

Studies on Bacterial Hypersensitivity in Man

Interrelationship between Skin Reactions to Bacterial Peptidoglycan and Serum Peptidoglycan Antibody Titers

J. V. MAYENBURG,¹ B. HEYMER,² H. DÜNGEMANN,¹ K. H. SCHLEIFER,³
P. H. SEIDL,³ A. NEIB⁴ and S. BORELLI¹

¹Dept. of Dermatology, Technical University of Munich, ²Dept. of Pathology, University of Ulm,

³Dept. of Microbiology, Technical University of Munich, ⁴Institute of Medical Statistics and Epidemiology, Technical University of Munich, West Germany

The nature and extent of bacterially induced allergies are difficult to define. Since peptidoglycan, the main component of the cell wall of almost all bacteria, has been available in a highly purified, chemically and immunologically well-defined form, investigation of the allergological significance of this cell component is feasible. Intracutaneous tests were carried out on 181 test subjects with five different peptidoglycan (PG) preparations from *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*. The results of the investigation were compared with the result of determination of serum PG antibodies and serum IgE concentrations. It was shown that test subjects with dual and late reactions to three different staphylococcal PGs displayed significantly higher PG antibody titers than test subjects with negative reactions. Such a relationship could not be found with the cutaneous reactions to streptococcal PG. The total serum IgE values were very much higher in test subjects with immediate reactions to staphylococcal PG than in test subjects with a negative reaction. Typical Arthus reaction or late granulomatous reactions were not observed. Humoral antibodies are involved at least in part in the elicitation of dual and late reactions. Thus, there are interesting parallels to allergy to fungal spores and organic dusts.

Key words: allergy to staphylococci and streptococci; bacterial cell wall; bacterial hypersensitivity; inflammation; intracutaneous test; peptidoglycan.

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CLINICAL ASPECTS

Hypersensitivity to cell wall antigens of staphylococci and streptococci is assumed to be an important pathogenetic factor in a series of diseases, e.g. infectious asthma, rheumatic fever, allergic vasculitis, erythema nodosum, microbial eczema, etc. Allergological research has recently devoted increasing attention to the complex reactions (type I–type IV) to mold spores and organic dusts, which has already been of use in practical allergology. Similar results may be expected from employment of defined bacterial substances.

In the present investigations, it was observed that there is a connection between raised levels of precipitating antibodies to PG and dual or late skin reactions to three different staphylococcal PG in the vast majority of the test subjects. From this it follows that PG skin tests and PG antibody assays in patients exhibiting symptoms of a bacterially induced type III reaction should be further investigated for their diagnostic utility and should be supplemented by additional methods. Test dose, particle size, degree of purity and immunochemical characterization of the test substances used are of crucial importance for the success of such investigations.

Allergic reactions to somatic antigens of staphylococci and streptococci have been investigated intensively in man only by a few authors so far (23, 25, 27, 29, 30, 31, 36, 38, 55, 56, 57, 62). The cell wall components of these bacteria constitute an exceedingly heterogeneous biologically and immunologically active material which induces complex reactions difficult to interpret (18, 32, 38, 55, 58, 63, 65).

New information can be expected from the use of isolated, chemically and immunologically well-defined bacterial components (31, 36, 55, 56). The cell wall skeleton of almost all bacteria consists of peptidoglycan (PG) (47). In Gram positive bacteria, PG constitutes 30–70%, and in Gram negative bacteria less than 10% of cell wall dry weight (46, 49). The chemical structure of many bacterial PGs is known today in all details (42, 43, 46, 49). With appropriate methods, it is possible to isolate PG in a highly purified form (24, 49). It is known from experimental investigations in animals that PG possesses both nonspecific toxic (1, 11, 17, 41) and immunologic properties (10, 19, 20, 24, 44, 45, 46, 48, 49). Thus, humoral antibodies to PG are formed by various animal species (13, 20, 21, 39). Antibodies to PG have also been demonstrated in numerous human sera, in substantial concentration (15, 16)). It is unknown whether these antibodies have a protective or rather a pathogenic effect. However, it is possible in principle that precipitating antibodies against PG induce allergic reactions which correspond to the type III reactions in the lung to fungal spore and organic dusts (7, 34, 35, 36).

