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Immunochemical and Biological Quantification of Peanut Extract

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Abstract

Biological standardization of allergen extracts is one of the steps in the characterization of an extract. The gold standard for determination of biological potency is the skin prick test, but histamine release (HR) has been used as a convenient *ex vivo* method for analyzing a large number of samples. We describe the use of rabbit basophils as a tool in biological standardization. Using peanut as a model allergen, it is described how rabbits immunized for production of antiserum may become sensitized and their basophils used for histamine release experiments. It is also possible to use rabbit antiserum to passively sensitize basophils derived from naive rabbits, but the sensitivity of this method is so far 100-1000 times lower than the direct histamine release. The rabbit histamine release results are compared to an ELISA developed by means of the same antisera and by passive sensitization of human basophils using serum from a strongly sensitized peanut-allergic patient. The overall sensitivity of the methods were ELISA > HR-human cells > HR-sensitized rabbit cells > HR-passively sensitized rabbit cells. The use of rabbit basophils for biological standardizations will allow for the use of rabbit antisera.

Introduction

Characterization of allergen extracts should ideally encompass both a quantification of the biological potency and description of the biochemical constituents of the extract (1). Methodologies for the latter have made large progress during the recent years due to development of monoclonal antibodies, and cloned allergens that can be used as standards. The principles for determination of biological potency i.e. the skin test of a specified population of allergic patients (2) has demonstrated less progress, however

(2). A number of examples such as cross-reactive carbohydrate determinants (3,4) as well as other food related cross-reactivities (5,6) have underlined the fact that the mere binding between an antibody and an antigen does not in itself constitute a clinical relevant allergen. Moreover, many patients react to more than one allergen and may still require an extract rather than a single allergen for diagnosis, and when it comes to risk evaluation of potential allergenic foods there is a growing need for establishment of biologically relevant measurements. Finally, since little is known about how more allergenic molecules contribute to the total biological activity of the combination there is a clear need for biological measurements.

Since it is neither feasible nor ethical to include a growing number of patients into routine analyses of biological potency, alternative methods should be developed. Histamine release from the basophilic granulocyte which are believed to be sensitized analogously to skin mast cells have been used extensively for many immunological studies of the allergic response (7,8). Being an *in vitro* method this technique has obvious advantages compared to the skin tests, since less strict requirements are posed on the test substance regarding non-toxicity and non-infectivity, albeit it should not be cytotoxic in the applied concentrations. We have previously demonstrated the applicability of the method for determination of biological activity of a number of extracts and allergens (9-11).

The large number of available rodent antibodies makes animal mediator release assays an interesting possibility and the RBL-2H3-cell line has previously been employed for standardization and characterization (12). The aim of the present study was to extend the basophil histamine release method to the rabbit system, in order to make use of the large number of polyclonal rabbit antibodies to allergen mixtures.

Materials and Methods

Extraction of peanut and peanut product

Fifty g of raw de-shelled peanuts were extracted for 120 sec in 100 ml isotonic saline in an automated extractor (Stomacher 80, Lab. System). After centrifugation (2000 g, 10 min) the lipid phase was removed and the extract re-centrifuged. Protein concentration was determined to 7.8 mg/ml by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as standard and by amino acid analysis to 9.9 mg/ml. The latter value was used for dilutions and calculations.

A similar extraction was performed at a later stage both of raw (extract #1, giving a protein concentration of 4.6 mg/ml) and—with distilled water—of roasted (extract #2, protein content 5.2 mg/ml) peanuts.

For peanut containing products, the product was extracted 1:10 w/v and heat treated (56 °C, 30-60 min), frozen overnight, and centrifuged at 18.000 g.

Immunization of rabbits with allergen extracts

Two rabbits were immunized with 2 mg/ml in Freund's incomplete adjuvant every 2 weeks for a period of 12 months. Blood was drawn for preparation of serum every 4

weeks (before the next immunization were pooled and stored in a sulfate preparation.

ELISA

A double sandwich assay was developed. Immune serum (0.75 µg/ml) with protein A-gelatin 1 mg/ml in phosphate buffered 0.1% Tween supplemented with 1.8 mg/ml). Detection antibody at 2 µg/ml in normal rabbit serum. Enzyme (Kirkegaard and Perry) was added out with 1,2-phenylene diamine as reference. The assay was run above. The inter-assay variation between 1 ng/ml and 400 pg/ml.

