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THE ANAPHYLACTIC IN PLAQUE-FORMING-CELL RESPONSE IN RATS SENSITIZED BY INHALATION OR INTRAPERITONEAL INJECTION OF OVALBUMIN

A Dissertation

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ABSTRACT

Rats were immunized with ovalbumin either by intraperitoneal injection (systemic) or by inhaling an ovalbumin aerosol (topical). The number of plaque forming cells (PFC) in the spleen, respiratory tract (RT) lymphnodes and lung lavage fluid was determined by direct (IgM) and indirect (IgG) plaque assays. Serum IgE was determined by passive cutaneous anaphylaxis (PCA) assay. Levels of serum hemagglutinins were determined by hemagglutinin microtitration against ovalbumin adsorbed to sheep erythrocytes. Immunization was achieved both with and without B. pertussis as adjuvant.

Administration of ovalbumin without adjuvant, regardless of whether exposure was by inhalation or by i.p. injection, was relatively ineffective in producing an immune response to ovalbumin. A minimum of five antigen treatments and an elapsed time of at least two weeks after the first antigen treatment were necessary to induce detectable serum hemagglutination titers. Maximum hemagglutination titers of 1:1280 were observed only after 13 antigen treatments and an elapsed time of 25 days.

When antigen was administered with B. pertussis as an adjuvant, serum hemagglutinins were detectable after either a single inhalation exposure or a single i.p. injection of antigen. Intraperitoneal administration of antigen induced an initially strong IgM response and a comparatively weaker but longer lasting IgG response in the spleen. Respiratory tract tissue contained only a minimal number of both IgM and IgG antibody producing cells. Conversely, after a single inhalation

exposure to antigen, RT tissue responded initially with a strong IgM reaction, which was subsequently followed two days later by an even stronger IgG response, but the number of both IgM and IgG immunoglobulin producing cells from the spleen was barely detectable. In contrast, a second antigen treatment four weeks after initial sensitization considerably enhanced the PFC response in both the spleen and RT tissue regardless of whether antigen administration was topical or systemic. Each site had earlier and enhanced production of both IgM and IgG antibodies. Production of IgG was enhanced eight to tenfold and was quantitatively higher than that of IgM which was increased two to threefold. Immunoglobulin E was found equally in RT tissue and in serum regardless of the mode (topical or systemic) of antigen administration.

The effect of splenectomy two weeks prior to sensitization essentially abolished the primary immune response. Splenectomy two weeks after primary sensitization greatly reduced but did not abolish the secondary immune response.

PREFACE

Under normal conditions an organism's first line of defense against potentially harmful agents is the protective barrier that the epidermis (and all its variations) provides. As protection against noxious agents that manage to transgress the epidermis, the immune response offers a second line of defense and is critical to the defense of the host against potentially harmful antigenic substances. There is substantive experimental evidence indicating that lymphocytes (both B and T lymphocytes) and their various functional roles are at the center of the immune response. There is also ample evidence that the functional role(s) of lymphocytes are, at least in part, regulated or enhanced by interaction with other cells, most probably macrophages.

Air that we breathe is laden with foreign particles, including immunogenic (i.e. pollen, bacteria, dander, etc.), that are inhaled and subsequently deposited in the respiratory tract. The fate of inhaled particles, including the immunogins, deposited in the respiratory tract depends on the site of deposition within the respiratory tract. The epithelial lining of the lung, because of its unique function in allowing for the exchange of gases, cannot function as an effective barrier to particles deposited in the lower respiratory tract. Instead, those particles deposited in the lower respiratory tract (i.e. alveolar region) are phagocytized by macrophages and removed from the lung. The majority of phagocytized particles are simply cleared from the lung to the gastrointestinal tract via the tracheobronchial tree.

Allergies, which are a form of immune response, are easily manifested by inhaled antigenic particles deposited in the lung. Since the systemic immune response is regulated in part by macrophages, it is logical to postulate that immune responses within the respiratory tract are mediated by macrophages which have ingested antigenic particles deposited in the respiratory tract. This concept is further developed and elaborated in the introduction. In addition, some principles of respiratory mechanics plus factors governing deposition and clearance of particles in the respiratory tract are discussed as they relate to immune responses in the respiratory tract.

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I. INTRODUCTION

All living systems must have a mechanism(s) of identifying and resisting agents which can be harmful to their health and survival. The major mechanism that enables mammals to respond to these agents is the immune system which must quickly recognize, "attack," and effectively destroy or otherwise neutralize foreign matter that enters the body. The immune system is complex and functionally dependent upon a wide range of highly specialized organs, cells and the substances they produce. The main organs involved in immunity are composed of lymphoid tissue (bone marrow, spleen, thymus, etc.) capable of producing different classes of lymphocytes, which are at the center of the immune response. As such, an understanding of lymphocyte function is at the center of understanding the major mechanisms of the immune system. The immune response is not only a defense mechanism against disease, but is also recognized as a cause of some, as in allergies and the auto-immune diseases.

The respiratory tract has a multitude of defense mechanisms to cope with inhaled material that deposit in the lung. One of these mechanisms is the immune response. As such, the immune response is critical to the defense of the host against potentially harmful antigenic materials deposited into the respiratory tract. There is evidence that these immunological mechanisms may contribute to the pathogenesis of pulmonary diseases by tissue-damaging reaction of hypersensitivity. Although much is understood about the fundamentals of immune responses in the

systemic lymphoid tissue, little is known about the mechanisms by which both the humoral and cellular immune phenomena are expressed in the respiratory tract. From current evidence it is not clear whether the respiratory tract functions autonomously as an immunologically competent organ, whether there is immunological cooperation between the lung and the systemic immunologically competent organs, or whether the lung is immunologically passive, responding only to systemically generated immune reactions.

This study describes a number of experiments designed to examine the immune response of the respiratory tract of specific-pathogen-free (SPF) rats following either inhalation or systemic exposure to antigens. The primary objectives of these experiments were threefold: (1) to determine whether antigens deposited in the respiratory tract of SPF rats effectively trigger an immune response; (2) to locate the site of this immune response; and (3) where the stimuli are for the control and production of responsible antibodies.

Fundamental Concepts

During the past 40 years, intensive research efforts on the mechanisms of induction and expression of immune responses have generated an enormous amount of primary data (Wakeman, 1971). The vast majority of these data has been derived from research efforts concerned with immune phenomena as manifested in organized systemic lymphoid tissue such as the spleen and lymph nodes. These data have shown that the immunological apparatus, when properly stimulated, may be divided

into two principle effector systems: the antibody-mediated, or humoral response, and the cell-mediated response. Cell-mediated immunity plays a critical role in mechanisms such as homograft rejection, while humoral antibody predominates in common acute bacterial infections.

Lymphatic tissue is responsible for expression of both types of immune reaction. There are two major subpopulations of lymphocytes which can be distinguished on the basis of their mode of differentiation, surface membrane characteristics, functional properties and whose biological functions parallel those of the two effector systems (Fig. 1). Bone marrow derived lymphocytes (B-cells) differentiate in the bursa of Fabricius in avian species or the "bursa equivalent" in mammals, concentrate in lymphoid follicles of lymph nodes and spleen and serve as precursors for antibody forming cells that effect humoral immunity. Thymus dependent lymphocytes (T-cells) which also arise from stem cells within the bone marrow, differentiate in or under the influence of the thymus gland. These lymphocytes populate the periarterial lymphatic sheath in the spleen and interfollicular regions of lymph nodes and carry out the functions characteristic of a cell-mediated immunity system. Upon antigenic stimulation, B-cells which are relatively sessile and migrate slowly within lymphatic tissues differentiate into plasma cells <u>in situ</u> and synthesize and begin to secrete soluble antibody that is disseminated via the circulatory system and mucosal secretions. In contrast, T-cells are a relatively mobile population of lymphocytes that continuously recirculate. T-cells migrate from the peripheral blood through venules into secondary

Figure 1. Diagrammatic representation of the differentiation, maturation, proliferation and effector functions of bone marrow derived immunocompetent cells. After antigenic stimulation macrophages and/or thymus dependent lymphocytes effect cell-mediated immune functions and "Bursa" dependent lymphocytes carry out humoral immune functions.

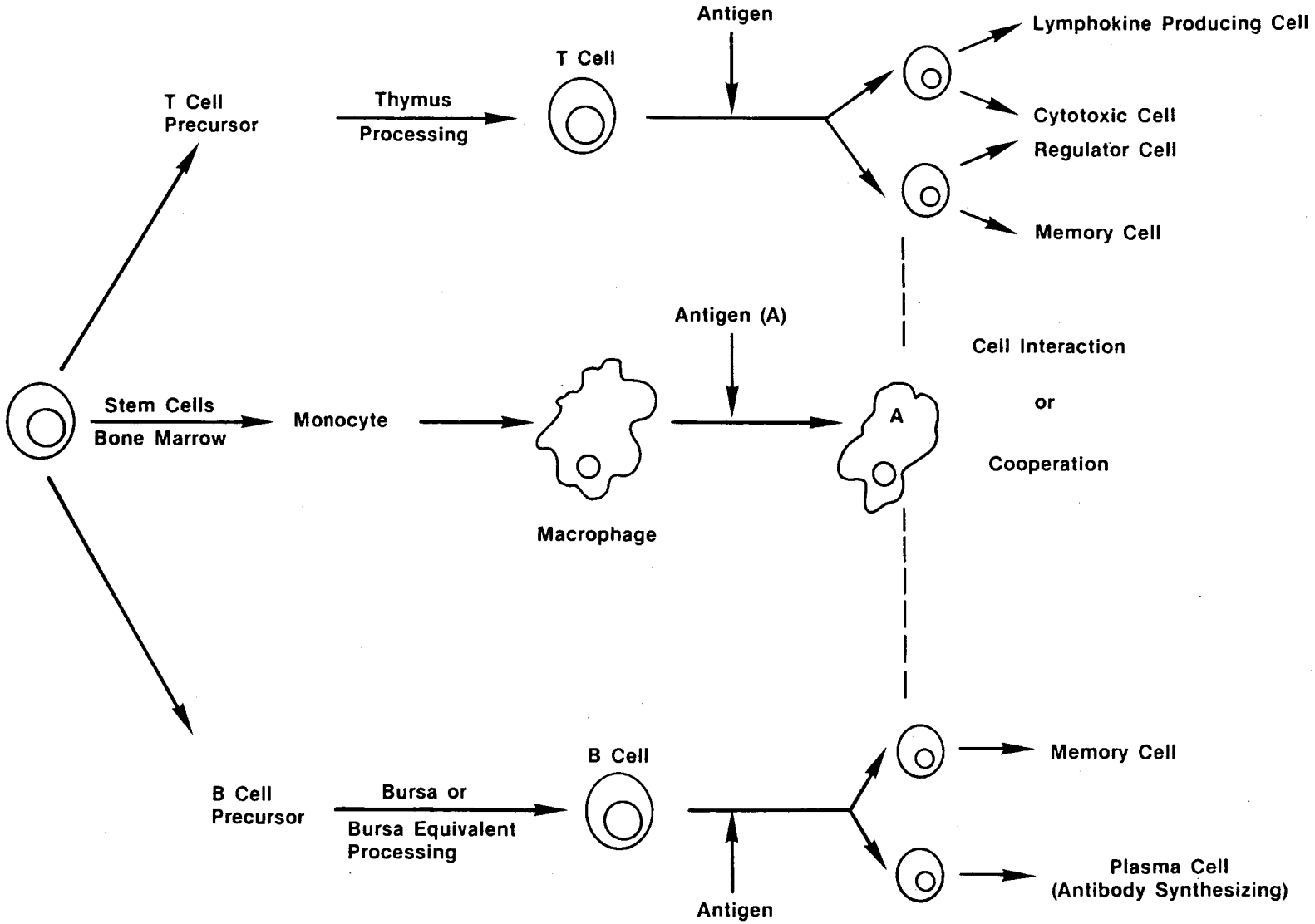
Antigen
Lymphokine Producing Cell
T Cell
T Cell
Precursor
Thymus
Processing
Cytotoxic Cell
Regulator Cell
Memory Cell
Antigen (A)
Cell Interaction
or
Cooperation
Stem Cells
Bone Marrow
Monocyte
A
Macrophage
B Cell
B Cell
Precursor
Bursa or
Bursa Equivalent
Processing
Memory Cell
Plasma Cell
(Antibody Synthesizing)
Antigen

Figure 1.

lymphatic tissues (lymph nodes, spleen, respiratory and gut submucosa) where they remain for variable periods before entering efferent lymphatic vessels such as the thoracic duct and eventually peripheral blood (Gowans and Knight, 1964). The recirculation of lymphocytes theoretically provides a pathway for the migration of sensitized T-cells from lymphatic tissue to the site of antigen depositions (i.e. tissue grafts).

Although the development of immunity is initiated by a reaction of an antigen with lymphoid tissue of the host, it remains unclear how an immunizing dose of antigen can trigger the observed changes in the lymphocytes encountered within the lymphoid tissue, and how events at the surface of a cell alter that cell's genetic expression. It has been suggested that antigen elicited sensitization processes are dependent either upon uptake of the antigen by macrophages, and/or antigen contact with T-lymphocytes (Fig. 2). The processed antigen is then able to react with lymphocytes known as antibody-producing cells of B-lymphocytes (bone marrow-derived lymphocytes), which have a complimentary combining site for the antigen on their surface (Raff, 1970; Singhal and Wiqzell, 1971). Contact of the processed antigen with B-lymphocytes alters the genetic expression of these cells, setting in motion a characteristic series of events during which the cell divides and differentiates along at least two lines: (1) short-lived plasma cells that produce antibody, and (2) committed long-lived small lymphocytes which are the carriers of immunological memory and which serve as mediators of a delayed hypersensitivity reaction (Gowans and Uhr, 1966). Therefore, following appropriate administration, the

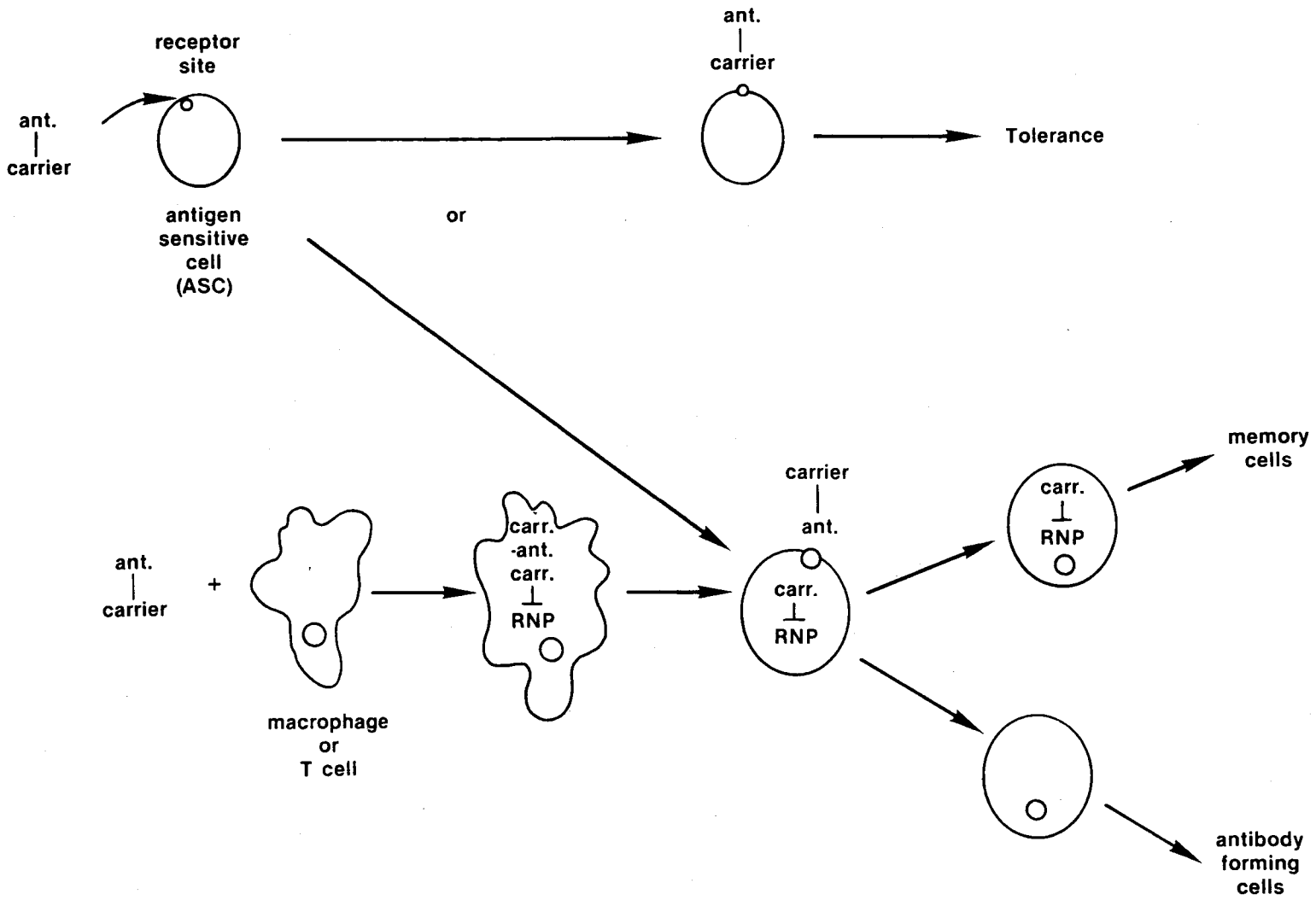

Figure 2. Diagrammatic representation of the maturation and proliferation of B-cells to serum antibody producing cells and the pathway sites and which T-cells and/or macrophages are active.

identical antigen can induce antibody formation, cellular immunity, and immunological memory (Leskowitz, 1967; Humpherey, 1965).

In summary, the cellular events outlined above can be characterized as follows: The inductive or latent phase which refers to the intervals between the introduction of antigen and the appearance of committed cells. At this stage the antigen is cleared from the circulation, becomes localized in lymph nodes, and may have been processed by certain cells. Subsequently, the proliferative/productive phase of the immune response occurs, which is characterized by rapid cell division. These cells then differentiate to form a population of lymphocytes committed either as effectors or delayed hypersensitivity and memory cells, or as immunoglobulin producing cells.

Immunoglobulin Sequence

Under appropriate conditions, two main, distinct classes of antibodies are sequentially formed following primary immunization with a specific antigen (Stelos et al., 1961; Svehag and Mandel, 1964). The first is a 19S type antibody known as immunoglobulin M(IgM), in turn followed by a 7S type antibody known as immunoglobulin G(IgG). The extent and duration of synthesis of these two classes of immunoglobulins varies depending upon the type of antigen, its mode of introduction, dosage and other factors (Bauer et al., 1963; Benedict et al., 1962; Fink et al., 1962; Stelos and Taliaferro, 1959; Uhr and Finkelstein, 1963). The 19S antibody is generally transient and is followed by the 7S type 2-3 weeks later, the latter type being the principal class of

specific antibody produced. A rise in the 7S immunoglobulin titer occurs concomitant with a fall in 19S antibody titer. These observations suggest that either different cells produce the two different species of antibody or that the same cell sequentially produces the 19S antibody first and then converts to the synthesis of the 7S antibody (Sahair and Schwartz, 1965).

In an attempt to elicit the mechanisms of the sequential immunoglobulin production, Sahair, et al. (1965) used the antimetabolite, 6-mercaptopurine (6-MP) to separate the process of 19S antibody synthesis from that of 7S formation. This agent is a powerful immunosuppressant having an especially potent action against early antibody synthesis. A subinhibitory dose of 6-MP was used to block the full maturation of the primary immune response to bovine gamma-globulin in rabbits. In animals thus treated the ordinarily transient 19S phase of antibody synthesis was prolonged, while the 7S phase of the response was incompletely developed. Prolongation of 19S production led them to conclude that 19S synthesis is controlled, in part, by the 7S antibody.

Production and Regulation of Antibodies

The production of antibodies can be regulated in a number of ways. One mechanism could involve the control of production, maturation or differentiation of antibody producing cells, while another could be to maintain a constant number of antibody producing cells but regulate the synthesis of antibodies. Experimental evidence appears to indicate that,

in vivo, the effective regulation of antibody production is accomplished by controlling both the maturation of antibody producing cells and regulation of antibody synthesis within these immunocompetent cells. Evidence in the literature indicates that the differentiation of B-cells into specific antibody producing cells is regulated, at least in part, by both T-cells and macrophages (M-cells) and that the regulation of antibody synthesis is accomplished by feedback inhibition of circulating antibodies.

The development and regulation of humoral immunity, including the maturation of B-cells to serum antibody producing cells and the pathway sites at which T- and M-cell factors are active is schematically presented in Figures 1 and 2. The major concepts embodied in these schematics are a constant migration of immature B-cells from the bone marrow to the spleen. These immunocompetent virgin B-cells have immunoglobulin M (IgM) class receptors (Strober, 1975). At this early stage, binding of antigen to receptor is insufficient for stimulation and a second signal is needed from an accessory cell (T- or M-cell). If the IgM-bearing B-cell is stimulated by both antigen and either T- or M-cell factors, synthesis of either immunoglobulin G (IgG), A (IgA) or E (IgE) is initiated. Whether the stimulated B-cell synthesizes IgG, IgA, or IgE probably depends on the site (circulating, Peyers patches, other lymphoid tissue, etc.) to which the B-cell has migrated from the spleen. The B-cells from each site form clones of cells which have a single homogeneous immunoglobulin on (in) the cell membrane which acts as an antigen specific receptor.

