

Correlates of skin test reactions to *Bacillus subtilis* enzyme preparations

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In a selected group of detergent industry workers, a close relationship was observed between cutaneous sensitivity and respiratory symptoms from exposure to air-borne B. subtilis enzyme-containing products. In intracutaneous tests with comparatively high concentrations of two B. subtilis products used commercially, prolonged responses which were generally maximal in diameter at 6 to 8 hours were present at the sites of 75 per cent of 35 "immediate" wheal and flare responses but not at skin test-negative sites. Precipitin reactions with human sera were nonspecific. Antibodies of the IgA and IgM classes were not detectable in the sera of most subjects with the responses observed at 6 to 8 hours. Antibodies of the IgG class were present in most of these sera but were also present in the sera of some subjects with immediate reactions without ensuing responses. Apart from the obligatory preceding "immediate" wheal and flare reaction, responses which evolved over 6 to 8 hours could not be related to any immunologic finding. Evidence is presented which illustrates that reactions observed at 6 to 8 hours did not have a necessary dependence upon the presence of proteinase activity in antigen preparations. Prausnitz-Küstner reactions to B. subtilis antigens and to ragweed antigen E evolved over a period of hours and were similar to gross appearance to those seen in actively sensitized industrial workers. In the discussion, these results are considered in relation to mechanisms underlying prolonged cutaneous allergic responses.

The times of onset, duration, and gross characteristics of cutaneous allergic reactions to antigen are considered useful in the identification of types of reactions and underlying immunologic processes.¹ The cutaneous reaction in reagin-dependent type I hypersensitivity² takes the form of a wheal and flare response which is maximal at 15 or 20 minutes and typically subsides in an hour or two. This "immediate" hypersensitivity is considered to be largely due to the interaction of antigen with antibody of the IgE class.³ Bronchopulmonary aspergillosis and pulmonary disease from the inhalation of certain organic dusts are associated with "dual" responses to inhalation and to intracutaneous antigen challenges.⁴ In the dual cutaneous response, resolution of the immediate response is described, followed by a "late" response which is generally maximal at 6 to 8 hours and is considered to be precipitin dependent.^{4, 5}

Industrial exposure to airborne *Bacillus subtilis* enzyme products is known to lead to respiratory disease in some workers.⁶ Nasal symptoms, breathlessness,

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and nonproductive cough occur during the course of the working day; in addition, affected persons report the presence of symptoms which persist after work and are troublesome at night. Tests for cutaneous sensitivity to *B. subtilis* enzyme products are positive in most symptomatic and in many asymptomatic exposed workers.⁹⁻¹⁰ Dual pulmonary and cutaneous responses from appropriate challenges with *B. subtilis* enzyme products have been described in symptomatic workers.^{10, 11}

The present report describes relationships between respiratory symptoms and the results of intracutaneous skin tests in a selected industrial population exposed to *B. subtilis* enzyme products. Immediate cutaneous reactions and responses observed at 6 to 8 hours are considered in relation to serum antibodies. Intracutaneous tests with enzyme preparations mixed with histamine and with histamine and bradykinin, to simulate allergic reactions of the wheal and flare type, and separate Prausnitz-Küstner (P-K) tests were performed in experiments designed to examine the possible significance of proteinase activity of the antigen preparations in the development of responses observed at 6 to 8 hours. Tests for serum antibodies of the various immunoglobulin classes were performed by labeled antigen-binding methods in an attempt to identify a reliable immunologic correlate of the observed cutaneous allergic responses.

MATERIALS AND METHODS

Antigen preparations

Crystalline preparations of the *B. subtilis* alkaline proteinases subtilopeptidase A (SPA) and subtilopeptidase B (SPB),^{12, 13} purified preparations of SPB and *B. subtilis* amylase, and samples of two *B. subtilis* enzyme preparations used industrially were employed. SPA was found to be the predominant *B. subtilis* alkaline proteinase in one of the industrial preparations and it is hereafter referred to as "nonpurified preparation A" (NPPA). SPB was the predominant alkaline proteinase of the other commercial preparation hereafter referred to as NPPB.¹⁴ Ragweed antigen E and a commercial preparation of whole ragweed extract (Omega Laboratories, Toronto), obtained as a 30,000 protein nitrogen units (PNU) per milliliter of solution and exhaustively dialyzed, were used in P-K tests.

