

Dual skin test reactions and serum antibodies to subtilisin and *Aspergillus fumigatus* extracts

O. ZETTERSTRÖM

Departments of Lung Medicine and Clinical Chemistry, University Hospital, Uppsala, Sweden

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Summary

Thirteen patients with an allergy to subtilisin were prick-tested with a solution of crystalline subtilisin A at 0.1 and 1 mg/ml, and the cutaneous reactions were noted.

Sera were examined by radioimmunoassay for IgE antibodies, double-binding antibodies—which seemed to be mainly IgG—and precipitins to subtilisin.

Sera from two subtilisin-tested patients showing immediate and late skin reactions and sera from two patients with allergic aspergillosis were chromatographed on Sephadex G-200, and IgE and IgG antibody peaks were collected and concentrated.

Passive sensitization of skin sites of healthy volunteers was performed with these sera and with IgE and IgG antibody fractions. The skin sites were challenged after 4 hr, 24 hr and 4-6 days.

All thirteen subtilisin-tested patients gave immediate skin responses, which in six patients developed into an oedematous late reaction. Patients with late skin reactions had higher serum levels of IgE antibodies to subtilisin. No correlation was found between the occurrence of late skin reactions and double-binding antibodies. No precipitins were found.

Both immediate and late skin test reactions were seen in skin sites passively sensitized with serum and IgE fractions. Histological examination of the skin with late reactions showed oedema and perivascular infiltration of mononuclear cells, and some eosinophils. Oral administration of prednisolone, 30 mg/day, inhibited the late skin test reaction to passive sensitization with three sera.

The observed late skin reactions seemed to be IgE-mediated and appeared to be dependent on a high level of specific IgE antibodies in the patient's sera or in the solutions used for passive sensitization.

Introduction

The immediate urticarial response elicited by a skin test in an allergic subject, a type I reaction (Coombs & Gell, 1975), is sometimes followed by a larger, longer-lasting,

Correspondence: Dr Olle Zetterström, Lungkliniken, Akademiska sjukhuset, S-750 14, Uppsala 14, Sweden.

oedematous response. This was noticed by Prausnitz & Küstner (1921), who also found such a late reaction after passive sensitization tests (P-K tests) with serum from one of their two investigated patients.

Pepys *et al.* (1959) described the occurrence of immediate and late skin reactions, and the inhibition of the late reactions by steroids, in some patients allergic to *Aspergillus fumigatus*. Late reactions to various allergens have since been reported by several authors.

The nature of the late skin test reactions was investigated by Pepys *et al.* (1968). They found that they were always preceded by immediate responses. Histologically, late reactions showed oedema and perivascular infiltration of mainly mononuclear cells, where complement and immunoglobulins were also found by immunofluorescence. Specific antibodies, measured as precipitins, were also observed in patients' sera and there was a close correlation between their presence and the occurrence of late skin reactions.

These findings support the theory that the late part of the dual skin reaction is complement-mediated via the precipitins—a type III (Arthus) reaction—and that a type I reaction is needed as an introductory mechanism (Pepys, 1969, 1973). This theory has, however, been questioned. Dolovich & Little (1972), in investigations of enzyme sensitivity in detergent industry workers, found no correlation between late skin reactions and the presence of IgG, IgA or IgM in the serum. Late reactions were seen after P-K tests with two sera challenged up to nine days after sensitization. In a further study, Dolovich *et al.* (1973) also observed immediate and late skin responses to testing with anti-IgE and F(ab')₂ of anti-IgE in healthy volunteers. These findings indicate that late skin reactions may be caused by IgE-mediated mechanisms.

Recently, Solley *et al.* (1976), in a study of skin reactions to ragweed, Timothy grass, *Alternaria* and guinea-pig extracts concluded that the late reactions were due to IgE antibodies. They also noted late skin reactions after passive sensitization with immunosorbent-purified IgE antibody to ragweed.

In a survey of allergic patients in Sweden for sensitivity to a detergent enzyme—subtilisin A—(Zetterström & Wide, 1974) skin tests were performed. All patients in whom serum IgE antibodies to subtilisin were found also had positive immediate prick test reactions. Some of these patients had late skin reactions with the same appearance as those seen in patients with allergic bronchopulmonary aspergillosis.

The aim of the present study was to determine whether: (a) the occurrence of dual skin reactions in subtilisin-sensitive patients was correlated to serum antibody levels; (b) late skin reactions to subtilisin and *Aspergillus fumigatus* could be elicited after passive transfer of serum; (c) late reactions after passive sensitization were due to IgE or IgG antibodies, or a combination of the two; (d) the reactions showed histological resemblance to those previously described; and (e) the reactions could be inhibited by steroids.

Materials and methods

Subjects

Thirteen female patients with a positive prick-test response and a positive RAST to subtilisin A were selected. None of them had been exposed industrially to detergent enzymes.

