ENHANCEMENT OF THE OPSONIZING AND AGGLUTINATING POWERS OF ANTIPNEUMOCOCCUS SERUM BY SPECIFIC PRECIPITATING SERUM.

By IDA W. PRITCHETT.

(From the Department of Immunology of the School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

(Received for publication, May 3, 1920.)

Bordet,[1] Ehrlich and Morganroth,[2] Pfeiffer and Friedberger,[3] and others have shown that it is possible to produce in animals, through the injection of certain immune or normal sera, antisera capable of inhibiting the action of antibodies. The action of such sera has been ascribed to specific substances designated as anti-antibodies, which are formed when animals of one species are injected with the serum of another species. The serum of the injected animals inhibits the action of antibodies derived from the species whose serum was used as antigen, but is ineffective against antibodies derived from another species. A number of anti-antibodies have been thus produced; *viz.*, antihemolysins, antiprecipitins, antihemagglutinins, anticomplement, and antibacteriolysins. Attempts to produce anti-antitoxins and antibodies for bacterial agglutinins have not been successful. Ehrlich and Morganroth[4] and Wassermann[5] maintain that anti-antibodies for bacterial antibodies cannot be formed in the animal body since animal tissues do not possess receptors suitable for bacterial antibodies. Pfeiffer and Friedberger,[3] however, report experiments in which they succeeded in obtaining a very effective inhibiting serum for cholera vibriolysins.

On the assumption that bacterial anti-antibodies may theoretically be formed in the animal body, the question becomes of interest in connection with the use of therapeutic sera in man, particularly when the same individual is given repeated injections of serum from the same animal species. This condition frequently arises, since therapeutic sera are almost exclusively derived from the horse.

[1] Bordet, J., *Ann. Inst. Pasteur*, 1904, xviii, 591.

[2] Ehrlich, P., and Morganroth, J., *Berl. klin. Woch.*, 1901, xxxviii, 598.

[3] Pfeiffer, R., and Friedberger, E., *Berl. klin. Woch.*, 1902, xxxix, 4; *Centr. Bakteriol., Ite Abt., Orig.*, 1903, xxxiv, 70.

[4] Ehrlich, P., and Morganroth, J., *Berl. klin. Woch.*, 1901, xxxviii, 569.

[5] Wassermann, A., *Z. Hyg. u. Infectionskrankh.*, 1903, xlii, 267.

The observations here reported are the results obtained in experiments on the production of pneumococcus antiopsonins. Second infections due to the same type of pneumococcus, though rare, have occurred. If antiopsonins should arise following serum treatment of the first infection, and persist in the patient's blood, they would tend to neutralize the opsonins in the immune serum employed during the second attack, thus lessening its value. The same effect might theoretically be observed if the patient had at any previous time received normal or non-specific immune horse serum.[1]

As will be seen from the following experiments, no evidence of the formation of pneumococcus antiopsonins could be obtained. On the contrary, the opsonizing and agglutinating properties of the immune horse sera were greatly enhanced in the presence of the sera which were expected to contain inhibiting substances.

EXPERIMENTAL.

Monovalent pneumococcus horse sera, Types I, II, and III, obtained from the Hospital of The Rockefeller Institute, were injected into rabbits. Each rabbit received intravenously 8 cc. of serum of one of the three types, and after 14 days, when the injected serum had disappeared, as indicated by opsonic tests, the animals were bled and their sera collected. Dilutions in salt solution were made of the antipneumococcus horse sera, each equal to one-fourth the dilution finally desired, since equal volumes of whole rabbit serum, pneumococcus culture, and leucocytic suspension were to be added to it. In each of a series of serological tubes was placed 0.1 cc. of the undiluted serum of the injected rabbits, 0.1 cc. of antipneumococcus horse serum dilution, and 0.1 cc. of a 24 hour blood broth culture of the homologous pneumococci. After their contents had been thoroughly mixed by shaking, the tubes were placed in a water bath at 37°C. for 1 hour to allow for sensitization of the pneumococci and interaction of the rabbit and horse sera. At the end of this time there was added to each tube 0.1 cc. of a fresh suspension of leucocytes, obtained by injecting a guinea pig on the previous day intraperitoneally with aleuronat. The contents of the tubes were again thoroughly mixed and incubated for another hour in the water bath, when a film

was made from the sediment in each tube and the slides were fixed in methyl alcohol and stained with Manson's stain. Control experiments were run at the same time in which normal rabbit serum was substituted for that of the rabbits previously injected with pneumococcus horse serum.

