

Demonstration of reaginic antibodies on human basophils by immune adherence to allergen-coated Sepharose beads

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Summary

Human basophils purified by gradient density centrifugation and differential glass bead adherence were interacted with grass pollen allergen in particulate form prepared by covalent coupling of *Dactylis glomerata* protein concentrate to Sepharose beads.

Basophils from allergic subjects were found to interact specifically with the allergen-coated bead surface in a manner so highly characteristic that false negatives with basophils from non-allergic subjects or subjects with unrelated allergies were not encountered. Microscopic examination indicated that the specifically stained malleable basophils had adapted in a multi-point attachment to the rigid bead surface by becoming one-sidedly flattened against it. When using basophils from highly pollen sensitive subjects all the beads carried basophils and sometimes as many as thirty per bead. The percentage of beads with basophils and the number of basophils per bead roughly correlated with clinical history and skin tests. Immunocytadherence of the basophils to the allergen-coated beads was specifically inhibited by anti-IgE and anti-allergen antibodies, including reagins.

Introduction

More tests are now available for measuring serum reagins than for probably any other type of antibody (Augustin, 1967, 1973). However, clinical reactions are not caused by free serum reagins, but by the release of vasoactive amines and other highly active substances during the interaction of allergens with reagins fixed to blood basophils and tissue mast cells. Allergen-mediated histamine release from sensitized chopped human lung (Augustin, 1967, 1973) and blood leucocytes (Lichtenstein & Osler, 1964) are well-established methods for demonstrating allergic hypersensitivity and it has been shown conclusively that of all the blood cells only basophils have receptors for IgE (Pruzansky & Patterson, 1970; Ishizaka *et al.*, 1972). A number of experimental findings have led to the assumption that allergens (and other agents) capable of initiating histamine release from basophils must be capable of bridging two or more of the IgE molecules on the cell surface (Ishizaka & Ishizaka, 1968; Stan-

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worth, 1973). We describe here the development of a test system which confirms this by direct visualization.

Materials and methods

Human blood

This was obtained by venepuncture from normal and allergic volunteers known to be clinically sensitive to grass pollens and giving positive prick tests to extracts of *Dactylis glomerata*. Medication with antihistamines and/or cromoglycate did not seem to interfere with our test, nor did it seem to matter whether the tests were done during or outside the hay fever season.

Reagents

Ficoll was obtained from Sigma Ltd and was prepared freshly each week as a 9% (w/v) solution in deionized water.

Triosil was obtained in 20 ml vials from Nyegaard & Co. A.S., Oslo. The contents of a vial were mixed with 24 ml of deionized water and the solution used the same week.

CNBr-activated Sepharose beads were purchased from Pharmacia, Uppsala, Sweden, *glassbeads* of about 0.7 mm diameter (Ballotini No. 9) from Jencons (Scientific) Ltd, Hemel Hempstead, Herts, and *methylcellulose* of viscosity 15 cP USP grade from Dow Chemicals, Cheshire.

Solutions

Tris A buffer was prepared according to Lichtenstein & Osler (1964). It contains 3.75 g Trisma 7-6, 6.95 g NaCl, 0.37 g KCl and 3 g human serum albumin (HSA) in 1 l of deionized water, adjusted to pH 7.4 with N HCl.

Buffered Ca⁺⁺ and Mg⁺⁺ free EDTA (referred to as R-buffer) was prepared according to Rabinowitz (1964). It contains 0.2 g disodium EDTA, 8.0 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄ and 0.2 g KH₂PO₄ in 1 l of deionized water (pH = 7.4).

Apparatus

Glassware. All glassware was carefully washed and siliconed as described by Rabinowitz (1964), including the glass beads, using water soluble 'Siliclad' (Clay-Adams). Used beads from the columns described below were first thoroughly rinsed in saline to remove residual cells and proteins, then boiled with dilute 'Teepol' detergent before finally rinsing and washing as described above (Shortman *et al.*, 1971).

