HEAT LABILE OPSONINS TO PNEUMOCOCCUS

I. Participation of Complement*

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Normal mammalian sera have long been known to contain heat labile factors that promote the phagocytosis of many bacteria, but the relation of these heat labile opsonins (HLO)[1] to "natural" antibodies, on the one hand, and to the complement (C) system, on the other, has been a matter of continuing controversy (1–13).

Hirsch and Strauss (14) recently concluded from studies on rabbit serum that HLO are distinguishable from antibodies by: (*a*) their heat lability, (*b*) their constancy of titer in different serum specimens against a variety of bacteria, (*c*) their rapid reaction with bacteria at 38°C, but not at 0°C, (*d*) their inactivation by hydrazine and ammonia, (*e*) their absence in the gamma globulin fraction of serum, and (*f*) their lack of specificity as demonstrated by cross absorption studies. In addition, their attachment to *Staphylococcus albus* was blocked by high concentrations of salt and did not require the presence of divalent cations in the medium. The latter finding led to the further conclusion that HLO are not identical to the hemolytic complement system.

In a confirmatory study, done with immunofluorescence techniques, Hirsch (15) reported that the HLO in guinea pig serum: (*a*) are adsorbed to the surfaces of staphylococci at 38°C, but not at 0°C, (*b*) are blocked in their adsorption by high salt concentration, but not by the absence of either Ca^{++} or Mg^{++}, (*c*) are altered or destroyed by exposure to 56°C, to hydrazine, or to trypsin, even when already adsorbed, and (*d*) are adsorbed independently of previously attached antibody. He concluded, therefore, that HLO are neither antibodies nor the complete hemolytic complement system.

The present studies concern HLO to both unencapsulated and encapsulated strains of *Diplococcus pneumoniae* (pneumococcus). The experiments described

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[1] Abbreviations used in this paper: BSS, balanced salt solution; C, complement; EDTA, ethylenediaminetetraacetate; HBG, modified Hanks' solution (see footnote 2); HLO, heat labile opsonins; NRS, normal rat serum; Pn1, fully encapsulated type 1 (A5) strain of pneumococcus; Pn25, fully encapsulated type 25 pneumococcus; PnR, unencapsulated (rough) pneumococcus strain R36NC; Ra-a-HuGG: HuGG, preformed immune aggregate (see footnote 11).

in this and the following paper (16) provide evidence: (*a*) that HLO to pneumococcus include multiple components of the hemolytic complement system, and (*b*) that the principal component involved is C3. A preliminary report has been published elsewhere (17).

Materials and Methods

Leukocytes.—Albino rats weighing from 200 to 225 g and obtained from Sprague-Dawley, Inc., Madison, Wis., and from Charles River Breeding Laboratories, North Wilmington, Mass., were injected intraperitoneally with 5 ml of autoclaved 3% starch and 5% aleuronat suspension diluted 1:2 with tryptose phosphate broth (18). After 21 hr they were killed with ether, and their peritoneal cavities were opened and washed with cold modified Hanks' solution (HBG)[2] containing 5 mg of heparin per 100 ml. The washings were centrifuged at 180 g for 5 min in the cold,[3] and the packed cells (about 70% granulocytes) were resuspended in fresh HBG, pooled, and counted by the standard hemocytometer technique before being transferred, in portions containing 2.5×10^8 cells, to 13 × 100 mm screw cap tubes. After centrifu-

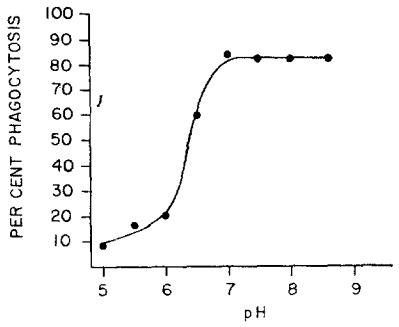

FIG. 1. Effect of pH on phagocytosis of unencapsulated pneumococcus (PnR) in normal rat serum diluted 1:20 with HBG and adjusted to the desired pH. Phagocytic tests done in dilute system; see Methods.

gation at 180 *g* for 5 min, the supernatants were drained from the tubes and the packed cells were held on ice until used.

The same procedures were used to obtain exudate leukocytes from white mice,[4] except that the volume of starch aleuronat suspension injected intraperitoneally was 1.5 rather than 5 ml.

Bacteria.—Four strains of bacteria were used in these studies: (*a*) an avirulent unencapsulated (rough) pneumococcus (PnR), strain R36NC (20); (*b*) a fully encapsulated type 1 (A5) strain (Pn1) which is highly virulent for both rats and mice (21), as well as for man; (*c*) a fully encapsulated type 25 strain of lesser virulence for rats (22), but of high intraperitoneal virulence for mice (LD_{50} < 10 organisms),[5] and (*d*) an avirulent laboratory strain of *Escherichia*

[2] Hanks' bovine albumin-glucose solution (19): NaCl, 128 mM; KCl, 10 mM; Na_2HPO_4, 5.6 mM; KH_2PO_4, 0.88 mM; K_2HPO_4, 2.8 mM; glucose, 5.5 mM; bovine albumin, 0.1 g per liter.