With the correct stimulus, these cells can differentiate into plasma cells that will secrete an immunoglobulin of identical specificity. Each sequential step leading up to the migration of B-cells out of the spleen is an antigen independent event (Strober, 1975; Vittetta and Uhr, 1975). After migration of B-cells from the spleen, their maturation or differentiation is not only antigen dependent but also greatly enhanced by T- and/or M-cell factors. Figure 1, page 4, which also incorporates the concept of tolerance, depicts the interaction of antigen, T- and M-cells with B-cells to produce antibody-secreting plasma cells.

Three distinct cell types, based on density gradient studies, are presumed to be involved in the primary antibody response in vitro (Haskill et al., 1970). These are: (1) a radiation resistant cell, probably a macrophage, (2) a radiation sensitive cell of higher density and (3) the antibody-forming cell precursor. Shortman et al. (1970) has carried out studies in the mouse which extend these observations and further point out the differential response of macrophages to different antigens.

In contrast, no such accessory cell appeared to be required for the response to polymerized flagellin (POF). Thus, such explanations that the accessory function, supplied by M- or T-cells, needed when sheep red blood cells (SRBC) are the antigen is some sort of nutrient or energy production by the M- or T-cell would appear to be excluded in view of the good response of lymphocytes to POF in the absence of the M-cells. Diener et al. (1970) have also noted that M-cells could be

inhibitory to the response to POF. These observations on POF are consistent with the results of Ada and Byrt (1969) in which it appears that POF antigen interacts directly with the lymphocyte surface. Interestingly, in the CBA mouse the response to POF is not thymus dependent (Armstrong et al., 1970). Shortman and Palmer (1971), in confirming the requirement of an accessory cell for an in vitro response of lymphocytes to SRBC, showed that the accessory cell could be blocked by antimacrophage antiserum. The antimacrophage antiserum did not prevent a response to POF. Further, it was shown that peritoneal macrophages function by releasing nonsedimentable antigenic material from sheep cells, and that this released material could initiate a response by direct interaction with lymphocytes. Macrophages were also capable of releasing a host of chemically undefined factors into the medium and these factors could release small sized antigenic material from whole red cells. A relevant but not yet independently verified observation in the same paper is that antigen size is indeed the essential factor in the inductions of antibody to SRBC and rendering SRBC smaller by mechanical procedures eliminates the macrophage requirement.

An observation of potential significance is that of Kolich (1970) which indicated that labelled M-cells injected intraperitoneally into mice accumulate rapidly and preferentially in the thymus. This would suggest that macrophage-borne antigen could in this way be transported to thymus-derived lymphocytes which were postulated to be the lymphocytes which first make contact with antibody forming cells. Kettermann and

Dutton (1971) have demonstrated the requirement for a thymus-derived lymphocyte in the response to SRBC conjugated to a TNP hapten. These investigators have shown that the number of anti-TNP plaques that arise after antigenic stimulation in vitro is greatly enhanced by prior immunization in vivo with carrier SRBC. The enhancement is antigen specific. Spleen cells from carrier-primed mice will enhance the anti-TNP response of normal cells and this enhancing effect is not abolished by exposure to 1000 R but it could be inhibited by anti-0 serum and complement. They suggest that the carrier primed cell is a thymus-derived cell, but its extreme radioresistance is suggestive of a macrophage. Katz et al. (1970) have demonstrated enhanced recognition for bovine gammaglobulin (BGG) attributed to a radioresistant cell. However, one should bear in mind that the thymus contains both T- and M-cells (Kolsch, 1970; Kostowiecki, 1963). Similar conclusions arrived at from other in vitro studies also suggest that three cell types must interact to give a successful antibody response against SRBC (Mosier, 1969; Mosier and Coppleson, 1968; Talmage et al., 1970); namely, a glass adherent cell (presumably a macrophage) and lymphocytes separately derived from thymus and bone marrow. When the nonadherent cell population was injected into X-irradiated mice, a strong immune response was noted, but these same cells were nonresponsive in vitro (Talmage et al., 1969a; Talmage et al., 1969b). A possible explanation is that the radioresistant macrophage survived in the irradiated mouse and was probably still functional.

Kennedy et al. (1970) have shown that in the mouse bone marrow cells and peritoneal exudate (PE) cells interact optimally in a 2:1

ratio in irradiated mice, producing a synergistic immune response to foreign red cell antigens (Kennedy et al., 1970). It was noted that comparable numbers of PE cells and thoracic duct lymphocytes produced comparable levels of synergistic immune response with marrow cells, in contradistinction to thymocytes alone which were only 2% as effective. Moreover, a synergistic immune response would occur if PE cells were replaced by an aqueous extract prepared by heating such cells (Kennedy et al., 1970).

The evidence is overwhelming that the maximum in vivo immune response (i.e. antibody production) is elicited by cooperation of B-, M- and T-cells. This does not preclude the responsiveness of each individual cell type to an antigen, but only implies that the maximum response is achieved by involvement of all three cell types. Strong circumstantial evidence exists that either T- or M-cells are a subpopulation of the other. However, this evidence may be artifactual in that the cells used in many of the studies could be inadequately defined. In the same light, there is good direct evidence that B-cells are a separate line of cells from either T- or M-cells (Moller and Coutinho, 1975).

It is postulated that the contact of native antigen, through its carrier determinants, with an antigen-sensitive cell (ASC) results in tolerance. This view of tolerance is conceptual rather than experimental. Regardless, the concept is consistent with observations (Ada and Parish, 1968) that a molecular fragment (A of flagellin) that remained bound to the surface of a reticular cell was responsible for maintenance of

tolerance. There is also evidence (Paley et al., 1975) that administration of tolerogens, through a cross-linking of cell surface receptor suppresses proliferation of IgG producing cells. Interaction of the ASC with antigen must be followed by presentation of fragmented or processed carrier determinants associated with a ribonucleoprotein (RNP) molecule for active immunity to occur (MacKaskil, unpublished data). Speculating, one view might be that processed antigens are antigenic fragments associated with the RNP molecule and under these conditions, proliferation of the respective clone of B-cells occurs. The RNP molecules bearing carrier determinants will be diluted out in the cell progeny and those cells lacking RNP will become antibody forming plasma cells. Cells which retain the RNP moiety would be responsible for immunological memory and may also be effector lymphocytes for responses of delayed hypersensitivity. In the secondary response, the memory cell would again be triggered to proliferate, but this could be accomplished by interaction of antigen alone bypassing the antigen processing step. The hypothesis permits an ASC to be either a T- or a B-cell. If the ASC is a maturing B-cell, the antigen would be presented to it either in free form or attached to the surface of a T-cell or macrophage.

In the same study referred to earlier, Kennedy et al., (1970) noted that an amplified response to foreign red cell antigens would occur if peritoneal exudate cells were added to mouse bone marrow cells. It is "presumed" that the active cells in the peritoneal exudate were thymus-derived T-cells. They also found that a supernatant factor from gently heated sensitized peritoneal cells could substitute for the peritoneal

cells themselves. Somewhat similar effect was observed (Hoffman and Dutton, 1971) in that the depression of the in vitro immune response of mouse spleen cells seen after removal of macrophages from such suspensions could be reversed by addition of a supernatant factor from peritoneal exudate cultures. This supernatant activity could be adsorbed by the red cell antigen. In addition, Bach et al. (1970) noted that a supernatant factor from adherent cells is required for proliferation of cells in a mixed lymphocyte reaction. Kishimoto et al. (1975) also presented data which indicates that there are two factors that induce B-cell proliferation and antibody synthesis. His results suggest that these two signals are first, cross linkage of IgG receptors by antigen and second, a B-cell response to soluble factor. Cross linkage of receptors on B-cells induces the differentiation of B-cells to a stage responsive to soluble factor and these differentiated cells proliferate and mature to antibody-forming cells under the influence of soluble factor.

A similar two-event model for triggering of B-lymphocytes has been postulated by Vittetta and Uhr (1975). They postulate that interaction of antibody-antigen on the cell surface exposes a site that allows for proteolytic cleavage of the antibody, further exposing a site for binding of a membrane protein. It is easy to modify this model wherein the membrane protein receptor site becomes the site for soluble factor reception. This model is in accord with the schematic representation in Figure 1, pages 4 and 5, in which cells bearing IgM receptor sites are induced to differentiate into antibody secreting cells by either an M- or T-cell.

There is also considerable evidence that macrophages, in addition to their role in the induction of immunity to a given antigen, act in a nonspecific way to suppress the development of the immune response. Caution should, therefore, be applied in the interpretation of experiments which claim to show either a macrophage-associated suppression or enhancement of the immune response to an antigen. A nonspecific suppressive effect of macrophages occurs when they are mixed with nearly equal numbers of lymph node cells. As the ratio of macrophages to lymphocytes in the in vivo lymph node or spleen is considerably less than one, some of the inhibitory activities of the M-cells may be related to such nonspecific suppression. Nevertheless, Harris (1965) has noted that when spleen cells from an immunized rabbit were incubated in the presence of M-cells from either an immunized or a nonimmunized animal, a high macrophage to lymphocyte ratio resulted in suppression of the ability of spleen cells to incorporate labelled thymidine into DNA. Similar results were noted regardless of whether or not the M-cells had been incubated with antigen. When the ratio of M-cells to lymphocytes was 1:10 or 1:100 a significant stimulation of DNA synthesis was observed. The same report noted that lymphocytes which clustered around M-cells did not divide, whereas lymphocytes which were not in contact with M-cells were found to divide actively. Similar observations were noted by Parkhouse and Dutton (1966) using sensitized and nonsensitized spleen cells in combination with different doses of peritoneal cells. Disrupted cells or culture supernatants of peritoneal cells were unable to cause suppression. Moreover, cells separated by a dialysis membrane were

ineffective, thus providing evidence that inhibition requires cell-cell interaction. However, two nondialyzable factor(s) could account for this data also. This observed phenomenon of cell-cell suppression might explain some of the contradictory data on the effect of M-cells on immune responses. M-cells derived from mouse spleen inhibited the formation of plaque forming cells (Diener et al., 1970). Mixing 10^7 peritoneal cells with 2×11^7 spleen cells in the presence of antigen led to inhibition of antibody-forming cells when compared to the response of spleen cell cultures exposed to free antigen alone (Diener et al., 1970). Using the incorporation of labelled thymidine as an index of cell proliferation, Dutton and Eady (1964) showed that the suppression of lymphocyte proliferation is reversible with time. Since the 24-48 hour depression of DNA synthesis in lymphoid cells exposed to antigen was then followed by a pronounced rise in thymadine incorporation, this lag period could have been caused by residual M-cells. If so, such a depression would be self limiting since lymphocytes activated by antigens would eventually exceed the number of macrophages in the culture and thereby abrogate the suppressive response. In contrast, Hersh and Harris (1968) have made the important observation that the blastogenic response of primed lymphocytes to antigen is prevented by the removal of M-cells from the leukocyte suspension before culturing with antigen. The blastogenic response to antigen was restored when column-purified lymphocytes were cultured with anitgen in the presence of macrophages. Cell-to-cell contact of the macrophage-lymphocyte type was required for the blastogenic response of lymphocytes to antigen

in vitro. Inhibition of DNA synthesis was not seen even at high macrophage to lymphocyte ratios. Harris (1967) also showed that macrophages from a tolerant rabbit took up the tolerizing antigen and stimulated a specific response in the DNA synthesis of spleen cells from an immunized animal.

Other studies (Sulitzeanu et al., 1971) have noted the clustering of lymphocytes around macrophages and large blastoid-type cells in antigen stimulated *in vitro* cultures. Auto-radiography of such clusters showed that only an occasional macrophage cluster had one or more labelled blast-like cells at the periphery. Both M-cells and the central type of blastoid-cell contained antigen (^{125}I BSA) but, unfortunately, no data was collected on how many cells around the central blast-cell were synthesizing DNA. It was suggested that antigen laden macrophages have a tactic effect leading to the production of macrophage clusters and then to the activation of antigen-reactive cells. These macrophage clusters might be related to the primary response and the central blast-like cell might be an activated memory cell that influences other clustering cells to produce antibodies. On the basis of these observations, enhancement and suppression of DNA synthesis in lymphocytes by macrophages are two aspects of a dose-response curve. Thus, a possible function of M-cells would be the modulation of immune responses leading to predominately delayed sensitivity in preference to humoral antibody response. The phenomenon of suppression suggests that macrophages may conceivably control the multiplication of cells following a stimulus, thereby regulating the formation of primed or memory cells.

Moreover, activated M-cells might nonspecifically depress the development of the humoral antibody response so as to insure the production of sufficient "fragmented" or "processed" antigen to induce delayed hypersensitivity. This bears out the dilemma described by Uhr et al. (1971) "that two months after primary antigen contact . . . considering the animal a test tube, the ratio of antibody to antigen is at least 10^7. Yet these handful of immunogenic molecules apparently can detect the differences between a ratio of 10^8 to 10^7 and stimulate antibody synthesis." As further evidence, Cerney et al. (1975) have presented data claiming two soluble factors derived from T-cells which activate M-cells. One of these T-cell factors enhances DNA synthesis while the other stimulated only antibody synthesis.

The phenomenon of suppression described above must be distinguished from suppression of the immune response by antibody. A study by Pierce (1969) has demonstrated that passively administered antibody decreases the in vitro response to SRBC by neutralizing the antigenic stimulus at the macrophage dependent phase of the response. Macrophages incubated with anti-SRBC antibody before or after exposure to SRBC were not capable of functioning in a reconstituted macrophage-lymphocyte response. Ryder and Schwartz (1969) carried out similar studies and concluded that passively administered antibody has an effect at the level of the macrophage, possibly on the processing step. They also indicated that memory cells were unaffected by the block that antibody places on unprimed cells because the memory cells contain a high affinity antibody-like receptor on its surface that can compete with circulating antibody for antigen.

An extremely convincing study on the regulation of antibody production by feedback inhibition of circulating antibodies has been reported by Uhr et al. (1971) who showed that when antibodies were passively (i.e. without antigen stimulation) administered to rabbits there was a decline in circulating antibodies to preexisting titers. In addition, if circulating antibodies were removed from a rabbit, there was an immediate increase in antibody production that raised the level of circulating antibodies to preexisting levels.

Experimental evidence indicates that regulation of humoral antibodies occurs through both the control of B-cell proliferation and B-cell synthesis of antibody. Early events in B-cell differentiation are probably antigen independent. Later, nonspecific antigen stimulation is responsible for the final events in B-cell differentiation. Macrophages and/or T-cells control, at least in part, the antigen dependent event(s) which are mediated through a soluble factor. In all likelihood an accessory cell (macrophage?) governs whether in differentiation a B-cell may be stimulated to produce antibodies or become a "tolerant" cell. M-cells can also suppress antibody production apparently by a tactic stimulus. Conceivably, this could result from interaction of M- and B-cell surface receptors thereby effectively preventing cross linking of IgG immunoglobulins by antigens. Finally, the level of circulating antibodies is maintained by feedback inhibition to the antibody producing cell by the circulating antibody.

These fundamental concepts assume that there is an interaction of antigen and lymphocyte, antigen and antibody and that the antigen-antibody

complex is with lymphocytes. These assumptions presuppose the ability of antigen material to somehow gain access to the interior of the organisms. Under normal conditions an organism's first line of defense against potentially harmful agents is the protective barrier that the epidermis (and all its variations) provides. However, because of the unique function of the respiratory tract, the epithelial lining of the lung cannot be an effective barrier against noxious agents. Therefore, other first line defense mechanisms have evolved against inhaled noxious agents that are deposited on the mucosal surfaces of the lung. These mechanisms are varied, and depend primarily on the biological, physical, chemical and other characteristics of the deposited material.

Respiratory Tract Immunoglobulins

IgM

IgM antibody is capable of compliment fixation and lysis of RBC and is mostly found in the circulatory system. Because of its relatively high molecular weight, little IgM diffuses out of vascular fluids. Although IgM increases in the respiratory tract (RT) during acute inflammation of RT tissue (Masson and Heremans, 1973), this finding could be the result of increased permeability of RT tissue induced by inflammation. However, other researchers (Hauptman and Tomasi, 1978) noting the presence of J-chain of IgM found in saliva argue for either local synthesis or selective transports in the respiratory tract. IgM antibody producing cells have been obtained by lavage from the lungs of both rabbits and dogs (Ford and Kuhn, 1973; Holub and Hauser, 1969;

Kaltreider et al., 1974). However, the act of introducing the lavage fluid into the respiratory tract could cause physical damage and allow extrarespiratory tract cells to cross the alveolar walls.

IgG

IgG is not the predominant antibody in the RT, yet substantial quantities are present in secretions from both the upper and lower RT (Kaltreider and Chan, 1978; Martinez-Tello et al., 1968; Fernald et al., 1972). Respiratory tract IgG is thought to be synthesized locally (Martinez-Tello, 1968). During acute inflammation enhanced exudation (or transudation) of IgG into the RT occurs but as with RT IgM this could result from an increased permeability of the alveolar-capillary walls due to inflammation. Respiratory tract IgG has been shown to agglutinate and to opsonize pseudomonas for phagocytosis by alveolar macrophages in humans (Reynolds et al., 1975) and rabbits (Reynolds and Thompson, 1973). Other evidence suggests that human alveolar macrophages have surface receptors for the Fc fragment of IgG and for complement but not for either IgA or IgM (Reynolds et al., 1975). If this is so, then this evidence suggests an active role for RT IgG in enhancing phagocytosis of inhaled antigen by macrophages. Since alveolar macrophages are known to penetrate RT tissue, it is possible that alveolar macrophages that have phagocytosed inhaled antigen may play an important role for localized sensitation of RT lymphoid tissue.

IgE

The role of IgE in immediate hypersensitivity is a topic that has been reviewed extensively (Johansson et al., 1972; Ishizaka, 1973;

Bennich and Johansson, 1971; Ishizaka, 1975). The cascade of events responsible for the clinical symptoms of RT hypersensitivity, such as asthma, requires the specific interactions of IgE, inhaled antigen and mediator containing mast cells. Respiratory tract IgE is synthesized locally (Tomasi and Grey, 1972; Taka and Ishizaka, 1970; Donovan et al., 1970), and resembles serum IgE although correlations between serum and RT concentrations of IgE are poor. This evidence is in agreement with local synthesis or specific transport. If RT IgE is synthesized in the RT but outside of the immediate RT mucosal tissue and specifically transported, then the intermediate role of antigen laden macrophages in immediate hypersensitivity described earlier is strengthened.

IgE mediated reactions may also control or modulate the manifestations of other immune reactions by regulating alveolar capillary permeability and consequently the availability of IgM and IgG to the RT.

IgA

Secretory IgA (SIgA) is the predominant class of immunoglobulin in mammalian external secretions, including those of the upper respiratory tract. The physiological and immunological activities of SIgA have been extensively reviewed by Hauptman and Tomasi (1978) and others (Masson and Heremans, 1973; Mastecky and Lawton, 1974 and Rossen, Morgan, Hsu, Butler and Rose, 1968). Secretory IgA provides the primary defense mechanism against local infections owing to its abundance in saliva, tears, prostatic fluid, vaginal secretions, mucus secretions in the small intestine, nasal mucosa and bronchi. Immunofluorescence studies

demonstrate IgA producing cells in the lamina propria and submucosa of the entire respiratory tract. Most respiratory tract SIgA is synthesized locally by these lymphoid cells. Immunoglobulin A is initially secreted from plasma cells as a dimer linked by J-chains but without secretory component. The secreted dimeric IgA binds to secretory component, which constitutes a receptor on the surface membrane of mucosal epithelial cells. The immunoglobulin molecule next enters the epithelial cell and covalently combines with the secretory component, forming SIgA, which is secreted onto the mucosal surface. The addition of secretory component to IgA apparently renders SIgA relatively resistant to enzymatic digestion by proteolytic enzymes present at mucosal surfaces.

There is ample evidence that specific SIgA antibody activity can be induced after antigenic stimulation. However, because of the predominance of SIgA in membrane secretions it is speculated that the principal function may not be to destroy antigen but rather to prevent access of the antigen to the general immunological system. The mechanism by which this occurs is thought to be via the "binding" of SIgA with soluble antigen, thus inhibiting or impeding the absorption of potentially immunogenic macromolecules across the mucosal surface of the respiratory tract. In the case for insoluble antigens which need to be phagocytized by macrophages before being cleared from the RT, SIgA differs functionally from IgM and IgG in its relative inability to opsonize these antigens for effective phagocytosis. Thus, in effect, SIgA constitutes a "barrier" between inhaled antigens and an organism's

immune system (including effector cells of that immune system). This is a protective effect in that it lessens the chance for systemic sensitivation and the possibility of interaction between antigen and complement fixing antibodies, thereby protecting against asthma type hypersensitivity and eventual alveolitis in the lung.

