AN ANALYSIS OF THE OPSONIC AND TROPIC ACTION OF NORMAL AND IMMUNE SERA BASED ON EXPERIMENTS WITH THE PNEUMOCOCCUS

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INTRODUCTION

Recent investigations (1, 2) on the pneumococcidal power of serum-leucocyte mixtures and of defibrinated human blood have demonstrated that the specific carbohydrate of the pneumococcus exerts a strong and type-specific antibactericidal action in such systems. As it is well known that ingestion of the pneumococcus by the leucocytes is essential for the destruction of this organism, the suggestion has been made by Sia (1) and Ward (2) that the specific carbohydrate inhibits the bactericidal properties of these systems by uniting with its corresponding antibody, and thus completely preventing the opsonization and subsequent phagocytosis of the organisms. For they thought it probable, in the absence of direct experimental evidence, that this antibody was an indispensable factor in the process of opsonization. We have found, however, that the addition of a quantity of the specific carbohydrate which will suppress the bactericidal action of defibrinated human blood does not prevent, under certain conditions, the phagocytosis of virulent pneumococci.

With this observation as a basis, we were led to reinvestigate the phagocytic mechanism in the case of the pneumococcus. The experimental results thus obtained not only served to clarify this special problem, but seemed to us to have a general application to the various theories of phagocytosis, in that they elucidate the function of a single well defined antibody in the mechanism of this phenomenon. Since even now there is not universal agreement concerning the nature and mode of action of the various serum constituents taking part in

phagocytosis, we give here a summary of the principal views on this subject.

Perhaps the majority would agree with Topley and Wilson's (3) description of the manner in which virulent bacteria are prepared for phagocytosis:

"Both normal opsonins and immune opsonins, or bacteriotropins, have a dual structure in a sense that they involve, or may involve, the combined action of a specific sensitizing antibody and complement; but that the very low concentration of the specific antibodies in normal serum necessitates the adjuvant action of a considerable amount of complement before its presence can be detected, so that the complementary action appears to dominate the picture, while the high concentration of the specific antibody in an immune serum reduces the adjuvant action of the complement to a mere enhancement of an effect which takes place in its absence."

There is not, however, universal agreement with this simple conception. Neufeld (4), one of the acknowledged authorities on this subject, maintains that there are two heat-stable phagocytic antibodies distinct from one another: (*a*) the tropin, either normal or immune, which does not require the aid of complement to bring about phagocytosis; (*b*) the opsonic amboceptor, either normal or immune, which does require the addition of complement to activate it. Muir (5) states that although specific opsonization (as distinct from tropinization) is always dependent on the combined action of a specific antibody and complement, nevertheless complement may unite directly with bacteria to bring about a non-specific opsonization.

Owing to the looseness with which the words "opsonin" and "tropin" have been applied to various phagocytic phenomena in the literature, we give here, to avoid confusion, the sense in which these terms have been employed in the ensuing description of our experiments: "Opsonin" is that property of unheated serum which prepares organisms for phagocytosis. "Tropin" is that property of heated serum which, by itself, prepares organisms for phagocytosis. We have used these terms in the description of our experiments because they are familiar to most workers in this field. Nevertheless, on the basis of our experimental results, in the discussion we shall urge the rejection of the term "tropin" and suggest a modification in the sense of the term "opsonin."

The experiments to be described deal primarily with the rôle of the anti-type-specific carbohydrate antibody (hereafter to be alluded to as the anticarbohydrate antibody) as an opsonic or tropic agent, and with a study of the properties of normal and immune sera which promote phagocytosis after this antibody has been eliminated by the addition of the specific carbohydrate in appropriate amounts. During the course of this work, the state of the organisms themselves has not been disregarded, and studies have been made on the phagocytosis of

cultures of varying age, and of pneumococci taken directly from the animal body.

Materials

The following materials were used in the course of these experiments.

Human blood defibrinated by shaking with beads and used on the same day it was withdrawn. In the most important experiments the blood of the authors was used, neither of whom had any history of pneumococcus infection. In certain other experiments, adult blood obtained from the Wassermann dispensary was employed. In two experiments, we made use of infants' blood, the infants being 7 and 12 months old, respectively. When washed corpuscles were required, the defibrinated blood was centrifuged, the supernatant serum removed, and the red and white corpuscles washed three times with normal saline solution. In the cases where inactivated serum was added, it was heated to 56°C. for 30 minutes.

Particular pains were taken to maintain the virulence of the three types of pneumococci employed. The organisms were passed through mice at least once a week and during most of the work, twice a week. Cultures for use in the experimental work were grown in rabbit blood infusion broth, and in order to eliminate any antiphagocytic effect of the soluble products of the pneumococci in the culture broth, the culture was centrifuged shortly before using, and the organisms suspended in sufficient fresh broth to yield a threefold concentration of the pneumococci. To obtain organisms fresh from the animal body, a mouse was injected with 0.5 cc. of a 1:200 dilution of a 24 hour broth culture. The mouse was killed after 12 hours, and the peritoneal cavity washed out with about 1.0 cc. of broth. The organisms were separated from the washings by centrifugation, and resuspended in the same amount of fresh broth. When organisms were used for absorption, they were grown in 0.1 per cent dextrose infusion broth at 37°C. for 18 hours. The quantity of serum to be absorbed was added to the organisms centrifuged from an equal quantity of the broth culture. The mixture of serum and organisms, after standing together at 37°C. for half an hour, was centrifuged at 2,000 R.P.M. for 45 minutes. The serum thus absorbed was removed from the organisms.

