA to coat the plates as determinants on the HA molecule common to all subtypes may be recognized after adsorption (Burlington et. al., 1983).

The standard ELISA-plaque assay as described in Section 2.7 was used in the experiments described in the succeeding chapters except for Section 5.5 where the modified assay was used to detect subtype specific ASCs during a heterotypic challenge.

SUMMARY

An ELISA-plaque assay is described which can reliably enumerate cells secreting influenza virus specific antibodies of the IgM, IgG and IgA isotypes. Influenza-specific ASCs were detected in significant numbers only in mice immunized with influenza virus. Cells producing antibodies of different subtype specificities could not be reliably distinguished, as with the indirect ELISA, unless the assay was modified by binding the virus to a monoclonal antibody on the solid phase rather than adsorbing the virus directly onto the solid phase.

CHAPTER FOUR

B CELL RESPONSES IN THE PRIMARY IMMUNE RESPONSE TO INFLUENZA

INTRODUCTION

Previous descriptions of the cellular events underlying the humoral response in the respiratory tract during viral infections have relied upon the demonstration by immunofluorescence of Ig-bearing cells. Using this approach, increases in Ig-bearing cells have been detected in the lungs of mice infected with Sendai virus (Blandford et al., 1971) and influenza virus (Scott & Walker, 1976). However, not only does this approach fail to demonstrate the viral specificity of the surface antibody on these cells, but it provides at best only indirect evidence that specific antibody in respiratory secretions is locally synthesized. More direct evidence for this would be provided by the demonstration of specific antibody secretion at the cellular level.

Previous reports of influenza-specific ASCs, detected by the haemolytic-plaque assay, have provided remarkably little information on events within the lung. There is a single finding of direct plaque-forming cells in tracheobronchial cell preparations from influenza infected ferrets (McLaren & Butchko, 1978).

In this chapter, the ELISA-plaque assay has been used to study the B cell response at the cellular level to primary influenza virus infection in mice.

4.1 Influenza-specific ASCs after Primary Intravenous Immunization

ASCs were enumerated in the spleen after primary intravenous immunization with 1000 HAU infectious A/Jap virus (Fig 4.1). IgM ASCs were first detected at 48h after injection and peaked at day 4. Thereafter the level of IgM ASCs declined rapidly and by one month only a small number of IgM ASCs were still present (10 ASCs/10^6 cells). IgG ASCs were first detected at day 4, peaked at day 6 and subsequently plateaued at a lower level; at one month approximately 50 ASCs/10^6 cells were still present. There was a minimal IgA response. At three months after injection the level of ASCs had not changed appreciably from that observed at one month.

ASCs were also enumerated in the lung after intravenous immunization to determine if an IgA response was induced in the lung in the virtual absence of an IgA response in the spleen. Mean lung virus titre at 3 days after intravenous injection was 4.5 $\log_{10}$ EID_{50}. The peak IgM response in the lung occurred 48h later than in the spleen and, in magnitude, was only one-third that of the splenic IgM response. As expected, IgA ASCs were more numerous in the lung than the spleen (Table 4.1).

4.2 B cell Responses after Primary Intranasal Inoculation

4.2.1 Influenza-specific ASCs

Mice were inoculated intranasally with 10^4 EID_{50} infectious A/Jap virus causing peak lung consolidation of 45±20% and 5-10% mortality. Mean lung virus titre measured at day 3 was 6.6 $\log_{10}$ EID_{50}. Class-specific ASCs cells to influenza virus were enumerated in the lung, spleen and blood (Fig. 4.2). ASCs were more numerous in the lung than in the spleen for each isotype; in each organ ASCs producing IgM were the predominant cell type found. IgM ASCs were first detected in the lung at day 5 and subsequently peaked at day 10. IgG and IgA ASCs were not detected in the lung until the second week and thence both peaked during the third week with slightly more IgG than IgA ASCs. At one month after infection, numerous ASCs of each isotype were still present in the lung.

The onset and peak of the splenic IgM response preceded the pulmonary IgM response by 24-48 hours. Splenic IgG ASCs were first detected at day 6 in very small numbers and peaked by day 18. A very small number of IgA ASCs (7 per 10^6 cells) was detected in the spleen during the third week. At one month, only IgG ASCs (30 per 10^6 cells) were detected in the spleen.

ASCs producing IgM also constituted the major response in the blood; IgM ASCs appeared at day 6 and had declined by the end of the second week. IgG and IgA ASCs also circulated during the second and third weeks in small numbers (< 20 per 10^6 cells).

In mice infected with a ten-fold lower dose of A/Jap virus, the onset of the response in both lung and spleen was delayed by 48 hours compared to mice infected with the higher dose. The pattern of the response was subsequently similar, though of lesser magnitude (Fig. 4.3). Peak responses in both the lung and spleen for each isotype were 40-60% lower than those seen with the higher primary dose.

In the preceding experiments, ASCs were enumerated using suspensions of pooled cells from several mice. To determine the extent of biological variation in the onset and magnitude of the ASC responses, IgM ASCs were enumerated using suspensions of cells from individual mice after intranasal inoculation (Table 4.2). There were no IgM ASCs detected in the spleen until day 5 or in the lung until day 6. The coefficients of variation of the mean number of ASCs detected at each time point averaged 40% with a maximum of 64%. In view of the extent of biological variation in the number of ASCs in individual mice, less than 2-fold differences in results obtained using suspensions of pooled cells could not be regarded as biologically significant.

4.2.2 Influenza-specific Antibodies

Influenza-specific antibody titres were measured, using an indirect ELISA, in the sera and tracheobronchial washes of mice infected with $10^4 EID_{50}$ infectious A/Jap virus (Fig. 4.4). In general, the onset and peak of the antibody response for each isotype coincided with or shortly followed the corresponding phase of the ASC response.

4.3 Generation of ASCs in vitro

The primary response to influenza was further evaluated by using lung and spleen cell cultures obtained from unprimed mice and stimulated in vitro with influenza virus to determine if precursors of influenza-specific ASCs were present in these tissues.

4.3.1 Conditions of Primary in vitro Cultures

To determine the optimal method for in vitro generation of ASCs, spleen and lung cell cultures were stimulated with either purified virus or virus-infected irradiated syngeneic spleen cells. The titre of purified virus or the amount of virus used to infect cells was varied to determine the optimal concentration for stimulation (Fig. 4.5). The maximum number of IgM ASCs detected in both spleen and lung cell cultures followed stimulation with purified virus at a concentration of 20 HAU/ml.

4.3.2 Influenza-specific ASCs in Primary in vitro Cultures

Spleen and lung cell cultures obtained from unprimed mice were stimulated in vitro with 20 HAU A/Jap virus per ml and the number of ASCs generated determined over the following week (Fig. 4.6). ASCs producing IgM were detected in spleen cell cultures after 48h incubation and in lung cell cultures at 72h. The peak IgM response developed in both cell cultures at day 4 and was slightly greater in the spleen cell culture. IgG ASCs were not detected in either cell culture, whereas a small number of IgA ASCs ($5/10^6$ cells) were detected in the lung cell culture.

ASCs were not detected in cultures of peripheral blood lymphocytes stimulated under the same conditions, indicating that the ASCs found in lung cell cultures were not derived to any major extent from precursors in the pulmonary vasculature.

4.3.3 Specificity of ASCs Generated in vitro

The specificity of the ASCs generated in vitro was determined using different viruses for stimulation (Table 4.3). The number of background IgM ASCs detected on plates coated with buffer only or from unstimulated cultures was greater than that observed using cell suspensions which had not been cultured in vitro (see Table 3.4). This most likely was related to the mitogenic activity of foetal calf

serum. This finding probably also underlies the apparently greater degree of cross-reactivity observed between the type A and B influenza viruses than that seen using immune cell suspensions obtained in vivo.

As influenza A viruses of the H2, H3 and H6 subtypes behave as T cell - independent B cell mitogens for murine lymphocytes (Anders et al., 1984), the generation of ASCs using A/Jap virus (H2 subtype) may have been related to nonspecific mitogenic activity. However, spleen cell cultures stimulated by a less mitogenic H1 subtype virus (A/PR8) produced similar results:- 486 ± 21 IgM ASCs/10^6 cells at day 4. Furthermore, in later experiments (see Table 5.1) depletion of $L3T4^+$ cells reduced the number of ASCs in spleen cell cultures by more than 95%.

FIG. 4.1 ASCs in spleen after primary i.v. immunization (1,000 HAU A/Jap). Values represent mean results from triplicate assays performed on suspensions of pooled cells from 3 to 4 mice.

Symbols: □ IgM, ○ IgG, △ IgA

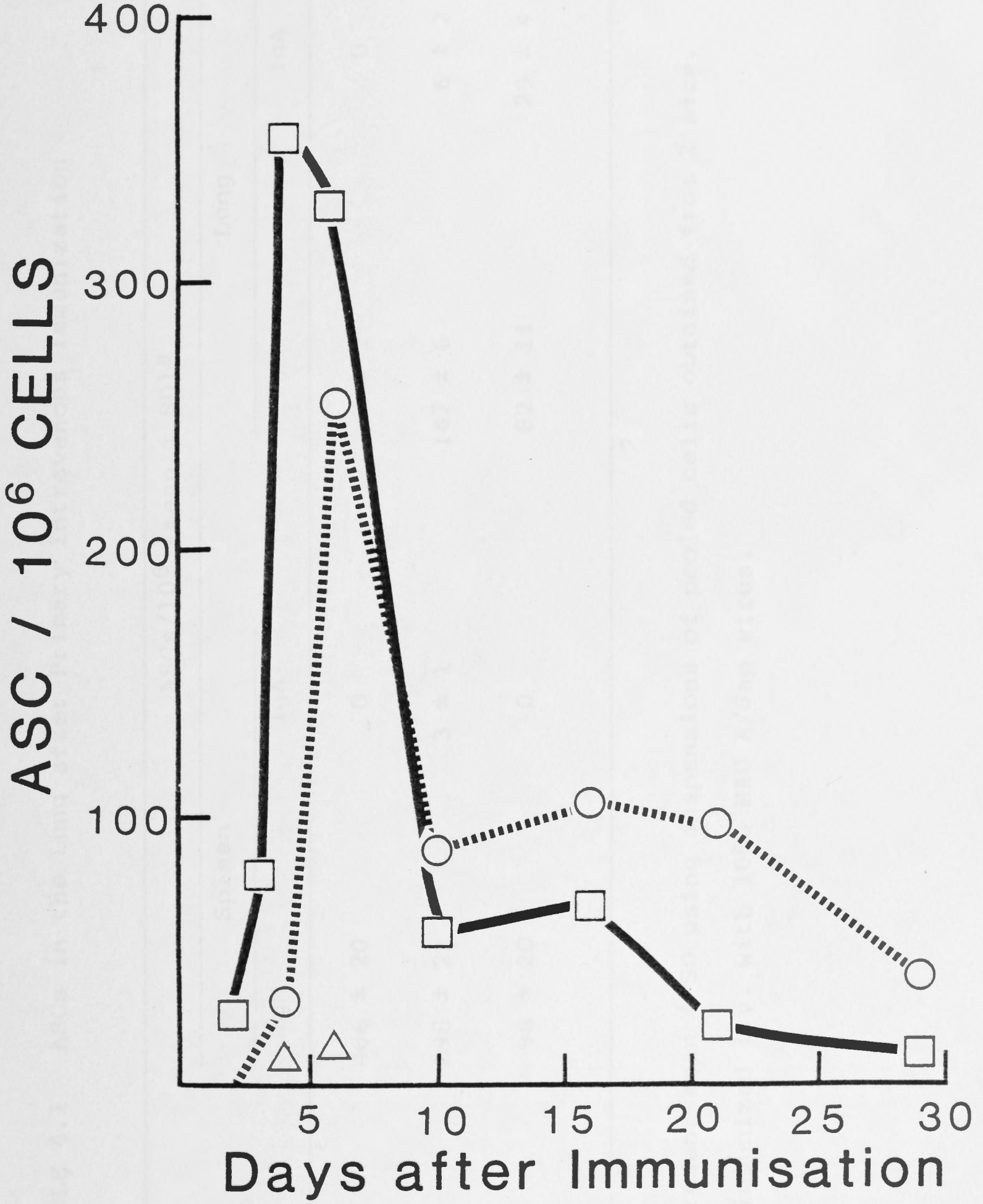

ASC / 10^6 CELLS
400
300
200
100
5
10
15
20
25
30
Days after Immunisation

TABLE 4.1 ASCs in the Lung after Primary Intravenous Immunization

Time after immunization[b]	ASCs/10^6 (mean ± SD)[a]			
	Spleen		Lung	
	IgM	IgA	IgM	IgA
Day 4	466 ± 20	0	8 ± 2	0
Day 6	298 ± 22	3 ± 1	147 ± 6	6 ± 2
Day 8	98 ± 20	0	82 ± 11	29 ± 4

a. Values represent mean ± SD using suspensions of pooled cells obtained from 2 mice.

b. Mice were immunized i.v. with 1000 HAU A/Jap virus.

FIG. 4.2 ASCs in lung, spleen and blood after primary intranasal inoculation (10^4 EID_{50} of A/Jap). Values represent mean results from triplicate assays performed on suspensions of pooled cells from 3 to 4 mice.

Symbols: □ IgM, ○ IgG, △ IgA

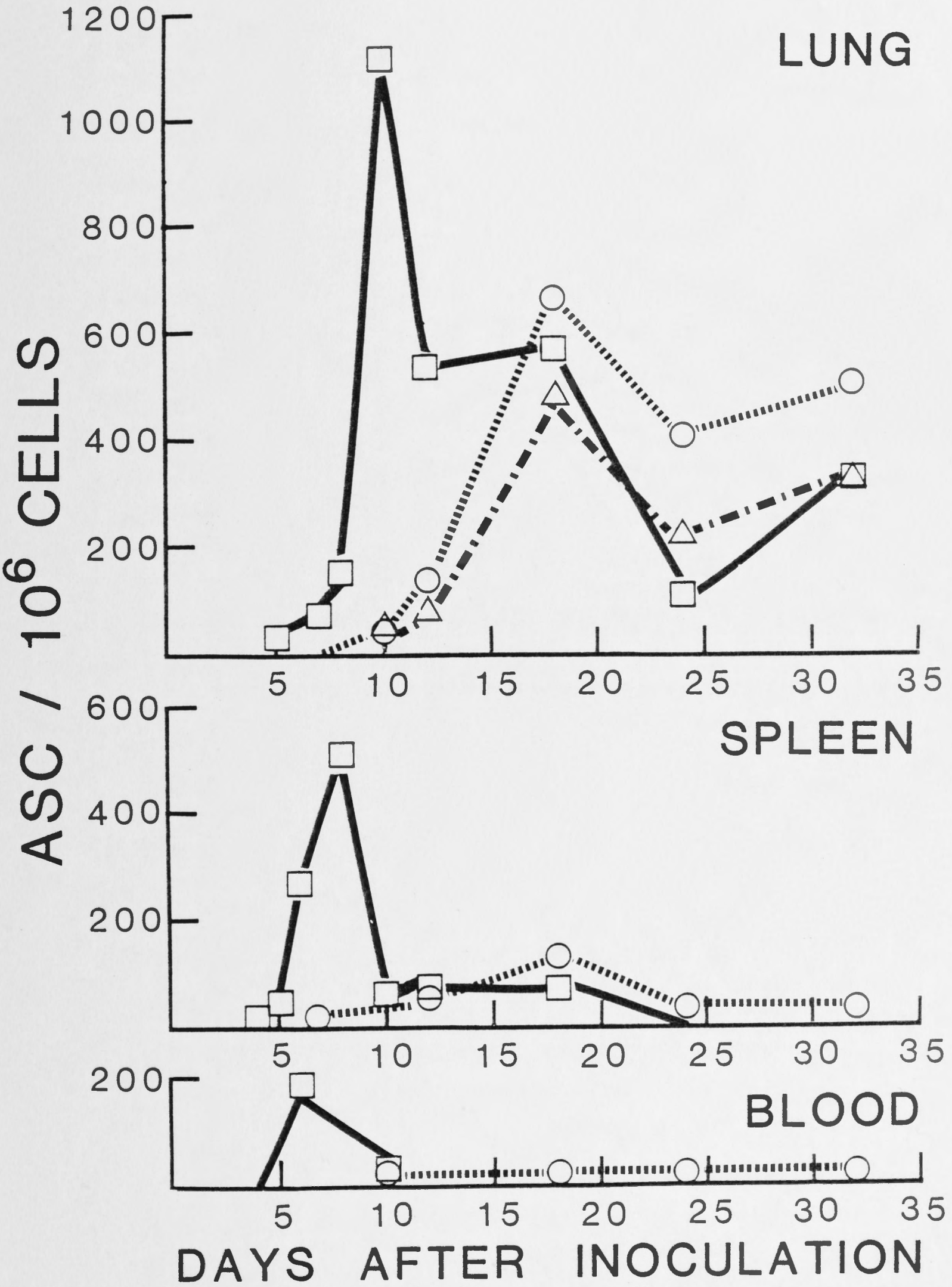

LUNG
SPLEEN
BLOOD
ASC / 10^6 CELLS
DAYS AFTER INOCULATION
1200
1000
800
600
400
200
5
10
15
20
25
30
35

FIG. 4.3 ASCs in lung and spleen after primary intranasal inoculation (10^3 EID_{50} of A/Jap). Values represent mean results from triplicate assays performed on suspensions of pooled cells from 3 to 4 mice.