[3] All centrifugations were performed at 4°C.

[4] Strain MBR/ICR, purchased from Hazleton-Carbia, Burtonsville, Md.

[5] Smith, M. R., and R. H. Drachman. Unpublished observations.

coli (9701).[6] The pneumococci were stored at 4°C in rabbit blood under vaseline (21). The encapsulated strains (Pn1 and Pn25) were passed through mice at monthly intervals to maintain virulence. Cultures were prepared by inoculating 4.5 ml of beef infusion broth (containing 10% sheep serum and 0.2% dextrose) with 0.5 ml of an overnight subculture from the stock and incubating for 4 hr at 37°C. The *E. coli* strain, maintained on trypticase soy agar slants at 4°C, was cultured in the same manner. Only sheep serum free of demonstrable HLO, even when undiluted, was used in the culture medium.

Cultures were processed as follows: the bacteria from several cultures that had first been

TABLE I

Heat Labile Opsonins to PnR in Normal Rat Serum

Suspending medium	Phagocytosis*
	%
HBG	17
NRS undiluted	85
NRS 1:2‡	92
NRS 1:5	93
NRS 1:10	91
NRS 1:20	88
NRS 1:40	77
NRS 1:80	30
NRS 1:100	17
Heated NRS undiluted	36
Heated NRS 1:2	35
Heated NRS 1:3	38
Heated NRS 1:5	31
Heated NRS 1:10	28
Heated NRS 1:20	22
Heated NRS 1:40	22
Heated NRS 1:80	18
Heated NRS 1:100	17

* In dilute phagocytosis system; see Methods.

‡ Diluted with HBG.

diluted 1:2 with HBG, were centrifuged in separate tubes at 1900 *g* for 30 min. The packed centrifugates were pooled, resuspended in 5 ml of HBG, and recentrifuged. The final centrifugate was suspended in a minimal volume of HBG, diluted 1:1000 for counting in a Petroff-Hausser chamber, and finally diluted with HBG to a concentration of 1.25×10^{10} organisms/ml. The standardized suspension was used immediately.

Serum.—Blood obtained by cardiac puncture, was allowed to clot on ice, and after the clots had been rimmed and the samples held at 4°C for 6 hr, the serum was recovered and cleared of cells by centrifugation (900 *g* for 15 min). The cleared serum was pooled and stored at −70°C. Samples were thawed at 4°C as needed.

Adsorption of serum.—The bacteria contained in 1 ml of the standardized suspension (i.e.,

[6] Obtained from the Naval Medical Research Laboratory, Bethesda, Md.

1.25×10^{10} organisms) were heat killed (100°C for 5 min),[7] packed by centrifugation at 1900 *g* for 30 min, and resuspended in 1 ml of 1:20 dilution of the serum (see Table I). After incubation in a 37°C water bath for 30 min the suspension was recentrifuged, and the serum was decanted. In some experiments it was adsorbed twice more with fresh organisms. Thereafter, it was either held on ice for immediate use or was stored at −70°C for later experiments.

Opsonization of Bacteria.—When bacteria preexposed to the serum were to be used in phagocytic tests, the same procedures were followed, except that the viable bacteria in 0.1 ml (rather than 1.0 ml) of the standardized suspension, i.e., 1.25×10^9 organisms, were incubated (once) in 1 ml of undiluted serum. In some experiments the serum treated organisms were subsequently washed in HBG.

Phagocytic Tests.—1 ml of the desired test medium and 0.1 ml of the standardized bacterial suspension containing 1.25×10^{10} organisms per ml were added to 2.5×10^8 leukocytes packed by centrifugation in the bottom of each of a series of 13 × 100 mm screw cap tubes. The final ratio of bacteria to cells was thus 5:1. After being thoroughly mixed and tumbled end over end at 12 rpm for 30 min at 37°C, the cells in each tube were diluted in 2 ml of HBG, centrifuged at 180 *g* for 5 min (to remove most of the extracellular bacteria), and examined microscopically in smears stained with methylene blue. The *per cent phagocytosis* was determined by counting the percentage of 400 polymorphonuclear leukocytes containing one or more bacteria.

This phagocytic system, hereafter referred to as the *dilute system*, was used in all experiments done with PnR, Pn25, and *E. coli*.

Since preliminary experiments revealed that Pn1 was too resistant to phagocytosis to be studied in the dilute phagocytic system, a more concentrated system was designed, where the phagocytic activity of the cells was enhanced by their proximity to one another (20). In this *concentrated system* 2×10^9 Pn1 in 0.025 ml HBG and 0.1 (rather than 1.0) ml of test medium were added to 2.5×10^8 packed leukocytes, making the final ratio of bacteria to cells 8:1. Samples containing 0.03 ml of the thoroughly mixed suspension of bacteria and cells were each spread on a 1 × 2 cm area of a carefully cleaned glass slide and were incubated for 30 min at 37°C in Petri dishes lined with moistened filter paper to prevent drying. After incubation the cells were washed from each slide with cold HBG, reconcentrated by centrifugation (180 *g* for 5 min), and examined in stained smears as already described.

