THE APPLICATION OF THE REACTION OF AGGLUTINATION TO THE PNEUMOCOCCUS.

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The following report is a part of the work on pneumonia as planned by the Commission for the Investigation of Acute Respiratory Diseases, and by the Department of Health.

Normal serum of various animals differs greatly in its tendency to agglutinate many strains of pneumococci. Thus rabbit serum generally gave negative results, while sheep and horse serum reacted slightly in a few instances, and the serum of one goat of four tested agglutinated a number of strains of pneumococci in dilutions of 1:10.

Of two normal human sera tested, one failed to react while the other agglutinated several of the organisms in dilutions as high as 1:10.

Neufeld, Clairmont, Landsteiner, Wadsworth, Heyrovsky, and others have succeeded in producing agglutinins for pneumococci in the animal body through immunization.

Gorgáno and Fattori state that agglutination of Diplococcus pneumoniae with the blood of patients suffering from infection with this organism is constant, that it persists for some time after recovery of the patient, and that the reaction is more marked if the homologous organism be used. The highest reaction obtained by them was, however, in a 1:10 dilution.

Glucose broth has been generally recommended as the medium best adapted for making agglutination tests with the pneumococcus, but as the organism quickly dies out in the presence of the excess of acid produced by the fermentation of the sugar, it can be carried through one generation only on this medium,
unless it is transferred before the acidity has increased sufficiently to destroy the growth. This fact makes the broth unsuitable for the work.

Marshall and Knox \(^1\) and Morello have shown that the typhoid bacillus loses its agglutinability when grown for some time in an active immune serum. Dr. Park and I have demonstrated the same to be true for the bacillus of dysentery when grown in its immune serum, and we were also able to show, further, that the agglutinability could be restored by long cultivation upon suitable media.

As the presence of serum constituents in the medium is required for the continuous growth of the pneumococcus, it might be assumed from the above facts that the agglutinability of the organism might at least be lowered by long cultivation upon a medium containing even very small amounts of these inhibitory substances. This assumption was borne out by several tests made with an organism taken on the one hand directly from a fresh rabbit-blood-agar culture, and on the other from a culture in calcium broth \(^2\) one generation old and several generations old. The last culture gave the best reaction, while the culture from the blood-agar gave the poorest reaction.

To eliminate this source of error, diluted sheep or hog serum, as suggested by Dr. Park, was boiled to destroy any inhibitory substances present, and added to broth or agar as the case required. Cultures obtained from media containing these sera, when transferred to calcium broth, usually gave a homogeneous growth with much less tendency to spontaneous agglutination than is seen with cultures grown in calcium-glucose broth. Heating the organism to \(70^\circ\) C. for 15 minutes does not affect its agglutinability. Heating the serum to \(85^\circ\) for 15 minutes,

\(^1\) The studies of Marshall and Knox, so far as I know, have not as yet been published.

\(^2\) The fact that the addition of calcium carbonate to culture media neutralizes the acid formed by the fermentation of sugar during bacterial growth has been recognized for some time. The application of this reaction to the growth of the pneumococcus was suggested independently by Bolduan and Hiss. The former recommended the addition of bits of marble to plain broth, the latter used calcium carbonate in the form of powder in glucose broth.
Reaction of Agglutination to the Pneumococcus

however, destroys the agglutinins both for the pneumococcus and the Pneumococcus mucosus.\(^3\)

Several methods of immunization were tried. The one which gave the best results is represented by the following example:

**Feb. 15.** A rabbit was given subcutaneously 3 c.c. of a culture grown for 48 hours in broth to which a few drops of defibrinated blood were added, heated previously to 60° C. for 30 minutes.

**Feb. 28.** 5 c.c. of a similar culture administered.

**March 8** 1/4 c.c. of a living culture of the same organism was given.

**April 1** 5 c.c. 1 c.c. 5 c.c. of a calcium-broth culture heated 70° C. for 15 minutes given.

**April 8** 5 c.c. 5 c.c. 5 c.c. 5 c.c. 5 c.c. 5 c.c. 5 c.c. 5 c.c.

**April 13** 5 c.c. 5 c.c. 5 c.c. 5 c.c. 5 c.c. 5 c.c. 5 c.c. 5 c.c.

**April 15** 10 c.c. 10 c.c. 10 c.c. 10 c.c. 10 c.c. 10 c.c.

**April 25** 10 c.c. 10 c.c. 10 c.c. 10 c.c. 10 c.c. 10 c.c.

**May 1** 10 c.c. 10 c.c. 10 c.c. 10 c.c. 10 c.c. 10 c.c. 10 c.c. 10 c.c.

**May 8** 15 c.c. 15 c.c. 15 c.c. 15 c.c. 15 c.c. 15 c.c. 15 c.c. 15 c.c.

**May 15** 15 c.c. 15 c.c. 15 c.c. 15 c.c. 15 c.c. 15 c.c. 15 c.c. 15 c.c.

**May 22** Animal bled and the serum tested with the homologous organism which it agglutinated in a dilution of 1:200.

**May 22** An emulsion heated to 70° C. for 15 minutes from four heated serum-agar plates was injected subcutaneously. Animal dead on the following day.

