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Detection of Allergen-Specific IgE Antibody Responses

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Allergen-specific IgE production is the central event in the pathogenesis of atopic disorders and increases in specific IgE serum antibodies are an indicator of immediate hypersensitivity responses in humans and in animal models of allergy. Consequently, accurate and user-friendly methods are needed to measure serum levels of allergen-specific IgE. This review examines historical and recent developments in *in vivo* and *in vitro* methods for the detection of allergen-specific IgE in humans and in animal models. Routinely, *in vitro* methods such as enzyme-linked immunosorbant assays or radioallergosorbant tests and *in vivo* methods such as the skin prick test (SPT) for humans and the passive cutaneous anaphylaxis assay (PCA) used in animals are utilized to detect allergen-specific IgE. While *in vivo* assays are usually more accurate than *in vitro* assays since they provide a functional readout of IgE activity, they are relatively costly and require considerable expertise. On the other hand *in vitro* assays are limited by the fact that the amount of allergen-specific serum IgG exceeds IgE antibody by several orders of magnitude, resulting in competition for allergen binding. Consequently, methods that use allergen as a direct capture step are limited by the availability of free allergen binding sites for IgE. In order to circumvent this problem, *in vitro* methods usually require prior depletion of IgG or use high amounts of allergen in order to facilitate availability of free binding sites for IgE detection. Clearly, these approaches are limited for small sample volumes and allergens that are in short supply. New methods such as protein microarray could potentially overcome this problem by providing high allergen concentrations in a relatively small reaction volume. Currently, *in vitro* methods are rarely used in isolation for prognosis but are used primarily to complement the information obtained from *in vivo* assays. With the emergence of new technologies it is conceivable that *in vitro* assays may in the future replace *in vivo* assays, however until then *in vivo* assays remain the gold standard of allergen-specific IgE detection.

INTRODUCTION

The increasing prevalence of atopic allergic disease to a variety of common and harmless environmental antigens in the western world highlights the importance of investigating the underlying mechanisms of the disease (Romagnani, 2004). Moreover, particularly in the case of food allergy, the emergence of modified crop plants containing novel proteins requires the development of reliable tests for the prediction of the potential allergenicity of such proteins. Although there are no animal models available that mimic every aspect of the pathophysiology of food allergy, there are nevertheless animal models that are suitable for studying the mechanisms of induction and repression of allergic reactions as well as for the development of therapeutic interventions to prevent such adverse reactions. One of the hallmarks of the allergic response to proteins is the induction of allergen-specific IgE antibodies that can usually be detected in the serum as well as locally (Ishizaka et al., 1966a, 1966b; Ishizaka and Ishizaka, 1967). IgE antibodies are the principal biological mediators of immediate hypersensitivity reactions including allergic asthma, rhinitis, urticaria, atopic dermatitis, and the majority of food allergic reactions. Circulating specific IgE binds to high and low affinity receptors ($F_{c\varepsilon}$RI and $F_{c\varepsilon}$RII or CD23) on mast cells and basophils. Subsequent contact with the allergen results in crosslinking of receptor bound allergen-specific IgE, culminating in the release of inflammatory mediators, such as histamine and leukotrienes. The physiological response to these substances includes dilation of local blood vessels, attraction of eosinophils and neutrophils to the reaction site, increased permeability of the capillaries, and loss of fluid into the tissues.

Tests for the presence of allergy in animals and humans are therefore based primarily on the detection of allergen-specific IgE in serum. In humans, diagnosis of allergic disease usually involves a combination of clinical history, physical examination, and *in vivo* and *in vitro* assays for the detection of allergen-specific IgE antibodies (Hamilton and Adkinson, 2004). IgE responses can be measured *in vivo* by introduction of allergens via a small cut in the skin (Skin prick test, SPT). In allergic individuals, this usually results in the development of a visible weal and flare reaction within minutes of challenge, which is indicative of the presence of allergen-specific IgE antibodies (Stenius, 1973).

In some experimental (animal) models of allergy, such as the dog or swine, it is also possible to perform similar active

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challenges of sensitized animals to characterize the immediate hypersensitivity response (Helm and Burks, 2002; Kimber et al., 2003). Currently available assays for the detection of allergen-specific IgE in serum from experimental animals or from man can be divided into antibody-based tests based on the principle of immunoabsorption and into bioassays, which measure biological activity of specific IgE either *in vitro* or *in vivo*. With all of these methods, consideration must be given to surmounting the technical challenges associated with the measurement of specific IgE antibody responses. The primary issue when measuring specific IgE antibody is that of competition with specific IgG antibody that generally exceeds IgE antibody concentrations by at least 3-log orders of magnitude (Plebani et al., 1986). Provision must be made against the blocking effects of IgG antibody, leading to potential underestimation of IgE antibody, and the cross-reactivity of anti-IgE antibody reagents with IgG antibody, leading to false positive results.

ANTIBODY-BASED ASSAYS FOR IgE DETECTION

Commercially Available IgE Antibody Assays

Given the importance of the measurement of specific IgE for the diagnosis of allergic disease, there are commercially available several methods and reagents for the detection of allergen-specific IgE antibody responses in human serum. Analogous reagents for the measurement of allergen-specific IgE in serum from other species are not available for the assays described next.

