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Agglutination of the Encapsulated Anthrax Bacilli.

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There is no convincing experimental evidence on the specific agglutination of anthrax bacilli. The rapid sedimentation of most strains of anthrax bacilli makes a systematic study somewhat difficult. We know however that although by using certain special strains a stable suspension can be secured, the addition of immune serum will not be followed by any particulation, in spite of its containing a high concentration of precipitating antibodies.

A way was opened for the reinvestigation of this question since Szongott and one of us had discovered in the anthrax serum a highly active antibody, hitherto unknown.[1] The antibody of the anthrax immune serum known up to now and used for the thermoprecipitation test acts on the somatic substance of the bacillus, which is a polysaccharide. According to these authors, another and a more potent antibody is obtained, when suitable strains are used for immunization. This antibody reacts probably with the capsular material which is a carbohydrate-free, proteinlike substance. The 2 antibodies were named anti C and anti P respectively.

An accidental observation led us to study the effect of the anti P immune body on the agglutination of anthrax bacilli. We had primarily intended to study the phagocytosis of the encapsulated anthrax bacilli *in vitro*. The strain used for this experiment was isolated of the so called "Carbozoo" vaccine prepared by Mazzucchi and utilized in Italy for prophylactic immunization of animals.[2] This strain was virulent for laboratory animals, it could easily be emulsified in physiologic salt solution and produced in 24 hours an abundant capsule when cultured on agar at 37° C. In this respect this strain appeared very much similar to the "mucoid" varieties, which are obtained when virulent anthrax bacilli are attenuated by repeated subcultures at 41° C. according to Pasteur's well known procedure. When the phagocytosis of this strain was studied in the presence of an immune serum containing both C and P antibodies, a very marked agglutination could be observed under the microscope.

The agglutination was then performed following the usual micro-

[1] Tomcsik, J., and Szongott, H., *Z. f. Immunitätsforsch*, 1933, **77**, 86.

[2] Mazzucchi, M., *La Clinica Veterinaria*, 1931, **9**, 3.

scopic technic. The result was quite surprising when 1:2 - 1:8 dilutions of the immune serum were added. Immediate total agglutination occurred and in an hour all of the bacilli were so firmly attached to each other in a transparent disc, that they could not be separated by intensive shaking. Higher dilutions of the immune serum (1:16-1:256) caused a typical floccular agglutination. This agglutination was strictly specific. No trace of agglutination occurred even when undiluted normal serum was added to the bacterial suspension.

Considering the peculiar nature of the agglutination, we had to weigh the possibility that we might be confronted with a special case of "agglutination by precipitin."[3] This suspicion seemed to us acceptable since we observed that a considerable quantity of P specific substance and a smaller quantity of C substance might be in solution after 24 hours in a broth culture of the encapsulated anthrax bacillus. Consequently we carried out a few agglutination tests with bacilli washed carefully before the performance of the test. No change occurred in the previously recorded results. This reaction therefore had to be regarded as a true agglutination.

The relation of precipitating antibodies to the agglutination. Our previous experiments concerning the antigenic structure of anthrax bacilli had made it very likely that the agglutination of the encapsulated anthrax bacillus had been caused by the same antibody which precipitated the P substance. Direct experiments had to be carried out to prove this supposition.

There was no difficulty in forming an opinion about the rôle of the C antibody since the commercial precipitating anthrax immune serum contains this antibody alone. Four sera were used in this experiment, each of which gave specific precipitation with anthrax polysaccharide up to 1:1,000,000 dilution of the latter and no precipitation whatsoever with P substance. No trace of agglutination was observed by using any of these sera in 1:1-1:1024 dilutions. An entirely negative result was observed likewise by performing this test with a commercial protective immune serum, which contained none of the precipitating antibodies.

