THE DEVELOPMENT OF ANTIBODIES TO PENICILLIN IN RABBITS*

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Clinical reactions following administration of penicillin have been noted since this antibiotic was first used (1, 2). These side effects are considered to be allergic, and both serological and cutaneous tests (3, 4) confirm the immunological activity of the drug. However, the characteristics of this compound as an antigen have not yet been defined.

Because of its low molecular weight, the binding properties of penicillin are important in its allergenic potential, since other compounds of similar size which possess antigenic activity have been shown capable of firmly coupling with large molecules *in vitro* and *in vivo* (5). Penicillin and serum albumin combine in a loose association (6), but this type of binding has been demonstrated to be ineffective in making other low molecular weight substances antigenic (7). The important combination has not been determined. Another unresolved question is that of the specificity of the immunological response, including the cross-reactivities of the various types of penicillin. These two problems, it was felt, could best be investigated in the experimental animal.

In this report the production of antibodies to penicillin in the rabbit is described, and data bearing on the binding capacity of penicillin and the specificity of the immunological response are presented.

Materials and Methods

Immunization Technique.—White rabbits of either sex and 2500 gm. at the start of the experiment were fed Purina chow and water *ad libitum*. Commercial aqueous penicillin G potassium (Lilly and Pfizer) was used for inoculation with complete Freund's adjuvant (Difco). Basic immunization procedure extended over a 4 week period. At the start of the 1st week each animal received 100,000 units of penicillin dissolved in 1 ml. of saline and emulsified with an equal volume of adjuvant. One-tenth ml. of the emulsion was injected into each foot-pad, and the balance injected intradermally in sixteen areas over the back. The second and third injections were performed at weekly intervals and consisted of the deposition of a similar quantity of emulsion, subcutaneously, usually in two separated areas. At the start of the 4th week each animal received 0.1 ml. of the emulsion in each foot-pad and 1.6 ml. subcutaneously. All animals were bled prior to the start of the procedure, 7 days after the last injection and at

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varying intervals thereafter. Many of the animals were given subsequent courses of penicillin in adjuvant subcutaneously.

Agglutination Technique.—Red cells for agglutination were prepared by the addition of 4 ml. of fresh rabbit blood obtained by cardiac puncture to 4 ml. of Alsever's solution containing 200,000 units of penicillin G or 0.[1] An equal volume of blood was added to Alsever's solution alone as a control. The cells were incubated for 1 hour at 37°C., centrifuged, and washed 3 times with normal saline, according to the method of Ley (3). Control and coated cells were handled identically throughout. One drop of a 2 per cent suspension of cells was added to 0.3 ml. of serum serially diluted in saline or 5 per cent human serum albumin. Serum and cells were allowed to stand for 2 hours at 37°C. at which time readings were made by lightly tapping the tubes. End points were taken as the last tube in which gross clumping was noted. Tubes preceding the last positive tube usually exhibited complete agglutination, the cells coming up as a solid clump.

Electrophoresis.—Zone electrophoresis on starch grain supporting medium was carried out following the method described by Kunkel (8). Barbital buffer, pH 8.6, ionic strength 0.05

TABLE I

Titers of Rabbits After Basic Course of Immunization

No. of rabbits	Titer
4	128
5	64
2	32
2	16
1	8
Total.....14	

was used. Twenty-one cuts were made at ½ inch intervals and the segments eluted with normal saline by displacement filtration. Protein concentrations were determined by a modified Folin technique (8) and aliquots of each eluate were immediately assayed for antibody content.

RESULTS

Serological Tests with Penicillin-Prepared Red Cells.—Fourteen successive animals showed agglutination titers of 1/8 or greater with coated red cells after the basic immunization course. Controls consisting of normal serum, drawn prior to immunization, tested with coated cells, and immune sera tested with uncoated cells, were negative. Table I shows the initial titers of these animals. The majority developed titers of 1/64 or greater. Titers were comparable whether the serum was diluted in saline or 5 per cent human albumin.