Symbols: □ IgM, ○ IgG, △ IgA

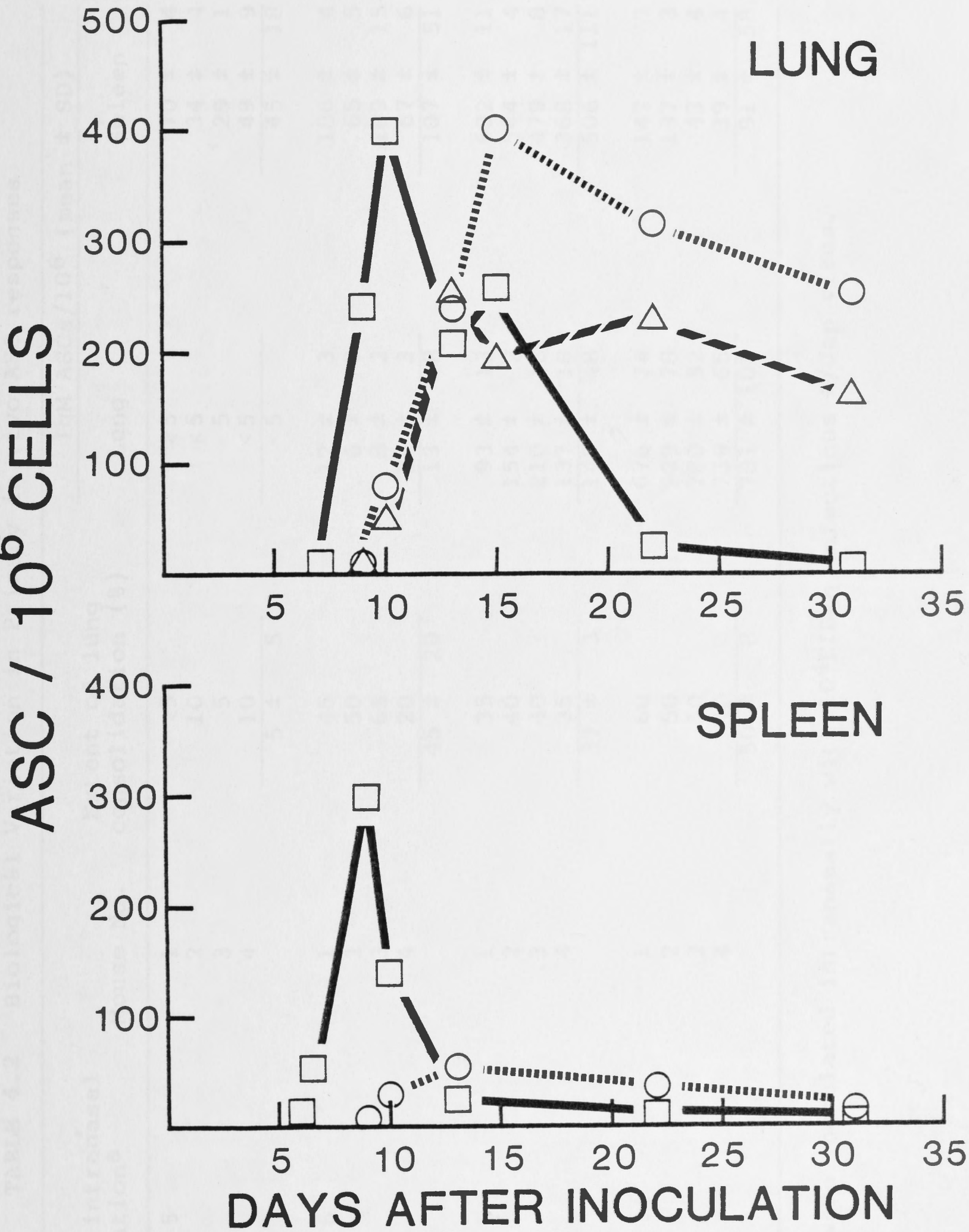

LUNG
SPLEEN
ASC / 10^6 CELLS
500
400
300
200
100
400
300
200
100
5
10
15
20
25
30
35
5
10
15
20
25
30
35
DAYS AFTER INOCULATION

TABLE 4.2 Biological Variation in Primary in vivo ASC responses

Time after intranasal inoculation[a]	Mouse No.	Extent of lung consolidation (%)	IgM ASCs/10^6 (mean ± SD)	
			Lung	Spleen
Day 5	1	<5	<5	70 ± 4
	2	10	<5	34 ± 4
	3	5	5	29 ± 1
	4	10	<5	49 ± 9
		5 ± 5	<5	45 ± 18
Day 6	1	45	12 ± 3	186 ± 4
	2	50	6 ± 3	65 ± 5
	3	65	8 ± 2	109 ± 15
	4	20	25 ± 3	67 ± 6
		45 ± 20	13 ± 8	107 ± 51
Day 8	1	35	93 ± 13	632 ± 11
	2	40	154 ± 2	544 ± 4
	3	40	210 ± 18	479 ± 8
	4	35	137 ± 18	368 ± 17
		37 ± 3	149 ± 48	506 ± 111
Day 11	1	60	676 ± 74	147 ± 7
	2	50	929 ± 78	137 ± 3
	3	50	780 ± 32	43 ± 4
	4	40	739 ± 65	39 ± 4
		50 ± 8	781 ± 107	91 ± 58

a. Mice were inoculated intranasally with $10^4 EID_{50}$ infectious A/Jap virus.

FIG. 4.4 Influenza-specific antibody titres after primary intranasal inoculation (10^4 EID_{50} A/Jap). Values represent reciprocal of ELISA titre (log_2) using tracheobronchial washes (TBW) and sera pooled from 3 to 4 mice.

Symbols: □ IgM, ○ IgG, △ IgA

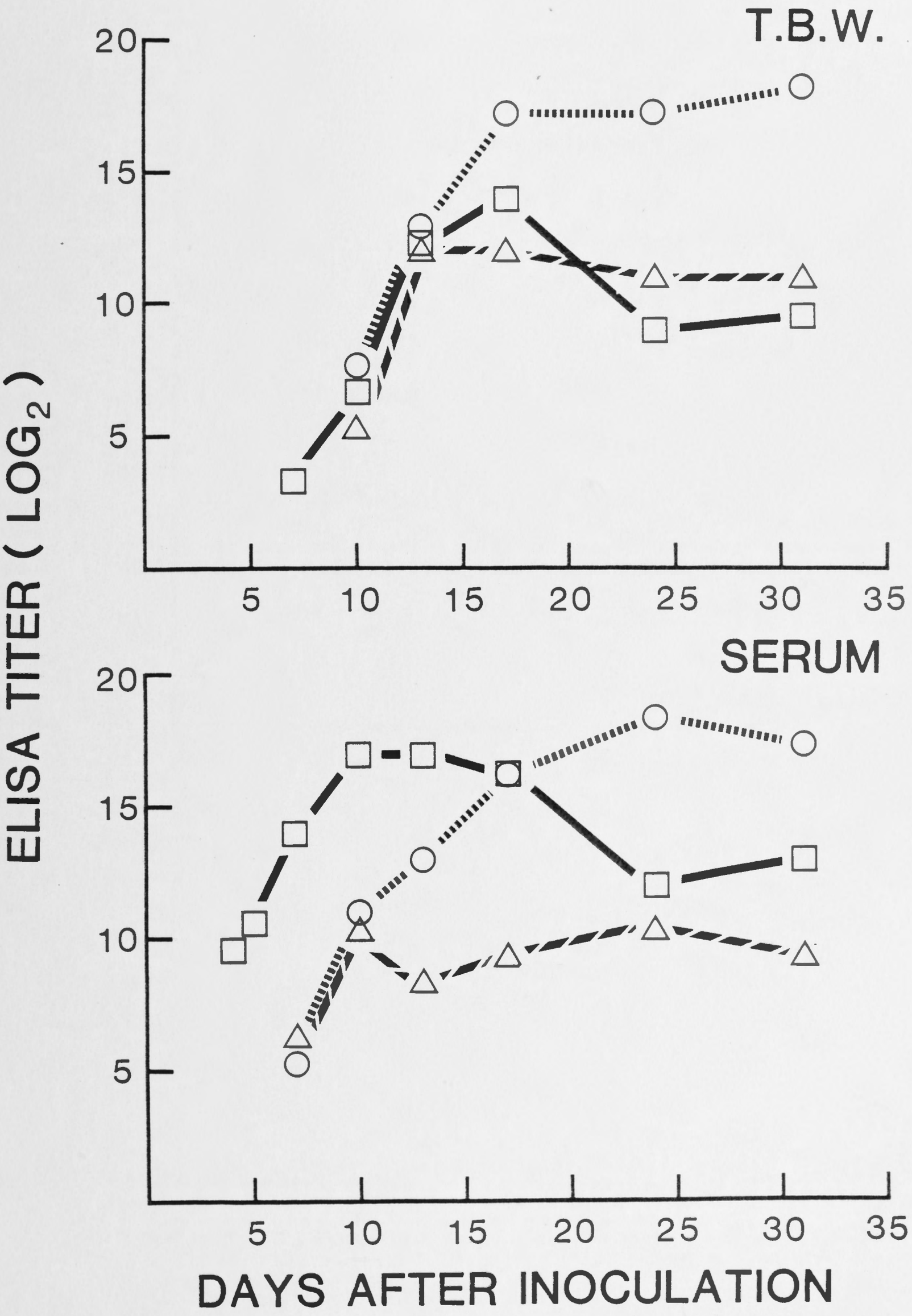

T.B.W.
SERUM
ELISA TITER (LOG2)
20
15
10
5
5
10
15
20
25
30
35
DAYS AFTER INOCULATION

FIG. 4.5 IgM ASCs in primary spleen and lung cell cultures stimulated with either purified virus (open symbols) or virus-infected cells (closed symbols). The virus concentration represents either the titre of purified virus (HAU/ml) or the amount of virus used to infect cells (EID_{50} per cell). Values represent mean results of triplicate assays using suspensions of pooled cells from 3 mice stimulated in vitro for 4 days.

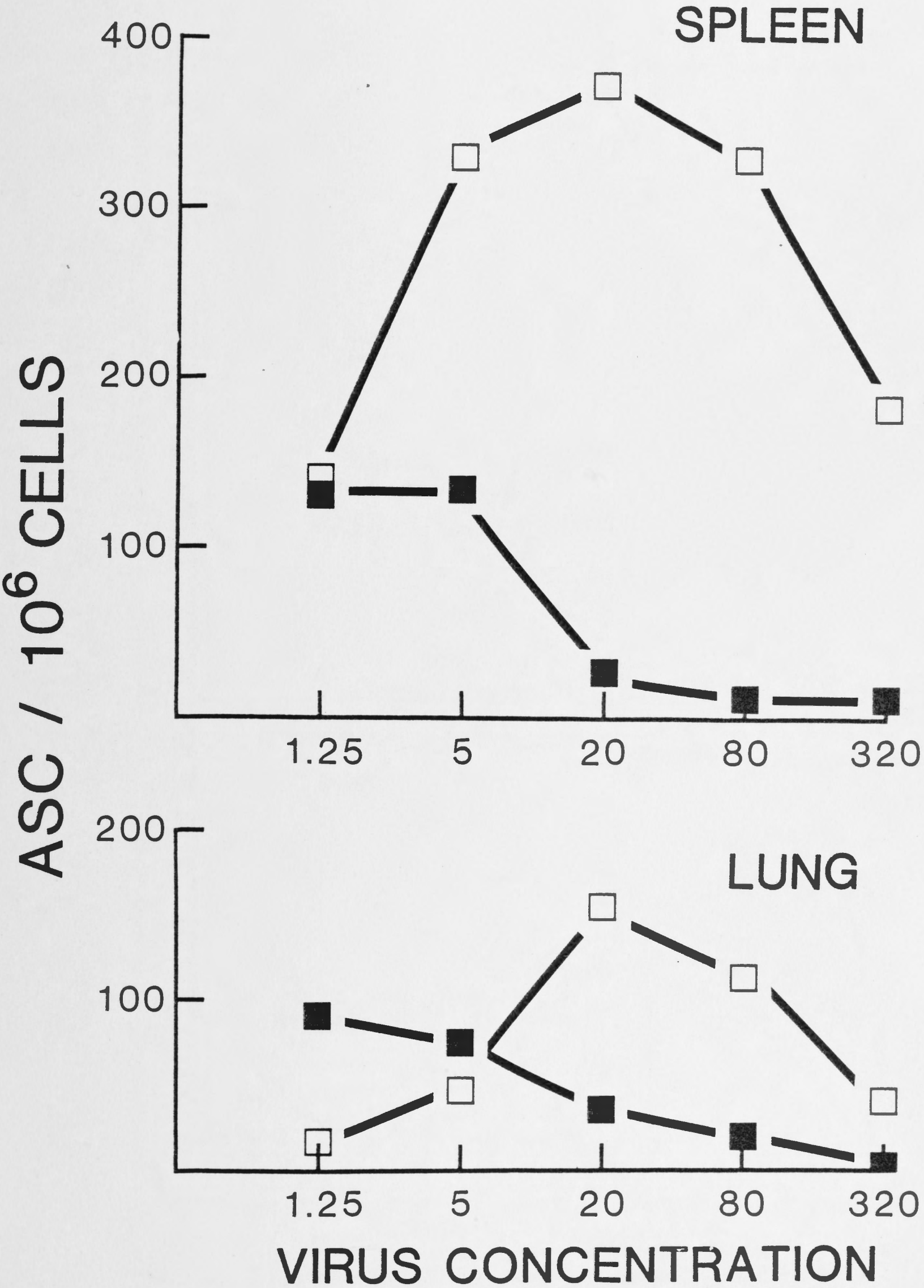

SPLEEN
400
300
200
100
ASC / 10^6 CELLS
1.25
5
20
80
320
LUNG
200
100
1.25
5
20
80
320
VIRUS CONCENTRATION

FIG. 4.6 IgM ASCs in primary spleen (solid line) and lung (broken line) cell cultures either stimulated with 20 HAU of A/Jap virus per ml (□) or unstimulated (■).

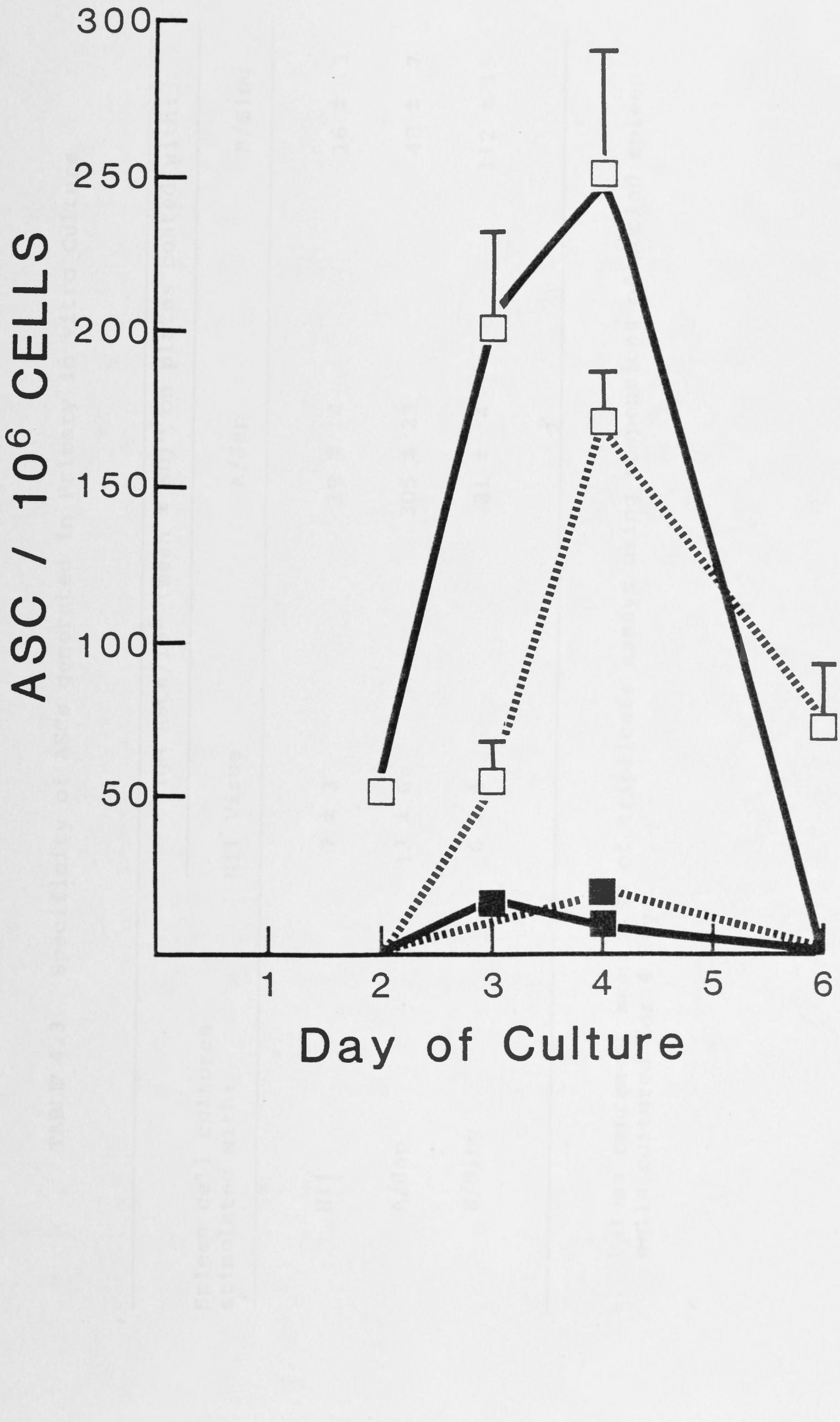

ASC / 10^6 CELLS
300
250
200
150
100
50
1
2
3
4
5
6
Day of Culture

TABLE 4.3 Specificity of ASCs generated in Primary in vitro Culture

Spleen cell cultures stimulated with:	IgM ASCs/10^6 (mean ± SD)[a] on plates coated with:		
	Nil Virus	A/Jap	B/Sing
Nil	7 ± 3	19 ± 4	16 ± 3
A/Jap	13 ± 6	305 ± 23	48 ± 7
B/Sing	6 ± 4	31 ± 4	112 ± 15

a. Values represent mean ± SD of triplicate assays using suspensions of pooled spleen cells cultured for 4 days.

DISCUSSION

The findings in this chapter provide the first detailed description of the appearance and development of specific ASCs in the lung during infection. The only previous detailed descriptions of specific ASCs in the lung were after local immunization with foreign erythrocytes (see Section 1.2.3.2). In contrast to previous failures to detect ASCs in the lungs of mice after primary local immunization with foreign erythrocytes (McLeod et al., 1978; Kaltreider et al., 1983) cells secreting influenza-specific IgM, IgG and IgA were readily detected in the lungs of mice during primary influenza infection. Thus an earlier report (Scott & Walker, 1976) of an increase in Ig-bearing cells, detected by immuno-fluorescent labelling, in the lungs of influenza-infected mice has not only been confirmed but extended to demonstrate their specificity for antigen. These results have provided the most direct evidence yet obtained that influenza-specific antibody of each isotype in respiratory secretions is most likely locally produced. This conclusion is further supported by the close temporal relationship between the appearance of class-specific ASCs in the lung and the corresponding antibodies in tracheobronchial washes.

