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**IgE response to *Bordetella pertussis* components**

**Diane Sarah Jane Lindsay**

**Presented for the Degree of Doctorate of Philosophy in the  
Faculty of Science, University of Glasgow.**

**Department of Microbiology, September 1991**

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" It is irrational to believe that people and things should be different from the way they are and that it is catastrophic if perfect solutions to the grim realities of life are not immediately found"

A. Ellis 1961

## DEDICATION

I'd like to dedicate this thesis to my parents, John and Catherine for all their love, support and encouragement. In particular, I'd like to thank them for always having faith in me and not listening to the doubters.

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## **DECLARATION**

**This thesis is the original work of the author except where otherwise stated.**

**Diane S. J. Lindsay**

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## ABBREVIATIONS

AC	Acellular
Adj	Adjuvant
Agg	Agglutinogens
Ag	Antigen
AP	Affinity purified
BG	Bordet Gengou
BGG	Bovine gamma globulin
BSA	Bovine serum albumin
bvg	virulence gene
DNP-KLH	Dinitrophenyl keyhole limpet haemocyanin
DTP	Diphtheria Tetanus Pertussis triple vaccine
EAE	Experimental allergic encephalomyelitis
EB	Evans blue
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. HCl
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
FHA	Filamentous haemagglutinin
GEF	Glycosylation enhancing factor
HA	Haemagglutination
HRP	Horse radish peroxidase
HS	Horse serum
HSF	Histamine sensitizing factor
IAP	Islet-activating protein
ICMPT	Intracerebral mouse potency test
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
i.p	intraperitoneal
i.v	intravenous
LAST	Latex-allergosorbent test
LPF	Lymphocytosis or leukocytosis promoting factor
LPS	Lipopolysaccharide
M.W	Molecular weight
MeBCD	Methylated $\beta$ -cyclodextrin
OPD	O-phenylene diamine

Oa	Ovalbumin
PCA	Passive cutaneous anaphylaxis
PNP	p-nitrophenyl phosphate
PT	Pertussis toxin
RAST	Radio-allergosorbent test
RBC	Red blood cell
RIA	Radio-immunoassay
RSV	Respiratory syncytial virus
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SM	Skimmed milk
SS	Stainer-Scholte medium
SS-C	Cyclodextrin stainer-scholte
s.c	subcutaneous
TNP	Trinitrophenyl
WC	whole cell

## S U M M A R Y

Pertussis vaccine and pertussis toxin (PT) are well known as IgE adjuvants for a variety of protein antigens. However, there is very little information on whether PT acts as an IgE adjuvant for the antigens of *B. pertussis* itself, as studied in this investigation.

Initial experiments with ovalbumin (Oa) showed that an anti-Oa IgE response in mice was produced after both one-dose and three-dose immunization schedules, with PT as adjuvant for the first doses only. The one-dose schedule involved injected Oa and bioactive PT as adjuvant on day 0, then bleeding on day 10, 21 or 28. The sera in the one-dose immunization were IgE-positive only when tested individually, and became unreactive when pooled. The mice, in this instance, thus acted as high or low responders for IgE production. The optimal concentration of PT in this one-dose scheme was 1 µg per mouse. This adjuvant dose of PT was kept constant in subsequent experiments with the three-dose immunization schedule.

A persistent boosterable IgE response was detected with a three-dose immunization schedule, which involved injecting antigen (Ag) and adjuvant (Adj) on Day 0, then boosting on days 28 and 56, with Ag alone, and bleeding on day 63. The sera, when pooled, contained a high IgE titre but only from mice given the 0.1 µg and 1 µg Oa immunization doses. These titrations for anti-Oa IgE were done by passive cutaneous anaphylaxis (PCA) test and by ELISA.

The PCA test, with anti-Oa IgE, was optimal with a two-day sensitization interval after the intracutaneous injection of the serum and with<sup>a</sup>/challenge dose of 1 mg of antigen with Evans blue. The usual 30 min heating at 56°C, which has been reported to destroy IgE in serum, caused 90% inactivation of the PCA titre. A high-titre standard anti-Oa IgE serum had to be heated for 1 h to remove all 48 h PCA activity.

The effect of injecting PT along with Oa in the challenge dose in the above PCA was also studied, as previous work had found that small doses of PT inhibited histamine release from mast cells, and therefore the PCA reaction. We found that very low (0.1 µg) and very high (500 µg) doses of PT had a fast-acting, inhibitory effect on the anti-Oa IgE PCA test when injected along with Oa in the challenge dose.

The anti-Oa IgE serum was also titrated *in vitro* by ELISA. A sandwich ELISA, with an initial affinity-antibody coating stage was found to be the most sensitive and specific method of detecting IgE. In contrast, the conventional ELISA was unsuitable because of high backgrounds and low specificity.

With filamentous haemagglutinin (FHA), as the antigen for IgE production there were only two mice from a total of 30 which were IgE positive in the 48 h PCA test after a one-dose immunization, with PT as adjuvant. In the three-dose immunization schedule the resulting anti-FHA IgE sera, in the 48 h PCA yielded a diffuse blueing over the entire skin surface. This was thought to be due to high IgG1 levels in the serum which also bind to mast cells and thereby interfered with the test.

When the sensitization interval in the PCA was extended, to 7-days, and the mice challenged with an optimal (500 µg) dose of FHA, a more distinct, localised blueing reaction was obtained with the sera from the three-dose protocol. Anti-FHA IgE responses were detected over a large immunizing dose range (0.05 - 20 µg), with 1 µg bioactive PT as adjuvant. The sera were also titrated by ELISA, for IgE and IgG1, and there was a correlation between high IgE levels and high IgG1 levels in these sera.

IgE responses to PT were investigated with purified and toxoided PT as antigen and bioactive PT as adjuvant. Anti-PT IgE was also detected in the 7-day PCA, when the mice were challenged with an optimal (500 µg) dose of bioactive PT. The anti-PT IgE response was only detected over a restricted PT toxoid immunizing dose range (0.05 - 1 µg). This was independent of animal strain (Ham/ICR and Balb/c), and toxoiding (glutaraldehyde or carbodiimide) procedure. There was a correlation between high IgE levels and low IgG1 levels in the sera.

In animals infected intranasally and sublethally with *B. pertussis* strain number 18323, neither an anti-FHA nor PT IgE or IgG1 response could be detected by PCA or ELISA, up to 35 days after initial infection.

The possible implications of the above responses are difficult to relate to the disease, whooping cough, or to pertussis vaccination. A possible host-beneficial effect of IgE production during the disease may result from mast cell degranulation near the site of *B. pertussis* attachment in the respiratory tract, with consequent release of vasoactive amines in the local area thereby helping to recruit immune cells to that area and aiding in removal of the bacteria.

This study has shown that IgE can be produced in mice against two of the major vaccine components of acellular pertussis vaccines, namely FHA and PT. These responses were detected by the PCA test and the ELISA.

## INTRODUCTION



## INTRODUCTION

The introduction is divided into two main sections. The first, entitled *Bordetella pertussis*, includes general background information on the organism, the disease and vaccination. Two of the antigens of *B. pertussis*, namely the filamentous haemagglutinin (FHA) and pertussis toxin (PT) are then reviewed in detail, with particular emphasis on the role of PT as an IgE adjuvant. This conveniently leads into the second section, Immunoglobulin E. This section includes the historical benchmarks in our understanding of IgE, how it is produced and detected and what factors influence the response. The introduction will be concluded with a discussion of IgE responses as a result of parasitic and microbial infections and after immunization.

### *Bordetella pertussis*

#### **Whooping cough - the disease**

The first mention of this unusual type of coughing disease was noted in Moulton's "The Mirror of Health" in 1540 (Lapin, 1943). However *Bordetella pertussis* the bacterium responsible for the whooping cough was first isolated in 1906 by Bordet and Gengou.

Initially, *B. pertussis* was placed in the genus *Haemophilus* until 1952 when Moreno-Lopez created the new genus *Bordetella*. Today there are 4 species in this genus: *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*, which cause respiratory infections in man and animals. *B. parapertussis* was also initially classified as a member of the *Haemophilus* (Johnson and Sneath, 1973) and causes a milder form of whooping cough in humans. *B. bronchiseptica* was placed in the genera *Bacillus*, *Alcaligenes*, *Brucella* then *Haemophilus* and causes respiratory infections in animals, such as kennel cough in dogs and atrophic rhinitis in swine; only rarely does it affect humans (Pittman and Wardlaw, 1981). *B. avium* once belonged to the *Alcaligenes* and is a pathogen of poultry and other birds and is best known as the causative agent of turkey coryza.

The major human pathogen, *B. pertussis* localizes on the mucosal cilia of the respiratory epithelial cells during pertussis infection. Pertussis is a highly transmissible disease and is usually spread via droplet infection and mainly affects infants and young children. There are three stages during typical infection: incubation; catarrhal and paroxysmal. The initial, incubation, stage is asymptomatic and usually lasts ten to fourteen days. The catarrhal stage is indistinguishable from non-specific respiratory tract infections and is characterized by low-grade fever and

mild coughing. The paroxysmal stage is characterized by spasms of coughing and the "whooping" being caused by a long inspiration of air after the breathlessness of a bout of coughing (Preston, 1988). Vomiting may also follow these paroxysms. The violent coughing may last for 4-6 weeks and sometimes persist for several months. Complications can arise due to the increase in venous pressure during these characteristic paroxysms, resulting in nose bleeds and bloodshot eyes. Bronchopneumonia may occur as a result of spread of *Bordetella* to the alveoli but more commonly it is due to secondary bacterial infections by *Streptococcus pneumoniae*, *Staphylococcus aureus* or occasionally *Haemophilus influenzae*.

The laboratory verification of whooping cough has encountered many problems in the past because of the fastidious nature of *B. pertussis*. Therefore attempts have been made to devise serological tests for its detection. The enzyme-linked immunosorbent assay (ELISA) has been used, but results with whole bacteria and crude extracts showed low sensitivity and specificity (Bradstreet *et al.*, 1972). A more specific test, using a purified antigen namely FHA, showed the assay to be specific for *B. pertussis*, except that it also measured antibody responses to *B. parapertussis* probably because of structural similarities of homologous components (Granstrom *et al.*, 1982). Onorata <sup>and Wassilak</sup> (1987) and Friedman (1988) reviewed a number of methods for laboratory diagnosis of pertussis. These included fluorescent antibody tests, agglutination reactions and the ELISA. There were difficulties with all the above, and at present there seems to be no single serological test, which is both sensitive and specific enough to detect infection in all individuals.

Between 1946 and 1949, whooping cough was the most common of the acute communicable diseases to affect children below the age of 1 year. The World Health Organisation estimated that the total number of cases of pertussis is 60 million annually, and that the disease is responsible for half a million deaths per annum mainly in underdeveloped countries (Muller *et al.*, 1986). Vaccination has been available since the 1940's, as a preventative measure in controlling the disease. The whole-cell (WC) pertussis vaccines used for this purpose have been produced by empirical approaches which involved the bacteria being killed and detoxified by treatment with formaldehyde, thiomersal, heating to 56° C or by a combination of heat and chemicals (Lapin, 1943). Today, pertussis vaccine is defined as a saline suspension of killed *Bordetella pertussis* consisting of a mixture of strains, so as to incorporate the three agglutinogens 1, 2 and 3 (Cherry *et al.*, 1988) and is usually given as part of the triple vaccine, with diphtheria and tetanus toxoids adsorbed to aluminium hydroxide or calcium phosphate as adjuvants (Wardlaw and Parton, 1983). The vaccination schedule currently in use in the UK involves a series of three DPT injections, given to children in the first few months of life, starting at two months of age (Department of Health, 1990).

More recently research has been focused on the development of acellular (AC) vaccines, because of local or systemic side effects produced by the WC vaccine. The first AC vaccine was used in Japan and consisted of PT and FHA co-purified from the culture supernate of the Tohama strain grown in static culture (Sato *et al.*, 1984). The potency of WC pertussis vaccine is tested by the intracerebral mouse protection test (ICMPT) which was developed by Kendrick *et al.*, (1947). This involves mice being injected with vaccine, and then challenged 14 days later intracerebrally, with live *B. pertussis*. The efficacy of the vaccine is related to the survival rate of the challenged animals. This test is regarded as the criterion for a protective pertussis vaccine. However, the new AC vaccines are found to be active in this system only when the mice are challenged 21 days after immunization, which is distinct from the 14-day ICMPT for the WC vaccine. PT is the only known virulence determinant of *B. pertussis* which confers protection in the ICMPT. An AC vaccine has also been developed at the Centre for Applied Microbiology and Research, Porton, UK which consists of separately purified PT, FHA and AGG's toxoided with formaldehyde and adsorbed to an aluminium hydroxide adjuvant (Robinson *et al.*, 1986). This vaccine has low potency in the ICMPT but is protective against pulmonary infection with *B. pertussis*. A new range of AC vaccines are now being developed and tested, and are at different stages in clinical trials, but they mostly consist of PT and mixtures of FHA, agglutinogens and the 69 kDa surface antigen which have been purified and detoxified where necessary, either chemically or genetically (Parton, 1991)

### **Growth and nutrition**

Bordet and Gengou (1906) used a solid blood-based potato glycerol agar (BG agar) to isolate the Gram-negative coccobacillus of *B. pertussis*. Later Hornibrook (1939) grew the bacterium in a liquid medium. This consisted of casein hydrolysate to which was added various salts, growth factors, and starch, to remove inhibitors produced during growth.

Stainer and Scholte, (1971) introduced a chemically-defined medium for growth of *B. pertussis*. This consisted of the two essential amino acids, glutamate and proline together with nicotinic acid and cysteine, and is suitable for large-scale vaccine production of phase I *B. pertussis*. Imaizumi *et al.*, (1983) then modified this medium with heptakis (2,6-O-dimethyl)  $\beta$ -cyclodextrin as a novel growth stimulant. This so-called cyclodextrin medium (SS-C) is used for growth of virulent *B. pertussis* which can be passaged a number of times without serological changes taking place.

In culture, alteration of expression of the virulence factors of *B. pertussis* known as antigenic modulation, can be induced by an increase in  $MgSO_4$  or nicotinic acid in the growth medium or by a decrease in incubation temperature. There are

three modes produced under different growth conditions: xanthic (X-mode) which is the normal virulent form; and cyanic (C-mode) which is avirulent and an intermediate (I-mode) has also been described (Lacey, 1951; 1960). The X-mode occurs after growth in the presence of NaCl, whereas the C-mode occurs after growth in medium containing high levels of MgSO<sub>4</sub>. Thus *B. pertussis* grown in medium containing MgSO<sub>4</sub> had a decreased adenylate cyclase activity, histamine-sensitizing activity and had reduced quantities of two cell-envelope polypeptides (Parton and Wardlaw, 1975; Brownlie *et al.*, 1985). Nowadays, modulation of *B. pertussis* is understood to be caused by an alteration in gene transcription of a range of virulence determinants in response to a variety of environmental factors and is controlled by a virulence regulatory locus *bvg* (Scarlat<sup>o</sup> *et al.*, 1990).

*B. pertussis* can alternate between virulent and avirulent forms either by phenotypic change (antigenic modulation) or by a genotypic change (phase variation). The biological activities of *B. pertussis* are dependent on the phase of growth of the organism which is distinct from antigenic modulation described above. Bordet and Sleswyck (1910) considered there to be two serological states of the pertussis bacillus depending on the growth medium. Later Leslie and Gardner (1931), analysed strains of *B. pertussis* by agglutination and postulated that there were four phases. Phase I appears after initial isolation on BG medium and possesses the major virulence determinants required for host infection and WC vaccine production. At the other extreme, Phase IV is completely degraded antigenically, is avirulent and usually results from repeated passage. Phases II and III are both intermediate. Weiss and Falkow, (1984), from avirulent phase IV variants, isolated haemolysin-positive revertants, which also expressed FHA and PT. Stibitz *et al.*, (1988) reported cloning of the vir (bvg) gene and that phase variation, could partly be explained by a frame-shift mutation at an unstable stretch of guanines in the vir (*bvg*) gene. Mutational events involving insertions and deletions of genetic material can also result in phase variation.

### **Biologically-active components**

The components of interest in this work are PT and FHA which are described later. The other virulence factors possess a range of biological activities which combine to make *B. pertussis* a complex microorganism from the standpoint of pathogenicity. A summary of the virulence components and their biological activities is presented in Table 1.

### **Filamentous haemagglutinin**

The haemagglutination (HA) activity of *B. pertussis* was first demonstrated by Keogh

**Table 1 : Biologically active components of *Bordetella pertussis***

Component	Biological properties	Location in the cell	Reference
Adenylate cyclase toxin	Increases cAMP levels	Extracytoplasmic	Wolff <i>et al.</i> (1980) Rogel <i>et al.</i> (1989)
Heat labile toxin	Dermonecrotic: Causes spleen atrophy Vasoconstrictive	Cytoplasmic	Nakase and Endoch. (1984)  Nakase and Endoh (1988)
Tracheal cytotoxin	Ciliostatic	Extracellular	Goldman <i>et al.</i> (1982) Gentry-weeks <i>et al.</i> (1988)
Agglutinogens	Adhesion	Cell surface	Cowell <i>et al.</i> (1987)
Lipopoly-saccharide	Endotoxic, Adjuvanticity	Cell surface	Peppler. (1984) Ibsen <i>et al.</i> (1988)
Filamentous haemagglutinin	Adhesion	Cell surface and extracellular	Ashworth <i>et al.</i> (1982)
Pertussis toxin	Multiple	Extracellular	Wardlaw & Parton (1983) <sup>b</sup> Burns (1988)

**Table 2: History of developments in knowledge of FHA**

Date	Event	Investigators
1947	Discovery of HA activity of <i>B.pertussis</i>	Keogh <i>et al.</i>
1952	HA not a protective antigen	Masry
1974	Two distinct components involved in HA: LPF-HA and FHA	Sato <i>et al.</i>
1982	Anti-FHA responses detected after vaccination and infection	Granstrom <i>et al.</i> , a & b
1984	FHA regarded as a protective antigen	Oda <i>et al.</i>
1985	Mutant FHA minus and PT minus strains exhibited loss of adherence to ciliated epithelia	Tuomanen & Weiss.
1988	Gene sequence of FHA	Stibitz <i>et al.</i>
1989	Nucleotide sequence	Relman <i>et al.</i>
1990	Amino acid sequence of FHA	Domenighini <i>et al.</i>

*et al.*, (1947), who observed that whole cells of *B. pertussis* agglutinated or clumped washed red blood cells. Later, Sato *et al.*, (1974) isolated and purified the two distinct components responsible for HA : FHA and LPF-HA now known as pertussis toxin (PT). The two could be distinguished by different HA inhibitors: cholesterol for FHA and haptoglobin for PT. FHA is the more potent in that it has five to seven times greater HA activity per mg of protein than PT. (Sato *et al.*, 1983). Table 2 summarises some of the important events in FHA literature.

FHA has a rod-shaped filamentous structure, 40-100nm long, and with subunits of Mr 220,000 and a MW of 200,000 (Sato *et al.*, 1983). FHA was widely thought to be related to pili and was previously known as fimbrial haemagglutinin, until experiments by Ashworth *et al.*, (1982) indicated that the electron microscopically-visible pili did not label with anti-FHA, but instead with antibody to one of the agglutinogens.

FHA is an adhesin and is involved in attachment of *B. pertussis* to respiratory cilia. Mutant strains, deficient in one or several bacterial virulence factors have been tested for their ability to adhere to single, human ciliated respiratory epithelial cells *in vitro*. Loss of adherence was associated with lack of secretion of either of two surface antigens; FHA or PT. The strains regained adherence if both components were later added back into the assay system (Tuomanen & Weiss, 1985). Exogenous addition of FHA to the FHA-minus strains and PT to the PT-minus strains also caused a significant increase in adherence. There was also a requirement for the two virulence determinants to be present for maximum adherence.

An anti-FHA response can be elicited in humans after vaccination with WC or AC vaccines and after infection. IgG, IgM and IgA responses have all been reported after vaccination and infection (Granstrom *et al.*, 1982). When mice are immunized with various doses of FHA, they were protected from aerosol infection after challenge with live *B. pertussis*. However intraperitoneal (i.p) injection of FHA did not protect mice from intracerebral (i.c) challenge (Oda *et al.*, 1984). Therefore FHA, unlike PT, is not protective in the ICMPT, but there is a requirement for FHA in the vaccine, because FHA reduces the amount of PT toxoid needed for the vaccine to pass both the i.c and aerosol challenge tests. In the Swedish acellular vaccine field trials, vaccination with PT toxoid alone was shown to protect against typical whooping cough with laboratory confirmation, but did not protect against infection and colonisation. However, there was evidence that the addition of FHA to the PT toxoid provides some protection against infection (Storsaeter *et al.*, 1990)

### **Pertussis toxin**

The molecular weight of PT is around 105,000. It is a subunit toxin consisting of two components: the A protomer (S1), with ADP-ribosyl transferase activity and the B

oligomer (S2-S5) which is thought to be involved in binding to receptor molecules. (Tamura *et al.*, 1982). The S1 subunit is an ADP-ribosyltransferase, and so PT belongs to a family of ADP-ribosylating toxins which includes cholera toxin (CT). However, the target proteins of PT and CT, the regulatory G proteins in eukaryotic cells are different. Whereas CT, ADP-ribosylates Gs, PT attacks Gi, Go and Gt. The result is that PT inhibits the ability of the G proteins to mediate signals from cell surface receptors to effector within the cell and thus alters the responsiveness of cells to regulatory molecules and other exogenous stimuli. Not all of the activities of PT depend on the enzymatic activities of S1. Unlike cholera toxin, the B subunit of PT consists of four dissimilar subunits, S2, S3, S4 and S5 (Tamura *et al.*, 1982). The B oligomer consists of two dimers (S2-S4 and S3-S4) connected by S5. This B oligomer alone acts as T cell mitogen and enhances glucose oxidation in adipocytes. These effects are due to cross-linking of cell surface receptors by divalent attachment of the B oligomer via the two dimers, which in turn triggers a cascade of intracellular signal events. On the basis of indirect evidence, leukocytosis promotion, adjuvanticity and histamine-sensitizing activity were also attributed to cross-linking (Burns, 1988). However, recently, it has been shown that specific mutations in the toxin gene, that reduce enzymatic activity also reduce these particular activities of PT (Black *et al.*, 1988)

Pertussis toxin was cloned and sequenced by Loch and Keith in 1986, and has a diverse range of biological activities which is reflected in the different names that have been assigned to it in the past such as pertussigen, HSF, LPF and IAP (Pittman, 1984). A list of some of the biological activities is provided in Table 3.

### **Lymphocytosis and Leukocytosis promotion**

Lymphocytosis was first observed by Frolich in 1897 (Wardlaw and Parton, 1983). In patients with pertussis, the peripheral leucocyte count may be greater than  $175,000/\text{mm}^3$ . It has also been shown that the active disease is not a prerequisite since lymphocytosis occurs following injection of WC vaccine (Sauer, 1933) or purified LPF (PT) (Morse, 1965, Morse and Morse, 1976). In these early studies it was suggested that lymphocytosis was caused by lymphocytes usually found in the lymphoid tissue entering the blood stream, rather than by increases in lymphocyte production. Lymphocytosis in whooping cough was best observed in patients over 6 months of age and less frequently in younger patients (Lagergren, 1963).

LPF (PT) is a T-cell mitogen for spleen and lymph node cells. It has also been suggested that LPF enhances production of IgE through the agency of lymphocytosis which prevents suppressor cells from exerting their effects on the production of this reaginic antibody (Tada *et al.*, 1972).



**Table 3. Biological activities of pertussis toxin in the mouse**

Biological activity	Previously designated name of substance	Minimum active dose (ng/ml)
Adjuvant for IgE	-	0.1
Sensitization to histamine and serotonin	Histamine sensitizing factor (HSF)	0.5
Enhancement of insulin secretion	Islet-activating protein (IAP)	2.0
Induction of leukocytosis and lymphocytosis	Leukocytosis/ Lymphocytosis-promoting factor (LPF)	8-40
Lethality	-	550
Protective antigen	-	-

Adapted from Munoz and Bergman, (1979) with additional information from Parton, (1989)

### **Histamine sensitization**

Parventjev *et al.*, (1947) found that WC vaccine increased the susceptibility of mice to histamine 100-fold. Similarly, *B. pertussis* extracts induced hypersensitivity to histamine in mice as well as leukocytosis and lymphocytosis. Clausen *et al.*, (1968) noticed that all these effects peaked around the 3rd day after treatment with *B. pertussis*, although animal species were found to differ widely in their sensitivity to the lethal effects of histamine. Mice and rats are naturally highly resistant to histamine, whereas guinea pigs and rabbits are highly susceptible. Guinea pigs and rabbits become if anything, more resistant to histamine after treatment with WC vaccine or PT, which is why mice are used for assay of HSF. Death is the main criterion of sensitization (Munoz and Bergman, 1968).

### **Islet activation**

Tabachnick and Gulbenkian, (1969) found that after WC vaccination, there was a correlation between hypoglycaemia (reduction in glucose levels) and hyperinsulinaemia, and suggested that these effects were linked. It is also known that PT acts on pancreatic B cells, blocking the inhibition of insulin release. Sumi and Ui, (1975) injected rats with *B. pertussis* cells and found a marked increase in the blood concentration of insulin in response to injection of insulin secretagogues such as arginine. Hypoglycaemia probably occurs due to the IAP component (i.e. PT) altering the B cells, resulting in increased secretion of insulin, but only after various insulin-releasing stimuli (Katada and Ui, 1977). Adrenaline is known to inhibit insulin secretion in normal pancreas cells but not in pertussis infected cells. Sidey *et al.*, (1987) suggested that the observed stimulatory effects of ether on insulin secretion in pertussis-treated mice, could be explained by the release of adrenal catecholamines leading to increased insulin secretion.

### **Pertussis vaccine and Pertussis toxin as immunological adjuvants**

In the 1940's, whole cells of *Bordetella pertussis* were first observed to alter certain biological systems in man and animals (Table 4). Greenberg and Fleming, (1947) demonstrated that mixtures of pertussis vaccine and diphtheria toxoid gave enhanced levels of antitoxin. Later, Mota, (1958) identified the mast cell-sensitizing effect of whole cells of *B. pertussis* in rats and mice. This effect caused an increase in the homocytotropic antibodies which were found to have similar characteristics to the human IgE-type reaginic antibody (Levine and Vaz, 1970). In the initial studies, Mota used horse serum as the protein antigen, but similar effects have been found with a variety of antigens including Ovalbumin (Oa), bovine serum albumin (BSA), sheep red blood cells (RBC), dinitrophenyl keyhole limpet haemocyanin (DNP-KLH) and bovine gamma globulin (BGG), using whole cells of *B. pertussis* as the adjuvant. Other

**Table 4: Literature summary on PT adjuvanticity**

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Observation	Investigator(s), Date
WC pertussis vaccine has adjuvant activity	Greenberg & Fleming, (1947)
Mast cell sensitizing effect of WC of <i>B. pertussis</i> - the production of homocytotropic antibody	Mota, (1958)
Alkaline saline extracts of <i>B. pertussis</i> had adjuvanting effect on IgG and IgE	Clausen <i>et al.</i> , (1969, 1970)
Main adjuvant effect attributed to HSF	Lehrer <i>et al.</i> , (1975, 1976)
Highly purified PT (0.1 ng) stimulated IgE antibody production to Oa	Munoz <i>et al.</i> , (1981)
PT causes an increased vascular permeability in brain tissue of mice resulting in experimental autoimmune encephalomyelitis (EAE)	Linthicum <i>et al.</i> , (1982)
An anti-PT IgE response could not be detected in mice after Oa and PT immunization	Munoz and Peacock, (1990)

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adjuvants that were similarly active were aluminium hydroxide and Freund's complete and incomplete adjuvants.

Clausen *et al.*, (1969, 1970) analysed alkaline, saline extracts of *B. pertussis* for adjuvant activity, with Oa as the antigen, in different strains of mice. Two types of homocytotropic antibodies were detected. The 2-h passive cutaneous anaphylaxis (PCA) antibody was a 7S immunoglobulin which did not persist in the skin of mice and was heat stable and resistant to reduction and alkylation. The other, 72-h PCA antibody was similar to the reaginic antibody of allergic persons, since it was inactivated by heating undiluted sera at 56° C for 30 min, and remained in the skin for long periods of time. *B. pertussis* alkaline saline extracts were found to be the best adjuvant for the production of the 72-h PCA, while Freund's adjuvant was superior in stimulating the 2-h PCA antibody. The main adjuvant activity for IgE was later attributed to the HSF of *B. pertussis* (Lehrer *et al.*, 1975, 1976) which is now known as pertussis toxin. Carswell and Oliver, (1978) found that when rats were sensitized i.p with DNP-Oa, with or without *B. pertussis* there was a definite increase in the serum IgE in the adjuvanted animals. There was also an increase in cutaneous immediate-type hypersensitivity but, surprisingly, no increase in immediate pulmonary reactivity to aerosol challenge. Munoz *et al.*, (1981) found that as little as 0.1 ng of a highly purified PT given i.v effectively stimulated the formation of IgE antibodies to Oa in mice.

While PT is the main adjuvant component of *B. pertussis* the LPS was also active in the guinea pig model. Guinea pigs were injected i.p with 10 µg Oa plus 5 µg of LPS which had been extracted by the phenol-water method. In the same experiment, a crude PT extract was also tested for IgE adjuvant activity. In this guinea pig system, the LPS of *B. pertussis* was the main active material responsible for the enhancement of IgE antibody production (Mota *et al.*, 1974).

Sekiya, (1983) investigated the effects of various *B. pertussis* components on the production of IgE and IgG1 responses. The effect of the K-agglutinogens, FHA, dermonecrotic toxin (HLT), LPS and pertussigen (PT) were all investigated for the stimulation of IgE and IgG1 to Oa in C57BL/6 mice (known to be high responders). The IgE was detected by heterologous PCA in the skin of Lewis rats, while IgG1 antibody was detected by PCA in the skin of mice. Among the *B. pertussis* components tested, pertussigen (PT) was found to be the most effective adjuvant for increasing both IgE and IgG1 antibodies to Oa. FHA only slightly increased the IgG1 titres, while the LPS increased both antibody levels moderately. HLT and the K-agglutinogens showed no increase in antibody levels. When each of the above components were then tested for there ability to modify the adjuvant action of pertussigen, HLT was the only component which interfered with the adjuvant action of pertussigen when

given on the day of immunization with Oa.

The adjuvant activity of the LPS of *B. pertussis* is shared by the LPS of most Gram-negative bacteria (Newburger *et al.*, 1974; Morrison and Ryan, 1979). Danneman and Michael, (1976) have investigated the ability of Gram-ve LPS from *Salmonella* species and *E. coli* to function as an adjuvant and as an antigen in IgE and IgG1 immune responses in mice. LPS failed to induce LPS-specific IgE or IgG1 under a variety of experimental conditions. However the isolated LPS and whole heat killed bacteria were capable of enhancing IgE and IgG1 antibody formation to the protein antigen Oa.

Experimental autoimmune encephalomyelitis (EAE) in mice is also dependent upon the use of *B. pertussis* suspensions as adjuvant. Intravenous administration of *B. pertussis* caused an increased vascular permeability in brain tissue and an increased vascular sensitivity to vasoactive amines which promoted the development of EAE (a disease of the CNS). PT was responsible for these adjuvant activities, whereas purified endotoxin was inactive (Linthicum *et al.*, 1982).

As seen in Table 3, only 0.1 ng/ml PT is required to elicit an adjuvant effect on anti-Oa IgE production in mice. The active component is PT since highly purified fractions of *B. pertussis* induced the production of IgE in mice (Munoz and Bergman, 1977). Wardlaw *et al.*, (1979) investigated the effect of antigenic modulation on adjuvanticity. They showed that X-mode vaccine was highly active in the induction of reaginic antibodies to Oa in mice, an effect consistent with the view that the adjuvant activity was due to PT. In C-mode cells, the effect was less and was transient and probably was due to the LPS. Mota *et al.*, (1974) had previously concluded that the LPS was the main active material responsible for the enhancement of IgE antibody production in guinea pigs.

There are numerous reports demonstrating that the IgE response is dependent on many variables including dose of antigen and adjuvant and whether boosting was given. IgE is only produced in certain strains of mice which were classed as high responders (Lehrer *et al.*, 1975). Table 5 shows a listing of known high, medium and low responder strains. Similar systems have been optimized for the rat (Jarrett *et al.*, 1980, 1981) and the guinea pig (Mota *et al.*, 1974).  
Jarrett and Hall,

In order to produce a substantial IgE response, it is important to consider several factors, including the dose of antigen and/or adjuvant, the timing of the injection(s) and of the bleeding times, and as emphasised, the mouse strain. Laboratory animals have been immunized using numerous combinations of the above factors to produce IgE. Table 6 details some of the investigations into IgE responses to Oa. The dose of adjuvant has been varied between 0.02 µg to 10,000 µg without having a dramatic effect on the IgE response. The antigen concentration (100 µg) seems to be

**Table 5 : High and low responding mouse strains**

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<b>High responders</b>	References
SWR/J C57BL/6 A/HeJ	Revoltella and Ovary. (1969)
Ham/ICR CBA/J	Lehrer <i>et al.</i> (1975, 1976)
Balb/C	Suko <i>et al.</i> (1977)
<b>Medium responder</b>	
AKR	Levine and Vaz. (1970)
<b>Low responders</b>	
C57L SWR SJL	Lehrer <i>et al.</i> (1975)

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**Table 6: Immunization procedures for the production of IgE in mice**

Antigen	Optimal dose ( $\mu$ g) Adjuvant (PT)	Boosting interval (days)	Time-course of expt. (days)	IgE titre Day No( )	Reference
125 Oa	50	-	80	512(10-60)	Clausen <i>et al.</i> , (1970)
100 Oa	10	56	63	640 (60)	Levine and Vaz, (1970)
100 Oa	10,000	21	28	806(28)	Sadowski <i>et al.</i> , (1979)
100 DNP-Oa	10,000	21	28	970(28)	
100 Oa	0.02	21	28	1470(28)	Sekiya, (1983)

constant in the four studies. Mainly, the immunization schedule involves boosting as primary immunization can sometimes result in a transient IgE response. In all cases, the titres were calculated by PCA and ranged between 500 and 1500.



# Immunoglobulin E

## General introduction

In 1921, Prausnitz and Kustner first identified reaginic activity in human serum and subsequently correlated this activity with skin disorder in atopic allergic disease. Ishizaka *et al.* (1966) found that this reaginic activity was antibody mediated and could not be precipitated with any of the antibodies specific for IgG, IgA, IgM and IgD. This new protein was designated IgE and responsible for most allergic diseases. An extensive list of the major discoveries in IgE understanding are explored in Table 7.

Identification of IgE was helped initially by the discovery of a E-myeloma protein (Johansson and Bennich, 1967). This discovery allowed the purification of IgE and established that the Fc fragment of IgE binds with high affinity to specific cell surface receptors (FceR) on basophils and mast cells (Ishizaka *et al.*, 1970). It was later observed that radiolabelled IgE binds also to human lymphocytes and macrophages via a low affinity receptor (Lawrence *et al.*, 1975).

The initial *in vivo* test used for detection of IgE was the passive cutaneous anaphylaxis (PCA) test developed by Ovary, (1958). The discovery of the IgE-myeloma also made it possible to raise antisera against human IgE, which in turn allowed measurement of total and specific IgE in serum. These anti-IgE sera were utilized in such *in vitro* tests as the radioallergosorbent test (RAST) for IgE, which has now been superceded by a range of immunoassays.

IgE is found only in very low concentrations in human serum and comprises less than 0.001% of the total circulating antibodies in normal individuals. Only a small proportion of the plasma cells in the body synthesize this immunoglobulin. IgE does not cross the placenta and has the capacity to passively sensitize mast cells for anaphylactic release of histamine and other vasoactive components. IgE in serum is destroyed by heating to 56° C for 30 min and is also lost when stored at 4° C, unlike IgG which remains unchanged by these treatments (Revoltella and Ovary, 1969).

## Structure and purification

The structure of IgE is like other immunoglobulins in being based on the four chain model originally proposed by Porter, (1962), and which is based on two types of polypeptide chain, heavy and light. IgE differs from this basic model by having four constant regions whereas most of the other immunoglobulins apart from IgM have three (Knauer and Adkinson, 1984). The IgE heavy chain has a molecular weight of 72,500 suggesting that there are approximately 550 amino acid residues in the four

**Table 7: Major benchmarks in the history of IgE**

Date	Discovery	Investigator(s)
1901	Phenomenon of anaphylaxis	Portier & Richet
1921	First description of immediate-type hypersensitivity	Prausnitz & Kustner
1958	Introduction of the PCA test for detection of reaginic antibody	Ovary <sup>b</sup>
1958	Mast cell sensitizing activity of whole cells of <i>B. pertussis</i>	Mota
1964	Presence of reaginic antibody in animals immune to helminth infections	Ogilvie
1966	New class of immunoglobulin, IgE	Ishizaka <i>et al.</i>
1967	"E-myeloma protein"	Johansson & Bennich
1967	RAST used for clinical diagnosis of IgE	Wide <i>et al.</i>
1970	High affinity IgE receptor on mast cells	Ishizaka <sup>a</sup>
1975	Low affinity IgE receptor on macrophages, lymphocytes and platelets	Lawrence <i>et al.</i> , <sup>a</sup>
1980	IgE potentiating and suppressing factors of T-cells	Suemura <i>et al.</i> , Hirashima <i>et al.</i>
1986	Sensitive ELISA for the detection of IgE	Kenemy <i>et al.</i>
1986	IL-4 involvement in IgE response	Coffman & Carty

<sup>a</sup> - reviewed by Speigelberg, (1984)

**Table 8 : Detection systems for mouse IgE .**

Assay Reference	Mechanism	Sensitivity (ng/ml)
Homologous PCA (Ovary, 1958)	vascular leakage of Evans blue dye following <i>in vivo</i> degranulation of sensitized <b>mouse</b> mast cells.	0.1 - 10
Heterologous PCA (Ovary <i>et al.</i> , 1975)	vascular leakage of Evans blue dye following <i>in vivo</i> degranulation of sensitized <b>rat</b> mast cells	10
ELISA (Lehrer <i>et al.</i> , 1979, Kenemy <i>et al.</i> , 1986)	quantitation of IgE bound to microtitre plates, followed by enzyme-dependent substrate colour development	0.01
RAST (Kelly <i>et al.</i> , 1980)	detection of antigen bound IgE (paper disc) with anti-IgE	0.1
RIA (Liu <i>et al.</i> , 1980)	detection of antigen (microtitre plate) bound IgE with radiolabelled anti-IgE	0.01
LAST (Gilles <i>et al.</i> , 1988)	detection of IgE with latex-bound antigen and enzyme-labelled anti-IgE	0.1

heavy chain constant regions (Roitt *et al.*, 1985).

The first attempt to purify reaginic antibody from mouse serum was done by gel filtration on a Sephadex G-200 column (Clausen *et al.*, 1969). A subsequent study demonstrated that the fraction positive in the 48-h PCA test, used routinely for detection of IgE, differed from the other immunoglobulin fractions (Clausen *et al.*, 1970). Partial purification of this reagin was later achieved by ammonium sulphate precipitation, gel filtration and ion-exchange chromatography (Lehrer *et al.*, 1976) and contaminating immunoglobulins were, in the case of IgG, removed by Protein A, or by immunoabsorption with appropriate antisera. The IgE was isolated by binding to, and elution, from an anti-Fab immunoabsorbent.

### Assay of IgE

A list of the mechanisms of some of the *in vivo* and *in vitro* tests used to detect IgE in mouse serum can be seen in Table 8, which will be described briefly in relation to sensitivity, specificity and operational difficulties. The PCA test is, at best, only semi-quantitative but is as sensitive as RAST (Karlsson *et al.*, 1979). On the other hand RAST may be easier to perform because of the *in vivo* operational difficulties of the PCA (Kelly *et al.*, 1980). RAST shows less intrinsic variation and is not influenced by serum IgE levels in the recipient animal. The latex allergosorbent test (LAST) is as sensitive as RAST, but does not use radioisotopes. The radio-immunosorbent assay (RIA) and ELISA are the most sensitive *in vitro* tests for IgE; as little as 0.01 ng/ml being detected. Moreover, there is not a requirement for radiolabelling in the ELISA, which is a major advantage over other *in vitro* tests.

### Passive cutaneous anaphylaxis

IgE and IgG1 can be detected in the mouse by the passive cutaneous anaphylaxis (PCA) test. Test serum is injected intradermally into the dorsal sides of a shaved laboratory mouse. After 2 h, for the detection of IgG1, and 48 h for the detection of IgE, the animals are challenged *iv* through the tail vein with a high dose of the specific antigen plus Evans blue dye. The actual mechanism depends on the mast cells becoming sensitized with either IgG1 or IgE. The degranulation of mast cells occurs following, in the case of IgE, cross-linking of the high affinity mast cell IgE receptor (Fc $\epsilon$ RI) with specific IgE antibody and antigen (Orange *et al.*, 1961). The cascade of events which follows the bridging of the Fc $\epsilon$ RI starts with the activation of a serine esterase and is rapidly followed by activation of adenylate cyclase, calcium ion influx, activation of methyltransferases and many other events, the last of which is the release of chemotactic factors and vasoactive amines such as histamine and serotonin

**Table 9: The different species of animal in which the PCA test may be done.**

Species of animal serum injected	Antibody detected	<u>Sensitization period (h) in recipient species</u>	
		Homologous	Heterologous
Mouse	IgG1	1.5-2	
	IgG2a	-	3-6 (guinea pig)
	IgE	48-72	24 (rat)
Rat	IgGa	4	-
	IgE	48	-
Guinea pig	IgG1a	4	-
	IgG1b	16-48	-
	IgE	48-72	-
Rabbit	IgG	-	-
	IgGa	4	-
	IgE	48-72	-
Human	IgG1	-	3-6 (guinea pig)
	IgG3	-	3-6
	IgG4	-	3-6
	IgE	-	48(monkey)

- no response

Data from Ovary. (1976)

(Ishizaka, 1983). These, in turn, primarily cause increased vascular permeability of the surrounding capillaries, in the mouse skin during the PCA test. Thus the blue dye enters the area of sensitization and can be seen as a distinct blue spot on the undersurface of the skin (Ovary, 1964).

The animal of choice for PCA is the albino guinea pig, but other species have been used. Strong PCA reactions may be obtained in the mouse, with rabbit or mouse antibodies (Ovary, 1958). Adult female albino mice are most suitable. However, the amounts of antibody needed for even weak reactions are about 25 to 50 times greater than that required in the guinea pig. The comparable test in humans is the Prausnitz-Kustner (PK) test which involves intradermal injection of antigen into an area of skin which has previously been sensitized with IgE antibody (Ovary, 1964). PCA has been performed in different animal species. Table 9 shows the PCA reactions of different animal sera in homologous and heterologous species.

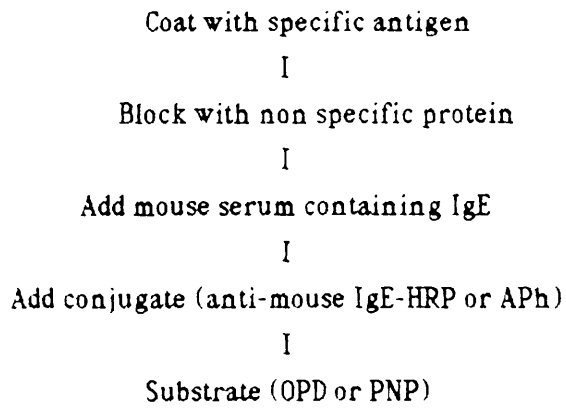
In the human, the first system for detection of skin-sensitizing antibody *in vivo* was the P-K reaction. Today, especially because of the danger of hepatitis and HIV and with the development of sensitive *in vitro* techniques, human skin testing is undesirable and is becoming obsolete. The PCA test was designed to detect homocytropic antibody (IgE) using a large excess of antigen. This is a limiting factor in the PCA test for IgE. When small amounts of antibody are injected i.d., more antigen is needed for the challenge dose (Ovary, 1964). The molecular weight of the antigen also has an effect on the PCA response (Ovary, 1958, 1976, 1986; Watanabe and Ovary, 1976). The larger the antigen, the more is required to produce a positive PCA response. This will be reviewed in the discussion section.

A frequent problem with 48-h PCA is that IgE sera with high concentrations of IgG<sub>1</sub> may cause generalized blueing, over the whole animal, which frustrates the observation of local blueing at the dorsal injection sites (Levine and Vaz, 1970). A number of modifications have been made to the PCA reaction first described by Ovary, (1958). For example with the mouse ear, the amount of extravasated dye can be determined colourimetrically (Inagaki *et al.*, 1988). PCA has also been found to be suppressed in rats by pretreatment with ether (Ovary, 1958) and by PT. The latter inhibits histamine release by a mechanism in which metabolism of cyclic AMP was not directly involved (Nakamura and Michio, 1983). Passive sensitization of mast cells with reaginic antibody is inhibited by simultaneous injection of a second reagin directed against another antigen, and also if the test animal is actively producing unrelated IgE (Jarrett *et al.*, 1971)

### **Enzyme linked immunosorbent assay**

The conventional ELISA first described by Engvall and Perlmann, (1972) involves

**Fig 1 : Flow diagram of the conventional ELISA for specific IgE in mouse serum**



HRP - horse radish peroxidase

APh - alkaline phosphatase

OPD - o-phenylene diamine

PNP - p-nitrophenylphosphate

antigen being coated onto a microtitre plate. Any free binding sites on the plate are then "blocked" with an unrelated protein, such as bovine serum albumin (BSA), whereupon the test antisera are added. The binding of any antibodies specific for the antigen are, in turn, detected by anti-immunoglobulin conjugated to an enzyme such as horse radish peroxidase or an alkaline phosphatase. Finally, a substrate is added, either O-phenylene diamine or p-nitrophenyl phosphate which completes the test (Fig 1). The intensity of the resultant colour reaction is thus related to the amount of antibody in the serum.

There are very few problems in measuring serum IgG using the ELISA because this immunoglobulin is usually the predominant antibody and there is little chance of interference from other antibody types. Reading and interpreting the absorbance values produced in the ELISA is more difficult. Bidwell *et al.* (1976) has reviewed the four main ways of expressing ELISA results: 1) as the extinction value, which is based on one serum dilution only but requires known positive and negative reference sera; 2) as the endpoint titre at a fixed absorbance; 3) with the latter as a percentage of a positive reference sera and 4) as the probability percentage which applies when either endpoint titres or extinction values are beyond the normal range.