Fate of Particulates Inhaled and Deposited in the Respiratory Tract

The fate of inhaled particles depends on their size and shape. Large particles are deposited on the mucociliary escalator of the nasal passages or respiratory tree and their subsequent eliminization is via the mucus transport by the mucociliary escalator. Smaller particles can penetrate to the alveoli where they usually either pass rapidly through the surfactant lining or are ingested by macrophages. Many inhaled dust particles are innocuous, but certain dusts, notably silica and asbestos, stimulate a fibrogenic reaction in the lung. Silica particles damage macrophages which then release a factor(s) that increases collagen synthesis by fibroblasts. Asbestos particles are long and thin and are not completely ingested by macrophages but can stimulate fibroblasts in culture to synthesize collagen. There is a wealth of data in the literature concerning the noxious effects of other particles inhaled, deposited in the respiratory tract and ingested by macrophages (Task Group on Lung Dynamics, 1966).

Respiratory Tract Deposition of Inhaled Particles

The health risk resulting from the deposition of toxic or infectious particles within the respiratory system need not be

proportional to the total quantity of particles trapped. For some diseases the risk varies in magnitude depending upon the site of deposition within the system and, in certain cases, there will be no risk whatsoever unless particles are deposited at particular sites. Silicosis, for example, develops only in consequence of dust deposition in the pulmonary air spaces, and for such dust upper respiratory trapping actually provides protection. In contrast, perforation of the nasal septum of chrome metal workers results from particulate deposition in the nose. Systemic poisoning, distinct from the respiratory system, can result secondarily either from absorption of the toxic agent from the gastrointestinal tract after the particles have been cleated from the upper respiratory tract and swallowed, or by direct transfer from the lungs into the blood stream. Thus, knowledge of the overall efficiency of respiratory deposition in relation to particle size and to the dynamics of air flow is insufficient to fully assess the toxicological importance of particles deposited in the respiratory system in disease etiology. Such relationships must be established for different sites at various depths within the system, taking into consideration the fact that the magnitude of deposition at a given depth depends upon the probability that a particle will penetrate to that depth as well as upon the efficiency of trapping at the site in question, and the degree of physiological interaction between deposited particle and tissue at the deposit site.

The Van-Wijk-Patterson curve of overall respiratory deposition is made up of the sum of deposition values at different depths (Hatch and

Hemeon, 1948). Since no more than 2/3 to 3/4 of the inhaled air is likely to reach the pulmonary air spaces, it follows that deposition here cannot exceed an equal fraction of the inhaled dust, even when the local efficiency of deposition is 100%. Hence, a probability of overall respiratory deposition which approaches 100% for particles > 5 mm can only mean that there is a high probability of upper respiratory deposition with consequent low probability of penetration to, and deposition of, these coarse particles in the pulmonary air space. The inherent decrease in the gravitational deposition force as particle size decreases is such that the magnitude of overall deposition of the fine particles must be accounted for largely by deposition in the pulmonary air spaces.

Employing such arguments in an analysis of the Van Wijk-Patterson curve of overall respiratory deposition, Hatch and Hemeon (1948) derived deposition efficiency curves, in relation to particle size, for the upper respiratory tract (UR) and the pulmonary air spaces (A), respectively. From these curves, the percentage penetration of inhaled particles into the pulmonary air spaces (P) was obtained and, finally, the percentage of inhaled particles which are deposited in the pulmonary air spaces, as the product of A and P.

A detailed discussion of those forces that determine particle deposition in the respiratory tract is beyond the scope of this introduction and the reader is referred to Task Group on Lung Dynamics (1966) for a detailed description. A brief summary of the pertinent forces without any supporting theoretical explanation is presented below.

The differing characteristics of dust trapping at various depths within the respiratory system may be summarized as follows:

1. Particles larger than 10 mm are removed in the nasal chamber and therefore have little probability of penetrating to the lungs. Upper respiratory efficiency drops off as size decreases and becomes essentially zero at about 1 (i.e., 100% of particles > 1 mm will pass through the nasal trubinates).

2. The efficiency of particle removal is high in the pulmonary air spaces, being essentially 100% down to around 2 mm. Below this size it falls off to a minimum at about 0.5 mm. It then increases again as the force of precipitation by diffusion increases with further reduction in size.

3. The percentage penetration of particles into the pulmonary air spaces rises from essentially zero at 10 mm to a maximum at and below about 1 mm where it equals the fraction of tidal air which reaches the lungs.

4. The percentage of inhaled particles which penetrate to and are deposited in the pulmonary air spaces has a maximum value between 1 mm and 2 mm. Particles larger than 2 mm are deposited in the lungs in lesser degree because they are trapped higher up in the respiratory tract. Lung deposition of finer particles falls off because the local efficiency of removal decreases as size goes down below 2 mm.

5. Below 0.5 mm, the probability of deposition in the pulmonary air spaces rises in proportion to the increase in the force of precipitation by diffusion with decreasing size.

6. Relative amount deposited and the distribution of the collected particles in the respiratory system changes with breathing frequency and tidal volume. Upper respiratory trapping increases as the rate of inspired air flow goes up with faster breathing frequency. Magnitude of deep-lung deposition increases with slow, deep breathing because of the larger fraction of tidal air which reaches the pulmonary spaces and the longer transit time of air into and out of the lungs.

Clearance of Particles Deposited in the Respiratory Tract

The preceding section described how the dust-trapping characteristics of the respiratory system operate to limit the deposition of inhaled particles in the lungs. Coarse particles are trapped in the nose, nasopharynx, or along the airways, thus protecting the lungs; and of the finer particles which do penetrate to the pulmonary air spaces, only a fraction are deposited, whereas the remainder are exhaled. The protection thus offered is limited, however, and other and more effective means must be sought to account for the fact that large quantities of particulate matter do not accumulate in the lungs over a lifetime of exposure. This is illustrated, for example, by the experience of a city dweller exposed to a moderately dirty community atmosphere which may contain as much as 1 mg of fine solid particles per cubic meter. Assuming a daily respiratory intake of only $10m^3$ of air (very light activity), this individual will inhale 10 mg per day or more than 3 g of particulate matter in a year. Over a lifetime, the quantity inhaled may amount to several hundred grams.

Despite this, only a few grams of mineral ash will be found in the lungs. Even the lungs of workers exposed to silica or other mineral dusts at the substantial concentrations encountered in the dusty trades are found to contain but a small fraction of the estimated quantity inhaled over a normal working life. Thus, among the South African gold miners exposed to the high levels of dustiness during the early years of operation (10 mg/m^3), McCrae (1938) at autopsy found the total mineral ash content of silicotic lungs to be no more than 20 g, which may be compared with an estimated intake of well over 1000 g. In contrast to this low percentage of retained material, one can assume from experimental findings presented that the initial pulmonary deposition amounted to at least 20% of the inhaled particles.

From this it follows that clearance mechanisms of considerable effectiveness must operate, even against cytotoxic dusts such as silica, to remove, over a long period of time, a very substantial fraction of particles deposited in the respiratory system. For aerosols which elicit physiological response immediately after deposition, the relative amounts of initial trapping along the upper respiratory tract and in the lungs may be the important factor for preventing disease. However, it is the subsequent clearance rather than the initial deposition that predominates in determining the harmful reaction from dusts like silica which produce ill effects only following prolonged periods of exposure. Clearance of particles from the lungs is a two-stage process, with the two mechanisms operating in series. The particles are first transported from sites of initial deposition within

the aveoli to the terminal bronchioles and are then carried with the moving blanket of mucus which covers the respiratory airways up to the throat from which point they are commonly swallowed. The rate of removal along the airways must obviously equal or exceed the rate of alveolar clearance if dust accumulation in the lungs is to be avoided. With light dust burdens a reasonable balance is maintained in healthy lungs between rate of initial deposition and pulmonary clearance. A slight excess of particles may be translocated from the alveoli to the lymph nodes which drain the lungs. If the load of particulates initially deposited upon the alveolar surfaces is large, however, or if the lung clearance mechanism is impaired, a sequestering tissue reaction (pneumonconiosis) occurs which may be followed by other pathological events. The fate of the deposited particle and the probable extent of lung damage are thus determined by the competition between these several factors: transport to the mucus blanket, sequestration, and translocation to lymph nodes. For soluble particles, the competition is between rate of pulmonary clearance, sequestration, and rate of solution.

Upper Respiratory Tract Clearance

The mechanism of upper respiratory tract clearance (exclusive of nasal passages) is dependent upon the proximal movement of a blanket of mucinous fluid which is maintained by the ciliary activity of the columnar epithelium lining the tracheobronchial mucus membrane. This ciliated surface extends from the inferior aspect of the epiglottis down to the terminal bronchiole. Not all the cells of this surface are

ciliated. In the rat, for instance, the ratio of nonciliated (goblet cells) to ciliated cells is 3:5 according to Antweiler. However, this ratio is variable not only from individual to individual or from species to species, but also to different portions of the tracheobronchial tree within the same individual.

Alveolar Clearance

The subsequent fate of a dust particle settling upon the alveolar surface is fourfold, namely: (1) transported to the mucus blanket of the tracheobronchial mucosa and thence to the pharynx where it is either swallowed or expectorated, (2) transported to the satellite lymph nodes draining the lungs (from which some may escape into the blood stream), (3) sequestrated within the lung by a tissue reaction (pneumoconiosis), and (4) dissolved and transferred in solution to the bloodstream or become bound to tissue substance in the lungs.

This process first requires that the local clearance mechanism be adequate for the magnitude of the lung burden. Adequacy of the clearance mechanism implies that the dust particles are removed from the alveolar membrane. The alveolar clearance mechanism, of course, must transport the dust particles to the region where the moving mucus blanket can take over their further removal. The second pathway of removal, to lymph nodes, comes into play only after particles have penetrated the alveolar membrane or otherwise enter the interstitium, and the likelihood of such an event increases as the effectiveness of removal to and with the moving blanket of the upper respiratory tract or in the alveoli. If in the latter, it may be due either to intrinsic

deficiencies in the mechanism or to excessive loads, or to both. The third fate, which is associated with the production if intrapulmonary dust deposits, is also dependent upon an inadequacy of the clearance mechanism but only in association with other factors which are little understood and, at present, must be considered conjectural. Direct transfer into the bloodstream, the fourth fate, is limited by particle solubility and solution rate; for a given solubility rate of any particulate substance, solubility increases as particle size decreases.

As noted previously, there are two pathways by which lung tissue may be cleansed of entrapped matter: one leading to the pharynx and the other to lymph nodes. The first pathway is physiological and by far the most effective in terms of the quantity of matter removed, whereas the second, having pathological implications, generally involves only a minor fraction of the total material that comes to rest upon the alveolar surface. There are two main components to the first process of alveolar clearance: phagocytosis of the particulates and transport of the cells and material to the moving mucus blanket.

A distinction exists between the cellular ingestion of a solid particulate as opposed to a droplet of fluid. Ingestion of solid particles is defined as phagocytosis, whereas ingestion of a droplet is defined as pinocytosis. In both processes, however, there are villus-like projections of cytoplasm which extend around each side of the particle or droplet. These cytoplasmic projections join and thereby enclose the particle or droplet within a vacuole. During this process a portion of the cytoplasmic membrane is brought into the interior of

the cell. What becomes of this interiorized membrane and what the physiochemical processes are by which the plasmalemma (cytoplasmic membrane) is replaced are not well understood.

The roles of phagocytosis in hygiene of the alveolus are threefold: (1) to render dust particles incapable of irritating or otherwise injuring the alveolar surface epithelium; (2) to prevent the penetration of the dust particles into the lung interstitium; and, (3) to facilitate the removal of the dust by the alveolar clearance mechanism.

Phagocytic cells have an ability to move about in an amoeboid manner. This motion is of random character except for that caused by specific attraction or repulsion, termed positive and negative tropism, respectively. Various types of tropism are recognized in biology, including phototropism, thermotropism, and chemotropism. It is generally agreed that the motion of phagocytic cells may be directed by positive chemotropism, and this is often illustrated by the demonstration of leucocytes moving toward a clump of bacteria and finally engulfing them. Despite the severely limited ability of phagocytic cells to initiate purposeful directional motion (such as caused by chemotropism) in the past, it has been generally assumed that alveolar macrophages migrate in one of two directions: (1) to the mucus blanket covering the terminal bronchioles or (2) through the intact alveolar wall and then to and into lymphatic vessels in the lung interstitium.

Phagocytosis and possible translocation of particles deposited in the respiratory tract to respiratory tract lymphatic systems is of prime concern to this study.

Respiratory Tract Deposition and Translocation of Antigenic Material

A host of investigations have been conducted on the relationship between deposition and clearance of various chemical classes of particulate substances, yet the fate of inhaled antigens and the role of macrophages in relation to inhaled antigens has received scant attention. Various intratracheally instilled protein (i.e., serum, hemoglobin, and albumin) have been found to migrate from the alveoli into the pulmonary blood capillaries (Drinker et al., 1937; Courtice and Simmonds, 1949). Albumin is not absorbed directly into the blood vessels but first enters into the lymphatics, a conclusion reached because of its delayed appearance into the blood. Intratracheally instilled radio-sodinated albumin is slowly absorbed into the blood circulation (Schultz et al., 1963; Schultz et al., 1964). Bensch et al. (1970) and Meyer et al. (1969) were able to show, however, that albumin could be observed by both the blood and lymphatic capillaries. Using horseradish peroxidase and the electron microscope, Bensch and Dominguez (1971) carried these studies further and investigated the pulmonary blood capillaries in both guinea pigs and dogs' lungs and found morphologic evidence for a transcellular transport of horseradish peroxidase from the lung toward the capillary lumen.

Nonorganic particulates deposited in the lung are treated differently. Very small particles such as iodine appear rapidly in the blood plasma (Gieseking, 1958) and therefore appear to pass directly from the alveoli to the capillary lumen. Larger particles such as

carbon first appear mainly in the lymph nodes, especially the lung hilar lymph nodes (Gieseking, 1958) and in fact remain there for months only slowly eluting from the pulmonary lymph nodes to the blood. The precise cellular pathways and mechanisms by which the particles finally get into the lumen of either the lymphatics or blood capillaries are unknown, although, Lauweryns and Boussauw (1969; Lauweryns, 1971; Lauweryns, 1975) have shown that both intratracheally instilled ferritin and carbon reach the lymphatic lumen through open intercellular junctions. The lymphatic endothelial cells endocytose numerous ferritin molecules but no carbon particles. The ferritin molecules, but not carbon, are also transported through capillary endothelial cells by means of micropinocytotic vesicles.

In general, it appears that the walls of the pulmonary blood capillaries appear to be less permeable than those of lymphatic walls, suggesting that lymphatics have a far more significant role in the alveolar clearance than do the blood capillaries. The latter are perhaps more important for the removal of water and electrolytes, to which their wall is freely permeable (Chinard et al., 1962), while the former are perhaps more important for the clearance of proteins and particulates deposited in the alveolar region. Still, the overall importance of the pulmonary lymphatics in alveolar clearance may be doubted because they do not normally occur in any great quantity in the alveolar walls and the interalveolar septa (Lauweryns and Boussauw, 1969; Lauweryns, 1970). The particles apparently have to travel a relatively long way through the interstitium or pulmonary airways

before entering the lymphatic capillaries. Alveolar macrophages play a vital role in both general alveolar clearance (i.e. via the tracheobronchial tree) and for the transport of material from the alveolar spaces to the lymphatics. These macrophages possess a pronounced endocytosis activity and a highly developed lysosome system (Taylor and Gaar, 1970; Sorokin, 1970). It is also known that they remain functional for long periods because they possess relatively stable heterolysosomes that retain their capacity of fusion with endosomes and of digestion of their contents (Sorokin and Brain, 1975; DeDuve and Wattiaux, 1966; Weiss, 1973). Activated alveolar macrophages depend primarily on oxidative phosphorylation to provide energy for endocytosis and amoeboid motion (Oren et al., 1963; Portugalov et al., 1973). The question remains unanswered, however, as to whether the transport of material by macrophage from the alveolar lumen to either the tracheobronchial tube or to the pulmonary lymphatic system is passive, random, or whether directed by some biological or physical property of the material being transported.

Specialized structures may exist at pulmonary mucosal surfaces that facilitate the transport of materials. The flattened, nonciliated lymphoepithelium overlying lymphoid nodules of large and medium bronchi presents a potential portal of entry into submucosa, thereby bypassing the mucociliary blanket (Bienenstock, 1973). Recent evidence suggests that this phenomena may occur in similar nodules of the gastrointestinal tract (Owen and Jones, 1974a; Owen and Jones, 1974b). Such a portal of entry would be important not only in the efficient transport of material

to mucosal lymphatic tissue but also might help to explain how allergens appear to gain access to submucosal mast cells rapidly as evidenced by such fast reacting conditions as hay fever and asthma.

Many studies have been directed toward defining mechanisms by which material is cleared from upper and lower respiratory surfaces, yet little work has been directed toward the fate of antigens, or the correlation between the site and mode of particle deposition with the consequent immune responses in lymphatic tissues associated with the respiratory tract. Kaltrider et al. (1975; 1978) has shown that sheep erythrocytes instilled into bronchoalveolar spaces of dog lungs resulted in pulmonary consolidation, rapid ingestion and degradation of the particles by phagocytic cells with a subsequent antibody mediated immune response that occurred in the hilar lymph nodes but not in systemic lymphoid tissue. These results suggest that since sensitization initiated via the respiratory tract does not manifest itself in a systemic reaction, the immune apparatus of the respiratory tract is independent of the systemic immune system. In the rat and mouse, however, the respiratory tract is relatively devoid of lymphoid tissue unless the animals are exposed to noxious environmental agents, including antigenic material.

Respiratory Mechanics

During breathing the respiratory muscles must move the "chest wall,"* the lungs, and the gas in the respiratory tract. In accomplishing this

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*It is difficult to find a single term to denote the structures outside the lungs which take part in respiratory movements. The term "chest wall" is used here and is meant to include the chest cage, the diaphragm, and the abdominal contents displaced by the diaphragm.

movement the respiratory muscles encounter two principal physical properties of the system: (1) the elastic properties of the lungs and chest wall, and (2) the flow-resistive properties of the lungs and chest wall. The flow-resistive properties include the viscous resistance offered by the lung and chest wall tissue and the gas flow-resistance within the air passage.

When all the respiratory muscles are relaxed the volume of the lungs is determined by the balance of elastic forces between the lungs and the chest wall. The elastic recoil of the lungs tends to collapse them. Collapse is prevented by opposing elastic forces developed in the chest wall. In terms of lung volume the point of equilibrium approximates the resting end-expiratory volume or the functional residual capacity.

During inspiration the respiratory muscles increase the volume of the thorax and lungs from the equilibrium volume. In this process the lungs are further stretched and their force of elastic recoil is increased. This increased elastic force is reflected in a fall in intrapleural pressure. That is to say the intrapleural pressure becomes increasingly subatmospheric as lung volume increases. At the end of inspiration, the muscles relax and the lungs and chest wall are driven back to the equilibrium position by their elastic recoil.

But in breathing, the respiratory muscles encounter opposition of another sort as well. They must supply the force necessary to produce flow of gas through the upper airways and the tracheobronchial tree, and additional force to produce "flow" of the lung parenchyma and the moving tissues of the chest wall. During quiet breathing, they need supply

these additional forces only during inspiration. The elastic recoil of the lungs and thorax is sufficient to overcome gas and tissue flow-resistance during expiration.

Just as the force required to oppose the elastic recoil of the lungs is reflected in the intrapleural pressure, so are the forces overcoming gas and tissue flow-resistance of the lungs and airways. For example, during a rapid inspiration the expanding chest wall may be thought of as a "pulling" on the lung surfaces, and the lungs resist the "pull" not only with their elastic recoil, but also with the flow-resistance within the airways. As a result, intrapleural pressure drops further below atmospheric pressure than can be accounted for by the lungs' elastic recoil alone. During a forced and rapid expiration the elastic recoil of the lungs is insufficient to produce so rapid a volume change. The chest wall may be thought of as "pushing" on the lung surfaces, and intrapleural pressure may be driven considerably above atmospheric pressure. Thus, the pressure fluctuations at the surface of the lungs are not simply a result of lung elasticity. They relate also to flow-resistance. This is true during quiet breathing as well as forced rapid breathing. To obtain a measure of the elastic and flow-resistance properties of the lungs, it is necessary to separate the intrapleural pressure fluctuations into elastic and flow-resistive components.

The central features of the analysis may be stated as follows: (1) the elastic component of intrapleural pressure depends on the degree of "stretch" of lungs, and hence the volume of the lungs. It is independent of the rate at which lung volume may be changing; (2) the

flow-resistive component of intrapleural pressure depends on the rate at which lung volume is changing and is independent of lung volume.

If the respiratory cycle is considered in the light of these two statements, it may be seen that at two points in the respiratory cycle, namely at the end of expiration and at the end of inspiration, all flow has stopped and the intrapleural pressure at these instants depends entirely on the elastic recoil of the lungs. Between these two instants the tidal volume has been produced and the lungs have changed volume. The associated change in intrapleural pressure between these two points may be related to the tidal volume to give an expression of the lungs' elastic properties in terms of the elastic pressure change associated with a given volume change of the lungs. This relationship is now commonly expressed as the volume change in liters per cm H_2O pressure change, and is termed "pulmonary compliance."

