

Evaluation of an in vitro method for the measurement of specific IgE antibody responses: the rat basophilic leukemia (RBL) cell assay

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Abstract

The evaluation of allergenic potential is a key parameter in the safety assessment of novel proteins, including those expressed in genetically modified crops and foodstuffs. The majority of allergic reactions to food proteins are immediate type hypersensitivity reactions in which the principal biological effector is IgE antibody; the accurate measurement of specific IgE antibody is therefore a critical factor in experimental systems designed to characterize protein allergenic potential. Due to the presence of much higher concentrations of other immunoglobulin isotypes, the assessment of specific serum IgE antibody poses substantial technical challenges. We have examined the utility of the rat basophilic leukemia (RBL) cell line for the measurement of murine IgE responses. RBL cells were sensitized with mouse monoclonal anti-dinitrophenyl (DNP) IgE antibody and challenged with DNP–albumin conjugates with various hapten substitution ratios (SR). Polyclonal anti-OVA IgE antisera were also assessed for activity in the RBL assay. Results were compared with titers measured in homologous passive cutaneous anaphylaxis (PCA) assay. Marked degranulation of RBL cells was induced by conjugates with SRs of between 16 and 32, whereas conjugates with lower SRs (of 10 or 3) failed to elicit significant serotonin release. All conjugates were able to induce mast cell degranulation in vivo in a PCA assay. Anti-OVA antisera with PCA titers of 1/32 to 1/64 failed to stimulate RBL cell degranulation, whereas high titer antibody (1/2048 to 1/4096 by PCA) induced a positive RBL cell response. Successful stimulation of RBL cell degranulation requires not only appropriate epitope densities but also high affinity antibody. These data indicate that this assay is inappropriate for the routine analysis of specific polyclonal IgE antibody responses such as those that are induced by exposure to complex protein allergens. © 2004 Elsevier Ireland Ltd. All rights reserved.

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1. Introduction

One of the key parameters in the safety assessment of novel proteins, including those expressed in genetically modified crops and foodstuffs, is an evaluation of

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allergenic potential. The majority of allergic reactions to food proteins, in common with allergic asthma, rhinitis and atopic dermatitis, are immediate type hypersensitivity reactions in which the principal biological effector is IgE antibody (Bush, 2002; Galli et al., 2003; Sampson, 2003; Sicherer, 2003). The accurate measurement of specific IgE antibody is therefore a critical factor in experimental systems designed to characterize the allergenic potential of proteins. It is necessary to measure specific IgE, rather than changes in the total serum concentration of IgE, because exposure to protein allergens alone (without the presence of adjuvant) is not usually associated with significant increases in total serum IgE levels (Hilton et al., 1994; van Halteren et al., 1997).

There are various methods available for the analysis of specific IgE antibody responses. As IgE antibody is present in the serum at concentrations that are orders of magnitude lower than other isotypes of immunoglobulin including IgG, precautions must be taken against the blocking effects of or cross-reactivity of reagents with such antibody. It is therefore necessary to employ steps that either deplete of non-IgE isotypes, or that are selective for IgE antibody. Many investigators agree that the demonstration of biologically active IgE is important, so homologous or heterologous passive cutaneous anaphylaxis (PCA) assays are commonly utilized (Kimber et al., 2003). In this assay, specific IgE antibody is measured by the adoptive transfer of serum by intradermal injection into naïve recipient mice or rats (Inagaki et al., 1988; Lehrer and Vaughan, 1976; Ovary, 1982). IgE antibody present in the transferred serum will bind to specific receptors (Fc ϵ receptors; Fc ϵ R) on tissue mast cells in the skin of the host animal. Recipient animals are then challenged with an intravenous injection of the test protein together with Evans Blue dye. If specific IgE antibody is present then it will be crosslinked causing local mast cell degranulation and vasodilation. The injected blue dye is therefore able to extravasate, resulting in a blueing of the skin and serum IgE antibody titer can be determined with respect to the maximum dilution of serum required to provoke a detectable reaction.

A possible alternative method for IgE detection is based on the measurement of IgE-induced degranulation *in vitro*. The rat basophilic leukemia (RBL) cell line possesses Fc ϵ R which can selectively bind murine IgE antibody and can be stimulated by crosslinking with protein allergen to degranulate, releasing granules

which have been preloaded with radiolabelled serotonin (Eccleston et al., 1973). This method has been utilized extensively to study the mechanisms of IgE interactions with mast cell Fc ϵ R (Vincent-Schneider et al., 2001; Wilson et al., 2000) and for the measurement of IgE antibodies induced by intranasal exposure to respiratory sensitizing detergent enzymes (Kawabata and Babcock, 1993). It has not yet been evaluated rigorously as an alternative to PCA assays for the measurement of serum IgE antibody responses, particularly those induced by exposure to protein allergens in the absence of adjuvant (Ballmer-Weber et al., 2002; Hoffmann et al., 1999; Yamanishi et al., 1995).

In the current investigations we have compared the sensitivity of the RBL assay and the homologous PCA assay for the detection of specific IgE antibody. A commercially available monoclonal IgE antibody (recognizing a single antigenic epitope; dinitrophenyl, DNP) and hapten protein conjugates that express multiple IgE binding sites of varying hapten: protein conjugate ratios have been utilized. In addition, the ability of polyclonal IgE antibody (directed at multiple epitopes on a complex protein allergen, ovalbumin [OVA]) to induce RBL cell degranulation or positive PCA responses has been examined.