Containers. Short round- or flat-bottomed polycarbonate centrifuge tubes, 10–12 ml and 1–2 cm diameter, were used as well as 25 ml plastic screw top 'Universal' bottles, all from Sterilin Products, Richmond, Surrey, from whom we also purchased the disposable Pasteur pipettes.

Methods

Grass pollen allergen concentrate. This was prepared essentially as described by Augustin & Hayward (1962) and Augustin (1973). As described there and detailed earlier (Augustin, 1959; Augustin & Hayward, 1962), large amounts of impurities together with up to 10% activity were removed by a preliminary 1 min extraction with water. The subsequent combined extracts were precipitated by 90% saturation with

ammonium sulphate. After desalting and freeze-drying, this preparation may be stored for prolonged periods at 4°C and as much as $1.5\text{--}2.0 \times 10^6$ Noon units (Noon, 1911) are recovered per 1 g of pollen.

Particularized allergen (Axen, Porath & Ernbach, 1967). A 0.4 ml aliquot of the desalted pollen protein concentrate freshly prepared by the method described above (freeze-drying omitted) and containing by skin test 10^7 Noon units/ml (Noon, 1911) was dialysed for 24 hr (magnetic stirrer) against 0.1 M NaHCO_3 /0.5 M NaCl, pH 8.0 (two changes) and then diluted to 5 ml with the same buffer. This was added to 1 g of washed swelled CNBr-activated Sepharose 4B beads in a 10 ml screw top test tube. After rotating at 4°C for 16 hr the suspension was filtered and washed with bicarbonate/NaCl buffer. The suspension was then incubated for 2 hr (rotation) at room temperature with 50 ml of 1 M ethanolamine, pH 8.0 and followed by five cycles of washing with 0.1 M acetate/1 M NaCl pH 4.0 alternating with 0.1 M borate/1 M NaCl pH 8.0, until the optical density 280 readings of the filtrates were negligible. The beads were finally suspended in 0.1 M borate/1 M NaCl pH 8.0 for storing at 4°C, NaN_3 being added at a concentration of 0.02% as a preservative. When required for the basophil immune adherence test (BIAT), a suitable aliquot of the suspension was washed four times in R-buffer to remove NaN_3 . Human serum albumin was similarly bound to Sepharose beads using 10 mg/g of dry gel.

Inactivated uncoupled beads were prepared by reacting with ethanolamine at pH 8.0 for several hours (end on end rotation) followed by several wash cycles alternating between pH 4.0 and 8.0.

Our conjugated beads have been stored in the cold room (4°C) in the presence of azide for over a year without deterioration.

Basophil enrichment. Fifty millilitres of blood drawn into a 50 ml plastic syringe containing 500 units of heparin (no preservative) and 5 ml of 1% methylcellulose in Hanks' balanced salt solution (HBSS) was gently mixed and allowed to sediment in an inverted position for 30–35 min at room temperature. The upper plasma layer containing the bulk of the leucocytes was then gently pushed out through a soft piece of narrow tubing into polypropylene tubes (May, Lyman & Alberto, 1970). After centrifugation for 10 min at 150 g (4°C), the combined cell sediments were resuspended in 4 ml of the supernatant plasma previously clarified by centrifugation at 1000 g. Of the leucocytes, 60–75% were recovered and the bulk of the polymorphonuclear cells were then removed by differential centrifugation according to Boyum (1968). This step gave basophil/leucocyte ratios of about 1.4–3.5 in 60–70% yield and the final fractionation was performed on glass bead column, essentially according to a method originally devised by Rabinowitz (1964) for the fractionation of lymphocytes. At a temperature of 37°C the cell plasma suspension was layered on to the glass bead column immediately after washing it with Tris-A buffer. After penetration, 30 min was allowed for equilibration and active adherence. For the first elution 30 ml of 20% of the reserved cell-free plasma in Tris-A buffer was used in place of Rabinowitz's HBSS, at a speed of 15 drops/min. The resulting first eluate contained non-adherent lymphocytes and the bulk of residual platelets, but no monocytes or basophils. This was followed by elution with 20 ml of Na^{2+} - and Mg^{2+} -free EDTA solution (R-buffer) at a speed of 25 drops/min and yielded the bulk of the basophils in the second eluate together with the more adherent lymphocytes and some monocytes, the monocytes and damaged cells emerging last. From 50 ml of blood $1.5\text{--}10 \times 10^5$ basophils were recovered, representing overall yields of 12–30%. Of the suspended cells 8–25% were basophils with

viabilities above 95% by dye exclusion. After washing four times in R-buffer the cells were suspended in 1 ml of the same buffer.

