CUTANEOUS REACTIVITY OF IMMUNE AND HYPERSENSITIVE RABBITS TO INTRADERMAL INJECTIONS OF HOMOLOGOUS INDIFFERENT STREPTOCOCCUS AND ITS FRACTIONS

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It is now well established that rabbits can be made hypersensitive by repeated intracutaneous injections of streptococci so that subsequent inoculations induce enormously exaggerated skin lesions (1-6); and it is also known that rabbits immunized by suitable intravenous injections of the same microorganisms show lesions smaller than normal at subsequent skin tests (7, 8). The purposes of the present study were: (a) to observe the types of dermal reactions induced in such bacterially hypersensitive and immune rabbits by different chemical fractions of the homologous cocci, and (b) to investigate the relationship between these reactions and the circulating antibodies. The work was undertaken in three separate experiments, but, since the basic technique was the same in each, the methods and results will be described together, with special notation where exceptions were encountered.

EXPERIMENTAL

The bacterial culture used throughout was a strain (Q 155/0/7) of Type I indifferent streptococcus (9) which had been extensively tested in this laboratory. Healthy adult Chinchilla rabbits were used; all in Experiment 1 were females, while all in Experiments 2 and 3 were males. In each experiment the animals were divided into five groups which were treated as follows: Group A comprised normal controls; Group B was sensitized by repeated intracutaneous inoculations of intact cocci; the remaining three groups were immunized by intravenous injections—Group C received intact cocci, Group D, the so called nucleoprotein fraction, and Group E an emulsion of mechanically ground bacterial bodies. The number of rabbits in each of the three experiments is summarized in Table I.

After test bleedings or skin tests had indicated that the animals were sufficiently immunized or sensitized, sera were collected for final titrations of agglutinins and
of precipitins against both type- and non-type-specific streptococcal fractions. Following depilation, each rabbit was skin-tested with intracutaneous injections of (a) 0.01 and 0.001 cc. of a living 20 hour broth culture of Streptococcus Q 155/0/7; (b) 0.5 and 0.1 mg. of Q 155 "nucleoprotein"; and (c) 2.5 and 0.25 mg. of Q 155 type-specific substance. In each instance the dilution was such that the injected material was contained in a volume of 0.1 cc. In Experiments 1 and 2 all test substances were injected simultaneously, but in Experiment 3 the tests with nucleoprotein were made 48 hours after the others. The volumes of all resultant lesions were calculated daily over a period of 2 weeks and were charted for comparison.

Preparation of Nucleoprotein Fraction.—The non-type-specific nucleoprotein fraction, here referred to as P, after Lancefield's terminology, was prepared from Streptococcus Q 155/0/7 by a slight modification of the method described by Lancefield (10). It was not obtained in dry form, however, but was standardized, instead, by micro Kjeldahl determinations. Furthermore, on the advice of Dr. Lancefield, N/1000 sodium hydroxide rather than N/100 was used for the extraction of the ground cocci. The yield was approximately 28 mg. of P from each liter of culture.

Preparation of the Type-Specific Carbohydrate.—The source of this material, referred to as S, was the clear supernatant left over in the preparation of P when NaOH extracts were acidified and the resultant precipitate centrifuged off. It was further alkalinized and precipitated several times to remove additional protein and the final supernatant was made neutral to phenol red. The clear yellow fluid thus obtained was concentrated to small volume by vacuum distillation at 25°C. This was then treated with three volumes of 95 per cent alcohol in the cold for 3 hours, and the white precipitate was redissolved in saline. As the supernatant revealed no S when tested with anti-S serum, it was discarded. Alcohol precipitation was repeated four times and the final precipitate was dried over P₂O₅ and weighed. In Experiment 3 an additional procedure was employed:
the supernatant after acid precipitation was passed through an ultrafilter. The filtrate thus obtained was concentrated by vacuum distillation at 25°C. and then submitted to alcohol precipitation as noted above. As shown by precipitin tests with anti-P serum (Table V), the S extract used in Experiment 2 contained minute traces of P, but that employed in Experiment 3 probably did not.

Preparation of Bacterial Emulsion (B. E.).—20 hour living cultures of Streptococcus Q 155/0/7 in 0.05 per cent dextrose broth were centrifuged and the frozen and dried sediment was ground in a ball-mill until only Gram-negative debris could be seen in stained films, and culture gave no growth. The powder was weighed and added to physiologic saline in standard amounts. Some went into solution, but much was in suspension so that it was necessary to mix thoroughly before it was used for intravenous injection.

