

ANTIGENICITY AND CROSS-REACTIVITY OF 6-AMINOPENICILLANIC ACID AND PENICILLIN G

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ALLERGIC reactions to penicillin are a major clinical problem.^{1, 2} Use of penicillin itself as a skin test antigen is not adequate to evaluate clinical sensitization, since a negative test does not exclude the existence of penicillin allergy and the possibility of a reaction to a subsequent administration.³

The identity of the antigenic components of penicillin which determine sensitization is of prime importance for the selection of reagents to diagnose sensitivity states accurately.

The molecular "nucleus" of penicillin is 6-aminopenicillanic acid (Fig. 1). It can be isolated in pure form from penicillin fermentations, and its formation from penicillin by enzymatic hydrolysis has been demonstrated.⁴

The purpose of this investigation was to evaluate the antigenicity of 6-aminopenicillanic acid in rabbits and to determine its cross-reactivity with penicillin, by use of the tanned-cell hemagglutination and passive cutaneous anaphylaxis techniques for measurement of antibody activity.

MATERIALS AND METHODS

Immunization Procedure.—Eight New Zealand albino rabbits weighing approximately 3000 grams each were used in this study. Half were immunized with 6-aminopenicillanic acid (lot No. 16033 Pfizer), the other half with crystalline potassium penicillin G.

Rabbits given 6-aminopenicillanic acid (6-APA) were immunized as follows. The animals were injected intramuscularly daily for 5 days with 10 mg. of 6-APA dissolved in 1.0 ml. isotonic phosphate-buffered saline. Twenty-five milligrams of 6-APA in 2.0 ml. of complete Freund's adjuvant (Difco) was then injected intramuscularly and repeated 7 days later. Following a rest period of 3 weeks, a booster dose of 24 mg. of 6-APA in 1.0 ml. of complete Freund's adjuvant was injected intramuscularly. All materials for injection were prepared fresh at the time of injection.

Rabbits immunized with penicillin G (Pnc G) were given 25 mg. dis-

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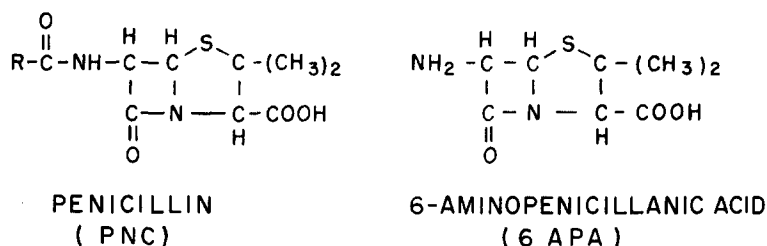


Fig. 1.—Structural formulas of penicillin and 6-aminopenicillanic acid.

solved in 1.0 ml. isotonic phosphate-buffered saline intramuscularly daily for 5 days, followed by 3 intramuscular injections of 125 mg. Pnc G in 2.0 ml. of complete Freund's adjuvant administered at 7 day intervals. Two of the animals died before the last injection. The rabbits were rested for 3 weeks, after which they were injected with Pnc G (50 mg. dissolved in 2.0 ml. isotonic phosphate-buffered saline intravenously) daily for 10 consecutive days. Trial bleedings were obtained at appropriate intervals.

Hemagglutination Technique.—The tanned-cell hemagglutination technique of Boyden,⁵ as modified by Feinberg,⁶ was used.

Erythrocytes.—Fresh sheep red blood cells were stored at 5° to 10° C. in sterile Alsever's solution for at least 3 days before use. These cells were found to be usable over a period of 6 weeks.

Tannic Acid.—A 1:220 solution (w/v) of tannic acid (Baker C. P.) was sterilized by passage through a Millipore filter. This stock solution was stored at 5° to 10° C. and proved stable and active for at least 4 weeks. Dilutions were made with isotonic phosphate-buffered saline, pH 7.2.