Qualitative Test of Motility.—To determine whether constituents of the medium grossly affected the motility of the leukocytes, hanging drop preparations containing the phagocytic test mixture of leukocytes and bacteria were incubated at 37°C for 30 min and immediately examined under the microscope. Whereas unaffected cells could be seen to migrate in large numbers into the originally clear zone of medium at the extreme periphery of the drop, damaged cells failed to do so. For illustrations of method, see Fig. 4 (22).

The *complement titrations* were performed as described in (23), except for the following modifications:[8] (*a*) Each ml of the standardized suspension of sensitized erythrocytes was further diluted with 5.5 ml of isotonic Veronal buffer before being used in the hemolytic titrations. (*b*) The titrations were done in Pyrex test tubes, 15 × 125 mm. (*c*) The reaction mixtures, which contained 0.5 ml of the sensitized erythrocytes and 1.0 ml of the appropriate serum dilution, were incubated at 37°C for 90 min. The contents of each tube were then diluted with 3.0 ml of 0.85% NaCl and centrifuged. The per cent lysis was estimated from OD readings made on the supernates at a wave length of 412 mμ.

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[7] When *viable* pneumococci or *E. coli* in such numbers were used for adsorption, their production of acid during the 30 min period of incubation lowered the pH of the diluted serum to levels (<6.5) at which phagocytosis was markedly depressed (see Fig. 1).

[8] Suggested by Dr. Abraham Osler, who together with Miss Betsy Hill, kindly assisted in these titrations.

RESULTS

I. Heat Labile Opsonins to Unencapsulated Pneumococci (PnR): Titer of HLO to PnR in Pooled Normal Rat Serum (NRS)

As shown in Table I, the substitution of undiluted NRS for HBG in phagocytic tests performed with PnR increased the per cent phagocytosis from 17 to 85. When the serum was heated at 56° for 30 min, the increase in per cent phagocytosis was substantially less, i.e. only 36. These findings, together with the observation that the motility of the leukocytes was not detectably depressed in the HBG medium, suggested that most of the stimulatory effect of the serum was due to the presence of heat labile opsonins. Furthermore, the phagocytosis-promoting activity of the serum was still present at fairly high

TABLE II

Comparative Opsonizing Activities of Homologous and Heterologous Sera on PnR

Suspending medium	Phagocytosis*
	%
HBG	17
Normal rat serum‡	87
Normal mouse serum	82
Normal guinea pig serum	77
Normal rabbit serum	93
Normal human serum	94

* In dilute phagocytic system containing rat leukocytes; see Methods.

‡ All sera were undiluted.

dilutions. Indeed, full activity of the unheated serum was retained in dilutions as high as 1:20.

Effectiveness of HLO to PnR in Heterologous Sera.—Phagocytosis of PnR by rat granulocytes in the dilute system was promoted to approximately the same degree by rat, mouse, rabbit, guinea pig, and human sera (Table II), revealing that, under the conditions employed, heterologous sera are just as effective as homologous sera in enhancing phagocytosis of the *unencapsulated* organism. It should be noted that this is not the case with *encapsulated* pneumococci (see below).

Lability of HLO to PnR as Compared to that of the Hemolytic Complement System (C).—Both the HLO to PnR[9] and complement activities of rat serum stored at −70°C for 8 years were found to be the same as that of fresh serum. There was also no loss of either after storage at −20°C for 11 wk or at 4°C for 24 hr. After 4 days at 4°C, however, the C titer dropped from 264 to less than 100 CH_{50} units, while the titer of HLO appeared to be unaffected. Simi-

[9] All determinations of HLO to PnR in these lability experiments were done on serum diluted 1:20 with HBG (see Table I).

larly, after 24 hr at room temperature (24°C) the titer of C was slightly depressed (170 CH_{50} units), whereas that of the HLO was unchanged. Neither was detectably diminished after incubation at 37°C for 30 min. Heating at 56°C for 30 min, on the other hand, lowered the hemolytic C titer to 0, but only partially inactivated the HLO (see Table I).

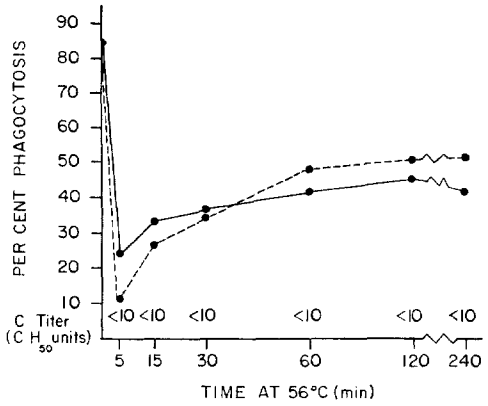

FIG. 2. Kinetics of inactivation of HLO and C at 56°C. Two curves depict HLO activities for PnR in two representative experiments. Figures at bottom of chart indicate C titers in a single experiment in which the original C titer of the serum was 434. All sera were undiluted, and phagocytic tests were performed in the dilute phagocytic system; see Methods.