In order to clarify this question, the effects of highly purified PG in the intracutaneous test was checked in patients with various respiratory tract and skin diseases. The cutaneous reactions observed from 20 min up to 48 h after injection were analyzed macroscopically, histologically, immunohistologically and electron microscopically. The results of these investigations have already been reported in detail elsewhere (26). Furthermore, the levels of antibodies against the pentapeptide determinant of PG were determined radioimmunologically in the serum of the test subjects. In the present paper, the result of the comparison of PG anti-

bodies and serum IgE concentrations of the test subjects with the result of the skin reactions will be presented.

MATERIAL AND METHODS

Test subjects

A total of 181 test subjects (92 female, 89 male, aged 12 to 87 years, mean: 37 years) were investigated with various bacterial preparations (see below) in the intracutaneous test. 163 test subjects were patients with respiratory tract diseases (asthma, rhinitis) or skin diseases (atopic dermatitis, urticaria, eczema) from the Allergy Division of the Department of Dermatology, Technical University of Munich. A group of 18 probands constituted clinically healthy control subjects. The patients were subdivided into four groups according to the cause of disease (26): 66 with atopic diathesis, 34 with chronic bacterial infections, 9 with atopic diathesis and chronic bacterial infections, plus 52 without one of the specified diseases. In addition, two patients with primary immunodeficiency were investigated. In all, serum samples were available from 152 test subjects for determination of the PG-(pentapeptide) antibody and total serum IgE levels.

Bacterial test preparation

The bacterial preparations used in the present study were obtained from the following strains: *Staphylococcus aureus* Cowan I, *Staphylococcus epidermidis* CCM 2495, *Streptococcus pyogenes* B 196. Isolation of cell walls, enzymatic treatment of the cell wall preparation with pepsin, trypsin and ribonuclease as well as the subsequent extraction of staphylococcal PG with trichloroacetic acid (46, 49) or of streptococcal PG with hot formamide (12, 13) was performed as already described in detail in an earlier publication (11, 42, 46, 49). The amino acid and amino sugar composition of all PG preparations was likewise determined as communicated earlier (17, 19, 42, 43). A contamination of the PG pre-

parations by other cell wall constituents could be excluded (11, 47). The lyophilized PG preparations were suspended in saline and brought into microsuspension by ultrasonication (12, 13). The solutions used in the skin test were prepared by dilution with 0.007 M PBS with addition of 0.45% phenol (26).

The following preparations and substance amounts were employed in the intracutaneous test:

1. PG-Sa = PG from *Staphylococcus aureus* Cowan I, test dose 10 µg;
2. PG-Se = PG from *Staphylococcus epidermidis* CCM 2495, test dose 1.4 µg;
3. PG-Str.F = formamide extracted PG from *Streptococcus pyogenes*, group A, type 17, strain B 196, test dose 1.3 µg;
4. PG-StrNaOH = as 3), additionally treated with dilute NaOH for removal of the O-formyl groups introduced into the PG by the formamide extraction (8, 49), test dose 1.4 µg;
5. RCW-Sa = ultrasonicated raw cell wall of *Staphylococcus aureus* Cowan I, test dose 20 µg;
6. PG-TA-Sa = PG-Teichoic acid complex obtained from preparation No. 5 by removal of protein A with trypsin, test dose 20 µg.