The radioallergosorbent test (RAST) as described by Wide et al. (1967) was historically the first immunoassay available for the measurement of IgE antibodies to specific allergens in human serum. In its original form it consisted of a paper disc to which allergen was covalently attached (allergosorbent) and binding of IgE was detected using radiolabeled polyclonal anti-human IgE antibodies. The results were reported as a function of arbitrary units relative to a reference curve. The relatively large surface area of the paper disc ensured that sufficient allergen was bound to prevent competition by allergen-specific IgG antibody. Subsequently, nonradioactive labels were developed based primarily upon chromogenic enzyme immunoassay (EIA) or fluorescence enzyme immunoassay (FEIA) techniques. For clinical routine purposes the most commonly used assay is currently the Pharmacia CAP RAST system (Bousquet et al., 1990; Brunnee et al., 1996). The solid phase material is a capsulated cellulose polymer (CAP), which is densely conjugated with allergen extracts or recombinant allergens. The CAP is capable of binding more antigen than conventional systems due to an enlarged surface area, which circumvents blocking by specific IgG antibody, and results in a high level of sensitivity for allergen-specific IgE (Ewan and Coote, 1990; Brunnee et al., 1996). Test serum is incubated with the solid phase material, allowing reaction of the specific IgE in the sample with bound allergen and fluorescently labeled anti-IgE antibody conjugate is added. Binding of the anti-IgE antibody is measured with a special fluorometer and expressed in units (kU/L) or in specific Ig classes (0–6) (Bousquet et al., 1990). Although the CAP RAST is very sensitive and its results approximate closely to the SPT (Ewan and Coote, 1990), it requires specialized equipment, which makes the procedure relatively costly and not applicable for use in all laboratories.

The ELItest is a similar system in which allergen is bound to paper rings and an alkaline phosphatase conjugated secondary antibody detects bound specific IgE. The advantage over the CAP method is that the ELItest is an enzyme-linked assay similar to a conventional enzyme-linked immunosorbant assay (ELISA) that can be conducted in a standard microwell plate format. Binding is detected by colorimetric analysis after addition of substrate using a standard ELISA plate reader, therefore eliminating the need for specialized equipment. However, due to the nature of the allergen carrier, the allergen density is somewhat lower than in the CAP system, resulting in reduced sensitivity particularly at high specific IgE concentrations and an increased potential for competition by specific IgG antibodies (Brunnee et al., 1996).

A modification of these solid-phased IgE detection methods is offered by the AlaSTAT assay (Diagnostic Products Corporation, LA, USA), which uses liquid allergen complexes instead (El Shami and Alaba, 1989; Ownby et al., 1993; Hollander et al., 1996). In this test, a solution of the allergen is covalently bound to a soluble polymer/copolymer matrix, which is subsequently labeled with a ligand. These ligand-labeled allergen complexes are added together with the test sample into wells of a microtiter plate coated with the same ligand. During a subsequent incubation step, endogenous IgE binds to the allergen and are bound to the ligand-coated wells through addition of a multivalent anti-ligand during a secondary incubation. Unbound IgE is removed by stringent wash and decanting procedures and allergen/IgE complexes are visualized by horseradish-peroxidase labeled monoclonal anti-IgE antibodies and subsequent addition of a chromogenic indicator. The rate of color development is measured as milli-Optical Density units per minute using a kinetic microplate reader and can be directly related to allergen-specific IgE concentrations. In comparison to solid-phase methods, the AlaSTAT has been reported to reduce the number of false positive results and to improve detectability of low amounts of IgE (Moneret-Vautrin et al., 1993). However, similar to solid-phase systems it requires specific allergen for detection, which poses problems, partly due to limited availability of certain allergens and partly since the quality of allergen preparations can differ between suppliers and batches.

Sandwich ELISA (Capture ELISA)

Capture or sandwich ELISA techniques are routinely used for the measurement of total serum antibodies in serum from both animals and humans (Plebani et al., 1986; Kemeny et al., 1989; Strouse et al., 1991; Larsson et al., 1997). The capture and detection antibodies are isotype specific, each recognizing a different epitope. Quantification is performed using standard curves generated with known concentrations of commercially available

purified polyclonal antibody of the appropriate isotype. Whereas measurement of total serum immunoglobulin concentration is relatively easy to perform, quantification of allergen-specific antibodies is more technically demanding. However, antigen specificity can be determined using antigen labeled with biotin or enzyme (Plebani et al., 1986; Hirano et al., 1989; Sakaguchi et al., 1989; Strouse et al., 1991; Olivieri et al., 1993).

