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Research Article

The phagocytic, bactericidal, and metabolic capabilities of circulating blood leukocytes from three adults (two males, one female) with hypogammaglobulinemia and recurrent pneumonia, chronic sinusitis, and intestinal giardiasis were studied. These functions were found to be normal when leukocytes from the patients were incubated in media containing normal human serum. Phagocytosis of *Staphylococcus albus* and polystyrene balls by both patient and normal leukocytes was diminished when the cells were incubated in hypogammaglobulinemic plasma. A similar defect in opsonization by patient plasma was also noted for pneumococci, *Escherichia coli* and variably with *Staphylococcus aureus*. Both patient and normal sera had equivalent levels of heat-labile *S. albus* opsonins; normal serum, however, contained heat-stable *S. albus*-specific absorbable opsonins in significantly greater quantities to account for its superior opsonic capacity. The addition of commercial gamma globulin or purified IgG to hypogammaglobulinemic sera restored full *S. albus* opsonic activity. The relevancy of these observations to the impaired host defenses in these patients will be discussed.

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Leukocytic Function in Hypogammaglobulinemia

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ABSTRACT The phagocytic, bactericidal, and metabolic capabilities of circulating blood leukocytes from three adults (two males, one female) with hypogammaglobulinemia and recurrent pneumonia, chronic sinusitis, and intestinal giardiasis were studied. These functions were found to be normal when leukocytes from the patients were incubated in media containing normal human serum. Phagocytosis of *Staphylococcus albus* and polystyrene balls by both patient and normal leukocytes was diminished when the cells were incubated in hypogammaglobulinemic plasma. A similar defect in opsonization by patient plasma was also noted for pneumococci, *Escherichia coli* and variably with *Staphylococcus aureus*. Both patient and normal sera had equivalent levels of heat-labile *S. albus* opsonins; normal serum, however, contained heat-stable *S. albus*-specific absorbable opsonins in significantly greater quantities to account for its superior opsonic capacity. The addition of commercial gamma globulin or purified IgG to hypogammaglobulinemic sera restored full *S. albus* opsonic activity. The relevancy of these observations to the impaired host defenses in these patients will be discussed.

INTRODUCTION

Repeated pyogenic infections, most frequently involving the respiratory tract, characterize the clinical course of patients with acquired hypogammaglobulinemia. Although this predilection to infection might be presumed to be on the basis of impaired antibody formation (1), it has been established that during the initial exposure to an organism, phagocytosis of the invading bacteria is the major host defense mechanism (2). Phagocytosis is facilitated by both heat-labile (3) and heat-stable serum opsonins (4). It has been reported that patients with Swiss type agammaglobulinemia have decreased phagocytic function (5, 6), but some of these patients are also complement deficient (7, 8). On the other hand, there have been no reports of systematic studies of phagocytic function in other types of hypogammaglobulinemia beyond several case reports in which leukocyte function appeared to be normal (9, 10). In the present investi-

gations, three adult patients with acquired hypogammaglobulinemia were studied to determine the phagocytic, bactericidal, and metabolic properties of their peripheral leukocytes, with particular attention to the influence of homologous or normal serum upon these functions. The results indicate that phagocytic function is impaired in the hypogammaglobulinemic state, and that the defect lies, not in the cells themselves, but rather in a lack of heat-stable serum opsonins in affected patients.

METHODS

Patients. All patients were hospitalized at the National Institutes of Health during the period of study. Pertinent clinical data concerning the patients are presented in Table I.

Leukocytes. Leukocytes were obtained by sedimenting heparinized¹ peripheral blood with 3% dextran (mol wt 124,000). The leukocyte-rich supernatant plasma was aspirated and centrifuged at 900 rpm for 6 min at 4°C. The leukocytes were then washed twice in modified Hanks' solution (MHS),² and cells were counted in an electronic particle counter.³ Differential counts of Wright's stained smears were performed and the percentage of phagocytic cells (granulocytes and monocytes) enumerated. Occasionally, patients were plasmaphoresed and the blood was centrifuged at 2500 rpm for 3 min. The buffy coat and homologous plasma were then aspirated, mixed, and the erythrocytes allowed to sediment at 37°C for 1 hr. The resulting leukocyte-rich plasma supernatant was centrifuged. The cells were washed twice in MHS and counted as described above. Normal leukocytes were obtained in a similar manner from blood anticoagulated with acid-citrate-dextrose (ACD).

Serum and plasma. Fresh serum was obtained from normal donors by centrifugation of whole clotted blood. The serum was stored at -70°C until used. Plasma was obtained from anticoagulated blood (10 U/ml heparin) from both patients and normal volunteers. It was stored at -70°C and before use was thawed and centrifuged at 3000 rpm for 20 min at 4°C to remove fibrin. Heat inactivation of serum and plasma was carried out at 56°C for 30 min.

Serum and heparinized plasma gave identical results and were used interchangeably. Citrated plasma was not used because the citrate ion which is known to inhibit several glycolytic enzymes (11) depressed hexose monophosphate shunt activities. For heat inactivation studies, only normal serum was used since heat-inactivated normal plasma in-

¹ 10 μ /ml Pan Heparin, Abbott Laboratories, Baltimore, Md.

² K_2HPO_4 , 1.568 g/liter; NaCl, 8.12 g/liter; glucose, 2000 mg/liter.

³ Coulter Electronics, Industrial Div., Hialeah, Fla.

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TABLE I
Clinical Data of Patients

Patient	Age	Sex	Age at onset of symptoms	Patient characteristics	Family history	Immunoglobulin*			Positive skin tests	Isohem-agglutinin titer	Serum complement	Clinical response to gamma globulin administration
						IgG	IgA	IgM				
R.S.	23	M	18	Intestinal giardiasis; frequent otitis media, pneumonia and sinusitis; mal-adsorption syndrome and nodular lymphoid hyperplasia of bowel†	No	0.7 1.0‡	0 0	0 0	Mumps and DNCB	Anti-A neg Anti-B neg	Normal	Good
G. W.	42	F	28	Intestinal giardiasis; bronchiectasis requiring prophylactic antibiotics and previous lobectomy; chronic sinusitis	Yes (nephew)	0.4 0.4‡	0 0	0 0	DNCB	Anti-A 1:1 Anti-B 1:1	Normal	Questionable
H. R.	36	M	20	Intestinal giardiasis; frequent otitis media and pneumonias	No	1.2‡	0	0	Tuberculin and histoplasmin	Anti-A neg Anti-B neg	Not done	Good

* Normal levels for the laboratory are: IgG 8.5 mg/ml (4.3–13.9), IgA 1.8 (0.8–4.7), IgM 1.3 (0.5–4.0).