Two patients with a typical clinical and immunological picture of allergic bronchopulmonary aspergillosis were also included in the study.

Skin tests

Prick tests were performed with a solution of crystalline subtilisin A (32 Anson units/g from Novo A/S, Copenhagen), at 0.1 and 1 mg/ml, on the forearms. Two opposite diameters of the cutaneous reactions were then measured, for the first 1–2 hr at the hospital and then by the patients themselves, in the same way, at specified intervals. Between 4 and 6 hr after testing the sites were inspected for the quality of the reactions and to check that the measurements had been made accurately. In some patients the test reactions were seen at several occasions.

In the two *Aspergillus*-sensitive patients intracutaneous skin tests were performed with various *Aspergillus* extracts. A batch of *Aspergillus fumigatus* from Vitrum, Sweden, was used in both patients, an extract from Bencard Ltd in one patient and a protein fraction of *Aspergillus fumigatus* (a gift from Professor Pepys, Brompton Hospital, London) in the other patient. The reactions were measured at the hospital. Typical immediate and late skin responses occurred. At the time of investigation serum samples were taken and stored at -20°C until analysed or used for passive sensitization.

Gel chromatography

Sera from the two *Aspergillus*-sensitive patients and from two patients with the largest late reactions to subtilisin skin testing were used. 3 ml of serum were chromatographed on a Sephadex G-200 column (93×2.6 cm) in 0.1 M Tris-HCl buffer (pH 7.4) with addition of 0.2% sodium azide, and 3 ml fractions were collected. The fractions were analysed and the IgE and IgG antibody peaks were pooled and concentrated at positive pressure, using a Diaflow UM 10 (Amicon Ltd), to approximately 3 ml. The solutions were rechromatographed on a Sephadex G-25 column with Coca's solution as eluent. The void volumes were then concentrated approximately twice by adding dry Sephadex G-25 coarse and collecting the excluded solutions (batch procedure), which were sterilized by filtration through a millipore membrane ($0.22 \mu\text{m}$) into sterile tubes. In the following text the fractions will be referred to as IgE and IgG fractions.

RAST

Serum levels of IgE antibodies were measured by the RAST method (Wide, Bennich & Johansson, 1967). The coupling of subtilisin A, in a freshly made solution, to microcrystalline cellulose particles and the RAST assay were performed as described previously (Zetterström & Wide 1974).

The *Aspergillus* antigens used in the RAST and double-binding antibody assays were a protein fraction of *Aspergillus fumigatus* (see above), in a concentration of 1 mg/ml, and an extract from a growth medium of *Aspergillus fumigatus* culture prepared in our laboratory, of comparable strength. In the coupling procedure 1 ml of extract was used per 100 mg of activated cellulose particles.

The RAST results were expressed in sorbent units (su) (Stenius & Wide, 1969).

Double-binding antibody assay

Serum antibodies were assayed by a solid phase sandwich technique according to

'system 4' as described by Wide (1971). In this assay the antigen to be analysed is coupled to a solid phase immunosorbent. This is then mixed with serum or test solution. If antibodies are present in the mixture they attach to the solid phase and remain there after washing. When radioactively labelled antigen is added it becomes bound to the immunosorbent-fixed antibodies if these are bi- or multi-valent. After final washing the amount of radioactivity bound to the solid phase is measured in a gamma counter.

In the double-binding antibody assay the same suspension of immunosorbent cellulose particles was used, and in the same concentration (1 mg/ml) as in the RAST assay. Aliquots of 0.5 ml were put into small plastic tubes and 50 or 100 μ l of serum were added. In analysis of the Sephadex G-200 fractions, 0.5–1 ml was used. The reagents were mixed by slow rotation of the tubes for 20 hr. After centrifugation and washing four times the labelled subtilisin A or *Aspergillus* antigen, diluted to about 70,000 ct/min in 100 μ l, was added and the reagents were mixed for another 20 hr. After the final washing, the amount of radioactivity bound to the cellulose particles was measured.

For the labelling of subtilisin A, 5–10 μ l of a freshly made solution, 1 mg/ml in physiological saline, was added to 10 μ l of 1 M phosphate buffer (pH 7.4) and 0.5 mCi ^{125} I in NaOH (Amersham, England). The solution was mixed with 10 μ g of chloramine T at room temperature for 45–60 sec and the reaction was stopped by the addition of 24 μ g of sodium metabisulphite. Unbound ^{125} I was separated from the labelled enzyme by passage through a Sephadex G-75 column (30 $\times$ 1 cm). About 1/3 of the radioactivity emerged in a single peak shortly after the void volume.

