A Physiological Basis for the Development of Opportunistic Infections in Man

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"There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes. Stimulate the phagocytes. Drugs are a delusion."

G. B. SHAW (1906)
The Doctor's Dilemma, Act I

INFECTION continues to be one of the major challenges in surgical practice,[1,2] and in many conditions, such as burn injury and organ transplantation, it remains the leading cause of death. While many problems of infection have been solved, new ones have arisen to take their place. The general use of modern antibiotic therapy, for example, was once thought to offer the promise of controlling all infectious problems, but instead it has compounded their complexity because of the emergence of opportunistic pathogens which have become increasingly difficult to treat, especially in the compromised host. Altemeier[11] recently brought the problem into focus during his presentation of the 1971 Scudder Oration on Trauma: "Antibiotic therapy has now been used for over a quarter of a century. Clinical and laboratory studies have indicated that it has failed to reduce the overall incidence of infection associated with surgical operations or other trauma." The analogy between Altemeier's and Shaw's remarks is striking, and it has become increasingly apparent that fresh approaches to opportunistic infections are required. The search for new and increasingly effective antibiotics seems misdirected; instead emphasis on host defense mechanisms and their regulatory control now provides the greatest promise for the future.

Immunological therapy to bolster an individual's resistance to selected infections has been utilized for almost two centuries,[21] but immunological deficiency states were neither well defined nor understood until the pioneering discovery of agammaglobulinemia by Colonel Ogden Bruton in 1952.[15] His finding stimulated an intense and ongoing world-wide investigation which has markedly increased our understanding of the role of the lymphoid system in infectious processes.

As a result of Metchnikoff's pioneering work[29] the neutrophilic system of defense has been recognized for over one-half of a century as having exceptional importance in host resistance, but critical examination of neutrophilic function was not made until 1959 when Cohn and Morse[17] extended the use of the *in vitro* neutrophil function test described by Maaløe[27] to the problem. Even so, specific abnormalities of neutrophilic function were not clearly defined until 1966 when, as a result of the broad based survey of immunological variables in the congenital disease, chronic granulomatous disease of childhood, a clear cut intracellular bactericidal leukocyte defect was described.[20] Since then, several investigators have shown that both congenital and acquired deficiencies of neutrophilic function may be asociated with infection.[23]

In 1967, a test of neutrophilic antibacterial function was described by Alexander *et al.*[9] which has been used to study a large number of patients considered to be susceptible to infection, applying it especially to the problem of opportunistic infections in burns and transplantation. This manuscript amalgamates our previously published data[4,6,10] with a series of new observations and additional clinical data to provide the basis for our hypothesis relating the importance of abnormalities of

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neutrophilic function to the genesis of opportunistic sepsis in man.

Materials and Methods

Patient Population

Two groups of patients were studied: those with burn injuries and those receiving immunosuppression following renal transplant. Serial measurements of neutrophilic function were made in each patient, and no data are included for patients with single or random measurements. Data for the multiple organism study, enzyme studies and separation of defects are listed separately.

Burn Patients: Twenty-seven male and 21 female patients with major burns were studied. Their ages ranged between 1 and 82, and the size of burn between 25% total/22% 3° and 93% total/90% 3°. The average patient age and size of burn was 15.6 years and 53.6% total/39.2% 3° respectively.

Transplant Patients: Similar measurements of neutrophilic function were made on three female and 11 male patients receiving kidney transplants. Their ages ranged from 17 to 52 (average 36.2).

Control Subjects

The normal controls consisted of laboratory personnel. To provide an accurate daily baseline for calculation of the neutrophil bactericidal index, tests of neutrophil antibacterial function were routinely performed on at least two normal individuals on each of the days that a patient was studied, although occasionally, only one control was available.

Method for Documentation of Sepsis

The documentation of life-threatening sepsis was usually straight forward, and only those septic episodes associated with positive blood cultures or instances of pneumonia or frank burn wound sepsis which the attending physician in charge felt were life-threatening were acceptable for inclusion in this series. To eliminate any bias on the part of the authors, a research assistant, who was not aware of the results of the neutrophil function tests, collected the clinical data which was later confirmed.

Tests of Neutrophilic Antibacterial Function

The test used for these studies was modified only slightly from that previously described.[9] For each subject, approximately 15 ml. venous blood was collected in a plastic syringe and discharged into a conical silconized glass centrifuge tube containing 3 ml. dextran-70 in saline and 1000 units heparin. After gentle mixing, the erythrocytes were allowed to sediment for 45 minutes, and the leukocyte rich plasma was withdrawn with a Pasteur pipette and centrifuged at 500 × g for 4 minutes. The leukocyte pellet was resuspended in 5 ml Hanks solution containing 1% gelatin, (Hanks-gel), and quantitative counts were made for neutrophils. After re-centrifugation, the supernate was discarded, and the cells were suspended in a sufficient volume of Hanks-gel solution to produce 10 $\times 10^6$ neutrophils per milliliter. Approximately 90% of the cells from such suspension were neutrophils with greater than 95% viability by trypan blue exclusion. *Staphylococcus aureus 502A* was used as the test organism for all of the antibacterial studies described herein except those in which the antibacterial killing capacity of neutrophils was measured against multiple organisms. An 18-hour culture of the test organism was washed twice with normal saline and resuspended to a concentration of approximately 1.5×10^7 organism per milliliter. A stock tissue culture medium in which the neutrophil function test was performed was made each day and was composed of 5 ml. Hanks-gel, 5 ml. fetal calf serum, and 0.01 ml. chicken embryo extract. Immediately prior to each experiment, 1.0 ml. of freshly thawed pooled normal human serum as a source of opsonins, was added to 3 ml. of the stock culture medium. The pooled normal human serum was prepared from a minimum of four normal adults and stored at −70°C until needed.

The test itself was performed with 0.5 ml. of the leukocyte suspension (5×10^6 neutrophils), 0.4 ml. stock medium containing human serum (10% final concentration), and 0.1 ml. of the bacterial suspension containing approximately 1.5×10^6 viable organisms as determined by pour plate assay. The leukocyte-bacterial mixture was incubated in a small plastic Wasserman tube on a tilting table aliquot mixture for 4 hours at 37°C. At the end of incubation, care was taken to obtain an evenly disbursed suspension, and 0.1 ml. was removed for a quantitative viable bacterial count by a standard pour plate assay. The plates were incubated for 48 hours before colonies were counted.

Calculation of the Neutrophil Bactericidal Index

The neutrophil bactericidal index (NBI) was calculated for each test by dividing the average of the numbers of bacteria not killed by the neutrophils from each normal control into the number of bacteria not killed by the subject during the 4 hours incubation period (Fig. 1). As an example, an NBI of ten would mean that ten times as many bacteria survived in the subject's test than in the normal control's test; an NBI of four indicated that four times as many bacteria survived; and an NBI of one represented equivalence with the controls. Numbers less than one were expressed as a negative reciprocal and represented better than average function for normals. A value of minus two, for example, indicated that the test for the experimental subject contained only half as many viable bacteria as did the controls on the

day of testing. Normal individuals have previously been determined to have NBI's of less than two in 93% of the tests.[4]

Using this calculation, minor variations in the inoculum can be disregarded, providing a much more accurate assessment of neutrophil function than percentage of kill.

Quantitative Cultures of the Burn Wounds

In 12 selected burn patients, quantitative cultures were made of the burn wound on a periodic basis. Specimens for cultures were taken from at least two sites by excising portions of the burn wound eschar or scraping the granulation tissue. When topical antibacterial agents were being used, these were removed by washing with Dreft and hydrogen peroxide in normal saline. Meticulously sterile technic was used throughout, and specimens were as representative of the entire burn wound as possible. The individual specimens were weighed, diced, and homogenized in 10 ml. of sterile saline in an ice bath for 5 minutes at 720 rpm using a 50 ml. Duall tissue grinder (Kontes Glass Co).[31] Bacteria were estimated by standard plate count methods using acetimide agar, and the count was expressed as the number of bacteria per gram of tissue submitted.