TABLE III

Comparative Heat Sensitivities of C and HLO for PnR

Temperature	Phagocytosis	C titer
°C*	%	CH_{50} *units*
0	87	400
37	86	500
45	88	309
50	49	200
56	39	<10
60	66	<10
65	serum clotted	—

* To which serum (undiluted) was exposed for 30 min.

Kinetics of Inactivation of HLO to PnR and of C at 56°C.—The effects on HLO to PnR and on C of heating normal rat serum at 56°C for varying lengths of time are shown in Fig. 2. It will be noted that the C titer became negligible after 5 min, whereas the opsonin activity fell abruptly in the first 5 min, only to rise again on continued heating. The bimodal character of the latter response suggests that the inactivation of the heat labile factor was followed by activation of a heat stable factor. Although the nature of the second factor has not

been determined, it is presumed to be heat denatured proteins capable of opsonizing the unencapsulated pneumococci. As is shown in Table XI, no such bimodal response is demonstrable with encapsulated pneumococci.

Comparative Heat Sensitivities of HLO to PnR and of C.—When the serum was heated at varying temperatures for 30 min, the same bimodal effect on the HLO to PnR was apparent (Table III). In contrast, the hemolytic C activity was fully destroyed at 56°C and above.

Site of Action of HLO to PnR.—Whereas preincubation (37°C) of rat leukocytes in normal serum for 30 min had no effect upon their subsequent phagocytic

TABLE IV

Opsonization of PnR by Preincubation at 37°C for 30 Min in Normal Rat Serum

Preincubation medium	Phagocytic test medium	Phagocytosis
		%
NRS*	NRS	90
NRS	HNRS‡	86
NRS	HBG	90
NRS (culture washed)	HBG	81
HNRS	NRS	85
HNRS	HNRS	65
HNRS	HBG	29
HBG	NRS	80
HBG	HNRS	33
HBG	HBG	12

* Undiluted.

‡ NRS heated at 56°C for 30 min.

activities in HBG, similar treatment effectively opsonized PnR, as shown in Table IV, even when the bacteria were washed in HBG after being incubated in the serum. It is therefore clear that the HLO act, not upon the phagocytic cells, but upon the bacteria.

Adsorption of HLO to PnR from Serum.—In accordance with the preceding findings, it was demonstrated that much of the HLO to PnR could be adsorbed from a 1:20 dilution of rat serum by incubating it for 30 min at 37°C with an appropriate quantity of the homologous heat-killed organisms (see Methods), but not with rat leukocytes, or with polystyrene beads.[10] Adsorption with heat-killed *E. coli* was also just as effective as with PnR, indicating that the HLO to PnR are not species-specific (Table V).

[10] Diameter 0.184 $\mu \pm 0.01 \mu$ SD (Dow Chemical Co., Midland, Mich.).

Failure of HLO to PnR to be Adsorbed at 0°C.—Even when huge numbers of rough pneumococci (up to 0.6 ml of packed organisms per ml of serum) were used, no adsorption of HLO from NRS could be demonstrated to occur at 0°C. Similarly, no opsonization of PnR could be effectuated with NRS at 0°C. Thus HLO to PnR do not behave like conventional antibodies.

Relation of PnR HLO to Hemolytic C Activity of Serum.—Because of the rela-

TABLE V

Adsorption of HLO to PnR from Normal Rat Serum

Particles with which serum* was adsorbed at 37°C for 30 min	Phagocytosis of PnR in adsorbed serum
	%
None	84
PnR‡	48
E. coli§	42
Polystyrene beads	82

* Serum diluted 1:20 with HBG; see Table I.

‡ Heat-killed (100°C for 5 min).

§ Heat-killed (100°C for 15 min).

TABLE VI

Effect of C Fixation on HLO to PnR

Preformed immune aggregate* added to undiluted normal rat serum	C titer	Phagocytosis
mg/ml	CH_{50} units	%
0	270	87
0.015	200	73
0.030	147	73
0.060	89	72
0.125	34	63
0.250	10	47
1.000	<10	16

* Ra-a-HuGG:HuGG; see text.

tive heat lability of the HLO to PnR and because of their interaction with the organisms at 37°C, but not at 0°C, experiments were performed to determine whether they were in any way related to components of the hemolytic C system. To diminish progressively the C titer of the serum, increasing quantities of a preformed immune aggregate (Ra-a-HuGG:HuGG)[11] were added as shown in Table VI. When the activity of HLO in each sample of the treated serum was measured, it was found that the opsonizing activity was roughly proportional

[11] Immune aggregate resulting from the interaction of human gamma globulin with specific antihuman gamma globulin serum prepared in rabbits, and kindly supplied by Dr. Abraham Osler.

to the titer of residual C. Conversely, adsorption of normal rat serum with PnR (at 37°C for 30 min) removed the hemolytic C activity from the serum. These results suggest that at least one component of the C system plays a role in the action of the HLO to PnR.

