HYPERSENSITIVITY TO PENICILLENIC ACID DERIVATIVES IN HUMAN BEINGS WITH PENICILLIN ALLERGY*, ‡

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In this paper we are concerned with the following question: Are the benzylpenicilloyl and the benzylpenicillenate groups specific determinants of allergic reactions to benzylpenicillin in man?[1] Since simple organic molecules combine covalently with proteins as a preliminary step in the induction of the hypersensitive state, it seems clear that penicillin itself, which is incapable of reacting in the required manner with protein, must be regarded as a precursor of some derivative which possesses this necessary capacity. Penicillenic acid could well be the derivative in question since it forms spontaneously and readily from penicillin in neutral aqueous solution and is a highly reactive molecule capable of coupling with protein sulfhydryl and amino groups forming *S*-penicillenate and *N*-penicilloyl substituents, respectively (1, 2). Moreover, penicillenate- and penicilloyl-protein conjugates, prepared *in vitro* and injected into experimental animals, have been shown to be potent antigens (1). Elicitation of wheal-and-erythema responses in humans with well documented histories of penicillin hypersensitivity seemed to offer the most direct means for evaluating the determinant role of penicillenate and penicilloyl groups. Our preliminary studies in man using penicilloyl- and penicillenate-proteins as test reagents showed an encouraging correlation between positive wheal-and-erythema responses and a

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[1] Benzylpenicillin and its derivatives are used in the studies described in this paper except where otherwise specified. (For structural formulas, see reference 6, Fig. 1 and reference 1, Fig. 1.)

history of penicillin hypersensitivity. Before extending these observations to a larger population it seemed prudent, however, to develop skin test reagents which are as effective as the protein conjugates but which are incapable of inducing antibody formation to these penicillin derivatives. The feasibility of preparing reagents which meet these requirements has been pointed out in a companion paper with reference to the dinitrophenyl system (4).

In the present paper we have examined a series of penicillin derivatives with respect to their propensity for stimulating antibody production in guinea pigs and their effectiveness in eliciting immediate type allergic skin responses in guinea pigs and in humans. Among the penicilloyl derivatives, penicilloyl-polylysine proved to be the most promising reagent for skin testing because of its effectiveness as an elicitor in guinea pig passive cutaneous anaphylaxis and lack of capacity to induce detectable antibody formation, specific for the penicilloyl group. In respect to the penicillenate derivatives, a satisfactory replacement for the protein conjugate has not been prepared. The results of skin testing over 1200 human subjects in a selected population with a high incidence of allergy to penicillin indicates that sensitivity to the penicilloyl determinant is very common in human beings who are allergic to penicillin. Sensitivity to the penicillenate determinant also commonly occurs but with a considerably lower frequency. In this study specific inhibition of positive wheal-and-erythema responses (5) with unifunctional penicilloyl and penicillenate haptens has proven of considerable value in establishing confidence in the specificity of the human skin responses and in the identification of the determinant groups involved.

Materials and Methods

Antigens, Haptens, and Rabbit Antisera.—In preceding papers (1, 6) we have described the preparation of penicilloyl- and penicillenate-proteins, bifunctional and unifunctional haptens, penicilloyl-polylysines, and rabbit antiserum specific for the penicillenate and penicilloyl determinants.[2]

6-aminopenicillanic acid and benzylpenicillin were generously furnished by Chas. Pfizer and Co., Brooklyn, New York.

Immunogenicity.—Each of the derivatives tested for capacity to induce antibody formation (Table I) was given to a group of 5 guinea pigs. Each animal received 1 mg of test material in Freund's adjuvant, distributed among the foot-pads (7, 8). Sera obtained 2 weeks later were assayed for antibody activity by passive cutaneous anaphylaxis in guinea pigs, as described by Ovary (9) and in the accompanying paper (4).

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[2] Abbreviations: HSA, human serum albumin; BγG, bovine gamma globulin; HγG, human gamma globulin; penicillenate S–BγG and penicillenate S–HSA, penicillenate conjugates prepared by reaction of penicillenic acid with BγG and HSA previously treated with *N*-acetyl-DL-homocysteine-thiolactone in order to increase sulfhydryl content; bis-PNCE-MMD, 3,6-bis-(penicillenate-mercurimethyl)-dioxane; penicillamine S–HSA, the product obtained by coupling penicillamine to HSA previously thiolated by reaction with *N*-acetyl-DL-homocysteine-thiolactone.

Purification of Anti-Penicillenate Antibody.—Antibody specific for the penicillenate group was recovered in purified form by 2 different methods from washed specific precipitates formed at equivalence with rabbit anti-penicillenate serum and penicillenate S–BγG (33 penicillenate groups per molecule of BγG). In the 1st method, the specific precipitate was dissolved in 0.05 M ice cold acetic acid and 0.8 volumes of ice cold 2-N HCl were added. The suspension was centrifuged immediately at 11,000 *g* for 3 minutes and the supernatant was dialyzed at 4°C against 0.15 M NaCl–0.01 M phosphate, pH 7.5, or was first partially neutralized with phosphate and then dialyzed. By this procedure antibody was recovered in 40 to 50 per cent yield and was 88 per cent precipitable with penicillenate S–BγG.

In the second procedure, the specific precipitate was dissolved in ice cold 0.05 M acetic acid and 1/4 volume of ice cold 5 M NaCl was added. After centrifugation at 11,000 *g* for 3 minutes, the supernatant was removed and dialyzed without delay at 4°C against 0.15 M NaCl–0.01 M phosphate, pH 7.4. Recovery: 20 per cent. Precipitability with penicillenate S–BγG was 77 per cent.

