

D. S. J. Lindsay^aR. Parton^bA. C. Wardlaw^b^a Department of Laboratory Medicine,
Ruchill Hospital, Glasgow,^b Department of Microbiology,
University of Glasgow, UK

Adjuvant Effect of Pertussis Toxin on the Production of Anti-Ovalbumin IgE in Mice and Lack of Direct Correlation between PCA and ELISA

Key Words

IgE enzyme-linked immunosorbent
assay

Ovalbumin

Passive cutaneous anaphylaxis

Pertussis toxin

Abstract

In studies designed to optimize the production and detection of anti-ovalbumin (anti-Oa) IgE in Ham/ICR mice, a range of doses of both Oa and pertussis toxin as the IgE adjuvant was explored. As determined by 48-hour passive cutaneous anaphylaxis (PCA) tests, the highest titre of anti-Oa IgE was obtained in a three-injection protocol with 0.1 µg Oa and 1 µg pertussis toxin for the priming dose, followed by two further doses of 0.1 µg Oa alone. In single-dose immunizations, the highest PCA responses were obtained in sera from mice given 20 µg Oa and 1 µg pertussis toxin. These data confirm that murine IgE production to Oa depends on particular combinations of immunization variables. There was no direct correlation between the PCA and anti-IgE enzyme-linked immunosorbent assay (ELISA) titres of the PCA-positive sera; indeed there was a significant *negative* correlation. This relationship was not due to interference by IgG1, as there was no correlation between the anti-Oa IgG1 and anti-Oa IgE ELISA titres of the sera. These results highlight the need for caution in assuming that serum IgE levels as measured by ELISA will necessarily correlate positively with IgE biological activity as measured by allergen challenge *in vivo*.

Introduction

The earliest report of an IgE response to ovalbumin (Oa) in mice was in 1969 by Clausen et al. [1] who used a passive cutaneous anaphylaxis (PCA) test for its detection. These and later authors [2–5] found that the highest titres of serum anti-Oa IgE in mice occurred after high doses of both the antigen and of the crude *Bordetella pertussis* extract which was used as the IgE adjuvant. IgE production was first detected 10 days after immunization, and the titres usually remained elevated for 60–80 days. With other antigens, the beneficial effect of multiple booster doses on IgE responses was demonstrated by Levine and Vaz [6] with Al(OH)₃ as

the adjuvant for the primary immunization. Peak responses usually developed 1 week after each booster injection, the successive boosters with repeated small doses of antigen giving progressively higher titres. With purified pertussis toxin (PT) as the IgE adjuvant for Oa in mice, Munoz and Bergman [7] obtained high PCA titres with a single booster dose of Oa alone, after the primary immunization with Oa and PT. In humans, there is no equivalent of the murine PCA test for allergenically active IgE, since safety considerations now preclude the use of the Prausnitz-Küstner reaction [8].

The first serological method for the detection and measurement of IgE was the radioallergosorbent test reported in 1967 by Wide et al. [9] which subsequently has been used

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Correspondence to: Dr. D. S. J. Lindsay
Department of Laboratory Medicine
Ruchill Hospital
Glasgow G20 9NB (UK)

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mainly to detect IgE in human and rat sera. Other *in vitro* methods include radioimmunoassay [10], enzyme-linked immunosorbent assay (ELISA) [11], and mast cell degranulation [12]. Of these, only ELISA has been reported for the measurement of IgE in mouse sera [13–15], but not with Oa as the antigen. A correlation between ELISA and PCA has been demonstrated in sera from rats and guinea pigs injected with various antigens [16, 17], but not with anti-Oa IgE in mice.

The primary objective of the present investigation was to define the optimum conditions for production of anti-Oa IgE in mice, with purified PT as the adjuvant, by exploring the effects of different primary doses of Oa and PT, of different bleeding times, and of booster injections. A second objective was to determine whether anti-Oa IgE as measured by PCA was correlated with anti-Oa IgE as measured by ELISA.

Materials and Methods

Mice

Ham/ICR mice were obtained from Charles River (Margate, UK) and bred in-house in temperature- and light-controlled rooms. They were fed with mouse chow and water *ad libitum*, and animals of either sex, ranging in age from 3 to 6 weeks, were used for immunization. The PCA tests were done only in females which were 6–8 weeks old.