† Radiological findings reported elsewhere (12).

‡ 1 wk following gamma globulin intramuscularly.

|| Denotes ability of patient to develop delayed hypersensitivity to dinitrochlorobenzene after sensitization. The method of sensitization has been described elsewhere (13).

creased hexose monophosphate shunt levels. On the other hand, phagocytic mixtures with heat-inactivated hypogammaglobulinemic plasma and sera gave identical hexose monophosphate shunt activities.

Phagocytic particles. Cultures of *Staphylococcus albus*, *Staphylococcus aureus*, and *Escherichia coli* isolated from patients were maintained in semisolid media. A strain of pneumococcus Type 25 was obtained from the Department of Preventative Medicine, Seattle, Wash., and stored in defibrinated rabbit blood under petrolatum jelly at -20°C until used. Before use, a loopful of bacteria was inoculated into trypticase soy broth (TSB) and cultured for 16 hr. Pneumococci were cultured in Todd-Hewitt broth for 16 hr and then subcultured for 4 hr. The bacteria were centrifuged at 2000 rpm for 10 min and washed once with MHS. When dead *S. albus* were required, the washed organisms were boiled for 45 min. The bacterial concentration was estimated turbidometrically at 650 $m\mu$ in a spectrophotometer (Coleman Junior) by comparison to a standard curve previously prepared by plating out serial dilutions of bacterial suspensions.

When indicated, *S. albus* were preopsonized by incubating 2×10^8 bacteria with 1 ml of serum at 37°C for 1 hr with agitation. The bacteria were resuspended in appropriate media at a concentration of 2×10^8 bacteria per ml.

Polystryrene balls⁴ were diluted 1:10 in Hanks' balanced salt solution (HBSS) which gave an approximate concentration of 2×10^8 balls per ml.

Measurements of phagocytosis. Serum or plasma, 10×10^6 phagocytic cells (PWBC), and 2×10^8 heat-killed *S. albus* (PWBC:bacteria ratio of 1:20) were added to

MHS or HBSS to bring the total volume to 2 ml. In these studies, the final concentration of serum or plasma was 10%. After 1 hr incubation at 37°C in a shaker incubator, the mixtures were spun at 750 rpm for 5 min. The cells were resuspended in serum and slides made and stained with Wright's stain. 100–200 cells were counted and the per cent of cells containing bacteria determined. Vacuolation and degranulation were also noted.

Opsonic titers. Whole serum opsonic titers were determined for *S. albus*, *S. aureus*, and pneumococcus Type 25 by twofold dilutions of patient and normal sera in HBSS. 5×10^6 PWBC and 5×10^8 bacteria (ratio PWBC:bacteria = 1:100) were suspended in 1 ml of the serum-HBSS mixtures and tumbled at 37°C for 30 min (pneumococci) or 60 min (*S. albus* and *S. aureus*). The opsonic titer was defined as the greatest dilution of serum which permitted ingestion of one or more organisms by 90% or more of the cells.

Leukocyte bactericidal assay. To assess bacterial killing by leukocytes as well as phagocytosis, a modification of the bactericidal assay described by Cohn and Morse (14) was employed. For each determination, four suspensions consisting of 10% serum, 10×10^6 PWBC, approximately 10×10^6 live bacteria, and enough MHS or HBSS to bring the volume to 2 ml were made. At 0, 30, 60, and 120 min, aliquots were obtained from the suspensions and tenfold serial dilutions in $\frac{1}{2}$ N saline were made. The appropriate dilutions were plated in trypticase soy agar, and the following day colony counts were performed to determine the total number of viable bacteria remaining at each time interval. The removal of extracellular bacteria by phagocytosis was also determined by centrifuging the mixtures at 100 g for 5 min at 4°C and plating serial dilutions of the supernatant fluid.

⁴ 1.099 μ , Dow Chemical Co., Midland, Mich.

Control bacterial cultures without WBC were made at 0, 60, and 120 min to rule out extracellular bactericidal activity.

The procedure was modified in later experiments by inoculating 10^{-8} -ml aliquots of the bacteria-white blood cell suspension with a calibrated platinum loop⁶ into 10 ml of distilled water at 0, 60, and 120 min. 1 ml of the distilled water-bacteria suspension was then plated in trypticase soy agar. When pneumococci were used, 1 ml of defibrinated rabbit blood was added to the trypticase soy agar. The resultant colonies represented the total bacteria remaining.

Measurement of phagocytosis by leukocyte hexose monophosphate shunt activity. Another measurement of phagocytosis employed was the assay of hexose monophosphate shunt activity as described by Skeel, Yankee, Spivak, Novilous, and Henderson (15). Suspensions consisting of 1 ml of 10×10^6 PWBC in HBSS, 1 ml of serum or plasma, 0.5 ml of $1 \mu\text{Ci/ml}$ glucose-1- ^{14}C ,⁸ 0.5 ml of phagocytic particles (i.e. PSB or heat-killed *S. albus*, both at a concentration of 2×10^9 , to attain a PWBC:particle ratio of 1:100), and enough HBSS to bring the volume to 3.5 ml were placed in a 25 ml Erlenmeyer flask. The flask was covered with a rubber cap⁷ from which a polyethylene centerwell⁷ was suspended. 0.2 ml of 10% KOH (made weekly) was injected with a tuberculin syringe through the cap into the centerwell. The flask was shaken at 60 oscillations per min in a shaker (Dubnoff) for 1 hr at 37°C after which 0.5 ml of 1 N HCl was injected into the mixture to stop the reaction and release CO_2 . The polyethylene well containing the labeled CO_2 trapped in the KOH was placed in a counting vial containing 20 ml of a scintillation cocktail.⁸ The samples were then counted in a liquid scintillation counter,⁹ with at least a 50% counting efficiency and at a standard deviation of less than 2.5%. The counts per minute were converted to disintegrations per minute from an appropriate quench curve. Studies were usually done in triplicate.

Gamma globulin. Gamma globulin at a concentration of 165 ± 15 mg/ml was obtained from commercial sources.¹⁰ Purified IgG was obtained by dialyzing gamma globulin in distilled water, redialyzing it in 0.02 M Tris buffer, pH 8.5, and then chromatographing it on a diethylaminoethyl cellulose column. The first peak eluted was shown by immunoelectrophoresis to contain only IgG. The protein concentration of this fraction was determined at 280 m μ in a spectrophotometer. The IgG was then dialyzed and equilibrated with MHS.