In the resting tidal range of lung volume change the compliance of the lungs is nearly constant in a given individual. That is to say if the lung volume were increased 0.5 liters from the resting end-expiratory volume with an associated pressure change of 2.5 cm H_2O, the compliance would be 0.2 liters per cm H_2O. If the volume change were twice as great, namely 1 liter, the associated pressure change would be nearly 5 cm H_2O; the compliance would be the same. This being so, it is possible to predict the elastic component of intrapleural pressure at any point in the respiratory cycle if the volume change up to that point is known. The flow-resistive component of pressure at such a point is then the difference between the predicted elastic pressure and

the observed pressure. For example, midway during inspiration the intrapleural pressure might be -8 cm H_2O, i.e., 8 cm H_2O below atmospheric pressure. If the predicted elastic pressure for this point were -6 cm H_2O, the flow-resistive pressure would be 2 cm H_2O. An expression for the flow-resistance of the lungs and airways could then be obtained by relating this flow-resistive pressure to the rate at which lung volume was changing at this instant. If the rate of volume change were 0.5 liter per second, the flow-resistance, expressed as the pressure necessary to produce a flow of 1 liter per second, would be 4 cm H_2O per liter per second.

Summary

In summary, strong experimental evidence from the literature has been presented indicating (1) lymphocytes and their function are at the center of the immune system; (2) lymphocyte function can be regulated through processing of immunogens by accessory cells including macrophages; and (3) particles deposited in the respiratory tract are phagocytized and processed by alveolar macrophages. There is definite lack of data in the literature, however, on the subsequent fate of immunogens phagocytized by alveolar macrophages and what role they may have in eliciting an immune response either systemically or locally (i.e. in the respiratory tract).

After immunogens are ingested by alveolar macrophages two possible options for the immunogen laden macrophages are to interact with lymphocytes of respiratory tract lymphoid tissue or migrate to and

interact with lymphocytes in the spleen. These options need not necessarily be exclusive of each other. However, if either option is chosen, questions still remain to be answered as to where the center of the immune response, which is manifested in the respiratory tract, is located. In other words, is the respiratory tract and its abundant lymphoid tissue an independently immunocompetent organ or does the spleen control the immune response of the respiratory tract or is there cooperation between both organs?

The studies described in the following text are designed to address these questions.

II. MATERIALS AND METHODS

Glassware

All glassware that was used was Pyrex. The glassware used was vigorously cleaned with detergent and chromic acid cleaning solution prior to rinsing several times in tap water and double glass distilled water. Where practical, disposable, nonpyrogenic, sterile, plastic laboratory equipment was routinely used.

Animals

Specific pathogen-free (SPF)* female Sprague-Dawley rats weighing 250-300 grams were used in this study. The rats were derived from a germ-free stock (rederived, Oak Ridge National Laboratory, Oak Ridge, Tennessee) and were maintained as SPF animals in a barrier facility (Brick et al., 1969) throughout the experiment to exclude the influence of common infectious diseases. The rats were kept five per cage and allowed free access to food and water. Animal feed was Wayne Sterilizable Lab-Blox (Allied Mills, Inc., Chicago, Illinois) and the water was filtered tap water chlorinated to 20 ppm with sodium hypochloride. No fluoride was detected in the drinking water. Bedding for the animals was hardwood chips. Lighting was standard fluorescent on a timed 12-hour light/dark cycle.

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*Sendai virus, MHV, LCM, Polyoma virus; Ectomelia; Salmonella; Endo and Ecto parasites.

Antigen

Ovalbumin (5X crystallized, Nutritional Biochemical Company, Cleveland, Ohio) was used as a sensitizing agent in this study. Sensitization of the rat by a single exposure of ovalbumin was found to be obligatorily dependent on the interperitoneal (i.p.) administration of heat-killed Bordetella pertussis vaccine as an adjuvant (Jarrett and Stewart, 1974). All animals in this study, sensitized by a single exposure of antigen, whether sensitized by inhalation or systemically, were i.p. treated with B. pertussis vaccine (0.5 ml; approximately 6.5×10^9 killed cells/ml).

Sensitization by Inhalation

The inhalation chamber used for airborne antigen exposure was developed (Creasia, 1974) at Oak Ridge National Laboratory, Oak Ridge, Tennessee. The exposure chamber was constructed entirely of plexiglass and essentially is a hexagon of approximately 0.5 m^3 volume with four ports for nose only exposure of rats on each side of the hexagon. The animals are held in a tubular plexiglass container designed with a neck collar to prevent the animals from removing its nose from the exposure port. The animal holders are attached to the aerosol exposure chambers by a telescopic pressure fit in which an airtight seal is maintained with butyl-rubber O-rings. This animal holding device does not alter pulmonary mechanics or normal respirations of the animals being held (Amdur and Mead, 1958; Creasia, 1966). The chamber is operated dynamically under negative pressure with ten changes per hour of

filtered air carrying the aerosol. The aerosol enters at the bottom of the chamber, is baffled to insure adequate mixing and even distribution of the aerosol to all exposure ports and exits from the top through an HA millipore filter (Millipore Corp., Bedford, Massachusetts). Negative pressure in the chamber was maintained at about 1 to 2 mm water and monitored with water manometer. Aerosol dilution was standardized by appropriate adjustments of calibrated flowmeters.

The antigen aerosol was generated by atomizing a solution (5.0% w/v) of ovalbumin in distilled water with a Lovelace atomizer. The solution reservoir was maintained at 3 to 5 ml and the atomizer operated at a Δp of 22 psi. Aerosol mass concentration and particle size was grossly regulated by adjusting the atomizer impact screw. Mass concentration of the antigen aerosol was 18 to 22 mg/m^3 and the geometric mean diameter was 0.35μ. The mass concentration was determined gravimetrically from grab samples taken on a PF-type Millipore filter. The aerosol size was determined optically from electrostatic precipitator samples.

Whenever multiple antigen exposures were used, animals were exposed to aerosol antigen for six hours per day, three days per week for the desired number of weeks. Whenever a single antigen exposure was used, the animals were exposed to a single six hour exposure. Just prior to each single antigen aerosol exposure, all animals were injected i.p. with 0.5 ml B. pertussis heat killed vaccine ($\sim 6.5 \times 10^9$ cells/ml) as an adjuvant. Blood was obtained by suborbital puncture of the animals at designated intervals after antigen exposure, serum was separated and stored at -70°C until used.

The quantity and site of deposition of the antigen in the respiratory tract was determined by mixing the ovalbumin with Cr_2O_3 particles (<0.1μ diameter) that were tagged with ^{51}Cr. Chromium-51 was chosen as the radiolabel because it has a relatively long half-life, its 497 Kev gamma emission allows for easy external monitoring and $^{51}Cr_2O_3$ is insoluble and physiologically inert (Creasia, 1973). The amount of deposited aerosol was determined by carefully isolating the appropriate tissue (or organ) immediately after aerosol exposure and comparing the amount of ^{51}Cr associated with the tissue (or organ) relative to that associated with a known amount of ^{51}Cr-ovalbumin (i.e. the 5% solution of ovalbumin to be aerosolized). The site of deposition of antigen was determined immediately after aerosol exposure by dissecting the respiratory tract (larynx, trachea, bronchi, and lung lobes as one; skinned nasal turbinates minus nares as a unit) from each rat and determining ^{51}Cr-ovalbumin deposition in the respective regions. Alveolar deposition was selectively determined by separating all lung lobes from other tissue and measuring the amount of ^{51}Cr retained in each lobe. In all procedures, care was taken to remove extraneous tissue and to minimize contamination of internal tissue from external ^{51}Cr-ovalbumin.

Systemic Sensitization to Ovalbumin

Rats were initially systemically sensitized to ovalbumin by i.p. injection three times per week for the desired number of weeks with 50 μg of antigen in 0.5 ml sterile saline. Whenever a single antigen

injection was used for sensitization 0.5 ml B. pertussis heat killed vaccine was used as an adjuvant and given i.p. prior to ovalbumin treatment. Antisera obtained by suborbital bleeding of the animals at designated intervals after the last antigen injection were stored at -70°C.

Collection of Cell Specimens

The number of plaque forming cells in spleens and lymph nodes were determined immediately after animals were killed. The spleen was first cut into narrow strips and the cells freed from the fibrous stroma by gently scraping with the blade of a scalpel in a small volume of Hanks balanced salt solution (HBSS) containing heparin and antibiotics. The cell suspension was allowed to sediment for 10 minutes to remove tissue fragments. The cells in the supernate were collected by centrifugation; hypotonic lysis was used to remove contaminating erythrocytes. Respiratory tract tracheal and cervical lymph nodes were dissected free of surrounding fat and connective tissue and pooled. Cells from the lymph nodes were freed from stroma, tissue debris was removed, and the cells were collected by centrifugation as described above.

Free respiratory tract cells were obtained by lavage (Brain and Frank, 1968). At least 12 washes were performed on each lavaged animal and pooled. The pooled washes from individual animals were centrifuged, the pellet resuspended, and the cells plated for determination of PFC.

The approximate number of viable cells obtained by the various procedures were 125×10^6 cells per spleen; 5×10^6 cells per pool

mediastinal lymph nodes, and 8-10 × 10^5 cells per pooled lung washings. Viability was determined by trypan blue exclusion and the number of cells determined by counting on a calibrated optical microscope grid.

Hemagglutination

Micro-titration with Takatsky plates was employed for all agglutination assays. Approximately 0.05 ml (one drop) was placed in each well of the plates except the first well. Into this well approximately 0.1 ml of the sera to be assayed was dispensed with a Pasteur pipette and serially diluted with microdiluters into sequential wells. Next, 0.1 ml of 0.5% SRBC tanned and coated with ovalbumin was added to each well, the plates gently shaken, covered, and incubated at room temperature for about four hours, positive evidence of antibody to ovalbumin was noted by agglutination of SRBC after about four hours. Data is presented as the reciprocal of the highest dilution showing positive agglutination.

Fixation of Ovalbumin to SRBC

Ovalbumin antigen was coated to SRBC for use in the indirect PFC assay and the agglutination assay with Takatsky plates. The fixation of ovalbumin to SRBC was accomplished by treating SRBC with dilute tannic acid and then incubating the SRBC in a solution of ovalbumin. Washed, packed SRBC were diluted (suspended) in a phosphate buffered saline. An aliquot of SRBC was then added to dilute (1/20,000) tannic acid in saline, incubated at 37°C for approximately 10 minutes, centrifuged,

washed with buffered saline (pH 7.2) and then suspended in saline. Ovalbumin in buffered saline (pH 6.4) was mixed with an aliquot of tanned SRBC and incubated at room temparature for about 10 minutes. The SRBC were then centrifuged, washed with inactivated rabbit serum and suspended in the rabbit serum. A control series of SRBC was also prepared by following all of the above steps with SRBC except for the step involving fixation with ovalbumin.

Plaque Assays

The hemolytic plaque techniques described by Jerne et al. (1953) was used to determine the number of antibody forming cells (PFC). Cell procedures were done in HBSS containing heparin and antibiotics. Portions of the various cell suspensions ranging from 50 to 600 µl were added to plastic tubes containing 2 ml of molten 0.7% agar, 0.1 ml of 1.0% DEAE-dextran and 0.1 ml of 20% (vol/vol) washed SRBC at 45°C. The contents of the tubes were mixed by inversion and poured rapidly into plastic petri dishes previously coated with 10 ml of 1.4% agar. The soft agar mixture was allowed to set at room temperature and the petri dishes were incubated for 45 minutes at 37°C in humidified air containing 5% CO_2.

Direct (IgM) plaques were detected by adding reconstituted lyophilized guinea pig complement (diluted 1:10 with HBSS, 10 ml per plate) after incubation at 37°C for 45 minutes. Indirect (IgG) plaques were determined as follows: agar layers were covered with the appropriate rabbit antisera to rat immunoglobulins and incubated for

45 minutes at 37°C. Antisera were decanted and guinea pig complement added as described above. All assays were performed in duplicate.

The agar layers were stained with benzidine hydrochloride-acetic, acid-hydrogen peroxide, and plaques were counted with the aid of a dissecting microscope. The number of indirect plaques was determined by subtracting from the total the number of direct plaques obtained from parallel assays of cells from the same animal. Results are expressed at PFC per 10^6 viable cells in each tissue. Background PFC as determined in five control animals were less than 5×10^6 for each cell specimen.

Splenectomy

Animals scheduled to be splenectomized were first anesthetized with pentobarbital. The abdomen was washed with 100% ethanol and splenectomy was performed through a lateral abdominal incision using a cauterizer to sever and coagulate the splenic vessels.

Passive Cutaneous Anaphylaxis (PCA)

Serial dilutions of antisera were made in 0.85% saline and 0.1 ml volumes were injected into the shaved dorsal surface of nonsensitized recipient rats to determine passive cutaneous anaphylaxis. Seventy-two hours later the animals were challenged intravenously (i.v.) with 1 ml of a saline solution containing 4 mg ovalbumin and 5 mg Evans blue. Spots of extravascular bluing were determined 30 minutes later. A 4 mm spot was considered the lower limit of detection. Titers are recorded

as the reciprocal of the lowest dilution showing a 4 mm spot. Each PCA assay was accompanied with a control group of animals in which sera from nonsensitized rats instead of sera from sensitized rats was used. All other procedures were conducted exactly as above.

Procedure for Measuring Pulmonary Functions

Measurements of total respiratory flow resistance were performed essentially by the principle described by Mead (1960). A modified pressure-vacuum pump imposes a sine-wave pressure pulse around the animal's body, while bleeding off the relatively smaller changes due to thoracic excursions. This cycled pressure-pulse causes an in-phase change in flow at the mask, which is superimposed upon the animal's natural respiratory flow pattern. Total respiratory flow resistance may be calculated by measuring on the oscillograph record the amount of imposed pressure required to produce a unit change in flow at the mask; the following formula is used: $R = \Delta P\rho/\Delta$ Vm where R = total respiratory resistance, $\Delta P\rho$ = change in pressure (cm H_2O) in the plethysmograph and ΔVm = change in flow rate (cc/sec) at the mask. For routine measurements of resistance, the cycled pressure changes in the plethysmorgraph are plotted electronically on the x-axis and flow-calibrated pressure changes in the mask are plotted on the y-axis of xy oscilloscope.

A series of vector lines or loops is displayed. The cotangent of the angle formed with the horizontal by the long axis of these loops is directly proportional to resistance [R(cot) = $\Delta P\rho$ (side adj.)/Δ Vm (side opp.)] and a cotangent scale is placed over the xy scope for

direct readings. The cotangent figures are converted to physiological units by use of a constant K, which is dependent upon the flow calibration for each individual mask screen. Thus, Cot × K = R in cm H_2O/ccsec; where K = kp/km, and kp equals the cm H_2O change in pressure in the plethysmorgraph per mm of deflection on the x-axis of the xy scope, and km equals the cc/sec change in flow at the mask per mm of deflection on the y-axis. A constant flow of air was established with a capillary tube (critical orifice) and supplied to the nose cone which was then supplied around the animal's nose. This constant flow assures a fresh supply of air and eliminates rebreathing due to dead space of the exposed nose cone. The constant flow to each exposed nose cone was sufficient so that during inspiration the pressure in the mask does not return to zero. The constant flow, positive-pressure baseline in the mask is reinforced or decreased proportionately to the flow rate at the nose during expiration or inspiration respectively. Calibrated pressure changes in the sealed plethysmorgraph that result from changes in body volume during respiration was used to measure respiratory rate and tidal volume (Amdur and Mead, 1958).

III. RESULTS

Sensitization following Administration of Ovalbumin without Adjuvant

It is generally accepted that without adjuvant rats are difficult to sensitize with soluble antigens such as albumin. Therefore, preliminary studies were conducted to determine optimum conditions for studying the immune response to ovalbumin in the RT of rats. In these preliminary studies, animals were subjected to various antigen exposure schemes and appropriate tissue sampled for their degree of response. Initially animals were injected i.p. with ovalbumin three times per week for four weeks and serum titers for antibodies to ovalbumin determined at 1, 3, 5, 10, 15, 20 and 25 days after either of 1, 3, 5, 7, 9, 11 or 13 antigen treatments. The results are summarized in Table 1. The data indicates that at least five antigen injection treatments are necessary to produce detectable antibody to ovalbumin and antibody titers were higher at four weeks than at two weeks after the last antigen administration. In a second set of preliminary experiments, animals were exposed to an ovalbumin aerosol three times per week for four weeks and serum titers for antibody to ovalbumin determined at 5, 10, 15, 20 and 25 days after either 1, 3, 5, 7, 9, 11 or 13 aerosol exposures. Results from these multiple inhalation exposures to ovalbumin aerosol are summarized in Table 2. As was noted following systemic sensitization (Table 1) very little effect is detected when antigen treatment is <5 times and <2 weeks has elapsed after the last antigen treatment.

Table 1. Serum humagglutinin response in rats systemically (i.p.) sensitized to ovalbumin.

Day After Treatment	Number of Ovalbumin Treatments						
	1	3	5	7	9	11	13
	HA Titer[2]						
1	<80	<80	<80	<80	80	80	80
3	<80	<80	<80	<80	160	160	320
5	<80	<80	80	80	160	320	640
10	<80	<80	80	160	320	640	640
15	<80	<80	160	160	320	640	1280
20	<80	<80	180	320	640	1280	1280
25	<80	<80	Not Obtained	320	1280	1280	1280

[2]Equal volume of pooled serum from 3-5 animals; duplicate determinations.

Table 2. Serum hemagglutinin response in rats sensitized to ovalbumin by inhalation of ovalbumin aerosol.

Days After Treatment	Number of Ovalbumin Aerosol Exposures						
	1	3	5	7	9	11	13
	HA Titers[2]						
5	<80	<80	<80	80	80	80	80
10	<80	<80	80	160	160	160	320
15	<80	<80	80	320	320	320	320
20	<80	<80	160	640	320	320	640
25	<80	<80	160	320	320	640	1280

[2]Equal volume of pooled serum from 5 animals; duplicate determinations.

Overall, sensitization to ovalbumin by inhalation results in considerably less of an immunological response than i.p. injection of ovalbumin.

These preliminary studies show that, although weak, an immune response against ovalbumin can be elicited in rats by either a systemic or inhalation exposure to ovalbumin. The positive finding that SPF rats can be sensitized to ovalbumin indicated that the experimental concept was feasible and that the principle of sensitization via the RT could be tested. The data from these studies, however, gave no evidence as to whether sensitization after inhalation exposure was due primarily to ovalbumin aerosol particles interacting directly with RT tissue or whether the response was due to ovalbumin particle interaction with extrarespiratory tract tissue after being cleared from the RT via the gastrointestinal tract or via the circulatory system. Therefore, after it was shown that rats can be sensitized by inhalation of ovalbumin, it was next necessary to estimate the amount of antigen deposited in the RT and to determine its subsequent distribution between the respiratory tract, gastrointestinal tract and blood. This was done by exposing three animals by nose only for six hours to an ovalbumin aerosol tagged with ^{51}Cr for detection. The albumin aerosol had a mass concentration of 21 mg/m^3. Data on the total quantity of aerosol deposited in the animal as well as data on respiratory frequency, tidal volume and minute volume are summarized in Table 3. Based on an aerosol concentration of 20 mg/m^3 (21 mg/m^3 actual) the animals were exposed to 0.02 µg aerosol per cc of "exposure air" or a total of 72.0 mg total for six hours.

Table 3. Deposition of ^{51}Cr in the rat after inhalation of ovalbumin aerosol tagged with ^{51}Cr*.

Animal	Respiratory Frequency (per min)	Tidal Volume (CC)	Minute Volume (CC/Min)	Total Whole Body ^{51}CR (CPM)	Total Ovalbumin Deposit (mg)**
1	104	0.92	96	43,694	219
2	88	1.02	90	39,868	200
3	96	0.95	91	40,233	201
Average	96	.96	92	41,265	20.7

*Data based on one 6 hr. exposure to ovalbumin aerosol; Mass conc 21 mg/m^3.

**Microgram equivalents based on 100,000 CPM ^{51}Cr/mg ovalbumin aerosolized solution.

The average minute volume of these animals was 92 cc/min. Therefore, total "respiratory tract exposure" was approximately 66.0 mg ovalbumin aerosol. Average total aerosol deposition was 20.7 mg albumin and deposition efficiency about 30%.

Within minutes of particle deposition in the upper respiratory tract (nasal turbinates, larynx, trachea, major bronchi), the particles are removed by various means from the RT to the gastrointestinal tract. Particles deposited in the lower respiratory tract reside at the deposition site longer, sometimes months, depending on the particles' physical and/or chemical properties (Creasia, 1973; 1976; Task Group on Lung Dynamics, 1966).

When the distribution of the deposited ^{51}Cr was determined by isolating selected organs or organ system, approximately 27% (5.6 mg ovalbumin) was deposited in the deep lung and 44% (9.1 mg ovalbumin) was found in the gastrointestinal tract. This data is tabulated in Table 4. Since the aerosol exposure was by nose only and grooming by the animals is prohibited by the constraints of the body holder, it is assumed that most, if not all, of the 73% of deposited aerosol was initially deposited in the upper respiratory tract and approximately 2/3 of this amount translocated to the gastrointestinal tract. Chromium 51 was not detected in blood taken from the dorsal aorta. This data is consistent with published results from other studies (Task Group on Lung Dynamics, 1966; Creasia et al., 1973; Creasia et al., 1976).

To summarize the data shown in Tables 3 and 4, after exposure to an aerosolized solution of 5% ovalbumin for six hours followed by

Table 4. Distribution of ^{51}Cr in the rat after exposure to ^{51}Cr tagged ovalbumin aerosol (Percent of deposited ^{51}Cr*).

