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The Occurrence of Haemagglutinating Antibody to Penicillin

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Summary. Certain sera from individuals receiving large doses of penicillin have been found to contain a factor which can cause agglutination of human red cells sensitized with penicillin. Such sera were detected by a preliminary white tile screening technique and subsequently titrated in tubes.

The haemagglutinating factor appeared to be heat stable (56·0° for 6 hours at least) and active over a pH range of approximately 4·8 to 9·0. In the presence of complement certain strongly positive sera caused haemolysis of the red cells. The addition of penicillin inhibited the haemagglutination reaction but this effect was reversible by the addition of penicillinase. Certain agglutinating sera gave positive complement fixation tests but no visible precipitates were obtained by tube or double diffusion agar gel techniques. Attempts to obtain active eluates from agglutinated sensitized cells were unsuccessful. It was also found that the haemagglutinating factor did not appear to have any inhibiting effect on the bactericidal action of penicillin.

Starch-gel electrophoresis of positive sera demonstrated haemagglutinating activity in two pre-albumin zones. One such zone, pre-albumin-2, has been shown to be acidic-alpha-1-glycoprotein whereas the nature of pre-albumin-1 remains obscure. In twenty-two of twenty-three sera so tested agglutinating activity was confined to these two zones. In the remaining serum, in addition to activity in the two pre-albumin zones, positive agglutination of sensitized cells was also brought about by the gamma-globulin fraction, and this serum was the only one which gave a positive antiglobulin test. The interpretation of these electrophoretic findings is not altogether clear and requires further elucidation.

INTRODUCTION

In spite of the widespread use of antibiotic agents, and particularly of penicillin, during the past decade, it is only recently that examples of sera containing antibodies directed specifically against the antibiotic, have been described. So far such antibody appears only to have been detected against penicillin, although the occurrence of hypersensitivity reactions with other antibiotics, e.g. streptomycin, indicates that antibody formation probably occurs with these also. Muelling, Beven, Samson, Jenevein and Guillory (1958) have claimed to demonstrate penicillin antibody by means of agar-gel diffusion methods, using sera from patients with a history of penicillin hypersensitivity. However, a more satisfactory technique using red cells sensitized with penicillin has been described by Ley, Harris, Brinkley, Liles, Jack and Cahan (1958). Antisera containing penicillin-reacting antibody produces agglutination of such cells.

We have previously reported some preliminary observations on positive sera detected by the red cell method (Watson, Joubert and Bennett, 1959). More detailed observations on the nature of the haemagglutination reaction involved are presented below.

MATERIALS AND METHODS

PREPARATION OF SENSITIZED CELLS

Four ml. of group 'O', Rh negative blood from a donor were added to 4·0 ml. of freshly prepared Alsever's solution and the contents of a vial, containing 500,000 units of crystalline penicillin G, were then dissolved in this mixture. After solution the mixture was incubated at 37·0° for 1 hour and then stored at 4·0° overnight. According to Ley *et al.* (1958) incubation of red cells for 10 minutes with 50,000 units/ml. of penicillin will give the same degree of sensitization as incubation for 24 hours with a concentration of 3000 units/ml. In our experience, however, the cells were much more reactive after 24 hours of storage at 4·0° and even more so after 48 hours. After this period the cells were well washed four times in twenty volumes of 0·85 per cent saline, and finally re-suspended in saline to an approximate 10 per cent concentration. Unless the cells are adequately washed in saline there may be sufficient residual free penicillin to inhibit the haemagglutination reaction. It was found that red cells sensitized in this way could be stored at 4·0° C. for about 10 days and still be satisfactory for use before haemolysis became too gross. Each batch of cells was washed in saline each morning before use and was checked against a known positive antiserum. Control non-sensitized red cells from the same donor were similarly treated but without the addition of the antibiotic.

Treatment of red cells with penicillinase or trypsin did not render them agglutinable with any of the sera investigated. Penicillinase-treated cells, however, were found to be abnormally liable to rouleaux formation.

ANTISERA

All sera were obtained by separation from clotted blood specimens as soon as possible after collection. Sera were stored in the deep-freeze till required for use. Freeze-dried preparations of positive sera were found to be satisfactory for all investigations.

