THE CHARACTERIZATION OF ANTIBODIES TO PENICILLIN *

By ALAN S. JOSEPHSON,† EDWARD C. FRANKLIN‡ AND ZOLTAN OVARY§

(From the Departments of Medicine and Pathology and the Rheumatic Study Group of the New York University School of Medicine and the Third and Fourth (N.Y.U.) Medical Divisions, Bellevue Hospital, New York, N. Y.)

(Submitted for publication August 5, 1961; accepted October 23, 1961)

Antibodies to penicillin, detectable by agglutination of coated red blood cells, have been described in the sera of humans and rabbits (1-4). The human sera contain antibodies of the 19S type which are "saline agglutinins" and 7S antibodies which may behave either as "saline" or as "incomplete agglutinins" (4).

A significant difference in one of the biological properties of 7S and 19S antibodies was recently demonstrated by use of the human B blood group system as well as the heterophile system (5). It was shown, with the passive cutaneous anaphylaxis (PCA) reaction, that only 7S antibodies can fix to the skin, a property that is not shared by the 19S γ -globulins. Similar studies of the penicillin-antipenicillin system have not, until now, been possible because of the lack of a soluble antigen of macromolecular size.

This communication will describe the preparation of such antigens and demonstrate their specificity by inhibition experiments. Data will be presented showing that antibodies of the 7S and 19S type are present in immune rabbit sera, and that these antibodies differ in that only the 7S γ -globulins sensitize guinea pigs' skin to PCA, while the 19S antibodies are responsible only for hemagglutination.

* Presented in part at the Forty-fifth Annual Meeting of the Federation of American Societies for Experimental Biology. Supported by Training Grant E.T.S. 2-E-5 from the National Institute of Allergy and Infectious Diseases; and in part by the Arthritis and Rheumatism Foundation, United States Public Health Service Grants A-2594, A-1431 (C2) and E-3075, and by the Health Research Council of the City of New York under contract 1-140.

† Present address: USPHS, Toms River Laboratory, S.E.C., Cincinnati, Ohio.

‡ Senior Investigator, Arthritis and Rheumatism Foundation.

§ New York Health Research Council Investigator.

MATERIALS AND METHODS

Rabbit antisera were produced by the repeated injection of commercial aqueous penicillin emulsified in Freund's adjuvant as previously described (3). Human sera were collected from Bellevue Hospital ward patients suspected of having reactions to penicillin, as well as from rheumatic adolescents attending the Irvington House prophylaxis clinic.¹

Starch block electrophoresis was carried out with barbital buffer used, pH 8.6, ionic strength 0.05, according to the technique described by Kunkel (6). The blocks were cut into 0.5-inch segments and the protein was recovered by displacement filtration. The protein content of the eluates was measured by a modified Folin technique, and the fractions were assayed for ability to agglutinate penicillin-coated red cells in 5 per cent human serum albumin, as previously described (3), and to elicit PCA reactions when injected into the skin of the guinea pig.

Density gradient ultracentrifugation was performed in the SW-39 rotor in a Spinco model L ultracentrifuge; 0.2 to 0.4 ml of whole serum was layered over a gradient formed with 21, 14, and 7 per cent saline as previously described (7). After centrifugation the fluid was allowed to run through a small hole in the bottom of the Celluloid tube and was collected in 10 to 12 tubes. The amount of protein in each fraction was determined by the modified Folin technique, after which certain tubes were pooled to simplify subsequent assays and dialyzed against several changes of 0.85 per cent saline. Localization of the 7S and 19S fractions in a simultaneously fractionated human serum was further checked, in most experiments, with specific antisera to these proteins. In general the bottom 3 or 4 tubes contained only 19S proteins and were essentially free of 7S contaminants, while the top 3 or 4 fractions were free of 19S proteins.

Agar gel diffusion was carried out by the method of Ouchterlony (8). Washed agar in concentrations of 0.6 to 0.7 per cent was used. The plates were incubated at 37° C for periods up to 1 week and some were kept at 4° C for several weeks thereafter.

Passive cutaneous anaphylaxis reactions were performed by the technique previously described (9). Guinea pigs weighing 200 to 250 g were carefully shaved and 0.1 ml of diluted whole serum or serum fraction was injected intradermally. After a latent period of 3 to 8

¹ By courtesy of Dr. A. Feinstein.

hours the guinea pigs were challenged intravenously with 1.0 ml of antigen mixed with 0.5 ml of 1 per cent Evans blue dye. The animals were killed after half an hour, skinned, and the diameter of the reaction measured on the internal surface of the skin.