The specific carbohydrates were prepared according to the procedures described by Avery and Heidelberger (6, 7).

The Type I and Type II antisera used were prepared in rabbits and the Type III antiserum in a horse.

Technique

In carrying out the experiments, the following technique was employed:[1] 0.5 cc. of defibrinated blood, or a mixture of serum and washed corpuscles was placed in a pyrex glass tube 10 cm. long, 7 cm. inside diameter, and the organisms and various reagents added in this order: antiserum, specific carbohydrate, organisms. The

[1] For more complete details of this technique, see article by Ward (2).

contents were mixed by rapid rotation of the tube after the addition of each substance. The tubes were then sealed and warmed by immersion in water at 37°C. for a few minutes. After being dried, the tubes were put into a revolving box (rotating at a rate of 15 R.P.H.) placed in a 37°C. incubator. At the end of 30 minutes, the tubes were opened, smears of the contents were made and stained with Wright's stain. In general, the organisms in 50 polymorphonuclear neutrophiles were counted, but where the number of phagocyted cocci was few, making the result doubtful, the bacterial content of 100 cells was recorded. In counting, a diplococcus was regarded as a single organism. The accuracy of the technique which has been described was checked in this manner: 5 smears were made from each of 2 tubes containing 0.5 cc. of defibrinated blood, and 1 drop of a suspension of pneumococci, after the tubes had been rotated at 37°C. for half an hour. The organisms in 50 leucocytes were counted in each smear. The counts of the 5 smears made from the contents of the 2 tubes were: 425, 447, 460, 451, 493, and 473, 479, 511, 502, 485. The maximum deviation from the mean of the 10 counts is 10 per cent, and the maximum difference between the highest and the lowest count is 20 per cent of the latter.

The investigation of the weak tropic action of heated normal human serum demanded a modification of the technique described above. To the serum, which had been heated to 56°C. for 30 minutes, were added the various materials to be tested and then the bacterial suspension. The tubes were then placed in the ice box at 4°C. for 18 hours, after which washed corpuscles were added in equal volume. The mixtures were rotated for 30 minutes at 37°C. in the usual manner, and the phagocytic counts made. In the study of the tropic action of immune serum, the dilution of antiserum chosen was added to a mixture of heated normal serum and washed corpuscles, and after the addition of the various reagents and organisms, the tubes were incubated for 2 hours in the rotating machine. Essentially the same procedure was adopted in investigating the opsonic action of immune serum, save that a dilution of unheated normal serum was also added. Heated normal serum was used to suspend the corpuscles instead of normal saline solution, since it was found that in the latter medium the leucocytes showed little or no capacity to ingest sensitized organisms.

Bactericidal tests with defibrinated blood were carried out by the technique described by Ward in a previous communication (2).

The Opsonic Action of Normal Human Serum

The Determination of an Amount of Specific Carbohydrate Necessary to Neutralize the Homologous Antibody in Normal Serum.—As a basis for these experiments on phagocytosis, it was necessary to demonstrate the presence of the small amount of anticarbohydrate antibody in normal serum. The most delicate test we have at our disposal for detecting the presence of this antibody is the determination of the

bactericidal titre of defibrinated blood against the virulent organism (8). The same test offers the most accurate method of ascertaining the quantity of specific carbohydrate required to neutralize this antibody in normal and immune serum. In Table I, the results recorded demonstrate the effect of the specific carbohydrate on the bactericidal power of the two bloods which have been used in most of the experiments. The type specificity of the antibactericidal action of these substances is not shown, since this has been pointed out in previous papers (1, 2). From Table I it can be seen that in the case of Normal Blood E, the bactericidal power of the blood against Type I

TABLE I

	Type of pneumococcus	Concentration of carbohydrate	Maximum No. of diplococci killed by 0.5 cc. blood
Normal Blood E	I	0	70,000
	I	Type I, 1:800	7
	II	0	40,000
	II	Type II, 1:1,600	0
	III	0	4,000
	III	Type III, 1:1,600	0
Normal Blood W	I	0	0
	I	Type I, 1:800	0
	II	0	40,000
	II	Type II, 1:1,600	0
	III	0	40,000
	III	Type III, 1:1,600	0

pneumococcus is reduced 10,000 times by the addition of an amount of specific carbohydrate which was used subsequently in the course of the phagocytic experiments. In the case of Types II and III, and in that of all three types with Normal Blood W, the bactericidal titre was reduced to nil. It will also be noted that Normal Blood W, without the addition of the carbohydrate, possessed no bactericidal power against Type I, which indicated that this blood was completely lacking in Type I anticarbohydrate antibody.

