UNIVERSITY OF ALBERTA

# EXPERIMENTAL ASTHMA IN GUINEA PIGS

by

**JANET WINSOME CAMPBELL**

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE.**

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The undersigned certify that they have read, and recommended to the Faculty of Graduate studies and Research for acceptance, a thesis entitled **EXPERIMENTAL ASTHMA IN GUINEA PIGS** submitted by **JANET WINSOME CAMPBELL** in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE** in **Pharmaceutical Sciences (Pharmacology)**

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"My help comes from the Lord who made heaven and earth"
(The Amplified Bible, Ps. 121:2)

## ABSTRACT

I developed three guinea-pig models of asthma using different active sensitization protocols: Model I - only OA (20 mg/kg, ip); Model II - OA (20 μg/kg + $Al(OH)_3$ [100 mg/kg], ip); and, Model III - cyclophosphamide (100 mg/kg, ip) 24 h prior to OA (20 μg/kg + $Al(OH)_3$ [2.0 mg], + *B. pertussis* [$10^{10}$ organisms]). I sought to determine which model most closely resembled the disease in humans; I used five criteria for comparisons: 1) The nature of the immunologic response; 2) The presence or absence of anaphylaxis upon antigen challenges; 3) The number and types of cells appearing in BAL fluid following challenges; 4) The morphology of the lungs following challenges; and, 5) The appearance of airways' hyper-responsiveness to agonists injected iv. I found that anti-OA IgG, $IgG_1$ and $IgG_2$ antibodies were detected in all models, but at different times. Anti-OA IgE antibodies were detected in Models II and III at days 21, 35 and 60, but they were absent in Model I. In all models, guinea pigs showed similar anaphylactic responses to antigen challenge 21 days after sensitization. In all models, lung sections from antigen challenged animals taken on days 35 and 60, showed morphologic changes, e.g., cellular infiltration, folding of the mucus membrane and reduced airway lumen diameter, that were typical of asthma; these changes were absent from controls. Emphysematous changes and edema were noted in Models II and III. In all models, on days 35 and 60, total cell counts from broncho-alveolar lavage fluid revealed significant increases from controls; differential counts revealed significant increases in eosinophils and macrophages; neutrophil numbers were significantly increased only in Models II and III. In Models II and III, dose-response curves to histamine and serotonin were shifted significantly to the left, compared to controls, at days 35 and 60, and at day 60, respectively, indicating AHR. Model I showed no AHR. I concluded that one can induce the features of features of asthma in guinea pigs, e.g., high serum IgE levels, anaphylaxis, histologic changes, eosinophilia and AHR. Model II, and possibly Model III, showed features which most closely mimicked allergic asthma in humans.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ag | Antigen |
| AHR | Airway hyperresponsiveness |
| $Al(OH)_3$ | Aluminium hydroxide |
| ANOVA | Analysis of variance |
| Anti-OA Ab | Anti-OA antibody |
| ASA | Acetylsalicylic acid |
| BAL | Broncho-alveolar lavage |
| C3a | Complement 3a |
| C5a | Complement 5a |
| ELISA | Enzyme-linked immunosorbent assay |
| ECF-A | Eosinophil chemotactic factor of anaphylaxis |
| Fc | Constant region of immunoglobulin |
| FcR | Receptor for constant region of immunoglobulin |
| H | Histamine |
| IgE | Immunoglobulin E |
| IgG | Immunoglobulin G |
| MCh | Methacholine |
| OA | Ovalbumin |
| PCA | Passive cutaneous anaphylaxis |
| PAF | Platelet activating factor |
| PMN | Polymorphonuclear cells |
| RAST | Radioallergosorbent test |

# Chapter I - INTRODUCTION

## I. Definition and classification of asthma

Asthma is an inflammatory disease of the lungs characterized by episodic attacks of bronchospasm which are reversible spontaneously or by bronchodilators. Patients may be asymptomatic between attacks or show signs of chronic (eosinophilic) bronchiolitis. Rarely, patients experience continuous attacks; this is termed *status asthmaticus*.

Airway hyperresponsiveness (AHR) is a characteristic feature of asthmatics. Their airways are much more responsive than non-asthmatics to agonists such as methacholine (MCh), histamine (H) or serotonin (5HT). Also, asthmatics show excessive mucus secretion which leads to airway plugging and mucosal edema.

Asthma can be classified according to its causative agents. Allergic (atopic) asthma is caused by the inhalation of allergens such as pollens, animal hair and dander. The cause of nonatopic (cryptogenic) asthma is unknown, but could be viral. Occupational asthma is caused by exposure to cedar dust, ozone and toluene diisocyanate. Asthma can also be induced by drugs, e.g., acetylsalicyclic acid (ASA) or propranolol.

Allergic asthma is the most common form of asthma. Patients usually have a positive family history of the disease and have increased serum IgE levels. Pathologic investigations of the lungs of these patients reveal

eosinophilia, airway inflammation and thickening of the basement membrane (Pagel, 1932; 1939). Despite the use of potent and selective bronchodilators, the incidence of, and mortality from, allergic asthma is increasing (Buist, 1988). Thus investigation of the pathogenesis of this disease in experimental models is of great importance.

## II. Definition of experimental asthma

Experimental asthma may be defined as the provocation of asthmatic or asthma-like episodes by asthmogenic substances in humans and experimental animals mainly, but not exclusively, by inhalation (Noelpp-Eschenhagen and Noelpp, 1954). Since 1910, many advances in the treatment of allergic reactions and asthma have been made using data from experimental models of asthma.

## III. Experimental asthma in humans

Early investigations of experimental asthma were carried out in human subjects both fortuitously and intentionally. For many years, it was known that exposure of industrial workers to dry antigenic substances induced sensitization. Thus, typically, veterinarians, grooms and cavalrymen become sensitized to horse dander, millers and bakers to meal dust and dealers in fur skins to hair. Also, pollens and environmental dusts may cause allergic reactions in many individuals (Ratner, 1951).

Causes of asthma in various communities have been extensively followed by various researchers. Ancona (1926) reported several cases of asthma among millers and granary workers in Italy. Cases of allergy have been reported among merchants handling grains in The Netherlands. Also, children who slept near bins containing "infected" grain showed signs of allergy. Similar observations were reported by Prausnitz (1936) among workers in the cotton industries in Manchester, England. Figley and Elrod (1928) cited cases of "endemic" asthma among workers in a castor oil factory and in peasants living in areas surrounding it. More recently, Thorpe (1989) showed that the toxic material in castor beans, ricin, is a potent stimulator of IgE production in humans.

Clarke and Leopold (1940) recognized that Europeans who came to America, and who had not been exposed previously to ragweed, developed the symptoms of bronchial asthma when re-exposed to this allergen after an initial contact. Methods of exposing asthmatics and other allergic patients to asthmogenic materials were first designed by Leopold and Leopold (1925) and later used by other investigators. These provided useful information about the diagnosis, etiology and treatment of asthma and related diseases.

The work of Cohen and his co-workers (1930) showed that dry antigen can be absorbed through the nasopharynx and that it was effective in producing sensitization. They used both normal and allergic subjects and reported severe local nasal reactions when ragweed pollen was insufflated in

patients sensitive to ragweed.

Asthmatic "attacks" may be induced in sensitized individuals not only by re-exposure to the respective allergen but also by inhalation of bronchospastic agents, e.g., histamine and methacholine (Schiller and Lowell, 1947; Herxheimer, 1952).

Human experimental asthma research, although interesting and informative, is limited by the possibility of dangerous accidental reactions and ethical considerations, and (hence) lack of volunteers. Therefore, the need for suitable experimental animal models soon became apparent. Any animal model of asthma should show: eosinophilia, airway inflammation, thickening of basement membrane and airway hyperresponsiveness (AHR).

## IV. Animal models

Studies in rabbits, cats and dogs show that re-exposure to the antigen to which animals had been previously sensitized produced reactions that were confined to organ systems such as the blood vessels and the gastro-intestinal tract (Noelpp-Eschenhagen and Noelpp, 1954).

Studies with guinea pigs showed that they could be sensitized more easily than any other species to allergens and that their respiratory system is exquisitely sensitive to antigenic stimuli. Thus, they are well-suited for research on anaphylactic reactions, asthma and dermal hypersensitivity reactions.

Recently, guinea pigs have been reported to exhibit early- and late-onset airway obstructions (Hutson *et al.*, 1988a) bronchial eosinophilia (Aoki *et al.*, 1988; Dunn *et al.*, 1988) and increased airway reactivity following exposure of sensitized animals to antigen (Aoki *et al.*, 1988; Daffonchio *et al.*, 1989).

## V. History of experimental asthma in guinea pigs

The history of experimental asthma in guinea pigs began with Meltzer (1910). He showed similarities between the pathophysiology of guinea-pig anaphylaxis and human bronchial asthma. A year later, Friedberger (1911) described the structural changes in the lungs of guinea pigs that had been sensitized to horse serum and challenged. He termed these changes "anaphylactic pneumonia". Also in that year, Busson (1911) was successful in actively sensitizing guinea pigs to diluted cattle serum by inhalation.

Sewell and Powell (1915), sensitized animals by intranasal instillation of horse serum and reported symptoms of bronchial asthma after repeated exposure. Besredka's work (1920) showed that serum introduced into the larynx of normal guinea pigs was harmless but was fatal in sensitized animals.

Petragnani's (1922) contributions to experimental asthma, are worthy of mention as he was successful in sensitizing and producing shock in guinea pigs using sheep serum introduced via the nares with the animals in an inverted position. This prevented seepage into the laryngotracheal passage.

In 1924, "true" bronchial asthma in guinea-pigs was successfully induced for the first time by Busson and Ogata. They exposed animals repeatedly to a heavy spray of horse dander which resulted in decreased temperature, anaphylaxis and eventually death in all the animals. This technique was further developed by Alexander and co-workers (1925), and Ratner and co-workers (1951). Ratner discovered that the classic anti-anaphylactic state, observed in sensitized individuals surviving intravenous or intraperitoneal antigen, will not occur within a certain time interval. Discussing the validity of experimental guinea-pig asthma as a model of human bronchial asthma, Ratner suggested that truly comparable conditions will only be obtained if dry antigens are used for sensitization and challenge in animals sensitized by inhalation. This was with particular reference to the mechanism of "regional sensitization" a term which he used to characterise an allergy functionally and histologically confined to a single organ.

The most important developments in experimental asthma research are due to the extensive investigations done by Kallos and co-workers (1937). In their experiments, guinea pigs, sensitized and re-exposed to aerosol, developed symptoms that resembled human bronchial asthma in many ways. The occurrence of respiratory symptoms was always dependent on previous contact with the antigen and was very specific. They showed similarities between the radiographic findings in human and guinea-pig lungs, i.e., occlusion of the middle sized (class 2) bronchi and hyperinflation of the lungs. Observation of

electrocardiographs revealed that, at the beginning of respiratory distress, the T waves were negative at all leads. Continued exposure to the antigen caused changes in the rhythm and conduction which indicated "contraction and hypoxia of the cardiac muscles". At the end of antigen exposure, electrocardiographs reverted to the original form within 3 h.

These authors were the first to report the benefits of adrenalin administration in experimental asthma. If adrenalin, in therapeutic doses was given just prior to antigen exposure, asthmatic episodes were eliminated. If adrenalin was given at the peak of an attack, symptoms of respiratory distress decreased and the recovery time was significantly shortened.

They listed the characteristic histological features of "true" allergic asthma as: alveolar emphysema, thickening of the bronchiolar and intra-alveolar walls and cellular (eosinophilic) infiltration. These findings were identical to those found in the lungs of persons who died in *status asthmaticus* (Pagel, 1939). They also observed marked differences in clinical symptoms and the histological changes between true allergic asthma and the asthma-like symptoms induced by inhalation of histamine or methacholine.

Kallos *et al.* (1937) also found that the nasal and bronchial secretions of guinea pigs with antigen-induced asthma were voluminous, viscous and contained a massive cellular infiltrate, i.e., epithelial cells, neutrophils and eosinophils. If animals were repeatedly exposed to the homologous antigens, eosinophils dominated. Similar observations were made in humans (Ehrlich,

1935; Ehrlich, 1945).

Neely (1941) was able to induce symptoms of human allergic asthma in guinea pigs by sensitizing them with ovalbumin and challenging them with ovalbumin aerosol. He noticed that the symptoms in the sensitized animals resembled those of human asthma, except that their onset and subsidence were more rapid.

After careful observations of animals placed in closed chamber and exposed to swine, rabbit, goat or horse serum or egg albumin aerosol, Hopps and Moulton (1943) concluded that active sensitization can be achieved by inhalation of finely atomized fluid antigen and that serious allergic reactions and fatal anaphylactic shock can occur when sensitized animals later inhaled aerosols of the same specific antigen.

## VI. Human vs guinea-pig asthma

Pagel (1935) who examined the lungs of sensitized guinea-pigs after exposure to antigen aerosol, observed that after the first exposure, which was terminated at the first signs of respiratory distress, itching and dyspnea were present. Eosinophilia appeared in the peribronchial vessels. After repeated inhalations, plugs consisting of mucus, degenerated epithelial cells and eosinophils appeared in the small and middle-sized bronchi. With repeated exposure twice a week, the animals showed signs of illness accompanied by coughing with viscous secretions containing eosinophils. Numerous goblet cells

appeared in the epithelial layer. Mucus degeneration of the epithelial layer was also present. There was also hypertrophy of the middle-sized bronchi. Granulomas formed by eosinophils appeared in the thickened and edematous peribronchial tissues. Atelectatic and emphysematous areas were found in the lung parenchyma. If exposure was continued until convulsions occurred and the animal died in shock, there was eosinophilic infiltration into the mucosal layer and bronchoconstriction. These findings were confirmed by Baumal (1981). The lungs of guinea pigs that died of antigen-induced asthma were maximally distended and porcelain white.

Changes were also observed in other organ systems. There was eosinophilia in the spleen and the heart and degeneration of the diaphragm. The histological changes were similar to those found in humans (Kallos, 1937; Rachemann & Greene, 1939; Lagendijk, 1940; Pagel, Miescher & Muller-Eberhard, 1976; Austen & Lichtenstein, 1977; Turner-Warwick, 1978). Similar findings in guinea pigs have been confirmed by other investigators (Ratner, 1951; Friebel, 1953a,b, 1954; Noelpp-Eschenhagen, 1954; Eastham & Muller, 1962; Businco *et al.*, 1978; Broder & Rogers, 1978, 1979; Cain *et al.*, 1980a,b; Karol *et al.*, 1980; Richerson, 1977; Yamamura *et al.*, 1973; Swanson & Reed, 1982).

These clinical and histologic findings provided convincing evidence that experimental asthma in the guinea pigs was similar to human allergic asthma. Some clinical studies suggest a strong correlation between airway eosinophilia

and increased airway reactivity (Metzgar *et al.,* 1986, Hakansson *et al.*, 1989, De Monchy *et al.*, 1985, Metzgar *et al.,* 1987). It has been proposed that eosinophils cause epithelium damage by releasing major basic protein (Filley *et al.*, 1982; Frigas and Gleich, 1986) which may lead to increased airway reactivity either by exposing sensory nerve endings (Barnes, 1986) or by removing the protective effects of an epithelial derived relaxant factor. However, other reports indicate that eosinophilia and increased airway reactivity may be unrelated (Thorpe *et al.*, 1987; De Monchy *et al.*, 1985).

## VII. **Recording techniques in experimental asthma**

The introduction of recording techniques in experimental asthma (Noelpp-Eschenhagen and Noelpp, (1954) was a major development. This allowed for the observation and evaluation of the course of asthmatic attacks in guinea pigs. Various ventilatory and respiratory parameters, such as airway resistance, elastance, tidal volume and dynamic lung compliance, were measured or calculated and compared with those obtained from human subjects. The evaluation of the recorded breathing patterns show that they are very similar to those regarded as characteristic of asthma in human subjects. The influence of drugs on the course of guinea-pig asthma has been extensively evaluated using these recording techniques (Koller, 1940; Friebel, 1953 a,b,c; Stein *et al.*, 1961; Douglas, 1971; Popa, *et al.,* 1973, 1974; Michoud, *et al.,* 1974; Souhrada, 1978; Karol *et al.*, 1980; Basold, 1981; Kallos, 1984).

## VIII. Immunologic studies

Similarities between human allergic asthma and experimental asthma in guinea pigs were also observed with immunologic studies. Immunoglobulins of the IgG and IgE classes have been causally associated with hypersensitivity reactions in humans and guinea pigs (Karol *et al.*, 1991). Immediate-onset hypersensitivity reactions in humans are mediated predominantly by IgE antibodies. These antibodies appear to play a major role in the pathogenesis of human asthma. IgE antibodies are able to sensitize respiratory tissues and to mediate the release of chemical mediators which contract bronchial smooth muscles or which are chemotactic for eosinophils. Bronchoconstriction in guinea pigs shows a common feature with bronchial asthma in that neuropeptides and histamine participate in the acute anaphylactic broncho-constriction. The involvement of IgE in late-onset hypersensitivity reactions has been reported (Ishizaka, 1984; Larsen *et al.*, 1984). Immunohistochemical studies done on the lungs of asthmatics demonstrated the specific location of IgE antibodies in the epithelium of the small bronchioles, on the basement membrane and within intrabronchial mucus. IgE was not detected on mast cells. These findings suggest that bronchial mucosal layer may be the target for reaginic (IgE) antibodies and that this is the site of the immunologic reaction in asthma (Gerber *et al.*, 1971). The importance of IgG antibodies to the pathogenesis of hypersensitivity reactions has also been reported (Wilson *et al.*, 1986).

The main homocytrotropic antibodies in the guinea pig have been characterized as IgE and $IgG_1$ and to a lesser degree $IgG_2$ (Benacerraf *et al.*, 1963, Ovary *et al.*, 1963, Bloch *et al.*, 1963).

Catty (1969) was the first to observe heat labile (reaginic or IgE) antibodies in guinea pigs infested with *Trichinella spiralis*. Their specific molecular weight was 185,000 daltons, substantially greater than $IgG_1$ (Dobson, 1971 a,b). The molecular weight of guinea-pig IgE is consistent with data obtained in humans (Johansson & Bennich, 1967).

### IX. Passive cutaneous anaphylaxis (PCA)

Passive cutaneous anaphylaxis (PCA) is the classical method for measuring IgE present in guinea-pig sera (Brocklehurst, 1978). This immunoglobulin is difficult to detect as its concentration in serum is in the order of μg/ml; other serum immunoglobulin isotypes are present in mg/ml concentration - a thousand-fold difference. The above technique is simple. Briefly, the skin of the animal is sensitized with an injection of serum containing IgE of unknown titre. This IgE binds to IgE specific Fc receptors present on the tissues' mast cells (mast cell sensitization). After a specified latent period, a mixture of antigen and dye (Evans Blue) is injected intravenously. If the IgE was directed against the injected antigen, the cross-linking of the membrane-bound antigen specific IgE by the antigen initiates the degranulation of the sensitized mast cells. The subsequent release of

histamine and other mediators alters the permeability of local blood vessels, causing leakage of dye into the tissue. Blue lesions appear on the skin and their diameters are measured.

$IgG_1$ class can be subdivided into two subclasses, $IgG_{1a}$ and $IgG_{1b}$ (Parish, 1970). $IgG_{1b}$ has a faster electrophoretic mobility and can be differentiated from $IgG_{1a}$ by passive cutaneous anaphylactic reactions as $IgG_{1b}$ persists longer in the skin than $IgG_{1a}$ (Parish, 1970; Ovary and Warner, 1972). $IgG_1$ antibodies are heat stable whereas IgE antibodies have been shown to be destroyed by heating at 56°C for 1 h. Also IgE antibodies persist in the skin much longer than $IgG_1$ antibodies (Catty, 1969; Parish 1970).

### X. Enzyme-linked immunosorbent assay (ELISA)

IgG antibodies can be detected by enzyme-linked immunosorbent assays (ELISA). The antigen is bound passively by incubation to the solid phase (microtitre plate) through an ill-defined process. The bound antigen then binds to specific antibodies in the test sample. Unbound material is removed by washing and bound immunoglobulin is detected by an enzyme-labelled anti-immunoglobulin.

Until sufficient amounts of purified guinea-pig IgE antibodies are isolated for sequencing or until an IgE- secreting guinea-pig myeloma is obtained for physicochemical and immunological characterization, one is forced to rely on less precise methods of identifying guinea-pig IgE antibodies.

## XI. Sensitization protocols and Ig development

The protocols for sensitization determine the predominance of immunoglobulins of the IgE or IgG subclass (Austen & Orange, 1975; Tada, 1975; Andersson, 1980). In guinea pigs, sensitization with large amounts of antigen produces $IgG_1$ and $IgG_2$ antibodies. Small amounts of antigen and adjuvants such as $Al(OH)_3$ and *Bordetella pertussis* vaccine yield high IgE titres (Benacerraf *et al.*, 1963; Ovary *et al.*, 1963, 1967; Bloch *et al.*, 1963). Similar findings have been noted in rabbits and mice (Revoltella and Ovary, 1969a,b).

The predominance of one or the other immunoglobulin may determine the preferential involvement of a group of mediators. Guinea pigs with high titres of circulating IgE and IgG are affected by anti-asthmatic agents after antigen challenge differently from those with high levels of only IgG antibodies (Andersson, 1980; Andersson & Bergstrand, 1981; Andersson & Brattsand, 1982). Antigen-dependent release of mediators from pulmonary tissues passively sensitized with purified antibodies varies according to the immunoglobulin class (Regal, 1984 & 1985; Graziano *et al.*, 1984; Undem *et al.*, 1985).

Using passively transferred heated and unheated serum, Desquand *et al.* (1990) showed that sensitizing activity of serum is not exclusively accounted for by IgE, as was previously suggested (Carcez *et al.*, 1986; Lagente *et al.*, 1987). Although the role of $IgG_1$ and $IgG_2$ in experimental asthma has not been clearly defined, these authors reported intense bronchoconstriction in

experiments *in vivo* and *in vitro* with purified $IgG_1$ and $IgG_2$. These findings were supported by Undem *et al.*, 1985, Cheng *et al.*, 1987. These results led the authors to conclude that $IgG_1$ and $IgG_2$ could account for the transferable sensitizing activity of serum from animals actively sensitized in a system that was thought to be mainly IgE-mediated. The ability of $IgG_2$ to mediate anaphylactic reactions could be due to activation of the complement cascade and thus lead to the generation of anaphylatoxins C3a and C5a that indirectly stimulate smooth muscle contraction via the release of histamine (Osler *et al.*, 1959; Oliviera *et al.*, 1970; Sanberg *et al.*, 1970;

There seems to be a correlation between allergic diseases of the Type I category, e.g., human allergic asthma and the presence of IgE and/or $IgG_1$ and/or $IgG_2$ immunoglobulins. These antibodies are homocytotrophic and therefore possess great affinity for surface receptors of mast cells and basophils within the species. Re-exposure of presensitized IgE-coated mast cells to the homologous or a cross-reacting antigen leads to antigen and mast-cell-bound antibody molecules. This results in mast cell activation and the release of mediators such as, histamine, eosinotactic peptides and platelet activating factor, which may play a major role in the pathogenesis of asthma.