Antigens.—Crystalline potassium penicillin G (lot No. 0X051 Pfizer) and 6-aminopenicillanic acid (lot No. 16033 Pfizer) were used for test reagents. Thirty milligrams of 6-APA was dissolved in 0.5 ml. of 5 per cent sodium bicarbonate solution and diluted with buffered saline, pH 7.2. Penicillin G was dissolved in buffered saline, pH 7.2.

Sera.—The rabbit serum samples were heated at 56° C. for 30 minutes. The sera were subsequently adsorbed by thrice-washed normal sheep red blood cells for 12 hours at a ratio of 0.05 ml. packed red blood cells per milliliter of serum.

Hemagglutination Tests.—Sheep red blood cells were washed 3 times with isotonic saline and resuspended in buffered saline, pH 7.2, to a concentration of 2.5 per cent. An equal amount of tannic acid diluted 1:22,000 was added and the mixture incubated in a water bath at 37° C. for 10 minutes. The cells were then washed with buffered saline, pH 7.2, with unbuffered saline, and resuspended to a concentration of 2.5 per cent in buffered saline, pH 6.4. Antigen was added to the cells (4 volumes of antigen solution to 1 volume of cell suspension, i.e., 3 mg. of 6-APA or 20 mg. of Pnc G per each milliliter of 2.5 per cent tanned cells) and the mixture incubated for 15 minutes at 37° C. After incubation, the tanned cells with adsorbed antigen were washed twice in normal rabbit serum (NRS) diluted 1:200 in saline. Cells were resuspended to a final

concentration of 1.5 per cent in 1:200 NRS. Centrifugation speed of 1000 to 1500 r.p.m. was used for preparations throughout the entire experiment.

Titration were done on twofold serial dilutions of test serum starting at 1:10, with NRS diluted 1:100 in saline as diluent. With the same 1 ml. pipette, 0.05 ml. of the 1.5 per cent suspension of antigen-coated cells was added to each tube. Tubes were left at room temperature for 2 hours and observed for agglutination. The dilution in the last tube showing visible agglutination was interpreted as the titer of a test sample. Appropriate known negative and known positive controls were included in each experiment as follows: (1) normal erythrocytes in NRS diluent, (2) unmodified tanned erythrocytes in NRS diluent, and (3) antigen-treated tanned erythrocytes in NRS diluent in serial dilution.

Hemagglutination cross-reaction tests were done, using 6-APA with anti-Pnc G serum, and Pnc with anti-6-APA sera.

Hemagglutination-Inhibition Tests.—Pnc G and 6-APA were alternately used to inhibit both anti-Pnc G and anti-6-APA antisera. Five-hundredths milliliter of each dilution of inhibitor was pipetted into 0.5 ml. of each serial dilution of antisera. These tubes were incubated at 37° C. for 1 hour and at 5° to 10° C. for 12 hours. Appropriately sensitized sheep erythrocytes were added according to the method of hemagglutination test described above. The tubes were left at room temperature for 2 hours, and presence of visible agglutination was noted.

Passive Cutaneous Anaphylaxis.—Passive cutaneous anaphylaxis (PCA) determinations were done, exactly following the procedure described by Ovary.⁷ Albino guinea pigs weighing 200 to 300 grams were injected intradermally with 0.1 ml. of a 1:5 dilution of either anti-6-APA or anti-Pnc G rabbit antiserum. Dilutions were made in Hank's balanced salt solution. For positive control, anti-bovine gamma globulin (anti-BGG) was employed in 1:100 dilution. After 5 hours, these animals were challenged intravenously with 2 to 3 ml. of either a solution of 20 mg. of 6-APA or 50 to 100 mg. of Pnc G, each with 10 mg. BGG (positive control) per milliliter of Evans blue solution. The 6-APA, Pnc G, and BGG solutions were prepared separately in isotonic phosphate-buffered saline, pH 7.2, and mixed with the Evans blue solution immediately

TABLE I. HEMAGGLUTINATION TITERS AND PCA REACTIONS OF RABBIT ANTI-6-APA SERUM

RABBIT NO.	HEMAGGLUTINATION TITERS (RECIPROCAL)						PCA REACTIONS (CM.)*					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
219	<10	5120	10240	5120	1280	5120	0	—	2.0	—	1.2	2.0
278	<10	10240	10240	2560	640	10240	0	—	2.0	—	1.2	1.6
280	<10	20480	20480	10240	1280	2560	0	—	1.8	—	Tr.	1.2
286	<10	10240	5120	2560	640	1280	0	—	1.5	—	Tr.	1.2