The specificity and sensitivity of this approach has been compared with the more conventional passive cutaneous anaphylaxis (PCA) assay, an *in vivo* approach that measures biologically active specific IgE as a function of the ability of serum to sensitize and induce degranulation of mast cells in naïve recipient animals (Sakaguchi et al., 1989; Birmingham et al., 2003). Sakaguchi et al. (1989) utilized a monoclonal anti-mouse IgE rat antibody in the capture step and binding of ovalbumin-specific IgE in sera from sensitized mice was detected using biotinylated antigen. Using this approach the authors reported specific IgE binding with no or little interference from other isotypes and a similar sensitivity for IgE to that observed for the PCA assay, with the advantage of requiring less antigen for detection than the PCA assay. The disadvantage, however, is that for each allergen, a separate labeled allergen preparation must be synthesized.

Reverse ELISA

A second strategy to allow the detection of allergen-specific IgE antibodies by ELISA employs the allergen as the specific capture step with visualization based on the use of an anti-IgE labeled antibody (reverse ELISA) (Wal et al., 1995). In the reverse ELISA, the antigen is coated directly to the plate. It has been shown previously that this approach is more sensitive than the capture ELISA (Silva et al., 2001). However in this approach all antigen-specific antibodies irrespective of their isotype will be bound to the plate. This is a significant problem when measuring specific IgE antibody since specific IgG antibody exceeds IgE by at least a 3-log order (Plebani et al., 1986). Therefore specific IgG competes with IgE for solid-phase binding, potentially leading to inaccurate assessment of IgE levels (Zimmermann et al., 1980; Gleich et al., 1981; Zeiss et al., 1981). Although it is generally recognized that allergens cannot be immobilized as densely on the ELISA microtiter plate as is possible with CAP/RAST systems, some authors have reported that simply increasing the concentration of antigen on the microtiter plate is sufficient to allow accurate detection of specific IgE antibody without competition from specific IgG antibody (Birmingham et al., 2003). However, the majority of investigators take active steps to circumvent this problem. The most obvious approach is to remove IgG from sera prior to analysis. Recently Kadooka et al. (2000) used a protein G (PG) immobilized gel to selectively remove IgG in order to improve specific IgE detection. Similarly, Lehrer et al. (2004) demonstrated a 3–5-fold increase in IgE detection by ELISA after IgG removal using immobilized PG. These findings suggest that depletion of IgG antibodies results in a significant increase in the sensitivity of IgE antibody measurements. However, it has also been shown that removal of IgG can result in the reduction of detectable IgE in some cases. Kadooka et al. reported that this reduction was reversed when PG was acid treated, suggesting that some IgE was bound to IgG and therefore escaping detection in the IgE-specific ELISA. Upon inclusion of this modification the authors could show that the 2-step process of removal of IgG and liberation of IgE from IgE-IgG complexes resulted in an increase in specific IgE measurements by direct ELISA in sera that previously only showed low levels of IgE. Their results suggest that low detection levels of allergen-specific IgE in sera by ELISA can be due to at least 2 mechanisms; first, IgG antibodies may compete with IgE for allergen binding and second, IgG autoantibodies may form complexes with IgE in the sera and prevent recognition of allergen-specific IgE by ELISA.

Although ELISA-based assays are an easy and convenient *in vitro* system for measuring total immunoglobulin concentrations, they require considerable modification in order to detect allergen-specific IgE antibodies. One substantial problem with such ELISAs is the lack of availability of commercial allergen-specific IgE antibodies required to generate the standard curve necessary to calculate the absolute amount of IgE in the test sample. Therefore absolute quantification is difficult, leading at best to a semiquantitative comparison of different sera. Moreover, use of allergen as a capture or detection step is often hampered by the lack of availability of the allergen in sufficient quantity.

Western Blotting for Specific IgE Antibody

Western blotting is an alternative antibody-based assay that has been applied for the measurement of specific IgE antibody responses in serum from experimental animals and humans (Jeep et al., 1996). In this system, allergen extracts are separated on the basis of molecular size by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and in sufficient amounts to largely avoid the issues of competition with specific IgG antibody. Following electrophoretic transfer of the separated proteins onto nitrocellulose membranes and incubation with test sera, IgE binding is detected by an anti-IgE monoclonal antibody labeled with a reporter enzyme. The allergen bands recognized by specific IgE antibodies are visualized by adding a chromogenic substrate and quantified by scanning. The advantage of the Western blot is that it allows identification and characterization of the IgE-inducing protein subunits in a crude allergen extract. Using sera from venom allergic patients, Zollner et al. (2001) compared the sensitivity and specificity of Western blots with a RAST-like method (AlaSTAT) and *in vivo* SPT. The authors reported that Western blot was sensitive and showed higher specificity in the detection of IgE to a specific known allergen in all cases and concluded that it was superior to AlaSTAT and skin tests. Western blotting techniques have also been utilized to characterize IgE binding profiles of serum derived from mice or rats exposed to pollen or to hens' egg and cows' milk allergens in comparison with serum from allergic patients (Knippels et al., 2000; Seitzer et al., 2003). In both investigations the patterns of IgE reactivity

induced were very similar to that found in sera isolated from allergic patients.