The Effect of the Specific Carbohydrates on the Opsonic Action of Unheated Normal Serum.—Allusion has been made already in this paper to our observation that an amount of the specific carbohydrate

which removes the bactericidal power of normal blood, does not invariably render ineffective the phagocytic mechanism. This is strikingly shown by the results recorded in Table II. In this experiment, where 24 hour cultures were employed, it will be observed that in no case did the addition of the specific carbohydrate completely suppress the phagocytosis, and in only one case was the phagocytosis by the normal blood alone reduced more than two-thirds by the presence of the carbohydrate. The results of an investigation into this residual phagocytosis will be presented subsequently.

TABLE II

	Type of pneumococcus	Concentration of carbohydrate	No. of organisms phagocyted by 50 cells	Cells taking part
				per cent
Normal Blood E	I	0	512	100
	I	Type I, 1:800	249	98
	II	0	583	100
	II	Type II, 1:800	43	38
	III	0	818	100
	III	Type III, 1:800	525	100
Normal Blood W	I	0	183	96
	I	Type I, 1:800	166	92
	II	0	271	98
	II	Type II, 1:800	125	78
	III	0	617	100
	III	Type III, 1:800	630	100

Another experiment, the details of which are not given here, showed that the reduction in the degree of phagocytosis produced by the specific carbohydrate is, as one would expect, type-specific.

When, however, instead of 24 hour cultures, 8 hour cultures were used in the same experimental procedure, the specific carbohydrate exerted a much stronger antiphagocytic effect, except in the case of Type I. When the organisms were taken directly from the mouse peritoneum, the influence of the specific carbohydrate was still more marked. The experimental data of these two experiments are assembled in Table III. In addition to showing the presence of an opsonic factor distinct from the anticarbohydrate antibody in the phagocytosis

of 24 hour cultures, these experiments indicate that as the organism approaches and attains to the state in which it exists in the animal body, the less effective is the residual factor, and the more necessary is the anticarbohydrate factor in causing phagocytosis. This generalization does not apply to the Type I pneumococcus, where even the organisms taken from the mouse are susceptible to the opsonic action of the residual factor.

Our findings in respect to the Type III organisms may explain the old observations of von Gruber and Futaki (9). These authors noted

TABLE III

	Type of pneumococcus	Concentration of carbohydrate	No. of organisms from 8 hr. culture phagocyted by 50 cells	Cells taking part	No. of organisms from mouse peritoneum phagocyted by 50 cells	Cells taking part
				per cent		*per cent*
Normal	I	0	408	98	339	98
Blood	I	Type I, 1:800	438	96	130	72
E	II	0	544	98	73	74
	II	Type II, 1:1,600	0	0	1	2
	III	0	500	100	4	6
	III	Type III, 1:1,600	11	8	0	0
Normal	I	0	269	95	14	12
Blood	I	Type I, 1:800	235	84	18	12
W	II	0	336	82	8	12
	II	Type II, 1:1,600	6	6	10	12
	III	0	329	96	4	6
	III	Type III, 1:1,600	104	54	4	4

that, whereas fully virulent but uncapsulated anthrax organisms were readily phagocyted both *in vitro* and *in vivo*, the same strain when capsulated was completely resistant to phagocytosis. One would venture to predict that it will be found that the virulent uncapsulated anthrax bacilli can be opsonized by a serum component corresponding to the residual factor mentioned above in the case of the pneumococcus, but that this factor is powerless to bring about the phagocytosis of the capsulated organisms. Robertson and Sia (10), in their work on the opsonization of Type I pneumococci by dog's blood, found an absolute difference in the degree of phagocytosis of old and young cultures.

Our own work has not borne this out, but the difference in technique may well account for the discrepancy.

The Analysis of the Opsonic Mechanism in Normal Human Blood.—It remained to determine whether the anticarbohydrate and residual factors were heat-stable and in conjunction with complement brought about immediate phagocytosis according to the description of Topley and Wilson (3), to which allusion has already been made. The experimental evidence on which this is based is largely derived from the work of Dean (11) and Cowie and Chapin (12). The latter authors showed that, in the case of the *Staphylococcus albus*, an amount of unheated normal serum, in itself practically without any opsonic effect, was able to reactivate completely normal serum, the opsonic activity of which had been almost entirely removed by heating. We have attempted, unsuccessfully, to confirm their results, employing Type I and Type II pneumococci as the test organisms. Our results showed that it was impossible to reactivate the heated normal serum with diluted unheated normal serum, and therein they agree essentially with those obtained many years ago by Wright and Douglas (13), working with the staphylococcus. Perhaps the success of Cowie and Chapin's procedure lay in their choice of an organism of low virulence. As we shall see, the conclusion of Cowie and Chapin, that opsonic action depended on the combined effect of a thermostable element with complement, is correct, but, to demonstrate its validity for the virulent pneumococcus, a much greater concentration of complement was found to be necessary in order to reactivate the heated normal serum. As a source of complement which could be used in the highest possible concentration and still be free of antibody, the serum of infants below the age of 12 months was employed. The complement titre of such sera for sensitized sheep red blood cells was in general as high as that of adult sera, but the opsonizing action was negligible. In Table IV, we present the results of an experiment in which the undiluted adult Sera E and W and undiluted infant's serum were used in equal proportions. Washed corpuscles and organisms were added to these mixtures.