Allergy skin tests

Preliminary tests in the industrial population by the scratch test method were performed with NPPA and NPPB solutions prepared by the separate addition of 0.5 mg. for each milliliter of 0.15M borate buffer, pH 8.0, followed by passage of the solutions through a Millipore filter.

Intracutaneous tests in nonexposed normal laboratory workers and in exposed industrial workers were performed by the injection of 0.02 ml. of the NPPA and NPPB solutions diluted fivefold. Despite the use of an antigen concentration which was tenfold higher than that which was used routinely to test for sensitivity, it was possible to readily distinguish the small pale papule at the injection site in nonexposed subjects from immediate reactions seen in sensitized persons. Reactions were measured in two perpendicular directions at 15 minutes, and those reactions in which the mean wheal diameter was greater than the value of the mean $\pm$ one standard deviation for the normal nonexposed population were considered to be positive. Test sites were again examined at 6 to 8 hours after intracutaneous challenge and, in some cases, during the intervening period. In many instances, the immediate reactions seemed to subside over an hour or two, but they did not disappear completely; there was a disappearance of the flare, but at the site of the wheal some swelling persisted. After 2 to 3 hours, the predominant alteration was a convex swelling at the site. At 6 to 8 hours the

lesions were less raised, but there was a pale to bright erythema of a greater diameter. A slight itchiness, tenderness to tapping, and a sensation of warmth were generally present. Diameters of the zones of erythema were measured in two perpendicular diameters at 6 to 8 hours, and the sizes of responses were expressed as mean diameters. Most responses were reported to have largely disappeared by the following morning. The largest responses tended to persist longest. The components of these reactions which evolved over a period of hours following the wheal and flare responses are designated "6 to 8 hour responses."

Histamine and bradykinin added to NPPA and NPPB were used in skin tests to simulate allergic wheal and flare reactions in subjects not sensitive to *B. subtilis* products. Histamine, 1:1,000, was mixed with an equal volume of the NPPA or NPPB solutions prepared for intracutaneous tests. When both histamine and bradykinin were used, histamine, 1:1,000, was mixed with an equal volume of bradykinin (BRS 640, Sandoz, Basle), 100 μ g per milliliter, and the mixture was added to an equal volume of NPPA or NPPB.

In P-K tests, 0.1 ml. volumes of reagin-containing sera were used to separately sensitize multiple skin sites of nonallergic recipients. Appropriately sensitized sites were challenged separately with about 0.02 ml. of a number of *B. subtilis* and ragweed antigen preparations. The NPPA and NPPB were those solutions prepared for intracutaneous tests. A dialyzed 30,000 PNU per milliliter solution of whole ragweed antigen was used (see above). SPB, *B. subtilis* amylase, and ragweed antigen E were used in concentrations of 100 μ g per milliliter.

Study populations

Tests of cutaneous sensitivity to NPPA and NPPB preparations by the scratch test method and tests for the presence of serum antibodies of the IgG class by radioimmuno-diffusion (RID) were done routinely on patients seen at the industrial health clinic. On the basis of the results of skin tests for immediate reactions and in the absence of any information relating to the symptomatic state or to prolonged allergic reactions at skin test sites, a group of subjects was selected to comprise approximately equal numbers of persons with and without cutaneous reactivity. In addition, the selection was performed in such a manner that in each group there would be persons with and without serum antibodies of the IgG class based upon the preliminary RID tests with sera previously taken: This was to potentially permit an effective analysis of relationships between immunologic findings and cutaneous responses. The 60 subjects chosen were among workers who had the greatest early exposure to *B. subtilis* products and the earliest evaluations for sensitivity to these products. The test population comprised a total of 37 of the selected subjects who happened to be at work on the day shift on one or other of two days, one week apart, in late 1969. On one or other of the two days, symptom histories were recorded, blood specimens were taken, and intracutaneous tests where NPPA and NPPB were performed.

Sera also were collected during 1968 and 1969 from 39 asthmatic subjects from a clinic population and from a group of 22 normal laboratory workers with no industrial exposure to *B. subtilis* preparations.

Two sera were used in P-K tests; one was from a subject with clinical sensitivity to *B. subtilis* products and strongly positive skin tests to NPPA and NPPB, and the second was from a clinic patient who was sensitive to ragweed but had not received antigen injection treatment. Corresponding antibodies of the IgE class were observed in RID tests. The ragweed reaginic serum was selected after examination by RID for the presence of antibodies to ragweed antigen E of the IgG, IgA, and IgM classes; antibodies of the IgG class were detectable at the limits of detection by this method, and antibodies of the IgA and IgM class could not be detected.