Histamine release from rabbit basophils

Twenty-five ml of whole blood was added to 10 mM, and enriched with 10% removed by three times centrifugation. Cells were either challenged from the immunized or from naive animals were challenged with serum (buffer) as control. Cells were reconstituted with dilutions described in the literature. Histamine release was measured as described.

Histamine release from rabbit basophils

HR was performed with 10% (RefLab, Copenhagen) tested as described. Cells were added to buffy coat leukocytes at 37 °C with 250 µl of placebo-controlled. Cells were added to histamine adsorbed to amine from disrupted cells on basis of total histamine.

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weeks (before the next booster was given). Serum samples from >3 months of immunization were pooled for later preparation. An IgG-fraction was purified by ammonium sulfate preparation.

ELISA

A double sandwich ELISA using the polyclonal rabbit antibody to peanut extract was developed. Immuno-Maxisorp plates (NUNC, Roskilde, Denmark) were coated (0.75 µg/ml) with protein G affinity-purified IgG. Non-attached sites were blocked with gelatine 1 mg/ml in phosphate-buffered saline (PBS). Samples were diluted in PBS + 0.1% Tween supplemented with 30% of an extract of wheat flour (stock concentration: 1.8 mg/ml). Detections were performed with a biotinylated preparation of the same antibody at 2 µg/ml in PBS + 0.1% Tween, supplemented with wheat extract and 1% normal rabbit serum (DAKO, Glostrup, Denmark) followed by streptavidin-peroxidase (Kirkegaard and Perry La., Gaithersburg, MD, USA). Development was carried out with 1,2-phenylendiamine dihydrochloride and measured at 490 nm with 630 nm as reference. The assay was calibrated with a standard extract of raw peanuts as described above. The inter- and intra-assay coefficients of variation for the concentration range between 1 ng/ml and 1 µg/ml were less than 15%. The sensitivity of the ELISA was less than 400 pg/ml.

Histamine release from rabbit leukocytes

Twenty-five ml of blood were drawn from each rabbit, anticoagulated with EDTA 10 mM, and enriched for basophils on a lymphoprep gradient. Thrombocytes were removed by three times wash followed by centrifugation for 5 min at 11°C at 600 g. Cells were either challenged directly with antigen (direct histamine release using cells from the immunized rabbits) and histamine measured as described below, or cells from naive animals were passively sensitized with the hyperimmune rabbit serum or no serum (buffer) as control for 60 min at 37°C. After removing serum by washing, the cells were reconstituted in PIPES buffer and challenged with allergen extract in the dilutions described under Results. Histamine was measured and expressed as percentage histamine as described below.

Histamine release from human basophils

HR was performed by the glass fiber technique according to the manufacturer (RefLab, Copenhagen, Denmark) (9). Each extract was diluted in PIPES buffer and tested as described under Results. Briefly, a lymphoprep gradient was used to enrich buffy coat leukocytes to monocytes and basophils. These were sensitized for one h at 37°C with 250 µl of serum from a patient reacting strongly to peanut in double-blind, placebo-controlled, food challenges (DBPCFC) made according to the EAACI-guidelines (18). Cells were then challenged with dilutions of the extracts, and the released histamine adsorbed on glass-fiber coated microtiter plates. Remaining cellular histamine from disrupted cells was measured and percentage histamine calculated on the basis of total histamine content in non-challenged cells.

Results

When rabbits were immunized with a peanut extract antibodies readily developed. Figure 1a shows a standard curve of an ELISA based on the use of these antibodies. When another extract of raw peanut (extract #1) was tested a parallel curve emerged, whereas an extract of roasted peanut (extract #2) produced a non-parallel curve with a lower maximal response which indicated a partial breakdown of some of the antigens (Figure 1a). The apparent deterioration was even more pronounced when extracts of peanut containing food items were tested (Figure 1b and 1c). Both peanut products #1 and #2 showed a very low response with dilution curves clearly not parallel to the extract of raw peanut.