2. Materials and methods

2.1. Animals

Young adult (6–12 weeks old) female BALB/c strain mice (Specific Pathogen Free Breeding Unit, Alderley Park, Macclesfield) were used throughout these studies. Mice were housed three to five per cage on flushing metal racks with materials provided for environmental enrichment. Food (SDS PCD pelleted diet; Special Diets Services Ltd., UK) and water were available *ad libitum*. The ambient temperature was maintained at $21 \pm 2^\circ\text{C}$ and relative humidity was $55 \pm 10\%$ with a 12 h light/dark cycle. All procedures were approved by the UK Home Office and carried out in compliance with the Animals (Scientific Procedures) Act, 1986 under a Home Office granted Project Licence.

2.2. Test materials

Commercial preparations of ovalbumin, human serum albumin, DNP–albumin commercial conjugate

(substitution ratio 32 mol of DNP per mole of albumin) and 2,4-dinitrofluorobenzene (DNFB) were obtained from Sigma Chemical Co., St Louis, MO.

2.3. Generation of dinitrophenol (DNP)–albumin conjugates

Albumin (30 mg/ml in distilled water) was incubated at room temperature for various periods of time with 2.5, 5 or 7.5 mg/ml DNFB added at hourly intervals. After 1–3 h, the solutions were dialyzed against phosphate buffered saline (PBS) for 24 h and against water for a further 24 h prior to lyophilization and storage at 4 °C. The degree of substitution of the DNP–albumin conjugates was assessed by absorbance at 360 nm as described previously (Makela and Sepala, 1986). The conjugate substitution ratios ranged from 3 mol hapten to 1 mol protein to 32 mol hapten to 1 mol protein.

2.4. Sensitization for anti-ovalbumin IgE antibody production

To generate low titer anti-OVA IgE antibody, groups of mice ($n = 5$) received 0.25 ml of 2% (w/v) of OVA in PBS by intraperitoneal injection. Seven days later treatment was repeated. Mice were exsanguinated 14 days after the initiation of exposure. To generate high titer anti-OVA IgE antibody, groups of mice ($n = 5$) received 0.25 ml of 0.02% (w/v) of OVA in PBS with 16 mg/ml aluminium hydroxide adjuvant (Sigma) by intraperitoneal injection on days 0, 14, 28 and 42 days. Mice were exsanguinated 49 days after the initiation of exposure. Pooled serum samples were prepared and stored at –20 °C until analysis.

2.5. Rat basophilic leukemia cell culture

Rat basophilic leukemia (RBL-2H3) cells were cultured at $(1-2) \times 10^6$ cells per 75 cm² flask at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cells were cultured in RPMI 1640 medium (GibcoRBL, Renfrewshire, UK) supplemented with 25 mM HEPES, 10% fetal calf serum, 2 mM glutamine and 10 µg/ml gentamycin. Cells were passaged every 4–5 days.

2.6. Rat basophilic leukemia cell assay

Cells were isolated using a cell scraper, resuspended in fresh medium at 10⁶ cells/ml and aliquoted (1 ml) into 24-well flat bottomed tissue culture plates. Cells were incubated at 37 °C for 16 h in the presence of ³H-hydroxytryptamine creatinine sulfate (³H-serotonin; specific activity 25.5 Ci/mmol; Amersham International, UK). Various concentrations of monoclonal anti-DNP IgE antibody (Sigma; serial doubling dilutions from 2000 to 0.12 ng/ml), normal mouse serum, anti-OVA serum or medium alone were added and cells incubated for a further 4 h. Cells were washed four times by centrifugation and resuspended in 1 ml of fresh medium. 100 µl aliquots of cells were transferred into 96-well microtiter plates. 100 µl of various concentrations of DNP–albumin conjugates of different substitution ratios (in duplicate) or OVA was added. In order to determine total serotonin content of cells, 100 µl of 5% Triton X100 (in quadruplicate) was added to separate aliquots of cells. Cells were incubated for 45 min and then the plates were spun at 1000 × *g* for 5 min. 100 µl of cell supernatant was transferred to 20 ml scintillation vials and 2 ml of scintillation fluid (Optiphase HiSafe 3; Wallac, Turku, Finland) was added. Serotonin content was assessed by β-scintillation counting.

The specific release (%) induced by IgE antibody was calculated according to the following equation

$$\text{specific release (\%)} = \frac{\text{test dpm} - \text{medium dpm}}{\text{Triton X100 dpm} - \text{medium dpm}} \times 100$$

