

Standardization of Allergens

Examination of Existing Procedures and the Likely Impact of New Techniques on the Quality Control of Extracts

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Allergens

Allergens are those antigens that induce an immediate hypersensitivity reaction in sensitive individuals following contact with the airways, gastrointestinal tract, skin or mucous membranes. Allergens are derived from diverse sources including pollens, mites, fungi, animal epithelia, insects, venoms, bacteria, foods and drugs (36, 55). The crude sensitizing material from most of these sources contains more than one allergen. For example, at least six allergens have been identified in ragweed (*Ambrosia elatior*) pollen (33, 36, 53) and other important allergen sources such as house dust mites, ryegrass and timothy grass pollens and animal epithelia are also multiallergen systems (12, 51, 54, 55).

Most allergens are proteins or glycoproteins although pure polysaccharides have been shown to cause immediate reactions in man (14, 35). Major inhalant and food allergens are generally acidic proteins with isoelectric points in the range 4-6 and a MW range of 5,000-70,000 daltons (36).

What makes an antigen an allergen is not known (2). It remains unclear why individual allergens vary in potency from one patient to

the next and why some antigens appear to demonstrate no allergenic properties at all. Examination of the physicochemical properties of the allergens so far purified has not clarified these questions. We should not be surprised at this, however, since amino acid sequence data is available for only a few purified allergens, namely codfish allergen M (22), ragweed antigen Ra5 (58), phospholipase and melittin from bee venom (32, 63) and β -lactoglobulin, serum albumin and caseins from cow's milk (18, 21). With the exception of codfish allergen M and ragweed antigen E, nothing is known of combinations of amino acids (or perhaps sugars or lipids) that make up various allergenic determinants. With drugs that cause a type 1 immediate hypersensitivity reaction, identification of allergenic determinants should be simpler than with proteins since structural analogues can usually be obtained for examination. Although reports of IgE antibodies that react with drugs are rare (60), significant progress has been made in identifying allergenic determinants on penicillins (6) and muscle relaxants. In the latter case, structure-activity studies designed to explore the molecular basis of specific IgE binding by the drugs, established that quaternary ammonium and substituted ammonium ions were

the complementary allergenic sites on the drugs (11). However, even if there are certain common structural features that impart allergenic properties to antigens or small pharmacologically-active compounds, the number of structurally defined allergens or allergenic determinants available at present is too few to draw definite conclusions.

"Major" and "minor" allergens

From crossed radioimmunoelectrophoresis (CRIE) studies, Løwenstein (50) has defined the term "major allergen" as "antigens which bind IgE from at least 50 percent of the sera of patients tested and show strong binding to at least 50 percent of these". In this context we believe that a more appropriate description of the antigens would be "major IgE-binding antigens". When the importance of an antigen has been unequivocally established by the use of methods that reveal the biological properties known to be possessed by allergens, the description "clinically important or clinically relevant allergens" would seem to be more appropriate than the presently used titles of "major" and "minor" allergens.

Why standardize allergen extracts?

Allergen extracts and a few purified allergens are routinely used in the diagnosis of IgE-mediated allergies. Some preparations, in particular from pollens, are used for desensitization of allergic subjects (59). Unlike other immunological products used for diagnosis and/or therapy, for example vaccines, antisera, toxoids, antigens for diagnostic tests, patch test materials and so on, allergen preparations are not required to conform to any present or past pharmacopoeial standard. However, due principally to the efforts of Aas who, for over a decade, has articulated the pressing need for authoritative regulations and controls (1, 4), there is now genuine and widespread awareness that controls are not only essential but inevitable.

In the absence of standards for both quality and quantity, a situation exists in which the

physician frequently has no guarantees that the allergen extracts he uses for diagnosis or immunotherapy satisfy even minimum requirements for potency, stability, absence of non-allergenic components and batch-to-batch variation. Variations that occur between preparations from different manufacturers and between different batches from the same manufacturer, will affect skin tests, radioallergosorbent test (RAST) investigations, bronchial provocation tests and hyposensitization therapy.

Why has progress towards allergen standardization been so slow?