Therefore it is evident that many of the characteristic features of human allergic asthma can be successfully reproduced in guinea pigs. In the laboratory, I sought to reproduce experimental asthma in sensitized guinea pigs challenged with aerosolized liquid antigens as demonstrated successfully

by others. Using various immunogens, I tried to create three guinea-pig models of human allergic asthma that showed the characteristic features of bronchospasm, eosinophilia and pathological changes such as thickened basement membrane and hypertrophy of airway smooth muscle, 35 and 60 days after sensitization. I sought to determine the time course of the appearance of antibodies in the sera of test animals that were actively sensitized. I characterized these antibodies by the two assay methods described above. The enzyme-linked-immunosorbent assay (ELISA) was used for guinea-pig IgG antibodies and passive cutaneous anaphylaxis (PCA) for the detection of IgE antibodies in the sera. I attempted to determine which of the three models showed features that most closely mimicked the conditions observed in human allergic asthma.

The criteria used for comparisons were: 1) The nature of the immunologic response; 2) The presence or absence of anaphylaxis upon antigen challenge; 3) The number and types of cells appearing in BAL fluid following challenges; and 5) The appearance of airways' hyperresponsiveness to agonists injected iv.

# Chapter II - MATERIALS AND METHODS

## I. Animals

Female, Hartley strain guinea pigs, (250-350 g) purchased from Charles River Inc., St. Constant, Québec, were used for all experiments. They were housed in laminar-flow units (Bioclean™, Hazleton, MD) in cages suspended over trays of rock salt. A maximum of 4 animals were placed in each cage. They were fed guinea-pig chow supplemented with apples and allowed water *ad libitum*. Animals were housed for at least 7 d before immunization.

## II. Experimental design

Six groups of 10 animals were used. Within each group, 5 were used as control and given only vehicle (saline); test animals were treated with an immunogen on day 1. Each group of 10 animals represented one model of asthma.

## III. Chemicals and solutions

The chemicals, drugs and immunoglobulins used in the experiments are listed below:

1 - Chemicals and Drugs

ABTS peroxidase substrate system (2,2'-azino-di[3-ethyl-benzthiazoline sulfonate (6)]) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD);

bovine serum albumin, ovalbumin (chicken egg, grade V), aprotinin, cyclophosphamide monohydrate, pyrilamine maleate, methacholine chloride, serotonin sulphate, and succinylcholine chloride (Sigma Chemical Co., St. Louis, MO); Carbowax$^{R}$ (PEG 400) and Evans Blue Dye (Fisher Scientific Co); formaldehyde solution U.S.P. (BDH Inc., Toronto, Ont.); pentobarbital sodium (Euthanyl$^{TM}$) (M.T.C. Pharmaceuticals, Cambridge, Ont.); *Bordetella pertussis* (Lot 3-04EII) (IFA BioVac Inc., Montreal, Qué.); epinephrine (adrenalin chloride injection) (Parke-Davis Canada Inc., Scarborough, Ont.); histamine dichloride (Fluka AG, Buchs SG, Switzerland).

2 - Immunoglobulins

Anti-goat IgG (whole molecule) peroxidase conjugate; anti-rabbit IgG (whole molecule) peroxidase conjugate; goat anti-guinea-pig IgG (whole molecule) (Sigma Chemical Co., St. Louis, MO); rabbit anti-guinea-pig $IgG_1$ serum and rabbit anti-guinea-pig $IgG_2$ serum (ICN Biomedical Inc., St-Laurent, Qué.)

## IV. Preparation of solutions

The following solutions were prepared in the laboratory:

1 - Acetate buffered formalin (10%, pH 7.2)

Neutral buffered formalin (pH 7.2) was made by dissolving sodium acetate (8 g) in 100 ml distilled water. Formaldehyde solution U.S.P. (37%, 100 ml) was then added to the mixture.

2 - Lavaging solution

The solution used for broncho-alveolar lavage (BAL) was prepared by dissolving bovine serum albumin (8 g) in aprotinin solution (15-30 TIU/ml, 5.6 ml). The solution was made up to 80 ml with PBS.

3 - Phosphate buffered saline (PBS) (pH 7.4)

Phosphate buffered saline (pH 7.4) was made by mixing mono-potassium phosphate ($KH_2PO_4$, 9.073 g/L) and disodium phosphate ($Na_2HPO_4.2H_2O$, 11.87 g/L).

4 - Sodium pentobarbital solution (35 mg/ml)

Sodium pentobarbital solution (35 mg/ml) was prepared by mixing (Euthanyl™, 14.6 ml), polyethylene glycol (PEG 400) (17.08 ml) and distilled water (68.32 ml).

5 - Scott's tap water

Scott's tap water was made by dissolving sodium bicarbonate (7 g) and magnesium sulphate (40 g) in tap water (2 L); 1% formalin (100 ml) was then added.

## V. Sensitization procedures

1 - Model I

A pre-immune bleed was done on all animals. Solutions of ovalbumin (OA) were made by dissolving various quantities of OA in saline (0.9% sodium chloride injection, U.S.P). Animals in Model I were sensitized with

OA (20 mg/kg, ip). Injection volume was 0.25 ml.

2 - Model II

A suspension of aluminum hydroxide, $Al(OH)_3$ was prepared by suspending aluminum hydroxide (0.166 mg) in saline (10 ml). The suspension was then mixed with OA solution and allowed to stand for 2 h before immunization. Each animal received OA (20 µg/kg, ip) and $Al(OH)_3$ (100 mg/kg, ip). Injection volume was 0.6 ml.

3 - Model III

Guinea pigs were given cyclophosphamide (100 mg/kg, ip). 24 h later, they received OA (20 µg), $Al(OH)_3$ (2.0 mg) and *B. pertussis* vaccine ($10^{10}$ organisms). Injection volume was 1 ml.

**VI. Procedure for obtaining serum samples**

Blood samples were collected into heparinized microhematocrit capillary tubes (FISHERbrand$^R$) from nicks made in animals' ears on days 1, 7, 15, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60. After centrifuging at 4000 rpm for 5 min, the serum (10 µL) was removed, diluted 1 in 100 with PBS and stored in microcentrifuge tubes at -20°C until assayed.

**VII. Aerosol challenges**

Twenty-one days after sensitization, all animals received an antihistamine, pyrilamine maleate (0.5 mg/kg, ip). This was used to prevent,

or reduce the severity of, anaphylactic reactions. After 30 min, test animals were exposed to OA aerosol (2% in saline, Vix ACORN™ nebulizer, compressed air @ 10 psi) for up to 4 min in a Plexiglass™ chamber on 10 consecutive days. Animals were carefully observed for signs of respiratory distress, and were given adrenalin chloride injection (0.1 ml) if anaphylaxis developed. Moribund animals were killed with an overdose of sodium pentobarbital (Euthanyl™, 1 ml). Control animals were exposed to saline aerosols using a similar protocol.

### VIII. Measurement of total pulmonary resistance and elastance

Resistance of the pulmonary system was measured on days 35 and 60. Guinea pigs were anesthetized with sodium pentobarbital (35-40 mg/kg, ip). Their necks were shaved, their tracheas cannulated (PE240), and attached to a small animal respirator (Harvard Apparatus, Model 681), tidal volume = 10 ml/kg, rate = 20 breaths/min. A jugular vein was cannulated (PE50) for injection of drugs. Spontaneous breathing was prevented by giving succinylcholine (0.1 mg/kg, iv). Changes in airway resistance were measured breath-by-breath using a computerized system (View-Dac™ software or MINC™ system).

### IX. Dose-response curves to agonists

Dose-response curves to histamine, serotonin and methacholine (iv)

were established. Increasing doses of each agonist were given to each animal in randomized order. Sufficient time was allowed for recovery to base line between doses until the curves had been established. Recovery to baseline was aided by briefly inflating the lungs with twice tidal volume.

### X. Broncho-alveolar lavage

After airway measurements were completed, the lungs were lavaged with 2 x 5 ml lavage solution; 7.5-8.0 ml were recovered and centrifuged (Dynac II centrifuge, Adams) at 2000 rpm. The cell pellet was resuspended in PBS (5.0 ml). Dead cells were stained with trypan blue and total (viable) cell counts were performed with a hemocytometer (Neubauer). Slides were prepared with a Cytospin$^R$ III using 125 µL of the resuspended cells. They were stained with Leukostat$^R$, allowed to dry and cover-slipped. Differential cell counts were performed on 200 cells.

### XI. Preparation of lungs for histology

Lung sections were removed and prepared for histology by first making a midline incision into the thoracic cavity. The esophagus and great vessels were severed and the lungs and heart removed *en bloc*. Bleeding was controlled by applying pressure and blotting with a cotton gauze pad. With the tracheal cannula still in place, the lungs were connected to a 5 ml syringe and manometer, inflated to 25 cm $H_2O$, then fixed in 10% buffered formalin.

A small segment was cut from a lobe, placed in a Histoprep$^{R}$ cassette (Fisher Scientific Co.) and placed in freshly prepared buffered formalin.

### XII. **Preparation of slides for microscopy**

Tissues were again placed for 24 h in 10% buffered formalin (Fisher Scientific, product number SF100-3), pH 6.9-7.1. They were dehydrated and infiltrated with paraffin in a Shandon Citadel 2000 tissue processor by sequential immersion in the following solutions.

Table 1

Tissue processing

| Position/step | Reagent(s) | Immersion time, h |
|---|---|---|
| Position 1 | 1% Zinc sulfate in formalin | 2.0 |
| Position 2 | 80% Isopropanol | 0.3 |
| Position 3 | 95% Isopropanol | 1.0 |
| Position 4 | 95% Isopropanol | 1.0 |
| Position 5 | 100% Isopropanol | 1.0 |
| Position 6 | 100% Isopropanol | 1.0 |

| Position/step | Reagent(s) | Immersion time, h |
|---|---|---|
| Position 7 | 100% Isopropanol | 1.0 |
| Position 8 | Xylene | 1.0 |
| Position 9 | Xylene | 1.0 |
| Position 10 | Xylene | 1.0 |
| Wax bath 1 (11) | Paraffin | 1.0 |
| Wax bath 2 (12) | Paraffin | 1.0 |

END OF CYCLE

Total Processing Time = 12.5 h

Full Program Time = 12.5 h + 11 min, allowing 1 min for every change of position.

Paraffin infiltrated tissues were embedded into a paraffin block using a Fisher Tissue Tec II embedding centre. A 5 µm section of tissue was trimmed from each block and floated onto a 3" x 1" x 1 mm glass microscope slide (FISHERbrand, Cat # 12-544-1) using a water bath. Slides were dried in an incubator at 60°C for 1 h.

Paraffin was removed from dry sections by rinsing for 3 min in two changes of xylene. Slides were then stained with hematoxylin and eosin by immersion in stain solutions in the following sequence:

Table 2

Hematoxylin and eosin staining.

| Reagents | Time | Purpose |
|---|---|---|
| 1. Xylene | 3 | hydrate slides |
| 2. Xylene | 3 | hydrate slides |
| 3. 100% Anhydrous alcohol | 2 | hydrate slides |
| 4. Alcoholic picric acid | 10 | remove formalin pigment |
| 5. Running tap $H_2O$ | 10-12 | remove picric acid |
| 6. Distilled $H_2O$ | 10 dips | hydrate slides |
| 7. Harris Hematoxylin | 7 | nuclear stain |
| 8. Running tap $H_2O$ | 3 | rinse excess stain |
| 9. 1% Alcoholic acetic acid | 2 | differentiate |
| 10. $H_2O$ | 30 dips, change 10 more dips | remove excess differentiator |

| | | |
|---|---|---|
| 10. $H_2O$ | 30 dips, change<br>10 more dips | remove excess differentiator |
| 11. Scott's tap $H_2O$ | 1 | bluing |
| 12. Running tap $H_2O$ | 5 | remove excess bluing agent |
| 13. Eosin Y | 3 | counterstain |
| 14. 95% Ethanol | 10 dips | dehydrate slides &<br>remove excess Eosin |
| 15. 100% anhydrous alcohol | 10 dips | dehydrate slides |
| 16. 100% anhydrous alcohol | 1 | dehydrate slides |
| 17. Xylene | 3 | clear slides |
| 18. Xylene | 3 | clear slides |
| 19. Xylene | 3 | clear slides |

Slides were checked microscopically following immersion in Scott's tap water. Sections should show blue nuclei with a well-defined chromatin pattern and nuclear membrane; cytoplasm should be almost colourless.

Slides were photographed with a Nikon camera (Nikon, FX-35DX, Labophot - 2A) and 25 ASA (Ektar™) film.

### XIII. Enzyme-linked immunosorbent assay (ELIZA)

Serum samples were assayed for IgG, $IgG_1$ and $IgG_2$ antibodies using an enzyme-linked immunosorbent assay (ELISA). Microtitre plates (96 well, Nunc-immunosorbent plates, MaxiSorp™, Gibco) were coated with ovalbumin (20 μg/ml, 75 μl) at 4°C for 48 h. After aspiration, non-specific sites were blocked with 100 μL undiluted skimmed milk at 22-24°C for 2 h. Plates were then washed twice with Wash Solution® (0.002 M imidazole buffered saline with 0.02% Tween 20, Kirkegaard & Perry Laboratories Inc.) using an automated washer (Molecular Devices, Maxline, model 4845-02). Doubling dilutions of serum sample were then incubated (50 μL, 1 h, 22-24°C). After aspiration, plates were incubated with rabbit anti-guinea-pig IgG, anti-guinea-pig $IgG_1$, or anti-guinea-pig $IgG_2$ (50 μL, 1 h, 22-24°C). Plates were washed twice, then incubated with anti-rabbit IgG-HRPO (50 μL, 1 h, 22-24°C). Plates were washed 3x, incubated with ABTS/$H_2O_2$ (50 μL, 0.5 h, 22-24°C) and optical densities were read in an automated plate reader (Molecular Devices, V max™, model 04662); SoftMax® software, Version 2.02).

XIV. **Passive cutaneous anaphylaxis (PCA)**

Karol *et al.*'s (1991) technique was used for PCA. Briefly, guinea pigs weight range 250-350 g, were lightly anesthetized with pentobarbital (20-30 mg/kg, ip). Their backs were shaved with clippers (Oster "Lucky Dog", Model 113 Series B, clipper head: fine, No. 913-6), then a commercial depilatory (Neet™) was applied, left for about 30 min, then gently wiped off with moist paper towels. After recovery from the anesthetic, animals were returned to their cages. About 24 h later, they were anesthetized as before, and dilutions of test and control guinea-pig sera in PBS were injected (0.1 ml, intradermally, tuberculin syringe attached to a ½ inch, 27 G needle, Pierce) within sections of a grid marked on the animals' backs. After recovery from the anesthetic, animals were returned to their cages. After 24 h, or after 7 d, the animals were anesthetized with pentobarbital (35-45 mg/kg, ip), the right jugular vein was isolated and cannulated (PE50), and a solution of Evan's Blue Dye (2%) and ovalbumin (0.1%) injected iv over about 1 min. After 30 min, the animals were killed with overdose of pentobarbital (iv), then skinned. The diameter of the areas showing extravasation of dye on the inside surface of the skin was measured with a ruler - two directions at 90° to each other. In some instances, if the areas of extravasation to be measured were irregular in shape, the skin was pinned out and the areas of extravasation traced onto a sheet of paper. A rating scale was devised to relate the activity of the diluted sera to the size of the PCA response (see Table 3).

Table 3

Grading of PCA reactions

| Grades | Diameter (mm) |
|---|---|
| - | no reaction |
| + | 1-5 |
| ++ | 5-10 |
| +++ | 10-15 |
| ++++ | 15-20 |
| +++++ | >20 |

In guinea pigs, IgE can be distinguished from other homocytotrophic antibodies by: a) heating the serum for 4 h at 56°C; or b) waiting >5 d post-injection before challenge (Karol *et al.*, 1991). Both methods of distinguishing IgE-mediated responses were used in these experiments.

### XV. Statistical methods

Data were expressed as mean ± S.E.M. and processed using SPSS-X on an Amdahl computer. UANOVA was used to compare dose-response lines to agonists, baseline measurements of resistance and elastance, cell counts, and antibody titres among controls and Models I, II and III. Multiple

comparisons of the means were done using the Student-Newmans-Keuls' tests. In all tests, significance was assumed at the 5% level. Student's *t* tests were used for some comparisons.

## PROTOCOLS FOR MODELS

### MODEL I

| | |
|---|---|
| **DAY 1** | Guinea pigs sensitized with OA (20 mg/kg, ip); blood sample taken |
| **DAY 7** | Blood sample taken |
| **DAY 15** | Blood sample taken |
| **DAY 21** | pyrilamine maleate (0.5 mg/kg, ip) 0.5 h later, exposed to OA (2%) aerosol for up to 4 min/d for 10 consecutive days, blood sample taken on **DAY 21** |
| **DAY 22** | As for **DAY 21** |
| **DAY 23** | As for **DAY 21** |
| **DAY 24** | As for **DAY 21** |
| **DAY 25** | As for **DAY 21** |
| **DAY 26** | As for **DAY 21** |
| **DAY 27** | As for **DAY 21** |
| **DAY 28** | As for **DAY 21** |
| **DAY 29** | As for **DAY 21** |
| **DAY 30** | As for **DAY 21** |
| **DAY 35** | Determine AHR, perform BAL, take tissue for histology, blood sample taken |
| **DAY 40** | Blood sample taken |
| **DAY 45** | Blood sample taken |
| **DAY 50** | Blood sample taken |
| **DAY 55** | Blood sample taken |
| **DAY 60** | Determine AHR, perform BAL, take tissue for histology, blood sample taken. |

**MODEL II**

| | |
|---|---|
| **DAY 1** | Guinea pigs sensitized with OA (20 μg/kg + $Al(OH)_3$ [100 mg/kg], ip), blood sample taken |
| **DAY 7** | Blood sample taken |
| **DAY 15** | Blood sample taken |
| **DAY 21** | pyrilamine maleate (0.5 mg/kg, ip)<br>0.5 h later, exposed to OA<br>(2%) aerosol for up to 4 min/d for<br>10 consecutive days (see Protocol for **Model 1**)<br>blood sample taken on **DAY 21** |
| **DAY 30** | Blood sample taken |
| **DAY 35** | Determine AHR, perform BAL, take<br>tissue for histology, blood sample taken |
| **DAY 40** | Blood sample taken |
| **DAY 45** | Blood sample taken |
| **DAY 50** | Blood sample taken |
| **DAY 55** | Blood sample taken |
| **DAY 60** | Determine AHR, perform BAL, take tissue<br>for histology, blood sample taken |

**MODEL III**

| | |
|---|---|
| **DAY 0** | Guinea pigs given cyclophosphamide (100 mg/kg, ip) |
| **DAY 1** | Guinea pigs sensitized with OA (20 μg/kg + $Al(OH)_3$ [2 mg], + *B. pertussis*, ip), blood sample taken |
| **DAY 7** | Blood sample taken |
| **DAY 15** | Blood sample taken |
| **DAY 21** | pyrilamine maleate (0.5 mg/kg, ip) 0.5 h later, exposed to OA (2%) aerosol for up to 4 min/d for 10 consecutive days (see Protocol for **Model I**) blood sample taken on **DAY 21** |
| **DAY 30** | Blood sample taken |
| **DAY 35** | Determine AHR, perform BAL, take tissue for histology, blood sample taken |
| **DAY 40** | Blood sample taken |
| **DAY 45** | Blood sample taken |
| **DAY 50** | Blood sample taken |
| **DAY 55** | Blood sample taken |
| **DAY 60** | Determine AHR, perform BAL, take tissue for histology, blood sample taken. |

## Chapter III - RESULTS

### I. Dose-response curves to agonists

Changes in airway hyperresponsiveness can be assessed from measurements of baseline resistance and elastance after bronchospastic drugs are injected (iv). Dose-response curves to agonists in test guinea pigs can then be compared with those from controls.

Dose-response curves to histamine, methacholine and serotonin were established for controls and animals in Models I, II and III, at 35 and 60 d after sensitization. Baseline measurements for control and test animals are set out in Tables 4 and 5.

Table 4

Baseline measurements: Day 35

| Model | Resistance (cm $H_2O$/ml/s) | Elastance (cm $H_2O$/ml) |
|---|---|---|
| Controls | 0.199 ± 0.007 | 1.616 ± 0.106 |
| 1 | 0.198 ± 0.034 | 2.415 ± 0.095* |
| 2 | 0.212 ± 0.006 | 2.227 ± 0.135* |
| 3 | 0.149 ± 0.010* | 2.518 ± 0.126* |

At day 35, baseline resistance values for Model III were significantly lower than controls, and baseline elastance values for Models I, II and III were significantly higher than controls.

Table 5

Baseline measurements: Day 60

| Model | Resistance (cm $H_2O$/ml/s) | Elastance (cm $H_2O$/ml) |
|---|---|---|
| Controls | 0.193 ± 0.008 | 2.04 ± 0.048 |
| 1 | 0.213 ± 0.020* | 1.851 ± 0.166* |
| 2 | 0.204 ± 0.063 | 1.493 ± 0.157* |
| 3 | 0.261 ± 0.019* | 1.669 ± 0.119* |

At day 60, baseline resistance values for Models I and III were significantly higher than controls, and baseline elastance values for Models I, II and III were significantly lower than controls.

Of the groups evaluated on day 35, two control animals and two animals from Model I, died during cannulation of either their trachea or jugular vein. During OA aerosol inhalation, test animals from all three models showed classic signs of anaphylaxis. The effects noted were:

**Day 21** All animals developed dyspnea after 20 s of exposure and some gave a 'croaking' sound which signalled severe bronchospasm,

and they appeared cyanotic and were immediately removed from the exposure chamber and given adrenalin (0.1 ml).

**Days 22-24** All animals tolerated 45 s of exposure before showing signs of respiratory distress. Animals that remained in the chamber > 45 s developed severe dyspnea, seizures and died. A total of (7) animals from Model I, (7) from Model II and (8) from Model III died during aerosol exposure (see Tables 6 & 7). Some animals from Models II and III bled profusely from the nose.

