

The use of monoclonal antibodies raised against a human IgE myeloma paraprotein for the study of allergen extracts and sera from allergic patients

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SUMMARY

Murine monoclonal antibodies have been produced against a single human IgE myeloma preparation. A single fusion yielded six different monoclonal antibodies which bound strongly to the immunogen. Two of these failed to react with 'normal' human IgE or with a second IgE paraprotein. The other four antibodies reacted with normal and myeloma-derived IgE and were found to be specific for antibodies of this class. Two of these antibodies were potent reagents for the detection of IgE by two site immunoradiometric assay, immunoblotting, and radioallergosorbent test. The other two antibodies were considerably less potent reagents when used in these assay systems. Our findings suggest that care should be taken in selection of suitable monoclonal antibodies for immunochemical study of allergens and sera from allergic patients.

Keywords IgE monoclonal antibodies immunoblotting allergens two-site IRMA

INTRODUCTION

Immunoglobulin E is now firmly implicated as a physiological mediator of allergic reactions (Ishizaka, Ishizaka & Hornbrook, 1966; Bennich & Johansson, 1970; 1971). The immunochemical study of allergen extracts and of the sera from allergic patients is strongly dependent on the use of specific anti-IgE antibodies, and considerable effort has been directed into the production of such reagents. A major problem has been that IgE is present in very low concentrations in normal serum and in relatively low concentrations (even when described as 'high') in sera from allergic patients (Johansson, 1967), and that genuine sources of high levels of this antibody, as found in patients suffering from IgE secreting myeloma, occur extremely rarely. Also IgE myeloma proteins may differ by virtue of their neoplastic and monoclonal origin from the IgE present in sera from allergic patients. Thus antisera raised using an IgE paraprotein as immunogen may contain an unpredictable proportion of myeloma paraprotein specific antibodies which do not react with IgE of non-myeloma origin.

The use of hybridoma technology (Köhler & Milstein, 1975) allows the preparation of monoclonal antibodies which may at least partially overcome this problem (Hassner & Saxon, 1983; Sanchez-Madrid *et al.*, 1984). Here we report the production and characterization of monoclonal antibodies against a human IgE myeloma paraprotein and investigate their use for the measurement of IgE in the sera from allergic patients and in the study of allergen extracts.

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MATERIALS AND METHODS

Production of monoclonal antibodies against human IgE

A Balb/c mouse was immunized subcutaneously with 50 μ g of an IgE myeloma paraprotein (a kind gift from Dr T. Platts-Mills) emulsified with Freund's complete adjuvant. Three weeks later the mouse was boosted intraperitoneally with 30 μ g of immunogen in phosphate buffered saline (PBS) and a further identical boost given 2 weeks after the first boost. Splenocytes from the mouse were fused 4 days later with the myeloma cell line NSO using the method described by Galfre *et al.* (1977). After fusion the cells were distributed into eight 24-well microtitre plates, and supernatants from confluent cultures were screened by solid phase radiobinding assay using the immunogen to coat the wells of flexible 96-well microtitre plates (1 μ g/well; Johnstone & Thorpe, 1982). For those antibodies binding to IgE, specificity was established by carrying out a similar immunoassay, except that human immunoglobulins of the other classes were used to coat the plates. Hybridomas from positive wells were cloned twice in soft agar and clones secreting monoclonal antibody specific for IgE either cryopreserved, grown in culture to produce monoclonal antibody containing supernatant, or injected into pristane-primed Balb/c mice to produce ascitic fluid. Monoclonal antibody class was determined by biosynthetic labelling of hybridoma secretion products with ^{3} H-leucine followed by analysis by SDS-PAGE and fluorography (Laskey & Mills, 1975), and IgG subclass established by double immunodiffusion against subclass-specific antisera.