A number of animals were given subsequent injections of penicillin, in adjuvant subcutaneously, or in solution intravenously. In Table II can be seen

[1] Supplied by William R. Jester, Food and Drug Administration, Department of Health, Education, and Welfare.

the immunization schedule of four rabbits, and the titers of successive bleedings. It may be seen that the titers remained at a fairly constant level for each animal if immunization was continued. If the animals were then permitted to rest for approximately 4 months, the antibody fell to a low level. At that time

TABLE II

Titers of Serial Bleedings of Four Rabbits Following Basic Course of Immunization Tested with G Coated Cells

Date	Dec. 11	Dec. 23*	Dec. 31*	Jan. 7
Injection		Penicillin in adjuvant	Penicillin in adjuvant	
Bleeding No	1	2	3	4
Titers				
Rabbit 8-2	128	128	128	128
Rabbit 8-3	32	16	32	16

Date	Jan. 22	Feb. 6	Feb. 14*	Feb. 24	Mar. 9	June 10	June 24	July 2	July 10
Injection			Aqueous penicillin intravenously				Aqueous penicillin intravenously		
Bleeding No.	1	2	3	4	5	6		7	8
Titers									
Rabbit 8-8	64	128	64	64	32	4		128	32
Rabbit 9-4	32	64	64	64	64	4		64	32

* Animals bled before being given penicillin.

two animals were given 100,000 units of aqueous penicillin G sodium without adjuvant, intravenously. Both showed anamnestic responses, their titers rising rapidly to approximate their previous level.

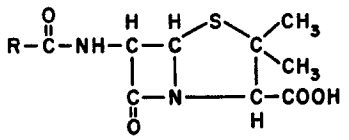
Effect of Antibody on the Antibiotic Action of Penicillin.—To determine if sera of immunized animals inhibited the antibiotic effects of penicillin, the concentrations of the drug needed to kill a standard number of known sensitive β-hemolytic streptococci in the presence and absence of immune sera were measured.

The sera were passed through a Seitz filter and dispensed in 0.5 ml. aliquots into small sterile test tubes. Three-tenths ml. of neopeptone broth was added as was 0.1 ml. of varying dilutions of penicillin and 0.1 ml. of broth containing a dilution of a culture of β-hemolytic streptococci, adjusted to give a concentration of 10^3 bacteria per ml. The tubes were incubated for 24 hours and loopfuls were then streaked on blood agar plates. End point of the assay was

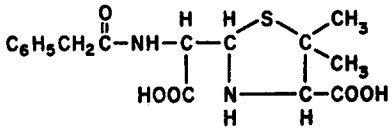
that tube in which growth could not be demonstrated by plating. Although the organism was destroyed by 0.004 units of penicillin per ml. in the culture tubes containing saline, it took at least 0.015 units of penicillin if 0.5 ml. of normal serum was used. Therefore, controls containing normal serum were used.

It was found that more penicillin was required to inhibit growth in the tubes containing the immune sera than in the controls. However, these increments never exceeded 0.01 units, even with the higher titer sera, and no attempt at correlation of agglutinating titer and inhibiting capacity could be made.

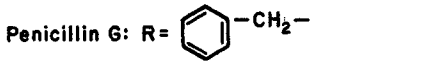
It was found, as will be described later, that between 5000 and 15,000 units of penicillin were needed to completely inhibit the agglutinating power of 1 ml.



PENICILLIN

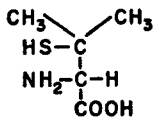


PENICILLOIC ACID



Penicillin G: R= $-CH_2-$

Penicillin O: $R=CH_2=CH-CH_2-S-CH_2-$



PENICILLAMINE

FIG. 1. Structure of penicillins and derivatives tested for inhibition of agglutination

of serum with titers of $\frac{1}{64}$ or $\frac{1}{128}$. It was concluded that either the antibody was not directed against that portion of the penicillin molecule which interacts with bacteria, or else that the antibody-antigen complex is easily dissociable. Evidence supporting the first hypothesis, but not eliminating the second will be presented below.

