Neutrophil Function in Gram-Negative Rod Bacteremia

THE INTERACTION BETWEEN PHAGOCYTIC CELLS, INFECTING ORGANISMS, AND HUMORAL FACTORS

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ABSTRACT To assess the phagocytic and bactericidal function of neutrophils in the acute stages of gram-negative rod bacteremia, cells from 30 nonleukopenic patients were studied in a test system utilizing plasma obtained simultaneously with culture-positive blood, the autologous infecting strain, and two laboratory test strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Results were compared to those obtained with normal neutrophils and plasma. Patient and control plasma were simultaneously tested with each source of phagocytic cells to localize any abnormalities. Four patients had a defect against their infecting strain, 33% of the inoculum phagocytized and killed versus 80% by controls. In these cases differences were localized to the patients' plasma, as normal plasma tested with patients' cells reversed the defect. Thus, four patients had impaired opsonization when compared to normal controls, but we also observed that 11 of 30 bacteremic isolates, all *Escherichia coli*, showed absolute or relative resistance to phagocytosis in the patient and control assay system. No intrinsic granulocyte killing abnormalities were noted. There was poor correlation between results obtained with infecting strains compared to laboratory test organisms. We conclude that in patients without evidence of an inherited neutrophil bactericidal disorder, recurrent infection, or treatment with cytotoxic drugs, intrinsic bactericidal defects are uncommon at the onset of gram-negative bacteremia, and impaired opsonization is the most commonly encountered cause of neutrophil dysfunction.

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INTRODUCTION

Abnormal human neutrophil function occurs in certain genetically transmitted syndromes (1–4). Most of these defects are expressed clinically by an increased incidence and severity of infection. Recently, several investigators have described other syndromes of neutrophil dysfunction in which a defect is presumably "acquired" before or during infection and is occasionally reversible. Leukocytes from some acutely infected patients have demonstrated diminished responses in studies of their leukotactic, phagocytic, metabolic, and microbicidal activity, but other studies have shown these functions to be normal or increased (5–10). The rapidly changing clinical circumstances in severely ill patients and differences in the methodology used in such studies have made it difficult to evaluate these divergent conclusions.

In view of the central role that polymorphonuclear neutrophilic leukocytes (PMNLs) [1] play in human host defenses and the major causative role of gram-negative bacilli in serious hospital-associated infections, we chose to study the function of morphologically normal neutrophils from adult patients with proven gram-negative rod bacteremia but without evidence of an inherited disorder of leukocyte bactericidal function, leukemia, or recent history of cytotoxic drug therapy. Specific attention has been focused on evaluating phagocytic and microbicidal capacity of neutrophils and the opsonic activity of plasma obtained from acutely infected patients. Further, a central feature of this study has been the comparison of results using the autologous infecting organism with those obtained using standard laboratory "test strains" to as-

[1] *Abbreviations used in this paper:* C3, Third component of complement; HBSS, Hanks' balanced salt solution; PMNL, polymorphonuclear neutrophilic leukocyte; (used interchangeably with neutrophil) PSS, saline solution; RBC, erythrocyte.

sess the specific host-parasite interaction. By examining these interactions at a point in time, i.e. near the onset of bacteremia, a clearer picture of in vivo occurrences has emerged. We have found that impaired neutrophil function against autologous infecting strains, if present, is usually caused by depressed plasma opsonic activity. A striking observation was that a significant proportion of bacteremic isolates of *E. coli,* the most common cause of gram-negative rod bacteremia, demonstrated resistance to phagocytosis by PMNLs from both infected and control subjects, and this may be a virulence property of bacteremic isolates of this species.

METHODS

Selection of patients. 30 patients hospitalized at the UCLA Center for the Health Sciences were selected for study using the following criteria: (*a*) Each had clinical evidence of bacteremia and was seen and examined by one of the authors. A gram-negative bacillus was isolated from at least one set of blood cultures; more than ¾ of patients had more than one positive culture "set." (*b*) The interval between drawing of blood and report of positive culture was less than 72 h (mean 36 h). (*c*) A plasma specimen was obtained simultaneously with the blood that was culture positive, stored at 4°C for no more than 18 h, and frozen at −20°C until use. (A system requiring submission of a heparinized tube of blood in parallel with blood inoculated into blood culture bottles was developed in cooperation with Dr. William J. Martin, Chief of the Clinical Microbiology Section). Patients were not studied if their plasma contained antibiotics other than cephalosporins or penicillins (these agents were enzymatically inactivated by cephalosporinase and penicillinase respectively). No patient had a history of multiple recurrent infections before adulthood, was leukopenic, had received radiation, had leukemia, or was receiving cytotoxic drugs. Patients were classified according to the nature of their underlying disease by the method of McCabe and Jackson (11).

Bacteria. Blood culture isolates were identified by standard criteria (12). For initial testing, organisms were subcultured for purity and generally used the same day they were identified. For follow-up studies, isolates were stored by freezing in human blood at −80°C. Laboratory strain *Staphylococcus aureus* 502a was obtained from Dr. Henry Shinefield, Kaiser Permanente Foundation Medical Center, San Francisco, Calif, and *Pseudomonas aeruginosa* 1829 (Fisher type VII.) (13) was a bacteremic isolate previously studied in this laboratory (14). Suspensions of bacteria in the logarithmic phase of growth were prepared at a concentration of approximately 2.5×10^7 bacteria/ml by matching turbidity to a $BaSO_4$ standard.