2.7. Measurement of IgE antibody by passive cutaneous anaphylaxis assay

IgE antibody was also measured by homologous PCA assay. Pooled serum samples isolated following intraperitoneal exposure to OVA were diluted to various extents with PBS injected (30 µl) into the dermis of the ears of naïve recipient mice ($n = 4$). Concurrent negative control serum samples (derived from naïve animals) diluted to an equivalent extent with PBS were incorporated into every PCA assay. Two days later, 0.25 mg of OVA, together with Evans Blue dye (Sigma; 1.25 mg), in 0.25 ml of PBS were injected intravenously. Thirty minutes following challenge, mice

were killed and the diameter of cutaneous reactions measured. A positive (IgE) response was recorded if the challenge resulted in a >3 mm blue lesion in the skin in the majority of recipient animals, with antibody titer recorded as the highest dilution of serum resulting in a positive PCA reaction. The ability of different DNP–albumin conjugates to induce positive PCA reactions was also assessed. Mouse monoclonal anti-DNP IgE antibody diluted to various extents with PBS (10–0.001 $\mu\text{g/ml}$) was injected (30 μl) into the dermis of the ears of naïve recipient mice ($n = 4$). Two days later, 0.25 mg of DNP–albumin conjugates, together with Evans Blue dye (1.25 mg), in 0.25 ml of PBS were injected intravenously. As described previously, 30 min following challenge, mice were killed and the diameter of cutaneous reactions measured.

2.8. Statistical analysis

RBL assay data (expressed in terms of % change relative to control values) were converted to positive values by the addition of a factor of 100 then log transformed to stabilize the variance. The significance of effects on serotonin release induced by changes in the concentration of anti-DNP IgE antibody relative to values recorded in the absence of anti-DNP IgE antibody was considered by analysis of variance using a two-sided Student's *t*-test, based on the error mean square in the analysis of variance. For conjugates of differing substitution ratios, differences between individual group means were compared using Scheffe's test based on the error mean square of the analysis of variance (Scheffe, 1953). For anti-OVA antisera, the data were analyzed by paired Student's *t*-test. Statistical analyses were conducted using Statistical Analysis System (SAS) User's Guide, Version 8 (SAS, 1999).

3. Results

3.1. Assay optimization: rat basophilic leukemia cell degranulation induced by monoclonal anti-dinitrophenol IgE antibody

In initial experiments, conditions were optimized for serotonin release by RBL cells following sensitization with a monoclonal anti-DNP IgE antibody and challenge with a commercially available DNP–albumin

conjugate with a substitution ratio of 32 mol of hapten per mole of protein. RBL cells were sensitized with serial doubling dilutions (2000–0.12 ng/ml) of monoclonal anti-DNP IgE antibody and challenged with DNP–albumin (Fig. 1). Dilution curves derived for IgE sensitized cells challenged with 80 ng/ml DNP–albumin in five independent assays are illustrated in Fig. 1a. In each experiment, a typical prozone effect was observed with a bell-shaped dose–response curve. Relatively low levels of serotonin release were recorded at high concentrations of anti-DNP IgE (2000–500 ng/ml), with RBL cell degranulation increasing to maximal levels between 250 and 7.8 ng/ml of anti-DNP IgE. At concentrations of 3.9 ng/ml and below, serotonin release approached background levels. Although there was little inter-experimental variation in the shape of the dilution curve there was, however, considerable variation in the maximal serotonin release recorded, with levels ranging from approximately 53% to 13%. A further five independent assays were conducted and mean (and S.E.) of the total of 10 experiments are displayed in Fig. 1b. The extent of serotonin release for each concentration of anti-DNP IgE tested (2000–0.12 ng/ml) has been compared with control levels observed for cells challenged with DNP–conjugate in the absence of anti-DNP IgE. In this series of experiments, control levels varied from 1.6% to –9.6% (mean – $1.0 \pm 1.0\%$). As observed previously, there was a marked prozone effect at high concentrations of IgE (2000–500 ng/ml), although levels of RBL degranulation were significantly higher ($P < 0.01$) than those recorded for control treated cells. Maximal serotonin release was observed at 125 ng/ml anti-DNP IgE, with a gradual decrease thereafter, returning to background levels at 1.95 ng/ml. The influence of the challenge concentration of DNP–conjugate upon RBL cell degranulation was investigated (Fig. 1c; representative experiment). RBL cells were sensitized with serial doubling dilutions of anti-DNP IgE (2000–0.12 ng/ml) and challenged with 40–320 ng/ml of DNP–conjugate. The prozone shape of the dose–response curve was unaffected by the challenge concentration of hapten–protein conjugate, with a bell-shaped profile recorded for each dose used. Similar levels of maximal serotonin release were observed for each concentration of DNP–conjugate (ranging from 18% to 33%) within a relatively narrow range of sensitization concentrations of anti-DNP IgE (125 to 15.6 ng/ml).