**Days 25-30** All test animals tolerated up to 4 min of exposure before respiratory distress developed, except one from Model II and one from Model III; both died after 4 min of exposure. Animals were usually quiet, limp, with a puffy, "porcupine-like" appearance after aerosol exposure.

A total of 22 out of 84 animals died during aerosol inhalation. Data are summarized in Tables 6 and 7.

Because of persistent increases in baseline values of resistance and elastance, some experiments were terminated before complete dose-response curves could be obtained to all three agonists.

Histamine, methacholine and serotonin induced dose-dependent increases in resistance and elastance in all groups of animals. Dose-response

Table 6

Day-35 Group: Duration of aerosol exposure in seconds

| Days | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|
| Model I | 20 (1/8) | 45 (1/7) | 45 (1/6) | 45 | 240 | 240 | 240 | 240 | 240 | 240 |
| Model II | 20 (1/8) | 45 (1/7) | 45 | 45 (1/6) | 240 | 240 | 240 | 240 | 240 | 240 |
| Model III | 20 (1/8) | 45 (1/7) | 45 (2/6) | 45 | 240 | 240 | 240 | 240 | 240 | 240 |

( ) Number of animals that died during aerosol exposure

Table 7

Day-60 Group: Duration of aerosol exposure in seconds

| Days | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|
| Model I | 20 (2/10) | 45 | 45 (1/8) | 45 (1/6) | 240 | 240 | 240 | 240 | 240 | 240 |
| Model II | 20 (1/10) | 45 (1/9) | 45 (1/8) | 45 | 240 (1/7) | 240 | 240 | 240 | 240 | 240 |
| Model III | 20 | 45 (2/10) | 45 (1/8) | 45 | 240 (1/6) | 240 | 240 | 240 | 240 | 240 |

( ) Number of animals that died during aerosol exposure

curves from control animals were compared with those obtained from animals in Models I, II and III at 35 and 60 d after sensitization.

On day 35, measurements of resistance showed that the dose-response curve for histamine was shifted to the left compared to control (increased responsiveness) in Models II and III. Model I was similar to control (Fig. 1A).

On day 60, dose-response curves for this agonist in Models II and III were also shifted to the left; again, Model I showed no difference from control (Fig. 1B). ANOVA confirmed these findings and indicated significant differences among the control and histamine dose-response curves of those from Models II and III on days 35 and 60. In all three models, dose-response curves to methacholine were not significantly different from control on days 35 and 60 (Fig. 2A,B). Although dose-response curves to serotonin on day 35 were not significantly different from control in any of the models (Fig. 3A), on day 60, curves from Models II and III were significantly shifted to the left (Fig. 3B).

Thus, in Model I, animals sensitized with OA (20 mg/kg) there was no increased airway responsiveness to any of the three agonists on day 35 or on day 60. Guinea pigs from Model II, sensitized with OA (20 μg/kg + $Al(OH)_3$ [100 mg/kg], ip) showed increased responsiveness to only histamine on day 35 and to histamine and serotonin on day 60. Animals given cyclophosphamide (100 mg/kg, ip) and sensitized with OA (20 μg/kg + $Al(OH)_3$ [2 mg] + *B. pertussis*, ip) showed similar changes to those seen in Model II.

Figure 1. Dose-response curves to histamine: A) 35 days (n = 3); and, B) 60 days (n = 5); post-sensitization. In Models II and III, test curves were significantly shifted to the left from control (* $p < 0.05$) at days 35 and 60.

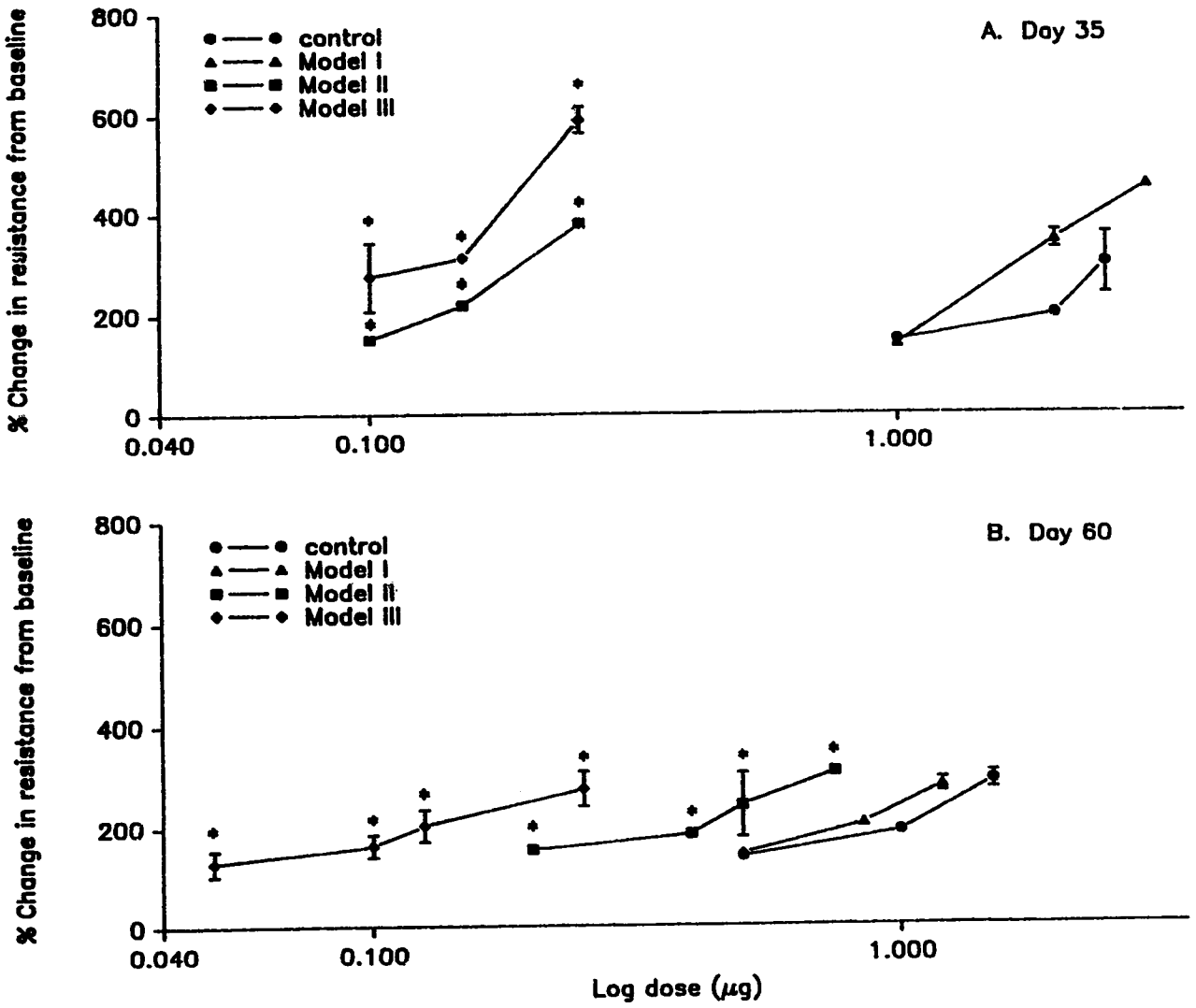

% Change in resistance from baseline
800
600
400
200
0
control
Model I
Model II
Model III
A. Day 35
0.040
0.100
1.000
% Change in resistance from baseline
800
600
400
200
0
control
Model I
Model II
Model III
B. Day 60
0.040
0.100
1.000
Log dose (μg)

Figure 2. Dose-response curves to methacholine: A) 35 days (n = 3); and, B) 60 days (n = 5); post-sensitization. In all models, there was no difference between the control and the test curves.

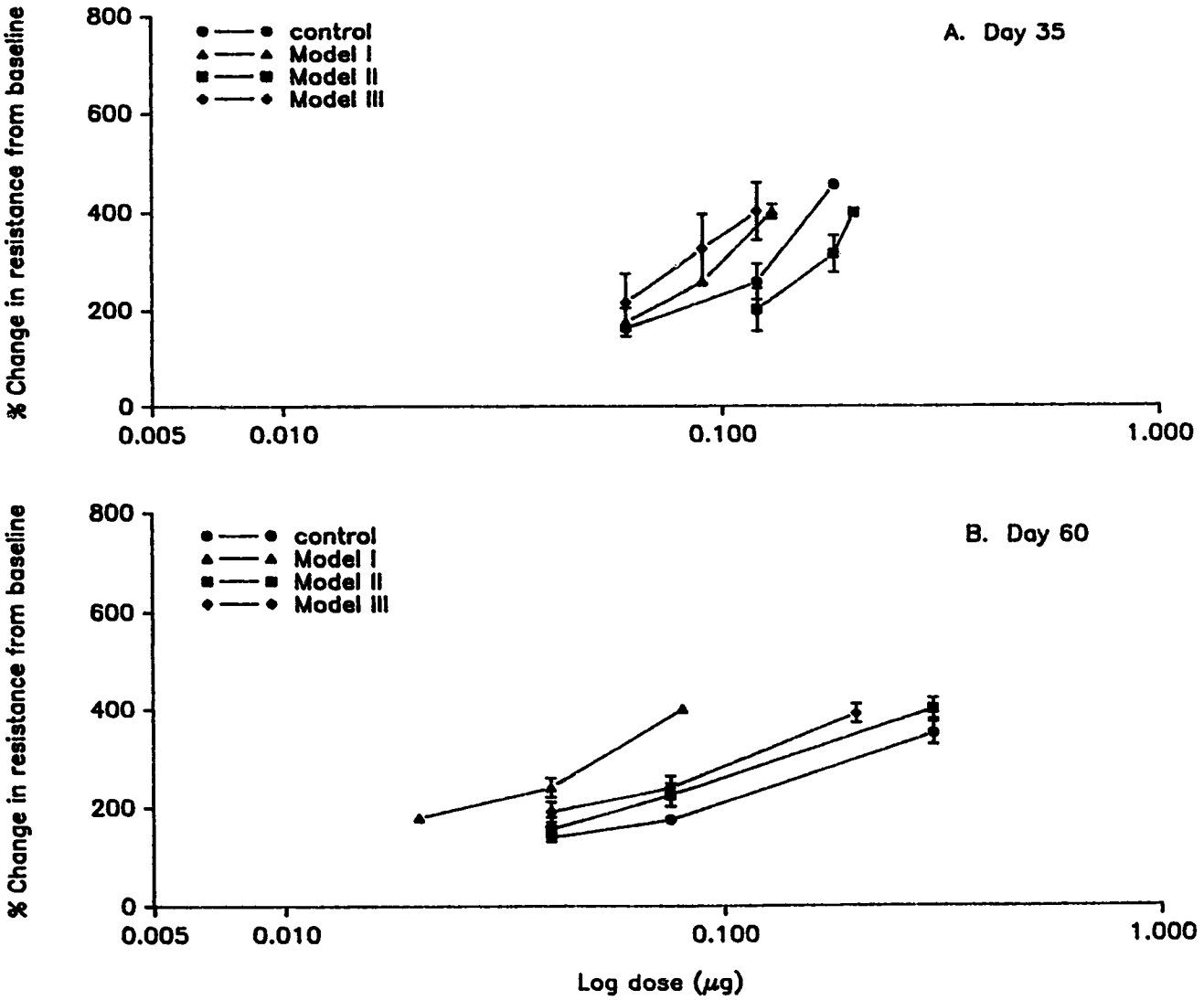
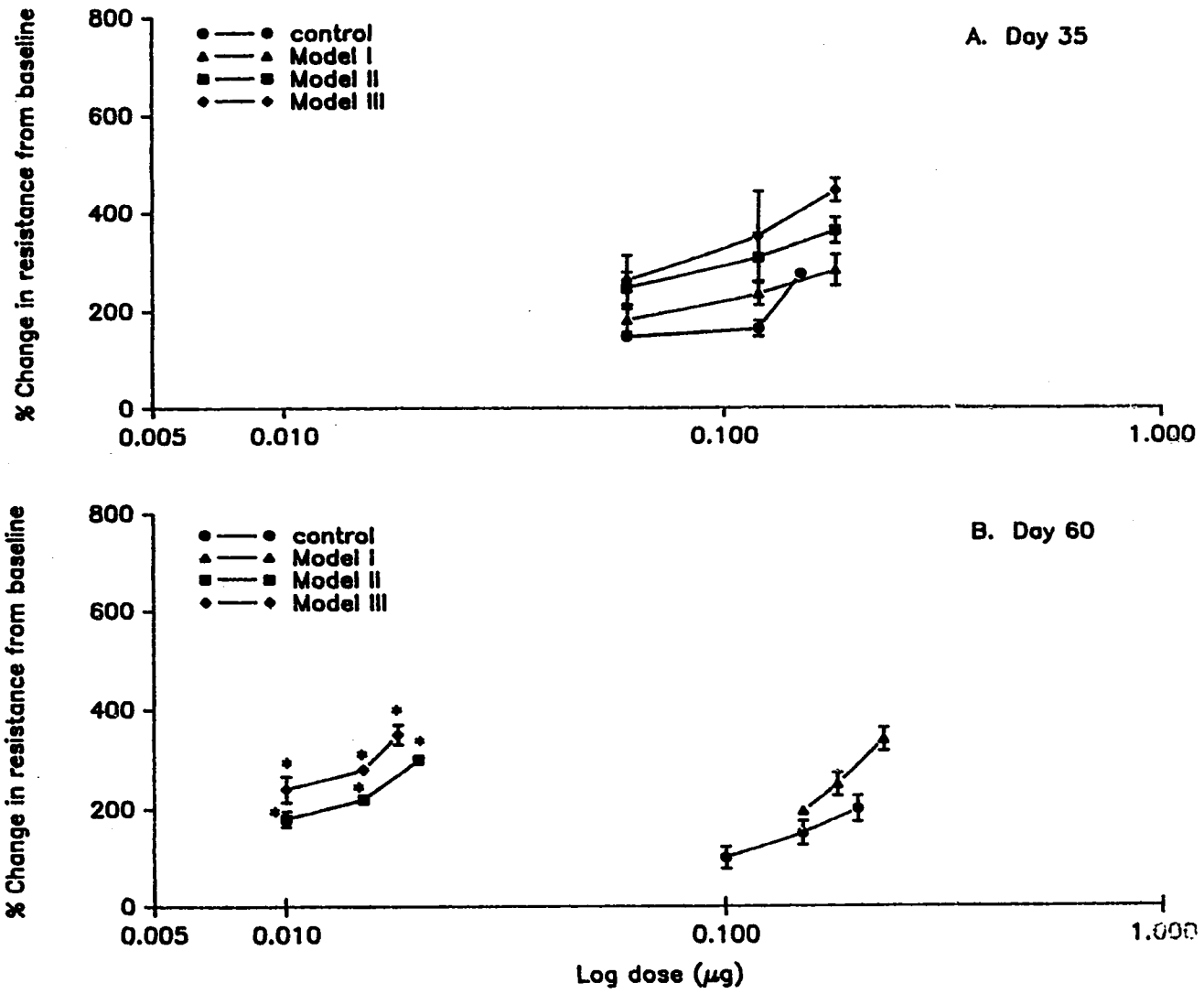

% Change in resistance from baseline
800
600
400
200
0
control
Model I
Model II
Model III
A. Day 35
0.005
0.010
0.100
1.000
% Change in resistance from baseline
800
600
400
200
0
control
Model I
Model II
Model III
B. Day 60
0.005
0.010
0.100
1.000
Log dose (μg)

Figure 3. Dose-response curves to serotonin: A) 35 days (n = 3); and, B) 60 days (n = 5); post-sensitization. In Models II and III, test curves were significantly shifted to the left from control (* $p < 0.05$) at day 60.


% Change in resistance from baseline
800
600
400
200
0
control
Model I
Model II
Model III
A. Day 35
0.005
0.010
0.100
1.000
% Change in resistance from baseline
800
600
400
200
0
control
Model I
Model II
Model III
B. Day 60
0.005
0.010
0.100
1.000
Log dose (μg)

II. Histology

Lungs of test animals showed noticeable histologic differences from control animals (Fig. 4A,B). On day 35, lung sections from animals from Model I showed infiltration of mononuclear cells with occasional macrophages free in the alveolar lumen. The bronchioles appeared to be constricted (Fig. 5A). By day 60, these animals showed alveolar emphysema and perivascular edema with less infiltration of mononuclear cells. Also, the bronchioles appeared to be constricted (Fig. 5B).

Lungs of animals from Model II showed periarterial edema, the area of the smooth muscle in the bronchi and bronchioles and in the arterial walls appeared to be increased compared to controls. There was accumulation of fluid in the alveoli with some alveolar macrophages. The bronchi and bronchioles appeared somewhat less constricted than those in Model I (Fig. 6A). On day 60, there seemed to be constriction of the bronchioles, but again less than that seen in Model I. There was also alveolar edema and occasional macrophages in the alveoli (Fig. 6B). Lung sections from animals in Model III showed mild alveolar emphysema at day 35. This was not present in the other models at this time. The area of the smooth muscle in the bronchi and bronchioles appeared increased. Constriction of the bronchi and bronchioles was comparable to Model I (Fig. 7A). On day 60, there was a greater degree of alveolar emphysema than was seen in the other models at this time. There was perivascular and peribronchial lymphoid infiltration and the area of the

smooth muscle appeared increased in the bronchioles. Macrophages were present in some alveoli (Fig. 7B).

### III. Total and differential cell counts

1 - Day 35

The results of total and differential cell counts done on lavage fluid obtained on day 35 are shown in Tables 8 and 9. ANOVA revealed marked increases in cell numbers in all three models, compared to controls, but no significant differences among the models (Fig. 8A).

Differential counts on 200 cells on day 35 showed an increase in eosinophils, macrophages and polymorphonuclear (PMN) cells in all three models. Multiple comparisons using Student-Newman Keuls' Test were carried out among cell types, models and days at $\alpha = 0.05$. Eosinophil numbers for Model II were significantly higher than for the other two models. Also, the eosinophil count for Model I was significantly higher than for Model III on day 35. Macrophage numbers for Model II were significantly higher than those for Models I and III. There was no difference between counts for Models I and III. Neutrophil numbers for Models II and III were similar but significantly higher than in Model 1 (Fig. 9A).

2 - Day 60

The results of total and differential cell counts on day 60 are shown in Tables 10 and 11. Total cell counts at day 60 were not significantly different

Figure 4. Histological sections from control animals' lungs at: A) day 35; and, B) day 60. Control animals were sensitized with saline and inhaled saline aerosols. Photographs show normal parenchyma and large, clear airway lumens. (Bar = 0.1 mm)

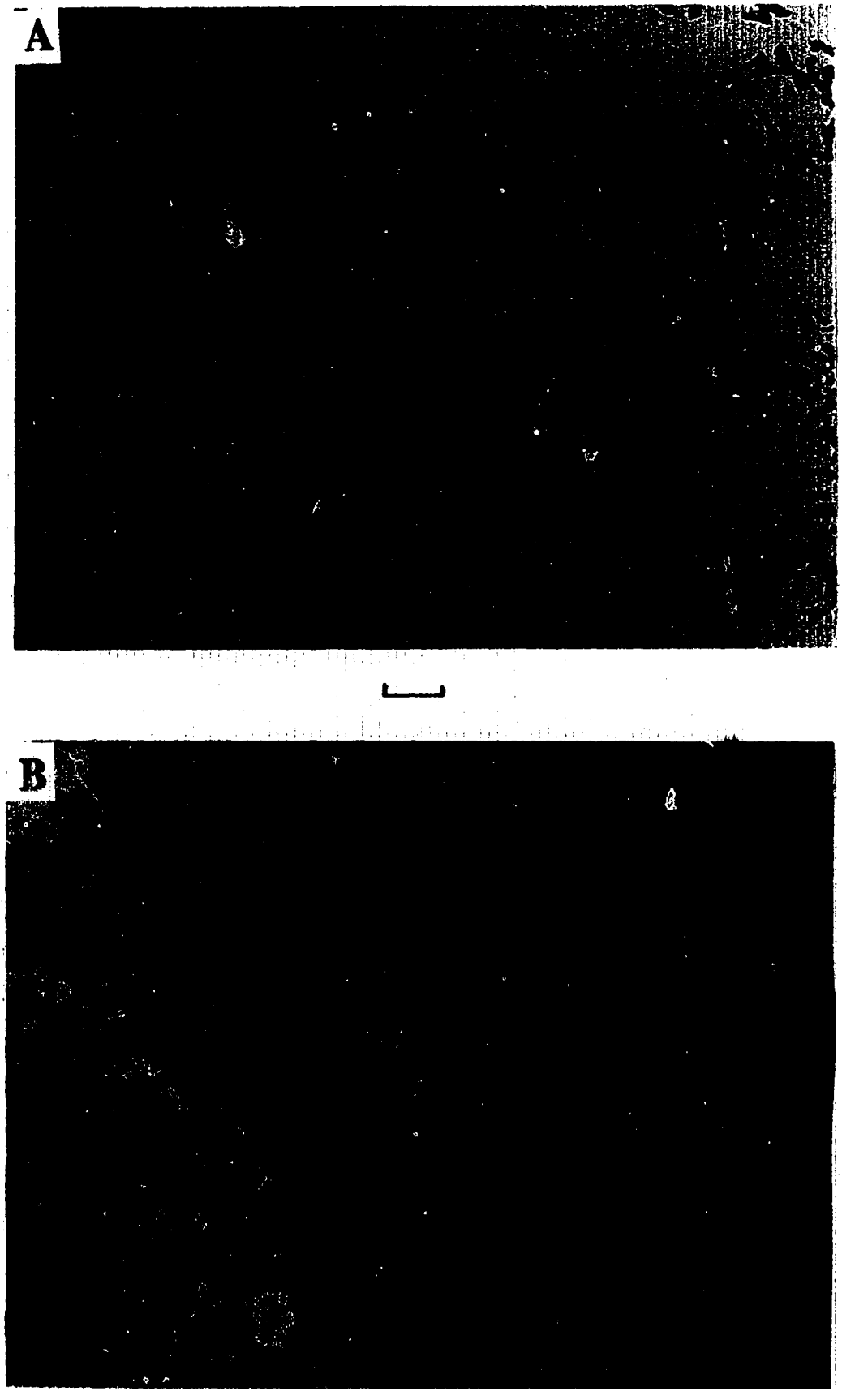

A
B

Figure 5. Histological sections of animals' lungs from Model I at: A) day 35; and, B) day 60. Guinea pigs were sensitized with OA (20 mg/kg, ip) and inhaled OA (2%) aerosols. Photographs show: A) cellular infiltration (➞), and the bronchioles appear constricted (▸); and B) alveolar emphysema (→), cellular infiltration (➞), and the bronchioles appear constricted (▸).

