# REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

## I. The Antibody Response

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During the course of studies on the effectiveness of Pneumococcus antigen under different conditions, unexpected results were observed following the introduction of the bacteria into the skin. It was found that the antibody response under this condition is strikingly different from that which is evoked by intravenous injection. The response of the animal to the intracutaneous injection of pneumococci has been subjected, therefore, to a thorough study. Some of the results have been stated in a preliminary communication (1). Further work is reported here.

### *Methods*

*1. Preparation of Vaccines.* Eight to ten hour broth cultures of Pneumococcus were centrifuged at a high rate of speed, the supernatant fluid was discarded, and the organisms were suspended in an amount of saline solution equal to one-tenth of the original volume of culture. Occasionally, the sedimented bacteria were washed 1 to 3 times before final resuspension. The concentrated emulsion of bacteria was then heated for one hour at 56–60°C. and sterility was determined by cultures and intraperitoneal injection of white mice.

*2. Preparation of the Skin.* In the earlier experiments the fur was removed by clipping and shaving, but later this was accomplished by gentle rubbing with a saturated solution of barium sulfite. The latter method proved more satisfactory, since, with care, it left a perfectly clear skin which remained free of fur for a number of weeks. The depilation was carried out on both sides of the body, and, since consecutive injections were made, it frequently became necessary to repeat the removal of fur.

*3. Method of Injection.* The suspensions of heat-killed pneumococci were injected intracutaneously once a week in quantities of 0.2 cc. This amount was equivalent to 2.0 cc. of the original culture. The injections were given alternately on the two sides of the body, each injection being made in a different area of the skin. The

number of injections in the different animals varied. In the majority of instances, the total amount of suspension given was equivalent to 20 cc., or more, of culture. The results obtained, therefore, could be roughly compared with those observed following intravenous immunization of rabbits with similar amounts of culture.

*4. Testing for Antibodies.* Some of the animals were tested for antibody production only after a complete series of ten injections and others at different intervals during the process. Usually the rabbits were bled once a week, immediately preceding each injection. Final specimens of blood were procured 10 days following the last injection. The sera were tested for the presence of type-specific antibodies (anti-S), which agglutinate the encapsulated, or S, cells and precipitate the soluble specific substance derived from them, and also for species-specific antibodies (anti-P or anti-R) which agglutinate only the capsule-free, or R, cells and precipitate the protein derived from any type of Pneumococcus.

## EXPERIMENTAL

Rabbits were injected into the skin with suspensions of heat-killed Pneumococcus Type I or Type III, or an R strain derived from Type II Pneumococcus. In addition, intracutaneous injections were made with solutions of derivatives of Pneumococcus, one of which consisted essentially of a solution of "nucleoprotein" (2) and the other of the supernatant fluid after acid precipitation of the "nucleoprotein" from a solution of pneumococci resulting from repeated freezing and thawing. The strain of Type I Pneumococcus employed was virulent for white mice and rabbits, and killed animals of both species when $10^{-7}$ cc. of culture was administered. The Type III culture killed mice in a quantity of $10^{-7}$ cc. of culture, but did not kill rabbits in amounts as high as several cc., although in these animals it was capable of producing a transient septicemia. In one experiment a rabbit-virulent strain of Type III Pneumococcus was used as antigen. The lethal dose of this strain varied from $10^{-3}$ to $10^{-4}$ cc. of culture.

### *Antibody Response to Type I (S) Pneumococcus*

Several groups of rabbits (sixty animals in all) were given repeated intracutaneous injections of suspensions Pneumococcus Type I. The development of antibodies was essentially the same in all the animals so that for the sake of brevity and clarity, one group will be described in detail as typical. The data obtained in the study of this group are represented graphically in Figure 1. The curves for the development of type-specific and species-specific agglutinins were plotted from the averages of the titres of the sera of a group of four rabbits. These rabbits had received 12 intracutaneous injections of 0.2 cc. each of a suspension of heat-killed pneumococci of Type I (S form), a total number of bacteria equivalent to those contained in 24 cc. of culture,

which is more than the amount required for the usual intravenous immunization. The astonishing result was the absence of type-specific agglutinins and type-specific precipitins in the sera of all the animals. In other words, the sera did not cause the agglutination of the bacteria employed in the immunization, nor precipitate the soluble specific substance derived from them. Moreover, this result was obtained not only in this particular group of animals, but in a total of 60 rabbits receiving repeated intracutaneous inoculations of Type I Pneumococcus only 7, or about 12 per cent, showed any evidence of type-specific response, and in these instances specific agglutinins were demonstrable only in low dilutions of the serum. In two