Antibody studies

Double-diffusion tests for precipitins were initially performed in 1.5 per cent agar in 0.15M phosphate buffer, pH 7.4. Unstained patterns were read at 1, 2, and 3 days. Tests were also performed in agar in citrate buffer, pH 7.0. The test serum was placed in 12 mm. central wells, and the antigen solutions (NPPA, 10 and 40 mg. per milliliter in borate buffer) placed

in peripheral wells 4 mm. in diameter and at a 4 mm. distance. After storage at 4° C. and daily reading for 3 days, they were washed at 4° C., dried, and stained with amido black.

Radioimmuno-electrophoresis (RIEP) and RID were performed by a two-step method as previously described,¹⁵ except that trays were stored at 4° C. after the addition of labeled *B. subtilis* preparations. Antisera to IgA and IgM were obtained from commercial sources and absorbed with lyophilized cord serum and a lyophilized DEAE-cellulose-purified serum IgG preparation, respectively, to render them monospecific. Antiserum to IgG was prepared by the injection of purified IgG in complete Freund's adjuvant into rabbits; the antiserum was not further treated. Antiserum to human IgE was prepared by the immunization of rabbits with purified IgE myeloma protein followed by absorption of the antiserum with normal human serum. The NPPA, NPPB, and ragweed antigen E were labeled with ¹²⁵I by the chloramine-T method.¹⁶ A successful labeling of all antigenic components of NPPA and NPPB was demonstrated with RIEP examinations performed with rabbit antisera to these preparations.

Tests for antibodies of the IgG class to *B. subtilis* products were performed by RIEP and RID, and tests for antibodies of the IgA and IgM classes were performed by RID. Results were found to be more distinct when a nonlabeled sample of the alternate *B. subtilis* preparation was added to the test pattern prior to the addition of the labeled NPPA or NPPB preparations. The tests were done with and without the prior addition of the alternate nonlabeled preparations. Serum specimens were tested in duplicate by each method. Serum antibody of a particular class was considered to be present if any one of the multiple tests indicated the presence of antigen binding which was greater than that of control sera tested simultaneously.

In a coprecipitation test, 0.1 ml. of the test serum diluted 1:40 in 0.15M phosphate-buffered saline, pH 7.4, was mixed with the ¹²⁵I-labeled NPPA. Preliminary titrations of labeled antigen assured that the quantity of labeled antigen was not a limiting factor in the tests. Following overnight incubation at 4° C., an optimal quantity of rabbit antiserum to IgG was added to the reaction mixture. It was mixed, stored at 4° C. for another 4 hours, and centrifuged. After four washes with precooled borate buffer, the precipitate was counted in a well-type scintillation counter. In the coprecipitation tests, the addition of a small quantity of nonlabeled NPPB, which contained less than 0.001 per cent SPA,¹⁴ prior to addition of NPPA resulted in an improved discrimination between sera from nonexposed subjects and sera from subjects with industrial exposure to *B. subtilis* in which antibodies of the IgG class were demonstrated by RIEP. The prior addition of NPPB could be expected to block nonimmunologic binding of the labeled NPPA to serum proteins.^{17, 18} Preliminary tests with serial dilutions of test sera in normal human sera revealed a linear relationship between the antibody content and the coprecipitated radioactivity.

RESULTS

Relationship between symptoms and skin test reactions

In intracutaneous tests in the normal population with no industrial exposure to *B. subtilis* products, the wheal diameter mean $\pm$ one standard deviation (S.D.) was 3.5 ± 1.5 mm. for the NPPA and 5.1 ± 1.4 mm. for the NPPB preparation. All tests with the NPPA and NPPB preparations with wheal diameters greater than one S.D. above the normal mean were considered to be positive.

Cutaneous responses to NPPA are illustrated in Fig. 1. There was a high frequency of positive tests only in the group of symptomatic exposed subjects. There were positive tests in two nonexposed subjects and one exposed industrial worker who denied symptoms. Three subjects who claimed to have respiratory symptoms from exposure to the *B. subtilis* products had negative skin tests to NPPA. Results with NPPB were similar. Table I illustrates that 18 of 20 symptomatic workers had immediate reactions to at least one of the antigen preparations.