Both purified antibody preparations were titrated at 30°C with *p*-penicillenate-mercuribenzoate by the fluorometric method previously described (10). In both cases, the association constant found was 3×10^6 liters per mole. Under the titration conditions used, the antibody concentration was 0.24 mg per ml, and 55 per cent of the antibody sites were titrated, assuming (*a*) that the protein preparations were 90 per cent pure, (*b*) that there are 2 combining sites per antibody molecule and (*c*) that the antibody molecular weight is 160,000. Despite the severity of the treatment of the antibody with 0.9 N HCl, papain digestion (11) of the purified product yielded a soluble fraction in 50 per cent yield which did not precipitate with antigen, but which was immunologically active as evidenced both by specific inhibition of precipitation and by fluorometric titration. Moreover, a small amount of crystalline material resembling Porter's fraction III (11) was obtained, approximately in 7 per cent yield.

γ-Globulin Fractions of Human Serum.—γ-Globulins were precipitated from human sera at 4°C with ammonium sulfate, pH 7.4, at a final concentration of 1.75 M. The precipitates were washed twice with 1.75 M ammonium sulfate, dissolved in 0.15 M NaCl–0.01 M phosphate, pH 7.4, and dialyzed for several days in the cold against the latter solvent.

Skin Testing in Human Subjects.—Materials used for testing were diluted with 0.15 M NaCl–0.01 M phosphate, pH 7.4. The final concentration of the stock solutions was determined following sterilization by filtration through Seitz filters. When penicillin and 6-aminopenicillanic acid were used, the solutions were freshly prepared. The penicilloyl-polylysine used for skin testing was the preparation designated B in the companion paper (6), unless otherwise specified.

Intradermal injections were made with a 26 gauge needle, the volume injected being generally about 0.07 ml. The size of the initial bleb was outlined on the skin for reference purposes, and the test sites were examined every 5 minutes for 20 minutes. After 20 minutes the area of urticaria and erythema were marked with a pen and transferred to a transparent tape as a permanent record (5). Subjective symptoms of the subject and impressions of the observers also were recorded.

Preliminary intradermal injections in normal human subjects showed the following compounds to be non-irritant: Penicilloyl-polylysine,[3] penicillenate S–HSA, ε-penicilloyl-aminocaproate, bis-penicilloyl-cystine, ε-penilloaldehyde-aminocaproate, *S*-(*N*-ethylsuccinimidyl)-penicillenate. *p*-Penicillenate-mercuribenzoate gave a slight but significant reaction in 4 out of 7 apparently normal subjects.

Since a substantial number of the individuals evaluated in this study gave unusually marked

[3] The non-specific urticarial responses produced in human skin by DNP-polylysines were eliminated by succinylation (4). The non-irritancy of penicilloyl-polylysines is probably due to their having multiple anionic substituents (6).

skin responses, the system employed elsewhere (5) for scoring wheal-and-erythema responses has been modified as follows: ±, an ambiguous response, the wheal being only slightly larger than the initial injection bleb; 1+, a distinctly positive response, wheal <8 mm in diameter; 2+, wheal, 8 to 12 mm in average diameter; 3+, wheal, 12 to 20 mm in average diameter; 4+, wheal >20 mm in average diameter.

Human Subjects.—More than 90 per cent of the subjects tested were patients at the Venereal Disease Clinic of the St. Louis City Health Division. Many of the patients had received multiple courses of penicillin therapy over a period of years, with an average of about 3 penicillin courses per year per patient. Patients with medical indications for penicillin treatment were routinely tested with penicilloyl-polylysine regardless of their history in respect to previous allergic reactions to penicillin. Skin testing with penicillenate S–HSA was limited to persons with a history of previous allergic reactions to penicillin, and those in whom a positive skin reaction had been evoked with penicilloyl-polylysine.

A history of penicillin allergy was considered reliable if any of the following symptoms had occurred within 1 week of penicillin treatment, provided no other drug had been given: angioneurotic edema, urticaria, maculopapular eruption, or anaphylaxis. At least three-fourths of the persons in the group with a positive history of penicillin sensitivity had been attending the Venereal Disease Clinic at the time their allergic reactions had occurred, and the diagnosis of penicillin allergy had been corroborated by an attending physician. In perhaps one-tenth of the patients listed in this group there was, however, serious doubt as to the validity of their history of penicillin hypersensitivity. The majority of sensitive subjects had had their reactions to penicillin within the preceding 2 years, but in a few instances more than 10 years had elapsed.

In the group with a history of penicillin allergy, penicillin was not given therapeutically. In the group with a negative history for penicillin hypersensitivity, those with a negative skin response to penicilloyl-polylysine were treated with parenteral penicillin, as required by their disease.[4] Many of the individuals who had negative histories for penicillin sensitivity but *positive* wheal-and-erythema skin responses to penicilloyl-polylysine were also treated therapeutically with penicillin: the dosage schedule and general procedures in this group were dictated by the specific medical condition under treatment.

All subjects who were given penicillin were instructed to report to the clinic any symptoms of penicillin allergy. Moreover, as a consequence of the medical follow-up required to insure eradication of disease, many of the tested subjects were seen at a later time, when additional specific inquiries were made with reference to symptoms of penicillin allergy. Previous experience at this clinic indicated that individuals who developed untoward symptoms returned in large measure for diagnosis and treatment of their own volition, even without the specific instructions noted above.