These data suggest that Western blot may be a suitable alternative to ELISA and RAST for the detection of allergen-specific IgE with the additional advantage of identifying and characterizing major allergens in a complex mixture. However, the major disadvantage is that SDS-PAGE separation of allergens occurs under denaturing conditions, resulting in the destruction of the three-dimensional structure of the allergen. This is a problem in so far as some allergen B cell epitopes are only recognized by the antibody in their conformational, 3-dimensional structure rather than in their linear, amino acid sequence; therefore, the denaturing conditions of SDS-PAGE may interfere with the recognition of these epitopes. The consequence of this could be either failure of the IgE antibodies to recognize their respective epitope or loss of specificity of interaction.

Allergen Microarray Assays

Although the assays described previously can be suitable for the measurement of allergen-specific IgE antibodies, these tests are relatively expensive, time consuming, and require a large volume of serum. There has therefore been considerable interest in the development of a fast and economic screening technology for the detection of allergen-specific IgE in serum samples, which would allow the simultaneous detection of hundreds of allergens in a single run. One strategy being explored currently is the use of protein microarrays. The assay is based on the established DNA microarray technology and was first developed by Lueking et al. (1999). It consists of microscope glass slides with an activated surface onto which allergen solutions are immobilized as small droplets on defined positions using a piezoelectric arrayer. The principle of the method is that of a reverse immunoassay with the allergens as capture molecules. The serum sample is screened for allergen-specific IgE binding with a fluorescently labeled anti-IgE antibody, and fluorescence is measured by using a special array scanner. In contrast to other tests for specific IgE, such as Pharmacia CAP RAST, allergen-specific IgE against a variety of allergens can be screened using 25 μl of serum sample and with automation can be performed in less than 1 hour. Sensitivity for IgE detection can be enhanced by using special fluorescence enhancers, which are linked to the secondary antibody (Bacarese-Hamilton et al., 2004). Protein microarrays are being used increasingly for the detection of allergen-specific IgE in human serum samples but so far have not been employed for measurement of specific IgE in animal sera (Kim et al., 2002; Suck et al., 2002). The protein microassay is generally comparable in specificity to methods such as CAP RAST and reverse ELISA. Moreover, in a study of sera from birch and grass pollen allergic subjects, the protein microarray assay was shown to be more sensitive that the CAP RAST for the detection of specific IgE to the major birch and grass pollen allergens (Jahn-Schmid et al., 2003). Similarly Fall et al. (2003) have used this approach to screen allergen-specific IgE from patients to a variety of allergen extracts and recombinant/purified allergens (24 allergens including rBet v 1, rBet v 2, PLA2, Der p 1, Der f 1, rPhl p 1, rAln g 1, rFel d 1, rAlt a 1). Using these slides, it was possible to distinguish reproducibly between patients with and without elevated levels of allergen-specific IgE as confirmed by CAP RAST. Similar to the Western blot technique, using protein microarrays it is possible to identify and characterize the allergenic components in an allergen extract. However, in contrast to the Western blot, a variety of single allergens or overlapping peptides can be probed for IgE binding simultaneously, potentially allowing the identification of single allergenic epitopes. This is important, since it enables assessment of the antibody reactivity profile of an allergic patient, and can help to identify the specific molecules that elicit the clinical manifestations of the disease with the aim of tailoring individual treatment options. Shreffler et al. (2004) used a microarray immunoassay to analyze sera from peanut allergic patients and detected a remarkable heterogeneity in the number and patterns of epitope recognition between individuals. Interestingly, a high frequency of epitope recognition was found in patients with a history of more severe allergic reactions; although these patients' sera did not exclusively recognize such epitopes. The same sera also induced greater effector responses in a biological assay measuring basophil activation. Furthermore, by using microassay technology the authors not only confirmed Ara h 1, Ara h 2, and Ara h 3 as the major peanut allergens but they also discovered an additional important epitope of Ara h 1. This suggests that quantitative and qualitative differences in epitope recognition might provide prognostic information about the patient.

The characterization of IgE epitopes is of fundamental importance for the understanding of the mechanisms of food allergy and for the design of therapeutic interventions, for example by modification of the dominant IgE epitopes in the food allergen. The advantage of the microarray-based IgE test is significant acceleration of the immune diagnosis by simultaneous, multiparametric determination of specific subclasses of antibodies directed against various antigens in parallel. Consequently, a large amount of information can be obtained using a relatively small amount of serum, which is of relevance for the analysis of individual animal sera and diagnosis of infants and young children. However, similarly to other *in vitro* methods based on limited amount of allergens as capture molecules for IgE detection, the excess of allergen-specific IgG in sera will potentially result in competition for allergen binding and could therefore compromise the sensitivity of the assay.