Neutrophil function studies. On the day of testing, 40 ml of venous blood was drawn from the patient and a normal control (healthy laboratory personnel). It was heparinized (final concentration, 10 U of aqueous sodium heparin/ml, Riker Laboratories, Northridge, Calif.) and combined with 6% clinical dextran (average mol. wt, 70,000) in isotonic saline (Abbott Laboratories, Chicago, Ill.) in a ratio of 2 vol blood to 1 vol dextran. After 1 h of erythrocyte (RBC) sedimentation at room temperature, the leukocyte-rich supernatant layer was separated, centrifuged, washed once, and suspended in 0.9% saline solution (PSS). RBC's were lysed by addition of 0.87% NH_4Cl. The preparation was again washed, centrifuged, and resuspended in Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, N. Y.) with 0.1% gelatin (Difco Laboratories, Detroit, Mich.), referred to as Hanks' gel. More than 95% of PMNLs appeared viable by their ability to exclude 0.4% trypan blue. Quantitative and differential leukocyte counts were made and the final suspension was adjusted to yield a concentration of 1×10^7 PMNLs/ml. Morphologic examination of Wright stained smears of all leukocyte preparations revealed no leukemic cells, $< 5\%$ metamyelocytes in the granulocyte population, and no cells with characteristics of an inherited leukocyte abnormality but some PMNLs from most study patients contained "toxic" granules and Döhle bodies. Since the latter reflect a normal response to an abnormal physiologic state we considered these PMNLs morphologically normal for the purpose of this study.

Quantitative PMNL phagocytosis and killing assays were modified after the method of Maaløe (15) and Hirsch and Strauss (16). Briefly, duplicate plastic tubes containing 0.1 ml Hanks' gel were rotated end over end (20 rpm) in a Roto-Rack (Model 340, Fisher Scientific, Pittsburgh, Pa.) for 60–120 min at 37°C. At time 0 and at intervals thereafter aliquots were taken, diluted with distilled H_2O, and the number of bacteria/milliliter was determined by the pour plate method using trypticase soy agar. The final concentration of PMNLs and bacteria was 1×10^6 and 2.5×10^6, respectively, yielding a ratio of bacteria to cells of 2.5 : 1 in 10% plasma.

Reaction mixtures containing plasma and bacteria without PMNLs were included to test for plasma sensitivity of the organism, and the percentage of bacteria killed by PMNLs was calculated using the difference in counts from tubes with and without PMNLs. The two laboratory test strains, *S. aureus* 502a and *P. aeruginosa* 1829, were controls in all experiments. In addition to homologous patient and control assay systems, control PMNLs were combined with patient plasma and patient PMNLs were combined with control plasma for each organism tested to localize the source of any defects.

Quantitation of viable intraleukocytic bacteria. Experiments using *S. aureus* 502a included studies to determine the number viable intracellular bacteria by a method using an extracellular staphylocidal enzyme, lysostaphin, as described by Tan et al. (17). After each incubation period, a 1-ml aliquot of the reaction mixture was combined with PSS (9 ml) and centrifuged at 200 *g* for 5 min to remove the noncell associated bacteria. The cell pellet was resuspended in 1 ml of PSS. Lysostaphin 0.9 ml (Mead Johnson Laboratories, Evansville, Ind.) at a concentration of 20 U/ml was added and the mixture incubated in a water bath (37°C) for 20 min. Lysostaphin was then inactivated by addition of 2.5% trypsin (0.1 ml) and after a 10-min incubation at 37°C the suspension was diluted in distilled water to disrupt the neutrophils before preparation of pour plates.

Experiments using the autologous strains were evaluated for the number of viable intracellular bacteria by a similar method with the following modifications: In place of lysostaphin, gentamicin sulfate (Schering Diagnostics Div., Schering Corp., Port Reading, N. J.) was added at a final concentration of 20 μg/ml. (This exceeded by more than fourfold the minimal bactericidal concentration for all strains used.) Tubes were tumbled for an additional 30 min at 37°C. The gentamicin was removed by centrifuging at 200 *g* and twice washing with 9 ml HBSS. The final cell pellet was diluted in distilled water to lyse the neutro-

phils, and pour plates were made to enumerate the viable intracellular bacteria.

Results were expressed as the percent of ingested organisms remaining viable by the following formula:

$$\frac{\text{no. of viable intracellular bacteria}}{\text{no. of bacteria killed} + \text{no. of viable intracellular bacteria}} \times 100.$$

Measurement of phagocytosis. A visual index of phagocytosis was determined by combining 5×10^6 granulocytes, 2.5×10^8 viable bacteria in 10% autologous serum and HBSS. After 20 min of continuous mixing at 37°C, a cell monolayer was prepared using a Shandon Cyto-centrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.), Wright stained, and the average number of organisms per granulocyte was determined by examining 200 cells.

