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Replacement of Murine Sera by Allergen-Specific Monoclonal IgE Antibodies: A New Approach for the Characterisation of Allergen Extracts

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Abstract: Allergen characterisation that is based on patients' sera or monoclonal allergen-specific IgG antibodies has several disadvantages. Current methods such as immunoblotting or allergen-specific ELISA are non-functional assays and cannot be used to evaluate the biological allergenic activity of allergen products. We have established an in vitro assay based on polyclonal murine IgE and allergen-dependent mediator release of rat basophilic leukaemia (RBL) cells as an alternative to passive cutaneous anaphylaxis (PCA), an animal model of IgE-mediated allergic reactions. The RBL assay is a functional in vitro test which enables the measurement of biological potency of allergen extracts to be made. Up to now, allergen-specific IgE-containing murine sera were used for sensitisation of RBL cells. Sensitisation with allergen-specific IgE monoclonal antibodies (IgE mAbs) would reduce the number of animals necessary for the production of allergen-specific IgE. In addition, IgE mAbs are better defined and will offer more exact determination of allergens. Since allergen-specific IgE mAbs were not available, the aim of this study was to produce such antibodies. As a new strategy to select IgE-producing hybridomas the RBL mediator release assay was used: the cells were incubated with hybridoma supernatant and stimulated with allergen and crosslinking allergen-specific polyclonal IgG antibodies. By this technology IgE mAbs specific for the birch pollen allergens Bet v 1 and Bet v 6 were produced. In conclusion, this novel strategy enables the production of panels of allergen-specific IgE mAbs by immunisation of a limited number of mice to be made. These IgE mAbs in combination with the RBL mediator release assay may serve as new tools for the evaluation of diagnostic and therapeutic allergen extracts.

INTRODUCTION

The characterisation and determination of the allergen content in allergen products are usually based on methods such as immunoblotting or ELISA using sera of allergic patients or monoclonal allergen-specific IgG antibodies. These

methods have several disadvantages. Patients' sera have individual patterns of antibody reactivities which prevent an accurate quantification of single allergens. This problem can be solved by the use of monoclonal allergen-specific IgG antibodies but these IgG mAbs may not bind to IgE-reactive epitopes. In addition, both assay types are non-functional and measure allergen concentrations in mass units that may not correlate with biological potency. The biological activity can only be measured in a biological assay, such as animal models or cellular in vitro assays. Passive cutaneous anaphylaxis (PCA) in rodents is a model system to study IgE-mediated allergic reactions. It is often used to assess the activity of anti-allergic drugs, the allergic potential of a variety of substances, and for parasitological investigations. To reduce the use of laboratory animals we have established an in vitro assay based on polyclonal murine IgE and allergen-dependent mediator release from rat basophilic leukaemia (RBL) cells as an alternative for PCA [1]. Since this RBL assay simulates the IgE-dependent activation and mediator release from mast cells, it is a functional test and enables the measurement of allergenic potency of extracts to be made [2,3]. The RBL assay is based on polyclonal IgE-containing murine sera which can only be produced in small quantities. As with patients' sera, these murine sera have individual polyclonal IgE patterns which makes it difficult to use them in allergen characterisation. Since allergen-specific IgE monoclonal antibodies (IgE mAbs) were not available, the aim of our study was to establish such antibodies to reduce the number of mice necessary for IgE production. In addition, IgE mAbs are better defined reagents for the RBL assay and will allow a more detailed determination of allergens. Since ELISA or RIA are not suitable for detecting functional IgE-producing hybridomas, previously identified murine IgE mAbs were always screened additionally by PCA [4-7]. To avoid PCA and thus reduce the number of laboratory animals we used the mediator release from RBL cells to select functional IgE-producing hybridomas. As a new strategy RBL cells were sensitised with hybridoma supernatants and mediator release was measured after stimulation with allergen and crosslinking by allergen-specific polyclonal IgG antibodies. This crosslinking is necessary to induce mediator release from RBL cells, sensitised with monoclonal IgE. Here we report the production and characterisation of birch pollen-specific IgE mAbs.