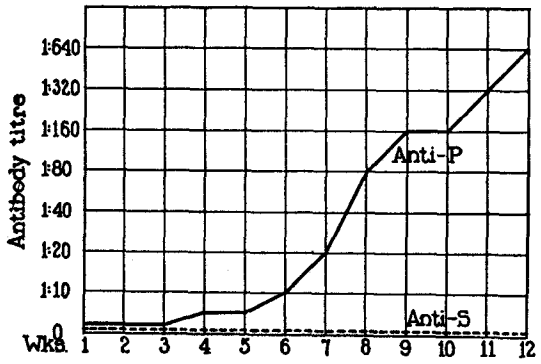


FIGURE 1. The development of antibodies in rabbits immunized intracutaneously to Pneumococcus, Type I.

instances agglutination occurred in a dilution of 1:1, in one instance 1:3, in three instances 1:5, and in one case 1:20. Obviously, these results are in marked contrast to those obtained with the sera of animals which have received similar quantities of organisms administered intravenously, in which case agglutination usually occurs in dilutions of 1:80 to 1:160. The lack of type-specific antibodies can be interpreted as evidence of the disintegration of the type-specific antigen after the introduction of the intact cell into the skin.

Despite the fact that type-specific (anti-S) antibodies were absent, the species-specific antibodies (anti-P or anti-R) were found to be present in high degree in the sera of all the rabbits. As illustrated in

Figure 1 the species-specific antibodies, as measured by the agglutination of R cells, are definitely detectable after the third or fourth injection, and after about the sixth injection there is a constant and rapid rise in titre which may reach a serum dilution of 1:640. In the sera of some rabbits, not included in the accompanying curves, the concentration of species-specific antibodies was even higher.

*Antibody Response to Type III (S) Pneumococcus*

In terms of its capacity to stimulate the formation of type-specific antibodies, Type III Pneumococcus is at best a poor antigen. The recent work of Tillett (4) has established the fact that Type III strains

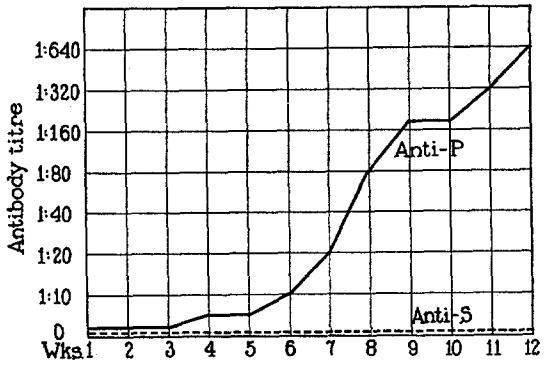


FIGURE 2. The development of antibodies in rabbits immunized intracutaneously to Pneumococcus, Type III.

of marked virulence for mice are usually not only avirulent for rabbits, but in only a small percentage of rabbits (15 per cent) do they elicit a type-specific antigenic response following intravenous inoculation. Moreover, his results indicate that with Type III Pneumococcus there is a parallelism between type-specific antigenicity and virulence. It was to be expected, then, that rabbits immunized intracutaneously with Type III pneumococci would respond very poorly in the production of type-specific antibodies.

In Figure 2, curves are given which illustrate the development of anti-S and anti-R agglutinins. The rabbits were given 12 injections of 0.2 cc. each of a suspension of Type III pneumococci, thus receiving

in all the equivalent of 24 cc. of culture. It will be seen that at no time were type-specific antibodies demonstrable either as agglutinins or precipitins. None of a total of 45 rabbits, studied at different times, possessed anti-S antibodies in their sera during or following repeated intracutaneous injections of Type III pneumococci.

On the other hand, in the sera of all the rabbits species-specific antibodies were demonstrable. As Figure 2 illustrates, the development of anti-R agglutinins in these rabbits closely parallels that observed in the group immunized intracutaneously to suspensions of Type I pneumococci.

*Antibody Response to R Pneumococcus*

That R organisms fail to incite the formation of type-specific antibodies has been the common experience of all workers. The intravenous administration of R cells leads only to the development of species-specific antibodies. In studying the antibody response in rabbits following intracutaneous injections, an R strain derived from Type II was employed as antigen, and in each instance the total amount of heat-killed suspension employed was equivalent to at least 20 cc. of culture. None of the rabbits immunized in this manner formed type-specific antibodies. The serum of all the rabbits, however, contained species-specific agglutinins and precipitins in titres comparable to those observed in rabbits immunized to Pneumococcus Type I and Type III.

The injection into the skin of the Pneumococcus cell, whether R or S, elicits an antibody response which is essentially or entirely of the species-specific variety. It seems likely, therefore, that the type specificity of the S form is destroyed when it is introduced into the skin.