A second method of measuring phagocytosis, similar to the one described by Root et al. (18), consisted of growing a suspension of bacteria in trypticase soy broth in 50 μl of thymidine ([^{3}H]Methyl) (New England Nuclear, Boston, Mass.) for 4 h on a tilt table at 37°C. Unincorporated radioactivity was removed by repeated washing and centrifugation at 4°C (Sorvall RC-2B. Ivan Sorvall, Inc., Norwalk, Conn.). The cell pellet was reconstituted with sterile PSS to an approximate concentration of 10^9 organisms/ml and a 0.1-ml aliquot was removed for enumeration of viable bacteria by the pour plate method. The remainder of the labeled bacterial suspension was refrigerated at 4°C overnight for use the following day except for a 1-ml aliquot which was used to determine the specific activity. After centrifugation and removal of supernate, 1.0 ml of 0.4 N $HClO_4$ was added to the pellet, mixed, and incubated in a 90°C water bath for 30 min. After cooling, 0.4-ml aliquots were added to 10 ml of counting solution [(15 g of 2,5 diphenyloxazole, 0.9 g of [p-bis(2)-(5-phenyloxazole) benzene], and 500 ml of Bio-Solv BBS-3)] dissolved in 3 liters of toluene and counted in an LS230 scintillation counter (Beckman Instruments Inc., Fullerton, Calif.). Results ranged from 4×10^5 to 1.5×10^6 cpm/ml of bacteria (1×10^9 organisms). The following day granulocytes were obtained at a concentration of 10^7 PMNLs/ml, as previously described. The reaction mixture contained 0.5 ml of granulocytes, 0.15 ml of autologous plasma, 2.5×10^8 labeled bacteria, and HBSS in a total volume of 1.5 ml in 13×100 mm plastic tubes (Falcon Plastics, Division of Bioquest, Oxnard, Calif.). All reactions were performed in duplicate including a control containing no serum, used to determine nonphagocytizing granulocyte associated radioactivity. After incubation at 37°C on a tilt table for 20 min, tubes were removed, immediately placed in ice, and 0.5 ml of cold HBSS was added. The mixture was twice washed with HBSS, centrifuged at 100 *g* for 10 min, and 1 ml of $HClO_4$ (0.4 N) was added to the pellet, mixed, placed in a 90°C water bath for 30 min, and cooled to room temperature. From this mixture, 0.4-ml aliquots were transferred to 10 ml of counting solution before counting. The cpm obtained from the control without serum was subtracted from the total count. Results were expressed as the mean per cent of radioactivity taken up by the granulocytes, from which the mean number of organisms per PMNL was then calculated.

Hemagglutinating antibody titers. Hemagglutinating antibody titers to "O" somatic antigens of infecting organisms were determined in duplicate by the technique of Neter et al.

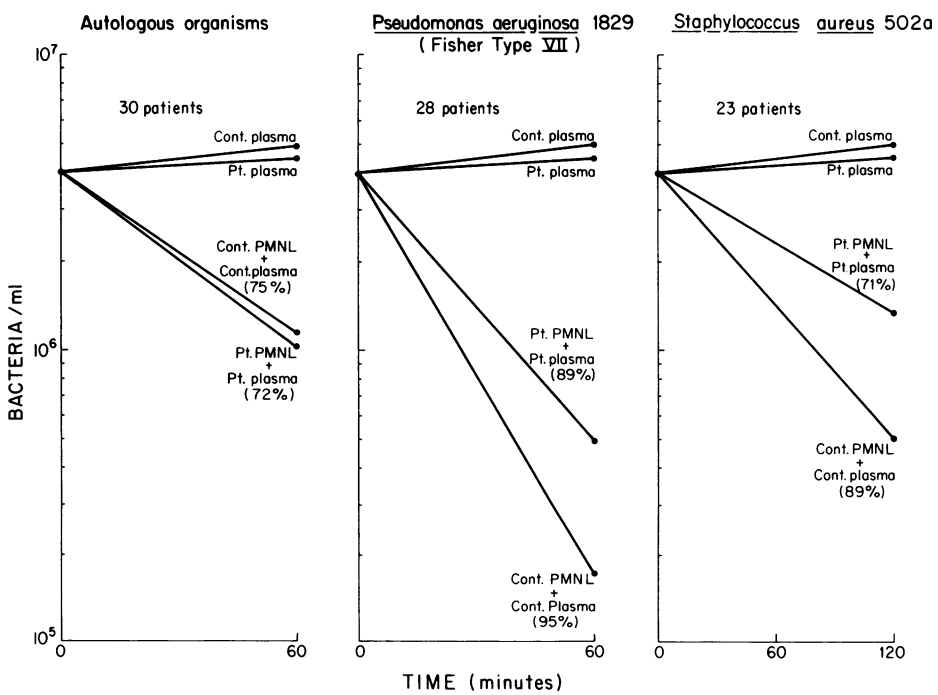

FIGURE 1 Summary of in vitro killing assay results vs. the autologous infecting organism, *P. aeruginosa* 1829 and *S. aureus* 502a. Numbers in parentheses indicate the percent of bacteria killed over the period of incubation. All assays contain a plasma control, i.e., without addition of phagocytic cells.