Method of Sensitization.—The rabbits were sensitized by intracutaneous injections of 0.001 cc. living culture of Streptococcus Q 155/0/7 diluted in Ringer’s solution so that the desired amount was contained in 0.1 cc. These inoculations were given daily until the animals showed hypersensitive reactions to them, which usually occurred in 10 to 15 days. Following this, sensitivity was maintained by single weekly injections.

Method of Immunization.—In Experiments 1 and 2 intravenous injections were given on 4 consecutive days each week, with no injections the remaining 3 days. In Experiment 3, injections were given daily every alternate week. The animals receiving the intact cocci were started on small doses of heat-killed vaccine and were continued on the centrifuged sediment of living 20 hour broth cultures resuspended in saline. Those immunized with P were started on doses of 10 mg. daily which was increased to 20 mg. The rabbits immunized with B. E. received 2.5 mg. daily at first which was increased to 25.0 mg. daily. The total amounts received by the individual animals of the three immune groups, together with the total time immunization was continued, are shown in Table II.

Technique of Agglutinin Tests.—0.5 cc. of each serum dilution was mixed with

### TABLE II

<table>
<thead>
<tr>
<th>Group</th>
<th>Material Injected</th>
<th>Route</th>
<th>Total amount received by each animal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Exp. 1</td>
</tr>
<tr>
<td>B</td>
<td>Intact cocci</td>
<td>Intradermal</td>
<td>0.121 cc.</td>
</tr>
<tr>
<td>C</td>
<td>&quot;</td>
<td>Intravenous</td>
<td>215.0 cc.</td>
</tr>
<tr>
<td>D</td>
<td>Nucleoprotein (P)</td>
<td>&quot;</td>
<td>Not done</td>
</tr>
<tr>
<td>E</td>
<td>Bacterial emulsion (B. E.)</td>
<td>&quot;</td>
<td>421.0 mg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days over which given</td>
<td>255</td>
<td>90</td>
<td>105</td>
</tr>
</tbody>
</table>
0.5 cc. of a 20 hour broth culture of Streptococcus Q 155/0/7, and readings were made after 1 hour at 56°C. and 20 hours in the ice box.

Precipitin Tests.—0.5 cc. of each dilution of P and S respectively was mixed with 0.1 cc. of serum and readings were made after 15 minutes for ring reactions and after 2 hours at 37°C. and 20 hours in the ice box.

Results of Serologic Study

Agglutinins.—The agglutination results are summarized in Table III. As expected, the rabbits immunized with intact cocci showed high titers. Those intracutaneously sensitized, and the P-immune and B.E.-immune groups all showed comparatively low titers which were, however, distinctly greater than normal except in the case of the three B.E.-immune rabbits of Experiment 1. These differed from the seven corresponding animals of Experiments 2 and 3 in that their sera gave negative, or essentially negative, agglutination reactions. The low agglutinin titers in these three animals is difficult to explain since each received a total of 421 mg. of B.E. compared with 380 mg. given in Experiment 2; possibly the reason lies in the fact that in Experiment 1 immunization was performed with smaller individual doses over a much longer period.
Anti-P Precipitins.—As seen in Table IV, the sera of normal rabbits and of those sensitized with small intracutaneous inocula of living culture gave no precipitation with P extract in any of the dilutions used. As has been noted by others (11), the animals immunized with large intravenous doses of intact cocci varied greatly in titer; eight gave negative results, while others showed relatively high titers. As expected, the P-immunized animals gave uniformly high titers; less expected was the finding that the B.E.-immunized group gave results of almost the same degree. All precipitates were of the flocculent type encountered with bacterial proteins.

Anti-S Precipitins.—The results of these tests are summarized in Table V. Here again, the normal and the intracutaneously sensitized animals were negative. In contrast, the animals immunized by means of intravenous injections of intact cocci showed high anti-S titers, the precipitates formed being of the disc type characteristic of carbohydrate antigens.