On the other hand positive agglutination was obtained with each of the 5 immune sera prepared by us in rabbits through immunization with encapsulated anthrax bacilli. Each of these sera contained an anti C immune body and gave at the same time precipitation with 1:500,000-1,000,000 dilutions of the P substance. Furthermore a certain correlation could be demonstrated between the P precipitin

[3] Jones, F. S., *J. Exp. Med.*, 1927, **46**, 303 ; 1928, **48**, 183.

titer and the agglutinin titer of these sera. The agglutinin titer of the immune sera varied, however, according to the strain used in the experiment.

It was found but once, that the serum of a rabbit bled before the end of full immunization showed agglutination up to a dilution of 1:16, without giving any precipitation with the P substance. The lack of correlation was however temporary since the serum of the same rabbit gave both precipitation and agglutination after 2 more injections. This is another instance that during the immunization the agglutinating property of the immune serum manifests itself earlier than its precipitating property, owing to the fact that different proportions of antibodies are necessary to bring forward the appearance of a visible reaction in the 2 tests.

Agglutination and capsule formation. Two sera were selected for further experiments in order to study the correlation of capsule formation and of the agglutinability in different strains. One of the sera possessed a high anti C and no anti P precipitating activity, the other a high anti P and a lower anti C activity.

Ten different anthrax strains were selected for the first part of these experiments. It was carefully established in wet India ink preparations that none of these strains grown on ordinary agar media ever exhibited a trace of capsule formation. Most of these were virulent, a few attenuated. The bacterial suspensions were prepared by emulsifying a 24 hour agar growth in saline solution and shaking them for varying length of time according to their tendency for sedimentation. In most cases a homogeneous suspension could be obtained though the rate of sedimentation was sometimes increased. The results of the agglutination tests were completely negative, neither of the 2 immune sera caused any agglutination.

An attempt was also made to sensitize these strains through absorption of purified P substance. The bacteria washed and resuspended after this procedure however were just as refractory toward agglutination as before.

On the contrary positive agglutination test was obtained with each of those strains which produced capsules on agar medium. We should emphasize here that not alone strains isolated of anthrax vaccines, or attenuated in our laboratory according to Pasteur's procedure belonged to this group. Almost 50% of our virulent old laboratory strains and some of the recently isolated fresh strains showed some capsule formation. It is true, that in many instances the capsule formation could be detected only by careful search in India ink preparations and extended but to one portion of some of

the long chains. The agglutination by anti P immune serum was without exception positive whenever capsule formation could be revealed by microscopic study. The difference between the agglutination of the virulent strains and the attenuated "mucoid" varieties was that the former gave a floccular type of agglutination, whereas the latter, when lower dilutions of the serum had been used, was agglutinated in the characteristic disc form described above.

We confine ourselves to the description of one strain in order to illustrate the behaviour of the virulent and encapsulated anthrax bacillus. Anthrax strain No. 4 was isolated 6 years ago from naturally infected sheep and kept on agar medium with monthly subcultures. During this time it was transferred only a few times in mice. It was virulent for rabbits. At the time of our study the capsule production of this strain on agar medium was distinct. Its 24 hour agar growth consisted of long chains, some of these completely surrounded by a sharply defined capsule, twice as thick as the bacillary body. Other chains in turn had but a few capsulated members with well defined or with partly dissolved capsules. Some were entirely bare. At this stage this strain gave total agglutination with anti P immune serum up to a dilution of 1:512. The agglutination had a floccular character and contrary to the behaviour of the mucoid strains some inhibition was observed in the lower serum dilutions (up to 1:8). It was then subcultured daily for 15 days. At the end of this period it ceased to produce any capsule on agar medium and its agglutinability was completely lost, that is neither the P nor the C antibody produced any agglutination when mixed to this subculture.

Summary. 1. Antianthrax immune serum containing both P and C precipitin agglutinates only the encapsulated anthrax bacilli. 2. Antianthrax immune serum containing only C precipitin does not give agglutination with any type of anthrax bacilli. 3. P antibody can be regarded as the anticapsular antibody. 4. The serological specificity of the capsules of attenuated "mucoid" strains is the same as that of the virulent anthrax bacillus, if the latter produces capsules on culture media.