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LABORATORY DIAGNOSIS OF THE ATOPIC CONDITION: EVALUATION OF A POSSIBLE METHOD BASED ON HUMAN SERUM COMPLEMENT CONSUMPTION

By

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Recent evidence indicates that the system of complement factors in human blood serum may differ in its reactivity from the guinea pig or rabbit counterparts usually employed in complement fixation studies. For example, Volanakis & Kaplan (19) reported that C-reactive protein complexed with C-polysaccharide or with choline phosphatides can activate fluid phase complement by engaging human, but not guinea pig, C1q. The sera of both symptomatic and asymptomatic patients with pigeon breeder's disease contain thermostable antibodies which selectively consume human haemolytic complement (6). As early as 1908, Hecht propagated fresh human serum instead of guinea pig complement for use in the serological test for syphilis (11). The reaction enjoyed some popularity at the time as the Hecht-Weinberg modification of the Wassermann reaction (12). Jaffé (13) appears to have been one of the first to apply the Hecht reaction to allergy; she also recommended using the natural anti-sheep red cell haemolysins in the same human serum sample as a source of amboceptor. This technique (since we were unaware of this early work) was duplicated in 1972 in this laboratory (4). Jaffé (13), Albus (2) and Cavelti (9) had already realized the potential usefulness of this complement method for the

improvement of allergenic extracts; a method for the standardization of house dust allergens along these lines has recently been developed (5).

Several of the common allergens have now been reaffirmed to be capable of consuming human, but not guinea pig, haemolytic complement *in vitro* (4, 7). In the course of these investigations, it was noted that haemolytic complement in the serum of patients classified as atopic on the basis of clinical criteria, was more sensitive to inactivation than it was in the serum of healthy donors. The present study was undertaken to develop a possible *in vitro* method for the diagnosis of the atopic condition founded on the inactivation of autologous haemolytic complement by a purified house dust allergen preparation. The possible involvement of natural antibodies of the ABO system and of some individual complement factors was also investigated.

MATERIALS AND METHODS

Sera. Blood samples of adult patients attending the out-patient Department of Allergy, of patients from other hospital departments and of healthy volunteers were obtained by venous puncture, and were allowed to clot in glass tubes at 4° C. After centrifugation, the fresh sera were aliquoted into 0.5 ml polythene vials and were stored at -90° C until use. Residues from samples thawed for investigations were discarded.

House dust allergen, fraction code 72/E, was employed throughout this work. The product was isolated from a large batch of house dust as previously described (13). Experimental justification for employing the purified dust fraction E has been put forward in a separate paper (5).

Complement inactivation tests were routinely carried out as follows. Samples of 15 μ l of unheated, fresh human serum were incubated in disposable polypropylene test tubes with quantities of dust allergen 72/E ranging from 0.25 to 4.0 μ g. Total incubation volume was 0.5 ml in $\text{Ca}^{++}/\text{Mg}^{++}$ -containing veronal-saline buffer (VSB $^{++}$) (Kabat & Mayer (14)). After 30 min at 37° C, 2.5 ml of ice-cold VSB $^{++}$ and 1.0 ml of optimally sensitized sheep red cells (EA, 2.5×10^8 cells/ml) were added; the sheep red cells E and rabbit amboceptor A were obtained from the State Institute of Health, Bilthoven. Incubation was continued for 60 min at 37° C, and then the tubes were centrifuged and the colour of the supernatant was read at 541 nm. Controls included a serum sample preincubated in the absence of allergens, and a water-lysed control for 100 per cent haemolysis.

Total haemolytic complement values of sera were determined in conventional

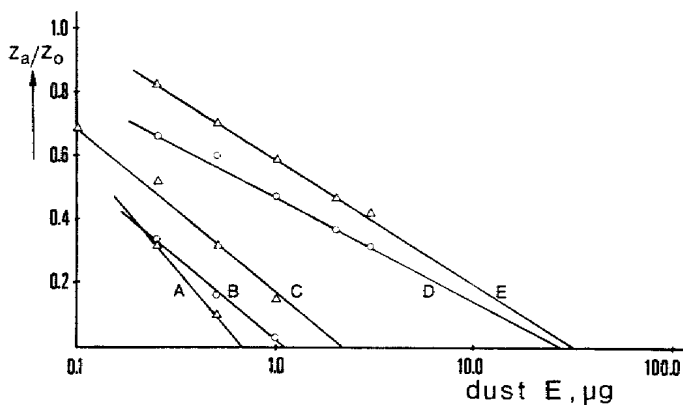


Fig. 1.