Iodination of monoclonal antibodies

Monoclonal antibodies 102 and 117 were purified from ascitic fluid by a combination of precipitation with ammonium sulphate (45% saturation) and HPLC using a Pharmacia Mono Q column (Cleazardin *et al.*, 1985). The purified material was dialysed against 0.1 M sodium phosphate buffer, pH 7.4, and the protein concentration adjusted to 3 mg/ml. Aliquots (30 μ l) were iodinated using 150 μ Ci Na ^{125} I (SA 100 mCi/ml; Amersham International, UK) and chloramine T as the catalyst (Johnstone & Thorpe, 1982). Unbound iodine was removed by application to a Dowex 1-X8 ion exchange column (bed vol 1 ml) equilibrated with 0.1 M sodium phosphate buffer, pH 7.4, containing 2% BSA and ^{125} I-labelled anti-IgE removed by washing through with 2 ml of this solution.

Two-site immunoradiometric assay

Monoclonal antibodies (50, 66, 102 and 117) were partially purified from ascitic fluid by precipitation with ammonium sulphate (45% saturation). The precipitates were resuspended with and dialysed against PBS, the protein concentrations adjusted to 2 μ g/ml and these solutions used to coat the wells of flexible 96-well microtitre plates (50 μ l/well). After incubation overnight at 4°C, the unreacted protein binding sites on the plates were blocked by incubation for 1 h with PBS containing 1% gelatin.

The microtitre plates were then washed by filling the wells with PBS containing 0.05% Tween 20, incubating for 3 min and discarding the solution. This was repeated five times. Nine serial 4-fold dilutions of the 2nd International Reference Preparation for human serum IgE (NIBSC 75/502) or of a pool of normal human serum were made in PBS and 50 μ l of each dilution of these were added to the plates in duplicate for each of the four monoclonal antibodies and incubated for 4–5 h in a humid box. The washing process was repeated.

Fifty microlitres (5×10^4 ct/min) of iodinated anti-human IgE antibody (either polyclonal obtained from Pharmacia or monoclonal antibodies 102 or 117, see above), diluted in horse serum diluent (20% horse serum in PBS), were added to each well, the plates were incubated overnight at 4°C, washed as above and the individual wells cut from the plate with a hot wire plate cutter. The counts bound to the wells were then estimated using a gamma counter.

Immunoblotting

Against IgE paraproteins. Immunoblotting using the different MoAb against IgE paraproteins

from myeloma patients was carried out exactly as previously described (Thorpe, Bird & Spitz, 1984).

To detect antigens recognized by IgE antibodies in allergic patient sera. Proteins from extracts of Cocksfoot grass pollen (NIBSC 75/506); Timothy grass pollen (NIBSC 82/520) and *Aspergillus fumigatus* (NIBSC 78/575) were separated on a 7.5 to 17.5% linear gradient polyacrylamide gel, and transferred onto nitrocellulose filters as previously described (Thorpe *et al.*, 1984). The nitrocellulose filters were blocked in 3% haemoglobin phosphate buffered saline (Hb/PBS) for 30 min to block non-specific protein binding. Blots of Cocksfoot pollen extract and Timothy grass pollen extract were incubated in 20 ml Hb/PBS containing 1 ml of serum pooled from patients with specific IgE to Cocksfoot pollen (NIBSC 77/503). Blots of *Aspergillus fumigatus* extract were incubated with 20 ml Hb/PBS containing 1 ml of serum pooled from patients with specific IgE to *A. fumigatus* serum (NIBSC 78/545). After 16 h incubation the filters were washed with Hb/PBS and incubated with ¹²⁵I-labelled monoclonal antibody 102 (1×10^6 ct/min per track) for 4 h. The nitrocellulose filters were then washed with PBS, dried and exposed at -70°C to pre-flashed (Laskey & Mills, 1977) X-ray film (Kodak X-OMAT) in a cassette containing one fast tungstate intensifying screen for 72 h.