Hanging drops were chiefly relied on for ascertaining the reactions, though in many instances these were controlled by the macroscopic method and the contents of the tubes examined microscopically after reaction had taken place.

The sources of error seem about equal in the two methods, while the hanging drop has the advantage of shorter time limit of reaction, and of easy recognition of contamination.

With the pneumococcus the tube method generally indicates a higher microscopical reaction than the hanging drop. This is contrary to tests made with the dysentery and typhoid bacilli, and is explained by the fact that in the former case the free organisms must be present in great numbers to cloud the supernatant fluid, whereas in the latter a comparatively small number of free bacilli may render the fluid turbid, so that in the case of the pneumococcus a good reaction viewed macroscopically may become only a fair reaction when viewed microscopically.

\(^3\) A description of this organism will be found in the article by Drs. Park and Williams in this volume.
Neufeld states that the various strains of the pneumococcus agglutinate alike, an observation probably due to the low reactions obtained by him, since his maximum reaction was 1:50. My work has shown great irregularity in this respect, the serum of an animal immunized with one strain of pneumococcus agglutinating only seven organisms out of seventy tested in dilutions equalling the reaction (1:200) with the homologous organism. Four strains reacted in dilutions of 1:10, eleven in 1:2, while the remaining organisms were entirely negative.

The serum of a second immunized animal agglutinated the homologous organism in dilution of 1:100, while other strains were affected in less dilutions or not at all.

TABLE I.
AGGLUTINATION TESTS WITH THE SERUM OF A SHEEP injected for a period of three months with pneumococcus No. 36.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>2</th>
<th>10</th>
<th>20</th>
<th>50</th>
<th>100</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical pneumococcus 36 . . . . . .</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+1</td>
<td>—</td>
</tr>
<tr>
<td>&quot; 14 . . . . . .  &quot;</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>&quot; 4 . . . . . .  &quot;</td>
<td>++</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>&quot; 16 . . . . . .  &quot;</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>&quot; 18 . . . . . .  &quot;</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>&quot; 33 . . . . . .  &quot;</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Atypical &quot; 2 . . . . . .  &quot;</td>
<td>++</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>&quot; 66 . . . . . .  &quot;</td>
<td>+1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Pneumococcus mucosus 47 . . . . . .</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

The remaining sixty-one organisms tested reacted some in dilution of 1:2 and others not at all, thus emphasizing the distinction of pneumococcus No. 36 from the other strains in regard to its power to produce agglutinins.

4 For the details of the immunization of the sheep, the paper of Drs. Park and Williams is to be consulted.
There is apparently a difference in the agglutinability of the pneumococci, some strains uniformly reacting much more readily than others in normal and immune sera.

A rather interesting fact that deserves further investigation is this: Two strains of pneumococci which showed good agglutination with several active sera failed to produce agglutinins to any extent in the animal body for themselves or other strains of pneumococci. A rabbit and a goat were inoculated without results with one strain, and a rabbit and a horse with the other; and as the same kind of animals was immunized under the same conditions with other strains with good results, this irregularity would seem to indicate a peculiarity of the organism rather than of the animals injected.

Another observation of interest, but one which has not been carried far enough on account of insufficient time to establish definite conclusions, is certain reactions obtained with a streptococcus serum and absorption experiments made with this organism.

A young goat which was immunized with a strain of streptococcus yielded a serum which agglutinated a few pneumococci and its own culture in dilutions of 1:10.

Pneumococcus 66, which coagulates inulin-serum water late, absorbs the agglutinins for several pneumococci from a typical pneumococcus immune serum. This culture is the only one of the pneumococci that has its agglutinins taken out of the above streptococcus immune serum by the streptococcus; it produces agglutinins in the animal body for itself and many of the pneumococci. These reactions suggest the possibility of the occurrence of intermediate types of organisms between pneumococci and streptococci.

EXHAUSTION EXPERIMENTS.

The extreme sensitiveness of the pneumococcus to changes of conditions not readily determined brings about variation in the behavior of this organism which proved a serious factor in the application of the agglutination reaction and in the interpretation of the results obtained. To eliminate as far as possible
any errors arising from this instability, the exhaustion experiments were conducted in groups, each group covering as many observations as practical, in order to insure uniform conditions for a number of tests.

In the exhaustion experiments a slight loss of agglutinins has generally been observed. This loss occurs whether the organism used for absorption is an homologous or a related one or of a foreign type. This fact points to the cause of the loss lying outside of the presence of the organism. The loss is readily estimated on account of its uniformity, and in no way affects the determination of the amount of absorption excepting where a strain reacts only in low dilutions. In this case the disappearance of the agglutinins cannot be ascribed with certainty to the organism used for absorption, and the establishment of relationship by the absorption method is not possible in these instances.

