

## HEAT LABILE OPSONINS TO PNEUMOCOCCUS

### II. INVOLVEMENT OF C3 AND C5\*

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Heat labile opsonins (HLO)<sup>1</sup> to pneumococci require participation of multiple components of the hemolytic complement (C) system (1). Among the components required are those inactivated by a purified cobra venom factor (VF) (1), which is capable of destroying C3-C9, without affecting C1, C4, or C2 (2). The present paper deals with the requirements for C3 and C5.

#### *Materials and Methods*

Phagocytic tests were performed as described in the preceding report (1). For reasons of technical convenience, all tests were done in the *dilute* phagocytic system (1). Guinea pigs were Hartley strain males purchased from Bar F Rabbitry, Baltimore, Md. 15 ml of starch aleuronat suspension was injected intraperitoneally into the guinea pigs to generate an acute granulocytic exudate (1).

Three strains of pneumococcus were used: (a) an unencapsulated rough strain (PnR) (1), (b) a fully encapsulated type 25 strain (Pn25) (1) of relatively high intraperitoneal virulence for mice ( $LD_{50} < 10$ ), and (c) an "intermediate" type 3 (III SIR6) strain (Pn3-int) with a relatively small capsule (3) and only a moderate intraperitoneal virulence for mice ( $LD_{50} = 10^{6.6}$ ) (4).

Studies dealing with C5 were done with two coisogenic strains of inbred black mice: B10.D2/Sn "old line", deficient in C5 (C5-deficient); and B10.D2/Sn "new line" not deficient in C5 (C5-normal) (5-7). Both strains were purchased from the Jackson Laboratory, Bar Harbor, Maine. These mice possess a high degree of histocompatibility (8) and, as far as is known, differ only in their production of C5.

Individual complement components were assayed by the method of Nelson et al. (9).

Monospecific anti-C3 serum was prepared by immunizing rabbits with purified guinea pig C3 (10).

Goat anti-rabbit globulin labeled with fluorescein isothiocyanate was purchased from Difco Laboratories, Inc., Detroit, Mich.

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<sup>1</sup> *Abbreviations used in this paper:* EDTA, ethylenediaminetetraacetate; HBG, modified Hanks' solution (1); HLO, heat labile opsonins; Pn3-int, an "intermediate" type 3 (III SIR6) strain of pneumococcus; Pn25, a fully encapsulated type 25 strain of pneumococcus; PnR, an unencapsulated rough strain of pneumococcus; VF, purified cobra venom factor.

Purified guinea pig C3 was radiolabeled with  $^{125}\text{I}$  according to the method of Greenwood et al. (11). The weight ratio of chloramine T to C3 was 1:200. Measurements of radioactivity were done with a well scintillation counter containing a thallium-activated NaI crystal (Tracerlab, Waltham, Mass.).

The methods used to purify C5 and the cobra venom factor are described elsewhere (12).<sup>2</sup>

#### RESULTS

*Consumption of Complement Components during Opsonization Process.*—When either encapsulated (Pn25) or unencapsulated pneumococci (PnR) (1) were opsonized by incubation in normal guinea pig serum, they consumed a larger proportion of the C3 than any of the other 8 complement components (Fig. 1).