MATERIALS AND METHODS

Preparation of polyclonal IgE containing mouse serum pools

Balb/c mice were immunised five times in intervals of two weeks with 0.5 ml birch pollen extract (8 µg/ml of protein) (Allergopharma, Reinbeck, Germany) adsorbed to aluminium hydroxide. Sera were tested by RBL assay as described previously [2] with slight modifications. Briefly, permanently growing RBL-2H3 cells were harvested in the stationary phase, transferred to 96-well flat bottom cell culture plates (Nunc, Wiesbaden, Germany) and cultivated overnight at 37°C in Eagle's minimal essential medium containing 5% foetal calf serum (MEM). The attached cells were passively sensitised for 1 h with mouse serum diluted 1:50 in MEM. After sensitisation, the cells were washed three times with Tyrode's buffer. Subsequently, the RBL cells were incubated with 100 µl birch pollen extract (10 µg/ml of protein) diluted in Tyrode's buffer for 1 h. After transfer of 30 µl of the supernatants to a second microtitre plate, the specific release was quantified by β-hexosaminidase activity by hydrolysis of p-nitro-phenyl-N-acetyl β-D-glucosaminide for 45 min. The total release was determined by treating the cells with Tyrode's buffer containing 1% Triton X-100. RBL cells incubated without (i) sensitising antibodies or (ii) sensitising antibodies and allergen were used as controls to measure antigen-induced and spontaneous release, respectively. Results were expressed as per cent of the total.

Production and selection of IgE antibody producing hybridomas

Balb/c mice were immunised according to the same immunisation schedule and serum IgE was tested with RBL mediator release as described above. Spleen cells of immunised mice were harvested and the hybridomas were raised by conventional hybridoma technology as described by Peters and Baumgarten using P3x63Ag8.653 cells for fusion [8]. IgE-secreting hybridomas were identified by RBL assay. RBL cells were incubated with hybridoma supernatants diluted 1:4 in MEM. To detect RBL cell-bound monoclonal IgE additional allergen-specific polyclonal IgG antibodies were added after incubation with allergen to crosslink and bridge the Fcε receptors, and thus, induce mediator release from RBL cells. This is necessary, because monoclonal IgE binds to a single epitope and fails to induce mediator release from RBL cells since the cell-bound IgE molecules are not bridged. This can be overcome by additional crosslinking by allergen-specific IgG leading to bridging the IgE molecules and thereby causes mediator release from RBL cells (Fig. 1). As additional crosslinking antibodies a mouse antiserum (Bet pool 8) containing birch pollen-specific IgG antibodies diluted 1:800 was used. The selected hybridomas were cloned twice.

Characterisation of birch pollen-specific IgE monoclonal antibodies (IgE mAbs) by immunoblotting and RBL mediator release assay

Birch pollen extract (5 µg/cm total protein), rBet v 1 (0.3 µg/cm) (Biomay, Linz, Austria) and rBet v 6 (0.3 µg/cm) [9] were separated by SDS-PAGE (T = 12.5 %, C = 2.7 %) under reducing conditions using a BioRad (Munich, Germany) Mini Protean cell [10]. Proteins were transferred on to nitrocellulose membranes (0.45 µm, Schleicher und Schüll, Dassel, Germany) [11]. The IgE mAb hybridoma supernatants were diluted to an IgE concentration of 0.5 µg/ml. As controls Bet v 1 and Bet v 6-specific IgG mAbs and TIB 141, a tri-nitro-phenol-(TNP)-specific IgE mAb without crossreactivity to birch pollen allergens were used, all applied as diluted hybridoma supernatants (0.5 µg/ml). Bound mAbs were detected with rat anti-mouse IgE alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, USA) or goat anti-mouse IgG(γ-chain) alkaline phosphatase conjugate (Sigma, Deisenhofen, Germany) using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) as substrate. The diluent for all reagents was 0.05 M Tris buffered saline (TBS) containing 0.1 % bovine serum albumin (BSA) and 0.05 % (v/v) Tween 20. pH 7.4. Blocking buffer was 0.3 % Tween 20 in TBS.