Separation of Phagocytic Defects from Intracellular Killing Defects

In studies of the first 42 patients, duplicate tests were done in which 100 μg. streptomycin and 100 units penicillin were added at the beginning of incubation to kill extracellular bacteria which were not rapidly phagocytized.[5,9] At the end of the 4-hour incubation period, all of the remaining viable bacteria were presumed to be intracellular. A phagocytosis-intracellular killing index (PIK index) was calculated for the tests in which antibiotics were used to kill extracellular bacteria in a manner similar to the NBI.[6] In a patient with an abnormally high NBI, high values for the PIK index indicated rapid phagocytosis, and low values indicated an impairment of phagocytosis.

The intracellular location of bacteria was confirmed in an additional seven tests on burn patients using lysostaphin, a specific staphylocidal enzyme supplied by Dr. James Tan and used in the manner he has described.[34] At the end of the 4-hour incubation period, the leukocyte-bacterial suspension was sampled, 20 units of lysostaphin was added, and the mixture incubated in a water bath at 37°C for an additional 20 minutes. One tenth ml. of 2.5% tryspin was added to inactivate the lysostaphin, and the mixture was sampled for viable bacteria. The lysostaphin kills all extracellular bacteria, therefore allowing separation of a bactericidal from a phagocytic defect.

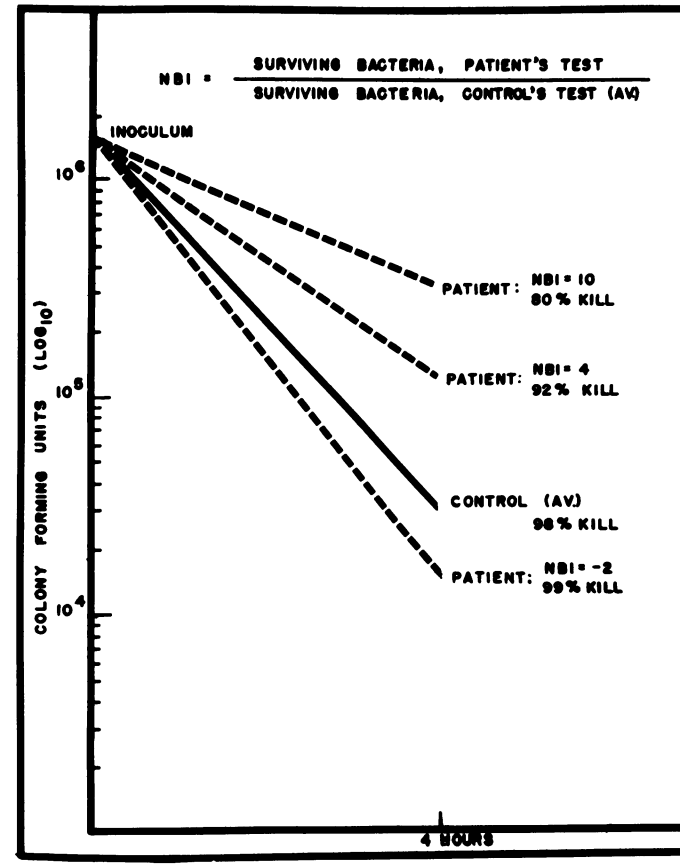

FIG. 1. Method for calculation of the neutrophil-bactericidal index with examples to show how the values are determined. See text for further explanation.

In addition, a series of electron micrographs were made of the neutrophils from a 15-year-old patient with a 51% burn and two controls. The cells were sampled at two points in the neutrophil function test, the first just prior to incubation on the aliquot mixer, and the second at the time of sampling for viable bacteria. These specimens, each containing about 4.5×10^6 cells, were centrifuged to make a pellet, the supernatant was removed, and 3% gluteraldehyde gently layered on the pellet which, when fixed, was cubed and placed in phosphate buffer. The specimens were postfixed in osmium tetroxide and embedded in Maraglas. Ultrathin sections were cut on a LKB-2 ultramicrotome and double stained with uranyl acetate and lead citrate. The sections were examined and photographed on an RCA-EMU-3G and a Model 101 Siemans Electron Microscope. To ensure that individual cells and engulfed bacteria could not be examined more than once, a ribbon of sections was cut for a single grid, and 25–30 thick sections were then cut before the next ribbon was prepared. To ensure that cells were not counted twice in the same plane, a limited number from each grid were examined, usually about 75, depending upon the size of the block face. The cells of

TABLE 1. *Patient Data*

Type of Patient	Number of Patients	Ages Range (Av)	Total & (Av) Days Studied	Total & (Av) Tests Performed	Total Episodes of Sepsis
Burn	48	1–82 (15.6)	1604 (33.4)	573 (12)	45
Transplant	14	17–52 (36.2)	1218 (87.0)	376 (27)	2
Total	62	1–82 (20.3)	2822 (45.5)	949 (15)	47

both control and burn neutrophils were evaluated for evidence of phagocytosis, the number of intracellular bacteria, and the number of paired bacteria.

The temporal dynamics of the intracellular killing defect were studied in four burn patients and two normal controls by preparing individual tests in quintuplicate with the same neutrophil-bacterial mixture, and sampling them in the described manner at 15, 30, 60, 120 and 240 minutes. A set of tests in the absence of neutrophils was performed at the same time.

Multiple Organism Study

In one series of tests on five burn patients, *Serratia marcescens, Streptococcus viridans, Pseudomonas aeruginosa* serotype 1, *Staphylococcus aureus* 502A and *Candida albicans* were utilized as the test organism in separate sets of tests done concurrently on the same patient's neutrophils to determine if selective antibacterial defects were present. The organisms were prepared in the same manner as described for *Staphylococcus aureus 502A* (*vide supra*), and the tests were performed identically. An NBI was calculated for each organism.

Enzyme Studies

Intraleukocyte myeloperoxidase (MPO) levels [22] were measured on three patients with severe burns in conjunction with neutrophil function tests. Six ml. blood was added to an equal volume of 6% dextran in normal saline with 0.1 ml. EDTA. The red cells were allowed to sediment, and the leukocyte rich plasma was withdrawn and washed sequentially in 2 ml. of normal saline, 6 ml. of cold sterile H_2O and 2 ml. 3.5% cold saline to lyse the red cells. Slow centrifugation produced a pellet of leukocytes, and the red cell stroma in the supernatant was discarded. The neutrophils, now free of erythrocytes, were counted and resuspended in saline to a concentration of 1×10^7 PMN/ml. The remainder of the test was done in an ultracentrifuge tube in which 1.2 ml. of the PMN suspen-

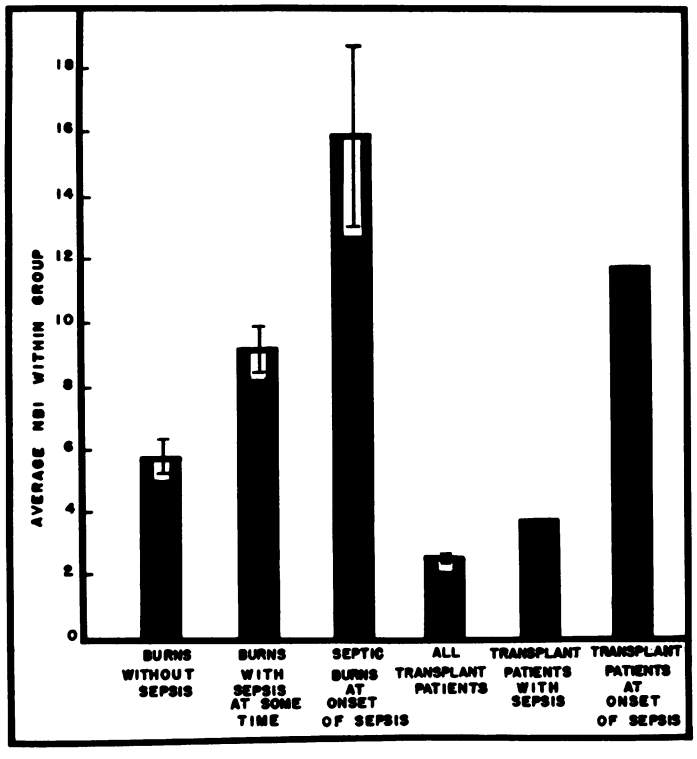

FIG. 2. Average of all determinations of NBI with standard error of the mean for patients within various categories. Because of the small number of determinations for transplant patients with sepsis and transplant patients at the onset of sepsis, standard error of the mean was not calculated for these groups.