Absorption of opsonins by *S. Albus*. Specific opsonic activity for *S. albus* was removed from heat-inactivated sera by repeated absorptions with 1×10^{10} heat-killed organisms per ml of serum at 4°C for at least 4 hr. After each absorption, the bacteria were removed by centrifugation at 3500 rpm at 4°C for 1 hr. An identical absorption procedure was used with heat-killed *E. coli*.

RESULTS

Phagocytosis. The white blood cells from both normal and hypogammaglobulinemic individuals displayed de-

⁶ Arthur H. Thomas Co., Philadelphia, Pa.

⁸ Glucose-1- ^{14}C , New England Nuclear Corp., Boston, Mass. SA 0.035, lot No. 292141.

⁷ Kontes Glass Co., Vineland, N. J.

⁹ 40 ml Liquifluor, New England Nuclear Corp., 660 ml toluene, and 300 ml methanol.

⁹ Packard 3375 Tri-Carb (Packard Instrument Co., Inc., Downers Grove, Ill.).

¹⁰ Hyland Div., Travenol Labs., Inc., Los Angeles, Calif.

TABLE II
Effect of Hypogammaglobulinemic Sera on
Phagocytosis of *S. albus**

Patient	Serum	Phagocytosis by leukocytes	
		Patient cells	Normal cells
		%	%
G. W.	Patient	18	43
	Control	67	80
R. S.	Patient	54	50
	Control	99	87
H. R.	Patient	73	85
	Control	88	91
Mean	Patient	48	59
	Control	85	86

* Results expressed as per cent of 200 phagocytic cells which contained one or more organisms. See text.

creased phagocytosis of *S. albus* when incubated in hypogammaglobulinemic sera. When leukocytes from either hypogammaglobulinemic patients or normal donors were incubated with bacteria in normal sera, the per cent of cells that phagocytized *S. albus* was similar (Table II) suggesting that the impaired phagocytosis was due to an opsonic defect of hypogammaglobulinemic serum rather than a cellular abnormality. Vacuolation and degranulation of both types of cells were comparable after phagocytosis.

The ability of hypogammaglobulinemic serum to support phagocytosis of *S. albus*, *S. aureus*, and pneumococcus Type 25 was expressed quantitatively as an opsonic titer (Table III). Each of the patient's serum had decreased opsonic titers for all of these organisms.

TABLE III
Opsonic Titers for *S. albus*, *S. aureus*, and *Pneumococcus*
Type 25 of Hypogammaglobulinemic and Normal Sera

Bacteria	Opsonic titer*			
	Normal sera	Hypogammaglobulinemic sera		
		R. S.	G. W.	H. R.
<i>S. albus</i>	64†	4	2	4
<i>S. aureus</i>	128	32	32	32
Pneumococcus Type 25	6§	4	Undil.	<Undil.

Undil. = undiluted.

* Greatest dilution of serum in which 90% or more cells exhibited phagocytosis (see text).

† Numbers are reciprocal of titer.

§ Average of two determinations with titers of 1:4 and 1:8.

Bactericidal assay. Consistent with the above findings, significantly more extracellular and therefore, total bacteria, remained after leukocytes were incubated with *S. albus* in hypogammaglobulinemic plasma than if incubated in normal plasma (Fig. 1). Both patient and normal cells displayed equal phagocytosis and killing of organisms when incubated with normal plasma (Fig. 2), indicating that intracellular killing was unimpaired in hypogammaglobulinemia. Since both normal and patient cells functioned similarly, the results using both cell types were pooled to compare the opsonic capabilities of normal and hypogammaglobulinemic plasma.

For comparison with the results using MHS, the bactericidal assays were repeated in HBSS which contains calcium and magnesium ions in physiologic concentrations. This was done to determine if repletion of these ions had a similar enhancing effect on phagocytosis in normal and hypogammaglobulinemic plasma. The results given in Table IV show that calcium and magnesium ions improved bactericidal activity of leukocytes in both normal and hypogammaglobulinemic plasma to a similar degree, yet the opsonic defect of the patient plasma remained.

Observations on the bactericidal function of leukocytes in hypogammaglobulinemic plasma were extended using *S. aureus*, pneumococcus Type 25, and *E. coli*. Similar to the findings with *S. albus*, a marked defect

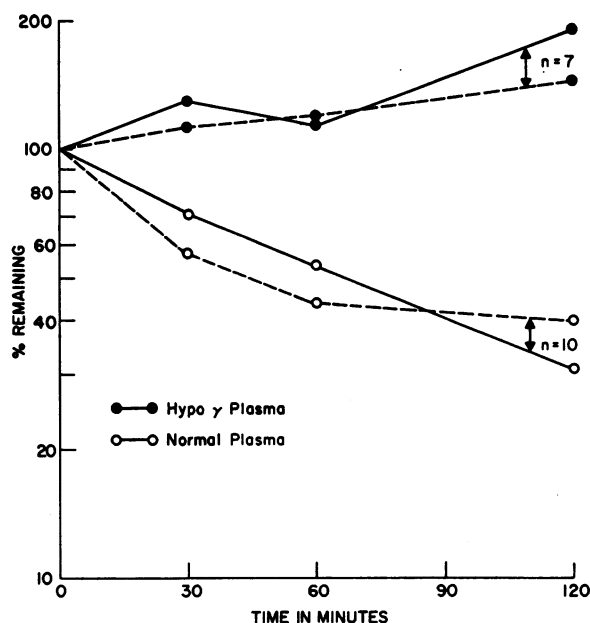


FIGURE 1 Leukocytes and *S. albus* at approximately a 1:1 ratio were incubated in 10% hypogammaglobulinemic plasma (closed circles) or normal plasma (open circles) in MHS. Solid lines represent the mean per cent of total bacteria remaining. Broken lines represent the mean per cent of extracellular bacteria remaining. n = the number of determinations.