HLO to PnR in Sera Lacking Individual Components of C System.—In order to determine whether all components of the hemolytic C system are required for heat labile opsonin activity for PnR, phagocytic tests were performed with (*a*) rat serum treated with zymosan to remove C3 (23), (*b*) rat serum treated with NH_4OH to inactivate C4 (23), (*c*) rat serum treated with a purified cobra

TABLE VII

Opsonization of PnR with Zymosan Treated, Ammonium Hydroxide Treated, and Cobra Venom Treated Rat Serum and with C5-Deficient Mouse Serum

Suspending medium	Phagocytosis
	%
NRS 1:20	88
NRS 1:20 + zymosan (1.76 mg/ml)*	74
NRS 1:20 + zymosan (25.0 mg/ml)	60
NRS + NH_4OH (30 mM), diluted 1:5‡	87
NRS 1:20 + venom (0.25 μg/ml)§	46
Normal mouse serum	82
C5-deficient mouse serum‖	90

* Zymosan was incubated with serum for 1 hr at 37°C (23) and removed by centrifugation. Remaining C titer was <10.

‡ See (23). Remaining C titer was <35.

§ Purified cobra venom factor (VF) (24) incubated with serum for 15 min at 37°C. This amount of VF was shown in control experiments with purified C3 to have no deleterious effect on the leukocytes (16).

‖ Obtained from B10.D2 *old line* mice (26) and kindly provided by the Jackson Laboratory, Bar Harbor, Maine. This serum has no detectable hemolytic C activity (26, 27) and lacks a protein analogous to the fifth component of human C (28).

venom factor (24) to inactivate C3–C9 (25), and (*d*) mouse serum deficient in C5 (26–28). As shown in Table VII, treatment with NH_4OH had no appreciable effect on the opsonizing action of the rat serum on PnR. Similarly the C5-deficient mouse serum was just as active as normal mouse serum. Zymosan treatment to block C3 and inactivation of C3–C9 with the cobra venom factor, on the other hand, significantly depressed the phagocytosis of PnR. Thus it appeared that at least C3 was required for opsonization of PnR under the conditions of these experiments.

Role of Divalent Cations in Interaction of PnR with HLO in NRS.—Since divalent cations are required for action of C1 and C2 in the complement system (23), experiments were performed to determine whether the interaction of HLO and PnR is Ca^{++} and/or Mg^{++} dependent. The binding of divalent cat-

ions by the addition of an appropriate amount of ethylenediaminetetraacetate (EDTA) to a 1:20 dilution of NRS was first shown to block the phagocytosis of PnR. The effect of the EDTA was readily reversed by the addition of Ca^{++} (Table VIII).[12] As a control in this experiment (14) the same phagocytic test was performed in a medium containing a physiological concentration of free Ca^{++} (Hanks' balanced salt solution, BSS, see Table VIII). Although the presence of this amount of Ca^{++} in the suspending medium appreciably raised the per cent phagocytosis (Table VIII, line 5), the amount of Ca^{++} equivalent to the concentration of ionized Ca^{++} in a 1:20 dilution of normal rat serum (about 0.06 mM) had only a minimal opsonizing effect (Table VIII, line 6). Thus the

TABLE VIII

The Effect of Divalent Cations on the Action of HLO to PnR

Suspending medium	Phagocytosis
	%
HBG	12
NRS 1:20	87
NRS 1:20 + 6.0 mM EDTA	4
NRS 1:20 + 6.0 mM EDTA = 6.0 mM $CaCl_2$*	90
BSS‡ (containing 1.27 mM $CaCl_2$)	81
BSS 1:20§ (containing 0.06 mM $CaCl_2$)	38

* When this amount of EDTA (6.0 mM) is reacted with 6.0 mM $CaCl_2$, the pH of the *diluted* serum falls from 7.4 to 6.2 (see Fig. 1). Accordingly, the medium was retitrated with NaOH to the original pH before the cells were added. Since the volume of NaOH added to each ml of the medium never exceeded 0.02 ml, the resulting dilution was ignored.

‡ BSS (Balanced salt solution; Microbiological Associates, Inc., Bethesda, Md.) contains: NaCl, 136.9 mM; KCl, 5.4 mM; $CaCl_2 \cdot 2H_2O$, 1.27 mM; $MgSO_4 \cdot 7H_2O$, 0.41 mM; $MgCl_2 \cdot 6H_2O$, 0.49 mM; $Na_2HPO_4 \cdot 7H_2O$, 0.34 mM; K_2HPO_4, 0.44 mM; D-glucose, 5.55 mM. To each liter of this solution was added 350 mg of $NaHCO_3$.

§ Diluted in HBG.

opsonizing action of the NRS 1:20, though dependent upon the presence of Ca^{++}, was clearly not due to the Ca^{++} per se. Indeed, the heat lability of the serum opsonizing factor had already excluded this possibility. Similarly, the adsorption of HLO from normal rat serum with PnR was found to require the presence of Ca^{++} and/or Mg^{++} (Table IX). And finally, opsonization of PnR with HLO resulting from pretreatment of the organisms with undiluted NRS was shown to be dependent upon the presence of divalent cations in the serum (Table X).