Specificity of the Antibody Combining Sites

A Comparison of Reactions with Penicillin O and G.—In an effort to determine the specificity of the antibody combining sites, reactions between sera and red cells coated with penicillin O and G, as well as the ability of each of these types of penicillin to inhibit agglutination were studied. Penicillin G has a benzyl side chain attached to the nucleus. Penicillin O, a semisynthetic form, has an allyl mercapto methyl side chain attached to the same nucleus, as shown in Fig. 1.

Inhibition data was obtained by the addition of 0.1 ml. of serially diluted penicillin to 0.1 ml. of a one to five dilution of serum. After incubation for one-half hour at 37°C., a drop of coated cells was added. The suspension was then treated by the standard procedure described

above. Concentrations of penicillin needed to inhibit agglutination completely were recorded as end points.

As is seen in Table III, the ratios of agglutination titers against the two types of penicillin varied when initial bleedings were compared, and to a

TABLE III

Comparison of Specificities of Sera Against Penicillin G and O

Rabbit	Bleeding	Agglutinating titer G/O	Inhibited by G/O*	
			vs. G cells	*vs.* O cells
8-2	1	128/128	6,250/25,000	6,250/1,560
	2	128/64	6,250/6,250	3,175/1,560
	3	128/128	12,500/200,000	6,250/6,250
	4	128/32	6,250/200,000	6,250/3,125
8-3	1	32/32	6,250/100,000	6,250/780
	2	16/16	780/—	780/350
	3	32/32	6,250/100,000	175/—
	4	16/16	3,125/100,000	—
8-8	1	64/64	12,500/50,000	1,560/3,125
	2	128/64	6,250/100,000	780/1,560
	4	64/64	12,500/400,000	780/12,500
	5	32/16		
	6	4/0		
	7	128/16		
9-4	1	32/8	12,500/6,250	—
	2	64/8	12,500/25,000	—
	3	64/16		
	4	64/8	12,500/50,000	—
	5	64/0	6,250/25,000	—
	6	4/0		
	7	64/32		

—, indicates concentrations below 175 units/ml.

* Expressed as units of penicillin per milliliter of undiluted serum.

much lesser extent, when several sera from the same animal were tested. For example, rabbits 8-8 and 9-4 received identical treatment (see Table II). The sera of rabbit 8-8 agglutinated O- and G-coated cells equally as well as initially, and tended to do so until its titer was permitted to drop. Sera from rabbit 9-4 consistently agglutinated G-coated cells in higher dilution than O-coated cells, the difference being less marked after the boosting injection.

If the inhibiting ability of penicillin G and O was measured, it was observed

that early in the course there was a tendency for the two types of penicillin to be equally as effective in blocking agglutination of G cells. Subsequent sera required increased amounts of penicillin O but not of penicillin G. Agglutination of O coated cells was efficiently blocked by both types.

It is to be noted that almost all sera of significant titer could agglutinate O-coated cells and that this type of penicillin inhibited each serum if used in sufficient concentration.

Inhibiting Power of Penicillamine.—It has been postulated by Eisen (7) that penicillamine, a probable metabolite of penicillin, containing a free sulfhydryl group (see Fig. 1), is the molecule responsible for the apparent antigenic properties of penicillin. To test this hypothesis, penicillamine, as the *d,l* form,[2] was used in an attempt to block the agglutination reaction.

Penicillamine in varying concentrations, up to those in considerable excess of twice an equimolar inhibiting concentration of penicillin was added to several sera. In no case was the agglutinating power of the serum reduced.

Inhibition with Penicilloic Acid.—Penicilloic acid is an hydrolysis product of penicillin obtained either by natural degradation or, more quickly, by reaction in the presence of a bacterial enzyme, penicillinase. It has already been noted by Ley (3) that this material is incapable of coating red cells for agglutination by penicillin antibodies. To further test the specificity of the antibody-combining site, inhibition experiments with penicilloic acid were performed.