To determine whether the order of combination would affect the result, three other methods of combining the various constituents were also employed as follows: (1) Pneumococcus horse serum and precipitating rabbit serum were incubated together for 1 hour before the addition of the pneumococci. After adding the pneumococci the mixture was incubated for another hour before adding the leucocytes. This method permitted considerable precipitation in the rabbit-horse serum mixture before sensitization of the bacteria was begun. (2) Pneumococcus horse serum and pneumococci were incubated for 1 hour before adding the precipitating rabbit serum. After adding the rabbit serum the mixture was incubated for 1 hour before adding the leucocytes. This method permitted sensitization of the bacteria before precipitation was begun. (3) Pneumococcus horse serum and homologous pneumococci were incubated together for 1 hour, when both the precipitating rabbit serum and the guinea pig leucocytes were added and the mixture was incubated for another hour.

No great difference in results was observed with these four methods, so that eventually only the first method, as described in detail above, was employed.

Experiment 1. Type I Pneumococcus Horse Serum.—Type I serum and pneumococci were combined, as described above, with the serum of rabbits previously injected with the Type I serum. The results obtained are presented in Table I.

From Table I it will be seen that the combinations described showed opsonization and microscopic agglutination in dilutions ten times as great as were effective when normal rabbit serum was substituted for that of the injected rabbits, while mixtures of the precipitating rabbit serum and normal horse serum gave uniformly negative results. When the pneumococcus horse serum was replaced by an equal volume of salt solution, neither normal nor precipitating rabbit serum was capable of causing the slightest opsonization or agglutination,

nor did these occur in control mixtures in which both rabbit and horse sera were replaced by salt solution. The Type I horse serum used in this experiment, when diluted with salt solution, showed microscopic agglutination and opsonization of Type I pneumococci in dilutions a little above 1:100.

TABLE I.

*Increased Opsonization and Agglutination of Type I Pneumococci When Combined with the Homologous Immune Serum in a Specific Precipitating Mixture.**

Tube No.	Type I antipneumococcus horse serum and normal horse serum dilutions.	Rabbit 1 (2 wks. after intravenous injection of 8 cc. of Type I antipneumococcus horse serum).				Normal rabbit serum.	
		With Type I antipneumococcus horse serum.		With normal horse serum.		With Type I antipneumococcus horse serum.	
		Agglutinins.	Opsonins.	Agglutinins.	Opsonins.	Agglutinins.	Opsonins.
1	1:100	++++	++++	−	−	++++	++++
2	1:300	++++	++++	−	−	++++	++++
3	1:500	++++	++++	−	−	−	−
4	1:1,000	++++	++++	−	−	−	−
5	1:2,000	++++	++++	−	−	−	−
6	1:3,000	++	++	−	−	−	−

* In this and succeeding tables the degree of opsonization and agglutination is represented by the number of plus signs, ++++ being complete, + slight but definite.

Experiment 2. Type II Pneumococcus Horse Serum.—The technique of Experiment 1 was employed, Type II serum and pneumococci being used. The results are given in Table II.

It will be seen from Table II that the results were essentially the same as those obtained in the experiments with Type I serum and pneumococci. In this particular experiment opsonization was marked in dilutions in which no microscopic agglutination was observed. This, however, was not always found, and was not peculiar to Type II, being sometimes encountered also in experiments with organisms and serum of Type I.