(Bar = 0.1 mm)

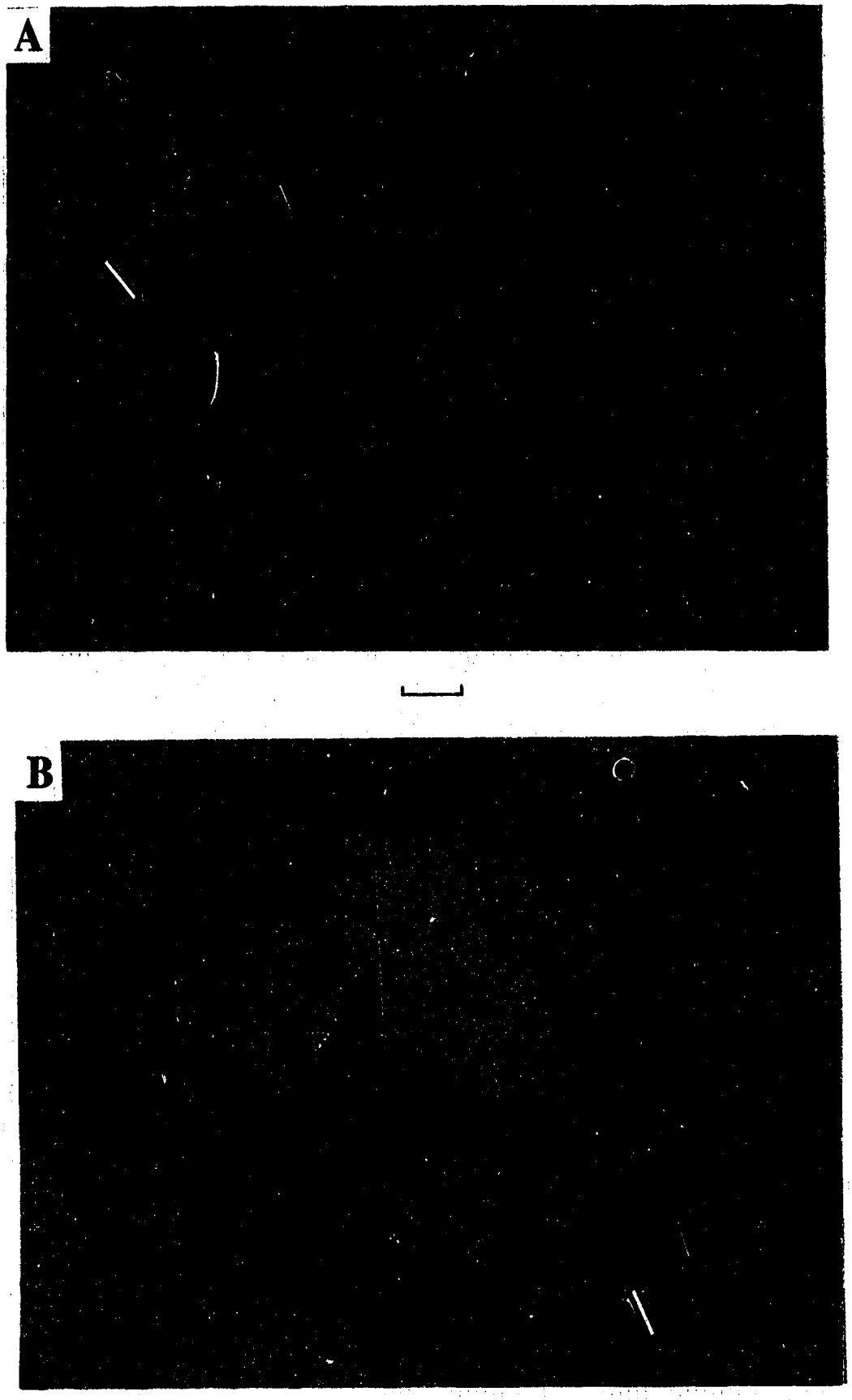

A
B

Figure 6. Histological sections of animals' lungs from Model II at: **A**) day 35; and, **B**) day 60. Guinea pigs were sensitized with OA (20 μg/kg + $Al(OH)_3$ [100 mg/kg], ip) and inhaled OA (2%) aerosols. Photographs show: **A**) increased area of smooth muscle in the airway and blood vessel walls (⇾), and the bronchioles appear constricted (▸); and **B**) the bronchioles appear constricted (▸).

(Bar = 0.1 mm)

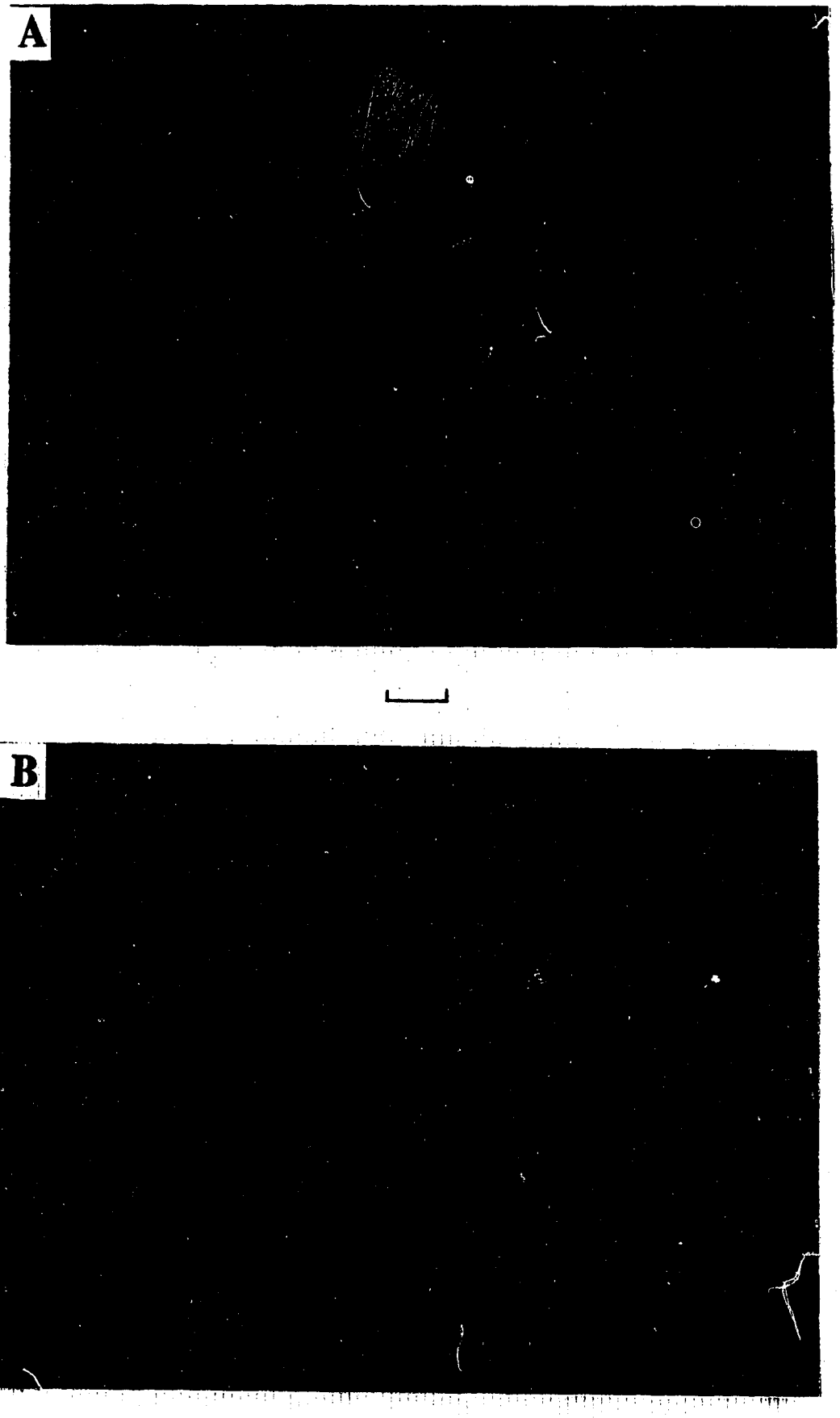

A
B

Figure 7. Histological sections of animals' lungs from Model III at: **A**) day 35; and, **B**) day 60. Guinea pigs were sensitized with OA (20 μg/kg + $Al(OH)_3$ [2 mg] + *B. pertussis*, ip) and inhaled OA (2%) aerosols. Photographs show: **A**) alveolar emphysema (→), increased area of smooth muscle in the airways' walls (→); and **B**) perivascular and peribronchial cellular infiltration (→). (Bar = 0.1 mm)


A


B

from those at day 35 for Model I. By contrast, total cell counts at day 60 were lower than at day 35 for Model II, but higher for Model III (Fig. 8B).

On day 60, differential counts showed a significant increase in eosinophils and macrophages in all three models compared to control, however, only PMN numbers were significantly increased in Models II and III. Eosinophil counts for Model II were significantly lower than on day 35, but were still significantly higher than Models I and III, which were similar. There was a significant increase in macrophages in Model I from day 35 to day 60. This increase in macrophage numbers was also much higher than that observed for Models II and III. Macrophage counts for Model II and III at day 60 were significantly less than on day 35. PMN counts for Model III were significantly higher than Model II which was significantly higher than Model I. PMN in all three models at day 60 were significantly higher than at day 35 (Fig. 9B).

Table 8

Day 35: Total cell counts $x$ $10^4$

| Controls | Model I | Model II | Model III |
|---|---|---|---|
| 31.7 ± 0.4 | 34.9 ± 27 | 35.2 ± 4 | 29.7 ± 3 |

Table 9

Day 35: Differential cell counts $x\ 10^4$

**Controls**

| Eosinophils | Macrophages | Neutrophils |
|---|---|---|
| 6.0 ± 0.08 | 25.0 ± 0.3 | 0.3 ± 0.004 |

**Model I**

| Eosinophils | Macrophages | Neutrophils |
|---|---|---|
| 214 ± 29 | 143 ± 15 | 10.53 ± 2.4 |

**Model II**

| Eosinophils | Macrophages | Neutrophils |
|---|---|---|
| 326 ± 9 | 184 ± 4 | 32.0 ± 10 |

**Model III**

| Eosinophils | Macrophages | Neutrophils |
|---|---|---|
| 145 ± 4 | 138 ± 21 | 32.3 ± 0.4 |

Table 10

Day 60: Total cell counts $x\ 10^4$

| Controls | Model I | Model II | Model III |
|---|---|---|---|
| 31.8 ± 0.4 | 35.2 ± 28 | 349 ± 18 | 330 ± 39 |

Table 11

Day 60: Differential cell counts $x\ 10^4$

**Controls**

| Eosinophils | Macrophages | Neutrophils |
|---|---|---|
| 0.028 ± 0.08 | 25 ± 0.3 | 0.001 ± 0.003 |

**Model I**

| Eosinophils | Macrophages | Neutrophils |
|---|---|---|
| 154 ± 12 | 193 ± 21 | 11.2 ± 1.9 |

**Model II**

| Eosinophils | Macrophages | Neutrophils |
|---|---|---|
| 203 ± 1 | 114 ± 5 | 33.4 ± 1.6 |

Table 11 continued

**Model III**

| Eosinophils | Macrophages | Neutrophils |
|---|---|---|
| 158 ± 19 | 125 ± 15 | 56 ± 0.7 |

## IV. ELIZA

1 - Standard curves

Levels of anti-OA IgG, and $IgG_1$ and $IgG_2$ anti-OA antibodies were estimated with ELISA. Antibody titres were taken from the respective standard curves (Figs. 10A,B,C; and Table 12). The general equation for the line of best fit was:

$$y = (a-d)/(1+x/c)^b + d$$

a = asymptote at low values of the x-axis
b = slope
c = mid-point between a and d
d = asymptote at high values of the x-axis

Figure 8. Total cell counts in broncho-alveolar lavage fluid: **A**) 35 days (n = 3); and, **B**) 60 days (n = 5); post-sensitization. All models showed significant increases (* $p < 0.05$) in total cell counts compared to control.

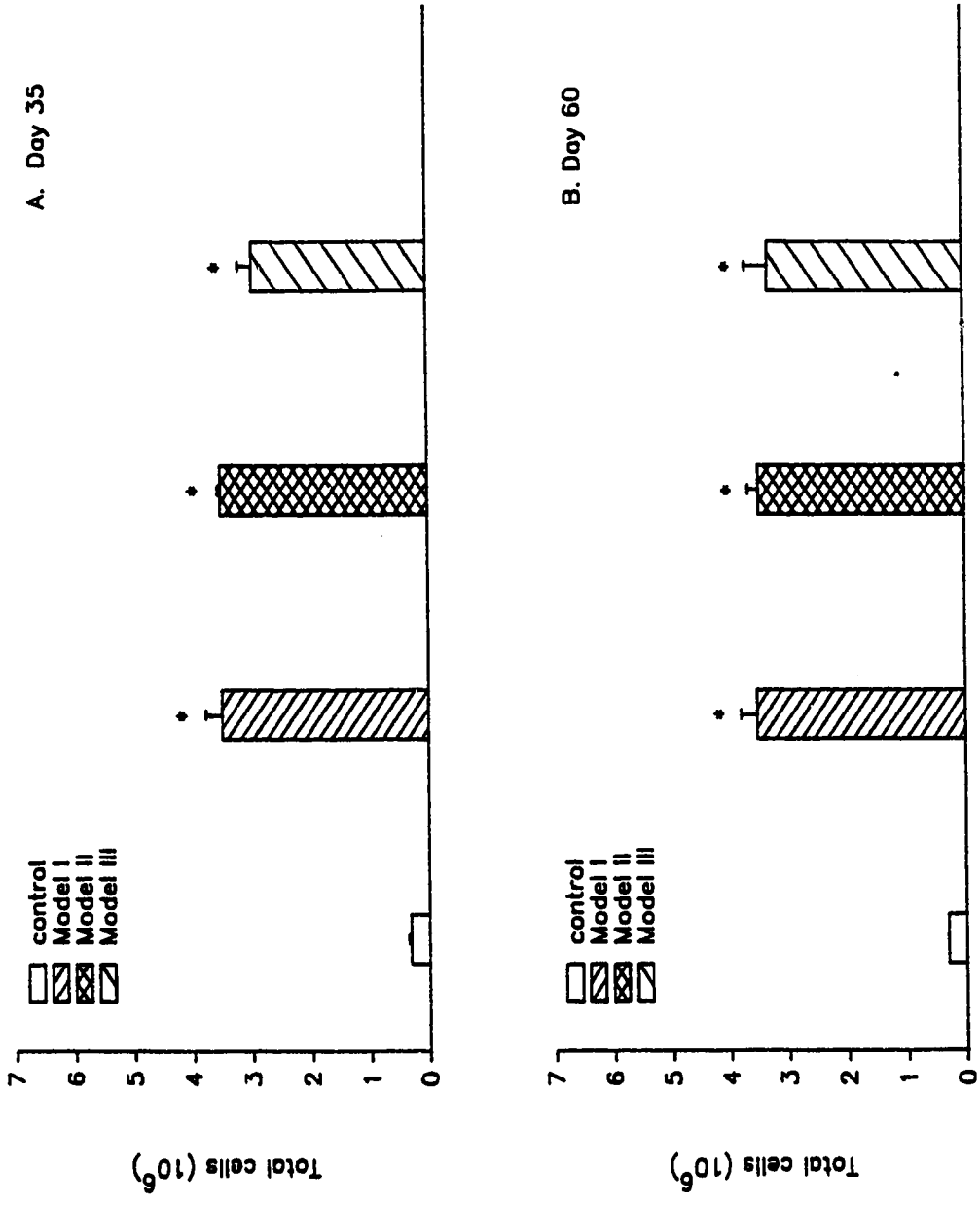

A. Day 35
B. Day 60
control
Model I
Model II
Model III
Total cells ($10^6$)
0
1
2
3
4
5
6
7
*

Figure 9. Differential counts of cells from broncho-alveolar lavage fluid: **A**) 35 days (n = 3); and, **B**) 60 days (n = 5); post-sensitization. (Significant differences: * from control; ◊ between Models I and II; ★ between Models I and III; ♦ between Models II and III. See text for a more detailed explanation.

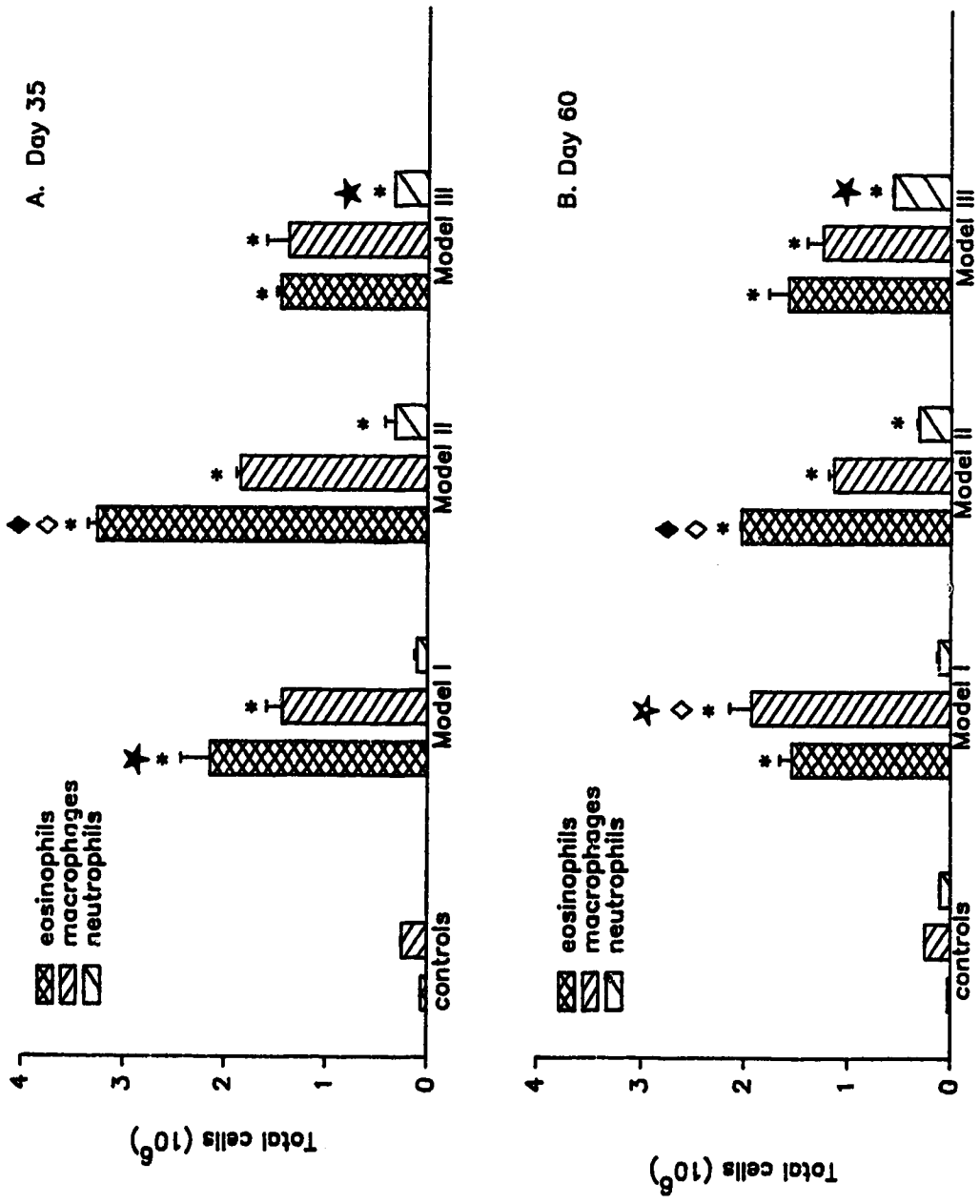
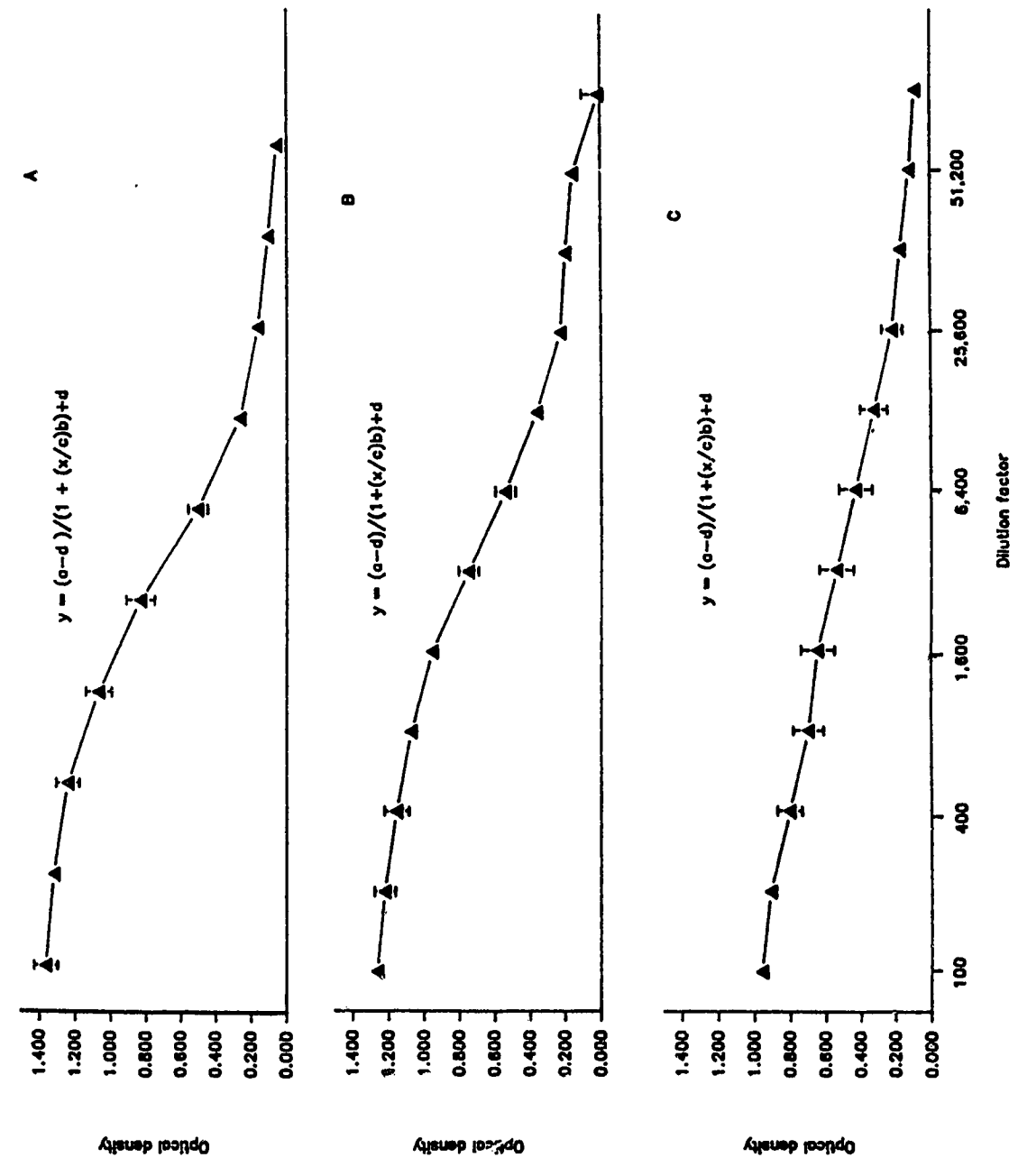

A. Day 35
B. Day 60
eosinophils
macrophages
neutrophils
controls
Model I
Model II
Model III
Total cells ($10^6$)
0
1
2
3
4

Table 12

Optical densities of serum dilutions. Values were used to construct standard curves.

| Dilutions | IgG | $IgG_1$ | $IgG_2$ |
|---|---|---|---|
| 100 | 1.36 ± 0.06 | 1.26 ± 0.47 | 0.96 ± 0.02 |
| 200 | 1.32 ± 0.05 | 1.22 ± 0.64 | 0.91 ± 0.04 |
| 400 | 1.24 ± 0.01 | 1.15 ± 0.77 | 0.80 ± 0.07 |
| 800 | 1.07 ± 0.07 | 1.07 ± 0.02 | 0.69 ± 0.09 |
| 1600 | [illegible] ± 0.08 | 0.95 ± 0.04 | 0.64 ± 0.09 |
| 3200 | [illegible] ± 0.06 | 0.75 ± 0.06 | 0.53 ± 0.10 |
| 6400 | 0.27 ± 0.04 | 0.54 ± 0.05 | 0.43 ± 0.10 |
| 12800 | 0.16 ± 0.02 | 0.36 ± 0.04 | 0.31 ± 0.08 |
| 25600 | 0.12 ± 0.02 | 0.23 ± 0.02 | 0.21 ± 0.06 |
| 51200 | 0.06 ± 0.01 | 0.20 ± 0.20 | 0.17 ± 0.04 |

Figure 10. Standard curves for estimation of **A**) anti-OA IgG; **B**) anti-OA $IgG_1$; and, **C**) anti-OA $IgG_2$ antibodies (n = 6, in each case).