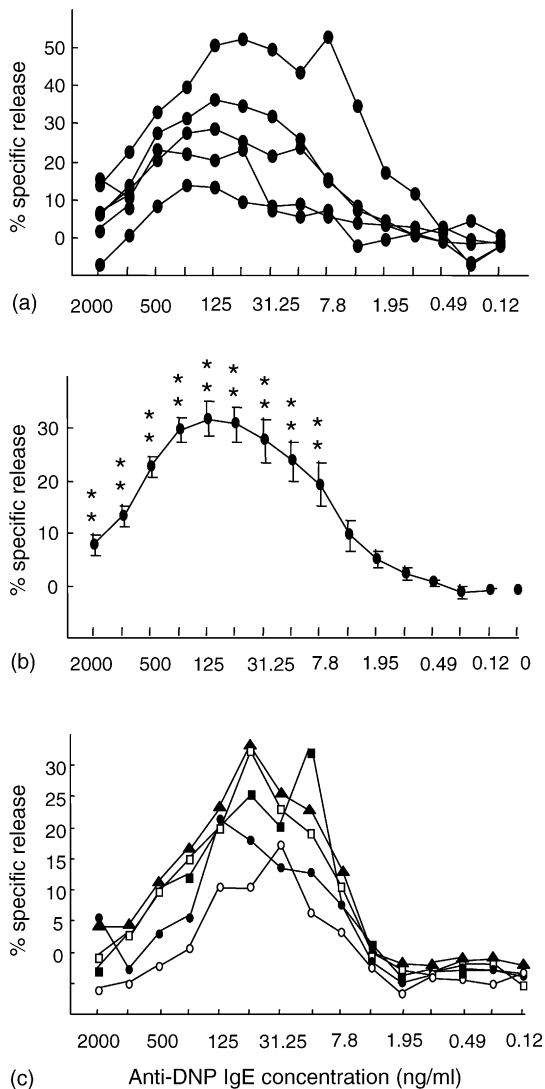


Fig. 1. RBL cell degranulation induced by anti-DNP IgE antibody: bell-shaped dose-response curve. RBL were incubated with serial doubling dilutions of monoclonal anti-DNP IgE antibody and challenged with 80 ng/ml DNP-albumin conjugate (substitution ratio 32 mol of DNP per mole of protein). Dose-response curves for five independent experiments are shown (a) and mean and S.E. for 10 independent experiments (b). The statistical significance of differences in serotonin release for each concentration of anti-DNP IgE antibody tested relative to that induced in the absence of anti-DNP IgE (0 ng/ml) was compared by analysis of variance using a two-sided Student's *t*-test, based on the error mean square in the analysis of variance. $**P < 0.01$. Dose-response curves were generated following challenge with 20 ng/ml (○), 40 ng/ml (●), 80 ng/ml (■), 160 ng/ml (□) or 320 ng/ml (▲) DNP-albumin conjugate (substitution ratio 32 mol of DNP per mole of protein) (c; representative experiment).

3.2. Rat basophilic leukemia cell degranulation induced by monoclonal anti-dinitrophenol IgE antibody: influence of conjugate substitution ratio

In subsequent experiments, the impact of conjugate substitution ratio on RBL cell degranulation was assessed. Following sensitization with anti-DNP IgE antibody (2000–0.12 ng/ml), RBL cells were challenged with five different DNP-albumin conjugates (80 ng/ml) with substitution ratios ranging from 32 to 3 mol of hapten per mole of protein (Fig. 2a; representative experiment). Conjugates with substitution ratios of 32–16 mol of DNP per mole of albumin induced typical profiles of serotonin release, with optimal RBL cell degranulation achieved at concentrations of between 250 and 31.25 ng/ml anti-DNP IgE. In contrast, conjugates with substitution ratios of 10 and 3 mol of DNP per mole of albumin failed to elicit serotonin release of above background (5%) at any concentration of anti-DNP IgE tested. Furthermore, the failure of the conjugates with low substitution ratios to stimulate RBL cell degranulation was not due to lack of responsiveness of the particular batch of RBL cells utilized in these assays as cells were tested concurrently with the commercial DNP-albumin conjugate and responded normally. These data were confirmed in subsequent assays in which RBL cells were sensitized with 125 ng/ml anti-DNP IgE, a concentration of antibody that was optimal for IgE-mediated serotonin release for conjugates with relatively high substitution ratios (32–16 mol of DNP per mole of albumin) (Fig. 2b; three to six independent experiments). Significant RBL cell degranulation was induced only in the presence of DNP-albumin conjugates with substitution ratios of 32, 19 and 16 mol of hapten to 1 mol of protein. Mean serotonin release in the presence of these conjugates varied from 25.7% to 28.4% whereas values for the conjugates with substitution ratios of 10 and 3 mol of hapten per mole of albumin were less than 5%.

Finally, it was demonstrated that increasing the challenge concentration of the conjugate with the lowest substitution ratio (3 mol hapten to 1 mol of protein) with doses ranging from 40 to 320 ng/ml, did not result in RBL cell degranulation, with levels of less than 7.5% recorded at all concentrations of anti-DNP IgE or DNP-conjugate examined (Fig. 2c). Concurrent control cells were challenged with the commercial DNP-conjugate and displayed the typical dose-response curve, with