Surface Plasmon Resonance Detection System

A recent development that may have applications for the analysis of allergen-specific IgE is the surface plasmon resonance (SPR) detection system. The detection principle, SPR, is an electron charge density wave phenomenon that arises at the surface of a metallic film when light is reflected at the film under specific conditions. The sensor chip consists of a glass surface, coated with a thin layer of gold to which a variety of molecules

such as protein allergens can be bound. Integrated micro-fluidics cartridges allow the sample to pass over the sensor and binding is detected by SPR as change in the refractive index. Analogous to an ELISA type assay, multiple binding steps can be performed and the extent of binding at each stage can be monitored by SPR. This simple principle forms the basis of the sensorgram, which provides quantitative information in real-time on specificity of binding, active concentration of molecules in a sample, kinetics, and binding affinity (Cullen et al., 1987; Karlsson et al., 1991; VanCott et al., 1992). The advantage of using this system for allergen-specific IgE antibody binding is that detection does not require fluorescent or radioactive labeling of the allergen. Moreover the system is quite economical in that the sensor chip can be regenerated and reused many times. Interactions between proteins, nucleic acids, lipids, carbohydrates, and even whole cells can be studied and most importantly molecules can be studied in their native state providing results that potentially reflect *in vivo* activity. The system has already been used for measurement of antigen-specific IgG and IgM antibody responses in human sera (van Remoortere et al., 2001; Canzani et al., 2004). With regard to IgE, to date SPR has mainly been used to investigate changes in binding affinity of human IgE to modified high or low affinity IgE receptors (Henry et al., 1997, 2000; Sayers et al., 1998; Chen et al., 2003). However, the system could also be used to monitor simultaneously allergen-induced increases in total serum IgE concentration and allergen-specific IgE antibody in sera from humans and animals. For this purpose the gold chip would be coated with anti-IgE antibody, or the high and/or low affinity IgE receptors, $F_{c\varepsilon}RI$ and $F_{c\varepsilon}RII$ in order to bind IgE specifically and to avoid competition with IgG in the serum. Although allergen is required to detect specific IgE, the quantities are less than needed for other assays such as ELISA and most importantly the allergen does not have to be labeled in order to detect the binding. Therefore, the assay should be suitable for a variety of allergens provided that they can be isolated and purified in appropriate amounts.

BIOLOGICAL ASSAYS FOR SPECIFIC IgE

The most substantial problem with *in vitro* antibody based assays for the detection of allergen specific IgE such as ELISA is that they often fail to correlate with tests that measure biological activity of specific IgE, such as PCA in animals or SPT in humans (Niederberger et al., 2001; Saevik et al., 2003). This emphasizes the importance of assessing biological activity as well as quantity of allergen-specific IgE for improved mechanistic understanding and diagnosis of allergy. Cross-linking of IgE on the surface of effector cells, such as mast cells or basophils, results in the release of inflammatory mediators, which contribute to the early phase response of the allergic elicitation reaction. In addition to *in vivo* methods, more recent studies have therefore focused on using allergen-induced activation of these IgE-bearing cells as surrogate *in vitro* assays to detect biological activity of allergen-specific IgE.

In vivo Assays for Specific IgE

As described previously, one of the common tests used for diagnosis of allergic disease in humans is the SPT for the detection of allergen-specific IgE antibodies. IgE responses are measured *in vivo* as a function of the weal and flare reaction induced by challenge (intradermal administration) of the specific allergen. In some animal experimental models of allergy it is possible to perform analogous experiments. In the high IgE responder dog experimental system used by Teuber et al. (2002) for example, animals are sensitized to allergen by subcutaneous injection, then at various time points after sensitization (typically 6–18 months) the development of allergen specific IgE antibody responses are assessed by challenge. Animals are challenged by intradermal injection of allergen preparations into the shaved skin and the induced inflammatory reaction visualized by concurrent intravenous injection of Evans Blue dye that extravasates into tissue containing degranulating mast cells.

In mice or rats, the mast cell binding activity of serum is measured usually in naïve recipient animals, "passive" cutaneous anaphylaxis (PCA) (Lehrer and Vaughan, 1976; Ovary, 1982; Inagaki et al., 1988; Dearman et al., 2003a, 2003b). Serum is administered intradermally and any IgE antibody present in the transferred serum will bind to $Fc\varepsilon R$ on the surface of 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 IgE antibody specific for the test protein is present, then it will be cross-linked, causing the local degranulation of mast cells and the release of inflammatory mediators, including agents that cause vasodilation. In such circumstances the blue dye injected with the protein antigen is able to extravasate, resulting in a blueing of the skin, which is the end point measured in this assay. In mice, both IgG1 and IgE antibody isotypes are homocytotropic (bind murine mast cells) (Lehrer and Vaughan, 1976; Inagaki et al., 1988). However, mast cell binding IgG1 and IgE antibodies have differential affinity for mast cells receptors with only IgE antibodies remaining bound after 48 hours, the incubation time of the assay, ensuring the specificity of the method (Lehrer and Vaughan, 1976). Similarly, the SPT in humans primarily detects homocytotrophic allergen-specific antibodies independent of their isotype. However, in contrast to the mouse model, the role of IgG as sensitizing antibodies is less clear. IgG antibodies have been shown to act as anaphylactic antibodies (van der Zee and Aalberse, 1991), while others have shown that allergen-specific IgG is able to block allergen-IgE binding to IgE-receptors on the cell surface (Wachholz et al., 2003). Although, cross-linking of IgG4 bound to leucocytes by polyclonal anti-IgG4 has been reported to result in histamine release, this occurred at much lower magnitude than when IgE antibodies were cross-linked (van der Zee and Aalberse, 1991). This suggests, that the majority of allergen-induced histamine release and therefore the cause of the weal and flare is attributable to allergen-specific IgE.