Immunization Procedures

PT was prepared and characterized as described by Sekura *et al.* [18]. Two immunization protocols were used to raise antisera against Oa (five times crystallized; Sigma). The first involved a single intraperitoneal injection of 0.5 ml containing various doses of Oa and PT, followed by bleeding on days 10, 21 or 28. In the second protocol, a primary intraperitoneal injection of 0.5 ml containing various doses of Oa mixed with a constant 1- μ g dose of PT was followed by further intraperitoneal injections of Oa alone on days 28 and 56 and bleeding on day 63. Both of these protocols were adapted from previous reports [1–5, 19]. All injection mixtures were prepared in phosphate-buffered saline (PBS: 8.0 g NaCl, 0.2 g KH_2PO_4 , and 0.2 g KCl in 1 litre distilled water and adjusted to pH 7.4 with NaOH).

Anti-Oa IgE Reference Serum

A high-titre anti-Oa IgE serum was prepared by pooling the sera from 25 mice which had been immunized by the three-injection protocol with 0.1 μ g Oa and 1 μ g PT. This serum was given an arbitrary unitage of 1,000 anti-Oa IgE PCA U/ml. Use of this serum as a reference in all tests enabled the PCA activity of the experimental anti-Oa sera to be expressed as PCA units per millilitre. This serum was also used as a reference in the ELISA studies.

Passive Cutaneous Anaphylaxis Test

The mice were sedated by intraperitoneal injection of 0.5 ml of a 1 in 10 dilution of Hypnorm (Janssen Animal Health). The hair was clipped from the dorsal surface and 50- μ l volumes of test sera were injected intradermally into normally four sites on each animal, two on

each side of the midline. Four such skin sites were used for each serum, each replication being in a different animal. Ten mice were used in a typical set of tests. After a 48-hour sensitization period, the PCA reactions were elicited by challenging the mice intravenously with a mixture of 1 mg Oa and 1 mg Evans blue (Sigma) contained in 0.2 ml PBS. After 30 min, the animals were asphyxiated with CO_2 and skinned so that the reactions could be viewed from underneath the skin. The diameter of blueing on the inner surface was measured in two directions at right angles and the average taken. Zones smaller than 5 mm were recorded as zero.

Preparation of Oa-Affinity Column

An Oa affinity column was used for the purification of anti-Oa for ELISA tests. Oa was coupled via its amino groups to cyanogen-bromide-activated Sepharose 4B (Sigma) according to the manufacturer's instructions. The Sepharose (5 g) was suspended in 1 mM HCl and washed with 1 litre of 1 mM HCl on a sintered glass funnel. Oa (25 mg) was dissolved in 5 ml of coupling buffer (0.1 M NaHCO_3 , pH 8.3, containing 0.5 M NaCl), and the washed Sepharose was added. This was mixed overnight at 4°C in an angled rotator to allow Oa to bind to the gel. Excess Oa was then washed away with coupling buffer and the gel poured into a Bio-Rad column (internal diameter 1.5 cm $\times$ length 15 cm) and allowed to settle. Any remaining active groups of the gel were blocked with 200 ml of 0.1 M Tris-HCl (pH 8.0) which was washed through the column for 2 h at room temperature. The Oa-Sepharose was further washed with three cycles of alternating pH, each cycle consisting of washing with 100 ml of 0.1 M acetate buffer (pH 4.0), containing 0.5 M NaCl, followed by 100 ml of 0.1 M Tris-HCl (pH 8.0), also containing 0.5 M NaCl.

Affinity-Purified Rabbit Anti-Oa

Rabbit anti-Oa serum was prepared by immunizing four NZW rabbits, each weighing approximately 2 kg, with 10 mg Oa in Freund's incomplete adjuvant (Sigma) injected subcutaneously in small aliquots at multiple sites, followed 6 weeks later by a booster dose injected similarly. The animals were bled (15 ml) at weekly intervals for 2 months thereafter, followed by terminal exsanguination, and all the sera pooled. A 25-ml portion of the serum was dialyzed in visking tubing against 1 litre of 0.1 M Tris-HCl (pH 8.0) overnight at 4°C and then pumped through the Oa-Sepharose column at 1 ml/min. The column was washed with 300 ml of 0.1 M Tris-HCl (pH 8.0) for 3 h to remove unadsorbed protein, followed by 100 ml of 0.1 M Tris-HCl (pH 8.0) containing 0.5 M NaCl to remove non-specifically bound protein. To elute specific anti-Oa, 0.1 M glycine-HCl (pH 3.0) was pumped through the column, 2-ml fractions collected, and the absorbance read at 280 nm. The peak fractions (8–10 ml) were pooled and, without delay, dialyzed for 18 h at 4°C against PBS. After use the column was washed with 500 ml PBS containing 0.1% (w/v) sodium azide and stored at 4°C.