TABLE I

In Vitro Defects Against the Autologous Infecting Organism in Four Patients

Patient	Patients' autologous strain			S. aureus 502a	P. aeruginosa 1829
	Control PMN + Control plasma	Patient PMN + Patient plasma	Control PMN + Patient plasma	Patient PMN + Control plasma	Patient PMN + Patient plasma	Patient PMN + Patient plasma
1	69* (62, 70, 75, 68)	0	0	63	67	90
2	55 (45, 64, 61, 50)	0	0	50	91	97
3	97 (93, 100, 99, 98)	58	65	92	83	99
4	99 (97.5, 99, 99, 99.9)	73	70	99	not done	85
Mean	80	33	34	76	80 (89)‡	93 (96)§

* This is a mean of the percent of organisms killed in four experiments using four different controls. The numbers in parentheses indicate the individual values used to determine the mean.

‡ Control PMNLs and control plasma killed 89% of *S. aureus* (mean of 23 determinations).

§ Control PMNLs and control plasma killed 96% of *P. aeruginosa* (mean of 28 determinations).

The columns summarize the percent of organisms killed in each test system. Defects are localized to the patients' plasma and are specific for the infecting strain.

using sheep RBC's (19). Washed suspensions of organisms (10^9/ml) grown overnight on trypticase soy agar were boiled in phosphate buffered saline (pH 7.2) for 1 h and the supernate after centrifugation at 2,400 *g* used as the crude antigen to sensitize sheep RBC's for 2 h at 37°C. Type specific antibody titers were determined in patient and control plasmas that were heat inactivated at 56°C for 30 min and absorbed with sheep RBC's for 2 h at 37°C. Plasma specimens were serially diluted in phosphate buffered saline (0.025 ml), using a semi-automated microtiter apparatus (Cooke Laboratory Products, Alexandria, Va.) and an equal volume of sensitized sheep RBC's (0.8%) added. Hemagglutination patterns were read after incubation at 37°C for 1 h and at 4°C for 18 h.

Levels of the third component of complement (C3). C3 levels were determined in duplicate by the standard Mancini radial immunodiffusion technique using a commercial antiserum (Meloy Laboratories Inc., Springfield, Va.) (20).

Statistical analysis. The reproducibility of neutrophil function assays was assessed by determining the SEM (21) of the percentage of bacteria killed by PMNLs. Significant variation in the residual viability of infecting organisms was inferred if the percentage phagocytized and killed was beyond 2 SD of the mean for laboratory test organisms (*S. aureus* 502a and *P. aeruginosa*). The significance of differences in geometric mean antibody titers was assessed using Student's *t* test for unpaired samples (21).

RESULTS

In Vitro phagocytosis and killing of autologous infecting organism, P. aeruginosa 1829 and S. aureus 502a. A total of 30 patients with gram-negative rod bacteremia were studied. Fig. 1 summarizes the mean per cent of autologous infecting organisms, *P. aeruginosa* 1829 and *S. aureus* 502a, phagocytized and killed by patient and control PMNLs in the presence of homologous plasma. The results for the control test system compared to the patients' PMNLs and plasma, when tested against the autologous infecting strains showed 75 vs. 72% killing, against *P. aeruginosa* 1829 95 vs. 89% killing, and against *S. aureus* 502a 89 vs. 71% killing, respectively. The quantity of plasma and phagocytic cells was not adequate to assess PMNL function in two patients with *P. aeruginosa* 1829 and seven patients with *S. aureus* 502a. Plasma controls were always included and no isolates were plasma-sensitive, i.e. killed by plasma in the absence of PMNLs.

Although there were small differences in the function of patients' PMNLs and plasma compared to normal controls and plasma, when tested with bacteremic isolates, we did find four patients with marked defects compared to controls (Table I). These four patients phagocytized and killed 33% of their infecting organisms compared to 80% by controls. There was a mean of 47% fewer organisms killed by patients than by controls (range 26–69%). The remaining 26 individuals killed 74% of their infecting organisms compared to 78% by controls (mean 4% fewer organisms killed by patients than by controls with a range of 0–13%). The second column (control PMNLs plus control plasma) shows the results of normal controls tested against each patient's bacteremic isolate. The differences in per cent killed by normal controls for each of the four patient isolates reflects the varying susceptibility of these organisms to phagocytosis and killing by normal PMNLs and plasma. The reproducibility of results using a given bacteremic isolate was tested by using a total of four different normal controls for each of the four organisms tested (numbers shown in parenthesis). Differences between

TABLE II

Reversibility of In Vitro Killing and Changes in Antibody and Complement Levels in Two Patients with Initially Decreased Plasma Opsonic Activity Against the Autologous Infecting Organism

Patient	Killed* acute/ convalescent	Ab Titer‡ acute/convalescent	C_3 Acute convalescent§
	%		*mg/100 ml*
1	0/97	1:32/1:512	105/110
3	65/99	1:64/1:2,048	108/140

* Patients' plasma and control PMNLs vs. the autologous infecting organism.

‡ Hemagglutinating antibody to "0" somatic antigen.