Basophil staining. Neutral red staining was performed according to Shelley (1965), using 6 mg of neutral red dissolved in 50 ml of 1% (w/v) phosphate buffer, pH = 5.4.

Formaldehyde fixative. A 0.5 ml aliquot of 40% (v/v) CH₂O were mixed with 3.5 ml of 1% phosphate buffer, pH 5.4.

Neutral red/saponin solution. One millilitre of the above neutral red solution was added to 0.025 ml of saponin solution prepared by dissolving 40 mg of saponin in 1 ml of the 1% pH 5.4 phosphate buffer.

Acridine orange (AO) staining. Two drops of the cell suspension (>10 000 cells/ml) were mixed with two drops of a 0.02% solution of AO in PBS, pH 7.2 and viewed under a fluorescent microscope (Fitzpatrick *et al.*, 1967; West & Augustin, 1970). Basophils can be distinguished very clearly from other polymorphonuclear cells, lymphocytes, monocytes and macrophages.

Skin testing was done by the prick method, using dilutions of a pollen standard containing 100 000 Noon units per ml (Augustin, 1967, 1973).

Tests for viability were done by dye exclusion according to Schreck (1936).

Cell counts. Total leucocytes were counted in a Neubauer counting chamber in the usual manner, and basophils in a Fuchs-Rosenthal chamber after staining according to Shelley (1965). In the absence of RBC, 0.05 ml of the cell suspension (>10⁵ basophils/ml) were mixed with 0.4 ml of neutral red solution while in the presence of RBC the saponin/neutral red solution was used in place of neutral red alone.