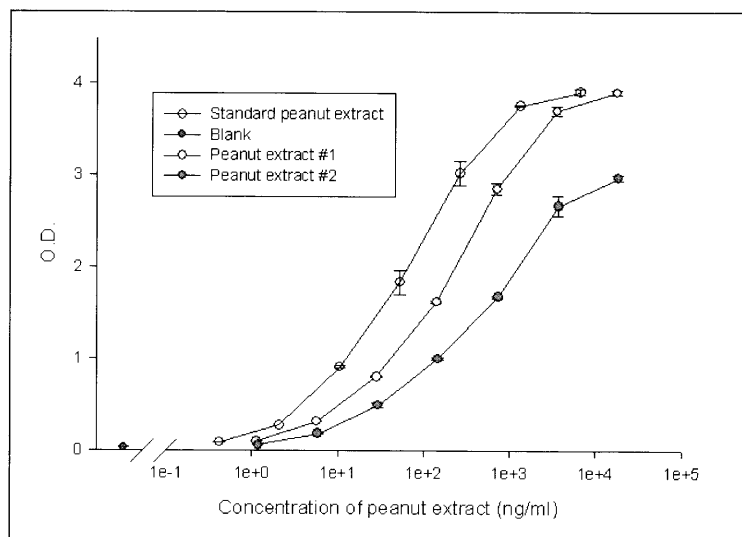


Fig. 1a.
Standard curves for
peanut ELISA. Protein
concentrations were
based on amino acid
analysis.

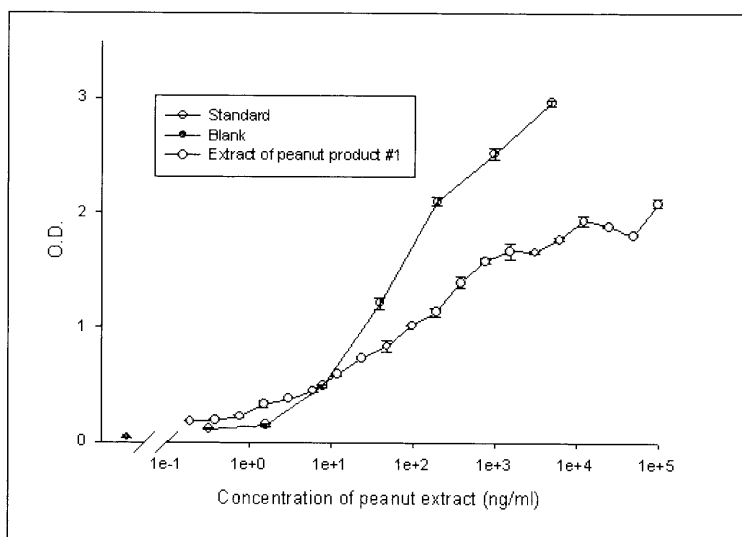
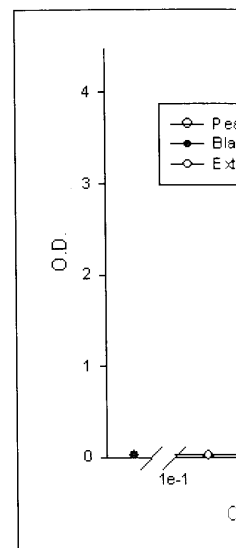
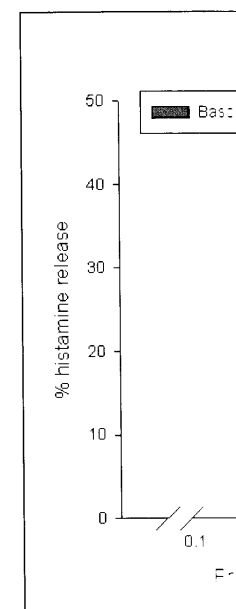


Fig. 1b.
ELISA dilution curve
of peanut product #1.
Undiluted extract
of product #1 was
arbitrarily set to 10^5
ng/ml.



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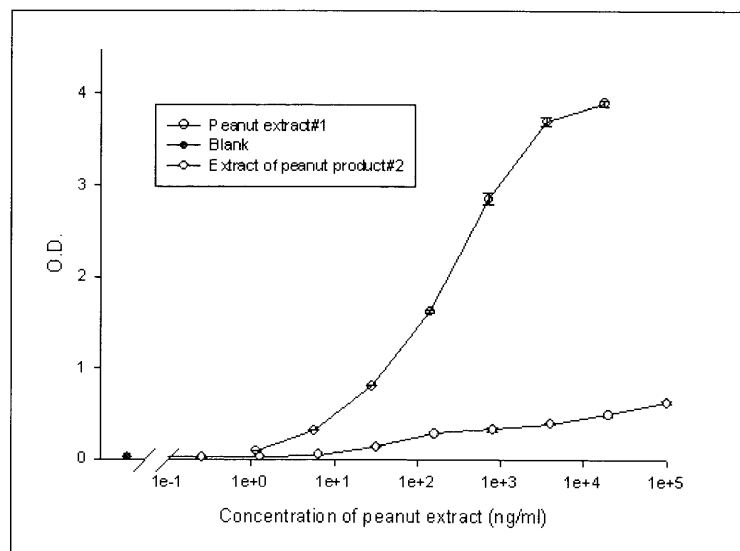


Fig. 1c.
ELISA dilution curve
of peanut product #2.
Undiluted extract
of product #2 was
arbitrarily set to 10^5
ng/ml.