For the labelling of *Aspergillus* antigen, 10–20 μ l of the extracts were used. On subsequent separation of labelled antigen from unbound ^{125} I on the Sephadex G-75 column, radioactivity was found in all fractions after the void volume without any specific peak activity, apart from the salt peak. The various fractions were therefore tested against known positive sera (with precipitins to *Aspergillus* antigen) and fractions showing the highest reactivity, usually with 30–50% binding and eluted midway between the void volume and the salt peak, were used in the assay.

Radioimmunoassay (RIA)

Tests for the presence of precipitins to subtilisin were made by a modification of the method used by Pepys *et al.* (1973). Seven 1 cm square glass slides were coated first with a thin film of agarose and then with a 1 mm thick layer of agarose in 0.025 M barbitone buffer (pH 8.6). Five groups of wells were cut, each with a central well, 4 mm in diameter, and four peripheral wells, 4 mm in diameter and equidistant, 2.5 mm apart. Aliquots of serial dilutions of radioactively labelled subtilisin A were placed in the central wells and the test sera in the peripheral wells, and they were allowed to diffuse for 24 hr at room temperature in a humidity cabinet. After washing for 48 hr the plates were dried and autoradiographs made.

A serum from an enzyme-exposed worker at Novo Industries was used as a positive control and reference serum in the double-binding assay and in the precipitin test.

Prausnitz-Küstner tests

Six healthy physicians volunteered as recipients. 0.1–0.2 ml of serum (sterilized by passage through a millipore filter) or 0.2 ml of IgG or IgE antibody fractions, or a

combination of these two fractions (0.1 ml of each), was injected into skin on the forearms. Challenge of sensitized skin sites was performed by placing a drop of subtilisin A solution, 10 mg/ml, over the sensitized area and then making five prick tests through the drop, or by injection of about 0.05 ml of *Aspergillus fumigatus* extract (Vitrum) in a concentration of 1:100 (w/v). The time interval between sensitization and challenge varied from 4 hr to 6 days. Measurements of skin test reactions were made by the recipients themselves at specified intervals.

Skin biopsies

3 mm punch biopsies were taken. The specimens were fixed in 10% formalin and stained by the van Gieson or haematoxylin-eosin methods.

Four biopsies were performed. In one recipient this was done 3.5 and 20 hr after challenge with subtilisin, 4 hr after sensitization with serum from patient G.B.J.; in another recipient, 6 hr after challenge, 4 days after sensitization with serum from the same patient; and in a third recipient, 6 hr after challenge with *Aspergillus* antigen, 4 days after sensitization with the IgE fraction from patient S.A.

Results

Skin tests

Prick-testing with subtilisin A elicited typical immediate urticarial reactions in all thirteen patients. Six of the patients showed distinct late skin reactions, while in five patients no late reaction was seen. In two patients the skin reaction diminished more slowly over several hours and the reaction was classified as intermediate between the two other types. In patients showing a late reaction the margins of the immediate weal became less and less well-defined, and after about 2 hr the swelling began to increase in size and became oedematous, slightly tender and warm, and erythematous. The oedema persisted for 1 to 2 days, and seemed to spread into the surrounding area while disappearing, becoming increasingly difficult to measure. Both immediate and late reactions were larger following challenge with 1 mg/ml of subtilisin A than with 0.1 mg/ml. A typical example is shown in Fig. 1.

The development of the skin reactions in the various patients is plotted against time in Fig. 2. To visualize the changes in area of the skin reactions the two diameters measured were multiplied and the root of the product, representing a 'mean geometric diameter', is given on the ordinate.

RAST

IgE antibodies to subtilisin A were found in all sera. The RAST titres varied widely from 2.4 to 101 su. The titre for each patient is given in Fig. 2.

Double-binding antibodies

The results of the assay were expressed in arbitrary units (au), using the reference serum which was given a value of 1000 as a provisional laboratory standard.

This serum gave about 60,000 ct/min undiluted and 1100-1700 ct/min when diluted 1:1000.

The reaction was considered positive when the radioactive uptake was twice that of the negative controls, corresponding to about 0.5 au.



Fig. 1. Prick test reactions to subtilisin. (a) Reactions to 0.1 and 1 mg/ml after 5 hr; (b) reaction to 1 mg/ml after 24 hr.

Only small amounts of double-binding antibodies could be found in six of the sera. The titres for the various patients are given in Fig. 2. The figures represent the mean of three different assays.

Radioimmunodiffusion

In the RID test a positive reaction was seen with the reference serum in a dilution of 1:100, while no positive reactions were noted with the patient sera.

The total serum IgE level, measured by the PRIST method (Phadebas IgE PRIST), for the various patients is also given in Fig. 2. The normal value of non-atopics with this method is 14 u/ml (plus two s.d. is about 120 u/ml). It is seen that this level was elevated in most patients.