From these three sets of observations, it may be concluded that the interaction of HLO with PnR is Ca^{++} and/or Mg^{++}-dependent, like the action of C1 and C2 in the hemolytic complement system (23).

[12] For consideration of the relation of Ca^{++} to phagocytosis, see Discussion.

II. Heat Labile Opsonins to Encapsulated Pneumococci (Pn25 and Pn1): Comparative Properties of HLO to PnR and Pn25

When the properties of rat serum HLO to Pn25 were compared in the dilute phagocytic system (see Methods) with those of HLO to PnR, the following differences were noted (Table XI):

In control experiments done in HBG and BSS (lines 1 and 2 of Table XI) Pn25 was found, as expected, to be more resistant to phagocytosis than PnR. Furthermore, its resistance to ingestion was not influenced by the presence of the Ca^{++} in the BSS, as was that of PnR.

Although the opsonizing effect of undiluted NRS on Pn25 was just as great

TABLE IX

The Effect of Divalent Cations on the Adsorption of HLO from NRS with PnR

Suspending medium	Phagocytosis
	%
NRS 1:20	84
NRS 1:20 adsorbed with PnR	45
NRS 1:20 + 0.6 mM EDTA	9
NRS 1:20 + 0.6 mM EDTA + 0.6 mM $CaCl_2$	88
NRS 1:20 + 0.6 mM EDTA adsorbed with PnR and then supplemented with 0.6 mM $CaCl_2$	94
NRS 1:20 + 6.0 mM EDTA	4
NRS 1:20 + 6.0 mM EDTA + 6.0 mM $CaCl_2$*	90
NRS 1:20 + 6.0 mM EDTA adsorbed with PnR and then supplemented with 6.0 mM $CaCl_2$*	88

* See *, Table VIII.

as it was on PnR (line 3), the titer of the opsonins to Pn25 was lower than the titer of opsonins to PnR (lines 4–6).

The opsonins to Pn25, like those to PnR, were destroyed by heating the serum to 56°C for 5 min (line 7); but unlike the results with PnR, no secondary opsonization of Pn25 occurred when the heating was continued to 30 min (line 8), i.e. the heat inactivation curve was not bimodal (cf. Fig. 2).

Whereas opsonization of PnR appeared not to require C4, opsonization of Pn25 was blocked by treating the serum with NH_4OH (line 10). The apparent difference, however, may be merely a quantitative one, since the treated serum still possessed considerable hemolytic activity (see footnote 8). It should be noted parenthetically that treatment of the serum with zymosan and with cobra venom depressed the phagocytosis of Pn25 (lines 9 and 11) just as it did PnR.

Phagocytosis of Pn25 was not promoted by heterologous sera, as was phago-

cytosis of PnR (lines 12–15); i.e., the more difficult task of ingesting the encapsulated organisms (Pn25) apparently could not be accomplished by the rat leukocytes in foreign serum. For this reason the possible C5 requirement for the phagocytosis of Pn25 could not be studied in the rat cell system. The neces-

TABLE X

The Effect of Divalent Cations on the Opsonization of PnR with NRS

Opsonizing medium	Phagocytosis*
	%
NRS (undiluted)	61
NRS + 6.0 mM EDTA	3
NRS + 6.0 mM EDTA + 6.0 mM $CaCl_2$‡	57

* In dilute phagocytic system (see Methods) containing HBG, after organisms had been washed once in HBG to remove the opsonizing medium.

‡ pH of *undiluted* NRS not significantly affected by acid from $CaCl_2$-EDTA reaction (cf. Table VIII).

TABLE XI

Comparative Properties of HLO to Pn25 and PnR

Suspending medium	Phagocytosis	
	Pn25	PnR
	%	%
HBG	0	17
BSS	0	81
NRS	90	88
NRS 1:5	90	93
NRS 1:10	25	91
NRS 1:20	3	88
Heated NRS (56°C for 5 min)*	0	12
Heated NRS (56°C for 30 min)*	2	36
Zymosan treated serum‡	2	60
NH_4OH treated serum§	8	87
Venom treated serum‡	0	64
Normal mouse serum	4	82
Normal guinea pig serum	1	77
Normal rabbit serum	2	93
Normal human serum	2	94

* Hemolytic C titer <10 CH_{50} units.

‡ Hemolytic C titer <10 CH_{50} units. Inactivation procedure as in Table VII.

§ Hemolytic C titer <35 CH_{50} units. Inactivation procedure as in Table VII.

sary experiments were therefore postponed for systematic study in a mouse cell system (16).

Dependence of HLO to Pn25 on Divalent Cations.—Like those to PnR, the HLO to Pn25 are Ca^{++}- and/or Mg^{++}-dependent, since their phagocytosis

promoting action (Table XII), their ability to preopsonize Pn25 (Table XIII), and their adsorption from serum with Pn25 (Table XIV) are all blocked by the presence of EDTA and are restored by the addition of $CaCl_2$. Thus the overall properties of the HLO to Pn25 suggest involvement of multiple components of the hemolytic complement system.