	Rat Anatomical Region					
Animal	Nasal Pharynx	Trachea (Includes Larynx and Stem Bronchi)	Lungs	Stomach	Intestine	Blood
1	31.5	<1	28.4	38.3	Negligible	Negligible
2	22.6	<1	26.1	49.2	Negligible	Negligible
3	26.7	<1	27.3	45.0	Negligible	Negligible
Average	26.9	<1	27.2	44.1		

*Average Total Deposited = 207 μG/animal.

immediate killing of the animals, the bulk of the radioactivity was found in the stomach. Approximately 25% of the antigen was retained in the nasal pharynx region. The larynx, trachea, bronchi, and lungs retained a little over 25% of the total deposited.

The possibility that the swallowed ovalbumin was the immunogen could not be excluded as an interpretation of the data in Tables 3 and 4. Therefore, rats were given 10.0 mg of ovalbumin by stomach intubation, three times per week for two weeks. Hemagglutination tests for antibody on days 10, 15, 20 and 25 after the sixth antigen exposure were negative. Passive cutaneous anaphylaxis tests at these times were also negative (data not shown). These initial tests were also used to study the effectiveness of antigenic stimulation via the lower RT in eliciting primary antibody response from cells of the RT, RT lymphnodes and the spleen as compared with results obtained after the more conventional method of sensitization by i.p. injection of antigen. Rats were exposed to ovalbumin (inhalation or i.p.) three times per week for three weeks. At days 5, 10, 15 and 20 after the last ovalbumin treatment the RT of the rats were first lavaged. Next the animals were killed and cells from the RT lymphnodes and spleen collected and the number of PFC determined from each cell sample. The results of this experiment are summarized in Table 5. A maximum number of plaque forming cells from the lungs of animals sensitized by inhalation was found at days 10 and 15; however, <100 direct and indirect plaque forming cells were detected. In contrast the spleen produced ∿2000 to 2500 direct and indirect plaque forming cells around

Table 5. Appearance of PFC in RT tissue and spleens of rats after inhalation or i.p. administration of antigen.

Days After Treatment	Mode of Administration	Direct PFC[1]			Indirect PFC[1]		
		RT Lymphnode	RT Lavage	Spleen	RT Lymphnode	RT Lavage	Spleen
5	Inhalation	0	0	642	0	0	218
	i.p.	0	0	21,921	0	0	22,618
10	Inhalation	9	5	1,831	3	0	323
	i.p.	3	3	140,000	6	2	101,000
15	Inhalation	32	18	1,942	27	18	610
	i.p.	23	19	240,000	38	29	275,000
20	Inhalation	3	0	448	9	5	118
	i.p.	1	6	3,027	4	2	7,491

[1]Pooled samples from 3 animals per time point; duplicate determinations.
Inhalation exposure was 6 hour exposures to ovalbumin aerosol 3×/wk for 3 weeks.
Systemic exposure was by i.p. injection of ovalbumin 3×/wk for 3 weeks.

days 10 to 15. The number of plaque forming cells harvested by lung lavage was also very small and slightly less than that found in RT lymph nodes.

Thus, in animals sensitized by multiple inhalation exposure, 30-40 times more plaque forming cells could be recovered from the spleen than from RT tissue of these animals at peak of response.

When animals were sensitized systemically, a similar response pattern was noted; but this time the spleen produced >250,000 PFC (vs ∿2000 to 2500 previously) while the number of PFC from RT tissue remained relatively the same. Thus, it appears that under the experimental conditions used, although serum IgM and IgG are detectable, the lung is not the major site of serum IgM or IgG production after sensitization by inhaled antigen.

Sensitization following Administration of Ovalbumin with Adjuvant

Multiple antigen exposures are unsatisfactory for some aspects of this series of experiments. Therefore a method was sought by which rats could be sensitized by one administration of ovalbumin. Sensitization of rats by a single antigen treatment was accomplished by using B. pertussis heat killed vaccine as an adjuvant just prior to ovalbumin exposure. When rats were pretreated with B. pertussis and then were exposed to an ovalbumin solution either by inhalation or i.p., the presence of serum antibodies was detected earlier and was more pronounced than in rats similarly exposed without adjuvant (Table 6).

Table 6. Sensitization to ovalbumin in rats exposed to ovalbumin aerosol with and without prior treatment with B. pertussis as an adjuvant.

Day After TREATMENT	Number of Ovalbumin Treatments With B. Pertussis 1 HA	1 PCA	2 HA	2 PCA	3 HA	3 PCA	W/O B. Pertussis 3 HA	3 PCA
5	0	0	0	0	0	0	<80	0
10	80	4	160	12	160	32	<80	0
15	160	36	160	16	320	32	<80	0
20	320	12	160	20	640	16	<80	0

Pooled samples from 4-5 animals per time point.

Each exposure to ovalbumin aerosol was for 6 hours; multiple exposure was every other day.

In addition, an anaphylactic response, implying production of IgE, was detected in these animals (Table 7). Single, double, and triple antigen exposures were studied. Differences in PFC responses following single or multiple exposures of ovalbumin consisted primarily of an earlier appearance of antibody coupled with an increased duration of production following three versus one exposure. It is also clear that the adjuvant B. pertussis was absolutely essential for antibody production with this small number of antigen exposures.

Next, an attempt was made to identify the major organ (or organs) involved in production of antibody to ovalbumin detected in the sera after the antigen was administered as a single dose either by inhalation or systemically. After exposure to antigens, tissues (spleen, RT lymph nodes) were harvested from the exposed animals and assayed for the presence of antibody forming cells by the plaque assay described by Jerne et al. (1953). These results, summarized in Figures 3 and 4, indicate that antigen stimulation via the RT is not as efficient as systemic stimulation for inducing antibody production.

After sensitization by i.p. injection of antigen, neither IgM nor IgG could be considered to have been a major antibody found in RT tissue. The "maximum" PFC counts from RT tissue occurred at day four for IgM and at day eight for IgG. In the spleen the largest number of PFC counted were of the IgM class and could be measured easily within four days after i.p. injection and the number of PFC peaked at day six. Production of IgG by cells of the spleen was also evident by day four and peaked at days six to eight.

Table 7. PCA response in the rat sensitized to ovalbumin by either inhalation or i.p. injection and respiratory function in sensitized animals challenged with an exposure to ovalbumin aerosol.

Day After Treatment	Animal No.	Inhalation Exposure PCA[1]	ΔResp. Resis	ΔResp. Freq.	ΔTidal Vol.	Systemic Exposure PCA[1]	ΔResp. Resis	ΔResp. Freq.	ΔTidal Vol.
10	1	14	+.308	-25	+.55	33	Respiratory Spasms		
	2	22	Respiratory Spasms			41	Respiratory Spasms		
	3	19	Respiratory Spasms			38	Respiratory Spasms		
	4	32	Respiratory Spasms			38	Respiratory Spasms		
	5	40	-.010	+2	+10	36	Respiratory Spasms		
15	1	19	Respiratory Spasms			36	Respiratory Spasms		
	2	23	Respiratory Spasms			38	Respiratory Spasms		
	3	23	Respiratory Spasms			42	Respiratory Spasms		
	4	28	Respiratory Spasms			48	Respiratory Spasms		
	5	38	Respiratory Spasms			46	Respiratory Spasms		

[1]Average of duplicate determinations.

Figure 3. Mean values of direct (IgM) and indirect (IgG) plaque forming cells in respiratory tract lymphatic tissue (——) and spleen (---) from animals sensitized by a single i.p. injection of ovalbumin. Values are mean ± 1 S.E. n = 3 animals per time point.

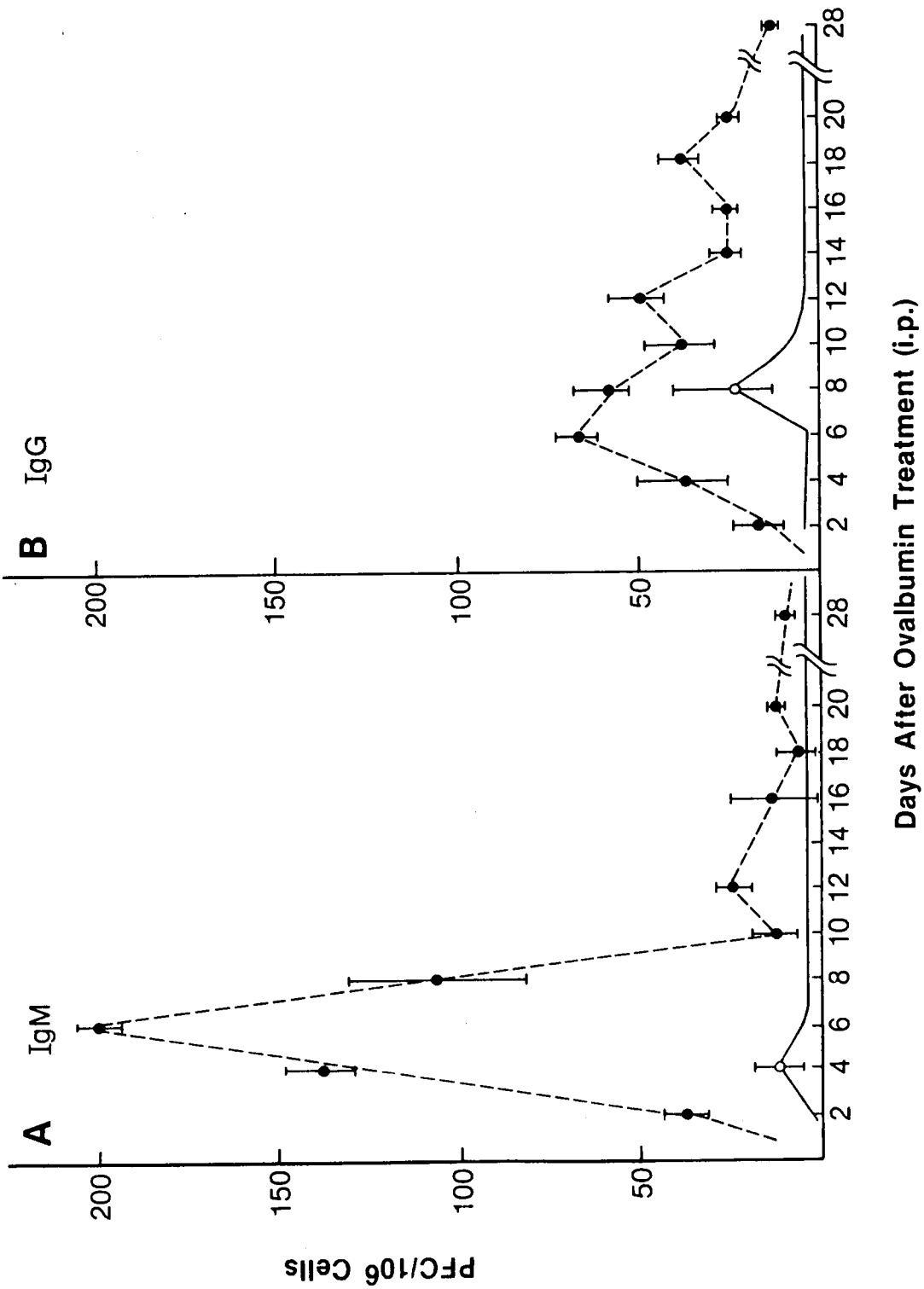
A
IgM
B
IgG
PFC/10⁶ Cells
200
150
100
50
2
4
6
8
10
12
14
16
18
20
28
Days After Ovalbumin Treatment (i.p.)

Figure 3.

Figure 4. Mean values of direct (IgM) and indirect (IgG) plaque forming cells in respiratory tract lymphatic tissue (——) and spleen (---) from animals sensitized by a single inhalation exposure to ovalbumin. Values are mean ± 1 S.E. n = 3 animals per time point.

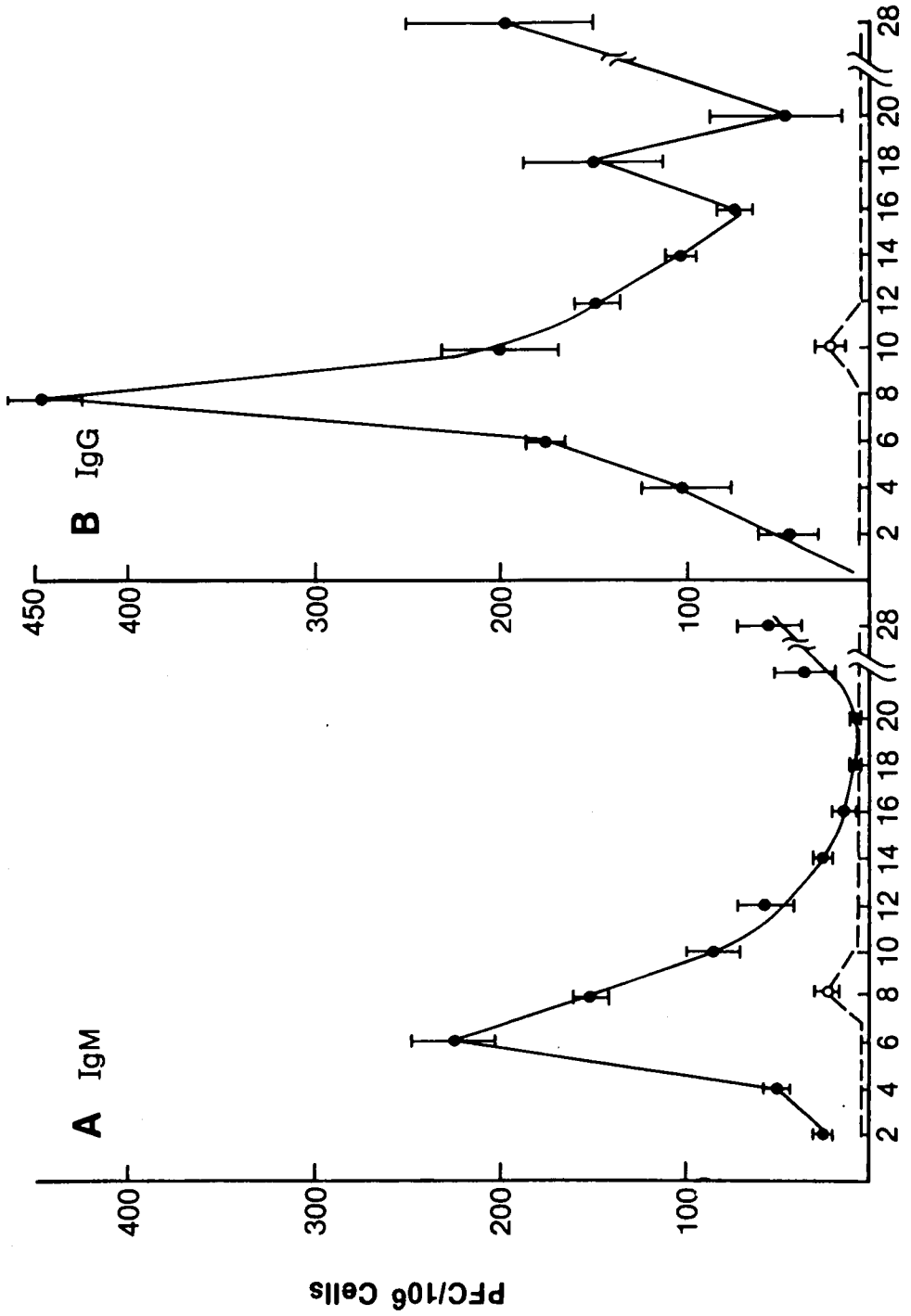
A IgM
B IgG
PFC/10⁶ Cells
Days After Ovalbumin Treatment (inhalation)
100
200
300
400
450
2 4 6 8 10 12 14 16 18 20 28

Figure 4.

After inhalation of antigen the greatest total response of RT tissue was in the production of antibodies of the IgG class (Figure 4). The peak response for this class of antibodies was at day eight. The number of PFC declined after this initial production until day 18-20 after which there was an inconsistent increase in PFC which reached a maximum on day 28. The peak response for IgM occurred at day six. Few PFC were observed in the spleen after inhalation of antigen. A slight increase in the number of PFC was observed at day eight for IgM and at day ten for IgG antibodies, but neither the IgG nor IgM response was paramount.

The next series of experiments were designed to study primary induction of IgE antibodies. In the presence of antigen, IgE antibody, fixed on mast cells and in the presence of antibody, is known to cause release of mediators. After interaction of IgE, antigen, and mast cell, a cascade of intracellular metabolic processes is triggered, resulting in the release of various mediators (depending on the species) that induce bronchoconstriction in the RT and/or alter vascular permeability. Therefore, it is reasonable to assume that following exposure to antigen, the presence of IgE in the respiratory tract should result in bronchoconstriction and serum IgE should be detectable by PCA assay. In these experiments that were designed to detect the presence of IgE, assays that would detect bronchoconstriction and/or vascular permeability were used.

Rats were exposed to ovalbumin by either inhalation or i.p. injection and then tested by PCA assay for the presence of IgE antibody

to ovalbumin (page 78). IgE antibody was detectable by the fifth day after treatment. Levels of IgE increased further by day 10 and then more or less plateaued through day 20. At this time the level of circulating IgE antibody to ovalbumin declined sharply by day 25, but remained detectable at low levels for at least 35 more days. This data is summarized in Figure 5. For brevity only the results from primary sensitization by inhalation are included in that figure.

Next, the effect of mode of antigen administration on the presence of IgE antibody in the RT was determined. Animals were first exposed once to ovalbumin either by inhalation or i.p. injection. After sensitization animals were arbitrarily chosen, bled by retroarbital bleeding and serum collected for determination of PCA titers. These animals were then challenged at either 10 or 15 days later by inhalation exposure to ovalbumin aerosol, at which time their respiratory resistance, respiratory frequency and tidal volume were measured. These results are summarized in Table 5, page 63. This data indicates that a primary IgE response can be illicited in the RT of the rat by a single antigen exposure. In addition, after primary sensitization IgE antibody is present in approximately equal amounts both locally (RT) and systemically (sera) regardless if the antigen exposure is by inhalation or by i.p. injection.

However, a definitive correlation between PCA titers and the respiratory response cannot be made because the respiratory response (i.e. measured respiratory mechanics) is highly irregular and unpredictable. The unpredictable respiratory response is not due to

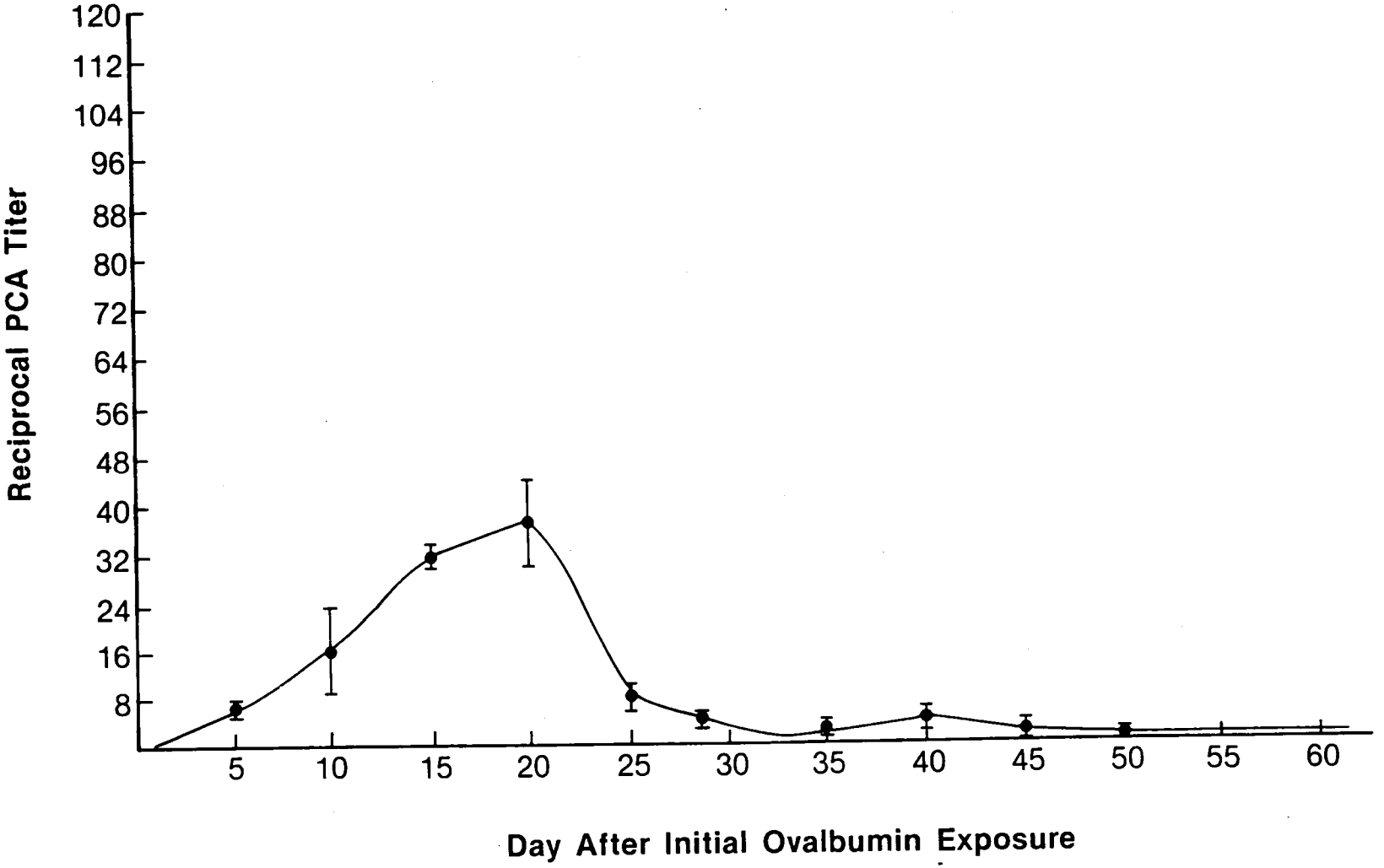

Figure 5. Mean values of PCA titers from animals sensitized by one six hour inhalation exposure to ovalbumin. Values are mean ± 1 S.E. n = 3 animals per time point.

differential sensitization to ovalbumin because PCA titers are rather reproducible, indicating adequate and relatively uniform sensitization. More likely, the irregular respiratory response is the result of the rat's poor tracheo-broncheal musculature. Since altered respiratory resistance is almost entirely due to bronchoconstriction, the poorly developed musculature of the RT of the rat will not permit readily quantifiable graded responses, and one effectively sees all-or-none responses exclusively (Table 5, p. 63).