ELISA for Measuring Anti-Oa IgE

Anti-Oa IgE in the mouse sera was measured using a sandwich ELISA in which affinity-purified rabbit anti-Oa was used to coat the wells, with sheep anti-mouse IgE conjugated to horseradish peroxidase (The Binding Site, Birmingham, UK) which was employed as the top layer. The 96 wells of flat-bottomed microtitre plates (Nunc Maxisorp F) were coated with 200 μ l of 0.175 μ g/ml affinity-purified anti-Oa in coating buffer (1.59 g/l Na_2CO_3 , 2.92 g/l NaHCO_3 , pH 9.6). This was left overnight at 4°C. The plate was then washed three times in PBS (pH 7.4) containing 0.05% (v/v) Tween 20 (PBS-T). To each

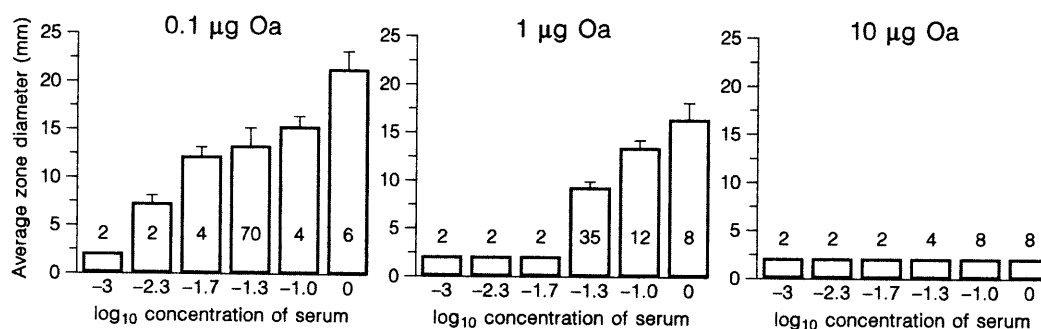


Fig. 1. Assay of IgE by serial dilution of pooled sera and determination of the average zone diameter of skin blueing in the 48-hour PCA test. The pooled sera were obtained on day 63 from three groups of 25 mice immunized with different doses of Oa (three injections of 0.1, 1, or 10 µg on days 0, 28, and 56) and constant PT (1.0 µg on the 1. day only). Each column shows the number of skin zones observed and the standard error of the mean of the zone diameter.

well was added 100 µl Oa (100 µg/ml) in PBS-T. The plates were kept at 4°C for 1 h and washed three times in PBS-T containing 1% (w/v) bovine serum albumin as blocking agent. Sera for IgE assay were diluted in ten-fold steps from 1 in 10 to 1 in 10,000, and 100-µl portions added to appropriate wells. The plates were kept at 4°C for 1 h and washed three times with PBS-T before treatment with 100 µl sheep anti-mouse IgE-horseradish peroxidase (1 in 100 dilution in PBS-T). After incubation at 4°C for 1 h, the plates were washed three times and treated with 100 µl substrate consisting of 200 µl of a 34-mg/ml solution of *o*-phenylene diamine (Sigma) in 100 ml of citrate-phosphate buffer (pH 5.0) to which 20 µl hydrogen peroxide had been added. The plates were left in the dark for 30 min and the reactions stopped with 50 µl of 12% (v/v) H₂SO₄ added to each well. The plates were read spectrophotometrically at 492 nm.

ELISA for Measuring Anti-Oa IgG1

Anti-Oa IgG1 in the mouse sera was titrated in the same type of microtitre plates as above, but by direct and not sandwich ELISA. Each well was coated with 200 µl Oa (100 µg/ml) in coating buffer overnight at 4°C. After washing three times with PBS-T and blocking with 2% (w/v) bovine serum albumin for 1 h at 37°C, ten-fold dilutions of the anti-Oa mouse sera were added (100 µl) and incubated for 1 h at 37°C. The plates were washed three times before adding 100 µl of 1/1,000 sheep antimouse IgG1 labelled with horseradish peroxidase (Sigma) and incubated for 1 h at 37°C. The rest of the procedure was as for IgE.