§ Range of normal values: 80 mg–150 mg/100 ml.

controls and patients could not be attributed to differences between normals. Recombining the patient and control plasma and PMNLs was carried out to isolate the source of the defect. Combinations using control PMNLs and patients' plasma continued to show defects, while patient PMNLs and control plasma yielded results similar to those using control PMNLs and plasma. In each case, these manipulations localized the defect to the patients' plasma, rather than to their PMNLs. The number of viable intracellular bacteria was less than 2% of the total bacterial count for both control and patient PMNLs.

A further objective was to determine whether the plasma defect was specific for the patient's own organism or was a more generalized abnormality such as an inhibitor of phagocytosis. The last two columns (Table I) give results for the four patients PMNLs and plasma tested against the two laboratory test strains *S. aureus* 502a and *P. aeruginosa* 1829. The plasma defects appeared to be specific for three of the four autologous infecting organisms, and except for patient 1 defects were not observed against any laboratory test strains.

Two of these four patients were available for further study at 14 days postonset of bacteremia. To further characterize the nature of the defect, acute and convalescent plasma were combined with normal PMNLs and were tested on the same day against the patient's autologous organism (Table II). The hemagglutinating antibody titer for each patient's bacteremic isolate as well as C3 levels were determined on paired specimens. The plasma defect was shown to be reversible, but C3 levels remained normal while specific antibody titers markedly increased, correlating with the improvement in the killing assay results.

Results analyzed by bacterial species tested. Results of the percent bacteria phagocytized and killed by normal PMNLs and plasma were analyzed by species (Table III). Results for *P. aeruginosa* 1829 and *S. aureus*

TABLE III

Phagocytosis and Killing of Each Bacterial Species By Normal Control PMNLs and Plasma

Organism	Strains tested	Mean killed (±SEM)	Range
	no.	%	
Staphylococcus aureus 502a	1	89*±1.2	75–97
Pseudomonas aeruginosa 1829 (Fisher type VII)	1	96‡±1.3	75–99.9
Serratia marcescens	3	95	87–98
Klebsiella pneumoniae	5	94	80–99.9
Citrobacter freundii	1	86	86
Enterobacter species	2	99.9	99.9
Escherichia coli ("sensitive")	8	93	83–99
Escherichia coli ("resistant")	11	43	0–69

* Mean of 23 separate determinations.

‡ Mean of 28 separate determinations.

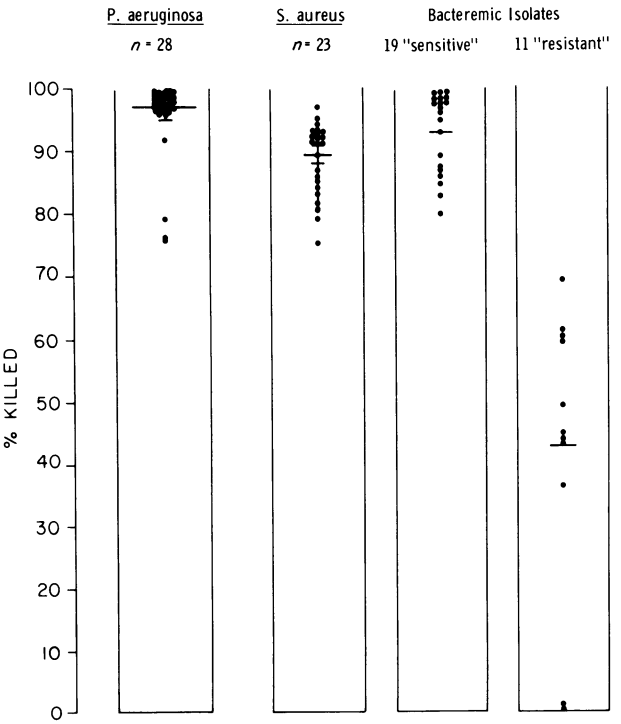

FIGURE 2 Percent of bacteria killed by normal control PMNLs and plasma. The mean percent of *P. aeruginosa* killed was 96%, range 75–99%. The mean percent of *S. aureus* killed was 89%, range 75–97%. Brackets are±SEM. The two right hand panels compare results of the 19 "sensitive" infecting strains (mean 93% killed, range 80–99%) and the 11 "resistant" infecting strains (mean 43%, range 0–69%). Results given for the 11 "resistant" strains represent the mean of four different controls.

502a were based on 28 and 23 observations, respectively. The day-to-day variation of this assay using the laboratory test strains is shown in the first two panels of Fig. 2. For *S. aureus* 502a the mean percent killed was 89% with a SD±5.5% and for *P. aeruginosa* the mean percent killed was 96% with a SD±5.7%. The two laboratory test organisms were thus designated as "sensitive" to phagocytosis and killing by normal PMNLs and plasma. 11 of the 30 bacteremic isolates, all *E. coli,* were found to be relatively or absolutely "resistant" because the percent of organisms killed by normal PMNLs and plasma was less than 2 SD below results using the standard laboratory strains. The mean percent killed of "resistant" organisms was 43% (range 0–69%). The remaining 19 isolates were considered "sensitive" with a mean 93% killed (range 80–99%). This assay was repeated (on another test day) using three additional normal control sources of PMNLs and plasma against the "resistant" isolates yielding similar results, thereby minimizing differences in controls as a variable.