RBL assay was carried out as described above. For the sensitisation of RBL cells we used dilutions of the IgE mAbs with IgE concentrations ranging from 1 µg/ml to 0.1 ng/ml. For additional crosslinking mouse serum containing birch pollen-specific IgG antibodies diluted 1:800 as well as a supernatant mixture of Bet v 1-specific IgG mAbs or Bet v 6-specific IgG mAbs diluted 1:50 were applied. Bet v 1 and Bet v 6 IgG mAbs were produced in our laboratory.

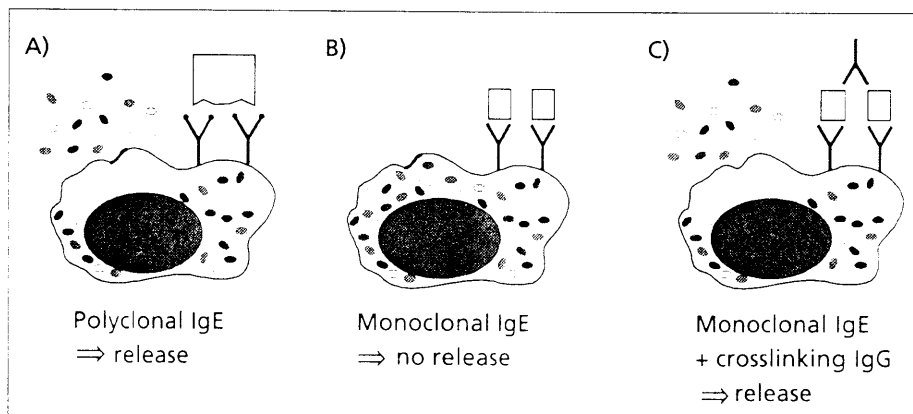


Fig. 1: A new concept of IgE hybridoma screening.

A) Polyclonal IgE antibodies bind to multiple epitopes, thus the IgE molecules are crosslinked by polyvalent allergen leading to mediator release from RBL cells. B) Monoclonal IgE, binding to a single epitope, fails to induce mediator release from RBL cells since the cell-bound IgE molecules are not bridged. C) Additional crosslinking by allergen-specific IgG leads to bridging IgE molecules and thereby causes mediator release from RBL cells.

RESULTS

Allergen specificity of IgE mAbs analysed by immunoblotting

With our new screening procedure nine birch pollen-specific IgE monoclonal antibodies were obtained. As isotype confirmation, immunoblotting with birch pollen extract was performed. Detection of bound IgE mAbs with anti-mouse IgE conjugate revealed bands whereas anti-mouse IgG conjugate did not show any reaction (data not shown).

Immunoblotting with birch pollen extract (Fig. 2) as well as with rBet v 1 or rBet v 6 revealed that six IgE mAbs are Bet v 1-specific (mP 30, mP 31, mP 33, mP 37, mP 39, mP 40), two IgE mAbs are Bet v 6-specific (mP 34, mP 35) and one IgE mAb is Bet v 6-specific with cross-reactivity for Bet v 1 (mP 36). These immunoblots indicated that the IgE mAbs not only reacted with Bet v 1 and Bet v 6 in natural birch pollen extracts but also with their recombinant counterparts.

RBL cell mediator release assay

As expected, most of the IgE mAbs required additional crosslinking by allergen-specific IgG to induce mediator release by RBL cells. As an example, the Bet v 1-specific IgE mAb mP 39 is shown in Figure 3. Neither stimulation with birch pollen extract alone nor with a mixture of Bet v 6-specific IgG mAbs as additional crosslinker induced mediator release. However, when crosslinked with polyclonal birch pollen-specific mouse serum or with a mixture of Bet v 1-specific IgG mAbs a mediator release was observed. This mediator release was IgE concentration-dependent.

Surprisingly, one of the IgE mAbs (mP 30) was able to induce allergen-specific mediator release of RBL cells without additional crosslinking IgG (Fig. 4). The IgE mAb mP 30 triggered mediator release with birch pollen extract alone and no enhancement was observed after additional crosslinking with IgG antibodies. This may be due to the recognition of repetitive epitopes or allergen dimers.