Intracutaneous test

The various test substances were injected i.c. in 0.02 ml volumes on the volar side of the forearms. The appropriate test doses (see above) had previously been determined in self-investigations. With the amounts of substance used (1.3–10 or 20 µg) no obligatory toxic cutaneous reactions were induced. The PG doses (1.3–10 µg) were mostly several powers of 10 below that employed in animal experiments (1–2 mg; (1, 11, 40). The diameters of the reactions were measured at 20 min, and 6, 24, 48 and 72 h after injection. In addition, the reactions were continuously registered in 25 test subjects every 60 min over a period of 48 h. The diameters of the weal, the erythema and the infiltrate were determined in each case. The "immediate reaction" was expressed as quotient of diameter of

the test reaction and histamine reaction (relative skin reaction = rsr; (53). Two threshold values were used for classification as positive or negative: in early reactions 0.4 or 0.28 rsr; in 6-h and late reactions in 12 or 6 mm.

Determination of peptidoglycan antibodies

Bacterial peptidoglycans possess several antigenic determinants (19, 20, 21, 24, 45, 47, 50). However, according to animal experiments the majority of PG antibodies are directed against the pentapeptide L-Ala-D-Glu-L-Lys-D-Ala-D-Ala which occurs especially in staphylococci and streptococci (21, 45, 47). This pentapeptide was synthesized (45, 47) and radioactively labelled with ¹²⁵I (14). In the present investigations, the binding capacity of the sera for ¹²⁵I-pentapeptide was determined as a measure of the PG antibody content (14, 16). The binding capacity of the sera expressed as percentage of the amount of ¹²⁵I-pentapeptide added was then converted into micrograms pentapeptide antibody per ml of serum by means of a standard antiserum with known antibody content (9). The amounts of antibody found were far in excess of the quantities of IgE and are hence likely to belong chiefly to the classes IgG and IgM. It was already observed earlier that the pentapeptide antibodies demonstrated radioimmunologically in human sera possess precipitating activity (16).

Total IgE determinations

Serum from each test subject was assayed for total IgE content with the Phadebas IgE kit (Pharmacia Diagnostics).

Statistical methods

For comparison of the titer values of the individual groups, the Wilcoxon test for unlinked random samples was used. The calculations were carried out with the program packet SPSS on the CDC Cyber equipment of the Leibniz Computer Center, Munich.

Table 1

Frequency of skin reactions of immediate type (20 min), late type (24–48 h), dual type (20 min + subsequent 24–48 h), of 6-h reactions (without other reactions) and of negative reactions to bacterial cell-wall antigens

Reaction type	RCW-Sa		PG-TA-Sa		PG-Sa		PG-Se		PG-Str. NaOH		PG-Str. F	
Immed. type	111	62	74	41.6	59	33.1	38	35.2	12	11.1	22	20.4
Late type	4	2.2	14	7.9	14	7.9	6	5.6	26	24.1	25	23.1
Dual type	17	9.5	29	16.3	22	12.4	11	10.2	40	37.0	40	37.0
6 h reaction	4	2.2	3	3.9	7	3.9	1	0.9	4	3.7	1	0.9
Negative reaction	43	24.0	54	30.4	76	42.7	52	48.1	26	24.1	20	18.5
Total	179	100	178	100	178	100	108	100	108	100	108	100

For explanation of abbreviations of the various antigens employed, see Material and Methods.

RESULTS

Cutaneous reactions

After i.c. application of the individual bacterial test preparations, cutaneous reactions occurred only in a proportion of the test subjects (Table 1). Reactions already manifested 20 min after injection were classified as "immediate reaction". However, it should be stressed that an interpretation in the immunopathological sense is not entailed by this designation. Furthermore, in the following hours after injection, erythematous-edematous, central infiltrative reactions painful to pressure were observed. These developed either subsequent to an immediate reaction ("dual reaction") or without a prior immediate reaction in the course of 6–24 h after injection ("late reaction"). Remarkably, these late inflammatory reactions, which were in the majority of cases most pronounced 24 h after injection, already subsided 48–72 h after injection. Thus for example, after injection of PG-Sa, an infiltration with an erythema of 12 mm diameter or more was still present in only eight out of 178 test subjects 48 h after injection. A residual erythema or residual infiltrate of 5 to 10 mm diameter was still found in 21 test subjects at this time. Three days after injection, all reactions had subsided as far as