At first sight, it is difficult to be tolerant of the laxity that has characterized the production and use of allergen extracts for so many years but, on reflection, the reasons are not hard to find. Firstly, as already discussed, few allergens from the multitude of sensitizing sources have been identified. Still less have been isolated and characterized. In these cases, how can standardization be effected with preparations that we know very little about? Secondly, what methods can be used to provide a qualitative and quantitative definition of the allergen preparation and a biological potency estimation? When these questions are considered, our present lack of progress in defining and establishing suitable standards for allergens is more easily understood.

The time has now come when it should no longer be permissible to use complex, unstandardized mixtures that frequently contain inert and even irritant components. Difficulties that appear to still threaten our aim of defining, and ultimately applying, suitable standards are now far from insurmountable.

In the following sections, existing methodology, already applied or suggested for the standardization of allergens, will be briefly reviewed. In addition, the relevance and likely impact of some newer methods will be examined with a view to assessing their likely contribution to our future endeavours in the field of allergen standardization.

Methods used to standardize allergen extracts

Methods based on weight per ml, protein content or N content are inadequate since they do not necessarily correlate with biological activity. Skin test titrations (4, 61) preferably with a histamine reference (3, 4), immunodiffusion (10, 37), histamine release assays (10, 23, 24, 49), RAST inhibition (5, 9, 23, 30), isoelectric focusing (68), crossed immunoelectrophoresis (CIE) (50, 70), lectin binding (16) and passive cutaneous anaphylaxis (7) have all been used to standardize allergen extracts. Use of combinations of some of the above methods, in particular skin tests, histamine release, RAST inhibition and CIE, has proved to be the most satisfactory approach to allergen standardization using the existing technology. In the following section the rationale for the selection and use of the presently favoured methods will be briefly discussed. This will be done with a view to highlighting the need for a unifying approach that might ultimately provide a better definition of allergen extracts used.

Currently favoured methodology

International biological standards are established for those biological substances used in man for diagnosis and therapy which have not yet been characterized physicochemically. As most allergens have not been characterized, or even isolated, biological standardization methods must be applied. It is the biological properties of allergens that are of interest but, unfortunately, biological standardization methods are often difficult in practice, especially if a quantitative estimation is sought. It is therefore often easier to use an *in vitro* method but only those methods that correlate with the biological assay are acceptable. For the present we are often advised to use RAST inhibition combined with at least one other method that gives an estimate of biological potency. For the biological potency measurement, skin prick testing is favoured (3). However, both procedures have inherent major defects and certainly cannot be viewed as ideal methods for standardization of poorly defined extracts. For

example, the RAST itself must be standardized in terms of the use of uniform discs, suitable sera and allergen preparations (3, 29). Unless careful attention is paid to these aspects of the test, consistency and reproducibility between different experiments and laboratories will not be achieved. Major problems with skin prick testing for allergen standardization are the difficulties and time spent in finding enough suitable patients and the variations one sees in skin prick test results. Patient reactions may vary greatly (9), the sizes of reactions depend on the injection site (66) and, even in the hands of an experienced tester, variations of 10–20% may be seen (3).

When all necessary steps have been taken to avoid all of the known sources of error with RAST inhibition and skin prick testing, the vulnerability of these two procedures still remains. As pointed out by Aas et al (3) both methods measure total allergenic activity without being able to assess contributions to total allergenicity made by different components in the extract. For example, a baker allergic to both wheat flour and wheat weevils may respond with a positive skin reaction when prick tested with an extract of insect-contaminated wheat wholemeal. Likewise, IgE antibodies in the patient's serum may react in the RAST with the contaminated cereal preparation. In both cases, however, the tests would not have discriminated between the two quite different allergic components present.