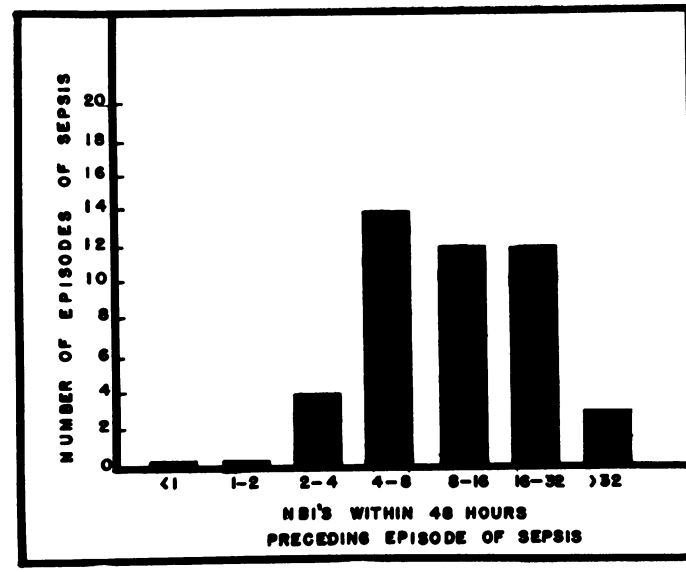

FIG. 3. Relationship of the onset of sepsis to the value of the NBI within the 48 hours immediately preceding the episode of sepsis. NBI's are grouped within ranges.

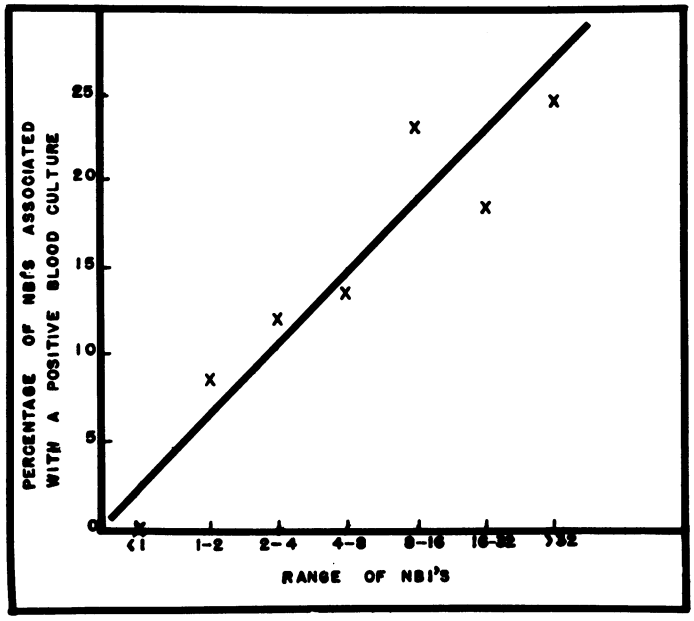

FIG. 4. Relationship of the values of NBI within specified ranges to recovery of positive blood cultures during the 48 hours following determination of the NBI. Note that the risk of having a positive blood culture was directly related to the value of the NBI.

sion was centrifuged, 3 ml. of acetate buffer (pH 3.7) was added to cell pellet, and the cells resuspended. The PMN were disrupted either by sonification or freeze-thawing (6 immersions in an acetone-dry ice bath). When the resulting suspension was centrifuged at 20,000 × G for 30 minutes in a preparative ultracentrifuge, the enzyme remained in the supernatant. MPO levels were measured in a recording spectrophotometer at 460 μ at 25°C. The substrate (0.3 ml. of H_2O_2 and 0.05 ml. of 0.02 M O-dianisidine in methanol) was added to 0.25 ml. of sample and 0.3 ml. phosphate buffer (0.1/M pH 6.0), and the volume adjusted to 3 ml. with H_2O. The final amount of enzyme was expressed as the equivalent per 1×10^6 PMN. The activity was recorded in units, a unit of MPO activity being defined as that activity causing an increase in absorbancy of 0.001/minute.

Intraleukocyte lysozyme determinations were done serially in eight patients with severe burns and four transplant patients, three of whom were also studied during the pre-transplant period while undergoing hemodialysis. The technic was identical to that used by Alexander[1] which is based upon the sample's lytic action on the cell walls of *Micrococcus lysodeikticus* as measured in a spectrophotometer at 540 μ at 25°C for 20 minutes. The results are tabulated in micrograms of lysozyme per 10^7 leukocytes.

Statistical Evaluations

Student's t test was used for all statistical measurements.

Results

Correlation of Neutrophil Function Tests with the Development of Sepsis

Nine hundred forty-nine individual tests for neutrophilic function were performed during 2,822 days of different from the average NBI for nine normal individ-patient observation in 62 patients. Forty-seven episodes of sepsis occurred during the periods of observation, 45 in burn patients and two in transplant patients (Table 1).

The average for all NBI's done on the transplant pauals studied concurrently with 265 tests[4] ($p < 0.01$). tients and on the burn patients (Fig. 2) were significantly Those 33 burn patients who had episodes of sepsis at some time had a significantly higher average NBI than the 15 burn patients who did not have sepsis during their post-burn course ($p < 0.005$). Likewise, the average NBI at the onset of sepsis was significantly higher

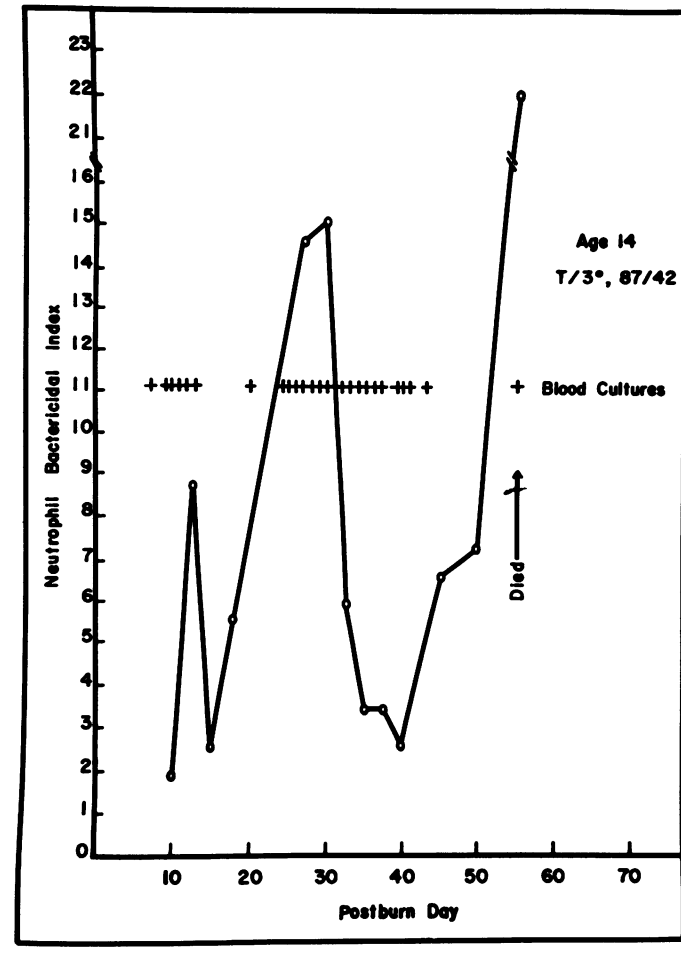

FIG. 5. Serial neutrophil function tests on a 14-year-old boy with an 87% burn (42% 3°). Only positive blood cultures are recorded. Note the cyclic variation of antibacterial function of this patient's neutrophils and the clear association of positive blood cultures with impaired neutrophilic function.