Examples of haemolytic complement consumption—in Z_a (in the presence of allergen) / Z_o (in the absence of allergen) ratios, where $Z = -\ln(1-y)$ and y represents per cent haemolysis—from 15 μ l of human serum as a function of house dust 72/E allergen concentration. The “sensitivity index” was read from the intercept of the dose-response graphs extrapolated to the horizontal axis. Sera were from: A, a severely asthmatic atopic patient with multiple skin-sensitivities; B, a healthy non-atopic control; C, recurrent atopic dermatitis; D, healthy non-atopic control; E, near-fatal bee sting anaphylactic shock.

fashion, using serial serum dilutions (14); CH_{50} values were read from Van Krogh plots.

Complement factor C_4 was determined by single radial immunodiffusion, using commercial antibody-containing agarose plates and a standard serum as prescribed by the manufacturer (Behringwerke, Germany).

Human anti A and anti B blood group titers of the sera were determined by agglutination at room temperature in 1 ml glass tubes, using 2 per cent suspensions of washed A and B erythrocytes from human donors and serial dilutions of the serum under investigation (1:5–1:1280). The titer was expressed as the reciprocal of the last serum dilution still causing agglutination.

RESULTS

Complement Tests

Complement in the serum of individual human beings differs appreciably in its susceptibility to inactivation by purified house dust allergen. This sensitivity was routinely, and arbi-

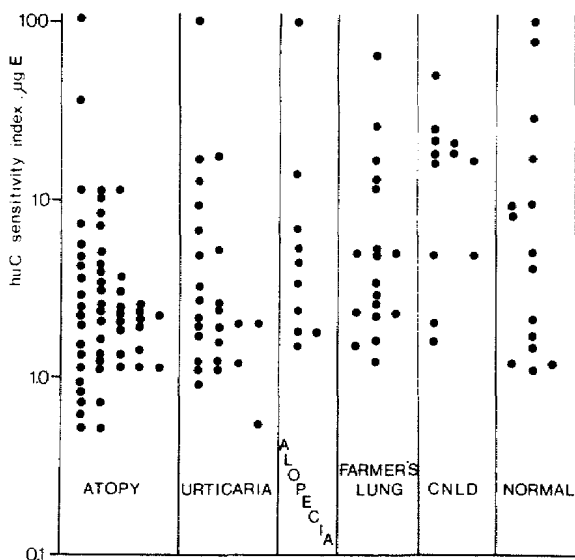


Fig. 2.

Collected results of complement sensitivity tests to house dust allergen 72/E in 137 serum samples arranged according to the patients' clinical diagnosis. Each point represents one patient. The atopy group comprised people with asthma, atopic eczema or hay fever with single or multiple skin-sensitivities to the common allergens ($N = 56$, mean = 5.4, S.D. = 13.8); the urticaria group comprised non-atopic subjects with Quincke oedema, urticaria *e causa ignota*, factitious, cold and heat urticaria ($N = 26$, mean = 7.9, S.D. = 19.4); sera from patients with clinically proven farmer's lung with precipitins to *M. faeni* culture filtrate antigens ($N = 18$, mean = 9.5, S.D. = 15.1); the group, chronic non-specific lung disease (CNLD), included cases of chronic bronchitis, emphysema and interstitial pneumonitis of unknown cause ($N = 13$, mean = 15.8, S.D. = 13.2); alopecia areata: $N = 10$, mean = 14.2, S.D. = 30.4; healthy controls: $N = 15$, mean = 18.8, S.D. = 29.8.

trarily, expressed as the amount of allergen 72/E required for total inactivation of haemolytic complement from 15 µl of serum under standard conditions. This allergen sensitivity "index" was found by expressing haemolytic complement in Z-values and by extrapolating graphs of the ratio of Z-values

TABLE 1

Sensitivity of Haemolytic Complement, in the Sera of Individuals with Various Diseases, to Inactivation by Incubation with Purified House Dust Allergen 72/E.