The main problem with identification and quantification of mouse IgE are a) the small quantities of IgE in relation to other antibodies present in serum b) the lack of suitable reagents for assay and c) the interfering effects of other antibodies (Zeiss *et al.* 1977, Hamilton *et al.* 1981). When performing the conventional ELISA to detect IgE such problems are still experienced and to overcome these difficulties Kenemy *et al.* (1985) devised a modified ELISA which involved an initial coating of the microtitre wells with a affinity-purified rabbit antibody directed against the chosen antigen. This allows a greater flexibility in the ELISA, as the antigen is bridged to the plate by antibody, and not just by hydrophobic interactions with the plastic, which could hide certain important epitopes. They found that this increased the sensitivity of the ELISA without significantly increasing background interference from other immunoglobulins. Other modifications have been made to the conventional ELISA for detection of IgE. Lee *et al.* (1988) developed an ELISA against ragweed antigen E (AgE). The plates were coated with a monoclonal anti-human IgE which was followed by dilutions of the test sera and finally AgE conjugated to alkaline phosphatase. This bound specifically to IgE and did not allow interference by ragweed-specific antibody of other classes.

### **Other tests**

The RAST developed by Wide *et al.* (1967) has been used to detect allergen-specific IgE



in human serum. The RAST involves crude extracts of allergens being covalently bound to solid phase particles such as paper discs, followed by incubation with the patients serum. In the second step, these particles are exposed to radiolabelled anti-IgE. The radioactivity count is directly proportional to the quantity of IgE reacting with the solid-phase allergen (Gleich and Jones, 1975). The RAST, or the new LAST, where antigen is bound to latex beads, have been reported to detect as little 100 pg of specific IgE/ml of human sera (Gilles *et al.*, 1988). RAST has also been developed for use in mice to quantify IgE and was found to be as sensitive as, and more accurate than the PCA test, for the quantitation of IgE antibody at low levels (Kelly *et al.*, 1980).

The original RIA procedure, by Berson and Yalow (1959), depends upon the competitive inhibition of binding of antibodies to specific radioactive antigen, by unlabelled antigen. Subsequently the RIA has been modified, incorporating new reagents, to detect very small amounts of IgE in serum of humans and rats (Karlsson *et al.*, 1979).

The ELISA is very similar to the RAST, but requires an enzyme labelled anti-IgE instead of a radiolabelled anti-IgE. The ELISA and RIA are both sensitive tests for IgE (Table 9). Although the RAST is a robust test, capable of working with crude allergen extracts, it is not as suitable as ELISA or RIA for measuring IgE antibodies to purified antigens, nor for detecting low levels of IgE antibodies in the order of 100 pg/ml (Kenemy *et al.*, 1983)

## **Regulation of the IgE response**

### **Genetic control**

There have been numerous studies of the effect of mouse strain, antigen and adjuvant dose, bleed time and boosting on the IgE response (Levine and Vaz, 1970; Vaz *et al.*, 1971; Lehrer *et al.*, 1976). Revoltella and Ovary (1969), and especially Levine and Vaz (1970) and Vaz *et al.*, (1971) showed that in mice, the reaginic antibody response was under genetic control and that under certain experimental conditions, the ability to produce an IgE response was associated with an MHC H-2 haplotype. Later however Levine and Vaz, (1972) disclosed that IgE responsiveness was not correlated with H-2 or immunoglobulin allotype and appeared to be specific for the antigens being tested. Bazin, (1976) also demonstrated with 1 µg Oa-DNP and pertussis vaccine as adjuvant in Hooded Lister and August rats, bearing the same haplotype Ag-B5, that there was a marked difference in the anti-Oa-DNP IgE response, in the two strains.

In man, the genetic influence is shown by the significant incidence of atopy in offspring from allergic parents. This correlation has been linked to certain HL-A

**Table 10: Determinants of the IgE antibody response.**

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Determinant	Reference
<b>Genetic predisposition</b>	
Genetic regulation of basal serum levels	Marsh <i>et al.</i> , (1974)
MHC gene regulation of specific IgE response	Marsh <i>et al.</i> , (1980)
<b>Environmental factors</b>	
Intensity of antigen exposure	Nagel <i>et al.</i> , (1977)
Duration of antigen exposure	Platts-Mills <i>et al.</i> , (1981)
<b>Host factors</b>	
Mucosal or skin permeability	Barbee <i>et al.</i> , (1981)
Sex, age, race	Orren and Dowdle, (1975)
<b>Cellular regulation</b>	
Macrophage processing of antigen	Ishizaka <i>et al.</i> , (1976)
T cell recognition of antigen	Ishizaka <i>et al.</i> , (1976)
Generation of T suppressor cells	Strannegard <i>et al.</i> , (1978)
<b>Physico-chemical characteristics of antigen</b>	
Molecular weight	Hayglass & Stefura, (1991)

---

haplotypes (Marsh and Bias, 1978). It is believed that the HL-A haplotype in man is the counterpart of the murine H-2 MHC (Roitt *et al.*, 1985). In atopic individuals, serum levels of IgE can be considerably elevated and may reach several thousand ng/ml (Marsh *et al.*, 1974, 1980) compared to normal individuals (around 100 - 200 ng/ml). However, certain environmental stimuli (e.g., parasite infestation) can induce pronounced increases in the total serum IgE levels of normal individuals. Serum IgE levels must therefore be dependent on the interplay of both genetic and environmental influences. Some of the determinants of the IgE antibody responses in humans are listed in Table 10. These include genetic predisposition, as allergic parents tend to have a higher proportion of allergic children than those who are not allergic; environmental factors such as high pollen counts in the summer months and cellular regulation which relates to the individual immune response.

### **T and B cell interactions**

The regulation of IgE antibody responses is among the most sophisticated and complex of the immune defense mechanisms. It involves an interaction of several cell types and cytokines. The spleen is known to contain IgE precursor cells, giving rise to IgE-producing cells, as identified by Kind and Macedo-Sobrinho, (1973) using the heterologous adaptive cutaneous anaphylaxis reaction. This method demonstrated that passively-transferred splenic lymphocytes which were secreting IgE antibody could be detected in the skin of recipient rats by a reaction similar to the heterologous PCA. If these splenic lymphocytes were injected into X-irradiated (immunosuppressed) mice the IgE response was replenished in the recipient animals. Reaginic antibody forming cells were apparent in the spleen of these immunized rats 6 days after injection. In general, however, the spleen may play only a minor role in serum IgE production; the work of Barnett and Wust, (1978) revealed no significant differences between the circulating levels of IgE in hereditary asplenic, or in splenectomized, mice compared with normal controls.

It is accepted that the IgE response is under T-cell regulatory influences. The cellular interactions resulting in the production of IgE are similar to those involved in the other immunoglobulins. Hamaoka *et al.*, (1973) demonstrated a cooperative interaction between specific T and B lymphocytes as necessary for IgE production in mice. For most protein antigens, the antibody response of B cells also depends on signals received from helper T cells, which do not interact with antigen in its native form, but instead recognize a complex of antigen-derived oligopeptides and a major histocompatibility complex (MHC)-encoded molecule on the surface of another cell, the antigen presenting cell (Roitt *et al.*, 1985). Okudaira and Ishizaka (1973, 1974)

showed that enhanced anti-hapten IgE antibody responses resulted from the interaction of antigen-specific memory cells with helper T cells. It is established that there exists a definite T-cell dependency for the reaginic antibody response as can be seen in studies where thymectomised or congenitally athymic mice were used (Ito *et al.*, 1979; Nomoto *et al.*, 1978). Ito *et al.* (1979) found that no specific IgE antibodies were induced in the serum of athymic mice, and suggested that this was due to the controlling effect of a suppressor T cell population (or their products) which selectively inhibited production of IgE. Chiorazzi *et al.* (1976) observed in mice that after low dose X-irradiation or cyclophosphamide treatment (both immunosuppressive), there was a substantial increase in the levels of IgE antibodies, but a reduction in the IgG response. Okumura and Tada, (1971) performed similar studies in rodents and showed that after thymectomy rats also failed to produce IgE antibodies upon antigenic stimulation. However, IgE bearing cells were present in thymectomized animals and increased after infection with *Nippostrongylus brasiliensis*. Human studies suggest that allergic individuals have weaker than normal T-suppressor cell function which selectively enhances the production of IgE (Leung *et al.*, 1984).

There are two distinct views on how IgE is regulated. One is that the IgE response involves immunoregulation through a mechanism of isotype switching induced by the T-helper cells. In particular, T-h1 cells produce gamma interferon (IFN) which selectively stimulate IgG2a but inhibit IgG1 and IgE synthesis by B-cells. T-h2 cells produce IL-4 and IL-5 which stimulate IgG1, IgE and IgA but inhibit gamma IFN-induced IgG2a synthesis (Ishizaka, 1988; Hagen *et al.*, 1989). In contrast, Ohmori, (1990) showed that IL-4 exerted an inhibitory effect on antigen-specific IgE response *in vitro*. When cultured antigen-primed spleen cells were mixed with specific antigen and recombinant mouse IL-4, a significant reduction in the antigen-specific IgE response occurred. However, Finkelman *et al.* (1989) demonstrated in *in vivo* studies that IL-4 is required for the generation of polyclonal primary IgE responses induced by injecting mice with the larvae of *N. brasiliensis* as well as a secondary TNP-specific IgE response in mice. Treatment with anti-IL-4 antibody during both primary and secondary inoculation in the above inhibited the development of the IgE response by greater than 99%. These observations suggested that blocking with anti-IL-4 may have a beneficial effect in treating IgE-mediated disease.

The second view of IgE regulation was developed after IgE-binding factors were isolated from T-cells, which either potentiated or suppressed the IgE response. Thus the IgE response may be regulated not only by antigen-specific helper and suppressor T cells, but also through isotype-specific mechanisms. Initial experiments

**Table 11: Comparison of IgE-potentiating factor and IgE-suppressor factor**

Properties	IgE-potentiating factor	IgE-suppressor factor
Source	Fc $\epsilon$ R <sup>+</sup> W 3/25 <sup>+</sup> T cells	W 3/25 <sup>+</sup> T cells
Molecular weight (kDa)	13-15	13-15
Affinity for IgE	+	+
Affinity for:		
Lentil lectin	+	-
Concanavalin A	+	-
Peanut agglutinin	-	+

Adapted from Ishizaka *et al.*, (1985)

by Ishizaka *et al.*, (1981) using *N. brasiliensis*-infected rats revealed that the T cells of these infected animals produced soluble factors which had affinity for IgE and selectively enhanced or suppressed the IgE response (IgE-potentiating factor and IgE-suppressing factor respectively). Thus when IgE synthesis was enhanced, IgE-potentiating factor was released by T cells and could be detected in the culture supernate. The production of IgE-binding factors is not restricted to *N. brasiliensis* infection. IgE potentiating factor was found in rat serum 5-7 days after *B. pertussis* treatment and peripheral blood T cells from treated rats spontaneously released IgE potentiating factors in culture (Iwata *et al.*, 1983; Ishizaka *et al.*, 1985). Table 11 summarizes some of the comparisons between the two factors.

Further analysis of these two factors suggested that IgE-potentiating factor contains both N-linked and O-linked oligosaccharide, whereas IgE-suppressor factor has only the O-linked oligosaccharide. The N-linked oligosaccharide in the IgE-potentiating factor is essential for enhancement of the IgE response (Yodoi *et al.*, 1982). Another important finding was that both IgE-potentiating and IgE-suppressor factors were produced by T cells and that the nature of these binding factors is determined by the environment. When rats were immunized with Freund's adjuvant, the IgE-suppressor factor was produced. Conversely, PT stimulated T cells to release a soluble factor glycosylation enhancing factor (GEF), which enhanced the assembly of the N-linked oligosaccharide to IgE-binding factor (Ishizaka *et al.*, 1985). GEF is a kallikrein-like enzyme which enhances the glycosylation of IgE-binding factors through an activation of a phospholipase. The production of GEF from T-cells with PT seems to explain the PT action on increasing IgE production. It was also found that GEF triggers normal mast cells to release histamine and arachidonate and could partly explain the role of PT in histamine-sensitization.

### **Interaction of IgE with cells**

The mast cell was first identified by Ehrlich in 1879 (Marone, 1988) and is now known to be the main cellular target for IgE. There are two basic types, mucosal and connective tissue mast cells. Mucosal mast cells appear to be dependent on T cells for proliferation, whereas the connective tissue mast cells are T-cell independent. Kitamura *et al.*, (1986) suggested that mast cells arise from common precursor cells that differentiate after they have reached their target sites, and that the final phenotype is dependent on the microenvironment. The two types of mast cell are easily distinguishable by their respective size, number of secretory granules, content of vasoactive amines and their staining characteristics (Lee *et al.*, 1985). It is now generally accepted that mast cells from different locations are functionally

**Table 12: Preformed and newly formed mediators in mast cells**

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Mediator	Physiological effect
<b>Preformed</b>	
Histamine	Vasodilation (increased capillary permeability)
Heparin	Anticoagulant
Chemotactic factors	Chemotaxis of
	Eosinophils
	Neutrophils
	Monocytes
	Basophils
	Lymphocytes
<b>Newly formed</b>	
SRS	Bronchoconstriction
Leukotrienes	Vasoactivity
Prostaglandins	Bronchial muscle contraction
	Platelet aggregation

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Adapted from Mygind. (1986)

heterogeneous within and between different animal species (Pearce *et al.*, 1985). Lowmann *et al.*, (1988) compared the ability of both immunological and non-immunological agents to stimulate histamine release from human skin, lung, adenoid, tonsil and colon mast cells. While they all secreted histamine in response to IgE and anti-IgE and a calcium ionophore, skin mast cells were also responsive to substance P, 48/80, poly-L-lysine and morphine. This was indicative of the functional heterogeneity within the human mast cell population. IgG1 can also sensitize mast cells and basophils, which are very similar to mast cells, and both release vasoactive amines upon degranulation. The 2 h PCA test in the mouse detects IgG1 bound to mast cells.

It is well established that immediate-type hypersensitivity reactions are produced by IgE binding to mast cells. The cross-linking of the IgE molecules on the surface of the mast cell by antigen, triggers secretion of granule-associated mediators (Table 12) such as histamine (Church *et al.*, 1982). Immunological stimulation by anti-IgE and antigen or by non-immunological stimulation from compounds such as 48/80, poly-L-lysine and morphine-like analgesics also cause degranulation of mast cells and basophils. When sodium cromoglycate (an anti-histamine) is administered to asthmatics before they are exposed to antigen, there is a reduction in histamine release from lung mast cells. Basophils, however, are less sensitive to sodium cromoglycate and continue to synthesise and release histamine.

The main vasoactive amine, histamine, was initially detected in biological samples by a fluorimetric assay developed by Shore *et al.*, (1959). This method involved histamine being extracted with *n*-butanol and added to *O*-phthalaldehyde to yield a product with a strong and stable fluorescence. This method has been superseded by radioenzyme assay (Snyder *et al.*, 1966) and an enzyme immunoassay (both reviewed by Chevrier *et al.*, 1986).

IgE binds with high affinity to specific cell-surface receptors (Fc $\epsilon$ R) on mast cells and basophils. For some time it was thought that IgE interacts only with receptors on basophils and mast cells. However, radiolabelled IgE has been shown to bind to human cultured lymphoblastoid cells, mouse lymphocytes, cultured human macrophage and rat macrophages (reviewed by Speigelberg, 1984). All these cells bind IgE with relatively low affinity when compared to mast cells and basophils. The receptors on monocytes and macrophage are thought to promote phagocytosis and killing of IgE-coated targets such as parasites. Aggregated IgE is known to induce the release of mediators of inflammation from rat and human macrophages.

Bringel *et al.*, (1982) also studied the *in vitro* biological activity of IgD



antibodies against a fraction of *Lolium perenne* thought to be responsible for certain cases of allergic pollenosis. They used three different methods: histamine release, basophil degranulation and the RAST inhibition test. In all the assays the data indicated a possible blocking effect of the IgD antibodies upon the IgE *in vitro* mediated mechanisms. While lack of skin-sensitizing properties of IgD has been demonstrated (Ovary, 1969), association between IgG and IgD has been reported in some clinical conditions, as in hyper-IgE syndrome and in undue susceptibility to infections (Buckley and Fiscus, 1975). Furthermore, when immunoglobulin levels were studied in atopic and non-atopic people, a statistically significant increase in IgD was found in the atopic groups (Kohler and Farr, 1967).

In 1964, Terry and Fahey discovered that human IgG could be further categorized into four subclasses IgG1, IgG2, IgG3 and IgG4. Analyses of responsiveness have suggested an association between IgG1 and/or IgG3 subclasses and the T-cell dependent response to protein antigens whereas the T-cell independent response antibodies to polysaccharide antigens including bacterial capsules are usually comprised of the IgG2 isotype. Various food, venom, inhalant and parasitic allergens induce IgE and IgG4-specific antibodies in man. It has been suggested that IgG4 represents a non-IgE antibody that can sensitize basophils for the release of histamine and, thus, contribute to clinical allergy (Heiner, 1984).

### **IgE responses in parasitic infection**

Elevated IgE levels have been reported in humans infected with a variety of parasites including *Toxocara canis* (Hogarth-Scott *et al.*, 1969) and *Schistosoma mansoni*, (Capron *et al.*, 1982).

Antigen-specific IgE is thought to be protective in parasitic infections. A number of investigators have looked at this in experimental parasitic infections, as demonstrated by the reduction in parasite burden (Table 13). Most work has been done in the rat. Ogilvie, (1966) passively immunized rats with IgE-rich serum, or heated serum where the IgE had been destroyed, and showed a marked reduction in the number of cercariae recovered from infected animals passively immunized with IgE when compared to the controls. Later, Capron *et al.* (1980) found similar results with a higher infective dose of the cercariae of *S. mansoni* than was originally used by Ogilvie. Gabriel and Justus (1979) demonstrated in *Trichinella*-infected mice a reduction in the recovery of larvae encysted in muscle, when IgE was passively administered before infection. Dessein *et al.* (1981) examined the effect of suppressing the IgE response by injecting neonatal rats *i.p.* with anti-epsilon chain serum or control serum (produced by injecting rabbits with FCA alone). At 7 weeks,

**Table 13. Protective activity of IgE in experimental parasitic infections.**

This was demonstrated by the reduction in parasite burden after passive immunization or by increase in parasite burden after IgE depletion.

Parasite	Host	Dose	IgE shown to be protective by:		Control serum	References
			Passive immunization	Inversely by IgE depletion		
<i>Schistosoma mansoni</i>	Rat	200 C <sup>a</sup>	+	N.T.	Heated <sup>c</sup>	Ogilvie, (1966)
<i>Schistosoma mansoni</i>	Rat	1000 C	+	N.T.	Heated	Capron <i>et al.</i> , (1980)
<i>Schistosoma mansoni</i>	Rat	1000 C	N.T.	+	Normal	Damonneville <i>et al.</i> , (1986)
<i>Trichinella spiralis</i>	Mouse	500 L <sup>b</sup>	+	N.T.	Heated	Gabriel & Justus, (1979)
<i>Trichinella spiralis</i>	Rat	1000 L	N.T.	+	Normal	Dessein <i>et al.</i> , (1981)

<sup>a</sup> - cerceriae

<sup>b</sup> - larvae

<sup>c</sup> - the same serum heated to 56<sup>0</sup> C for 30 min to destroy the IgE.

+ - protective effect

N.T - not tested.

the rats were infected with *T. spiralis* larvae and injected with either anti-epsilon or control serum every 4 days thereafter. Suppression of the IgE antibody response was accompanied by marked reduction in tissue eosinophilia and very brief blood eosinophilia. Moreover, IgE-suppressed rats were much less resistant to infection by the larvae than the control groups. In *S. mansoni* infection, Damonville *et al.* (1986) noted that schistosoma-release products (SRP) induced an IgE response to the cercariae. If IgE depleted anti-SRP serum was injected into the infected animals there was a significant decrease in the degree of protection. Most of these studies suggest that the role of IgE antibodies in certain parasitic infections is a protective one. Other investigators have suggested a role for IgE in helminth infections. Firstly, there are very high human IgE serum levels in areas where these parasites are endemic (Johansson *et al.* 1968). Secondly, with *in vitro* animal models, Capron *et al.* (1975) demonstrated IgE-dependent binding of macrophages and eosinophils to schistosomal organisms. Furthermore, others have proposed that IgE mechanisms cause mast cell discharge of mediators such as histamine in the gastro-intestinal mucosa and that these either directly or indirectly serve to expel helminthic infections (Dineen *et al.* 1973). Contrary to this is more recent evidence that mice, genetically-deficient in mast cells, can expel intestinal parasites as efficiently as mice with normal mast cells (Uber *et al.* 1980)

### **IgE responses after microbial infection**

In contrast to the above, the possible role of IgE in microbial diseases has so far been largely speculative. In several investigations, antigen-specific IgE responses have been detected with both viral and bacterial infections (Table 14).

The IgE response has been explored for two main reasons: as a diagnostic tool indicating infection, and for a possible role in the disease process. This latter could be either detrimental or protective to the host. Detrimental effects were suggested by Welliver *et al.* (1980) regarding respiratory syncytial virus (RSV) infection. IgE was found bound to exfoliated nasopharyngeal epithelial cells in most patients during the acute phase of infection. The continual presence of cell-bound IgE was more common in patients with RSV-induced bronchiolitis or asthma than patients with mild upper respiratory tract illness or pneumonia due to RSV. It was suggested that the production of IgE and subsequent release of chemical mediators of bronchospasm may contribute to the pathogenesis of the acute illness. Motala *et al.* (1986) also noted that in *Staphylococcus aureus*-induced atopic dermatitis, the anti-*S. aureus* IgE-positive adults had a more severe and prolonged disease than the IgE-negative patients. As well as adults, children found to be IgE-positive, had a more severe

**Table 14: Reported IgE responses in infectious diseases and their interpretation.**

Infective agent	Reason for assaying of IgE			Author(s)
	Possible diagnostic usage	Possible role in disease of host		
		Protective	Detrimental	
Respiratory syncytial virus	NT	NC	S	Welliver <i>et al.</i> ; (1980)
Rubella	D	NC	NC	Salonen <i>et al.</i> (1985)
<i>Pseudomonas aeruginosa</i>	NT	S	S	Shen <i>et al.</i> (1981)
<i>Staphylococcus aureus</i>	NT	NC	S	Motala <i>et al.</i> (1986)
<i>Bordetella pertussis</i>	D	S	S	Finger <i>et al.</i> ; Wirsing von König (1985)

S- Speculative

D - Demonstrated

NT - Not tested

NC - No comment

disease, with a greater prevalence of cutaneous *S. aureus* infections, and a greater prevalence of specific IgE responses to food allergens. A possible protective role for IgE in disease has also been proposed. Shen *et al.* (1981) detected IgE responses to *Pseudomonas aeruginosa* in patients with cystic fibrosis. They suggested that respiratory allergy may provide a degree of protection against pulmonary infection but also claimed that atopy caused a higher incidence of infection.

Finger *et al.* (1985) employed an IgE ELISA against whole cells of *B. pertussis* and tested the sera from whooping cough cases. Only a small percentage (3%) of those tested showed measurable quantities of IgE to whole cells of *B. pertussis*. The presence of IgE did not seem to affect the clinical course of the disease.

This single observation by Finger *et al.* (1985) is the only work involving an IgE response to *B. pertussis* which had been published before the start of this investigation.

**OBJECT OF RESEARCH**

It is well documented that PT is an IgE adjuvant and that only minute doses are needed to elicit an IgE response against protein antigens such as Oa. The possibility that PT may serve as an IgE-adjuvant for the antigens of *B. pertussis* including PT itself, has not so far been explored.

The object of this research was therefore to detect and quantify the IgE response against two components of *B. pertussis* namely FHA and PT. These were chosen as the targets for IgE-adjuvanting because of their importance as components of present-day acellular vaccines and as virulence factors in pertussis disease.

To provide an experimental basis for this work, Oa was taken as a model antigen for IgE induction, in order to determine optimal conditions for inducing and detecting IgE specific for FHA and PT.

**M A T E R I A L S**  
**&**  
**M E T H O D S**



## Bacterial culture

### Strains

*B. pertussis*, BP 18334, was obtained from the departmental culture collection. It came originally from Connaught Laboratories, Toronto, Canada where it had been used for many years as a strain for whole-cell vaccine production. *B. pertussis* BP 18323 was obtained from the departmental culture collection, as the standard mouse intracerebrally virulent strain, used in potency testing of pertussis vaccine. *B. pertussis* BP 353 strain is a transposon mutant lacking FHA. *B. pertussis* BP 357 is a transposon mutant lacking PT. These were originally gifted by Dr A. A Weiss.

All strains were either stored in vacuum sealed glass ampoules at R.T, or as frozen in a mixture of casamino acids and glycerol (Appendix 1) at 70° C.

### Culture media

Bordet Gengou (BG) agar base and defibrinated horse blood were obtained from Oxoid and Gibco respectively and used to prepare BG agar plates for propagation of *B. pertussis* strains. When culturing BP 353 and BP 357 the agar was supplemented with 50 µg/ml kanamycin (Sigma).

Stainer-Scholte liquid medium (SS) was used as described by Stainer and Scholte, (1971). Cyclodextrin-modified Stainer-Scholte liquid medium (SS-C) contained heptakis 2,6-O-dimethyl-β-cyclodextrin which was a gift from Teijin Ltd, Chiyoda-ku Tokyo, Japan. The solid agar was used for growing primary cultures from freeze dried or frozen stock cultures, and the liquid media were employed in large-scale liquid culture. The composition of the media are listed in Appendix 1.

### Growth of organisms from frozen stock cultures

Frozen stock suspensions in casamino acids and glycerol were thawed at R.T for 30 min. One loopful of the suspension was streaked on to each of two to three plates BG plates which were incubated in a moistened box in a 37° C incubator (Laboratory Thermal Equipment) for 2-3 days, and checked daily for growth. The resultant growth was checked by Gram stain to confirm the presence of Gram-negative coccobacilli and absence of contaminants.

### Growth of large scale liquid cultures

The growth was scraped from the BG plates and transferred into 2 L dimpled flasks (Rasotherm, Germany) containing 1 L of liquid culture either SS or SS-C medium (formulae in Appendix 1). The flasks were incubated at 37° C without shaking for 48 h. For aerated cultures they were placed on an orbital shaker (L.H Engineering), on

medium speed setting, at 37° C for 48 h. The cells were pelleted by centrifugation (Sorvall RC-5B) at 13,700 g for 30 min at 4° C and the supernate decanted and saved for extraction of FHA and PT.

### **Extraction of FHA and PT from Culture Fluids**

FHA and PT were extracted from membrane filtered supernates by exactly the same procedure but with BP 357 and BP 353 suspensions respectively as starting cultures. The bacteria was grown in SS-C at 37° C for 48 h with shaking, and either PT or FHA was extracted from the supernate by a dye-ligand affinity chromatography method modified from Sekura *et al.*, (1983). This method involved absorption at low ionic strength and elution at high salt ionic strength.

To culture supernate of BP 357 (10 L), 100 ml of Blue Sepharose CL-6B (Pharmacia LKB) was added and adjusted to pH 6.0 at R.T by the addition of about 100 ml of 2.5N HCl. The mixture was stirred overnight at 4° C in the presence of 1 mM PMSF (Sigma) as a protease inhibitor. The gel supernate was filtered through a G1 sintered glass funnel (Gallenkamp) and packed into an LKB 2137 chromatography column (2.6 x 35 cm, Pharmacia LKB) and washed with 0.05M Tris-HCl pH 8.0 using a peristaltic pump. The FHA was eluted with 0.05M Tris-HCl pH 8.0 containing 1.0M NaCl, and 10 ml fractions collected with an Ultratrac fraction collector connected to a uvicord II-UV absorptiometer (Pharmacia LKB) to monitor the elution peak. The exact same method was used for extraction of PT but the culture supernate came from BP 353. The preparations were dialysed overnight against physiological saline (9% w/v NaCl) and membrane filter sterilised before injection into mice. The packed gel was regenerated with 0.1M Tris-HCl, pH 8.0 containing 0.5M NaCl and 6M urea, to remove any strongly bound material, followed by alternate washing with high and low pH buffers. The gel was washed alternately with 0.1M Tris-HCl pH 8.5 containing 0.5M NaCl and 0.1M sodium acetate pH 4.5 containing 0.5M NaCl. The gel was then finally washed with 0.05M Tris-HCl pH 8.0 and stored at 4° C with thiomersal at a final concentration of 0.01% (w/v). Formulae for all the buffers can be found in Appendix 2.

### **Toxoiding of PT**

#### **a) with Glutaraldehyde**

The method of Munoz *et al.*, (1981) was followed. Briefly, a solution of 100 ml of PT 100 µg/ml was suspended in 20mM sodium phosphate with 0.5M NaCl, pH 5.0. Glutaraldehyde 0.2% (v/v) was added to bring the final concentration of glutaraldehyde to 0.05% (v/v). The mixture was incubated at room temperature (RT) for 2 h and then enough 0.2M L-Lysine was added to dilute the mixture by 1 in 10 and the final concentration of L-lysine to 0.02M. The mixture was incubated for 2 h at RT and then dialysed for 2 days

against 20mM sodium phosphate buffer pH 5.0 containing 0.5M NaCl and 0.02M L-Lysine.

#### **b) with Carbodiimide**

PT (3 ml of 2 mg/ml) was dialysed overnight at 4° C against 0.5M NaCl in 20mM sodium phosphate buffer pH 5.0. The toxoiding reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. HCl (EDAC, Sigma) at a concentration of 4 mg/ml was prepared in 117 ml of phosphate buffer, pH 5.0. The PT and EDAC were mixed so that the resultant mixture contained 50 µg/ml of PT and 4 mg/ml of EDAC. This was incubated at 37° C for 24 h without shaking then dialysed at 4° C for 3 days against daily changes of the buffer, 20mM sodium phosphate with 0.5M NaCl pH 7.6 containing 0.01% (v/v) thiomersal daily.

### **Pertussis Vaccine Preparation**

*B. pertussis* BP 18334 was grown in 1 L of SS-C at 37° C for 48 h and cells were harvested by centrifugation at 13,700 g in a Sorvall RC-5B centrifuge (6 x 500 ml rotor head) for 30 min. The pellet was resuspended and washed twice in physiological saline. The opacity of the resultant solution was compared to the international opacity standard (WHO 5th International reference preparation, 1975, NIBSC, Holly Hill, Hampstead, London) to which *B. pertussis* suspensions are matched. The number of opacity units (ou) was determined on the cell suspension before converting it into vaccine. The whole cells were then heated to 56° C for 30 min in a stirred water bath. Care was taken to ensure efficient heat transfer by making the level of water in the bath higher than that in the bottle, and also by swirling the bottle. The bottle was cooled and thiomersal (BDH) at a final concentration (0.01% v/v) added as a preservative. The final preparation was stored at 4° C.

### **Animals and Animal procedures**

#### **Mouse strains**

Stocks of Ham/ICR and Balb/c mice were obtained from Charles River UK Ltd (Manston Rd, Margate, Kent) and bred in house in temperature and light controlled rooms. They were fed ad libitum.

#### **Raising antisera**

Groups ranging between 5 to 25 Ham/ICR and Balb/c mice were injected intraperitoneally i.p. with 0.5 ml of the immunization mixture. After a time interval indicated by the immunization protocol the mice were killed and the blood collected as below.

**Table 15 : Immunization schedules used for raising IgE antisera**

Scheme No.	Injection mixture	Bleed day
2	Ag + Adj (1) <sup>a</sup>	10
	Ag + Adj (1)	21
	Ag + Adj (1)	28
3	Ag + Adj (1)	
	Ag (28)	
	Ag (56)	63

<sup>a</sup> - Injection day

Ag-Antigen; Adj-Adjuvant

### **Raising IgE antisera**

A variety of different immunization protocols was used to raise IgE antisera to Oa, FHA and PT in mice of the two strains. Both sexes were used, as available. The general procedure was to take groups of either the same age or ranging from 3 - 6 weeks and house them in cages of 15 - 20 animals. Antigen and adjuvant were administered i.p. in dose of 0.5 ml per animal (Table 15). In the early experiments, a single immunization dose (Scheme 1) was used and groups of mice were bled on different days. In later experiments, the mice received a total of three antigen (Ag) doses of which only the first contained adjuvant (Adj) as in (Scheme 2). The exact concentrations of Ag and Adj are outlined in the results section.

### **Preparation of sera**

The mice were asphyxiated with carbon dioxide. Blood was collected by cardiac puncture and kept on ice. The whole blood was then incubated at 37° C for 30 min to accelerate clotting. The blood was then centrifuged at 2,000 g for 20 min at 4° C. The serum was extracted from the clot using a glass pasteur and stored at -20° C.

### **Histamine sensitizing activity (HSA)**

The PT sample was diluted in PBS and 0.5 ml were injected i.p. into 6-8 week old Ham/ICR mice. After 5 days the mice were challenged i.p. with 3 mg of histamine dihydrochloride (Sigma) contained in 0.5 ml of sterile saline. Survivors were counted 2-3 h after challenge. The approximate LD<sub>50</sub> for each batch of PT was calculated (Appendix 5).

### **Mouse weight gain**

Groups of 5, 3 week old Ham/ICR mice were injected i.p. with graded doses of the test sample. The weight of each group was recorded using a flat pan balance (Sartorius) on Day 0 and recorded each day thereafter and the weight loss or gain was noted. The number of deaths if any were recorded over the 7 day period of the experiment.

### **Intranasal infection**

A group of 23 mice, 3 week old female Ham/ICR mice, were infected intranasally (i.n.) with live *B. pertussis* BP 18323. A concentration of  $2 \times 10^8$  cfu/ml was prepared in 1% (w/v) casamino acids and 25  $\mu$ l amounts were dropped onto the anterior nares of an ether anaesthetised animal using a gilson automatic pipette. This meant that each animal was infected with  $5 \times 10^6$  cfu. A control group of 13 were instilled i.n. with 1% (w/v) casamino acids alone. The weights and deaths in each group were recorded over a 32-day period. At approximately 5-day intervals some of the animals from the infected and control group were sacrificed and the extent of the lung infection scored.

## **Passive Cutaneous Anaphylaxis**

PCA tests were done in 6 to 8-week old Ham/ICR mice, of either sex and body weight more than 40 g, by a method based on that of Ovary (1958). One significant difference was the use of four intradermal (i.d) injection sites per mouse so as to have a positive control site (anti-Oa serum) and three experimental sites for *B. pertussis* sera. Ten mice were used in a typical set of PCA tests. With each mouse, hair was clipped from the dorsal surface and 50  $\mu$ l doses of the test sera were injected i.d into each animal under Hypnorm (Janssen Animal Health) analgesia. The injection site of the positive control serum was varied systematically between mice. Each experimental serum was usually tested in quadruplicate, each replication being in a different animal. To elicit the PCA reactions, the sensitized mice were challenged i.v at intervals ranging from 2 to 7 days with a mixture of either FHA or PT mixed with Oa and Evans Blue (Sigma). The challenge mixture was made in PBS so that each 0.2 ml contained 0.5 mg FHA or PT plus 1 mg each of Oa and the dye. At 30 min after challenge, the animals were killed and the skins removed so that the diameter of blueing, where the dye had entered, could be measured on the inner surface. The diameter of each blue zone was measured in two directions at right angles and the average taken. The averages of zones from different mice were summarized as arithmetic means with 95% confidence limits. On the rare occasions when the anti-Oa site gave an unexplained negative result, all the observations from that animal were discarded.

## **Enzyme Linked Immunosorbent Assay**

### **Reagents**

Oa was obtained from Sigma and the PT and FHA were prepared as described earlier by the method of Sekura *et al.*, (1983). Three conjugates were used a goat anti-mouse IgE, ICN Immunobiologicals, P.O. Box 1200, Lisle, IL 60532; sheep anti-mouse IgE conjugated to a horse-radish peroxidase, The Binding Site, 97 Vincent Drive, Edgbaston, Birmingham B15 2SQ, England; and a rabbit anti-goat IgG conjugated to horse-radish peroxidase (HRP), Sigma. Hydrogen peroxide and sulphuric acid were obtained from May Baker. O-phenylene diamine was purchased from Sigma.

### **ELISA for IgE**

The 96 wells of a flat bottomed microtitre plate (Nunc Maxisorp F) were coated with 100  $\mu$ g/ml of Oa in coating buffer pH 9.6. All the solutions are described fully in Appendix 3. This was incubated overnight at 4<sup>o</sup> C. The plate was then washed 3 times in washing/incubation buffer pH 7.4 and blocked with 2% (w/v) BSA in incubation buffer

pH 7.4 for 1 h at 37° C. The plates were then washed three times in wash buffer. Ten-fold dilutions of mouse anti-Oa IgE serum were added and incubated at 37° C for 1 h. The plates were washed with wash buffer containing 1% BSA. Goat anti-mouse IgE (1 in 100 dilution prepared in incubation buffer pH 7.4) was added and incubated at 37° C for 1 h. Plates were washed three times, then rabbit anti-goat HRP (1 in 1000) (Sigma, Lot No. 77F-8866) in incubation buffer pH 7.4, was added and incubated for 1 h at 37° C. The developing substrate consisted of 200 µl of a 34 mg/ml soln of O-phenylene diamine (OPD) in 100 ml of citrate-phosphate buffer pH 5.0. Hydrogen peroxide (20 µl) was added just before use. The plates were left in the dark for 30 min, and terminated by adding 50 µl of 12% (v/v) H<sub>2</sub>SO<sub>4</sub>. Modifications to this ELISA system are discussed in the Results section.

The above ELISA method was also used for measuring IgG1 and total IgG. However HRP-sheep anti-mouse IgG1 and IgG were the conjugates in the IgG1 and IgG ELISA's respectively.

### **Modified IgE ELISA**

#### **Preparation of affinity columns.**

Protein antigens Oa, FHA or PT were coupled via their amino groups to cyanogen bromide activated Sepharose 4B (Sigma). The Sepharose 5 g was suspended in 1mM HCl and the gel was washed with 1 L of 1mM HCl on a sintered glass funnel to wash away additives used in the freeze drying process. A 25 mg amount of protein antigen/ligand was dissolved in coupling buffer (0.1M NaHCO<sub>3</sub>, pH 8.3 containing 0.5M NaCl) and added to the washed Sepharose. The mixture was rotated overnight at 4° C in an angled rotator to allow all the ligand to bind to the gel. The excess ligand was then washed away with coupling buffer using a sintered glass funnel attached to a vacuum pump. The absorbance at A280 of the filtrate was checked to ensure that most of the ligand had bound to the gel. Any active groups of the gel remaining were blocked with 2 L of 0.1M Tris-HCl pH 8.0 which washed through the gel column for 2 h at RT. The gel ligand matrix was finally washed with three cycles of alternating pH. Each cycle consisted of a wash of 0.1M acetate buffer pH 4.0 containing 0.5M NaCl followed by a wash with 0.1M Tris-HCl pH 8.0 containing 0.5M NaCl. The gel was then poured slowly into a Biorad column (Internal diameter 1.5 cm x length 15 cm) allowed to settle and stored at 4° C.

#### **Preparation of affinity-purified antibody**

Rabbit anti-Oa serum (25 ml) was dialysed in visking tubing against 1 L of 0.1M Tris-HCl pH 8.0, overnight at 4° C. The serum was pumped through the Oa affinity column using a peristaltic pump (LKB) with a flow rate of 1 ml/min and fractions were collected with

an Ultrotrac fraction collector connected to a uvicord II-UV absorptiometer (Pharmacia LKB) to monitor the elution peaks. After the serum, 0.1M Tris pH 8.0 was pumped through the column for 3 h to elute unadsorbed protein. The column was washed again for 1 h with 0.1M Tris-HCl pH 8.0 containing 0.5M NaCl to elute any bound non-adsorbed protein. To elute the affinity-purified antibodies, 0.1M glycine-HCl pH 3.0 was pumped through the column and 2 ml fractions were collected. The peak fractions were then dialysed immediately against PBS (8 g NaCl, 0.2g  $\text{KH}_2\text{PO}_4$ , 1.15g  $\text{Na}_2\text{HPO}_4$ , 0.2g KCl all prepared in one litre of distilled water) pH 7.4. The column was washed with 500 ml of PBS containing 0.1% sodium azide and stored at 4° C. A similar procedure was also used to prepare affinity purified anti-FHA

### **ELISA for IgE**

The 96 wells of a flat bottomed microtitre plate (Nunc F maxisorp) were coated with 200  $\mu\text{l}$  affinity-purified anti-Oa in coating buffer pH 9.6. This was incubated overnight at 4° C. The plate was then washed 3 times in washing buffer pH 7.4. A 100 $\mu\text{l}$  of Oa (100  $\mu\text{g}/\text{ml}$ ) was prepared in incubation buffer pH 7.4, and added to each well. Then the plate was incubated at 4° C for 1 h and washed 3 times, 5 min each, in wash buffer containing 1% (w/v) BSA. The test sera containing anti-Oa IgE was diluted in tenfold steps from 1 in 10 to 1 in 10,000 and 100  $\mu\text{l}$  was added to appropriate wells and incubated at 4° C for 1 h. The plate was washed and the sheep anti-mouse IgE HRP conjugate was added and incubated for 1 h at 4° C. The developing substrate consisted of 200  $\mu\text{l}$  of a 34 mg/ml soln of O-phenylene diamine (OPD) in 100 ml of citrate-phosphate buffer pH 5.0. Hydrogen peroxide (20  $\mu\text{l}$ ) was added just before use. The substrate was added and incubated at 37° C for 30 min and terminated by adding 50  $\mu\text{l}$  of 12%  $\text{H}_2\text{SO}_4$ . A similar procedure was also used for measuring anti-FHA IgE. However, in the PT ELISA instead of a AP anti-PT, fetuin was used to coat the PT to the plate. All subsequent incubations were as above. Modifications of this ELISA system are discussed in the Results section.

## **Miscellaneous**

### **Assay of FHA**

The assay was performed in plastic round bottomed microtitre plates (Sterilin) with Titertek micro droppers (Flow). Horse erythrocytes were washed 4 times in sterile saline and the packed cell suspension diluted to give a 0.5% (v/v) suspension which was stored at 4° C. Test samples were serially diluted in saline (50  $\mu\text{l}$ ) in the microtitre wells and two drops (50  $\mu\text{l}$ ) of the horse erythrocyte suspension was added to each well. Controls consisted of sterile saline alone and the horse red blood cells. The plates were kept at R.T for 4-6 h or at 4° C overnight. The HA titre was the reciprocal of the highest



dilution showing complete agglutination of the erythrocytes.

### **Protein estimation (Lowry)**

The method of Lowry *et al.*, (1951) was used. Standard protein samples of BSA (Sigma) were prepared in distilled water, so that the final concentration of protein in the tubes ranged from 50 - 500 µg/ml. Test dilutions of samples (0.5 ml) were prepared, and to both the standards and the unknown, 0.5 ml of 0.1N NaOH (BDH) was added. The tubes were then placed in a boiling water bath for 5 min and allowed to cool. A reagent was prepared by adding 1 ml of 1% (w/v) CuSO<sub>4</sub> and 1 ml of Sodium potassium tartarate to 50 ml of 5% (w/v) Na<sub>2</sub>CO<sub>3</sub>, of which 2.5 ml was added to each tube, mixed and left at R.T for 10 min. Then 0.5 ml of 1N Folin-Ciocalteus phenol reagent was added and the colour reaction was allowed to develop for 30 min. The reaction was measured spectrophotometrically at 750nm against a reagent blank.

### **Protein A extraction**

A *Staphylococcus aureus* formalin-killed cell suspension (Sigma) of 200 µl was added to 600 µl of 0.05M potassium phosphate, pH 7.5. To this 200 µl of serum was mixed by inversion in a 1 ml eppendorf tube. The mixture was incubated at 37<sup>o</sup> C for 30 min and the cells pelleted by centrifugation at 4000 rpm in a fixed angled head for 15 min. An aliquot of 600 µl of once-absorbed serum was used in the IgE and IgG ELISA, and the remaining 200 µl was absorbed again following the same cycle as above. The serum was, in all, absorbed a total of 3 times. This meant at each absorption, the serum was diluted 1 in 5. This was taken into account in the final results.

### **Immunodiffusion**

1% purified agar (BDH) was dissolved in 0.3M phosphate buffer pH 8.0 containing 0.01% (w/v) sodium azide. Gel bond (FMC Corp., Rockland, Maine) cut to size was placed on top of a microscope slide and hot agar poured on through a plastic syringe (4 ml/slide). Wells were punched in the agar using a 2 mm well punch and the agar plugs were removed using a needle. After dispensing the solutions to be tested, the slide was incubated overnight in a moist level box at 4<sup>o</sup> C in the cold room. The slides were washed in PBS for 24 h to remove any excess protein followed by 24 h in distilled water. After drying, they were stained in coomassie brilliant blue (Gurr) to show precipitin lines. Generalized background blueing was destained using immunodiffusion wash buffer (40% v/v methanol, 10% v/v acetic acid and 50% v/v water).

## **SDS-PAGE**

SDS-polyacrylamide gel electrophoresis separates proteins on the basis of molecular size, as they move through a polyacrylamide gel matrix towards the anode (Ames, 1974). The proteins are solubilized first by boiling in buffer in the presence of SDS and 2-mercaptoethanol (ie solubilizing buffer) and the resulting polypeptides separate into individual bands electrophoretically.

Gel moulds were prepared from two glass plates separated by 1.5 mm plastic spacers and sealed with yellow tape. The separating gel was prepared as described in Appendix 4 and pipetted into the cassette to 14 cm from bottom of the plates. An overlay of 5% ethanol was added which removes the meniscus from the gel and leaves a perfectly flat surface. The gel was left for 20 min, the overlay removed, and the stacking gel was added whereupon a teflon comb was placed between the glass plates. The gel was allowed to polymerise before the comb was removed. Tris-glycine buffer pH 8.3 was added to the lower electrode vessel. The sealing tape from the base of the gel plate was removed. The plate was inserted into the top electrode vessel using a liberal amount of grease around the gasket to prevent leakage. The upper electrode vessel was filled with Tris-glycine buffer pH 8.3 so that it covered the top of the gel and filled the wells with buffer (all recipes for solutions in Appendix 4). Protein samples of between 20 and 35  $\mu$ l were pipetted into the gel lanes with a Hamilton syringe. The tank was connected to a power pack and the gel was run at a constant current of 15mA/gel for 1-2 h. Once the dye in the samples had left the stacking gel, the current was boosted to 40mA. The current was switched off when the dye line reached the bottom of the gel. The gel was removed from the plates and stained.

## **Staining of SDS-PAGE gels**

### **Coomassie blue**

The gel was placed in a plastic tray and coomassie blue stain (Appendix 4) was poured over so that the gel was completely covered and left for 1 h at RT. The gel was then destained, by pouring of the stain and repeatedly washing with destain (Appendix 4) at 37<sup>o</sup> C for 1 day.