This example, and the fact that most extracts are multiallergen systems, illustrates very well the need to supplement RAST and biological potency estimations (whether they be skin prick test or histamine release assays) with analytical methods designed to reveal the number and nature of antigens and allergens present in an extract. Methods based on electrophoretic separation or the isoelectric points of proteins must be combined with RAST determinations to detect the IgE-binding components. However, CRIE (50) is a more precise and efficient method for detecting reactivity of fractionated components with IgE antibodies. The method shows extraordinary resolving power and has already been successfully applied to a wide range of allergen

sources (12, 43, 47, 50), although like the other favoured methods, CRIE is not without some limitations and disadvantages. CIE and CRIE would appear to lend themselves to quantitation since the areas enclosed by the precipitin loops are proportional to the antigen-antibody ratio used but, at best, the techniques can only be described as semiquantitative. Although the excellent analytical properties of the methods are due to specific precipitation of extract components by antibodies, the antibodies also represent the main weakness of the method. The quality of the antiserum used is vitally important. Unless it contains a sufficiently high titre of precipitating antibodies to each of the antigens present in the extract, some antigens and allergens may escape detection if CIE and CRIE alone are used. Other deficiencies and potential sources of error have been discussed (50, 70) and include the need for large quantities of antisera, binding of antiimmunoglobulins to immunoprecipitates, the failure of small antigens to precipitate, masking of allergen sites by the antiserum so that IgE antibodies in patient's sera cannot bind and the non-specific binding of labelled antihuman IgE.

Despite these potential problems, when CIE and CRIE are combined with skin prick tests and/or histamine release estimations and RAST, one has the best combination of techniques from those that are currently applicable to the problem of quality control of allergen extracts. A detailed consideration of this combined approach is well illustrated in the so-called Danish Allergen Standardization 76 (DAS 76) procedure of Løwenstein (52) and in the recent study of "allergoprints" in horse-allergic children by Wahn et al. (69).

Recent international initiatives for quality control of allergen extracts

Despite emphasis in the foregoing discussion of the poor progress over the years in standardizing allergen extracts, some recent developments have been reassuring. In February 1981, the Allergen Standardization Subcommittee of the International Union of Immunological Societies under the chairmanship of Dr H. Lø-

wenstein (Copenhagen) undertook the task of ultimately preparing standards that could be used by manufacturers and control authorities. The Subcommittee defined the following goals:

"In consultation with WHO, to coordinate at the international level on a permanent basis all efforts presently being undertaken throughout the world in the field of allergen standardization.

1. Generate and make available to investigators, manufacturers and control authorities allergen reference preparations (with assigned arbitrary international units), standards and reagents.
2. Develop and publicize, on the basis of the appropriate collaborative trials, tests enabling assessment of 1) allergenic activity, and 2) composition and content of important allergenic molecules.
3. Provide an international collaborative framework for immunochemical allergen research."

Draft specifications were prepared by subcommittees for short ragweed pollen extract (March 1981), timothy pollen extract (late 1981) and house dust mite extract (*Dermatophagoides pteronyssinus*) (April 1982). These specifications are being reviewed and allergen preparations are being compared by a range of procedures in current collaborations.

Ideals for allergen standardization

In theory, the most scientifically desirable and satisfying approach to allergen standardization is to identify each of the different components in an extract, distinguish those that are probably allergens and isolate them. In practice, this is done by progressively combining antibody and electrophoretic procedures with biochemical fractionation and biological test methods. Following purification of individual allergens, comprehensive clinical and laboratory examinations should be undertaken to establish their biological potencies. Once the sensitivity spectrum of an allergen has been determined in a large number of patients, all that remains is to quanti-

tate levels of the allergen in unstandardized extracts. This can be achieved by the development of specific immunoassays.

In the absence of formulations of purified, biologically-characterized allergens, it is essential to remove irritant, toxic or large amounts of inert components from extracts. In practice, this may be done by identifying the offending agents and removing them from the extract or by identifying the allergens and reformulating them as a new extract.

Table 1 summarizes such an "ideal" approach to allergen standardization. Frequently, the task facing the research investigator may not be as arduous as it first appears since in practice the greater part of the allergenic activity of an extract will be accounted for by a relatively small number of clinically important allergens. Also, although the approach outlined in Table 1 may seem at first sight to be unrealistic, recent developments in the production of monoclonal antibodies (38, 39, 40, 57, 62) and application of the developments to allergen research (12, 42, 45, 46), has made, I believe, the listed aims achievable. This conclusion will be expanded in a following section, but first the standards necessary for quantitative analysis of allergens will be briefly discussed.