Heat Labile Opsonins to Pn1.—As shown in Table XV, most of the character-

TABLE XII

*Requirement of Divalent Cations for Promotion of Phagocytosis of Pn25 by HLO in NRS**

Suspending medium	Phagocytosis
	%
NRS	94
NRS + EDTA (6.0 mM)	<1
NRS + EDTA (6.0 mM) + $CaCl_2$ (6.0 mM)‡	93

* The serum was undiluted except for reagent additions which never exceeded 0.1 ml per ml of serum.

‡ pH of *undiluted* NRS not significantly affected by acid from $CaCl_2$–EDTA reaction (cf. Table VIII).

TABLE XIII

*Requirement of Divalent Cations for Opsonization of Pn25 by Pretreatment with HLO in NRS**

Opsonizing medium‡	Phagocytosis§
	%
NRS	63
NRS + EDTA (6.0 mM)	<1
NRS + EDTA (6.0 mM) + $CaCl_2$ (12.0 mM)‖	66

* See *, Table XII.

‡ Organism incubated in medium for 30 min at 37°C and then washed once in BSS to remove opsonizing medium before being transferred to phagocytic system.

§ In BSS, dilute phagocytic system; see Methods.

‖ See ‡, Table XII.

istics of the HLO to Pn1 are similar to those of HLO to Pn25, although the Pn1 opsonins have to be studied in the concentrated, rather than the dilute, phagocytic system (see Methods). It should be noted, however, that the titer of Pn1 opsonins in rat serum, as measured in the concentrated system (lines 3–6), is too low for use of the standard NH_4OH method to inactivate C4, since the method requires a 1:5 dilution of the serum (23). Similarly, heterologous sera will not promote phagocytosis of Pn1 in the rat cell system (lines 11–13), and hence tests for the requirement of C5 could not be performed.

Failure of Action of HLO to be Blocked by Homologous Capsular Polysaccharide.—The addition of a large excess of type 1 capsular polysaccharide to

the phagocytic test medium (100 μg/ml of undiluted NRS) had no demonstrable effect upon the action of HLO on Pn1. This finding suggests that anticapsular antibody is not involved in the opsonic activity of the normal serum.

TABLE XIV

Effect of Divalent Cations on Adsorption of HLO from NRS with Pn25

Suspending medium	Phagocytosis
	%
NRS 1:5	84
NRS 1:5 adsorbed with Pn25*	<1
NRS 1:5 + EDTA (1.2 mM) adsorbed with Pn25* and then supplemented with $CaCl_2$ (1.2 mM)‡	92

* Heat killed (100°C for 5 min).

‡ To restore Ca^{++} concentration needed for optimal phagocytosis. pH of NRS 1:5 was not significantly affected by acid from $CaCl_2$–EDTA reaction; see ‡, table XII.

TABLE XV

Properties of HLO to Pn1

Suspending medium	Phagocytosis*
	%
HBG	3
BSS	3
NRS	35
NRS 1:2	17
NRS 1:4	8
NRS 1:8	3
Heated NRS (56°C for 5 min)	1.5
Heated NRS (56°C for 30 min)	3
Zymosan treated serum‡	5
Venom treated serum‡	6
Normal guinea pig serum	3
Normal rabbit serum	2
Normal mouse serum	2
NRS decomplemented with immune aggregate‡	4
NRS + EDTA (8.0 mM)	1
NRS + EDTA (8.0 mM) + $CaCl_2$ (12.4 mM)	26

* Concentrated phagocytic system; see Methods.

‡ C titer <10 CH_{50} units. Inactivation procedure as in Table VII.

Presence of HLO in Normal Serum from Germfree Rats.—Phagocytic tests done in the concentrated phagocytic system (see Methods) with type 1 pneumococci and undiluted serum revealed that the HLO activity of serum from germfree rats[13] was the same as that of serum from the conventional strain of

[13] Kindly supplied by Dr. Albert Einheber, Walter Reed Army Institute of Research, Washington, D. C.

(naturally infected) rats used in these studies. The complement titers were likewise comparable.

DISCUSSION

The confusion that exists regarding the relation of complement (C) to phagocytosis has recently been reviewed by Boyden, North, and Faulkner (13). Most of the conflicting data can be traced to differences in the conditions of the phagocytic experiments and in the methods used to inactivate the various components of the C system. The present studies of the HLO to *Diplococcus pneumoniae* were initiated in an attempt to define more precisely the putative role of C in antibacterial defense.

Pneumococcus was selected as the test organism because its pathogenicity is relatively well understood (29). Its virulence is known to depend primarily, if not solely, on the antiphagocytic properties of its capsule; once ingested by either granulocytes or monocytes, it is promptly destroyed. At least two different mechanisms of phagocytosis are involved in its destruction within the tissues of the host: (*a*) *surface phagocytosis*, which operates in the preantibody[14] phase of the infection and may occur in the total absence of opsonin; and (*b*) *immune phagocytosis*, which occurs late in the infection when sufficient specific anticapsular antibody has accumulated to react with the capsules of the invading organisms and thus opsonize them. The first of these mechanisms is quantitatively less efficient than the second (30). Since HLO are present in normal mammalian serum (1–15), they too may play a role in the preantibody phase of pneumococcal and other acute bacterial infections by increasing the efficiency of early phagocytosis through their action as nonspecific opsonins.