Results

Selection of Serum for Use as an Anti-Oa IgE Reference

It was decided early during the investigation to prepare a house standard mouse serum containing anti-Oa IgE for use as a reference in PCA assays. Preliminary experiments with single-injection immunizations indicated that 1 µg PT was a

suitable adjuvant dose to stimulate anti-Oa IgE in Ham/ICR mice, provided that it was used in conjunction with an appropriate dose of Oa. This dose of PT was, therefore, mixed with doses of 0.1, 1.0, and 10 µg of Oa for primary immunization of groups of 25 mice. Other studies [6, 20, 21] had indicated a beneficial effect of booster doses of the antigen alone for stimulating high titres of IgE. After the primary immunizations, the animals were, therefore, boosted with the same doses of Oa alone on days 28 and 56, with final bleeding on day 63. The three pools of serum, each of about 10 ml volume, were then titrated for IgE in the 48-hour PCA test.

Figure 1 shows that the dose of antigen at each injection time had a strong effect on the IgE response as measured by PCA. The lowest-dose schedule (3×0.1 µg Oa) gave the most potent pooled serum, while the highest dose of 3×10 µg Oa gave a serum with no detectable PCA activity. The intermediate immunizing dose of 3×1 µg Oa gave a serum which had appreciable PCA activity, but less than that given by the lowest dose. The pooled serum from the 0.1-µg Oa immunizations was, therefore, selected as the reference serum.

PCA Doses-Response Relationship with Anti-Oa IgE Reference Serum

To establish a dose-response curve for the anti-Oa IgE reference serum in the 48-hour PCA test, the serum was tested undiluted and in serial dilutions to 1 in 1,000, with each of the dilutions being injected into at least five skin sites on different mice. Figure 2 shows that there was an approximately linear relationship between zone diameter and

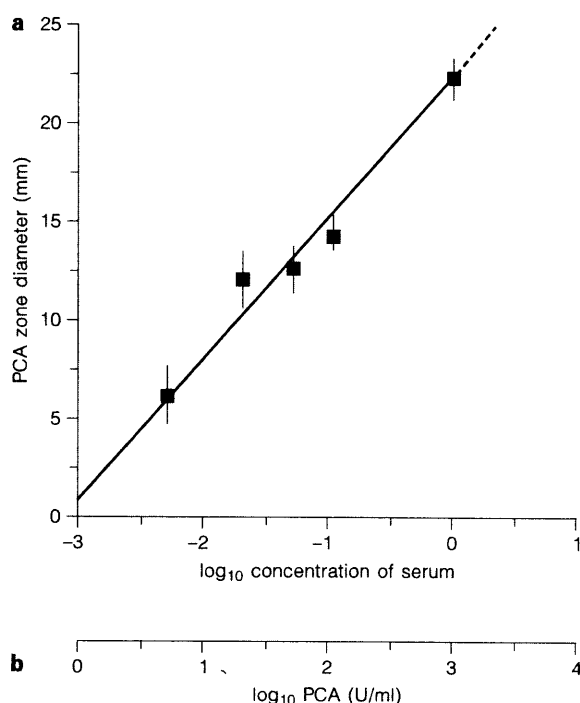


Fig. 2. Dose-response line of the reference anti-Oa IgE mouse serum in the 48-hour PCA test. The lower abscissa scale is for converting $\log_{10}$ concentration of serum (a) into $\log_{10}$ PCA units per millilitre (b). The dotted extension of the line was used for estimating the titres of sera which were more potent than the reference.

the $\log_{10}$ concentration of the serum. Arbitrarily, the serum was assigned a potency value of 1,000 anti-Oa PCA U/ml. Interpolation of PCA zone diameters, obtained with other sera, onto the straight line thus allowed their anaphylactic potency to be expressed in $\log_{10}$ PCA units per millilitre.