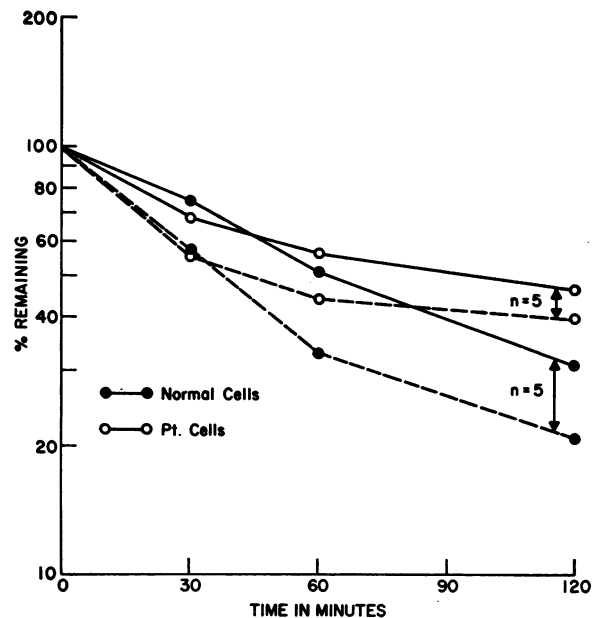


FIGURE 2 Leukocytes from patients with hypogammaglobulinemia (open circles) or normal donors (closed circles) incubated with *S. albus* and 10% normal plasma in MHS. Solid lines represent the mean per cent of total bacteria remaining. Broken lines represent the mean per cent of extracellular bacteria remaining. n = the number of determinations.

in opsonization of *E. coli* and pneumococcus was noted in plasma from all of the hypogammaglobulinemic patients. The mean per cents of *E. coli* and pneumococcus remaining at 1 hr in mixtures using patients' plasma were 76% and 49%, respectively, compared to 0.9% and 5.5% in mixtures using normal sera. In contrast, only one out of the three plasma samples tested displayed decreased opsonic activity for *S. aureus*. In bactericidal mixtures with patients' plasma, 45, 8, and 2% of the total *S. aureus* remained, whereas 3% *S. aureus* re-

TABLE IV
Effect of Divalent Cations on Leukocyte Bactericidal Activity of *S. albus*

Medium	Plasma	Mean survival (% \pm SE)	
		1 hr	2 hr
MHS*	Normal†	54 \pm 10	39 \pm 7
	Hypo γ §	114 \pm 18	189 \pm 21
HBSS	Normal	6 \pm 4	4 \pm 3
	Hypo γ	37 \pm 14	33 \pm 17

* Modified Hanks' solution (no divalent cations present).

† Plasma from normal donors.

§ Plasma from hypogammaglobulinemic patients.

|| Hanks' balanced salt solution (divalent cations present). See text.

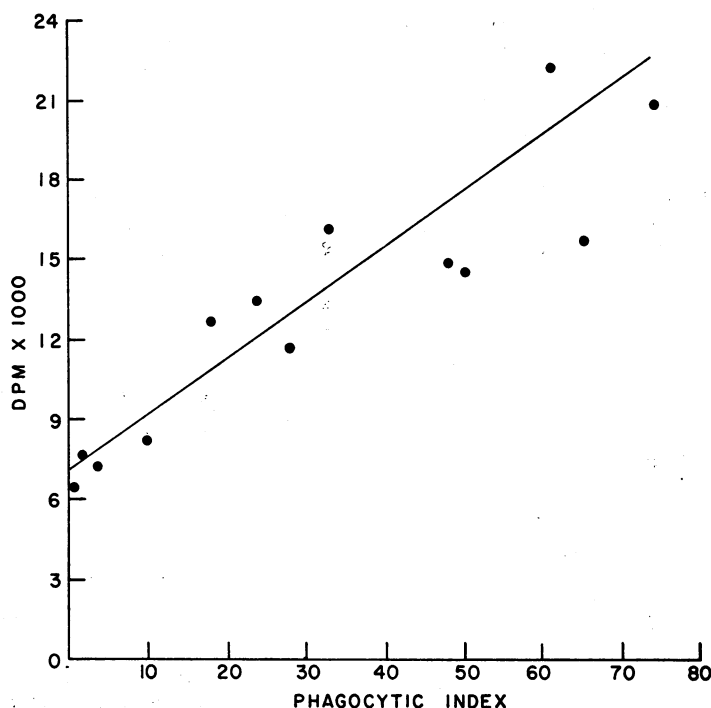


FIGURE 3 Relationship between HMS activity as determined by the release of $^{14}\text{CO}_2$ from glucose-1- ^{14}C (disintegrations per minute) and phagocytic index (particles per 100 cells) of leukocytes incubated with polystyrene balls for 60 min in 28.5% normal serum and HBSS. HMS activity is expressed as the mean of triplicate samples and phagocytic index was determined on a fourth identical sample. When balls filled the cytoplasm but not the nucleus of a cell an estimate of 50 intracellular balls was made; when the nucleus of the cell was also obscured by balls an estimate of 100 intracellular balls was made.

mained in mixtures using normal serum. Having thus demonstrated that the serum of hypogammaglobulinemic patients lacks opsonins for a variety of bacteria, detailed studies using *S. albus* as the phagocytic organism were undertaken to further characterize the nature of the defect.

Hexose monophosphate shunt activity. By varying the ratio of polystyrene balls to cells, a standard curve was constructed to depict the relationship between hexose monophosphate shunt activity (HMS) and phagocytic index (number of particles per cell). A direct relationship was demonstrated (Fig. 3) which confirms previous work (15). This relationship was consistent on a day-to-day basis provided the HMS activity of resting mixtures was less than 8000 dpm. Therefore, all data used represent incubation mixtures whose resting HMS activities were less than 8000 dpm.

Although there was considerable stimulation of HMS activity above resting levels with phagocytosis of either *S. albus* or PSB in hypogammaglobulinemic plasma, it was significantly less than when normal sera was used in the incubation medium (Table V). Since there was

day-to-day variation of the HMS activities using various sera and because the difference between paired cultures of normal serum and hypogammaglobulinemic plasma remained relatively constant, a paired-sample *t* test was used to evaluate statistical significance whenever appropriate. As expected, there was no difference in HMS activities when comparing resting and phagocytizing normal and hypogammaglobulinemic cells provided they were incubated in the same serum or plasma (Table VI). Therefore, the results obtained for both types of cells were combined to compare the opsonic activities of normal and patient sera. Of peripheral interest was the finding that the HMS activity was significantly increased when PSB's were phagocytized in normal serum rather than no serum at all (Table V).