Diagnosis or Major symptoms	Sensitivity index, μ g
Non-hereditary angioedema, cold urticaria	2.0
Heat urticaria	99.6
Hereditary angioedema	17.3
Systemic lupus erythematosus (SLE)	6.4
Chronic mucocutaneous candidiasis	13.6
Pyoderma gangrenosum	29.0
Onychomycosis	10.0
Endogenous eczema	0.6
Endogenous eczema	15.0
Baker's rhinitis, non-atopic	3.4
Near-fatal bee sting anaphylaxis	33.0
Pigeon-breeder's lung, acute	2.0
Pigeon-breeder's lung, chronic	4.4

Sensitivity expressed as μ g allergen preparation 72/E required for complete loss of haemolytic complement from 15 μ l of serum. The serum of the patient with hereditary angioedema contained no detectable functional or structural C1 esterase inhibitor. The SLE serum was C2 deficient; sensitivity in this case was assayed in the unheated serum in the presence of guinea pig complement.

in the presence and absence of allergen (Z_a/Z_o) to complete loss of measurable complement; Z equals the calculated value of $-\ln(1-y)$, where y represents percentage haemolysis (16). Though, strictly speaking, this expression must be reserved for molecular titrations of individual complement components, its employment for comparative studies seemed permissible because the C-inactivation test by allergen largely measures the depression of the rate-limiting C2-component (Berrens et al., in preparation). Some examples of complement-inactivation graphs of individual sera have been collected in Fig. 1.

Considerable problems arise on attempting to relate the clinical data to this laboratory index of complement sensitivity. One major source of uncertainty is the highly divergent symptomatology of the population of patients attending an allergy clinic. Some people attend with syndromes of unknown aetio-

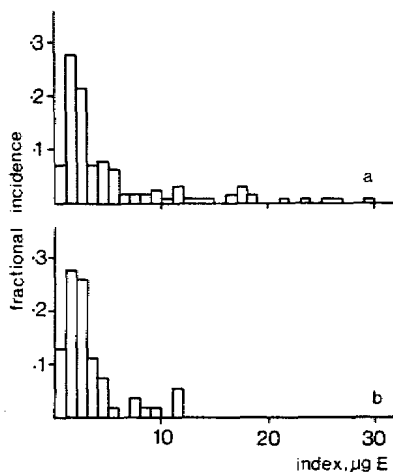


Fig. 3.

- a. Histogram of complement-sensitivities up to 30 μg 72/E in a group of 130 sera taken from Fig. 2.
- b. Frequency distribution of 72/E allergen-sensitivity index in a series of 54 atopic sera.

logy (e.g. urticaria), and very often different hypersensitivity phenomena overlap. However, as shown in Fig. 2, haemolytic complement in the sera of (house dust sensitive) atopic patients with asthma and/or eczema is usually quite sensitive to inactivation by purified dust allergen. As shown in the same figure, the sera of some perfectly healthy volunteers proved extremely sensitive as well; similar results were obtained on screening the blood sera of patients with farmer's lung (acute form) or with chronic nonspecific lung disease. Similarly, no differentiation of atopic sera was possible from sera of patients with various forms of urticaria; nor from alopecia areata, a condition considered to feature a high incidence of atopy (15). Examples of the C-sensitivity to house dust inactivation in some other diseases have been listed in Table 1.

Despite the unmistakable tendency of most atopic sera to

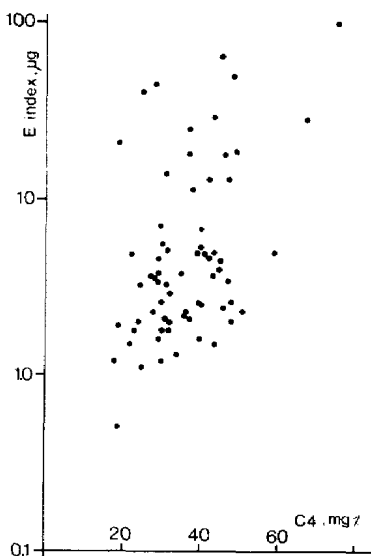


Fig. 4.