The results of these studies indicate that the HLO to pneumococcus in normal rat and normal mouse serum involve components of the C system. This conclusion is based on the demonstration that the HLO: (*a*) have approximately the same heat lability as C, (*b*) are active at 37°C but not at 0°C, (*c*) are inactivated proportionately to hemolytic C by the addition of immune aggregates to the serum, (*d*) are adsorbed from serum nonspecifically by bacteria at 37°C, but not 0°C, and (*e*) are Ca^{++}- and/or Mg^{++}-dependent in their action.

Clearly the requirement for divalent cations is a crucial link in the evidence that HLO to pneumococcus are related to C. The failure of Hirsch and Strauss (14) to demonstrate this requirement for the action of HLO to *Staphylococcus aureus* in normal rabbit serum is not apparent from the present studies. It should be emphasized, however, that the conditions of their experiments were very different from ours. Not only were the organisms and sera dissimilar, but the methods of measuring phagocytosis were not comparable. In the technique used by Hirsch and Strauss the concentration of leukocytes in the phagocytic tests were only 10–20 $\times$ 10^6 per ml, as compared to about 20 $\times$ 10^7

[14] Acquired anticapsular antibody.

(dilute system) and 20 × 10^8 (concentrated system) in the present studies. Furthermore, the ratio of bacteria to cells was 1:8 as compared to 5:1 and 8:1 (20).

The relation of divalent cations to phagocytosis is not simple. For example, Ca^{++} may itself act as an opsonin for some organisms, like PnR, and not for others, like Pn1 and Pn25, depending upon their surface constituents (cf. BSS, Tables VIII and XI). Also, the complete absence of Ca^{++} in the medium may depress the general phagocytic activity of the leukocytes (14, 31). Obviously, such factors must be fully considered in investigating divalent cation requirements.[15]

Although it is clear that HLO to pneumococci are not conventional antibodies, it is possible that they involve the participation of "natural" antibodies which initiate the fixation of C and thereby act indirectly as opsonins. In fact, there is evidence that such a combined action of C and natural antibodies is required for the optimal in vitro phagocytosis of starch granules (6), erythrocytes (32), *Staphylococcus aureus*, and *Shigella sonnei* (33) in normal guinea pig serum.

If natural antibodies are involved in the action of HLO to pneumococci, they are probably directed against antigens in the cell wall rather than the capsule, since homologous capsular polysaccharide does not inhibit the phagocytic reaction. But there is as yet no proof that cell wall antibodies participate in the opsonizing process. In fact, the failure of both PnR (Table IX) and Pn25 (Table XIV) to adsorb detectable amounts of HLO from NRS in the presence of EDTA (to prevent fixation of C), and the finding that the serum from germfree rats contains just as much HLO to Pn1 as the serum from "normal" rats suggest that the critical C component(s) may somehow become fixed to the organisms without the participation of antibody. Neither of these arguments, however, is compelling, since complete adsorption of cell wall antibodies may be difficult to achieve, and antibodies that cross-react with bacteria may be present in the sera of axenic animals. Nevertheless, it is of interest that Šterzl (34) has reported that complement opsonizes S-phase strains of *E. coli* in newborn piglet sera which contain no demonstrable antibodies to the organisms. The possibility, therefore, cannot be excluded that C3 may become activated by some alternative pathway that does not involve the participation of antibodies.

Collectively, the results of the present experiments suggest the involvement of C1, C4, C2, and C3 in the opsonization of pneumococci by normal serum. Inactivation of HLO by purified cobra venom factor, however, which in appropriate doses inactivated C3–C9 without affecting C1, C4, and C2, clearly

[15] For example, it should be noted that the phagocytic tests summarized in Table XIII were done in BSS to provide the cells with optimal Ca^{++} for phagocytosis. In the analogous tests summarized in Table X, on the other hand, BSS could not be used because of the opsonizing action of Ca^{++} on PnR (Table XI).

indicates that C3 and/or one or more of the later acting components (C5–C9) play a critical role in the opsonization process (16).

SUMMARY

Heat labile opsonins (HLO) in normal rat serum to both encapsulated and unencapsulated pneumococci (*a*) have the same heat lability as complement (C); (*b*) are active at 37°C but not at 0°C; (*c*) are inactivated proportionately to hemolytic C by the addition of immune aggregates to the serum; (*d*) are adsorbed from serum nonspecifically by bacteria at 37°C but not at 0°C; (*e*) are Ca^{++}- and/or Mg^{++}-dependent in their action; and (*f*) are inactivated by zymosan and a purified cobra venom factor, and in the case of encapsulated pneumococci, at least, by NH_4OH. Like other opsonins, HLO to pneumococci act primarily on the bacteria rather than on the phagocytes. Their combined properties indicate that they involve multiple components of the hemolytic C system.

Since HLO are immunologically polyspecific, they presumably play a broad protective role in the early (preantibody) phase of acute bacterial infections.

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