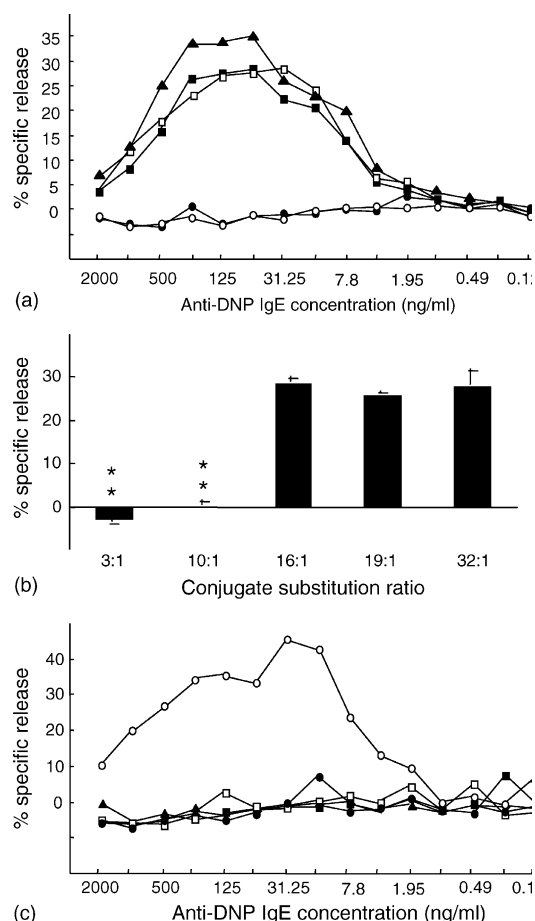


Fig. 2. Influence of conjugate substitution ratio on RBL cell degranulation induced by anti-DNP IgE antibody. RBL were incubated with serial doubling dilutions of monoclonal anti-DNP IgE antibody and challenged with 80 ng/ml DNP–albumin conjugate with a substitution ratio of 32 mol of DNP (▲), 19 mol of DNP (■), 16 mol of DNP (□), 10 mol of DNP (●) or 3 mol of DNP (○) per mole of protein (a; representative experiment). Serotonin-labelled cells were incubated with 125 ng/ml of monoclonal anti-DNP IgE antibody and challenged with 80 ng/ml of DNP–albumin conjugate with a substitution ratio of 32 mol of DNP, 19 mol of DNP, 16 mol of DNP, 10 mol of DNP or 3 mol of DNP per mole of protein. Mean and S.E.M. of $n = 3$ –6 experiments are displayed (b). The statistical significance of differences in serotonin release for DNP–albumin conjugate tested relative to that induced by the conjugate with the substitution ratio of 32:1 was compared by analysis of variance using Scheffe's test, based on the error mean square in the analysis of variance. $**P < 0.01$. RBL were incubated with serial doubling dilutions of monoclonal anti-DNP IgE antibody and challenged with 80 ng/ml DNP–albumin conjugate with a substitution ratio of 32 mol of DNP per mole of protein (○), or with 40 ng/ml (●), 80 ng/ml (■), 160 ng/ml (□) or 320 ng/ml (▲) DNP–albumin conjugate with a substitution ratio of 3 mol of DNP per mole of protein (c; representative experiment).

maximal levels of serotonin release of approximately 45% recorded at 31.25 ng/ml anti-DNP IgE. Thus, even increasing the effective dose of DNP to levels that are equivalent to those achieved with the more highly substituted conjugates is not sufficient to provoke RBL cell degranulation.

3.3. Measurement of monoclonal anti-dinitrophenol IgE antibody by passive cutaneous anaphylaxis assay

The same conjugates were tested for their ability to act as substrate in an homologous PCA assay. BALB/c strain mice ($n = 4$) received intradermal injections into the dorsum of both ears of monoclonal anti-DNP IgE antibody (10,000–1 ng/ml) diluted in PBS or PBS alone. Forty-eight hours later, separate groups of mice were challenged with each of the five DNP–albumin conjugates and the cutaneous reaction measured (Fig. 3). There was no evidence of a bell-shaped dose–response curve in the PCA reaction. All five conjugates provoked the maximal response when tested against 10,000 ng/ml anti-DNP IgE. There was some variation in the minimum concentration of anti-DNP IgE that was capable of stimulating a significant cutaneous response, with in general the more highly substituted conjugates able to elicit positive PCA reactions at lower doses of anti-DNP IgE. The DNP–albumin conjugate with the lowest substitution ratio (3:1) failed to stimulate mast cell degranulation at 200 ng/ml anti-DNP IgE, whereas the most highly substituted conjugate still provoked positive PCA reactions at 50 ng/ml anti-DNP IgE. This cut off is clearly less sensitive than the RBL assay, where significant serotonin release was recorded at concentrations as low as 1.96 ng/ml following challenge with the most highly substituted conjugate.

3.4. Rat basophilic leukemia cell degranulation for the detection of polyclonal IgE: comparisons with the passive cutaneous anaphylaxis assay

Finally, the ability of the RBL cell assay to detect polyclonal IgE antibody has been compared with that of the PCA assay. Pooled serum samples were isolated 14 days after intraperitoneal exposure to the common food allergen OVA (2%), or 49 days after repeated intraperitoneal exposure to low dose OVA (0.02%) in the