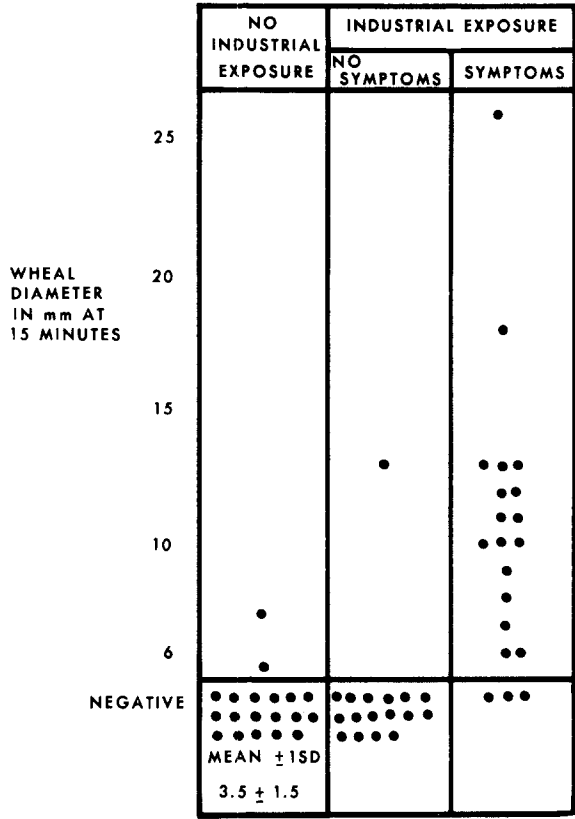


FIG. 1. "Immediate" reactions in intracutaneous tests with the *B. subtilis* preparation, NPPA. The wheal diameters in normal adults with no industrial exposure to *B. subtilis* products and in industrial workers with and without respiratory symptoms are illustrated. A mean wheal diameter greater than 5 mm. at 15 minutes after the injection is taken to indicate a positive test. Almost all symptomatic workers had positive skin tests.

TABLE I. Immediate cutaneous reactions to the *B. subtilis* products NPPA and NPPB

Test	No industrial exposure	Industrial exposure	
		No symptoms	Symptoms
Positive test with A and/or B preparation	3	3	18
Negative test with A and B preparations	16	14	2

There was a close relationship between positive skin tests and the presence of symptoms.

Responses were present at 6 to 8 hours at the sites of 75 per cent of the skin tests which exhibited immediate reactions but were almost invariably absent at sites at which no immediate reaction occurred. The relationship between immediate reactions and 6 to 8 hour responses to NPPB is illustrated in Fig. 2. No 6 to 8 hour responses were identified in nonexposed subjects. There were similar results in tests with NPPA. There was no apparent relationship between the presence or absence of 6 to 8 hour responses and a predominance of nasal symptoms or of chest symptoms.

In one subject, immediate reactions to NPPA and NPPB subsided within

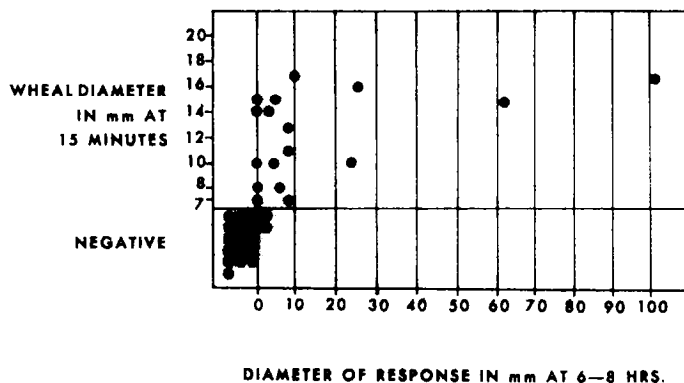


FIG. 2. Immediate reactions and "6 to 8 hours responses" to NPPB. Mean diameters of the wheals are plotted on the vertical axis and 6 to 8 hours responses on the horizontal axis. Responses present at 6 to 8 hours after intracutaneous injection of antigen occurred at the sites of 15 of 18 immediate reactions. In the absence of immediate reactions, there were generally no 6 to 8 hour responses.

2 hours and were followed on the second day by large red indurated reactions which gradually subsided over a period of 7 to 10 days. He claimed that shortly after industrial exposure to "enzymes" there were nasal symptoms which subsided over a period of hours, but he did not develop respiratory symptoms which corresponded in time to the delayed reactions observed at the skin test sites.