Penicilloic acid was prepared by dialyzing 2,000,000 units of penicillin contained in 18 ml. of a 1 molar phosphate buffer, pH 7.2, against 800,000 units of penicillinase (neutrapen, Schenley Corp., New York) dissolved in 2 ml. of the same buffer and placed in a dialysis bag. The bag and its contents were discarded after 48 hours of gentle agitation at 37°C. The pH of the remaining material was readjusted to 7.2 with 5 N NaOH and the completeness of the reaction confirmed by the loss of all antibiotic activity when assayed with an extremely sensitive β-hemolytic streptococcus.

When compared with penicillin dissolved in the same buffer, using the inhibition technique described above, it was found that penicilloic acid was as effective or more effective in blocking the agglutination reaction.

Electrophoretic Properties of the Antibody.—Because of a recent report describing a human penicillin antibody traveling in the prealbumin fraction of one serum (9), starch block electrophoresis was performed using high titer rabbit sera. The eluate of each section was diluted in saline and titered in the usual manner. No agglutination was noted using this technique, although a moderate amount of non-specific hemolysis was present in all zones except the albumin. The titrations were then repeated, this time using a 5 per cent solution of human serum albumin as diluent. Specific agglutination was now noted in the faster moving γ- and β-globulin zones, as shown in Fig. 2, and the non-specific hemolysis was no longer in evidence. Dextran in a 6 per cent *W/V*

[2] Obtained from the Aldrich Chemical Company, Milwaukee.

solution, used as diluent restored some of the agglutinating power of the globulin fractions, but was not as effective as serum albumin.

Penicilloic Acid as an Immunizing Agent.—As penicilloic acid has been shown to be incapable of attaching to red cells, although at least as effective as penicillin in combining with the antibody, it was felt that a study of this material as an immunizing agent might be productive. To this end a group of rabbits were injected with penicilloic acid in Freund's adjuvant by the usual method. Another group of animals were immunized with the same batch of

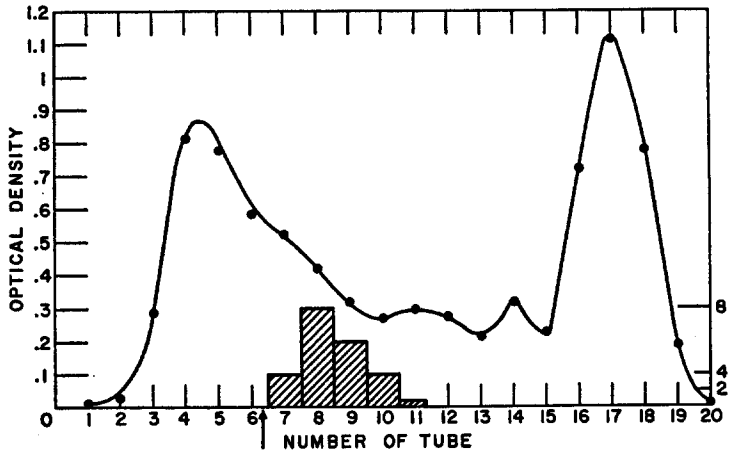


FIG. 2. Starch block electrophoresis of serum with titer of 1/64. Bar graph and ordinate on right indicate titer of fractions tested against G coated cells in 5 per cent human serum albumin. Arrow indicates origin. Albumin peak is to the right.

penicillin as was used to make the penicilloic acid, dissolved in an identical buffer.

Of the five animals that received penicilloic acid, none developed detectable agglutinating antibodies against penicillin-coated red cells. All three animals injected with penicillin as specific controls developed a titer of 1 to 64. It is to be remembered that all of fourteen animals given penicillin by this technique had developed antipenicillin antibodies. Subsequent bleedings of the penicilloic acid group yielded no positive sera, although the titers of the rabbits injected with penicillin had either risen or remained the same.

DISCUSSION

Antibodies to penicillin in the human being, demonstrable by agglutination and by skin sensitization have been reported on several occasions (3, 4, 9). Aside from a few early reports of anaphylactic sensitization in the guinea pig (10, 11), animal experimentation has received little comment in the literature.