Primary and secondary standards for allergens

After careful physicochemical, immunological and clinical assessments, a purified allergen may be viewed as a primary standard. Complementary reagents that specifically react with the primary standard, namely antibodies and lectins, then assume the status of secondary standards and it is with the latter tools that the mechanics of standardization may be effected.

As emphasized above, in the absence of purified, characterized allergens, biological potency assessments must be carried out in parallel with *in vitro* methods for allergen detection. Hence, if one has the purified and biologically characterized allergen, that is the primary standard, side-by-side measurements of the amount of purified allergen and its biological activity would appear to be unnecessary in every standardization procedure. It would be prudent,

Table 1

Ideals for the quality control of allergen extracts

For allergen standardization we should aim to:

1. Identify all of the IgE-binding components in the extract.
2. Isolate the components in pure form and characterize them physicochemically.
3. Comprehensively examine the purified components for biological activity (skin prick tests, challenge tests, histamine release from sensitized leukocytes).
4. Quantitate the different allergens in the extract.
5. Identify non-allergenic components (inert, irritant, toxic, etc.) present in the extract.

however, to adopt a cautionary approach and use skin prick test titrations in parallel with assays employing the secondary standard. Skin test reactivity should be assessed in at least one patient of known sensitivity. In most cases retention of a test for biological activity will be unnecessary but its routine use guards against the possibility that the allergen being measured may have lost its *in vivo* activity while retaining its antibody- or lectin-binding capacity.

"New" techniques and their application to allergen standardization

Within the limits imposed by the available methodology and the all too recent international initiatives for the preparation of standards (see above), a combination of *in vivo* and *in vitro* methods has evolved and been generally accepted as the approach of choice for the quality control of allergen extracts. As the foregoing reviews in the sections on methods reveal, skin prick tests, histamine release from sensitized leukocytes, RAST inhibition and CRIE are the methods that currently offer the best means of defining allergens in qualitative and biological terms. However, few would argue that the existing situation is ideal and a more precise and less complicated approach should be sought. In addition, any approach that purports to be an improvement on the existing combination of methods must include procedures that will provide a quantitative definition of the allergens in an extract. Some recently introduced techniques

are particularly well suited for application to allergen identification, isolation and quantitation. These methods, together with an analysis of their potentials and limitations, will be discussed in the following four subsections.

a. Standardization of allergens using monoclonal antibody-based immunoassays

Following the purification and physicochemical and biological characterization of an allergen, it is usually a simple matter to inject the allergen into a rabbit, sheep or goat and prepare a complementary antibody. Allergen-specific antibodies may then be used to detect and quantitate allergens in unstandardized extracts. Unfortunately, conventional antibodies are invariably heterogeneous with respect to class, subclass, binding affinity, combining site size and specificity (34). This means that antibodies to the same allergen produced at different times or in different laboratories, are far from ideal as secondary standard reagents. Monoclonal antibodies, on the other hand, are each homogeneous in chemical composition, structure, combining site size and specificity (28, 31, 34, 57). This means therefore, that the same protein can be made available as a standard in many different laboratories.

The development of immunoglobulin-secreting mouse hybridomas (38, 39, 40) has opened the way for the production at will of monoclonal antibodies with specificities for virtually any antigenic determinant. Not only does the method provide monoclonal antibody standards for use in the quality and quantitative control of extracts, but the antibody-secreting cell may be passaged in mice or maintained in culture to provide continuous supplies of antibody. In addition, hybridomas can be stored in a repository of standards under liquid nitrogen and sent, when required, to any laboratory. Such immortalization of an antibody-producing cell would permit, for the first time, widespread dissemination of identical allergen-specific antibodies ideally suited for comparative studies on unstandardized extracts.

In order to illustrate the value, simplicity and general applicability of the monoclonal anti-

body approach to allergen standardization, we have prepared a ragweed (*A. elatior*) antigen E-specific IgG monoclonal antibody derived from a mouse lymphocyte hybridoma and used it in an enzyme immunoassay to detect and quantitate levels of antigen E in ragweed pollen extracts (45). An important finding in the study was that the monoclonal antibody did not react with false ragweed (*Franseria acanthicarpa*) pollen extract. This demonstrated a level of specificity that had previously not usually been attainable with conventional antibodies. Previous RAST inhibition studies had revealed cross-reactivity between short and false ragweed pollens but did not establish whether this was due to the presence of antigen E in both pollens (17, 48).