Although ASCs were detected earlier in the spleen than the lung, the subsequent response in the lung was relatively greater for each isotype, particularly the IgA component.

The primary response to infection was also characterized by the more rapid decline in numbers of ASCs in the spleen compared to the lung. This was probably related to the persistence of viral antigen in the lung after infection (Astry et al., 1984) compared to the short pulse of antigenic stimulation in the spleen. The onset and magnitude of the primary response was also dose-dependent.

Influenza-specific ASCs have previously been detected by a haemolytic-plaque assay in the spleens and intrathoracic lymph nodes of influenza infected mice and ferrets. The kinetics of the splenic responses following intravenous immunization (McLaren & Butchko, 1978) and intranasal inoculation (McLaren et al., 1978; Reiss & Schulman, 1980b) were similar to those described here.

In contrast to the considerable difference observed in the sizes of the pulmonary and splenic primary in vivo responses, the primary IgM response generated in vitro in lung cell cultures was only slightly less in magnitude than that in the spleen cell cultures. However, as in vivo, ASCs appeared earlier in the spleen cell cultures than in the lung cell cultures. Previous work comparing the immune response in the spleens and lungs of mice inoculated intranasally with parental and cold-adapted preparations of influenza virus (Mak et al., 1984) indicated that a splenic response was due to antigen spillover of virus replicating in the lung (i.e., the antigenic challenge in the spleen is

both delayed and of smaller magnitude in the spleen compared to the lung). These findings taken together indicate that the antigen-presenting mechanisms in the spleen are qualitatively and/or quantitatively different from those in the lung.

What is the origin of ASCs in the lung during influenza infection? The proposition that these cells may arise from influenza-specific B cell precursors in the lung is directly supported by two findings in this study: (1) The generation of cells secreting specific IgM in stimulated lung cell cultures from unprimed mice; (2) The virtual absence of an IgA response in the spleen despite the prominent response in the lung after both intranasal and intravenous immunization. Do any circulating B cell precursors or ASCs derived from extra-pulmonary lymphoid tissue contribute to the lung response? The only evidence which might support this is the finding in vivo that the IgM response develops earlier in the spleen compared to the lung, thus allowing an opportunity for some migration to the lung. If this does occur, then further migration is arrested as indicated by the persistence of ASCs in the lung. Previous studies on the response to foreign erythrocytes has led to the suggestion, based on indirect evidence, that the major mechanism for the appearance of ASCs in the lung was through the recruitment of sensitized cells (Section 1.2.3.2), though it has also been shown that whilst the spleen

contributes to the pulmonary response, it is not essential (Kaltreider et al., 1983). Of course, migration of other cell types such as helper T lymphocytes from the spleen and other extra-pulmonary lymphoid tissue could occur.

SUMMARY

The development of influenza-specific ASCs during the primary immune response to influenza was characterized. Following intranasal inoculation cells secreting influenza-specific IgM, IgG and IgA were detected in greatest numbers in the lung and the data presented indicated that these cells may have originated from specific B cell precursors in the lung which were demonstrable in vitro. Influenza-specific ASCs were also detected in the spleen and blood. ASCs appeared earlier in the spleen than in the lung and declined more rapidly in the spleen.

CHAPTER FIVE

PERSISTENCE OF INFLUENZA-SPECIFIC B CELL RESPONSES AND B CELL MEMORY AFTER PRIMARY INFLUENZA VIRUS INFECTION

INTRODUCTION

Protection following influenza virus infection is long-lived as evident by the observation that persons infected with the H1N1 subtype before the 1950s were subsequently protected when this subtype reappeared in 1977 (Glezen et al., 1982). Since the principal mediator of protection is antibody to the HA molecule, persistence of HA specific antibody must occur to account for long-term protection. The finding of HA-inhibition (HI) antibody to H3 in 1968 in the serum of persons born before 1892 and to H1 in 1976 in persons born before the early 1950s confirms this (Couch & Kasel, 1983). Although there is a correlation between serum HI levels and protection, neutralizing antibody must be present within the respiratory tract to prevent infection of the respiratory epithelium. The relative importance of local and systemic B cell responses in maintaining a persistent level of protective antibody within the respiratory tract has not been previously addressed.

This chapter describes the persistence of B cell responses and the development of B cell memory after primary influenza infection and the effect of re-infection on these parameters.

RESULTS

5.1 Persistence of Influenza-specific ASCs

Influenza-specific ASCs were quantitated in the lung and spleen of mice at different times after primary influenza virus infection. Cell suspensions were obtained from mice immunized intranasally with either of two different doses of infectious A/Jap virus. The number of ASCs detected in the first 40 weeks after infection was determined in mice primed with $10^3 EID_{50}$ A/Jap virus intranasally (group A). The level of IgM-secreting cells in the lung declined shortly after infection, whereas the level of IgG- and IgA-secreting cells remained elevated for the first two months after infection (Fig. 5.1). Thereafter there was a gradual decline of ASCs of each isotype in the lung, with IgG ASCs remaining the predominant type throughout. Whereas in the spleen the level of ASCs declined within the first month after infection and remained fairly constant thereafter with less than 30 ASCs, principally IgG-secreting, per 10^6 cells. In a second separate group of mice primed with $5x10^2$ EID_{50} A/Jap virus (group B), ASCs were detected in the lung up to 18 months after priming (Fig. 5.1). Similarly, a small number of IgG-secreting cells (<10 per 10^6 cells) were present in the spleen at 18 months.

The level of ASCs was further examined at more frequent time intervals within a period of approximately six weeks in several experiments at different times after primary inoculation. In each experiment, cyclical variations in the number of ASCs in the lung were observed, as exemplified by the results obtained in mice from group A primed three months earlier (Fig. 5.2). Peak levels of IgG ASCs occurring every one to two weeks were significantly greater than trough levels ($p < 0.01$). The overall variation in the number of IgG ASCs detected in the lung over this period was 36%, considerably greater than the 12% interassay variation of the ELISA-plaque assay. Similarly, the number of circulating ASCs varied, although the significance of this finding was uncertain as only a small number of peripheral blood lymphocytes were assayed. However, there was no consistent correlation observed between the cycles in the lung and in the blood. The number of ASCs detected in the spleen was so small that a significant variation could not be detected.

5.2 Persistence of Influenza-specific Antibody

In parallel with the decline in ASCs in the lung, there was an 8- to 16-fold fall in antibody titres in TBW over the initial 40 weeks in group A mice (Fig. 5.3). IgG was the predominant isotype in TBW although there was a proportionately higher level of IgA in TBW compared to serum. Serum antibody titres remained remarkably constant

during the first 40 weeks following infection in group A mice (Fig. 5.3). Furthermore, there was little change in serum titres by 18 months in group B mice.

5.3 B Cell Memory after Primary Influenza Infection

5.3.1 Generation of Influenza-specific ASCs by Secondary in vitro stimulation

The level of B cell memory following primary influenza virus infection was determined indirectly by measuring the number of ASCs generated in cultures of lung and spleen cells derived from primed mice and stimulated in vitro with purified influenza virus. The two groups of primed mice used to detect persistence of ASCs were also used in the following experiments.

The maximum number of ASCs was generated in both lung and spleen cell cultures stimulated with 6 to 25 HAU purified virus per ml (Fig. 5.4). In subsequent experiments, cultures from primed mice were stimulated with 10 HAU/ml.

Lung and spleen cells obtained from group A mice twenty weeks after priming were stimulated in culture with 10 HAU purified A/Jap virus per ml and the number of ASCs enumerated over the following week. Cells continuing to secrete antibody spontaneously were enumerated in

unstimulated cultures (Fig. 5.5). ASCs increased in stimulated lung cell cultures slightly by day 3 and by day 5 there was over a 10-fold difference between the number of ASCs of each class detected in stimulated and unstimulated lung cell cultures ($p < 0.001$). The predominant isotype detected in stimulated lung cell cultures was IgG. ASCs increased in stimulated spleen cell cultures by day 2 and by day 5 there was an approximately 1000-fold increase in the number of IgM and IgG ASCs detected in stimulated spleen cell cultures ($p < 0.001$). The similar proportion of IgM- and IgG-secreting cells reflect the short duration of culture as the relative proportion of IgG- to IgM-secreting cells increases with the duration of culture.

5.3.2 Persistence of B Cell Memory

The level of B cell memory was determined at different times after primary infection. The number of ASCs generated by in vitro stimulation was calculated at day 5 of culture by correcting for the number of ASCs detected in unstimulated cultures. The total number of ASCs, including IgM-, IgG- and IgA-secreting cells, generated in lung cell cultures obtained from mice 3 months after infection was 50-100 fold higher than the number of ASCs in primary lung cell cultures (Fig. 5.6). Thereafter, there was a gradual decline in the number of ASCs generated in lung cell cultures derived from mice at later times after the primary infection. By 18 months after priming, the total number of

ASCs in cultures from primed mice was comparable to that found in primary lung cell cultures, though in the primed mice the principal cell type was IgG-secreting.

Within three months of the primary infection there was a 100-200 fold increase in the total number of ASCs, principally IgM and IgG, detected in spleen cell cultures. Thereafter, in contrast to the lung, the level of ASCs generated in spleen cell cultures did not change significantly.

5.3.3 Role of T Helper Cells in Declining Level of B Cell Memory in the Lung

The decline in the number of ASCs in lung cell cultures from long-term primed mice may have been due to a fall in the level of either B memory cells or Th cells. To determine whether the limiting factor was the level of Th cells, lung cell suspensions were co-cultured with additional Th cells. Gamma-irradiated (2000 rads) spleen cells obtained from long-term primed mice were able to provide help to spleen cell suspensions depleted of Th cells, but had only a moderate effect on increasing the number of IgG ASCs in lung cell cultures obtained from the same mice (Table 5.1). In separate experiments using short-term primed mice the addition of gamma-irradiated spleen cells similarly resulted in less than a 2-fold increase in the total number of ASCs generated in lung cell

cultures. Spleen Th cells were, however, able to increase the response in lung cell suspensions depleted of $L3T4^+$ cells, e.g. IgG ASCs in cultures depleted of $L3T4^+$ cells increased from 510 ± 16 to 2000 ± 13 per 10^6 cells when co-cultured with irradiated spleen cells. Similar increases in IgM- and IgA-secreting cells were also detected, suggesting that the ability of ASCs in intact lung cell cultures to respond to spleen Th cells was not being suppressed. As the level of Th cells in the lungs of long-term primed mice was not a limiting factor in the generation of ASCs, the decline in the number of ASCs in lung cell cultures predominantly reflected a reduction in the level of B memory cells in the lung.

5.4 Effect of in vivo Homotypic Challenge

5.4.1 Effect on ASCs and Antibody Levels

Mice from group A were initially challenged with a 10-fold higher inoculum (10^4 EID_{50}) of infectious A/Jap virus intranasally. However, re-infection was completely prevented as no B cell response, determined by either the ELISA-plaque assay or an indirect ELISA on serum or TBW for influenza-specific antibodies, was elicited. The mice were then challenged at six months after priming with $7.5x10^4$ EID_{50} of a recently mouse-adapted A/Jap virus. There was no virus detected in lung homogenates at day 3 after challenge. In unprimed mice inoculated with the same

inoculum, mean lung virus titre at day 3 was 6.3 $\log_{10}$ EID_{50}.

The number of ASCs was determined after the challenge and compared with the number of ASCs persisting in primed mice which were not challenged. In the lungs of challenged mice there was a significant increase in ASCs of each class ($p < 0.01$), greater for IgG- and IgA-secreting cells, by day 5 (Fig. 5.7). This response persisted until the end of week 3. In contrast, there was no response detected in the spleens of challenged mice.

Antibody titrations were performed before and after homotypic challenge. There was no significant change in serum antibody titres 10 days after challenge (Table 5.2). By day 23 there had been a 4-fold rise in influenza specific IgG in serum (data not shown). In contrast, there was an 8-fold rise in IgG and IgA levels in TBW at day 10 after challenge. There was no significant change in antibody titres in primed mice which were not challenged.

5.4.2 Effect on B Cell Memory

The effect of homotypic challenge on the level of B cell memory was determined 5 weeks after challenge with the larger inoculum (Table 5.3). There was a significant increase in the total number of ASCs generated in both lung (5-7 fold) and spleen cell (2-3 fold) cultures obtained from the challenged mice ($p < 0.01$).

5.5 Effect of in vivo Heterotypic Challenge

5.5.1 Effect on ASCs and Antibody Levels

In contrast to the large dose of homotypic virus required to elicit a secondary in vivo response, mice were successfully challenged with a relatively lower dose of heterotypic virus. Mice from group A were challenged 4 months after priming with $2x10^3$ EID_{50} A/PR8 virus intranasally.

In order to study the effect of heterotypic challenge, it was necessary to use the modified ELISA-plaque assay (Section 3.5.3) so that subtype-specific ASCs could be detected.

The number of ASCs specific for A/Jap and A/PR8 were determined at day 18 after challenge and compared with non-challenged primed mice (Table 5.4). There was a significant increase in the number of IgG and IgA ASCs directed to the A/Jap virus in the lung ($p < 0.05$) and of IgG ASCs in the spleen ($p < 0.05$). However, these ASCs were predominantly specific for the internal viral proteins as there was no significant increase in ASCs specific for the A/Jap virus using plates coated with antibody and virus. The number of ASCs formed on plates coated with A/PR8 virus tended to be smaller in mice primed with A/Jap virus compared with unprimed mice.

There was a 4- to 8-fold increase in influenza-specific IgG levels in serum and TBW, using plates coated with A/Jap virus, at day 18 after challenge (Table 5.5). Consistent with the specificity of the ASC response, there was, however, no significant increase in the level or A/Jap-specific antibodies in either serum or TBW determined by either haemagglutinin-inhibition or an indirect ELISA using the same modification for detecting subtype-specific antibodies. The antibody responses to A/PR8 virus were similar in the primed and unprimed mice.

5.5.2 Effect on B cell memory

The effect of heterotypic challenge on the level of B cell memory was determined one month after challenge (Table 5.6). Further in vitro stimulation with purified A/Jap virus of lung cell cultures, obtained from mice primed with A/Jap and subsequently challenged with A/PR8, generated a slightly greater number of ASCs (2-3 fold) compared with cultures from primed mice which were not challenged. This was evident for all ASCs detected on A/Jap-coated plates, but not ASCs specific for A/Jap virus.

The number of ASCs generated in spleen cell cultures stimulated with A/Jap virus was similar in cultures obtained from challenged or unchallenged mice irrespective of specificity.

The number of ASCs generated in cultures stimulated with A/PR8 virus was similar in cultures obtained from either mice primed with A/Jap and challenged with A/PR8 or mice primed with A/PR8 only (Table 5.7).

FIG. 5.1 ASCs in the lung after primary intranasal inoculation in Group A and Group B mice. Values represent mean results of triplicate assays performed on suspensions of pooled cells from 3 mice.

Symbols: □ IgM, ○ IgG, △ IgA

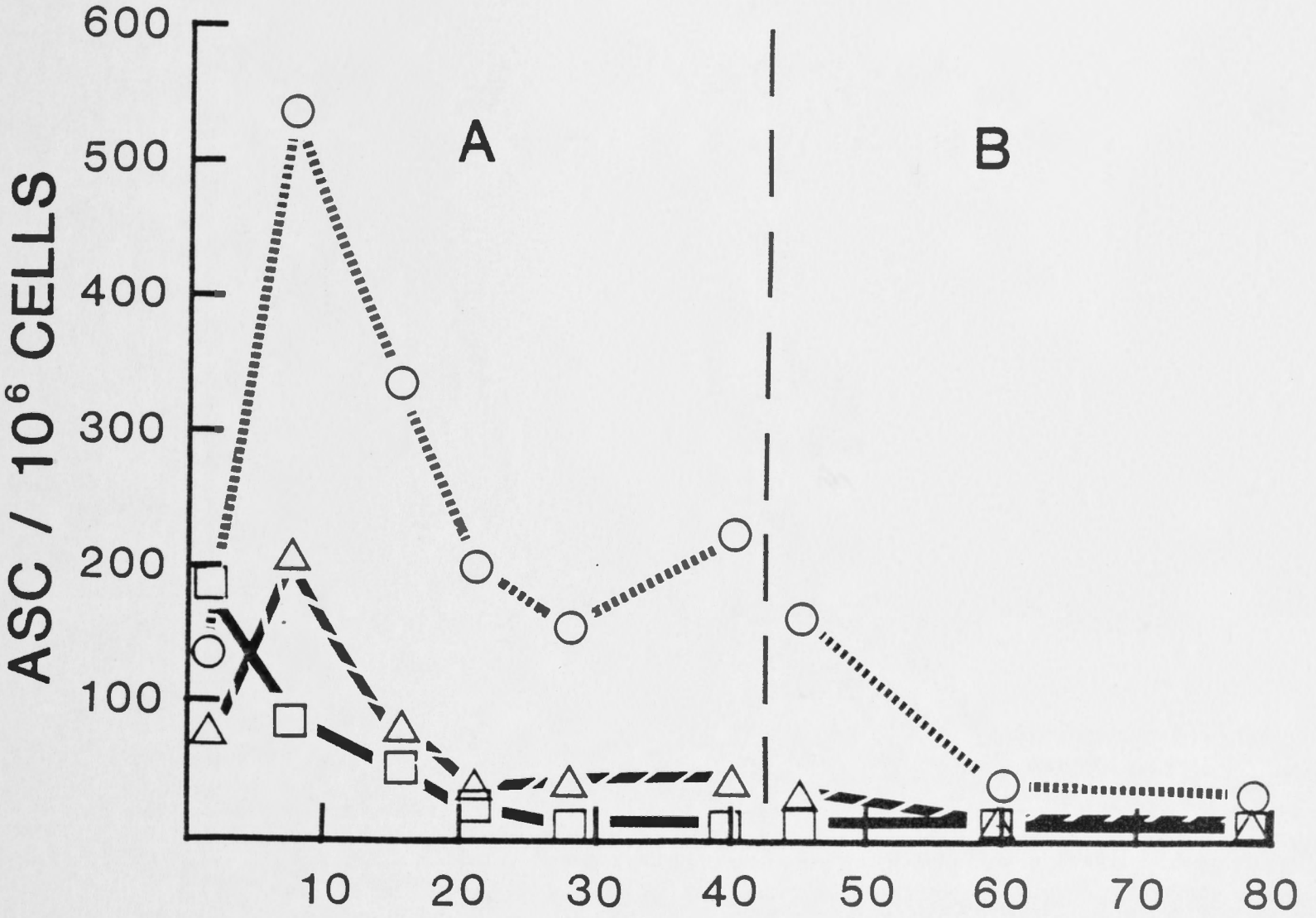

ASC / 10^6 CELLS
600
500
400
300
200
100
A
B
10
20
30
40
50
60
70
80
Weeks after Primary Inoculation

FIG. 5.2 ASCs in the lung after primary intranasal inoculation in Group A mice. Values represent mean ± SD of triplicate assays performed on suspensions of pooled cells from 3 mice.