RAST and RAST inhibition

Paper discs (Schleicher and Schüll No 589/3) were activated with cyanogen bromide (Ceska, Eriksson & Varga, 1972) and coupled with Cocksfoot pollen extract (NIBSC 75/506) at a dry weight of 20 μg per disc in 0.1 M sodium bicarbonate buffer for 24 h at 4°C . Discs were washed in this buffer for 10 min at 4°C and unreacted sites blocked with 0.1 M β -ethanolamine in buffer for 1 h. The discs were washed with 0.01 M sodium acetate buffer pH 4.0 (2×10 mins) followed by PBS (2×10 mins), excess liquid was removed by blotting on filter paper and then the discs were freeze dried overnight.

Direct RAST

Eight serial 2-fold dilutions of the Cocksfoot allergic serum pool (NIBSC 77/503) and of a normal human serum pool were made in horse serum diluent in duplicate in 96-well microtitre plates so that the final volume in each well was 200 μl . One paper disc coupled with Cocksfoot pollen extract was added per well and the plates incubated overnight at room temperature in a humid box. The discs were then washed in batches of 96 with 1 litre of RAST wash (0.9% NaCl + Tween 20) in a continuous flow washing device (Kemeny & West, 1983) and transferred to a clean plate.

Fifty microlitre volumes of iodinated anti-IgE antibodies (either monoclonal antibodies 102 & 117 or polyclonal anti-IgE from Pharmacia) diluted in horse serum diluent to contain 50,000 ct/min were added to each well. The plates were incubated overnight at room temperature in a humid box, the discs washed as before and the activity bound to each disc was determined using a gamma counter.

The results from these assays were used to calculate the 50% endpoint of the titration for each label used, by taking the counts bound by a 1/5 dilution of human anti-Cocksfoot serum as 100%.

RAST inhibition

Seven serial 3-fold dilutions of Cocksfoot pollen extract in horse serum diluent were made in duplicate in microtitre plates. The final volume in each well was 100 μl . Cocksfoot allergic serum and normal human serum were each diluted in horse serum diluent to a concentration twice that of the 50% endpoint value determined by direct RAST. One hundred microlitres of the Cocksfoot allergic serum were added to all the wells containing Cocksfoot pollen extract and to the wells containing diluent alone (representing uninhibited binding). One hundred microlitres of the normal human serum pool were added to two more wells containing diluent alone (representing background binding). The plates were incubated for 1 h at 37°C in a humid box and then one paper disc coupled with Cocksfoot pollen extract was added to each well and the plate further incubated overnight at room temperature. The discs were then washed as before and transferred to a clean microtitre plate. The remainder of the procedure was as for direct RAST using either labelled polyclonal anti-IgE or the labelled anti-IgE monoclonal antibodies.

The percentage inhibition of binding of the Cocksfoot allergic serum pool was calculated for each dilution of inhibiting antigen as follows:

$$\left(\frac{\text{uninhibited binding} - \text{inhibited binding}}{\text{uninhibited binding} - \text{background binding}} \right) \times 100$$

RESULTS

Monoclonal antibodies. As assessed by solid-phase radiobinding assay, a single fusion yielded 120 master wells containing antibody directed against the IgE myeloma protein used as immunogen. Of these, 28 were shown to be specific for IgE, i.e. did not bind to immunoglobulins of the other classes. After cloning, eight master wells yielded clones which bound strongly to the IgE myeloma protein. After recloning and expansion in culture, two of these clones bound only very weakly to the antigen and so were discarded; the other six clones were used for the immunochemical study described in this report. All hybridoma lines were stable in culture and, when injected into the peritoneal cavity of pristane-primed Balb/c mice, produced ascitic fluid containing a high concentration of monoclonal antibody. All monoclonal antibodies were of the IgG class, four being IgG₁ (102, 105, 66, 50) and two being IgG_{2a} (117, 86).