Sera Raised by One-Injection Immunizations

The above results had all been obtained with the three-injection protocol extending over 63 days. Attention was next given to the possibility that anti-Oa IgE could also be raised in a single-injection scheme and in less time. In this one-injection immunization protocol, three variables were explored in various combinations, in groups of 2–4 mice: the effects of antigen dose (20 or 200 μ g Oa), adjuvant dose (PT from 0 to 5,000 ng), and time of bleeding (days 10, 21, and 28 after immunization). Individual mouse sera were tested, undiluted, by PCA and the zone diameters converted into $\log_{10}$ PCA units per millilitre. Figure 3, which summarizes the results of tests on individual sera from 143 mice,

shows that all three variables affected the production of IgE. The most favourable combination for consistency of production and high PCA unitage was 20 μ g Oa with 1,000 ng of PT and bleeding on day 21. The ten-fold higher dose of antigen was, in general, less satisfactory. The dose of PT was fairly critical in that a ten-fold decrease gave markedly inferior IgE production, while doses of 2,500 ng and above were toxic. The dose of antigen also influenced the optimum time for bleeding in that day 21 appeared to be best with 20 μ g Oa and day 28 with 200 μ g Oa. There was considerable variation in the responsiveness of individual mice within the groups. Three of the sera had PCA titres greater than the 1,000 U/ml of the reference; the titres of these sera were estimated from the extrapolated portion of the reference line shown in figure 2.

Measurement of Anti-Oa IgE in PCA-Positive Sera by ELISA

In the initial experiments to measure anti-Oa IgE by ELISA, the wells were coated with Oa and then reacted first with sera that were known to contain IgE and then with sheep anti-mouse IgE (conjugated to horseradish peroxidase). However, this system proved to be unsatisfactory because of the high background absorbancies in wells containing either normal mouse serum or no serum. These problems were overcome by the sandwich ELISA technique in which affinity-purified rabbit anti-Oa IgG was used for the initial coating of the wells. Here the ELISA dose-response curve (fig. 4) with the reference anti-Oa IgE had a satisfactory slope in the middle region and a low background which allowed titres to be read from the $A_{492} = 0.50$ interpolation point. Comparison of figures 2 and 4 shows that the ELISA test with a detection limit at a $\log_{10}$ serum concentration around -3.5 was about ten times more sensitive than PCA, with a detection limit at a $\log_{10}$ serum concentration near to -2.5.

Further evidence for the IgE specificity of this reference serum was provided by demonstrating that its ELISA activity was abolished by heating at 56°C for 30 min and by treatment with mercaptoethanol (data not shown).

Correlation of PCA and ELISA for Anti-Oa IgE

The correlation between anti-Oa IgE PCA and ELISA titres was explored with 18 PCA-positive sera (fig. 5a). Two of these were pooled sera from the three-injection protocol, and 16 were individual-mouse sera from the one-injection immunizations. There was a highly significant negative correlation between the two sets of titres (Kendall coefficient -0.4; $p = 0.006$). Further analysis of the data was done in relation to antigen dose with the sera from the one-injection