EXPERIMENTAL PROCEDURES AND RESULTS

PRELIMINARY SCREENING OF SERA

Positively reacting sera were found to cause haemagglutination by mixing a drop of the serum and a drop of cell suspension on a white tile at room temperature. Agglutination usually appeared in a matter of ½ to 4 minutes and was arbitrarily scored from ± to ++++. By this method large batches of sera could be tested in a short space of time. In this way we obtained some forty positive sera from some 3000 specimens from hospitalized patients. These were routine unselected sera and were not exclusively obtained from patients receiving penicillin therapy. Many of the investigations reported here were carried out with one particular serum, obtained from a patient who had had large amounts

of penicillin over a period of several months and who was receiving 3 mega units daily at the time of detection. This serum was scored as ++++ on preliminary screening.

HAEMAGGLUTINATION TITRATION

Serial dilutions of patient's sera, inactivated at 56·0° for 30 minutes, were prepared in 0·85 per cent saline in 3 inch by $\frac{3}{8}$ inch tubes. These were incubated for 2 hours with an aliquot volume of an approximate 5·0 per cent suspension of sensitized red cells, at 37·0°. The cells were then spun at 1000 rev./min. for 2 minutes and the haemagglutinin titre was noted by gently tapping the tubes. Control non-sensitized group 'O' Rh negative cells were included. Final dilutions of positive sera causing agglutination varied from $\frac{1}{2}$ to 1/250. Only one serum with as high a titre as 1/250 was found. This was from the patient already referred to who was receiving 3 mega units of penicillin daily. Seven days after therapy was discontinued there was a rise in titre to 1/1000 presumably due to the fact that some of the antibody was combined with penicillin during the period of its administration.

Haemagglutination also took place at 4·0° but the final titres obtained at this temperature were usually one or two tubes lower than at 37·0°.

HAEMOLYSIN TITRATION

It was noted that incubation of fresh positive sera at 37·0° with penicillin-sensitized cells resulted in haemolysis in some cases, but not in all. The haemolytic titre of the serum showing agglutination to 1/250 dilution was 1/64, using 2 M.H.D.s. of guinea-pig complement. Further investigation of the haemolysin showed it to be of 'warm' type, although haemolysis also took place slowly at room temperature. No haemolysis of normal cells was observed.

EFFECT OF TEMPERATURE ON HAEMAGGLUTININ

Heating positively reacting sera for a period of 6 hours at 56·0° did not affect their ability to cause agglutination of sensitized cells nor result in a diminution of the titre.

EFFECT OF ALTERATION OF HYDROGEN-ION CONCENTRATION

Alteration of the pH of the sera over a range of approximately 4·8 to 9·0 did not affect haemagglutination. Outside this range there was inhibition presumably due to denaturation of the antibody.

EFFECT OF PENICILLIN ON THE REACTION

Serial dilutions of crystalline penicillin-G solution in saline were prepared in 3 inch by $\frac{3}{8}$ inch tubes in 0·25 ml. amounts. Equal volumes of an undiluted positive haemagglutinating serum (titre 1/250) were added to each tube. These were then allowed to stand at room temperature for 30 minutes and two drops of a 5·0 per cent suspension of sensitized red cells were then added. The presence or absence of haemagglutination was then observed after 2 hours' incubation in a water bath at 37·0°. Results are detailed in Table 1.

From Table 1 it can be seen that a concentration of penicillin as high as 1562 units/ml. was insufficient to prevent the haemagglutinating property of this particular serum. A

further experiment on the same lines was designed to show the highest concentration of penicillin required to just allow agglutination to take place at varying serum dilutions. Results are shown in Table 2.

TABLE 1
EFFECT OF PENICILLIN ON HAEMAGGLUTINATING ANTIBODY

Conc. of penicillin (units/ml.)	*Haemagglutination*
50,000	−
25,000	−
12,500	−
6,250	−
3,125	−
1,562	+
781	++
390	++++

− = no agglutination.
+ to ++++ = agglutination.