CELL-BASED ASSAYS FOR IgE

Basophil Histamine Release Assay

Although mast cells are the major source of allergen-IgE induced histamine release *in vivo*, human mast cells are not easily available for *in vitro* analysis since they mainly reside in tissue. Their peripheral homologous counterparts, polynuclear basophils, are therefore commonly used to measure allergen-induced histamine release *in vitro*. IgE-cross-linking by allergens on basophils leads to activation and can be analyzed by measuring the release of histamines (Wasserman, 1983; MacGlashan, 1995). The basophil histamine release assay is used frequently for routine diagnosis of allergy in humans, especially for allergy to insect venom (Engel et al., 1988). In the conventional basophil histamine release assay cells are isolated from each individual patient and histamine release is detected upon incubation with a test allergen. In each assay, an anti-IgE antibody is run in parallel as a positive control, to show that cross-linking of IgE receptor is sufficient to cause histamine release in cells isolated from a particular patient. Histamine concentrations in the supernatants are measured subsequently by ELISA or by fluorimetry. In addition to being quite laborious this approach also has several additional problems. The most substantial disadvantage of the basophil histamine release assay is that release of histamine in response to an IgE-dependent stimulus is dependent upon a number of variables, such as the severity of the allergy (Lichtenstein and MacGlashan, 1986). Moreover, release of histamine by basophils is also influenced by previous drug intake, cytokines, and hormones causing high variability in spontaneous histamine release.

Furthermore, about 20% of individuals are nonresponders with regard to IgE-mediated histamine release, despite having clinical symptoms of allergy, which may lead to false-negative results (van der Zee et al., 1988). In addition to diagnosis in humans, the antigen-induced histamine assay has also been used in some animal models. Originally, basophils from sensitized guinea pigs were analyzed for histamine release *in vitro* upon exposure to the sensitizing protein. In later studies, immune serum was used to passively sensitize basophils *in vitro*, resulting in comparable histamine release as achieved by *in vivo* sensitization (Lett-Brown et al., 1981, 1983). Pfeffer et al. (1997) compared the basophil histamine release assay to PCA tests by analyzing blood and sera from louse (*Bovicola ovis*) infected sheep. Interestingly, histamine release from washed whole blood cells was significantly higher in louse-infected sheep and correlated significantly with louse and cockle scores. In contrast, PCA tests were negative with sera from louse-infected sheep, suggesting that the basophil histamine-release assay is more sensitive that the PCA test in detecting homocytotropic antibodies in louse-infected sheep.

Stripped Basophil Histamine Release

In order to overcome some of these variables, an alternative approach is to use basophils from a normal nonatopic donor, with confirmed histamine releasability, and to strip the cells of endogenous IgE using an acidic buffer. The cells are subsequently sensitized by incubation with serum from allergic subjects, stimulated by allergen and histamine release is measured as before. Klein Budde et al. (2001) have compared the sensitivity of the stripped basophil histamine release assay with the RAST assay using sera from atopic house dust mite (HDM) allergic donors. The investigators showed that results obtained in the bioassay were comparable to those obtained by RAST when measuring IgE to HDM. However, the basophil assay was more sensitive in detecting IgE against minor allergenic components in the culture medium than the RAST. Although the basophil histamine release assay can complement the RAST assay especially when there is a discrepancy between allergic symptoms and specific IgE levels measured by RAST, the assay is rather more laborious and generally less sensitive than antibody-based assays.

Basophil Activation Tests

Basophils not only secrete and generate quantifiable bioactive mediators such as histamine in response to allergen-induced crosslinking of surface-IgE, but expression of different cell surface markers (CD45, CD63, CD69, CD203c) can also be upregulated. Changes in basophil phenotype can be detected by multicolor flow cytometry using specific monoclonal antibodies conjugated with fluorochromes. These fluorochromes are excited by laser light and the emitted fluorescence is detected and quantified by a flow cytometer. Various flow cytometry based methods have been developed for basophil identification and activation analysis (Valent and Bettelheim, 1992; Takahashi, 1993; Fureder et al., 1994). At present the most commonly used markers in basophil activation studies are CD63 and CD203c.

In resting basophils CD63 is generally weakly expressed on the surface membrane both in normal subjects as well as in allergic individuals (Valent et al., 2001). Upon stimulation of these cells by cross-linking of surface IgE, expression of CD63 is increased and parallels histamine release (Moneret-Vautrin et al., 1999; Lambert et al., 2003). In the assay, whole blood is stimulated with allergen, anti-IgE (positive control) or buffer alone to measure spontaneous CD63 expression (negative control). Cells are stained subsequently with a fluorescently labeled anti-CD63 antibody and, after lysis of red blood cells, analyzed by flow cytometry. Detection of surface expression of CD63 has been used successfully for the study of common allergens (Paris-Kohler et al., 2000). In a rat model of ovalbumin induced anaphylactic shock, Bellou et al. (2003) used human basophils to investigate changes in expression of CD63 in response to rat sera. The investigators compared leukocyte histamine release (LHR) with rat blood collected from sensitized animals with human basophil activation (HBA) in the presence of sera from the same animals. In contrast to a negative LHR assay, the HBA showed increase in the percentage of cells expressing CD63 in the presence of sera from immunized rats. They concluded that the HBA test using human basophils is a useful method to detect induction of experimental allergy in animals.