Type III Pneumococcus Horse Serum.—The opsonization and agglutination of Type III pneumococci by the homologous immune

horse serum was not augmented by the addition of the serum of rabbits injected with Type III horse serum, these organisms remaining unopsonized and unagglutinated except in very low dilutions of Type III serum, usually below 1:50.

TABLE II.

Increased Opsonization and Agglutination of Type II Pneumococci When Combined with the Homologous Immune Serum in a Specific Precipitating Mixture.

Tube No.	Type II antipneumococcus horse serum and normal horse serum dilutions.	Rabbit 2 (2 wks. after intravenous injection of 8 cc. of Type II antipneumococcus horse serum).				Normal rabbit serum.	
		With Type II antipneumococcus horse serum.		With normal horse serum.		With Type II antipneumococcus horse serum.	
		Agglutinins.	Opsonins.	Agglutinins.	Opsonins.	Agglutinins.	Opsonins.
1	1:100	++++	++++	−	−	++++	++++
2	1:300	+++	++++	−	−	+	+
3	1:500	++	+++	−	−	−	−
4	1:1,000	+	+++	−	−	−	−
5	1:2,000	−	++	−	−	−	−
6	1:3,000	−	+	−	−	−	−

Experiment 3. Normal Horse Serum.—All the experiments described above have in common the mixture of an immune pneumococcus horse serum with its homologous pneumococci in the presence of a specific precipitating serum. To determine whether the increase in opsonization and agglutination observed in these cases was due to some special property conferred upon the rabbit serum by the injection of the immune pneumococcus serum, or was merely dependent on the presence of the specific precipitating mixture, control experiments were run with the serum of rabbits injected with normal horse serum instead of the immune horse serum. One of these anti-horse rabbit sera had been prepared some months before, but still showed gross precipitins for horse serum in dilutions above 1 : 1,000. Two other anti-horse rabbit sera were prepared with two different samples of normal horse serum, and all three rabbit sera were tested with Type I and Type II pneumococcus horse serum and the homologous pneumococci. The results of these tests were similar for both Type I and Type II. A summary of the findings in an experiment with serum and organisms of Type I is given in Table III which will also serve to illustrate the results obtained with Type II serum and pneumococci.

It will be seen, from a comparison of Tables I and III, that the serum of Rabbit 3, injected some months previously with normal horse serum (Sample A) was but little better than normal rabbit serum in promoting the opsonization and agglutination of Type I pneumococci by the homologous serum, even though a specific precipitating mixture was here present. The other two rabbits, Nos. 4 and 5, injected with Samples B and C of normal horse serum, showed heightened opsonization and agglutination entirely comparable to that observed when Type I horse serum was combined with the serum

TABLE III.

Opsonization and Agglutination of Type I Pneumococci by the Homologous Immune Serum When Combined with the Sera of Rabbits Injected with Normal Horse Serum.

Tube No.	Type I pneumococcus horse serum dilutions.	Rabbit 3 (several mos. after intravenous injection of normal horse serum, Sample A).		Rabbit 4 (2 wks. after intravenous injection of normal horse serum, Sample B).		Rabbit 5 (2 wks. after intravenous injection of normal horse serum, Sample C).	
		Agglutinins.	Opsonins.	Agglutinins.	Opsonins.	Agglutinins.	Opsonins.
1	1:100	++	++	+++	++++	++	++++
2	1:500	+	+	++	++++	+	++++
3	1:1,000	−	−	+	++++	+	++++
4	1:2,000	−	−	+−	++	+	++

of rabbits previously injected with the same immune horse serum. Moreover, it was found that the sera of rabbits injected with Immune Horse Serum Type I, II, or III were equally effective in promoting the opsonization and agglutination of Type I and Type II pneumococci by the homologous horse sera, while organisms of Type III remained unaffected by any of the horse-rabbit serum combinations.

The failure of the serum of Rabbit 3 to produce the usual reaction is not understood. The serum of another rabbit immunized at the same time with the same sample of normal horse serum also failed to promote opsonization and agglutination of Type I and Type II pneumococci by the homologous immune sera.