DISCUSSION

Until now, only a few monoclonal mouse IgE antibodies have been produced [4-7]. This may be due to difficulties in inducing sufficient amounts of IgE-producing B-lymphocytes or to screening procedures that are not sensitive enough to detect minute amounts of IgE and thus to identify all IgE-producing clones. In addition, these assays cannot determine the biological activity (e.g. mediator release) of IgE antibodies. Using the RBL mediator release assay as new screening method we obtained nine birch pollen-specific IgE monoclonal antibodies without the need to use additional animals to screen hybridoma supernatants by PCA. The IgE isotype of these IgE mAbs was clearly shown by inducing allergen-specific and anti-mouse IgE triggered mediator release from RBL cells, as well as by immunoblotting. We showed that six of the IgE mAbs are specific for the birch pollen allergen Bet v 1 and two are Bet v 6-specific. One IgE mAb which predominantly showed reactivity to Bet v 6 also crossreacted with Bet v 1. This cross-reactivity points to similar epitopes on Bet v 6 and Bet v 1 which may be due to structural similarities. These

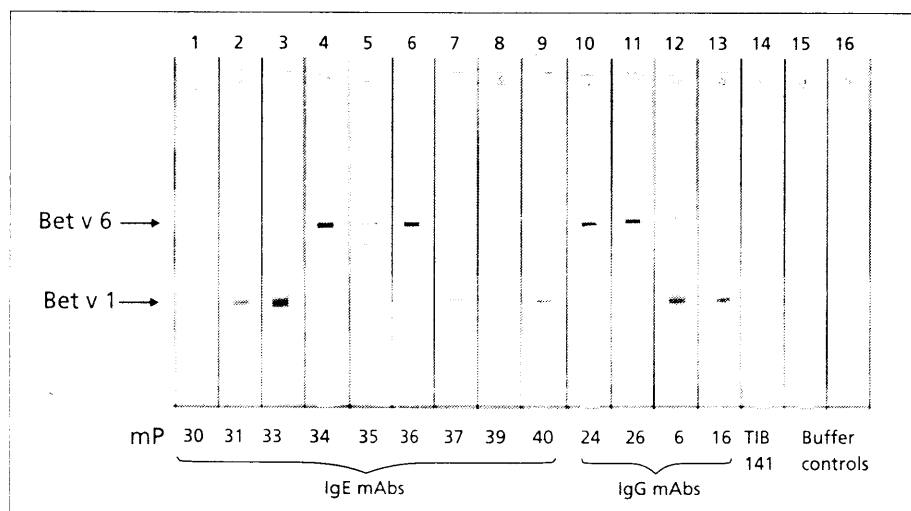


Fig. 2: Detection of birch pollen allergens by IgE mAbs.

The IgE mAbs detected birch pollen allergens Bet v 1 (lanes 1-3, 6-9) and Bet v 6 (lanes 4-6). As positive controls two Bet v 1 and two Bet v 6-reactive IgG mAbs (lanes 10-13) and as negative controls a TNP-specific IgE mAb and buffer were used (lanes 14-16).