Effect of gamma globulin on phagocytosis. To see if the reduced phagocytosis in hypogammaglobulinemic plasma was improved by replacement gamma globulin therapy, incubation mixtures were made using plasma obtained 1 week after 20 ml of parenteral gamma globulin to patients R. S. and G. W. Using either PSB or *S. albus* as the phagocytic particles (Table VII), the

TABLE V

Effect of Hypogammaglobulinemic Plasma on Phagocytosis as Measured by Hexose Monophosphate Shunt Activity*

Serum or plasma	Phagocytic particle	$\overline{\text{dpm}}\ddagger$	SE	n§	P
Hypo γ	<i>S. albus</i>	15,923	891¶	9	<0.05**
Normal	<i>S. albus</i>	18,218			
Hypo γ	PSB††	14,994	787¶	9	<0.01**
Normal	PSB	17,945			
Hypo γ	None	3,906	371§§	17	>0.05
Normal	None	4,024	458§§	16	
None	PSB	13,418	2,100¶	6	<0.05**
Normal	PSB	19,415			

*Hypogammaglobulinemic and normal cells were used and the results in each category combined. See text.

‡ Mean value of disintegrations per minute.

§ Number of determinations. Each determination done in triplicate.

|| Hypo γ = plasma from hypogammaglobulinemic patients.

¶ Standard error of the mean difference.

** Paired sample *t* test.

†† PSB = polystyrene balls.

§§ Standard error of the mean.

||| Two sample *t* test.

HMS activity increased only slightly when plasma obtained 1 wk after parenteral gamma globulin was used in the incubation mixtures. It should be noted that there was only a minimal increase in serum IgG levels following parenteral gamma globulin (Table I). On the other

TABLE VI

Comparison of Phagocytosis by Cells from Normal and Hypogammaglobulinemic Patients as Measured by Hexose Monophosphate Shunt Activity

Cells	Serum or plasma	Phagocytic particle	$\overline{\text{dpm}}^*$	n‡	P§
Hypo γ	Hypo γ ¶	<i>S. albus</i>	15,975 \pm 2407	4	>0.05
Normal	Hypo γ	<i>S. albus</i>	14,373 \pm 1375	8	
Hypo γ	Normal	<i>S. albus</i>	17,203 \pm 3367	3	>0.05
Normal	Normal	<i>S. albus</i>	18,726 \pm 2104	6	
Hypo γ	Hypo γ	PSB	13,408 \pm 2431	5	>0.05
Normal	Hypo γ	PSB	15,896 \pm 2144	5	
Hypo γ	Normal	PSB	17,040 \pm 2102	4	>0.05
Normal	Normal	PSB	18,607 \pm 2030	5	
Hypo γ	Hypo γ	None	3,848 \pm 698	7	>0.05
Normal	Hypo γ	None	4,333 \pm 534	10	
Hypo γ	Normal	None	3,301 \pm 494	4	>0.05
Normal	Normal	None	4,265 \pm 582	12	

* Mean disintegrations per minute \pm SEM.

‡ Number of determinations. Each determination done in triplicate.

§ Two sample *t* test.

|| Cells from patients with hypogammaglobulinemia.

¶ Plasma from patients with hypogammaglobulinemia.

TABLE VII

Effect of Parenterally Administered Gamma Globulin on Phagocytosis as Measured by Hexose Monophosphate Shunt Activity*

Time since last γ -globulin‡	Serum or plasma	Phagocytic particle	$\overline{\text{dpm}}\S$	n	P¶
4 wk	Hypo γ **	<i>S. albus</i>	17,574 \pm 1909	9	>0.05
1	Hypo γ	<i>S. albus</i>	18,893 \pm 1415	10	
4	Hypo γ	PSB††	12,200 \pm 626	5	>0.05
1	Hypo γ	PSB	14,550 \pm 1610	5	

* Results with normal and hypogammaglobulinemic cells in each category were combined. See text.

‡ 20 ml gamma globulin intramuscularly.

§ Mean disintegrations per minute \pm SEM.

|| No. of single determinations.

¶ Two sample *t* test.

** Hypo γ = plasma from patients with hypogammaglobulinemia.

†† PSB = polystyrene balls.

hand, if gamma globulin was added to plasma from hypogammaglobulinemic patients at a concentration of 9 mg/ml, the HMS activity with *S. albus* and PSB significantly improved (Table VIII) and was equivalent to values obtained with normal serum. Addition of 9 mg/ml gamma globulin to normal serum did not enhance HMS activity. As can be seen in Fig. 4, increasing amounts of gamma globulin up to a concentration of 2 mg/ml, when added to mixtures of leukocytes and *S. albus*, progressively increased HMS activity of phagocytizing cells. It might be concluded then, that the system was not sufficiently sensitive to detect the slight increments in IgG levels which followed parenteral administration. Gamma globulin at a final concentration of

TABLE VIII

Effect of Gamma Globulin Added In Vitro on Phagocytosis as Measured by Hexose Monophosphate Shunt Activity*

Gamma globulin‡	Serum or plasma	Phagocytic particle	$\overline{\text{dpm}}\S$	n	P¶
No	Hypo γ **	<i>S. albus</i>	13,258 \pm 801	5	<0.01
Yes	Hypo γ	<i>S. albus</i>	17,611 \pm 530	6	
No	Hypo γ	PSB††	20,065 \pm 398	3	<0.05
Yes	Hypo γ	PSB	24,504 \pm 1079	3	
No	Normal	<i>S. albus</i>	17,966 \pm 699	5	>0.05
Yes	Normal	<i>S. albus</i>	16,269 \pm 1202	6	
No	Normal	PSB	23,109 \pm 911	3	>0.05
Yes	Normal	PSB	23,818 \pm 2450	3	

* Results with normal and hypogammaglobulinemic cells in each category were combined. See text.

‡ Gamma globulin added in vitro at a concentration of 9 mg/ml.

§ Mean disintegrations per minute \pm SEM.

|| No. of single determinations.

¶ Two sample *t* test.

** Hypo γ = plasma from patients with hypogammaglobulinemia.

†† Polystyrene balls.