Convalescent plasma was available from 8 of these 11 patients and when combined with control cells, the percent of organisms killed increased to 88% as compared to 26% using control plasma and cells (Table IV). This correlated with the increase in specific antibody titers seen in convalescent plasma compared to normal controls; the geometric mean titer of convalescent plasma was 1:694 compared to the mean titer of control plasma of 1:42 ($P < 0.001$), as determined by passive hemagglutination.

Confirmatory studies of phagocytosis and intracellular killing. Phagocytosis by control PMNLs and plasma was quantitated by the uptake of five representative strains of both "resistant" and "sensitive" labeled bacteremic isolates and by visual indices, and results were compared to in vitro clearance studies utilizing the same organisms and controls (Table V). Granulocytes incubated with organisms found "resistant" to clearance (38.8% killed), were noted to contain far fewer bacteria by both direct visual observation (4.6 vs. 23.6 organisms/PMNL) and the radiolabeled technique (4.2 vs. 18 organisms/PMNL) than granulocytes incubated with "sensitive" organisms (94.6% killed).

The mechanism of resistance of bacteria to clearance in this assay was studied by the ability of control neutrophils to kill ingested microorganisms. The total number of bacteria killed plus the surviving intracellular bacteria represented the total number of ingested organisms. The percent of ingested organisms surviving intracellularly was compared to the number of bacteria killed (Table VI). For nine "resistant" strains (53% killed), a mean of 5% of ingested organisms remained viable and for the 13 "sensitive" strains (94% killed) a mean of 2.4% of ingested bacteria were viable after 60 min of incubation.

The decreased clearance of these bacteremic isolates

TABLE IV

Comparison of Control and Convalescent Plasma Type Specific Antibody Titers Correlated With Plasma Opsonic Activity for These Eight "Resistant" E. coli Isolates

Patient	Killed by control plasma and control PMNLs	Antibody titer control plasma	Killed by convalescent plasma and control PMNLs	Antibody titer* convalescent‡ plasma
	%		%	
5	45	1:64	97	1:512
6	18	1:128	99.9	1:2,048
7	62	1:32	85	1:256
8	0	1:128	80	1:512
9	0	1:8	89	1:128
10	44	1:8	92	1:1,024
11	43	1:128	83	1:1,024
12	52	1:16	75	1:2,048
Mean	26	1:42§	88	1:694§

* Hemagglutinating antibody to specific "0" somatic antigen.
‡ 14±3 days after documented sepsis.
§ Geometric mean titers significantly different ($P < 0.001$) by Student's *t* test.

TABLE V

Phagocytosis of "Sensitive" and "Resistant" Bacteremic Isolates of E. coli by Normal Granulocytes and Serum Comparison of In Vitro Clearance Assay and Phagocytosis As Determined By Uptake of Radiolabeled Bacteria and Visual Observation

	Uptake of labeled bacteria		Visual index
	Total cpm Ingested by PMNLs	Bacteria* per PMNL	Bacteria‡ per PMNL
	%	*no.*	*no.*
"Sensitive" strains			
4,298 (97)§	52	27	31
4,412 (93)	46	23	22
4,273 (89)	36	17	27
4,327 (95)	26	10	27
4,522 (99)	26	13	11
Mean±SEM (94.6%±1.7)	37.2±5.2	18±3.1	23.6±3.5
"Resistant" strains			
4,193 (46)	3	1	1
3,781 (68)	22	11	12
4,310 (37)	11	5	3
4,037 (0)	4	2	6
4,137 (43)	2	2	1
Mean±SEM (38.8%±11)	8.4±3.7	4.2±1.8	4.6±2.0

* This is a calculated value based on the uptake of radioactivity, the specific activity of the labeled bacteria, and the number of granulocytes in the assay.
‡ 200 cells counted after cytocentrifuge preparation.
§ Numbers in parentheses refer to the percent of organisms killed by control granulocytes and plasma in 60 min. For "resistant" strains this represents the mean results using four different sources of granulocytes and plasma.

TABLE VI

Total Bactericidal Activity and Percent of Viable Intracellular Bacteria After Ingestion by Normal Granulocytes

	9 "resistant" autologous strains		12 "sensitive" autologous strains	
	Killed*	Viable intracellular bacteria‡	Killed*	Viable intracellular bacteria‡
	%		%	
	62	7.4	99	0.2
	44	2.8	89	3
	43	2.9	87	3
	45	4.6	96	4
	49	9.2	99	1
	61	5.7	98	1
	37	4.9	87	1
	62	6.6	86	1
	69	1.0	99	5
			99	1
			98	2
			98	6
Mean±SEM:	53±3.7	5±0.8	94±1.6	2.4±0.5

* Percent of bacteria killed using control PMNLs and plasma after 60 min of incubation.

‡ After exposure of PMNLs to gentamicin for 20 min and twice washing the cell pellet with HBSS.

by normal granulocytes and plasma was corrected by substitution of convalescent plasma, correlated with diminished phagocytosis as measured by two methods, and was not explained by abnormal intraleukocytic killing.