Four of the eight P-immunized rabbits showed negative reactions, and in the remainder the precipitates merely formed slight whorls on agitation; these reactions were probably due to traces of P in the S

<table>
<thead>
<tr>
<th>Group</th>
<th>Precipitin titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rabbits</td>
</tr>
<tr>
<td>A Normal controls</td>
<td>6 6</td>
</tr>
<tr>
<td>B Sensitized intracutaneously with intact cocci</td>
<td>9 9</td>
</tr>
<tr>
<td>C Immunized intravenously with intact cocci</td>
<td>15 8 1 2 1 2 1</td>
</tr>
<tr>
<td>D Immunized intravenously with nucleoprotein (P)</td>
<td>8 3 1 4</td>
</tr>
<tr>
<td>E Immunized intravenously with bacterial emulsion (B. E.)</td>
<td>10 1 1 4 2 1</td>
</tr>
</tbody>
</table>

Figures indicate the number of rabbits in the respective groups showing precipitation to the titer designated.
extract. In this regard it is important, for reasons to be mentioned later, to point out that in Experiment 3 positive precipitation with S extract occurred with the serum of only one rabbit immunized with P, and this reaction was questionable (see footnote to Table V). The results in the B.E.-immunized group were negative in most instances; the serum of only one animal gave a reaction which in titer or character suggested the presence of anti-S precipitins.

TABLE V

Summary of Results of Precipitin Tests Using S as Antigen

<table>
<thead>
<tr>
<th>Group</th>
<th>Precipitin titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rabbits</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>A Normal controls</td>
<td>7</td>
</tr>
<tr>
<td>B Sensitized intracutaneously with intact cocci</td>
<td>12</td>
</tr>
<tr>
<td>C Immunized intravenously with intact cocci</td>
<td>8</td>
</tr>
<tr>
<td>D Immunized intravenously with nucleoprotein (P)</td>
<td>7</td>
</tr>
<tr>
<td>E Immunized intravenously with bacterial emulsion (B. E.)</td>
<td>7</td>
</tr>
</tbody>
</table>

Figures indicate the number of rabbits in the respective groups showing precipitation to the titer designated.

* Includes the only animal of Experiment 3 giving a positive reaction and this was merely ±.

Results of Skin Tests

The results of the skin tests of Experiment 3 are recorded graphically in Chart 1, in which the curves represent the average volumes of the lesions in each group of animals from day to day. The results were of the same general character in all three experiments, but in Experiments 1 and 2, because of the simultaneous intradermal injection of all the test substances, the lesions of the intracutaneously sensitized animals were less striking than in Experiment 3.

1 It has been found in work still in progress that injection of P, even intradermally, results in the desensitization of intracutaneously sensitized rabbits.
Chart 1. Volume curves of dermal reactions induced by Streptococcus Q 155 and its fractions in rabbits of the different groups of Experiment 3.
Intact Cocci.—In Chart 1 A are shown the averages of the cutaneous lesions induced in the animals of Experiment 3 by the intradermal injection of 0.01 cc. of living 20 hour broth culture of Streptococcus Q 155/0/7. The exaggerated skin reactions of the intracutaneously sensitized and the decreased reactions of the bacterially immune groups as compared with those of the normal animals are clearly apparent and were expected since these contrasting reactivities have become familiar through the studies of Swift and his coworkers. The P-immune animals likewise gave lesions smaller than the normal.

The results in the B.E.-immune groups of the three experiments were conflicting. In Experiments 1 and 2 (not shown in chart) the lesions were almost as small as those of the animals immunized intravenously with intact cocci, but in Experiment 3 (as shown in Chart 1 A) they were larger than normal. No explanation was found for this unexpected reaction in the four rabbits of Experiment 3.

Skin Tests with P.—The skin reactions induced by intradermal injections of P in the rabbits of Experiment 3 are shown in Chart 1 B. The enormous lesions of the intracutaneously sensitized animals are striking when compared with those of the normal controls. The average lesions of the intact coccus-immune group were similar in size to those of the normal animals, while those of B.E.-immune animals were smaller and very much more transient. The P-immune rabbits showed an immediate response more than twice as great as that of the normal animals, although less than that of the intracutaneously sensitized rabbits. The lesions in these two groups of animals illustrated well the differences between dermal reactivity of the anaphylactic and of the tuberculin types. Those of the former group had reached their maximal size by the end of 24 hours, were chiefly edematous in character, and subsided rapidly; in contrast, those of the intracutaneously sensitized group reached their maximal size only after 48 to 72 hours, were indurated as well as edematous, and subsided slowly.

In three of the seven normal rabbits the lesions induced by test injections of P showed a secondary increase in size about 4 days after their first appearance. This phenomenon was similar to the “secondary reaction” which occurs in normal rabbits injected intradermally with living bacterial cultures (2) except that it appeared several days earlier; and in this regard it should be noted that Schultz and Swift (12)
have observed the same early type of secondary reaction in normal rabbits injected with small amounts of horse serum.