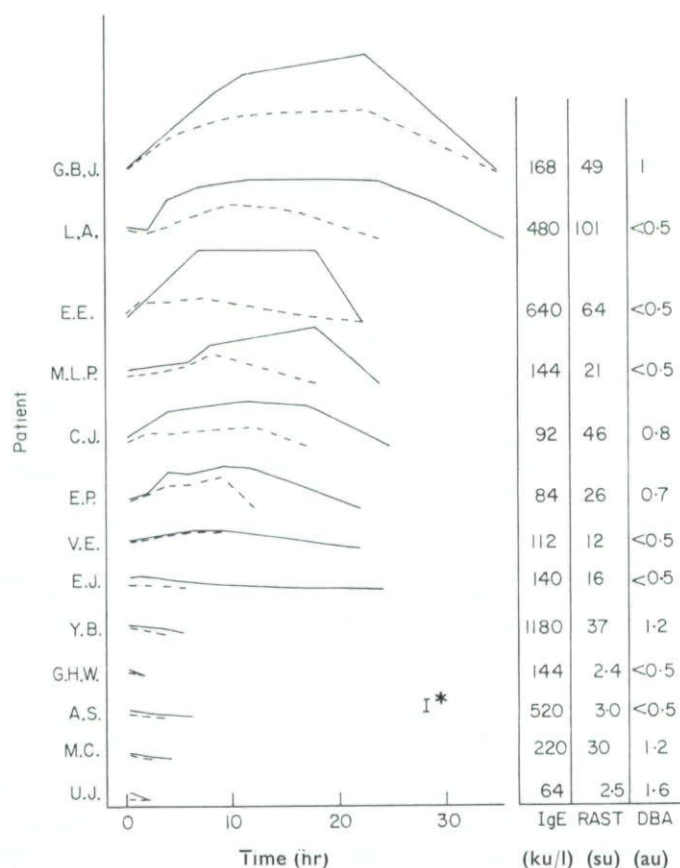


Fig. 2. Mean diameters of skin reactions to 0.1 and 1 mg/ml subtilisin in individual patients at various intervals after challenge. Serum levels of total IgE and of IgE antibodies (RAST) and double-binding antibodies (DBA) to subtilisin for the various patients are given to the right in the figure. * Shows 1 cm. (—) Test with 1 mg/ml; (---) test with 0.1 mg/ml.

Correlation between skin reactions and antibody titres

As seen in Fig. 2, patients with late skin test reactions showed higher levels of specific IgE antibodies to subtilisin. The geometric mean of the RAST values for the six patients with dual skin reactions to subtilisin in a concentration of 1 mg/ml was 45 su, while that for the five patients with only immediate reactions was 7.3 su. This difference is statistically significant ($P < 0.02$; Student's *t*-test).

The difference between the ratios of specific IgE to total IgE for these two groups reached a higher level of significance ($P < 0.002$). No correlation between the occurrence of late skin test reactions and double-binding antibody titres was found.

Gel chromatography

The result of chromatography on serum from patient G.B.J., on Sephadex G-200, is presented in Fig. 3. It is seen that the specific IgE and double-binding antibodies were eluted separately and that the double-binding antibodies were positioned in the IgG peak. Fractions that were concentrated and used for passive sensitization are also shown in the figure.

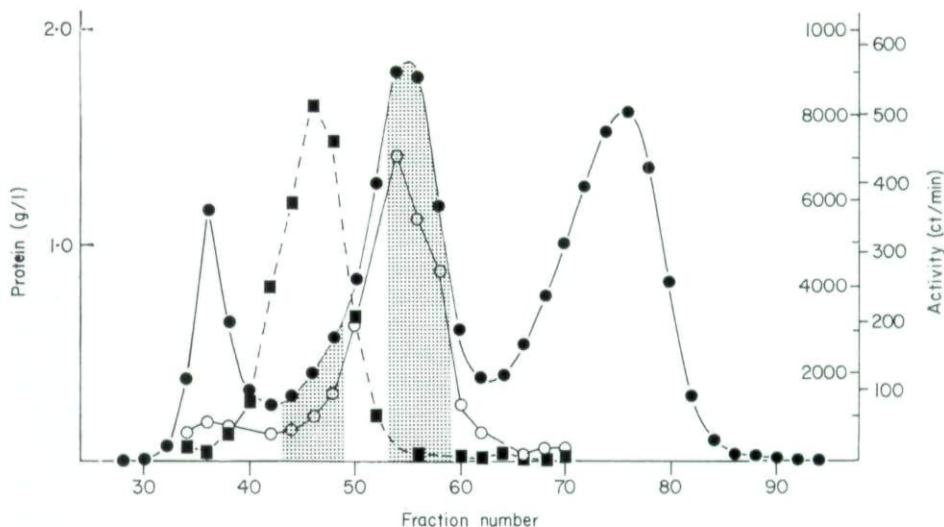


Fig. 3. Chromatogram (Sephadex G-200) of serum from patient G.B.J. The protein concentration in the fractions showed three main peaks. The IgE antibody activity was found in the fractions just before and in the first part of the second protein peak. Double-binding antibody activity was eluted parallel with the second protein peak. Fractions 43-49 and 53-59 were concentrated and used in P-K tests as 'IgE fraction' and 'IgG fraction' respectively (shaded areas). (●) Protein; (○) DBA (outer right ordinate); (■) RAST (inner right ordinate).