Secondary Antibody Response

The following series of experiments were designed to determine whether prior sensitization via the respiratory tract, which induces only a weak primary antibody response, would prime the rat for a secondary antigen challenge. Animals were first sensitized (primed) by exposure to an ovalbumin aerosol and then at the designated time challenged with ovalbumin either by aerosol exposure or i.v. injection. Only the primary antigen exposure was accompanied by i.p. injection of B. pertussis. The results are summarized in Figure 6 and Figure 7.

Inhalation exposure

Respiratory tract tissue. Following a secondary challenge with an ovalbumin aerosol antigen, the prime response of RT tissue was in IgG PFC (Figure 6) although the number of IgM PFC was increased relative to that observed after primary sensitization. The number of IgM PFC after primary sensitization was approximately 200 while after secondary antigen

Figure 6. Mean values of A. direct (IgM) and B. indirect (IgG) plaque forming cells in respiratory tract lymphatic tissue (——) and spleen (---) from animals challenged with a single six hour ovalbumin aerosol four weeks after primary sensitization by a single six hour inhalation of ovalbumin. Values are mean ± 1 S.E. n = 3 animals per time point.

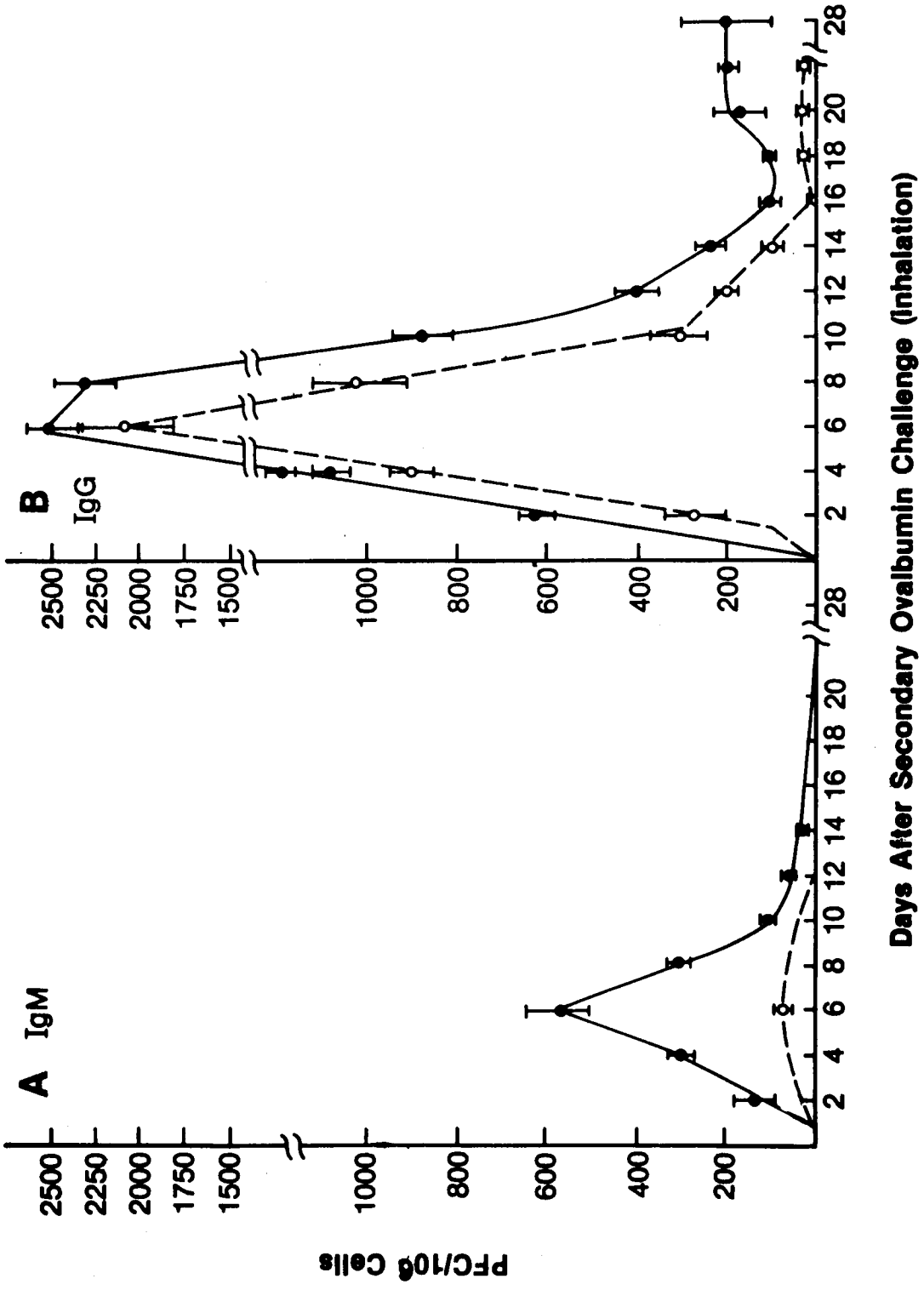
A IgM
B IgG
PFC/10⁶ Cells
2500
2250
2000
1750
1500
1000
800
600
400
200
2 4 6 8 10 12 14 16 18 20 28
Days After Secondary Ovalbumin Challenge (Inhalation)

Figure 6.

Figure 7. Mean values of A. direct (IgM) and B. indirect (IgG) plaque forming cells in respiratory tract lymphatic tissue (——) and spleen (---) from animals challenged with an i.p. injection of ovalbumin four weeks after primary sensitization by a single six hour inhalation exposure to ovalbumin. Values are mean ± 1 S.E. n = 3 animals per time point.

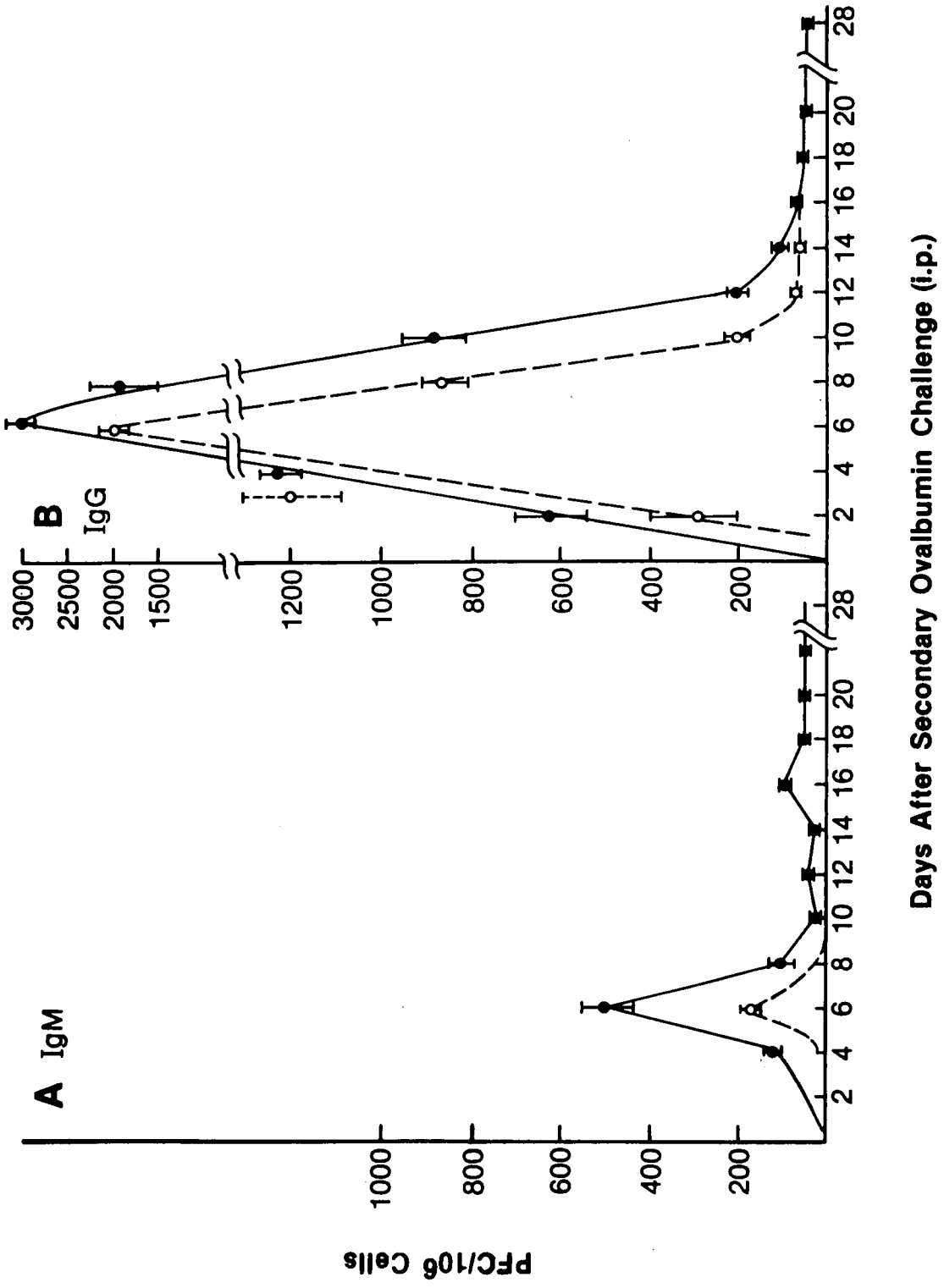
A IgM
B IgG
PFC/10⁶ Cells
Days After Secondary Ovalbumin Challenge (i.p.)

Figure 7.

challenge the number of IgM PFC was approximately 600. The time of the peak response by IgM PFC remained the same for both primary and secondary antigen exposure; about day six. In contrast, the IgG response, in addition to being more pronounced, was somewhat accelerated. After the first antigen exposure the number of IgG PFC was ∿450 and the number peaked at day eight. After the second antigen exposure the number of IgG PFC was ∿2500 and peaked at day six.

Spleen. Although the IgM response in the spleen was similar whether the antigen challenge was primary or secondary, the IgG response was significantly more pronounced after the second challenge. After primary sensitization, the greatest number of IgG PFC is observed at day 10 after sensitization but is <50 cells. After the secondary antigen challenge the number of IgG PFC is >2000 and shows a definite peak at day six.

Systemic exposure

The response of both the RT and spleen tissue after secondary antigen exposure indicates that elicitation of a primary response via the respiratory tract results in priming of the sensitized animal both locally (RT) and systemically (spleen) (Figure 7).

Respiratory tract tissue. The number of IgM PFC in RT tissue is essentially the same whether measured after the inhalation or i.p. secondary antigen treatment; values of ∿600 PFC after primary sensitization and ∿500 PFC after secondary i.p. antigen exposure.

After primary i.p. challenge the IgM response of the RT is minimal (<15 PFC) and the IgG response, although greater than the IgM, is also slight with <25 IgG PFC (Figure 3, p. 67). By contrast, after secondary i.p. antigen challenge the number of IgM PFC in RT tissue is increased to approximately 200 and there is a definite peak in the number of cells at day six. Although this secondary IgM response of RT tissue is less than the secondary IgM response of the spleen (∿500 IgM PFC) it is equal to the primary IgM response of the spleen after initial sensitization. In even greater contrast, the number of IgG PFC in RT tissue after secondary i.p. antigen challenge is ∿2000 cells versus ∿25 cells after primary i.p. sensitization. The secondary IgM response of RT tissue is also greater than the peak IgG response of the spleen after primary sensitization (∿65 versus 2000 IgG PFC).

Spleen. As in the RT, the number of IgM PFC is approximately the same after primary and secondary i.p. antigen exposure. After primary i.p. antigen exposure the number of IgM PFC is slightly >200 and after secondary i.p. antigen exposure slightly <200. The number of IgG PFC after primary i.p. antigen exposure was ∿60, peaking on day eight but after secondary i.p. antigen exposure there were ∿2000 PFC which number peaked on day six.

IgE. The response of primed animals receiving a second challenge with ovalbumin was essentially the same as observed after primary sensitization, except that PCA titers were somewhat higher and peaked somewhat earlier (Figure 8). For convenience, this data together with

Figure 8. Mean values of PCA titers from animals sensitized by one six hour inhalation exposure (——) and from animals challenged by one six hour inhalation exposure (---) 28 days after primary sensitization. Values are mean ± 1 S.E. n = 3 animals per time point.

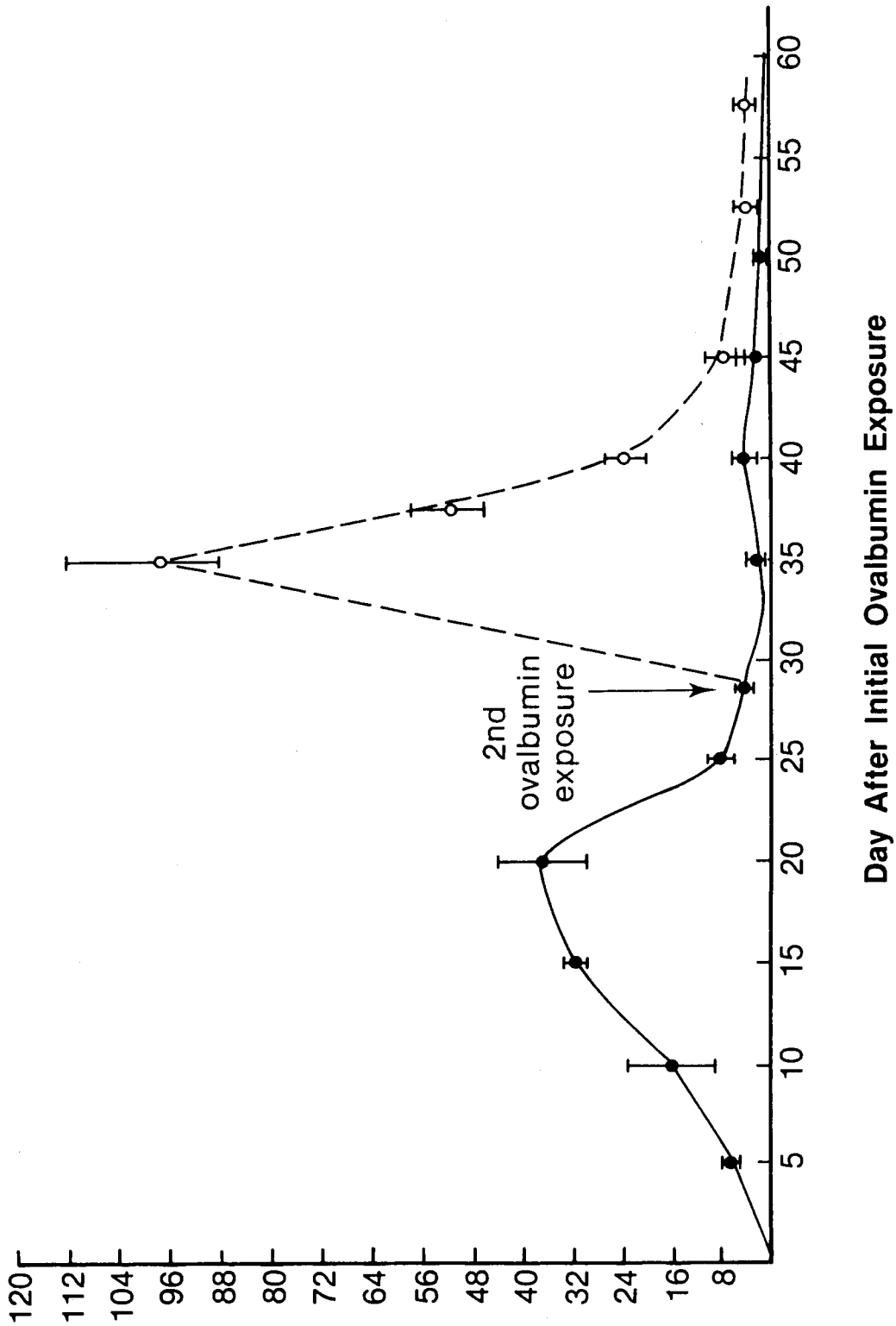
Reciprocal PCA Titer
120
112
104
96
88
80
72
64
56
48
40
32
24
16
8
5
10
15
20
25
30
35
40
45
50
55
60
2nd ovalbumin exposure
Day After Initial Ovalbumin Exposure

Figure 8.

PCA data from primary sensitization is summarized in Figure 8. After primary sensitization, IgE can be detected in rats by day five. Fifteen days after sensitization, the PCA titers have tripled, remain elevated through day 20 and by day 30, have decreased to levels observed at day five. Animals that are rechallenged with antigen 30 days after primary sensitization have an increase in PCA titers at day five after the secondary challenge that is triple the maximum peak response after primary antigen exposure. This is followed by a rather sharp decline in PCA titers to prechallenge levels by day 15 after secondary challenge. Rats not rechallenged show a gradual decline in PCA titers through day 55. As was observed after primary sensitization, respiratory parameters could not be measured after secondary antigen challenge because of respiratory spasm (data not shown).

Splenectomized Animals

In an effort to determine, in part at least, the major organ(s) responsible for the primary and secondary immune responses, rats were splenectomized. Some thirty rats were splenectomized two weeks before primary stimulation and 30 others were splenectomized two weeks after primary (but before secondary) stimulation. In a preliminary study, five extra splenectomized rats were injected i.p. with ovalbumin as a control against the possibility that RT or other lymphoid tissue might be responsible for any detected response after systemic antigen exposure. No PFC from RT tissue nor serum HA titers against ovalbumin were detected (data not shown). Animals were killed at 4, 7, 11, 14 and 21

days after exposure to the ovalbumin aerosol and direct and indirect PFC from RT lymph nodes plus serum HA titers to ovalbumin determined. Neither serum HA titers against ovalbumin nor PFC from RT tissue could be detected.

Next, animals were initially primed either by inhalation or i.p. injection of ovalbumin and splenectomized two weeks later. They were then challenged two weeks after splenectomy with an ovalbumin aerosol and RT tissue PFC and serum HA titers determined 4, 7, 11, 14 and 21 days later. This data is tabulated in Table 8. Splenectomy significantly reduced both the serum HA and PFC response but did not abolish it. The pattern of the immune response is similar. Peak response occurs between days 7 and 14 in both splenectomized and nonsplenectomized rats but the response is only about 1/3 for both serum HA titers and PFC from RT tissue.

Table 8. Effect of splenectomy on secondary immune response in RT lymphatic tissue from animals initially primed via respiratory tract.

Day After 2nd Antigen Treatment	Nonspleenectomized Animals			Spleenectomized Animals		
		PFC (RT)			PFC (RT)	
	HA Titer	Direct	Indirect	HA Titer	Direct	Indirect
4	80	32	800	<80	<5	58
7	320	409	3200	<80	52	800
11	640	20	700	320	8	25
14	640	18	110	320	<5	<5
21	320	No	No	160	<5	<5

Data is from pooled samples; 3-5 animals per time point.

IV. DISCUSSION

It is generally accepted that rats are difficult to sensitize with soluble antigens, such as ovalbumin, without adjuvant (Mota, 1964). Rats used in this study were SPF rats which normally have a respiratory tract sparsely populated with lymphoid tissue, but are capable of producing a full complement of RT lymphoid tissue if exposed to respiratory disease. This situation is extremely advantageous for a study of the immunology of the respiratory tract, since respiratory tract tissue of SPF rats is initially relatively devoid of immunocompetent tissue. Ovalbumin, a soluble protein antigen, was used in this study because a water soluble material with a relatively low density and low vapor pressure was needed to produce aerosols with a narrow size range.