could be evaluated macroscopically. Isolated 6-h reactions were observed extremely rarely (Table 1). Six-hour reactions (12 mm or more) occurred together with other reactions in only 3–7% of the test subjects. A typical Arthus reaction with hemorrhage and tissue necrosis could not be demonstrated in any cases. Granulomatous late reactions, which were observed in animal experiments days up to weeks after i.c. application of PG-carbohydrate polymers in relatively high concentrations (54), did not occur.

Correlation between PG-induced cutaneous reactions and serum PG antibody levels

To detect possible interrelationships between the PG antibody concentration in the serum of the test subjects and the cutaneous reactions induced by PG, groups of test subjects were formed according to the reaction type in the i.c. test. The results of these test subject groups were compared with the aid of statistical techniques. Significant correlations between skin reaction and serum antibody level could be demonstrated only in the reactions induced by staphylococcal PG (PG-Sa, PG-TA-Sa, PG-Se). Considerably higher pentapeptide antibody titers were consistently found in test subjects

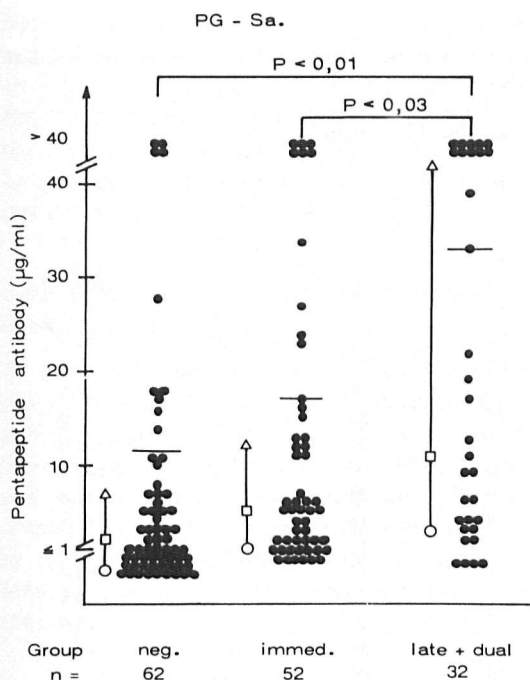


Fig. 1. PG (pentapeptide-) antibody titers in groups of test subjects with negative, immediate and late or dual cutaneous reactions to PG from *Staphylococcus aureus* Cowan I (PG-Sa). $\circ$ = 25%; $\square$ = 50%; $\triangle$ = 75% (fractiles) of the test subjects. — = mean values.

with dual and late reactions to all three preparations as compared to test subjects with negative reactions. Fig. 1 shows the pentapeptide antibody levels (single values, mean values, 25% fractiles) of the subjects tested with PG-Sa subdivided into three groups according to the reaction type (negative, immediate, late and dual). A large scatter of the values is evident from the Figure. However, in the Wilcoxon test a statistically significant difference ($P < 0.01$) of the PG antibody concentration between test subjects with negative and test subjects with dual or late reactions could be demonstrated.