Analysis of the fractions obtained after chromatography of serum from patient L.A. showed a single peak of IgE antibody activity in the beginning of the second protein peak, as in patient G.B.J. As in whole serum, no double-binding activity was found. From this patient, fractions from the second protein peak were pooled, concentrated and used in passive sensitization tests.

Serum antibodies to *Aspergillus* from the two aspergillosis patients (S.A. and S.R.) were eluted from the column in the same positions as the antibodies from patient G.B.J. (Fig. 3), with a peak of IgE antibodies shortly before, and a peak of double-binding antibodies coincident with, the second protein peak.

In the IgE fractions from the subtilisin-sensitive patients G.B.J. and L.A., the activities, measured as sorbent units, were 6.6 and 23 su respectively. No double-binding antibody activity was found in these fractions. The activities in IgE fractions from the two *Aspergillus*-sensitive patients S.A. and S.R. were higher, 90 and 52 su respectively. In these fractions a small amount of double-binding antibody activity was found.

The IgG fractions from subtilisin-sensitive patients contained a small amount of IgE antibodies, about 1 su, while the IgG fractions from *Aspergillus*-sensitive patients contained about 3 su.

Prausnitz-Küstner tests

In preliminary tests, challenge with subtilisin was made by intracutaneous injection of 0.05 ml of a 1 mg/ml solution. Relatively large dual reactions were seen. However, control tests made in unsensitized skin resulted in non-specific weals which persisted for several hours and became erythematous, but did not increase in size. After prick

tests with the subtilisin solution at 10 mg/ml, virtually no reactions were seen in unsensitized skin or in control sites sensitized with normal serum. With *Aspergillus* antigen small, slightly red weals persisting for more than 12 hr were found both in unsensitized skin and in skin previously injected with normal serum.

When patient sera were used, typical immediate reactions accompanied by itching occurred after challenge in all instances. The diameters of the weals were 6–7 mm and the flares 20–50 mm. The development of the test reactions resembled that in the allergic patients, but the reactions were smaller. The margins of the weals became less sharp and the flares subsided, but the reactions did not disappear completely. After 2–4 hr the now softer swellings began to increase in size and became erythematous. The smaller late reactions peaked at about 4 hr and the larger ones later, up to 10 hr after challenge. The reactions were not painful but were sensitive to touch, and some of them were almost hyperaesthetic.

Challenge tests made in skin sites sensitized with IgE antibody fractions resulted in immediate reactions. In tests made on sites sensitized with IgG antibody fractions from the subtilisin-sensitive patients, no immediate reactions occurred. Challenge with *Aspergillus fumigatus* in sites sensitized with IgG fractions from patients S.A. and S.R. gave small erythematous weals, 7–10 mm in diameter, i.e. slightly larger than in the controls. The reactions did not itch, there was no typical flare and they were

Table 1. Mean diameters (mm) of immediate and late reactions to Prausnitz-Küstner tests with sera and antibody fractions

Patient sera and antibody fractions	Period of sensitization					
	4 hr		24 hr		4–6 days	
	Immediate	Late	Immediate	Late	Immediate	Late
Sera						
G.B.J.	6	17	14*	31*	13†	22†
L.A.	12	42	17	42	15	16
S.A.	11	32	11	35	n.d.	n.d.
S.R.	12	49	n.d.	n.d.	15	37
Antibody fractions						
G.B.J. E	12*	33*	n.d.	n.d.	6†	0/8‡
E+G	8	8				
G	0	0				
L.A. E	10	39	n.d.	n.d.	15	15
E+G	9	6				
G	0	0				
S.A. E	13	37	12	27	16†	35†
E+G	9	46				
G	0	0	0	0		
S.R. E	12	40	17	37	10	10
E+G	8	30			14	15
G	0	0	9	0		

n.d. = Not done; E=IgE fraction, G=IgG fraction.

* Mean of three tests

† Mean of two tests.

‡ This test gave no visible late reaction in one case and a weak late reaction in the other (diameter 8 mm).

considered non-specific. These challenge tests were repeated 24 hr after sensitization. In one of the tests a weak immediate reaction with a flare was seen, and was regarded as positive.

Late reactions similar to those obtained with serum were also observed after P-K tests with IgE antibody fractions, and in some instances with a combination of IgE and IgG antibody fractions.