The conclusions to be drawn from these results are as follows:

1. The infant's serum exerted a negligible degree of opsonic action for Types I and II pneumococcus (Tubes 1 and 6). These small counts in the presence of abundant complement indicate that Muir's

(5) hypothesis, which states that complement alone can bring about phagocytosis, is untenable. Experiments with the blood of a different infant, which was incapable of exerting any phagocytic action whatever, establish the fact that complement by itself, at least in the case of the virulent pneumococcus, is incapable of inducing phagocytosis.

TABLE IV

	Tube No.	Type of pneumococcus	Concentration of carbohydrate	No. of organisms phagocyted by 50 cells	Cells taking part
					per cent
Heated infant's serum + unheated infant's serum + washed corpuscles	1	I	0	3	5
Heated Serum E + unheated infant's serum + washed corpuscles	2	I	0	93	75
Heated Serum E + unheated infant's serum + washed corpuscles	3	I	Type I, 1:800	63	61
Unheated Serum E + heated infant's serum + washed corpuscles	4	I	0	90	69
Heated Serum E + saline + washed corpuscles	5	I	0	0	0
Heated infant's serum + unheated infant's serum + washed corpuscles	6	II	0	14	16
Heated Serum W + unheated infant's serum + washed corpuscles	7	II	0	109	77
Heated Serum W + unheated infant's serum + washed corpuscles	8	II	Type II, 1:1,600	70	60
Unheated Serum W + heated infant's serum + washed corpuscles	9	II	0	90	72
Heated Serum W + saline + washed corpuscles	10	II	0	0	0

2. The opsonic property of Sera E and W was removed by heating (Tubes 5 and 10) and then restored by the addition of the fresh infant's serum (Tubes 2 and 7).

3. That this restoration is complete is shown by the phagocytic counts when the same quantity of unheated adult serum was added to heated infant's serum (Tubes 4 and 9).

4. The restoration of the opsonic activity of the heated adult serum by the unheated infant's serum eliminates the possibility of any

adjuvant action of a thermolabile constituent, other than complement, in the adult serum.

5. The residual factor is thermostable (Tubes 3 and 8); and the differences between the counts of Tubes 2 and 3 and of Tubes 7 and 8 indicate, without proving conclusively, that the anticarbohydrate antibody is also heat-stable. Other experiments that we have performed leave no doubt that this antibody, as it occurs in normal serum, is resistant to heat.

The Nature of the Residual Factor.—The last experiment demonstrated that part of the phagocytosis of Types I and II pneumococcus by normal blood was due to a factor, other than the anticarbohydrate

TABLE V

	Type of pneumococcus	Concentration of carbohydrate	No. of organisms phagocyted by 50 cells	Cells taking part
				per cent
Unabsorbed unheated normal serum + washed corpuscles	I	0	392	100
	I	Type I, 1:800	185	86
	II	0	351	100
	II	Type II, 1:800	141	86
Type I-absorbed unheated normal serum + washed corpuscles	I	0	21	18
	I	Type I, 1:800	8	8
	II	0	186	82
	II	Type II, 1:800	79	60

antibody, acting in conjunction with complement. It further made clear that this factor was resistant to a temperature of 56°C. This characteristic suggests its inclusion among the natural antibodies of the serum. Were it possible to show that it is type-specific in its action, it would strengthen the probability of its identification as an antibody. Our evidence in regard to its type specificity is not entirely conclusive. In our hands, the type-specific absorption of the opsonins of normal serum proved difficult, although Sia (14) reported successful results in this kind of experiment. We, however, observed a marked reduction of the opsonic content of the serum for a heterologous type, and this with practically no diminution in the complement content of the absorbed serum. We give in Table V the results of one of the

more successful absorption experiments, in which a normal serum was absorbed with Type I pneumococcus. Here, in addition to showing a type-specific reduction in the total opsonic effect (Type I from 392 of 21; Type II from 351 to 186), it will be noted that there is also a type-specific reduction in the phagocytosis, due to the residual factor (Type I from 185 to 8; Type II from 141 to 79). 4 per cent only of the residual factor remains in the case of the homologous organism, whereas 56 per cent was left for the heterologous pneumococcus. Although this is not a conclusive experiment, we believe that this opsonizing agent is type-specific. Additional evidence in support of this opinion may be found in the quantitative variation in the phagocytic activity of Bloods E and W against the three types of pneumococcus after the anticarbohydrate factor has been eliminated (Tables II and III). For if the residual factor was non-specific, the titre of the residual phagocytosis for all three types of pneumococcus would presumably be higher or lower in Blood E than it is in Blood W, but this is not the case.