A
y = (a−d )/(1 + (x/c)b)+d
B
y = (a−d)/(1+(x/c)b)+d
C
y = (a−d)/(1+(x/c)b)+d
Optical density
Optical density
Optical density
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
100
400
1,600
6,400
25,600
51,200
Dilution factor

2 - Plate reading

The optimal time for reading assay plates was obtained from kinetic studies; optical density was plotted against time for 40 min using Softmax$^R$, with readings taken every 17 s. The results are summarized in Figs. 11, 12 and 13.

Kinetic plots were done at three different dilutions for each antibody type. The rate of reactions and the shape of the curves varied with dilution. From the kinetic studies, $V_{max}$ for IgG was recorded at 1:100, 1:800 and 1:6,400 dilutions (Fig. 11). $V_{max}$ varied with dilution: range 9.6-16.68 mOD/min. The rate of reaction decreased with increasing dilution. At all dilutions, the reactions were essentially complete after 25 min. From the kinetic studies of $IgG_1$ at 1:100, 1:1,600 and 1:25,600, $V_{max}$ varied with dilution: range 14.99-25.76 mOD/min. The rate of reaction decreased with increasing dilution. At all dilutions, the reactions were essentially complete after 25 min (Fig. 12). From the kinetic studies of $IgG_2$ at 1:100, 1:1,600 and 1:12,800, $V_{max}$ varied with dilution: range 11.09-33.15 mOD/min. The rate of reaction increased from 1:100 to 1:1,600 dilutions and decreased from 1:1,600 to 1:12,800 dilutions. At all dilutions, the reactions were essentially complete after 25 min (Fig. 13). All assay plates were read 30 min after the addition of the ABTS/$H_2O_2$ substrate. This ensured that the reactions were complete even at very high serum dilutions.

Figure 11. Kinetic plot for anti-OA IgG ELISA. Reaction rate recorded at **A**) 1:100; **B**) 1:800; and, **C**) 1:6,400 dilutions.

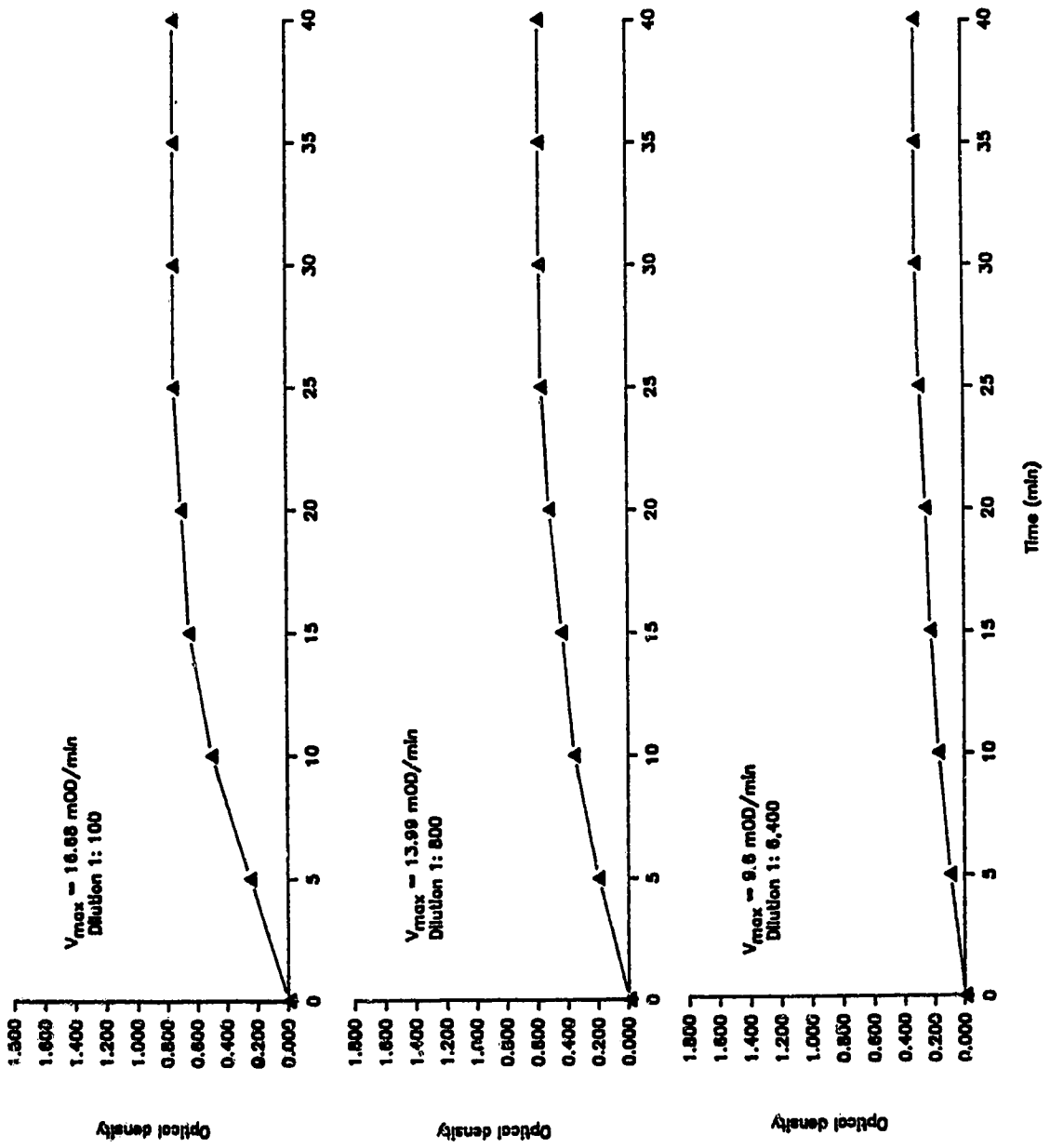

Optical density
$V_{max}$ = 16.88 mOD/min
Dilution 1: 100
$V_{max}$ = 13.99 mOD/min
Dilution 1: 800
$V_{max}$ = 9.8 mOD/min
Dilution 1: 6,400
Time (min)

Figure 12. Kinetic plots for anti-OA $IgG_1$ ELISA. Reaction rates recorded at **A**) 1:100; **B**) 1:1,600; and, **C**) 1:25,600 dilutions.

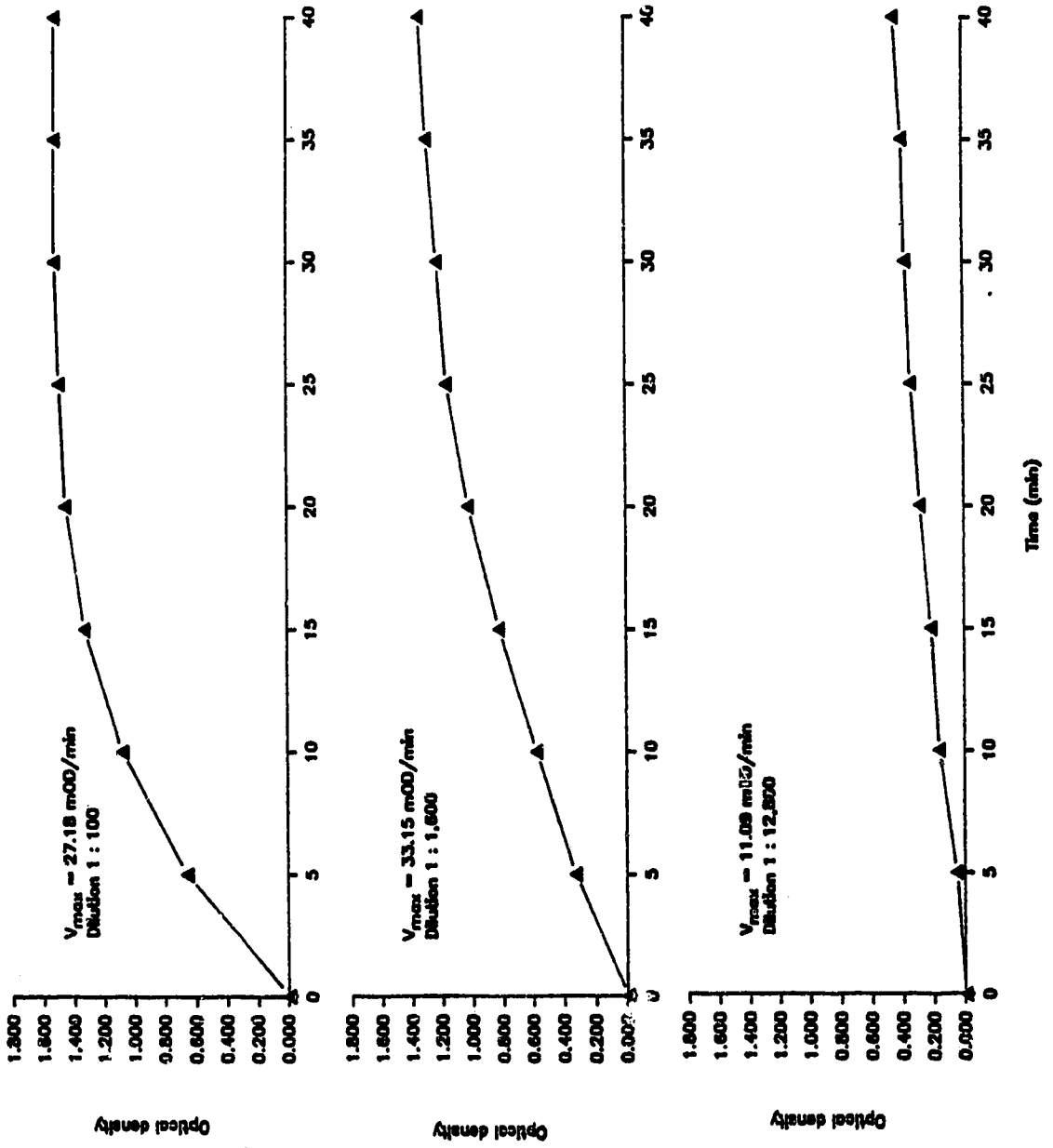
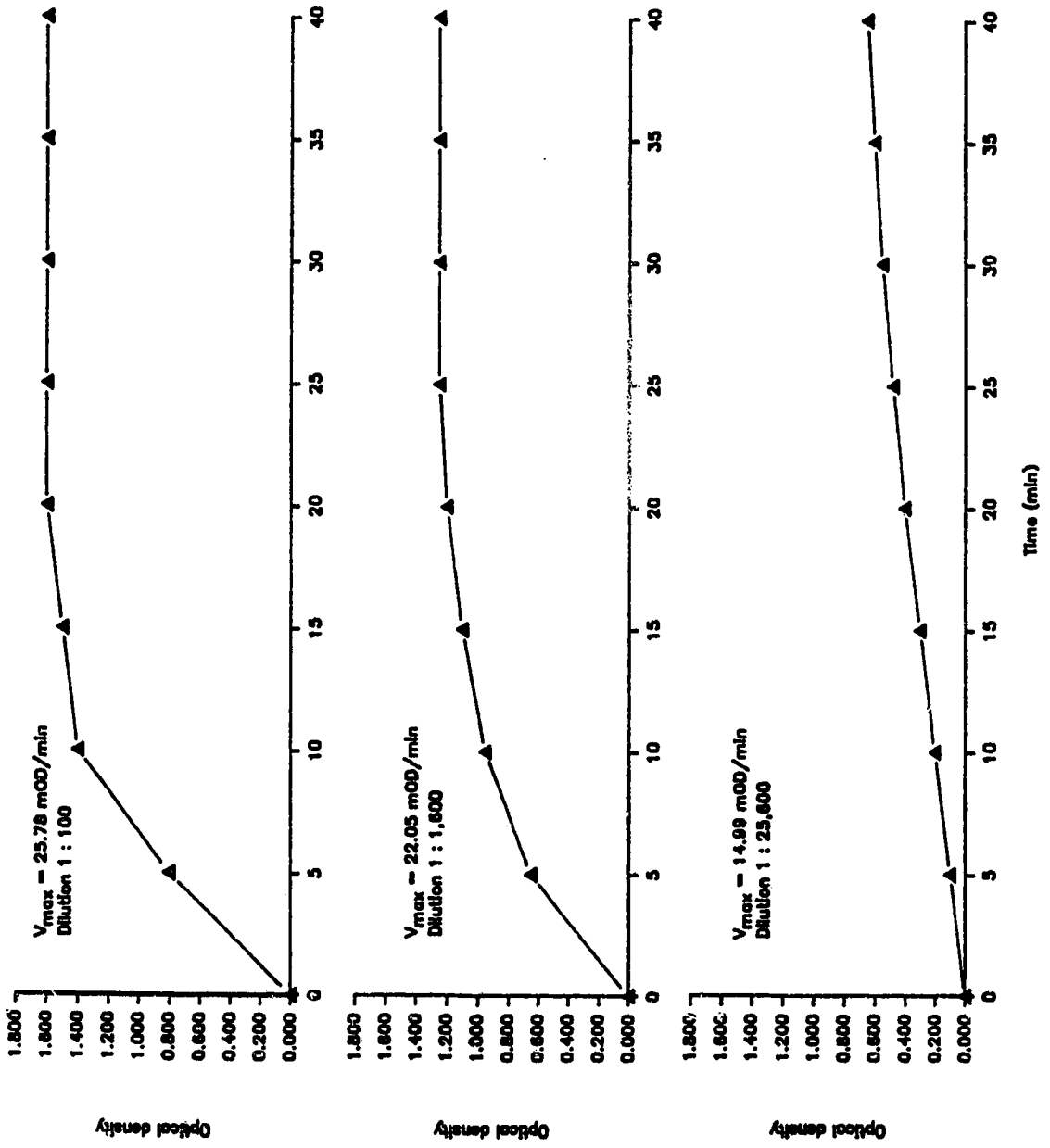

Optical density
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
20
25
30
35
40
Time (min)
Vmax = 27.18 mOD/min
Dilution 1 : 100
Vmax = 33.15 mOD/min
Dilution 1 : 1,600
Vmax = 11.09 mOD/min
Dilution 1 : 12,800

Figure 13. Kinetic plots for anti-OA $IgG_2$ ELISA. Reaction rates recorded at **A**) 1:100; **B**) 1:1,600; and, **C**) 1:12,800 dilutions.


Optical density
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
20
25
30
35
40
Time (min)
$V_{max}$ = 25.78 mOD/min
Dilution 1 : 100
$V_{max}$ = 22.05 mOD/min
Dilution 1 : 1,600
$V_{max}$ = 14.99 mOD/min
Dilution 1 : 25,600

3 - Coefficients of variation for ELISA

The intra-assay coefficients of variation for anti-OA IgG, $IgG_1$ and $IgG_2$ antibody assays were < 5% for dilutions up to 6,400. At higher dilutions, the CVs were ≤ 9% (Fig. 14A,B,C). The inter-assay variation was between 2-13% (Figs. 15A,B,C).

4 - Detection limits

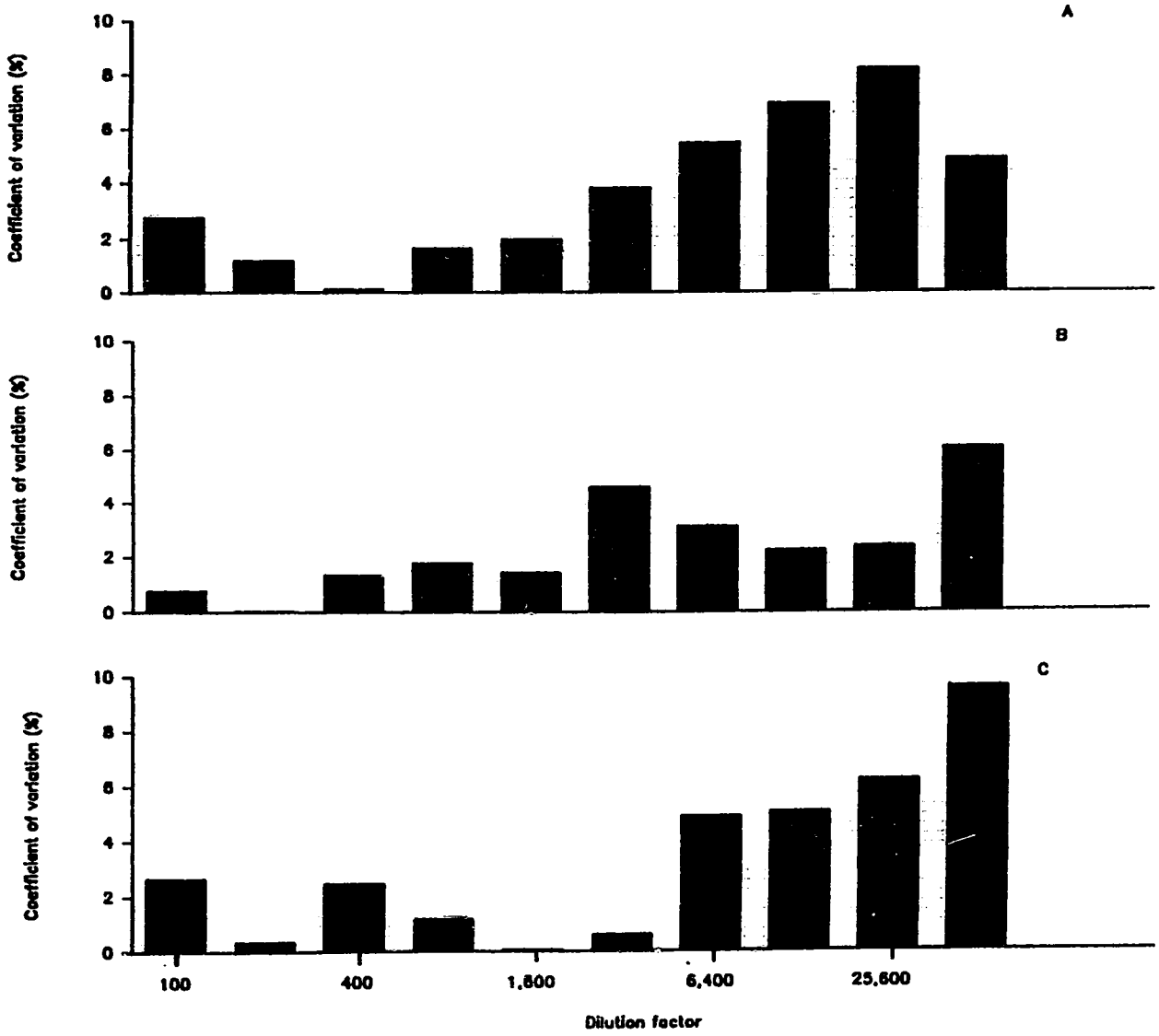
The detection limits for the assays varied with antibody subtypes. The detection limit for IgG, $IgG_1$ and $IgG_2$ in sera were approximately 1:6,400, 1:12,800 and 1:12,800, respectively (Fig. 16A,B,C). The detection limit was taken as twice the standard deviation.

**V. Assay results**

ELISA results for guinea-pig anti-OA IgG, $IgG_1$ and $IgG_2$ antibodies in the sera of animals from Models I, II and III are shown in Tables 13, 14, and 15.

Model I: anti-OA IgG antibodies were first detected in the sera after 15 d and titres ranged from 327 to 496. The highest titre (12,170 ± 4,077), was observed 35 days after sensitization and 5 d after aerosol exposure. On day 35 titres ranged from 10,600 to 21,100 (n = 4). Titres gradually decreased to day 60 (720 ± 321), ranging from 215 to 1,650 (Fig. 17A).