Symbols: □ IgM, ○ IgG, △ IgA

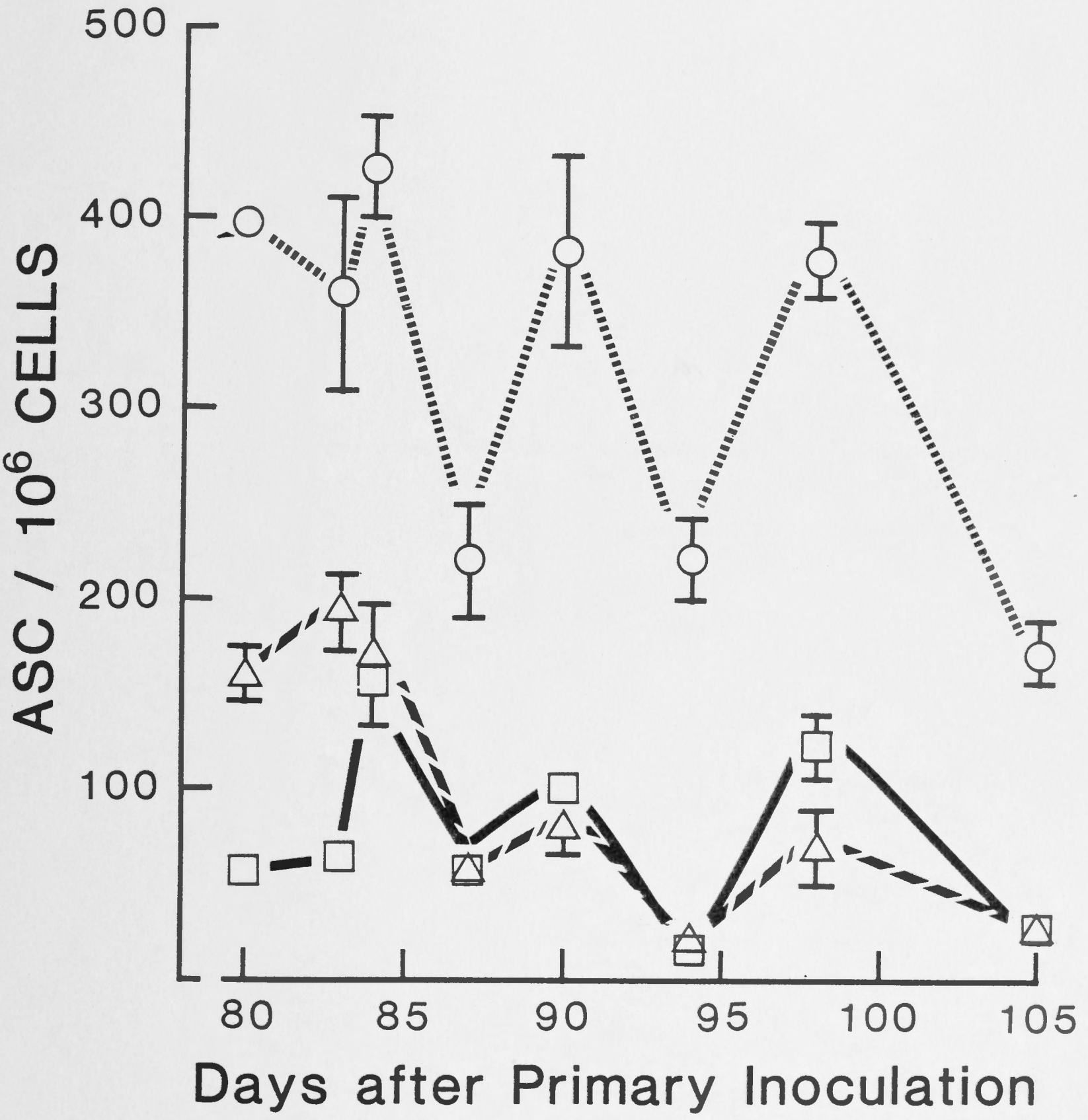

500
400
300
200
100
ASC / 10^6 CELLS
80
85
90
95
100
105
Days after Primary Inoculation

FIG. 5.3 ELISA antibody titres in tracheobronchial washings (TBW) and sera using pooled samples from 3 mice in either Group A or B. Values represent the reciprocal (log_2) of end point titre.

Symbols: □ IgM, ○ IgG, △ IgA

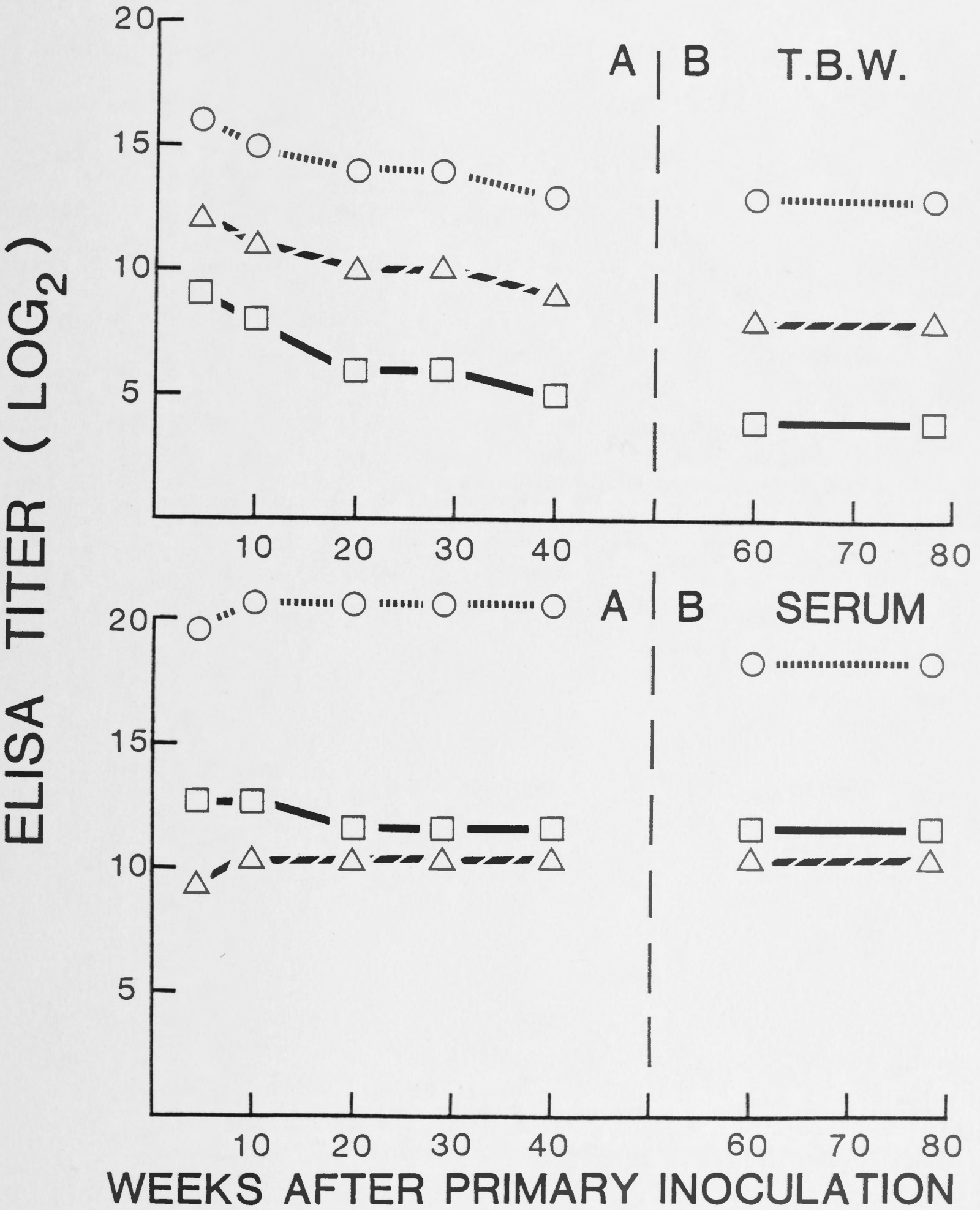

ELISA TITER (LOG2)
20
15
10
5
A
B
T.B.W.
10
20
30
40
60
70
80
SERUM
WEEKS AFTER PRIMARY INOCULATION

FIG. 5.4 IgG ASCs in lung (broken line) and spleen (solid line) cell cultures stimulated with variable concentrations of purified A/Jap virus. Values represent mean results of triplicate assays using suspensions of pooled cells from 4 Group A mice stimulated in vitro for 5 days. Lung cell cultures were prepared at 4 weeks after priming and spleen cell cultures at 20 weeks after priming.

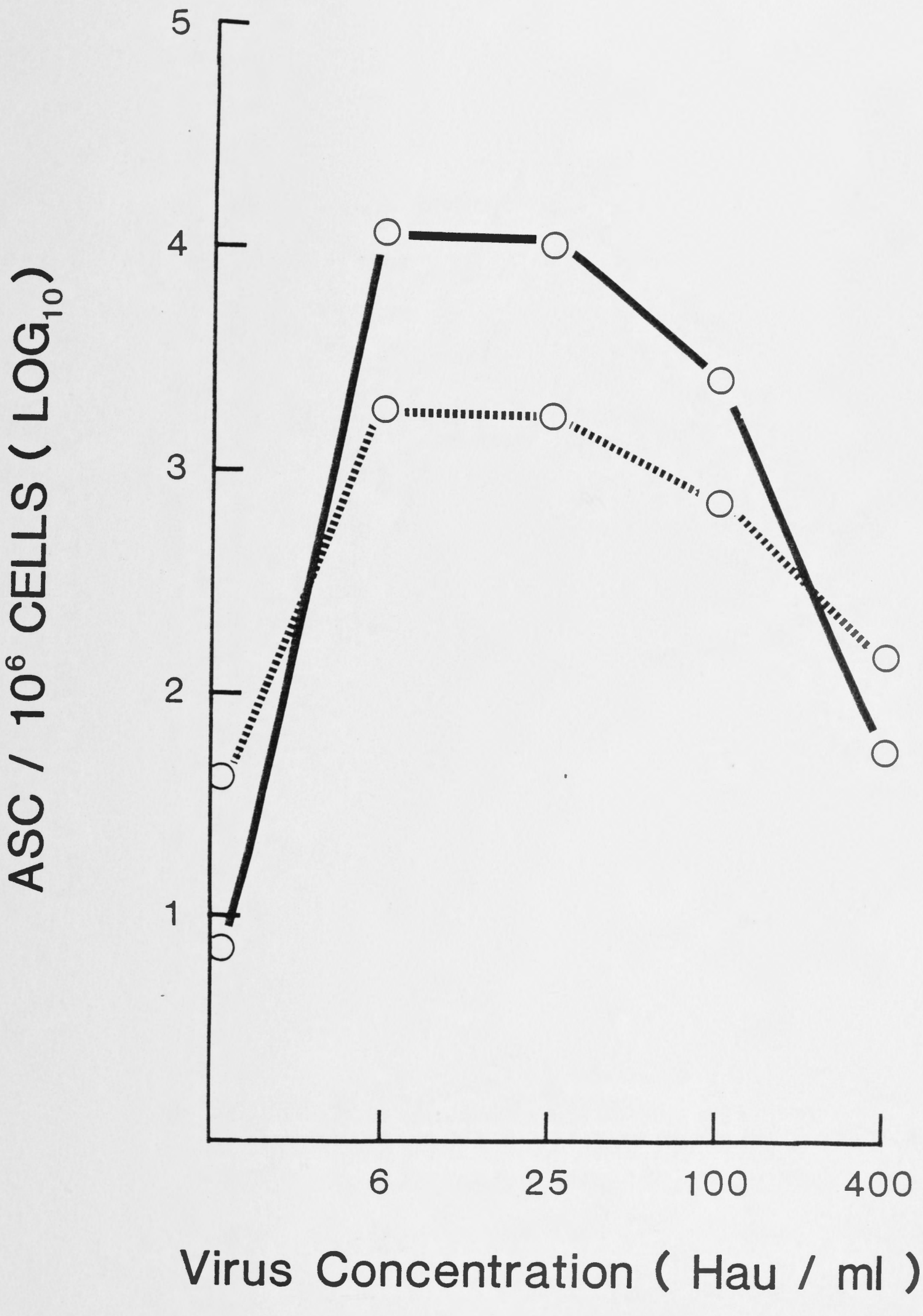

ASC / 10^6 CELLS (LOG_{10})
5
4
3
2
1
6
25
100
400
Virus Concentration (Hau / ml)

FIG. 5.5 ASCs in spleen and lung cell cultures either stimulated with 10 HAU of A/Jap virus per ml (open symbols) or unstimulated (closed symbols). Cell suspensions obtained from Group A mice 20 weeks after priming. Values represent mean results of duplicate assays.

Symbols: □ IgM, ○ IgG, △ IgA

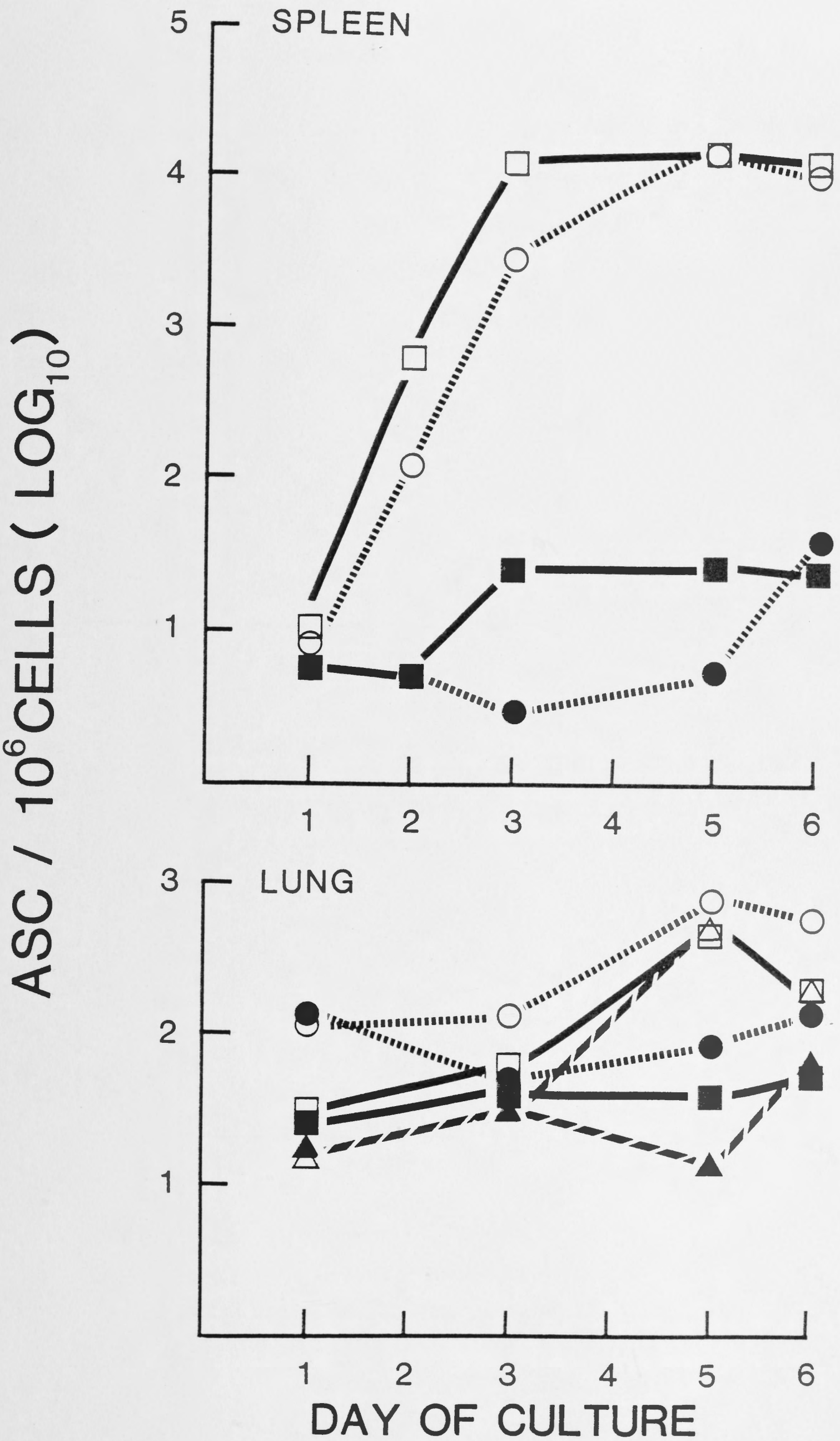

SPLEEN
LUNG
ASC / 10^6 CELLS (LOG10)
5
4
3
2
1
1 2 3 4 5 6
DAY OF CULTURE

FIG. 5.6 ASCs generated in spleen and lung cell cultures on day 5 of culture. Cell suspensions obtained from 3 mice in either Group A or B. Values represent mean results of duplicate assays.