This report offers a reproducible method which may be employed to investigate some aspects of penicillin as an antigen. No animal given the full course of immunization failed to demonstrate agglutinating antibodies, and once immunized, the animals may be boosted with aqueous penicillin alone, omitting adjuvant.

It has been shown that individual animals will react to a given antigenic stimulus with the production of antibodies showing a variety of specificities (12). To demonstrate that this is also true in the rabbit antipenicillin situation, advantage has been taken of the availability of several types of penicillin. These types, including O and G, have identical nuclei with side chains of various configurations. Should an animal's serum agglutinate cells coated with either type equally as well, it would be expected that the antibody was directed, for the most part, against the nucleus. Animals immunized with G and showing a high titer against that type, with only weak activity against O, would be presumed to have antibodies more specific for the G side chain.

The data presented here indicate differing specificities among rabbits at the same stage of immunization, and, considering inhibition of agglutination, a change in specificities during a course of immunization. It is of particular note that all animals show some specificity to the nuclear portion. None of the sera examined failed to be inhibited by penicillin O, although extremely high concentrations were necessary in some cases. The lack of parallelism between specificities as measured by agglutination and by inhibition of agglutination cannot be explained at this time. That the high concentrations of penicillin O act by displacing penicillin G from the red cell rather than by combination with the antibody has not been excluded.

More information on the specificity of the antibody is given by the failure of immune sera to neutralize the antibiotic activity of penicillin and the ability of antibiotically inactive penicilloic acid to inhibit the sera. It is likely, then, that the antibody is directed against the portion of the penicillin molecule which is not required for interaction with bacteria. Also in support of this is Ley's observation that red cell–bound penicillin is not bacteriocidal, suggesting that the antibacterial portion of the molecule is involved in the linkage to the red cell and thus unavailable as a microbicide. The exposed portion of the molecule, not primarily concerned with antibiotic activity, then acts as a receptor site to the antibody.

Because of its low molecular weight penicillin can be compared to 2,4-dinitrobenzene derivatives or to picric acid as an antigen. Both these materials have been shown capable of eliciting skin sensitivity (13, 14). This activity has been correlated with the ability of the dinitrophenyl compounds to react with endogenous proteins (5) and to the production of reactive metabolites from the picric acid. Gell, using other immunization techniques, has shown several low molecular weight substances capable of provoking production of

circulating antibody (15) and has also correlated this with chemical reactivity (16). Either penicillin or a derivative of penicillin should then be expected to bind to an endogenous material. Our data would indicate that penicillamine-protein binding is not the important linkage.

Penicillin binds tightly to red cells and can provoke antibody formation. Penicilloic acid inhibits these antibodies, thus showing the same determinant groups, but can neither bind to the erythrocyte nor cause antibody formation. It is thus possible that the important linkage is the same or similar to the bond between penicillin and the red cell.

Two other hypotheses are yet to be excluded. A metabolite of penicillin intermediate between the original compound and penicilloic acid may exist. If this material has a free sulfhydryl or other reactive group, it might be the material involved in *in vivo* binding. That the step from penicillin to penicilloic acid involves only the hydrolysis of one peptide linkage makes this unlikely. In addition, it must be considered that an alternate pathway for penicillin degradation may exist, completely bypassing the penicilloic acid step and resulting in a highly reactive metabolite.

The electrophoretic studies show that penicillin antibodies migrate in the same zone as other, well studied, antibodies and need small amounts of serum albumin or dextran to effect agglutination.

SUMMARY

A method for the production of antibodies specifically directed against penicillin is described.

The inability of this antibody to significantly reduce the antibiotic activity of penicillin is noted.

Evidence to show the variability of specificities of various sera, some directed for the most part against the side chain, others against the nucleus is presented.

Studies on serum fractions separated electrophorectically indicate that the antibody migrates in the fast γ-globulin and β-globulin fractions and requires dextran or albumin to effect agglutination.

The inability of penicilloic acid, an hydrolysis product of penicillin to provoke antibody formation despite its ability to inhibit the antibody is shown and the implications of this observation are discussed.

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