When leukocytes were drawn from the immunized rabbits, a dose-response curve for histamine release was obtained (Figure 2). Preliminary experiments (results not shown) demonstrated the importance of removing the histamine-containing platelets to avoid excessively high background values.

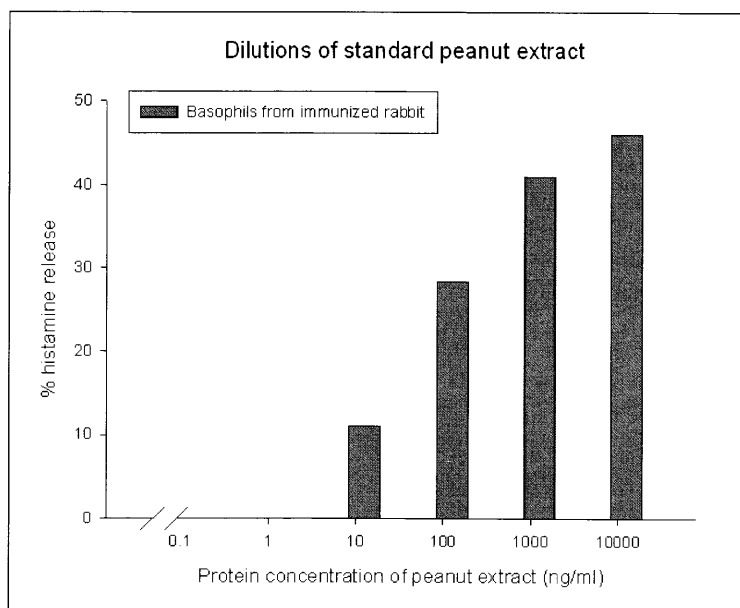


Fig. 2.
Direct histamine
release from rabbit
leukocytes.
The animal was
immunized with
peanut in Freund's
incomplete adjuvant,
and leukocytes were
isolated from a blood
sample. Cells were
challenged with the
standard peanut
extract.

Fig. 1a.
Standard curves for
peanut ELISA. Protein
concentrations were
based on amino acid
analysis.

Fig. 1b.
ELISA dilution curve
of peanut product #1.
Undiluted extract
of product #1 was
arbitrarily set to 10^5
ng/ml.

The histamine release method in rabbits would be considerably more versatile if it could be used in the form of passive sensitization, where an antiserum is used to sensitize leukocytes from naive animals. Thus we prepared leukocytes from a naive rabbit

and sensitized these with the antiserum obtained by immunization described above. The results in Figure 3a demonstrates that it was indeed possible to obtain a response when passively sensitizing rabbit leukocytes with the antiserum. The system was clearly less sensitive than the direct histamine release giving a positive response to the extract only in concentration of 10 $\mu\text{g/ml}$ and above. On the other hand, neither non-sensitized basophils nor IgG fraction of the antiserum was able to cause the same response.

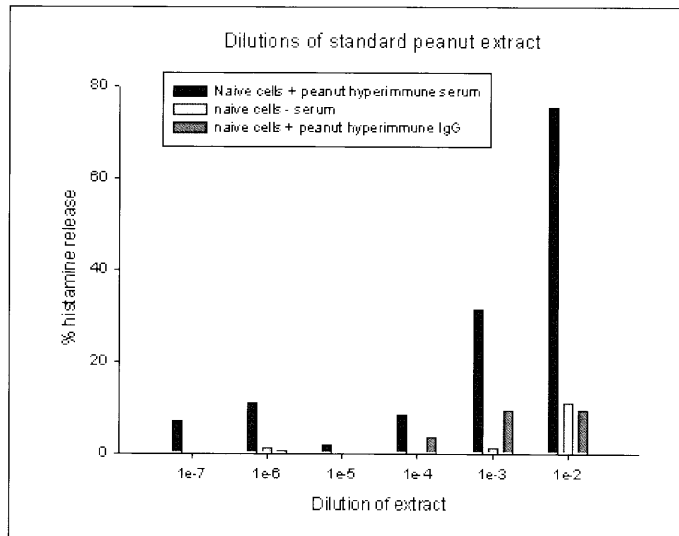


Fig. 3a.