Symbols: □ IgM, ○ IgG, △ IgA

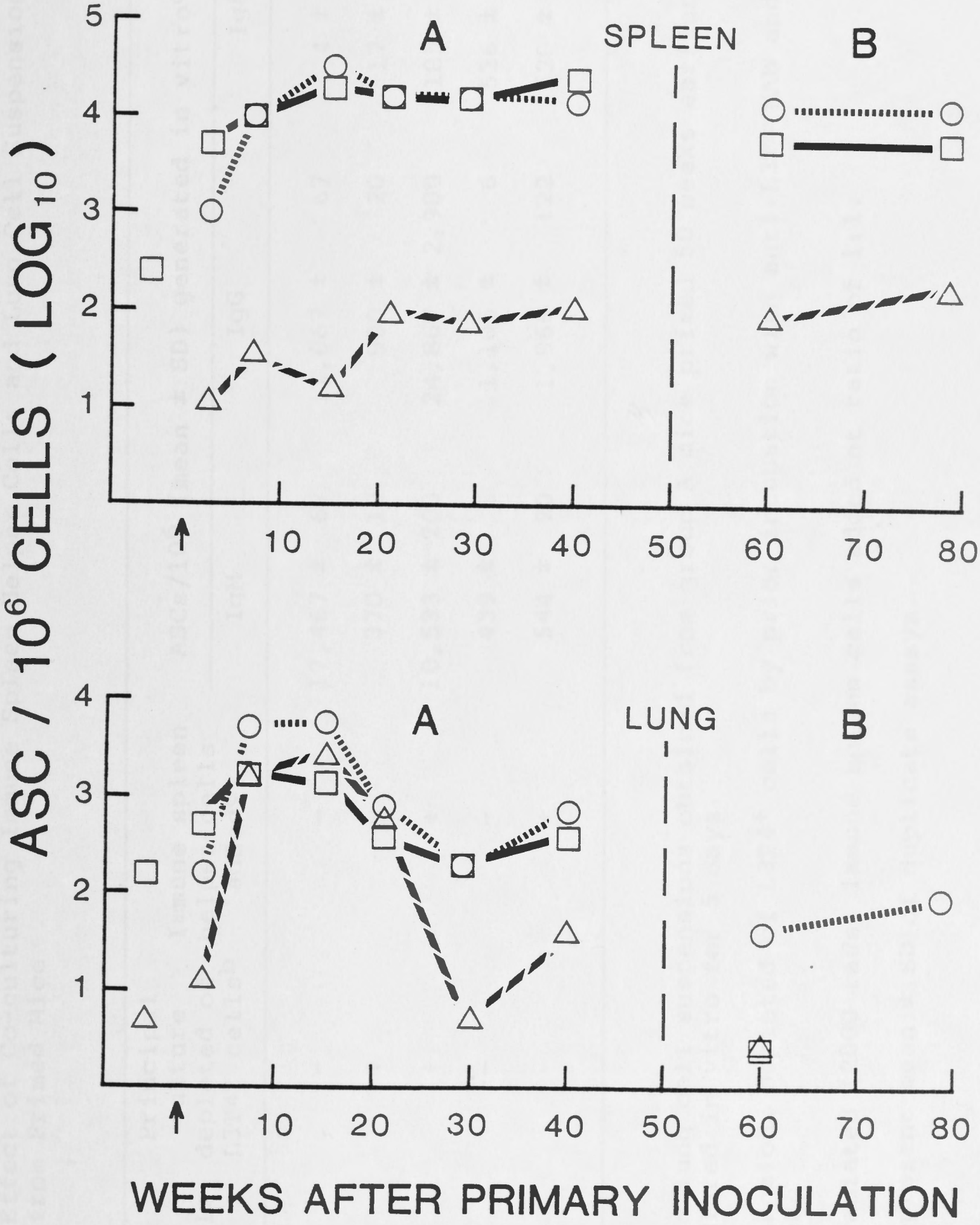

ASC / 10^6 CELLS (LOG_{10})
5
4
3
2
1
A
SPLEEN
B
10
20
30
40
50
60
70
80
4
3
2
1
A
LUNG
B
10
20
30
40
50
60
70
80
WEEKS AFTER PRIMARY INOCULATION

TABLE 5.1 Effect of Co-culturing Immune Spleen Helper Cells and Lung Cell Suspensions from Primed Mice

Principal cells cultured[a]	Principal culture depleted of $L3T4^+$ cells[b]	Immune spleen helper cells added[c]	ASCs/10^6 (mean ± SD) generated in vitro[d]		
			IgM	IgG	IgA
Spleen	-	-	17,467 ± 67	11,067 ± 67	4 ± 2
Spleen	+	-	370 ± 10	900 ± 20	17 ± 2
Spleen	+	+	10,533 ± 200	24,867 ± 2,900	185 ± 21
Lung	-	-	439 ± 6	1,105 ± 6	526 ± 53
Lung	-	+	544 ± 20	1,966 ± 122	520 ± 31

a. Spleen and lung cell suspensions obtained from group A mice primed 50 weeks earlier and stimulated in vitro for 5 days.

b. Cell suspensions depleted of $L3T4^+$ cells by prior incubation with anti-L3T4 MAb and complement.

c. Gamma-irradiated (2000 rads) immune spleen cells added at ratio of 1:1.

d. Values represent mean ± SD of duplicate assays.

FIG. 5.7 ASCs in the lung in primed mice either challenged with 7.5 x 10^4 EID_{50} A/Jap virus (A) or not challenged (B). Mice were challenged on day 173 (↑) after primary inoculation (10^3 EID_{50} A/Jap virus). Values represent mean results of triplicate assays performed on suspensions of pooled cells from 3 mice.

Symbols: □ IgM, ○ IgG, △ IgA

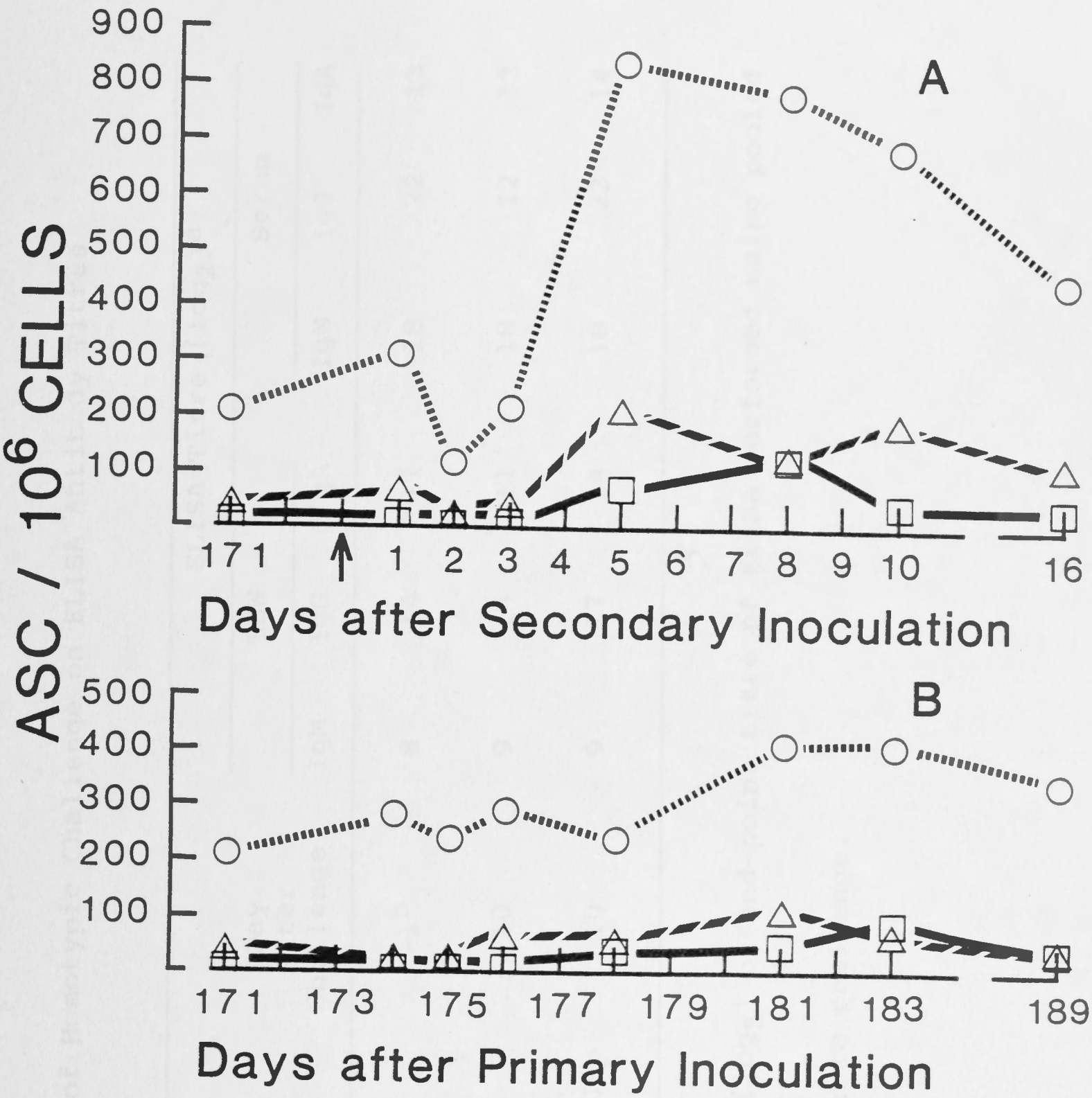

ASC / 10^6 CELLS
900
800
700
600
500
400
300
200
100
A
171 1 1 2 3 4 5 6 7 8 9 10 16
Days after Secondary Inoculation
500
400
300
200
100
B
171 173 175 177 179 181 183 189
Days after Primary Inoculation

TABLE 5.2 Effect of Homotypic Challenge on ELISA Antibody Titres

Primary Intranasal Inoculation	Secondary Intranasal Inoculation	Day After Challenge	ELISA Titre (log_2)[a]					
			TBW			Serum		
			IgM	IgG	IgA	IgM	IgG	IgA
10^3EID_{50} A/Jap	....	-1[b]	8	14	11	18	22	13
10^3EID_{50} A/Jap	Nil	10	9	14	11	18	22	12
10^3EID_{50} A/Jap	$7.5x10^4EID_{50}$ A/Jap	10	9	17	14	18	23	14

a. Values represent reciprocal (log_2) of end-point titre of ELISA performed using pooled sera and TBW from 3 mice.

b. ELISA performed one day prior to challenge.

TABLE 5.3 Effect of Homotypic Challenge on B Cell Memory

Primary Intranasal Inoculation	Secondary Intranasal Inoculation	ASCs/10^6 cells (mean ± SD) generated in vitro[a]					
		Lung			Spleen		
		IgM	IgG	IgA	IgM	IgG	IgA
10^3EID$_{50}$ A/Jap	Nil	74±14	498±23	12± 4	17,267± 867	13,600± 733	34±2
10^3EID$_{50}$ A/Jap	$7.5x10^4$ EID$_{50}$ A/Jap	1,039±60	1,990±60	590±82	50,667±2,933	32,600±1,333	57±2

a. Values represent mean ± SD of duplicate assays performed on suspensions of pooled cells from 3 mice obtained 5 weeks after secondary inoculation and stimulated in vitro with 10 HAU/ml for 5 days.

TABLE 5.4 Effect of Heterotypic Challenge on ASCs in vivo

Primary Intranasal Inoculation	Secondary Intranasal Inoculation	Plates Coated With:	ASCs/10^6 cells (mean ± SD)[a]				
			Lung			Spleen	
			IgM	IgG	IgA	IgM	IgG
10^3EID$_{50}$ A/Jap	Nil	A/Jap	61±12	258±23	105± 36	14± 3	12± 5
"	"	Anti-A/Jap MAb & A/Jap	17	75	15± 10	7± 1	11± 3
"	"	A/PR8	1	3	1	1	0
10^3EID$_{50}$ A/Jap	2x10^3EID$_{50}$ A/PR8[b]	A/Jap	108±22	804± 6	894±141	32± 2	110±13
"	"	Anti-A/Jap MAb & A/Jap	27±20	135±38	18± 9	12± 2	18± 6
"	"	A/PR8	30	138±40	36± 20	14± 4	20± 1
2x10^3EID$_{50}$ A/PR8[b]	Nil	A/PR8	140± 6	146±16	104± 26	64±18	86±17

a. Values represent mean ± SD of duplicate assays using suspensions of pooled cells obtained from 3 mice at day 18 after secondary inoculation.

b. Lung virus titres at day 3 were 6.5 ± 1.1 $\log_{10}$EID$_{50}$. In unprimed mice lung virus titres at day 3 were 7.6 ± 0.5 $\log_{10}$EID$_{50}$.

TABLE 5.5 Effect of Heterotypic Challenge on Antibody Titres[a]

Primary Intranasal Inoculation	Secondary Intranasal Inoculation	HI Titre[b] (log_2)		ELISA plate coated with:	IgG ELISA Titre (log_2)	
		TBW	Serum		TBW	Serum
10^3EID_{50} A/Jap	Nil	7	8	A/Jap	13	19
"	"			Anti-A/Jap MAb and A/Jap	9	16
10^3EID_{50} A/Jap	$2x10^3EID_{50}$ A/PR8	7	8	A/Jap	16	21
"	"			Anti-A/Jap MAb and A/Jap	10	17
"	"			A/PR8	15	20
$2x10^3EID_{50}$ A/PR8	Nil	ND	ND	A/PR8	15	18

a. Assays performed using pooled sera and TBW from 3 mice obtained at day 18 after secondary inoculation.

b. Haemagglutinin-inhibition (HI) titre determined using A/Jap virus only.

TABLE 5.6 Effect of Heterotypic Challenge on B Cell Memory (A)

Primary Intranasal Inoculation	Secondary Intranasal Inoculation	Cultures stimulated with:	Plates coated with:	ASCs/10^6 cells (mean ± SD)[a] generated in vitro				
				Lung			Spleen	
				IgM	IgG	IgA	IgM	IgG
10^3EID$_{50}$ A/Jap	Nil	A/Jap	A/Jap	707±21	1387± 80	293± 8	10370±428	9536±110
"	"	"	Anti-A/Jap MAb and A/Jap	253±12	250± 36	61± 7	2662±258	1430±147
"	"	"	A/PR8	5± 2	3± 1	3± 2	20± 3	10± 3
10^3EID$_{50}$ A/Jap	2x10^3EID$_{50}$ A/PR8	A/Jap	A/Jap	1060±86	2816±174	1068±21	16800±100	10967±300
"	"	"	Anti-A/Jap MAb and A/Jap	443±18	487± 70	107± 6	3696± 90	1206±234
"	"	"	A/PR8	3± 1	3± 2	0	64± 4	8± 2

a. Values represent mean ± SD of duplicate assays using suspensions of pooled cells obtained from 3 mice five weeks after secondary inoculation and stimulated in vitro for 5 days.

TABLE 5.7 Effect of Heterotypic Challenge on B Cell Memory (B)

Primary Intranasal Inoculation	Secondary Intranasal Inoculation	Cultures stimulated with:	Plates coated with:	ASCs/10^6 cells (mean ± SD)[a] generated in vitro				
				Lung			Spleen	
				IgM	IgG	IgA	IgM	IgG
10^3EID$_{50}$ A/Jap	$2x10^3$EID$_{50}$ A/PR8	A/PR8	A/Jap	43± 6	18± 5	6± 2	440± 40	2480± 40
"	"	"	Anti-A/Jap MAb and A/Jap	0	0	0	12± 3	16± 1
"	"	"	A/PR8	344±16	216±12	70±10	10933±525	3133±216
$2x10^3$EID$_{50}$ A/PR8	Nil	A/PR8	A/Jap	24± 4	33± 7	27± 3	490± 40	2500± 10
"	"	"	Anti-A/Jap MAb and A/Jap	0	0	0	6± 3	1
"	"	"	A/PR8	313±20	193± 7	40± 2	9467± 83	3533±495

a. See Table 5.6.

DISCUSSION

The duration of the antibody response in either serum or respiratory secretions has in the past been the principal parameter used for assessing the duration of B cell immunity after influenza virus infection. By quantitating influenza-specific ASCs, the duration of both local and systemic B cell immunity at the cellular level has been analyzed.

Cells secreting influenza-specific antibodies, predominantly of the IgG isotype, persisted after influenza infection in both the lung and spleen for essentially the lifetime of the laboratory mouse, providing a continuous, though diminishing, source of specific antibody within the respiratory tract to prevent or minimise re-infection. The persistence of ASCs in the lung and spleen could be due to one or both of two mechanisms: (i) the presence of long-lived ASCs; and (ii) the recruitment and maturation of B memory cells. Evidence for long-lived ASCs after antigenic stimulation has been obtained using two approaches. Firstly, the incorporation of tritiated thymidine into dividing cells and the subsequent detection of individual labelled specific ASCs (Miller, 1964). Secondly, by preventing replication of B memory cells by whole-body irradiation after antigenic stimulation and confirming that cells from such hosts, upon adoptive transfer, could not give rise to hapten-specific B cell

responses (Okudaira & Ishizaka, 1981; Holt et al., 1984). These approaches have demonstrated the presence of long-lived ASCs after antigenic stimulation, but in variable proportions. It is probably reasonable to suggest that in most situations, the persistence of ASCs is due to the existence both of long-lived ASCs and continuing recruitment and maturation of specific ASCs.

The extent to which either mechanism contributes to the persistence of influenza-specific ASCs in the present experimental model was not determined, but two observations may be relevant. The first is the cyclical variation in the level of ASCs observed in the lung after influenza infection. This effect is reminiscent of the pattern seen after immunization particularly with bacterial lipopolysaccharide and sheep erythrocytes (Nossal, 1975) which has previously been interpreted as evidence for B memory cell recruitment (Stavisky, 1980). The second aspect concerns the relative levels of specific B memory cells in the lung compared to the spleen.