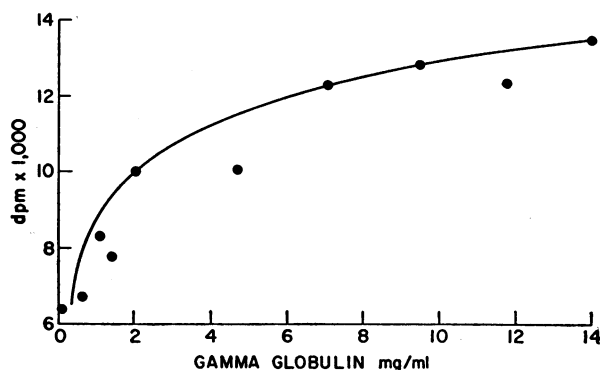


FIGURE 4 Relationship of gamma globulin concentration in vitro (mg/ml) and hexose monophosphate shunt activity ($\text{dpm} \times 10^3$ of C^{14}O_2 from glucose-1- ^{14}C) of leukocyte suspension phagocytizing *S. albus*.

8 mg/ml also restored full bactericidal activity to leukocytes suspended in hypogammaglobulinemic plasma (Fig. 5). Purified IgG (see Methods) at a concentration of 4 mg/ml was as effective as pooled gamma globulin in restoring hypogammaglobulinemic plasma opsonic activity to normal.

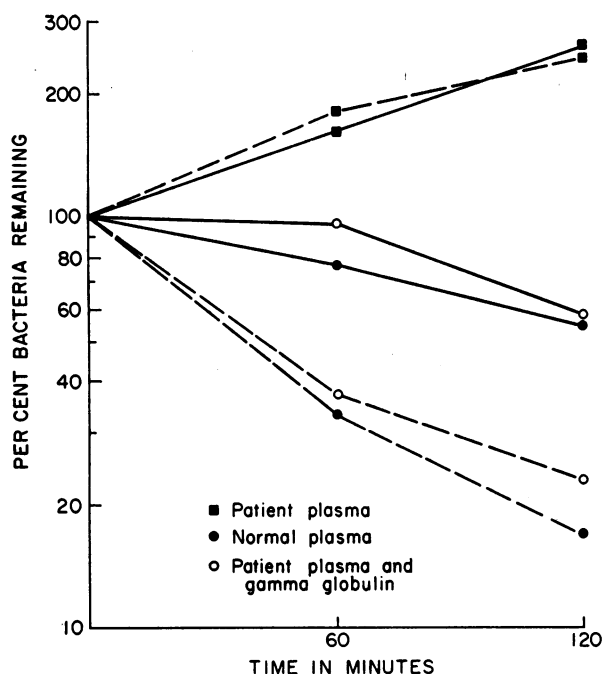


FIGURE 5 Effect of gamma globulin on bactericidal activity on hypogammaglobulinemic plasma. Leukocytes and *S. albus* were incubated in 10% hypogammaglobulinemic plasma (squares) or normal plasma (closed circles) in MHS. Gamma globulin (final concentration 8 mg/ml) was added to phagocytic mixtures of hypogammaglobulinemic plasma, leukocytes, and *S. albus* (open circles). Solid lines represent the percentage of total bacteria remaining. Broken lines represent the per cent of extracellular bacteria remaining.

TABLE IX

Effect of Heat Inactivation of Serum or Plasma on Phagocytosis as Measured by Hexose Monophosphate Shunt Activity*

Serum or plasma	Heat inactivation	Phagocytic particle	$\overline{\text{dpm}}\ddagger$	SE	n§	P
Hypo γ	No	<i>S. albus</i>	14,337	859¶	10	<0.001**
Hypo γ	Yes	<i>S. albus</i>	4,835			
Hypo γ	No	PSB‡‡	10,958	1248¶	6	>0.05**
Hypo γ	Yes	PSB	8,204			
Normal§§	No	<i>S. albus</i>	21,576	2468¶	4	<0.02**
Normal	Yes	<i>S. albus</i>	8,430			
Normal	No	PSB	23,109	911	3	>0.05¶¶
Normal	Yes	PSB	25,537	947	3	

* Results with normal and hypogammaglobulinemic cells in each category were combined. See text.

‡ Mean disintegrations per minute.

§ No. of determinations.

|| Hypo γ = plasma from patients with hypogammaglobulinemia.

¶ SEM difference.

** Paired sample *t* test.

‡‡ PSB = polystyrene balls.

§§ Normal = sera from normal patients.

||| SEM.

¶¶ Two sample *t* test.

Comparison of opsonins in normal and hypogammaglobulinemic plasma. Heat inactivation (56°C for 30 min) of both normal serum and hypogammaglobulinemic plasma significantly reduced phagocytosis of *S. albus* as measured by HMS activity (Table IX). The degree of reduction was similar for both the patient plasma and normal serum, suggesting that the content of heat-labile opsonins in both was similar. On the other hand, heat inactivation did not affect phagocytosis of polystyrene balls, so it would appear that all of the opsonins for polystyrene balls provided by serum are heat stable.

Comparison of hexose monophosphate shunt activity in phagocytic mixtures using pneumococcus Type 25, *S. aureus*, and *E. coli*, and heat-inactivated sera from normal and hypogammaglobulinemic patients shows a significant reduction in heat-stable opsonins for each of these organisms in hypogammaglobulinemic sera (Table X).

Adsorption of opsonins onto *S. albus*. To further study the relative contributions of heat-stable and heat-labile opsonins to phagocytosis of *S. albus*, washed organisms were preincubated with sera containing either primarily heat-labile opsonins (hypogammaglobulinemic serum), heat-stable opsonins (heat-inactivated normal serum), or both types of opsonins (normal serum). Following this, the *S. albus* were incubated with leukocytes in heat-inactivated hypogammaglobulinemic plasma which contains minimal amounts of opsonins and functioned primarily as a supporting medium. Control mixtures in these experiments consisted of washed cells incubated with washed *S. albus* in either normal serum,

TABLE X

Comparison of Opsonic Activity of Heat-Inactivated Sera for *Pneumococci*, *S. aureus*, and *E. coli* as Measured by Hexose Monophosphate Shunt Activity*

Serum or plasma	Bacteria	dpm \pm SE†	n‡	P
Hypo γ	<i>Pneumococcus</i> Type 25	4,112 \pm 191	15	<0.01
Normal	<i>Pneumococcus</i> Type 25	5,303 \pm 330	6	
Hypo γ	<i>S. aureus</i>	11,378 \pm 510	3	=0.01
Normal	<i>S. aureus</i>	14,224 \pm 917	3	
Hypo γ	<i>E. coli</i>	5,302 \pm 209	3	<0.01
Normal	<i>E. coli</i>	6,453 \pm 59	3	

* Mixtures consisted of 10% heat-inactivated serum, 0.5 μ Ci glucose-1-¹⁴C, 5×10^6 phagocytic cells, 5×10^8 heat-killed bacteria in a total volume of 2 ml.