### **Silver**

As a precautionary measure when silver staining, the gels were handled only with plastic gloves as this staining procedure is very sensitive to small amounts of protein. The gel was prefixed with 50% (v/v) ethanol containing 10% (v/v) acetic acid for 30 min and then fixed with 10% (v/v) glutaraldehyde for 30 min. Overnight, the gel was rinsed in a large volume of distilled water. The gel was then soaked in 5  $\mu$ g/ml

dithiothreitol for 30 min. The solution was poured off and, without rinsing, 0.1% (w/v) silver nitrate was added and left for 30 min. Developer was prepared immediately before use and consisted of 50  $\mu$ l of 37% (v/v) formaldehyde in 100 ml 3% (w/v) sodium carbonate. The gel was rinsed twice quickly in developer and allowed to soak in a third volume of developer until the desired level of staining was reached. Whereupon 5 ml of 2.3M citric acid was added and allowed to mix for 10 min. Finally the gel was washed in distilled water and preserve in 0.03% (w/v) sodium carbonate and heat sealed in cellophane. All the solutions that have been referred to are in 100 ml amounts, unless otherwise stated, the volumes of sodium carbonate and citric acid are especially critical.

## RESULTS

The object of this investigation was to determine whether pertussis toxin (PT) could act as an IgE adjuvant for the antigens of *B. pertussis* namely FHA and PT itself. Before attempting to answer this question, it was necessary to set up, with a well-researched antigen, appropriate experimental systems for the production and testing of IgE. For this purpose ovalbumin (Oa) was chosen as the model antigen and the mouse as the experimental animal.

### **IgE Responses to Ovalbumin, with IgG1 Measured in Parallel**

Three test systems were developed to detect and quantitate specific IgE responses to Oa in mice: passive cutaneous anaphylaxis (PCA), and enzyme-linked immunosorbent assay (ELISA) employed in both the "conventional" and "sandwich" mode. Experiments were done to establish immunization protocols in mice for the production of IgE-containing sera. Since IgG1 is also PCA-positive (with a shorter sensitization interval) and might therefore interfere with the detection of IgE, it was necessary to assay the sera for anti-Oa IgG1 as well as for anti-Oa IgE.

### **Measurement of IgE by PCA**

#### **Preliminary experiments**

The initial studies were done with two interrelated objectives: namely to develop an immunization protocol for the stimulation of anti-Oa IgE, and also to develop the PCA test for detection of these antibodies. Initially, Oa as the antigen was used at two dose levels of 20 and 200  $\mu\text{g}$  per mouse, based on previous experience in this laboratory (Wardlaw *et al.*, 1979). Both PT and PV were used as the IgE adjuvants and were mixed with the Oa before i.p. inoculation into groups of 5 or 10 mice. The effect of booster doses of antigen, without adjuvant, was also studied for its possible beneficial effect on IgE production. The various combinations of doses of Oa, PT and PV, and the number of mice, are set out in Table 16 which also shows the results of the 2 h PCA test for IgG1, and the 48 h PCA test for IgE on sera obtained at 21 days. There were deaths in the mice given 5 and 10  $\mu\text{g}$  doses of PT. In experiment 1, the serum was a single pool made from the blood of the surviving animals after 21 days. However, in experiments 2 and 3, the individual sera were collected and tested. The data in the table show that although IgG1 responses were regularly elicited, IgE responses were detected in only 4 sera out of the 31 obtained. There was an indication in preliminary experiments, that anti-Oa IgE was best produced in a) individual high responding mice b) with 20  $\mu\text{g}$  Oa plus 5  $\mu\text{g}$  PT, c) with no booster and d) with bleeding on day 21. Moreover, the adjuvant dose level had to be reduced to get rid of the toxicity problem.

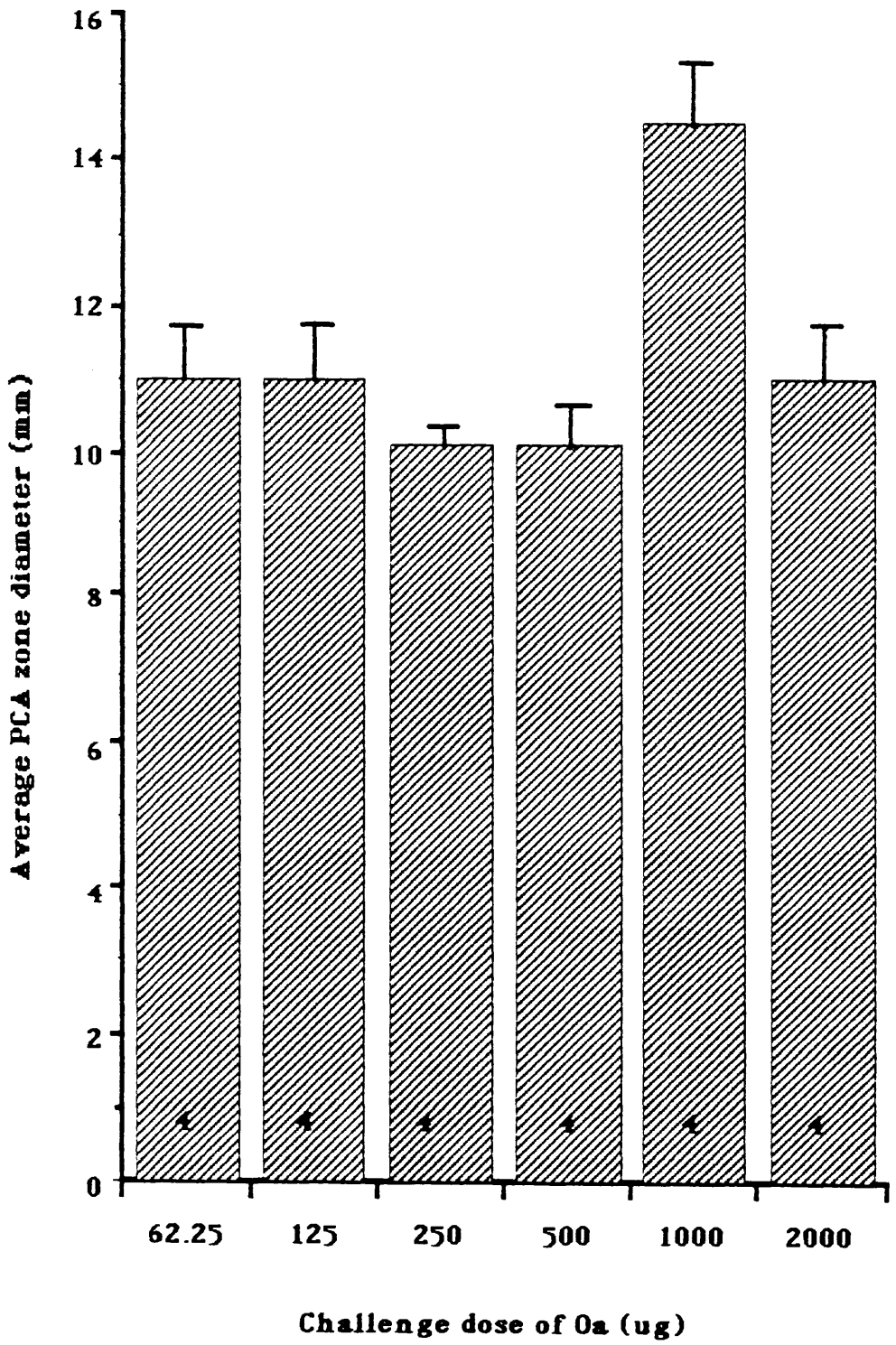
**Table 16. Results of preliminary experiments to obtain IgE against Oa in mice.** Immunization was by protocol 2 <sup>a</sup> and the sera were tested by 48 h PCA for IgE and 2 h PCA for IgG1. In experiment 1 the sera from the surviving mice were pooled and tested in duplicate in the 2 h PCA and in triplicate in the 48 h PCA. In expts. 2 and 3 the individual sera were tested once in the 48 h PCA.

Immunization mixture on day zero <sup>a</sup>			No. of mice surviving/ no. tested	No. of positive sera/ no. tested	
Oa (μg)	PT (μg)	PV (o.u)		2 h PCA	48 h PCA
<b>Expt 1: with booster</b>					
20	-	-	10/10	2/2	0/3
20	10	-	2/10	2/2	0/3
20	-	2.5	10/10	2/2	0/3
200	-	-	10/10	2/2	0/3
200	10	-	7/10	2/2	0/3
200	-	2.5	10/10	2/2	0/3
<b>Expt 2: with booster</b>					
20	-	-	2/2	nt	0/1
20	5	-	9/10	nt	1/9
20	10	-	7/10	nt	0/7
<b>Expt 2: without booster</b>					
20	5	-	8/10	nt	3/8

nt - not tested

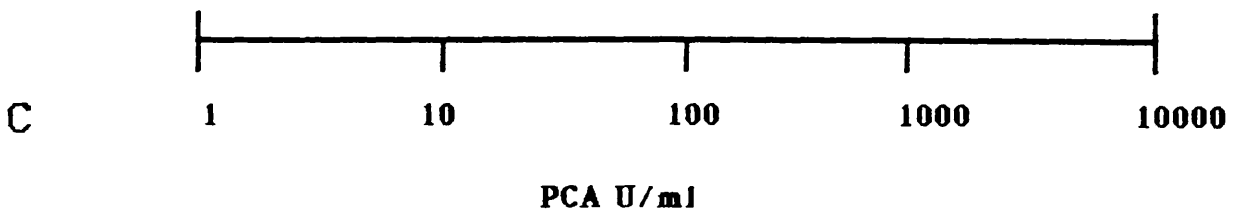
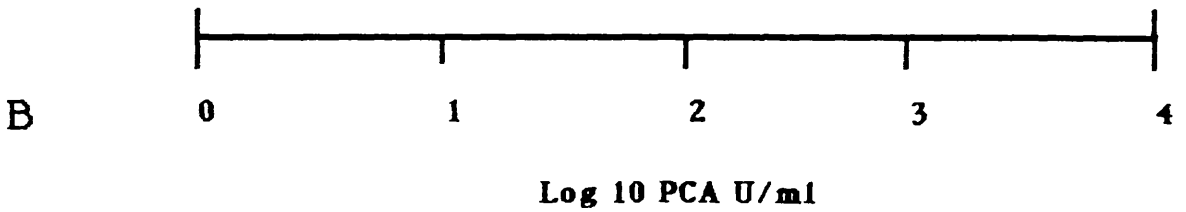
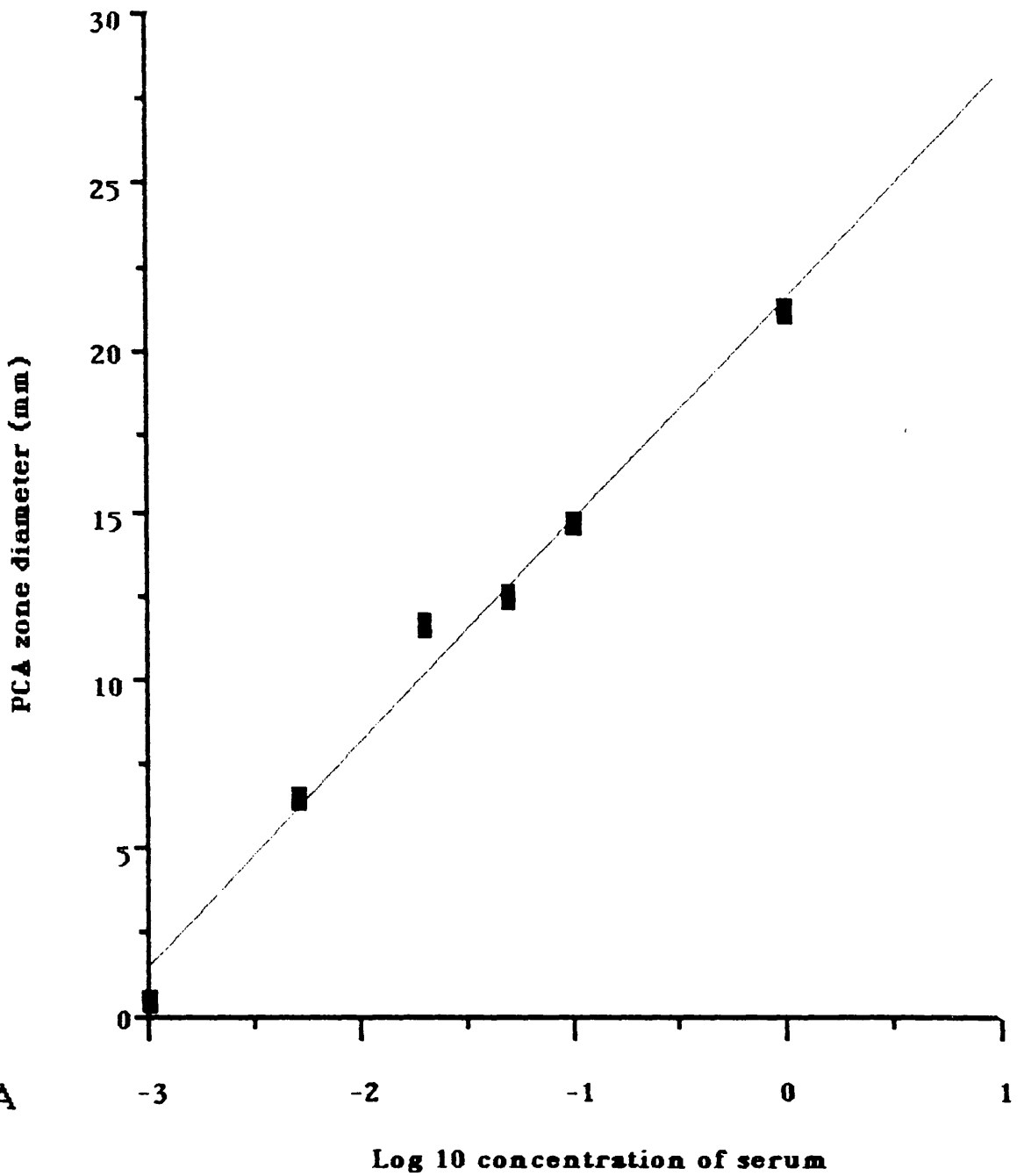
<sup>a</sup> - Protocol 2 consisted of the shown mixtures injected on day zero followed up where indicated by a booster dose of 10 μg Oa without adjuvant on day 14 and bleeding on day 21.

**Fig 2: Effect of varying the challenge dose of Oa in the 48 h PCA test.** Sera raised by the three-dose protocol and known to be IgE-positive were injected i.d at 1 in 10 dilution into each mice. After 48 h, pairs of mice were challenged i.v with graded doses of Oa. The bars indicate the average zone diameter (mm) and SEM with the number of replicates shown inside the bars.





**Fig 3: Titration of standard IgE-containing antiserum in the 48 h PCA test.** The serum had been produced by the three-dose protocol, and was a pool of 25 mouse bloods. The points indicate the average zone diameter and the SEM. Scale A shows the  $\log_{10}$  relative concentration of serum, scale B is the  $\log_{10}$  PCA U/ml and C the PCA U/ml.



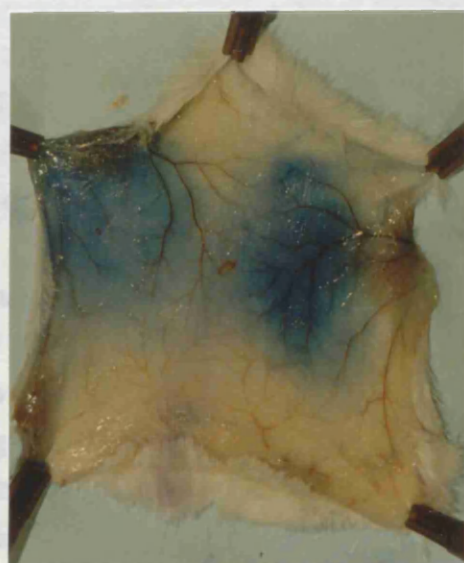
**Plate 1: Layout of the PCA test with 2 sites per mouse.** At positions A and B, anti-Oa IgE rich serum was injected i.d. The animals were challenged i.v with 1 mg of Oa and 1 mg Evans blue, 2 days after sensitization.

## Factors Influencing the PCA test

To try to optimize the conditions for IgE detection by PCA, it was necessary to generate a large pool (about 1 ml) of IgE-rich serum. This serum was obtained from a group of 25 mice and prepared as described below. By the three-day immunization protocol which eventually proved to be satisfactory. Using this "standard" anti-Oa IgE, the effect of such variables as challenge dose in the PCA, number of injection sites, effect of 56° C heating of the serum, and incorporating PT in the challenge injection, were studied.

*Effect of Oa challenge dose.* The standard anti-Oa serum was diluted 1 in 10, and two doses of 50 µl of this dilution were injected into each of two young adult Swiss-Webb mice. The mice were bled 24 hr later to compensate for occasional injections failures. The mice were then challenged with doses of Oa ranging from 0.1 to 1000 µg. The diameter of bluing was measured 30 min later.

The results accumulated in Table 1. Overall, there was little change produced by challenge dose over a 52-fold range, in that 62.5 µg Oa gave the largest diameter of bluing (2.000 µg). However, the 1000 µg dose appeared to be slightly better than either of the adjacent doses. This dose was used in all subsequent experiments. Plate 1 illustrates the underside of the skin of a mouse challenged with the 1 mg dose of Oa.



*Effect of serum dilution on assay response.* To generate a dose-response curve, the standard serum was tested undiluted and at serial dilutions from 1 in 10 to 1 in 1000, with each dilution being tested in at least 10 mice using different mice. Exceptionally, the 1 in 20 dilution was used altogether about 70 times since it was used as a positive control in many later experiments. Fig. 3 shows that there was an approximately linear relationship between log<sub>10</sub> relative diameter and the log<sub>10</sub> relative concentration of the standard serum. The y-axis is marked off in 3 scales, the lower two emerging from the serum being respectively assigned a value of 1000 PCA units per millilitre (U/ml).

The slope of the line is such that a 10-fold increase in serum concentration gives a 0.6 mm increment in zone diameter. Note that the dose-response line is extrapolated to zone diameters greater than 20 mm in order to allow estimation of serum potencies above 1000 PCA U/ml, as occurred occasionally.

## **Factors influencing the PCA test**

To try to optimize the conditions for IgE detection by PCA, it was necessary to generate a large pool (about 12 ml) of IgE-rich serum. This serum was obtained from a group of 25 mice and prepared as described below by the three-dose immunization protocol which eventually proved to be satisfactory. Using this "standard" anti-Oa IgE, the effect of such variables as challenge dose in the PCA, number of injection sites, effect of 56° C heating of the serum, and incorporating PT in the challenge injection, were studied.

***Effect of Oa challenge dose:*** The standard anti-Oa IgE serum was diluted 1 in 10, and two doses of 50 µl per intracutaneous injection site administered to each of two young adult Ham/ICR mice. The animals were used in pairs to compensate for occasional injection failure. Forty-eight hours later, the animals were challenged with doses of Oa ranging from 62.5 to 2000 µg, with a constant amount of Evans blue (1 mg). The diameter of blueing on the undersurface of the skin was measured 30 min later.

The results accumulated from 12 mice were presented in Fig 2. Overall, there was little change produced by varying the challenge dose of antigen over a 32-fold range, in that 62.5 µg Oa gave the same average zone diameter as 2000 µg. However the 1000 µg dose appeared to give significantly higher responses than either of the adjacent doses. This dose was therefore adopted in all subsequent experiments. Plate 1 illustrates the underside of the skin of a mouse tested by 48 h PCA with the 1 mg challenge dose of Oa.

***Effect of serum dilution on zone diameter:*** To establish a dose-response curve, the standard serum was tested undiluted and in serial dilutions from 1 in 10 to 1 in 1000, with each dilution being tested in at least 5 skin sites on different mice. Exceptionally, the 1 in 20 dilution was tested altogether about 70 times since it was used as a positive control in many later experiments. Fig 3 shows that there was an approximately linear relationship between zone diameter and the  $\log_{10}$  relative concentration of the standard serum. The abscissa is marked off in 3 scales, the lower two emerging from the serum being arbitrarily assigned a value of 1000 PCA units per millilitre (U/ml).

The slope of the line is such that a 10-fold increase in serum concentration gives a 6.6 mm increment in zone diameter. Note that the dose-response line is extrapolated to zone diameters greater than 20 mm in order to allow estimation of serum potencies above 1000 PCA U/ml, as occurred occasionally.

**Table 17. Effect of heating anti-Oa IgE sera on the subsequent 48 h PCA test.** The undiluted sera were heated to 56° C for 30 min and diluted 1 in 5 with PBS before i.d injection into mice. Positive controls consisted of the same sera unheated.

Immunization protocol	Dose		PCA activity of serum, before and after heating, from bleed day :					
	Oa (µg)	PT (ng)	10		21		63	
			U <sup>a</sup>	H <sup>b</sup>	U	H	U	H
	20	250	+ <sup>c</sup>	- <sup>d</sup>	nt <sup>e</sup>	nt	nt	nt
	20	1000	+	-	nt	nt	nt	nt
	200	250	+	-	nt	nt	nt	nt
1	20	250	nt	nt	+	-	nt	nt
	20	2500	nt	nt	+	-	nt	nt
	200	2500	nt	nt	+	-	nt	nt
	0.1	1000	nt	nt	nt	nt	+	+
3	1	1000	nt	nt	nt	nt	+	-
	10	1000	nt	nt	nt	nt	-	-

<sup>a</sup> Unheated

<sup>b</sup> Heated 56° C for 30 min

<sup>c</sup> 48 h PCA reaction: positive i.e > 5 mm in diameter

<sup>d</sup> 48 h PCA reaction: negative

<sup>e</sup> not tested

***Effect of heating sera at 56° C:*** An early priority in setting up the 48 h PCA test was to try to confirm that the positive results were due to IgE. For this purpose, the effect of heating a variety of anti-Oa IgE sera at 56° C for 30 min was investigated. These sera had been obtained by one-dose and three-dose protocols with various combinations of Oa and PT. Altogether 9 anti-Oa sera which had previously been found positive in 48 h PCA were examined before and after heating (Table 17). Each heated and unheated sample was tested in the same mouse to strengthen the comparison.

Of the 9 sera tested, only 8 were negative in 48 h PCA test after heating. One serum - the standard, raised with an immunizing dose of 0.1 µg Oa, was still positive after heating had nevertheless had its activity greatly reduced - from 13.5 mm average zone diameter down to 6 mm. Referring, to the above dose-response curve of the standard serum, the 13.5 mm reaction can be read as 80 PCA U/ml of a 1 in 10 dilution of unheated serum and the 6 mm reaction as 8 PCA U/ml of heated serum diluted 1 in 10. This corresponds to 90% inactivation, and is consistent with the 56° C-lability of IgE.

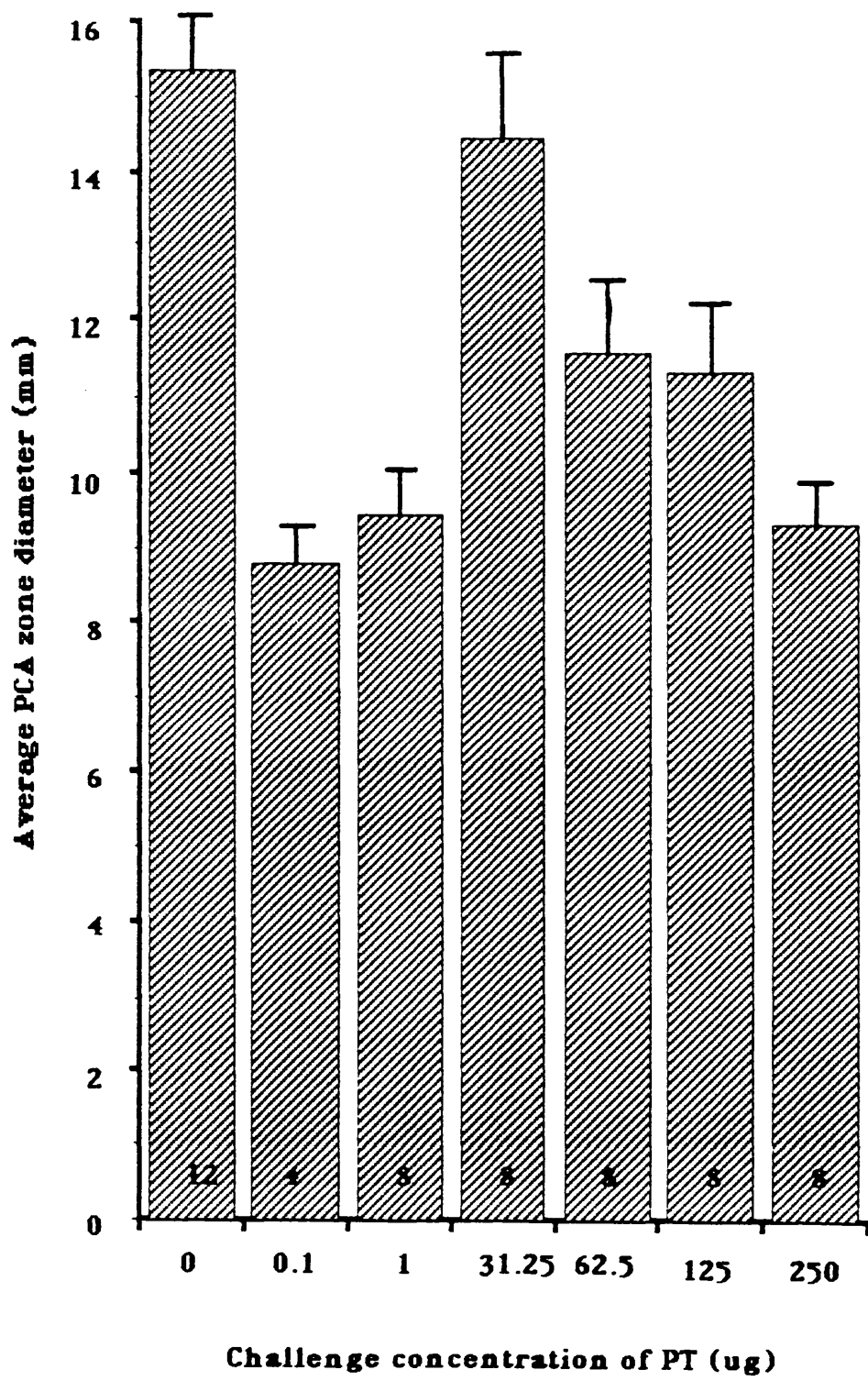
In order to increase the inactivation, further experiments were done with the heating period increased from 30 min to 1 h. This completely abolished the 48 h PCA of the standard serum, even when undiluted, and therefore corresponds to less than 5 PCA U/ml. The effect of heating for 60 min was to bring about 94% inactivation. This result was used in the studies on IgE production to PT and FHA where a 1 h treatment at 56° C was routinely used to inactivate IgE in serum.

***Effect of PT in the challenge dose:*** The inhibitory effect of PT on histamine release in the PCA test was previously described by Nakamura and Michio, (1983). They treated mice with bioactive PT, prior to antigen challenge, and found an inhibitory effect on histamine release and 48 h PCA reactivity. The degree of inhibition was dependent on the dose (0.1 µg to 3 µg) and injection time of PT. The inhibition of PCA was associated with a decrease in histamine release from peritoneal mast cells. Much less histamine was discharged in vitro, in response to IgE receptor stimulation. However, this present experiment was done to observe the effect of large doses of PT, mixed with the Oa (1 mg) challenge dose. Previously, the animals had been injected intracutaneously 48 h earlier with a 1 in 10 dilution of standard anti-Oa IgE-containing mouse serum.

Fig. 4 show that there was a significant depression of 48 h PCA reactivity and histamine release with low (0.1 and 1 µg) and high (125 and 250 µg) doses of PT in the challenge mixture. The average PCA zone diameters with the 0.1 µg and 250 µg doses

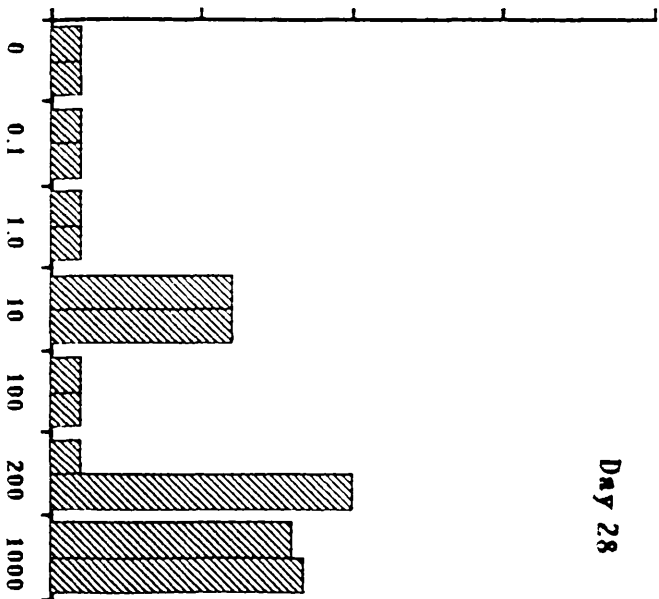
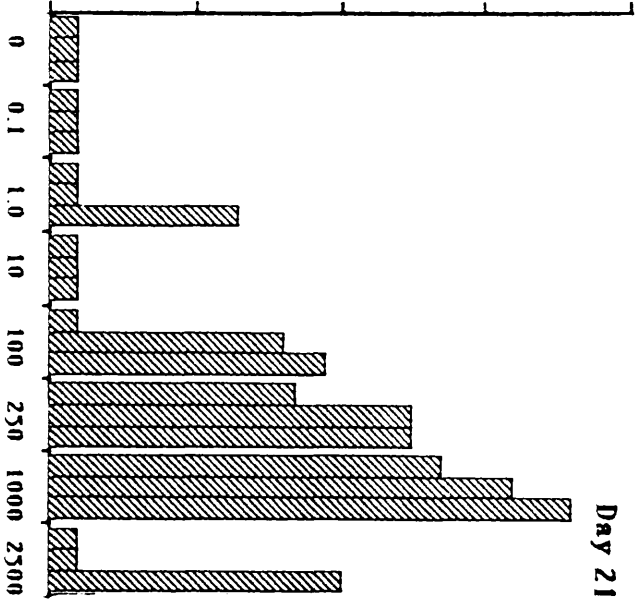
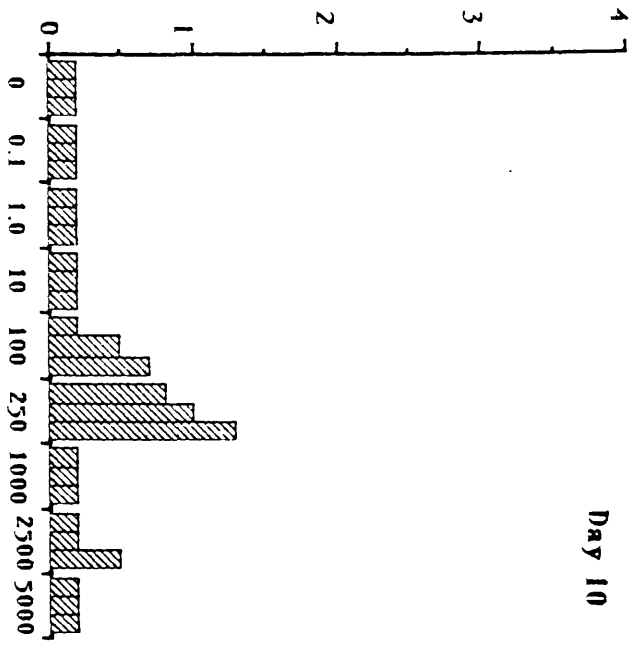
**Fig 4: Possible inhibitory effect of injecting PT simultaneously with Oa in the anti-Oa IgE 48 h PCA.** The challenge mixture contained a constant amount of Oa (1 mg) and Evans blue (1 mg) with various concentrations of PT. The bars indicate the average PCA zone diameter (mm) and the SEM with the number of replicate observations inside the bars.





**Fig 5: Effect of a range of adjuvant doses of PT, when mixed with a constant single dose of Oa (20 µg), on the development of anti-Oa IgE in mice.** Sera were obtained on days 10, 21 and 28 and tested by the 48 h PCA. Each bar represents the  $\log_{10}$  PCA U/ml of a single mouse serum compared with the standard serum which was defined as having  $3 \log_{10}$  U/ml.

Log<sub>10</sub> PCA U/ml



Adjuvant dose of PT (ng)

of PT were 8.8 and 9.4 mm respectively, while the control without PT gave a zone diameter of 15.4 mm. Referring to the dose-response curve (Fig. 3) when 0.1  $\mu$ g PT was added to the challenge mixture, there was a reduction in PCA U/ml from 200 to 15. Similarly with 125  $\mu$ g challenge dose of PT, there was a drop from 200 to 25 PCA U/ml. These correspond respectively to a 92.5% and 87.5% reduction in 48 h PCA reactivity. Surprisingly, these results suggest that PT may have a rapid pharmacological effect on mast cells in causing the suppression of histamine release and a significant reduction in 48 h PCA, over a wide challenge dose range of PT.

### **IgE responses to Oa after one and three dose immunizations**

In order to find a satisfactory protocol for raising IgE, a number of variables had to be considered. These included antigen and adjuvant dose, effect of boosting, the time of bleeding and the effect of pooling the sera.

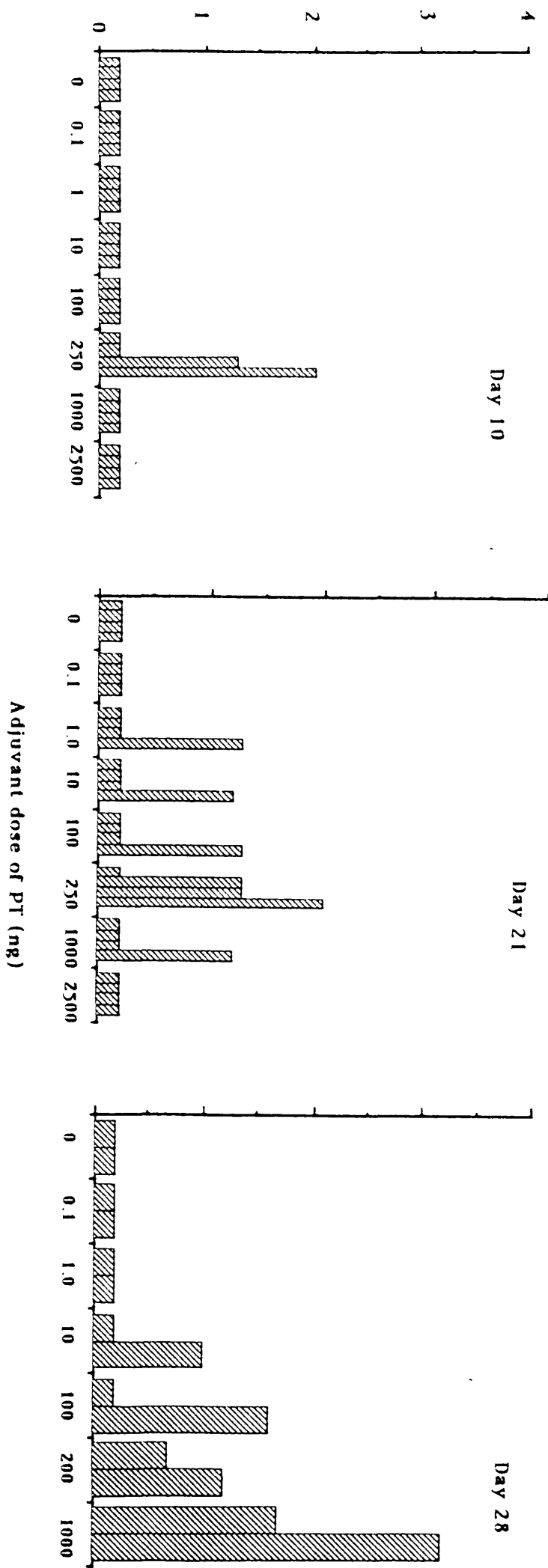
***Anti-Oa IgE responses after one dose immunization.*** Reference has already been made to preliminary experiments (Table 16), indicating that anti-Oa IgE might be best produced in individual high-responding mice, with no booster and bleeding on day 21. This was investigated further in a second experiment on a one-dose immunization.

Groups of mice were immunized with 20  $\mu$ g Oa mixed with various adjuvant doses of bioactive PT. The individual sera were collected after immunization intervals of 10, 21 and 28 days. The results, shown in Fig. 5, are expressed in  $\log_{10}$  PCA U/ml, obtained by interpolating the PCA zone diameter against the standard curve (Fig. 4). In Day 10 sera, the IgE responses to Oa tended to be clustered around the 100 and 250 ng PT adjuvant dose range, with doses on either side appearing to be unsuitable for IgE production. On bleed day 28, the anti-Oa IgE responses were scattered over the 10, 200 and 1000 ng adjuvant doses. However, on day 21, there were substantial responses over a ten-fold range from 100 to 1000 ng. The maximum response occurred when an adjuvant dose of 1000 ng was used in the Oa immunization.

Additionally, Fig 6 shows the effect of a high Oa dose (200  $\mu$ g), with varying PT adjuvant dose, on the subsequent IgE responses. On Day 10, only two of the four sera tested contained anti-Oa IgE with 200  $\mu$ g Oa and 250 ng PT, while the other dose levels of PT were unproductive of IgE. There was a scatter of results on day 21, with only a small number of mice responding at the 1, 10, 100 and 1000 ng PT levels. The strongest responses occurred at the 250 ng PT adjuvant concentration. The 28-day sera showed increasing IgE production from low to high as the PT dose increased from 10 to 1000 ng.

**Fig 6: As Fig. 5 except that the dose of Oa was increased to 200  $\mu\text{g}$  (from 20  $\mu\text{g}$ ). Sera were obtained on days 10, 21 and 28 and tested by PCA. Each bar represents the  $\log_{10}$  PCA U/ml of a single mouse serum compared with the standard serum which was defined as having 3  $\log_{10}$  U/ml.**

Log<sub>10</sub> PCA U/ml



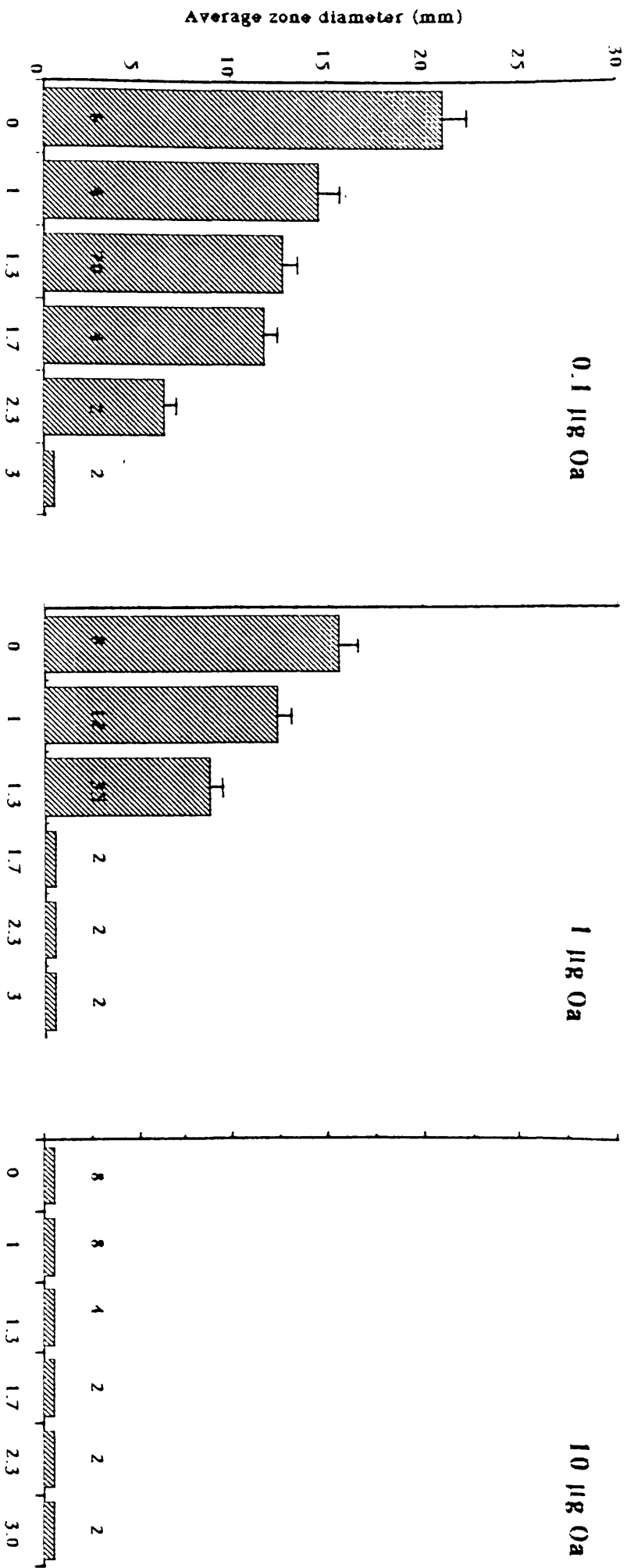
Day 10

Day 21

Day 28

Adjuvant dose of PT (ng)

**Fig 7: The effect of different immunizing doses of Oa (0.1, 1 and 10 µg) in the three-injection protocol and tested in the 48 h PCA.** The mice had received the indicated doses of Oa on days 0, 28 and 56, the first dose being mixed with 1 µg PT. The serum was from a pool of 25 mouse bloods. The bars indicate the average zone diameter (mm) and the SEM with the number of replicates shown inside the bars.





In summary, the low Oa (20  $\mu$ g) doses seem to produce the strongest IgE response at 21 days with an immunization protocol involving 20  $\mu$ g Oa and 1000 ng PT. In Fig 6, the Oa dose seems to have an erratic effect on IgE production, but there is a high response on day 28, with the 1000 ng PT dose. In subsequent experiments the 1000 ng PT adjuvant dose was preferred. Overall, the maximum responses occurred on day 21 with 20  $\mu$ g Oa as antigen and 1000 ng PT as adjuvant.

***Anti-Oa IgE responses after 3-dose immunizations.*** IgE responses can be transient in their duration, if a one-dose immunization protocol is used as shown in Fig 5 and 6. Levine and Vaz, (1970) showed that by altering a number of factors a prolonged IgE response could be elicited.

A protocol was used whereby antigen and adjuvant were injected on day 0 and the animals then boosted on days 28 and 56 with antigen alone. The final bloods for test (as pools) in the 48 h PCA were collected on day 63 (Fig 7).

When dose of 0.1  $\mu$ g Oa was used in the initial immunization with 1000 ng of PT, a high titre serum was produced which was adopted as the standard serum. The 1  $\mu$ g dose of antigen also gave strongly positive IgE serum. However when 10  $\mu$ g was used, no IgE anti-Oa response could be detected. This suggested that small repeated doses of Oa with 1000 ng bioactive PT (administered with the initial injection) gave high IgE titre serum. When the one-dose immunization protocol was used, the IgE response was erratic and could only be detected in individual sera, not in pools. In the three-injection protocol, however the sera raised against 0.1 and 1  $\mu$ g Oa, with 1000 ng bioactive PT in the initial injection, when pooled, produced a good IgE response as tested by 48 h PCA.