Passive sensitization of rabbit leukocytes with rabbit hyperimmune serum, no serum or the IgG fraction of the hyperimmune serum. Cells were challenged with a peanut extract diluted from 9 mg/ml.

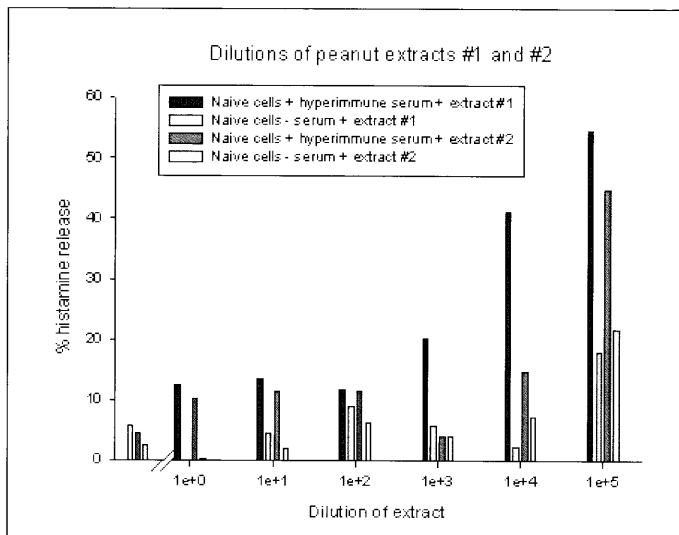


Fig. 3b.

Passive sensitization of rabbit leukocytes with rabbit hyperimmune serum or buffer controls. Cells were challenged with a peanut extract #1 and #2 (cf. materials and methods).

Figure 3b shows two other extracts compared. In the highest concentration there is a tendency to non-specific stimulations of the controls whereas the next dilution step illustrates that extract #1 (raw peanuts) but not #2 (roasted peanuts) is positive. Although much less sensitive compared to ELISA-results (Figure 1a), this method also

suggests that less active peanut-containing products. The second was only

For comparison a passive sensitization system in the order of 10 n strongly sensitized p

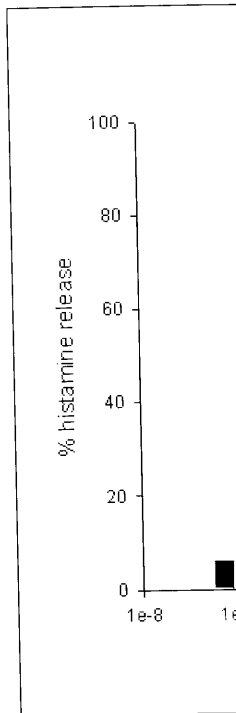


Fig. 4. Human leukocytes from an allergic patient or a positive control. Cells were challenged with a peanut extract diluted from 9 mg/ml.

Discussion

For studies of in vitro with other measures of specific IgE in plasma the technique for histamine release from sensitized rabbit system has n

The finding that the immunology c

on described above. To obtain a response the system was clearly responsive to the extract rather than non-sensitized cells. The same response.

suggests that less activity is present in the extract of the roasted peanuts. When the two peanut-containing products were tested, #1 were positive in a dilution of 1:25, whereas the second was only positive in the highest concentration (results not shown).

For comparison a titration of the standard peanut allergen extract in the human passive sensitization system is shown. Figure 4a shows that this system has a detection limit in the order of 10 ng/ml, i.e. a 10^6 times dilution of the extract, when serum from a strongly sensitized peanut-allergic is employed.