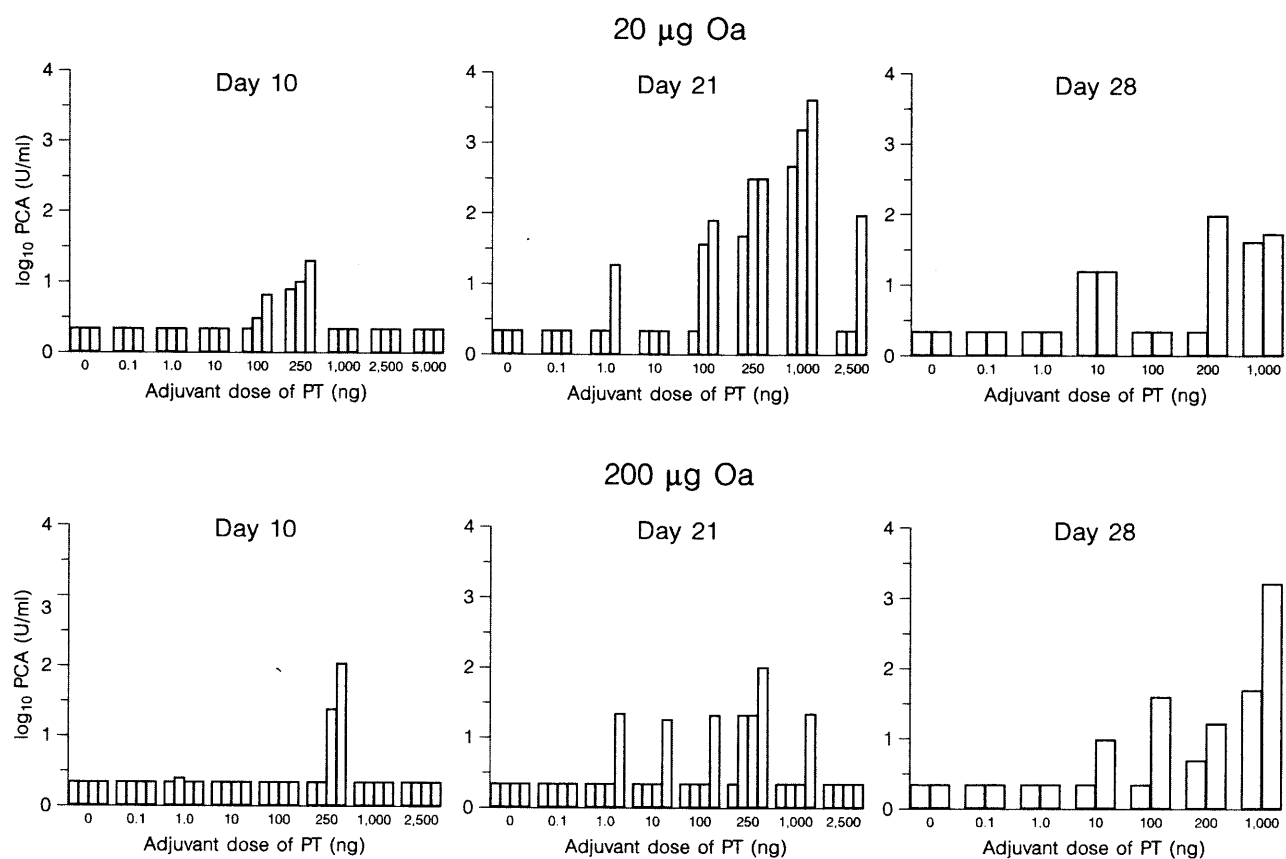


Fig. 3. Combined effects of varying the adjuvant dose of PT (0–2,500 ng), the immunizing dose of Oa (20 or 200 µg), and the time of bleeding (10, 21, or 28 days) on the PCA activity of sera obtained from mice in the one-injection protocol. Each column represents the serum from a single mouse.

immunizations. In figure 5b, which presents the seven sera obtained after the 20-µg immunizing dose of Oa, there was a positive but non-significant relationship between PCA and ELISA (Kendall coefficient 0.2; $p = 0.32$). However, with the 200-µg dose of Oa (fig. 5c), there was a significant negative correlation between the two types of assay (Kendall coefficient -0.5 ; $p = 0.042$).

Effect of Anti-Oa IgG1 on the Anti-Oa IgE Titres in ELISA

Anti-Oa IgG1 in the mouse sera was titrated to explore whether it might have been influential in the above negative correlation between the PCA tests and the ELISA for IgE. Figure 6 presents the results for individual mouse sera assayed for the two types of anti-Oa immunoglobulin. The sera had been obtained with the one-injection protocol and the 20- or 200-µg doses of Oa for immunization. There was no

indication that low titres of anti-Oa IgE were associated with high titres of anti-Oa IgG1 (Kendall coefficient 0.19; $p = 0.16$). Increasing the immunizing dose from 20 to 200 µg had a similar elevating effect on both anti-Oa IgE and anti-Oa IgG1.

Discussion

Despite the 25-year usage of Oa as an experimental antigen for raising anti-Oa IgE in mice [1–7], no two laboratories seem to have adopted the same procedure, as being superior to others, for reliability of production of high titres of this immunoglobulin. For example, both single-injection [1, 2, 4, 5] and multiple-injection [6] protocols have been described, without emergence of a consensus on which is best. There has, however, been agreement that an adjuvant is nec-

essary, notably $\text{Al}(\text{OH})_3$ [6] or materials derived from *B. pertussis*, such as the whole-cell vaccine [16], cell extracts [1–7], or purified pertussis toxin [22]. The strain of mouse is also an important determinant [6].