The Tropic Action of Heated Normal and Immune Serum

The Effect of the Specific Carbohydrate on the Tropic Action of Heated Normal Human Serum.—So far as we are aware, there is no reference in the literature concerning the occurrence in heated normal human serum of tropins for the pneumococcus, although Robertson and Sia (10) have demonstrated that heated swine serum promotes phagocytosis of the Type I pneumococcus. Employing the technique described at the beginning of the paper, it was possible to show, as will be seen from an examination of Table VI, that some tropic action can be exerted by normal heated human serum on 24 hour cultures of pneumococci. It will be noted that a marked difference exists in the amount of normal tropins in the sera of the two individuals. Further, the addition of the specific carbohydrate brings about a significant reduction in the phagocytic count. That this reduction is unquestionably type-specific has been demonstrated in two experiments which are not given here in detail. Nevertheless, in the case of Types I and III, the specific carbohydrate, as in the case of the normal opsonins, does not completely prevent phagocytosis. In two other experiments, the presence of a factor causing this residual phagocytosis

has been confirmed. It is logical to think that this residual tropic factor is the same as the residual opsonic factor, and that in the heated normal serum this factor acts by itself, and in the unheated normal serum its effect is markedly enhanced by the presence of complement.

In parallel experiments in which 8 hour cultures and organisms from the mouse peritoneum were used, no tropic action could be demonstrated except that the anticarbohydrate tropin of Serum E was able to bring about phagocytosis in both instances in the case of Type II.

The Effect of the Specific Carbohydrate on the Tropic Action of Immune Serum.—The results of an experiment planned to determine the effect

TABLE VI

	Type of pneumococcus	Concentration of carbohydrate	No. of organisms phagocyted by 50 cells	Cells taking part
				per cent
Heated Serum E + washed corpuscles	I	0	81	22
	I	Type I, 1:800	17	4
	II	0	174	32
	II	Type II, 1:1,600	3	4
	III	0	451	52
	III	Type III, 1:1,600	36	44
Heated Serum W + washed corpuscles	I	0	2	4
	I	Type I, 1:800	0	0
	II	0	0	0
	II	Type II, 1:1,600	1	2
	III	0	197	50
	III	Type III, 1:1,600	53	50

of the specific carbohydrate on the tropic action of the immune serum are summarized in Table VII. Here it will be seen that the addition of the homologous carbohydrate completely abolishes the tropic action of the immune serum, except in the case of the Type I antiserum. In the latter, the failure of the Type I carbohydrate completely to nullify the phagocytosis is probably due to the comparative weakness of this carbohydrate in its neutralizing properties. It is also evident that the action of the various carbohydrates is strictly specific. With the possible exception of Type I antiserum, there is no suggestion that a factor analogous to the residual factor in the normal serum is present

in the immune serum in the dilutions that were used. Were it possible to test stronger concentrations of antiserum, a residual factor might be revealed, but the very great amount of carbohydrate required for neutralization of large quantities of the homologous antibody makes this impractical. It must therefore remain undetermined for the present whether the residual antibody is increased as a result of immunization.

The Enhancement of the Tropic Action of Immune Serum by the Addition of Complement.—We have already mentioned in the introduc-

TABLE VII

	Type of pneumococcus	Concentration of type-specific antiserum	Concentration of specific carbohydrate	No. of organisms phagocyted by 50 cells	Phagocytes taking part
					per cent
Heated normal serum + washed corpuscles	I	1:400	0	357	58
	I	1:400	Type I, 1:800	30	20
	I	1:400	Type II, 1:800	238	68
	I	1:400	Type III, 1:800	412	74
	I	0	0	4	2
	II	1:32	0	414	44
	II	1:32	Type I, 1:800	369	72
	II	1:32	Type II, 1:800	5	6
	II	1:32	Type III, 1:800	413	66
	II	0	0	12	4
	III	1:800	0	182	22
	III	1:800	Type I, 1:800	326	40
	III	1:800	Type II, 1:800	367	46
	III	1:800	Type III, 1:800	0	0
	III	0	0	1	1

tion the view held by Neufeld (4), who maintains that the tropin of immune serum is a non-complex substance, the action of which is not increased by the addition of complement, whereas any increment in the amount of phagocytosis observed on the addition of complement to immune serum is to be attributed to an immune opsonic amboceptor, which is distinct from the tropin and, like a lytic antibody, entirely inert in the absence of complement. This hypothesis of Neufeld was formulated to explain the results of certain experiments in which the

addition of complement did not increase the amount of phagocytosis due to the antiserum, in contrast to those in which an adjuvant action of the thermolabile substance was demonstrated. However, this distinction has long somewhat confused our conception of the factors concerned in the mechanism of phagocytosis, and on the basis of the evidence presented below, is in our opinion unnecessary.