I. Preimmunization sera. II. Serum after intramuscular injections of 6-APA in saline. III. Serum 7 days after first injection of 6-APA in Freund's adjuvant. IV. Serum 7 days after second injection of 6-APA in Freund's adjuvant. V. Serum after 3 weeks' rest period. VI. Serum after booster injection of 6-APA in Freund's adjuvant.

*Antisera diluted 1:5; antigen (6-APA), 20 mg.

prior to injection. After 30 minutes, test sites were observed for maximum skin reactions. The animals were then sacrificed, the skin was reflected, and the reactions on the inner surface were measured by diameter and intensity of color.

RESULTS

Hemagglutination Reactions.—It was apparent that antibody response to 6-APA was rapid and strong (Table I). After the series of 5 daily intramuscular injections of aqueous 6-APA (without Freund's adjuvant), an antibody titer of from 1:5,120 to 1:20,480 was measurable. These titers were maintained 7 days following the first booster injection of 6-APA with Freund's adjuvant. Titers continued to drop (period V, Table I) after a rest period of 3 weeks, but an anamnestic response was obtained 7 days after a booster injection of 25 mg. 6-APA in Freund's adjuvant.

The antibody response of rabbits immunized with Pnc G was not as marked as in those injected with 6-APA. The initial series of intravenous injections of 25 mg. aqueous Pnc G for 5 days did not evoke measurable antibody response. Titers of 1:80 were obtained only after 2 weekly injections of 125 mg. of Pnc G in Freund's adjuvant. In an attempt to increase the titer, 2 rabbits were given an additional series of daily intravenous injections of 50 mg. aqueous Pnc G for 10 days after being rested for 3 weeks. Titers measured 7 days after the last intravenous injection were at 1:1280 levels. (Table II)

TABLE II. HEMAGGLUTINATION TITERS AND PCA REACTIONS OF RABBIT ANTI-PNC G SERA

RABBIT NO.	HEMAGGLUTINATION TITERS (RECIPROCAL)						PCA REACTIONS (CM.)*					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
223	<10	<10	<10	80	80	1280	0	—	—	0	0	0
253	<10	<10	<10	80	Died	—	0	—	—	0	—	—
260	<10	<10	40	80	Died	—	0	—	—	0	—	—
290	<10	<10	<10	10	160	1280	0	—	—	0	0	0

I. Preimmunization serum. II. Serum after intramuscular injections of Pnc G. III. Serum 1 week following intramuscular injection of Pnc G in Freund's adjuvant. IV. Serum 1 week following second intramuscular injection of Pnc G in Freund's adjuvant. V. Serum 1 week following third intramuscular injection of Pnc G in Freund's adjuvant. VI. Serum 1 week after restimulation with aqueous Pnc G (given daily for 10 days intravenously).

*Antisera diluted 1:5; antigen (Pnc G), 50 mg.

TABLE III. HEMAGGLUTINATION CROSS-REACTIONS OF RABBIT ANTI-6-APA SERA AGAINST PNC G

RABBIT ANTI-6-APA SERA	HEMAGGLUTINATION CROSS-REACTIONS (RECIPROCAL OF TITER)					
	I	II	III	IV	V	VI
219	<10	<10	<10	40	—	80
278	<10	80	80	40	—	160
280	<10	<10	20	80	—	320
286	<10	10	20	40	—	80

I. Preimmunization sera. II. Serum after intramuscular injections of 6-APA in saline. III. Serum 7 days after first injection of 6-APA in Freund's adjuvant. IV. Serum 7 days after second injection of 6-APA in Freund's adjuvant. V. Serum after 3 weeks' rest period. VI. Serum after booster injection of 6-APA in Freund's adjuvant.