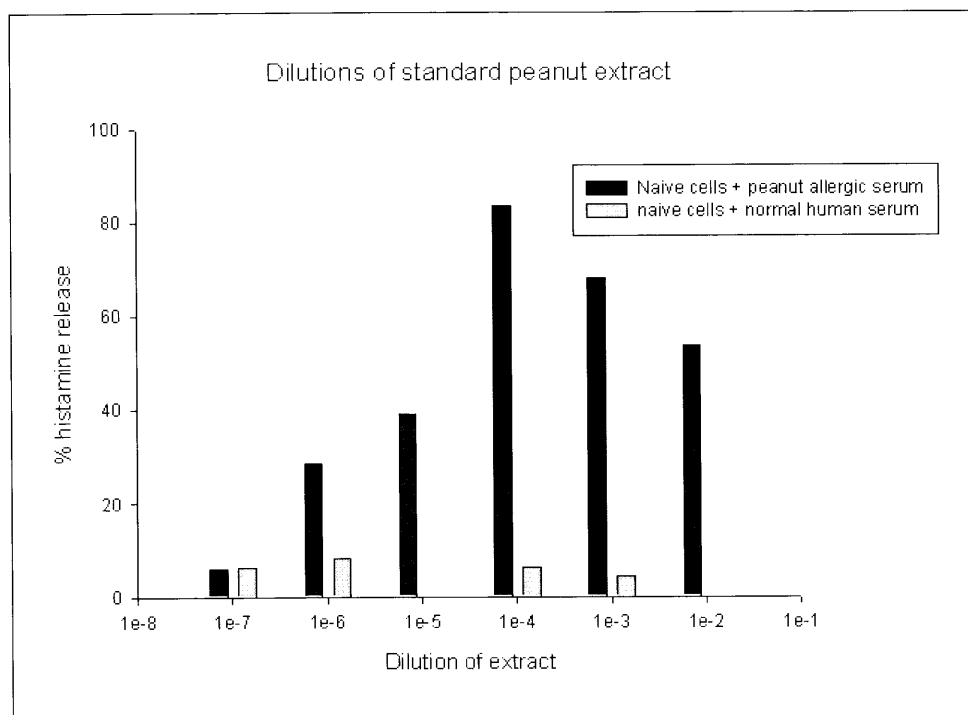


Fig. 4. Human leukocytes isolated from buffy coats were passively sensitized with serum from a peanut-allergic patient or a pool of sera of non-allergic persons. Cells were challenged with a standard peanut extract diluted from 9 mg/ml.

3a. Sensitization of leukocytes with rabbit immune serum, no IgG fraction of immune serum. Cells were challenged with peanut extract diluted from 9 mg/ml.

3b. Sensitization of leukocytes with rabbit immune serum or normal serum. Cells were challenged with a peanut extract diluted from 9 mg/ml and #2 (cf. material and methods).

concentration there is a response at the next dilution step (peanuts) is positive. This method also

Discussion

For studies of inhalation and food allergy histamine release tests are correlating well with other measures of IgE sensitization, such as skin prick tests or determination of specific IgE in plasma (13-16), and based on these findings it has been suggested to use the technique for biological standardization of allergen extracts including food allergens. Several studies exist in the human system and for small rodents, but so far the rabbit system has not been employed.

The finding that the rabbit has many basophils that are easily triggered, goes back to the immunology of the 1960's and 1970's. At this stage the rabbit was used as a

model for studies of IgE-induced anaphylaxis, and it was found that leukocytes – most likely to be basophils – were responsible for the activation of platelets via secretion of platelet activating factor, PAF. It was also demonstrated that platelets are responsible for a large fraction of the cell-associated histamine found in rabbit blood.

It became clear that if one should take advantage of the rabbit as an experimental animal model for histamine release, it is necessary to carefully eliminate the platelets from the preparations. Initial experiments (not shown) demonstrated that large amounts of histamine are present in the supernatants from the initial preparations and washings of the rabbit leukocytes. We speculate that the platelets are very easily activated either by mechanical stress during the cell preparations or via a low spontaneous activation of the basophils, perhaps via a low secretion of PAF, which serves as a potent platelet activator.

Immunization schemes have been described leading to a high level of rabbit IgE, but it seems that even an immunization aiming at producing IgG antibodies will also lead to homocytotropic antibodies with the ability to mediate basophil histamine release. At present we are not able to conclusively determine the isotype involved, but the fact that whole serum, but not a protein G-purified IgG-preparation, could passively sensitize naive rabbit basophils indicate that there is in fact specific IgE in the hyperimmune serum produced by means of Freund's incomplete adjuvant.

In the human system, the high affinity IgE-receptors on basophils are believed to be occupied to a 80-90% degree even in subjects with moderate levels of total IgE (17). Thus protocols have been developed for stripping (8) IgE of human basophils before passive sensitization. We tried a similar approach before passively sensitizing the rabbit basophils, but this did not induce the response compared to non-stripped basophils (results not shown). This indicates that the receptor occupancy in the rabbit system may be less critical compared to the human system.

In conclusion, we have indicated by means of the peanut allergen system, that rabbit basophil histamine release may be used to biologically characterize allergen extracts. The rabbit is a convenient experimental allergen, since there is already large experience with the production and use of polyclonal antisera. The initial results with the passive sensitization method indicates that this can be used to employ the large amount of polyclonal allergen-specific antisera to further characterize allergen extracts.

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