The sizes of the reactions to P-K tests are given in Table 1. It can be seen that large immediate reactions tended to give large late reactions. The time that had elapsed after sensitization seemed to influence the relationship between the immediate and late reactions. Immediate responses occurring after challenge 4 hr following sensitization were slightly smaller than those obtained after 24 hr and 4-6 days. The late reactions, however, tended to be larger in tests made 4 and 24 hr after sensitization.

Two challenge tests at sites sensitized 4 and 6 days previously with the IgE fraction from G.B.J. serum gave weak immediate reactions. In one test a weak late response and in the other no late response was seen.

A combination of IgE and IgG antibody fractions for sensitization did not give larger late reactions than IgE fractions alone.

Histological picture

In one P-K test made 4 hr after sensitization with serum from patient G.B.J., specimens were taken 3.5 hr and 20 hr after intracutaneous challenge with 0.05 ml of 1 mg/ml subtilisin A (this was one of the preliminary tests). The 3.5 hr reaction consisted of a swollen vascular endothelium and a slight perivascular infiltration of mainly lymphocytes and also granulocytes, most of which were eosinophils. After 20 hr there were denser infiltrates of lymphocytes, histiocytes and also eosinophilic and sparse neutrophilic granulocytes. Another recipient sensitized with the same serum was challenged by prick-testing after a sensitization period of 6 days. In the specimen taken from the late reaction 6 hr after challenge, oedema and a similar picture of perivascular infiltration of cells was seen. Lymphocytes predominated and abundant eosinophils and a few neutrophils were also observed (Fig. 4).

In the specimen taken 6 hr after challenge with *Aspergillus* antigen in a site sensitized with an IgE fraction from patient S.A., the same type of perivascular infiltration of mononuclear and eosinophilic cells was seen. In this case the neutrophils were slightly more numerous than in the other specimens (Fig. 4).

Prednisolone test

Passive sensitization was performed with serum from patients G.B.J., L.A. and S.R. Prednisolone in a dose of 10 mg was then given at noon and in the evening, and three times the next day, on which challenge was performed the next morning. This recipient had previously shown late skin test reactions after challenge following sensitization with sera from patients G.B.J. and L.A. After challenge this time the late skin test response was markedly inhibited (Fig. 5). After 1 and 6 months new P-K tests with sera from patients G.B.J. and L.A., respectively, were carried out in the same recipient without medication. Again an immediate and late skin reaction was seen.

Discussion

The dual skin test reactions seen in some of the subtilisin-sensitive patients were similar to those occurring in the two patients with allergic bronchopulmonary aspergillosis. The late component of these reactions also had the same characteristics and