Transfer of Purified Rabbit Anti-Penicillenate Antibody to Human Skin.—The procedures used to sensitize human skin sites with purified rabbit antibody, and to evoke wheal-and-erythema responses in such sites, have been described previously (5). When γ-globulin fractions of human sera were used to sensitize skin sites in normal subjects, the procedure followed was unchanged, except that the sensitized sites were not tested with antigens until 1 to 5 days later.

RESULTS

The capacity of various penicillenate and penicilloyl derivatives to induce antibody formation are given in Table I. Only the protein conjugates were

[4] The penicillin preparations usually employed therapeutically were benzathine–penicillin G (*N*,*N'*-dibenzylethylenediamine dipenicillin G) and penicillin–aluminum monosterate in 2 per cent sesame oil.

effective. The bivalent derivatives and penicilloyl-polylysine had no detectable immunogenic capabilities. As noted in the preceding paper (6), passive cutaneous anaphylaxis responses, with homologous antisera, were evoked feebly with bifunctional derivatives (bis-penicillenate-MMD and bis-penicilloyl-cystine) and intensely with penicilloyl-polylysine.

In human skin sites prepared passively with purified rabbit anti-penicillenate antibody, wheal-and-erythema responses were not evoked with bis-penicillenate-MMD, using 40 mμeq penicillenate, but with 400 mμeq positive responses were obtained. Penicillenate S–HSA elicited a positive response in these

TABLE I

Antibody Formation Induced by Various Penicilloyl and Penicillenate Derivatives as Indicated by Passive Cutaneous Anaphylaxis in the Guinea Pig

Serum obtained from guinea pigs injected with*	Skin responses‡ in guinea pig No.									
	1	2	3	4	5	6	7	8	9	10
bis-PNCE-MMD	0	0	0	0	0					
$Penicillenate_{50}$ S–BγG§	3+	4+	3+	4+	4+					
bis-Penicilloyl-cystine						0	0	0	0	—
Penicilloyl–polylysine A						0	0	0	0	0
Penicilloyl–polylysine B						0	0	0	0	0
$Penicilloyl_{60}$-BγG§						4+	4+	4+	4+	4+

* Each guinea pig received 1 mg of one of the substances listed in Freund's adjuvant (see Materials and Methods). Each recorded response corresponds to an individual animal's serum.

‡ Penicillenate S–HSA and penicilloyl-HγG in 1 mg amounts were used to evoke responses with sera from animals immunized with penicillenate and penicilloyl derivatives, respectively.

§ All sera from animals injected with penicillenate S–BγG and penicilloyl-BγG gave strongly positive ring precipitin tests with penicillenate S–HSA and penicilloyl-HγG respectively, using 50 μg antigen/ml. All other sera gave negative ring precipitin tests.

passively prepared sites when injected at a level corresponding to 5.6 mμeq penicillenate. Thus, the bivalent compound was much less effective than the protein conjugate as an elicitor of the wheal-and-erythema response, even allowing for the fact that the antibody specificity in this instance may be directed in part toward the *N*-acetyl-DL-homocysteine groups which are present in the protein conjugate but not in the bifunctional penicillenate derivative (6).

1250 individuals were skin tested with penicilloyl-polylysine, and of these, 64 were also tested with penicillenate S–HSA. Of the entire group, 15 subjects with well established penicillin allergy have been studied in detail. The responses in 5 of these latter subjects, summarized in Tables II to VI, will serve to illustrate the essential features of the responses in the hypersensitive population as a whole.

Penicilloyl-polylysine and penicilloyl-protein conjugates were about equally effective in eliciting wheal-and-erythema responses, and were clearly very much more effective than bis-penicilloyl-cystine (see Table II, subject T. S.; similar results were obtained in 2 other individuals). The failure of penicillenate S–HSA to evoke a response in subject T. S. (Table II) is striking: 1.5 mμeq penicillenate groups were ineffective although the corresponding penicilloyl derivatives were reactive when only 0.0005 mμeq of penicilloyl was injected. 2 other individuals who reacted positively to penicilloyl-polylysine

TABLE II

Wheal-and-Erythema Responses in a Penicillin-Sensitive Subject. Titration of Reagents*

Test No.	Substances injected	Quantity		Result
		(As total weight)	(As penicilloyl or penicillenate)	
		μg	mμeq	
1	Penicillenate S–HSA	6.6	1.5	0
2	Penicilloyl-HγG	3.8	0.4	4+
3	"	0.38	0.04	4+
4	"	0.038	0.004	3+
5	"	0.0048	0.0005	2+
6	Penicilloyl–polylysine A	0.0005	0.0005	2+
7	Penicilloyl–polylysine B	0.00036	0.0005	2+
8	Penicilloyl-BγG	0.0015	0.0005	2+
9	Penicillamine S–HSA	0.002	—	0
10	bis-penicilloyl cystine	0.024	0.05	1+
11	" "	0.0024	0.005	±
12	" "	0.00024	0.0005	0

* Subject: T. S. For passive transfer with subject's serum protein (γ-globulin fraction) and hapten inhibition of transfer reaction, see Table VI.

were tested with this derivative after it had been treated with 2-mercaptoethanol in order to eliminate penicillenate groups (6): the mercaptan-treated penicilloyl-polylysine derivative and the untreated derivative were equally effective.