It has been shown already (Table VII) that the tropic action of a diluted Type III antiserum is caused by the Type III anticarbohydrate antibody. If it could be demonstrated that a small amount of complement increased the amount of phagocytosis, and that this increase

TABLE VIII

	Type of pneumococcus	Concentration of type-specific antiserum	Concentration of carbohydrate	Concentration of unheated normal serum	No. of organisms phagocyted by 50 cells	Cells taking part
						per cent
Heated normal serum	III	1:800	0	0	182	22
+ washed corpuscles	III	1:800	Type I, 1:800	0	326	40
	III	1:800	Type II, 1:800	0	367	46
	III	1:800	Type III, 1:800	0	0	0
	III	0	0	0	1	2
	III	1:800	0	1:16	740	66
	III	1:800	Type I, 1:800	1:16	1,097	96
	III	1:800	Type II, 1:800	1:16	886	80
	III	1:800	Type III, 1:800	1:16	56	34
	III	0	0	1:16	38	25

was due to the anticarbohydrate antibody acting in conjunction with complement, then Neufeld's contention, that there were two distinct antibodies involved, would be proved to be incorrect. Table VIII contains the results of an experiment in which a Type III antiserum is shown to exert a type-specific tropic action by virtue of its anticarbohydrate antibody. Here it may also be seen that this tropic action is markedly increased by the addition of a small amount of unheated normal serum. In both cases, however, when the specific carbohydrate is added, the effect of the anticarbohydrate antibody is removed, showing clearly that the same antibody can act as an immune tropin or as an immune opsonin, to employ the terminology of Neufeld.

The Rôle of Complement in Phagocytosis

It is evident from the observation already presented concerning the absence of phagocytosis in unheated infants' serum (Table IV) that complement without antibody cannot induce phagocytosis in the case of the virulent pneumococci. What, then, is the function of complement when acting conjointly with antibody? In the foregoing experiments we had been impressed with the rapidity of the phagocytic reaction when complement was present. This fact suggested that possibly the only rôle of complement in phagocytosis was to increase the speed of the combination of the antibody with the organism. An experiment was therefore designed to test the validity of this hypothesis:

Two sets of 4 tubes were prepared, containing the following materials.

Set I

Heated normal human serum, 0.125 cc.
Washed human cells, 0.125 cc.
Type II antiserum—concentration, 1:128.
Unheated normal human serum—concentration, 1:32.
Type II pneumococci, concentrated 8 hour culture killed by heating to 60°C. for half an hour.

Set II

Identical with Set I, save that saline solution was substituted for the unheated normal human serum.

The tubes were sealed and placed in the rotating box at 37°C. One tube from each set was removed at the intervals of ½ hour, 2 hours, 4 hours, and 8 hours. Smears were made of the contents, and the organisms in 100 cells were counted in each preparation. The opsonic effect of the heated normal serum together with the unheated normal serum was controlled by examining smears made from such mixtures at the end of 8 hours. The heated normal serum induced no phagocytosis at this time interval, while the slight phagocytosis due to the mixture of unheated and heated normal serum was deducted from the count of the corresponding mixture containing antiserum. The same control at 4 hours showed negligible phagocytosis.

An important detail in this experiment is the concentration of antiserum that is chosen. If the concentration is too strong, the phagocytosis due to the antiserum alone will be rapid enough to mask to a great extent the enhancing effect of the complement; if too weak, the incubation would have to be prolonged indefinitely to demonstrate the full effect of the antiserum acting by itself.

It had been determined previously that the phagocytic effect of the antiserum in the concentration chosen for the experiment was entirely removed by the addition of an appropriate amount of the specific carbohydrate. This fact clearly indicates that the phagocytic antibody involved in the experiment is the anticarbohydrate antibody.