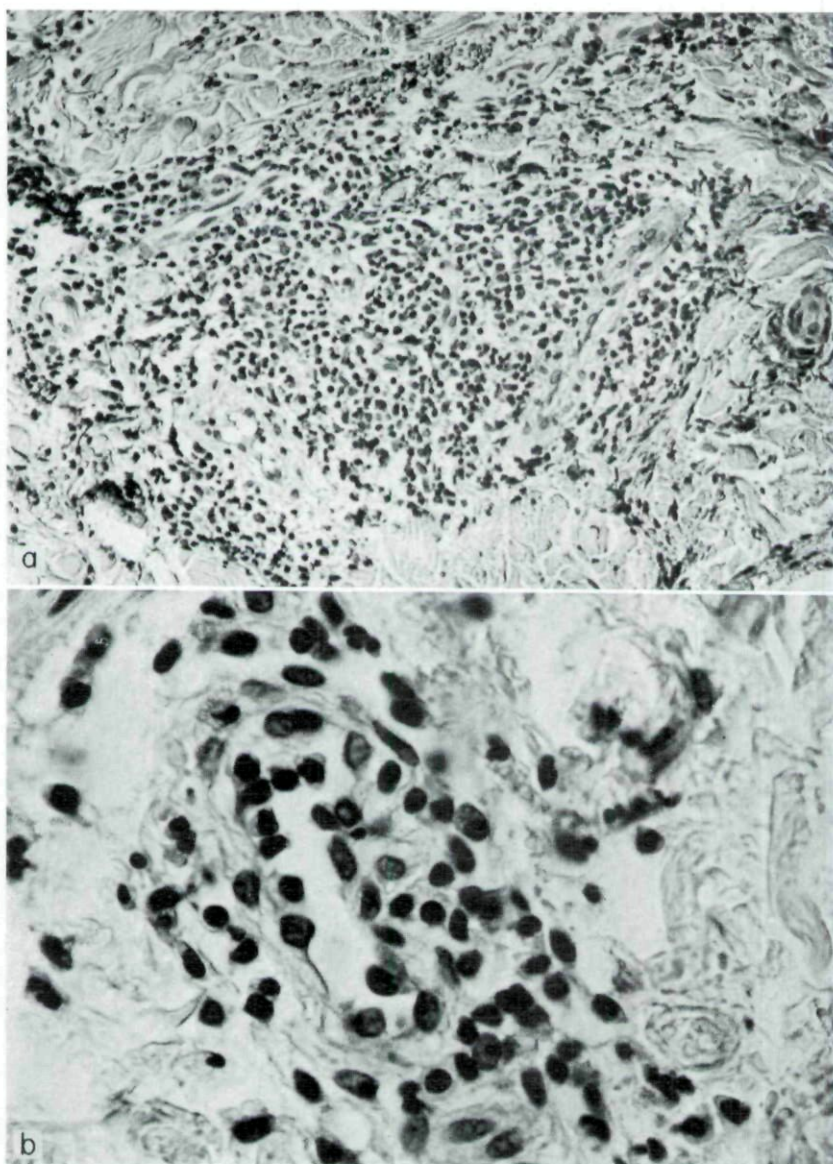


Fig. 4. Histology of late skin test reactions after Prausnitz-Küstner tests. (a) Biopsy taken 6 hr after challenge of a skin site sensitized 6 days previously with 'IgE fraction' of serum from patient S.A. There is oedema in the dermis and infiltration, mainly of mononuclear cells. The infiltration is most marked around blood vessels. Haematoxylin-eosin was the stain used (magnification $\times 220$). (b) Biopsy taken 6 hr after challenge of a skin site sensitized 6 days previously with serum from patient G.B.J. Perivascular infiltration of lymphocytes and some eosinophils. Haematoxylin-eosin was the stain used (magnification $\times 700$).

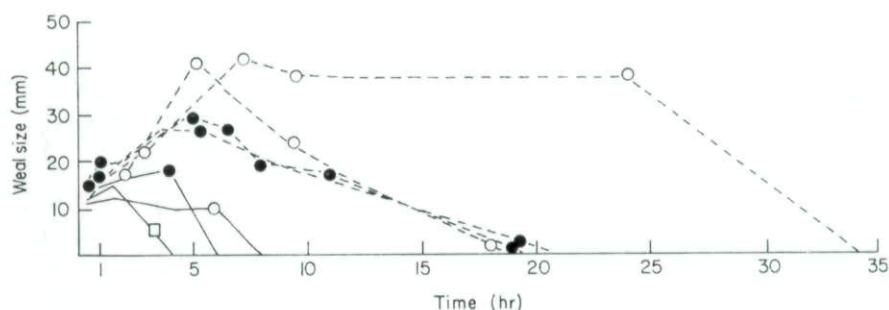


Fig. 5. Late reactions after Prausnitz-Küstner tests with serum from the subtilisin-sensitive patients G.B.J. and L.A. were inhibited when the recipient had received prednisolone. The skin reaction to *Aspergillus* antigen after sensitization with serum from patient S.R. had no late component. The same test in another recipient not treated with prednisolone resulted in both immediate and late reactions. (—) Reactions during prednisolone (30 mg/day); (---) reactions in the same recipient without treatment; (○) L.A.; (●) G.B.J.; (□) S.R.

general appearance as late reactions described by Pepys (1968, 1975) as a type III reaction. In one of the patients (L.A.) the initial response diminished and was then followed by an increasing oedematous late reaction, whereas in the other patients it changed continuously, to become a late reaction. This latter type of evolution has been described by Dolovich *et al.* (1973) and Solley *et al.* (1976), and was reported by Pepys (1969) to occur after intracutaneous tests. One explanation why prick-testing here gave this type of development might be that the crystalline subtilisin A is a very potent allergen because of its high degree of purification.

The occurrence of a late reaction does not seem to be an all-or-none response. Larger late responses were seen when patients were challenged with a 1 mg/ml solution than with 0.1 mg/ml. Two of the patients had a slight reaction to 1 mg/ml of subtilisin which persisted for several hours. When one of them was challenged with a 10 mg/ml solution a more typical late response developed. The importance of high doses of allergen for the occurrence of late skin reactions has also been stressed by McCarthy & Pepys (1971), Dolovich *et al.* (1973) and Umemoto *et al.* (1976).

Late cutaneous reactions to the chosen concentrations of subtilisin occurred in patients with high levels of specific IgE antibodies and strong immediate prick test responses. This corresponds to the finding of Robertson *et al.* (1974) of higher levels of IgE-related sensitivity and larger prick test reactions in patients with late skin and asthmatic responses to ragweed. Of the two patients with relatively high serum levels of specific IgE antibodies to subtilisin who did not have a late cutaneous reaction, one was very atopic and had a high serum titre of total IgE and specific IgE antibodies to many allergens. Since the ratio of specific IgE to total IgE in the serum correlated more closely than the specific IgE level to the occurrence of late reactions, it is possible that not only high levels of IgE antibodies, but also a high fraction of specific IgE antibodies is of importance for the development of late skin test reactions.