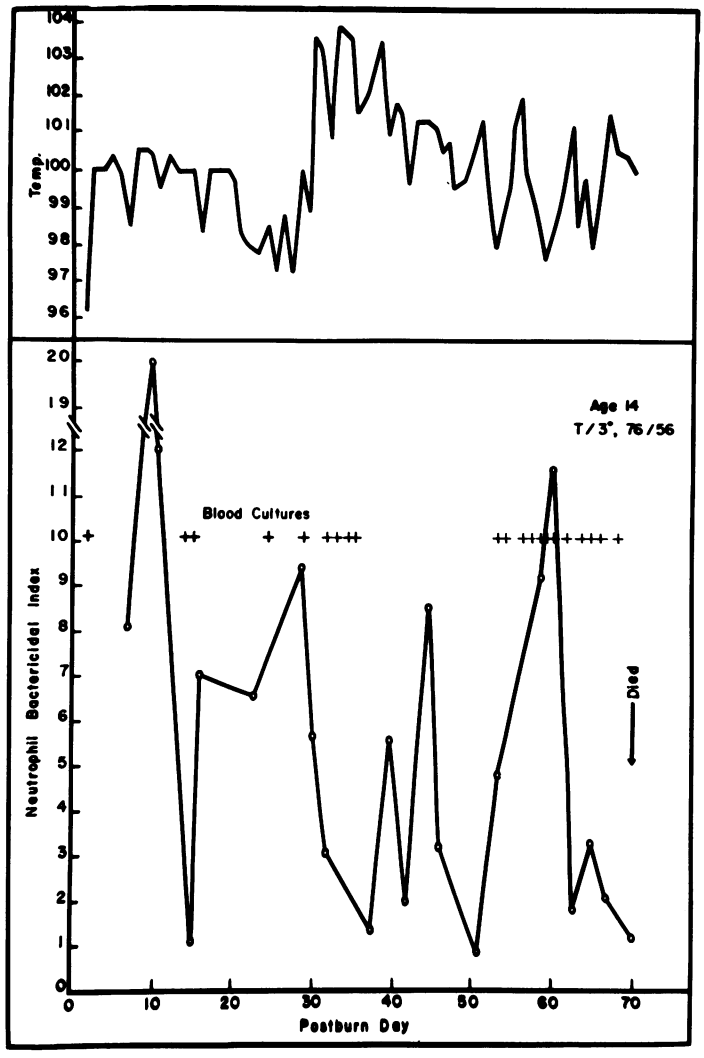

FIG. 6. Three distinct periods of sepsis were observed in this 14-year-old girl with a 76% burn (56% 3°). Each was associated with a relatively poor antibacterial function of neutrophils. It was interesting that positive blood cultures were not encountered when neutrophilic function was relatively better. The cyclic nature of the variation in neutrophil function is well demonstrated in this patient.

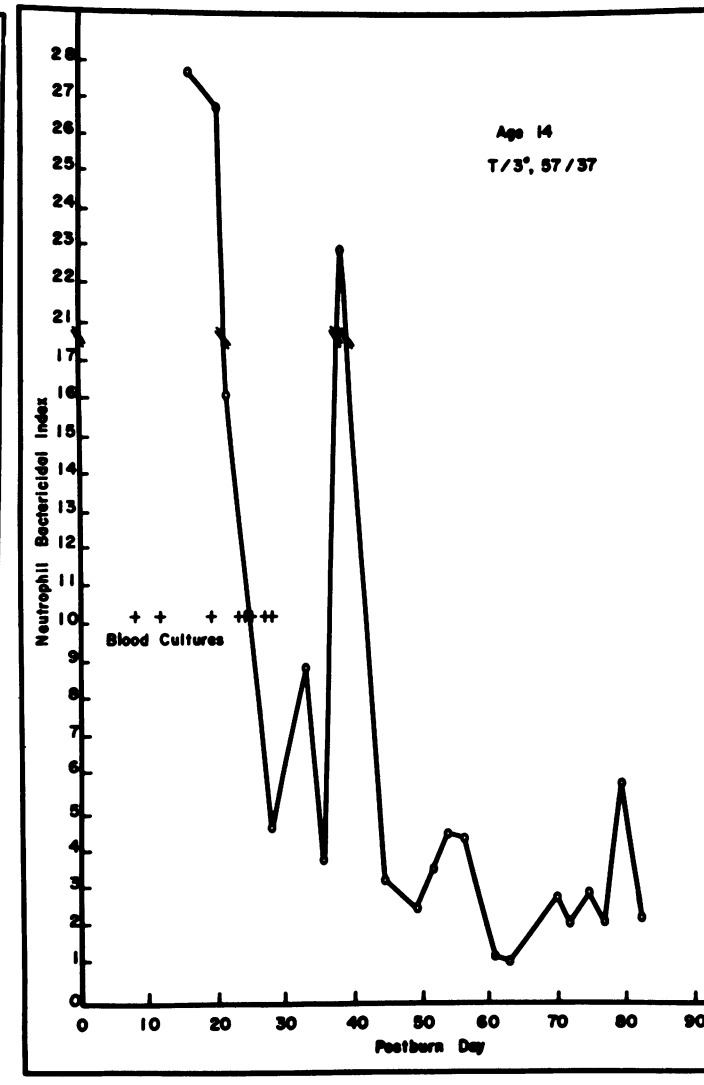

FIG. 7. This 14-year-old girl with a 57% (37% 3°) burn had poorly functioning neutrophils during the early period of observation. With recovery from burn injury, neutrophil function gradually returned toward normal, but the cyclic variation of function persisted. Positive blood cultures were observed only during the early portion of this patient's course.

than the average NBI obtained from those same patients during the entire period of observation ($p < 0.005$). Non-septic burn patients had significantly worse NBI's than transplant patients ($p < 0.001$). Since only two transplant patients developed sepsis, a statistical value for the difference of their NBI's compared to the NBI's of all transplant patients or in the same patients at the onset of sepsis could not be calculated. However, the same trend was seen in the transplant group as was observed in the burn group, relating the importance of the NBI to the development of sepsis.

Since a retrospective analysis of the development of sepsis was made in these patients, the tests for neutrophilic function were not always performed at the exact onset of sepsis, generally being done each Monday, Wednesday, and Friday. However, upon examination of the data, it was apparent that the abnormalities of neutrophilic function preceded the onset of septic episodes rather than followed them, suggesting that sepsis occurred as a consequence of an abnormality of neutrophil function rather than the reverse. The values for the NBI within the 48 hours preceding each episode of sepsis are shown in Figure 3. In our study, sepsis was not associated with NBI's considered to be within the normal range (less than two), and only four of the 45 septic episodes were related to a preceding NBI less than four. Therefore, it appeared that the risk of sepsis in a burn patient was not great until an NBI of four or more was encountered.