The latter group of test subjects showed significantly higher pentapeptide antibody titers. On the other hand, surprisingly, no difference in antibody levels was found between test subjects with a positive 6-h reaction and test subjects with a negative 6-h reaction. With PG-TA-Sa (which apart from PG also contains teichoic acid), the group with dual or late reactions also

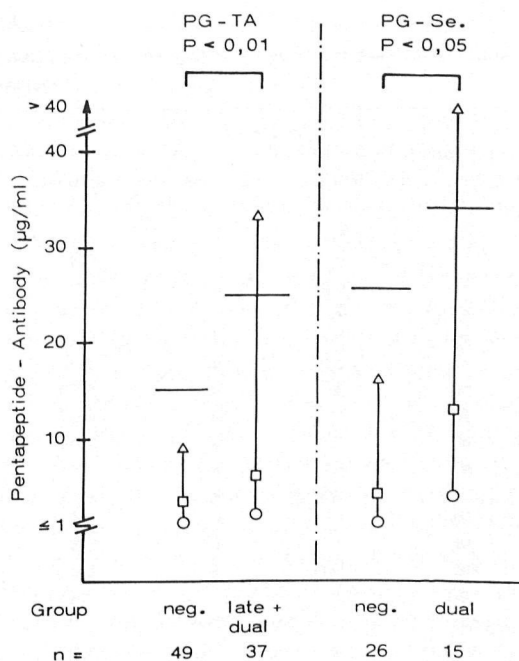


Fig. 2. PG (pentapeptide-) antibody titers in groups of test subjects with negative and late or dual cutaneous reactions to PG teichoic acid complex (PG-TA-Sa) and PG from *Staphylococcus epidermidis* (PG-Se). Representation of the 25% fractiles and mean values. For explanation of the abbreviations and symbols, see legend to Fig. 1.

showed higher titers ($P < 0.01$) than the group with negative reactions (Table 2). In the subjects tested with PG-Se, the group with dual cutaneous reactions likewise displayed higher antibody concentrations (Fig. 2). In contrast to the staphylococcal peptidoglycans, no correlation between cutaneous reaction and pentapeptide antibody concentration of the test subjects concerned could be demonstrated with the streptococcal peptidoglycans. No difference in the titer between the groups was shown as expected even with raw cell wall material (RCW-Sa) from *Staphylococcus aureus* Cowan I. In the patient with immunodeficiency (26), no pentapeptide antibodies could be demonstrated; after injection of staphylococcal PG, no cutaneous reactions developed. On the other hand, the two patients showed slight 24 h reactions against streptococcal peptidoglycans.

In Table 2, the data from 18 selected test subjects with extremely intense late reactions

Table 2

Comparison of skin reactions and PG (pentapeptide-) antibody titers of 18 test subjects with very intense late and dual reactions to PG-Sa

Proband No.	Diagnosis		Skin reaction Ø (mm)				Titer µg/ml
	Sympt.	Cause	20 min.	6 h	24 h	48 h	
4	b.	i.	0.27	10	25	—	1.7
5	b.	a.i.	0.33	10	22	—	8.5
11	b.r.	a.i.	0.26	7	30	—	190.0
39	b.	i.	—	25	30	—	2.6
45	b.r.	a.	0.48	25	20	16	44.3
48	r.	a.	1.0	25	20	10	9.6
50	b.	a.	0.53	—	25	—	19.0
74	r.	a.	0.57	20	35	12	< 1.0
83	r.	a.	0.64	—	20	—	< 1.0
88	r.	a.	—	10	20	—	> 200.0
92	nc.	a.	—	9	25	—	21.8
93	r.	i.	0.5	10	18	—	12.2
96	b.	i.	0.4	—	12	30	67.0
109	b.nc.	a.	0.4	4	25	—	88.0
128	r.		0.4	10	30	—	5.8
134	r.	n.n.	1.0	6	25	5	3.9
135	h.	—	1.0	9	35	—	48.5
140	r.	a.	0.27	10	20	10	45.0

Symptoms (sympt.): rhinitis (r.), bronchitis/asthma (b.), neurodermitis constitutionalis (atopic dermatitis) (nc.). Cause: chronic infection (i.), atopic diathesis (a.), neither chronic infection nor atopic diathesis (n.n.), relative skin reaction (r.s.r.).