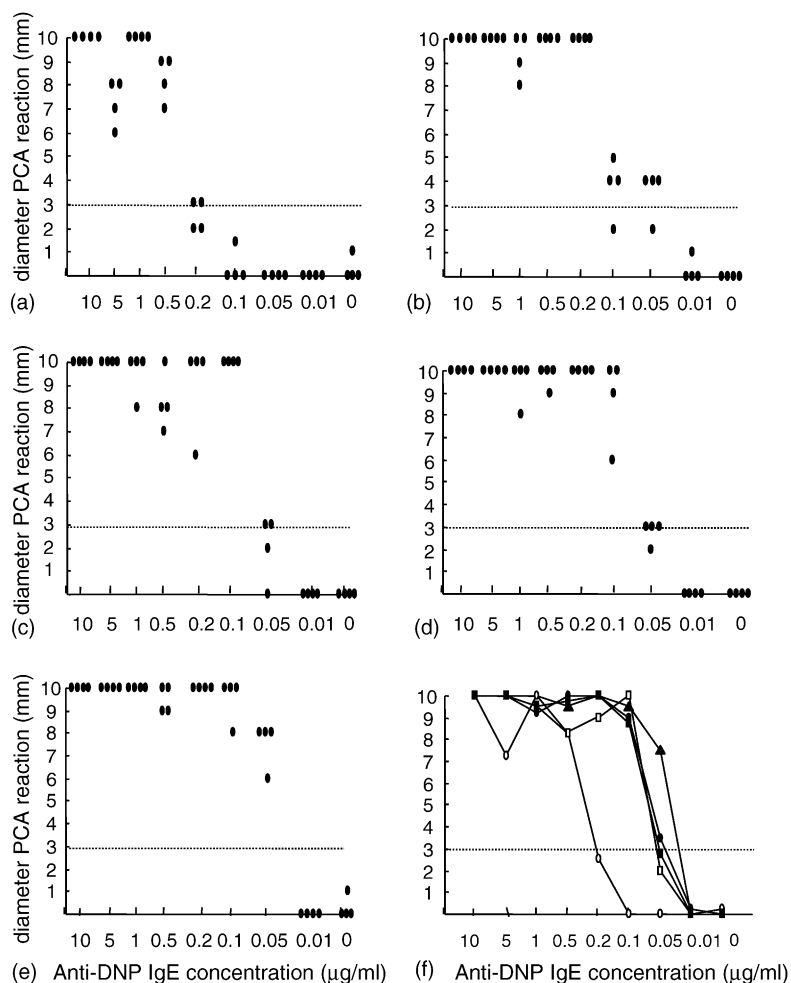


Fig. 3. Influence of conjugate substitution ratio on reactivity in the PCA assay. Naïve recipient mice ($n = 4$) received intradermal injections into the dorsum of both ears of monoclonal anti-DNP IgE antibody (10–0.01 $\mu\text{g/ml}$) diluted in PBS. Control intradermal injections of PBS were tested concurrently. The diameter (mm) of PCA reactions induced in individual animals ((●) represents response in an individual animal) are displayed for each concentration of anti-DNP IgE tested following challenge with conjugate with a substitution ratio of 3 mol of DNP (a), 10 mol of DNP (b), 16 mol of DNP (c), 19 mol of DNP (d) or 33 mol DNP (e) per mole of protein. Results are displayed also as the mean diameter of the PCA reaction ($n = 4$) for each concentration of anti-DNP IgE antibody tested with each conjugate (f): a substitution ratio of 32 mol of DNP (▲), 19 mol of DNP (■), 16 mol of DNP (□), 10 mol of DNP (●) or 3 mol DNP (○) per mole of protein. Dotted line represents the threshold for positivity.

presence of the adjuvant aluminium hydroxide. Serum samples were analyzed for IgE anti-OVA content by PCA (Fig. 4a; three independent experiments). In each of the three experiments, exposure to 2% OVA induced positive PCA reactions with serum dilutions of 1/8, 1/16 and 1/32, with cutaneous responses also recorded in one assay only at a 1/64 dilution of serum, equating to anti-OVA IgE titers of 1/32, 1/32 and 1/64. Exposure to low dose OVA in the presence of adjuvant resulted

in a very vigorous PCA response in each experiment, with positive PCA reactions recorded with serum diluted from 1/32 down to 1/2048 or 1/4096, equating to anti-OVA IgE titers of 1/4096, 1/2048 and 1/4096. The capacity of the relatively low titer anti-OVA antisera to provoke RBL cell degranulation was investigated. At every serum dilution and challenge concentration of OVA tested, levels of serotonin release were less than 5% (data not shown). However, significant levels