The existence of influenza-specific B memory cells in both the lung and spleen was demonstrated by stimulating resting B memory cells into ASCs in vitro. The level of B memory cells was greater in the spleen than in the lung and whereas the level of the spleen remained constant throughout the lifetime of the mouse, there was a decline in the pulmonary level within six months of infection. The

generation of B memory cells depends on the presence of complexes of antigen, antibody and the C3 component of complement attached to C3 receptors on follicular dendritic cells in germinal centres of the spleen and other lymphoid tissues (Klaus et al., 1980). These complexes may persist in the germinal centres for many months, leading to the continued generation of new B memory cells (Mandel et al., 1980). Whereas influenza-specific IgA memory cells may have developed in the follicles of the bronchus-associated lymphoid tissue (BALT), it is likely that other B memory cells found in the lung were derived from extrapulmonary lymphoid tissue because of the paucity of organized lymphoid structures within the lung parenchyma. The subsequent decline in the level of B memory cells in the lung may reflect the continual generation of ASCs within the lung, stimulated by persisting influenza viral antigens (Astry et al., 1984).

The persistence of ASCs after primary influenza contrasts with the dynamics of the response of effector T cells. Tc and Td cells reach peak levels between 6 and 8 days in both the lung and spleen, but are no longer detectable after 2 weeks in either organ (Yap & Ada, 1978a; Leung & Ada, 1980). However, memory for Tc cell responses has been demonstrated in murine lung and spleen at six weeks after intranasal inoculation (Mak et al., 1984) and in the spleen 2 years after intravenous immunization (Ashman, 1982). Thus Tc effector cells do not develop after

infection resolves despite the presence of Tc memory cells. Assuming that the persistence of ASCs may be partly due to recruitment from the pool of B memory cells, these findings suggest a disparity between the ability of either persisting viral antigens or anti-idiotype antibodies to stimulate the differentiation of B and Tc memory cells. Tc memory cells may not be stimulated if viral antigens are either sequestered on follicular dendritic cells and inaccessible to Tc memory cells or are not presented in association with class I MHC restricting elements. It is unknown whether anti-idiotype antibodies may stimulate Tc memory cells, although T cell specific anti-idiotype antibodies may induce cell-mediated responses in unprimed mice (Finberg & Ertl, 1986).

The effect of further exogenous antigenic stimulation was also determined in challenges using either the homotypic strain of influenza virus or a different subtype. Homotypic challenge using a large inoculum of virus given six months after priming increased the level of ASCs, principally IgG- and IgA-secreting, in the lung during the first week after challenge. There was no comparable response observed in the spleen - presumably insufficient antigen reached the spleen to stimulate the generation of ASCs from resident B memory cells. Accordingly, homotypic challenge increased influenza-specific antibody levels in TBW to a greater extent than in serum. There was an increase in the number of ASCs generated in both lung and spleen cell cultures

obtained from mice 5 weeks after challenge.

Heterotypic challenge similarly increased the level of ASCs in the lung, but also elicited a splenic response, presumably because viral replication was not limited in the initial stages of infection in the heterotypically primed mice. The cells in both the lung and spleen were secreting antibodies predominantly directed to the common internal proteins of the influenza A virus, as reflected by the antibody responses in both serum and TBW. Similarly, there was no increase in the level of ASCs specific for A/Jap virus generated in either stimulated lung or spleen cell cultures obtained from challenged mice.

These findings may provide some rationale for understanding the persistence of B cell immunity in humans. HA-specific antibodies, capable of preventing re-infection, may persist for decades in humans after the original influenza A virus subtype is no longer circulating in the population. Although the phenomena of original antigenic sin is well described during successive infections with drift viruses within the same subtype, there is little evidence that this occurs to a significant extent when different subtypes are involved. Heterotypic responses occur infrequently after either infection or immunization with different subtypes (Masurel, 1976; Kilbourne, 1976), although individual B cell precursors in limiting dilution cultures may be stimulated by viruses of one subtype to

produce antibodies specifically directed to the HA of a different subtype (Yarchoan & Nelson, 1984). However, there is no evidence that these heterotypic antibodies possess neutralizing capacity. In the absence of significant effect from subsequent exogenous antigenic exposure, it is likely that the persistence of antibody in humans relates to the persistence of ASCs and B cell memory as described in the murine model.

SUMMARY

Influenza virus-specific ASCs, enumerated using an ELISA-plaque assay, were found in the lung and spleen up to eighteen months after primary murine influenza infection. The number of ASCs generated in stimulated lung and spleen cell cultures increased 50-200-fold after influenza infection. Whereas the level of response did not change in spleen cell cultures up to eighteen months after infection, there was a gradual reduction in ASCs in lung cell cultures obtained more than six months after infection, predominantly due to a reduction in B memory cells. Homotypic re-infection increased ASCs in the lung only, whereas B cell memory increased in both the lung and spleen. Although ASCs increased in both the lung and spleen after heterotypic challenge, ASCs and B cell memory specific for the original subtype were not increased.

CHAPTER SIX

CELLULAR IMMUNE RESPONSES TO INFLUENZA VACCINATION

INTRODUCTION

Vaccines currently in use in the Western world against influenza A virus consist of inactivated whole or disrupted virus or of preparations (subunit vaccines) containing the two surface glycoproteins, the HA and NA. The major obstacle to successful vaccination is the unpredictable antigenic changes in the HA molecule which occur during antigenic drift and shift. The protective efficacy of inactivated vaccines is further limited by their low immunogenicity in unprimed hosts and their inability to induce Tc responses. The latter is thought to be important primarily because of murine studies which clearly demonstrate that a cross-reactive Tc response rapidly reduces lung virus titres. The available data from human studies also support the concept that development of a Tc cell response aids recovery from infection (Section 1.3.7.4).

Cold-adapted (ca) reassortant vaccines are promising candidates for future influenza vaccines. The immunogenicity of ca-variants compared with wild-type parental strains has been previously determined largely on the basis of antibody responses. Ca-reassortant vaccines were less immunogenic than wild-type virus in seronegative, though presumably primed, adult volunteers as judged by both serum and nasal wash antibody responses (Murphy et al., 1980; Clements et al., 1983). In murine studies, ca-

variants were less immunogenic than parental strains at inducing both primary serum antibody responses and primary Tc cell responses (Mak et al., 1982b). There is limited data on the ability of ca-variants to induce immunological memory. Evidence for B cell memory is suggested by the development of secondary nasal wash IgA responses in children previously immunized with a ca-reassortant virus and challenged intranasally with inactivated virus vaccine (Wright et al., 1983). Evidence for Tc cell memory has been demonstrated in murine studies by analyzing Tc cell precursor frequency after immunization (Mak et al., 1984).

An alternative approach to successful influenza vaccination is to improve the efficacy of inactivated vaccines. This could be achieved by increasing the immunogenicity so that high neutralizing antibody levels are produced, especially in unprimed hosts, and by using a vaccine formulation which is effective at inducing cross-reactive Tc cell responses.

One such approach could be based on the amphiphilic nature of the surface glycoproteins of the influenza virus and other enveloped viruses (Morein & Simons, 1985). The hydrophobic membrane anchors of the surface glycoproteins may aggregate to form protein micelles or be reconstituted with phospholipids to form liposomes in which the glycoproteins are exposed on the external surface. Micelle preparations and liposomes containing the influenza HA and

NA have induced higher antibody responses than protein monomers alone (Morein & Simons, 1985; Almeida et al., 1975; Oxford et al., 1981b). Recently a related structure has been described which consists of multimeric matrices formed by the interaction of the hydrophobic portion of membrane proteins with the glycoside Quil A (Morein et al., 1984). These complexes referred to as immuno-stimulatory complexes (iscoms) have been prepared with the membrane proteins of parainfluenza, measles and rabies viruses and have induced higher antibody titres than protein micelles.

In the first part of this chapter local and systemic B cell responses after immunization with a ca-variant virus have been compared with those to the parental virus and inactivated virus. In the second part the ability of influenza-specific iscoms and micelles to induce local cellular immune responses and immunological memory in both unprimed and primed mice, has been examined.

RESULTS

6.1 B Cell Responses after Immunization with Wild-type, ca-variant and Inactivated Influenza Viruses

6.1.1 ASCs after Primary Immunization with Parental and ca-variant viruses

Mice were inoculated intranasally with graded doses, ranging from 10^3 to 10^6 EID_{50} per mouse, of either the parental strain or ca-variant of A/AA virus. The ca-variant virus was given as either a single inoculum or two inocula three weeks apart. ASCs were measured in the lung and spleen during the primary response and ten weeks later (Table 6.1). Immunization with a single inoculum of 10^6 EID_{50} of ca-variant virus produced a level of ASCs in the lung during the primary response comparable to that following immunization with a 100 fold lower dose of the parental strain (non mouse-adapted). There were virtually no ASCs persisting in the lung ten weeks after immunization with a single inoculum of 10^6 EID_{50} of ca-variant virus whereas ASCs were still present in the lungs of mice immunized with a 100-fold lower dose of parental virus. Persistent ASCs were, however, detected in the lungs of mice immunized with two inocula of 10^6 EID_{50} of ca-variant virus.

Whereas immunization with high doses of parental virus resulted in the appearance of a small number of ASCs in the

spleen (12 IgG ASCs/10^6 cells at day 12), there was no splenic response following immunization with the ca-variant virus.

6.1.2 B Cell Memory after Primary Immunization with Parental and ca-variant Viruses

The number of ASCs generated in lung and spleen cell cultures obtained from primed mice and stimulated in vitro with purified A/AA virus was determined 15 weeks after primary intranasal inoculation (Table 6.2). Immunization with the parental virus, even at the lowest dose, resulted in a significant increase in the number of ASCs generated in both lung and spleen cell cultures compared to cultures from unprimed mice. Immunization with 10^6 and $10^5 EID_{50}$ of ca-variant virus resulted in a secondary in vitro IgM response in both lung and spleen cell cultures comparable to that obtained with the corresponding dose of parental virus. In contrast the number of IgG and IgA ASCs were smaller than that obtained with the parental virus. The number of IgG and IgA ASCs generated in cultures from mice immunized with lower doses of ca-variant virus was not significantly greater than the number obtained from unprimed mice. Immunization with two inocula of the ca-variant virus did not appreciably alter the number of ASCs generated in vitro.

6.1.3 ASCs and B Cell Memory following Primary Immunization with Different Viral Preparations

The number of ASCs in the lung and spleen were compared in 4 groups of mice immunized with either (A) 10^6 EID_{50} of parental A/AA virus intranasally; or (B) 10^6 EID_{50} of ca-variant A/AA virus intranasally; or (C) 10^3 HAU of ultraviolet-inactivated A/AA virus intranasally; or (D) 10^3 HAU of ultraviolet-inactivated A/AA virus intraperitoneally (Table 6.3). As above, immunization with the parental strain resulted in the appearance of ASCs during the primary response in both the lung and spleen whereas the ca-variant virus elicited a smaller pulmonary response only. Inactivated virus, given either intranasally or intraperitoneally, failed to elicit a primary response in the lung although intraperitoneal inoculation did elicit a splenic response.

At 12 weeks after immunization, a small number of IgG and IgA ASCs were still present in the lung - 14 ASCs/10^6 cells in mice primed with the parental virus and 6 ASCs/10^6 cells in ca-variant primed mice. The largest number of ASCs, particularly IgG and IgA ASCs, generated in stimulated lung cell cultures, 12 to 15 weeks after primary inoculation, was found in mice immunized with the parental virus (Table 6.4). Secondary in vitro responses were also obtained, in decreasing order of magnitude, in lung cell cultures from mice immunized with the ca-variant virus,

inactivated virus given intranasally and inactivated virus given intraperitoneally. Secondary in vitro splenic responses were comparable in mice immunized with the parental virus or inactivated virus given intraperitoneally. The IgG responses in spleen cell cultures from mice immunized with the ca-variant virus or inactivated virus given intranasally were of lesser magnitude, though still significantly higher than in cultures from unprimed mice.

6.1.4 Correlation of B Cell Responses with Protection

Immunized mice in the four groups described above were challenged 15 weeks after priming with 1.5×10^7 EID_{50} of the parental A/AA virus intranasally. Lung virus titres were measured in 4 mice from each group at day 3 after challenge (Table 6.5A). Lung virus titres in each of the 4 immunized groups of mice were significantly lower ($p < 0.05$) than the titres in unprimed mice. Furthermore lung virus titres in mice primed with the parental virus were significantly lower ($p < 0.05$) than in the other 3 immunized groups. Correlation coefficients were calculated comparing the degree of protection afforded by immunization, as assessed by the difference in lung virus titres between unprimed and immunized mice, and parameters of the B cell response measured prior to challenge (Tables 6.5 A & B). Overall there was a trend towards higher correlation coefficients with pulmonary responses. In particular, there

was a significant correlation between the degree of protection and the number of IgG and IgA ASCs in the lung at the time of challenge ($p < 0.05$) (Table 6.5A). In addition, protection also correlated significantly with the number of IgG and IgA ASCs generated in lung cell cultures obtained before challenge ($p < 0.01$) but, in contrast, there was no correlation with the number of ASCs generated in spleen cell cultures. Furthermore there was no correlation with serum antibody levels irrespective of isotype (Table 6.5B). Although a significant correlation was observed with IgM levels in tracheobronchial washings, the titres were very low and unlikely to have any functional significance. Despite the correlation observed with IgG and IgA ASCs in the lung, there was no correlation with IgG and IgA levels in tracheobronchial washings. This discordance is probably due to the variable effect of diluting secretory antibody levels whilst obtaining tracheobronchial washings.

6.2 Cellular Immune Responses to Influenza A Virus Glycoproteins in Iscoms and Micelles

6.2.1 In vivo Responses after Primary Immunization

Normal mice were inoculated intranasally with either (i) 10^3 EID_{50} infectious A/PR8 virus; or (ii) HANA iscoms, administered either as a single 5ug dose or two 5ug doses, one week apart; or (iii) HANA micelles, similarly administered either once or twice in 5ug doses. There was

no pulmonary or systemic toxicity following intranasal inoculation with either iscoms or micelles.

Influenza-specific ASCs were enumerated in lung cell suspensions obtained fourteen days after the initial inoculation (Table 6.6). There was no ASCs detected in the lungs of mice inoculated intranasally with micelles in either one or two doses. In contrast, inoculation with a single dose of iscoms resulted in the appearance of a moderate number of IgG ASCs and inoculation with two doses of iscoms resulted in numbers of cells secreting IgM and IgG, but not IgA, of comparable order to those observed in mice primed with the infectious virus. The lack of an IgA response was confirmed in two subsequent experiments.

Tc cell responses and NK cell responses were determined at six days after immunization; in mice inoculated twice, cytotoxicity assays were performed six days after the second inoculation (Table 6.7). There was no Tc cell response detected in the lungs of mice receiving either micelles or iscoms even after two inoculations. There was an increase in NK cell activity above the level in normal lungs in mice immunized with both micelles and iscoms.

6.2.2 B Cell and Tc Cell Memory after Primary Immunization

The development of B cell and Tc cell memory in the lung after primary immunization was determined at four weeks

after intranasal inoculation. The number of ASCs generated by in vitro stimulation with purified A/PR8 virus was compared in lung cell cultures obtained from either immunized mice or from normal mice (Table 6.8). In lung cell cultures from mice primed with infectious virus there was approximately a 10-fold increase in the total number of ASCs generated, including IgG- and IgA-secreting cells not found in primary lung cell cultures. A similar increase was found in cultures from mice immunized with iscoms, independent of the number of doses given. In contrast, there was no increase in the number of ASCs generated in lung cell cultures obtained from mice inoculated with either one or two doses of micelles, although a small number of IgG- and IgA-secreting cells were detected in cultures from mice given two doses of micelles.

The level of Tc cell memory present after primary immunization was determined by estimating Tc cell precursor frequency by limiting dilution analysis. Tc cell precursor frequency in the lung increased approximately 100-fold in mice primed with infectious virus. Immunization with two doses of iscoms increased the frequency of Tc cell precursors 10-fold. In contrast, inoculation with either a single dose of iscoms or two doses of micelles had little effect on the Tc cell precursor frequency.

6.2.3 In vivo Responses after Secondary Immunization

Mice were initially primed with $3x10^2$ EID_{50} A/PR8 virus intranasally and 3 weeks later were inoculated intranasally with either (i) 10^4 EID_{50} A/PR8; or (ii) 5ug HANA iscoms; or (iii) 5ug HANA micelles. The effect of this schedule of secondary in vivo immunization on the level of ASCs, Tc cells and NK cells in the lung was determined six days after the secondary inoculation.

Secondary inoculation with the homologous virus failed to elicit an increase in the level of ASCs persisting in the lung after primary inoculation (Table 6.9). In contrast, secondary inoculation with either iscoms or micelles significantly increased the level of ASCs, of each isotype, in the lung. The increases in IgM- and IgA-secreting cells were similar after inoculation with either iscoms or micelles, whereas iscoms induced a greater increase in IgG-secreting cells.

Similarly, in vivo challenge with the homologous virus failed to elicit a secondary Tc cell response (Table 6.10). Whereas both iscoms and micelles did induce a Tc cell response, the level of Tc cells induced by iscoms was about 4-fold higher than the level induced by micelles. The Tc cells exhibited cross-reactivity by lysing target cells infected with an influenza virus bearing both different HA and NA proteins. There was little NK cell activity after

either homologous virus challenge or secondary inoculation with either iscoms or micelles (Table 6.10).

6.2.4 Effect of Secondary in vivo Immunization on B Cell and Cytotoxic T Cell memory

The effect of secondary in vivo immunization on B cell and Tc cell memory was determined six weeks after secondary inoculation by re-stimulating lung cell suspensions in vitro. Neither homologous virus challenge nor immunization with either iscoms or micelles had a significant effect on the number of ASCs generated in vitro or on Tc cell precursor frequency (Table 6.11).