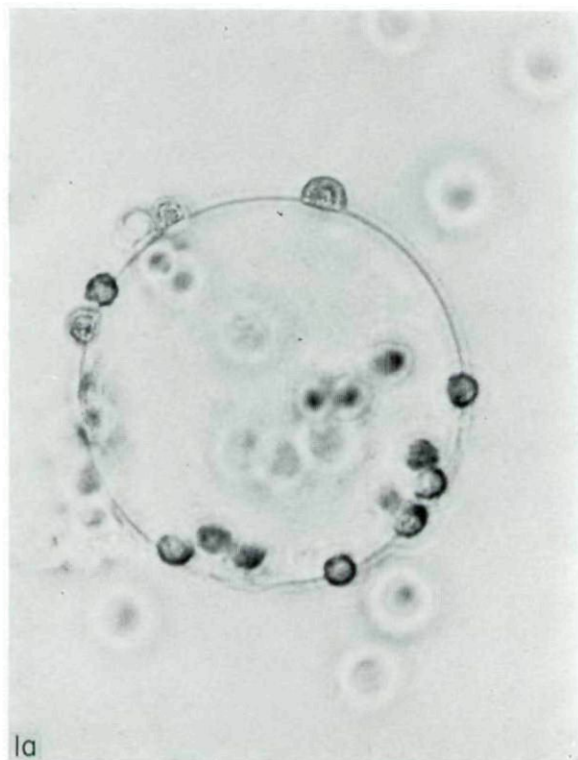


FIG. 1a

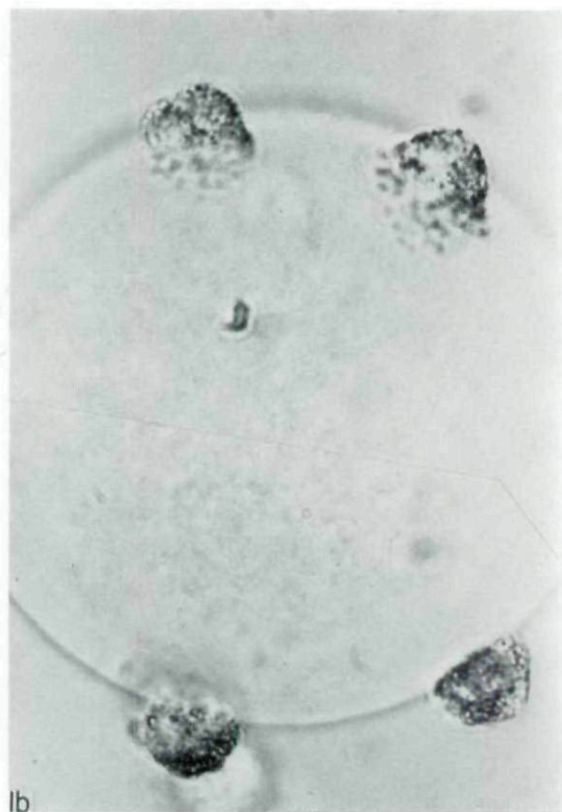


FIG 1b

Fig. 1. Specific immune adherence of basophils from a strongly grass pollen sensitive subject to Sepharose 4B beads irreversibly coated with the relevant pollen allergens (*Dactylis glomerata*). (a) $\times 300$. By varying the plane of focus twenty-five basophils could be seen to be characteristically attached to the upper surface of one single bead. (b) $\times 500$. Close-up of a bead with four attached basophils to show more clearly the very characteristic manner of basophil attachment.

Results

Immune adherence test procedure

If at all possible the immune adherence test was done immediately the basophils had been purified although it has been possible to keep the cells in good shape in the EDTA buffer overnight at 4°C and use them then. However, viability may then drop and bead adherence was never quite as high as when the basophils were used right away. One millilitre of the washed cell suspension containing a minimum of 10^5 basophils in R-buffer were incubated with a volume of washed allergen-coated beads in R-buffer giving a ratio of beads to basophils of 1:30. Usually the selected number of the appropriate washed beads were sedimented in 10 ml siliconed short, wide round- (or flat-) bottomed tubes, overlaid with the basophil suspension and then very gently mixed by carefully rotating and rolling the tube by hand. This was followed by a 5 min incubation at 37°C and the gentle mixing repeated. The tubes were then spun for 4

min at 150 g (bucket swing-out type of head) and this was followed without further disturbance by a 40 min incubation at 4°C. The supernatants were then drawn off with a Pasteur pipette and beads and cells very gently suspended in the remaining last drop of supernatant by gently rocking to and fro. The cells were then stained with neutral red (or AO) by gently mixing them with 0.4 ml of the neutral red (and 0.05 ml of the fixative solution). Basophil-bead adherence may then be determined quantitatively in a Fuchs-Rosenthal chamber. Under the given conditions basophils could be quite clearly distinguished from all the other cells since quite apart from their characteristic grape-like appearance no other cells but basophils stain pink with neutral red. After staining with AO the orange fluorescing granules cover more or less completely all other structures and these orange fluorescing grape-like balls are again very different from the other mononuclear cells which show distinctive green fluorescing nuclei. A minimum of fifty beads were each time evaluated by scanning the whole field at a $\times 100$ magnification, followed by checking at higher magnifications whenever desirable. Figure 1 shows the highly characteristic manner in which the basophils firmly cling in an obviously multivalent attachment to the bead surface. This can be already appreciated under the $\times 100$ magnification, but is seen much more clearly on changing to a higher magnification. At $\times 400$, and still better under oil ($\times 1000$), it can be seen that the basophils actually become deformed and flattened against the bead surface in their multivalent attempt to accommodate to the rigid bead surface. The basophil membrane appears to become broken to some extent in the region of attachment, with some spilling of granules which however appear also to remain attached to the bead surface. It is therefore very easy to distinguish between true basophil-bead interaction and the accidental presence of non-reacted basophils and other cells near or on the bead