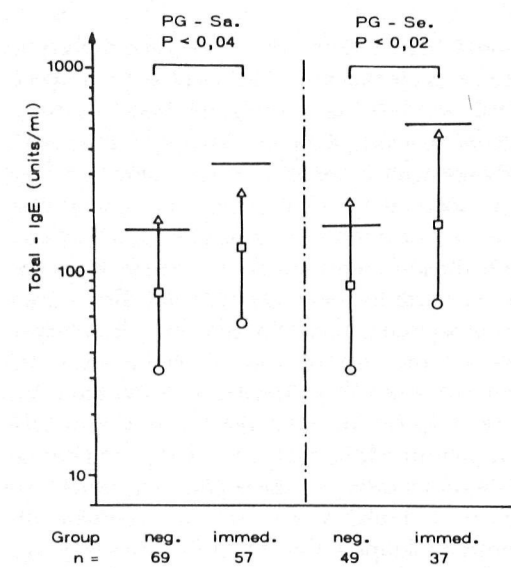


Fig. 3. Total IgE in the groups of test subjects with negative and immediate reactions to PG-Sa and PG-Se. Representation of the 25% fractiles and mean values. For explanation of the abbreviations and symbols, see legend to Fig. 1.

(≥ 18 mm) to PG-Sa are reproduced in a synoptic presentation. This shows that the late reactions reached a maximum 24 h after injection (exception: No. 96), and that in most cases the reactions have already subsided 48 h p.i. Residual erythemas or infiltrates were found only in individual cases at this time. The PG (pentapeptide-)serum antibody concentrations of the test subjects listed in Table 2 were mostly raised markedly (mean: 42.8 µg; median: 12.2 µg) and were statistically (Wilcoxon test) significantly ($P < 0.001$) above those of the group with negative reactions (mean: 12.1 µg/ml; median: 1.9 µg/ml).

Correlations between PG-induced cutaneous reactions and total IgE level

The total IgE concentration in the sera of the test subject groups classified according to reaction type was likewise analyzed. Once more, a significant correlation was shown only with the

staphylococcal peptidoglycans (Fig. 3). An appreciable variation of the values was present in all groups; nevertheless, a statistically significant difference ($P < 0.05$) could be observed in the Wilcoxon test between test subjects with immediate reaction and test subjects with negative reaction.

Correlations between PG (pentapeptide-) antibody levels and the illness of the test subjects

A statistically substantiated correlation could not be detected between the PG antibody titers and the illnesses of the test subjects (1. atopic diathesis, 2. chronic bacterial infections, 3. control group without these conditions). The increased occurrence of late cutaneous reactions in the test subjects with chronic bacterial infections has already been reported elsewhere (26).

DISCUSSION

Cutaneous test reactions with more or less well-defined somatic antigens from staphylococci and streptococci are difficult to interpret from a pathogenetic point of view (23, 31, 32, 37, 56, 62, 63). However, they are of particular interest with regard to bacterial hypersensitivity. The cutaneous reactions induced for the first time in man with purified and defined PG preparations also proved to be complex and difficult to interpret (17, 26). By statistical analysis of a large number of test reactions and comparison with the serum PG antibody titers, however, an insight into the pathomechanisms on which they were based could be obtained.

First of all, the result of our investigation indicates that *immunological factors* are involved in the genesis of at least a proportion of the cutaneous reactions observed. These factors are likely to involve primarily precipitating PG antibodies of the IgG and IgM classes. This is indicated by the significantly higher antibody levels in test subjects with dual or late cutaneous reactions compared to the test subjects with negative cutaneous reactions to PG-Sa, PG-TA-Sa and PG-Se. The trend to higher total IgE values in the groups with isolated immediate

reactions to PG-Sa or PG-Se can also be understood as an indication of a participation of humoral immune factors.