By generating enough clones of antigen-specific monoclonal antibodies it always remains possible that an antibody with the desired specificity and affinity will be found. This is an entirely different situation to the production of polyclonal antibodies where mixed populations of molecules with different specificities for the same antigen are produced.

Results with the ragweed antigen E-specific monoclonal antibody reinforced our belief (12, 13) that there is nothing to prevent the immediate introduction of hybridoma-derived, allergen-specific monoclonal antibodies to quantitate and thus standardize biologically characterized purified allergens such as those from ragweed pollen, timothy and birch pollens, bee venom and so on.

b. Hybridoma-derived antibodies for the analysis of complex allergen extracts

The value of hybridoma technology is even more apparent when one examines the potential of the procedure for the identification, isolation and ultimate standardization of allergens in complex mixtures. This was illustrated by our experiments in which monoclonal antibodies to clinically important allergens from the house dust mite (*D. pteronyssinus*) were obtained following immunization of mice with allergenic fractions (12). The great advantage of the hybridoma approach is that purified allergens are not

required for immunization of the mouse. Following cell fusion, clones of antibody-forming hybrids, each specific for a single antigenic determinant, are produced. The generation of antigen-specific monoclonal antibodies may be monitored by radioimmunoassay using a solid phase coupled to allergenic fractions, and by CRIE with crude allergen extract and ^{125}I -labelled antiserum to mouse immunoglobulins. To identify allergen-specific monoclonal antibodies, the CRIE patterns are compared with CRIE patterns obtained using sera from allergic patients. Monoclonal antibodies that react with antigens that bind human IgE antibodies are purified and used to isolate the complementary antigens by affinity chromatography (41, Krilis, Baldo and Basten, in preparation). The IgE-binding antigens prepared in this way are then subjected to skin prick tests, RAST and histamine release examination to identify the clinically important allergens. These will serve as primary standards while the allergen-specific mouse monoclonal antibodies may be used as secondary standards either with a radioactive, enzyme or biotin tag or as a first antibody in a sandwich immunoassay system.

c. Purification of allergens by combined use of hybridoma and immunoadsorption methods

This procedure, which may be referred to as the hybridoma cascade, can ultimately provide a complete definition of the antigenic components in a complex extract (56). The cascade of alternating hybridizations and affinity chromatographic separations using monoclonal antibodies is continued until all antigens in the extract have been isolated. This means that each new immunization is carried out with an extract containing previously unrecognized antigens. The method is summarized in Fig. 1.

Advantages of the cascade procedure are:

1. Immunodominant antigens are removed first.
2. Repeated production of monoclonal antibodies to the same antigen is avoided.
3. Ultimately, a definition of the components of the whole extract is possible.

The hybridoma cascade procedure offers a unified approach to the purification and standardization of allergens. Starting with a crude, unfractionated allergenic extract, an antibody library directed toward individual allergens can be progressively assembled. This approach should now be pursued using extracts from important allergen sources such as mites, pollens, fungi, danders and foods.

*d. Binding of proteins to nitrocellulose membranes
Applications to allergen studies*

(i) Simplified preparation of allergen discs

In the short term at least, RAST inhibition will continue to be one of the preferred methods for allergen standardization. Hence, any refinements of existing procedures or elimination of defects, would add to the test's worth as a standardization method. As already stated, the test itself must be standardized with careful attention being paid to the production and use of uniform discs and the selection of allergen preparations. In fact, both of these requirements can present difficulties especially when comparisons of results are to be made over a long period and between different laboratories. Although CNBr-activated paper discs (20) have proved to be both a convenient and versatile solid phase, any preparation that is at least as good but which is simpler, would be readily accepted. Nitrocellulose in the form of discs appears to be such a preparation.

The production of nitrocellulose allergen discs for use in the RAST requires three simple steps:

1. Drying a solution of the allergen(s) onto the discs.
2. Soaking the discs in 3% bovine serum albumin or Tween to block any sites left free after addition of allergen.
3. Washing in buffer.