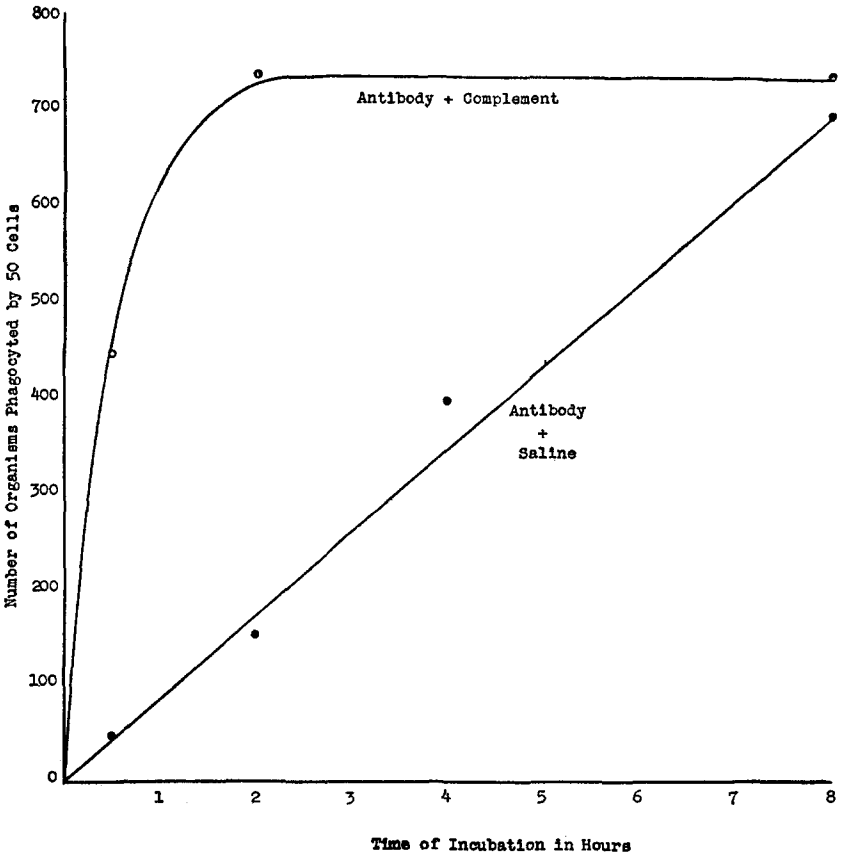

FIG. 1. Phagocytosis with and without complement.

The results obtained in the experiment are summarized in the form of a graph (Fig. 1). It will there be noted that the phagocytosis due to the antiserum alone increases at a uniform rate throughout an interval of 8 hours, but where complement is present the curve of the phagocytic count rises steeply and attains its maximum after 2 hours at the latest.

Thus at the end of ½ hour, the phagocytic effect of the antiserum is increased ninefold by the addition of complement, five times at an interval of 2 hours, twice at 4 hours, whereas at 8 hours the counts are approximately the same, whether complement be present or absent. From these results it would appear that, if the reaction is allowed to continue for a sufficient period of time, a given quantity of antibody promotes the same amount of phagocytosis, whether or not complement takes part in the process.

Complement, then, cannot increase the absolute capacity of a given concentration of antiserum to promote phagocytosis, but merely appears to do so by accelerating the velocity with which the organism is prepared for ingestion by the cells. The acceleration is probably caused by an increase in the rate at which the antibody enters into effective combination with the antigen under the influence of complement. This conception is supported by the experiments of Wright and Douglas (15), Bulloch and Atkin (16), and Sellards (17), which showed that if organisms were exposed to the action of unheated normal serum and subsequently heated to 60°C., they were phagocyted almost as readily as organisms subjected simply to the action of the unheated serum. In contrast, as is well known, organisms which are placed in normal serum which has previously been heated to 60°C., are not taken up by the phagocytes. The probable explanation of these results seems to us to lie in the rapid union of the available antibody with the organism under the influence of complement. When once this union has taken place, the subsequent destruction of the complement has no effect on the phagocytic process.

Another effect which we have observed repeatedly and which seems to be dependent on the accelerating action of the complement is seen in the proportion of cells taking part in phagocytosis. In the unheated serum, between 90 and 100 per cent of the phagocytes usually take part. On the other hand, in heated serum the same number of phagocyted organisms is distributed among a much smaller percentage of cells. For example, it was found in one experiment that after 2 hours' incubation, 50 polymorphonuclear leucocytes contained 182 organisms distributed among 45 out of the 50 cells when complement was present; whereas in the absence of this substance 151 organisms were distributed among 18 of the 50 leucocytes counted. It is probable that the reason for this difference lies in the fact that organisms in heated serum are agglutinated before they are ingested, unlike organisms in unheated serum, which are phagocyted before agglutination can take place.

For if the organisms are dispersed and yet prepared for ingestion, it is obvious that the chance of contact with the leucocytes is greater than where the organisms are aggregated, as occurs when phagocytosis is delayed. This reasoning is supported by an experiment in which the organisms were allowed to agglutinate in unheated and heated serum for some hours at 4°C., and then exposed to the action of washed leucocytes. The distribution of the organisms among the phagocytes was typical in both cases of that which is found in delayed phagocytosis.

DISCUSSION

It has been clearly demonstrated in the foregoing experiments that the type-specific anticarbohydrate antibody is capable by itself of preparing the pneumococcus for phagocytosis in heated normal and immune serum,—in other words, it may act as a normal or immune tropin. With the effect apparently increased in the presence of complement, the same antibody may be responsible for the opsonic action of unheated normal and immune serum. In the case of immune serum, at least, we have presented evidence which indicates that this augmentation due to complement is rather an acceleration in the rate at which phagocytosis takes place than any absolute enhancement of the phagocytic count. For it is apparent from the experiment presented that if this antibody is allowed to act on the organism for a sufficient length of time, the degree of phagocytosis reaches the same approximate level, whether complement be present or absent. The practical difficulty of prolonged exposure has made similar experimentation with normal serum impossible, but by analogy it is reasonable to suppose that complement has no other function than that of accelerating the phagocytosis of pneumococci by unheated normal serum.