Immunoblotting with IgE myeloma paraproteins. Immunoblotting studies showed that all six monoclonal antibodies bound strongly to the IgE myeloma protein used as immunogen when it was blotted from SDS-PAG run under non-reducing conditions, but none of the antibodies bound to this antigen when blotted from reducing SDS-PAG. In similar experiments carried out using a

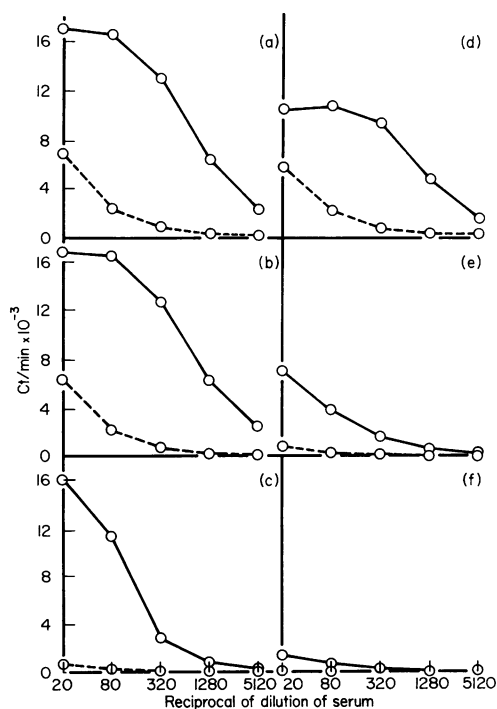


Fig. 1. Two-site immunoradiometric assay for human IgE. (a), (b) and (c) using MoAb 102 as catching antibody and (a) polyclonal anti-IgE serum, (b) MoAb 102 and (c) MoAb 117 as developing antibodies. (d), (e) and (f), using MoAb 117 as catching antibody and (d) polyclonal anti-IgE serum, (e) MoAb 102, and (f) MoAb 117 as developing antibodies: (O—O) using the International reference preparation for human serum IgE (75/502) (10,000 IU/ml); (O---O) using pooled normal human serum. See text for details.

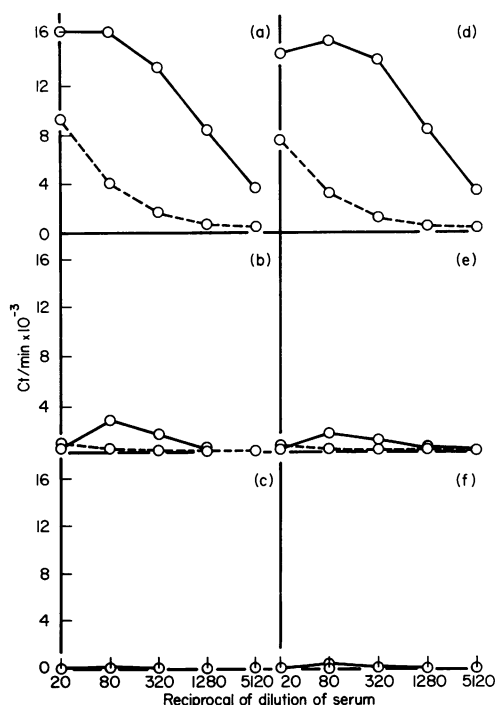


Fig. 2. Two site immunoradiometric assay for human IgE. (a), (b) and (c), using MoAb 50 as catching antibody and (a) polyclonal IgE serum, (b) MoAb 102 and, (c) MoAb 117 as developing antibodies. (d), (e) and (f), using MoAb 66 as catching antibody, and (d) polyclonal IgE antiserum, (e) MoAb 102, and (f) MoAb 117 as developing antibodies. (O—O) using the International reference preparation for human serum IgE (10,000 IU/ml); (O---O) using pooled normal human serum. See text for details.

different IgE myeloma protein and non-reducing gels, only four of the monoclonal antibodies were able to recognize the antigen (102, 117, 66, 50).