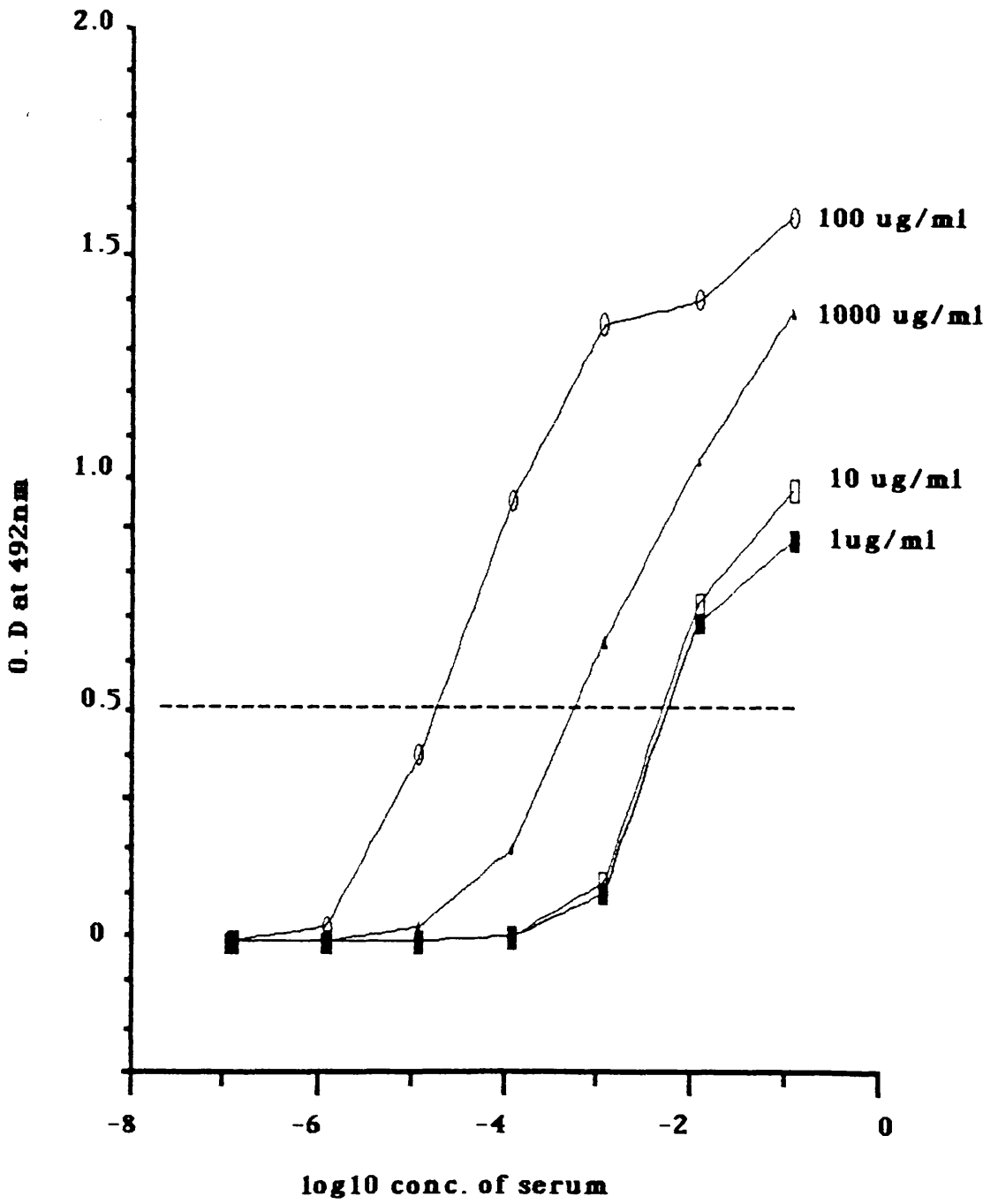
## **Measurement of IgG1 by conventional ELISA**

### **Preliminary experiments with IgG1.**

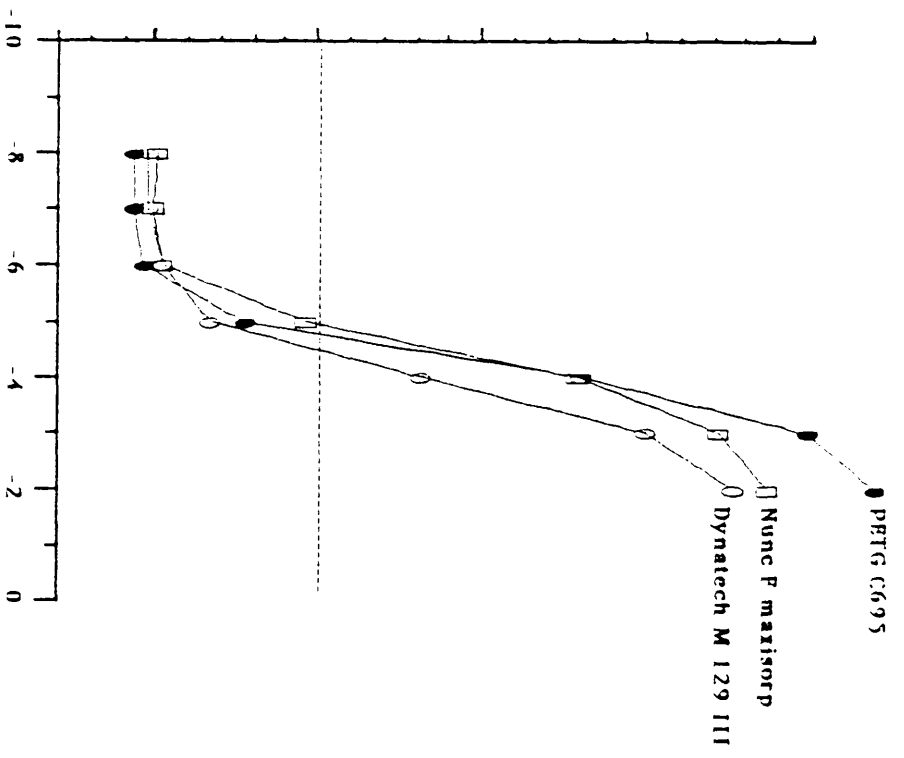
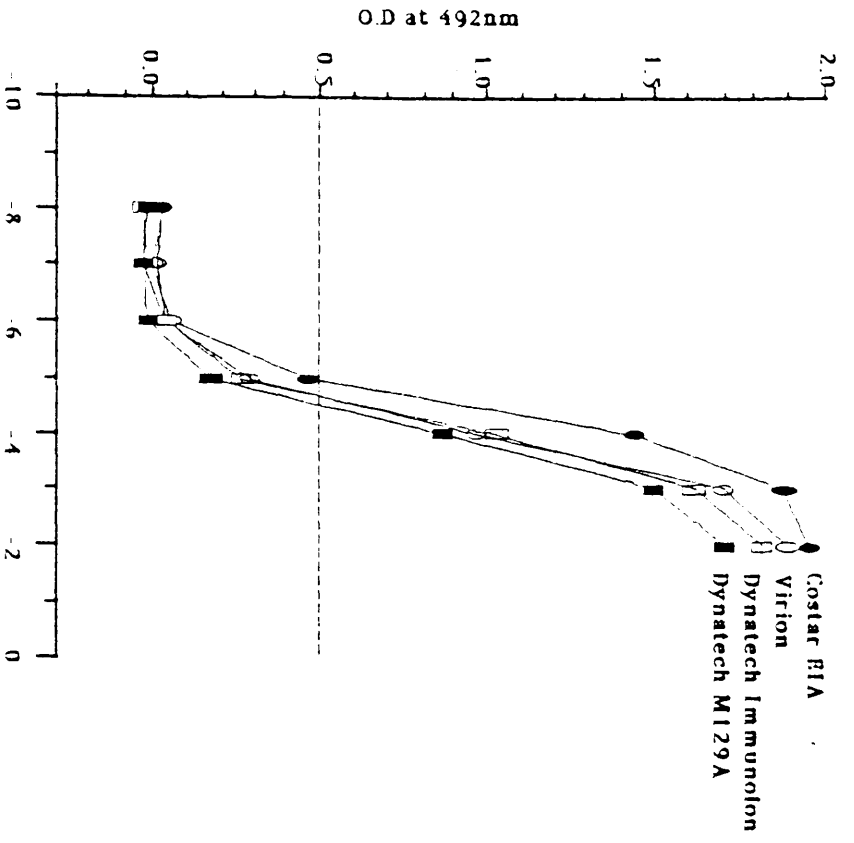
To provide experience with ELISA methodology, it was decided to establish an anti-Oa IgG1 ELISA system before attempts to measure anti-Oa IgE by ELISA.

***Effect of Oa coating concentrations.*** A variety of coating concentrations of Oa were tested with a 2 h PCA-positive serum in the IgG1 ELISA (Fig. 8). The most satisfactory coating concentration was 100  $\mu$ g/ml or 10  $\mu$ g/well. The serum endpoint titres after coating with 100  $\mu$ g/ml and 1000  $\mu$ g/ml Oa were 78,600 and 1,400 respectively. Thus there was a 56-fold reduction in the antibody titre when the Oa coating concentration was raised from 100 to 1000  $\mu$ g/ml. In the subsequent conventional IgG and IgE ELISA experiments, the coating concentration of Oa was kept constant at 100  $\mu$ g/ml.

**Fig 8: IgG1 ELISA: Effect of the Oa coating concentration on the measurement of IgG1 in a mouse serum.** A 2 h PCA-positive anti-Oa IgG1 serum was used, with the sheep anti-mouse IgG1-HRP conjugate at 1 in 1000 dilution. The Oa concentration in the coating mixture is shown on the right. The dotted line at O.D 0.5 represents the endpoint absorbance.



**Fig 9: IgG1 ELISA : Effect of different types of ELISA plate.** An IgG1-positive anti-Oa serum tested by 2 h PCA was used with the sheep anti-mouse IgG1-HRP conjugate at a 1 in 1000 dilution. The type of plate is shown on the right. The dotted line at O.D 0.5 represents the endpoint absorbance.



log<sub>10</sub> conc. of serum

**Type of plastic for ELISA plates.** A variable which is rarely reported in the ELISA literature is the suitability of different plastics in the microtitre plates. Seven types of plates were tested in parallel, the Oa coating concentration and anti-Oa serum dilutions being replicated on each. Fig. 9 shows that there was very little variability. However Costar EIA and Nunc F maxisorp produced, slightly higher endpoint titres than the other 5 plates. In all subsequent experiments Nunc F maxisorp plates were used.

### **Factors influencing the IgE ELISA**

There was a requirement for the sera raised by the one and three-dose protocols to be titrated in an *in vitro* assay for IgE. To do this, a number of factors had to be considered when planning the IgE ELISA. These included the titration of the conjugates, the choice of blocking agent and the possible requirement for Protein A extraction, of the test serum, to reduce IgG which might interfere with the assay.

### **Measurement of IgE by conventional ELISA**

The conventional ELISA was explored because it offered a simple and sensitive assay for titrating large numbers of sera. Its advantages over the PCA are sensitivity, avoidance of use of animals, technical simplicity and convenience, and economy of antigen.

**Titration of conjugates** In initial studies, the secondary antibody employed was a goat anti-mouse IgE. Since it was not labelled with horseradish peroxidase (HRP), there was the need for a further antibody. This was donkey anti-goat IgG HRP conjugate which served to amplify the signal and generate a colour from the OPD and hydrogen peroxide substrate. Table 18 shows a chequerboard titration with different dilutions of both antibodies. The dilution combination of the two antibodies giving the most satisfactory results was 1 in 100 of goat anti-mouse IgE and 1 in 1000 dilution of the HRP-labelled donkey anti-goat IgG. In experiment 1 (Table 18), it appeared that the 1 in 100 dilution of the first antibody and 1 in 1000 of the second gave satisfactory result. Therefore, in experiment 2 (Table 18), the goat anti-mouse IgE was diluted further in order to economise on its use. This was not successful and the 1 in 100 dilution was adopted for the subsequent titration of sera. A summary of experiment 2 can be seen in Fig 10 with the 1 in 100 dilution of goat anti-mouse IgE and 1 in 1000 donkey anti-goat IgG HRP producing a good titration curve with the standard anti-Oa IgE mouse serum. The endpoint titre was taken at O.D 0.5. Since the higher dilutions of 1/250 and 1/500 of the goat anti-mouse IgE all had O.D's which were  $<0.5$ , they were not used in the subsequent experiments.

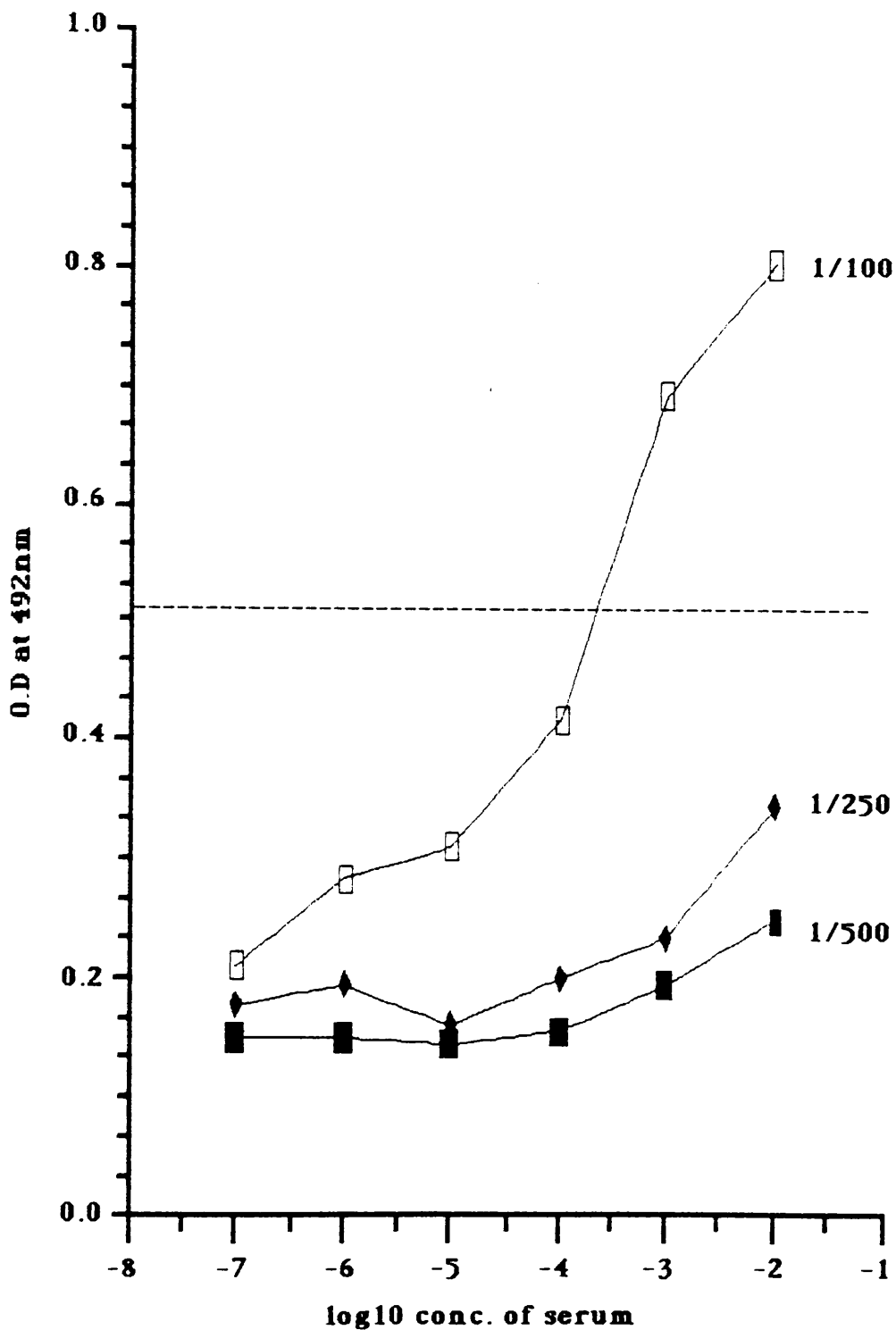
**Table 18 : Effect of different combinations of the ELISA reagent sera for assaying anti-Oa IgE in standard mouse serum.** The reagent sera were goat anti-mouse IgE (source: ICN) and donkey anti-goat IgG HRP conjugate (source: SAPU). The combinations giving satisfactory results are highlighted in bold

Expt No.	Reciprocal diln. of goat anti- mouse IgE	Range of absorbancy values (A492nm) given by standard IgE serum when tested with donkey anti-goat IgG HRP at reciprocal diln.		
		100	1000	10,000
1	10	all > 3.0	all > 3.0	1.1- > 3.0
	100	0.5- >3.0	<b>0.2 - 0.7</b>	0.03 - 0.1
2	100	n.t	<b>0.2 - 0.8</b>	n.t
	250	n.t	0.2 - 0.3	n.t
	500	n.t	0.18 - 0.2	n.t

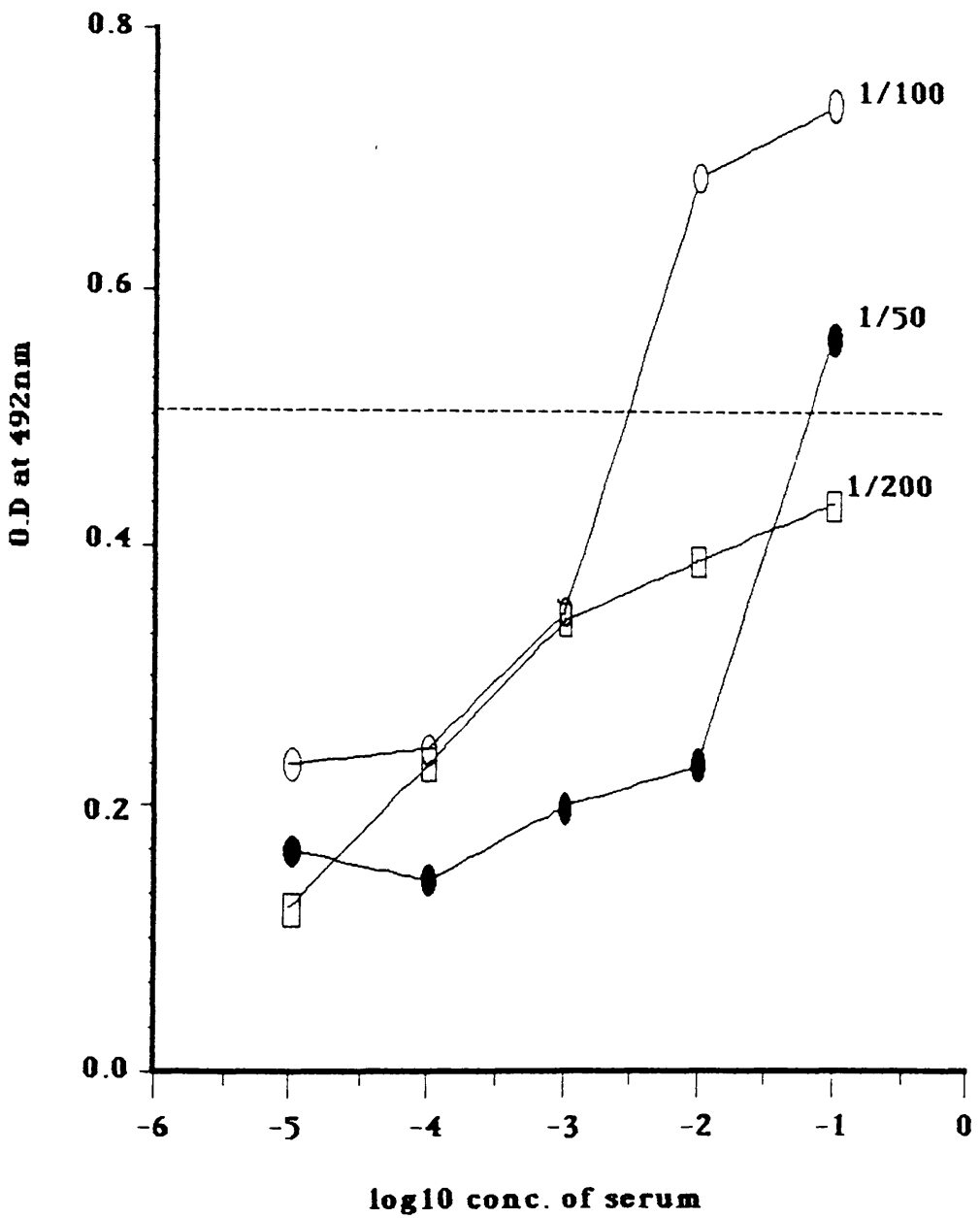
Footnote : The standard IgE serum was raised against Oa in mice by the three injection protocol.

**Fig 10: IgE ELISA: Effect of goat anti-mouse IgE (ICN) concentration on the measurement of IgE.** A 48 h PCA-positive anti-Oa IgE serum was used with the donkey anti-goat IgE HRP conjugate at a 1 in 1000 dilution. The dilution of the goat anti-mouse IgE are shown on the right of the graph.





**Fig 11: IgE ELISA: Effect of sheep anti-mouse IgE (The Binding Site) concentration on the measurement of IgE.** A 48 h PCA-positive anti-Oa IgE serum was used. The dilution of sheep anti-mouse IgE HRP conjugate is shown on the right.



**Table 19 (A): Effect of different blocking agents on IgE ELISA.** The tests were done on an IgE-positive serum, before and after heating at 56° C, and a normal serum, all at 1/100 dilution. The ELISA reagents (goat anti-mouse IgE and donkey anti-goat IgG HRP) were also used at fixed dilutions of 1/100 and 1/1000 respectively.

Mouse serum	O.D with blocking agent (2%)				
	Horse serum	Gelatin	Foetal calf serum	Bovine serum albumin	Skim milk
IgE-positive	0.432	0.350	0.550	0.572	0.417
IgE-positive(56°C)	0.420	0.312	0.560	0.369	0.455
Normal	0.121	0.122	0.180	0.101	0.094

**Table 19 (B) : Control results relating to the data in Table 19(A), and in which there was stepwise omission of one or more reagents in the ELISA on the IgE-positive serum.** The desired results are shown for comparison, and the troublesome observations are highlighted in bold.

ELISA step/reagent omitted	Desired result	O.D with blocking agent (2%)				
		H.S	G	FCS	BSA	SM
A nil	> 0.5	<b>0.432</b>	<b>0.350</b>	0.550	0.572	<b>0.417</b>
B coating	< 0.1	n.t	n.t	n.t	0.124	n.t
C blocking	> 0.5	0.530	<b>0.475</b>	0.701	0.548	0.582
D IgE-positive mouse serum	< 0.1	<b>0.217</b>	<b>0.185</b>	<b>0.227</b>	0.096	0.083
B + C	< 0.1	<b>0.111</b>	<b>0.134</b>	<b>0.255</b>	<b>0.137</b>	0.067
B + D	< 0.1	0.053	0.072	<b>0.170</b>	0.083	0.035
B + C + D	< 0.1	0.072	<b>0.409</b>	<b>0.257</b>	0.075	<b>0.193</b>

Latterly, an anti-mouse IgE conjugate raised in sheep and already labelled with horseradish peroxidase (The Binding Site) was tried in the conventional ELISA. This avoided the need for a further antibody. As seen in Fig. 11, the best dilution of the sheep anti-mouse IgE-HRP conjugate in the ELISA was 1 in 100. Therefore in subsequent experiments the preferred conjugate (sheep anti-mouse IgE-HRP) was used at 1 in 100 dilution.

***Effect of different blocking agents*** A major problem found in this study, in conventional IgE ELISA was the high background O.D. This was thought to be due partly to incomplete blocking, thereby allowing binding of non-specific serum proteins, which augment the final O.D readings. In an initial experiment, the O.D readings at test serum dilution 1 in 100 was recorded with a) an IgE-positive serum, b) the same serum heated to 56° C for 1 h and c) normal mouse serum. Five different blocking agents were used all at 2%. The results in Table 19a showed that heating serum at 56° C, which inactivates IgE had little effect on the final O.D readings, when compared with unheated IgE-positive serum, with any of the blocking agents. However with normal serum, there was generally a three to four-fold decrease in O.D throughout

In Table 19b, control wells, were set up where one or more of the steps or reagents in the ELISA had been omitted. The desired results are tabulated with highlighting of the troublesome observations. When no steps were omitted, and where a high O.D was expected with the IgE-positive serum, only FCS and BSA gave readings > 0.5 i.e around the endpoint value. Omission of blocking, in the ELISA, had no significant effect on the resultant O.D readings. An important control, the no-serum well, which should always be < 0.1, for assay validity, yielded higher than expected O.D's with horse serum, gelatin and foetal calf serum. When blocking and coating were omitted altogether the O.D still remained high. When no coat or serum was added, only FCS gave higher than the minimum desired result and, in the one of the wells without coat, block and serum, there was an unusually high background reading of 0.409, which may represent the conjugate sticking to the plastic.

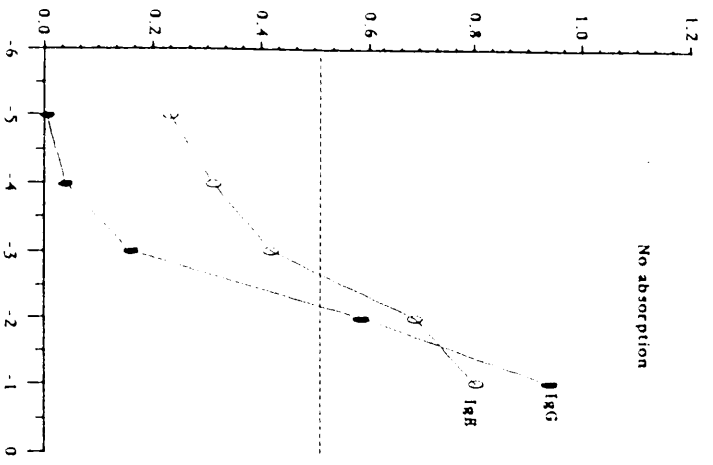
The blocking agent chosen was 2% BSA, since background levels with this blocking agent were usually low, while still allowing high O.D readings in experimental wells with IgE-positive serum.

***Effect of absorbing serum with Staphylococcus aureus (Cowan strain)***

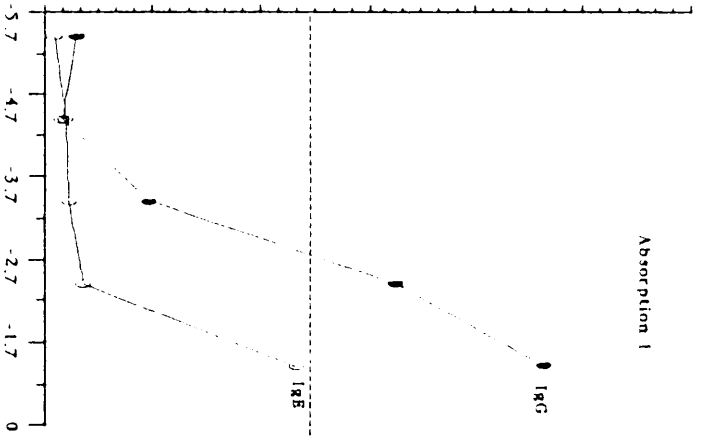
Protein A on the surface of *S. aureus* binds to the Fc receptor of IgG and removes it from serum. This might have been advantageous, since IgG has been reported

**Fig 12: IgG and IgE ELISA: Effect of absorbing the standard mouse serum with Protein A (Sigma).** The serum was tested before absorption and after 3 successive absorptions and the IgE and IgG content of the serum was measured by the conventional ELISA. The different scales on the abscissa refer to the absorption steps which resulted in dilution of the serum

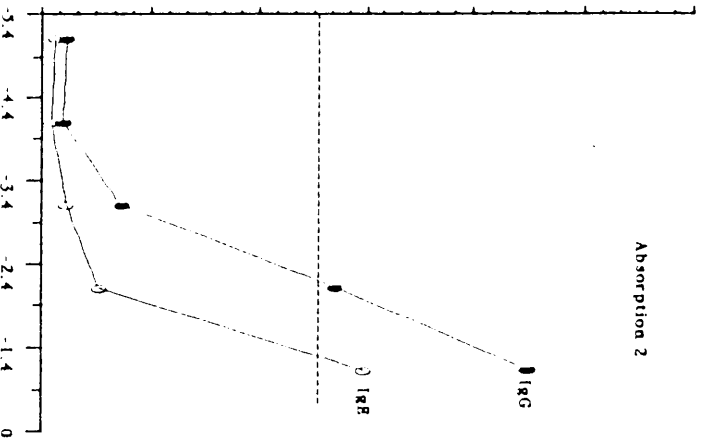
O.D at 492nm



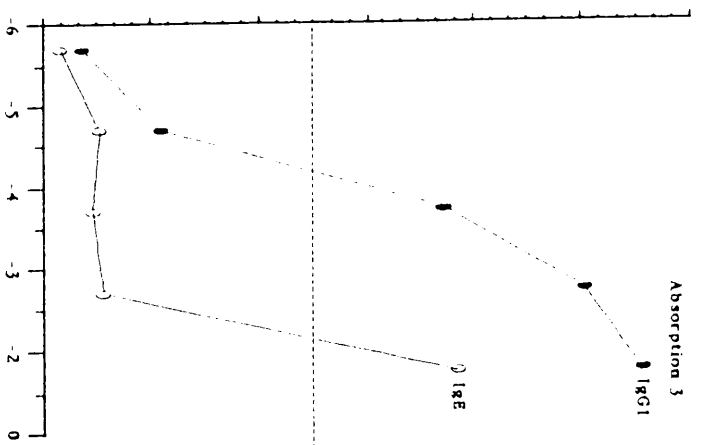
Titre  
IgG 1875  
IgE 5625



Titre  
IgG 500  
IgE > 50



Titre  
IgG 163  
IgE > 125

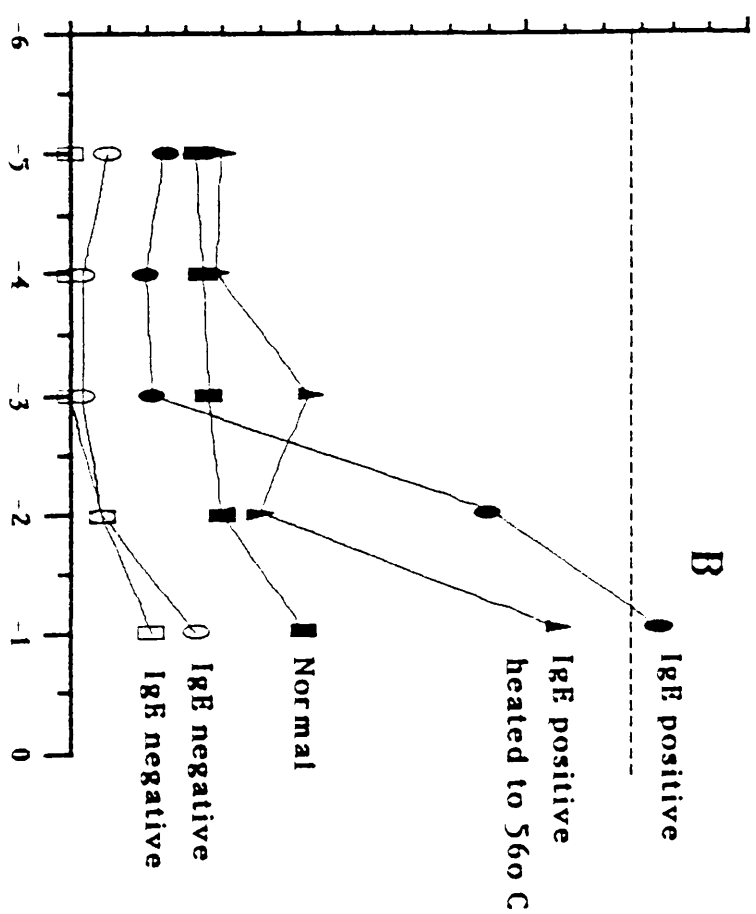
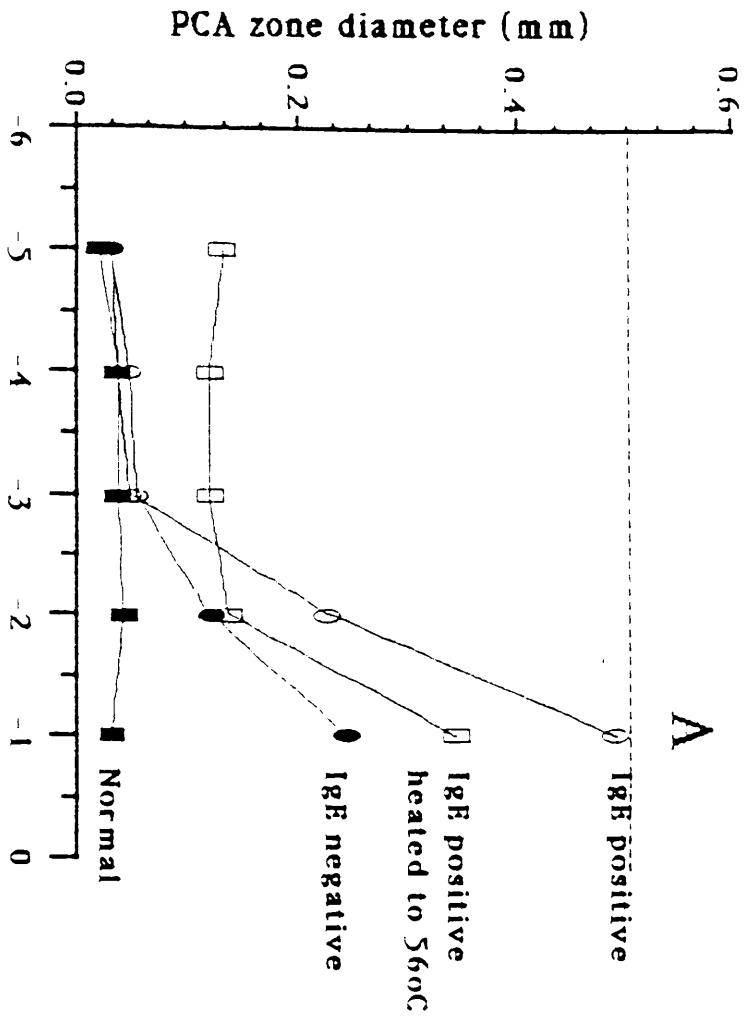


Titre  
IgG 22,726  
IgE 272

log 10 conc. of serum

**Fig 13: IgE ELISA: Titration of mouse sera of known PCA reactivity.** The reagent sera used in A was goat anti-mouse IgE (ICN) 1 in 100 dilution and donkey anti-goat IgG HRP (SAPU) 1 in 1000 dilution and in B sheep anti-mouse IgE HRP conjugate (The Binding Site) 1 in 100 dilution.





log 10 conc. of serum

(Hamilton *et al.*, 1981, Finger and Wirsing von Koenig., 1985) to interfere with the IgE ELISA, by competition for the antigen coating on the plate. Therefore 48 h PCA-positive serum was tested for IgE and IgG before and after absorption with formalin-killed whole cells of *S. aureus*.

In the IgG ELISA, the antibody titres were low in untreated serum and after the 1st and 2nd absorptions, were reduced 4 and 3-fold respectively (Fig. 12). Surprisingly, after the third absorption, there was a massive apparent increase in detectable IgG. Conversely, the reciprocal IgE titre started high in untreated serum and then dropped 100-fold after the 1st absorption, and only increased in 2.5 and 2-fold steps in the 2nd and 3rd absorptions. In subsequent experiments, the sera were therefore not absorbed with *S. aureus* since absorption seemed to have a detrimental effect on both the IgE and IgG titration.

### **Titration of mouse sera of known PCA reactivity**

Having explored some factors that might influence the performance of the conventional ELISA for IgE, a series of control sera were assayed. These were the standard PCA-positive mouse serum, before and after heating to 56° C, known PCA-negative sera from Oa-immunized mice and normal mouse serum. Each series was tested with two reagent sera, viz HRP-conjugated sheep anti-mouse IgE (The Binding Site) and goat anti-mouse IgE (ICN) used in conjunction with HRP-conjugated donkey anti-goat IgG (SAPU).

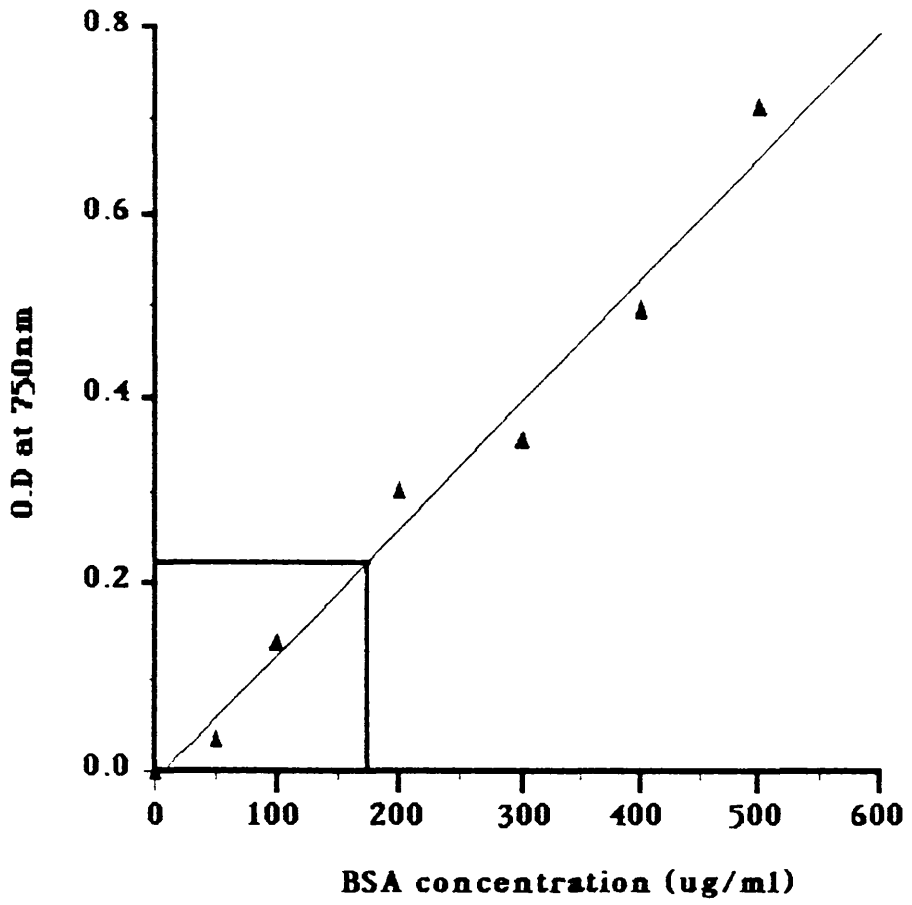
As shown in Fig 13, the two reagent systems gave broadly similar results. With each, the standard PCA-positive mouse serum was the most active, although the dose response line only barely reached an absorbancy of 0.5 with 1 in 10 serum. This was considerably less active than expected from previous experiments (Fig. 10, 11). The reason for the reduction in activity was not uncovered but might have been due to use of a different batch of reagent or the detrimental effect of storage and repeated freezing and thawing.

Despite the low initial titre of the untreated PCA-positive serum, the 56° C treatment produced a significant reduction although less than expected from the PCA test. Normal serum and PCA-negative sera were much less reactive.

### **Measurement of IgE by sandwich ELISA**

Because of the low IgE titres obtained with known PCA-positive sera in the "conventional" ELISA it was decided to explore the possible beneficial effect of the "sandwich" ELISA technique. This involved coating the plates with affinity-purified

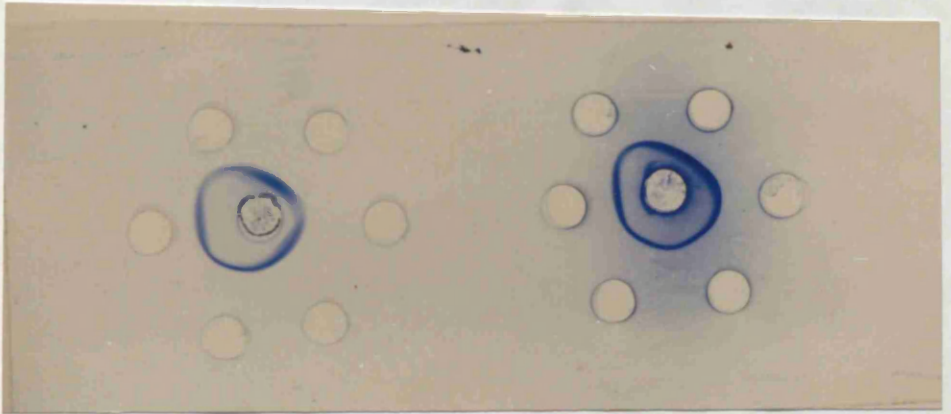
**Fig 14: Standard curve for measuring protein content in affinity purified anti-Oa.** By the method of Lowry *et al.*, (1953) outlined in the materials and methods.



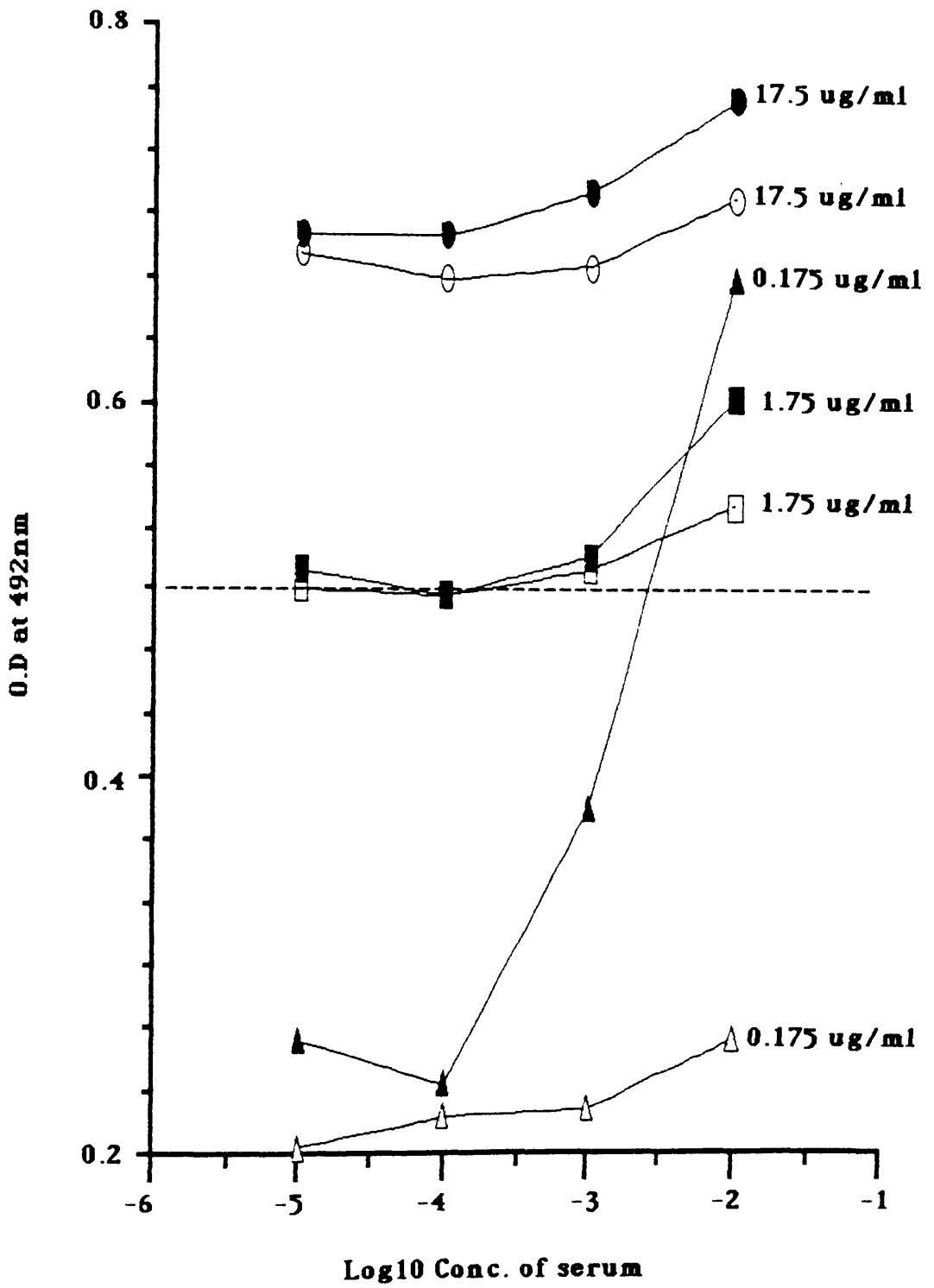
**Plate 2: Immunodiffusion pattern of anti-O<sub>a</sub> serum before and after extraction on a O<sub>a</sub>-sapharose column.** The serum was raised in a rabbit with O<sub>a</sub> and FCA.

**After  
extraction**

**Before  
extraction**



**Fig 15: IgE ELISA: Effect of the concentration of affinity-purified rabbit anti-Oa as a coating agent in the sandwich ELISA.** The ELISA was done on IgE-positive serum, before (solid points) and after heating at 56° C (open points). The Oa for binding was used at a constant concentration of 100 µg/ml and the sheep anti-mouse IgE HRP at 1 in 100 dilution. The concentration of AP anti-Oa in the initial coating stage is shown on the right.





(AP) rabbit anti-Oa, instead of with Oa itself. However before describing these experiments it was necessary to determine the concentration and purity of the AP anti-Oa.

### **Characterization of affinity-purified anti-Oa**

Starting with 25 ml of the anti-Oa serum which had been produced by immunizing rabbits with Oa in Freund's incomplete adjuvant, the anti-Oa antibodies (irrespective of immunoglobulin class) were removed by adsorption onto, and elution from an Oa-sepharose column. The eluate was then concentrated to 2 ml by dialysis against Aquacide, to give a sample with a protein content of 1.75 mg/ml by the Lowry method estimated by extrapolation from the standard curve (Fig 14). The yield of anti-Oa was estimated roughly by immunodiffusion with the results shown in Plate 2.

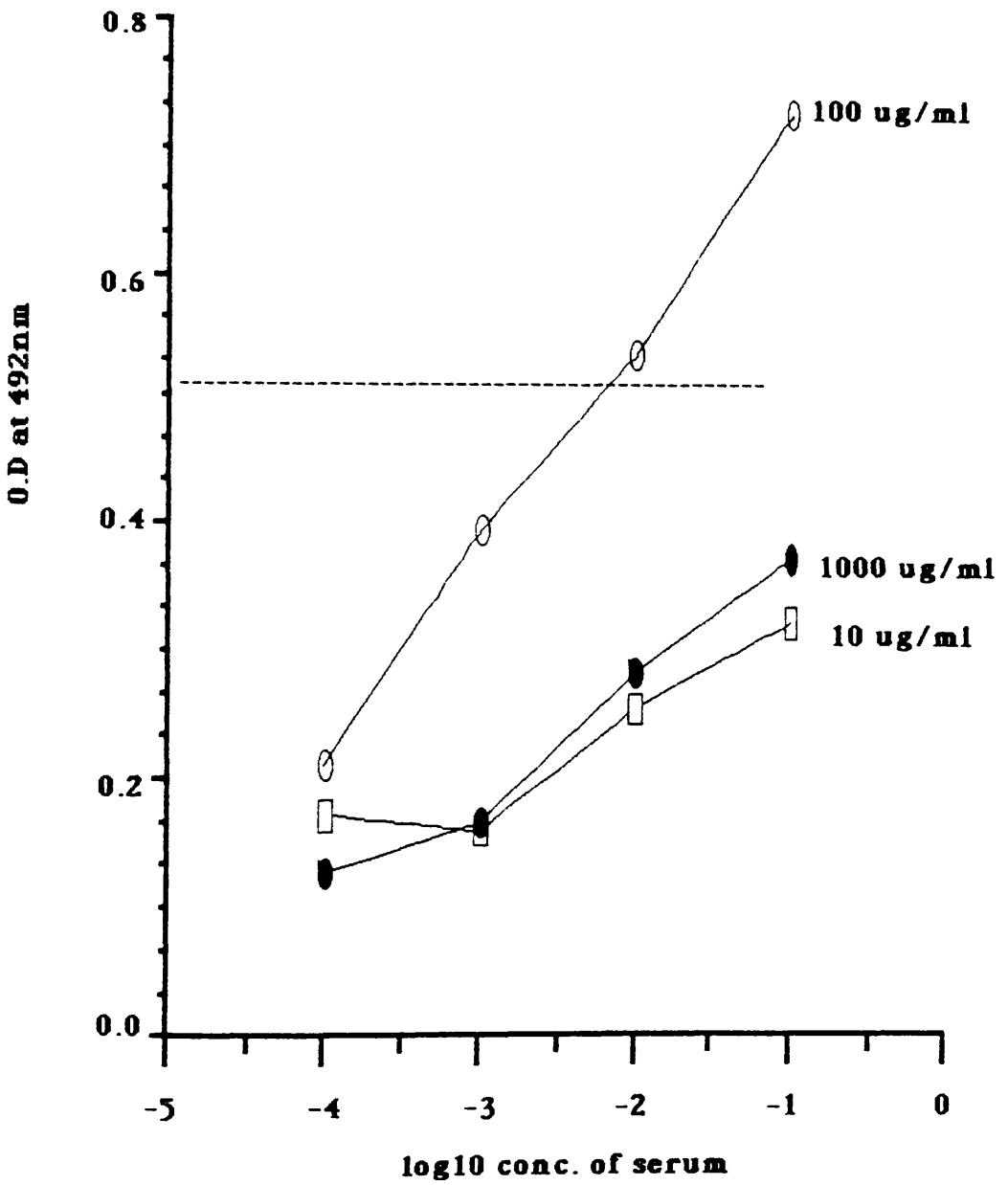
### **Factors influencing the ELISA**

**Effect of concentration of affinity-purified anti-Oa.** Ten-fold dilutions of 1.75 mg/ml AP anti-Oa were prepared in coating buffer and incubated at 4° C overnight. In this initial experiment the Oa concentration was kept constant at 100 µg/ml and bound to the plate via the AP antibody (Fig. 15). Throughout this sandwich ELISA technique all incubations were done at 4° C for 1 h apart from the substrate-incubation step which was done at 37° C for 30 min. Thus, the procedure differed from the conventional ELISA where incubations were performed at 37° C and had been adapted from the method of Kenemy *et al.*, (1985). Both unheated and heated anti-Oa sera, produced by the one-dose protocol, were tested in this system. Dramatically, the wells coated with 0.175 µg/ml AP anti-Oa produced a satisfactory dose-response curve with an antibody titre of 600. Moreover, when heated test serum was used in the above system, the absorbance values of the lowest dilution of the test sera (1 in 10) with the 0.175 µg/ml AP anti-Oa coating concentration was very low, giving an absorbance of < 0.3. It was therefore clear that the sandwich ELISA technique with AP anti-Oa as coating reagent would overcome the earlier difficulties of high backgrounds.