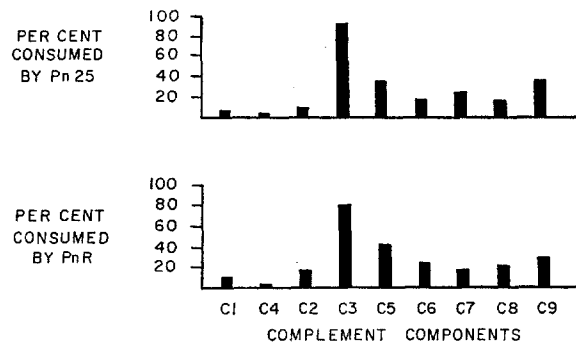


FIG. 1. Per cent of complement components in pooled normal guinea pig serum consumed by interaction with encapsulated (Pn25) and unencapsulated (PnR) pneumococci in opsonization reaction. The organisms were harvested in the log phase, washed twice, and resuspended in Hanks' bovine albumin-glucose solution (HBG) (1) at a concentration of  $1.25 \times 10^{10}$  per ml. 0.05 ml of the suspension was added to 1 ml of the serum and incubated for 30 min at  $37^\circ\text{C}$ . After removal of the organisms by centrifugation the serum was titrated for the residual complement components (9). Their titers are expressed as percentages of the respective components contained in normal serum that had been similarly incubated with 0.05 ml of HBG.

Since C3 is the most plentiful of the C components in guinea pig serum,<sup>3</sup> it is clear that the principal component consumed in the reaction was C3.

*Fixation of C3 to the Organisms.*—The results of each of the following experiments indicated that C3 is fixed to the encapsulated organisms during opsonization.

Type 25 pneumococci (Pn25), opsonized by being incubated twice in undiluted normal guinea pig serum for 30 min at  $37^\circ\text{C}$ , were washed in HBG (modified Hanks' solution) (1) and reincubated in monospecific anti-C3 serum

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obtained from rabbits immunized with purified guinea pig C3 (10). The organisms so treated promptly agglutinated, showing that they carried C3 on, or near, their surfaces.

The same treatment caused a capsular swelling reaction, which was difficult to distinguish from the classical quellung reaction produced by homologous anticapsular antibody (Fig. 5). The capsular swelling caused by the anti-C3 antibody was readily blocked by ethylenediaminetetraacetate (EDTA) (1) and clearly indicated the presence of C3 in the capsules.

Similarly, the capsules of Pn25, pretreated first with normal guinea pig

TABLE I  
*Ability of Purified C3 to Restore Opsonic Activity of Normal Guinea Pig Serum (NGS) Pretreated with a Purified Factor of Cobra Venom (VF)*

Suspending medium	Phagocytosis
	%
NGS	82
VF-treated NGS	0
VF-treated NGS + C3	85
C3 in HBG	0
HBG	0

To inactivate C3 without appreciably affecting C5 (see Table II), 0.86  $\mu$ g of VF (12) was added to 1 ml of NGS and incubated at 37°C for 15 min. To restore C3 activity, 1.2 mg of purified C3 in 0.2 ml of Hanks' bovine albumin-glucose solution (HBG) (1) was added to the 1 ml of VF-treated serum. When the C3 was tested alone as an opsonin, the same amount was dissolved in 1 ml of HBG. The phagocytic tests were done with encapsulated pneumococci (Pn25) and with guinea pig exudate leukocytes. Although the quantity of VF required to inactivate most of the C3, without inactivating C5 (Table II), varied somewhat with different lots of NGS and VF, the results recorded in this table were highly reproducible in repeated experiments.

serum and then with the monospecific anti-C3 rabbit serum, stained specifically with fluorescein-labeled goat antiserum to rabbit globulin (Fig. 6). This finding further substantiated the presence of C3 in the capsules of the serum-treated organisms.

Finally, to demonstrate more directly the capsular uptake of C3,  $6.25 \times 10^8$  Pn25 were twice incubated at 37°C for 30 min in 1 ml of normal guinea pig serum containing 10  $\mu$ g of purified  $^{125}$ I-labeled C3 (11). After being washed in HBG, the organisms retained 5.3% of the total radioactivity of the serum; whereas the uptake from heated serum<sup>4</sup> was 1.3% and from serum containing 0.01 M EDTA (1) was only 0.9%.