It appears from the foregoing experiments that no special property is conferred upon the serum of rabbits by the injection of immune

horse serum, since no demonstrable antiopsonins are produced by such injections. On the contrary, the serum of such rabbits, as well as that of rabbits injected with normal horse serum, is capable, *in vitro*, of augmenting many fold the opsonizing and agglutinating power of Type I and Type II horse sera for the homologous pneumococci. These findings are in accord with the observations of Moreschi[6] on typhoid agglutinins. He found that when typhoid bacilli were subjected for 2 hours at 37°C. to the action of specific agglutinating serum from various sources (goat, man), and were then washed and resuspended in salt solution, the addition of a small quantity (0.05 cc.) of the serum of rabbits previously injected with the same agglutinating serum raised the titer of the immune serum five- to tenfold. His explanation for this phenomenon was that the serum of the injected rabbits, while itself incapable of agglutinating typhoid bacilli, combined with and precipitated the agglutinating serum already absorbed by the bacteria, thus causing agglutination. Non-specific bacteria, such as the cholera vibrio and *Bacillus dysenteriæ*, he found to be unaffected by the serum mixtures, while the precipitating serum had no affinity for unsensitized typhoid bacilli. The last two observations were also borne out by the present experiments, since in no case were Type I and Type II pneumococci opsonized or agglutinated by a heterologous or normal horse serum, even in the presence of a specific precipitating serum.

Protection Experiments.

Since the efficacy of therapeutic pneumococcus serum (Type I) depends largely on its opsonizing power, and since this power is so greatly enhanced *in vitro* by the addition of a specific precipitating serum, it was hoped that a corresponding increase in protective power might be produced by the same means. Experiments were accordingly carried out for the purpose of testing this point, mice being chosen as the test animals. The results obtained were disappointing. In this instance opsonization in the test-tube was not paralleled by protection in the animal, since no very marked increase in protection could be demonstrated. The impossibility of maintaining *in vivo*

[6] Moreschi, C., *Centr. Bakteriol., Ite Abt., Orig.*, 1908, xlvi, 456.

the same quantitative relations between bacteria and serum that exist *in vitro* may account for the failure. Dilution of the opsonizing and precipitating sera with peritoneal exudate and unequal absorption of the various constituents would tend to upset the balance very quickly, and no doubt would markedly affect the outcome. Incubation of the mixtures before injection served only to confuse the results, since the pneumococci were sometimes injured by the broth and died, while they multiplied in the tubes containing serum, so that not infrequently the control animals outlived the other mice. Only once was

TABLE IV.

*Protection Afforded to Mice from a Combination of Type I Pneumococcus Horse Serum with Specific Precipitating Rabbit Serum.**

Mouse No.	Weight.	Type I antipneumococcus horse serum.	Normal horse serum.	Normal rabbit serum.	Serum of rabbits previously injected with Type I horse serum.		Broth.	Type I pneumococcus broth culture.	Result.
					No. 1.	No. 6.			
	gm.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	
1	20	0.0005		0.25				0.01	Died in 33 hrs.
2	20	0.0005			0.25			0.01	Survived.
3	20	0.0005				0.25		0.01	"
4	20	0.0005					0.25	0.01	Died in 22 hrs.
5	20		0.0005		0.25			0.01	" " 22 "
6	20		0.0005			0.25		0.01	" " 22 "

*The total volume of each dose was made up to 1 cc. and injected as soon as mixed into the peritoneal cavity, without incubation. The serum samples of Rabbits 1 and 6 were a month old at this time.

a successful experiment performed, in which the mice survived that had received a mixture of pneumococcus horse serum and specific precipitating rabbit serum, while all the other mice died. The protocol is presented in Table IV.

DISCUSSION.