Another way of examining the significance of the data

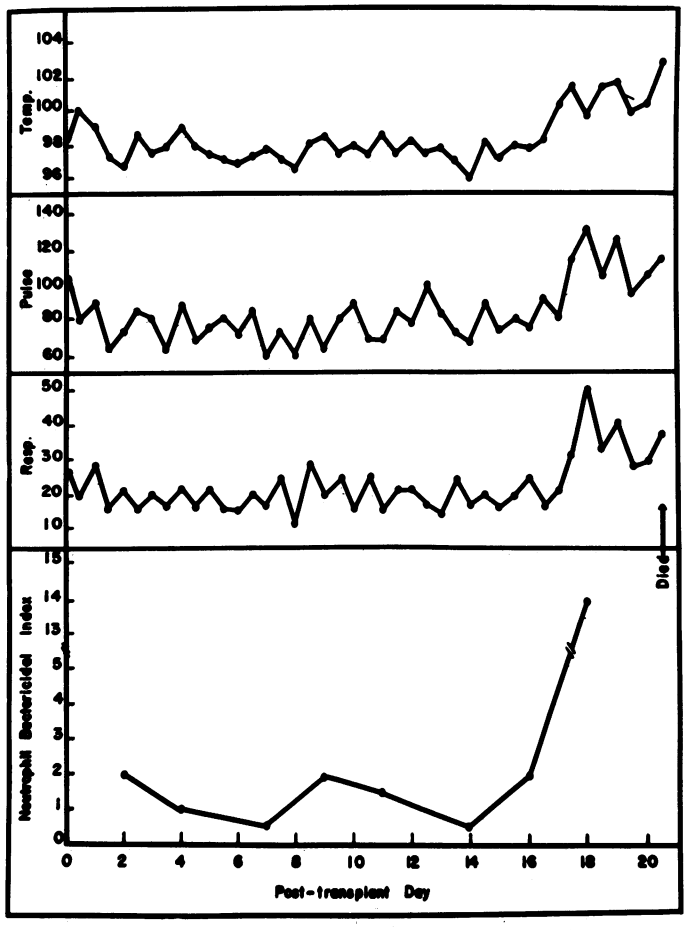

FIG. 8. This 52-year-old woman received a cadaveric renal transplant. Neutrophilic function was within normal range until last determination on the 18th post-transplant day. Concurrent with a rather pronounced abnormality of neutrophilic function, she developed extensive pneumonia from which she died. Note the changes in pulse, temperature, and respiration which accompanied the alteration of neutrophilic function.

was to calculate the percentage of tests within specified ranges of NBI which was associated with a positive blood culture during the 48 hours following the determination of the NBI (Fig. 4). Since positive blood cultures sometimes persisted for a few days after improvement of the NBI, this method of examination was not felt to be as accurate as evaluating specific septic episodes; nevertheless, a striking correlation was noted. Of the 75 positive blood cultures in these patients, none was associated with an NBI which was better than normal, but the risk progressively increased until one-fourth of the NBI determinations greater than 32 were associated with a positive blood culture during the subsequent 48 hours.

Examples of the relationship of the neutrophil-bactericidal index to septic episodes in the burn patients are shown in Figures 5–7. In each of these patients, there was clear evidence of a cyclic variation of neutrophil function. Positive blood cultures often persisted for a brief period after neutrophil function had begun to improve, indicating that the presence of sepsis itself did not adversely affect neutrophilic function. In fact, in none of our studies were we able to demonstrate a significant alteration of the intrinsic cyclic variation of neutrophilic function by any natural event or infectious disease that we have studied. However, improvement in neutrophil function was seen to occur as the burns healed. (Fig. 7).

Considerably less data was available concerning the relationship of abnormal neutrophilic function to sepsis in the transplant patients since only two patients became septic during the period of observation. However, both episodes were associated with a significant abnormality

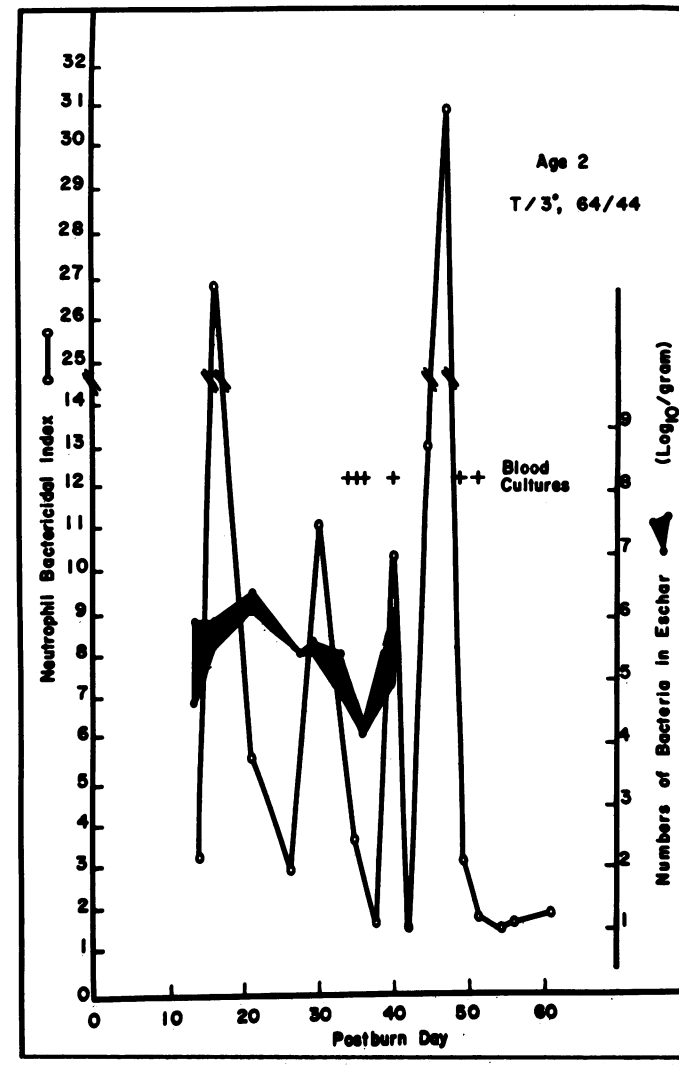

FIG. 9. Quantitative cultures from the burn wounds and measurement of neutrophilic function were made serially in this 2-year-old boy with a 64% burn (44% 3°). Again note that positive blood cultures were associated with preceeding abnormalities of neutrophilic function. The fluctuations in the numbers of bacteria in the burn eschar varied in relation to the cyclic changes in neutrophil function. The scale for the neutrophil-bactericidal index is different from previous figures.