Approximately 50 per cent of all individuals with positive cutaneous responses to penicilloyl-polylysine failed to react to penicillenate S–HSA, although the comparison in the group as a whole was generally made with equivalent amounts of penicilloyl and penicillenate groups, rather than with the 3000-fold difference illustrated in Table II.

Penicillenate and penicilloyl conjugates are both contaminated by a significant number of penamaldate or penilloaldehyde groups as judged by absorption at 280 mμ (6). The penamaldate:penicillenate ratio is greater in penicillenate S–HSA than is the penamaldate:penicilloyl ratio in penicilloyl-polylysine.

Hence, in testing with equimolar amounts of penicilloyl and penicillenate conjugates (penicilloyl-polylysine and penicillenate S–HSA, respectively), a negative response to the penicillenate-protein conjugate might be construed to mean that groups of the penamaldate-penilloaldehyde type make little or no contribution to the response to penicilloyl-polylysine. As pointed out in the preceding paper, however, the nature of the penamaldate substituents in these 2 kinds of conjugates may well be different (6).

TABLE III

Hapten Inhibition of Wheal-and-Erythema Response to Penicilloyl-Polylysine in Penicillin-Sensitive Subjects

Subject	Test No.	Substances injected	Quantity (as penicilloyl or penicillenate)	Result
			mμeq	
J. A.	1	Penicilloyl–polylysine B	4	4+
	2	Penicilloyl–polylysine B + ϵ-penicilloyl-aminocaproate	4 400	1 to 2+
	3	Penicilloyl–polylysine B + ϵ-penilloaldehyde-aminocaproate	4 400	0
	4	Penicillenate S–HSA	4	1 to 2+
P. L.	1	Penicilloyl–polylysine B	0.7	4+
	2	Penicilloyl–polylysine B + ϵ-penicilloyl-aminocaproate	0.7 100.0	0
	3	Penicilloyl–polylysine B + ϵ-penicilloyl-aminocaproate	0.7 25.0	±
	4	Penicilloyl–polylysine B + ϵ-penicilloyl-aminocaproate	1.7 100.0	±
	5	Penicilloyl–polylysine B + ϵ-penilloaldehyde-aminocaproate	0.7 100.0	0
	6	Penicilloyl–polylysine B + ϵ-penilloaldehyde-aminocaproate	0.7 25.0	1+
	7	Penicilloyl–polylysine B + ϵ-penilloaldehyde-aminocaproate	1.7 100.0	±
	8	Penicilloyl–polylysine B + *N*-ethylsuccinimidyl-penicillenate	0.7 90.0	4+
	9	Penicillenate S–HSA	3.5	0
	10	Benzylpenicillin	2.0	0

Cutaneous responses to penicilloyl-polylysine were readily inhibited by ϵ-penicilloyl-aminocaproate, provided a sufficient excess of the latter univalent hapten was used and the level of penicilloyl-polylysine employed gave a 2 to 3+ response rather than the maximal 4+ response. Partial or complete inhibition with this combination of univalent hapten and penicilloyl-polylysine was obtained in 39 out of the 43 subjects in whom it was attempted (*i.e.*, gen-

erally with penicilloyl-polylysine as 4 mμeq penicilloyl and 400 mμeq ε-penicilloyl-aminocaproate). In 4 subjects, little or no inhibition was obtained; but in these no attempt was made to reduce the penicilloyl-polylysine to a level more favorable for demonstrating inhibition by the univalent hapten. ε-Penicilloyl-aminocaproate was clearly more effective as an inhibitor in some sub-

TABLE IV

*Hapten Inhibition of Wheal-and-Erythema Response to Penicilloyl-Polylysine and Penicillenate S-HSA in a Penicillin-Sensitive Subject**

Test No.	Substances injected	Quantity (as penicilloyl or penicillenate)	Result
		mμeq	
1	Penicilloyl–polylysine B	0.7	3+
2	Penicilloyl–polylysine B +	0.7	0
	ε-penicilloyl-aminocaproate	100.0	
3	Penicilloyl–polylysine B +	0.7	0
	ε-penilloaldehyde-aminocaproate	100.0	
4	Penicilloyl–polylysine B +	0.7	2+
	N-ethylsuccinimidyl-penicillenate	360.0	
5	Penicilloyl–polylysine B +	0.7	3+
	N-ethylsuccinimidyl-penicillenate	90.0	
6	Penicillenate S–HSA	0.7	3+
7	Penicillenate S–HSA +	0.7	2+
	ε-penicilloyl-aminocaproate	400.0	
8	Penicillenate S–HSA +	0.7	2+
	ε-penilloaldehyde-aminocaproate	400.0	
9	Penicillenate S–HSA +	0.7	0
	N-ethylsuccinimidyl-penicillenate	360.0	
10	Penicillenate S–HSA +	3.5	0
	N-ethylsuccinimidyl-penicillenate	360.0	
11	Penicillenate S–HSA +	0.7	2+
	p-penicillenate-mercuribenzoate	400.0	
12	*p*-Penicillenate-mercuribenzoate	400.0	2+
13	bis-Penicillenate-MMD	1.2	0
14	Benzylpenicillin	2.0	1+

* Subject: S. G.

jects than in others. For example, in subject J. R. (not illustrated in a Table), complete inhibition of the cutaneous response to penicilloyl-polylysine was produced by this univalent hapten in 35-fold molar excess over the penicilloyl groups in the polylysine derivative; and considerable inhibition with hapten excesses of this order of magnitude have been the rule where titrations were carried out, rather than the exception. With subject J. A., however, a 100-fold molar excess of ε-penicilloyl-aminocaproate over the penicilloyl residues in the polylysine conjugate only partially inhibited the cutaneous response (see