EXPERIMENTAL RESULTS

Production of a challenging antigen specific for penicillin. Several recent reports have indicated that penicillin, when mixed with various protein solutions, produces an antigen capable of shocking guinea pigs sensitized with penicillin, or eliciting skin reactions in sensitive humans (10, 11). Since it was found that penicillin alone was unable to elicit a PCA reaction, an attempt was made to produce an effective antigen by binding penicillin to whole normal rabbit serum and fractions of normal human serum.

One million U of commercial aqueous penicillin G was added to 15 ml of whole normal rabbit serum or electrophoretically separated human albumin, α_2 -, β - and γ -globulin. The mixtures were brought to pH 8.5 with 5 per cent Na_2CO_3 and incubated at 37° C with gentle agitation for 18 hours. The solutions were then dialyzed against six changes of saline, buffered to pH 7.2 with 0.015 M phosphate buffer to remove free penicillin. The solutions were centrifuged at 15,000 rpm for 0.5 hour, the supernate being used for PCA reactions as outlined above.

As is shown in Table I, whole serum, as well as each of the serum fractions mentioned above, when mixed with penicillin and dialyzed, was capable of eliciting a positive PCA reaction in animals previously given intradermal injections of sera from sensitized rabbits. Serum 1-18-3, diluted 1:10 and 1:50 was injected into the skin of three or four guinea pigs to test each antigen. All guinea pigs showed positive reactions, with the usual variation in size of the reaction (9) among different guinea pigs similarly treated. Little difference could be observed in response to the antigens made from various serum fractions.

In all subsequent experiments a stock antigen was prepared by the addition of 5 million units of aqueous penicillin G to 1 g of Cohn fraction II (human γ -globulin) dissolved in 50 ml of buffer. The mixture was incubated, dialyzed, and centrifuged as described, and kept frozen in small aliquots.

TABLE I
Passive cutaneous anaphylaxis (PCA) reactions of serum 1-18-3 using several penicillin-protein conjugates as antigens

Antigen-penicillin coupled to	Size of PCA reaction	
	Dilution of serum 1/10	Dilution of serum 1/50
	mm	mm
Whole serum	15, 30, 20	14, 20, 18
γ -globulin	20, 23, 22, 28	12, 16, 20, 18
β -globulin	20, 20, 13	15, 15*, 10*
α_2 -globulin	25, 20	10, 12
Albumin	20*, 15, 12, 18	Trace, 12*, 0, 10*
Cohn Fr. II- γ -globulin	30, 35, 30	20, 15, 20

* Pale reaction.

It was found that sera from all rabbits immunized with penicillin were capable of sensitizing guinea pigs' skin for PCA reactions with penicillin-protein complexes. The sera regularly gave reactions in dilutions of 1:100, and end points of 1:1,000 or over were noted. Cohn fraction II, which had not been penicillinated, was unable to provoke the reaction, and normal rabbit sera were unable to sensitize the guinea pigs' skin. Three human sera, all from patients suffering recent penicillin reactions, characterized by fever, urticarial eruptions, and joint pains after penicillin therapy of approximately 1 week, were able to sensitize the animals. None of these human sera gave PCA reactions in dilutions greater than 1:50. Many other sera from patients said to have had recent reactions of varying nature, but always including a skin eruption, as well as 23 sera from rheumatic adolescents who had been on prophylaxis for over 3 years with monthly injections of benzathine penicillin, were found to be negative. None of these rheumatic patients had suffered a reaction to their prophylaxis.

Specificity of the antigen-antibody reaction. In order to demonstrate that the PCA reaction was specific for penicillin, blocking experiments were performed. Since penicilloic acid, as well as penicillin, has been shown capable of blocking the penicillin agglutinin (3), both of these materials were used in an attempt to block the PCA reaction.

All experiments were carried out in triplicate

or quadruplicate. Guinea pigs were injected intradermally with the highest dilution of antisera of both human and rabbit origin which had been found capable of *regularly* producing positive reactions. After the usual latent period the animals were injected intravenously with 100,000 U of sodium penicillin-G, or sodium benzyl penicilloate, prepared enzymatically as previously described (3). Evans blue dye was mixed with penicillin or penicilloate before injection. Control animals were injected with dye alone. At the end of 20 minutes the animals were examined and then injected intravenously with 1 ml of the globulin antigen.

It was found that both penicillin and penicilloate, while unable to elicit a PCA reaction, were capable of blocking the dermal reaction, demonstrating the specificity of the antibody-antigen reaction for penicillin.