TABLE IV. HEMAGGLUTINATION CROSS-REACTIONS OF RABBIT ANTI-PNC G SERA AGAINST 6-APA

RABBIT ANTI-PNC G SERUM	HEMAGGLUTINATION CROSS-REACTIONS (RECIPROCAL OF TITER)					
	I	II	III	IV	V	VI
223	<10	80	80	80	—	160
253	<10	10	10	40	—	Died
260	<10	10	20	320	—	Died
290	<10	<10	<10	<10	—	20

I. Preimmunization. II. Serum after intramuscular injections of Pnc G. III. Serum 1 week following intramuscular injection of Pnc G in Freund's adjuvant. IV. Serum 1 week following second intramuscular injection of Pnc G in Freund's adjuvant. V. Serum 1 week following third intramuscular injection of Pnc G in Freund's adjuvant. VI. Serum 1 week after restimulation with aqueous Pnc G (given daily for 10 days intravenously).

TABLE V. INHIBITION OF RABBIT ANTI-PNC G SERUM BY PNC G AND 6-APA

INHIBITOR (MG./ML.)	HEMAGGLUTINATION TITERS*			
	1:10	1:20	1:40	1:80
Pnc G				
2.5	—	—	—	—
1.25	+	—	—	—
0.625	+	+	—	—
0.312	+	+	+	—
6-APA				
0.312	—	—	—	—
0.156	+	—	—	—
0.078	+	+	—	—
0.039	+	+	+	+
None	+	+	+	+
Control (NRS)	—	—	—	—

*Serum sample used from rabbit No. 223; anti-Pnc G titer, 1:1280.

Hemagglutination Cross-Reactions.—Cross-reactivity of 6-APA with anti-Pnc G sera and Pnc G with anti-6-APA sera was clearly demonstrable. With Pnc G as the antigen against anti-6-APA sera, cross-reaction titers up to 1:320 were noted (Table III). With 6-APA as the antigen against anti-Pnc G sera, cross-reaction titers up to 1:320 were measured (Table IV).

Hemagglutination Inhibition.—In order to determine specificity of antibody produced by immunization of rabbits to 6-APA and Pnc G, hemagglutination-inhibition studies were done with homologous and heterologous antigens for test inhibitors. The Pnc G—anti-Pnc G reaction was found to be inhibited both by Pnc G and 6-APA. Either 0.05 ml. of a 2.5 mg. per milliliter solution of Pnc G or 0.05 ml. of a 0.312 mg. per milliliter solution of 6-APA completely inhibited the reactivity of 0.5 ml. anti-Pnc G serum at 1:10 dilution (Table V).

The 6-APA—anti-6-APA reaction was also inhibited both by 6-APA and Pnc G. The reactivity of 0.5 ml. anti-6-APA serum at 1:10 dilution was completely inhibited by 0.05 ml. of a 20 mg. per milliliter solution of 6-APA, while 0.05 ml. of a 100 mg. per milliliter solution of Pnc G was able to inhibit

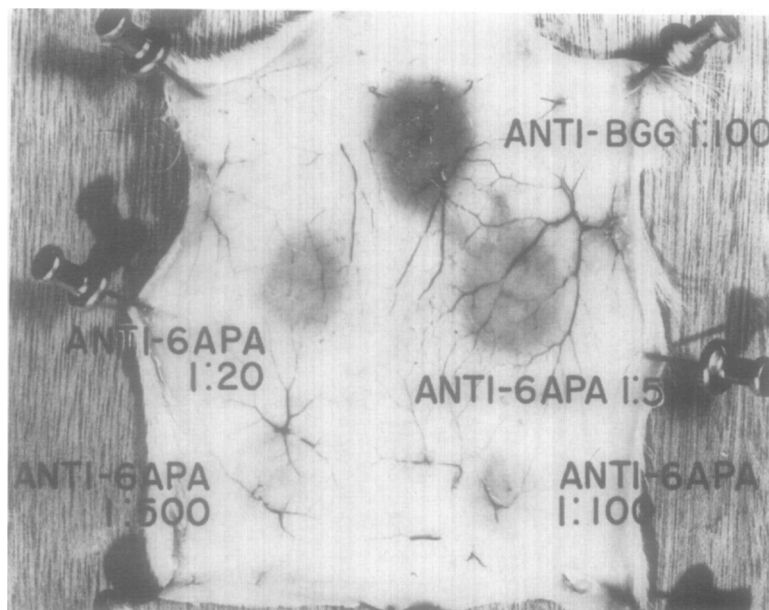


Fig. 2.—PCA reactions decreasing in intensity with increasing dilutions of anti-6-APA sera.