Relationship between serum antibodies and skin test results

Tests for precipitins were positive in approximately one third of the serum specimens from persons with and without industrial exposure to *B. subtilis* preparations. Results did not relate to the presence or absence of serum antibody as determined by labeled antigen-binding techniques. The lines did not have the sharp quality of usual immunologic precipitin lines and were not observed in washed, stained preparations.

Results of tests for serum antibodies to NPPA of the IgG, IgA, and IgM classes in normal subjects, in nonexposed asthmatic subjects, and in the pre-selected population of exposed industrial workers are illustrated in Table II. Tests for the presence of antibody of the IgG class were performed by RID and RIEP. The ^{125}I labeled NPPA binding by IgG of the serum of one normal and one nonexposed asthmatic subject was confirmed by repeated tests. Neither had cutaneous reactivity to NPPA. The method of selection of subjects (see Materials and methods) explains the presence of antigen binding by the IgG of the sera of 11 of 18 exposed industrial workers with no cutaneous responses to NPPA; this high frequency does not represent the prevalence in the total industrial population.¹⁹ There was antigen binding to the serum IgG of 13 of 14 subjects with both immediate and 6 to 8 hour responses. Tests with one serum specimen were repeatedly negative. In the sera of 3 of 4 subjects with immediate reactions but no 6 to 8 hour responses, antibodies of the IgG class to NPPA were present. Antibodies of the IgA and IgM classes to NPPA (Table II) and NPPB were not demonstrable in most of the sera. In the coprecipitation

TABLE II. Serum antibodies to NPPA

	Patient	Coprecipitation with IgG*	Radioimmunodiffusion		
			IgG	IgA	IgM
Immediate and 6-8 hour cutaneous responses	T. B.	147	+	+	+
	J. C.	78	+	+	-
	A. G.	777	++	+	-
	D. H.	89	+	+	-
	P. H.	451	++	-	-
	K. L.	673	+++	+	-
	E. S.	70	+	-	-
	W. W.	149	+	-	-
	G. Y.	501	++	-	-
	B. C.	ND	+	-	-
	W. M.	127	+	-	-
	G. P.	202	+	+	+
	J. S.	90	-	-	-
	R. T.	151	+	-	-
Immediate reaction only	4†	54-420	3/ 4‡	2/ 4	0/ 4
No reaction	17	74-450	11/18	6/18	0/18
Nonexposed asthmatic patients		ND	1/39	0/36	0/36
Nonexposed normal subjects		Mean $\pm$ 2 S.D. 114 $\pm$ 47.5	1/22	ND	ND

The results illustrated are of tests of antibodies in the sera of the selected population of exposed industrial workers who exhibited immediate and 6 to 8 hour, immediate only, and no responses in intracutaneous tests with NPPA and of nonexposed normal and asthmatic subjects. Due to the method of selection of the test population (see Materials and methods), the results do not indicate the prevalence of serum antibodies in the total industrial population. Tests for antibodies of the IgA and IgM classes were by RID and of the IgG by RID and RIEP. Antibodies of the IgG class to NPPA were present in persons with immediate reactions with and without 6 to 8 hour responses. A single subject with an immediate reaction and a large 6 to 8 hour response had no detectable antibody of the IgG class. The mean counts per minute $\pm$ 2 standard deviations for normal sera in the coprecipitation test was 114 ± 47.5 . None of the tests provided a reliable correlate of 6 to 8 hour responses in subjects with immediate sensitivity.

*Counts per minute of coprecipitated ^{125}I -labeled NPPA.

†Number of subjects.

‡Number of sera with detectable antibodies/number tested.

test there was a wide distribution of antigen binding in the sera of exposed industrial workers with immediate and 6 to 8 hour, immediate only, and no cutaneous responses.

Fig. 3 illustrates relationships in skin test-positive subjects between cutaneous responses and serum antibody levels determined by the coprecipitation procedure. In persons with immediate cutaneous reactions to NPPA, there was no apparent relationship between the observed serum levels of antibody to NPPA and the development of 6 to 8 hour responses.