Currently a CD63-based assay is commercially available from Becton Dickinson (Basotest). Paris-Kohler et al. have used this test to measure sera from cypress pollen allergic patients and compared sensitivity to the conventional CAP RAST system. While the Basotest was more sensitive than the CAP system in the diagnosis of cypress pollen allergy, both failed to correlate fully with symptoms as assessed by SPT or nasal challenge. Another disadvantage of using CD63 as a surrogate marker for basophil activation is that it is also expressed on other cells such as mast cells, macrophages, and platelets (Nieuwenhuis et al., 1987; Metzelaar et al., 1991). Recently, Boumiza et al. (2003) presented a paper reporting marked improvement of the basophil activation test using CD203c instead of CD63 as the activation marker.

CD203c is a specific activation marker for basophils that is rapidly up-regulated in response to allergen-specific and anti-IgE induced IgE cross-linking, similar to CD63 (Hauswirth et al., 2002; Buhring et al., 2004; Valent et al., 2004). Boumiza et al. (2003) analyzed sera from latex allergic patients using either the CD63 or the CD203c basophil activation test. Their results demonstrated an increased sensitivity when CD203c was used compared with CD63 with similar specificity in both assays.

Flow-cytometry based assays such as the CD63 or CD203c tests have the advantage of providing information about basophil responsiveness to an allergen relatively rapidly. Moreover, in contrast to the basophil histamine release assay that also detects nonspecific histamine release from other sources such as platelets, the flow-cytometry based assays are capable of detecting specifically activated basophils based on expression of surface markers. The flow cytometric analysis of *in vitro* activated basophils is, however, highly dependent on stimulation conditions and requires carefully controlled experimental conditions in order to provide for reproducibility. Moreover, baseline CD63 and CD203c expression is highly variable and may differ significantly among individuals.

Rat Basophilic Leukemia Cell Assay (RBL)

Based on a similar principle as the basophil assays, the rat basophilic leukemia cell (RBL) assay can be used to measure IgE-mediated histamine release. The RBL cell line possesses FcεR, which can selectively bind rat or murine IgE antibody and can be stimulated by cross-linking with protein allergen to degranulate, releasing granules, which have been preloaded with radiolabeled serotonin (Eccleston et al., 1973). Alternatively, allergen-specific degranulation can be detected by measuring release of hexosaminidase using a nonradioactive ELISA (Hoffmann et al., 1997, 1999). The RBL has been utilized extensively to study the biochemical mechanisms of IgE interactions with mast cell receptors (Wilson et al., 2000; Vincent-Schneider et al., 2001). The RBL assay has also been used to measure IgE antibodies induced in mice by exposure to protein allergens in the presence of adjuvant (Yamanishi et al., 1995; Hoffmann et al., 1999; Ballmer-Weber et al., 2002). Recently, the relative sensitivity of the RBL assay, and of the homologous PCA assay, for the measurement of specific IgE antibody has been investigated (Dearman et al., 2004). RBL cells were sensitized with mouse monoclonal anti-dinitrophenyl (DNP) IgE antibody and challenged with DNP-albumin conjugates with various hapten substitution ratios.

Polyclonal anti-OVA IgE antisera were also assessed for activity in the RBL 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 therefore not only requires appropriate epitope densities but also high affinity antibody. The RBL assay was unable to detect polyclonal antibodies that have low affinity or that have a low epitope density, and thus lacks the necessary sensitivity for the detection of anti-protein antibodies derived in the absence of adjuvant. These data demonstrated that there are serious limitations for the use of the RBL assay as an *in vitro* replacement for the PCA assay as it is insufficiently sensitive for the routine analysis of specific polyclonal IgE antibody responses such as those that are induced by exposure to complex protein allergens.

In addition, the method has been adapted to measurement of IgE in human sera. RBL cells have been stably transformed to express the human high-affinity receptor for IgE FcεRI, which can bind sera from allergic individuals and can be activated in an allergen-specific manner resulting in histamine release (Ecclestone et al., 1973; Barsumian et al., 1981; Wiegand et al., 1996). Contrary to human basophils, which have to be freshly isolated for every assay, these cells can be simply maintained in culture, which reduces the interassay variability (Saito et al., 1996; Kepley et al., 1999). However, the assay is not very sensitive. Dibbern et al. (2003) reported that sera must contain at least 200 IU/ml of total IgE and concluded therefore that it is mainly suitable to detect allergen-specific (in their case peanut allergen) IgE in highly active sera. Moreover, a similar limitation to the human basophil-based assays is that not only IgE but also IgG is capable of triggering histamine release. At least in rodents it has been shown that allergen-specific IgG antibodies are anaphylactic, inducing histamine release by crosslinking allergen-specific IgG present on rodent mast cells (Nussenzweig et al., 1964; Oettgen et al., 1994). Moreover, similar to the human basophil assays, a clear correlation between results from the RBL assay and *in vivo* clinical responses has not been found, probably due to the low sensitivity (Dibbern et al., 2003). The most likely explanation for the reduced sensitivity of RBL assay compared to the basophil histamine release assay is that naturally occurring basophils contain about 5×10^5 receptors per cell (MacGlashan et al., 1998a, 1998b, 1997) whereas a rat RBL cell line transfected with the human FcεRI receptor