**Skin Tests with S.**—Chart 1D records the results obtained when 0.25 mg. of S was injected intradermally into the rabbits of Experiment 3. Again, the largest lesions were shown by the bacterially hypersensitive group and the peak of the reaction was reached in 48 hours. Although smaller than the above, the lesions induced in the rabbits immunized with living culture were striking when compared with those of the normal controls. At the end of 24 hours these lesions were pink, edematous areas almost 25 times as voluminous as those of the normal rabbits, but they then rapidly decreased, showing the same size as those of the normal animals after 48 hours. The B.E.-immune animals showed essentially negative reactions, while those of the P-immune group became negative after 48 hours in spite of an initial response slightly greater than normal.

When a skin test dose of S ten times as large (2.5 mg.) was used, the results (Chart 1C) were essentially an exaggeration of those just described except in the case of the normal control group. The latter animals showed the expected small lesions after 24 hours; but a very pronounced increase in size then occurred, and reached its peak at 72 hours. The character of the lesions and the early occurrence of the enlargement make it improbable that it represented a secondary reaction of the hypersensitive type, but rather that the increase in size was due to some irritant in S solutions of this strength. This view is supported by the observation that similar effects are obtained in normal rabbits following intradermal injection of xylol in high concentration but not in low concentration (13). Furthermore, it is interesting to note that the irritant was probably of specific antigenic nature because the animals immunized with P and with B.E. were completely protected against this later reaction. So also were the rabbits immunized with living culture, in spite of the fact that they showed initial responses almost five times as great as those of the normal controls.

**DISCUSSION**

The differences between the reactivity to tuberculin of guinea pigs immunized with tuberculo-proteins and those inoculated with tubercle bacilli have long been known through the studies of Baldwin (14),
Krause (15), Zinsser (16), Dienes and Mallory (17), and many others. It is accepted that reactivity of the sort first mentioned is dependent upon an antigen-antibody reaction of the classical anaphylactic or Arthus type, but that true tuberculin hypersensitivity is independent of obviously demonstrable antibodies; and it is probable that these reactivities represent phenomena which can be elicited by bacteria in general. Various aspects of immunization and sensitization with streptococci and their fractions have been reported by a number of investigators, and it has been clearly shown that treatment with the various soluble fractions leads to the anaphylactic type of response on subsequent injection (18, 19), but that suitable intracutaneous inoculation with intact cocci induces sensitivity of the tuberculin type (3). In the work here reported, analogies to the studies with the tubercle bacillus are obvious.

For purposes of comparison, the results of serologic and dermal tests have been shown diagrammatically in Chart 2. As expected, there was a certain degree of correspondence. Thus, the animals immunized with P gave high anti-P precipitin titers and reacted with large lesions when cutaneously tested with P. These lesions reached their peaks comparatively early, were largely edematous in character, and subsided rapidly, as indicated by the sloping line in Chart 2. Similar lesions were induced by intradermal injections of S into the rabbits having high anti-S precipitin titers. As has been noted by Francis and Tillett (20), these probably represent carbohydrate antigen-antibody reactions wholly comparable to the familiar Arthus phenomenon obtained with proteins. That the correspondence between precipitin titer and dermal reaction was not complete, however, is indicated by the responses of the rabbits immunized with B.E. This group showed skin lesions even smaller than those of normal controls when injected intradermally with P, in spite of the presence of circulating anti-P precipitins in titer as high as that of the P-immune group.

Worthy of comment also was the limited antigenic capacity of the bacterial emulsion. Immunization of rabbits by intravenous injections of living, formalinized or heat-killed cultures led to the production of agglutinins and type-specific precipitins in high titer and of anti-P precipitins in varying amounts. Similar immunization using the
bacterial emulsion, in which the cocci had been killed by mechanical grinding, induced no demonstrable antibodies until relatively massive doses had been injected; and then only anti-P precipitins and agglutinins of only low titer were found, while anti-S precipitins were doubtful. In other words, the ground bacteria acted antigenically like the extracted nucleoprotein fraction. A possible explanation for this (21) is that the type-specific fraction of the antigenic complex was split off during the grinding, leaving S merely as haptene and P as the only effective antigen. Although the mechanism underlying this change is not yet clear and is still under investigation, the fact that pulverized streptococci had such limited antigenic capacity would lead one to question the validity of Krueger's suggestion (22) that bacterial antigens be prepared by mechanical

![Chart 2: Schematic comparison of cutaneous and serologic reactions of rabbits treated in different ways.](chart2)
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grinding in order to obtain more efficient immunizing agents. From the results described here it would seem that, at least in the case of the ndifferent streptococcus, the mechanically ground antigen is efficient in calling forth non-type-specific antibodies, but is of very little value where type-specific immunization is desired.