0.5 ml. of a 1:20 dilution of the antiserum (Table VI). The titer of this anti-6-APA antiserum to the homologous antigen was 1:2560.

Passive Cutaneous Anaphylaxis.—Antibody response of rabbits to injections of 6-APA and Pnc G was also measurable by the passive cutaneous anaphylaxis technique. An almost uniform skin response was observed with sera from rabbits immunized with 6-APA (Table I). It was also found that the degree of test site skin reactivity, as measured by the diameter of reaction and intensity of blueness, paralleled the hemagglutination titer. By increasing the dilution of antisera, concomitant diminution in degree of intensity and size of the skin reaction was noted (Fig. 2).

Attempts to elicit PCA reactions with sera from rabbits immunized with Pnc G were unsuccessful.

Attempts also were made to cross-react anti-6-APA and anti-Pnc G sera with Pnc G and 6-APA, respectively, by means of the PCA technique. Anti-6-APA sera did not give positive reactions with Pnc G, even when undiluted antisera and as much as 100 mg. of Pnc G were employed. One anti-Pnc G serum produced a weak reaction with 6-APA. Sample VI, rabbit No. 223, at 1:2 and 1:4 dilutions reacted weakly (maximum, 0.75 cm.) with 50 mg. of 6-APA.

Precipitin Reactions.—Though considered *a priori* as unlikely to be positive, precipitin tests were carried out by the ring and Landsteiner precipitin and the Ouchterlony gel diffusion techniques. Each attempt to demonstrate precipitating antibodies in sera of rabbits immunized with 6-APA and Pnc G proved unsuccessful.

DISCUSSION

The intramuscular injection of small amounts of 6-APA in rabbits elicited strong antibody response measurable by both tanned-cell hemagglutination and passive cutaneous anaphylaxis techniques. In contrast to this, antibody response to the whole penicillin molecule could be obtained only after repeated injections of the drug incorporated in complete Freund's adjuvant. The specificity of these reactions was shown by inhibition studies. Furthermore, it was demonstrated that 6-APA can inhibit the reactivity of anti-Pnc G antibody to Pnc G. Also, Pnc G was capable of inhibiting the reaction of anti-6-APA to 6-APA, although a large amount of Pnc G was required for inhibition. Cross-reaction between 6-APA and Pnc G was also demonstrated; namely, rabbit anti-6-APA serum agglutinated Pnc G-coated red cells, and rabbit anti-Pnc G serum agglutinated 6-APA-coated red cells.

The ability of 6-APA to induce strong antibody response, to cross-react with Pnc G, and to inhibit the hemagglutination reaction of rabbit anti-Pnc G antibodies with Pnc G clearly demonstrated its antigenic specificity in the laboratory. These facts, in addition to the observation that these properties are shared to a much lesser degree by Pnc G, suggest that 6-APA might be one of the major antigenic determinants in penicillin hypersensitivity.

Penicillin, *in vivo*, undergoes degradation to 6-APA. When penicillin was given orally and intravenously to mice and orally to man, dogs, rabbits, and rats, 6-APA was subsequently detected in the urine.⁸

The isolation of 6-APA led to the commercial preparation of various "synthetic" penicillins by combination of the nucleus with various side chains. Allergic reactions to these preparations have been reported, as well as the ability to evoke positive cutaneous reactions in penicillin-sensitive subjects.⁹ Subsequently, it was shown that 6-APA is cross-reactive with Pnc G and synthetic penicillin by skin testing techniques.¹⁰ The fact, then, that 6-APA is common to