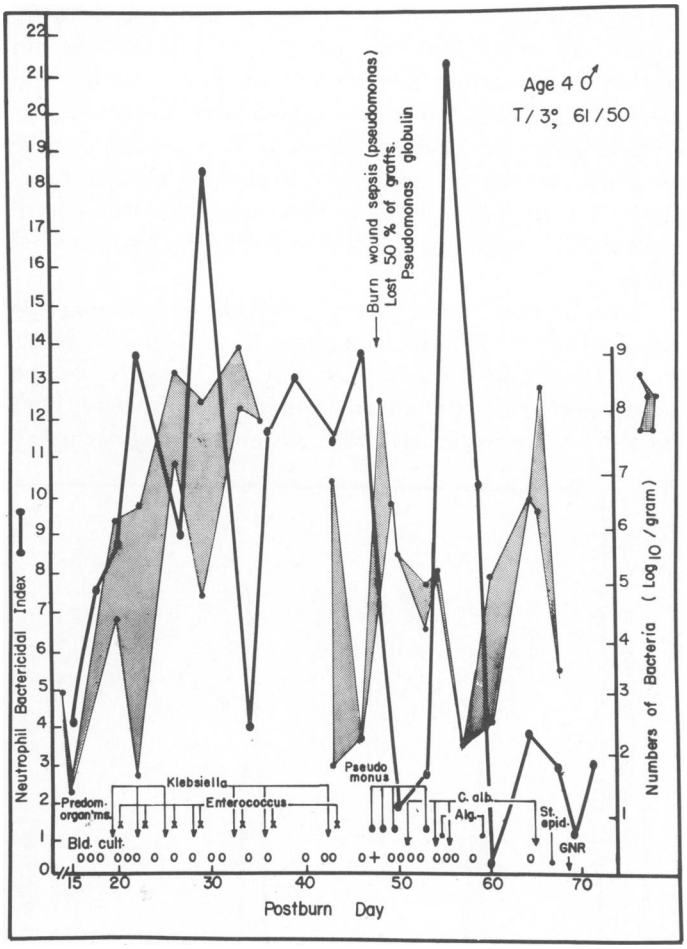

FIG. 10. Burn course of a 4-year-old boy with a 61% burn (50% 3°). The predominant organisms in the burn wound eschar are shown at the bottom of the figure. Only one positive blood culture was recovered, on the 47th post-burn day. It was at this time that he developed classical burn wound sepsis from *Pseudomonas aeruginosa*. While the onset of his septic episode was associated with a markedly abnormal NBI, this returned to normal by the 50th post-burn day, and the patient made a rapid and uneventual recovery. Correlation of the quantitative cultures in the burn wound with the neutrophil-bactericidal index was better during the early post-burn period, possibly because all cultures after the 50th post-burn day were taken from granulating wounds without eschar.

of neutrophilic function, and both patients died of their sepsis. The clinical course of one of these patients is shown in Figure 8.

Correlation of NBI with Quantitative Cultures in Burn Wounds

Continuation of sepsis in burn patients for 1 to 4 days after improvement of neutrophil function was at first perplexing, but determinations of the quantitative wound bacteriology helped to clarify this observation (Figs. 9, 10). Prominent fluctuations in the microbial density in the burn wound eschar occurred in association with deteriorating and improving neutrophilic function. High bacterial counts tended to lag the points of maximal neutrophilic abnormality or improvement by 2–3 days. Adjusting for this lag period, the relationship was statistically significant ($p < 0.01$). Since high bacterial counts in the burn wound act as a potential continuous source for seeding of the blood stream, this lag period could account for many of the positive blood cultures seen during the 2–3 day period following improvement in neutrophilic function. Details of this study are to be published elsewhere,[8] but this summary of findings is being presented to provide substantiating data for our hypothesis.

Separation of Phagocytic Defects from Intracellular Killing Defects

The phagocytosis-intracellular killing index (PIK index) was calculated in all of the studies on the first 42 patients. At no time was there evidence of an intrinsic defect in phagocytosis in either the burn or transplant

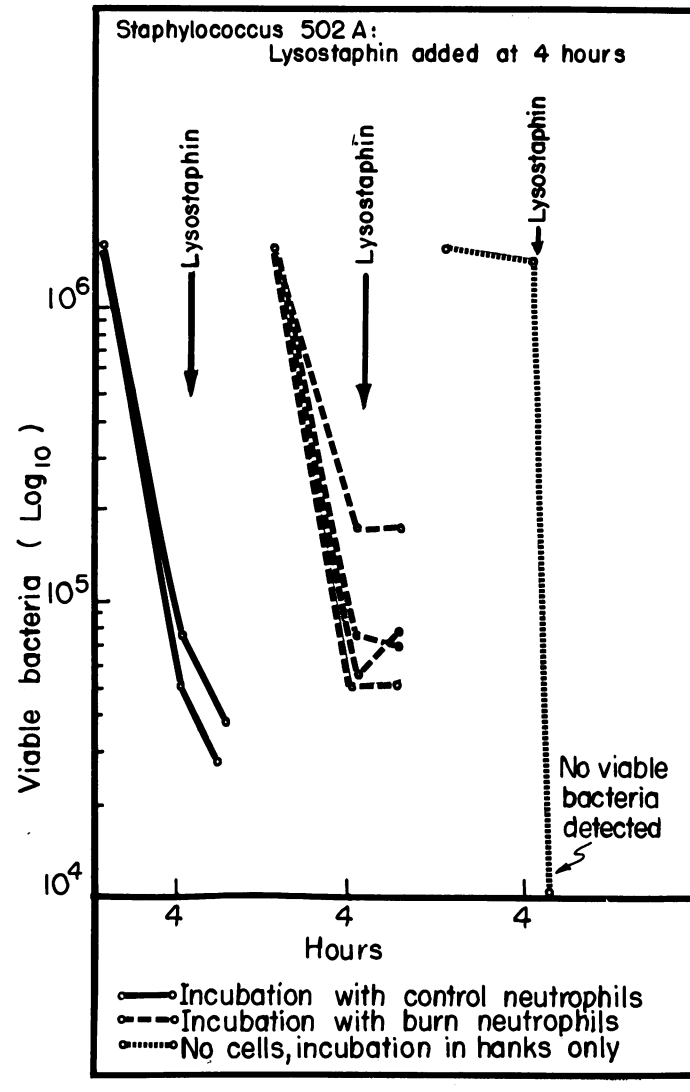

FIG. 11. Neutrophil function with lysostaphin added at 4 hours. See text.

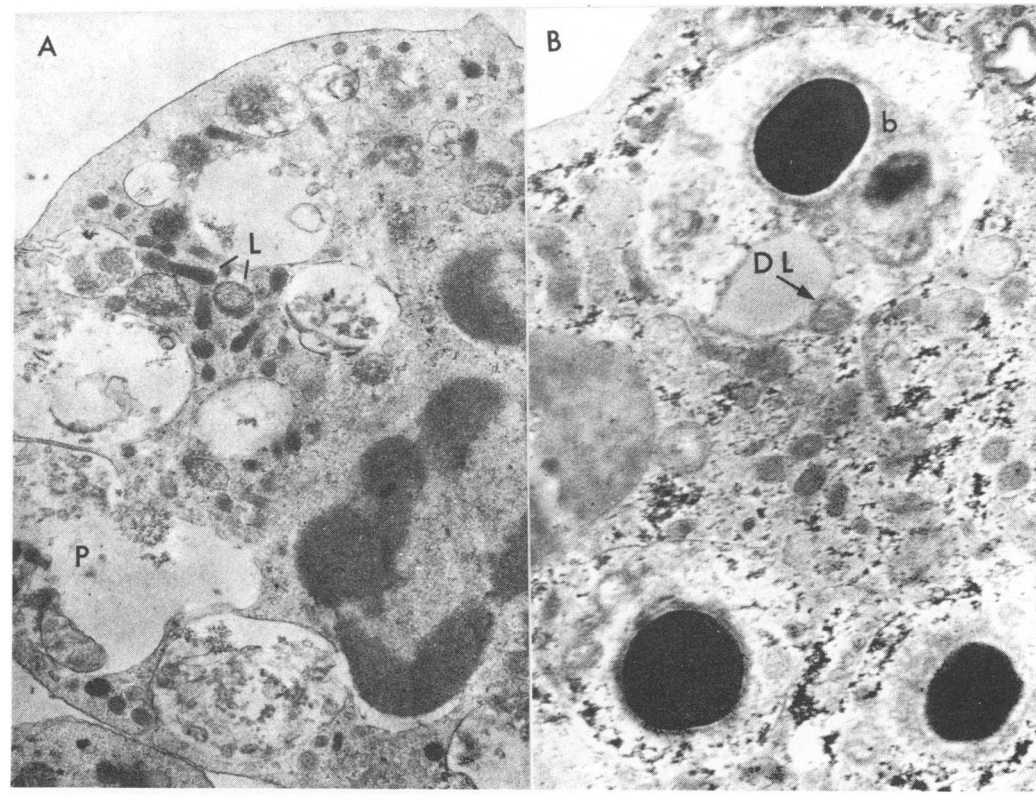

FIG. 12. A. Electron micrograph of a normal neutrophil: P is example of one of several phagolysosomes containing debris of digested bacteria, L indicates 2 types of lysosomes, specific granules on left and azurophilic granules on the right. B. Electron micrograph of a burn neutrophil: b indicates one of two clearly intact bacteria within phagolysosomes, a third and fourth bacterium are tangentially cut. DL represents a recently degranulated lysosome. Mag. 23,500×.

patients. Instead, consistently high values for the PIK index associated with a high NBI indicated that there was rapid phagocytosis with a deficiency of the neutrophil's ability to kill ingested bacteria. PIK indexes were not calculated for the remaining 20 patients since other technics were used to provide confirmatory evidence for the presence of an intracellular killing defect.