Defective clearance of S. aureus. Analysis of results using *S. aureus* 502a showed that 12 of 23 patients killed 89% of organisms vs. 91% for controls (mean difference 2%, range 0–9%). 11 of 23 patients were able to kill

TABLE VII

Total Bactericidal Activity and Percent of Viable Intracellular Bacteria in Patients' Cells Studied with Staphylococcus aureus 502A

	8 patients with defects against *S. aureus* 502a		9 patients with normal staphylocidal activity	
	Killed*	Viable intracellular bacteria‡	Killed*	Viable intracellular bacteria‡
	%		%	
	73	1.7	91	7.0
	57	3.9	96	3.7
	70	13.3	91	1.5
	57	3.0	92	4.8
	5	9.3	94	10.0
	65	8.9	90	2.0
	3	8.0	81	4.9
	65	2.0	93	4.7
			91	3.2
Mean±SEM:	49.4±10	6.3±1.5	89.8±1.6	4.6±0.9

* Percent of bacteria killed using patient PMNLs and plasma after 60 min of incubation.

‡ After use of lysostaphin to kill extracellular nonphagocytized cocci.

TABLE VIII

Clinical Data on 30 Patients Studied

	All patients (30)	Four patients with plasma opsonic defects against their autologous infecting organism
Severity of underlying disease*		
Rapidly fatal	2	—
Ultimately fatal	14	3
Nonfatal	11	1
Predisposing clinical factors		
Renal disease	5	—
Diabetes mellitus	3	2
Carcinoma	5	—
Liver disease	2	1
Lupus erythematosis	3	—
Lymphoma	3	1
Postsurgical complications	10‡	1‡
Source of sepsis		
Pulmonary	6	1
Urinary tract	20	3
Wound	6	—
Abscess	2	—
Biliary	2	—
Other	5	—
Shock	7	1
Corticosteroid therapy	9	1

* Reference 11.

‡ One patient underwent surgery and had liver disease.

only 51 vs. 90% for controls (mean difference between control and patient 39%, range 20–86%). Only one of the four patients with a defect against his infecting organism had a defect against *P. aeruginosa.* The nature of the defect to *S. aureus* 502a was further characterized by studying the ability of patient neutrophils to kill ingested bacteria. The percent of ingested *S. aureus* surviving intracellularly was compared to the bactericidal activity of patient PMNLs and plasma (Table VII). The efficiency of intraleukocytic killing, comparing those patients with and without defects against *S. aureus* (6.3 vs. 4.6% viable intracellular organisms, respectively), did not explain the differences in the percent of organisms killed (53 vs. 94%). The abnormality against *S. aureus* was corrected by combining patients' PMNLs with normal plasma indicating differences between patients and controls were due to plasma defects rather than intrinsic granulocyte microbicidal mechanisms.

Clinical data. Table VIII summarizes clinical and prognostic information on all cases studied as well as the four patients with opsonic defects. Two of the four patients with opsonic defects had diabetes, one had lymphoma and was on 40 mg/day of prednisone, one had undergone surgery and had liver disease, three patients had urinary tract infections, and one had pneumonia. Shock was noted in the patient with liver disease. None of the four patients with decreased plasma opsonic activity against their infecting organism had abnormal serum immunoglobulins.

DISCUSSION

Quantitative deficiencies in the populations of phagocytic cells circulating in the blood have long been known to be associated with serious bacterial and fungal infections (22). More recently, increased susceptibility to infection has been reported in patients with morphologically normal neutrophils and normal or elevated leukocyte counts. Impaired functional capacity of PMNLs has been observed in studies of patients with genetically inherited syndromes (1–4), neoplastic diseases (23, 24), metabolic diseases (25, 26), immunologic disorders (27, 28), surgical diseases (29), extensive burns (30), and severe infection (5–9). The extent to which this occurs and the nature of the underlying defects remain controversial.

To test the hypothesis that PMNLs may be functionally deficient in their phagocytic and bactericidal capacity during severe infection, we chose to study nonleukemic PMNLs from patients with gram-negative rod bacteremia. The design of this work differed from that of others in that we simultaneously tested for plasma opsonic activity and neutrophil function. In some other studies, metabolic inhibitors such as cyanide and azide, inhibitors of intracellular killing such as phenylbutazone, antimicrobials to kill extracellular nonphagocytized bacteria, and cytochalasin B to inhibit cytokinesis have been added to in vitro test systems to assess abnormalities of neutrophil function (7, 29, 31, 32). It has been argued that some of these additives are both unphysiologic and nonspecific and have introduced new and difficult to control variables to the assessment of neutrophil function (31, 32).

By using various combinations of neutrophils and plasma (bacteria opsonized with patient plasma and combined with control PMNLs, and bacteria opsonized with control plasma and combined with patient PMNLs) we were able to characterize the abnormalities we observed without the introduction of these other variables. The criterion used for severe infection, i.e. gram-negative rod bacteremia, was more rigorous than that used in similar studies and enabled us to test the invading pathogen in our assay. We noted marked differences in clearance of various bacteremic isolates, even within a single species, a variable which may be extremely important in studies of host-microbial relationships.