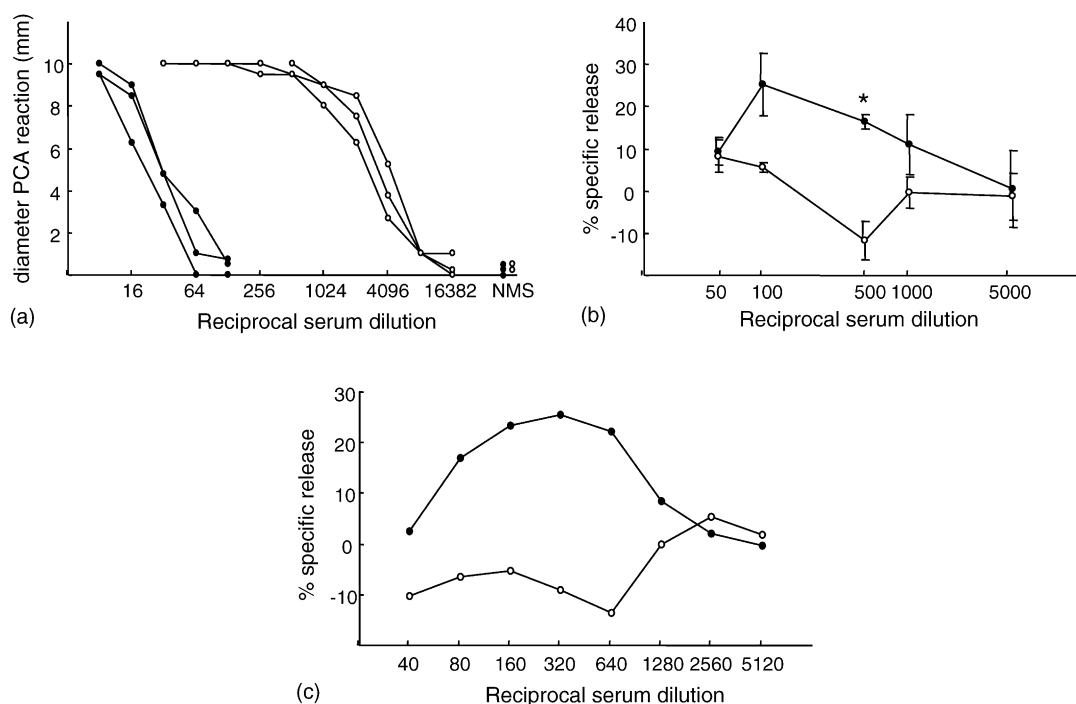


Fig. 4. Comparison of RBL cell degranulation and PCA assay for the detection of anti-OVA polyclonal IgE antibody. Naïve recipient mice ($n = 4$) received intradermal injections into the dorsum of both ears of high titer IgE anti-OVA antisera (derived following intraperitoneal exposure 0.02% OVA in the presence of alum) (●) or of low titer IgE anti-OVA antisera (derived following intraperitoneal exposure to 2% OVA) (○) serially diluted (1 in 4 to 1 in 16,384) in PBS. Control intradermal injections of pooled normal mouse serum (NMS) were tested concurrently. Results are displayed as the mean diameter of the PCA reaction ($n = 4$) for each concentration of anti-OVA antisera tested (a; three independent experiments). RBL cells were incubated with serial dilutions (1 in 50, 1 in 100, 1 in 500, 1 in 1000, 1 in 5000) of pooled serum derived after repeated intraperitoneal exposure to 0.02% OVA in the presence of alum (●) or with equivalent concentrations of pooled normal mouse serum (NMS) (○) and challenged with 1000 ng/ml OVA (b; mean and S.E. of three independent experiments). The statistical significance of differences in serotonin release for each dilution of anti-OVA antisera tested relative to that induced by an equivalent dilution of normal mouse sera was compared by paired Student's t -test. * $P < 0.05$. Further aliquots of RBL cells were incubated with serial doubling dilutions (1 in 40 to 1 in 5120) of pooled serum derived following intraperitoneal exposure to 0.02% OVA in the presence of alum (●) or with equivalent concentrations of pooled normal mouse serum (NMS) (○) and challenged with 1000 ng/ml OVA (c; representative experiment).

of RBL cell degranulation were achieved with the high titer anti-OVA antisera (Fig. 4b and c). RBL cells were sensitized with various concentrations of anti-OVA antiserum or with equivalent concentrations of serum isolated from naïve (untreated) mice (1/50–1/5000) and challenged with the optimal concentration of OVA (data not shown; 1000 ng/ml). As observed for anti-DNP monoclonal IgE sensitized cells, there was a marked prozone effect with high concentrations of anti-OVA serum (1/50) failing to induce significant serotonin release. Optimal RBL cell degranulation was achieved at 1/100 dilution of sera with levels approaching control values at lower serum dilutions, suggesting

a titer of between 1/500 and 1/1000. This was confirmed in subsequent assays in which RBL cells were sensitized with serial doubling dilutions of anti-OVA antisera (1/40–1/5120) (Fig. 4c). A similar prozone effect was observed and a titer of 1/640 was recorded.

4. Discussion

The relative sensitivity of the RBL assay, and of the homologous PCA assay, for the measurement of specific IgE antibody has been investigated. Using a monoclonal anti-DNP IgE antibody for sensitization,

the RBL assay was more sensitive than the PCA assay (threshold IgE concentrations of 3.9 and 50 ng/ml, respectively). However, the epitope density of the conjugate was found to have a marked effect on RBL cell degranulation. Conjugates with hapten: protein substitution ratios of 10: 1 and below failed to provoke significant serotonin release under any conditions examined here. In contrast, the conjugate substitution ratio had little impact on activity in the PCA assay, with all conjugates tested (substitution ratios from 32 to 3 mol of hapten per mole of protein) eliciting vigorous reactions. Furthermore, a differential ability of polyclonal IgE antibody (directed at multiple epitopes on a complex protein allergen, OVA) to induce RBL cell degranulation or positive PCA responses was observed. Low titer anti-OVA IgE antibody, raised in mice in the absence of adjuvant, failed to stimulate RBL cell serotonin release, although IgE titers of 1/32 and 1/64 were recorded in the PCA assay. High titer anti-OVA IgE antibody (raised by intraperitoneal exposure of mice in the presence of alum adjuvant) induced RBL cell degranulation with a titer of 1/640 observed. This was somewhat lower than the titers of 1/2048 and 1/4096 that were recorded in the PCA assay, suggesting that the PCA is more sensitive than is the RBL assay for the detection of both high and low titer polyclonal IgE antibody. The lower sensitivity of the RBL assay for anti-OVA IgE may be a reflection of the fact that in the absence of adjuvant there are likely to be relatively low numbers of allergenic epitopes recognized by the induced polyclonal IgE antibody and/or low affinity antibody. In the presence of adjuvant, both the number of epitopes and the affinity of the antibody are likely to be increased. It has been reported previously that RBL cell degranulation is affected markedly by antibody affinity, such that a combination of low affinity and low epitope density fails to cross link Fc ϵ R effectively on the surface of RBL cells (Collins et al., 1995). It is also of some significance that the RBL assay, as used herein, is a heterologous assay, relying on the interaction of mouse IgE with rat Fc ϵ R. There is some evidence that the heterologous PCA assay is not as effective as the homologous PCA for mouse polyclonal IgE (Lehrer and Vaughan, 1976), suggesting that there may be some species selectivity in the interaction between IgE antibody and Fc ϵ R.