No precipitating antibodies to subtilisin were found in the sera of the investigated patients, all of whom were only exposed to detergent enzymes as consumers. The titre of double-binding antibodies found in some of the sera was also very low, in the order of 1/10 to 1/1000 of the amounts found in the sera of exposed workers in detergent

industries (unpublished data). There was no correlation between the level of double-binding antibodies and the occurrence of late skin reactions. This supports the findings of Dolovich & Little (1972) and seems to indicate that antibodies of the IgE class are of major importance for the development of late skin reactions.

The fact that dual skin reactions could be elicited in nonatopic subjects by P-K tests performed several days after sensitization supports the theory that dual responses are mediated by IgE antibodies. Parish (1973) has described the existence of IgG antibodies capable of sensitizing human skin for 4-6 hr in some allergic sera. Various time-periods between sensitization and challenge were therefore chosen in the P-K tests in an attempt to determine whether such antibodies contributed to the late reactions. Challenges made 4 hr after sensitization gave slightly smaller immediate reactions than those made after longer periods, but nevertheless they gave relatively large late reactions. Augustin (1967) performed P-K tests with varying periods of sensitization and noted that when a short period of 2 hr was used the reactions to challenge were not so well defined as ordinary P-K reactions, and they continued to increase in size 4 hr after the challenge test. She ascribed this to poor fixation of reagins in the first hours after sensitization. Another possible explanation is that small amounts of IgG antibodies persisting in the skin influenced the development of the reactions. However, immediate reactions were not seen after challenge tests made 4 hr after sensitization with IgG fractions, so no direct evidence of short-term sensitizing IgG antibodies were found.

Chromatography of sera did not give a complete separation of IgE and IgG antibodies. The relative amounts of IgE and IgG antibodies in the fractions were, however, different from those in the original sera. The P-K tests in which the various fractions were used resulted in late reactions only in skin sites where IgE fractions, i.e. relatively large amounts of IgE antibodies, had been injected before challenge. Tests in which a mixture of IgE and IgG fractions were used did not give rise to larger late reactions than tests with IgE fractions alone. This seems to indicate that the late responses were dependent only on the IgE antibodies. However, it can not be ruled out that small amounts of IgG in the IgE fractions contributed to the development of the late skin responses via a complement-mediated mechanism. This especially applies to the tests with the IgE fractions of serum from the *Aspergillus*-sensitive patients in which small but measurable amounts of IgG antibodies to *Aspergillus* were found. In the late reactions observed after P-K tests with IgE fractions from the subtilisin-sensitive patients, an IgG-mediated mechanism is less likely to have occurred. In these fractions, IgG antibodies to subtilisin were below the level of detection and the IgE fraction from patient L.A. must have contained very minute amounts of IgG antibodies, since such antibodies could not be demonstrated, even in the IgG fraction or in the whole serum, before chromatography.

This suggests that high titres of IgE antibodies alone might suffice to induce late skin test responses in some instances. The fact that P-K tests resulted in larger late reactions after sensitization with whole serum than after sensitization with the corresponding IgE fraction might be explained by the higher IgE antibody titre in the original sera and is not necessarily an effect of the presence of other antibodies in the sera.

The histological picture of the late reactions fits with the description given by Pepys *et al.* (1968), Taylor & Shivalkar (1971) and Solley *et al.* (1976). Furthermore, the late part of the dual reaction was inhibited by steroid treatment, as reported by

Pepys *et al.* (1959, 1968) and Poothullil *et al.* (1975). This supports the assumption that the dual skin reactions resulting from the passive transfer of serum are of the same type as those previously described.

The immediate skin reaction is probably caused mainly by histamine release from mast cells. It is possible that if the amount of specific IgE antibodies bound to these cells is large and the antigen introduced into the skin is potent, the resulting intense reaction of the mast cells causes a release of other mediators, which will be manifested as the characteristic late oedematous skin reaction. These mediators may derive from the mast cell itself or their formation may be initiated by the mast cell reaction, which can be started either by allergens, anti-IgE or by compound 48/80 (Solley *et al.*, 1976). A possible mediator system involved in the pathogenesis of the late part of the reaction is the kallikrein-kinin system. Skin tests with kallikrein gave late reactions (Juhlin & Michaelsson, 1969). These were inhibited by prednisone (Michaelsson, 1970) and were similar in appearance to those described here (Michaelsson, personal communication). After strong skin tests and P-K reactions, kinin-like activity has been found in dermal perfusates (Michel *et al.*, 1970). Newball, Talamo & Lichtenstein (1975) have also reported the release of kallikrein from leucocytes treated with allergen and anti-IgE.

Whatever the mechanism may be, it seems probable that the late part of the dual skin response can result from a strong IgE-mediated reaction.

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