Two groups of investigators previously have observed neutrophil bactericidal defects from patients with bacterial infections using techniques similar to those described here (6, 7). Both of their reports showed only slight differences between patients and controls (approximately 5%), a defect not considered significant in our laboratory. Three patients with well documented transient bactericidal defects during infection also have been reported (8). These patients differed from those presented here in that two had *S. aureus* bacteremia and both were studied more than 10 days after acute onset of infection. Of interest, the one patient with gram-negative rod bacteremia, in the latter series, had a defect for *S. aureus* but not against his infecting strain, an observation consistent with our findings. In another study a slight decrease in the capacity of "toxic" neutrophils to phagocytize and kill *Staphylococcus albus* was reported (5). Clearance of *S. albus* by normal serum and patient PMNLs was 83%, compared to 81% clearance of *S. aureus* 502a in our experiments. Further, in that study normal serum and PMNLs cleared 96% of *S. albus* vs. 89% of *S. aureus* 502a in our report, and this slight difference may be accounted for by strain differences among staphylococci. Although virtually all of our patients had some PMNLs with morphological characteristics typical of toxic neutrophils, i.e., toxic granules and Döhle bodies, the presence of these abnormalities did not appear to affect the functional capacity of these phagocytic cells in our assay. Results using *S. aureus* 502a as the test strain in this study showed decreased clearance of this organism in about one-half of patients tested. Studies using lysostaphin eliminated an intraleukocytic killing defect as the cause of this abnormality, and cross over studies localized the abnormality to the patient's plasma. An adequate explanation for this observation with staphylococci is not apparent in this or other reports when similar findings have been observed (5, 8). What seems clear is that results using such standard test organisms may not parallel results using the infecting organism. Thus, we question the validity of studies ascribing neutrophil function defects in gram-negative rod bacteremia based on studies of the *Staphylococcus.*

Our major finding was that a significant proportion of blood culture isolates, 11 of 19 *E. coli,* demonstrated relative or absolute resistance to clearance by normal plasma and neutrophils. This resistance was overcome by substitution of convalescent plasma from patients infected with these organisms, and evaluation of intraleukocytic killing showed no differences between "sensitive" and "resistant" strains. Direct assessment of phagocytosis by two techniques showed decreased ingestion of isolates that were "resistant" to clearance, as compared to those which were "sensitive." This indicates that the mechanism was impaired opsonization rather than a defect in intracellular killing. Certain gram-negative bacteria contain antiphagocytic surface determinants (33, 34), and we observed that convalescent plasma containing high titers of type specific antibodies when combined with PMNLs supported enhanced clearance of these organisms. Interestingly, none of the control plasmas used on the day of the experiment or the triplicate confirmatory studies differed significantly in their ability to

promote clearance of "resistant" strains. This suggests that among normal individuals there is a widespread lack of opsonizing antibodies against the antiphagocytic antigenic determinants of these invasive organisms.

Bacteria can trigger either the alternate or classic pathway of complement activation, and Forsgren and Quie have reported that different bacterial species may require different pathways of complement activation for opsonization (35). More recently Fine was able to demonstrate that different serotypes of a single bacterial species, *Streptococcus pneumoniae,* can differ in their interaction with complement (36). Lack of antibody for *S. aureus* 502a in certain patients and the requirement of the classic complement pathway for opsonization of this organism may explain the defects. Similarly, interspecies differences among *E. coli* in their interaction with complement and the amount of opsonizing antibody present in normal plasma for each specific organism may relate to our observations.

From this study of 30 patients, no evidence of an acquired, intrinsic bactericidal defect in neutrophils from patients with acute, severe gram-negative bacillary infection was observed. Four patients were noted to have defective in vitro clearance of their autologous strains when compared to normal controls. "Cross over" studies in which normal and control plasma and PMNLs were combined, localized this defect to the patients' plasma. One consideration which might explain this observation is that the patients' plasma contained an inhibitor of phagocytosis. This seems unlikely since in most instances the patients' plasma, although unable to promote clearance of the infecting organism, supported phagocytosis of both *S. aureus* and *P. aeruginosa.* A reversal of the opsonic defect was seen in both patients available for follow-up study. Further, this reversal correlated with a marked increase in the specific hemagglutinating antibody titer and we speculate that low titers of opsonizing antibodies are a likely explanation for the defect noted in these patients. These observations are consistent with the view that the most frequent reason for defective ingestion of PMNLs is diminished serum opsonic activity (37). 11 of 30 patients were bacteremic with "resistant" strains, and 4 of 30 had defects compared to normals. Two patients with defects were also infected with relatively "resistant" organisms. Thus, approximately 40% of patients, 13/30, studied in the acute stages of gram-negative bacteremia demonstrated impaired clearance of their infecting pathogen. In a few instances the cause was diminished plasma opsonic activity compared to normal controls, but in most cases this decrease in opsonic activity was seen both in infected patients as well as normal controls and was due to a resistance to phagocytosis of certain bacteremic isolates, all of which were *E. coli.* The specific properties of these *E. coli* conferring this resistance, even in presumably normal in vitro systems, need further characterization.

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