Testing the reaction of the meningitis coccus in antipneumococcus serum, Sorgente failed to obtain agglutination with a number of strains. We failed to absorb the agglutinins from a serum agglutinating several strains of pneumococci in dilution of 1:200 with a culture of the diplococcus of meningitis.

As shown in Table II, the power of the serum to agglutinate pneumococcus Nos. 14 and 72 in equally high dilutions with the homologous organism, and by absorption that the agglutinins are group agglutinins in the case of the former, and both group and specific agglutinins in the case of the latter organisms, are readily explained by the fact that the two types of agglutinins constantly vary in ratio both in different animals and at different periods of inoculation, the group agglutinins exceeding even at times the specific ones.

The increase of agglutinins for different strains of Pneumococcus mucosus in the serum of an animal inoculated with one of the typical pneumococcus strains, and the results obtained by the absorption of these agglutinins, separate them into a distinct variety from the majority of other pneumococci.

The similar results obtained, as indicated in the following table, by absorption with Pneumococcus mucosus No. 47 and
### TABLE II.

**Serum of a Sheep Injected with Pneumococcus Mucosus (Williams), Showing the Agglutination Index Before and After Absorption with Its Own Group, Typical Pneumococci, a Strain of Streptococcus, and Bacillus Typhosus, Respectively.**

<table>
<thead>
<tr>
<th></th>
<th>Before Exhaustion</th>
<th>After Exhaustion with Pneumococcus No. 14</th>
<th>After Exhaustion with Streptococcus longus</th>
<th>After Exhaustion with B. typhosus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>10</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Pneumococcus mucosus</td>
<td>25</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Atypical Pneumococci</td>
<td>47</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Typical Pneumococci</td>
<td>4</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Typical Pneumococci</td>
<td>14</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Typical Pneumococci</td>
<td>11</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Typical Pneumococci</td>
<td>36</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Typical Pneumococci</td>
<td>16</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Typical Pneumococci</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Part of the remaining organisms tested reacted in dilutions of 1:2 and part failed to react even in this low dilution.

+ + complete agglutination; + i not quite complete; + good; 1 trace; + fair; - negative.
<table>
<thead>
<tr>
<th>Typical Pneumococcus</th>
<th>Before Exhaustion</th>
<th>After Exhaustion with Pneumococcus No. 66</th>
<th>After Exhaustion with Streptococcus longus</th>
<th>Before Exhaustion</th>
<th>After Exhaustion with Pneumococcus No. 22</th>
<th>After Exhaustion with Pneumococcus No. 72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>20</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>Control</td>
</tr>
<tr>
<td>&quot;</td>
<td>4</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>&quot;</td>
<td>14</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Normal</td>
<td>&quot;</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Atypical</td>
<td>46</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>&quot;</td>
<td>66</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>&quot;</td>
<td>73</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>i</td>
</tr>
</tbody>
</table>

The remaining organisms tested reacted as in Table II. The above tests were made simultaneously, the same cultures being used throughout.

The irregularity of the pneumococci in their behavior to the agglutination reaction is amply indicated by the tables. The interaction of typical and atypical cultures does not bear out the classification based upon the morphological and cultural characteristics of the organisms. Atypical pneumococci 46, 66, and 73 differ constantly from each other, and yet correspond with several of the typical pneumococci in their agglutination reactions.

The different degrees of partial exhaustion of agglutinins for normal culture No. 46 suggest the existence of agglutinins common to this culture and several other strains of pneumococci, as well as to the Streptococcus longus.
pneumococcus No. 4 (the latter organism when studied not producing the characteristic capsule and gelatinous colonies), suggest that these cultural attributes may be lost while the agglutinative affinity is still retained.

The ability of the Pneumococcus mucosus group to produce common agglutinins for some pneumococci, and the fact that the streptococcus failed to affect through absorption their agglutinins, would indicate a closer relation of this variety to the pneumococci than to the streptococci.

CONCLUSIONS.

Owing to unavoidable circumstances only a limited amount of time was available for the work on agglutination. The foregoing report is therefore preliminary only and the following conclusions are provisionally offered:

I. Pneumococci by reason of their agglutinating properties exhibit a tendency to separate into numerous groups similar to streptococci.

II. Pneumococcus mucosus forms a distinct and consistent variety. The production by it of common agglutinins for some pneumococci and the resistance of the agglutinins produced by it to absorption by the streptococcus indicate a nearer relation to the former than to the latter organism.

III. The agglutinating substances in the serum of immunized animals were demonstrated by absorption tests to consist of specific and group agglutinins in cases where the agglutinins were sufficiently developed to make use of this method.

IV. The pneumococci seem to show marked differences in their ability to undergo agglutination.

V. There was considerable uniformity of reaction of the various strains in low dilutions, but this uniformity is not continued as the animal becomes more highly immunized.

VI. At present it is not possible to establish a definite relation between the agglutination reaction and the other characteristics of the pneumococcus excepting in the case of the Pneumococcus mucosus.
The above work has been conducted under the direction of Dr. Wm. H. Park, Director of the Research Laboratory of the Department of Health of New York City, to whom I desire to express my appreciation of the interest he has shown in the work.

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