If it is permissible to generalize from the data obtained by the study of one organism, it appears that the fundamental reaction, which occurs when an organism is prepared for phagocytosis either by normal or immune serum, is the union of a specific antibody with its antigen. This is essentially the conception held by the majority of observers and expressed by Topley and Wilson (3) in the passage quoted in the introduction. Further, Mudd and his associates (18) have shown that this is the underlying principle also in the phagocytosis of colloidal particles coated with protein and subsequently treated with homologous protein antiserum. However, divergent views and experimental data not strictly in accordance with this generalization have served to

set apart the subject of phagocytosis as something which is still somewhat mysterious and obscure. The demonstration that the anticarbohydrate antibody can be the one essential serum factor in all the phenomena of phagocytosis definitely relates phagocytosis to the other serum reactions, since it is known that this antibody can be responsible for agglutination, precipitation, fixation of complement, anaphylaxis, and at least is an essential factor in the intracellular digestion and death of the pneumococcus in the blood of resistant animals. It may be pointed out in passing that this universal participation of the anticarbohydrate antibody in all the serum reactions strongly supports the unitarian conception of antibodies advanced by Dean (19) and Zinsser (20).

It is true that in normal human serum we have shown that there is another heat-stable substance which under certain conditions can bring about phagocytosis in the absence of the anticarbohydrate antibody, but like the latter, this substance exerts a tropic and opsonic action, and therefore is presumably a separate and distinct antibody reacting with a separate and distinct antigen in the pneumococcus. Attention should be drawn here to the fact that this phagocytic factor by itself is incapable of leading to the destruction of pneumococci in a bactericidal system. This fact shows that the phagocytic capacity of a normal blood may give no indication whatever of the resistance of the animal to infection, at least as far as this resistance is correlated with the bactericidal power of the blood.

Some, at least, of the obscurity that still hangs over this subject can be laid at the door of the terminology. In our opinion, the word "tropin" should be discarded as both confusing and unnecessary. "Opsonin," however, adequately expresses the idea of preparing an organism for phagocytosis, and we would propose that this term be reserved for the antibody in the serum—whether normal or immune, heated or unheated—which unites with the organism and leads to its phagocytosis. At the same time, it would be understood that the rôle of complement in apparently increasing the degree of phagocytosis is that of a catalyst accelerating the rate of the reaction.

In concluding our discussion, we wish to emphasize the importance of the state of the organism in the phagocytic process. As an example, it has been found that Type III pneumococci growing in culture

medium were readily taken up by the leucocytes, whereas these organism when derived directly from the animal body were wholly resistant to phagocytosis by normal blood. In this study we have concerned ourselves rather with the mechanism of phagocytosis than with the correlation of this phenomenon with resistance to infection, but it is obvious that in any attempt to link up phagocytosis with infectious processes, more attention should be paid to the organism as it exists in the body than to the same strain growing in a test-tube.

CONCLUSIONS

1. In normal unheated human serum, virulent pneumococci may be prepared for phagocytosis by two separate antibodies, acting in conjunction with complement. One of these is the type-specific anticarbohydrate antibody reacting with the carbohydrate fraction of the pneumococcus. The other is probably also a type-specific antibody, but quite distinct from the former, and therefore must react with a different antigenic constituent of the bacterium.

2. In the normal human serum heated to 56°C., these two antibodies may, after prolonged contact with the organism, promote phagocytosis of the pneumococcus without the adjuvant action of complement.

3. Although these two antibodies are equally effective in the phagocytosis of 24 hour culture organisms by normal blood, the anticarbohydrate antibody tends to become the predominant factor as the pneumococci approach the state in which they exist in the animal body.

4. In so far as we have been able to show, the anticarbohydrate antibody is the only antibody in immune serum which can induce phagocytosis. This substance by itself is active in a phagocytic system, but just as in the normal serum, complement enhances its effect. The failure to demonstrate the presence in the immune serum of an antibody, distinct from the anticarbohydrate antibody, analogous to that found in the normal serum, may be due to the experimental difficulty of removing all the anticarbohydrate antibody from a concentrated immune serum.

5. Thus it is seen that a single well defined antibody (the anticarbohydrate antibody) may be responsible for the phagocytic action of normal unheated serum, normal heated serum, inactivated immune serum, and immune serum activated by complement. These facts

appear to us to invalidate Neufeld's division of the phagocytic antibodies into (*a*) bacteriotropins (antibodies, the phagocytic titre of which is not raised by the addition of complement); (*b*) opsonic antibodies (antibodies, comparable to the lysins, which are only active in the presence of complement).

6. Complement alone is incapable of inducing phagocytosis of the pneumococcus. In the phagocytic process, it appears simply to increase the speed at which the reaction takes place. Its rôle may be compared to that of a catalyst in a chemical reaction.

7. On the basis of these findings, it is proposed that the term "tropin" be discarded as misleading and unnecessary, and that the term "opsonin" be retained to denote any heat-stable antibody which prepares bacteria for phagocytosis. Contrary to current usage, it would not suggest a combination of antibody with complement.

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