Two-site immunoradiometric assay. The two-site IRMA was used to assess the ability of the monoclonal antibodies to bind to IgE present in sera from individuals suffering with allergy or from apparently normal individuals. Using this technique four of the monoclonal antibodies (102, 117, 50 and 66) bound to IgE present in sera from allergic patients (Figs 1 & 2) and this could be detected using radio labelled polyclonal anti-IgE. The antibodies were also able to detect IgE present in sera from non-atopic people, although in all cases far lower counts were obtained when compared with sera from allergic patients (Figs 1 & 2). Antibodies 105 and 86 and the irrelevant monoclonal antibody gave no counts above background with any of the serum samples tested.

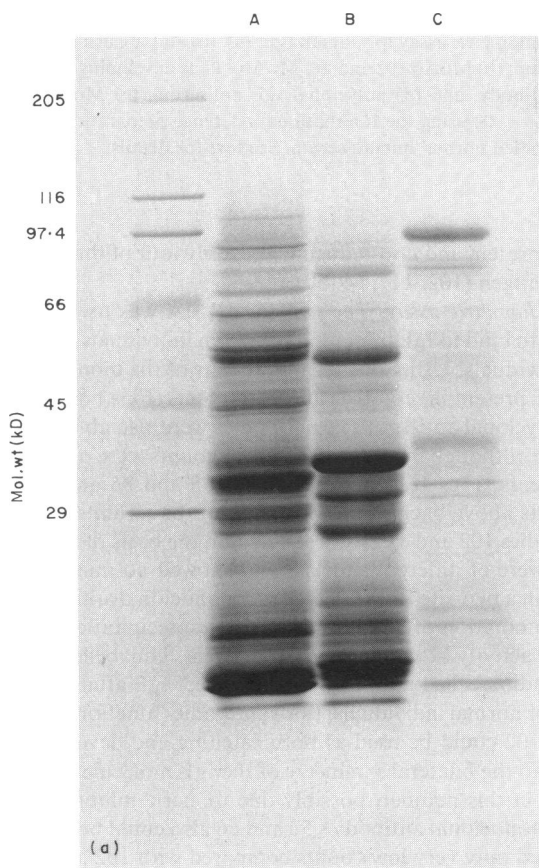
Monoclonal antibodies 102 and 117 were selected on the basis of ease of production and being clearly different (they were of different subclass and showed no mutual inhibition in solid phase binding assays) for use in a two-site IRMA using two monoclonal antibodies. Both antibodies were easily purified by a combination of ammonium sulphate precipitation and HPLC and radioiodinated to a high specific activity (4×10^7 ct/min/ μ g protein). Unlabelled antibody was used to coat microtitre plates and radiolabelled antibody used to detect IgE after incubation with serum from either allergic patients or normal individuals. Both antibodies functioned well in this system (Fig. 1). Monoclonal antibody 102 could be used as both catching and developing antibody in the same assay, presumably due to the bilateral symmetry of the IgE molecule (Fig. 1b). However antibody 117 could not be used in this manner, possibly due to steric inhibition of the second antigenic determinant (Fig 1f). Monoclonal antibodies 50 and 66 also could be used as catching antibody in this system but produced only very low counts compared with 102 and 117 (Fig. 2).

Immunoblotting with allergen extracts. Immunoblotting studies using allergen extracts as antigens showed that four of the monoclonal antibodies (102, 117, 50 and 66) could be used to detect IgE antibodies in sera from allergic patients which bound to proteins present in the extracts. Although an identical immunoblot pattern was produced with each of the four antibodies a marked difference in potency was noted; monoclonal antibodies 102 and 117 bound strongly to antigen whereas a much weaker reaction was observed with antibodies 50 and 66. The other two monoclonal antibodies (86 and 105) did not recognize any antigen IgE complexes.

For all three allergen extracts Coomassie Blue staining of SDS-polyacrylamide gels showed that they contained many protein components (Fig. 3 (a)). Immunoblotting showed that only some of these proteins were recognized by IgE present in sera obtained from allergic patients (Fig. 3 (b)).