*Role of C3 in Opsonization.*—Addition of less than 1  $\mu$ g of a purified fraction

<sup>4</sup> Labeled C3 was added *after* serum had been heated at 56°C for 30 min.

of cobra venom (VF) (12) to 1 ml of guinea pig serum destroyed more than 95% of the C3 activity of the serum without causing a detectable loss of C1, C4, or C2 (2, 12) and without significantly depressing the activity of C5 (see Table II). The opsonic activity, which was completely suppressed by the treatment, was fully restored by the addition of 1.2 mg of purified C3 (Table I, lines 1-3). This finding indicates that C3 is critically involved in the opsonization process. The failure of C3 alone to act as an opsonin (Table I, line 4) suggests that it must be "activated", presumably to C3b (13), before it can participate in the reaction.

TABLE II  
*The Effects of Larger Doses of Purified Cobra Venom Factor on the Activities of C3, C5, and HLO in Normal Guinea Pig Serum*

VF added to NGS	C3 inactivated	C5 inactivated	C3 added	Phagocytosis
$\mu\text{g}$	%	%	mg	%
0	0	0	0	83
0.86	95	10	0	0
0.86	—	10	1.2	85
2.57	95	30	0	0
2.57	—	30	1.2	51
7.70	95	73	0	0
7.70	—	73	1.2	3

Where blanks are left in the table, large excesses of C3 were added to the serum, and hence per cent inactivation of normal C3 levels could not be determined. The experiments were performed as described in Table I.

As the dosage of VF was raised, increasing amounts of C5 were inactivated, in addition to the C3 (Table II). Under these circumstances, the added C3 was proportionately less effective in restoring the opsonic activity of the serum (Table II, lines 3, 5, and 7). Kinetic measurements of C3 inactivation, however, revealed that these higher doses of VF rapidly destroyed the C3 added to the serum (Fig. 2). Since the added C3 may therefore have been inactivated before it could participate in the opsonization process, no conclusion could be drawn about the possible involvement of later acting components (C5-C9).

*Requirement for C5.*—To test for possible participation of C5-C9, experiments were performed with C5-deficient mouse serum (5-7). Its promotion of phagocytosis, when tested with Pn25 (Table III), was slightly less than that of coisogenic mouse serum, and the deficiency was at least partially reversible with purified C5. A similar deficiency could be demonstrated in vitro with Pn3-int (Table IV).

The participation of C5 in antipneumococcal defense could also be demonstrated in vivo, provided the pneumococcal strain inoculated intraperitoneally

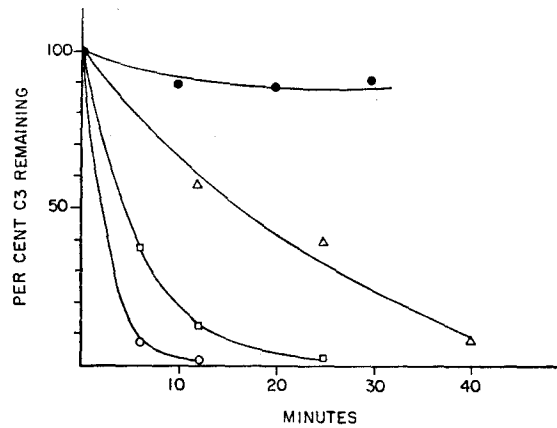


FIG. 2. Kinetics of C3 inactivation by varying doses of purified venom factor (VF). The VF was dissolved in 0.15 M NaCl and added to 3 volumes of serum. The four serum samples were treated with 0 (●—●), 0.86 (Δ—Δ), 2.57 (□—□), and 7.70 (○—○)  $\mu\text{g}$  of VF. To the treated samples at 0°C was added 0.1 part of purified C3 dissolved in 0.15 M NaCl buffered with 0.005 M phosphate at pH 7.5. The reaction mixtures were incubated at 37°C, and the reactions were terminated at the times indicated in the chart by diluting the samples 1000-fold in ice cold glucose-gelatin veronal buffer containing 0.15 mM  $\text{Ca}^{++}$  and 1.0 mM  $\text{Mg}^{++}$  (9).