Relationship between proteinase activity of antigen and 6 to 8 hour responses

Skin tests in industrial workers exposed to *B. subtilis* products suggested an apparent dependence of 6 to 8 hour responses on preceding wheal and flare reactions. It was hypothesized that if proteinase activity of the antigen could, in some way, produce 6 to 8 hour responses, this had to occur at the site of wheal and flare reactions. Histamine and bradykinin were used to simulate allergic wheal and flare responses. NPPA and NPPB separately mixed with histamine and used in a total of 7 tests in nonsensitive subjects produced wheal and flare responses in which the mean of the wheal diameters at 15 minutes

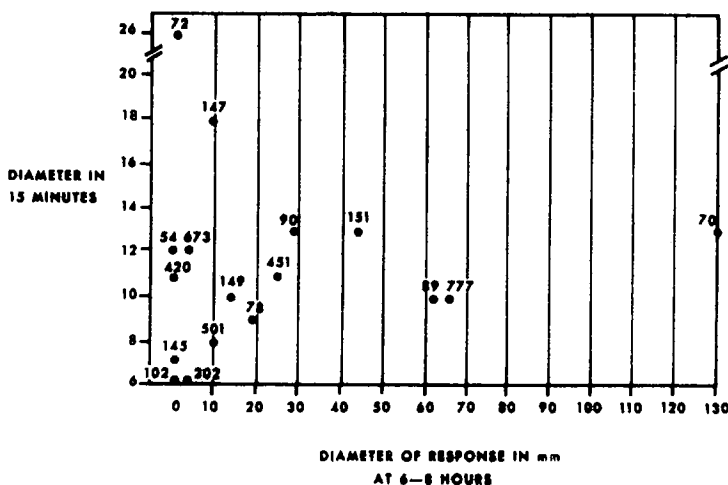


FIG. 3. Coprecipitation test results in subjects with positive skin tests to NPPA. ^{125}I -labeled NPPA was coprecipitated from serum specimens with an antiserum to IgG. The mean counts per minute $\pm$ 2 standard deviations for normal sera was 114 ± 47.5 . The numbers indicate the mean counts per minute of three simultaneous tests performed with the individuals' sera; there was no apparent relationship to the 6 to 8 hour responses.

was 11 mm. The reactions subsided at the same time as reactions induced by histamine alone. A total of 14 tests in 7 nonsensitive subjects were performed with a histamine-bradykinin mixture added to NPPA or NPPB. The mean wheal diameter at 15 minutes was 10 mm. There were no 6 to 8 hour responses.

In order to compare the *B. subtilis* antigen preparations with an antigen which contained no detectable proteolytic activity, P-K tests were performed in two recipients at separate cutaneous sites passively sensitized with a *B. subtilis*-reaginic serum and a ragweed-reaginic serum. The sensitized sites were separately challenged with 0.1 mg. per milliliter solutions of NPPA, NPPB, SPB preparation, *B. subtilis* amylase, and the dialyzed 30,000 PNU per milliliter ragweed antigen solution. Each of the *B. subtilis* preparations was reactive in skin sites sensitized with the *B. subtilis*-reaginic serum and non-reactive in sites sensitized with the ragweed-reaginic serum. Following immediate reactions to the *B. subtilis* and ragweed antigens at sites sensitized with the corresponding reaginic sera, the reactions to *B. subtilis* and ragweed antigens evolved with typical gross appearances of 6 to 8 hour responses (see Materials and methods).

Relationship between IgG antibodies and 6 to 8 hour responses

A mixture of histamine and NPPA was injected intracutaneously in two nonsensitive industrial workers with serum antibodies of the IgG class to NPPA. There were the expected histamine-induced wheal and flare responses but there was no component which could be observed 6 to 8 hours later. Thus, by this method, it was not possible to create conditions for the demonstration of a relevance of circulating antibodies of the IgG class to the 6 to 8 hour responses.

Cutaneous sites of a nonallergic subject were sensitized with a ragweed-

reaginic serum which contained antibody of the IgG class at the limits of detectability and no detectable antibody of the IgM or IgA classes to ragweed antigen E in RID tests. The serum of the recipient contained no detectable antibody to ragweed antigen E. After a latent period of 9 days, the sites were challenged with a 100 μ g per milliliter of solution of ragweed antigen E. There were allergic reactions with 6 to 8 hour components.