Table III); in this same subject, ϵ-penilloaldehyde-aminocaproate completely inhibited the response to penicilloyl-polylysine. As a general rule, however, penilloaldehyde-aminocaproate was about equal in effectiveness to penicilloyl-aminocaproate in respect to inhibition of responses evoked with penicilloyl-polylysine. Quantitative comparison between these two unifunctional inhibitors has not been attempted on an extensive scale since detailed interpretation of the inhibition is limited by a number of unevaluated side reactions; for example

TABLE V

*Hapten Inhibition of Wheal-and-Erythema Response to Penicilloyl–Polylysine and Penicillenate S–HSA in a Penicillin-Sensitive Subject**

Test No.	Substances injected	Quantity (as penicilloyl or penicillenate)	Result
		mμeq	
1	Penicilloyl–polylysine B	0.7	2+
2	Penicilloyl–polylysine B + ϵ-penicilloyl-aminocaproate	0.7 400.0	0
3	Penicilloyl–polylysine B + ϵ-penilloaldehyde-aminocaproate	0.7 400.0	0
4	Penicilloyl–polylysine B + *N*-ethylsuccinimidyl-penicillenate	0.7 360.0	2+
5	Penicillenate S–HSA	0.7	2+
6	Penicillenate S–HSA + ϵ-penicilloyl-aminocaproate	0.7 400.0	1+
7	Penicillenate S–HSA + ϵ-penilloaldehyde-aminocaproate	0.7 400.0	±
8	Penicillenate S–HSA + *N*-ethylsuccinimidyl-penicillenate	0.7 360.0	2+
9	Penicillenate S–HSA + *p*-penicillenate-mercuribenzoate	0.7 400.0	2+

* Subject: J. M.

(*a*) the possibility that the penilloaldehyde hapten might react with free amino groups in the skin, and (*b*) the possibility that one or both of these inhibitors are bound to a considerable degree by proteins in skin, *e.g.*, by serum albumin. As expected (6), unifunctional penicillenate haptens are, at best, poor inhibitors of responses evoked by penicilloyl-polylysine (see Tables III, IV, and V). 6-aminopenicillanic acid failed also to inhibit responses to penicilloyl-polylysine in each of 4 subjects in whom this was tested (see also Fig. 5 in reference 6).

Ten subjects have been tested in respect to the capacity of *S*-(*N*-ethyl-succinimidyl)-penicillenate to inhibit wheal-and-erythema responses evoked with penicillenate S–HSA. In 4 of the 10, inhibition was essentially complete, in 3 it was partial, and in 3 there was little or no inhibition. In 2 of 4 subjects

with complete inhibition by *S*-(*N*-ethylsuccinimidyl)-penicillenate, hapten inhibition of the response to penicillenate S–HSA was also attempted with ϵ-penicilloyl-aminocaproate and ϵ-penilloaldehyde-aminocaproate and, as expected, the latter haptens were ineffective inhibitors (see illustrative group of responses in Table IV, S. G.). The responses of J. M., however, (Table V), illustrate the converse situation, where unifunctional penilloaldehyde and penicilloyl haptens inhibited more effectively than *S*-(*N*-ethylsuccinimidyl)-penicillenate. Thus despite its promise as a monospecific test reagent for penicillenate sensitivity as indicated by passive cutaneous anaphylaxis studies (6),

TABLE VI

*Wheal-and-Erythema Responses Following Passive Transfer of Serum Globulins from a Penicillin-Sensitive Subject**

Test No.	Test substance	Quantity (as haptenic sites)	Result
		mμeq	
1	Penicilloyl–polylysine B	4	3+
2	Penicilloyl–polylysine B + ϵ-penicilloyl-aminocaproate	4 1040	0
3	Penicilloyl–polylysine B + ϵ-penicilloyl-aminocaproate	4 104	0
4	Penicilloyl–polylysine B + ϵ-penilloaldehyde-aminocaproate	4 600	0
5	bis-penicilloyl cystine	40	±
6	Benzylpenicillin	10	±

Donor: T. S. (see Table II for active responses of this subject).

Recipient: C. W. P.

* Sites were prepared with 0.1 ml of a γ-globulin fraction containing 5 mg protein in 0.15 M NaCl–0.01 M PO_4 buffer, pH 7.4. Sites were challenged 5 days later.

it appears that contaminating groups of the penilloaldehyde-penamaldate type in penicillenate S–HSA may contribute significantly to positive responses evoked with this conjugate, at least in some human subjects. Positive reactions evoked in man with penicillenate S–HSA have been inhibited poorly with the unifunctional hapten *p*-penicillenate-mercuribenzoate, inhibition being obtained in only 4 out of the 22 subjects examined. In some instances, in fact, *p*-penicillenate-mercuribenozate seemed even to augment the response to penicillenate S–HSA despite the fact that in normal skin this unifunctional hepten is relatively inert. Moreover, even when *p*-penicillenate-mercuribenozate was given alone to some penicillin-sensitive individuals it produced typical wheal-and-erythema responses (Table IV, subject S. G.). It is possible that the Hg-S bond in this hapten is cleaved in the skin by cutaneous sulfhydryl groups with the subsequent formation of protein-linked penicillenate which can then, in turn,

evoke specific wheal-and-erythema responses. The significant reaction blank in some normal subjects makes this possibility difficult to evaluate.