Figure 14. Mean intra-assay coefficients of variation for: **A**) IgG; **B**) $IgG_1$; and, **C**) $IgG_2$ ELISA (n = 2, assays in duplicate).


A
B
C
Coefficient of variation (%)
0
2
4
6
8
10
100
400
1,600
6,400
25,600
Dilution factor

77

Figure 15. Mean inter-assay coefficients of variation for: A) IgG; B) $IgG_1$; and, C) $IgG_2$ ELISA (n = 6, assays in duplicate).

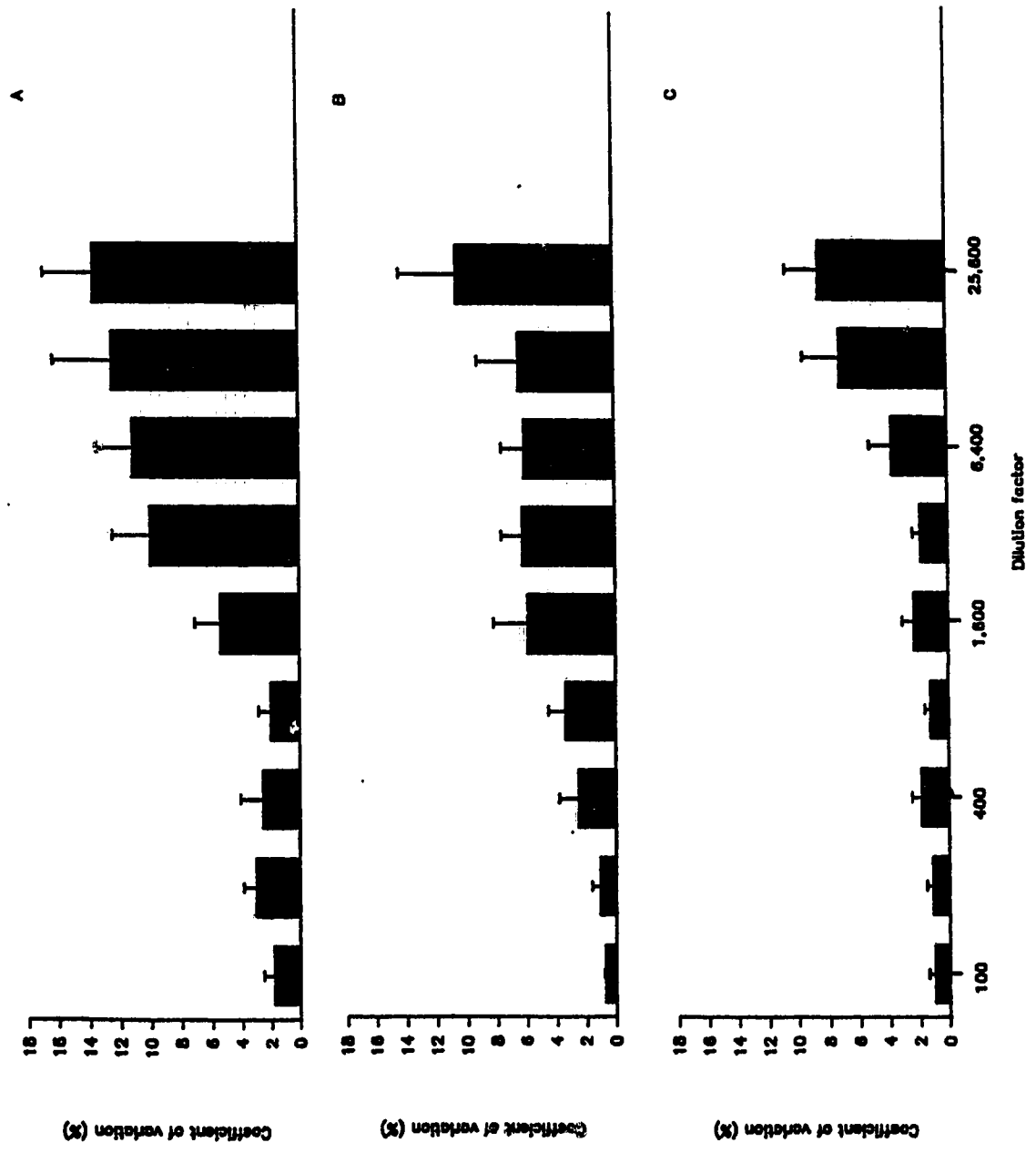

A
B
C
Coefficient of variation (%)
Coefficient of variation (%)
Coefficient of variation (%)
0
2
4
6
8
10
12
14
16
18
100
400
1,600
6,400
25,600
Dilution factor

Figure 16. Detection limits for: A) IgG; B) $IgG_1$; and, C) $IgG_2$ ELISA (n = 6, assays in duplicate).

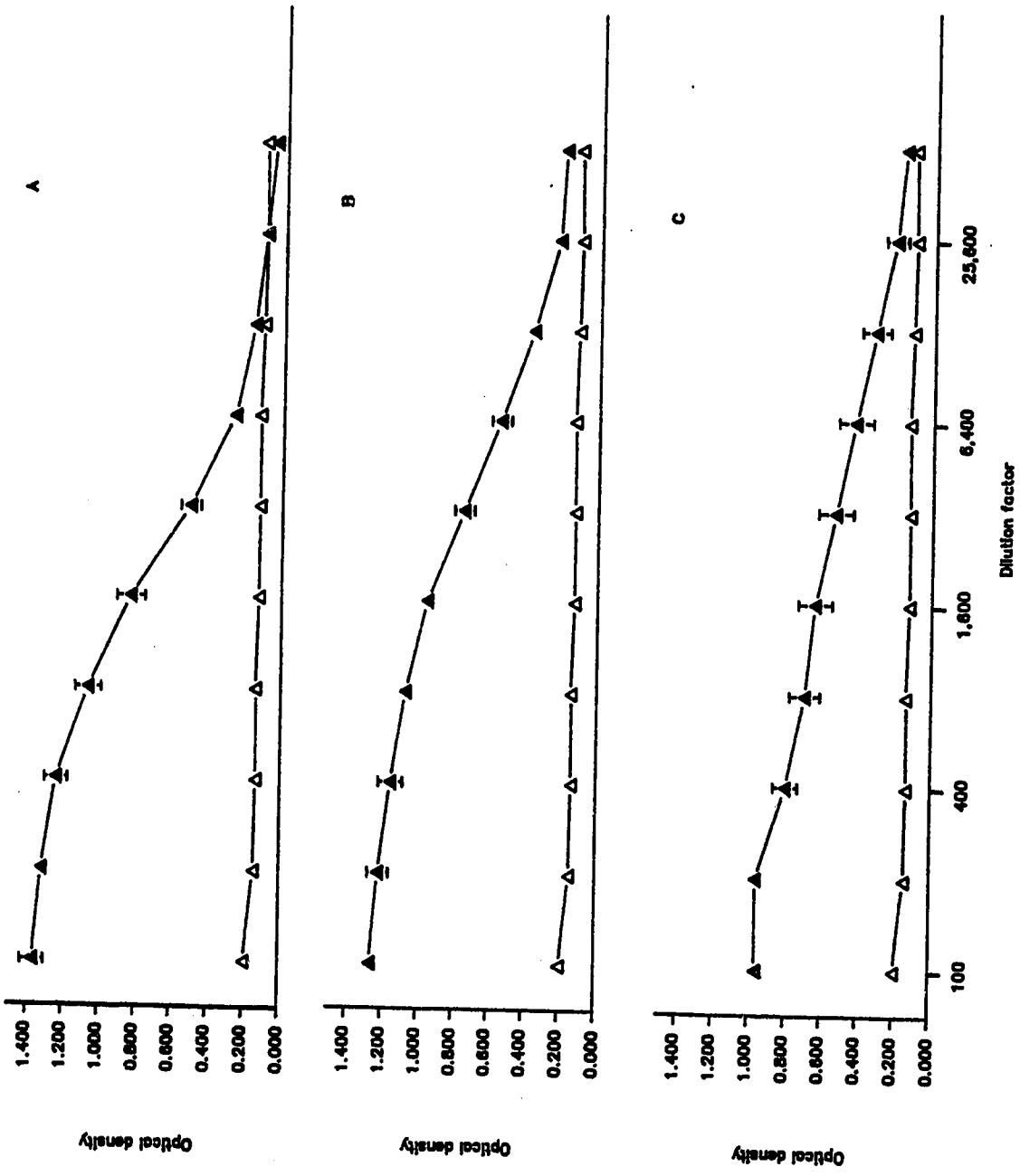

A
B
C
Optical density
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
100
400
1,600
6,400
25,600
Dilution factor

Table 13

Model I: Anti-OA antibody titres in guinea-pig sera

| Time d | IgG | $IgG_1$ | $IgG_2$ |
|---|---|---|---|
| 15 | 390 ± 53 | 0 | 129 ± 64 |
| 21 | 951 ± 56 | 1100 ± 200 | 1120 ± 551 |
| 30 | 8966 ± 2480 | 7980 ± 2270 | 1150 ± 443 |
| 35 | 12170 ± 4077 | 7383 ± 3610 | 5720 ± 1355 |
| 40 | 2719 ± 900 | 3400 ± 967 | 5310 ± 1287 |
| 45 | 1985 ± 717 | 3180 ± 1416 | 2760 ± 310 |
| 50 | 1436 ± 406 | 2230 ± 832 | 2850 ± 404 |
| 55 | 744 ± 487 | 1390 ± 713 | 2666 ± 870 |
| 60 | 720 ± 321 | 1280 ± 736 | 829 ± 275 |

n = 4

Table 14

Model II: Anti-OA antibody titres in guinea-pig sera

| Time d | IgG | $IgG_1$ | $IgG_2$ |
|---|---|---|---|
| 15 | 0 | 0 | 0 |
| 21 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 |
| 35 | 202§ | 132§ | 0 |
| 40 | 1181 ± 418 | 2640 ± 350 | 1906 ± 561 |
| 45 | 6726 ± 2848 | 4961 ± 2500 | 2000 ± 351 |
| 50 | 11390 ± 3309 | 6400 ± 3200 | 2100 ± 316 |
| 55 | 10750 ± 5142 | 3066 ± 1540 | 2576 ± 577 |
| 60 | 8000 ± 3000 | 2500 ± 1200 | 3500 ± 500 |

n = 4, except: § n = 1

Table 15

Model III: Anti-OA antibody titres in guinea-pig sera

| Time d | IgG | $IgG_1$ | $IgG_2$ |
|---|---|---|---|
| 15 | 0 | 0 | 0 |
| 21 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 |
| 35 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 |
| 45 | 6950 ± 2840 | 4960 ± 2507 | 4636 ± 1817 |
| 50 | 10800 ± 1000 | 11750 ± 698 | 2400 ± 610 |
| 55 | 14200 ± 1600 | 14250 ± 850 | 2293 ± 903 |
| 60 | 11900 ± 1200 | 8500 ± 647 | 1946 ± 544 |

n = 4

Model II: anti-OA IgG antibodies were only detected after aerosol challenge, with the highest titre (11,390 ± 3,309) noted after 50 days. On day 50, titres ranged from 3,000 to 25,600 (n = 4). Antibody titres were still very high (8,000 ± 3,000) at day 60 and ranged from 4,000 to 20,000 (Fig. 17B).

IgG antibody production was delayed in animals from Model III. There was no IgG antibody development until 15 d after aerosol challenge. Highest mean titres (14,200 ± 1,600) were obtained on day 50 and ranged from 5750 to 14,000. At day 60, the highest mean titre was 11,900 ± 1,200, antibody titres and ranged from 680 to 5,470 (Fig. 17C).

Multiple comparison tests were carried out among antibody titres, times, and models at $\alpha = 0.05$. Predictably, significant differences were found in the time for antibody development among the three models. At day 15, only Model I showed antibody titres. At day 35, titres were highest in Model I, very low in Model II and undetectable in Model III. By day 60, IgG for Model I was very much reduced, whereas Models II and III had high titres.

Guinea-pig $IgG_1$ antibodies were detected in all models, but titres varied with time. For Model I, $IgG_1$ antibodies (1,100 ± 300) were first detected at day 21 and ranged from 183 to 2,970 (n = 4) (Fig. 18A). After 3 days of aerosol exposure there was a significant increase in both $IgG_1$ and $IgG_2$ antibody titres (Fig. 19). There was no significant increase in titres between day 23 and 29 for these subtypes.

For Model II, $IgG_1$ antibodies were detected after 35 days with highest mean titre (6,400 ± 3,200) at day 50, and ranged from 9,380 to 16,100 (Fig. 18B). $IgG_1$ antibodies were detected after 45 days for Model III and ranged from 2,727 to 12,000 (n = 4). Highest titre (14,250 ± 850) was observed on day 55 (Fig. 18C).

The time course of development of IgG, $IgG_1$ and $IgG_2$ antibodies were similar in all models; $IgG_2$ titres were always significantly lower than those for IgG and $IgG_1$. In Model I, anti-OA $IgG_2$ antibodies were first detected after 15 d and ranged from 3 to 59. Highest mean titre (5,720 ± 1,255) was observed after 35 days (Fig. 20A). For Model II, highest mean titre (3,500 ± 500) was obtained after 60 d and ranged from 1,500 to 1,843 (Fig. 20B). Highest mean titre (4,636 ± 1817) was observed after 45 days for Model III, ranging from 2,100 to 8,160 and gradually decreased to day 60 (Fig. 20C).

### VI. Passive cutaneous anaphylaxis tests (PCA)

In these experiments, PCA was performed 2 and 7 days after the injection of sera to determine the presence of IgE. For Model I, sera from days 21, 35 and 60 showed PCA activity at 2 days, but no activity at 7 days, at 1:100, 1:400 and 1:800 dilutions.

Sera taken from animals in Model II showed PCA activity at days 21, 35 and 60 at low and high dilutions at both 2 and 7 days. This indicated the presence of homocytotrophic antibodies of the IgE type. For Model III, PCA activity was present at very low dilutions (1:100) in 21-day sera at 2 days, but not at 7 days. Sera taken on days 35 and 60 gave PCA activity at 2 days even at high dilutions (1:800), but activity at 7 days was present only at the lowest (1:100) dilution (Table 16).

Figure 17. Time course of appearance of anti-OA IgG antibodies in guinea-pig sera from: **A**) Model I; **B**) Model II; and, **C**) Model III (n = 4).

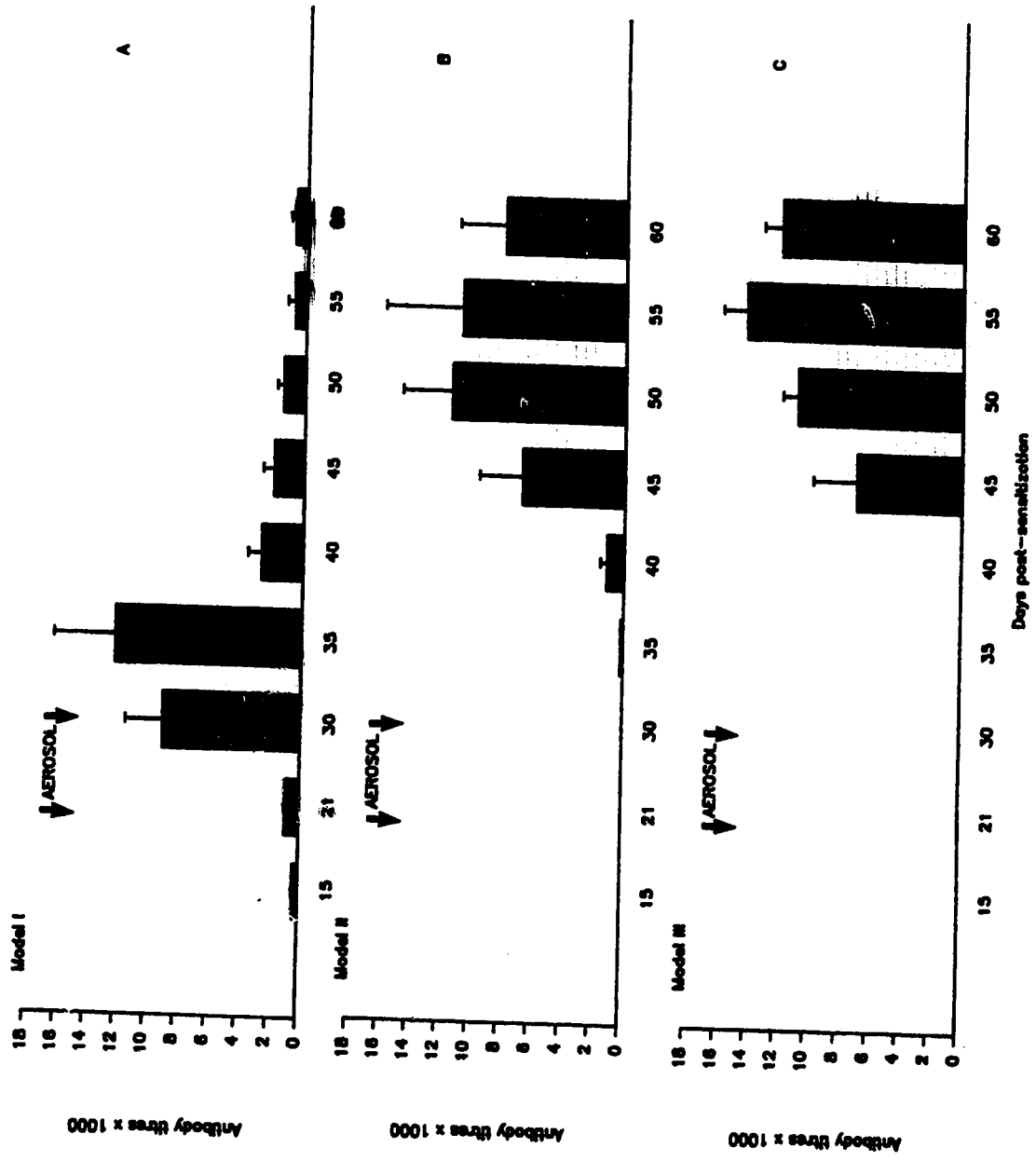

A
B
C
Model I
Model II
Model III
AEROSOL
AEROSOL
AEROSOL
Antibody titres x 1000
Antibody titres x 1000
Antibody titres x 1000
Days post-sensitization

Figure 18. Time course of appearance of anti-OA $IgG_1$ antibodies in guinea-pig sera from: A) Model I; B) Model II; and, C) Model III (n = 4).

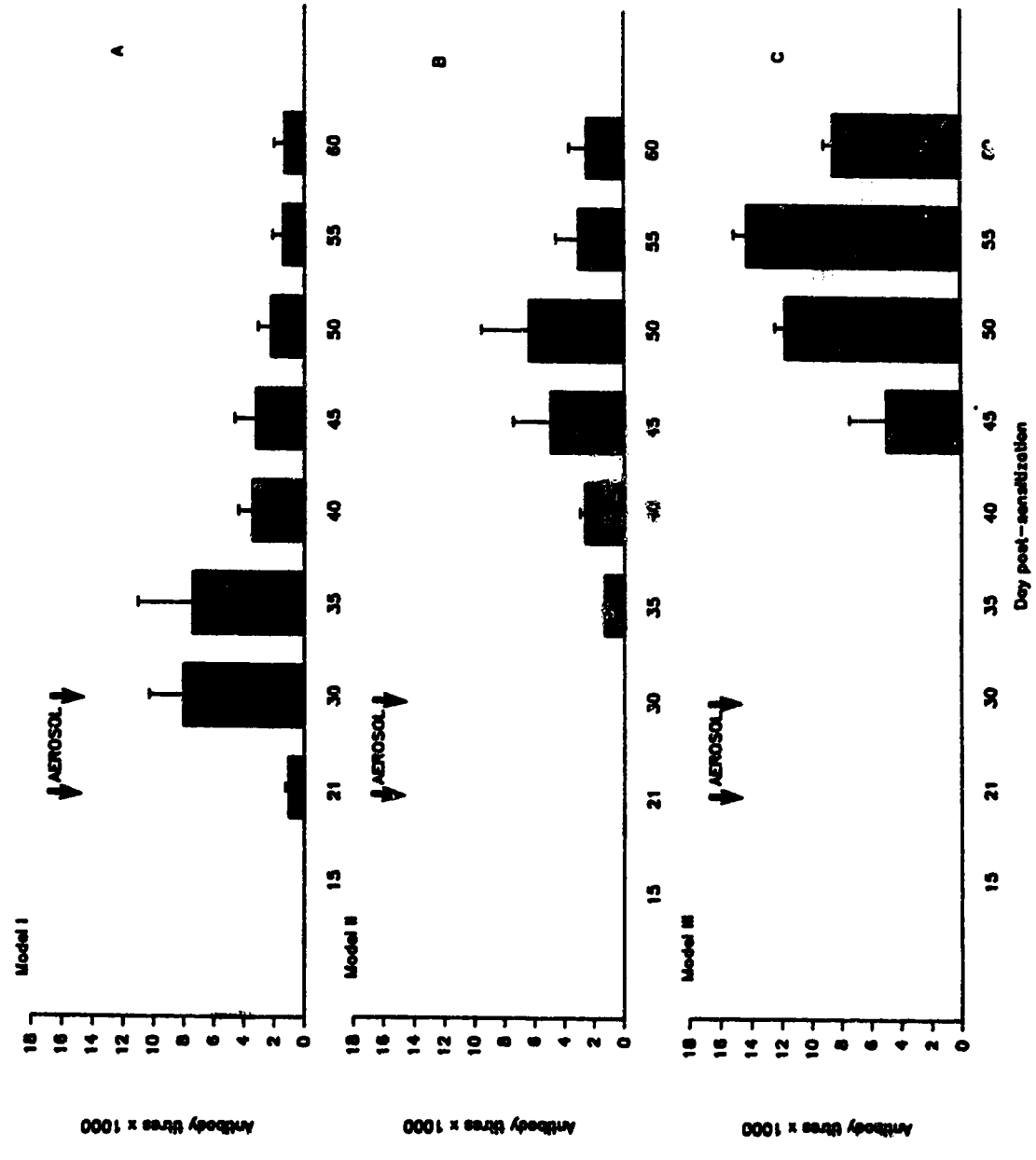

A
B
C
Model I
Model II
Model III
AEROSOL
Antibody titres x 1000
Day post-sensitization

Figure 19. Time course of appearance of anti-OA IgG, $IgG_1$ and $IgG_2$ antibodies in guinea-pig sera obtained during OA aerosol exposure (days 21, 23, 25, 27, 29, and 30) from: A) Model I; B) Model II; and, C) Model III (n = 4).