Besides greater simplicity, the advantages of nitrocellulose discs include the capacity to test allergens that are poorly soluble or insoluble in water, the simultaneous evaluation of one serum

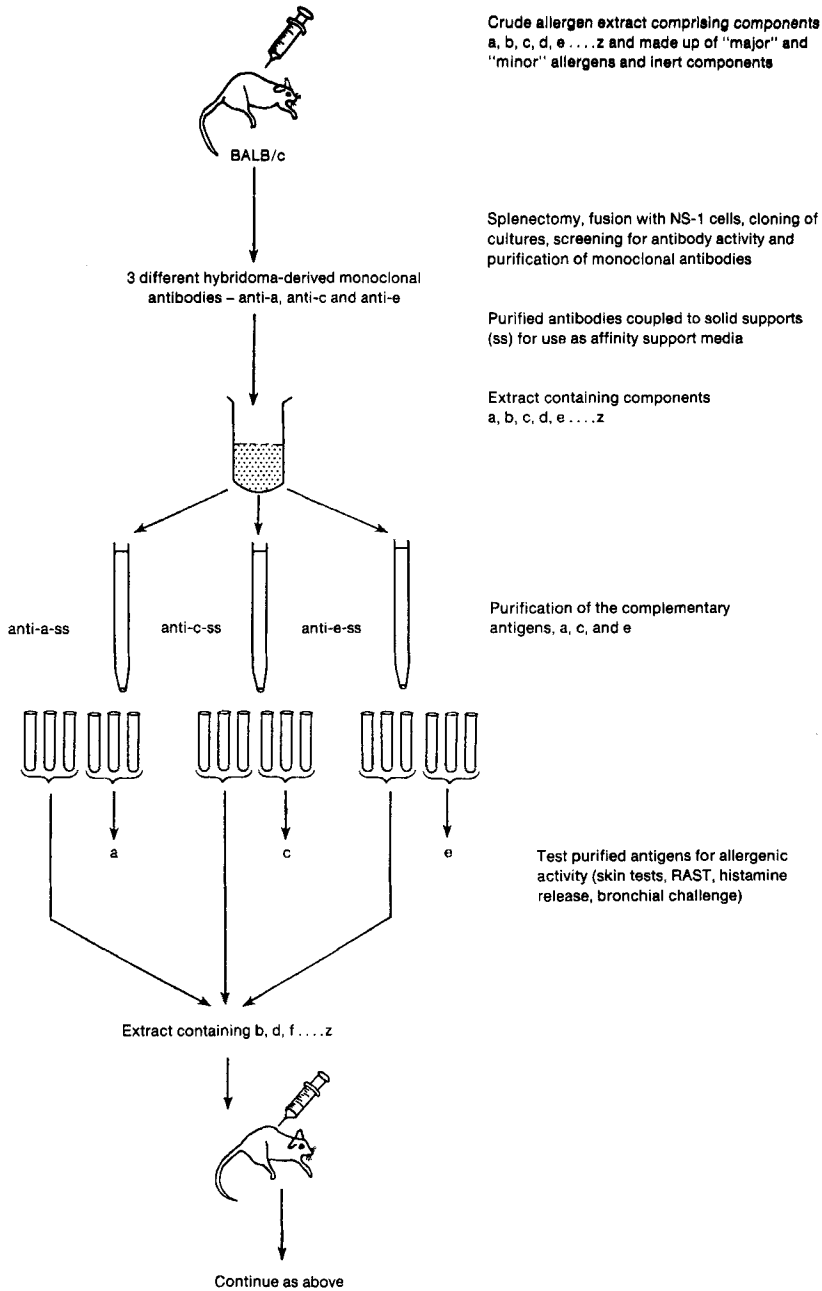


Fig. 1. Protocol for cascade purification of allergens in a crude extract. Combined use of hybridoma-derived monoclonal antibodies and affinity column chromatography.

sample with several different allergens on the one disc and a significant cost saving. Unlike with CNBr-activated paper, organic solvents, acids or strong alkalis can be used to attach

insoluble material (for example, wheat gluten proteins) to nitrocellulose discs (Walsh, Wrigley and Baldo, in preparation).
Use of sheet nitrocellulose as a solid phase in

the RAST also promises considerable advantages in the standardization of discs used in the assay. Differences in the degree of CNBr-activation of paper discs and hence differences in the amount of allergen coupled, are not a problem with nitrocellulose since very large numbers of discs can be punched from sheets of a single batch. In addition, of course, no chemical activation step is involved.