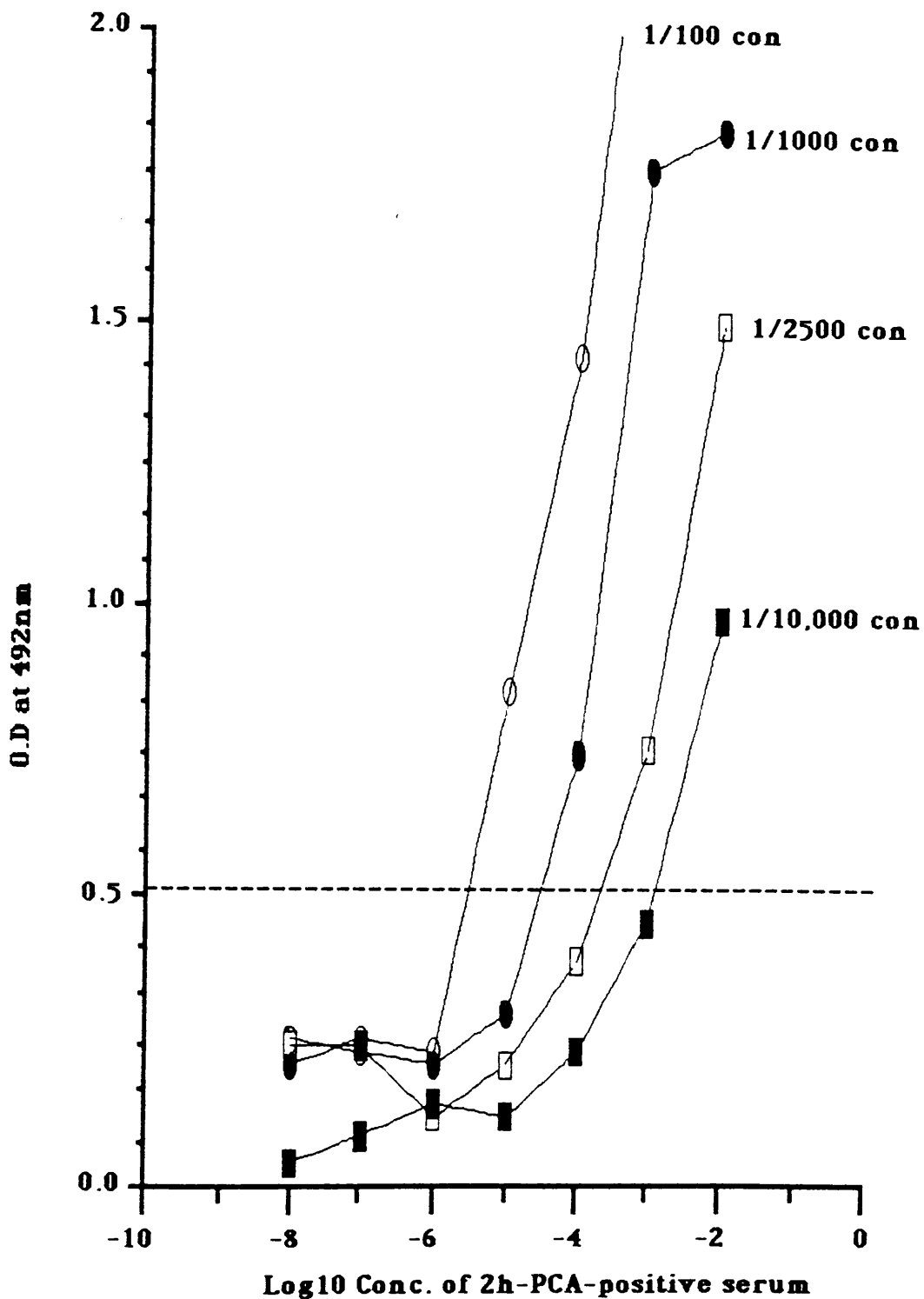
With the higher concentrations (1.75 and 17.5 µg/ml) of AP anti-Oa, heating the IgE-positive serum caused a reduction in the dose-response line when compared with unheated serum (Fig. 15), but all the absorbances were > 0.5 and titres could not be calculated. Throughout the following experiments therefore, the coating concentration of AP rabbit anti-Oa was 0.175 µg/ml.

**Effect of concentration of Oa in sandwich layer.** In the previous Fig. 15, it was shown that different concentrations of AP rabbit anti-Oa had a marked effect on

**Fig 16: IgE ELISA: Effect of Oa concentration in the sandwich layer on the measurement of IgE by sandwich ELISA.** The ELISA was done with standard anti-Oa IgE serum with the initial coating of the plate with AP anti-Oa at a fixed concentration of 0.175  $\mu\text{g/ml}$ . The concentration of Oa in the sandwich layer is shown on the right.



**Fig 17: IgG1 ELISA: Effect of the dilution of sheep anti-mouse IgG1 HRP conjugate on the measurement of IgG1.** The dilutions of which are shown on the right. A 2 h PCA-positive anti-Oa IgG1 serum was used. The coating concentration of Oa was 100 µg/ml.



anti-Oa IgE detection, with a constant dose of Oa (100 µg/ml) in the sandwich layer. Subsequently, the AP anti-OA was kept constant at 0.175 µg/ml and the Oa conc. was varied (Fig. 16). Taking the endpoint absorbance as 0.5, the 100 µg/ml Oa in the sandwich layer gave a titre of 1400 with the standard IgE serum. Whereas the 10 µg/ml and 1000 µg/ml Oa concentrations, although showing a similar trend, the absorbance readings at all the serum dilutions was < 0.5, which frustrated reading the endpoint. Routinely, therefore the concentration of Oa in the sandwich layer was 100 µg/ml with the initial AP anti-Oa coating being 0.175 µg/ml.

### **Measurement of IgG1 by conventional ELISA**

The IgG1 content of serum was measured because of its potential to interfere with both the PCA test and the ELISA. It was also studied for possible correlation with IgE content.

### **Titration of conjugate**

As before, in the conventional ELISA, the conjugate had to be titrated to find a suitable dilution for the ELISA (Fig. 17). A range of dilutions from 1 in 100 to 1 in 10,000 of the sheep anti-mouse IgG1-HRP conjugate were tried. A 2 h PCA-positive serum was used in the ELISA to evaluate the effect of the different dilutions of conjugate. The 1 in 100 dilution at low dilutions of test serum produced absorbances > 2.0. The 1 in 1000 diln. gave a satisfactory response with a calculated IgE titre of 40,000. The titres obtained with 1 in 2500 and 1 in 10,000 dilutions of conjugate were 7300 and 1000 respectively, a 7.5 and 55 fold decrease from that obtained with the 1 in 1000 dilution of conjugate. In subsequent experiments, the sheep anti-mouse IgG1-HRP conjugate was used at a 1 in 1000 dilution.

### **Correlation of 48h PCA and ELISA for detection of IgE and IgG1**

Having raised a large number of anti-Oa sera by various immunization protocols, and having tested them for IgE and IgG1 by 2 different methods, it was then appropriate to compare the accumulated results, Table 20 sets out the PCA values for 26 sera, or pools of sera, together with the corresponding IgE ELISA data. One of the main features to emerge was that the  $\log_{10}$  IgE ELISA titres tended to range from about 15 to 350-fold higher than the titres found in the PCA test for the same sera. Unexpectedly, this range of differences was only found in sera raised using the "high" dose of Oa (200 µg). However, they did vary with respect to adjuvant dose and day of bleeding. Conversely in the remaining serum, the  $\log_{10}$  PCA U/ml values for the test sera

**Table 20 : Comparison of anti-Oa sera by 48 h PCA, conventional<sup>ELISA</sup> for IgG1 and sandwich ELISA for IgE.** Details of the three methods can be found in the Materials and Methods.

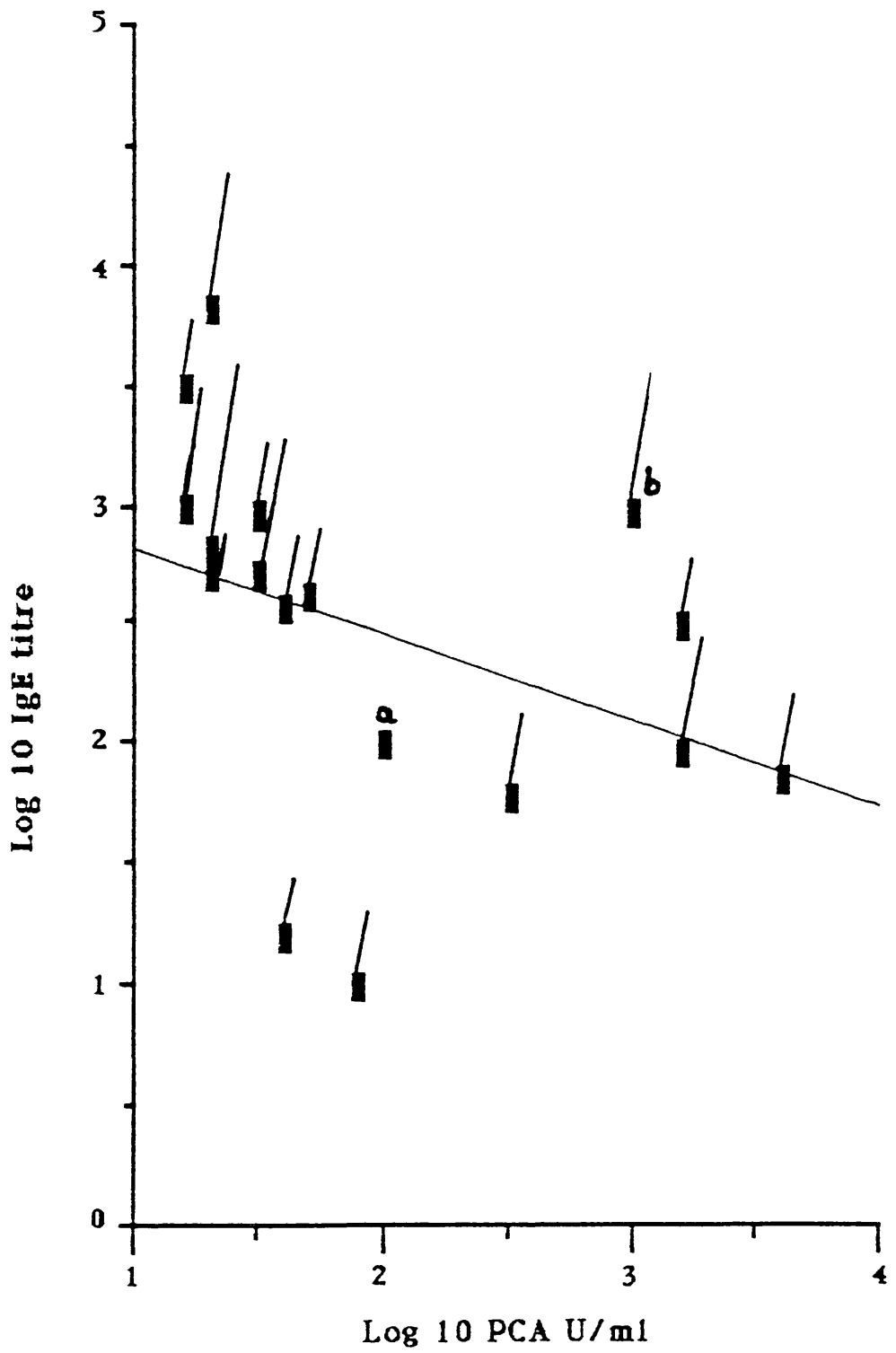
Serum no. <sup>a</sup>	log10 48h PCA U/ml	log10 IgE titre	log10 IgG1 titre
4f	0.9	n.t	4.7
3f	1.2	3.0	3.9
4e	1.2	n.t	3.9
5a	1.2	3.5	2.9
2e	1.3	n.t	3.7
1a	1.3	2.7	2.6
3e	1.3	2.9	4.6
3g	1.3	3.8	3.6
4d	1.5	3.0	3.9
6b	1.5	2.7	3.8
3c	1.6	1.2	2.7
4b	1.6	2.6	2.8
2b	1.7	n.t	3.5
5c	1.7	2.6	2.7
3d	1.9	<1	2.9
1b	2.0	2	2.1
2a	2.0	n.t	3
2c	2.5	1.8	3
2d	2.5	<1	3
6a	3.0 <sup>b</sup>	3	3.3
3b	3.2	2.5	2.7
4c	3.2	2.0	3.6
3a	3.6	1.9	2.9
4a	n.t	n.t	<1
4g	n.t	3.3	2.7
6c	n.t	<1	4.9

<sup>a</sup> - for further particulars, see Appendix 7. Sera with serial numbers from 1 to 5 were individual mouse sera obtained by protocol 2 and those with serial number 6 were pools of 25 obtained by protocol 3.

<sup>b</sup> - Standard defined as having 1000U/ml, log10 value 3 in PCA and IgE ELISA

**Fig 18: Correlation of anti-Oa IgE, measured by PCA and ELISA, with IgG1 measured in parallel.** The bars represent the log<sub>10</sub> IgG1 titre where 1 cm is equivalent to 1 log<sub>10</sub>. The line starts at a log<sub>10</sub> IgG1 titre of 2 as the 1 in 100 dilution was the lowest used in the IgG1 ELISA





tended to range from about 2 to 550-fold higher than the log<sub>10</sub> IgE titres for the same sera. In this instance, the immunizing dose was mainly 20 µg Oa, the "low" dose. There did seem therefore to be a correlation between the 200 µg Oa immunizing dose and high IgE titres in the ELISA while the PCA results remained low in comparison. The "low" 20 µg Oa immunizing dose sera produced high PCA titres, when compared with log<sub>10</sub> IgE titres in ELISA, which was invariably lower.

Before the data could be analysed statistically, a judgement on the normality of the data had to be made. For this purpose, the rankit test was applied. In appendix 9, the rankit plots of log<sub>10</sub> PCA U/ml, log<sub>10</sub> IgE and IgG1 titres can be seen. The rankit plot of the log<sub>10</sub> PCA U/ml showed a deviation from normality in that the observed rankit line (solid) did not zig zag in a random fashion fairly closely around the fitted theoretical line (dashed). From these rankit plots, it was calculated that the log<sub>10</sub> PCA U/ml and the log<sub>10</sub> IgG1 titres were not normally distributed. The log<sub>10</sub> IgE titres however appeared not to depart from normality. Therefore the data as a whole were analysed by the Kendall rank correlation test, a non-parametric procedure that is applicable to populations which are not normally distributed. A graph of the negative correlation of log<sub>10</sub> PCA U/ml and log<sub>10</sub> IgE titre can be seen in Fig. 18. Kendall statistics show that there was a highly significant negative correlation with a p value of 0.006. In Fig 18, the log<sub>10</sub> IgG1 data are also incorporated into the graph (vertical lines) where 1 cm is equivalent to 1 log<sub>10</sub>. The lines start at a log<sub>10</sub> IgG1 titre of 2 as the 1 in 100 dilution was the lowest used in the IgG1 ELISA. There did not seem to be any real trend in IgG1 production at different antigen and adjuvant immunizing concentrations, as most of the sera seemed to have similar amounts of IgG1. Exceptionally, a and b on the graph in Fig. 18, whose PCA and IgE ELISA results give equivalent titres also had low IgG1 titres when compared with the other sera. Therefore there may be a requirement in these assay systems that the IgG1 should be low, in order to give a good comparison between PCA and IgE ELISA titres. A possible reason for the discrepancies between the PCA and ELISA titres could well be the interference of IgG1 antibodies in these systems.

### **IgE Responses to FHA, with IgG1 Measured in Parallel**

With all the groundwork already investigated in the Oa system, it was easier to quantify IgE responses to the FHA of *B. pertussis*. In the Oa work, a protocol for

**Table 21 : Characterization of 15 batches of FHA, purified by gel-affinity chromatography.** The protein content of each batch was measured by Lowry with BSA as standard and the batches were variously examined for haemagglutination activity and/or 200 kDa and other proteins by SDS-PAGE. The batches highlighted in bold type were used for immunizations.

Batch	Vol. culture (L)	Vol. (ml)	Protein content (µg/ml)	HA units	SDS-PAGE 200 kDa	approx. % contam.	Specific activity HAU/µg	Yield HAU/L
1	6	20	300	308	-	5	1.03	1023
<b>2</b>	<b>6</b>	<b>30</b>	<b>1000</b>	<b>19607</b>	+	<b>10</b>	<b>19.6</b>	<b>98035</b>
<b>3</b>	<b>6</b>	<b>20</b>	<b>600</b>	<b>9900</b>	+	<b>10</b>	<b>16.5</b>	<b>33000</b>
4	5	20	250	4805	n.t.	n.t.	19.20	19.220
5	6	30	600	4805	+	10	8.0	24.025
6	5	15	100	615	+	10	6.15	1846
7	6	20	100	308	+	10	3.08	1023
8	6	15	50	n.t.	n.t.	n.t.	n.t.	n.t.
9	6	28	900	n.t.	+	10	n.t.	n.t.
10 <sup>a</sup>	6	30	200	n.t.	+	50	n.t.	n.t.
<b>11</b>	<b>8</b>	<b>40</b>	<b>700</b>	<b>9900</b>	+	<b>10</b>	<b>14.14</b>	<b>49500</b>
12	6	40	600	n.t.	+	5	n.t.	n.t.
13	6	20	300	n.t.	+	15	n.t.	n.t.
14	6	24	300	n.t.	+	15	n.t.	n.t.
15	6	20	200	n.t.	+	10	n.t.	n.t.

n.t - not tested

<sup>a</sup> - discarded

raising IgE antibodies and the PCA and sandwich ELISA were all studied for producing and measuring IgE. However, the PCA had to be altered in the FHA system, as discussed below.

## **Measurement of anti-FHA IgE by PCA**

### **Characterization of FHA**

From a 6L culture supernate of *B. pertussis* strain BP 357, FHA was purified by blue sepharose gel affinity chromatography. The FHA fraction was eluted by high salt and had a volume of 20 - 40 ml. Some of these batches (Table 21) were analysed for protein content by Lowry, haemagglutination and the presence of a 200 kDa protein band on SDS-PAGE. The batches highlighted in bold were used for immunizations because they had high protein values and good haemagglutination activity titres, and only 10% contamination with other proteins or degraded FHA. Batch 10 was discarded because of a very high percentage contamination as revealed by SDS-PAGE. The other batches were either used in challenge mixtures for PCA or as coating in the ELISA.

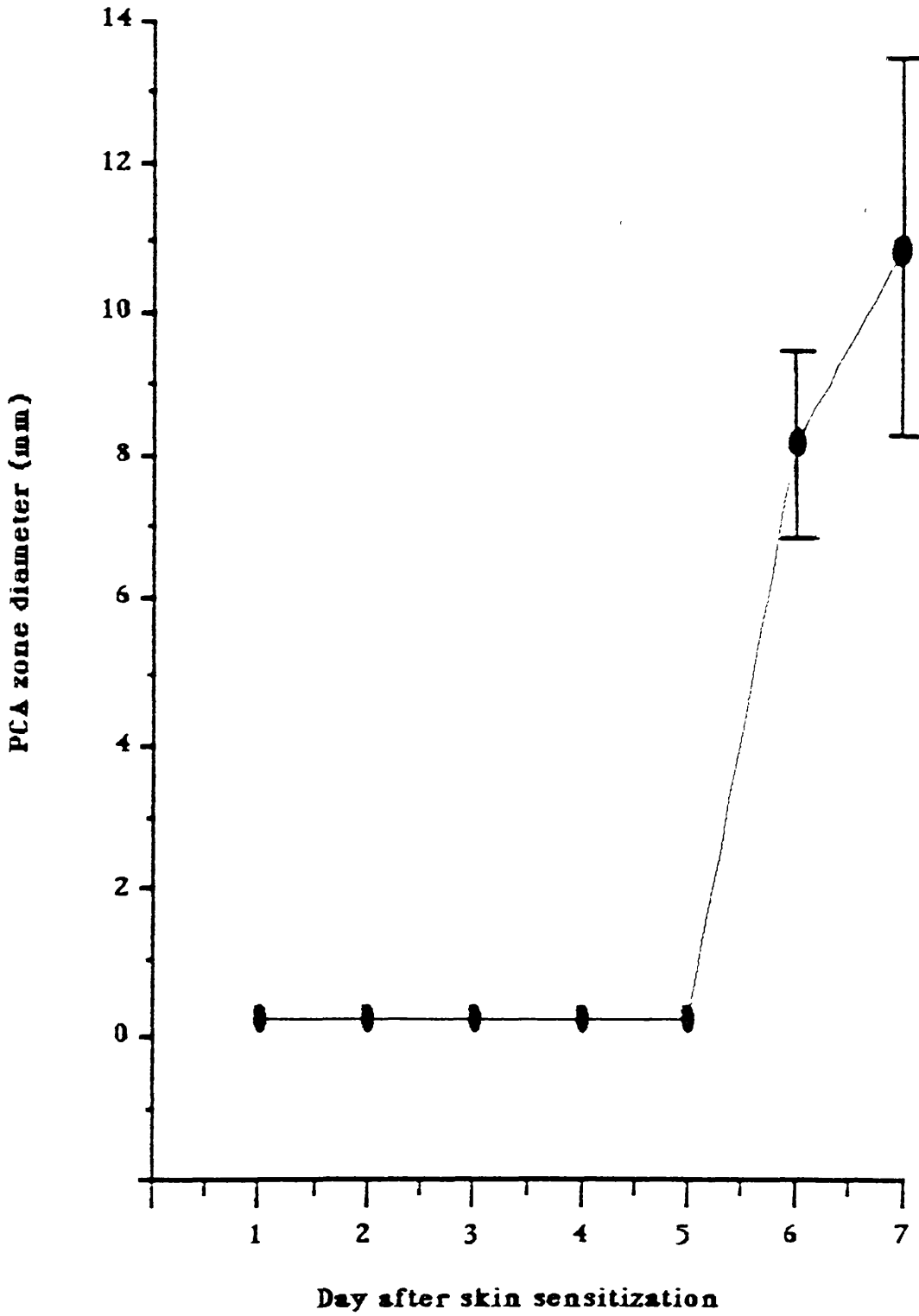
### **Factors influencing the PCA test**

A number of factors although already optimized in the Oa system, had to be checked and in some cases altered in the FHA system. These included the a) increasing the sensitization interval b) FHA challenge dose c) and the effect of four sites per mouse.

***Effect of increasing the sensitization interval*** In preliminary experiments in the 48 h PCA with anti-FHA and FHA, there were problems of diffuse blueing on the undersurface of the skin which obscured the blue zone at injection sites. The diffuseness disappeared with dilution of the test serum, but so did the positive 48 h PCA responses, although in some animals there was a slightly darker region of blueing at the site of injection. A possible reason for this diffuseness could have been the interfering effect of high levels of IgG1 reacting throughout the vasculature after challenge. It was therefore decided to increase the sensitization interval up to 7 days, and to challenge groups of four mice at one-day intervals during this period. A 7-day interval was taken as the longest, since IgE has a half-life of 6 days.

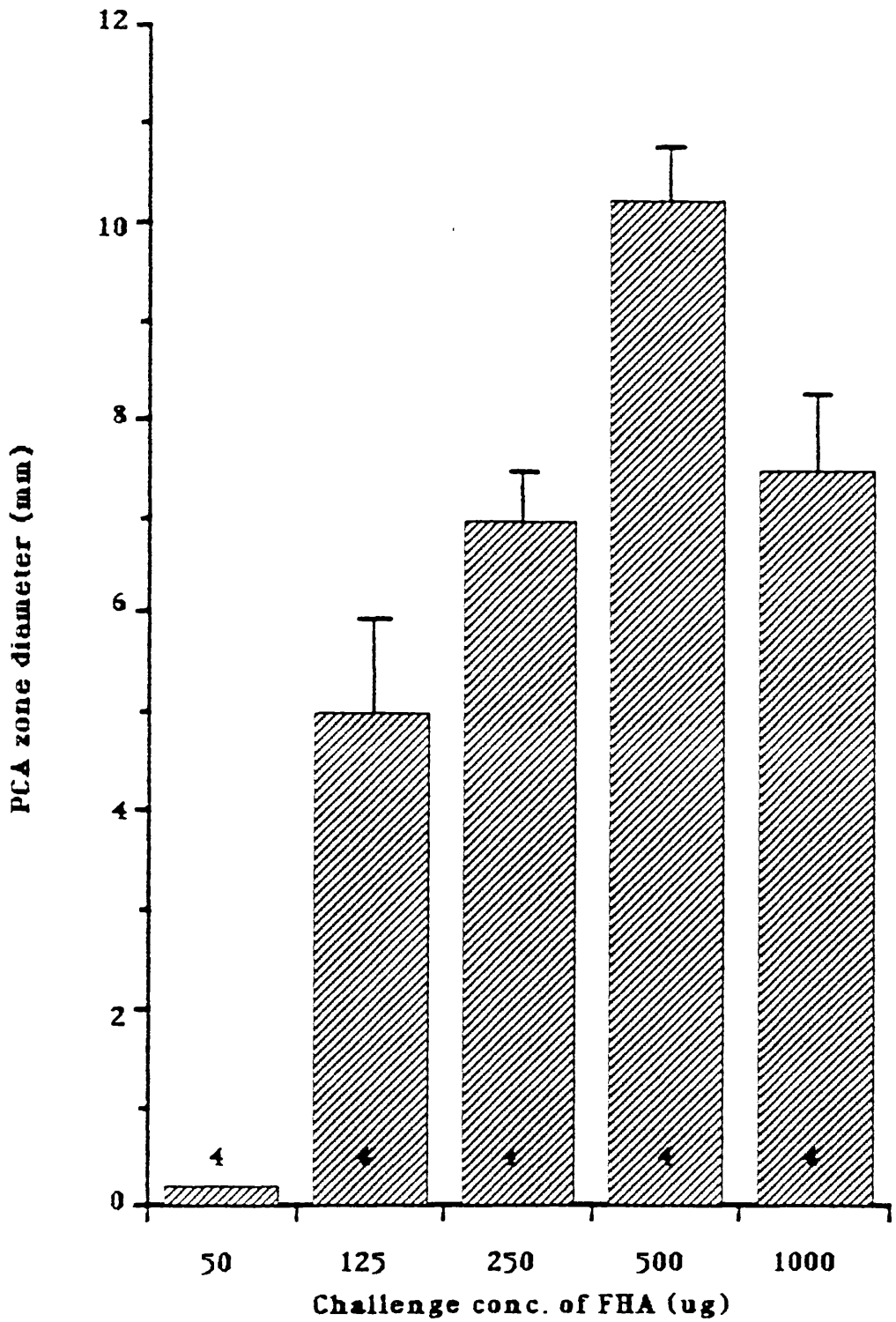
As shown in Fig. 19, with sensitization intervals between 1 and 5 days there was no detectable PCA response, in that the whole surface of the skin turned blue. At day 6, there was a zone of blueing which could be measured and, surprisingly, on day 7 this PCA zone diameter had increased to 12 mm with undiluted test serum. In subsequent experiments, a sensitization interval of 7 days was adopted.

**Fig 19: The beneficial effect of a 7-day interval between sensitization and challenge in PCA tests with FHA.** Mice were sensitized with anti-FHA serum and groups of 4 mice were challenged i.v with FHA after different sensitization intervals. The points indicate the average zone diameter and the SEM.



**Fig 20: Effect of varying the challenge dose of FHA in the 7-day PCA test.**

Undiluted serum, raised by the three-injection protocol, and known to be IgE-positive was injected i.d into all mice; 7 days later, pairs of mice were challenged i.v with FHA and Evans Blue. The bars indicate the average zone diameter (mm) and SEM with the number of replicates shown inside the bars.



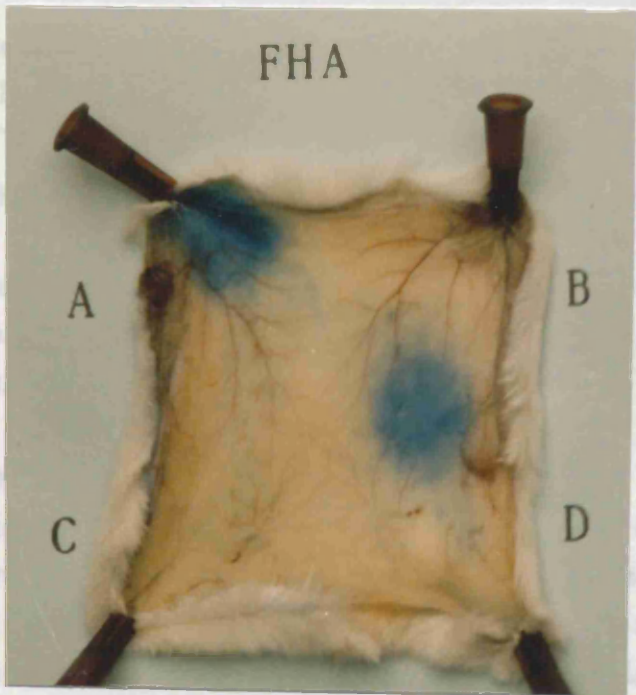


**Plate 3: Layout of the PCA test with 4 sites per mouse.** At positions A and D, anti-FHA IgE rich serum was injected i.d. The animals were challenged i.v with 500 µg FHA and Evans blue, 7 days after sensitization. Positions C and B were injected with the same sera heated to 56° C for 1h.

*Effect of FHA challenge dose.* The FHA challenge dose was chosen in the first PCA to see if it would behave differently from the previous studies. The optimal concentration was 1 µg/mouse. In this experiment, the dose range was limited, with a PCA-positive anti-FHA IgE serum. The optimal dose range of FHA challenge doses were 100 µg/mouse. The results showed that there was no detectable IgE response in the 7-day PCA. The results showed that mice given similar zone diameters, but the 500 µg/mouse dose gave a significantly larger average zone diameter than the doses of 100 µg/mouse. In this experiment, the dose of FHA was set at 500 µg/mouse.

*Effect of 4 sites per mouse.*

A commercially-available challenge involved in preparing purified tested in each challenged mouse. be tested in the one animal. A diagram is shown. Plate 3 shows sites A, B, C, and D injected i.d. and the same sera he was challenged with 500 µg FHA at sites A and D only. This was after the



Anti-FHA IgE responses after in the Os system, the one-dose reactions from individual high-doses and two strains of mice, the 7-day PCA. The 48 h PCA was content of the serum was presented. In the PCA, as had happened with the sera, there was no detectable PCA reactivity was given after a 48 h sensitization course. It had been encountered earlier was therefore investigated as B6/J mice and low responding animals. Balb/c mice were (Sako *et al.*, 1977).

In pools of anti-FHA sera, the reactivity at either 48 h or 7 days from antigen-alone injected mice. On the other hand, there were 4 positive

**Effect of FHA challenge dose.** The FHA challenge dose was altered in the 7-day PCA to see if it would behave differently from the Oa system, where the optimal concentration was 1 mg/mouse. In these experiments pairs of mice were i.d injected, with a PCA-positive anti-FHA IgE serum and challenged 7 days later with FHA. A wide range of FHA challenge doses were used (Fig. 20). When 50  $\mu$ g FHA was used, there was no detectable IgE response in the 7-day PCA. The 250  $\mu$ g and 1000  $\mu$ g doses both gave similar zone diameters, but the 500  $\mu$ g challenge dose produced a significantly higher average zone diameter than the doses either side of it. In subsequent experiments, the dose of FHA was set at 500  $\mu$ g/mouse.

**Effect of 4 sites per mouse.** In the Oa system, there was an abundant source of commercially-available challenge antigen. However because of the lengthy process involved in preparing purified FHA, there was a requirement for more sera to be tested in each challenged mouse. Therefore the PCA was modified to allow four sera to be tested in the one animal. A diagrammatic representation of the four injection sites is shown. Plate 3 shows sites A and D where anti-FHA IgE-containing serum was injected i.d and the same sera heated to 56<sup>o</sup> C was injected at sites B and C. The mouse was challenged with 500  $\mu$ g FHA and blueing indicative of IgE can be seen at positions A and D only. This was after the extended sensitization interval of 7 days.

#### **Anti-FHA IgE responses after one-dose immunization**

In the Oa system, the one-dose immunization protocol only gave positive 48 h PCA reactions from individual high-responding mice. With FHA at different immunizing doses and two strains of mice, the sera obtained were pooled and tested in the 48 h and 7-day PCA. The 48 h PCA was reintroduced in this experiment because the IgG1 content of the serum was presumed to be low and therefore would not interfere in the PCA, as had happened with the sera raised in the three-dose protocol. In fact, there was no detectable PCA reactivity with these anti-FHA sera and with an FHA challenge given after a 48 h sensitization interval. Moreover, the generalised blueing that had been encountered earlier was not a problem. Another strain of mouse was therefore investigated, as Ham/ICR may have contained a mixture of high and low responding animals. Balb/c mice are known to be a reliable IgE-responding strain (Suko *et al.* 1977).

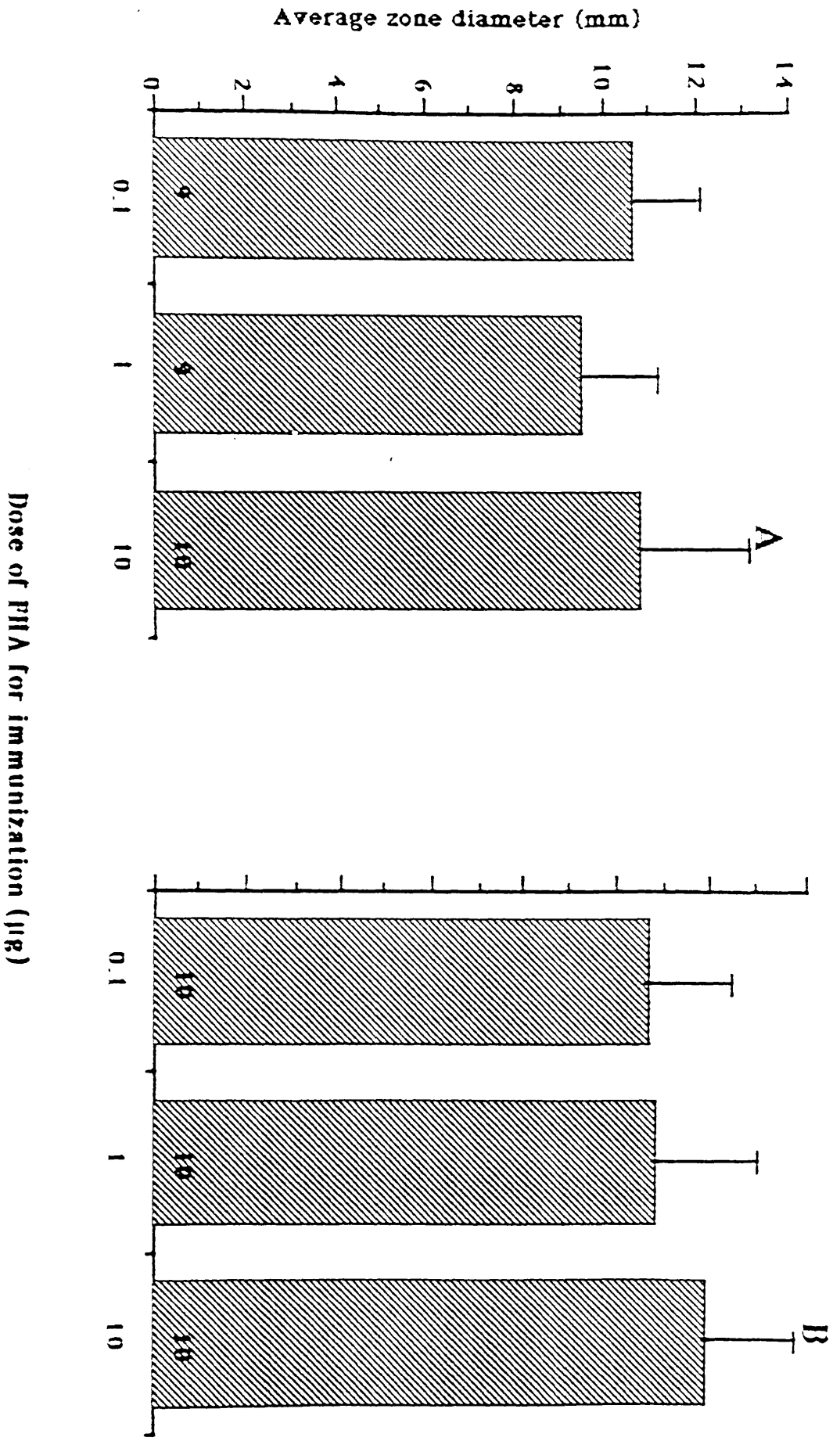
In pools of anti-FHA sera raised in Ham/ICR mice, there was no PCA reactivity at either 48 h or 7 days (Table 22). The controls which consisted of sera from antigen-alone injected mice did not elicit a IgE response at days 10, 21 and 28. On the other hand, there were 4 positive 7-day PCA responses with pooled sera raised in

**Table 22 : Effect of a range of immunizing doses of FHA, when mixed with a constant adjuvant dose of PT (1  $\mu$ g), on the development of an anti-FHA IgE response by the one-dose protocol in both Ham/ICR and Balb/c mice. Sera were pooled from 5 individual mouse bloods and were tested undiluted in both 48 h and 7-day PCA tests. The positive anti-FHA IgE responses are shown in bold type.**

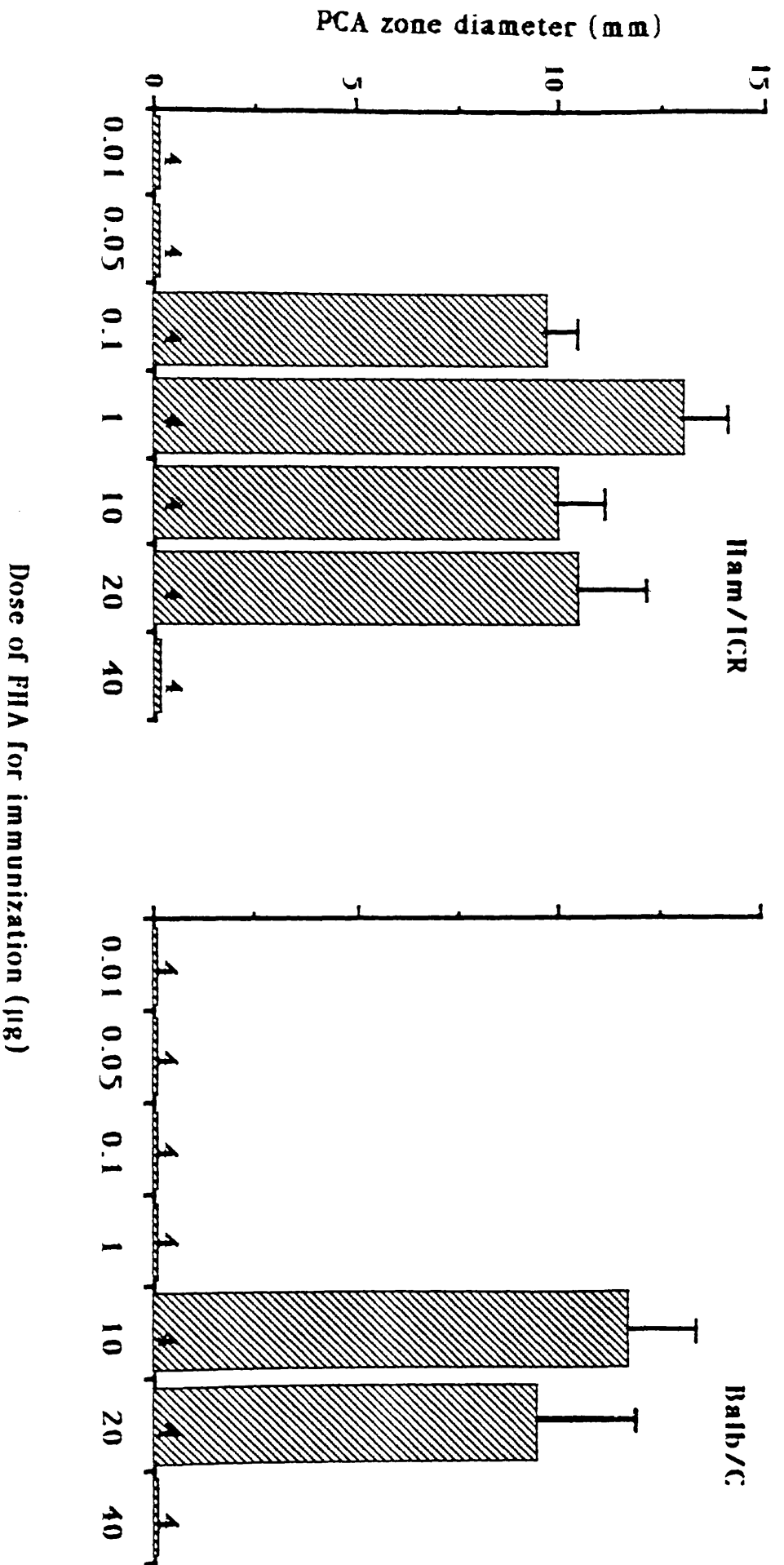
Immunizing dose of FHA ( $\mu$ g) on day zero	Adjuvant dose of PT ( $\mu$ g)	No. of PCA positive pooled sera tested in 48 h and 7-day PCA test on bleed day:					
		10		21		28	
		48 h	7-day	48 h	7-day	48 h	7-day
<b>Ham/ICR</b>							
0.05	+	0/2	0/2	0/2	0/2	0/2	0/2
0.5	+	0/2	0/2	0/2	0/2	0/2	0/2
2.5	+	0/2	0/1	0/2	0/2	0/2	0/2
5	+	0/2	0/2	0/2	0/1	0/2	0/2
10	+	0/2	0/2	0/2	0/2	0/2	0/2
25	+	0/2	0/2	0/2	0/2	0/2	0/2
50	+	0/2	0/2	0/2	0/2	0/2	0/2
0.05	-	0/1	n.t.	0/1	n.t.	0/1	n.t.
5	-	0/1	n.t.	0/1	n.t.	0/1	n.t.
50	-	0/1	n.t.	0/1	n.t.	0/1	n.t.
<b>Balb/c</b>							
0.05	+	0/2	0/2	0/2	0/2	0/2	0/2
0.5	+	0/2	0/2	0/2	0/2	0/2	<b>2/2</b>
2.5	+	0/2	0/2	0/1	0/2	0/2	0/2
5	+	0/2	<b>2/2</b>	0/2	0/2	0/2	0/2
10	+	0/2	0/2	0/2	0/2	0/2	0/2
25	+	0/2	0/2	0/2	0/2	0/2	0/2
50	+	0/2	0/2	0/2	0/2	0/2	0/2
0.05	-	0/1	n.t.	0/1	n.t.	0/1	n.t.
5	-	0/1	n.t.	0/1	n.t.	0/1	n.t.
50	-	0/1	n.t.	0/1	n.t.	0/1	n.t.

n.t. - not tested

**Fig 21: Application of the 7-day PCA test to detect IgE antibodies to FHA in mice.** Sera were raised by the three-injection protocol. A and B were replicate experiments, with the immunizing doses indicated on the abscissa. The bars indicate the average zone diameter and the SEM with the number of replicate observations inside the bars.



**Fig 22: Effect of different immunizing doses of FHA on the development of anti-FHA IgE in Ham/ICR and Balb/c mice.** Sera were raised by the three-dose protocol and tested undiluted in the 7-day PCA test. The bars indicate the average zone diameters (mm) and SEM with the number of replicates inside the bars.





Balb/c, with a 0.5  $\mu$ g immunizing dose of FHA and a 28-day bleed, and with 5  $\mu$ g FHA immunizing dose and a 10-day bleed, both with 1  $\mu$ g bioactive PT as adjuvant. Two pools, each of 5 mouse bloods were positive in the 7-day PCA performed on > 6 wk old Ham/ICR mice. These observations are outlined in bold in Table 22. Generally however the one-dose immunization protocol with FHA as antigen, PT as adjuvant and two different mouse strains, yielded very few PCA-positive responses, as judged by observations with pooled serum. Perhaps, with hindsight, if the sera had been tested individually, any high responders could have been detected.