An important feature of RBL cell degranulation induced by monoclonal or polyclonal antibody was

a marked prozone effect (bell-shaped dose–response curve). There was no evidence of a similar prozone effect for mast cells challenged in vivo, with maximal levels of cutaneous responses observed at the highest concentrations of IgE antibody utilized, suggesting that these effects may be an in vitro phenomenon. Indeed, such has been noted previously for freshly isolated peritoneal murine mast cells (Coleman, 1988; Holliday et al., 1993) and human basophils (Hoffmann et al., 1999). It has been hypothesized that this is attributable to the fact that efficient crosslinking of IgE molecules on the surface of mast cells or RBL cells is required for degranulation. Under circumstances where IgE antibody is saturating, there will be insufficient conjugate available to crosslink receptor bound IgE and optimal degranulation will not be induced, resulting in a potential underestimation of IgE levels. This finding has obvious implications for the use of RBL cells for the routine assessment of IgE content of sera; detailed dose–response analyses will be required to confirm that a given serum sample is indeed negative. There are other considerations that limit the use of the RBL assay for IgE measurements, including the considerable inter-experimental variation observed, even in the response of these cells to monoclonal IgE anti-DNP. In addition, it is apparent that, unlike the mouse PCA method, the RBL assay lacks the sensitivity to measure polyclonal IgE antibody responses induced in the absence of adjuvant. The RBL cell assay was unable here to detect IgE anti-OVA antibody with a titer of 1/32 or 1/64 as determined by homologous PCA assay. In other experiments designed to assess the intrinsic sensitization hazard of proteins, it has been reported that proteins that lacked significant sensitizing activity failed to induce detectable IgE antibody, or induced low titer (<1/2) IgE only, whereas proteins with sensitizing activity such as OVA or peanut lectin, induced IgE antibody production with titers ranging between 1/32 and 1/128, measured by PCA assay (Dearman et al., 2003). Clearly, based on the experiments described here, the RBL assay would be insufficiently sensitive to discriminate between such materials with differential intrinsic abilities to induce IgE antibody. Indeed, in laboratories where the RBL assay is used to measure IgE antibody responses, serum samples are derived using repeated exposure protocols in the presence of adjuvant (for example, injections every fortnight for 8 weeks with alum

(Ballmer-Weber et al., 2002; Yamanishi et al., 1995) or biweekly injections for 3 weeks with alum (Hoffmann et al., 1999)), protocols that will ensure high titer (and presumably high affinity) IgE antibody. The need for a highly substituted or multivalent antigen for the detection of low affinity antibody could be obviated by challenge of RBL cells with an anti-IgE antibody. However, this approach is unlikely to be successful for the measurement of IgE responses induced by protein allergens in the absence of adjuvant as increases in specific IgE antibody are not paralleled by increases in the total serum concentration of IgE (Hilton et al., 1994).

As described above, the most appropriate and sensitive method for the detection of polyclonal IgE antibody induced by proteins in the absence of adjuvant appears to be the homologous PCA assay. It is important to consider therefore the relevance and potential utility of this method. In mice, both IgG1 and IgE antibody isotypes are homocytotropic (bind murine mast cells) (Inagaki et al., 1988; Lehrer and Vaughan, 1976). IgG1 and IgE antibodies can be distinguished on the basis of their differential affinity for mast cell receptors (Lehrer and Vaughan, 1976). Not only do IgG1 antibodies dissociate more quickly than do IgE antibodies (active after 2 and 48 h, respectively) (Lehrer and Vaughan, 1976), but also the minimal amount of IgG1 antibody required to elicit an homologous PCA reaction is orders of magnitude higher than the minimal amount of IgE required (500–700 and 15 ng, respectively) (Ovary, 1982). The PCA protocol used herein should be largely selective for murine IgE antibody, as a 48 h incubation step is utilized. In addition, it has been demonstrated that differential IgE antibody production is detected by homologous PCA assay under conditions where equivalent IgG1 responses are stimulated (measured by ELISA) (Dearman et al., 2003). The PCA assay is therefore apparently selective for IgE antibody, and furthermore, is sensitive enough to detect relatively low titer antibody.

In conclusion, these data suggest that there are serious limitations for the use of the RBL assay as an in vitro replacement for the PCA assay. The RBL assay is not appropriate, at least in its present configuration, for the detection of polyclonal antibodies that have low affinity and/or epitope density, and thus lacks the necessary sensitivity for the detection of anti-protein antibodies derived in the absence of adjuvant.

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