Passive transfer to normal human skin of γ-globulin fractions of sera of three penicillin-sensitive human subjects has been attempted. Transfer was successful in one instance, using the γ-globulin fraction from subject T. S. whose skin responses were summarized in Table II. As shown in Table VI, 5 days after skin sites in a normal subject had been injected with about 5 mg γ-globulin from T. S., injection of penicilloyl-polylysine evoked a wheal-and-erythema response, and this response could be completely inhibited with ϵ-penicilloyl-aminocaproate.

TABLE VII

Correlation Between History of Previous Allergic Reactions to Penicillin and Wheal-and-Erythema Responses to Penicilloyl-Polylysine and to Penicillenate S-HSA

History	Response to					
	Penicilloyl-Polylysine		Penicillenate S-HSA			
			In persons giving positive responses to penicilloyl-polylysine		In persons giving negative responses to penicilloyl-polylysine	
	Positive	Negative	Positive	Negative	Positive	Negative
Previous allergic reaction to penicillin	45	14	12	10	0	6
No previous allergic reaction to penicillin	44	1147	12	12	0	12

The results obtained thus far in screening 1250 subjects are summarized in Tables VII and VIII. Of the persons having a history of previous allergic reaction to penicillin approximately 75 per cent (45 of 59) had positive skin reactions to penicilloyl-polylysine. On the other hand, only 4 per cent of individuals with *no* previous history of penicillin allergy gave positive skin reactions to this penicilloyl conjugate. About 50 per cent of individuals who gave positive skin responses to penicilloyl-polylysine also gave positive responses to penicillenate S-HSA.

Of the 14 individuals with positive histories of previous penicillin allergy and negative skin responses to penicilloyl-polylysine, 6 were tested with penicillenate S-HSA, and none reacted (Table VII).

Reactions to subsequent penicillin therapy in relation to skin test responses to penicilloyl-polylysine were particularly striking. In 1191 subjects without a previous history of penicillin allergy, 44 gave positive skin reactions to penicilloyl-polylysine (Table VII). Of these 44 individuals, 23 were treated with

penicillin shortly after the skin test had been performed. 9 of these 23 persons exhibited systemic allergic reactions (Table VIII). On the other hand, of the 1147 subjects in this group (*i.e.*, with no history of previous penicillin allergy) with *negative* skin reactions to penicilloyl-polylysine, only 1 subject reacted to

TABLE VIII

Correlation between Wheal-and-Erythema Responses to Penicilloyl-Polylysine and Allergic Reactions to Subsequent Penicillin Therapy in Individuals with No Previous History of Penicillin Allergy

Skin reaction to penicilloyl-polylysine B	Response to penicillin therapy		Incidence of allergic reaction
	Allergic reaction	No allergic reaction	
			per cent
Positive	9*, ‡	14§	39
Negative	0‖	1146	<0.1

* 4 non-fatal anaphylactic reactions; 1 immediate urticarial reaction; 4 late urticarial reactions (onset 4 to 48 hours after injection).

‡ 4 of the 9 had been tested with penicillenate S–HSA: In 1 (anaphylactic reaction), a positive wheal-and-erythema response had been obtained. In 3 (late urticarial reactions), the cutaneous reaction had been negative.

§ 6 of the 14 were tested for hapten inhibition. In 5 the response to penicilloyl-polylysine was inhibited by ε-penicilloyl-aminocaproate.

‖ There were actually 2 subjects with a negative history of previous penicillin allergy and a negative skin test response to penicilloyl-polylysine who had systemic allergic reactions to subsequent penicillin therapy. Further evaluation, however, provided the basis for excluding these 2 subjects from this category: thus, subject C. C. had an anaphylactic reaction after injection of dicrysticin (400,000 units procaine penicillin plus 0.5 gm streptomycin). Subsequently intradermal injection of 3.5 μg streptomycin produced massive local edema and urticaria and intradermal tests with penicilloyl-polylysine and with penicillenate S–HSA were negative. Moreover, 1.2 million units penicillin given later, therapeutically, were tolerated without any reaction. This subject's reaction to dicrysticin was presumably due, therefore, to streptomycin rather than to penicillin. The 2nd subject in question (S. B.) had known systemic lupus erythematosis and developed urticaria, arthritis, and fever 3 days after penicillin therapy. On repetition of the skin tests after the systemic reaction, positive responses were evoked with penicilloyl-polylysine and penicillenate S–HSA. Moreover, re-evaluation of the history indicated a probable penicillin reaction in the past.

subsequent therapy, but on further investigation it seemed very likely that this reaction was due to streptomycin which had been included in the therapeutic injection (see footnote, Table VIII). There is, therefore, in individuals with no previous history of penicillin allergy more than a 400-fold difference in the incidence of systemic reaction to penicillin therapy between those individuals who give positive wheal-and-erythema responses to penicilloyl-polylysine and those who do not.

Ten of the subjects who gave positive skin responses to penicilloyl-polylysine

were tested with penicillin; each test consisted of an intradermal injection of 1 to 22 units of penicillin corresponding to about 2 to 40 mμmoles, or on a molar basis, 2 to 10 times more penicillin than the penicilloyl substituents in the penicilloyl-polylysine tests which gave strongly positive responses. 9 of the 10 subjects thus tested gave negative, or at most, feeble responses (see Tables III, IV, VI for representative results). In 1 of these 10 subjects, who was tested with 11 mμmoles of penicillin O (allylthiomethylpenicillin), a strong reaction was obtained. Examination of the penicillin O solution revealed, however, an absorbance of 0.050 at 322 mμ, corresponding to 0.13 mμmoles penicillenic acid as a contaminant in the intradermal injection.