† Mean disintegrations per min \pm SEM.

‡ No. of determinations.

|| Two sample *t* test.

hypogammaglobulinemic plasma, or their heat-inactivated equivalents. As noted in Table XI, preincubation of *S. albus* in the various sera restored HMS activities to their control levels suggesting that all types of serum opsonins, both heat labile and heat stable, can be absorbed onto the surface of the *S. albus*. Moreover, washing the *S. albus* after preincubation did not reduce the HMS activity, indicating that the opsonins are firmly bound to the cell surface.

Having shown that all of the opsonins are capable of binding to the bacterial cell surface, an attempt was made to fully deplete heat-inactivated serum of *S. albus* opsonins by exhaustive absorptions with bacteria. Hypogammaglobulinemic and normal sera were absorbed six times with heat-killed *S. albus* and as a control for specificity, with *E. coli*. The relative abilities of the absorbed sera to support phagocytosis of *S. albus* were then compared. Phagocytosis as measured by HMS activity was equivalent for leukocytes incubated in both normal and hypogammaglobulinemic heat-inactivated sera exhaustively absorbed with *S. albus*, and was significantly less than in *E. coli*-absorbed sera (Table XII). These results indicate that the differences in opsonic activity of normal and hypogammaglobulinemic sera following heat inactivation could be accounted for by their content of heat-stable opsonins which were apparently specific for *S. albus*.

To determine the relationship of these heat-stable opsonins to gamma globulin, 9 mg/ml of the commercial preparation was added to phagocytic mixtures incubated with heat-inactivated hypogammaglobulinemic plasma. The HMS activity of these mixtures was restored to that

TABLE XI

Effect on Phagocytosis of Preopsonization of *S. albus* in Various Sera as Measured by Hexose Monophosphate Shunt Activity*

Serum or plasma source	Heat inactivation	Control‡	Preopsonized <i>S. albus</i> §
Hypo γ	Yes	4,872 ¶	4,835
Hypo γ	No	18,721	19,620
Normal**	Yes	7,354	10,386
Normal	No	22,396	23,294

* Normal cells used.

† Washed *S. albus* incubated with sera or plasma listed under Serum or Plasma Source, and normal cells.

‡ *S. albus* preopsonized with sera or plasma listed under Serum or Plasma Source and incubated with normal cells in heat-inactivated plasma from hypogammaglobulinemic patients.

|| Hypo γ = plasma from hypogammaglobulinemic patients.

¶ Mean disintegrations per minute from six single determinations.

** Normal = sera from normal patients.

of phagocytic mixtures containing heat-inactivated normal sera (Table XIII).

DISCUSSION

Although the immunological reactivity of patients with hypogammaglobulinemia has been the subject of extensive investigation, another important parameter of host responsiveness to infection, that is phagocytosis, has received relatively little attention. In the present studies, phagocytic and bactericidal activity of leukocytes from patients with hypogammaglobulinemia as well as those of normal individuals were significantly impaired for several strains of bacteria when the incubation medium contained plasma or serum from hypogammaglobu-

TABLE XII

Ability of Heat-Inactivated Sera to Support Phagocytosis of *S. albus* as Measured by Hexose Monophosphate Shunt Activity following Absorption of the Sera with *S. albus* or *E. coli**

Serum source	Resting mixtures, sera absorbed with <i>S. albus</i> , dpm†	Phagocytic mixtures	
		Sera absorbed with <i>S. albus</i> , dpm	Sera absorbed with <i>E. coli</i> , dpm
Control	3092	8101	15,202
R. S.§	2907	7961	10,487
G. W.§	2691	8084	9,828
H. R.§	2566	6001	9,851

* Normal cells and washed *S. albus* used in all incubation mixtures. Sera absorbed six times with the bacteria as indicated.

† Mean disintegrations per minute for three determinations.

§ Sera from hypogammaglobulinemic patients.

TABLE XIII

*Effect on Phagocytosis of the Addition of Gamma Globulin to Heat-Inactivated Plasma from Patients with Hypogammaglobulinemia as Measured by Hexose Monophosphate Shunt Activity**

	Experiment 1		Experiment 2	
	dpm†	P‡	dpm†	P‡
Normal	9315 ± 364		14,469 ± 476	
Hypo γ¶	6065 ± 741		7085 ± 585	
Hypo γ and γ-globulin**	8494 ± 538	0.06	13,905 ± 317	<0.001

* Normal cells and *S. albus* used in all mixtures.

† Disintegrations per minute ± SEM.

‡ Two sample *t* test.

|| Normal = heat-inactivated sera from normal patients.

¶ Hypo γ = heat-inactivated plasma from hypogammaglobulinemic patients.

** Heat-inactivated plasma from hypogammaglobulinemic patients and gamma globulin (9 mg/ml) added in vitro.

linemic donors. These effects were most pronounced when *S. albus*, *E. coli*, or pneumococci were used, whereas only one of three hypogammaglobulinemic sera demonstrated impaired opsonic activity for *S. aureus*. The explanation for these differences is unknown. Reversal of the abnormality by incubation of patient or normal leukocytes in medium containing normal serum demonstrated that the hypogammaglobulinemic serum failed to provide adequate opsonization. Addition of calcium and magnesium ions improved bactericidal function of cells in both normal and hypogammaglobulinemic serum, although the relative differences in the effectiveness of the patients' and normal sera remained.

Since quantitation of phagocytosis by examination of smears is laborious and relatively inaccurate, a technique employing hexose monophosphate shunt activity was used to study the *S. albus* opsonins and the opsonic defect in hypogammaglobulinemic serum. Sbarra and Karnovsky (16) have demonstrated a sevenfold increase in the conversion of carbon 1-labeled glucose to CO₂ during phagocytosis by guinea pig exudate cells. This measurement has proved to be the most sensitive metabolic indicator of phagocytosis, and in previous studies (15), confirmed here, a direct correlation between increase in hexose monophosphate shunt activity and phagocytic index was observed. Since studies of patients with chronic granulomatous disease of childhood (17) have shown that hexose monophosphate shunt activity may be more relevant to the bactericidal events within the cell subsequent to phagocytosis rather than the ingestion process itself, it was necessary to first demonstrate that with the bactericidal assay, no intracellular killing defect by hypogammaglobulinemic cells existed before using metabolic activity as an indicator of phagocytic function. No such intracellular defect was

observed, and consistent with the observations on phagocytosis in normal and hypogammaglobulinemic plasma, HMS activity of leukocytes was significantly increased in the presence of normal serum.