### **Anti-FHA IgE responses after three-dose immunization.**

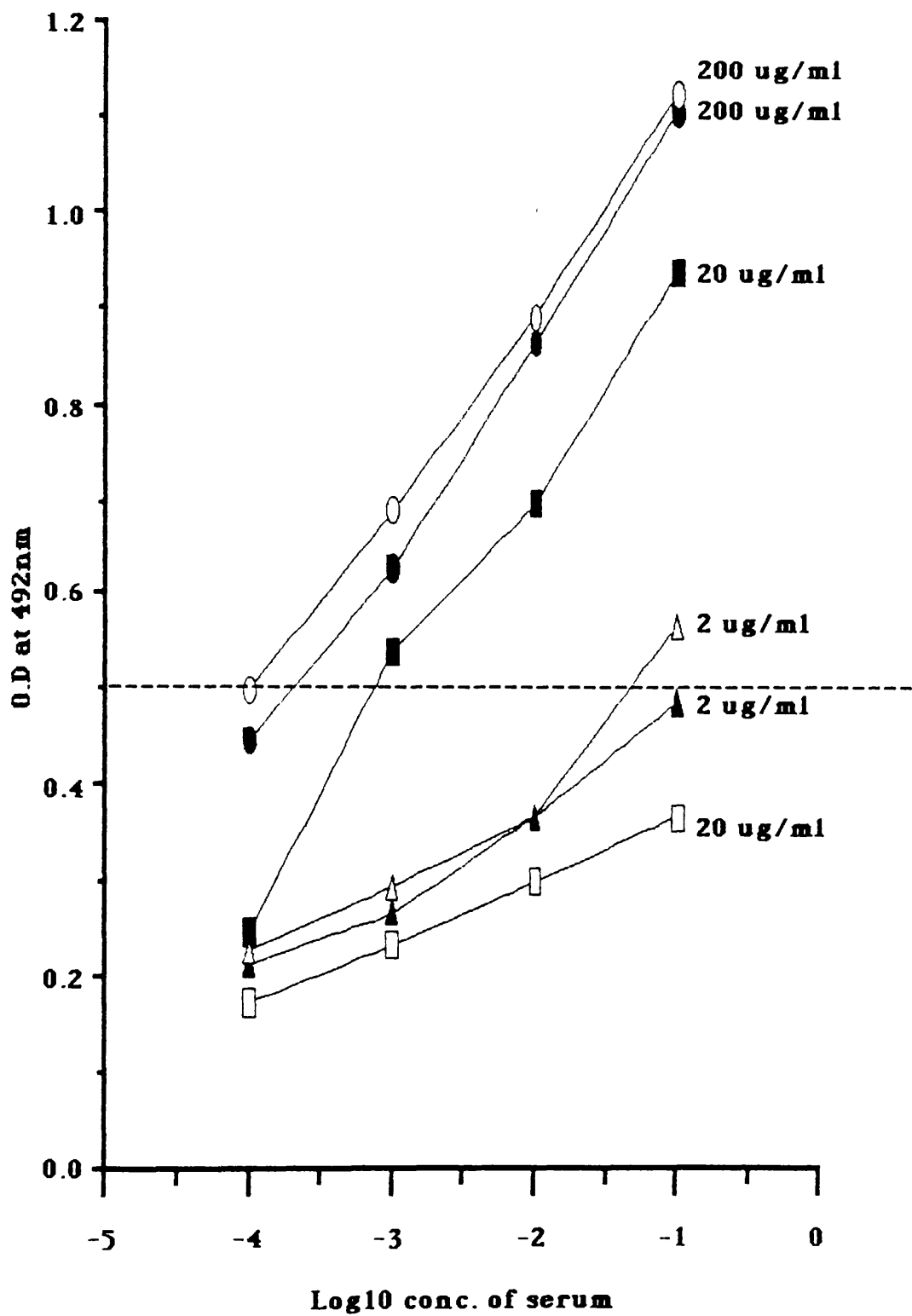
Before attempting to raise IgE-rich sera to FHA, prior studies were done in Ham/ICR mice with Oa as the model antigen. Strongly IgE-positive anti-Oa sera were obtained using the 3-dose protocol described in Materials and Methods. These anti-Oa sera were used as a pooled positive control at 1 in 20 dilution in the FHA PCA tests which required a 7-day interval for detection. The recipient mice in these tests received a mixed challenge of FHA and Oa with Evans blue.

In the 7-day PCA tests, the effect of different doses of a carbodiimide toxoid of PT/FHA was investigated as a stimulus for the production of IgE antibodies to FHA. As shown in Fig 21A, IgE was produced over a 100-fold range of immunizing doses of FHA/PT toxoid from 0.1  $\mu$ g to 10  $\mu$ g. The actual amount of FHA in this carbodiimide toxoid was one-half the dose inferred on the abscissa, as PT was present in equal amount in the toxoided mixture. There seemed to be no significant difference in 7-day PCA reactivity, with the different immunizing doses of FHA. In Fig 21B the entire experiment was independently replicated and the same trend as Fig 21A in the IgE response was found. Thus an anti-FHA IgE response was elicited over the entire range of FHA immunizing doses.

Because a PCA-positive reaction was produced so widely, subsequent experiments focussed on the effect of different immunization doses of FHA when mixed with a constant dose of PT, in the first injection, in the 3-dose protocol. Two mouse strains were used: Ham/ICR and Balb/c. In Fig 22, Ham/ICR produced a convincing IgE response, as detected by 7-day PCA, with immunizing doses of FHA ranging from 0.1 to 20  $\mu$ g and with a peak at the 1  $\mu$ g dose. There were no detectable responses at 0.01, 0.05 or 40  $\mu$ g immunizing doses of FHA. This showed that the IgE response was limited to a particular immunizing dose range and that the animals would not respond to very low or high immunizing doses of FHA.

A different picture appeared with Balb/c mice (Fig. 22), in that an anti-FHA IgE response was observed only at the 10 and 20  $\mu$ g immunizing doses. These

**Fig 23: IgE ELISA: Effect of the concentration of affinity-purified rabbit anti-FHA as a coating agent on the performance of the sandwich ELISA.** The ELISA was done on IgE-positive serum, before (solid points) and after heating at 56° C for 1 h (open points). The FHA for binding was used at a constant concentration of 100 µg/ml and the sheep anti-mouse IgE HRP conjugate at 1 in 100. The concentration of AP anti-FHA is shown on the right.



**Plate 4: Immunodiffusion pattern of anti-FHA serum before and after extraction on a FHA-sepharose column.**

Serum provided by A.C. Wardlaw and prepared as per the method of M. Aprile (1972)

experiments highlighted some of the limitations of the current system. However, the ELISA system was found to be a more suitable system, in that the latter was significantly more sensitive.

### Measurement of IgE in the presence of IgG

#### Factors influencing the assay

Initial studies using microtitre plates showed that the assay was sensitive to the concentration of the antigen.

Therefore, the concentration of the antigen was varied and the effect on the assay was determined.

The results showed that the assay was sensitive to the concentration of the antigen.

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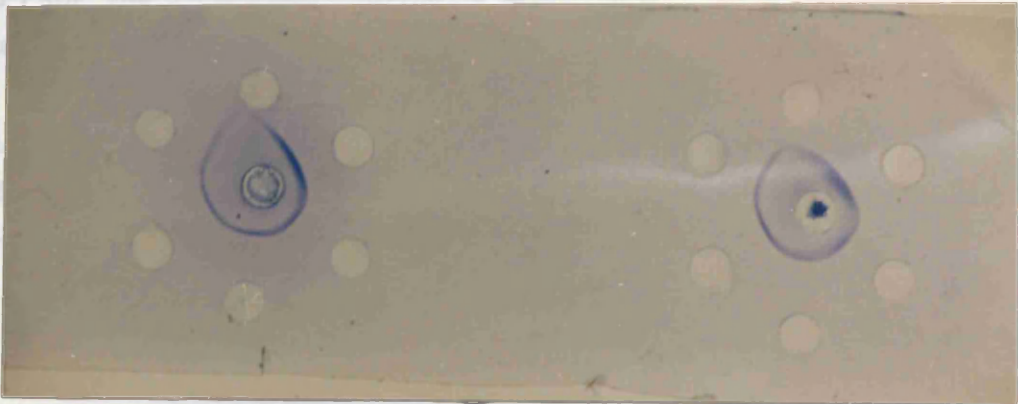
The assay was found to be sensitive to the concentration of the antigen.

The assay was found to be sensitive to the concentration of the antigen.

The assay was found to be sensitive to the concentration of the antigen.

**Before  
extraction**

**After  
extraction**



#### Effect of concentration of IgE on the assay

The effect of the concentration of IgE on the assay was studied.

The results showed that the assay was sensitive to the concentration of IgE.

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The assay was found to be sensitive to the concentration of IgE.

experiments highlighted major differences in IgE-responsiveness between mouse strains. However, the FHA responses were different from those produced in the Oa system, in that the latter were restricted to the 0.1 and 1  $\mu$ g doses.

### **Measurement of anti-FHA IgE by sandwich ELISA**

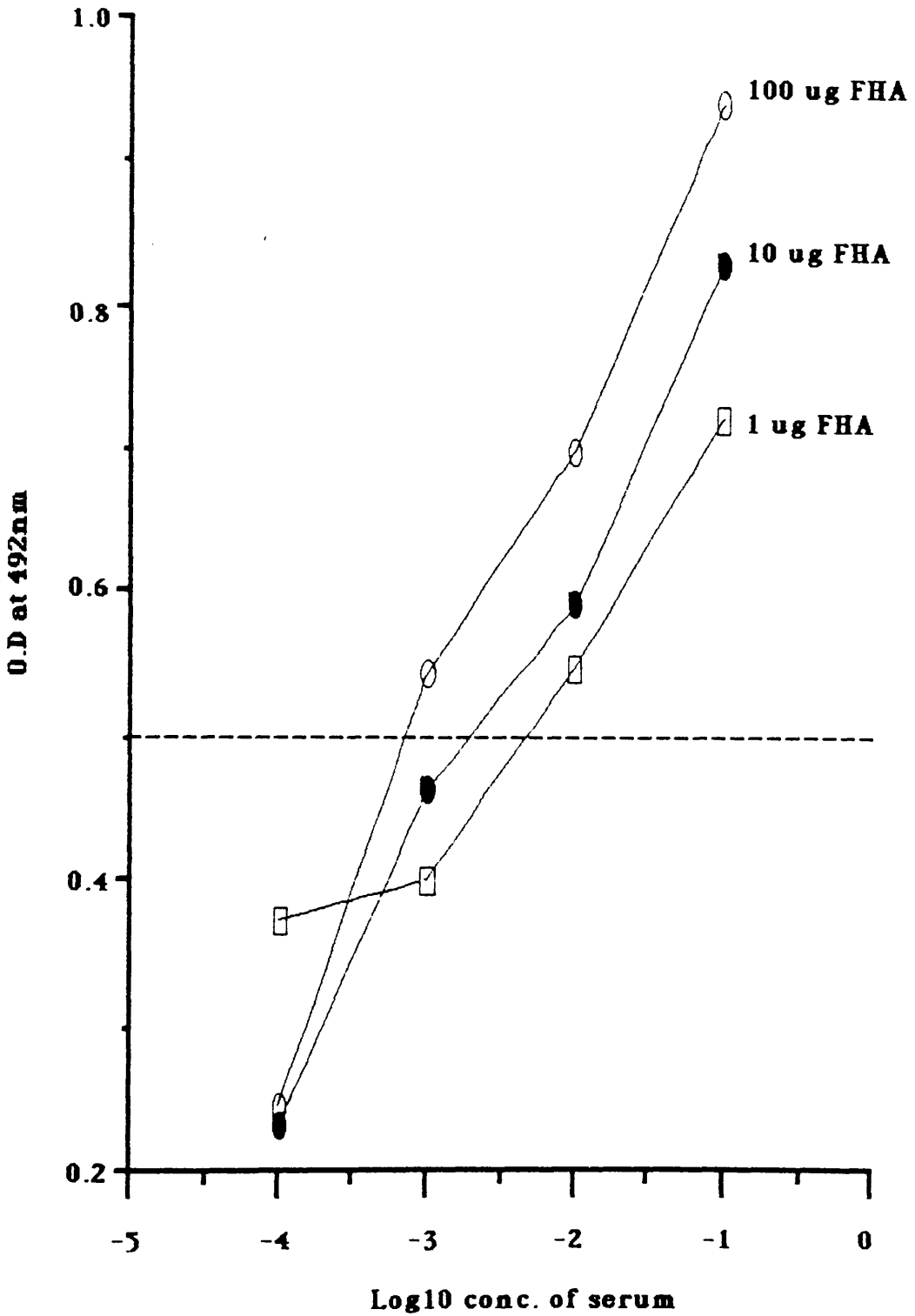
#### **Factors influencing the ELISA**

Initial studies using conventional ELISA to detect anti-FHA IgE were unsuccessful. Therefore, the sandwich ELISA technique was developed for the Oa system. For application of the sandwich method to anti-FHA, an affinity-purified rabbit anti-FHA was required. This was done by affinity purification on an FHA-sepharose column. The anti-FHA was then characterized by Lowry, immunodiffusion against FHA (Plate 4) and SDS-PAGE. The AP anti-FHA coating and the FHA in the sandwich layer were both titrated to optimize the overall procedure.

*Effect of concentration of affinity-purified rabbit anti-FHA in the sandwich layer* The AP anti-FHA was prepared from 25 ml rabbit serum raised as per the method of Aprile, (1972). The resultant fraction of 3 ml eluted from the sepharose-FHA column and concentrated by dialysis against Aquacide, contained 2 mg/ml protein. This purified antibody was diluted in coating buffer, pH 9.6 to a final concentration of 2, 20 and 200  $\mu$ g/ml and used in the sandwich ELISA as the initial coating layer (Fig. 23). The FHA in the sandwich layer was kept constant at 100  $\mu$ g/ml. With an endpoint absorbance of 0.5, the best coating concentration was 20  $\mu$ g/ml (Fig. 23). The graph shows that a satisfactory dose response line was obtained with unheated test serum and that this was reduced to a baseline level after heating the IgE-containing serum at 56<sup>o</sup> C for 1 h. The 2  $\mu$ g and 200  $\mu$ g coating concentration of AP anti-FHA showed no significant difference in dose response line produced with IgE-positive serum and after heating at 56<sup>o</sup> C for 1 h to destroy IgE. Subsequently, the AP anti-FHA coating concentration was used at 20  $\mu$ g/ml, as this allowed the mouse anti-FHA serum to give its highest titre of 1360, which was reduced upon heating at 56<sup>o</sup> C to a titre of < 10.

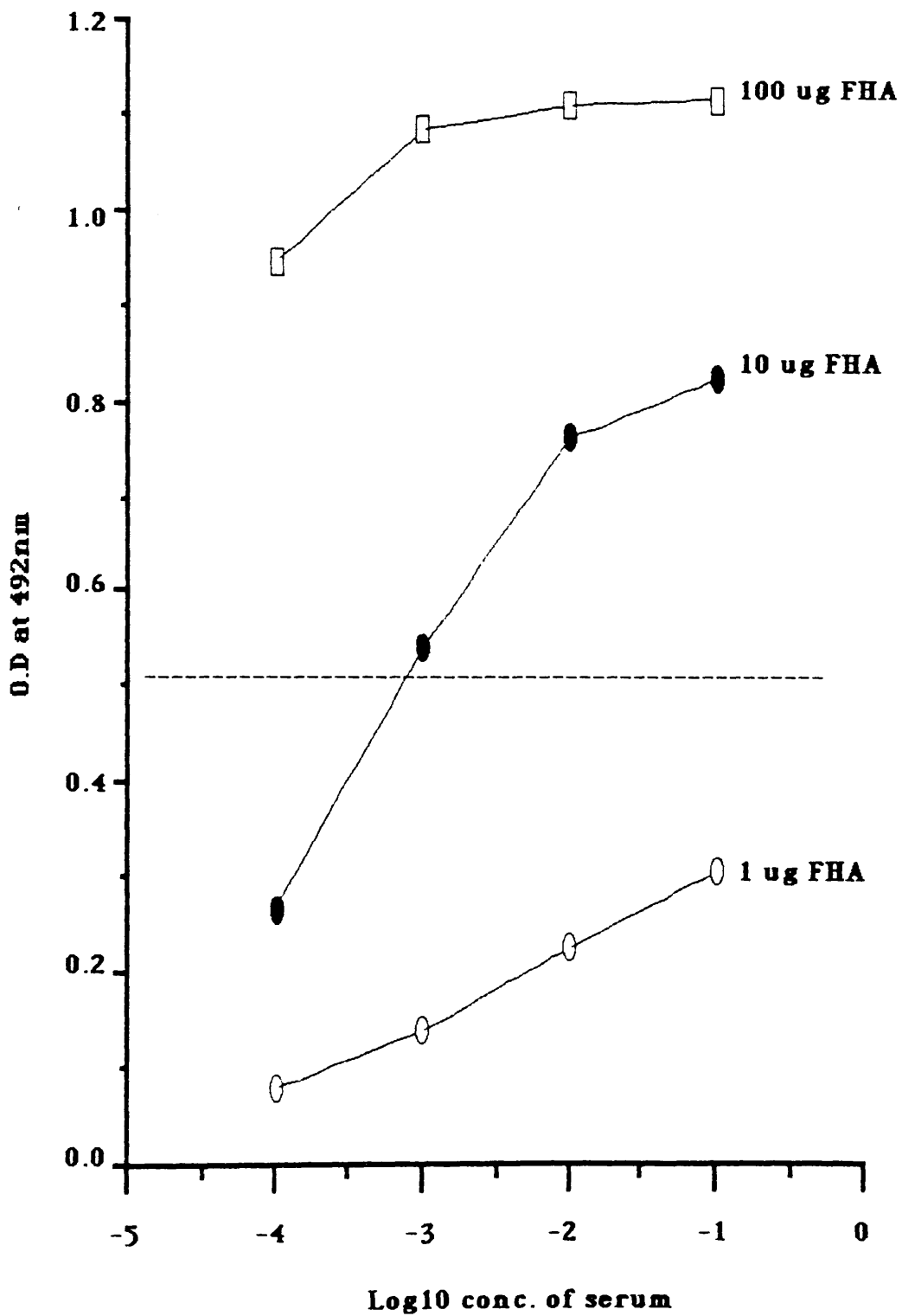
*Effect of concentration of FHA in the sandwich layer.* With the FHA being scarce, time-consuming and expensive to prepare, the 100  $\mu$ g/ml concentration in the sandwich layer was thought to be excessive. Therefore the FHA in the sandwich layer was varied, so as to find the lowest concentration which would give an adequate dose-response line. In Fig 24, this was shown to be the 100  $\mu$ g/ml FHA coating which

**Fig 24: IgE ELISA: Effect of varying the FHA concentration in the sandwich layer, with a constant AP anti-FHA coating concentration (20 µg/ml) in the sandwich ELISA. An anti-FHA IgE positive serum raised by the three-injection protocol was used. Sheep anti-mouse IgE HRP conjugate was used at 1 in 100 dilution.**





**Fig 25: IgG1 ELISA: Effect of FHA coating concentration on the measurement of IgG1 by conventional ELISA.** The ELISA was done with a known anti-FHA IgG1 positive serum with the sheep anti-mouse IgG1 HRP at 1 in 1000 dilution.



allowed the mouse anti-FHA serum to yield a titre of 1300. The 10  $\mu\text{g/ml}$  coating yielded a titre of 700, and the 1  $\mu\text{g/ml}$  a titre of 260. There was an approximate two-fold difference between the titres of 100  $\mu\text{g/ml}$  FHA and 10  $\mu\text{g/ml}$  FHA. The dose response with 10  $\mu\text{g/ml}$  was satisfactory for further work. Therefore, to be economical with reagent, this dose was chosen. This allowed a large number of sera to be titrated in the sandwich ELISA without using large amounts of a scarce reagent.

### **Measurement of IgG1 by conventional ELISA**

#### **Effect of FHA coating concentration**

In the conventional ELISA, there was a requirement for the FHA to be titrated to find a suitable concentration for coating. The 100  $\mu\text{g/ml}$  FHA coating concentration was most promising, but still unsatisfactory since over the mouse serum dilutions which were used, the dose-response line did not intercept the 0.5 absorbance endpoint (Fig. 25). Conversely, the 1  $\mu\text{g/ml}$  FHA coating was too weak even with the 1 in 10 dilution of IgG1-positive serum. However, the 10  $\mu\text{g/ml}$  FHA gave a good response line in that it intersected the absorbance 0.5 and also allowed economical use of the FHA. This concentration was chosen for subsequent experiments.

#### **Comparison of anti-FHA sera tested in PCA, sandwich and conventional ELISA.**

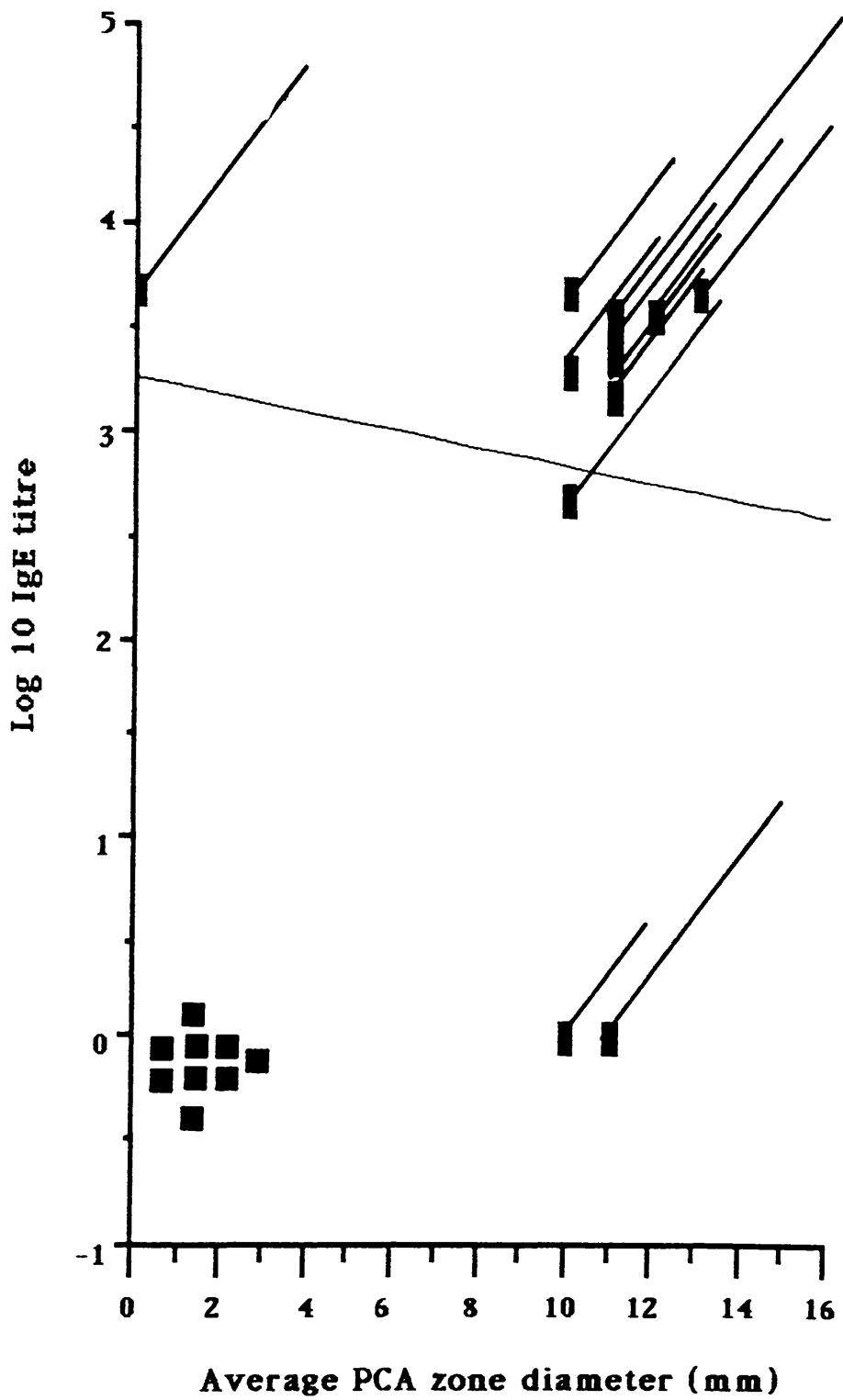
Having raised anti-FHA sera, using a three-dose protocol and in two different mouse strains, it was important to detect and titrate the IgE and IgG1 that might be present. Two methods were used: the 7-day PCA for detection of IgE; the sandwich ELISA for IgE titration, and the conventional ELISA for IgG1 titration. Table 23, shows the results obtained in each of these systems. In the 7-day PCA, a unitage or titre could not be ascribed to the sera, as the PCA reactivity diminished to  $> 5\text{mm}$  after even a 1 in 5 dilution. In this table however, the 7-day PCA and  $\log_{10}$  IgE titre were correlated, in that where there was a positive PCA reaction, IgE could usually be detected by ELISA and *vice versa*. Only in one case (serum G) was this correlation absent. Fig 26, shows the average zone diameters plotted against the  $\log_{10}$  IgE titres. The lines, coming from the points, represent the IgG1 titre of the particular serum. A point to note is that sera which were PCA-negative were ELISA-negative as regards IgE titre and did not elicit an IgG1 response. Conversely, the high IgE titre sera also had high IgG1 responses. Thus there seemed to be a correlation between high IgG1 levels and high IgE levels in serum. The average PCA zone diameter and  $\log_{10}$  IgE titres showed an

**Table 23: Comparison of anti-FHA pooled sera by 7-day PCA, by conventional ELISA for IgG1 and by sandwich ELISA for IgE. All sera consisted of pools of mouse bloods. Experiments 38 and 39 were pools of 10 and expts. 78 and 79 were pools of 5.**

Experiment no. and mouse strain <sup>a</sup>	Serum no.	7 day PCA (mm)	log10 IgE titre	log10 IgG1 titre
38	3	11	3.6	6.8
Ham/ICR	1	10.75	3.4	4.3
	2	9.5	2.7	5.3
39	3	12	3.6	4.8
Ham/ICR	2	11	3.5	4.9
	1	10.75	3.2	3.9
78	D	13	3.7	4.7
Ham/ICR	E	10	3.7	4.0
	F	10	<1	3.9
	A	0	<1	<1
	B	0	<1	<1
	C	0	<1	<1
	G	0	3.7	5.0
79	F-2	11	<1	6.0
Balb/c	E-2	10	3.3	4.0
	A-2	0	<1	<1
	B-2	0	<1	<1
	C-2	0	<1	<1
	D-2	0	<1	<1
	G-2	0	<1	4.0

<sup>a</sup> - The sera were obtained by immunization protocol 3 with the mixtures listed in Appendix 7. The sera were tested undiluted in the 7-day PCA test.

**Fig 26: Correlation of anti-FHA IgE, measured by PCA and ELISA, with IgG1 measured in parallel.** The bars represent the log<sub>10</sub> IgG1 titre where 1 cm is equivalent to 1 log<sub>10</sub>. The line starts at a log<sub>10</sub> IgG1 titre of 2 as the 1 in 100 dilution was the lowest used in the IgG1 ELISA



**Table 24 : Characterization of 9 batches of PT purified by gel-affinity chromatography.** The protein content of each batch was measured by the method of Lowry *et al.*, (1952) with BSA as standard, and the batches were variously examined for histamine sensitization activity and/or the 5 distinct bands characteristic of PT or, other proteins, by SDS-PAGE. The batches highlighted in bold type were used for immunizations.

Batch	Vol. culture (L)	Vol. purified PT (ml)	Protein content ( $\mu\text{g/ml}$ )	SDS-PAGE		HSA test
				PT <sup>a</sup> subunits	approx. % contamination	
1	6	15	800	+	5	+
<b>2</b>	<b>6</b>	<b>20</b>	<b>1600</b>	+	<b>5</b>	+
3	6	10	400	+	5	n.t
4	6	40	500	n.t	n.t	n.t
5	6	45	200	n.t	n.t	n.t
<b>6</b>	<b>8</b>	<b>40</b>	<b>700</b>	+	<b>15</b>	+
<b>7</b>	<b>8</b>	<b>50</b>	<b>600</b>	+	<b>20</b>	+
8	6	50	400	+	20	n.t
9	6	20	600	+	25	n.t

n.t - not tested

<sup>a</sup> - The five bands characteristic of PT in SDS-PAGE have molecular weights of 9, 12, 22, 23 and 28 kDa.

almost significant positive correlation  $p=0.057$  using the Kendall correlation statistics. In conclusion, anti-FHA IgE responses seemed to occur only in association with high anti-FHA IgG1 responses and there is the suggestion of correlation between the two.

## **IgE Responses to Pertussis Toxin, with IgG1 Measured in Parallel**

### **Measurement of anti-PT IgE by PCA test**

As before, with Oa and FHA, it was important to develop assays for detecting and measuring the possible IgE responses to PT in sera of PT-immunized mice. These involved, as before, a 7-day PCA test and a sandwich ELISA for IgE, and conventional ELISA for IgG1. In the sandwich ELISA, instead of AP anti-PT antibody used at the coating stage, fetuin from foetal calf serum which is known to bind PT, via sialic residues, was used as initial coating. In addition, the effect of different toxoided preparations of PT were investigated for their IgE-inducing abilities alone and with bioactive PT as adjuvant.

**Characterization of PT.** All batches of PT were examined by the method of Lowry, (1951) for protein content, and some batches were examined for the typical 5-band appearance of PT in SDS-PAGE. In important batches, viz those highlighted in Table 24, tests for histamine sensitization activity were done.

Nine batches of PT were purified in all. Batch numbers 2, 6 and 7 were used for immunizations and, in particular, batch 2 was used for toxoiding with either glutaraldehyde or carbodiimide. The yield was usually high but yields for batches 4 and 5 were low. This was thought to be due to the lowering of the blue sepharose binding capacity with repeated use. A new batch of blue sepharose was used in the batches 6 to 9, and the yields increased markedly. The percentage contamination with other protein tended to be low (around 10%). However there seemed to be an increase with increasing batch number, perhaps due to the FHA-minus strain reverting on subculture. Thus the later batches started to show a faint line of FHA at 200 kDa. Histamine-sensitizing activity (HSA) tests were carried out on the batches used in immunizations. The HSA test involved groups of 4 mice being injected with graded doses of PT from 0.11  $\mu\text{g}$  to 3  $\mu\text{g}$ /mouse. The animals were challenged after 5 days with 3 mg histamine per mouse, the survivors scored 2-3 h later and the LD 50 estimated. In appendix 6, the approx. LD 50 of batches 1, 2, 6 and 7 were 0.1, 1.5, 0.5 and 0.5  $\mu\text{g}$ /mouse respectively. When the PT was toxoided with glutaraldehyde and carbodiimide, the LD 50 was  $>3$  when compared with the untoxoided PT which had a



LD 50 of 1.5  $\mu\text{g}/\text{mouse}$ . This meant that the toxoiding of the PT had reduced its activity at least 2-fold and probably much more.

These toxoids were also tested in the mouse weight gain test (MWGT), which involved a large doses of toxoid being administered i.p and the weight gain recorded over a 6-day period and compared with a control group which received only the diluent, PBS. In appendix 6, shows that even when large doses of toxoids were injected the mice gained weight in a similar fashion to the controls, with the weights consistently increasing over the 6-day time interval. From preliminary experiments in the Oa system (Table. 16), when bioactive PT at 5  $\mu\text{g}$  and 10  $\mu\text{g}$  were given as adjuvant in immunization a large percentage of the mice (15% and 50%) died within the first few days. With the much higher doses of 25  $\mu\text{g}$  carbodiimide-PT per mouse and 50  $\mu\text{g}$  glutaraldehyde-PT per mouse, there was no toxicity in any of the animals and they gained weight progressively. Therefore, in conclusion, by toxoiding PT, the HSF activity was reduced more than two-fold and when large concentrations of toxoid were used in the MWGT there was no detrimental effect on the animals as they gained weight similarly to controls.

#### **Factors influencing the PCA test**

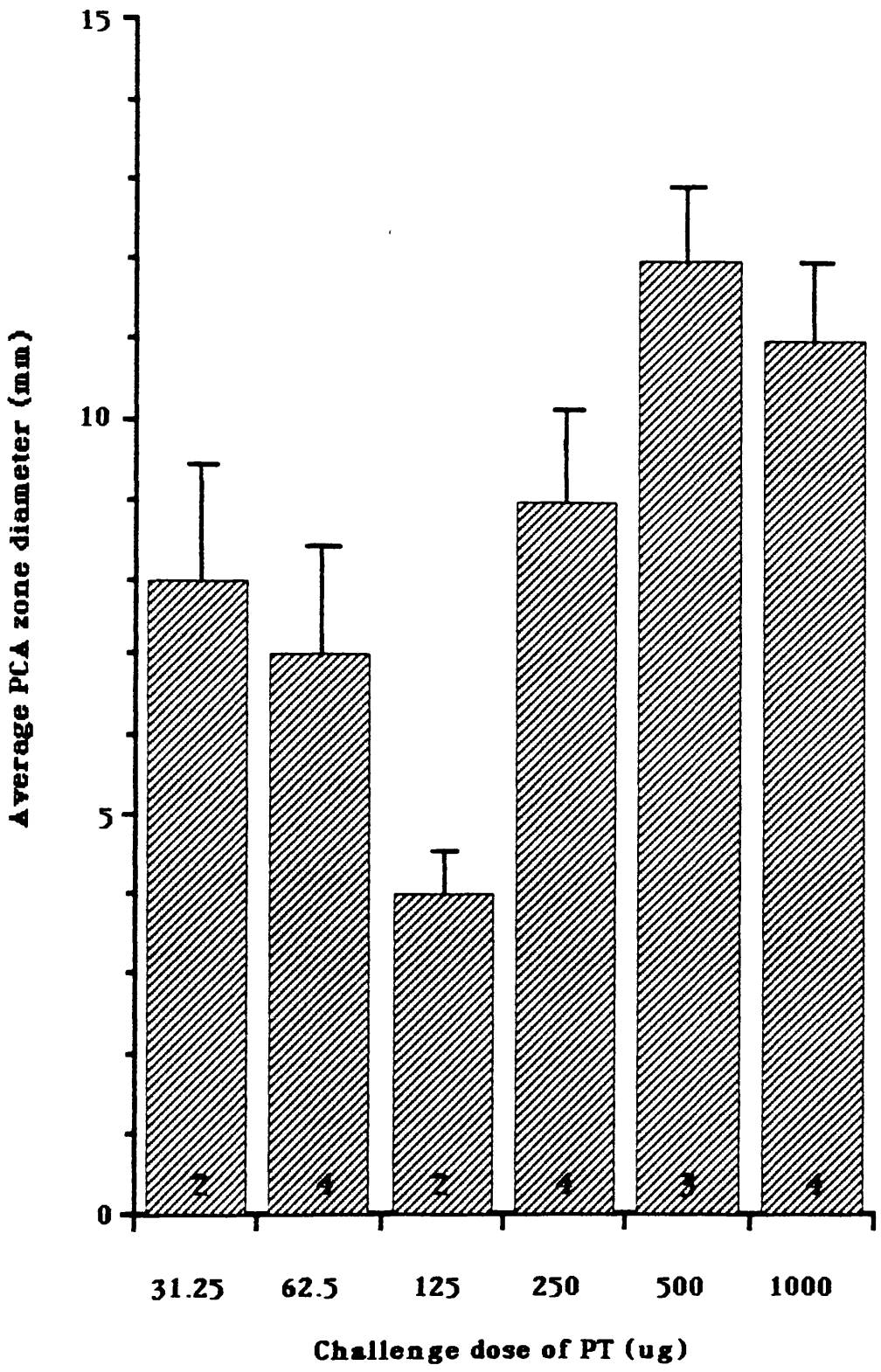
Most of the factors and problems encountered in the PCA test had been resolved in the Oa and FHA systems. In the PT system, it only remained for the effect of having bioactive PT in the challenge dose and an illustration of four sites per mouse to be looked at in detail.

***Effect of PT in the challenge dose.*** There is evidence (Fig. 3) in the Oa section that PT may have a fast-acting inhibitory effect on histamine release, resulting in significant reduction in the average PCA zone diameters. This effect was prominent at low (0.1 and 1.0  $\mu\text{g}$ ) and high (250  $\mu\text{g}$ ) dose levels of PT, when injected along with Oa, in the anti-Oa IgE 48 h PCA.

Using undiluted IgE-containing anti-PT serum for skin sensitizing and a 7-day sensitization interval, mice were challenged with Evans blue mixed with bioactive PT in doses varying from 31.25  $\mu\text{g}$  to 1000  $\mu\text{g}$  in two fold steps (Fig. 27). The most strongly positive PCA reactions occurred with 500  $\mu\text{g}$  and 1000  $\mu\text{g}$  PT doses in the challenge mixture. The animals challenged with 125  $\mu\text{g}$  PT dose produced a PCA zone diameter of only 4 mm, a 3-fold decrease from that obtained with the 500  $\mu\text{g}$  challenge dose of PT. When analysing these results, the possible inhibitory effect of PT has to be taken into account. If the 500  $\mu\text{g}$  is the preferred challenge dose of PT in the 7-day PCA, then this amount of PT may also be having an inhibitory effect on anti-PT IgE

**Fig 27: Effect of varying the challenge dose of PT in the 7-day PCA test.**

Sera raised by the three-dose protocol and known to be IgE-positive were injected into all mice; 7 days later, mice were challenged i.v with bioactive PT. The bars indicate the average zone diameter (mm) and the SEM with the number of replicates shown inside the bars.



**Plate 5: Layout of the PCA test with 4 sites per mouse.** At positions B and C, anti-PT IgE rich serum was injected i.d. The animals were challenged i.v with 500 µg PT and Evans blue, 7 days after sensitization. Positions A and D were injected with the same sera heated to 56° C for 1h.

**Table 25: Effect of a single dose of glutaraldehyde PT vaccine on the one dose protocol in mice from 5 individual mouse lines with positive anti-PT IgE response.**

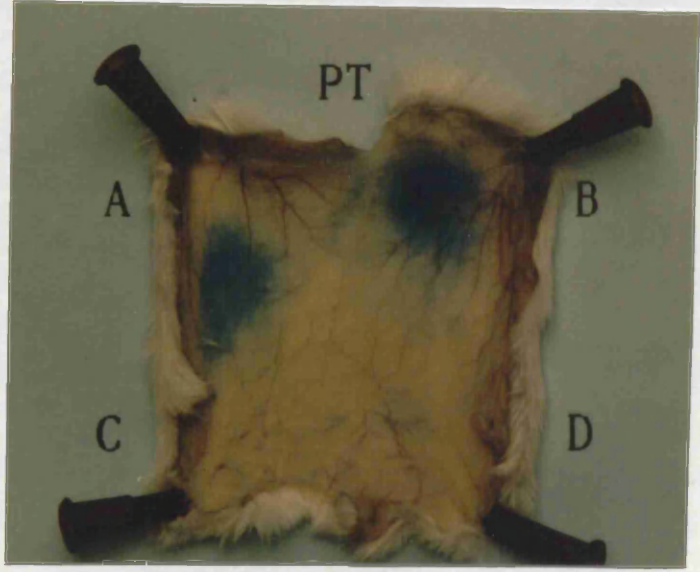
Immunization dose of PT (µg)	Adjuvant dose of PT (µg)
zero	zero

Strain	ICR
0.1	0.1
1	1
5	5
10	10
20	20
50	50
0.1	0.1
10	10

Strain	ICR
0.1	0.1
1	1
5	5
10	10
20	20
50	50
0.1	0.1
10	10

Strain	ICR
0.1	0.1
1	1
5	5
10	10
20	20
5	5

0.1 - not tested



**Table 25: Effect of a range of immunizing doses of carbodiimide and glutaraldehyde PT toxoids, when mixed with a constant single dose of bioactive PT (1 µg), on the development of an anti-PT IgE response by the one dose protocol in both Ham/ICR and Balb/c mice. Sera were pooled from 5 individual mouse bloods and were tested undiluted in 7-day PCA tests. The positive anti-PT IgE responses are shown in bold type.**

Immunization dose of PT toxoid on day zero	Adjuvant dose of PT (1 µg)	No. of PCA positive pooled sera on bleed day, tested in 7-day PCA, using either a carbodiimide or a glutaraldehyde PT toxoid for immunization					
		10		21		28	
		Carb.	Glut.	Carb.	Glut.	Carb.	Glut.
<b>Ham/ICR</b>							
0.1	+	0/1	0/1	0/1	0/1	0/1	0/1
1	+	0/1	0/1	0/1	<b>1/1</b>	0/1	0/1
5	+	<b>1/1</b>	0/1	0/1	0/1	0/1	0/1
10	+	0/1	0/1	0/1	0/1	0/1	0/1
20	+	n.t	0/1	n.t	0/1	n.t	n.t
50	+	n.t	0/1	n.t	0/1	n.t	0/1
0.1	-	0/1	n.t	n.t	0/1	n.t	n.t
10	-	n.t	0/1	0/1	n.t	n.t	0/1
<b>Balb/c</b>							
0.1	+	0/1	0/1	0/1	0/1	0/1	0/1
1	+	0/1	0/1	0/1	0/1	0/1	0/1
5	+	0/1	0/1	0/1	0/1	0/1	0/1
10	+	n.t	0/1	n.t	0/1	n.t	0/1
20	+	0/1	0/1	0/1	0/1	0/1	0/1
5	-	0/1	n.t	n.t	0/1	n.t	0/1

n.t - not tested

detection in serum, indicated by the blueing reaction. Plate 5 illustrates the 7-day PCA test. An anti-PT IgE serum was injected i.d at positions B and C and the same serum heated to 56° C for 1 h at positions A and D, as indicated by the arrows on the sketch. The animal was challenged with 500 µg PT i.v 7 days after sensitization. A positive 7-day PCA response can be seen at positions C and B only, illustrating that heating at 56° C destroys IgE and the PCA reactivity disappears.

### **Anti-PT IgE responses after a one-dose immunization with carbodiimide and glutaraldehyde toxoids.**

A one-dose immunization protocol involving different doses of two PT toxoids and a constant dose of bioactive PT was investigated for possible IgE responsiveness in the 7-day PCA. Two mouse strains were used : Ham/ICR and Balb/c. The bleed days were, as before, days 10, 21 and 28 days. Similarly to the FHA system, when the sera were pools of blood from 5 mice, the 7-day PCA yielded only 2 positive responses out of 64 sera tested (Table 25). Those protocols producing an IgE-positive serum involved 5 µg carbodiimide-PT and a 10-day bleed, and 1 µg glutaraldehyde-PT and a 21-day bleed. Each of the above sera was raised with injection mixtures that contained 1 µg bioactive PT. When PT toxoid alone was for immunization, there was no detectable IgE response with either of the toxoids, on any bleed day, nor with either the Ham/ICR or Balb/c strains of mice.

The only positive anti-PT IgE sera were raised in Ham/ICR mice, the Balb/c sera were all negative. However there may be a requirement for the sera raised in Balb/c to be tested in Balb/c.

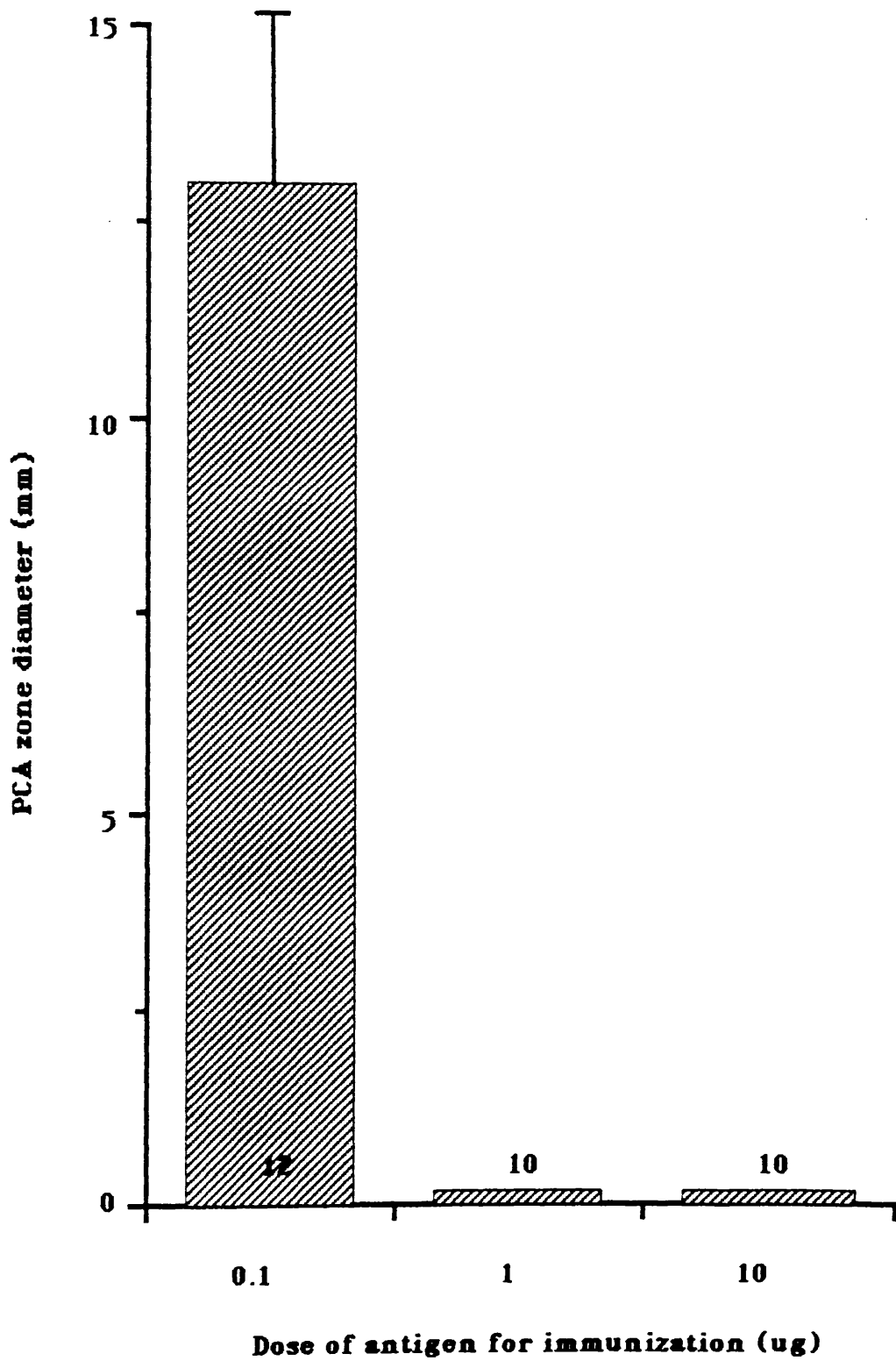
### **Anti-PT IgE responses after a three-dose immunization**

Initially, a mixed PT and FHA carbodiimide toxoid was used in the 3-dose immunization procedure. However in subsequent experiments, only PT-alone toxoids (without FHA) prepared by carbodiimide or glutaraldehyde treatment were tested for PCA reactivity.