From the foregoing experiments it appears that a simple precipitating mixture is capable of augmenting the opsonization and agglutination of Type I and Type II pneumococci by their homologous immune

sera. Similar observations have been made by other workers with other types of antibodies. Friedberger and Moreschi[7] found that rabbit red cells sensitized with goat amboceptor were much more readily hemolyzed in the presence of a small quantity of the serum of a rabbit previously injected with the goat amboceptor. Arkwright,[8] in his experiments on the agglutination of *Bacillus typhosus*, found that in a specific serum precipitating mixture, with bacterial extract as antigen, not only the specific bacteria, but even non-specific bacteria and inanimate particles were agglutinated. Nicolle[9] also reports that non-specific bacteria are carried down when present in a specific serum precipitating or agglutinating mixture. He found that in mixed suspensions containing equal numbers of *Bacillus coli* and *Bacillus typhosus*, the addition of an agglutinating serum specific for either would completely agglutinate both. The phenomena of non-specific opsonization and agglutination have not been observed in the present study, since neither Type I nor Type II pneumococci were opsonized or agglutinated in the presence of the heterologous immune serum or of normal horse serum, even though the addition of the anti-horse rabbit serum furnished a specific precipitating mixture. Arkwright's explanation for his results is that the coagulum formed in the serum precipitating mixture drew together and clumped the bacteria, specific and non-specific, a mechanical explanation that could hardly be applied to opsonization and phagocytosis. Since the reaction of an antigen with its specific antibody results in lowering the surface tension of the mixture,[10] and since it is thought that phagocytosis may be a surface tension phenomenon,[11] the explanation for the increased opsonization of Type I and Type II pneumococci by their homologous immune sera, in the presence of a specific precipitating serum, may perhaps lie in the changes in surface tension thus produced, though why a general lowering of the surface tension of the

[7] Friedberger, E., and Moreschi, C., *Centr. Bakteriol., Ite Abt., Orig.*, 1908, xlv, 346.

[8] Arkwright, J. A., *J. Hyg.*, 1914, xiv, 261.

[9] Nicolle, C., *Ann. Inst. Pasteur*, 1898, xii, 161.

[10] Wells, H. G., Chemical pathology, Philadelphia and London, 3rd edition, 1918, 208.

[11] Wells,[10] pp. 266–275.

medium should render the leucocytes more actively phagocytic is not clear. Another possibility is that precipitation takes place in the serum already absorbed by the sensitized bacteria, or that the previously precipitated substance is adsorbed by the bacteria together with the specific opsonins, and that these adsorbed substances render the pneumococci more susceptible to phagocytosis. It seems probable that the effect, whatever it may be, is exerted upon the bacteria, but at present no satisfactory explanation for the phenomenon has been reached.

SUMMARY.

1. No demonstrable antiopsonins are formed in rabbits following the intravenous injection of monovalent pneumococcus horse sera, Types I, II, and III.

2. The serum of rabbits injected with immune pneumococcus horse serum, Type I, II, or III, or with normal horse serum, when mixed in the proportion of 1:4 with Type I or Type II pneumococcus horse serum, can greatly augment, *in vitro*, the opsonization and agglutination of Type I and Type II pneumococci by the homologous immune horse sera. No similar effect is obtained with Type III serum and pneumococci.

3. The increase in opsonization and agglutination is dependent upon (*a*) specific sensitization of the pneumococci by the homologous immune serum and (*b*) the presence of the precipitating serum. In the absence of sensitization, as when a heterologous or normal horse serum is employed, opsonization and agglutination do not occur, even though a precipitating mixture is provided. The substitution of normal rabbit serum for the precipitating rabbit serum gives opsonization and agglutination in dilutions slightly higher than are effected with salt solution only, due possibly to the more favorable medium created for the leucocytes by the addition of 25 per cent of whole rabbit serum.

4. Different methods of combining the immune horse serum, precipitating rabbit serum, and pneumococci yield very similar results, preliminary sensitization of the bacteria before precipitation, or precipitation in the rabbit-horse serum mixture before the addition

of the pneumococci for sensitization causing little if any difference in result from that obtained when immune horse serum, precipitating rabbit serum, and pneumococci are all mixed and incubated together.

5. This increased opsonization in the test-tube does not seem to be paralleled by increased protective power, or at any rate such protection is not readily demonstrated.