TABLE III

*Phagocytosis of Encapsulated Pneumococci (Pn25) in Sera of Coisogenic C5-Deficient and C-Normal Mice*

Suspending medium	Phagocytosis					
	Exp:	1	2	3	4	5
		%				
C-normal serum		35	36	35	51	33
C5-deficient serum		22	26	25	34	19
C5-deficient serum + purified guinea pig C5		27	39	33	41	26

The phagocytic tests were done with both isologous and homologous exudate leukocytes. The purified guinea pig C5<sup>2</sup> used to restore the activity of the C5-deficient serum was added in a concentration of 15  $\mu\text{g}$  per ml in the first four experiments and 150  $\mu\text{g}$  per ml in the fifth.

was not too virulent (see Discussion). In a preliminary experiment using ten-fold dilutions of a 16 hr culture of Pn3-int (3) and inoculating 6 mice at each dilution, it was found that the LD<sub>50</sub> for the C normal mice was about 10 times greater than that for the C5-deficient mice. This difference was confirmed in a second more complete experiment in which the intraperitoneal inocula were varied two-fold and the mice were infected in groups of 10 (Table V and Figs.

TABLE IV  
*Phagocytosis of Pn3-int in C-Normal and C5-Deficient Mouse Serum*

Suspending medium	Phagocytosis			
	Exp:	1	2	3
			%	
C-normal serum		31	32	24
C5-deficient serum		21	11	8

TABLE V  
*The Effect of C5 Deficiency on Survival of Mice Challenged Intraperitoneally with Varying Doses of Pn3-int*

Dose	No. of mice	C5	Survivors on days:							Dead/ total*
			2	3	4	5	6	7	8	
$1.3 \times 10^8$	10	+	9	4	3	2	2	2	2	8/10
	10	-	4	0	0	0	0	0	0	10/10
$6.4 \times 10^7$	10	+	10	10	10	7	7	7	7	3/10
	10	-	8	1	1	1	1	1	1	9/10
$3.2 \times 10^7$	10	+	10	10	10	5	3	3	3	7/10
	10	-	9	3	1	1	1	1	1	9/10
$1.6 \times 10^7$	10	+	10	10	8	7	7	7	7	3/10
	10	-	9	5	2	2	2	2	2	8/10
$8.0 \times 10^6$	10	+	10	10	10	10	10	10	10	0/10
	10	-	10	7	2	1	1	0	0	10/10
$4.0 \times 10^6$	10	+	10	10	10	10	10	10	10	0/10
	10	-	10	8	4	0	0	0	0	10/10
$2.0 \times 10^6$	10	+	10	10	10	10	10	10	10	0/10
	10	-	10	10	8	6	6	6	6	4/10
$1.0 \times 10^6$	10	+	10	10	10	10	10	10	10	0/10
	10	-	10	10	10	10	10	10	10	0/10
$5.0 \times 10^5$	10	+	10	10	10	10	10	10	10	0/10
	10	-	10	10	9	9	9	9	9	1/10

\* Pn3-int was cultured from the blood of every mouse that died during the experiment.

3 and 4). The  $LD_{50}$  values for the C5-normal and C5-deficient mice, as calculated by the Reed-Muench method (14, 15), were  $10^{7.6}$  and  $10^{6.6}$ , respectively. It should be noted that at two of the inoculation levels (Table V, lines 5 and 6), all of the C5-deficient mice died, whereas all of the C-normal mice survived.

## DISCUSSION

It is clear from the present studies that activated C3 (i.e., C3b) plays a major role in the opsonization of encapsulated pneumococci by normal mammalian sera.

This finding is of great theoretical interest in relation to the recent detection

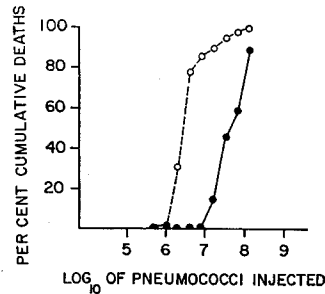


FIG. 3. Comparative dose-response curves of C-normal (●—●) and C5-deficient (○—○) mice to intraperitoneal injections of Pn3-int.