The results in this study are consistent with previous reports in showing that IgE production, as detected by the 48-hour PCA test on sera from the immunized animals, occurs only within the confluences of relatively narrow ranges of certain experimental variables. In single-injection immunizations, the titres were strongly influenced by the doses of both Oa and PT and the day of bleeding. The highest PCA titres were observed after immunization with 20 μg Oa plus 1 μg PT and bleeding on day 21. In contrast, the 200- μg dose of Oa, with 1 μg PT, gave an erratic IgE response over the 3 bleed days (10, 21, and 28). In the previous reports on one-injection immunizations, Clausen et al. [1, 2], Lehrer et al. [4], and Lehrer [5] found that high doses of both antigen and adjuvant were needed for the highest IgE responses. Moreover, the peak response was usually on day 10 and was long lasting. We found, in contrast, that the high antigen and adjuvant doses in one-injection immunizations produced only transient IgE responses at times later than 10 days. It seems likely that the quantitative differences between the present and previous work are due to differences in the IgE-producing abilities of different strains of mice. As regards the dose of purified PT for IgE adjuvant activity, Munoz et al. [22] found 0.75 μg to be best. This is very close to the 1- μg PT dose which was optimal in our studies.

Detection systems for IgE are numerous and diverse, but fall into the two main categories of serological procedures

and mast cell degranulation tests, the latter either in vitro [14] or in vivo [23]. Of the various serological tests for antigen-specific IgE, radioallergosorbent test [9] and ELISA [11] have been the most widely reported. The latter was chosen in this study so as to avoid the inconvenience of a radio-

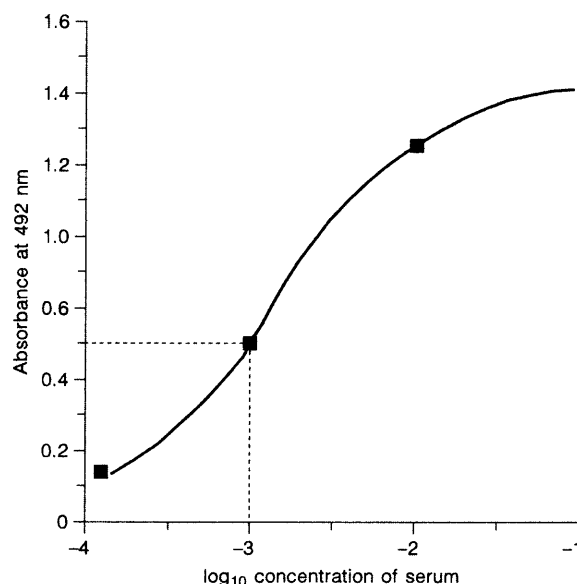


Fig. 4. Sample dose-response curve of the reference anti-Oa IgE mouse serum in the IgE ELISA. An absorbance end point of 0.5 at 492 nm was used for reading the titre of this and other sera.

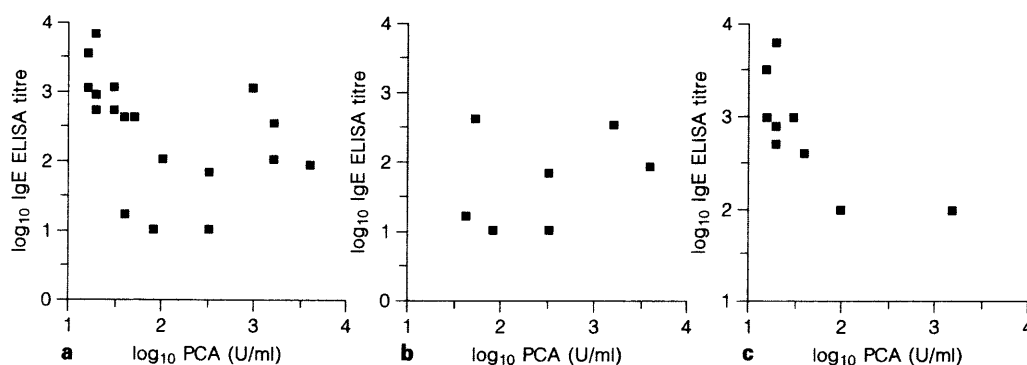


Fig. 5. Correlation between 48-hour PCA and IgE ELISA of PCA-positive mouse sera obtained by different injection protocols, but all with PT as the IgE adjuvant. **a** All available PCA-positive sera which were tested by ELISA, **b** Sera obtained on different days after 20 μg Oa and various doses of PT. **c** Sera obtained on different days after 200 μg Oa and various doses of PT.

active reagent. A variety of ELISA procedures was explored, but only the sandwich method gave satisfactory specificity without high background levels. The qualities of the coating antibody (purified rabbit anti-Oa IgG) and of the anti-mouse IgE (sheep) were crucial in both this and our previous [24] studies.