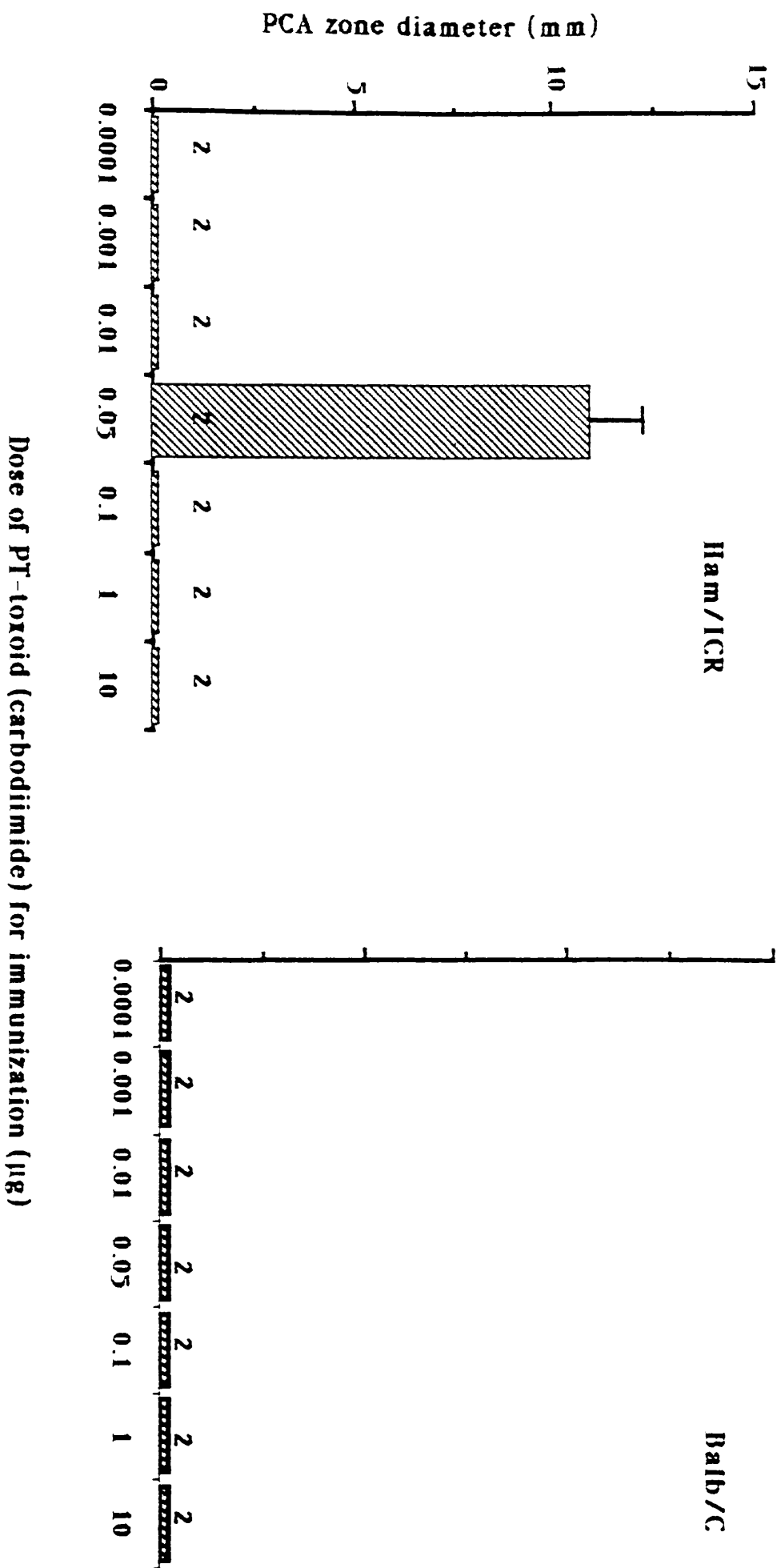
***Carbodiimide toxoid of FHA/PT.*** The effect of different doses of a carbodiimide toxoid of PT and FHA was investigated for the production of IgE antibodies to PT and tested by 7-day PCA (Fig. 28). It will be seen that an IgE response was produced only at the lowest immunizing dose of 0.1 µg. The actual amount of PT in the 0.1 µg dose of PT/FHA toxoid was 0.05 µg, as the PT and FHA in the toxoid were present in approximately equal amounts (Christodoulides *et al.*, 1987). Therefore the IgE response to PT differs from that found to Oa and FHA, in that it was produced only at very low immunizing doses. Surprisingly, no detectable IgE response could be found after the 1 µg and 10 µg immunizing doses of the PT/FHA toxoid.

**Fig 28: Application of the 7-day PCA test to detect IgE antibodies to PT in mice after FHA/PT carbodiimide immunization.** Sera were raised by the three-dose protocol with the immunizing doses indicated on the abscissa. The bars represent the average zone diameter and SEM of observations with undiluted serum. Figures inside the bars indicate the number of zones observed.

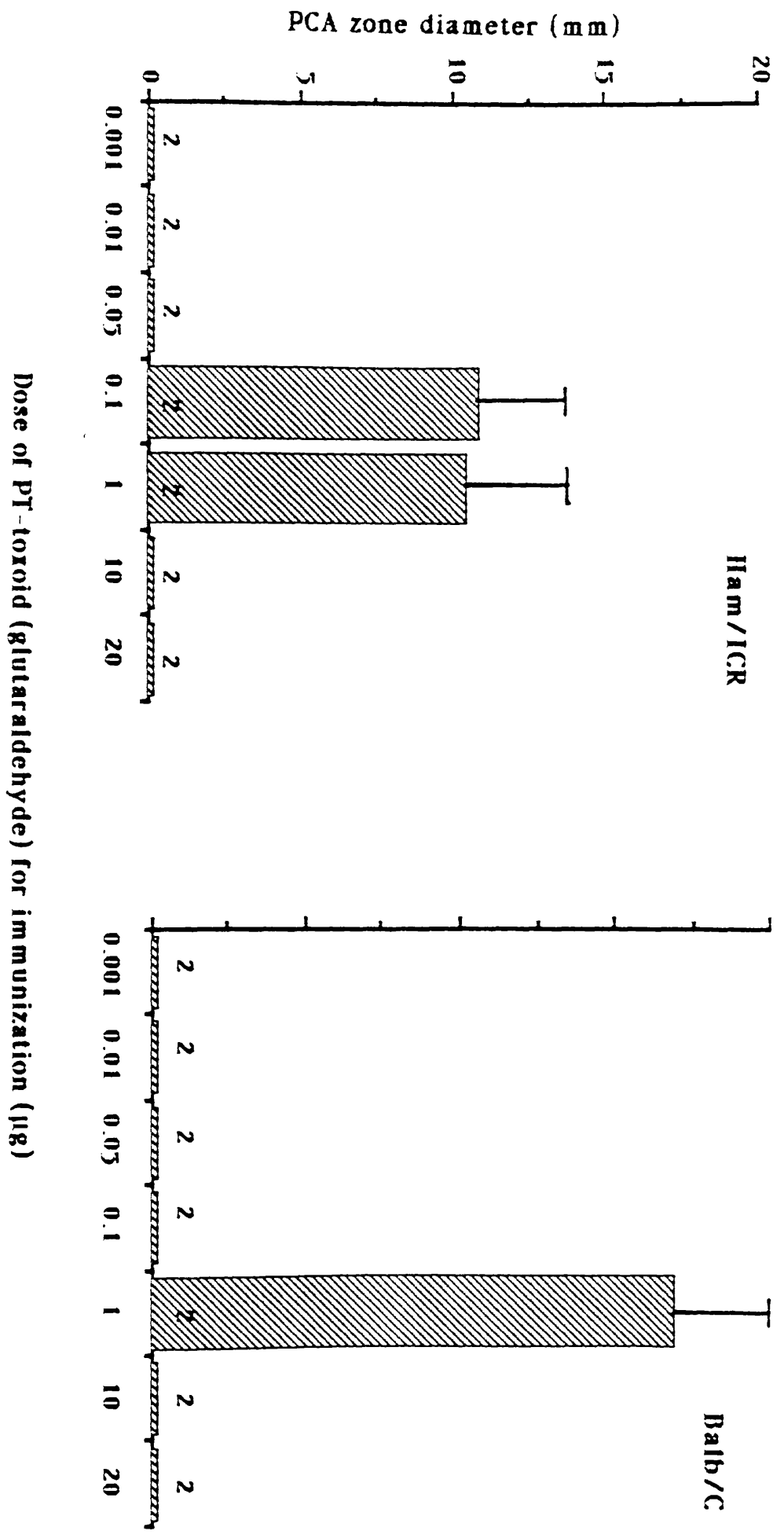




**Fig 29: Effect of different immunizing doses of carbodiimide toxoids of PT, when mixed with a constant dose of bioactive PT (1 µg), on the development of anti-PT IgE in Ham/ICR and Balb/c mice. Sera were obtained by the three-dose protocol and tested undiluted in the 7-day PCA test. The bars indicate the average zone diameter (mm) and SEM with the number of replicates inside the bars.**



**Fig 30: Effect of different immunizing doses of glutaraldehyde toxoids of PT, when mixed with a constant dose of bioactive PT (1  $\mu$ g), on the development of anti-PT IgE in Ham/ICR and Balb/c mice. Sera were obtained by the three-dose protocol and tested undiluted in the 7-day PCA test. The bars indicate the average zone diameters (mm) and SEM with the number of replicates inside the bars.**



Dose of PT-toxoid (glutaraldehyde) for immunization (µg)

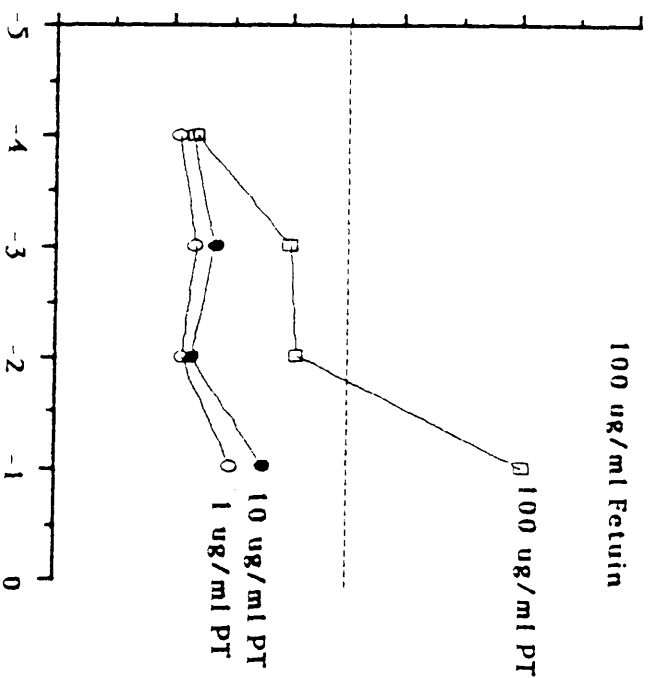
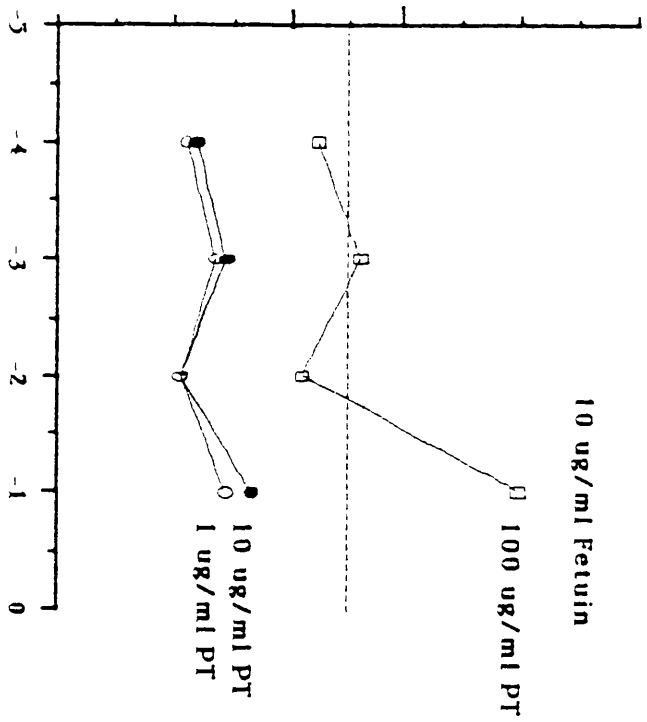
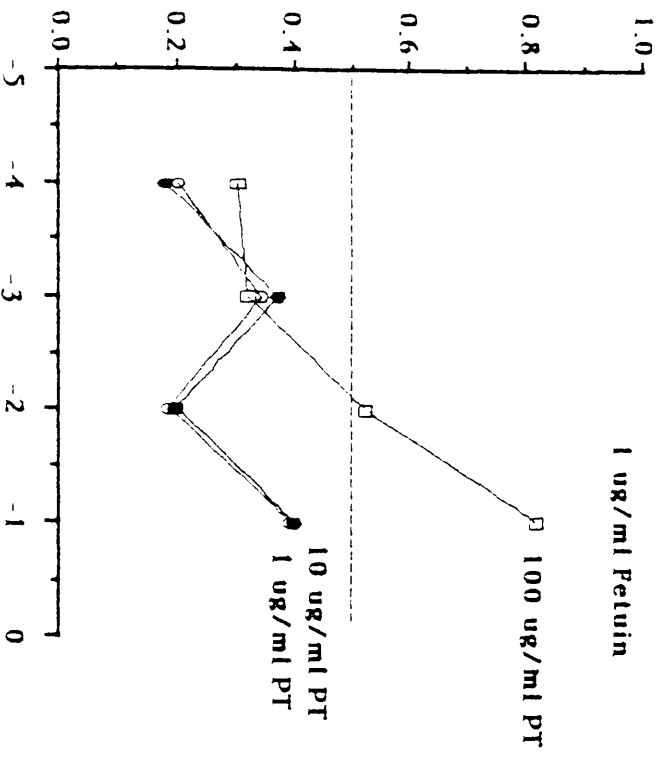
***Carbodiimide toxoid of PT*** In the above system, only three different immunizing doses of PT/FHA toxoid were used. In Fig. 29, a range of immunizing doses were explored using a carbodiimide toxoid of PT alone, which did not contain any detectable FHA. Using the 7-day PCA, an anti-PT IgE response was elicited at only one dose of toxoid (0.05  $\mu$ g). Conversely, with sera raised in Balb/c mice, there was no detectable 7-day PCA reactivity. The results in Ham/ICR were similar to those found in the initial experiment with PT/FHA toxoid. The PT toxoid in the mixture there was at a dose, only of 0.05  $\mu$ g. This showed that the channelling of anti-PT IgE occurred only at one particular dose of the immunizing antigen and could not be detected in any of the 6 other doses tested. The significance of this observation may be difficult to evaluate but may be a result of the diverse biological properties of PT and PT toxoids.

***Glutaraldehyde toxoid of PT*** In previous work (Hayglass and Strejan, 1983), glutaraldehyde "toxoiding" of Oa was found to diminish IgE antibody production. Therefore PT was toxoided with glutaraldehyde to observe if it had a similar effect. This toxoiding was done by the method of Munoz *et al.*, (1981) and the product was used for immunizing Balb/c and Ham/ICR mice, with bioactive PT (1  $\mu$ g) as adjuvant in the three-injection protocol. As with the carbodiimide results, Fig. 30 shows an anti-PT IgE response was produced only over a very narrow immunizing dose range. The glutaraldehyde PT differed from the carbodiimide PT in that the highest IgE responses were detected in sera raised in Balb/c mice with the 1  $\mu$ g immunizing dose of toxoid. These sera gave average PCA zone diameter of 16 mm. Ham/ICR mice responded with an anti-PT IgE response at 0.1 and 1  $\mu$ g immunizing doses of PT in the three-injection protocol. If anything, the glutaraldehyde treatment of PT seemed to be stimulating the IgE response when compared with the carbodiimide treatment, instead of suppressing the IgE response as reported in the Oa system. However, the carbodiimide toxoids produced the IgE response at 0.05  $\mu$ g immunizing toxoid dose, whereas the glutaraldehyde toxoid stimulated IgE both at 0.1 and 1  $\mu$ g. The carbodiimide PT toxoid seemed to be more potent in that less was required to elicit an IgE response.

### **Measurement of IgE and IgG1 by sandwich ELISA**

Previously, the sandwich ELISA had involved an affinity-purified antibody directed against the antigen in question, either Oa or FHA. In the anti-PT sandwich IgE and IgG1 ELISA, the AP antibody was replaced by fetuin, a protein with a large number of sialic acid residues and which binds avidly to both PT and to plastic. Without initial coating of the plates with fetuin, the PT does not bind well. In the forthcoming

**Fig 31: Effects of altering the fetuin coating and PT binding concentrations in the sandwich ELISA for anti-PT IgE.** The serum was known to be IgE-positive by 7-day PCA and the sheep anti-mouse IgE HRP conjugate was used at a constant dilution of 1 in 100.



log 10 conc. of serum



section, the effect of varying the PT and fetuin concentration in the IgE sandwich ELISA is reported.

### **Effect of concentration of fetuin and PT in the sandwich layer**

Initially, microtitre plates were coated at pH 9.6 with different concentrations of fetuin (1  $\mu\text{g}$ , 10  $\mu\text{g}$  and 100  $\mu\text{g}/\text{ml}$ ). After an overnight exposure at 4<sup>o</sup> C, PT was added at concentrations ranging from 1  $\mu\text{g}/\text{ml}$  to 100  $\mu\text{g}/\text{ml}$ .

As shown in Fig 31, the coating concentration of fetuin seemed to have little effect on the anti-PT IgE ELISA, as the same pattern of responses was observed with all the concentrations of fetuin. However it should be noted that the dose-response line only intercepted the endpoint absorbance of 0.5 when the 100  $\mu\text{g}/\text{ml}$  PT was used. With the 1  $\mu\text{g}$  and 10  $\mu\text{g}/\text{ml}$  PT, and irrespective of the fetuin concentration, the absorbance values of the test sera were always below 0.5, at all dilutions. Therefore in this system, the PT had to be used at a concentration of 100  $\mu\text{g}/\text{ml}$  but since all the fetuin concentrations gave similar results, the lowest fetuin concentration of 1  $\mu\text{g}/\text{ml}$  was favoured.

In appendix 8, there is a calculation to determine whether solution of fetuin at 1  $\mu\text{g}/\text{ml}$  could coat the surface of a microtitre well with a protein layer that was at least one-molecule thick. This was done by taking the surface area of the microtitre well and the molarity of fetuin at 1  $\mu\text{g}/\text{ml}$ , and assuming that the diameter of the molecule would be similar to Oa, which has a similar molecular weight. The outcome was that  $2 \times 10^{13}$  molecules of fetuin would be required to coat the well one molecule thick. In 100  $\mu\text{l}$  of 1  $\mu\text{g}/\text{ml}$  fetuin, there would be approximately  $10^{18}$  molecules, a 300,000-fold excess. Therefore in 1  $\mu\text{g}/\text{ml}$  fetuin, there should be a massive excess of molecules able to bind to the plastic and take part in the ELISA.

### **Comparison of anti-PT sera tested in PCA, sandwich and conventional ELISA.**

Anti-PT IgE sera were raised in both Ham/ICR and Balb/c mice and against three different toxoids of PT using the three-injection protocol (Table. 26).

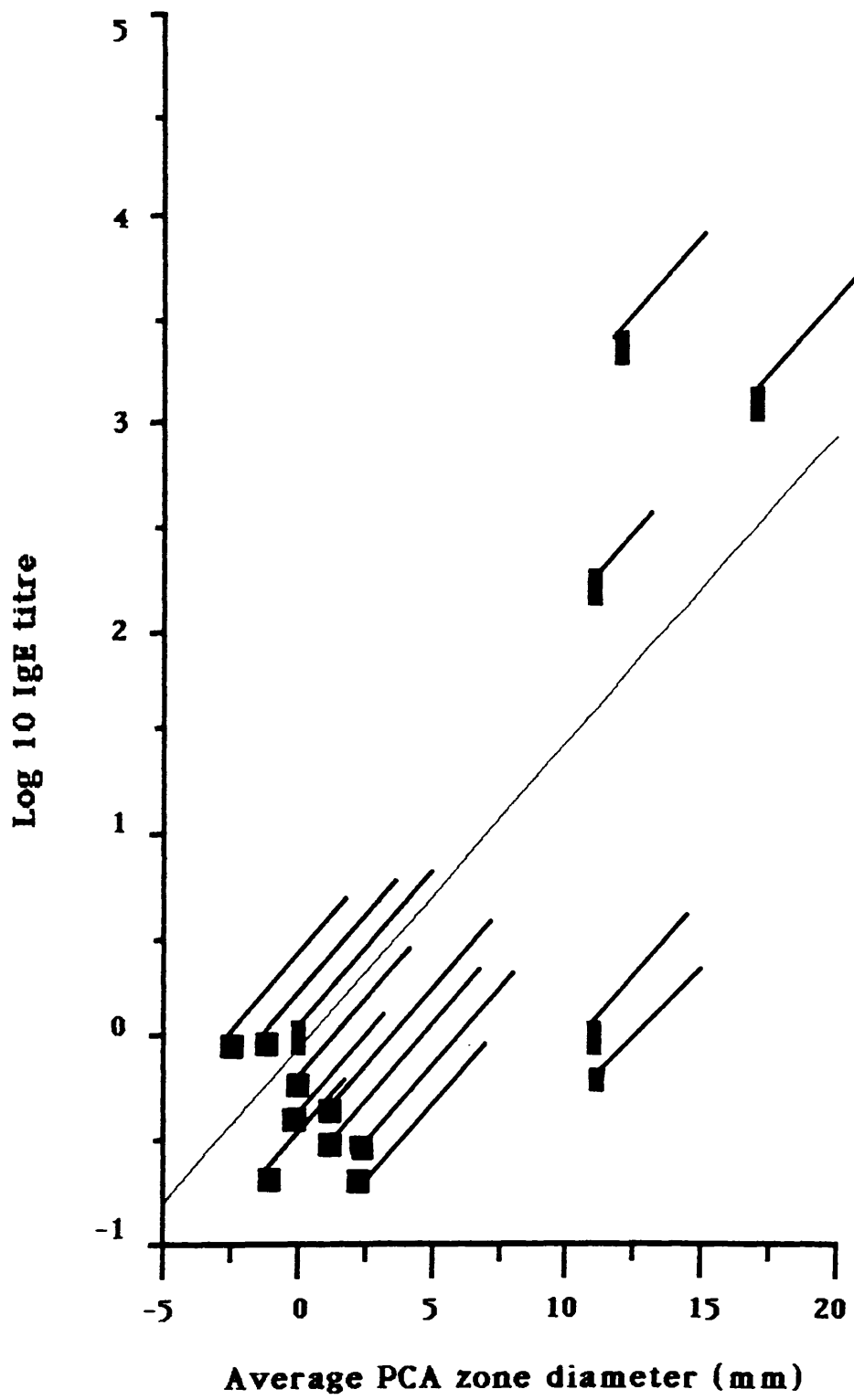
The sera were tested in the 7-day PCA test and in the sandwich ELISA. As in Figs. 28, 29, 30 an anti-PT IgE response was produced only over a very narrow immunizing dose range, and was independent of the toxoid or the mouse strain. However the fetuin PT ELISA was not as sensitive or specific as the AP sandwich ELISA for Oa and FHA, and therefore there were discrepancies between PCA and ELISA. In all, five sera reacted positively in the 7-day PCA, but of those only 3 gave a definite titre in the IgE ELISA. Conversely to the FHA system, anti-PT IgE antibodies

**Table 26: Comparison of anti-PT pooled sera tested by 7-day PCA, by conventional ELISA for IgG1 and by sandwich ELISA for IgE. All sera consisted of pools of mouse bloods. Experiment 43 was a pool of 10 and expts. 78 and 79 were pools of 5.**

Experiment no. and mouse strain	Serum no. <sup>a</sup>	7 day PCA (mm)	log10 IgE titre	log10 IgG1 titre
43 Ham/ICR	1	12	3.4	3.4
	2	0	<1	4.5
	3	0	<1	4.8
78 Ham/ICR	K	11	<1	4.0
	L	11	<1	4.0
	H	0	<1	1.9
	I	0	<1	3.6
	J	0	<1	2.8
	M	0	<1	5.0
79 Balb/c	L-2	16.8	3.1	4.0
	H-2	0	<1	4.8
	I-2	0	<1	3.7
	J-2	0	<1	4.0
	K-2	0	<1	3.5
	M-2	0	<1	5.0
	N-2	0	<1	5.0
78 Ham/ICR	R	10.5	2.2	3.3

<sup>a</sup> - The sera were obtained by immunization protocol 3 with the mixtures listed in Appendix 7. The sera were tested undiluted in the 7-day PCA test.

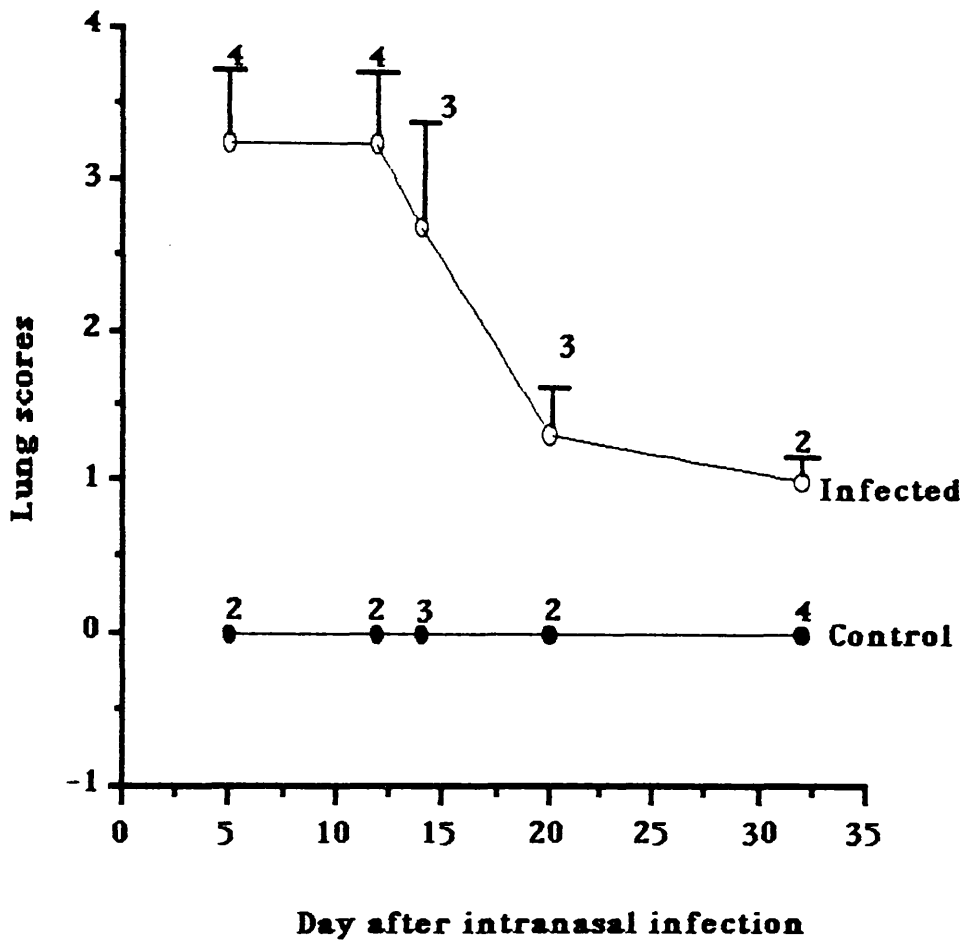
**Fig 32: Correlation of anti-PT IgE measured by PCA and ELISA with IgG1 measured in parallel.** The bars represent the log<sub>10</sub> IgG1 titre where 1 cm is equivalent to 1 log<sub>10</sub>. The line starts at a log<sub>10</sub> IgG1 titre of 2 as the 1 in 100 dilution was the lowest used in the IgG1 ELISA



**Table 27: Comparison of sera from intranasally infected mice tested in the conventional and sandwich ELISA. All pooled sera were tested for IgE by sandwich and IgG1 by conventional ELISA.**

Bleed day (after infection)	log10 IgE titre		log10 IgG1 titre	
	PT	FHA	PT	FHA
	5	<1	<1	<1
12	<1	<1	1.5	<1
14	<1	<1	1.2	<1
20	<1	<1	<1	<1
32	<1	1.5	<1	<1

**Fig 33: Lung scores from Ham/ICR mice infected i.n with *B. pertussis*BP 18323.** The lungs were scored by the number of lobes which were infected. The open points indicate the infected group and the solid points indicate the control animals that only received casamino acids. The points show the average lung scores and the SEM along with the number of mice.



were only produced when the IgG1 content of the serum was low (Fig. 32). The non-responding serum in the PCA and ELISA all showed very high IgG1 titres. Using the Kendall correlation test, and comparing the IgE detected by PCA and by ELISA, there was a definite trend but no significant correlation in that IgE to PT was only produced over a narrow immunizing dose range and that high levels of IgG1 may play an important role in suppression of anti-PT IgE.

**Comparison of sera from intranasally infected mice tested in the conventional ELISA for IgG1 and in the sandwich ELISA for IgE.**

Mice were infected i.n with *B.pertussis* strain BP 18323. Their weight after infection was recorded, and groups of 2 to 5 mice were sacrificed at approximately 5-day intervals for serum and for lung examination for signs of infection. Table 27, gives an indication of the low immunogenicity of FHA and PT as regards IgG1 and IgE production under these conditions. There were slight detectable IgG1 responses at days 12 and 14, but the  $\log_{10}$  IgG1 titres to PT reached only 1.48 and 1.18 respectively. A small anti-FHA IgE response was detected at day 32 by ELISA. At all the other bleed days, neither IgE nor IgG1 responses to FHA or PT could be detected. The lung pathology scores of the animals showed that significant infection had taken place (Fig. 33). The infected group had widespread lung infection (4 lobes) at 12 days after the i.n challenge. The severity of the disease decreased thereafter but was still apparent at day 32. All the controls, who received only casamino acids, but were kept in the same cages as the infected animals, were all clear of any lung infection. Perhaps the secretory IgA and IgM response might be more prominent during the short duration of the experiment and IgG and IgE might be more prevalent in the convalescent stage.



## DISCUSSION

## DISCUSSION

This investigation has shown that in mice, IgE antibodies to at least two of the major virulence factors of *B. pertussis*, namely FHA and PT can be induced under appropriate conditions of antigen and adjuvant dose, time of bleeding and mouse strain. The significance of IgE production in *B. pertussis* infection and after pertussis vaccination will be discussed.

### Detection of IgE by PCA

Although the 48 h PCA test for IgE had been in existence for 30 years (Ovary, 1958), at the start of this investigation it proved inadequate for detecting IgE responses to FHA and PT of *B. pertussis*. Thus the detection of anti-FHA and anti-PT IgE was crucially dependent on extending the sensitization period from 48 h to 7 days. In fact, early in this work with Oa as the model antigen, it was necessary to explore various aspects of the conduct of the PCA test in order to apply it to IgE quantitation.

### Mouse strain

It is important, in the PCA test, to find a recipient animal species and strain which will react well when injected with serum raised in another individual. The species of choice for PCA is stated to be the guinea pig (Ovary and Warner, 1972) but the PCA test has also been done in the rabbit, the rat and the mouse (Ovary, 1976). According to Ovary (1964), less antibody was required to produce a positive reaction in the guinea pig than that required for the same reaction in the mouse.

In the present work, for reasons of convenience and availability, female Ham/ICR albino mice (6-8 weeks old) were used for the PCA test. Generally females were preferred because males tend to fight and cause scarring of the dorsal skin with consequent non-specific blueing. Justus and Saelinger, (1976) examined three different mouse strains CFW, ICR and C57Bl/6J. They found that ICR mice were most sensitive to histamine, whereas C57Bl/6J were least sensitive. CFW proved to be the best PCA recipients of test sera, when the sera had been raised in the CFW mouse strain. This seems to contradict Ovary *et al.*, (1975) who had previously shown that CFW mice were inadequate for mouse IgE titration. As well as differences between strains, there may be marked variability in the PCA-reactivity of individual animals within a strain (Ovary, 1986). Therefore it may be necessary for sera to be tested in more than one recipient. All sera in the present study were therefore tested more than once, to overcome the problem of between-animal variability.

### Route of injection

Ham/ICR mice were clipped, as chemical depilation was known to irritate the skin and lead to non-specific reactions (Ovary, 1976). The clipping was done on the dorsal sides

of the back before the intra-dermal injection of test sera. The midline of the back contains slightly fewer mast cells than the dorsal sides of the back. Injections were therefore not done on the midline of the back because of the reported low reactivity in this area (Watanabe & Ovary, 1977). Anti-Oa IgE sera were usually serially diluted in 2-fold steps and injected i.d into 2 sites on the dorsal sides of different mice. Four injection sites (Levine and Vaz, 1970) and up to a maximum of 6 sites (Watanabe, 1976) have been reported. With 6 sites per mouse, the size of the animal and the size of the response make this difficult, as each injection site should be at least 1 cm or more from the others. In the FHA and PT PCA tests, the antigen was costly to prepare and was needed in large amounts for PCA challenge. Therefore the number of injection sites on each animal was increased to 4. One of these sites was assigned to a standard anti-Oa IgE serum which was used as a positive control in the PCA test. The animals, after the sensitization interval, were challenged i.v with a mixture of Evans blue and Oa and either FHA or PT depending on the test serum. The Oa reacted with the anti-Oa IgE positive serum which produced a blueing reaction in the mouse. This meant there was the expectation of a positive blueing reaction in all the animals with which to compare the test sera and also to check the PCA-reactivity of the individual recipient mouse. In the literature, there seems to be no mention of this particular method of PCA, although other types of control mice are common. These involve positive sera being injected into one animal and the PCA reaction compared with test sera injected into other animals of the same batch (Justus and Saelinger, 1976; Ito *et al.*, 1988). The advantage of the method developed in the present study is that the individual recipient mouse is checked for reactivity, as also is the success of the i.v challenge injection.

### **Sensitization period**

The sensitization period (SP) in the mouse PCA, between i.d injection of test serum and i.v challenge with antigen is reported to be optimal at 2 h for IgG1 and at 48-72 h for IgE (Ovary, 1958, 1976). However in my work, I found that for IgE, the SP for detection of IgE could usefully be extended to 7 days.

In the Oa system, with anti-Oa serum, a 48 h SP produced distinct PCA reactions with well defined spots of blueing on the undersurface of the skin. Moreover these reactions were confirmed as being due to IgE by showing abolition of the reaction with 56° C heated serum. However when anti-FHA sera with FHA challenge, and anti-PT sera with PT challenge, were tested in the PCA, the 48 h SP gave a diffuse blueing on the whole undersurface of the skin with only a slightly darker region at the site of injection. When these sera were diluted, the diffuse blueing disappeared but so also did any positive PCA reactivity. Clausen *et al.*, (1969) also noted this diffuse blueing in the skin with a 72 h SP and Oa as the challenge antigen. A possible explanation is that IgG1 antibodies, responsible for the 2 h PCA in mice, may still be

interfering in the 48h PCA (Lehrer *et al.*, 1975, 1976; Clausen *et al.*, 1968, 1969). The actual mechanism of interference is unclear but may be a result of antigen-specific IgG1 in the serum. IgG1 binds to mast cells for a few hours, but may remain in the skin for 24 to 48 h (Ovary, 1982), presumably when present in high concentrations. When the challenge antigen is injected, it may interact with IgG1 in the circulation and perhaps also fixed to mast cells throughout the body causing an overall histamine-releasing effect and therefore the diffuse blueing. Lehrer, (1977) reported that in some instances IgE was not detected, due to a masking effect of IgG1. In studies with IgE myeloma protein, mouse IgE and IgG1 had different receptor sites on mast cells. Therefore the masking effect of IgG1 is not a result of competing for the same receptor sites. In contrast, Binaghi and Badoux, (1976) observed that in rats, both IgE and IgGa antibodies acted in an additive way in the resultant PCA when IgE antibody was injected at the same site 24 h before IgGa, and challenged with antigen 2 h later. Apart from IgG1 involvement in the 48 h PCA, Watanabe *et al.*, (1983) also suggested that pre-existing IgE molecules on the mast cells in the skin interfere in the PCA.

As was stated previously, IgG1 can remain in the skin for 24 to 48 h (Ovary, 1982) and this may have had a detrimental effect on observing a positive 48 h PCA reaction. In the present study, the SP was increased from between 2 to 7 days in the hope that such interfering antibody would diffuse out of the skin. In fact, the effect of increasing the SP allowed a positive reaction to be scored at 6 days, which increased slightly after 7 days. Thus the diffuse blueing ceased to be a problem. This is interpreted as the interfering antibodies diffusing away, after the long SP, leaving only the IgE tightly bound to mast cells. Mota (1964) had previously shown that anti-Oa IgE could still be detected in the skin at 31 days, but activity peaked at 72 h. Hirano *et al.*, (1983) reported that the half-life of i.d injected murine IgE antibody was 6 days. In my work, by extending the interval of the PCA test to 7 days, there was a marked beneficial effect on the detection of IgE and a resolution of the problem of diffuse blueing.

### **Dose of Antigen**

The dose of challenge antigen in the PCA test was also a crucial factor for consideration, as it determines the amount of IgE-specific antibody detectable in the test sera. High levels of specific antibody require less antigen to elicit a 48 h PCA-positive reaction. In the 48 h PCA test for anti-Oa IgE, and the 7-day PCA tests for anti-FHA and anti-PT, the optimal concentration of challenge dose was found to be 1 mg, 0.5 mg and 0.5 mg/mouse respectively. Ovary *et al.*, (1983) also observed that the best dose of antigen was around 0.5 mg/mouse in most PCA systems. Specifically, Watanabe *et al.*, (1983) observed that 0.5 mg of *Ascaris* extracts was enough to elicit an anti-*Ascaris* IgE response in the rat PCA. The amount given i.v is also affected by the

**Table 28: The effect of molecular weight of antigen in the PCA test.**

Antigen	Molecular weight (kDa)	Minimal amount of challenge antigen for PCA, with Ab nitrogen ( $\mu\text{g}$ ) *	
		1	0.025
Ribonuclease	13	0.05	0.63
Egg albumin	45	0.31	10
Bovine serum albumin	70	0.63	31
Haemocyanin	650	14.0	100

Data from Ovary, 1958, 1976

\*Ab N ( $\mu\text{g}$ ) is amount injected i.d and challenge antigen is the amount of challenged i.v in the PCA test for IgE

molecular size of the antigen. The larger the molecular weight of the antigen, the more was required for a PCA response even with large amounts of specific antibody (Table 28). On this basis, haemocyanin with a molecular weight of 650 kDa would be required at a dose of 14  $\mu$ g per mouse to elicit a positive PCA response with a large (1  $\mu$ g) dose of specific antibody, whereas Oa would be required at only 0.31  $\mu$ g to elicit the same response with 1  $\mu$ g anti-Oa. In our study, the Oa (45 kDa) challenge was found to be optimal at 1 mg, but the PT (100 kDa) and FHA (200 kDa) challenge doses were optimal at 500  $\mu$ g although the amount of specific antibody used were not standardized. In the Oa system the optimal dose was found by injecting a 1 in 20 dilution of anti-Oa IgE positive serum into mice and challenging them with different concentrations of Oa. In the FHA and PT system, undiluted sera were used. This may relate to the optimal concentration differences between Oa and FHA and PT because the anti-Oa sera was diluted and so there would be a requirement for more Oa in the challenge. Undiluted sera were used in the FHA and PT system because with the 7-day sensitization period, a positive reaction could not be elicited when the sera were diluted. However, without knowing the amount of specific antibody in the test serum this hypothesis is difficult to prove.

### **Effect of heating**

Previously it has been reported that when serum is heated to 56<sup>o</sup> C for 30 min, the 48 h PCA reactivity is abolished (Mota and Piexoto, 1966; Clausen *et al.*, 1969, 1970). On this basis that this treatment destroys IgE, it has been used to provide IgE-negative controls in the 48 h PCA. However, in the present work, a particularly high titre anti-Oa IgE serum, heated to 56<sup>o</sup> C for 30 min, did not lose all of its 48 h PCA activity. In fact, approximately 10% of the initial activity was still detectable. The heating interval was therefore increased to 1 h, which caused apparently complete lose of 48 h PCA reactivity.

The actual mechanism involved in this inactivation has been studied by Dorrington and Bennich, (1973) who found that after a 30 min heating, the intact IgE showed changes in both the aromatic side chains and peptide bond spectral regions. They also noticed that these effects were partially reversed upon cooling to 25<sup>o</sup> C. The irreversible transition of IgE was localised in the Fc region, viz. the site that interacts with mast cells, but the Fab remained intact as the heated molecules still reacted with specific antigen.

In our study, the heating procedure was always performed on undiluted serum as diluting serum in a solution of high solute concentration has a preventative effect on thermolability (Binaghi and Demeulemester, 1983). They observed that 50-100% of the 48 h PCA activity of rat and mouse antisera is recovered after heating to 56<sup>o</sup> C for 1.5 to

5 h, in solutions of 1M MgSO<sub>4</sub> or 2M glucose respectively. This protection is thought to be a result of steric hindrance, between molecules of IgE in solutions containing high concentrations of ions or hexose molecules.

The failure to inactivate completely our high titre IgE serum by heating to 56° C for 30 min was not due to high ionic protection of the IgE molecule, since the serum was heated undiluted. It is more likely to have been due to the initial high potency of the serum. Our results suggested that there was 90% inactivation after 30 min; therefore after 1 h, the inactivation would be expected to reach 99%.

### **Effect of PT in the Oa challenge**

PT has a vast array of biological activities. It was important therefore to ascertain any possible effects of injecting large doses of PT, as part of the challenge dose, in the 48 h PCA with the Oa system before exploring PCA tests with PT in the challenge injection and anti-PT used for sensitization. An inhibitory effect of PT, incorporated in the Oa challenge was noted with anti-Oa serum in the 48 h PCA test. Very low (0.1 and 1 µg) and high (125 and 250 µg) concentrations of PT, when mixed with 1 mg Oa, caused a significant reduction in the recorded PCA zone diameters. This effect took place within the normal 30 min observation period and if caused by PT, is a very fast acting biological activity of PT. Munoz and Anacker, (1959) had previously found that bioactive PT had no effect on PCA induced with anti-Oa and Oa. More recently, Nakamura and Michio, (1983) observed that when small concentrations (0.1-3 µg) of PT were administered 3 days before *Ascaris* antigen challenge, the resultant PCA was suppressed. They did not explore injecting PT in the actual challenge dose, but did find that by injecting small doses of PT, just before the antigen challenge, had no effect on the resultant 48 h PCA zone diameters when compared to controls which had been injected with *Ascaris* alone.

Our results are contrary to the above, in that as little as 0.1 µg PT had a significant inhibitory effect on the 48 h PCA, when injected with the Oa challenge. The actual mechanism causing this suppression is still unknown, as PT usually requires a longer lag time, of the order of hours, for its biological activities to be manifest (Katada and Ui, 1980). This is due to the time required for the binding of the B subunit to target cell surface and for the A component of PT to traverse the plasma membrane of cells and to ADP-ribosylate key molecules within the cell which, in turn, alter the cells responses to various exogenous stimuli. Although, Karlsson *et al.*, (1985) reported that PT blocked noradrenaline-induced pigment aggregation in fish melanophores. This effect had a maximal effect in 10 minutes.

In the 7-day PCA test with anti-PT and PT, an IgE response was detected but the possible inhibitory effects of the 500 µg PT in the challenge dose was not investigated.

In view of the findings with Oa, a suppression of the PCA is most likely. This could have been investigated with toxoids of PT as challenge, mixed with different amounts of bioactive PT. Therefore the PCA test may not be the most sensitive test for detecting IgE against PT antigen. In the following section, an *in vitro* test, the enzyme linked immunosorbent assay (ELISA) will be discussed as an alternative to the PCA test for detection and quantitation of IgE.

### **Detection of IgE and IgG1 by ELISA**

Although *in vitro* tests for detection of IgE have been available since 1967, with the initial discovery of the radioallergosorbent test by Wide *et al.*, (1967), the first report of an IgE ELISA was by Zeiss *et al.*, (1977)

A main aim of our study was to detect IgE antibodies to components of *B. pertussis* using either the PCA test or the *in vitro* test ELISA. However, before study of *B. pertussis* antigens, extensive preparatory work was done with Oa as the model antigen. In parallel IgG1, which will be discussed first, was detected with a conventional ELISA but this ELISA was not sensitive or specific enough for IgE, which eventually was assayed successfully by sandwich ELISA. IgG1 antibody is thought to play a role in IgE regulation and as a masking antibody in both PCA and ELISA detection systems.

### **Detection of IgG1 by conventional ELISA**

The ELISA was devised by Engvall and Perlmann, (1972) as a simple and sensitive method for quantifying antibodies in serum. The first antigen used in this system was dinitrophenyl-ovalbumin (DNP-Oa). In our work, Oa was considered as a suitable model antigen in the conventional IgG1 ELISA. However there was no information on the measurement of IgG1 responses to Oa in mice by this method.

The first step in the conventional ELISA involved coating microtitre plates with the protein antigen. The type of plate used can be a crucial limiting factor in very sensitive systems, such as for IgE. Kenemy *et al.*, (1986) reported that, in an allergen IgE ELISA, the type of plate was critical and that irradiated plates generally gave best results. In our studies, the Nunc F maxisorp plate was used routinely, after testing a variety of other plates. In fact, they all acted similarly in the conventional IgG1 ELISA with Oa. However, Kenny <sup>and Dunsmoor,</sup> (1983) observed that adsorption of BSA varied with the type of plate, and that certain plates were ineffective for measuring anti-BSA IgG antibodies. The differences in BSA adsorption between plates may be a result of detachment of the coating protein during incubation and washing. Proteins bind by hydrophobic interactions with the plastic, and overcoating can lead to a reduction in binding of the protein because a monolayer of antigen cannot be maintained and antigen molecules already absorbed can become detached during the test, and hence



give reduced sensitivity and reproducibility (McLaren *et al.*, 1981)

It is important that different coating concentrations are explored in the ELISA so as to find the one most suitable. In our system, the optimal Oa and FHA coating concentration, for detection of IgG1, was 100 µg/ml. Kenny *et al.*, (1983) also found that rabbit anti-Oa IgG bound optimally to Oa when the Oa coating concentration was 100 µg/ml, whereas BSA coating was optimal at 0.1 µg/ml. These concentrations were also dependent on the type of plate. McLaren *et al.*, (1981) have reviewed the literature and found that generally the best coating concentrations in the ELISA were in the range of 1 µg to 10 µg/ml, although they stated that higher concentrations have been used. Bidwell *et al.*, (1976) advised that in all systems, the best antigen concentration should be established in preliminary experiments.

### **Detection of IgE by conventional ELISA**

In the present work, the conventional ELISA was first investigated as a method to quantitate IgE levels in serum. Initially, a major problem was high background levels with normal serum and with control wells which had not had any test serum added. These problems were thought to be partly due to incomplete blocking of unreacted sites on the plastic of the microtitre plates. A number of different blocking agents were therefore tested in the system including foetal calf serum, horse serum, skimmed milk and gelatin. Although BSA at 2% (w/v) gave the best results. Other investigators, using different antigen/antibody systems have used BSA as the blocking agent in the IgE ELISA (Hamilton *et al.*, 1981; Gaveriaux *et al.*, 1986; Hirano *et al.*, 1988) but the effect of different blocking agents was not reported. If a blocking agent is not incorporated into the ELISA, sites on the plates can remain uncoated, which allows non-specific serum proteins or even conjugate to bind and affect assay sensitivity and specificity.