Semilogarithmic scatter diagram of relationship between complement-sensitivities to house dust 72/E and levels of the fourth component of complement, determined by single radial immunodiffusion.

display high complement sensitivities (Fig. 2), the histograms in Fig. 3a and 3b demonstrate that discrimination from other sera on this basis alone is not possible. Fig. 3 also shows that the allergen sensitivity index within these groups of sera is not normally distributed.

In a preliminary attempt to correlate the allergen sensitivity of haemolytic complement to more specific parameters of human complement, the levels of the fourth component were determined immunochemically by single radial immunodiffusion. The scatter diagram of Fig. 4 demonstrates that, in a selection of sera from the disease groups mentioned in Fig. 2, no positive or negative correlation between the 72/E index and C4 levels was apparent. Also, C4 levels within the group of atopic sera were within the normal range; this was also true

for the CH₅₀ values for total haemolytic complement in fresh serum samples.

Haemolysins and Blood Group Antibodies

Although the serum factor reacting with the allergen preparation is extremely thermolabile (10 min at 56° C), it was considered that possible immune complexes with naturally-occurring antibodies could not be excluded as a cause of the observed complement consumption. However, no relationship was apparent between titers of natural haemolysins for sheep red cells in the human blood samples, and the allergen sensitivity index. Nor were human Forssman antibody titers in atopic sera either elevated or depressed in comparison to groups of control sera of non-atopic individuals. Absorption of human sera with unsensitized sheep red cells had no influence on the complement-consuming serum factor(s) activated by the allergen. In these experiments, human serum

TABLE 2

Influence of Natural Haemolysin Absorption upon Human Fluid Phase Complement Activation by Purified House Dust Allergen.

Absorption step (SRBC $\times 10^8$)	Activation step (μ g house dust E preparation)				
	0	0.5	1.0	2.0	4.0
0	0.845	0.700	0.615	0.450	0.228
0.5	0.805	0.655	0.590	0.440	0.230
1.0	0.830	0.690	0.595	0.425	0.242
2.0	0.805	0.665	0.560	0.420	0.230

Human allergic serum (2 ml) with high haemolysin titer was diluted 1:10 in veronal-saline buffer containing 0.002 M disodium-EDTA, in the presence of 0, 0.5, 1.0 or 2.0 $\times 10^8$ sheep red cells SRBC, respectively. Absorption proceeded for 30 min at 37° C, and then the cells were removed by centrifugation. Supernatants (2 ml) were recalcified with 0.88 mg CaCl₂; then 4 ml guinea pig complement (1:50) in VSB⁺⁺ was added, followed by 0, 0.5, 1.0, 2.0 and 4.0 μ g dust allergen 72/E, respectively. After 30 min at 37° C, 2 ml VSB⁺⁺ and 1.0 ml SRBC (2.5 $\times 10^8$ cells) sensitized with rabbit amboceptor were added. After 60 min at 37° C, haemoglobin in supernatants was read at 540 nm; results expressed as extinction at 540 nm.

was diluted 1:10 in veronal-saline buffer, containing 0.002 M EDTA as chelating agent; aliquots were then incubated for 30 min at 37° C with increasing amounts of unsensitized sheep erythrocytes. After centrifugation, the supernatants were recalcified and guinea pig complement was added, followed by allergen in different concentrations. It has previously been shown that a factor in unheated human serum, once activated by allergen, is subsequently capable of engaging guinea pig complement (7). As demonstrated by the example in Table 2, activation of the human factor proceeded undiminished in the absorbed serum depleted of its natural haemolysins.

Rimington et al. (18) have described inhibition of A- and B-isoagglutination by their purified house dust allergen preparation. Working with the purified fraction E from house dust extracts, Brocteur & Versie (18) confirmed the presence of components with A, B, H, Le^a and P₁-specificity. By agglutination-inhibition, the highly skin-active dust preparation 72/E employed in the present work was likewise found to exhibit appreciable A- and B-blood group activity (Berrens et al., in preparation). It was, therefore, a distinct possibility that the observed human complement consumption induced by the allergen is mediated by blood group antibodies of the ABO-system.