Table 1. Correlation of basophil adherence with skin tests and clinical history. Severity of clinical sensitivity to grass pollen indicated by number of plus signs, ? indicates probable lack of clinical sensitivity to pollen. Note that skin sensitivity is the higher the lower are the Noon units (Noon, 1911)

Patient	Clinical sensitivity to pollen	Skin tests: lowest pollen concentration giving +ve reaction (Noon units/ml)	Allergies other than to pollen	Drugs taken	% Beads with basophils
TOD	+++++	1	No	Antihistamines, cromoglycate	100
BROO	+++++	10	Yes	Antihistamines, ACTH	100
RO	+++	100	No	Antihistamines	80
BERN	+++	10	No	Antihistamines	50
RES	+++	1000	No	Antihistamines	37
WES	++	100	Yes	Antihistamines	15
PHIL	?	1000	Yes	Antihistamines	3
CART	?	100	Yes	Antihistamines, ACTH	0
JON	?	10 000	Yes	Antihistamines, ACTH, cromoglycate	0

surface. Non-reacted cells do not conform to the bead surface and remain spherical.

In a positive test where the basophils have been derived from a donor with reaginic antibodies for the allergen attached to the beads, 10–100% of the beads may have one to thirty basophils attached to them in this very characteristic manner. Table 1 shows that the percentage of beads with basophils attached to them and the number of basophils per bead increases with clinical sensitivity and was also roughly correlated with the severity of the skin test reaction to the allergen.

Specificity of the basophil immune adherence test

Three negative controls were used; in one the specific allergen-covered beads were replaced by unconjugated beads (inactivated with ethanolamine as described under Methods) and in the other by beads coupled to an unrelated antigen such as human serum albumin or haemocyanin: not in one single instance was a false positive obtained nor did we ever obtain a false positive result when reacting the basophils of non-allergic subjects with our pollen allergen-conjugated beads. In all three negative controls, all the beads were always completely free of cells, demonstrating clearly the specific nature of the reaction.

Furthermore, incubation of the basophils with anti-IgE serum (kindly supplied by the Drs Ishizaka) prior to reacting with the specific beads, inhibited the immune adherence, confirming the IgE specificity of the basophil-attached antibody. Similarly, allergen-coated beads failed to collect basophils on their surface when reacted previously with antisera for the allergens and this included some reaginic sera.

Discussion

The test described here may be regarded as a variant of the basophil rosette test described by Wilson *et al.* (1971, 1972) in which the allergen-coated RBC have been replaced by allergen-coated Sepharose beads.

Although rosette tests involving lymphocytes are by now well-established methods, basophil rosette tests pose special problems, firstly because usually only the very dilute and impure commercial allergen solutions used in skin testing are available and secondly because of the very low concentration of basophils in whole blood. The necessary intimate contact between the particularized reactants may therefore be prevented simply by crowding out with impurities. Tests are therefore subject to the availability of at least partially purified antigen concentrates and highly enriched basophil suspensions. A rapid method for the preparation of grass pollen allergen concentrates is indicated in the text. The iron carbonyl method used by Wilson *et al.* (1972) for basophil enrichment is only suitable for bloods with exceptionally high basophil counts and is replaced here by a three-step purification similar to the combination of methods used independently by Pruzansky & Patterson (1970) and Ishizaka *et al.* (1972), both groups applying the leucocytes to glass bead columns as a suspension in HBSS/plasma (containing Ca^{2+} and Mg^{2+}), a step considered necessary by Rabinowitz (1964) for the promotion of active adherence to the glass beads. We found, however, that this 30-min incubation and the subsequent elution of non-adherent cells with HBSS/plasma was detrimental to the basophils. By replacing HBSS in the 20% plasma mixture with the EDTA and glucose containing R-buffer no interference with basophil attachment resulted and this yielded a 10–30% pure basophil suspension in 15–25% overall yield and in excellent morphological condition. The viability of these cells by dye exclusion was always in the region of 94–98%. Preservation of histamine content was not tested

for, but surface IgE antibodies appear to have remained intact. At any rate, the basophils remained capable of specific immune adherence to the corresponding allergen-covered beads and failed to stick non-specifically to beads, even when stored overnight (4°C). Under appropriate conditions, the human basophil may thus prove more stable than hitherto expected although preservation of histamine content may prove to be a more stringent test for this than the retention of reagins, for instance Ishizaka & Ishizaka (1974) have shown that IgE molecules can be dissociated from their receptors at pH 4.0 without greatly damaging these receptors, although histamine release had been triggered off in the process.