Using *A. fumigatus* extract as antigen and processing immunoblots with serum pooled from patients with specific IgE to *A. fumigatus* the antibodies showed four major bands to be antigen IgE complexes with approximate M_r values of 81 K, 78 K, 38 K and 22 K. A number of much weaker immunoreactive bands were also evident (Fig 3 (b) C). Using Cocksfoot pollen or Timothy grass pollen extracts as antigens and processing immunoblots with a pooled Cocksfoot allergic serum showed that a number of antigen IgE complexes could be detected (Fig 3 (b) A & B). With Cocksfoot grass pollen a group of four closely spaced proteins were the major immunoreactive proteins with approximate M_r values of 35 K, 30 K, 28 K and 26 K. A much weaker reaction was also evident with a protein of M_r 52 K (Fig 3 (b) B). The cross reaction with Timothy grass pollen was mainly directed against two proteins of M_r 33 K and 28 K with a weaker reaction against a protein with an M_r of 52 K (Fig 3 (b) A).

Direct RAST. Using the direct RAST technique two monoclonal antibodies (102 and 117) were



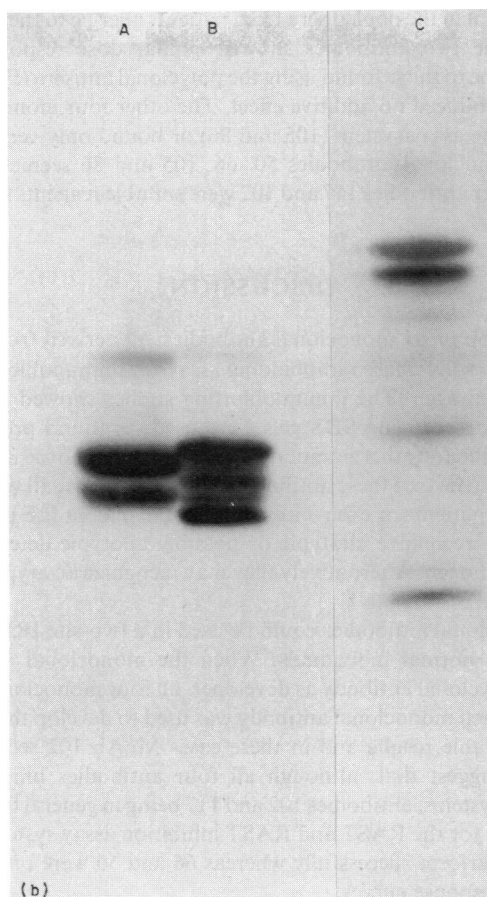


Fig. 3. (a) Allergen extracts analysed by SDS polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. A, Timothy grass pollen; B, Cocksfoot grass pollen; C, *Aspergillus fumigatus*. (b) Immunoblot of an identical gel. A, Timothy grass pollen and B, Cocksfoot grass pollen developed with Cocksfoot allergic serum pool and then ^{125}I labelled MoAb 102. C, *Aspergillus fumigatus* developed with *A. fumigatus* allergic serum pool and ^{125}I labelled MoAb 102. See text for details.

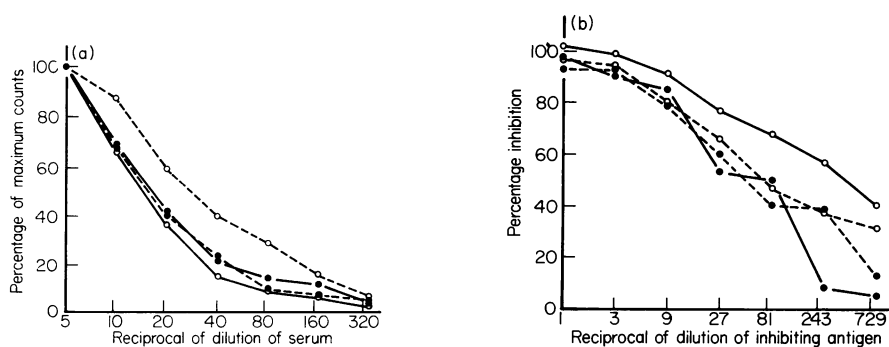


Fig. 4 (a) Direct RAST; (b) RAST inhibition. See text for details. (○—○)=Mixture of ^{125}I labelled monoclonal antibodies 102 and 117; (○---○)= ^{125}I labelled polyclonal antiserum; (●—●)= ^{125}I labelled MoAb 102; (●---●)= ^{125}I labelled MoAb 117.