TABLE 2
SERUM DILUTIONS AND THE EFFECT OF PENICILLIN ON HAEMAGGLUTINATION

Dilution of serum	*Conc. of penicillin in first tube showing agglutination (units/ml.)*
1/2	1500
1/5	750
1/10	375
1/20	190
1/40	95
1/60	47
1/80	47
1/100	47

If the serum dilutions in Table 2 are plotted against the final penicillin concentrations an exponential type curve is obtained showing that proportionally more penicillin is required to prevent the haemagglutinating property of the antibody at lower dilutions of serum. This mode of inhibition is in many ways similar to the inactivation of penicillin by penicillinase.

It can also be demonstrated that inactivation of the antibody by penicillin appears to be a reversible phenomenon since the addition of penicillinase to the reacting mixture restores the haemagglutinating property of the antibody to its original titre.

EFFECT OF PENICILLINASE ON THE REACTION

When a positive haemagglutinating serum (titre 1/250) was allowed to react with penicillin-sensitized cells, in the presence of an equal volume of 'Wellcome' penicillinase, there was found to be a marked delay in the time required for agglutination to occur. However, the ultimate degree of agglutination appeared to be similar to that observed in the absence of penicillinase. Two explanations of this phenomenon appear possible. It may be that penicillinase by acting on the pencillin adsorbed on to the red cell surface can produce a sufficient degree of desensitization of the cell to prolong the reaction time. If this is so the reaction is presumably a reversible one since the final degree of haemagglutination was not affected. This can be clearly shown by incubating penicillin-sensitized cells with penicillinase solution for 2 hours followed by three washings in 0·85 per cent saline, and finally resuspending to make a 5·0 per cent suspension. Such cells are agglutinated by positively reacting sera to the same titre and at the same speed as ordinary sensitized cells. The second explanation may be that penicillinase may in some way delay the combination of the antibody with the penicillin adsorbed on the cell surface. It may be that penicillinase becomes loosely adsorbed on to the cell surface independently of the pencillin and may prevent antibody combination with the latter due to some mechanism of spatial crowding.

COMPLEMENT FIXING PROPERTY OF ANTIBODY

Three serum dilutions (neat, 1/5 and 1/10) were used in complement-fixation tests using two and four M.H.D.s respectively of guinea-pig complement for each dilution. The antigen consisted of a solution of penicillin containing 12,500 units/ml. Appropriate antigen, serum, haemolytic system and cell controls were included. Results showed fixation of both doses of complement with neat serum and of two M.H.D.s (but not four M.H.D.s) with both 1/5 and 1/10 dilutions. Higher antigen concentrations were found to have an anti-complementary effect.

AGAR GEL DIFFUSION TESTS

These were carried out by a double diffusion technique using 1·0 per cent clarified agar. Varying concentrations of penicillin from 1000 to 100,000 units/ml. were placed in the peripheral cups and positive sera in the central cups. None of the positive sera so tested were found to give precipitation zones in the agar after 10 days' observation. Similarly, precipitation tests carried out in tubes were also negative.

ELUTION OF ANTIBODY

Ley *et al.* (1958) found that sensitized cells exposed to the action of positive sera could be heated at 54·0° for 15 minutes in saline with subsequent elution of the antibody. We have been unable to confirm this observation and all attempts to obtain active eluates have so far proved fruitless.

EFFECT OF ANTIBODY ON THE BACTERICIDAL ACTION OF PENICILLIN

An investigation was carried out to determine whether the combination of antibody with penicillin would result in some neutralization of the bactericidal effect of the latter.

Replicate reacting systems were prepared containing inactivated positive serum (haemagglutinating titre 1/250) neat and diluted 1/10. Dilution of the serum before inactivation largely removes bactericidal effects due to normal serum factors (Mackie and Finkelstein, 1932). Final penicillin concentrations consisted of 10, 20 and 50 units/ml. respectively. Complement was added in the form of guinea-pig serum suitably absorbed with *Staphylococcus pyogenes* (N.C.T.C. 6571), on two occasions. The bacterial inoculum consisted of approximately 350 organisms per tube (*S. pyogenes*, N.C.T.C. 6571). Final serum concentrations were 1/2 and 1/20 and the final volume of mixture in each tube was 2·0 ml. Tests were carried out by allowing the sera to react with the penicillin solutions for varying periods of time from 1 to 24 hours at both room temperature and at 4·0° before the addition of complement and bacterial inoculum. All tubes were then incubated at 37·0° and surface viable counts were performed at intervals of incubation according to the technique of Miles and Misra (1938). Results seemed to indicate clearly that initial incubation of serum and penicillin for periods between 1 and 24 hours did not affect the bactericidal properties of the penicillin, at least of the concentrations of antibiotic employed in the tests. This may mean that the antibody-penicillin complex is such as to allow of free dissociation with release of active penicillin, in much the same way that toxin-antitoxin complexes may dissociate with release of free toxin.