TABLE 6.1 ASCs in the Lung after Primary Intranasal Inoculation with Graded doses of Parental or ca-variant Virus

Primary Intranasal Inoculation	Dose (EID_{50})	ASCs/10^6 cells (mean ± SD)[a] Day after inoculation					
		15			70		
		IgM	IgG	IgA	IgM	IgG	IgA
Parental A/AA	10^6	192 ± 30	300 ± 25	176 ± 12	<5	58 ± 6	2 ± 1
	10^5	70 ± 2	48 ± 3	2 ± 2	6 ± 1	44 ± 2	2 ± 1
	10^4	34 ± 9	37 ± 1	3 ± 1	<5	8 ± 2	1
	10^3	8 ± 5	10 ± 6	2 ± 2	<5	3 ± 1	1
Ca-variant A/AA	10^6	35 ± 3	22 ± 3	2 ± 2	<5	2	<1
	10^5	<5	<1	<1	<5	<1	<1
	10^4	<5	<1	<1	<5	<1	<1
	10^3	<5	<1	<1	<5	<1	<1
Ca-variant A/AA	10^6x2[b]	ND	ND	ND	23 ± 8	32 ± 1	13 ± 4
	10^5x2	"	"	"	<5	1	<1
	10^4x2	"	"	"	<5	<1	<1
	10^3x2	"	"	"	<5	<1	<1

a. Values represent mean ± SD of triplicate assays using suspensions of pooled lung cells obtained from 3 mice.

b. Two doses given, 3 weeks apart, on day 0 and day 21.

ND = Not determined.

TABLE 6.2 B Cell Memory in Lung and Spleen after Primary Intranasal Inoculation with Graded Doses of Parental or ca-variant Virus.

Primary Intranasal Inoculation	Dose (EID_{50})	ASCs/10^6 cells (mean ± SD) generated in vitro[a]				
		Lung			Spleen	
		IgM	IgG	IgA	IgM	IgG
Nil	-	231 ± 11	<1	<5	859 ± 65	<1
Parental A/AA	10^6	590 ± 50[b]	198 ± 12[b]	326 ± 21[b]	2580 ± 280[b]	2520 ± 20[b]
	10^5	550 ± 63[b]	65 ± 10[b]	76 ± 6[b]	1660 ± 120[b]	1990 ± 130[b]
	10^4	610 ± 30[b]	34 ± 7[b]	22 ± 7[b]	1475 ± 80[b]	1450 ± 20[b]
	10^3	587 ± 38[b]	5 ± 1[b]	35 ± 7[b]	1620 ± 160[b]	1950 ± 110[b]
Ca-variant A/AA	10^6	742 ± 63[b]	112 ± 6[b]	127 ± 4[b]	2267 ± 93[b]	1146 ± 90[b]
	10^5	708 ± 40[b]	8 ± 3[b]	45 ± 6[b]	1933 ± 267[b]	312 ± 60[b]
	10^4	182 ± 23	4	8 ± 2	1247 ± 140[b]	56 ± 11[b]
	10^3	234 ± 13	4 ± 1	9 ± 4	ND	ND
Ca-variant A/AA	10^6x2	477 ± 23[b]	148 ± 13[b]	115 ± 7[b]	2160 ± 130[b]	1104 ± 106[b]
	10^5x2	737 ± 61[b]	3	20 ± 2[b]	1090 ± 80[b]	658 ± 104[b]
	10^4x2	570 ± 14[b]	2	29 ± 4[b]	1100 ± 130[b]	80 ± 16[b]
	10^3x2	273 ± 21	3	24 ± 6[b]	640 ± 40[b]	58 ± 5[b]

a. Values represent mean ± SD of duplicate assays on suspensions of pooled cells from 3 mice obtained 15 weeks after primary inoculation and stimulated in vitro with purified A/AA virus for 5 days.

b. Significant increase ($p < 0.05$) in ASCs generated in vitro over cultures from unprimed mice.

ND = Not determined.

TABLE 6.3 ASCs in the Lung and Spleen after Variable Primary Immunization Protocols

Primary Inoculation			Lung virus titre[a] ($log_{10}EID_{50}$)	ASCs/10^6 cells (mean ± SD)[b]				
				Lung[c]			Spleen	
Virus	Dose	Route		IgM	IgG	IgA	IgM[d]	IgG[c]
Parental A/AA	10^6 EID_{50}	i.n.	5.25 ± 0.25	82	189 ± 8	43 ± 19	104 ± 15	15 ± 7
Ca-variant A/AA	10^6 EID_{50}	i.n.	2.0 ± 0.5	16 ± 2	48 ± 5	13 ± 2	<5	0
Inactivated A/AA	10^3 HAU	i.n.	<2	6 ± 4	0	0	<5	0
Inactivated A/AA	10^3 HAU	i.p.	<2	0	0	0	25 ± 2	7 ± 3

a. Mean ± SD of triplicate titrations obtained at day 3.

b. Mean ± SD of triplicate assays using suspensions of pooled cells from 3 mice.

c. Day 16 after inoculation.

d. Day 7 after inoculation

TABLE 6.4 B Cell Memory in the Lung and Spleen after Variable Primary Immunization Protocols

Primary Inoculation			ASCs/10^6 cells (mean ± SD)[a] generated in vitro				
			Lung			Spleen	
Virus	Dose	Route	IgM	IgG	IgA	IgM	IgG
Nil	-	-	125± 25	1	4 ± 1	1033±260	1
Parental A/AA	10^6 EID_{50}	i.n.	1775±126[b]	280±52[b]	127±30[b]	5240±720[b]	2300±420[b]
Ca-variant A/AA	10^6 EID_{50}	i.n.	1167± 81[b]	30±10[b]	25± 7[b]	4260± 20[b]	580±200[b]
Inactivated A/AA	10^3 HAU	i.n.	755± 19[b]	7± 2[b]	20± 2[b]	4220±660[b]	400± 80[b]
Inactivated A/AA	10^3 HAU	i.p.	460± 27[b]	12± 1[b]	12± 2[b]	7240±340[b]	1290±150[b]

a. Values represent mean ± SD of duplicate assays using suspensions of pooled cells obtained from 3 mice 12-15 weeks after primary inoculation and stimulated in vitro for 5 days.

b. Significant increase ($p < 0.05$) in ASCs generated in vitro over cultures obtained from unprimed mice.

TABLE 6.5A Correlation of ASCs and B Cell Memory with Protection

Primary Inoculation (Route)[a]	Lung virus titres[b] ($log_{10}EID_{50}$)	Number of IgG & IgA ASCs in Lung	Mean no. of ASC's generated in vitro				
			Lung			Spleen	
			IgM	IgG	IgA	IgM	IgG
Nil	6.9 ± 0.2	0	125	< 1	4	1033	< 1
Parental Virus (i.n.)	2.9 ± 1.4	14	1775	280	127	5420	2300
Ca-variant Virus (i.n.)	5.1 ± 1.2	6	1167	30	25	4260	580
Inactivated Virus (i.n.)	5.3 ± 0.8	2	755	7	20	4220	400
Inactivated Virus (i.p.)	5.5 ± 0.4	0	460	12	12	7240	1290
Correlation Coefficient:		0.96	0.92	0.99	0.99	-0.05	0.84
Probability Value:		<0.05	NS	< 0.01	<0.01	NS	NS

a. Same protocol for primary inoculation as in Tables 6.3 and 6.4.

b. Lung virus titres measured in 4 mice at day 3.

NS = Not significant

TABLE 6.5B Correlation of Antibody Levels with Protection

Primary Inoculation (Route)[a]	Lung virus titres[b] ($log_{10}EID_{50}$)	Reciprocal of ELISA Titre[c] (log_2)					
		Serum			TBW		
		IgM	IgG	IgA	IgM	IgG	IgA
Nil	6.9 ± 0.2	< 3	< 3	< 3	< 3	< 3	< 3
Parental Virus (i.n.)	2.8 ± 1.4	14	22	13	5	13	10
Ca-variant Virus (i.n.)	5.1 ± 1.2	14	20	11	3	11	9
Inactivated Virus (i.n.)	5.3 ± 0.8	14	20	12	3	10	3
Inactivated Virus (i.p.)	5.5 ± 0.4	13	20	9	3	7	< 3
Correlation Coefficient:		0.44	0.74	0.81	0.99	0.81	0.72
Probability Value:		NS	NS	NS	< 0.01	NS	NS

a. & b. As in Table 6.5A.

c. Sera and tracheobronchial washings (TBW) pooled from 3 mice.

TABLE 6.6 ASCs in the Lung after Primary Immunization with Iscoms and Micelles

Primary Intranasal Inoculation	Dose	ASCs/10^6 cells (mean ± SD)[a]		
		IgM	IgG	IgA
Nil	-	3 ± 1	0	0
A/PR8 virus	10^3 EID_{50}	391 ± 14	109 ± 28	295 ± 36
HANA iscoms	5ug	0	24 ± 2	0
HANA iscoms	5ug x 2	147 ± 1	250 ± 3	16 ± 6
HANA micelles	5ug	0	0	0
HANA micelles	5ug x 2	0	1	0

a. Values represent mean ± SD of triplicate assays on suspensions of pooled lung cells obtained from 3 mice 2 weeks after primary inoculation.

TABLE 6.7 Tc Cells and NK Cells in the Lung after Primary Immunization with Iscoms and Micelles

Primary Intranasal Inoculation	Dose	% Specific ^{51}Cr release from:[a]			
		A/PR8-P815[b]		YAC-1	
		100:1[c]	50:1	100:1	50:1
Nil	-	11 ± 0.4	7 ± 0.2	31 ± 0.6	11 ± 0.3
A/PR8 virus	10^3 EID_{50}	83 ± 1	66 ± 3	70 ± 2	67 ± 1
HANA iscoms	5ug	3 ± 0.2	2 ± 0.1	65 ± 2	59 ± 2
HANA iscoms	5ug x 2	9 ± 0.3	11 ± 0.2	39 ± 1	39 ± 1
HANA micelles	5ug	3 ± 0.2	2 ± 0.2	69 ± 2	62 ± 1
HANA micelles	5ug x 2	11 ± 0.4	10 ± 1	40 ± 1	29 ± 1

a. Values represent mean ± SD of quadruplicate assays on suspensions of pooled lung cells obtained from 3 mice 6 days after inoculation. Background medium release from infected and uninfected P815 and YAC-1 cells was 7.8, 7.5 and 7.4% respectively.

b. Specific release from uninfected P815 cells was <1% except in the presence of cells from A/PR8 primed mice when specific release was 11% (E/T ratio 100:1).

c. Effector cell:Target cell ratio (E/T).

Table 6.8 B Cell and Tc Cell Memory after Primary Immunization with Iscoms and Micelles

Primary Intranasal Inoculation	Dose	ASCs/10^6 cells (mean ± SD) generated in vitro:[a] IgM	IgG	IgA	Tc Cell precursor frequency[b] ($x10^{-4}$)
Nil	-	235 ± 20	0	0	.16
A/PR8 Virus	10^3 EID_{50}	1200 ± 43	577 ± 63	257 ± 83	28.6
HANA Iscoms	5ug	1167 ± 110	130 ± 20	253 ± 63	.39
HANA Iscoms	5ug x 2	1230 ± 210	273 ± 33	177 ± 17	2.74
HANA Micelles	5ug	137 ± 60	0	0	.15
HANA Micelles	5ug x 2	187 ± 43	7 ± 3	3 ± 2	.43

a. Values represent mean ± SD of duplicate assays on suspensions of pooled lung cells obtained from 3 mice 4 weeks after immunization and stimulated in vitro with purified A/PR8 virus for 5 days.

b. Limiting dilution assays on suspensions of pooled lung cells obtained from 3 mice 4 weeks after immunization.

TABLE 6.9 Effect of Secondary in vivo Immunization with Iscoms and Micelles on ASCs in the lungs of primed mice

Secondary Intranasal Inoculation[a]	Dose	ASCs/10^6 cells (mean ± SD)[b]		
		IgM	IgG	IgA
Nil	-	34 ± 7	62 ± 8	32 ± 3
A/PR8 Virus	10^4 EID_{50}	15 ± 9	95 ± 18	10 ± 4
HANA Iscoms	5ug	314 ± 32	920 ± 62	232 ± 8
HANA Micelles	5ug	318 ± 44	470 ± 62	200 ± 2

a. Mice were primed 3 weeks earlier with $3x10^2$ EID_{50} A/PR8 virus intranasally.

b. Values represent mean ± SD of triplicate assays on suspensions of pooled lung cells obtained from 3 mice 6 days after secondary inoculation.

TABLE 6.10 Tc Cells and NK Cells in the Lung after Secondary in vivo Immunization with Iscoms and Micelles

Secondary Intranasal Inoculation[a]	Dose	% Specific ^{51}Cr release from[c] A/PR8-P815 50:1[b]	A/PR8-P815 25:1	A/PR8-P815 12:1	A/PC-P815 50:1	A/PC-P815 25:1	A/PC-P815 12:1	YAC-1 50:1
Nil	-	16	14	10	12	11	7	11
A/PR8 Virus	10^4 EID_{50}	14	13	10	13	12	9	10
HANA Iscoms	5ug	53	48	35	26	23	17	13
HANA Micelles	5ug	32	31	22	18	18	13	7

a. As in table 6.9.

b. Effector cell:Target cell ratio (E/T)

c. Values represent mean of quadruplicate assays on suspensions of pooled lung cells obtained from 3 mice 6 days after secondary inoculation. Background medium release from all target cells <10%. Specific release from uninfected P815 cells <1% (E/T ratio 50:1).

TABLE 6.11 Effect of Secondary in vivo Immunization with Iscoms and Micelles on B Cell and Tc Cell Memory

Secondary Intranasal Inoculation[a]	Dose	ASCs/10^6 cells (mean ± SD) generated in vitro:[b] IgM	IgG	IgA	Tc Cell precursor frequency[c] ($x10^{-4}$)
Nil	-	2467 ± 27	1133 ± 53	1120 ± 147	5.0
A/PR8 Virus	10^4 EID_{50}	3019 ± 150	2004 ± 77	1427 ± 253	2.1
HANA Iscoms	5ug	3478 ± 266	2334 ± 326	1950 ± 80	7.0
HANA Micelles	5ug	3049 ± 49	1736 ± 22	1197 ± 22	3.3

a. As in Table 6.9.

b. Values represent mean ± SD of duplicate assays on suspensions of pooled lung cells obtained from 3 mice 6 weeks after secondary immunization and stimulated in vitro for 5 days.

c. Limiting dilution assays on suspensions of pooled lung cells obtained from 3 mice 6 weeks after secondary immunization.

DISCUSSION

Influenza virus-specific ASCs were detected in the lung but not in the spleen following immunization with a ca-variant virus, whereas primary infection with the parental virus induced both pulmonary and splenic responses. The discordance between pulmonary and splenic B cell responses to ca-variant viruses has also been observed with Tc cell responses (Mak et al., 1984) and is probably related to the restricted replication of the ca-variant virus in the murine lung with a resulting lack of antigen "spillover" to the spleen. Ca-variant virus was 100 fold less efficient than the parental virus in the induction of primary ASC responses in the lung. This difference in immunogenicity is similar to that previously reported for serum antibody responses and primary Tc cell responses (Mak et al., 1982b).

B cell memory, assessed as the ability to prime for secondary in vitro responses, was established in both the lung and spleen after immunization with sufficiently high doses of the ca-variant virus. However the number of IgG and IgA ASCs generated in vitro was less than that detected in the corresponding cultures obtained from mice immunized with the parental virus. Thus B cell memory, but not a primary ASC response, was detected in the spleen after administration of the ca-variant virus (as well as after lower doses of the parental virus).

The low immunogenicity of ca-variant viruses in unprimed mice parallels the lesser degree of protection afforded against challenge with homotypic or heterotypic wild-type viruses as compared with the parental virus (Mak et al., 1984; Tannock et al., 1984). In previous studies protection against homotypic challenge, which is principally mediated by neutralizing antibody, correlated poorly with serum antibody responses (Tannock et al., 1984). The ability to accurately localize B cell responses in mice immunized with different priming strategies provided the opportunity to determine whether protection correlated with local or systemic B cell responses. Accordingly protection has been shown to correlate with the development of pulmonary B cell responses rather than splenic responses. In particular the presence of ASCs in the lung and the number of IgG and IgA ASCs generated in stimulated lung cultures obtained at the time of challenge correlated significantly with protection. In contrast serum antibody levels and the number of ASCs generated in stimulated spleen cell cultures did not correlate with protection.

ASCs persisted in the lung for less than ten weeks after immunization with a single inoculum of ca-variant virus. In contrast ASCs persisted in the lung for up to eighteen months after primary infection with a mouse-adapted wild-type virus (Section 5.1). However immunization with two inocula of ca-variant virus prolonged the duration of ASCs in the lung, although the levels were still lower than

the results obtained with mouse-adapted virus. Although immunization with two doses of ca-variant virus did not appear to appreciably expand the pool of B memory cells, a larger amount of residual viral antigen in the lung may have lead to increased recruitment of B memory cells. Consistent with the observation that the presence of ASCs in the lung correlated with protection, immunization with two doses of ca-variant virus has previously been shown to afford greater protection in murine studies (Tannock et al., 1984) and is recommended for future use of ca-variant vaccines in unprimed humans.

The immunogenicity of iscoms and related structures has previously been assessed almost solely on serum antibody titrations following parenteral administration. In this chapter, cellular immune responses in the lung involving NK cells, influenza virus-specific ASCs and Tc cells were analyzed following local immunization with either iscoms or micelles containing the influenza virus glycoproteins.

In unprimed mice, only intranasal inoculation with two doses of iscoms induced a level of IgM and IgG ASCs in the lung comparable to that following primary inoculation with infectious virus. However, there was no in vivo IgA response following immunization with iscoms possibly because aerosols of iscoms were predominantly delivered to the peripheral zones of the lung. Local B cell memory was also established only after immunization with iscoms but was less

dependent on dose and involved the generation of IgA memory cells as well.

In contrast to the ASC responses, neither iscoms nor micelles even after two inoculations induced primary in vivo Tc cell responses. However local Tc cell memory was established after immunization with two doses of iscoms although the frequency of Tc cell precursors was only 10% of that following inoculation with infectious virus. An analysis of Tc cell precursor frequency after primary immunization with a ca-vaccine produced similar results (Mak et al., 1984).

In mice primed with infectious virus homologous virus challenge failed to elicit both secondary B and Tc cell responses presumably because viral replication was prevented by neutralizing antibody. In contrast secondary immunization with either iscoms or micelles induced significant elevations in the level of ASCs in the lung and the appearance of cross-reactive Tc cells. This difference probably reflects the greater antigenic load delivered in the iscom and micelle preparations. There was no significant change in the level of either B cell or Tc cell memory after secondary immunization with either iscoms or micelles.