Out of the 1250 individuals who have so far been tested in the present work, only two systemic reactions to the skin tests have been observed. The first occurred in a subject (M. L.) who had received two intradermal injections of penicillenate S–HSA and one injection of penicilloyl-HγG and within a few minutes developed generalized itching and urticaria. The second systemic reaction occurred in a subject (D. W.) 48 hours after skin testing with penicillenate S–HSA and *p*-penicillenate-mercuribenzoate; it consisted of nausea, generalized itching, and a marked urticarial reaction at the skin test site which had been recorded initially as a negative response.

DISCUSSION

Penicillin derivatives and dinitrophenyl derivatives exhibit striking similarities in respect to dependency of their biological activities on molecular size, character, and multiplicity of determinants per molecule: (*a*) Derivatives containing multiple determinant groups per molecule evoke specific wheal-and-erythema responses and univalent haptens specifically inhibit these responses. (*b*) The polyfunctional polylysine derivatives are about as effective as the corresponding protein conjugates in eliciting the cutaneous responses. (*c*) Small bivalent derivatives, *i.e.*, those with 2 determinants per molecule and molecular weight less than 1500, are relatively ineffective elicitors of the cutaneous responses; moreover, in the dinitrophenyl system these derivatives give variable responses and this variability seems to reflect dependency on antibody affinity (see reference 4). (*d*) In contrast to the well known effectiveness of protein conjugates, small bivalent derivatives and multivalent polylysine conjugates fail to produce antibody in a quantity sufficient to be detectable by passive cutaneous anaphylaxis, even when given in Freund's adjuvant.

The results of testing over 1000 human subjects demonstrates clearly a striking correlation between penicillin allergy and specific cutaneous reactions to the penicilloyl and penicillenate determinants. Of the two, the responses to penicilloyl were the more frequent. Although hapten inhibition demonstrates clearly that the specificity of the wheal-and-erythema response to penicilloyl-

polylysine is directed towards the penicilloyl group, detailed evaluation of the contributions made by component segments of this group cannot yet be made. It is highly probable, however, that C^5-C^8, and the α-carbonyl and the lysyl side chain to which it is attached in amide linkage, are particularly significant (see reference 6, Fig. 1). Thus, penilloaldehyde-aminocaproate was ordinarily just as effective as penicilloyl-aminocaproate in inhibiting skin responses to penicilloyl-polylysine, and 6-aminopenicillanic acid was entirely ineffective. Similar results were obtained *in vitro* by hapten inhibition of the precipitation of rabbit antisera prepared against penicilloyl-proteins (6). The contribution made by the various R substituents in different penicillins (*i.e.*, attached to C8; see reference 6, Fig. 1) must await further investigation, as with only rare exceptions in the present work all of the substances examined were derivatives of benzylpenicillin.

The results obtained by hapten inhibition of skin responses to penicillenate S–HSA are less clear-cut with respect to establishing the nature of the determinants involved here. The few tests so far made with *S*-(*N*-ethylsuccinimidyl)-penicillenate encourage the view that the penicillenate group is the major determinant in about one-half of the responses to penicillenate-proteins. The fact that in subject J. M. (Table V) skin responses to the latter conjugate were instead inhibited by ϵ-penilloaldehyde-aminocaproate, suggests that substituents of the penilloaldehyde-penamaldate type make a major contribution to skin reactivity to penicillenate S–HSA in some instances. As in the case of the responses to penicilloyl-polylysine, the contribution of the R group to the responses evoked with penicillenate S–HSA will have to await study with derivatives made from penicillins other than benzylpenicillin.

Since one-half of all humans who responded to penicilloyl-polylysine reacted also to penicillenate S–HSA, which lacks the penicilloyl group, it is apparent that determinants other than penicilloyl are frequently involved in penicillin allergy. Nevertheless, it is abundantly clear that all of the determinants for which there is now clear or suggestive evidence are formed by the reaction of penicillenic acid with proteins. Penicillenic acid appears, therefore, to be the immediate precursor of the protein conjugates which induce antibody formation and which elicit the allergic responses to penicillin.

From the foregoing point of view the search for penicillins with little or no potential for inducing allergic reactions might well revolve about those which do not degrade to penicillenic acid, or do so at a very slow rate. Whether such penicillins will be effective antibacterial agents remains uncertain, however, as it is conceivable that the antibacterial activity of penicillin is a consequence of degradation to penicillenic acid in the bacterial cell.

Although the data obtained by skin-testing guinea pigs and human subjects with conjugates are reasonably clear and consistent, the injection of penicillin itself has highly variable consequences. Intradermal injection of penicillin has,