The use of lysostaphin to kill extracellular bacteria was another means to provide confirmatory evidence. Each of the seven tests on neutrophils from burn patients showed that the remaining viable bacteria at the end of the 4-hour incubation period were all intracellular whereas one-half of the organisms were extracellular in the control tests (Fig. 11).

In the electron microscopic study, 1,167 neutrophils from control subjects and 2,132 burn neutrophils were examined (Fig. 12). The number of neutrophils with phagolysosomes, as an indicator of phagocytosis, was not different between burn and control. However, 17.4% of the neutrophils from the burn patients contained intact bacteria (22.5/100 cells) compared to 8.2% of the neutrophils from control patients (9.3/100 cells) ($p < 0.001$). Likewise, the number of neutrophils with paired bacteria was significantly higher in burn patients than in controls, 4.2% versus 0.8% ($p < 0.005$). Not surprisingly, the number of neutrophils with intact bacteria and paired bacteria in the burn patients correlated well with the neutrophil-bactericidal index. These observations provide additional strong evidence that bacteria not only survive in the abnormal neutrophils of burn patients, but they multiply intracellularly.[28]

Temporal dynamics of the intracellular killing defect were examined by sequential sampling of the neutrophil-bacterial mixtures performed on four burn patients and two normal controls (Table 2). This series of tests showed that the burn patients killed normally during the first hour of incubation, but subsequently there was no bactericidal activity. In contrast, neutrophils from normal controls continued to kill bacteria for at least four hours.

Multiple Organism Study

Although there was a consistently decreased activity of the burn neutrophils against *Candida albicans,* compared to controls, there were factors in the test which may have made the results unreliable,[25] and the results were deleted from final analysis. Neither the burn nor control neutrophils killed the four different bacteria with the same efficiency, and in all but one patient, the order of increasing bactericidal efficiency was for *Staphylococcus, Pseudomonas, Streptococcus* and *Serratia.* Killing of *Staphylococcus* and *Serratia* appeared to vary periodically in phase with each other, whereas the NBI's for *Streptococcus* and *Pseudomonas* were markedly similar and cycled in phase. The patient shown in Figure 13 was a remarkable exception to this general observation since

TABLE 2. *Bacterial Counts after Incubation Showing Dynamics of the Defect in Burn Neutrophils*

	15 Min Mean ± SE × 10^4	30 Min Mean ± SE × 10^4	60 Min Mean ± SE × 10^4	120 Min Mean ± SE × 10^4	240 Min Mean ± SE × 10^4
Burn	57 ± 6.3	37 ± 4.5	21 ± 3.0	31.0 ± 6.5	22.0 ± 2.8
Control	71 ± 9.2	32 ± 3.7	16 ± 2.0	8.5 ± 0.6	6.6 ± 0.9
Probability	N.S.	N.S.	N.S.	$p < 0.005$	$p < 0.001$

there appeared to be no correlation between the capability of his neutrophils to kill one bacterium with the capability to kill another.

This patient was studied from the 29th to the 67th post-burn day, during which time he had eight positive blood cultures, three with *E. coli*, beta hemolytic *Streptococcus* and *Proteus mirabilus*, two with *Bacteroides*, one with *Staphylococcus* alone and one together with *Bacteroides*, and one with *Staphylococcus epidermidis* which was probably a contaminant. The changes for his NBI for each organism were independent of one another and he frequently presented with a normal bactericidal capacity against one organism or another. When neutrophil function studies were done at a time near the positive blood cultures, the NBI was abnormal for two or more organisms. The positive blood cultures for *Staphylococcus* were associated with a high NBI for *Staphylococcus*, and for *Bacteroides*, with a high NBI for *Staphylococcus, Streptococcus*, and *Serratia*. The blood cultures which were positive for multiple organisms involving *Streptococcus* and *E. coli* were associated with an elevated NBI for *Streptococcus, Pseudomonas* and *Staphylococcus*. The patient ultimately survived.

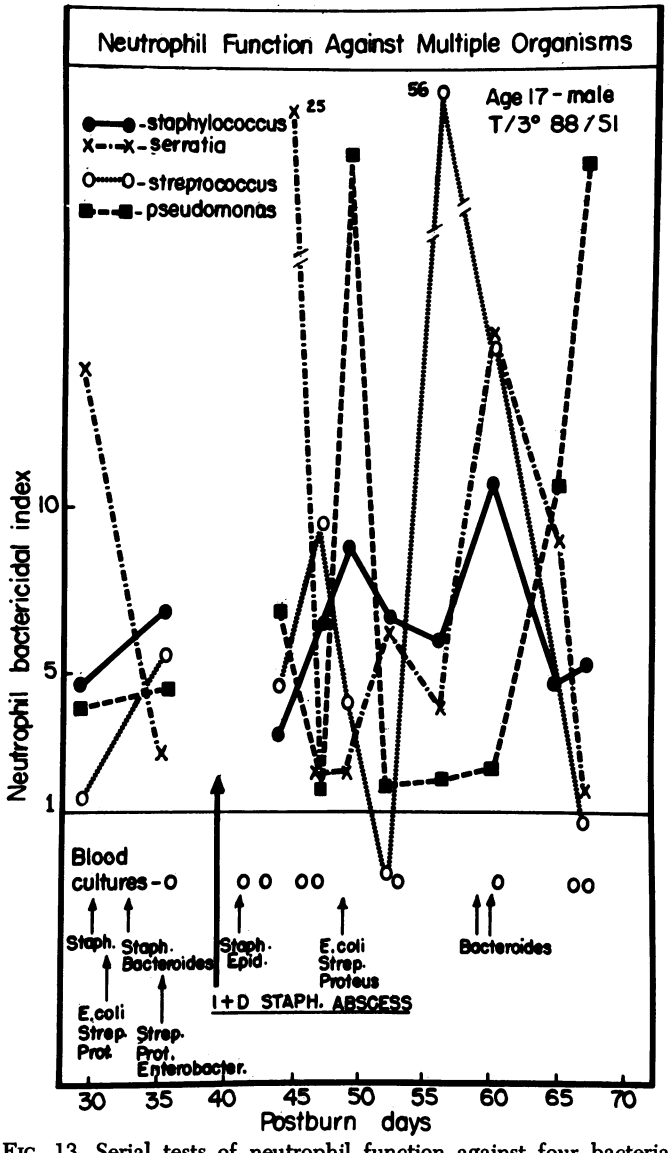

FIG. 13. Serial tests of neutrophil function against four bacteria are shown as indicated for this 17-year-old boy with an 88% burn (51% 3°). Positive blood cultures are listed by organisms at the bottom of the figure whereas negative blood cultures obtained during the same period of observation are indicated by a O. See text for further explanation.

Enzyme Studies

Intraleukocyte lysozyme values and their correlation with the NBI are shown in Tables 3 and 4. Those patients who died of sepsis had significantly lower intraleukocyte lysozyme levels than the patients who did not die of sepsis or the normal controls, and extremely low values of intraleukocyte lysozyme tended to correlate with sepsis in these patients. However, there was no significant correlation of the value of NBI with the intraleukocyte lysozyme level, and it was concluded that variations in the intraleukocyte lysozyme level were not related to the cyclic variation of neutrophil-bactericidal index.