In confirmation of earlier studies, Eriksson (10) and Rasmuson et al. (17) detected no deviation in the frequency distribution of ABO blood group specificity in groups of allergic individuals relative to the normal population, though the latter authors observed a preponderance of A-specificity in animal dander sensitive patients. In the present study, 105 patients from the disease groups shown in Fig. 2 were typed for A, B, AB and O specificity by determining the titer of A- and B-agglutinins in their sera. From the collected results in Fig. 5, it is apparent that the allergen sensitivity index of haemolytic complement in these samples is not related to the ABO type of the serum donors. Also, the distribution of blood group specificities within these groups of patients was as expected for the normal population (Fig. 5).

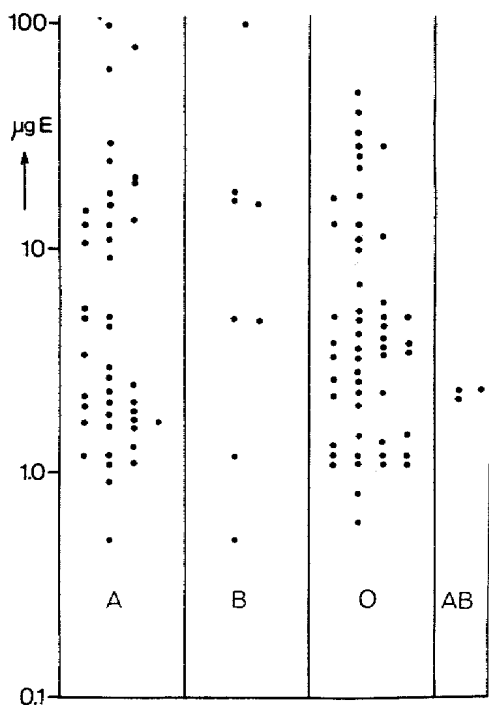


Fig. 5.

Relation between complement sensitivity to dust allergen 72/E and ABO type of the serum donor in 105 samples taken from the disease groups summarized in Fig. 2. Incidence: type A = 40.00 per cent, B = 7.62 per cent, O = 49.52 per cent and AB = 2.86 per cent.

House dust allergen preparation 72/E exhibited both A and B specificity. Consequently, even if the type of blood group antibody in the serum is unrelated to the extent of allergen-induced complement depletion, the sensitivity of haemolytic complement in the serum might still be related to the *titer* of natural iso-agglutinins. This was verified in a collection of 90 serum samples, mainly from allergic donors; in these sera, the 72/E sensitivity index of haemolytic comple-

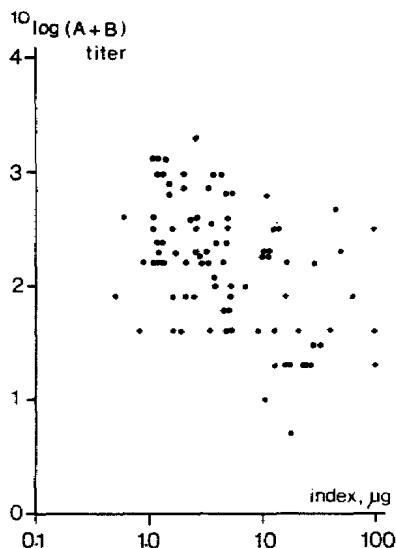


Fig. 6.

Bilogarithmic scatter diagram of the relation between complement-sensitivities of 90 sera (taken from disease groups shown in Fig. 2) *versus* the sum of anti A and -B isoagglutinin titers in A, B and O-type patients.

ment was also determined. The scatter diagram of Fig. 6 demonstrates that the highest anti (A+B) titers do tend to occur in the highly allergen-sensitive sera; the combination of high titered sera with low complement sensitivity is less frequent. However, the association is, at best, very weak. For this reason, and also because the allergen-sensitivity index is not normally distributed (Fig. 3), correlation coefficients were not calculated.