Other antibody classes have also been reported to interfere with the IgE ELISA, by competing for the antigen coating on the plate (Zeiss *et al.*, 1977; Kenemy *et al.*, 1985; Lee *et al.*, 1988). IgG is usually the predominant immunoglobulin in serum whereas IgE is found in minute quantities, which can be increased by helminth infection (Capron *et al.*, 1975; Dessein *et al.*, 1981) or immunization (Clausen *et al.*, 1969, 1970). Therefore other classes of specific antibody in an IgE ELISA can be a major problem. Protein A of *S. aureus* binds to the Fc region of IgG and has been used to deplete IgG from serum. Conversely, however our experiments showed that instead of decreasing IgG, upon the final absorption, the IgG titre apparently was dramatically increased. This could possibly have been due to the commercially-available protein A becoming detached from the *S. aureus*, and remaining soluble in serum, with IgG bound to it and thereby increasing the IgG titre detected in the ELISA instead of decreasing it. Protein A adsorption should only have affected the IgG titre but it also caused a decrease in the IgE titres. Perhaps, the IgE molecules were being masked by

**Table 29 :Problems encountered by various investigators with ELISA**

Detrimental effect on:	Source of problem	References
Sensitivity	Choice of plastic for plates	Kenemy <i>et al.</i> , (1986)
	Coating antigen concentration	Kenny <sup>and Donsmaer,</sup> (1983)
	Low titre conjugates	McLaren <i>et al.</i> , (1981)
	Insufficient blocking	Hamilton <i>et al.</i> , (1981)
Specificity	Quality of antigen	Wong and Skelton, (1988)
	Interfering antibodies	Finger and Wirsing von Koenig (1985)
	Choice of plastic	Kenemy <i>et al.</i> , (1986)
	Incubation period	Engvall and Perlmann, (1972)
	Quality of conjugate	McLaren <i>et al.</i> , (1981)

the increase in IgG in the system. This method of IgG reduction was abandoned in our experiments although it has been shown to have a beneficial effect in some types of ELISA (Finger and Wirsing von Konig, 1985). However, another commercially-available form of Protein A was used in this study. Hamilton *et al.*, (1981) found it difficult to detect IgE to *Brugia malayi* antigens. They extracted IgG from immune serum and found this procedure did not affect detection of IgE by RIA, but it did enhance the IgE ELISA. Thus IgG may interfere with the IgE ELISA. In a sense, the effect of IgG in the ELISA may be similar to that in the diffuse blueing problems encountered in the PCA, thought to be due to IgG1.

The conventional ELISA was tried with 48 h anti-Oa serum which was PCA-positive, and therefore should have contained anti-Oa IgE. The serum was assayed before and after heating for 30 min at 56° C. Earlier, the secondary antibody goat anti-mouse IgE and the sheep anti-mouse IgE-HRP conjugate had been titrated. All the absorbance readings at 492 nm were very low. These antibodies may have deteriorated on storage which may be a possible reason for the titres being lower than expected. Kenemy *et al.*, (1986) also observed very low absorbance readings in the conventional ELISA for IgE. They gave no explanation for this effect but did suggest that the coating stage was critical. They also explored a sandwich ELISA in parallel and found it to be much more sensitive.

Thus the conventional ELISA for IgE was abandoned because of high background levels and massive day to day variability, which reduced its sensitivity and specificity.

Some of the factors causing these problems are highlighted in Table 29. The conventional IgG1 ELISA, however, was found to be sensitive, specific and did not suffer from high background levels which were found to be a problem in the IgE ELISA.

### **Detection of IgE by modified ELISA**

Underestimation, or masking, of antigen-specific IgE and which is caused by specific antibody of other immunoglobulin classes has been documented in RAST (Zeiss *et al.*, 1981) and in the ELISA (Reid *et al.*, 1985; Vassella *et al.*, 1990).

To overcome this problem, Kenemy *et al.*, (1985) devised an IgE ELISA which involved an initial coating with an affinity-purified (AP) rabbit antibody directed against mite or grass pollen. They found that the AP antibodies bound antigen to the plate and greatly increased the assay sensitivity without increasing background absorbances. Our study showed that by binding Oa and FHA to the plate with AP rabbit anti-Oa and anti-FHA respectively, specific IgE antibodies in the test serum could be readily quantified. The 56° C control and the normal serum controls were always at baseline levels. An absorbance at 492nm of 0.5 was taken as the endpoint and used to calculate the serum titre. This method of quantitation has been used previously

(Bidwell *et al.*, 1976, Granstrom *et al.*, 1982).

The concentration of AP antibody was titrated to find the most appropriate dilution for IgE detection. In both Oa and FHA IgE ELISA, the concentration of AP antibody was critical and the assay only worked when the coating was within certain ranges. As little as 0.175  $\mu\text{g}/\text{ml}$  of AP anti-Oa and 20  $\mu\text{g}/\text{ml}$  of AP anti-FHA were optimal for coating, in conjunction with 100  $\mu\text{g}/\text{ml}$  Oa and 10  $\mu\text{g}/\text{ml}$  FHA for the sandwich layer. Kenemy *et al.*, (1985) found that 10  $\mu\text{g}/\text{ml}$  of mite antigen was optimal in the sandwich layer for binding monoclonal anti-P1 or poly-specific rabbit anti-mite at 1 in 1000 dilution. However, they did not give the actual concentration of the monoclonal or AP polyspecific antibodies. These authors used a very high concentration of anti-IgE conjugate (1 in 30 dilution) whereas a 1 in 100 dilution of the sheep anti-mouse IgE-HRP was sufficient in our ELISA. They also noted that the addition of large amounts of antigen did not affect the assay, although I found that the concentration of both AP antibody and antigen were crucial.

In the PT IgE and IgG1 ELISA, fetuin was used to bind the PT to the microtitre plates. Fetuin was used in place of the AP antibodies, and successfully bound the PT with high affinity (Wong and Skelton, 1988). The coating concentration of fetuin was not critical in that as 1  $\mu\text{g}/\text{ml}$  and 100  $\mu\text{g}/\text{ml}$  gave similar results, but only when the PT concentration was 100  $\mu\text{g}/\text{ml}$ . In Appendix 8, there is a calculation for the area of a microtitre plate and the number of fetuin molecules required to coat it. Even with the 1  $\mu\text{g}/\text{ml}$  fetuin solution, there was roughly a 300,000-fold excess of molecules for coating the plastic with a complete layer of protein one-molecule thick. Therefore with the 10 and 100  $\mu\text{g}/\text{ml}$  fetuin concentrations, there was a vast excess.

PT is known to bind to fetuin via sialic acid residues on the glycoprotein. There is not a requirement in the fetuin ELISA for highly purified PT, as the fetuin binds PT specifically. Burstyn *et al.*, (1983) performed an IgG ELISA with purified PT, which did not involve an initial fetuin coating stage, but reported that to do an ELISA with purified PT routinely for diagnosis of pertussis would be difficult and expensive. The fetuin ELISA does not suffer from these problems as a partially purified PT can be used. Wong and Skelton, (1988) coated plates with 25  $\mu\text{g}/\text{ml}$  fetuin and required only 2.5 - 5  $\mu\text{g}/\text{ml}$  of PT in the sandwich layer for an IgG, IgA and IgM ELISA. In our study, we needed less fetuin (1  $\mu\text{g}/\text{ml}$ ) but more PT (100  $\mu\text{g}/\text{ml}$ ) in both the IgG1 and IgE ELISA. Our work differed in some respects from that of Wong and Skelton, (1988). They used a different strain (NIH) of *B. pertussis* which may have given better yields of PT in the partially purified preparations. They also modified the cyclodextrin medium to prevent extraction of unwanted cellular antigens, which may mean that their PT was purer than ours and therefore less was required in the coating stage of the ELISA.

With different sera, when we compared PCA and ELISA there was a positive correlation between reactivity in the PCA and high titres in the modified ELISA for Oa

and FHA. However, the fetuin ELISA for PT, occasionally yielded positive results in the PCA but not in the ELISA and also *vice versa*

In conclusion, the conventional ELISA suffered from a variety of problems including high backgrounds, low specificity and sensitivity and massive day to day variability. However the conventional IgG1 ELISA worked well and did not suffer from the problems encountered in the IgE ELISA. IgG1 was measured because of the possibility of an interfering, or contributory effect in the 48 h PCA. Moreover, the problems of the conventional ELISA were overcome with the sandwich ELISA which involved an initial coating stage with AP-rabbit antibody. This AP-antibody was employed because it bound the Oa, to the plates, and gave the antigen "flexibility", perhaps showing epitopes specific for the IgE antibodies, which may have been hidden in the conventional ELISA, where antigen was bound directly to the plate. Heating the test sera caused a massive reduction in the subsequent IgE sandwich ELISA titres. Heating is known to dramatically affect the Fc region of the antibody. Therefore when conjugate was added it could not bind to the epsilon chain, and the resultant absorbances remained low.

In summary, the fetuin-PT ELISA was applied to measure IgE and IgG1, as fetuin preferentially binds PT to the plate. This particular ELISA was not as sensitive or specific as the AP-modified ELISA used for Oa and FHA, but did show some positive correlation with the corresponding PCA results. The AP-ELISA was the best *in vitro* system, for measuring IgE antibodies, to Oa and FHA. There were no problems in measuring IgG1 by conventional ELISA.

### **Production of IgE**

In order to elicit an IgE response, a number of factors have to be taken into account including strain of animal, timing of injections, dose of antigen and adjuvant and the effect of boosting. A large and varied number of protein antigens can induce IgE antibodies in a range of mammalian species (Jarrett *et al.*, 1974, 1980; Mota *et al.*, 1974; Lehrer *et al.*, 1977).

Factors that favour IgE production include the use of minute but repeated doses of antigen, admixture of the first dose of antigen with Al(OH)<sub>3</sub>, the use of pertussis vaccine and, in particular, PT as adjuvants (Levine and Vaz, 1970; Clausen *et al.*, 1969, 1970). In the present study, a three-injection protocol was best for raising IgE, but the initial one-dose experiments with Oa as the model antigen allowed optimization of the adjuvant PT dose.

In preliminary experiments, it was found that boosting animals on day 14 with antigen alone, after initial immunization with Ag and PT on day zero and bleeding on

day 21 did not give a good IgE response against Oa in Ham/ICR mice. A more successful protocol involved bleeding at day 21, without boosting. However, boosting is thought to provoke an increased secondary IgE immune response. Ishizaka and Okudaira, (1972) reported that with Oa as the antigen and Al(OH)<sub>3</sub> as the adjuvant, boosting increased two-fold the IgE response as detected by PCA. In their experiment, adjuvant was administered as part of the boosting mixture whereas in our experiments the antigen alone was used for boosting. However, the effect of boosting without adjuvant has also been studied. Jarrett *et al.*, (1981) noticed that boosting enhanced the IgE response in rats. Lehrer *et al.*, (1975) examined the variability of different mouse strains in producing IgE after primary stimulation with Oa and HSF (PT). They found that with 100 µg Oa and 10 µg HSF, there was a marked increase in the PCA titre when animals were boosted with as little as 10 µg Oa. These responses were strain-dependent, which probably relates to the genetic make-up, environmental conditions and sex and age of the animals. CBA/J mice were the best IgE-responding strain. The initial treatment of animals with antigen and HSF resulted in the production of memory cells since subsequent immunization with Oa evoked a secondary reagenic antibody response. Lehrer *et al.*, (1976) also speculated that factors similar to PT, which acts as an adjuvant, could be produced by other microorganisms to modulate the immune response of individuals to common allergens.

Relating to allergy, Ishida *et al.*, (1989) repeatedly challenged guinea pigs with Oa and *B. pertussis* intranasally, then subsequently challenged with Oa. This produced cyanosis and breathing difficulties. They also showed that there were increased numbers of eosinophils in the epithelium and airway wall areas. However, they did not quantify or test for IgE, which would have been interesting, to determine whether the above factors related to an increase in IgE production.

Our own single-boosting experiment may not have increased the IgE response because, for example, of the non-optimality in the dose of antigen and adjuvant or the timing of the boost. The boosting experiment was found to be unsuitable for IgE production, therefore a one-dose immunization was tried with bleeding at 10, 21 and 28 days. Two levels of antigen, a low (20 µg) and a high (200 µg) dose were used, and the PT adjuvant doses were varied to find a suitable value for IgE production. Individual sera were tested, since pooling had the potential effect of diluting the IgE-positive sera. The best adjuvant dose was found to be 1 µg PT, with the lower dose of Oa (20 µg) giving the best responses on day 21. The large dose of Oa (200 µg) produced an erratic IgE response over the three bleed days but there was a peak of activity on day 28 with the 1 µg PT adjuvant dose.

Other workers have explored the effect of a one-dose system, and usually there was a primary increase in IgE antibodies at days 10 to 14, as illustrated by Lehrer *et al.*,

(1975, 1976), when  $\text{Al}(\text{OH})_3$  was used as adjuvant. This also caused an increase in IgG1, which appeared after the IgE response. They also noted that the IgE response to Oa with 1  $\mu\text{g}$  HSF (i.e PT), showed an initial peak at day 10, and there was a plateau of activity between days 20 and 40. This seemed to be independent of antigen concentration although the adjuvant doses administered were high (10  $\mu\text{g}$  and 40  $\mu\text{g}$ ), enough to kill an animal. This suggests that their preparation was not just HSF but contained a large amount of contaminating factors. However Suko *et al.*, (1977) found that the IgE antibody response to *Salmonella* - DNP with *B.pertussis* vaccine as adjuvant was highest at 21-30 days with no initial increase at 10 days.

Thus IgE responses are dependent on antigen and adjuvant dose, animal and strain, boosting and even the route of injection. Gerbrandy and Bienenstock, (1976) reported that intratracheal and intraperitoneal immunization routes were most effective for the production of IgE.

One of the main aims of this investigation was to see if it was possible to produce an IgE antibody response against components of *B. pertussis* itself. The one-dose immunization protocol with FHA and PT toxoid as antigens, showed no detectable IgE. Therefore a multiple boosting experiment which had been devised by Levine and Vaz, (1970) was tried in an attempt to induce persistent and boosterable reagin formation in mice, similar to that in allergic humans. In the three-injection scheme, the adjuvant dose of PT (given only on day zero) was kept constant at 1  $\mu\text{g}$ , as it was found to be the most suitable in the one-dose immunization system. The 3-dose schedule involved antigen and adjuvant being injected i.p on Day 0, then the animals boosted on days 28 and 56 with antigen alone and bled on day 63. Initially, the dose range of antigen was kept within the range (0.1 - 10  $\mu\text{g}$ ). The benefit to the IgE response of minute amounts of Oa (0.1  $\mu\text{g}$ ) (Vaz *et al.*, 1971; Schwartz and Levine, 1973; Jarrett and Stewart, 1974; Bazin, 1976) was confirmed. The resulting high IgE titre serum was used as a positive control serum in all experiments with FHA and PT. Pooling the serum did not dilute out the IgE reactivity in the PCA, as had been seen in the one-dose experiment. Therefore a large pool of serum from 10 mice was prepared.

Our immunization protocol differed from that of Levine and Vaz, (1970), because PT was used as adjuvant instead of  $\text{Al}(\text{OH})_3$ , but we also produced high titre IgE sera with Oa as antigen at doses of 0.1  $\mu\text{g}$  and 1  $\mu\text{g}$ . They found that mouse strain was important as animals could be divided into low and high responders. We also found similar results within the Ham/ICR mouse strain where some animals behaved as low and others as high responders.

An anti-FHA IgE response was elicited after the three-injection protocol with all 3 doses (0.1, 1 and 10  $\mu\text{g}$ ) of antigen. Further experiments confirmed this and IgE was also produced at the 20  $\mu\text{g}$  antigen dose level. In a large number of studies by other

workers, usually, only one immunization dose has been used. For example, Sydbom and Karlsson,(1979) immunized rats with one dose (20 µg) of Oa, Suko *et al.*(1977) immunized Balb/c mice with  $2 \times 10^{10}$  DNP-*Salmonella* and Imada *et al.*(1977) 1 µg Oa, all using PT as adjuvant. None reported the effect of varying the antigen dose on the IgE response. In our study the dose effects seemed to fall into 2 distinct categories. The anti-Oa and anti-PT IgE responses were elicited over a narrow dose range, whereas anti-FHA IgE was produced over a wide dose range. This may relate to the channeling system for IgE production in mice, and may be genetically determined, because with both glutaraldehyde and carbodiimide toxoids of PT, Balb/c mice react differently to Ham/ICR. The former strain behaved as non-responders when immunized with the carbodiimide PT toxoid. They did not produce an IgE response at any of the dose levels tested. Ham/ICR mice responded to the 0.05 µg dose of the toxoid. This corresponded precisely to the previous experiment, where a mixed toxoid of FHA and PT (ratio 1:1) only elicited an IgE response at 1 µg viz 0.05 µg FHA and PT. The effect of glutaraldehyde toxoiding was studied, because glutaraldehyde polymers of Oa were found to suppress anti-Oa IgE responses (Hayglass and Strejan, 1983). This time the Balb/c mice responded with an anti-PT IgE response at the 1 µg PT toxoid immunizing dose. Ham/ICR mice only reacted at doses of 0.1 µg and 1 µg glutaraldehyde toxoid and not at 0.05 µg level as in the carbodiimide experiment. Glutaraldehyde treatment apparently therefore destroyed some of the immunogenicity of PT toxoid, ie. more glutaraldehyde toxoid was required to elicit an IgE response. This disagrees with the findings of Hayglass and Strejan,(1983) who reported that an anti-Oa response showed a 8-fold reduction in PCA titre after 14 days when groups of mice were injected i.p on day 0 with 80 µg Oa-polymer (glutaraldehyde) when compared with those animals injected with 80 µg Oa (untreated). In contrast Attallah *et al.* (1975) found that low molecular weight ragweed allergen became immunogenic in rats after glutaraldehyde polymerisation. These differences may relate to the toxoiding process, particularly to the spectrum of molecules produced and their interaction with the animal's immune system. Some molecular clustering may suppress while others may induce IgE production. With the Balb/c mouse, carbodiimide toxoiding may have altered the PT protein structure so much that the antibodies produced could not react with native PT.

Throughout the PT toxoid immunizations, an anti-PT IgE response was only produced within a very narrow immunizing dose range. This may relate to antigen processing of PT toxoid molecules and how they are recognised by T-cells, as IgE production is T-cell dependent for protein antigens. Takatsu and Ishizaka, (1976) reported that anti-Oa IgE antibody formation was depressed if Oa or urea-denatured Oa was injected i.v during the course of the experiment. Using spleen cell transfer, evidence was obtained that this treatment suppressed the expansion of IgE-B memory cells, and when T-suppressor cells from the spleen were transferred to normal mice,



**Table 30: Summary of the IgE and IgG1 responses in mice.** The sera were all produced by the three-dose immunization protocol and tested as pools of five sera in the PT and FHA systems and as a pool of 25 in the Oa system. The antigen dose range is shown. Throughout, bioactive PT (1  $\mu$ g) was used as adjuvant

Antigen	Molecular weight (kDa)	Antigen dose ( $\mu$ g) range producing IgE	Levels of	
			IgE	IgG1
Oa	45	0.1 - 1.0	High	High
PT	100	0.05 - 1.0	High	Low
FHA	200	0.1 - 20	High	High

the primary antibody response to Oa was suppressed.

The antibody responses we detected could be explained by antigen size and IgG1 production. A summary of our results can be seen in Table 30. IgE production may be dependent partly on the molecular weight of the antigen. In particular, a relatively low molecular weight protein antigen such as Oa, with PT as adjuvant, may trigger the immune response to produce high levels of both IgE and IgG1 class antibodies. Similarly with a high molecular weight antigen such as 200 kDa FHA, there was a relationship between high IgE and high IgG1 titres after the three-dose immunization schedule. It has been proposed that IgG production can turn off the synthesis of IgE in rats (Tada et al., 1972). Lehrer *et al.*, (1975, 1976) did not support this conclusion since mice immunized with high doses of Oa and a crude extract of HSF (ie. PT) produced high titres of both IgE and IgG1 antibodies. Okudaira and Ishizaka, (1973) similarly did not observe a suppression of IgE synthesis by IgG in the mouse.

In our work, an anti-Oa IgE and IgG1 response was produced over a 10-fold antigen dose range (0.1 - 1.0  $\mu$ g). Whereas anti-FHA IgE and IgG1 was produced over a much wider (400-fold) antigen dose range (0.05 - 20  $\mu$ g). Lehrer *et al.*, (1975), reported that large doses of Oa were required to elicit both an IgE and IgG response after primary immunization. However Bazin (1976) stated that large primary doses were inhibitory to the production of secondary reaginic responses. Our system of immunization and range of antigen dose relate more to those allergy systems where repeated small doses of the allergen stimulated the IgE response in man.

An anti-FHA IgG response in children has also been detected after pertussis vaccination (Baraff *et al.*, 1983) and also after *B. pertussis* infection (Granstrom *et al.*, 1981, 1988). To date, however, there have been no reports on anti-FHA IgE responses after disease or immunization in either animals or man, although IgE responses to whole cells of *B. pertussis* have been found. Using the RAST assay, Haus *et al.*, (1988) showed there was an IgE response to whole-cells of *B. pertussis* after DTP vaccination. They speculated that, because of the high risk of allergy in certain individuals, the administration of vaccine should be delayed. In 1989, Wirsing von Koenig and Finger devised an ELISA against whole cells of *B. pertussis* and tested convalescent sera for IgE. They detected IgE antibodies in only 3% of individuals and concluded that IgE did not have any effect on the course or outcome of the disease. The particular components of *B. pertussis* responsible for the response were not identified.

In our study, the PT toxoids acted in a completely different manner to the Oa and FHA, since IgE could only be detected when IgG1 levels were low (when compared with IgE-negative sera where IgG1 levels were high). Hedenskog *et al.*, (1989), using the RAST, detected an anti-PT IgE response during whooping cough and also after both AC and WC vaccination. During pertussis infection and after immunization, 65% and 35% respectively of individuals tested exhibited an anti-PT IgE response. There was no

information given on the corresponding IgG response. Anti-FHA and anti-PT IgG responses have been detected after DTP vaccination (Baraff *et al.*, 1983), acellular vaccination (Storsaeter *et al.*, 1990) and infection (Wisnes *et al.*, 1985) in man. In our work, intranasally infected mice were bled at intervals, for 35 days, but no anti-FHA or anti-PT IgG1 response was detected. Sato and Sato, (1984) found that a significant antibody response was not found in mouse sera until five weeks after i.n infection.

### Conclusions and perspectives

IgE antibodies can be produced in mice against two of the *B. pertussis* antigens namely FHA and PT, which are major components of the new acellular vaccines. The IgE was successfully detected and quantified in two systems: the PCA and the ELISA. There was a requirement for the conventional tests to be modified for detection of anti-Oa, FHA and PT IgE. The usual 2-day sensitization period in the PCA test was adequate for titrating anti-Oa IgE but had to be extended to 7-days for detection of anti-FHA and anti-PT IgE. Future work could involve extending the PCA sensitization interval beyond 7 days to see if the test sensitivity could be improved. The IgE ELISA involved an initial coating stage with an affinity-purified antibody directed against the antigen. This increased the sensitivity of the IgE ELISA. It would be interesting to investigate the effect of this modified ELISA on the sensitivity of the IgG1 ELISA.

In the present study, in the three antigen systems i.e with Oa, FHA and PT, an IgE response was produced using the 3-dose immunization schedule of Levine and Vaz, (1970). However, with each antigen, a different immunizing dose was needed. Oa and PT produced an anti-Oa and anti-PT IgE response over a narrow immunizing dose range and FHA produced an anti-FHA IgE response over a wide range of immunizing doses. IgE and IgG1 production also seemed to be related. With Oa and FHA as antigen, IgE and IgG1 were produced to high levels after immunization. Whereas with PT toxoid as antigen, a high IgE response was produced in conjunction with a low IgG1 response. It would be interesting to see if other protein antigens could be slotted into one of the above groups with relation to IgE production, with high and low molecular weight antigens eliciting both a high IgE and IgG1 response but intermediate molecular weight antigens such as PT toxoids preferentially producing IgE.

In infected mice we found that the IgE or IgG1 response could not be detected up to 35 days after infection, even though the lung scores reduced rapidly after 10 days. If this experiment had been extended, IgE might have been detected in those mice with a more persistent infection.

Future work could involve measuring the levels of all the other immunoglobulin classes, after immunization and infection, to get an overall picture of the immune response concomitant with IgE production. It would also be important to investigate the types of T-cell populations involved in this IgE antibody response.

The disease, whooping cough is a localised respiratory infection manifesting in the characteristic paroxysmal cough. In certain individuals the initial inflammation of the bronchi, larynx and nasopharyngeal cavity may extend to deeper structures and result in diffuse bronchopneumonia. There may be numerous small areas of atelectasis, causing obstruction of the lower airways with mucus plugs, and increased fibrous tissue about the bronchi due to leukocytic infiltration.

What role does IgE play in the pathogenesis of whooping cough? In *Schistosoma mansoni* infections, IgE seems to have a beneficial effect for the host by reducing the worm burden (Ogilvie 1966; Capron *et al.*, 1980; Damonville *et al.*, 1986). In a few instances, mostly seasonal rhinitis, it has been possible to detect a significant correlation between the quantity of specific IgE and the intensity of the patient symptoms (Lichtenstein *et al.*, 1973). Such correlations provide indirect evidence that the IgE antibody also plays a role in the pathogenesis of atopic respiratory diseases and suggests that quantitation of IgE-specific antibodies may be useful in the assessment of IgE-mediated respiratory symptoms. Welliver *et al.*, (1980) hinted at a correlation between IgE production and increased pathogenesis of acute illness, caused by respiratory syncytial virus, which may explain the recurrent episodes of wheezing after infection.

Most microbial agents and environmental macromolecules gain access to the human host via the mucosa of the intestinal and respiratory tracts. Adjuvants are known to stimulate the immune responses to antigens administered with them. The basis behind allergy and sensitization involves exposure to an antigen, IgE-specific antibodies being produced and, upon subsequent challenge with the same or related antigen, the release of pharmacologically-activated mediators from specific IgE coated mast cells and basophils.

In infants suffering from whooping cough, and after vaccination, an IgE response has been detected against whole-cells of *B. pertussis* and particularly against PT, but there is no evidence that specific IgE is either beneficial or detrimental to the outcome of the disease. One tentative suggestion by Bjorksten and Ahlstedt, (1984) was that exposure to *B. pertussis* bacteria or other adjuvants, could possibly induce IgE to unrelated antigens in allergic patients.

IgE is known to bind with high affinity to mast cells and basophils which are distributed throughout the body. In pertussis, IgE may therefore be involved in specific degranulation at the sites of infection i.e. the lung, causing the release of a number of pharmacological agents into the respiratory mucosa. Sedgwick and Holt, (1985) repeatedly exposed rats to Oa and found that the parathymic and posterior mediastinal nodes draining the lower lung were the major sites of specific IgE and IgG production. The positive effects of mast cell degranulation at this area would be a) chemotaxis of immune cells such as leukocytes into the site of sensitization, which

may help in clearance of viable *B. pertussis* and b) histamine release, which could cause bronchospasm and help in the "paroxysmal coughing up" of the organism from its site of infection. On the other hand, the IgE response may be detrimental to the host by causing hypersensitivity reactions, resulting from an over stimulation of mast cells and release of large amounts of vasoactive amines, which locally may aid in removal but which, more generally, might damage the mucosa. This damage may, in turn, lead to some of the complications found in the clinical course of the disease.

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## APPENDICES

## Appendix 1 - Culture Media and Bacterial Diluents

### Casamino acids (CA) solution

To prepare 1 L of CA solution, the following ingredients were dissolved in 950 ml distilled water and adjusted to pH 7.1 with approximately 10 ml of 2.5N NaOH. The volume was made up to 1 L and the solution autoclaved at 121°C for 15 min.

	g
Casein hydrolysate (Gibco)	10.0
MgCl <sub>2</sub> . 6H <sub>2</sub> O	0.1
CaCl <sub>2</sub>	0.016
NaCl	5.0

### Casamino acids-glycerol

Glycerol (10% v/v) was added to CA solution and the mixture autoclaved as above.

### Bordet Gengou agar

BG agar base was normally prepared in 500 ml aliquots in 1 L bottles by first dissolving two portions, each of 18 g, of BG agar base (Oxoid) in 500 ml of distilled water containing 20 ml of glycerol. The bottles were placed in a steamer at 100°C for 30 min and the dissolved agar sterilized by autoclaving at 121°C for 15 min. After sterilization, the agar base was allowed to cool to 56°C in a water bath and 100 ml defibrinated horse blood at R.T. was added to each 500 ml BG base. The blood was carefully mixed into the agar by rotating the bottle. Approximately 25 ml of the completed medium was poured into 10 cm diameter petri dishes in a laminar flow cabinet. Air bubbles on the agar surface were removed by flaming.

### Bordet Gengou agar with kanamycin

For BG-kanamycin plates, a 10 ml stock solution of 10 mg/ml kanamycin in water was prepared and sterilized by passage through a 0.45 µm pore size, 25 mm diameter Acrodisc syringe filter (Gelman Sciences). The filtrate was dispensed directly (2.5 ml per 500 ml) into the melted and cooled BG agar before addition of the blood. The final concentration of kanamycin in the agar was 50 µg/ml.

## **Stainer Scholte liquid medium**

	g
L- glutamate (monosodium salt; BDH)	10.72
L-proline (Sigma)	0.24
NaCl	2.50
KH <sub>2</sub> PO <sub>4</sub>	0.50
KCl	0.20
MgCl <sub>2</sub> · 6H <sub>2</sub> O	0.10
CaCl <sub>2</sub>	0.02
Tris	6.10
Casein hydrolysate	0.50

### *Supplement*

L-cysteine mono HCl	0.04
FeSO <sub>4</sub>	0.01
Ascorbic acid	0.02
Niacin	0.004
Glutathione	0.15

All ingredients except those listed in the supplement were dissolved in 900 ml of distilled water. The pH of the solution was adjusted to pH 7.4 with 2.5N HCl, and made up to a total volume of 990 ml, then autoclaved at 121° C for 15 min and allowed to cool. The supplement was dissolved in 10 ml of distilled water and sterilized by passage through a 0.45 µm pore size, 25 mm diameter Acrodisc syringe filter (Gelman Sciences). The supplement (10 ml) was added to 990 ml of the autoclaved medium.

### **Cyclodextrin modified Stainer Scholte liquid medium**

The only modification to the above to obtain cyclodextrin modified Stainer Scholte medium involved 1.0 g of MeBCD being added to the above ingredients before autoclaving.

## **Appendix 2- FHA and PT Extraction.**

### **Tris-HCl buffer**

0.1M Tris (50 ml) and 0.1M HCl (26.8 ml) were mixed and a total volume of 200 ml was made up with distilled water. The pH of the resultant solution was 8.0. For 0.05M

Tris-HCl, 1M NaCl, pH 8.0, 11.7 g of NaCl was dissolved in the 0.1M Tris and 0.1M HCl and the total volume, once the NaCl was dissolved was made up to a total of 200 ml with distilled water. 0.1M Tris-HCl, pH 8.0 was prepared as above except 50 ml of 0.2M Tris and 26.8 ml of 0.2M HCl were added together and made up to 200 ml with distilled water.

### **1mM phenyl methyl sulphonyl fluoride(PMSF)**

To prepare 1 mM PMSF, 1.74 mg of PMSF (Sigma) was dissolved in 1ml of 0.1M iso-propanol and made up to a total volume of 10 ml with distilled water. For each litre of culture, 1 ml of 1 mM PMSF was added. The final concentration was 1µM/ml PMSF.

### **Physiological saline**

NaCl (9 g) was dissolved to a total volume of 1 L with distilled water. The pH of the resultant solution was 7.0.

## **Appendix 3 - Enzyme Linked Immunosorbent Assay**

### **Coating buffer**

	<b>g</b>
Na <sub>2</sub> CO <sub>3</sub>	1.59
NaHCO <sub>3</sub>	2.92

Both reagents were dissolved in 1 L of distilled water and kept at 4° C. The pH of the solution was approximately 9.6.

### **Wash/Incubation buffer**

	<b>g</b>
NaCl	8.0
KH <sub>2</sub> PO <sub>4</sub>	0.2
Na <sub>2</sub> HPO <sub>4</sub>	1.15
KCl	0.2

All the ingredients were dissolved in 1 L of distilled water. Tween 20 (0.5 ml) was then added to a final concentration of 0.05%. The pH of the solution was 7.4.

### **Citrate-phosphate**

Solution A: 0.1M citric acid (21.01 g/l of distilled water)

Solution B: 0.2M Na<sub>2</sub>HPO<sub>4</sub> (35.6 g/l of distilled water)

To prepare a 0.15M solution of citrate-phosphate pH 5.0, 49 ml of solution A and 51 ml of solution B were added together just before use.

### **Substrate**

A stock solution of O-phenylenediamine (34 mg/ml in citrate phosphate) was prepared and 200 µl was added to 100 ml of citrate phosphate along with 20 µl of H<sub>2</sub>O<sub>2</sub>.

## **Appendix 4- SDS Polyacrylamide Gel Electrophoresis**

### **Acrylamide**

	g
Acrylamide	30.0
Bis acrylamide	0.8

The ingredients were dissolved and made up to 100 ml in distilled water. The solution was filtered through a 18.5 cm diameter Whatman 1 filter and store at 4° C in the dark.

### **Tris HCl pH 8.8**

	ml
2 M Tris	50
1 M HCl	16.2

The solutions were added together with 0.4 g of sodium dodecyl sulphate (SDS) and made up to a total volume of 100 ml with distilled water. The buffer was aliquoted into 20 ml amounts and stored at -20° C.

### **Tris HCl pH 6.8**

	ml
1 M Tris	50
1 M HCl	45

The solutions were added together with 0.4 g of SDS and made up to a total volume of 100 ml with distilled water. The buffer was aliquoted into 20 ml amounts and stored at -20° C.



### **Tris Glycine pH 8.3 (10x)**

	g
Glycine (Chromatography grade)	144.13
Tris	30.28

The above were dissolved in 900 ml of distilled water. The pH was adjusted with 2.5N HCl. The volume was made up to 1 L with distilled water and stored at 4<sup>o</sup> C. The solution was diluted 1 in 10 in distilled water prior to use.

### **Ammonium persulphate**

A 1% w/v stock solution of ammonium persulphate (0.01 g/l of distilled water) was prepared just prior to use.

### **Solubilizing buffer**

	ml
Tris HCl pH 6.8	2
SDS	3.2
$\beta$ -mercaptoethanol	1.6
Glycerol	3.2
Bromophenol	0.32
Distilled water	5.68

All ingredients were added together and kept at R.T.

### **Coomassie blue stain**

Coomassie blue R250	1.25 g
50% v/v methanol	454 ml
Glacial acetic acid	46 ml

The coomassie blue was dissolved in methanol and acetic acid. The stain was filtered through a 18.5 cm Whatman paper filter Number 1.

### **Destain solution**

	ml
Methanol	50
Glacial acetic acid	75
Distilled water	875

The solutions were mixed in the order that they appear above.

**Lower gel**

	ml
Lower gel buffer	10
Distilled water	13.4
Acrylamide solution	16.6
Ammonium persulphate	0.2
TEMED	0.02

The solutions were added without aeration and used immediately.

**Upper gel**

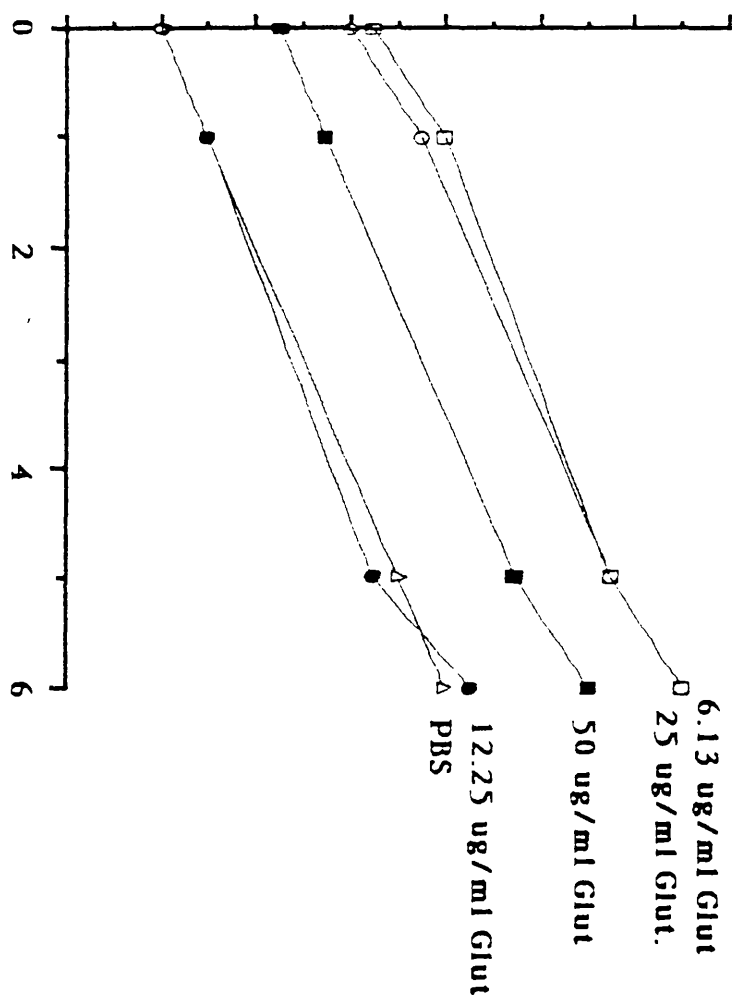
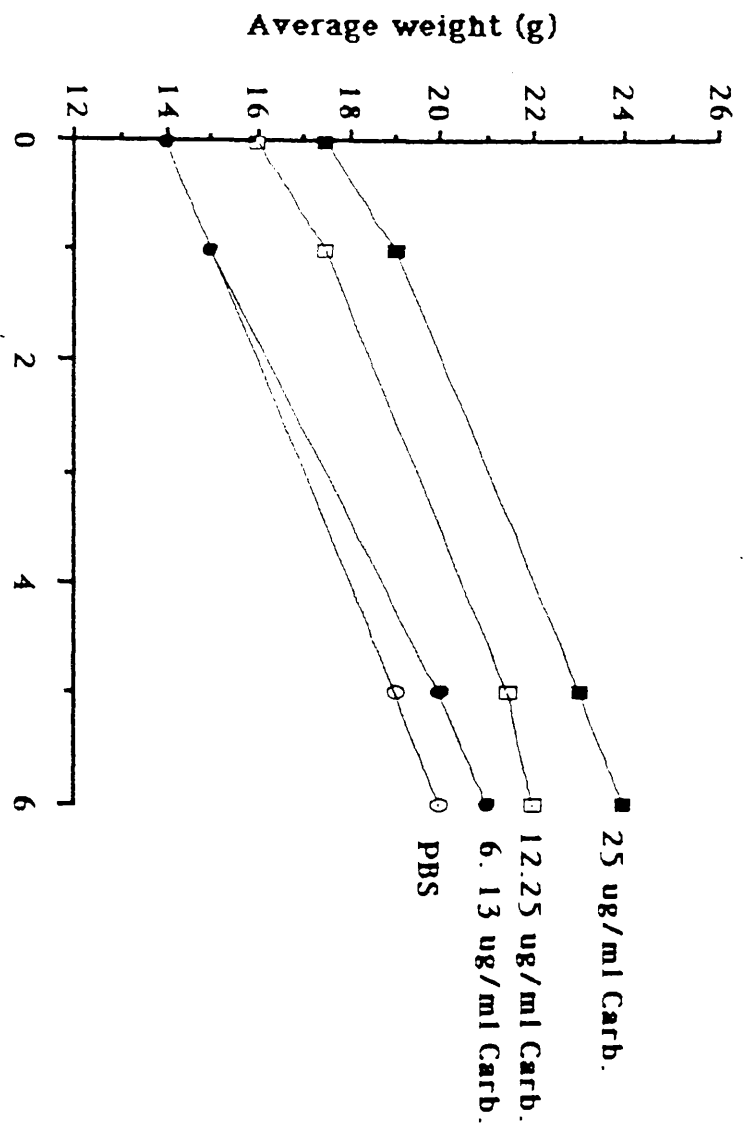
	ml
Upper gel buffer	2.5
Distilled water	6.0
Acrylamide solution	1.5
Ammonium persulphate	0.03
TEMED	0.02

The solutions were added carefully to avoid aeration and used immediately.

**Appendix 5 : Histamine sensitizing activity of PT used in immunizations and for toxoiding.** Groups of 4 mice were injected i.p with various concentrations of PT or carbodiimide and glutaraldehyde toxoids of PT. After 5 days, the mice were challenged with 3 mg of histamine and the survivors scored after 2 h.

Toxin batch and treatment	Dose of PT or toxoid ( $\mu\text{g}/\text{ml}$ )	No. of survivors/ no. of mice challenged.	Approx. LD <sub>50</sub> /mouse ( $\mu\text{g}$ )
PT			
1	3	0/4	
	1	0/4	
	0.33	1/4	
	0.11	3/4	0.1
2	3	2/4	1.5
	1	3/4	
	0.33	3/4	
	0.11	4/4	
6	3	0/4	
	1	2/4	0.5
	0.33	3/4	
	0.11	4/4	
7	3	1/4	
	1	2/4	0.5
	0.33	4/4	
	0.11	4/4	
Glut. PToxoid			
2	3	4/4	> 3
	0.3	4/4	
	0.1	4/4	
	0.01	4/4	
Carb. PToxoid			
2	3	4/4	> 3
	0.3	4/4	
	0.1	4/4	
	0.01	4/4	

**Appendix 6 : Effect of challenging Ham/ICR mice with known concentrations of carbodiimide and glutaraldehyde toxoids in the mouse weight gain test.**



**Appendix 7 : Plan of immunization mixtures used for raising anti-Oa sera for testing in PCA and ELISA for IgE and IgG1.**

Serum No.	Immunizing dose		Bleed day
	OA ( $\mu$ g)	PT (ng)	
1a	200	250	10
1b	200	250	10
2a	20	2500	21
2b	20	250	21
2c	20	250	21
2d	20	250	21
2e	200	2500	21
3a	20	1000	21
3b	20	1000	21
3c	20	100	21
3d	20	100	21
3e	200	100	21
3f	200	10	21
3g	200	1	21
4a	20	1000	28
4b	200	1000	28
4c	200	1000	28
4d	200	100	28
4e	20	10	28
4f	200	10	28
4g	200	1	28
5a	200	200	21
5b	20	200	21
5c	20	200	21
6a	0.1	1000	63 <sup>a</sup>
6b	1	1000	63 <sup>a</sup>
6c	10	1000	63 <sup>a</sup>

<sup>a</sup> - The sera were obtained by Protocol 3 and were pools from 25 mice. The others were obtained by Protocol 2 and consisted of individual mouse sera.

**Appendix 7 : Plan of immunization mixtures used for raising anti-FHA sera for testing in PCA and ELISA for IgE and IgG1.**

Experiment number and sera code	Immunizing dose of FHA( $\mu$ g) with a constant dose of PT (1 $\mu$ g) in Ham/ICR and Balb/c mice	
	Ham/ICR	Balb/c
38	1	0.1
	2	1
	3	10
39	1	0.1
	2	1
	3	10
78	A	0.01
	B	0.05
	C	0.1
	D	1
	E	10
	F	20
	G	40
79	A-2	0.01
	B-2	0.05
	C-2	0.1
	D-2	1
	E-2	10
	F-2	20
	G-2	40

**Appendix 7 : Plan of immunization mixtures used for raising anti-PT sera for testing in PCA and ELISA for IgE and IgG1.**

Experiment number and sera code	Immunizing dose of toxoid ( $\mu\text{g}$ ) with a constant dose of PT ( $1 \mu\text{g}$ ) in Ham/ICR and Balb/c mice					
	Ham/ICR			Balb/c		
	Carb. FHA/PT	Carb. PT	Glut. PT	Carb. FHA/PT	Carb. PT	Glut. PT
43	1	0.1				
	2	1				
	3	10				
78	H		0.001			
	I		0.01			
	J		0.05			
	K		0.01			
	L		1			
	M		10			
79	H-2					0.001
	I-2					0.01
	J-2					0.05
	K-2					0.01
	L-2					1
	M-2					10
	N-2					20
78	R				0.05	



**Appendix 8 : The ability of fetuin at a concentration of 1 µg/ml to coat a microtitre plate well surface one molecule thick.**

Surface area of microtitre well

Radius - 3 mm

Height - 4 mm (containing 100 µl of fetuin)

$$\begin{aligned}\text{Surface area} &= 2\pi rh + \pi r^2 \\ &= 103 \text{ mm}^2 \\ &= 103 \times 10^{12} \text{ nm}^2\end{aligned}$$

Molecular weight of fetuin = 48,700 take as 50,000 (assume it is a globular protein similar to Oa which has a molecular radius of 24Å, which is the same as 2.4nm)

Molarity of fetuin soln

50000 g/l - 1M

1 µg/ml - 0.00002M or 20 µM

No. of molecules in 1µg/ml fetuin is equivalent to

$$\begin{aligned}20 \mu\text{M} \times \text{Avogadros number} (6.022 \times 10^{23}) \\ = 1.2 \times 10^{19} \text{ molecules/ml} \\ = 1.2 \times 10^{18} \text{ molecules/100 } \mu\text{l}\end{aligned}$$

If the hypothetical radius of fetuin is the same as Oa 24Å or 2.4nm take the diameter as twice that number to account for the circular nature of the molecule and the binding properties to the plate. The diameter is 5 nm. The dimensions of the square are 25 nm

To find the number of molecules required to coat the well one molecule layer thick :

$$\frac{\text{Surface area of well}}{\text{Radius of one molecule}} = \frac{103 \times 10^{12} \text{ nm}}{25 \text{ nm}} = 4.12 \times 10^{12} \text{ molecules}$$

Therefore  $4.12 \times 10^{12}$  molecules of fetuin is required to coat the well one molecule thick. In a 100 µl solution of 1 µg/ml fetuin there is  $1.2 \times 10^{18}$  molecules, a 300,000 fold excess.

**Appendix 9: Rankit plots of the log<sub>10</sub> PCA U/ml and the log<sub>10</sub> titres of IgE and IgG1 respectively, in the Oa immunization system.**