When an animal is exposed to an aerosol, only that portion of the aerosol actually deposited in the RT is effective in illiciting a physiological response. In an attempt to estimate the effective dose of ovalbumin in these studies, rats were exposed to an ovalbumin aerosol radiolabeled with $^{51}Cr_2O_3$. The ^{51}Cr was used as a marker for deposited ovalbumin and also used to trace any translocation of ovalbumin particles from the RT to other tissue. When rats were exposed to the $^{51}Cr_2O_3$ tagged ovalbumin aerosol, it was determined that approximately 20.0 mg (range 20.1 mg to 21.9 mg) of ovalbumin was deposited in the exposed animals (Table 3, page 59). Calculations based on an aerosol mass concentration of 20 mg/m^3 and an animal minute volume of 90 cc/min show that a deposition of 20.0 mg of ovalbumin is approximately 30% deposition

efficiency, which is in agreement with other studies (Task Group on Lung Dynamics, 1966; Creasia et al., 1973; Creasia et al., 1976). Of the total deposited, approximately 6.0 mg of ovalbumin is deposited in the deep lung. The remaining 14.0 mg (70%) of ovalbumin is deposited in the upper RT, mainly the nasal pharynx area, where it is quickly cleared and translocated to the gastrointestinal tract. Since ovalbumin that was translocated to the gastrointestinal tract could have been the source of antigen responsible for sensitization, rats were gastric intubated with 10.0 mg each of ovalbumin and the animals tested for sensitization against ovalbumin. No evidence for sensitization was observed when these animals were tested by either HA or PCA assays for sensitization to ovalbumin. Thus it is assumed that ovalbumin deposited in the lower RT (i.e. deep lung) is responsible for sensitization. This data, indicating that rats can be sensitized by either systemic or topical (RT) administration of ovalbumin without adjuvant, is summarized in Tables 1 and 2, pages 56 and 57. Sensitization, however, required a relatively large dose of antigen and repeated exposures for at least two weeks. This treatment regime produced a minimal immunological response with serum antibody titers usually in the range of 1:400-600 and almost always <1:1000. When tissue from these same animals were tested for the presence of PFC, both direct and indirect PFC could be detected in cells from RT lymphnodes, from the spleen and in free RT cells obtained by lavage (Table 5, page 63). The number of PFC from the spleen were quantitatively higher than the number of PFC obtained from either RT lymphoid tissue or by RT lavage. Neither cells secreting IgM nor cells secreting IgG were predominant in any of the tissue tested.

When rats were first primed with killed B. pertussis vaccine prior to ovalbumin exposure, a positive antibody response to ovalbumin could be detected even after a single antigen exposure whether the exposure was by inhalation or by i.p. injection. Consistent with the responses observed after multiple antigen exposures, however, the serum antibody titers were relatively low although sufficient for these studies (Table 6, page 65).

When B. pertussis was used as an adjuvant just prior to i.p. injection of ovalbumin, direct and indirect PFC were observed in both the spleen and RT lymphatic tissues. The number of PFC/10^6 cells from the spleen were quantitatively higher than the number of PFC from the RT. The number of spleen cells secreting IgM also predominated over the number of spleen cells secreting IgG. In the RT, although the actual number of IgG secreting cells is greater than that secreting IgM, the number of cells of both are so low as to preclude any meaningful interpretation except to note their presence. These results are qualitatively similar to those of comparable studies using whole SRBC (insoluble antigen) (Sell et al., 1970; Wortis et al., 1969). Sensitization with soluble ovalbumin did, however, influence the time course of the immune response, since peak formation of IgM and IgG, as observed in the studies reported here, occurred two to three days later than that reported for whole SRBC.

The same studies with SRBC also describe a phasic response within the 7s immunoglobulin classes (IgG and IgA). Although fluctuations in IgG secreting PFC were observed in this study, a true phasic response could not be attributed to the data following a single injection of

ovalbumin. Differences in data between the studies using SRBC and these studies could be attributed to the physical state (solubilized) as well as the concentration of the antigen. Additionally, the lack of a phasic response could also be due to the fact that PFC determinations were not made on a daily basis but it is felt that this is unlikely.

Similar to the response observed in rats sensitized to ovalbumin by i.p. injection, direct and indirect PFC were also observed in both the spleen and RT lymphatic tissue from rats sensitized by exposure to an ovalbumin aerosol. However, in contrast to the response observed after i.p. administration of ovalbumin, the overwhelming majority of PFC observed after aerosol administration was found in RT tissue rather than in the spleen. Also in contrast, inhalation exposure induced the production of predominantly IgG secreting PFC rather than the IgM secreting PFC observed after i.p. injection of ovalbumin. In the above described animals sensitized by inhalation to ovalbumin differences in the number of direct and indirect PFC from the spleen are too low to be interpreted with meaning except to note their presence.

Thus it appears from these studies that the major site (RT lymphatic tissue versus spleen) of serum antibody production is dependent on the conditions under which rats are sensitized to ovalbumin. When rats are sensitized without adjuvant to ovalbumin by repeated exposure (necessary for sensitization) to ovalbumin, the spleen and not RT lymphatic tissue is the major site of serum antibody production, regardless of whether ovalbumin exposure is local (inhalation) or systemic (i.p. injection). Additionally, rats that are sensitized by repeated

inhalation exposure do not exhibit obvious respiratory distress in any of the repeated inhalation exposures for up to 13 exposures. On the other hand, when rats are sensitized to ovalbumin by a single ovalbumin treatment with adjuvant, the predominant site of serum antibody production is dependent on the route of ovalbumin administration. Respiratory lymphatic tissue is the major site for serum antibody production in rats that have been sensitized by inhalation of ovalbumin and comparatively few PFC are found in the spleen, rather than RT lymphatic tissue, from rats sensitized to ovalbumin by a single i.p. injection of ovalbumin and adjuvant. Also, in contrast to observations following multiple ovalbumin treatment, rats sensitized by a single ovalbumin treatment exhibit very obvious respiratory distress if exposed to an ovalbumin aerosol after sensitization.

Regardless of the mode and site of ovalbumin administration in these studies, the measured sensitization to ovalbumin was, overall, relatively mild. The most effective sensitization both for RT tissue and for the spleen was obtained by a single treatment (inhalation or i.p. injection) of ovalbumin with adjuvant. With this mode of antigen administration the number of PFC in the immunologically stimulated spleen was quantitatively higher than in immunologically stimulated RT tissue. It is possible therefore, that failure to detect PFC in RT tissue following multiple inhalation exposures to ovalbumin without prior treatment was not due to an inability of RT tissue to be sensitized but because ovalbumin treatment remained below the threshhold for detectable sensitization. This reasoning is consistent with the observation of no

respiratory distress in rats that are being exposed to an ovalbumin aerosol and known to be immunized (HA titer) against ovalbumin.

The data, therefore, both from the multiple (without adjuvant) and single (with adjuvant) ovalbumin administration is indicative of and consistent with the concept of a primary separation of RT and systemic (spleen) immunocompetent tissue. The findings in these studies are consistent with the findings reported from other studies (Holub and Hauser, 1969) in which SRBC (insoluble antigen) was injected directly into pulmonary tissue.

Although a true phasic PFC response as reported by other authors (Holub and Hauser, 1969) using nonsolubilized antigen was not noted, numbers of indirect PFC (IgG) from both RT lymphatic and spleen tissue did exhibit secondary "peaks" on days 12 and 18 after injection. While it is possible that these peaks could be the result of homeostatic control mechanisms (Crabbe et al., 1969; Bazin et al., 1970), leakage of intact molecules from the lung (Nash and Holle, 1973; Shultze et al., 1963; Meyer et al., 1964), as well as a slow redistribution of phagocytized material from the RT (Holub and Hauser, 1969; Drinker et al., 1937), also remain as distinct possibilities. The lack of a heightened response in this "second response," in contrast to the classical secondary response, suggests that the amount of antigen being released either directly or indirectly is minimal. Increases in both IgM and IgG immunoglobulin classes occur at about the same time, suggesting an accelerated response in both the RT and spleen for IgG production.

Levels of serum hemagglutinins were quantitatively different when induced by either an inhalation (lower) or i.p. (higher) dose of antigen. These results are in apparent contradiction to other similar designed studies using intratracheal intubation methods instead of inhalation. This apparent difference is conceivably due to the different doses and distribution of antigen imparted by the intratracheal intubation method as compared to inhalation exposures. More likely, however, is the distinct possibility that the intratracheal intubation method, in which antigen is suspended in a liquid media, created abnormal transpulmonary pressures between alveolar walls and pulmonary capillaries, allowing the "escape" of antigen directly from the lungs to the circulatory system. Sensitization by aerosol inhalation does not suffer from these possible methodological artifacts.

Findings of PFC in RT tissue, plus evidence of serum antibodies indicate that antibody formed in the RT lymphatic tissue contributes to the total serum antibody concentration. The data from these studies do not rule out, however, that stimulation of PFC occurs in the spleen and that these stimulated cells "home" to RT lymphatic tissue.

Another series of experiments were designed to test for antibody directly related to anaphylaxis. Rats have the capability to synthesize an immunoglobulin, termed mast cell sensitizing (Mota, 1961), which has been defined by its immunochemical analogy to and unique functional characteristics similar to human IgE. In the human, IgE is generally believed responsible for the skin sensitization and respiratory distress (anaphylaxis) due to exposure to antigen. This portion of the study

reports on and describes the elaborations of rat IgE antibody to ovalbumin immunization after either aerosol or i.p. antigen administration. The IgE antibody produced after ovalbumin immunization was heat-labile and possessed long latency in skin sites. It persisted in the circulation of the sensitized rat for a relatively long period (up to 60 days), and IgE antibody production was enhanced on subsequent resensitization by ovalbumin aerosol. Initiation of IgE antibody production required i.p. administration of B. pertussis vaccine as an adjuvant.

When rats were exposed to ovalbumin by either inhalation or i.p. injections, circulating IgE antibodies to ovalbumin were detectable at about the same levels regardless of the route of antigen administration. The fact that PCA titers were equivalent regardless of the mode of antigen administration suggests that either IgE antibody synthesized at one site contributes to total serum IgE (and that there is no "barrier" to subsequent antibody) or, that the quantity of antigen administered was sufficient to stimulate multiple sites. Based on the rate of production of IgM and IgG antibodies to ovalbumin observed in these studies, the latter hypothesis is less tenuous, unless one postulated further that cells responsible for IgE production are much more sensitive to ovalbumin than are cells responsible for either IgM or IgG production.

When rats initially primed by either inhalation or i.p. administration of ovalbumin were subsequently challenged 10 or 15 days later by inhalation and measurement of selected respiratory parameters attempted, it was found that, at least in the rat, it was almost impossible to measure a graded response to antigen challenge. The inability to illicit a graded

bronchoconstriction response is believed to be due to the poor tracheobronchial musculature in rats. This sparse musculature precludes development of graded bronchoconstriction, developing into an all-or-none response to the release of bronchoconstricting mediators by mast cells.

When similar rats were challenged by i.p. administration of ovalbumin 28 days after primary sensitization by inhalation, PCA assays indicated enhanced IgE production. This secondary response is more rapid than that observed after primary sensitization and is at least triple the magnitude of the primary response. As observed 10 and 15 days after primary sensitization, respiratory parameters could not be measured on day 28.

Taken as a group, these observations indicate that IgE antibody is distributed rather uniformly throughout the rat. That the initial burst of IgE production after primary sensitization is followed by a rapid return to low levels characteristic of unchallenged SPF rats indicates either that the rat is extremely sensitive to release of "mediators" from only a few mast cells, or that memory cells primed for the quick production of specific IgE antibody migrate and relocate throughout the organism. This hypothesis could be easily tested with radiolabeled precursors to IgE antibody.

Secondary IgM and IgG Response

Rats previously sensitized to ovalbumin exhibit a true, typical secondary immune response when reexposed to ovalbumin antigen. Indirect

PFC appear sooner and the number of PFC peak earlier than after primary sensitization. This phenomenon occurs in both the spleen and RT regardless of whether or not the second antigen challenge is via the RT (aerosol) or systemic (i.p.). Of particular interest in the secondary immune response is the large PFC response in the RT when the second antigen challenge is systemic and the large spleenic PFC response when antigen challenge is via the RT. This is in direct contrast with the small PFC response in the RT when primary stimulation is systemic and to the small spleenic PFC response when primary stimulation is via the RT. These observed differences between primary and secondary immune responses are indicative of memory cells in both the spleen and RT tissue.

Two critical observations should now be reemphasized and considered together. First, a PFC response in RT tissue and the spleen was detected only when primary sensitization was by inhalation for the former and by i.p. injection for the latter. Second, a secondary PFC response is observed in both RT tissue and spleen in sensitized rats when reexposed to antigen regardless of the route of antigen administration (i.e., i.p. injection or inhalation). The first observation appears to indicate a clear separation of RT immunocompetent tissue and spleen while the second point appears to indicate cooperation between the two immunocompetent sites. The two observations need not be contradictory, however, and may even be consistent with each other if either of at least two events have occurred after primary stimulation: (1) memory cells initially in one organ have migrated to the other, or (2) a given route of antigen administration produces a minimal immune response in one or the other

organ that though initially undetected gives rise to a committed cell population. These events need not be all inclusive nor necessarily exclusive of each other. If migration of committed cells (memory cells) is responsible for the observed phenomena, then splenectomized sensitized animals should abolish the secondary immune response. On the other hand if there is a resident population of committed cells (but below detection limits) splenectomy should have little effect.

Splenectomized Rats

If rats are splenectomized two weeks prior to primary antigen stimulation via the RT, the immune response, with regard to both the PFC response and serum HA titer is minimal. When rats are splenectomized two weeks after primary sensitization, the secondary immune response is also much reduced as compared to that observed in nonsplenectomized rats, but it is still detectable in both the number of PFC from RT tissue and from serum HA titers. These observations are consistent with the concept that some memory cells are located in RT lymphoid tissue. Further, antibody formed in the RT lymph nodes after stimulation of these memory cells (secondary response) contributes to the total serum concentration of immunoglobulin. The fact that the primary immune response following inhalation of ovalbumin is reduced in splenectomized SPF rats suggests that "stem" cells for RT lymphoid tissue is, in part at least, derived from the spleen. Additionally, the observation that the secondary immune response is also reduced in splenectomized rats suggests that although some memory cells are produced and remain in RT lymphoid tissue, control

of the number of secondary PFC in RT tissue is under control of the spleen.

The data from this series of experiments have both physiological and clinical implications. As stated earlier, inhaled antigens which are deposited in the respiratory tract are capable of stimulating both a systemic and local pulmonary immune response. However, while a wide variety of airborne antigens stimulate detectable immune responses after deposition in the lung, it appears that only some antigens produce lung disease (hypersensitivity pneumonitis) and/or respiratory distress (anaphylactic response). Susceptibility to these respiratory problems occurs in a random, unpredictable fashion among individuals at risk in a given environment. Classical examples of this group of respiratory disorders are "farmer's lung," "pigeon breeder's disease," and the susceptability of certain young children to respiratory infection followed by respiratory complications. Epidemiological studies with the above groups have invariably indicated SIgA deficiency in the young children and, although equivocal, data which suggests an SIgA deficiency (or low normal levels) in persons suffering from farmer's lung or pigeon breeder's disease. When medical complications develop in individuals suffering from these respiratory disorders, examination invariably reveals immune complex, most often of the IgG-antigen type, deposits in lung tissues. It is postulated that the lack, or deficiency, of SIgA in the RT allows inhaled antigen to stimulate a systemic immune response of IgM and IgG antibodies which are capable of fixing and activating complement. These circulating antibodies encounter antigen deposited in the RT, complex

with the antigen and activate complement. The activation of complement results in local inflammation and clinical symptoms generally classed as "extrinsic allergic alveolitis."

The general hypothesis concerning this condition is that nonsusceptible individuals have the capacity for production of sufficient SIgA to protect the systemic immune system from external antibody stimulation. Susceptible individuals lack normal capacity for production of SIgA. This condition allows for stimulation of the systemic immune response by antigen deposited in the RT (external antibody) and results in a pathogenic condition in the lung.

This mechanism of protection afforded by SIgA has been fruitfully studied and verified as operative in the gastrointestinal tract. Until very recently, however, attempts to develop animal models to study the protective role of SIgA in the RT has often either proven unsuccessful or investigators have had to resort to extremely unphysiological conditions such as intratracheal intubation or transpulmonary injections for the deposition of antigen in the RT. Even in studies in which aerosolized antigens are used, the method of aerosol generation is usually one that produces relatively large particles which primarily deposit in the upper RT leaving the alveolar area relatively free of antigen. The studies described in this report suffer none of the above mechanical deficiencies and, in addition, the use of SPF rats (relatively devoid of RT lymphoid tissue and hence immunologically naive in the RT) minimized the possibility of extraneous data resulting from cross-reacting antibodies. The data from these studies also suggest, under the conditions of these studies, a

minimal protective role against respiratory disease for SIgA in the RT.

In theory, antigenic stimulation in the RT should result in increased production of SIgA. The SIgA combines with the inhaled antigen, thus effectively neutralizing, externally, the inhaled allergin and inhibiting stimulation of the systemic immune response. If SIgA is effective, the theory implies that sensitization via the RT should not stimulate production of IgM and IgG PFC in the spleen and serum antibodies of the IgM and IgG class should be absent or at least at minimal levels. Conversely, the degree of sensitization by i.p. injection should not be affected by the level of RT SIgA. The data (Table 5, page 63) from this study indicate that stimulation of cells from local RT tissue to produce IgM and IgG is essentially the same whether antigen exposure is by inhalation or by i.p. injection. The number of splenic cells, however, producing IgM and/or IgG is increased if the route of antigen exposure is systemic rather than by inhalation; this difference is attributed more to concentration of antigen rather than protection by SIgA, although a protective role for SIgA cannot be ruled out. It should be noted that, although the site and number of PFC is altered when antigen stimulation is systemic, the level of circulating IgM and IgG antibodies is only slightly less when antigen exposure is by inhalation rather than i.p. injection. Thus when ovalbumin antigen is administered either by inhalation or by i.p. injection <u>without adjuvant</u> (1) an immune response, represented by IgM and IgG but not IgE antibodies, is stimulated; (2) the number of stimulated PFC in RT lymphoid tissue is independent of the route of antigen

administration while the number of stimulated PFC in the spleen is dependent on the route of antigen administration and (3) no local inflammation or tissue reaction in the RT of sensitized animals was observed. While the absence of local inflammation and an anaphylactic response could easily be attributed to the protective action of RT SIgA, it is difficult to concieve of a mode of action for SIgA that would allow for nonlocal sensitization to ovalbumin (increased RT and spleen PFC; circulating IgM and IgG antibodies to ovalbumin) but protect from local response. A more plausible explanation is the protection afforded by alveolar macrophages which quickly phagocytize inhaled particles deposited in the alveolar region and transport them from the RT. The majority of antigen would thus be immunologically neutralized within the alveolar macrophage, transported to the gastrointestinal tract and removed from the organism. The small amount of antigen transported within macrophages to lymphoid tissue could account for the low grade immune response that was observed.

When animals were sensitized by either inhalation or i.p. injection of ovalbumin antigen plus adjuvant (B. pertussis) the site (RT vs spleen) at which the greatest number of PFC were observed was dependent on the route of antigen exposure after primary sensitization but independent following secondary antigen challenge. From the results observed during initial sensitization (i.e. primarily a local response) it is easy to speculate on some sort of immunologically protective mechanism, such as that afforded to SIgA, by which a systemic immune response is inhibited. However, if the observations are carried through to the results after

secondary sensitization (i.e. local plus systemic response) it is difficult to properly evaluate a physiological system that would initially inhibit an immune response and then enhance the response upon rechallenge. Logically, it would seem that if ovalbumin-specific SIgA was to antigenically neutralize the ovalbumin, then, after primary sensitization, and presumably enhanced ovalbumin-specific SIgA production capability, a secondary challenge with ovalbumin would result in less production of circulating antibodies and less (or the same) number of PFC in the spleen. Considering all the data as a whole, although ovalbumin-specific SIgA was not measured in these studies and there is no reason to assume that the animals did not respond to inhaled ovalbumin aerosol by increased SIgA production, it is possible (probable) that the age and initial SPF status of the animals (immunocompetently naive and extremely sensitive to RT infection) did not allow for the initial and prompt SIgA response to inhaled antigen. This in no way should detract, regardless of the presence or absence of SIgA, from the observation that the anaphylactic response in the RT is obligatorally dependent on, and that both the anaphylactic response and location of the humorial (IgM and IgG) response of the RT is modified by a nonspecific adjuvant (B. pertussis in these studies) administered at an entirely nonrelated site to antigen administration; and that the action of the adjuvant is manifested through or by the spleen. Thus it is plausible from the data from these studies to speculate that individuals not susceptible to RT distress from inhaled antigens are nonresponders because they have not been exposed to airborne antigen at a critical

time of lymphocyte proliferation. Conversely, susceptible individuals conceivably were exposed either simultaneously to airborne antigen plus phytomitogen or antigen alone at a time of nonrelated lymphocyte proliferation. There is no reason to believe the above hypothesis is either independent of or is exclusive of the role of SIgA in the RT. However, the above hypothesis could explain the sudden onset of susceptibility of previously nonsusceptible individuals to a specific antigen. The data from these studies could also help to explain why some individuals have serum antibodies of the IgM and IgG class to inhaled allergins while others have only IgE (or IgA). The latter individuals would be free from chronic inflammatory lung damage (hypersensitivity pneumonitis) but may undergo an anaphylactic response while the former may or may not undergo anaphylaxsis but could suffer chronic inflammatory disease from IgG-antibody complexes in the lung.

REFERENCES

REFERENCES

Ada, G., and Parish, C. R. 1968. Low dose tolerance to bacterial flagellin in adult rats: A possible role for antigen localized in lymphoid follicles. Proc. Nat. Acad. U.S. 61:556.

Ada, G., and Byrt, P. N. 1969. Specific inactivation of antigen-reactive cells with ^{125}I labelled antigen. Nature 222:1291.

Amdur, M. O., and Mead, J. 1958. Mechanics of respiration in unanesthetized guinea pigs. Amer. J. Physiol. 192:364-368.