Based on opsonic requirements, Hirsch and Strauss divided bacteria into three classes (18). One group requires no serum factors, another requires either heat-labile or heat-stable factors, and a third always requires heat-stable factors and may also require heat-labile factors. Polystyrene balls have been reported to require no serum factors at all for phagocytosis (16); however, as shown in the present investigation, phagocytosis of polystyrene balls was significantly improved by the addition of normal or hypogammaglobulinemic serum and gamma globulin. These findings are consistent with those of Skeel (15) and Strauss and Stetson (19). Phagocytosis of these particles was unimpaired in heat-inactivated serum, indicating that heat-labile substances are not involved in the opsonization of polystyrene balls.

The opsonins for the strain of *S. albus* employed in our studies were found to be primarily heat labile, which is similar to previously published data on other strains of the same organism (18). Evidence for this was provided by the finding that phagocytosis as measured by hexose monophosphate shunt activity was markedly reduced in mixtures containing heat-inactivated serum when compared to unheated serum. The level of heat-labile opsonins for *S. albus* in the patients' plasma as measured by the degree of reduction of HMS activity with heat inactivation was equivalent to control serum levels, which is consistent with the finding that complement levels were normal.

Despite the fact that heat-labile opsonins were the major type required, significant amounts of heat-stable *S. albus* opsonins were also found in normal sera. When heat-inactivated normal serum was compared to similarly treated patient serum, phagocytosis as measured by hexose monophosphate shunt activity was significantly enhanced in the normal serum, indicating that a deficiency of heat-stable opsonins in hypogammaglobulinemic serum must be responsible for the observed depression of phagocytosis. Further characterization of the heat-stable opsonins was undertaken by studying the ability of heat-inactivated serum which had been absorbed with *S. albus* to support phagocytosis. This provided an indication of the relative amounts of *S. albus*-specific heat-stable opsonins present in each type of sera. Following three absorptions with *S. albus*, the difference in opsonic activity between hypogammaglobulinemic and normal sera remained, although it was less pronounced. However, after exhaustive (six) absorptions of heat-inactivated normal and hypogammaglobulinemic sera with *S. albus*, both sera were equally ineffective in opsonizing *S. albus*. The specificity of these opsonins for

S. albus was shown by comparison with serum that had been similarly exhaustively absorbed with *E. coli*. Moreover, in vitro commercial gamma globulin supplied sufficient presumably specific heat-stable opsonins for *S. albus* to restore opsonic function of hypogammaglobulinemic sera to normal. A nonspecific ability of gamma globulin to enhance phagocytosis is demonstrated by the significant improvement of phagocytosis of PSB in hypogammaglobulinemic plasma to which gamma globulin had been added.

Hypogammaglobulinemic patients such as those reported here are susceptible to pyogenic infections against which the primary defense is provided by phagocytosis. Our studies indicate that the sera of such patients often lack sufficient heat-stable opsonins necessary for effective phagocytosis. Gamma globulin added in vitro in sufficient quantities is capable of restoring normal opsonic activity to sera from hypogammaglobulinemic patients. In contrast, parenteral administration of standard amounts of gamma globulin to two hypogammaglobulinemic patients was sufficient to only slightly improve in vitro serum opsonizing activity for *S. albus* and polystyrene balls, although susceptibility to infection in vivo was definitely reduced. This discrepancy may be due to a limitation in the tests employed or more likely, a reflection of the minimal increase in serum IgG levels attained in these patients receiving monthly gamma globulin injections.

Using a variety of organisms and techniques, the present study has demonstrated deficient opsonic activity of hypogammaglobulinemic sera attributable to the low levels of circulating gamma globulins. Phagocytosis, a major host defense mechanism, is compromised because of the inadequate opsonic activity provided by hypogammaglobulinemic sera which may in part explain the frequency of infections in patients with hypogammaglobulinemia.

ADDENDUM

In addition to the detailed studies reported here, a fourth patient with acquired hypogammaglobulinemia had phagocytic studies performed while one of the authors (R. R.) was at the University of Washington, Seattle. The patient, a 54 yr old white female (KCH No. 46-34-66), has been followed at the King County Harborview Hospital, Seattle, since July 1967, after an admission for pneumococcal pneumonia. There was a history of 12 episodes of acute bacterial pneumonia preceding the July 1967 hospitalization and, despite prophylactic gamma globulin over the succeeding 29 months through December 1969, 11 hospital admissions occurred for acute pneumococcal pneumonia. Positive blood cultures for pneumococci were documented on 7 of 11 episodes and specific serological typing on three occasions revealed the infecting organism to be a Type 25 pneumococcus. Response to antibiotic therapy was prompt on each occasion. Laboratory studies revealed absent IgA and IgM and "very low" levels of IgG on qualitative immunoelectrophoresis. She failed to respond to DPT immuni-

zation. Other studies included stools positive for *Giardia lamblia* on two occasions. One 72 hr fecal fat excretion was 9.1 g and small bowel X-rays were normal. She was not lymphopenic. Phagocytic studies were performed on 4/69 with a Type 25 pneumococcus, a *Staphylococcus albus*, and a *Lactobacillus acidophilus*, at a ratio of two to four organisms per phagocyte. Shown below are the per cent leukocytes which phagocytized bacteria when incubated in 10% serum and HBSS medium 30 min. In parentheses are the per cent leukocytes which ingested more than five organisms.

	Type 25 pneumococcus	<i>S. albus</i>	<i>Lacto- bacillus</i>
Normal cells and serum	46.5 (28)	88 (65)	82 (54)
Patient cells and serum	29.5 (6.5)	43 (6)	74 (12)
Patient cells and normal serum	60 (17)		

The findings are consistent with an opsonic defect for Type 25 pneumococcus and, by analogy, for *S. albus* and *Lactobacilli*. Encapsulated pneumococci are known to require both heat-stable and heat-labile opsonins for optimal phagocytosis in human serum (20-22). There are no measurements available of serum complement activity in this patient; however, in view of the similarity of the clinical features of her case with the other patients described, this is presumably normal. The major heat-stable component for phagocytosis of pneumococci is specific antibody (20, 22). Consistent with a lack of heat-stable opsonins in the patient serum, negligible levels of specific antibody against Type 25 pneumococcus (28 ng antibody N/ml) were found in a convalescent serum sample, despite repeated exposure to this organism.

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