It has previously been observed (3) that rabbits injected with penicilloic acid in Freund's adjuvant failed to produce antibodies that could agglutinate red cells coated with penicillin, despite the ability of this material to block the agglutination reaction. The sera of five of these animals were tested for skin-sensitizing ability. Two of the five sera from animals immunized with benzyl-penicilloic acid were found capable of producing

the PCA reactions with penicillin-conjugated fraction II, but only in dilutions under 1:40. When penicilloic acid was incubated with Cohn fraction II, dialyzed, and centrifuged, the resultant supernate was capable of eliciting weakly positive PCA reactions in guinea pigs given low dilutions of antipenicillin sera intradermally.

Physical characterization of penicillin antibodies. It was previously reported that human and rabbit antipenicillin saline agglutinins travel in fast γ - or β -globulin regions of immune sera separated by starch zone electrophoresis (3, 4). Similar experiments were performed in order to compare the mobility of the agglutinating and the PCA-provoking antibody.

Figure 1 illustrates the results obtained with the electrophoretically separated fractions from a typical rabbit immune serum. In each of five sera studied, the agglutinating activity migrated ahead of the PCA antibody and this activity was frequently localized in a relatively sharp peak in the fast γ -globulin and β -globulin regions. The antibody responsible for the PCA reaction was broadly distributed throughout the γ -globulin region, and the peak of activity corresponded to the γ -globulin protein peak. Although some overlap of the two activities was noted after most elec-

FIG. 1. STARCH BLOCK ELECTROPHORESIS OF A RABBIT ANTIPENICILLIN SERUM. Solid bar graph and ordinate to the right indicate the titer of agglutinin of penicillin-coated red cells of each fraction. Hatched bar indicates the fractions demonstrating PCA activity to penicillin-protein complex. The arrow is at the origin.

TABLE II

PCA and agglutination reactions of fractions of rabbit serum 1-23-7; separated by ultracentrifugation in a saline density gradient

Fraction	Protein conc.	Agglut titer	PCA reaction
	mg/ml		
6	5.5	0	15,* 15, 14
5	8.8	0	20, 18, 19
4	5.9	±	15, 16, 15
3	2.3	1/4	12, 13, 0
2	1.25	1/8	0, 0, 0
1	0.6	1/32	0, 0, 0

* Diameter of blue spot in mm.

trophoretic separations, the peaks of activity were always separable.

In order to characterize the antibodies further, they were separated on the basis of size by centrifugation in a density gradient. Eight high-titer rabbit antipenicillin sera were separated, and the resulting fractions were tested for both agglutinating and PCA activity. As is shown in Table II, the direct agglutinating activity of a representative rabbit antipenicillin serum is confined to the more rapidly sedimenting fractions containing the 19S γ -globulins, while the antibody responsible for PCA sediments with those fractions containing the 7S γ -globulins. Again, although overlap of the functions was occasionally noted, separation of the two antibodies could be achieved readily by ultracentrifugation in a saline gradient.

In order to corroborate the conclusion that the agglutinin is a 19S γ -globulin, while the antibody responsible for PCA is a 7S γ -globulin, the effect of 2-mercaptoethanol on their activity was studied. It has been shown (12) that the treatment of 19S γ -globulins with this reagent results in their breakdown into smaller units and is accompanied by loss of antibody activity. The 7S antibodies, on the other hand, are not affected by this sulfhydryl compound. Thus, preservation or destruction of antibody activity after treatment with 2-mercaptoethanol can be used as a presumptive test of antibody weight.

To test the effect of this reagent on the rabbit antipenicillin antibodies, 20 rabbit sera were dialyzed against 0.1 M 2-mercaptoethanol for 24 hours, and then against three changes of buffered saline to remove the reducing agent. In each case the agglutinating capacity of the antiserum was completely destroyed, while its ability to sensitize

FIG. 2. DOUBLE DIFFUSION AGAR STUDY OF RABBIT ANTI-PENICILLIN SERUM 1-23-3 IN WELL MARKED Sa AND VARIOUS DILUTIONS OF PENICILLINATED HUMAN γ -GLOBULIN IN WELLS P₁, P₂, AND P₃. Antigen of highest concentration is in well P₁; P₂ and P₃ contain twofold dilutions of this antigen.

guinea pigs for the PCA reaction was not diminished. This offers corroborative evidence that the saline agglutinin is a 19S γ -globulin, and the antibody detected by PCA is a 7S γ -globulin.