EFFECT OF PENICILLIN ON PATIENT'S RED CELLS

Red cells of patients, whose sera were initially scored as +++ or ++++ haemagglutination, were found to be agglutinable, after washing in three changes of saline, by a solution of penicillin containing 1000 units/ml. Such agglutination was strongly positive in some cases, and suggested that the antibody fraction had been adsorbed on to the cell surface. In one instance where the patient had been receiving 1 mega unit of penicillin every 6 hours the addition of the patient's own serum to her washed red cells produced weak agglutination presumably as the result of the presence of free penicillin in her plasma, since the reaction was abolished by preliminary treatment of the serum with penicillinase.

In spite of this evidence that antibody had been adsorbed on the red cell surface we have only found one example where a positive result has been obtained with the use of an antiglobulin technique. Varying batches of antiglobulin sera have been tried since it is known that certain antiglobulin sera may fail to produce a positive result for some reason whilst another batch will prove satisfactory. According to Ley *et al.* (1958) a positive haemagglutination reaction could only be obtained with some sera by means of the antiglobulin method, and although it is not entirely clear from their paper it seems that they found the antiglobulin method to be a satisfactory indicator of the presence of antibody. The reason for this discrepancy with our findings is not clear but our observations on the nature of the antibody obtained by the use of starch-gel electrophoresis seem to explain our failure with the antiglobulin method.

STARCH-GEL ELECTROPHORESIS OF THE SERA

Starch-gel electrophoresis was carried out according to the method of Smithies (1955). The starch used was a mixture of three parts of soluble starch and two parts of potato starch (both obtained from E. Merck & Co., Darmstadt, West Germany). To date no batch variation has been observed. We preferred this procedure to the more laborious methods of obtaining suitable starch described by Poulik and Smithies (1958). Total starch concentration was 15·0 g. per 100 ml. of gel buffer. A constant current power source was preferred to constant voltage supply.

Sera were inserted into slots, cut in the gels, by suspension on starch particles. A current density of 4·0 milliamp. per gel strip was used and development was continued for 6 to 7 hours.

On completion of development of the electrophoretic pattern, the starch in the gel slots was transferred to stoppered round-bottomed centrifuge tubes and snap frozen in dry ice-ether mixture. The gels were freed from the trays and thoroughly washed with distilled water to remove any serum which might have migrated along the gel-tray interfaces, or which might be adherent to the gel-slot surfaces. The gels were then sliced and the bottom portion transferred to a Petri plate containing moist filter paper in the cover portion. The top half of each gel was stained for 30 seconds with Amido-black-10-B in the usual way and was then transferred to the clearing fluid consisting of methanol, water and acetic acid (25 : 75 : 10 v/v). The high concentration of water prevented shrinkage of the gel during clarification. As a rule the gels were sufficiently cleared within 30 minutes to recognize the developed electrophoretic protein patterns. Each stained section was removed from the clearing fiuid and accurately matched against the corresponding unstained portion. With the aid of a sharp blade, fractions were cut from the unstained section corresponding to the visualized fractions in the stained section. These were then transferred to stoppered round-bottomed centrifuge tubes and snap frozen in dry

ice-ether mixture, to break up the gel. Overnight storage at −20·0° C. served equally well in breaking up the gels without apparent irreversible adsorption of the contained protein. After thawing, 2·0 ml. of isotonic saline was added to each tube, irrespective of the volume of the fraction in the tube, and the contents were thoroughly triturated with a glass rod. The tubes were then left at 37·0° C. for 2 to 3 hours. They were then centrifuged for 10 minutes at 2500 rev./min. and the supernatants were then tested for antibody activity against sensitized red cells by the tube dilution method, after adjustment of the pH to approximately 7·0. Standard practice was to test the slot starch for unmigrated antibody and to slice approximately 1·0 mm. of gel from the leading edges of the slots to test these for antibody which failed to enter the gel. This is a necessary precaution to avoid confusion since it is common experience that, from time to time, the protein remains in the slot or fails to enter the gels.