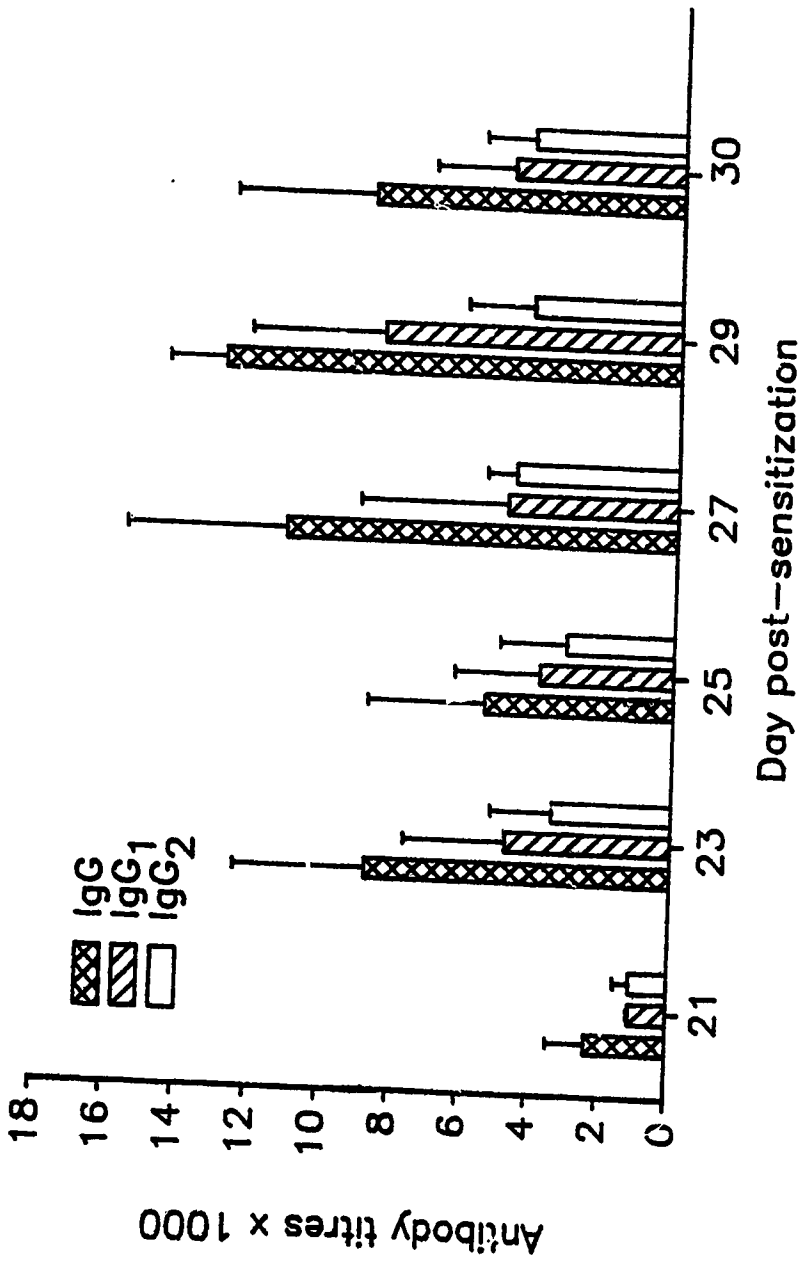

Antibody titres x 1000
0
2
4
6
8
10
12
14
16
18
IgG
IgG1
IgG2
21
23
25
27
29
30
Day post-sensitization

Figure 20. Time course of appearance of anti-OA $IgG_2$ antibodies in guinea-pig sera from: **A**) Model I; **B**) Model II; and, **C**) Model III (n = 4).

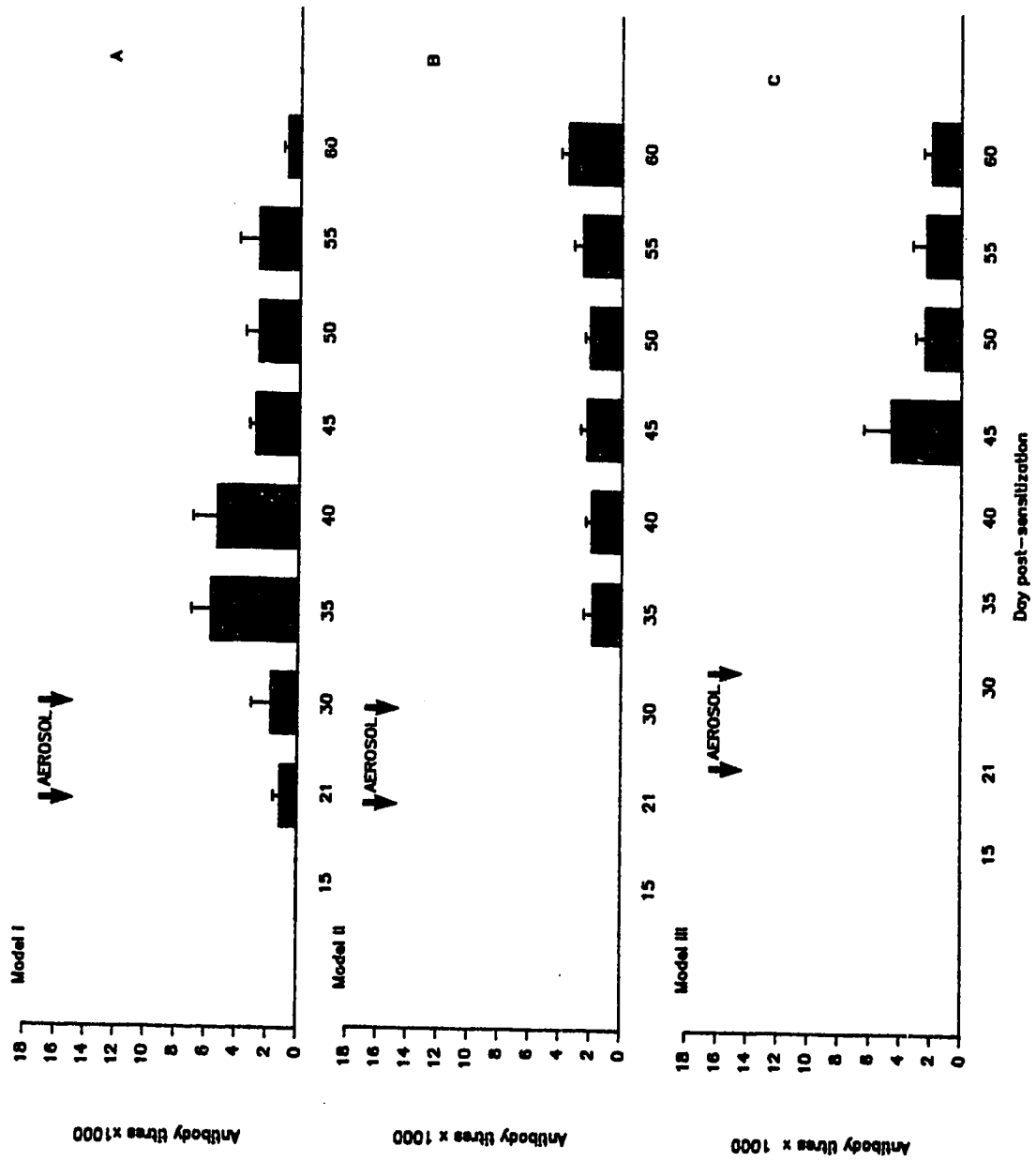

A
B
C
Model I
Model II
Model III
AEROSOL
AEROSOL
AEROSOL
Antibody titres x1000
Antibody titres x 1000
Antibody titres x 1000
Day post-sensitization
0
2
4
6
8
10
12
14
16
18
15
21
30
35
40
45
50
55
60

Table 16

Passive cutaneous anaphylaxis (PCA) with unheated sera 21, 35 and 60 days post-sensitization

**Sera taken 21 days post-sensitization**

| | Model I | | Model II | | Model III | |
|---|---|---|---|---|---|---|
| Dilution | 2 d | 7 d | 2 d | 7 d | 2 d | 7 d |
| 1 in 800 | ++ | - | ++ | ++ | - | - |
| 1 in 400 | +++ | - | ++++ | ++++ | - | - |
| 1 in 100 | +++ | - | +++++ | +++++ | +++ | +++ |

**Sera taken 35 days post-sensitization**

| | Model I | | Model II | | Model III | |
|---|---|---|---|---|---|---|
| Dilution | 2 d | 7 d | 2 d | 7 d | 2 d | 7 d |
| 1 in 800 | + | - | + | + | + | - |
| 1 in 400 | +++ | - | +++ | +++ | +++ | - |
| 1 in 100 | +++++ | - | +++++ | +++++ | +++++ | ++ |

Table 16 continued

**Sera taken 60 days post-sensitization**

| | Model I | | Model II | | Model III | |
|---|---|---|---|---|---|---|
| Dilution | 2 d | 7 d | 2 d | 7 d | 2 d | 7 d |
| 1 in 800 | + | - | + | + | + | - |
| 1 in 400 | +++ | - | +++ | +++ | + | - |
| 1 in 100 | +++++ | - | +++++ | +++++ | +++++ | + |

IgE antibodies can be distinguished from IgG antibodies by heating sera for 4 h at 56°C. If IgG is present, heated sera should show PCA activity at 2 days, but not at 7 days (Table 17).

These results showed that sensitization with large amounts of OA (20 mg, ip, Model I), induced high titres of $IgG_1$ and lower titres of $IgG_2$ as early as 15 days after sensitization, but very little IgE.

Model II - animals sensitized with low quantities of OA (20 μg + $Al(OH)_3$ [100 mg/kg]) developed $IgG_1$, $IgG_2$ and IgE antibodies that were detected up to 60 days after sensitization.

Model III- Animals treated with cyclophosphamide and sensitized with low doses of OA (20 μg + $Al(OH)_3$ [2 mg] + *B. pertussis*) developed very high titres of $IgG_1$, but low titres of $IgG_2$ and IgE.

Table 17

Passive cutaneous anaphylaxis (PCA) with heated sera 21, 35 and 60 days post-sensitization

**Sera taken 21 days post-sensitization**

| | Model I | | Model II | | Model III | |
|---|---|---|---|---|---|---|
| Dilution | 2 d | 7 d | 2 d | 7 d | 2 d | 7 d |
| 1 in 100 | + | - | + | - | ++ | - |

**Sera taken 35 days post-sensitization**

| | Model I | | Model II | | Model III | |
|---|---|---|---|---|---|---|
| Dilution | 2 d | 7 d | 2 d | 7 d | 2 d | 7 d |
| 1 in 100 | +++ | - | ++++ | - | +++ | - |

**Sera taken 60 days post-sensitization**

| | Model I | | Model II | | Model III | |
|---|---|---|---|---|---|---|
| Dilution | 2 d | 7 d | 2 d | 7 d | 2 d | 7 d |
| 1 in 100 | +++ | - | +++ | - | + | - |

## Chapter IV - DISCUSSION

I used three guinea-pig models of asthma for the work described in this thesis, and I sought to determine which model most closely resembled the disease in humans. I used five criteria to make comparisons: 1) The nature of the immunologic response; 2) The presence or absence of anaphylaxis upon antigen challenge; 3) The number and types of cells appearing in BAL fluid following challenges; 4) The morphology of the lungs following challenges; and 5) The appearance of airways' hyperresponsiveness to agonists injected iv.

### I. Immunologic responses

Of the models selected for examination in this study, Model I was expected to generate mainly anti-ovalbumin antibodies (anti-OA Ab) of the IgG isotype, and Models II and III to generate anti-OA Ab of the IgE isotype. It is generally accepted that allergic asthma is mediated via IgE (Nakagawa, 1991). However, in many guinea-pig models of asthma, responses are mediated via IgG antibodies (Kallos & Kallos, 1984). Predominantly IgE-mediated guinea-pig models of the disease have been described (Catty, 1969, Parish, 1970; Dobson *et al.*, 1971; Perini & Mota, 1972, 1973; Andersson, 1980a,b; Kallos & Kallos, 1984); the protocol described by Andersson (1980a,b) formed the basis for Model II. Model III was based on the procedures described by Andersson (1981) and Sanjar *et al.* (1990); it, too, was

expected to generate an IgE-mediated form of the disease.

Anti-OA Ab of the IgE and IgG isotypes interact with specific receptor sites on target cells. Cells' receptors for the Fc region of immunoglobulins are involved in mediating interactions between several cell types and the immunoglobulins IgE, IgG and its subtypes, IgA and IgM (Fridman, 1989). The high affinity ($K_A \geq 10^8\ M^{-1}$) receptor (FcεRI) for IgE found on rat mast cells and basophils has been characterized and its structure is known (Blank *et al.*, 1989). It is this receptor that mediates these cells' degranulation and release of mediators. Low affinity ($K_A = 10^6\text{-}10^7\ M^{-1}$) Fc receptors (FcR) expressed on lymphocytes, macrophages, polymorphonuclear cells (PMN) and mast cells bind at least three IgG subclasses and even bind IgE (Unkeless *et al.*, 1988; Fridman, 1989; Anderson, 1989). On mast cells, FcγR are capable of mediating degranulation and the release of inflammatory mediators (Daëron *et al.*, 1980; Fridman, 1989). In rats, the isotypes IgE and $IgG_{2a}$ appear to interact with FcεRI and FcγR on mast cells to induce the release of histamine and the production of leukotrienes (LTs) (Morse *et al.*, 1968; Bach *et al.*, 1971; Halper & Metzger, 1976; Bazin & Pauwels, 1982). Whereas in mice, IgE and $IgG_1$ isotypes are involved (Daëron *et al.*, 1980).

Studies of the specific types of antibodies involved in anaphylactic responses in guinea pigs indicate that both IgE and IgG isotypes are capable of mediating them (Perini & Mota, 1973; Desquand *et al.*, 1990; Karol *et al.*, 1991). Interestingly, unlike rats or mice, in addition to IgE, both $IgG_1$ and

$IgG_2$ subclasses have been reported to mediate reactions (Perini & Mota, 1973; Desquand *et al.*, 1990). I used commercially available antibodies to develop ELISA techniques for total anti-OA IgG, anti-OA $IgG_1$ and anti-OA $IgG_2$ Ab. I used these assays to monitor serum concentrations of these anti-OA Ab. Antibody titres were estimated from standard curves. The curves obtained for IgG and $IgG_1$ were steep, while that for $IgG_2$ less so. Kinetic ELISA also showed that the rate of the reaction during color development varied with antibody dilution and with antibody type. For IgG and $IgG_1$ the rate of reaction decreased with increasing dilution whereas for $IgG_2$ the highest rate of reaction was after 4-fold dilution. At low dilution of sera (antibody excess), it would be expected from the Law of Mass Action, that high affinity antibody would bind preferentially to antigen on the solid phase, whereas at high dilutions of sera (antigen excess) low affinity antibody should also bind. Therefore, affinity may influence the slope of the linear part of the curve, which would be steep with high affinity antibodies and gradual with low affinity antibodies. However, with sera containing polyclonal antibodies, the relative amounts of the different affinity populations will also influence the shape of the curves.

Serum IgE anti-OA Ab concentrations were difficult to measure. Serum concentrations would be expected to be around 1-10 μg/ml. Preliminary experiments using anti-mouse or anti-human IgE Ab in a "sandwich-type" assay based on the radioallergosorbent test (RAST) for the presence of

specific IgE were unsuccessful. Thus, as specific anti-guinea-pig IgE sera were not available commercially, I was compelled to use PCA to monitor serum IgE anti-OA Ab concentrations. At best, this technique is semi-quantitative! However, I was able to distinguish IgE- from IgG-mediated responses by performing PCA at 2 and 7 d after injection of serum (Ovary *et al.*, 1976; Karol *et al.*, 1991). Also, IgE- and IgG-mediated responses were distinguished by incubating sera at 56°C for 4 h (Bloch, 1967, 1969; Ovary *et al.*, 1976; Karol *et al.*, 1991).

**Model I** - My data for IgG anti-OA Ab are summarized in Figs. 17A, 18A, 19 and 20A, and for IgE, Tables 16 and 17. These findings show that IgG, $IgG_1$ and $IgG_2$ anti-OA Ab are the only anti-OA Ab detected in this model from 15 to 60 d after sensitization. Twenty-one days after immunization, the first day on which the guinea pigs were challenged with an OA (2%) aerosol, significant titres of IgG, $IgG_1$ and $IgG_2$ anti-OA Ab were detectable in animals' serum (Figs. 17A, 18A, and 20A). Animals in this group showed a "classic" secondary immune response (Figs. 17A, 18A, 19 and 20A) that was apparent 3 d after the first exposure to OA aerosols (Fig. 19) and which persisted for various times after aerosol inhalations were stopped on day 30 - total IgG < $IgG_1$ < $IgG_2$. Via PCA, homocytotrophic (reaginic) Ab were detected in the sera of these animals at 21, 35 and 60 d post sensitization. PCA tests at 2 and 7 d after the intradermal injection of sera indicated that these Ab were of the IgG isotype as they yielded a postive PCA

result at only 2 d (Table 16). Also, heating at 56°C failed to prevent their activity, confirming this conclusion. Thus, the immunization protocol for this model appears to induce formation of only IgG anti-OA Ab, or levels of IgE anti-OA Ab are below the detection limit of PCA, the most sensitive biologic test available. I had hoped to refine my ELISA techniques by evaluating their specificity for $IgG_1$ and $IgG_2$ and by quantifying them with appropriate authentic guinea-pig $IgG_1$ and $IgG_2$ standards. Unfortunately, time did not permit me to complete this work. As a result, the titres quoted can only be regarded as an indication of change of concentration and it is not possible to determine actual amounts of each type of anti-OA Ab.

My findings of only IgG anti-OA Ab in Model I are in good agreement with those of others. Thus, it is well known that the choice of antigen, the size of its dose, its route of administration and the presence or absent of a suitable adjuvant are critical to the development of a particular immune response (Tada, 1975; Jarrett and Stewart, 1978; Bazin & Pauwels, 1982). Benacerraff *et al.*, (1968); Hicks & Okpaka (1968); Ovary (1976) all reported that sensitization with a large dose of antigen in the absence of an adjuvant induced preferential formation of $IgG_1$ Ab. By contrast, Bazin & Pauwels (1982), who used rats, reported that a large dose of antigen (OA, 1 mg, im) failed to induce formation of $IgG_{2a}$. Large doses of sensitizing antigen have been reported to induce only short-lived, evanescent IgE Ab responses (Ishisaka & Ishisaka, 1978; Jarrett & Stewart, 1974; Jarrett, 1978) and may

reduce or prevent the production of IgE Ab (Andersson, 1980).

Interestingly, in Model I, although the end product of the interaction of the IgG anti-OA Ab with (for example) mast cells is their degranulation, the release of preformed mediators like histamine, and the production of (other) mediators of inflammation, the mechanism of interaction with cells' FcγR may be quite different from that for IgE. Monomeric IgE has a high affinity for FcεRI on mast cells (Ishizaka *et al.*, 1973; Kulczycky & Metzger, 1974; Conrad *et al.*, 1975; Metzger & Kinet, 1988). By contrast, monomeric IgG binds poorly, if at all, to FcγR on rat and mouse mast cells (Prouvost-Danon *et al.*, 1966; Vaz & Ovary, 1968; Bach *et al.*, 1971; Halper & Metzger, 1976). However, complexed (Ag/Ab) mouse IgG can bind to these cells (Tigelar *et al.*, 1971; Warner & Ovary, 1972, Daëron *et al.*, 1980). Thus, complexation with antigen may be one of the first steps in the IgG-mediated degranulation sequence in rats, mice and guinea pigs. In support of this suggestion, Tamoto *et al.* (1974) and Tamoto & Koyama (1976), who immunized guinea pigs with large or small doses of OA in Freund's complete adjuvant (FCA), reported that large doses yielded $IgG_1$ and $IgG_2$ Ab that failed to precipitate with OA as they were only able to recognize a limited number of epitopic sites on the antigen. By contrast, the animals immunized with small doses of OA yielded "precipitating" $IgG_1$ and $IgG_2$ Ab. Unfortunately, these authors provided no information as to the susceptibility of their two groups of guinea pigs to OA-induced anaphylaxis. Thus, the

involvement of OA/anti-OA IgG, $IgG_1$ or $IgG_2$ Ab complexes in anaphylaxis *in vivo* is unknown. There is other evidence of the importance of Ag/IgG complexes in activating cells. Monoclonal $IgG_1$ complexes activate human PMN via FcγRIII receptors (Hundt & Schmidt, 1992). Monoclonal $IgG_1$ complexed by antigen or heat aggregation induce electrophysiological changes on allogenically activated mouse T cells via FcγR (Huckel *et al.*, 1988); monomeric $IgG_1$ or $IgG_{2a}$ or complexes of $IgG_{2a}$ have no effect. Also, in guinea-pig PMN, although OA-complexed $IgG_1$ Ab failed to activate the arachidonic acid cascade, OA-$IgG_2$ complexes were very effective (Sato *et al.*, 1987); it was concluded that this effect is mediated via FcγRII rather than FcγRIII sites on the PMN. Sato *et al.* (1987) findings are in contrast to those of Desquand *et al.*, (1990), who showed that "anaphylactic contraction of lung strips from guinea pigs passively sensitized with $IgG_1$ was mediated by histamine and arachidonate derivatives, whereas that of lung strips sensitized with $IgG_2$ was mostly mediated by histamine." Others (Regal, 1984, 1985; Graziano *et al.*, 1984, Undem *et al.*, 1985) have shown that the types of mediators released after passive sensitization with purified Ab then challenge of pulmonary tissues is dependent on the class of Ab used. These findings are reflected in the differential effects of anti-asthmatic agents after antigen challenge in animal models that generate different classes of Ab (Andersson, 1980; Andersson & Bergstrand, 1981; Andersson & Brattsand, 1982). These data suggest that IgE titres, not IgG titres, are critical to the development of

the signs of "human" disease in animal models. However, IgG Ab can mediate human allergies (Fagan *et al.*, 1982; Nakagawa, 1991; Nakagawa *et al.*, 1983). Thus, which type of antibody that is produced may not be that critical.

Lastly, Ag/IgG complexes may activate the complement pathway with formation of anaphylatoxins C3a and C5a. Thus, the possibility exists that in IgG-mediated anaphylactic responses, Ag/IgG complexes rather than monomeric IgG (IgG-STS) are responsible for the physiologic effects seen. This does not appear to have been explored in guinea pigs, *in vivo*.