These findings differ in several respects to the murine and human responses described after immunization with

earlier influenza HANA subunit vaccines. The most important difference is the ability of HANA iscoms to induce Tc cell responses and Tc cell memory. Previous studies showed that earlier subunit vaccines failed both to prime mice for secondary Tc cell responses (Reiss & Schulman, 1980b; Webster & Askonas, 1980) and also failed to elicit secondary Tc cell responses, in mice and in humans, whether administered in vivo or in vitro (Reiss & Schulman, 1980b; McMichael et al., 1981; Ennis et al., 1981b). Accordingly immunization with HANA iscoms may afford broader heterotypic protection mediated by cross-reactive Tc cell responses than current inactivated vaccines. There are no comparable data on the development of ASCs or B cell memory in the lung with earlier subunit vaccines, but using an influenza specific haemolytic-plaque assay, direct plaque-forming cells were not detected in the spleens of mice after a single intraperitoneal injection (Reiss & Schulman, 1980b). Data on antibody responses have shown that it is necessary to give two inoculations of subunit vaccines to achieve serum titres comparable to levels obtained after immunization with inactivated whole virus (Potter, 1982). Similarly a single inoculation of iscoms was poorly immunogenic in unprimed mice.

The basis for the enhanced immunogenicity of HANA iscoms over earlier HANA subunit vaccines may relate to the ability of iscoms and other amphiphilic-based structures to interact with the cell membrane of antigen-presenting

cells. The homogenous preparations of HANA micelles and iscoms used in this study contrasts with earlier subunit vaccines which were composed of heterogenous complexes of glycoproteins, lipids and residual detergents (Morein & Simons, 1985). In this study HANA iscoms were also more immunogenic than HANA micelles, particularly in unprimed mice. The basis for this observation has not been determined although in other systems it is not merely a function of the adjuvant effect of Quil A (Morein & Simons, 1985).

Both murine and human studies have shown that the HA is recognized by only 10-15% of Tc cells and that there is little cross-recognition between the HA proteins of the H1 and H3 subtypes (Section 1.3.2.2). Although it is likely that some Tc cells recognize the NA, there are no reports unequivocally demonstrating this. Thus although neither the HA or NA proteins appear to be major determinants recognized by Tc cells, iscoms containing only these proteins were able to induce cross-reactive secondary Tc cell responses. This illustrates the marked immunopotentiating effect of iscom-based preparations. Whilst it is probable that most if not all the influenza viral proteins are recognized by Tc cells, the nucleoprotein (NP) appears to be a major Tc cell determinant. Hence a subunit vaccine containing the HA and NP has recently been proposed as an effective means for inducing both neutralizing antibody and cross-protective Tc cell responses (Wraith & Askonas, 1985). However it has

also been reported that there is considerable variation in the frequency of NP-specific Tc cells between mice of different MHC haplotypes (Pala & Askonas, 1986) and even between individual mice within the same inbred strain (Pala et al., 1986). Accordingly, the effectiveness of a vaccine containing NP would be limited if similar Ir gene effects occurred in humans. It would be interesting to determine if an iscom-based preparation of NP would be recognized by mice of low responder haplotypes.

SUMMARY

The development of regional B cell responses was studied in mice immunized intranasally with different influenza virus vaccines. The ca-variant virus was 100 fold less efficient than the parental virus in the induction of influenza virus-specific ASCs in the lung and failed to induce ASCs in the spleen. The ca-variant virus was also less efficient in priming for secondary IgG and IgA responses generated in vitro in both lung and spleen cell cultures. Protection against homotypic challenge in mice immunized by different vaccine strategies correlated with the development of pulmonary B cell responses rather than splenic responses. In particular, protection correlated with the presence of ASCs and IgG and IgA memory in the lung at the time of challenge.

Primary immunization with a single inoculum of either micelles or iscoms containing influenza A virus glycoproteins failed to induce either B or Tc cell responses. In contrast, immunization with two inocula of iscoms, but not micelles, resulted in the appearance of influenza virus-specific ASCs but not Tc cells in the lung. There was a 10-fold increase in Tc cell precursor frequency and an increase in ASCs generated by secondary in vitro stimulation of lung cell cultures obtained from mice primed with iscoms but not micelles. In mice primed with infectious virus, secondary immunization with either

micelles or iscoms increased the number of ASCs in the lung and elicited cross-reactive Tc cell responses. In contrast, homologous virus challenge failed to induce either detectable secondary B or Tc cell responses.

CHAPTER SEVEN

CONCLUSION

B cell responses to infection or immunization with viral respiratory pathogens have been previously characterized by measurements of serum and secretory antibody levels. This approach however does not assess the contribution of each target or lymphoid organ to the total response. The development of the ELISA-plaque assay, described in Chapter 3, provided a reliable method, previously unavailable, for quantitating influenza virus-specific ASCs following both in vivo and in vitro stimulation. Using this approach the responses of the lung, the target organ of influenza infection, and of the spleen, a major lymphoid organ, have been characterized after both murine influenza infection and immunization.

The development of B cell responses to murine influenza virus infection were described in Chapters 4 and 5 and address the aims of the thesis outlined in Section 1.4.

7.1 ASCs In Murine Influenza Infection (Table 7.1)

Influenza virus-specific ASCs secreting specific IgM, IgG and IgA, were detected in the lung during primary influenza virus infection. Subsequently cells continuing to secrete influenza specific antibodies, predominantly IgG, persisted in the lung, in the absence of any further exogenous antigenic stimulation, for at least 18 months after primary infection. These findings provide the most direct evidence yet available that specific antibodies in

respiratory secretions, in response to influenza infection, are derived from local antibody synthesis.

The magnitude of the ASC response relative to the number of lymphocytes assayed was greater in lung cell suspensions compared to spleen cell suspensions obtained during and after primary infection. However, since a greater number of lymphocytes were recovered from the spleen than from the lung the magnitude of the ASC response was greater in absolute terms in the spleen.

The characteristics of secondary ASC responses were dependent on whether the virus used for challenge was homotypic or heterotypic relative to the priming virus. The ASC response developed earlier in the lung after secondary homotypic challenge compared to primary infection and heterotypic challenge. The presence of pre-existing neutralizing antibody to the homotypic virus in the lung limited the extent of viral replication, thus necessitating the use of a relatively large dose of virus to elicit a secondary ASC response and restricting the response to the lung. In contrast, viral replication was not significantly affected in mice primed with a heterotypic virus. The ASC response developed at the same time as in unprimed mice and was not restricted to the lung. Furthermore, there was no evidence that challenge with a heterotypic virus elicited a B cell response to the original priming virus.

7.2 Influenza-specific B Cell Precursors in Murine Lung

That influenza-specific B cell precursors are present in the lungs of normal, non-immunized mice is evident by the finding of specific IgM-secreting cells in stimulated lung cell cultures obtained from unprimed mice. This finding indicates that the specific ASCs which appear in the lung during primary influenza infection may well have originated from specific B cell precursors already resident in the lung. However the appearance of ASCs in the spleen 24-48h earlier than in the lung during primary infection suggests that ASCs derived from the spleen and recruited into the lung may have also contributed to the pulmonary response.

7.3 B Cell Memory after Murine Influenza Infection (Table 7.2)

B cell memory was established in the lung as well as the spleen following primary influenza infection. Overall the level of memory was of lesser magnitude and was less durable in the lung compared to the spleen. The decline in the level of B cell memory in the lung was probably due to the recruitment of pulmonary B memory cells into ASCs due to continual stimulation from persisting viral antigen in the lung.

Homotypic virus challenge increased the level of B cell memory in both the lung and spleen. Heterotypic virus

challenge did not signicantly affect the level of B cell memory to the original priming virus. However memory to the challenge virus was established in both the lung and spleen at a level comparable to that developed in unprimed mice.

7.4 Intrapulmonary Localization of B Cell Responses

Although ASCs were detected in cell suspensions from whole lungs, either when assayed immediately after preparation or after in vitro stimulation, this approach has not provided adequate information to determine whether intra-pulmonary B cell responses were occurring within the mucosal and/or parenchymal divisions of the lung. Studies utilizing immunofluorescence have similarly not provided this information as cell suspensions were assayed (Scott & Walker, 1976). An attempt to study this aspect further by in situ immunohistochemical localization of the B cell response in the lung was undertaken. However this approach was unsuccessful because of insurmountable technical difficulties (Appendix).

Where are the influenza-specific B cell precursors, demonstrated in cultured cell suspensions, localized in the lungs? There was no evidence that these precursors were derived to any extent from circulating lymphocytes in the pulmonary vasculature. It is probable that influenza-specific IgA ASCs originated in the BALT where appropriate IgA T switch cells reside. The origin of ASCs of other

isotypes is unknown. The relative predominance of IgG over IgA ASCs in both primary and secondary responses suggest that the B cell responses detected were principally occurring within the parenchymal division (Fig. 7.1).

As the induction of B cell memory is dependent on a well organized lymphoid architecture it is likely that the non-IgA B memory cells detected in the lung were derived from extrapulmonary lymphoid tissue in view of the paucity of organized lymphoid tissue in the lung parenchyma.

7.5 Protective Role of Lung Responses

Protection against re-infection by influenza virus and other viral respiratory tract pathogens is mediated by neutralizing antibody within the respiratory tract - the predominant isotype involved being determined by the site of initial infection within the respiratory tract (Section 1.3.6.4). It has been previously assumed that influenza virus specific IgG in the respiratory tract is derived from serum. The data presented in this thesis shows that IgG ASCs may persist in the lung after infection providing a continual source of IgG in the respiratory tract. Furthermore protection against re-infection was shown to correlate with the presence of IgG ASCs in the lung rather than serum IgG. Protection also correlated with the level of B cell memory in the lung rather than the spleen. This correlation probably relates to the continual recruitment of

ASCs from the pool of B memory cells after primary infection. As it is necessary for antibody to be already present at the time of virus challenge to prevent infection, the activation of B memory cells at this point would not contribute to preventing infection. Furthermore, given the short incubation period, it is unlikely that ASCs, induced from B memory cells at the time of virus challenge, would significantly affect the onset of disease.

7.6 Effect of Antigen on Induction of B Cell Responses

The induction of ASCs was shown to be dose-dependent after both intravenous and intranasal inoculation. Interestingly however there appeared to be differences in the dose requirements for the generation of ASC responses compared to the generation of B cell memory. Over a range of priming doses with wild-type virus, ASC responses declined, whereas the subsequent level of B cell memory did not significantly differ. Similarly when viral replication was restricted after immunization with a high dose of ca-variant virus ASC responses were diminished whereas the level of B cell memory was comparable to that obtained with the parental virus. Furthermore, intranasal inoculation with a single dose of influenza-specific iscoms resulted in the generation of B cell memory in the absence of a significant ASC response whereas two inoculations induced both responses. These findings suggest that the generation

of B cell memory is more efficient, i.e. requires less antigenic mass, than the generation of ASCs.

There were also differences observed in the ability to generate B cell responses in different sites. ASCs were generated in the lung but were not detectable in the spleen after inoculation with low doses of virus or when viral replication in the lung was restricted e.g. during homotypic virus challenge and after immunization with ca-variant virus. Whereas ASCs were not detected in the spleen under these conditions, there appeared to be no effect on the generation of B cell memory. Again these results suggest that less antigen is required for the generation of B cell memory. Furthermore ASCs are generated more efficiently in the region of antigen deposition presumably because less antigen is subsequently delivered to distal sites. This is also supported by the differences in regional responses observed after inoculation with inactivated virus vaccine by either the intranasal or intra-peritoneal routes.

7.7 Implications for Vaccination

The conclusions drawn so far have important implications in the development of a successful vaccination strategy against influenza.

B cell responses in the respiratory tract, which have been shown to correlate with protection, are more

effectively induced by local immunization. Hence, an influenza vaccine should preferably be administered locally rather than parenterally.

Secondly, the degree of antigenic stimulation should be comparable to that of natural infection. In this regard it would appear that ca-variant viruses may induce adequate B cell responses when administered in two doses to unprimed subjects. Furthermore, it has been previously shown that ca-variant viruses effectively induce cross-reactive Tc cell responses, at least in murine studies (Mak et al., 1984). These findings suggest that attenuated virus vaccines would be suitable for immunizing unprimed subjects. There is no evidence to date however that ca-variant virus vaccines are more effective in adults, previously primed by different influenza strains, than inactivated virus vaccines. It may be that in the presence of cross-reacting antibodies to different subtype variants, attenuated viruses are less immunogenic. Enhancing immunogenicity by either decreasing the degree of attenuation or using higher doses of attenuated viruses would induce greater reactogenicity.

In Chapter 6 an alternate approach to immunization has been explored by enhancing the immunogenicity of influenza virus proteins. B cell responses and surprisingly Tc cell responses were enhanced by incorporating the influenza virus HA and NA into iscoms. Further work on this approach has shown that the influenza virus NP can also be incorporated

into iscoms (Morein, B, personal communication). Work is in progress to determine if iscoms containing the HA, NA as well as NP will induce even greater Tc cell responses.

7.8 Implications for Other Viral Respiratory Pathogens

Although the broad conclusions presented in this chapter are based on data pertaining only to influenza virus, the methodology especially the ELISA-plaque assay, and the experimental approaches taken to provide these data should be applicable to other viral respiratory pathogens as well. At present there is no comparable data on the development of ASCs or B cell memory in the lung after infection with any of the other respiratory viruses. Although it is likely that the conclusions drawn in the influenza model would be applicable to other respiratory viruses, it would be important to establish the relative roles of local and systemic immune responses in different infections where the level of viral replication within the respiratory tract differs. Assuming that effective vaccines will be available in the future, as is almost the case for RSV, the most effective method of vaccination should be chosen based on knowledge of the nature of regional immune responses.

TABLE 7.1 ASC Responses in Murine Influenza

ASC responses following:	Lung			Spleen		
	IgM	IgG	IgA	IgM	IgG	IgA
Primary Inoculation	+++	++	++	+++	++	-
Secondary Inoculation						
(a) Homotypic challenge	+	++	++	-	-	-
(b) Heterotypic challenge						
(i) Type specific ASCs	+	++	++	+	++	-
(ii) Subtype specific ASCs to priming virus	-	-	-	-	-	-

TABLE 7.2 B Cell Memory in Murine Influenza

ASCs generated in vitro following:	Lung			Spleen		
	IgM	IgG	IgA	IgM	IgG	IgA
Primary Inoculation	++	++	++	+++	+++	+
Secondary Inoculation						
(a) Homotypic challenge	++	++	++	++	++	-
(b) Heterotypic challenge						
(i) Type specific ASCs	-	+	+	-	-	-
(ii) Subtype specific ASCs to priming virus	-	-	-	-	-	-

FIG. 7.1 Structural and functional divisions of the respiratory tract.

STRUCTURAL and FUNCTIONAL DIVISIONS

of the RESPIRATORY TRACT

	LYMPHOID TISSUE	MAJOR Ig ISOTYPE
MUCOSAL	• Bronchus-associated lymphoid tissue (BALT) • Submucosal lymphoid aggregates	IgA
	Broncho-alveolar Junction	
PARENCHYMAL	• Lymphocytes and macrophages scattered throughout interstitium and alveolar spaces	IgG

APPENDIX

IMMUNOHISTOCHEMICAL LOCALIZATION OF B CELL RESPONSES IN INFLUENZA-INFECTED LUNGS

Although specific immuno-competent cells can be detected in lung cell suspensions obtained from virus infected mice, the precise location of the local immune responses cannot be determined by this approach. Localization of antibody-containing cells by immuno-fluorescence in infected lungs (Cassell et al., 1974) has provided very limited data which has not answered the question as to where the local B cell responses detected in the lung are generated. This question could be addressed more precisely if the localization of the cells involved in the induction and expression of B cell responses, namely APCs, Th cells and B cells, in the lung could be determined during the course of a viral infection. Using an immuno-histochemical technique, described previously for the localization of class II antigens in murine tissues (Hume, 1985) (see Section 2.13), an attempt was made to characterize the expression of viral antigens, class II antigens and surface markers on pulmonary macrophages, Th cells and B cells in influenza infected lungs. This method was chosen because of improved antigen preservation during fixing and embedding and enhanced sensitivity using the avidin-biotin-peroxidase complex (ABC).

In normal uninfected lungs, non-specific staining was negligible and specific antigens could be satisfactorily identified. In contrast, there was extensive non-specific staining in the absence of a primary antibody, in influenza infected lungs, especially in the bronchial epithelium, at

various times after intranasal inoculation. Non-specific staining developed only when the ABC was included in the assay irrespective of whether the biotinylated secondary antibody was used indicating that the ABC was binding non-specifically to probably virus damaged epithelium.

The non-specific binding of ABC may have been related to either of the following mechanisms:

(i) Avidin is a basic glycoprotein with a high isoelectric point (pHl0) which may bind non-specifically by electro-static interactions at neutral pH and physiologic ionic concentrations (Guesdon et al., 1979).

(ii) Avidin may bind to endogenous biotin which is an important co-enzyme for transcarboxylation and is widely distributed in mammalian tissues (Hsu et al., 1981).

In order to minimize the extent of background staining due to these mechanisms several modifications to the assay were tried, including:

(i) Buffering the ABC solution in a high salt alkaline buffer e.g.

carbonate/bicarbonate buffer, pH 9.4

0.2M glycine/0.2M NaOH/0.5M NaCl, pH 9.0

(ii) Pre-treating the sections with a synthetic basic polypeptide e.g.

poly-L-Lysine 0.1% (Bussolati & Gugliotta, 1983).

(iii) Blocking binding to endogenous biotin by prior incubation with excess avidin; the remaining biotin-binding sites on the avidin being blocked by incubation with excess biotin effectively closing the endogenous system to further interaction (Wood & Warnke, 1981).

Although background staining was reduced to some extent with these modifications, the level of background staining remained too high, even when combining these modifications, to allow accurate definition of specific staining. It is possible that the non-specific binding of ABC to the respiratory epithelium is similar to the binding of avidin to cell surface receptors on liver cells which are unrelated to the biotin receptor (Chalifour & Dakshinamurti, 1982). The possibility that avidin was binding to viral proteins expressed on the infected cell surface was not supported by the observation that ABC did not bind to P815 cells expressing the HA glycoprotein (determined by haemabsorption).

In view of the great difficulties encountered in obtaining satisfactory definition of specific staining patterns, it was not possible to proceed with this approach using this technique.

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