only has about 1×10^4 (Wiegand et al., 1996). Consequently the allergen-specific IgE must be relatively high for the cells to respond to allergen exposure.

IgE-Facilitated Allergen Presentation

The role of antigen presenting cells is to capture protein antigens, internalize, and degrade them into small peptides. Studies have shown that IgE is able to bind antigen and focus it to the surface of antigen-presenting cells via binding to the high affinity IgE receptor, $F_{c\varepsilon}RI$, or the low affinity IgE receptor, CD23 (Kehry and Yamashita, 1989; Maurer et al., 1995). This CD23-mediated IgE-dependent antigen presentation is able to induce proliferation of antigen specific T-cells at 100-fold lower antigen concentrations than is required normally (Kehry and Yamashita, 1989; Pirron et al., 1990). In addition, although IgG binds to the antigen with similar affinity, the antigen-IgG complexes are presented less efficiently. IgE-facilitated allergen presentation can be measured *in vitro* by using B cells as antigen presenting cells. The B cells are incubated with a mixture of allergen and serum and facilitated presentation of allergen at low concentrations is measured by proliferation (thymidine incorporation) of an allergen specific T-cell clone. This assay was first introduced by van der Heijden et al. (1993), who showed that elevated serum-IgE antibody levels in house dust mite allergic patients were able to increase antigen presentation by EBV-transformed B cells to a T-cell clone.

Although this method is capable of detecting biological activity of allergen-specific IgE antibodies in sera of sensitized subjects it is quite costly and time consuming due to the fact that allergen-specific T-cell clones provide the readout. In order to circumvent this problem a flow cytometric assay was developed that measures binding of allergen-IgE complexes to the surface of CD23-expressing EBV-B cells using a fluorescently labeled polyclonal anti-human IgE antibody (Wachholz et al., 2003). Using this assay, it was shown that binding of allergen-IgE complexes was enhanced in the presence of relatively low allergen concentrations and the extent of binding correlated with enhanced stimulation of allergen-specific T-cell clones. This suggests that binding of complexes translates functionally into enhanced antigen processing and presentation and is therefore a suitable surrogate marker for biological activity of allergen-specific IgE. Additionally, allergen-enhanced IgG binding was not detected on these cells suggesting that this system is capable of discriminating between allergen-specific IgG and IgE, selectively detecting the latter.

This assay has been applied successfully to the measurement of specific IgE in human serum samples, but it is in theory applicable also to the assessment of IgE in serum from other species. Preliminary investigations have been conducted in our laboratory using splenic B cells from BALB/c mice isolated by magnetic bead separation. IgE-allergen and IgG_1-allergen binding could be detected when B cells were incubated with serum derived from allergen (ovalbumin) sensitized mice, ovalbumin, and fluorescently labeled anti-isotype antibodies. Although IgE-allergen binding was detectable at high serum concentrations, IgG1-allergen binding was considerably more vigorous. In addition, it was only possible to detect IgE-allergen binding using serum containing high titer anti-ovalbumin IgE (titers of 1 in 1024 or 1 in 2048 determined by PCA assay). In its current format, therefore, IgE-facilitated antigen presentation lacks the sensitivity necessary for the accurate measurement of specific IgE in mouse serum samples. It may be that an EBV transformed B cell line is required to ensure appropriate levels of CD23 expression and hence the required sensitivity for low titer antibody.

CONCLUSIONS

The SPT in humans and the PCA in experimental animals remain the "gold standard" assays for the assessment of biologically active specific IgE antibody for diagnosis of disease and for characterizing allergic responses in animal models of allergy, respectively. Considerable progress has been made in the development of alternative *in vitro* methods, including antibody-based and cell-based assays, for the measurement of serum IgE. Many of these are gaining favor as adjuncts to the SPT in the diagnosis and management of allergic disease, coupled with an understanding of their potential limitations. There have also been encouraging advances in methods for the *in vitro* measurement of specific IgE antibody in animal models of allergy, although these have generally been developed for the determination of IgE antibody responses to a single particular allergen. Such assays have proved useful research tools for increased understanding of the underlying immunobiological mechanisms of allergic responses. To date, however, these approaches have not yielded an assay that is sufficiently selective and sensitive enough to replace the PCA assay for the routine measurement of specific IgE antibody for many allergens. It is this last application that is necessary in the context of the safety evaluation of novel proteins; a method that is applicable to the assessment of specific IgE across a wide spectrum of potential allergens.

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