found to bind to IgE present in the pooled sera from patients allergic to the antigen used (Cocksfoot grass pollen extract). The two antibodies showed similar dose response curves, which were somewhat different in shape to those found using the polyclonal antisera (Fig. 4 (a)). Mixing the two monoclonal antibodies produced no additive effect. The other four monoclonal antibodies either showed no reactivity in the assay system (105 and 86) or bound only very weakly (50 and 66).

RAST inhibition. Monoclonal antibodies 50, 66, 105 and 86 seemed inactive in the RAST inhibition system. However antibodies 117 and 102 were suitable reagents for this assay especially if mixed 1:1 (Fig 4. (b)).

DISCUSSION

We describe the production of six monoclonal antibodies, all derived from a single fusion, which reacted equally strongly in solid phase radiobinding assays or immunoblots with the IgE myeloma paraprotein used as immunogen. The immunoblotting studies showed that the antibodies only reacted with antigen if non-reducing SDS gels were used, as found previously for monoclonal antibodies which are specific for other immunoglobulin classes (Thorpe *et al.*, 1984). A somewhat unexpected finding was that two of these antibodies failed to react at all with IgE derived from the sera of normal or allergic patients or even with a different myeloma IgE protein. This implies that these two antibodies may recognize idiotypic or possibly allotypic determinants present on the paraprotein used as immunogen. Alternatively they may recognize an atypical structure peculiar to this myeloma paraprotein.

The four other monoclonal antibodies could be used in a two-site IRMA to detect IgE in sera from allergic patients or normal individuals. When the monoclonal antibodies were used as catching reagents and polyclonal antibody as developer, all four monoclonal antibodies performed equally well. However, when monoclonal antibody was used to develop the system only MoAb 102 and 117 produced acceptable results and in these cases MoAb 102 seemed the better catching reagent. These results suggest that, although all four antibodies bind to IgE, they perform differently in these assay systems, antibodies 102 and 117 being in general far better reagents than 66 or 50. This is also the case for the RAST and RAST inhibition assay systems in which monoclonal antibodies 102 and 117 perform successfully whereas 66 and 50 were only marginally active and produced no clear dose response curves.

Using the immunoblotting technique, MoAb 102 and 117 were found to be ideal reagents for identification of antigens which are recognized by IgE antibodies present in sera from allergic patients. These experiments showed that although the allergen extracts seemed equally complex when analysed by SDS-PAGE and Coomassie blue staining, they gave characteristic binding patterns when analysed by immunoblotting. Cross reactivity was detected between Cocksfoot grass pollen and Timothy grass pollen using sera from patients who were known to be sensitive to the former material. The immunoblotting pattern produced by the allergic serum pool differed according to which species of pollen was used suggesting that either common epitopes are present on different molecular species in the two different grass pollens, or, more probably, that the serum contains IgE molecules specific for some Timothy grass proteins as well as the expected Cocksfoot pollen antigens. Monoclonal antibodies 50 and 66 could also be used in these immunoblotting studies, but were very much less potent than antibodies 102 or 117.

It can be concluded from our results that a single fusion has yielded a number of monoclonal antibodies specific for IgE, but that a surprisingly high proportion (one-third) of these only recognize the myeloma paraprotein used as immunogen. The other antibodies show differing potency in the immunochemical systems described, and this heterogeneity is probably best attributed to differing avidity for antigen. Our results suggest that polyclonal antisera raised against IgE will probably contain a range of antibody species, a considerable proportion of which either bind normal IgE poorly or not at all. This may explain the difficulty in raising potent polyclonal antisera against IgE.

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