Gel diffusion studies. In order to study the nature of the antigen-antibody reaction further, experiments were carried out by double diffusion in agar. Undiluted rabbit antisera and varying dilutions of human γ -globulin-penicillin complexes were placed in opposing wells in Ouchterlony plates. Within 3 to 7 days two lines developed between the antigen and antibody. The line closest to the antibody was concave toward the antibody well; the other line was straight. Figure 2 illustrates the appearance of such a pair of lines when a central well is filled with antiserum and the peripheral wells with dilutions of antigen.

Prior treatment of the antisera with soluble penicillin prevented the formation of these lines, indicating their specificity for the penicillin-antipenicillin system. If the antisera were treated first with 2-mercaptoethanol, the line closest to the antibody cup failed to develop, while the

FIG. 3. PENICILLIN γ -GLOBULIN COMPLEX IS IN WELL Pb. Well Sm contains antipenicillin serum dialyzed against 2-mercaptoethanol. Well Ss contains the same serum dialyzed against saline. The arrow indicates the curved line adjacent to the antibody well.

straight line remained unchanged. This result is illustrated in Figure 3. The observation that 2-mercaptoethanol treatment prevented the appearance of one line and not the other suggests that one of the lines observed involves a 19S antibody, the other a 7S antibody.

DISCUSSION

Antibodies to penicillin have been detected by means of skin tests and by agglutination of coated red cells (1, 2, 13). Levine (14) has more recently reported delayed skin reactions in guinea pigs to various derivatives of penicillin. These reactions are of the contact type, as described by Chase (15). Donomae and co-workers (10) described the sensitization of guinea pigs to penicillin-albumin and penicillin-abscess protein antigens and were subsequently able to shock the animals with these agents. While their experiments indicate that the guinea pigs were sensitized to a determinant group formed when the protein was incubated with penicillin, they were unable to sensitize guinea pigs by using penicillin alone as an immunizing agent. The present communication confirms the observation that penicillin can bind to protein to form an effective antigen, but also demonstrates that the sensitizing antibody can be induced by immunizing rabbits with unconjugated penicillin in adjuvant.

It has been demonstrated repeatedly that a low molecular weight compound must have the ability to bind to a macromolecule to be an effective antigen (16, 17). Penicillin (1) and its chemical nucleus, 6-amino penicillanic acid (18), have been shown capable of binding to red cells and inducing the formation of agglutinating antibodies. Penicilloic acid, on the other hand, although an effective agent in inhibiting agglutination of penicillin-coated red cells by immune serum, is incapable of binding to red cells, and when injected with adjuvant into rabbits fails to provoke the formation of agglutinating antibodies (3). However, penicilloic acid does appear able to bind to protein to form a weak antigen capable of eliciting the PCA reaction in animals sensitized with immune sera. Moreover, two of five rabbits immunized with penicilloic acid produced a low titer of antibody detectable by PCA. While the observation that penicilloic acid can induce the

formation of the PCA antibody and not the agglutinin may simply reflect the weakness of the antigenic stimulus and the greater sensitivity of the PCA technique, it may also result from this small molecule's inability to bind to particulate endogenous material, such as red cells, which may influence the type of antibody produced. This problem is now under investigation.

Previous studies of human antibody to penicillin by Fudenberg and German (4) have shown that the complete, saline agglutinin is usually a 19S γ -globulin. The incomplete agglutinin was associated with the 7S γ -globulins, and only one of five sera tested showed direct agglutinating activity in this fraction. The present study indicates that rabbits also produce two biologically different types of antibodies to penicillin, both of which migrate with the γ -globulins. One of these, that has a sedimentation rate of 7S, can be detected by the PCA technique and thus behaves similarly to other antibodies (5). The other, an agglutinating antibody, falls into the 19S class of γ -globulins and, like other members of this group, cannot fix to the skin and consequently fails to give the PCA reaction. Studies have not been performed to show that the skin-sensitizing antibodies of the rabbit are also incomplete agglutinins.

The agar gel studies indicate that the two antibodies belonging to the 19S and 7S class are capable of giving independent lines with the penicillinated protein. Two possibilities for such a finding arise. The first is that each of the antibodies has a differing specificity, and that the antigen has determinant groups specific for one type of antibody or the other. Observations by Levine and Ovary (19) indicate that this is not the case. The second possibility is that either antigen or soluble antigen-antibody complexes can migrate through the area where the 7S precipitin line has been laid down, to the region that is rich in 19S antibody protein and result in the formation of a second precipitin line. That such a phenomenon is indeed possible has been shown by Korngold and van Leeuwen (20).