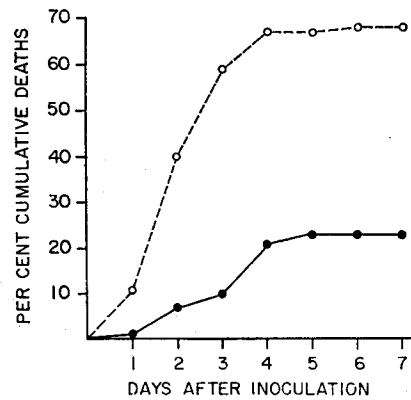


FIG. 4. Comparative combined cumulative deaths (%) occurring at all dose levels (Table V) in C-normal (●—●) and C5-deficient (○—○) mice inoculated intraperitoneally with varying doses of Pn3-int.

of C3 reactive sites (or more specifically C3b reactive sites) on the surfaces of both granulocytes (16) and monocytes (16, 17). These sites have been shown to participate in the immunoadhesion of sensitized erythrocytes to phagocytic cells (18) and in the erythrophagocytosis that follows. In this system, antibodies to surface antigens of the erythrocytes fix the complement components to the surfaces of the erythrocytes and thus render them "sticky" to the phagocytes. The principal ligand between the erythrocytes and the phago-

cytes appears to be C3b, since sensitized erythrocytes carrying C1, 4, 2a, 3b are opsonized, whereas those carrying only C1, 4, 2a are not (19).

The results of the present experiments indicate that the same ligand (C3b) is critical in the pneumococcus-HLO system. As already noted (1), however, the antibodies that initiate fixation of the complement components to the bacterial surfaces have not been positively identified. Presumably they are natural antibodies to cell wall antigens that are shared by many bacterial species. In keeping with this presumption are the following facts: (a) such natural antibodies are known to exist in mammalian sera (20); (b) HLO have consistently been shown to be immunologically polyspecific (20); and (c) their action is not blocked by an excess of homologous type specific capsular antigen (1). Nevertheless, the possibility must also be considered that C3 is fixed and activated by an alternative pathway that does not involve an antigen-antibody reaction. Regardless of the precise mechanism by which the C3 is fixed to the pneumococcus, once enough C3b molecules have accumulated in its capsule, opsonization results.

The role of C5 in promoting the phagocytosis of encapsulated pneumococci seems to be relatively minor as compared to that of C3. Nevertheless, a definite C5 effect was demonstrated both *in vitro* and *in vivo*.

The observation that the C5-deficient mice were more susceptible to pneumococcal peritonitis than the coisogenic C-normal mice indicates that C5 is somehow involved in the mouse's antipneumococcal defense. Demonstration of this difference depended upon the selection of a pneumococcal strain (Pn3-int) with just the right degree of virulence. An attempt to perform the experiment with Pn25 was unsuccessful because most of the C-normal mice succumbed to even the smallest intraperitoneal inoculum ( $LD_{50} < 10$ ).

As has already been emphasized elsewhere (21), significant differences in antibacterial resistance often go undetected when the challenge strain is either too virulent or too easily phagocytized. In analogous experiments with the same two mouse strains, Caren and Rosenberg (22) have recently found that the C-normal mice are slightly more resistant than the C5-deficient mice to intravenous injections of *Corynebacterium kutscheri*. The delicate host-parasite balance in this experimental model is complicated by the common occurrence of latent *C. kutscheri* infections in mice (23); but despite this complication, C5 appears to play a definite role in the host's defenses. In contrast, Stiffel et al. (24) and Glynn and Medhurst (25), using the same two strains of mice, were unable to detect any difference in their ability to clear relatively large intravenous doses of radiolabeled *Salmonella typhi* and *Escherichia coli*. Such models, of course, do not simulate bacterial disease and, therefore, are of limited value in studying many phases of phagocytosis and antibacterial immunity (21, 26, 27).