(ii) Detection of allergens after electrophoretic transfer from gels

Our use of nitrocellulose as an insoluble carrier for allergens stemmed from use of the material in "Western blotting" (19, 67). In this procedure, zones transferred electrophoretically from a separation gel to sheet nitrocellulose are subsequently detected by autoradiography, enzyme staining, biotin-avidin coupling and so on. "Western blots" continue to find wide application in many areas of biomedical research (8, 25, 26, 27) since protein zones transferred to a membrane are not subject to diffusion and are immediately accessible to detecting reagents. This is in contrast to gels used for electrophoretic and isoelectric point separations where the relatively small pore size, at least for polyacrylamide, restricts diffusion of labelled detection probes into the gel. In addition, nitrocellulose membranes have the advantage that gels can be dried and used more easily for autoradiography or staining with reagents that detect zones of biological activity.

We applied the electrophoretic transfer technique to the identification of wheat and house dust mite antigens that bind IgE antibodies from the sera of allergic subjects (15, 44, 65). Proteins separated in polyacrylamide gels by isoelectric focusing, gradient, SDS or 2-dimensional electrophoresis, were transferred to nitrocellulose before IgE-binding proteins were identified by incubation with patient's sera and then ^{125}I -labelled anti-human IgE. The method is proving to be a viable alternative to CRIE and, in some important respects, the electrophoretic transfer procedure is superior. Although CRIE can readily demonstrate identity or cross-reactivity of antigens, a good quality

antiserum must be produced for each allergen extract studied. Also, antigens that do not precipitate remain undetected. The transfer technique requires less time than CRIE, it can be more readily applied to the study of new allergens and it is ideally suited for side-by-side comparisons of cross-reacting allergens from taxonomically-related species. As well as detecting the IgE-binding bands, additional information about the proteins can be obtained from the gel at the same time – for example, molecular weight or isoelectric point of the allergen being studied.

CONCLUSIONS

In the short term, hybridoma-derived monoclonal antibodies will not replace the currently favoured techniques for the standardization of complex allergen mixtures. However, if the hybridoma technology is intelligently applied, and combined with affinity chromatographic and electrophoretic transfer procedures, rapid advances in the provision of purified allergens and their complementary monoclonal antibody standards are to be expected.

Ultimately, it is theoretically possible to develop a panel of hybridoma cells producing antibodies capable of reacting with all of the clinically important allergens in allergen extracts from mites, pollens, fungi, danders and so on. The time taken to reach that goal will depend very much on the persistence and dedication of those who seek to apply hybridoma technology to the analysis of complex allergen extracts, and on the supporting techniques harnessed to assist these studies.

Finally, it is suggested that future meetings organized to examine the problem of allergen standardization should consider the possibility of using allergen-specific monoclonal antibodies from murine lymphocyte hybridomas as international standards for allergens. This might involve the creation of a panel of hybridoma cells that could be stored under liquid nitrogen and sent, in response to requests, to laboratories for passage in mice or growth *in vitro*. Such a panel of reference tumour cell lines could be continually enlarged as more allergens are puri-

fied, assessed for biological activity and used to produce murine lymphocyte hybridomas.

ADDENDUM

Since submitting this article, predictions concerning the value of allergen-specific monoclonal antibodies to allergen standardization have been supported by results of recent studies with monoclonal antibodies specific for pollen and fungal allergens (64; see also abstract numbers 25, 31 and 285, *J. Allergy Clin. Immunol.* 71, No. 1, part 2, Jan. 1983). The methods employed by Smart et al (64) should prove generally applicable to a variety of unfractionated allergen sources. ELISA was used both to screen hybridoma supernatants and for the detection of IgE-binding activity while specificities of monoclonal antibodies were examined by reaction with ryegrass pollen antigens following electrophoretic transfer to nitrocellulose sheets.

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