It can be regarded as a further argument for the pathogenetic role of immunological mechanisms that the two patients with antibody deficiency syndromes did not develop any cutaneous reactions to staphylococcal PG. In the cutaneous reactions induced by streptococcal PG, no correlation with the PG (pentapeptide-) antibody titers in the serum could be demonstrated. It was already communicated in an earlier publication (26) that the two staphylococcal peptidoglycans (PG-Sa and PG-Se) induced an identical reaction pattern on the skin and that this pattern differed substantially from that induced by streptococcal PG. This might be connected on the one hand with differences in the primary (chemical or immunological) structure of staphylococcal and streptococcal PG (24, 46), or might on the other hand be due to the different preparation techniques for the two kinds of peptidoglycan (49). Differences in the optimal test dose for PG-Sa and PG-Se determined in skin titrations with rising doses (26) are possibly based on differences in fragment size. Identical preparations with regard to fragment size are difficult to obtain.

The question furthermore arises as to the *kind of immunological reactions* on which the different cutaneous reactions observed are based. IgE antibodies with specificity for PG were found in patients with hyperimmunoglobulinemia E syndrome (52). Demonstration of IgE antibodies to "whole bacterial cell bodies" of staphylococci was communicated by two authors (57, 61). The greatest difficulty in such investigations is to exclude nonspecific binding of IgE to the bacterial antigen preparation concerned (33). In animal studies, immediate reactions (20 min after injection) to PG were investigated by only one author (28) and interpreted as a nonspecific reaction due to an enhancement of vascular permeability by PG.

It is furthermore known that PG is able to activate the complement system via both the classical and the alternative pathway (2, 51, 59, 60, 64). The complement activation mediated by the antigen-antibody complex via the clas-

sical pathway is much more pronounced, however (2). These investigations were also performed in man, employing PG preparations as in the present study (PG-Se; (2)). A correlation between raised PG antibody titers in the serum and positive 6-h cutaneous reactions, which was to be expected according to the observations mentioned above, was surprisingly not observed. The observation of a correlation between raised PG antibody levels and dual or late reactions was thus all the more interesting. The 24-h reaction to PG observed in animal experiments has been regarded up to now as a typical delayed-type hypersensitivity (4, 5, 22). However, in the opinion of other authors (66) 24-h reactions after prior early reactions are not necessarily to be interpreted as delayed-type hypersensitivity.

Our results indicate that PG antibodies participate in the pathogenesis of the 24-h reactions, especially in those with prior immediate reactions. Such 24-h reactions correspond to attenuated type III reactions such as are triggered also in patients with fungal spore allergies by using low doses of allergen extracts of these fungi (35, 36). The prior early reaction observed in the PG investigations, which is of particular importance for the development of type III reactions (3, 36), reinforces this hypothesis. The macroscopic characteristics (35) described for fungal spore type III skin reaction observed also apply to the late cutaneous reactions induced by PG: "soft, often extensive, with ill-defined borders accompanied by an erythematous flare", and "... there may be a small area of central induration" (36).

Unusual in the dual (and late) reaction to PG is the delayed occurrence of the late peak with maximum reaction only after 12 to 24 h. It is also noteworthy that these late reactions to PG had mostly subsided 48 h after injection or were in the process of subsidence. Granulomatous late reactions with a slow regression over days or weeks were not observed. The papular infiltrates regressed after 3 days at the latest. With the low doses of staphylococcal PG used, type III reactions can evidently be triggered, but not pronounced type IV reactions lasting for several days. Accordingly, besides the degree of sensi-

zation to PG and the PG fragment size (12, 22), above all, the test dose used is likely to be of crucial importance for the nature and extent of the reactions. An unequivocal assignment of raised PG antibody titers to one of the groups of test subjects or diseases which we investigated was not possible. However, the results presented here now suggest investigations in patients displaying symptoms giving rise to the suspicion of type III reactions to bacterial antigens.

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Address:

J. v. Mayenburg
Dept. of Dermatology
Technical University of Munich
Biedersteinerstraße 21–29
8 München 40
W. Germany

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