DISCUSSION

Intradermal skin tests with *B. subtilis* antigens were negative in the majority of subjects with no industrial exposure to *B. subtilis* preparations and in industrial workers with no symptoms resulting from exposure. In this pre-selected industrial population, the tests were highly sensitive and selective as indicators of symptoms. This relationship between symptoms and cutaneous responses is much closer than was observed in larger surveys of exposed industrial populations.^{7, 8, 19} The close relationship is probably determined by a number of factors. Workers knew when "enzymes" were being processed and were specifically asked if symptoms were present at times corresponding to times of exposure. In addition, the method of selection resulted in the inclusion of workers who were among those with the greatest initial exposure to *B. subtilis* preparations. This might be expected to reduce the frequency of sensitivity unaccompanied by symptoms. Improved industrial methods with resulting reductions in levels of exposure have greatly reduced the frequency of symptoms among sensitized individuals.

Six to 8 hour cutaneous responses more than a few millimeters in size were invariably preceded by immediate reactions. The various tests of serum antibodies failed to provide a reliable correlate of 6 to 8 hour responses in persons with immediate reactions. Apparently positive tests for serum precipitins did not relate to industrial exposure or to the antibodies contained in the area. The observed lines are considered to be nonimmunologic in origin.²⁰ The failure to detect immunologic precipitins could have been due to the proteolytic activity of the preparations or the nonimmunologic binding of the alkaline proteinase antigens.^{17, 18} The use of concentrated sera might possibly have elicited the presence of precipitins in some instances.

The possible relationship of serum antibodies of the IgG, IgA, and IgM classes and 6 to 8 hour responses was examined. Antibodies of the IgA and IgM classes were not detectable in the sera of most sensitive subjects with 6 to 8 hour responses. Antibodies of the IgG class were present in the sera of most of these subjects, but there was no clear relationship between the 6 to 8 hour responses and results of qualitative or quantitative assays of antigen binding by these antibodies.

The proteinase activity of antigen preparations could not be shown to be responsible for 6 to 8 hour responses. Cutaneous reactions to mixtures of *B. subtilis* preparations with histamine and with histamine and bradykinin produced only typical mediator-induced wheal and flare reactions. Passive cutaneous reactions to *B. subtilis* enzyme products and ragweed antigens in which no proteolytic activity could be demonstrated followed time courses which were

similar to each other and to those observed in actively sensitized workers. In both instances, high antigen concentrations were used, and a persistence of antigen might explain the prolonged responses. The absence of a necessary dependence upon proteinase is, however, clearly illustrated by the tests with ragweed antigen.

Intracutaneous injections of histamine mixed with NPPA in nonsensitive workers with serum antibodies to NPPA of the IgG class failed to demonstrate a contribution of these antibodies to 6 to 8 hour responses. Moreover, Prausnitz-Küstner tests in which a latent period of 9 days was allowed prior to antigen challenge exhibited 6 to 8 hour responses. The ragweed-reagin serum used in sensitization and tested by RID and RIEP tests contained detectable antibodies to ragweed antigen E of the IgG class but not the IgA or IgM classes. The long latent period was to reduce the possibility that antibodies of classes other than IgE might be retained at the sites of passive sensitization.^{21, 22}

The described observations illustrate an apparent dependence of 6 to 8 hour responses upon the presence of "tissue sensitization." It was not possible to demonstrate that these responses require the participation of antibodies of multiple immunoglobulin classes. On the contrary, the findings suggest that the evolution of responses over a period of many hours may, in some way, be intrinsic to the type I allergic reactions. There was no direct information relating to the possible participation or nonparticipation of complement or complement-fixing antibody in the cutaneous responses; this would probably best be obtained by immunofluorescent studies of biopsies of cutaneous reactions. However, complement fixation is a property of antibodies of the IgG and IgM classes²³ but not the IgE class.³ Indirect evidence based upon assays of antibodies of the various classes suggest that the prolonged cutaneous allergic responses observed do not require a complement-dependent component. Even if this is the case, it does not mitigate the relevance of complement-dependent processes in "late" allergic responses. Indeed, these prolonged cutaneous responses which appear to be reagin-dependent might, in fact, be the necessary condition for the superimposition of additional immunopathologic processes. Regardless, from a practical standpoint, it would presently appear that it is not possible to assume a simple and direct relationship between allergic responses which evolve over a period of hours and the presence of serum precipitins or the involvement of complement-dependent Arthus reactions.