for example, given erratic responses when used in efforts aimed at detecting human penicillin allergy (for example, see reference 12). Since polyfunctional conjugates are required for elicitation of wheal-and-erythema responses (4, 5), it is likely that in those instances where penicillin itself has elicited such a skin reaction it is a consequence of penicillenic acid (which presumably can form rapidly *in vivo*, or may even be present in the solution injected) coupling with skin protein to form the required conjugates. Considerations of this type probably account also for some of the apparent discrepancies between systemic reactions to penicillin therapy and positive wheal-and-erythema responses to penicilloyl-polylysine. Although 40 per cent of individuals with positive wheal-and-erythema responses to penicilloyl-polylysine who were treated with penicillin exhibited systemic allergic reactions, the majority of these individuals did not (Table VIII). The failure to observe systemic reactions more frequently in this group may be ascribed to one or more of the following possibilities: (*a*) the level of humoral antibody may have been too low; (*b*) the rate of degradation of penicillin to penicillenic acid may have been too slow to permit the accumulation of polyfunctional protein conjugate at a sufficient rate; (*c*) penicillin itself, and also free penicilloic acid formed by hydrolysis of penicillenic acid, are probably specific inhibitors of the reaction between penicilloyl-protein conjugates and anti-penicilloyl antibody (see reference 6). Thus the outcome of the injection of penicillin in respect to the development of local or systemic allergic responses probably reflects the balance of a series of competitive reactions; *i.e.*, diffusion and excretion of penicillin, rearrangement of penicillin to penicillenic acid, hydrolysis of penicillenic acid to penicilloic acid, coupling of penicillenic acid to proteins, etc. Penicillenic acid reacts rapidly with proteins and with water at physiologic pH values (1, 2, 6), and it is likely that the rate-limiting step in the formation of protein conjugates of any of the types discussed herein is the rate of formation of penicillenic acid from penicillin. If the accumulation of protein conjugate is sufficiently slow to avoid rapid and extensive complex formation with antibodies, then the conjugates may actually succeed in specifically removing antibodies, particularly those of high affinity for the determinants involved. Hence temporary desensitization, at least to some extent, is actually an expected consequence of the administration of therapeutic amounts of penicillin to sensitive individuals, whether or not immediate systemic responses are observed. We have, in fact, observed loss of skin reactivity to penicilloyl-polylysine in a few of the sensitive patients who were given penicillin in therapeutic amounts.

The fact that some subjects with histories of previous penicillin allergy have given negative skin responses to intradermal injection of penicilloyl-polylysine and to penicillenate S–HSA (Table VII) may be ascribed to one or more of the following possibilities: (*a*) the determinants involved in the previous systemic allergic reactions may have been generically different from those in the present

skin test reagents; (*b*) humoral antibody levels may have declined below the threshhold value, either as a consequence of the long time elapsed from previous therapy, or as a consequence of desensitization by the penicillin therapy itself (see above); (*c*) the threshold concentration of antibody required for some systemic allergic reactions might be lower than the threshold required for the wheal-and-erythema response; (*d*) the variable R substituents of different types of penicillin may make a significant contribution to the interaction with antibody, and may have been different in the therapeutically administered penicillin which evoked a previous systemic reaction and in the conjugates used for skin testing in the present study; (*e*) previous histories in some subjects may have been inaccurate or inaccurately interpreted.

Although the preliminary results so far obtained are encouraging, it is obvious that considerably more information is required before penicilloyl-polylysine can be considered a safe and practical skin-test reagent for the diagnosis of penicillin allergy. Thus if the variable R substituent of penicillins makes a significant contribution to the specific interaction with antibody, a correspondingly large number of conjugates, differing from each other in respect to the R group, will ultimately be required. Moreover, although our preliminary findings have failed to reveal antibody formation as a consequence of injecting guinea pigs with penicilloyl-polylysines in Freund's adjuvant, it is obvious both from the limited sensitivity of the assay used (passive cutaneous anaphylaxis; see reference 4) and from differences in immune responses between humans and guinea pigs, that the immunogenic potency of penicilloyl-polylysine will have to be extensively evaluated in man.

The successful transfer, in one instance, of cutaneous reactivity to penicilloyl-polylysine with a serum globulin fraction of a penicillin-sensitive individual is noteworthy. In this experiment (Table VI), the wheal-and-erythema response to penicilloyl-polylysine was elicited 5 days after passive sensitization of the skin site. Hence, the specific globulin involved may correspond to the class of human antibodies known as "reagins." In fact, quite aside from their implications for the practical detection of penicillin allergy, the findings reported herein seem to us particularly significant because they give promise of making available an immune system which may permit the isolation and characterization of those human antibodies ("reagins") which seem to have distinctive features in respect to skin sensitivity.

The results of the present study, taken as a whole (see also references 1, 4–6, 13, 14), provide gratifying confirmation that the principles established in the study of hypersensitivity to simple chemicals in laboratory animals are applicable also to drug hypersensitivity in man.

SUMMARY

Multifunctional derivatives of penicillenic acid are effective elicitors of wheal-and-erythema skin responses in humans allergic to penicillin. Of the effective

derivatives, penicilloyl-polylysines are particularly attractive as skin test reagents because they appear to be incapable of inducing antibody formation. The skin responses are specifically inhibitable in most instances by homologous unifunctional haptens. The penicillenic acid derivatives which appear to be determinants of human allergic reactions to penicillin are: penicilloyl, penicillenate, and groups of the penamaldate-penilloaldehyde type. Of these, the most significant appears to be the penicilloyl-lysyl determinant.

Note Added in Proof.—Since submission of this and the accompanying papers (4, 6), Levine and Ovary (*J. Exp. Med.*, 1961, **114,** 875), have reported positive wheal-and-erythema responses induced with penicilloyl-HγG (and inhibited with ϵ-penicilloyl-aminocaproate) in 3 penicillin-sensitive human subjects. In 1 of 5 sera from penicillin-sensitive humans, passive cutaneous anaphylaxis reactions were elicited with a penicilloyl-protein and inhibited with ϵ-penicilloyl-aminocaproate.

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