The PCA test involves antigen challenge of skin mast cells of an experimental animal and is similar to the Prausnitz-Küstner reaction once performed in humans. In our study, a pool of high-titre anti-Oa IgE serum was prepared for use as a reference by a three-dose immunization protocol and assigned an arbitrary unitage of 1,000 PCA U/ml. This allowed the assignment of PCA units to other sera by the use of a standard dose-response line, a procedure not reported by previous investigators. The 48-hours PCA test has the major advantage of detecting antigen-specific and biologically active IgE; it does, however, have the disadvantages of needing experimental animals and being subject in some instances to interference by non-specific IgE and by antigen-specific IgG1 [25]. For example, in our previous PCA studies [24] to detect IgE to *B. pertussis* filamentous haemagglutinin, a 7-day interval between skin sensitization and challenge overcame the problem of general blueing of the whole skin of the animal after antigen challenge. In the present work, however, the 48-hour PCA was satisfactory for demonstrating IgE to Oa.

When the PCA titres of PCA-positive anti-Oa sera were compared with the IgE ELISA titres of the same sera, not only was there no direct correlation between the two methods, but there was a highly significant *inverse* relationship. However, when the sera were segregated according to the dose of antigen used to raise them, those from immunizations with the 20- μ g Oa dose showed a small but non-significant positive correlation between PCA and ELISA. In contrast, the sera from the 200- μ g Oa immunizing dose showed a significant negative correlation between the two types of assay. These observations do not correspond with previous reports, mainly with antigens other than Oa, in which ELISA and PCA were compared directly. For example, Maekawa and Ovary [13], with two polyspecific sera to dinitrophenyl hapten, reported that PCA and ELISA titres were parallel. Ito et al. [17], while measuring guinea pig antibodies to mite antigen, found a highly significant correlation between PCA and ELISA. However, the ELISA results were derived only from optical density readings at a fixed dilution and were not actual titres as in our study. Hirano et al [15], with four polyspecific and six monoclonal antibodies to dinitrophenyl, found a good correlation between PCA and ELISA, but the polyspecific sera all gave low values, and all the monoclonal antibodies were of high titre. This differs from

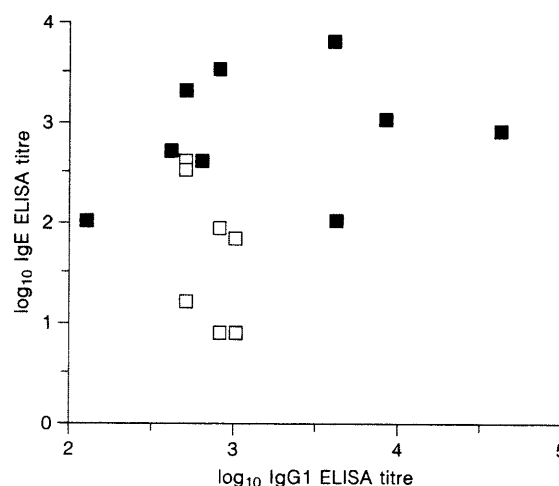


Fig. 6. Correlation between the ELISA titres of anti-Oa IgG1 and anti-Oa IgE in mouse sera raised by the one-injection protocol with 20 μ g Oa (open squares) and 200 μ g Oa (closed squares).

our study where the sera were all polyspecific and, as a collection of 18 samples, exhibited a broad range of both PCA and ELISA titres.

The only report, other than ours, in which there was no overall correlation of IgE measurements by PCA and ELISA is that of Astorquiza and Cisternas [26] which happened also to be with Oa. As in our work, this lack of correlation was only an incidental observation peripheral to the main study on the effect of an adjuvant (phytohaemagglutinin) on the anti-Oa IgE response in mice. There was no evidence in our work that the lack of correlation between PCA and ELISA was due to high levels of anti-Oa IgG1, which was possible on theoretical grounds. In conclusion, it would seem wise to exercise caution before assuming that the measurement of IgE by ELISA necessarily correlates positively with IgE biological activity as measured by PCA. Many factors might contribute to such discrepancies, and perhaps Oa, in the light of our and the previous reports, may be well suited to exploring them.

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