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REFERENCES

- 1 Pepys, J.: Skin tests in diagnosis, in Gell, P. G. H., and Coombs, R. R. A., editors: *Clinical aspects of immunology*, ed. 2, Oxford, 1968, Blackwell Scientific Publications, p. 189.
- 2 Coombs, R. R. A., and Gell, P. G. H.: Classification of allergic reactions responsible for

- clinical hypersensitivity and disease, in Gell, P. G. H., and Coombs, R. R. A., editors: Clinical aspects of immunology, ed. 2, Oxford, 1968, Blackwell Scientific Publications, p. 575.
- 3 Ishizaka, K., and Ishizaka, T.: Biological function of γ E antibodies and mechanisms of reaginic hypersensitivity, Clin. Exp. Immunol. **6**: 25, 1970.
 - 4 Pepys, J.: Hypersensitivity diseases of the lungs due to fungi and organic dusts. Monographs in allergy, vol. 4, Basel, 1969, S. Karger AG, p. 39.
 - 5 Salvaggio, J. E.: Hypersensitivity pneumonitis: "Pandora's box," N. Engl. J. Med. **283**: 314, 1970. (Edit.)
 - 6 Flindt, M. L. H.: Pulmonary disease due to inhalation of derivatives of *Bacillus subtilis* containing proteolytic enzyme, Lancet **1**: 1177, 1969.
 - 7 Greenberg, M., Milne, J. F., and Watt, A.: Survey of workers exposed to dust containing derivatives of *Bacillus subtilis*, Br. Med. J. **2**: 629, 1970.
 - 8 Newhouse, M., Tagg, B., and Popecock, S. J.: An epidemiological study of workers producing enzyme washing powders, Lancet **1**: 689, 1970.
 - 9 Shapiro, R. S., and Eisenberg, B. C.: Sensitivity to proteolytic enzymes in laundry detergents, J. ALLERGY **47**: 76, 1971.
 - 10 Franz, T., McMurray, K. D., Brooks, S., and Bernstein, I. L.: Clinical, immunologic, and physiologic observations in factory workers exposed to *B. subtilis* enzyme dust, J. ALLERGY **47**: 170, 1971.
 - 11 Pepys, J., Hargreave, F. E., Longbottom, J. L., and Faux, J.: Allergic reactions of the lungs to enzymes of *Bacillus subtilis*, Lancet **1**: 1181, 1969.
 - 12 Guntelberg, A. V., and Ottesen, M.: Purification of the proteolytic enzyme of *Bacillus subtilis*, C. R. Trav. Lab. Carlsberg **29**: 36, 1954.
 - 13 Ottesen, M., and Spector, A.: A comparison of two proteinases from *Bacillus subtilis*, C. R. Trav. Lab. Carlsberg **32**: 63, 1960.
 - 14 Debanne, M. T., Wicher, V., and Dolovich, J.: Antigenic compositions of enzyme-containing preparations of *Bacillus subtilis* origins, J. ALLERGY **48**: 212, 1971.
 - 15 Dolovich, J., Tomasi, T. B., Jr., and Arbesman, C. E.: Antibodies of nasal and parotid secretions of ragweed-allergic subjects, J. ALLERGY **45**: 286, 1970.
 - 16 McConahey, P. J., and Dixon, F. J.: A method of trace iodination of proteins for immunologic studies, Int. Arch. Allergy Appl. Immunol. **29**: 185, 1966.
 - 17 Wicher, V., and Dolovich, J.: Interactions of *Bacillus subtilis* alkaline proteinases with α_2 -macroglobulin and α_1 -antitrypsin, Int. Arch. Allergy Appl. Immunol. **40**: 779, 1971.
 - 18 Dolovich, J., and Wicher, V.: The binding of *Bacillus subtilis* alkaline proteinases to α_2 -macroglobulin, J. Lab. Clin. Med. **77**: 951, 1971.
 - 19 Dolovich, J., and Little, D. C.: Unpublished observations.
 - 20 Wicher, V., and Dolovich, J.: Proteinase-dependent false positive precipitin tests, J. ALLERGY **49**: 59, 1972.
 - 21 Fireman, P., Boesman, M., and Gitlin, D.: Disappearance of intradermally administered plasma immunoglobulins and skin-sensitizing antibodies, J. ALLERGY **40**: 304, 1967.
 - 22 Ishizaka, K., Ishizaka, T., and Tomoika, H.: Demonstration of passive sensitization of monkey mast cells by IgE, J. ALLERGY **47**: 89, 1971. (Abst.)
 - 23 Ishizaka, T., Ishizaka, K., Borsos, T., and Rapp, H.: C'I fixation by human isoagglutinins: Fixation of C'I by γ G and γ M but not by γ A antibody, J. Immunol. **97**: 716, 1966.