SUMMARY

Penicillin, when mixed with serum proteins, forms an antigen capable of reacting with serum from rabbits sensitized with penicillin, as shown

by the passive cutaneous anaphylaxis and agar gel diffusion techniques.

The antibody capable of directly agglutinating penicillinated red cells is a 19S γ -globulin, while the skin-sensitizing antibody is a 7S γ -globulin. These two antibodies are also electrophoretically separable.

Two lines are noted when rabbit immune sera are reacted with the penicillin-protein complex in a double diffusion system. Abolition of one of these lines by treatment of the sera with mercaptoethanol demonstrates that one line is formed with a 19S antibody and the other with a 7S antibody.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Dr. William S. Tillett for his constant support and encouragement. The technical help of Frances Prelli and Csaba de Szalay is gratefully acknowledged.

REFERENCES

1. Ley, A. B., Harris, J. P., Brinkley, M., Liles, B., Jack, J. A., and Cahan, A. Circulating antibody directed against penicillin. *Science* 1958, **127**, 1118.
2. Watson, K. C., Joubert, S. M., and Bennett, M. A. E. The occurrence of haemagglutinating antibody to penicillin. *Immunology* 1960, **3**, 1.
3. Josephson, A. S. The development of antibodies to penicillin in rabbits. *J. exp. Med.* 1960, **111**, 611.
4. Fudenberg, H. H., and German, J. L. Certain physical and biological characteristics of penicillin antibody. *Blood* 1960, **15**, 683.
5. Ovary, Z., Fudenberg, H., and Kunkel, H. G. Anaphylactic reactions in the skin of guinea pigs with high and low molecular weight antibodies and gamma globulins. *J. exp. Med.* 1960, **112**, 953.
6. Kunkel, H. G. Zone electrophoresis *in* *Methods of Biochemical Analysis*, D. Glick, Ed. New York, Interscience, 1954, vol. 1, p. 141.
7. Edelman, G. M., Kunkel, H. G., and Franklin, E. C. Interaction of the rheumatoid factor with antigen-antibody complexes and aggregated gamma globulin. *J. exp. Med.* 1958, **108**, 105.
8. Ouchterlony, O. Diffusion-in-gel methods for immunological analysis. *Progr. Allergy* 1958, **5**, 1.
9. Ovary, Z. Immediate reactions in the skin of experimental animals provoked by antibody-antigen interaction. *Progr. Allergy* 1958, **5**, 459.
10. Donomae, I., Kumagai, A., Doi, H., Yoshida, S., and Hiramatsu, S. Experimental studies of penicillin shock. *Med. J. Osaka Univ.* 1958, **9**, 1.
11. Rajka, G., Jr., and Vincze, E. Penicillin combined with gamma globulin as a diagnostic agent in urticaria of the serum-sickness type due to penicillin. *Ann. Allergy* 1958, **16**, 291.
12. Deutsch, H. F., and Morton, J. I. Dissociation of human serum macroglobulins. *Science* 1957, **125**, 600.
13. Harris, J., and Vaughan, J. H. Immunological reactions to penicillin. *J. Allergy* 1961, **32**, 119.
14. Levine, B. B. Studies on the mechanism of the formation of the penicillin antigen. I. Delayed allergic cross-reactions among penicillin and its degradation products. *J. exp. Med.* 1960, **112**, 1131.
15. Chase, M. W. Models for hypersensitivity studies *in* *Cellular and Humoral Aspects of the Hypersensitive States*, H. S. Lawrence, Ed. New York, Hoeber, 1959, p. 251.
16. Eisen, H. N. Hypersensitivity to simple chemicals *in* *Cellular and Humoral Aspects of the Hypersensitive States*, H. S. Lawrence, Ed. New York, Hoeber, 1959, p. 89.
17. Gell, P. G. H., Harrington, C. R., and Michel, R. The antigenic function of simple chemical compounds; correlation of antigenicity with chemical reactivity. *Brit. J. exp. Path.* 1948, **29**, 578.
18. Chisholm, D. R., English, A. R., and MacLean, N. A. Immunologic response of rabbits to 6-aminopenicillanic acid. *J. Allergy* 1961, **32**, 333.
19. Levine, B. B., and Ovary, Z. Studies on the mechanism of the formation of the penicillin antigen. III. The N-(ν - α -benzylpenicilloyl) group as an antigenic determinant responsible for hypersensitivity to penicillin-G. *J. exp. Med.* 1961, **114**, 875.
20. Korngold, L., and van Leeuwen, G. The formation of multiple zones of precipitate by one antigen; an immunological explanation. *Int. Arch. Allergy* 1959, **15**, 278.