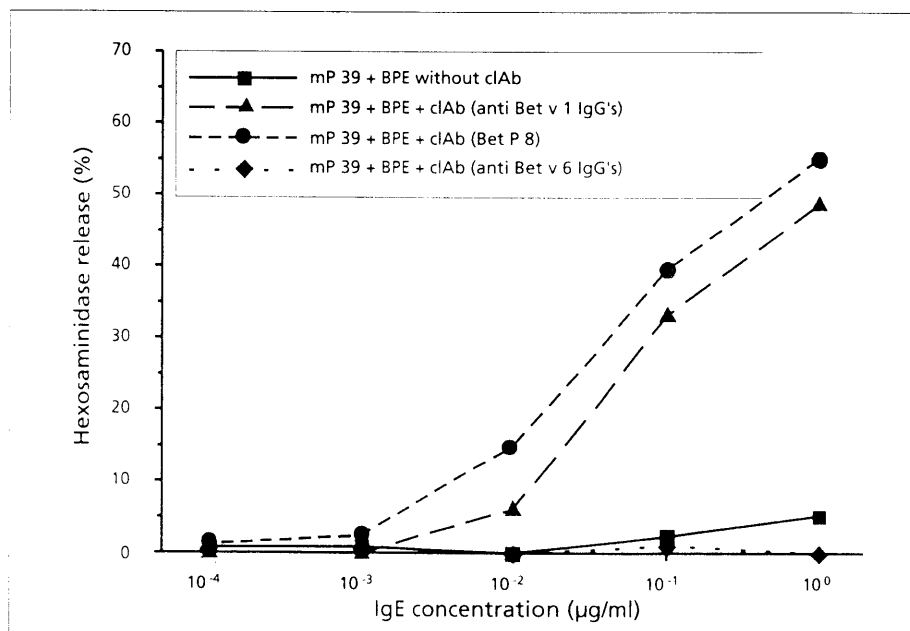


Fig. 3: Dependency on additional crosslinking IgG antibodies for mediator release.

RBL cells sensitised with Bet v 1-specific IgE mAb mP 39 were stimulated with birch pollen extract (BPE) without additional crosslinking IgG (cAb) (-■-), with a mixture of Bet v 1-specific (-▲-) or Bet v 6-specific IgGs (-◆-) or with mouse serum containing birch pollen-specific antibodies (Bet P 8) (-●-). Mediator release was only observed when Bet v 1 was crosslinked by additional IgG antibodies.

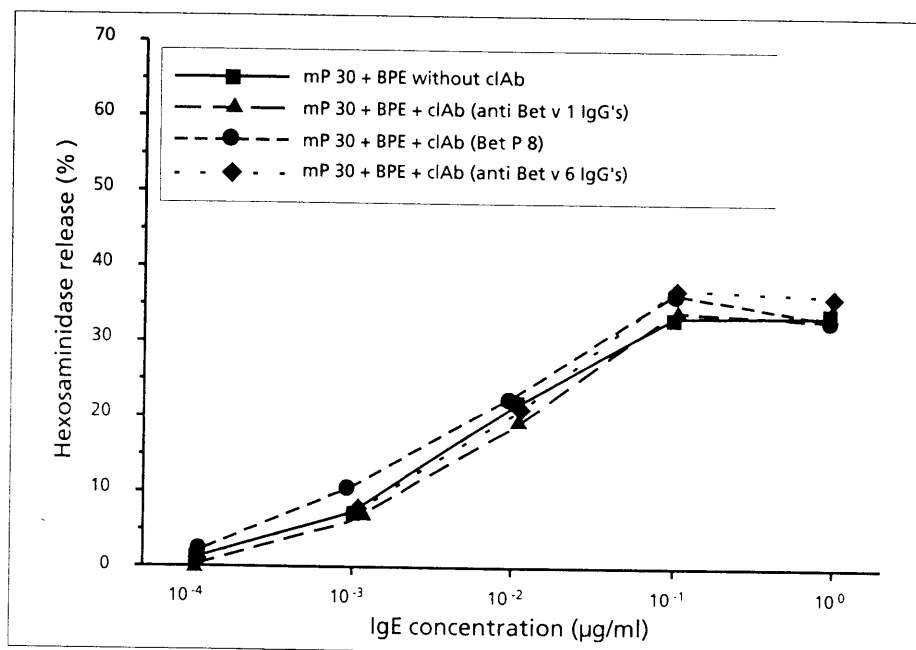


Fig. 4: The IgE mAb mP 30 react independently of additional crosslinking IgG antibodies.

IgE mAb mP 30-sensitised RBL cells were stimulated with birch pollen extract (BPE) without additional crosslinking IgG (cIAb) (-■-), with a mixture of Bet v 1-specific (-▲-) or Bet v 6-specific IgGs (-◆-) or with mouse serum containing birch pollen-specific antibodies (Bet P 8) (-●-). Similar mediator release was observed with all stimulations.

IgE mAbs in combination with the RBL mediator release reveals allergen-specific information combined with biological activity and thereby allows the determination of single allergens in allergen extracts. Thus, this method combines the advantages of allergen-specific ELISA with mediator release by polyclonal antibodies.

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