TABLE VI. INHIBITION OF RABBIT ANTI-6-APA SERUM BY 6-APA AND PNC G

INHIBITOR (MG./ML.)	HEMAGGLUTINATION TITERS*				
	1:10	1:20	1:40	1:80	1:160
6-APA					
20	-	-	-	-	-
10	+	-	-	-	-
5	+	+	-	-	-
2.5	+	+	+	-	-
Pnc G					
100	+	-	-	-	-
50	+	+	-	-	-
25	+	+	+	-	-
12.5	+	+	+	+	+
None	+	+	+	+	+
Control (NRS)	-	-	-	-	-

*Serum sample used from rabbit No. 286; anti-6-APA titer, 1:2560.

all penicillins, including synthetic preparations, strongly suggests that modification of the benzyl side chain does not abolish the allergenicity of penicillin.

Antigenicity of 6-APA in rabbits was first reported by Chisholm and associates,¹¹ who used the hemagglutination technique as described by Ley.¹² Unmodified penicillin-coated erythrocytes were used to measure anti-6-APA activity. They found that efforts to conjugate 6-APA to erythrocytes resulted in little attachment of 6-APA to the surface of the red cell. Using the tanned-cell hemagglutination technique, we found that 6-APA can be attached easily to sheep RBC, making it possible to measure antibody activity of anti-6-APA or anti-Pnc G serum using 6-APA as antigen. By this method the sensitivity of the test was greatly enhanced, as can be seen by the significantly elevated titers that were measured. The specificity of the reaction was validated by the hemagglutination procedure of Ley.¹² Rabbit anti-6-APA sera gave strongly positive passive cutaneous-anaphylaxis reactions in the guinea pig, while negative reactions were noted with the anti-Pnc G sera.

The observation that hemagglutination activity paralleled PCA activity in test sera was of considerable interest. This finding by no means proves that both reactions are due to a single antibody.

Although the data in this study and those reported by DeWeck¹³ indicate that 6-APA is an important antigenic determinant in penicillin, it must be recalled that there are other known derivatives of penicillin, and perhaps unknown potential products of *in vivo* degradation, that might also function as antigenic determinants. DeWeck and Eisen¹⁴ have demonstrated that benzylpenicillenic acid (a derivative of benzyl penicillin) conjugated to native protein is a potent antigen capable of inducing the formation of antibodies specific for penicillenic acid. Levine and Ovary¹⁵ later showed that rabbit antipenicillin antibodies were directed against the diastereomeric mixture of N-(D- α -benzylpenicilloyl) groups, predominantly the diastereomeric mixture of ϵ -N-(D- α -benzylpenicilloyl)-lysine groups. Parker and co-workers,^{16, 17} using penicillininate and penicilloyl derivatives conjugated with proteins to immunize rabbits, demonstrated antigenicity of these groups. They also showed that these derivatives of penicillenic acid are capable of eliciting wheal and erythema skin reactions in humans presumably allergic to penicillin.

DeWeck and Eisen¹⁴ showed the inability of 6-APA to inhibit antipenicillenic rabbit antisera and suggested that the antibodies induced by benzylpenicillenic acid conjugates might have slight specificity for the benzyl group. Because of the absence of inhibition by 6-APA, they felt that antipenicillenic antibodies do not have affinity for the β -lactam ring and the thiazolidine ring of penicillin. In conjunction with our data, this would indicate that penicillenic acid and 6-APA may well function independently as antigenic determinants.

SUMMARY AND CONCLUSIONS

1. 6-Aminopenicillanic acid is shown to be a potent antigen. When injected (with or without adjuvant) into rabbits, high specific antibody titers were ob-

tained, as determined by tanned-cell hemagglutination and passive cutaneous anaphylaxis techniques.

2. Cross-antigenicity of 6-aminopenicillanic acid with penicillin G was demonstrated.

3. 6-Aminopenicillanic acid was shown to inhibit the reactions mediated by both anti-6-aminopenicillanic acid and anti-penicillin G antibodies.

4. It is suggested that 6-aminopenicillanic acid may play an important role in the etiology of penicillin hypersensitivity states.

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