Distinct from the findings in the immune groups were those of the intracutaneously sensitized rabbits. Of all the groups, these animals showed by far the largest lesions to each of the four skin test substances used although their sera gave negative precipitin tests; and these dermal responses were delayed in reaching their peaks and persisted as large indurated nodules for many days. Whatever the mechanism underlying the exaggerated dermal reactions of intracutaneously sensitized animals may eventually prove to be, it is clear that, with the streptococcus as well as with the tubercle bacillus, the local reactions are not dependent upon the presence of demonstrable circulating precipitins of either the species- or type-specific varieties.

A difference between the present study and those dealing with tubercle bacilli exists in the apparent ability of S to call forth the tuberculin type of reaction in the intracutaneously sensitized animals. Zinsser at first (23) observed typical reactions following the intradermal injection into tuberculous guinea pigs of both protein and carbohydrate ("residue") fractions of the tubercle bacillus, but later (24), using more highly purified materials, found only the protein capable of inducing the reaction. Similarly, Laidlaw and Dudley (25), Long and Seibert (26), Mueller (27), Dorset, Henley and Moskey (28), and Dienes (29) obtained local responses following intradermal injection of tuberclo-protein into tuberculous guinea pigs but did not observe them following similar injections of tuberculo-carbohydrate. Only Petroff (30) found tuberculo-carbohydrate active in the tuberculin sense, and it is interesting to note that the animals which he used were rabbits rather than guinea pigs. In the present study, the streptococcal carbohydrate used was highly potent in inducing dermal reactions in rabbits intracutaneously sensitized with the homologous streptococcus. Whether this represents a difference between the skin reactivity of rabbits and guinea pigs, or whether bacterial sensitivity

2 Not all these authors called their material carbohydrate but, from the methods of preparation, it may be assumed that all were dealing with essentially the same substances.
to streptococci differs from that to tubercle bacilli in this regard, cannot be stated. Another possible explanation is that the carbohydrate extract employed by Petroff and that used in the present study, contained enough contaminating nucleoprotein to give rise to reactions in bacterially sensitized animals. Against this explanation, however, is the fact that the S extract used in Experiment 3 had been subjected to ultrafiltration through a collodion membrane and contained only doubtful traces of P, for the serum of only one P-immune rabbit gave precipitation with S extract and this reaction was merely a questionable one in the lowest dilution (see Table V). Actually the lesions resulting from the injection of 0.25 mg. of S into intracutaneously sensitized rabbits were comparable in size to those induced by 0.1 mg. of P, and it seems hardly possible that traces of P in the S extract could have approached this amount. Nevertheless, this remains an important possibility, and experiments are in progress to check the results with even more highly purified S.

There appear to be three possible explanations of the wide hypersensitivity of the intracutaneously sensitized rabbits to all the test substances used: (a) that the skin responses are due to an antigen-antibody reaction involving a fraction common to all the test substances, (b) that they represent antigen-antibody reactions to each of various fractions used in testing, or (c) that they are due to a general heightening of tissue reactivity independent of antigen-antibody interaction and hence are non-specific in nature. Further studies are in progress to test these possibilities.

SUMMARY AND CONCLUSIONS

Rabbits were immunized intravenously with intact indifferent streptococci, with homologous P fraction, and with an emulsion of mechanically ground cocci; others were sensitized by intravenous injection of the intact microorganisms. Their serologic and dermal reactions to these materials and to the homologous S fraction were compared with those of normal animals. The dissociation, in certain instances, between circulating antibody and dermal reactivity was noteworthy. From the results the following conclusions were drawn.

1. Intradermal injection of a soluble streptococcal protein into a rabbit immunized intravenously with that protein leads to the immediate anaphylactic type of skin response; while similar dermal testing
of a rabbit sensitized by intracutaneous inoculation of the intact microorganism induces the delayed (tuberculin) type of response.

2. The induction of the immediate type of dermal reaction to streptococcal protein requires more than the mere presence of a high serum precipitin titer to that protein.

3. Lesions of the immediate type can be induced by the intradermal injection of a streptococcal carbohydrate into rabbits immunized intravenously with intact cocci and showing a high serum precipitin titer to that carbohydrate.

4. Intravenous immunization of rabbits with an emulsion of mechanically ground indifferent streptococci leads to the production of only non-type-specific antibodies.

5. It is possible that carbohydrate as well as protein fractions of indifferent streptococci are capable of eliciting the delayed type of dermal response in rabbits intracutaneously sensitized with that microorganism.

BIBLIOGRAPHY
11. Lancefield, R. C., personal communication.