Armstrong, W. D., Diener, E., and Shellam, G. R. 1970. Antigen-reactive cells in normal, immunized and tolerant mice. J. Exp. Med. 129:393.

Bach, F. H., Alter, B. J., Solliday, S., Zoschke, D. C., and Janis, M. 1970. Lymphocyte reactivity in vitro. II. Soluble reconstituting factor permitting response of harmful lymphocytes. Cell. Immun. 1:219.

Bauer, D. C., Mathies, M. J., and Stavitsky, A. B. 1963. Sequences of synthesis of macroglobulin and gamma-globulin antibodies during primary and secondary responses to proteins, Salmonella antigens, and phage. J. Exp. Med. 117:889.

Bazin, H., Levi, G., and Doria, G. 1970. Predominant contribution of IgA antibody-forming cells to an immune response detected in extraintestinal lymphoid tissues of germ-free mice exposed to antigen by the oral route. J. Immunol. 105:1049.

Benedict, A. A., Brown, R. J., and Ayengar, R. 1962. Physical properties of antibody to bovine serum albumin as demonstrated by hemoglutination. J. Exp. Med. 115:195.

Bennich, H., and Johansson, S. G. O. 1971. Structure and function of human immunoglobulin E. Adv. Immunol. 13:1.

Bensch, K. G., Dominguez, E., Meyer, E. C., and Liebow, A. A. 1970. Studies on the pulmonary air-tissue barrier: Absorption of macromolecules by the alveolar wall. Am. Rev. Respir. Dis. 101:439.

Bensch, K. G., and Dominguez, E. A. 1971. Studies on the pulmonary air-tissue barrier. Part IV. Cytochemical tracing of macromolecules during absorption. Yale J. Biol. Med. 43:236.

Bienenstock, J., Johnston, N., and Perey, D. Y. E. 1973. Bronchial lymphoid tissue. I. Morphologic characteristics. Lab. Invest. 28:686.

Brain, J. D., and Frank, N. R. 1968. Recovery of free cells from rat lungs by repeated washings. J. Appl. Physiol. 25:65.

Brick, J. O., Newell, R. F., and Doherty, D. G. 1969. A barrier system for a breeding and experimental rodent colony: Description and operation. Lab. Anim. Care 19:92-97.

Cerny, J., Waner, E. B., and Rubin, A. S. 1975. T-cell products activating stem cells: Further studies on the origin and action of the factor(s). J. Immunol. 115:513.

Chinard, F. P., Enns, T., and Nolan, M. F. 1962. Permeability characteristics of the alveolarcapillary barrier. Trans. Assoc. Am. Physicians 75:253.

Courtice, F. C., and Simmonds, W. J. 1949. Absorption from the lungs. J. Physiol. 109:103.

Crabbe, P. A., Nash, D. R., Bragen, H., Eyssen, H., and Heremans, J. F. 1969. Antibodies of the IgA type in intestinal plasma cells of germ-free mice after oral or parenteral immunication with ferritin. J. Exp. Med. 130:723.

Creasia, D. A. 1966. Unpublished data.

Creasia, D. A., Nettesheim, P., and Hammons, A. S. 1973. Impairment of deep lung clearance by influenza virus infection. Arch. Environ. Health 26:197.

Creasia, D. A. 1973. Increased effective half-life of intratracheally administered $^{51}CR_2O_3$ by respiratory infection. In: Radionuclide Carcinogenesis. (Eds.) Sanders, C. L., Busch, R. H., Ballou, J. E., and Mahlum, D. D. U.S.A.E.C. Office of Information Services, Washington, D.C.

Creasia, D. A. 1974. Impairment of pulmonary clearance of inhaled particles by respiratory infection. Biology Division Annual Progress Report ORNL 4993, Oak Ridge National Laboratory, NTIS.

Creasia, D. A., Poggenburg, J. H., Jr., and Nettesheim, P. 1976. Elution of Benzo(a)pyrene from carbon particles in the respiratory tract of mice. J. Tox. Environ. Health 1:967.

DeDuve, C., and Wattiaux, R. 1966. Functions of lysosomes. Annu. Rev. Physiol. 28:435.

Diener, E., Shortman, K., and Russell, P. 1970. Induction of immunity and tolerance *in vitro* in the absence of phagocytic cells. Nature 225:731.

Donovan, R., Johansson, S. G. O., Bennich, H., and Soothill, J. F. 1970. Immunoglobulins in nasal polyp fluid. Int. Arch. Allergy 37:154.

Drinker, C. K., Warren, M. F., and MacLanaham, M. 1937. The absorption of protein solutions from the pulmonary alveoli. J. Exp. Med. 66:449.

Dutton, R. W., and Eady, J. D. 1964. An *in vitro* system for the study of the mechanism of antigenic stimulation in the secondary response. Immunology 7:40.

Fernald, G. W., Clyde, W. A., Jr., and Bienenstock, J. 1972. Immunoglobulin-containing cells in lungs of hamsters infected with Mycoplasma pneumoniae. J. Immunol. 108:1400.

Fink, C. W., Miller, W. E., Dorward, B., and Lo Spalluto, J. 1962. The formation of macroglobulin in antibodies. II. Studies on neonatal infants and older children. J. Clin. Invest. 41:1422.

Ford, R. J., Jr., and Kuhn, C. 1973. Immunologic competence of alveolar cells. I. The plaque-forming response to particulate and soluble antigens. Am. Rev. Respir. Dis. 107:763.

Gieseking, R. 1958. Aufnahme und Ablagerung von Fremdstoffen in der Lunge nach elektronenoptischen Untersuchungen. Ergeb Allerg. Pathol. Anat. 38:92.

Globerson, A., and Feldman, M. 1970. Macrophages and the bicellular mechanism of antibody response. In: Mononuclear Phagocytes. (Ed.) Van Furth, R. F. A. Davis Co., Philadelphia.

Gowans, J. L., and Knight, E. J. 1964. The route of recirculation of lymphocytes in the rat. Proc. Roy. Soc. Lond. (Biol.) 159:257.

Gowans, J. L., and Uhr, J. W. 1966. The carriage of immunological memory by small lymphocytes in the rat. J. Exp. Med. 124:1017.

Harris, G. 1965. Studies on the mechanism of antigen stimulation of DNA synthesis in rabbit spleen cultures. Immunology 4:529.

Harris, G. 1967. Macrophages from tolerant rabbits as mediators of a specific immunological response *in vitro*. Immunology 12:159.

Haskill, J. S., Byrt, P., and Marbrook, J. 1970. *In vitro* and *in vivo* studies of the immune response to sheep red cells using partially purified cell populations. J. Exp. Med. 131:57.

Hatch, T., and Hemeon, W. C. L. 1948. Influence of particle size in dust exposure. J. Ind. Hyg. Toxicol. 30:172.

Hauptman, S. P., and Tomasi, T. B., Jr. 1978. The secretory immune system. In: Review of Basic and Clinical Immunology. (Eds.) Fudenberg, H. H., Caldwell, J. L., Stites, D. P., and Wells, J. V. Lang Medical Publications, Los Altos, California.

Hersh, E. M., and Harris, J. E. 1968. Macrophage-lumphocyte interaction in the antigen-induced blastogenic response of human peripheral blood leukocytesk. J. Immunol. 100:1184.

Hoffman, M., and Dutton, R. W. 1971. Immune response restoration with macrophage culture supernatants. Science 172:1047.

Holub, M., and Hauser, R. E. 1969. Lung alveolar histiocytes engaged in antibody production. Immunology 17:207.

Humpherey, J. H. 1965. The suppression of immune responses by nonspecific agents. In: Immunological Diseases. Little, Brown, Little and Co., Boston.

Ishizaka, K. 1973. Chemistry and Biology of Immunoglobulin E in Antigens, Vol. 1. (Ed.) Sela, M. Academic Press, Inc., New York.

Ishizaka, K. 1975. Function of IgE antibody response. In: New Directions in Asthma. (Ed.) Stein, M. American College of Chest Physicians, Park Ridge, Illinois.

Jarrett, E. E. E., and Stewart, D. C. 1974. Rat IgE production. Effect of dose of antigen on primary and secondary reaginic antibody responses. Immunology 27:365.

Jerne, N. K., Nordin, A. A., and Henry, C. N. 1953. The agar plaque technique for recognizing antibody-producing cells. In: Cell Bound Antibodies. (Eds.) Amos, B., and Koprowski, H. Wistar Institute Press, Philadelphia.

Johansson, S. G. O., Bennich, H. H., and Berg, T. 1972. The clinical significance of IgE. In: Progress in Clinical Immunology, Vol. 1. (Ed.) Schwartz, R. S. Grune and Stratton, Inc., New York City.

Kaltreider, H. B., Kyselka, L., and Salmon, S. E. 1974. Immunology of the lower respiratory tract. II. The plaque-forming response of canine lymphoid tissues to sheep erythrocytes after intrapulmonary or intravenous immunization. J. Clin. Invest. 54:263.

Kaltreider, H. B., Adam, E., and Turner, F. N. 1975. Bronchaolveolar clearance of an organic particulate antigen after intrapulmonary immunization (abstract). Clin. Res. 23:348A.

Kaltreider, H. B., and Chan, M. K. L. 1978. The class-specific immunoglobulin composition of fluids obtained from various levels of the canine respiratory tract. J. Immunol. 117:396.

Kaltreider, H. B., Turner, F. N., Adam, E., and Chan, M. K. 1978. Bronchoalveolar clearance and immunologic reaction to an organic particulate antigen instilled into alveolar spaces of dog lungs. Chest 69:282.

Katz, D. M., Paul, W. E., Goidl, E. A., and Benacerraff, B. 1970. Radioresistance of cooperative functions of carrier specific lymphocytes in antihapten antibody responses. Science 170:642.

Kennedy, J. D., Treadwell, P. E., and Lennox, E. S. 1970. Antigen-specific synergism in the immune response of irradiated mice given marrow cells and peritoneal cavity cells as extracts. J. Exp. Med. 132:353.

Kettmann, J., and Dutton, R. W. 1971. Radioresistance of the enhancing effect of cells from carrier-immunized mice in an in vitro primary immune response. Proc. Nat. Acad. Sci. U.S. 68:699.

Kishimoto, T., Miyaki, T., Nishizawa, Y., Watanabe, T., and Yamamura, Y. 1975. Triggering mechanism of B—Lymphocytes I. Effect of anti-immunoglobulin and enhancing soluble factor on differentiation and proliferation of B-cells. J. Immunol. 115:1179.

Kolsch, E. 1970. Patterns of handling and presenting antigen by macrophages. In: Mononuclear Phagocytes. (Ed.) Ban Furth, R. F. A. Davis Co., Philadelphia.

Kostowiecki, M. 1963. The thymic macrophages. A. Mikrosk. Anat. Forsch. 69:585.

Lauweryns, J. M., and Boussauw, L. 1969. The ultrastructure of pulmonary lymphatic capillaries of newborn rabbits and of human infants. Lymphology 2:108.

Lauweryns, J. M. 1970. The juxta-alveolar lymphatics in the human adult lung. Am. Rev. Respir. Dis. 102:877.

Lauweryns, J. M. 1971. The blood and lymphatic microcirculation of the lung. Pathol. Annu. 6:365.

Lauweryns, J. M. 1975. The blood and lymphatic microcirculation of the lung. In: Cardiopulmonary Pathology Decennial 1966-75. (Ed.) Sommers, S. C. Appleton-Century-Crofts, New York.

Leskowitz, S. 1967. Tolerance. Ann. Rev. Microbiol. 21:157.

MacKaskil, L. 1976. Unpublished data.

MacLaurin, B. P., and Humm, J. A. 1969. Macrophage specificity of antiserum against thymus lymphocytes. Aust. J. Exp. Biol. Med. 47:105.

Martinez-Tello, F. J., Braun, D. G., and Blanc, W. A. 1968. Immunoglobulin production in bronchial mucosa and bronchial lymph nodes, particularly in cystic fibrosis of the pancreas. J. Immunol. 101:989.

Masson, P. L., and Heremans, J. F. 1973. Sputum proteins. In: Sputum: Fundamentals and Clinical Pathology. (Ed.) Dulfano, J. J. Charles C. Thomas, Springfield, Illinois.

Mastecky, J., and Lawton, A. R. 1974. The immunoglobulin A system. In: Advances in Experimental Medicine and Biology. Plenum Press, New York.

McCrae, J. 1939. The ash of silicotic lungs. Publs. S. African Inst. Med. Rec. 3:17.

Mead, J. 1960. Control of respiratory frequency. J. Appl. Physiol. 15:325.

Meyer, E. C., Dominquez, E. A., and Bensch, K. G. 1964. Pulmonary lymphatic and blood absorption of albumin from alveoli: A quantitative comparison. Lab. Invest. 20:1.

Meyer, E. C., Dominguez, E. A., and Bensch, K. G. 1969. Pulmonary lymphatic and blood absorption of albumin from alvecoli: A quantitative comparison. Lab. Invest. 20:1.

Moller, G., and Coutinho, A. 1975. Factors influencing activation of B-cells in immunity. Ann. N.Y. Acad. Sci. 244:68.

Mosier, D. E., and Coppleson, L. W. 1968. A three-cell interaction required for the induction of the primary immune response in vitro. Proc. Nat. Acad. Sci. 61:542.

Mosier, D. S. 1969. Cell interactions in the primary immune response in vitro: A requirement for specific clusters. J. Exp. Med. 192:351.

Mota, I. 1961. Mast cell lytic antibodies. Nature (London) 192:1201.

Mota, I. 1964. The mechanism of anaphylaxis. I. Production and biological properties of "Mast cell sensitizing" antibody. Immunology 7:681.

Nash, D. R., and Holle, B. 1973. Local and systemic cellular immune response in guinea-pigs given antigen parenterally or directly into the lower respiratory tract. Clin. Expt. Immunol. 13:573.

Oren, R., Farnham, A. E., Saito, K., Milofsky, E., and Karnovsky, M. L. 1963. Metabolic patterns of three types of phagocytizing cells. J. Cell Biol. 17:487.

Owen, R. L., and Jones, A. L. 1974a. Specialized lymphoid follicle epithelial cells in the human and non-human primate: A possible antigen uptake site. In: Scanning Electron Microscopy, Part III. (Ed.) Orn, Johari. ITT Research Institute, Chicago, Illinois.

Owen, R. L., and Jones, A. L. 1974b. Epithelial cell specialization within Peyer's patches: An ultrastructural study of human intestinal lymphoid follicles. Gastroenterology 66:189.

Paley, R. S., Leskowitz, S., and Borel, Y. 1975. Effect on tolerance induction of the mode of attachment of the hapten to the carrier. J. Immunol. 115:1409.

Parkhouse, R. M. E., and Dutton, R. W. 1966. Inhibition of spleen cell DNA synthesis by autologous macrophages. J. Immunol. 97:663.

Pierce, C. W. 1969. Immune responses in vitro. I. Cellular requirements for the immune response by non-primed and primed spleen cells in vitro. J. Exp. Med. 130:345.

Policard, A. 1950. Sur quelques caractieres histophysiologiques des formation lymphoid bronchiques des certaines mammifers. Bull. Histol. Appl. 27:118.

Portugalov, V. V., Durnova, G. N., Kaplansky, A. S., and Kolganova, N. S. 1973. Cytochemical investigations of the metabolism of alveolar and peritoneal macrophages. Acta Histochem. 46:216.

Raff, M. C. 1970. Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. Immunology 19:637.

Reynolds, H. Y., and Thompson, R. E. 1973. Pulmonary host defenses. I. Analysis of protein and lipids in bronchial secretions and antibody responses after vaccination with Pseudomonas aeruginosa. J. Immunol. 111:358.

Reynolds, H. Y., and Thompson, R. E. 1973. Pulmonary host defenses. II. Interaction of respiratory antibodies with Pseudomonas aeruginosa and alveolar macrophages. J. Immunol. 111:369.

Reynolds, H. Y., Kazmierowski, J. A., and Newball, H. H. 1975a. Specificity of opsonic antibodies to enhance phagocytosis of Pseudomonas aeruginosa by human alveolar macrophages. J. Clin. Invest. 56:376.

Reynolds, N. Y., Atkinson, J. P., Newball, H. H., and Frank, M. M. 1975b. Receptors for immunoglobulin and complement on human alveolar macrophages. J. Immunol. 114:1813.

Rossen, R. D., Morgan, C., Hsu, K. C., Butler, W. T., and Rose, H. M. 1968. Localization of 11S external secretory IgA by immunofluorescence in tissues lining the oral and respiratory passages in man. J. Immunol. 100:706.

Ryder, R. J. W., and Schwartz, R. S. 1969. Immunosuppression by antibody. Localization of site of action. J. Immunol. 103:970.

Sahir, K., and Schwartz, R. S. 1965. The immunoglobulin sequence. I. Arrest by 6-mercaptopurine and restitution by antibody, antigen, or splenectomy. J. Immunol. 95:345.

Schultz, A. L., Grismer, J. T., and Grande, F. 1963. Absorption of radioactive albumin from the lungs of normal dogs. J. Lab. Clin. Med. 61:494.

Schultz, A. L., Grismer, J. T., Wada, S., and Grande, F. 1964. Absorption of albumin from alveoli of perfused dog lungs. Am. J. Physiol. 207:1300.

Sell, S., Park, A. B., and Nordin, A. A. 1970. I. Localized hemolysis in agar plaque-forming cells belonging to five immunoglobulin classes. J. Immunol. 104:483.

Shortman, K., Diener, E., Russell, P., and Armstrong, W. D. 1970. Role of nonlymphoid accessory cells in the immune response to different antigens. J. Exp. Med. 131:461.

Shortman, K., and Palmer, T. 1971. The requirement for macrophages in the in vitro immune response. Cell. Immun. 2:399.

Shultze, A. L., Grismer, J. T., and Grande, F. 1963. Absorption of radioactive albumin from the lungs of normal dogs. J. Lab. Clin. Med. 61:494.

Singhal, S. K., and Wigzell, H. 1971. Cognition and recognition of antigen by cell associated receptors. Progr. Allergy 15:188.

Sorokin, S. P. 1970. Properties of alveolar cells and tissue that strengthen alveolar defenses. Arch. Intern. Med. 126:450.

Sorokin, S. P., and Brain, J. D. 1975. Pathways of clearance in mouse lungs exposed to iron oxide aerosols. Anat. Rec. 181:581.

Stelos, P., and Taliaferro, W. H. 1959. Comparative study of rabbit hemolysins to various antigens. II. Hemolysins to the Forssman antigen to guinea pig kidney, human type A red cells and sheep red cells. J. Inf. Dis. 104:105.

Stelos, P., Taliaferro, L. G., and Dalesandro, P. A. 1961. Comparative study of rabbit hemolysin to various antigens. III. Chromatographic analysis of Forssman hemolysin induced by various antigens. J. Inf. Dis. 108:113.

Strober, A. 1975. Maturation of B-lymphocytes in the rat: II. Subpopulation of virgin B-lymphocytes in the rat: II. Subpopulation of virgin B-lymphocytes in the spleen and thoracic duct lymph. J. Immunol. 114:877.

Sulitzeanu, D., Kleinman, R., Benezia, D., and Gery, I. 1971. Cellular interactions and the secondary response in vitro. Nature 229:254.

Svehag, S. E., and Mandel, B. J. 1964. The formation and properties of polio virus neutralizing antibody. I. 19S and 17S antibody formation: Differences in kinetics and antigen dose requirement for induction. J. Exp. Med. 119:1.

Tada, T., and Ishizaka, K. 1970. Distribution of IgE-forming cells in lymphoid tissues of the human and monkey. J. Immunol. 104:377.

Talmage, D. W., Radovich, J., and Hemmingsen, H. 1969a. Cell interaction in antibody synthesis. J. Allerg. 43:323.

Talmage, D. W., Radovich, J., and Hemmingsen, E. 1969b. Wistar Inst. Sympos. 9:151.

Talmage, D. W., Radovich, J. and Hemmingsen, H. 1970. Cell interaction in antibody synthesis. Advances Immun. 12:271.

Task Group on Lung Dynamics. 1966. Deposition and retention models for internal dosimetry of the human respiratory tract. Health Phys. 12:173.

Taylor, A. E., and Gaar, G. A. 1970. Estimation of equivalent pore radii of pulmonary capillary and alveolar membranes. Am. J. Physiol. 218:1133.

Tomasi, T. B., Jr., and Grey, H. M. 1972. Structure and function of immunoglobulin A. Prog. Allergy 16:81.

Uhr, J. W., and Finkelstein, M. S. 1963. Antibody formation IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ϕx174. J. Exp. Med. 117:457.

Uhr, J., Bystryn, J-C., and Graf, N. W. 1971. The regulation of antibody formation. In: Morphological and Fundamental Aspects of Immunity. (Eds.) Lindahl-Kiessling, Alm, and Hanna. Plenum Press, New York.

Vittetta, E. S., and Uhr, J. W. 1975. Immunoglobulin—Receptors revisited. Science 189:964.

Wakeman, B. H. 1971. Presidential address: Immunology as a basis and applied science. J. Immunol. 107:617.

Weiss, L. 1973. The cell. In: Histology. (Eds.) Greep, R. O., and Weiss, L. McGraw-Hill Book Co., New York.

Wortis, H. H., Dresser, D. W., and Anderson, H. R. 1969. Immunology 17:93.

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