In summary, from my studies, the immunologic response in Model I shows minimal resemblance to that seen in allergic asthma. The mechanism by which IgG Ab mediate anaphylactic responses via FcγR has been the subject of little or no direct research. This may be because of its lack of immunologic relevance to anaphylaxis and asthma in humans.

**Model II** - My data for IgG anti-OA Ab are summarized in Figs. 17B, 18B, and 20B, and for IgE, in Tables 13 and 14. These data indicate that IgG anti-OA Ab are not detected until about 35 d after the initial sensitization. Presumably, their appearance represents the immune response to OA aerosol inhalation as 10 inhalations of aerosol (2%) would represent a large dose of antigen that would be expected to evoke the formation of Ab of this class. In humans, immunotherapy for the treatment of allergies uses large doses of antigen that could work by: a) Suppression of IgE formation; b) Production of IgG blocking Ab that are of the $IgG_1$ subclass in the early part of therapy

and the $IgG_4$ subclass in later phases of therapy; c) Production of anti-idiotypic Ab; and, d) Activation of antigen-specific T suppressor cells (Nakagawa, 1991). Is there evidence that similar mechanisms are operative in this model? Data presented in Tables 12 and 13 summarize the findings with PCA using untreated and heat-treated serum. Sera taken on day 21 show clearly that the majority of the PCA response is mediated via IgE Ab - responses were similar in size at all three dilutions on days 2 and 7 post-injection. Also, heated sera gave only a small response on day 2 but no response at all on day 7 after injection. Thus, there can be only a small amount of IgG, $IgG_1$ and $IgG_2$ anti-OA Ab present. These levels are below the detection limit of my ELISA techniques. If sera from day 35 or 60 post-sensitization are used, the data suggest that more IgG anti-OA Ab are present as heat-treated sera yielded bigger responses than sera from day 21. Also, there is no indication IgE anti-OA Ab are "boosted" by the inhalation of OA-aerosols as was seen with IgG anti-OA Ab in Model I. In fact, my findings suggest that IgE anti-OA Ab titres may actually have fallen. This finding supports the suggestion that large does of antigen may reduce the formation of IgE Ab. Jarrett *et al* (1980) suggested that use of $Al(OH)_3$ as adjuvant led to greater proliferation of both memory B cells and memory T cells. Therefore, a second exposure to antigen could activate an enlarged suppressor T cell population to become more dominant and result in dimished IgE formation. IgG anti-OA Ab are present in sera from days 35 and 60, and titres of $IgG_1$ are readily detectable at this

time. As $IgG_1$ can passively sensitize guinea pigs (see above), it is unlikely that $IgG_1$ anti-OA Ab contribute to the anaphylactic response in a negative way as would be expected of a "blocking antibody." The data do not allow determination of the involvement of $IgG_4$ Ab as my ELISA cannot be used for quantitative measurements for comparing among subclasses of Ab. I made no measurements of anti-idiotypic Ab, nor did I evaluate T-cell functioning in my animals. Thus, the analogy between immunotherapy in humans and the effects of aerosols in these guinea pigs has not been evaluated completely.

The above discussion may well represent a simplistic view of the events that occur in this model. Desquand *et al.*,(1990) reported consistently higher titres of $IgG_2$ than $IgG_1$ in all animals sensitized with a similar protocol. These authors also showed that incubation of purified $IgG_1$ and $IgG_2$ with parenchymal lung strips, transferred passive sensitization as serum did. This was always accompanied by the release of histamine. This confirmed other's data (Anderson & Bergstrand, 1981; Regal, 1984; Graziano *et al.*, 1984. Undem *et al.*, 1985). Desquand *et al.*, also reported similar contractions when either $IgG_1$ or $IgG_2$ were used in contrast to Regal's (1984) observation of different sensitizing abilities for $IgG_1$ and $IgG_2$. Desquand *et al.* (1991) also noted sensitization of guinea pigs, *in vivo,* in experiments using either $IgG_1$ or $IgG_2$. Similar results were obtained by other investigators (Cheng *et al.*, 1987; Regal,1984; Undem *et al.*, 1985). Therefore, $IgG_1$ and/or $IgG_2$ may contribute to active sensitization of guinea pigs. As mentioned above, hypersensitivity

responses involving $IgG_2$ may involve the activation of the complement system and may lead to the production of anaphylatoxins, C3a and C5a that stimulate smooth muscle contraction and cause the release of histamine.

$IgG_2$ antibodies may have a "blocking" effect in asthma. Nobukazu et al., 1986, found that antigen-induced asthma mediated via IgE was completely blocked by intravenous injection of $IgG_2$ 1 h before inhalation of antigen. These results suggest that IgG antibodies induced via specific immunotherapy in asthmatic patients could prevent asthmatic attacks mediated via IgE antibodies and allergen inhalation. Yagura *et al.* (1971) reported that $IgG_2$ antibodies were able to block $IgG_1$ antibody-induced asthma in guinea pigs.

Recently, Nakagawa (1991) reported that in humans, the IgG antibody response during allergen-specific immunotherapy is due mainly to $IgG_1$ and $IgG_4$ however low levels of $IgG_2$ and $IgG_3$ were detected. In most cases $IgG_1$ antibodies seemed to dominate early in the course of treatment, but $IgG_4$ antibodies developed with continued therapy.

Human $IgG_1$ is believed to neutralize allergen and thus play an important role in immunotherapy. $IgG_4$ antibodies are believed to play a major role in the blocking of IgE antibodies by binding to Fcγ receptors on mast cells and basophils (Nakagawa & Weck, 1983; Stanworth, 1986). They may even protect the host from immunological damage induced by complexes composed mainly of $IgG_1$ antibodies and the relevant allergen by competing for $IgG_1$.

These data from humans were different from studies in guinea pigs that show that only $IgG_1$ and IgE can sensitize pulmonary tissue and that $IgG_2$ does not fix to homologous tissue (Ovary *et al.*, 1963; Graziano *et al.*, 1984; Parish, 1970; Benacerraf *et al.*, 1963; Oliviera *et al.*, 1970). Desquand *et al.* (1991) found that mepyramine an antagonist of the histamine $H_1$ receptor failed to block the contractions of lung strips from guinea pigs sensitized with $IgG_1$ or whole serum. This confirmed results of Carcez do Carmo *et al.* (1986), who showed that the contractions of lung strips from guinea pigs sensitized with serum are not blocked by mepyramine. However, Desquand *et al.* (1991) reported that mepyramine blocked the early phase of the contractions of lung strips from guinea pigs sensitized with $IgG_2$. If isolated lung strips passively sensitized with $IgG_1$ or $IgG_2$ or serum were exposed to WEB 2086, a platelet activating factor (PAF) or NDGA, an inhibitor of cyclo-oxygenase and lipoxygenase, the contraction of the lung strips was unchanged. If mepyramine and NDGA were administered together, there was inhibition of the contraction of the lung strips sensitized by $IgG_1$ - indicating that the response is mediated by histamine and arachidonate derivatives. This was supported by Cheng *et al.* (1987), Undem *et al.*(1985) and Regal (1984, 1985) who showed that histamine and leukotrienes are important to antigen-induced contraction of IgG-sensitized lung strips, but play a minor role for IgE sensitized lung strips.

PAF is known to contract the guinea-pig lung parenchymal strips

(Stimler & O'Flaherty, 1983; Detsouli *et al.*, 1985), and may account for the antigen-induced effects. However, if WEB 2086, a PAF antagonist, was administered alone it failed to block the inhibition of the contractions of lung strips sensitized with $IgG_1$ or with $IgG_2$. When administered together, NDGA and mepyramine blocked the early phase of the contraction and slightly reduced OA-induced contraction of lung strips sensitized with whole guinea-pig serum. These results are in agreement with Pretolani *et al.* (1978) who reported that WEB 2086 will not inhibit the contraction of lung strips from actively sensitized guinea pigs, unless it is used in association with other antagonists of other mediators.

The limited usefulness of the combined antagonists, particularly against contractions of lung strips of guinea pigs sensitized by serum in Desquand *et al.* (1991) study confirmed findings of Detsouli *et al.* (1985) and Carcez do Carmo *et al.* (1986), who had shown that combinations of inhibitors of different mediators only slightly reduced OA-induced lung-strip contractions. This implies that contraction of parenchymal lung strips is controlled by several mediators.

In summary, compared to Model I, the immunologic response in Model II shows a much closer resemblance to that seen in allergic asthma. The response appears to be dominated by the production of IgE anti-OA Ab. However, although inhalation of OA aerosols does result in a secondary immune response, as judged from PCA responses in which untreated and

heated sera were compared, IgE anti-OA Ab titres appear to fall rather than rise following exposure to these aerosols. IgG anti-OA Ab appear in sera collected at day 35 post-sensitization, 5 d after the last OA aerosol exposure. Titres of total IgG and $IgG_1$ anti-OA Ab peak around days 45-55. Titres of $IgG_2$ anti-OA Ab remain more or less constant from days 35-60. My findings with this model agree, in part, with those of Tada (1975) and Andersson (1980) who concluded that in this model, anaphylactic responses to antigen were mostly mediated by IgE and that IgG made only a small contribution.

**Model III** - My data for IgG anti-OA Ab in this model are summarized in Figs. 17C, 18C, and 20C, and for IgE, in Tables 16 and 17. These data indicate that titres of IgG anti-OA Ab detectable by ELISA were not present until about 45 d after the initial sensitization - about 20 d after the animals were first exposed to OA aerosols. Titres persisted through day 60, the last day on which sera were taken for determination of anti-OA Ab. For IgE, PCA yielded some interesting findings. Data for sera taken at day 21 post-sensitization indicate the presence of low titres of IgG anti-OA Ab, which were below the detection limit of my ELISA. Sera taken on day 35 confirmed the presence of IgG and indicated the presence of IgE anti-OA Ab which appeared to persist in sera taken on day 60. Heat treatment confirmed these conclusions, except the PCA response 2 d after injection of the 60-d sera was much less than expected. The PCA and ELISA findings did not agree with one another when 35-d sera were used. Thus, although PCA strongly

indicated the presence of IgG anti-OA Ab, ELISA did not detect them. Interestingly, the sera from day 60 yielded somewhat similar results. I have no simple explanation for these findings. The delay in the appearance of IgG anti-OA Ab may well have been due to the fact that the animals were pretreated with cyclophosphamide, or to the presence of *B. pertussis* as adjuvant, or both. Alternatively, the IgG response may simply be a primary immune response. However, the PCA data make this possibility unlikely.

Cyclophosphamide was used because it is believed to reduce suppressor T-cell responses. Thus, in mice, Katz (1978a) noted that cyclophosphamide treatment, converted "low" to "high" responders and yielded significantly higher levels of serum IgE than controls. Others (Chiorazzi *et al.*, 1976, 1977; Watanabe *et al.*, 1976; Katz, 1978b, Graziano & Askenasa, 1979) reported similar findings in mice and other species, and confirmed the observation that increased serum IgE levels result from the inhibitory effect of cyclophosphamide on antigen specific, or antigen non-specific, suppressor T-cells. Andersson (1980) noted that guinea pigs treated with cyclophosphamide 2 d before, and challenged 21 d after sensitization, developed increased titres of IgE-like Ab. However, cyclophosphamide's effects may not be specific; Debre *et al.* (1976) and Gagnong & Maclennon (1979) reported increased serum IgG levels in response to antigen given after cyclophosphamide treatment.

Jarrett *et al.* (1974) found that absorption of the *B. pertussis* to $Al(OH)_3$

appears to promote IgE production. My findings show that animals pretreated with cyclophosphamide and sensitized with OA, *B. pertussis* and $Al(OH)_3$ produced more IgE than animals sensitized with only a large dose of OA (Model I), but the IgE levels were very much less than those seen in Model II.

In summary, Model III failed to yield high titres of IgE anti-OA Ab and only low titres of IgG anti-OA Ab were detected by PCA, and not by ELISA, in sera taken 21 d after sensitization. This model's immune response to antigen does not appear to resemble that seen in asthma.

**II. Presence or absence of anaphylaxis**

Data are summarized in Tables 6 and 7. In each of the three guinea-pig models of asthma, the response to inhaled antigen (OA), anaphylaxis, is probably the best indicator of sensitization. In all experiments, guinea pigs were pretreated with mepyramine (0.5 mg/kg, ip) before exposure to OA (2%) aerosols. In preliminary experiments, even with mepyramine pretreatment, on first exposure, all animals developed severe signs of acute anaphylactic shock and mortality rates approached 100%. Accordingly, I modified my protocol to prevent mortalities and to reduce animals' suffering; I arbitrarily elected to terminate the first exposure to the OA aerosols at 20 s. Similarly, the second, third and fourth exposures were limited to 45 s. Thereafter, (exposures 5-10) animals tolerated a full 4 min of exposure to OA aerosols without developing

severe signs of anaphylaxis. There was one exception: guinea pigs in the groups taken through to 60 d experienced severe anaphylaxis on the fifth exposure and 2/13 animals died. Originally, I had planned to expose all animals to OA (2%) aerosols for 8 min/d. The 2% concentration of OA was selected because it appeared to "work" satisfactorily by inducing significant airways' hyperresponsiveness, cellular infiltrations and pulmonary inflammation in a model similar to my Model I (Ladenius, personal communication).

All guinea pigs in Model I, II, and III developed anaphylactic responses that clearly indicate that the animals were "sensitized" by each of the immunization protocols used. Thus, Model I, in which immunization induced IgG anti-OA Ab, yielded similar biologic responses to Model II, in which immunization induced predominantly IgE anti-OA Ab. In Model III, in which PCA indicated only low titres of IgG anti-OA Ab, below the detection limit of my ELISA, there was a severe anaphylactic response. Thus, the correlation between IgG and IgE anti-OA Ab titres and the anaphylactic response is poor among the models. Also, it was not possible to distinguish among the various models by gross observation of the anaphylactic response. In hindsight, it might have been better to have measured respiratory parameters during induction of anaphylaxis in order to examine the response more carefully. However, this was not done in these experiments.

The "exhaustion" of the anaphylactic response observed was noteworthy. This phenomenon has been reported by others (see Nakagawa,

1991). By the fifth exposure, the majority of animals tolerated 4 min of exposure to OA aerosol. All models behaved similarly in this respect. I did not seek an explanation for this observation. Possible explanations include: a) depletion of mediators from target cells; b) depletion of IgG and/or IgE mediating the anaphylactic response; c) Down regulation of the FcεR and FcγR responsible for antibody binding to target cells; and, d) Production of "blocking" IgG Ab via the secondary immune response (see Fig. 19). For a): I made no measurements of mediators in targets. For b): My ELISA data (Fig. 19) for Model 1 indicate increased IgG anti-OA Ab titres rather than depletion. However, depletion of IgE may have occurred in Model II, but I did not monitor for this. Titres of IgG and IgE anti-OA Ab were so low in Model III at day 21 that I cannot comment on their possible depletion. For c): I made no measurements of FcR on target cells. For d): It is possible in Models I and II that significant titres of "blocking" Ab were induced during aerosol exposures. My data for Model I showed clearly (Fig. 19) that total IgG, $IgG_1$ and $IgG_2$ titres rise rapidly during exposure to OA aerosols. Some of these Ab may well interfere with the anaphylactic response. With Model II, the data are less easily interpreted. Significant titres, well above the detection limit for ELISA, of IgG, $IgG_1$ and $IgG_2$ were present in sera on day 35; these may have contained significant levels of "blocking" Ab. As these Ab were undetectable with ELISA on day 21, I elected not to take sera during exposure to OA aerosols. My findings with Model III suggest that the

hypothesis involving "blocking" Ab production is unlikely to be correct. IgG, $IgG_1$ and $IgG_2$ anti-OA Ab titres were detected only after day 45 with ELISA. However, PCA indicated the presence of low titres of IgG homocytotrophic Ab in sera on day 21, and increased concentrations of these IgG Ab on day 35. A small amount of IgE was detectable with PCA in day 35 sera, too. It is possible that none of the above possibilities offers an adequate explanation for my findings of "exhaustion" of the anaphylactic response in these experiments.

In summary, as qualitatively similar anaphylactic responses occurred in all three models, the presence or absence of anaphylaxis cannot be used to distinguish among them.

**III. Appearance of cells in broncho-alveolar lavage (BAL) fluid**

Data for total and differential cell counts at day 35 are summarized in Figs. 9A, 10A and Tables 8 and 9 and data for total and differential cell counts at day 60 in Figs. 9B, 10B and Tables 10 and 11. In all three models, an increase in total cell numbers was noted both at 35 d and 60 d after sensitization. Differential cell counts done on 200 cells revealed a marked increase in eosinophils and macrophages in all three models on these days; PMNs were only significantly increased in Models II and III. Similar findings have been reported by others (Kallos, 1935; DeMonchy *et al.*, 1985 ; Thorpe *et al.*, 1987). Cellular infiltration (especially by macrophages) is a characteristic feature of inflammation and this increase in cell numbers could

be attributed to the inhalation of OA aerosol and the subsequent anaphylactic responses.

The increase in eosinophil numbers observed in all three models is typical of allergic reactions. This effect was greatest in Model II. Studies with guinea-pig lung have shown that eosinophil chemotactic factor of anaphylaxis (ECF-A) is among the mediators released following antigen challenge. This material is chemotactic for homologous eosinophilic leucocytes (Austin, 1972).

Although all three sensitization protocols were effective in inducing high eosinophil numbers, the use of the adjuvants ($Al(OH)_3$ for Model II; $Al(OH)_3$ + *B. pertussis* for Model III) may have contributed to the significant increase in PMN seen in these two models, as this increase was not observed in animals from Model I which were sensitized with OA only.

In summary, all three models showed a significant increase in total cell counts. Differential cell counts showed that this increase was due mainly to eosinophils, (highest in Model II at day 35). Therefore, although all models showed this characteristic feature of asthma, cell counts of BAL did not differentiate among the models.

### IV. Lung morphology

Pictures of lung sections are seen in Figs. 5A,B, 6A,B, 7A,B, 8A,B. Sections from all three models at 35 and 60 d showed invagination of the mucosal layer, constriction of the lumen and cellular infiltration, (Figs. 6A,B,

7A,B, 8A,B). It should be noted that the invagination seen could be due to a fixation artefact rather than a result of any treatments given. Verification of this statement would require morphometery which was beyond the scope of this present study.

Hypertrophy of the bronchial smooth muscle was more pronounced in Models II and III than in Model I. Emphysematous changes were noted in Model I at day 60 and Model III on day 35. Similar morphological changes were noted by other investigators who used OA for sensitization and challenge (Noelpp-Eschenhagen & Noelpp, 1954; Broder, 1979), and in cases of human asthma (Pagel, 1935; Kallos & Kallos, 1983).

In summary, all animals within the three models developed histologic features characteristic of asthma. There was no difference among the models for any of the sensitization protocols. Therefore, these features appeared in all models and the inflammatory responses closely resemble those in human disease.

**V. Airways' hyperresponsiveness**

The baseline measurements obtained for Models I, II, III were very similar to control values at 35 d and 60 d (see Tables 4 and 5). However, observation of histologic sections from Models I, II and III show definite invagination of the mucosal layer and the appearance of constricted lumens and would therefore lead to a change in baseline values.

Dose-response curves to the agonists at day 35 are outlined in Figs. 1A, 2A and 3A and dose-response curves to agonist at day 60 are summarized in Figs. 1B, 2B and 3B. Animals from Model I sensitized with large doses (20 mg/kg) of OA showed airway reactivity to increasing doses of the three agonists at 35 d and 60 d. However, the dose response curves for animals from this model were similar to control animals. Model II, animals sensitized with small amounts of antigen (20 μg/kg) and $Al(OH)_3$ showed selective hyperresponsiveness. Dose-response curves for histamine were significantly different from controls on 35 d and 60 d. However, dose-response curves for methacholine and serotonin were very similar to controls. Animals from Model III, given cyclophosphamide and low doses of antigen (20 μg/kg) with $Al(OH)_3$ and *B. pertussis* also showed selective airway hyperresponsiveness to histamine at days 35 and 60 and to serotonin at day 60. Again there was no response to methacholine at day 35 and 60. Although the measurement of airway hyperresponsiveness is a useful parameter for monitoring the sensitivity of the airways and has been used extensively by other investigators (Drazen & Austin, 1975; Mills & Widdicombe, 1970; Popa *et al.*, 1973; Stanworth *et al.*, 1961), my results were somewhat different from expected. I expected to find non-selective hyperresponsiveness with the agonists used. I intended to repeat the AHR measurements, but due to lack of time I was not able to do so.

## VI. Limitations and problems

AHR was always measured as the increase in resistance from baseline. Some experiments were terminated before complete dose-response curves were obtained for all three agonists due to persistent increase in baseline values of resistance and elastance. This increase from baseline could be due to overinflation of the lungs between experiments or to excessive mucus secretion caused by the agonists. Another limitation of using actively sensitised animals is that this approach is difficult to standardize, since the immune response varies between animals, both quantitatively and qualitatively; the alternative would be to employ passive sensitization.

## VII. Conclusions

In conclusion, it is important to note that the development of an animal model of bronchial asthma is crucial in order to fully understand the pathogenesis of the disease and the development of new drugs for the management of the disease. Although the guinea pig is a good choice and tremendous advances have been made in experimental asthma research, it is difficult to reproduce a model with all the characteristic features of human bronchial asthma. These experimental models will fall short of the ideal, as they lack such common features of the human conditions as a genetic predisposition, bronchial irritability to multiple irritants and mild continuous obstruction. However, any animal model of asthma should show the

characteristic features of airway hyperresponsiveness (AHR), hypertrophy of the bronchial smooth muscles, airway inflammation, eosinophilia, and at least, partly be mediated reaginic antibodies hence, high levels of antibodies in the sera. Human asthma is a chronic condition and an ideal model should have the capability of the precipitating multiple episodes in individual animals. In my experiments, anaphylaxis and cellular infiltration (mainly macrophages and eosinophils) were successfully induced in Models I, II and III. Morphological changes noticably different from controls were also observed in all models. AHR was only observed in Models II and III to histamine and to a lesser extent serotonin but not to methacholine. However, a secondary immune response resulting in a significant increase in serum IgE was only observed in Model II. These findings would suggest that Model II show features which most closely resembles human allergic asthma.

Table 18

Comparison of characteristic features of human allergic asthma with models.

| | IgE | Anaphylaxis | Eosinophilia | Histology: ↓ lumen ⌀ | AHR |
|---|---|---|---|---|---|
| Model I | | *** | *** | *** | |
| Model II | *** | *** | *** | *** | *** |
| Model III | * | *** | *** | *** | *** |
| Asthma | *** | *** | *** | *** | *** |

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