*Antibody Response to Soluble Derivatives of Pneumococcus*

In the preceding experiments rabbits were immunized by the inoculation of the intact, formed cells. The studies of Reimann (5) have revealed that the antigenic response of animals to injection of the "nucleoprotein" of Pneumococcus is identical with the response to the intact R cells. It was interesting, therefore, to compare the antigenic response of rabbits to the intracutaneous injections of soluble derivatives of Pneumococcus with the response to the similar injection

of the intact cells. The soluble derivatives were obtained from an S strain of Type II Pneumococcus. The cells were frozen and thawed repeatedly, and from the resulting solution of bacterial substance, by precipitation with acetic acid in the cold, a material designated for convenience as "nucleoprotein" (2) was obtained. The residual supernatant fluid, after precipitation with acetic acid, was also studied. This supernatant fluid contains, in addition to other substances, the purpura producing material of Pneumococcus (3, 6). The two soluble derivatives were inoculated consecutively once a week into the skin of rabbits in a volume of 0.2 cc. each. The substances were standardized on the basis of protein nitrogen, and rabbits received in toto at least 70 mgm. of the first substance and at least 30 mgm. of the latter considered as protein.

As was to be expected, none of the rabbits injected with either of these two soluble derivatives showed anti-S in their sera. This is in agreement with previous results (7) from this laboratory. On the other hand, all of the rabbits possessed precipitins for the "nucleoprotein" and agglutinins for the R cells but in somewhat lower titre than that demonstrated in the sera of rabbits immunized by either the intact R or S forms of Pneumococcus.

### *Antibody Response to Intravenous Injections after Previous Intracutaneous Immunization*

The marked differences in antibody responses to type-specific antigens, depending upon whether the intravenous or intracutaneous route of administration was employed, raised the question whether the property of inducing disintegration or dissociation of the type-specific antigen is possessed only by certain tissues, such as the skin, or whether all tissues of the animal body are potentially able to induce this disintegration, and whether, as a result of repeated intracutaneous injections, this property might be so exalted that subsequent intravenous introduction of the bacteria would fail to elicit a type-specific antibody response. In order to determine the answer to this question the following experiment was made. Twelve rabbits were selected which, following repeated intracutaneous immunization, did not show type-specific antibodies in their sera. Six had been immunized with a suspension of heat-killed Type I pneumococci, and six with a suspension

of Type III pneumococci. All twelve were then immunized by intravenous injections of a suspension of heat-killed Type I pneumococci. Following the intravenous immunization, the sera of all 12 rabbits were found to agglutinate Type I cells specifically. The titre in each case reached as high as 1:160. This titre is slightly higher than that usually found in normal rabbits after intravenous immunization alone.

## DISCUSSION

The significant result of the present study is that the type-specific antigen of pneumococcus does not stimulate the production of antibodies when it is injected into the skin. This is in marked contrast with the results obtained when the injections are made intravenously. Following intracutaneous injections of the whole organisms the resulting antibodies are of the species-specific type. While in the skin the type-specific substances of the bacterial cell, therefore, become ineffective, the remaining constituents of the pneumococcal cell retain the power of stimulating antibody formation. The antibody response to intracutaneous inoculations is the same whether the type-specific cells (S forms), the cells which do not possess type-specific characters (R forms), or the soluble protein constituents of the cell are injected.

Why the type specific antigen of the cell, when injected into the skin, becomes ineffective in stimulating antibody response is not entirely clear. It is possible that in the skin the bacteria are mechanically localized and that, as a result, the cells undergo a disintegration, comparable to autolysis, during which the type-specific antigen is destroyed. It is more likely that the skin possesses a peculiar property of causing disintegration or dissociation of the compound type-specific antigen, so that the latter is no longer able to stimulate the production of antibodies.

Similar failure of pneumococci to stimulate the production of type-specific antibodies when the bacteria are injected into the skin has been noted by others. Goodner (8) found that when live virulent cultures of Type I pneumococci were injected into the skin of rabbits half of the animals failed to develop type-specific antibodies in their sera. Gross (9) has also found that following the intracutaneous injections of small amounts of live pneumococci in rabbits, no agglutinins appeared in the sera. Bull and McKee (10) found that type-specific antibodies

frequently failed to appear in the sera of animals after pneumococci had been instilled into the nose. It is of much significance that in the experiments of these three sets of observers antibodies failed to develop following the injection of live virulent pneumococci, in two cases into the skin, and in the other case into the nose.

## SUMMARY AND CONCLUSIONS

1. Sixty rabbits were immunized by the repeated injections into the skin of small doses of suspensions of heat-killed Type I pneumococci. In 53 of the rabbits no type-specific antibodies appeared in the serum, and in the remaining seven the titre of these antibodies in the serum was very low. In all cases, however, the sera possessed a high titre of species-specific antibodies.

2. Forty-five rabbits similarly immunized by injections of heat-killed Type III pneumococci also failed to form type-specific antibodies but did form species-specific antibodies.

3. Suspensions of heat-killed R pneumococci and solutions of bacterial substances when injected into the skin stimulated the production of species-specific antibodies, although they failed to stimulate the production of any type-specific antibodies.

4. Animals which had been immunized by intracutaneous injections still possessed the ability to form type-specific antibodies when they were subsequently given intravenous inoculations of type-specific pneumococci.

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