During preliminary studies, the level of house dust specific IgE antibody was determined in 33 sera from atopic donors by radio-allergosorbent test (RAST), using allergen preparation 72/E coupled to microcellulose. The scatter diagram, RAST *versus* complement 72/E sensitivity, featured a weak tendency towards correlation, similar to the pattern displayed

in Fig. 6. However, because it has since been discovered that these RAST-results must have included a considerable proportion of non-immune IgE binding, definite conclusions will have to await the introduction of a modified RAST technique (1).

Despite the observed tendency of high complement-allergen sensitivity to be associated with elevated levels of isoagglutinins (Fig. 5), absorption studies with human red cells and experiments using A- and B-substance for complement consumption, have indicated that blood group antibodies cannot be held responsible for the phenomenon of human complement consumption by house dust allergens. These results will be discussed in a separate communication.

DISCUSSION

The results of these investigations leave no doubt about the conclusion that the *in vitro* complement-test in its present form is unsuitable for the routine laboratory selection of atopic people. Apart from the highly susceptible sera of some perfectly healthy non-atopic individuals, the test very frequently gave a "positive" result with other samples, particularly those from (non-atopic) patients with acquired cold urticaria. One of the reasons for the failure of the complement test may be that the method is founded on the measurement of total haemolytic complement. Inactivation of fluid phase complement by purified house dust allergen has been observed with every human sample examined so far. Current studies indicate that complement depletion is mediated by an as yet unidentified protein (complex) in the serum, which is excluded in the macromolecular fraction on Sephadex® G200 filtration. Even if only very little of this factor is being activated by the allergen in a normal serum, any partial deficiency, lability or modification of subsequent factors in the complement cascade may therefore contribute to an apparent primary allergen-sensitivity. Examination of sequential monthly samples also showed that the allergen-sensitivity index of the serum of a

single individual is variable. In a trial group of 15 subjects, serum haemolytic complement displayed high allergen-sensitivity in autumn, whereas relative resistance to inactivation occurred in early spring.

Molecular titration of individual C components has shown that inactivation by allergen involves the "classical" sequence by way of C1 engagement. No evidence has so far been obtained for the participation of IgE or IgG class antibodies (Berrens et al., in preparation). Absorption experiments have shown that neither naturally-occurring Forssman haemolysins, nor A/B isoagglutinins can be held responsible for the effect of allergen on complement. In this connection, the weak tendency of titers of A/B agglutinins (and possibly also of IgE) to be associated with the more allergen-sensitive sera may, as a working hypothesis, be interpreted as a reflection of an overall increased reactivity of the immune apparatus in atopic people.

Except for the sera of patients with farmer's lung and other chest conditions, which were obtained on first consultation, no special care was taken to restrict the investigation to the sera of untreated patients. Most patients with allergy symptoms had in fact received medication, some of them by immunotherapy with house dust extract. It is a distinct possibility that drugs may influence the haemolytic efficiency of the complement system. Moreover, both Walker (20) and Albus (2) pointed out that the complement-"fixation" reaction becomes negative by hyposensitization treatment. Taking these variables into account, it is conceivable that a more strictly controlled "Hecht"-reaction applied to allergy may enable a sharper distinction to be made between atopic and non-atopic individuals.

SUMMARY

Purified house dust allergen has been employed for screening the susceptibility to inactivation of haemolytic complement in the blood serum of atopic individuals and control subjects. Though the atopic sera displayed a tendency to higher sus-

ceptibility, no practical distinction was possible from the sera of patients with various forms of chronic pulmonary disease, extrinsic allergic alveolitis, or urticaria. CH₅₀ and C₄ levels were unrelated to allergen-sensitivity of haemolytic complement, nor was there a correlation with the titers of naturally-occurring sheep red cell haemolysins. Although blood group specificity within the ABO system was distributed over the patients as expected for the normal population, the titers of A- and B-type antibodies displayed a trend to correlate with the allergen-sensitivity of fluid phase complement.

ACKNOWLEDGEMENTS

The proficient technical assistance of Miss A. G. van Dijk and Mr. C. L. H. Guikers is gratefully acknowledged. The author is also indebted to Drs. W. J. Koers (Utrecht) and De Boer (Heerenveen) and their patients for providing the serum samples.

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