Twenty-three sera which gave positive haemagglutination by the tile method were submitted to starch-gel electrophoresis as well as nine sera which gave negative haemagglutination by the same method.

In our hands the starch-gel method gives very clear definition of the pre-albumin zone. The pre-albumin-1 fraction gives a narrow band whereas pre-albumin-2 (now proved to be acidic-alpha-1 glycoprotein (Poulik and Smithies 1958) is wider (3–5 mm.), but clearly differentiated from the albumin and pre-albumin-1 fractions. In all sera examined the pre-albumin zones were well defined. The twenty-three positive sera (all from African or Indian patients) all had grossly abnormal protein patterns (both on starch-gel and paper electrophoresis), of the type associated with infectious disease, i.e. increased gamma-globulin, and alpha-2-globulin concentrations and decreased albumin concentrations. In African patients these changes were superimposed on the local African type of protein pattern described by Joubert *et al.* (1959). All described haptoglobin types were found in these patients.

In twenty-two of the twenty-three positively reacting sera haemagglutinating antibodies, after starch-gel electrophoresis, were exclusively associated with the fractions corresponding to acidic-alpha-1-glycoprotein and what Smithies (1955) termed pre-albumin-1. The remaining serum showed haemagglutinating antibodies in the gamma-globulin fraction as well. This last serum was the only one which gave a positive reaction with the antiglobulin test.

To be quite sure that the antibody activity was associated with both pre-albumin fractions and did not represent migration and/or technique artefacts, the pre-albumin zones were differentiated by serial section at 1·0 mm. intervals and the resultant fractions were assayed by tube dilution for haemagglutinating antibody. Two clear-cut zones of activity corresponding to the two pre-albumin fractions were demonstrated. In the serum showing activity in the gamma-globulin fraction as well it was found that antibody was evenly distributed throughout the gamma-globulin zone in addition to being present in the pre-albumin zones.

Eight of the nine sera which gave negative haemagglutination by the tile method showed no haemagglutinating activity in any starch-gel fraction. The ninth specimen showed haemagglutinating antibodies in the two pre-albumin zones. When the whole serum was re-examined by the tube technique it was found to give weakly positive haemagglutination. This experience indicated that the simple technique of saline extraction of the starch probably was efficient, as it is obviously impossible by the starch-gel procedure to obtain reliable quantitative data.

DISCUSSION

The nature of the antibody substance involved in the haemagglutination of penicillin-sensitized red cells is not clearly understood. The well-known ability of penicillin to produce hypersensitivity reactions in certain individuals might suggest that the antibody involved may be responsible for both haemagglutination and for skin sensitization. This, however, appears unlikely. In none of the patients investigated in this series was any history obtained of sensitivity reactions following administration of the antibiotic. This agrees with the findings of Ley *et al.* (1958). In addition we investigated the sera from a number of individuals with well-established drug hypersensitivity to penicillin, but none of these gave a positive haemagglutination reaction. The injection of positive haemagglutinating sera intracutaneously in volunteers followed 24 hours later by the injection of penicillin solution did not result in any observable skin reaction of either immediate or delayed type. According to Swift (1954) penicillin sensitivity can be passively transferred, though the experience of most workers with passive transfer tests in drug sensitivity states has been that they are usually unsuccessful. It appears probable that penicillin acting as a hapten may result in the production of two different types of antibody one of which is responsible for classical hypersensitivity responses. Kuhns and Pappenheimer (1952a, 1952b), for example, have shown that diphtheria toxoid can give rise to the formation of two types of antibody. One is precipitating and non-skin sensitizing and the other is non-precipitating and skin sensitizing to both toxin and toxoid. The latter may remain at the site of injection for weeks whereas the former leaves the injection site in a matter of 100 minutes or so. They have shown that the non-precipitating antibody can be identified in the fast-moving T-fraction by paper electrophoresis and the precipitating type appears as a slow moving gamma-2 fraction.