How C5 contributes to antibacterial immunity is not known. It may con-



ceivably act as an accessory opsonin after reacting with C3 on the bacterial cell, or the chemotactic C5a fragment that is released during the reaction (28) may stimulate the phagocytes. Both possibilities are being investigated.

It is evident from the present studies that components of the complement system, acting as HLO, participate in antipneumococcal defense, particularly in the preantibody phase of pneumococcal disease. When the organisms first gain a foothold in the tissues, the resulting inflammatory response brings granulocytes and plasma to the lesion. Although the granulocytes alone can destroy some of the invading organisms by surface phagocytosis (21, 26), the efficiency of phagocytosis is greatly increased by the HLO in the plasma. Since the action of HLO is immunologically polyspecific, it does not require the presence of specific anticapsular antibodies. When these antibodies eventually do arrive in the lesion, however, phagocytosis becomes still more efficient (29, 30). Even at this stage complement is evidently involved, for its presence has long been known to accelerate the phagocytosis of pneumococci opsonized with anticapsular antibody (31). Very recently, the complement components required for this acceleration have been shown to be the same as those that promote the phagocytosis of sensitized erythrocytes, namely C1, C4, C2, and C3 (32).

The synergistic action of IgG and C3b molecules as opsonins must now be considered in relation to the corresponding reactive sites that have recently been demonstrated on the surfaces of phagocytes (16, 17). In early pneumococcal disease, before enough anticapsular antibody has been generated to neutralize the capsular antigen in the fluid phase of the lesion (30), the C3b reactive sites are apparently involved in the HLO-promoted phagocytosis. Once enough anticapsular IgG has accumulated, on the other hand, both sets of reactive sites may participate in the phagocytic process. The IgG reactive sites are presumably to the Fc portion of the specific IgG molecules that are acting as opsonins (33). Determination of the relative degrees to which the two kinds of reactive sites participate in the ingestion of pneumococci, when both anticapsular IgG and complement are present in the system, will require further quantitative studies *in vitro*.

#### SUMMARY

When encapsulated type 25 pneumococci (Pn25) were opsonized with normal guinea pig serum, they consumed much more C3 than other complement (C) components. Fixation of C3 to the organisms was demonstrated by radiolabeling techniques, and its capsular localization was established by the use of monospecific anti-C3 antibody.

Treatment of the serum with an appropriate dose of a purified cobra venom factor (VF) destroyed C3 and all of the opsonic activity, without appreciably affecting the other C components. Addition of purified C3 completely restored

the opsonic activity of the VF-treated serum, indicating a requirement for C3. Since purified C3 alone had no opsonic activity, it was concluded that the C3 molecules had to be cleaved (to C3b) to function as opsonins.

Experiments with C5-deficient mice revealed that C5 also plays a definite, but quantitatively less impressive, role in antipneumococcal defense.

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FIG. 5. Capsular swelling reaction caused by incubating type 25 pneumococci (Pn25) at 37°C for 30 min, twice with normal guinea pig serum and once with mono-specific anti-C3 rabbit serum (10). The quellung reaction thus induced is shown in the right upper panel. The organisms in the panel below (right lower) were treated in the same manner except that enough EDTA (0.01 M) was added to the guinea pig serum to block the uptake of the heat labile opsonins (1). In the left upper panel, for comparison, is shown the capsular swelling of Pn25 caused by treatment with anti-Pn25 rabbit serum, and in the left lower panel are Pn25 treated with normal rabbit serum ( $\times 2000$ ).

FIG. 6. Type 25 pneumococci pretreated with normal guinea pig serum and mono-specific anti-C3 rabbit serum (as in Fig. 5) and then exposed to goat anti-rabbit globulin conjugated with fluorescein isothiocyanate. Before the fluorescein-labeled anti-rabbit globulin was added to the pneumococci, they were washed twice in HBG to remove the residual anti-C3 rabbit serum. (Fluorescence microscopy,  $\times 2000$ ).