Several advantages accrued from our replacing the RBC indicator cells with inert beads. Sepharose beads are stable and available commercially in an already activated form which makes coating with protein antigen a very easy and quick operation while antigen coating of mammalian cells has required great ingenuity (Coombs & Franks, 1969) and can easily result in incipient damage with subsequent non-specific stickiness and non-specific adherence. Moreover accidental juxtaposition of cells can sometimes mimic specific adherence and is not possible in the BIAT described here which is a kind of inverted rosette test in which the basophils surround the much larger antigen-coated beads. This led to a very characteristic mode of attachment which cannot be confused with chance non-specific adherence or juxtaposition. We found that the obviously multiple attachment of the malleable basophils to the rigid bead surface caused a characteristic deformation of the basophils and visually supports the previously postulated multivalent triggering of basophils (Ishizaka & Ishizaka, 1968; Stanworth, 1973). False positives were never encountered. On the other hand, the relatively large size is probably also the reason why the test is not as sensitive as one would wish and using beads of a size similar or even smaller than the basophils may be expected to improve sensitivity. Subjects with positive prick tests to not less than 1000 Noon units/ml reacted poorly in the present BIAT.

Contact with the appropriate allergen may cause not only histamine release from the basophils (or mast cells), but may also lead to degranulation and it has been reported that fewer than usual blood basophils are found during the hay fever season (Wilson *et al.*, 1971). We, on the other hand, have not become aware of such a decrease although we have isolated basophils from four of our volunteers on three and more occasions, some inside and some outside the hay fever season. One reason for this may be that most of our more sensitive volunteers appear to have been under corticoid and/or cromoglycate treatment when tested during the hay fever season. Although this must have affected allergen-mediated histamine release, it in no way interfered with our test which depends solely on maintaining surface IgE attachment. Even if histamine had been released from basophils collected during the hay fever season, this would not have interfered with our test although actual degranulation would have done so. Moreover, under our conditions, basophil membranes were well maintained, possibly stabilized by the drugs taken by the patients.

Serum IgE contents can vary between <100 and over 10 000 ng/ml. This is equivalent to about 4×10^{14} to 4×10^{16} molecules of free IgE/ml. And as there are usually between 5000 and 10 000 basophils/mm³ of blood and about 20 000 IgE receptors/basophil (Ishizaka *et al.*, 1973), there can only be between $5 \times 10^6 \times 2 \times 10^4$ ($= 10^{11}$) and 2×10^{11} basophil-bound IgE molecules/ml blood, i.e. there are probably 1000–100 000 as many free IgE molecules in the blood as are bound to the basophils. Of course, only some of these are antibodies and it may even be that the qualitative

antibody composition in serum differs from that on the basophils. Yet antigen-mediated histamine release requires the participation of cell-bound reagins (i.e. either blood basophils or tissue mast cells). From this it follows clearly that, once no more free IgE receptors are available (on basophils or mast cells), free reagins will protect just as well against allergen-mediated histamine release as do IgG and other non-IgE classes of antibodies of the relevant allergen specificity—although only the latter are usually referred to as ‘blocking’ antibodies. Yet the reaction of allergen entering the blood stream with basophil-fixed antibodies will be competed with—and therefore ‘blocked’—by any allergen-specific free antibodies, including free serum reagins. Excess reagins produced during hyposensitization treatment in addition to non-IgE antibodies may therefore be not always as deleterious to the patient as is generally thought.

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