We have not been able to determine fully the relationship between penicillin administration and the production of the haemagglutinating-type antibody, apart from the observation that all patients with positive sera, with one exception, were receiving large doses of penicillin per diem, usually amounts of at least 1 mega unit. In addition practically all had received the antibiotic for a period of at least 3 weeks. The exception was a patient hospitalized for 6 months who had received no penicillin for the last 4 months of that period, but who had had the antibiotic before that. This patient's serum gave a strong positive response with sensitized red cells. On the other hand, the patient whose serum agglutinated sensitized red cells to a titre of 1/250 showed a drop in titre to 1/12 1 month after cessation of penicillin therapy.

Muelling *et al.* (1958) were able to demonstrate precipitation in agar gels using sera from individuals with a history of penicillin hypersensitivity. In this way they found no less than thirty-four positive reactions in seventy-one sera. However, some of their positive results only became so after the period of test had been prolonged to as many as 21 days and in some instances more than one band of precipitate was visible. These features make it difficult to interpret their findings with any confidence. We have investigated the sera of individuals with histories of penicillin hypersensitivity, and also sera showing positive haemagglutination reactions, but in no case, using double diffusion methods in agar, have we been able to obtain visible precipitates. In view of the relatively small size of the penicillin molecule it may be that there is only one reactive group and that consequently the complexes formed are too small to be thrown out of solution.

The presence of haemagglutinating activity in the two pre-albumin fractions obtained

by starch-gel electrophoresis is puzzling. However, it is well known that not all antibody activity is associated with what is classically described as gamma globulin. We have cited the case of diphtheria antitoxin which in the horse appears in the T-fraction intermediate between the beta and the gamma fractions. Seibert and Nelson (1942) have shown that antibody to tuberculo-protein can exist in immune sera as a component with a mobility similar to or very close to that of an alpha globulin. Koprowski *et al.* (1947) have shown that in anti-viral sera neutralizing substances may appear in different electrophoretic fractions depending on the viruses employed. Similarly, part of the Wassermann antibody has a high molecular weight and migrates electrophoretically between the beta and gamma fractions. It is also well known that in the course of immune antibody production a change in the globulin association may take place as immunization proceeds, e.g. in the case of antipneumococcal sera produced in horses. However, we cannot offer any explanation for the observation that, with a single exception, the sera examined had antibody activity associated with the pre-albumin fractions only whereas in the sera examined by Ley *et al.* (1958) the positive antiglobulin tests suggest that antibody activity would be associated with the gamma-globulin fraction in most of their cases. The single example of a serum showing a positive antiglobulin test, which we examined, showed antibody activity associated with both the gamma-globulin and the pre-albumin fractions.

Further identification of the antibody was attempted with the following proposed scheme: de-ionized or dialysed serum containing penicillin antibody is passed through a column of a strong anion exchange resin in the penicillin phase in order to fix the antibody on the resin. This is followed by displacement of the penicillin/antibody complex and recovery of the protein moiety from the eluate with the aid of a strong cation synthetic or cellulose exchanger. If sufficient material proves recoverable from the cation exchanger this can be used for immunological studies. Rather large volumes of serum are required for this type of investigation. However, with one serum investigated we were able to show that antibody activity was removed after passage through an anion exchanger in the penicillin phase and that it was present in the eluate after displacement of the penicillin. Unfortunately investigations along these lines have so far been hampered by the difficulty of obtaining sufficient serum from patients with high titre haemagglutinating sera.

We have conducted a preliminary investigation into the possibility of detecting similar haemagglutinating antibody produced by the administration of streptomycin. Red cells were treated with streptomycin sulphate in varying concentrations in the same way as with penicillin and the sera employed were obtained from patients who had been undergoing streptomycin therapy for pulmonary and other forms of tuberculosis. Some 300 sera were tested in this way but no positive results were obtained. This investigation is still under way. However, it may be that red cells cannot be sensitized with streptomycin in the same way as with penicillin since the antibiotic may be readily washed off with 0·85 per cent saline. Such is certainly the case with chloramphenicol in which case the adsorption of the drug to the red cell surface is of a very loose type (Glazko *et al.* 1949; Watson, 1958).

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