Intraleukocyte myeloperoxidase levels, expressed as units/million neutrophils, averaged 17.94 ± 3.1 units for the controls and 17.38 ± 3.2 for the burn neutrophils.

Discussion

We have previously shown that abnormalities of the ability of neutrophils to kill ingested bacteria are related temporally to the onset of sepsis in humans.[4,6,10] The data presented in this communication confirm and extend these previous impressions, and form the basis for the hypothesis that abnormalities of the antibacterial function of neutrophils are the major reason for the development of opportunistic sepsis in man. It must be emphasized, however, that neutrophilic dysfunction is not the only factor of importance, and numerous im-

TABLE 3. *Intraleukocyte Lysozyme Levels and the NBI in Burn Injury*

Control		Burn Patients															
		Age 14 M (T/3°—51/24)			Age 6 M (T/3°—67/29)			Age 4 M (T/3°—61/50)			Age 15 M (T/3°—52/40)			Age 12 F (T/3°5—4/41)		
Age 30 M Lys	Age 27 M Lys	PBD	Lys	NBI	PBD	Lys	NBI	PBD	Lys	NBI	PBD	Lys	NBI	PBD	Lys	NBI
4.4		8	9.0	3.5												
3.9	9.0	10	9.0	3.8												
14.5	3.1	12	2.2	7.1												
4.0	12.8	15	22.0	6.9												
6.0	12.8	17	4.1	14.7												
11.2	14.0	19	16.2	13.6												
5.6		22	18.0	6.5												
70.0†		24	5.0	12.8												
3.8		26	20.0	4.5												
6.4		29	13.3	10												
		31		25.4												
4.6		33	9.6	2.7	10	11.4	4.7	15	1.5	3.7						
5.3					13	5.4	3.8	18	4.1	7.4						
3.4					15	4.3	4.5	20	1.9	8.6						
7.8					17	6.0	11.5	22	1.9	13.5						
13.3					22	6.2	6.3	27	3.3	8.8						
					24	9.3	9.4	29	3.0	18.3						
5.3					27	7.0	3.5	32	2.5	8.9						
6.0					29	2.9	10.2	34	8.0	3.9						
3.0					31	4.3	3.4	36	4.7	11.5						
3.5					34	14.0	4.4	39	3.0	13						
10.0					38	>10	2.8	43	2.6	11.2						
4.0					41	>10	−1.5	46	3.2	13.6						
			**N.S.			N.S.			$p<0.01$							
16								67	55	1.8	8	28	1.2			
10								69	3.7	1	10	4	3.0			
								71	3.6	3.2	12	1.3	3.0			
								76	11.6	−1.2	17	5.5	2.9			
20								78	60	−2.6	19	1.0				
5.9											26	2.0	23.6	16	5.5	12.9
1.4									‡		29	1.8	1.7	19	1.3	3.6
2.1											31	5.0	3.8	21	1.3	18.9
13.6											33	1.4	3.7	23	1.5	25.3
3.8											36	2.8	3.7	26	1.2	15.6
10.8											40	1.4	3.7	Died		
											$p < 0.05$*			N.S.		

‡ Statistically greater than control, days 67-78, values of 55, 60 unexplained
* Calculated without value 28, which is clearly out of order with respect to other values
† Value of 70, unexplained and excluded from statistical calculation
** Control data used for statistical evaluation are those for the same dates as burn data

munological variables may contribute to the development of infection.[7] Burn injury, in particular, may be associated with a variety of immune defects including abnormal responses to antigens, delayed rejection of grafts, abnormal vascular responses, impairment of delayed hypersensitivity reactions, and diminished uptake of particles by the reticuloendothelial system.[2] Nevertheless, among the immunological variables presently known to contribute to host defense, functional abnormalities of neutrophils appear from our data to be foremost in rendering a patient susceptible to the development of opportunistic infection.

Several investigators have provided additional evidence that acquired abnormalities of the antibacterial function of neutrophils may contribute in a major way to the development of sepsis in man. In a study of the phagocytic and killing capabilities of leukocytes from lymphoproliferative diseases in 1964, Sbarra *et al.*[32] found several patients for whom there was evidence of abnormal intracellular killing which seemed to be related to clinical infections. Later, Rosner *et al.*[30] studied leukocyte function in patients with leukemia with similar findings. In 1969, Coen *et al.*[16] reported that neutrophils of newborn infants had a functional deficiency in almost half of the subjects studied during the first half hour of life, a time of particular susceptibility to sepsis. At the opposite extreme of life, Douglas *et al.*[18] a year later, described a 75-year-old man with a reversible neutrophil bactericidal defect, but their observations may only have been related to the normal cyclic variation in neutrophilic function which we have seen so many times in our patients. They incorrectly suggested that their case was the first reported incidence of a transient neutrophil bactericidal defect, since these had been documented several

TABLE 4. *Intraleukocyte Lysozyme Levels and the NBI in Burn Injury and Renal Transplant Patients.*

Burn								Renal Transplant Patients							
Control		Age 46 F			Age 41 F			Age 24 M		Age 45 M		Age 52 M		Age 48 F	
Age 31 M	Age 25 F	T/3° 33/33			T/3° 75/58			Pre & Post Tx.		Pre & Post Tx.		Dialysis		1 Yr. Post. Tx.	
Lys	Lys	PBD	Lys	NBI	PBD	Lys	NBI	Lys	NBI	Lys	NBI	Lys	NBI	Lys	NBI
9.6					15	2.0	3.9	13.5	2.4						
5	12.2	44	1.3	17.7	18	4.3	6	4.2	4.2	12	3				
10	21	46	1.9	2.9	20	4.7	6.1	5.2	1.5	30.4	1.4				
2	12.2	48	1.3	1.2	22	2.7	5.8	3.2	1	14.5	−1.4				
4.3	12.5	51	1.1	1.7	25	2.1	9.2	9.0	1.6	12.5	1.2				
5.3	9.6				27	3.2	3.1	3.2	1.5	13.6	1.8				
3.5	12.8				29	1.6	—	6.4	—	16.2	—				
6.0	13.2				32	3.2	—	Transplant		13.5	—				
4.0	12.7							5.1	1.3	—	1.4				
—	12.0							2.9	−1.6	13.3	−2.5				
—	10.8	$p < 0.005$			$p < 0.001$			3.4	2	14.3	−1.3				
5.6	—							3.3	7.1	—	3	12.4	6.4		
4.2	13.5							2.9	2.5	7.5	2.8	6.2	1		
										Transplant					
	15.2								2.1	14.5	−1.3	19.8	−6.9		
	14.0							2.5	10	15.2	4.5	14.0	1.6		
	9.0							1.6	2	10	1	12.7	−3.7		
5.4	15.2							3.4	1.0	8.0	1.2			15	−1.3
3.5	8.7							3.2	1.5	5.6	1.4	13.5	−2.3	13.1	1
	17.0							4.0	1.1	13.5	1.5			13.0	−1.3
3.3	11.2							5.8	1.1					15.0	1.5
3.3								4.4	1.6	14.8				10.0	1.1
								N.S.*		N.S.†		N.S.		N.S.	

* The pre- and post transplant means are 6.4 and 3.5 respectively and are not significant at the 0.05 level.
† The pre- and post-transplant means are 14.7 and 10.5 respectively and are not significant at the 0.05 level.
N.S. Not significant

times previously.[6,9,32] In 1971, McCall *et al.*[26] studied the function of human neutrophils from patients with severe infections and inflammation. They found that neutrophils sometimes had a decreased ability to kill *Staphylococcus albus*, but sequential studies on their 11 patients apparently were not made. Not all patients were abnormal implying that infection *per se* was not the source of the abnormality. Our observation that abnormalities of neutrophilic function are related to the development of sepsis has most recently been supported by Grogan *et al.*[19] who used *Pseudomonas aeruginosa* as the test organism to study patients with burn injuries and these receiving major non-thermal trauma. In contrast, Balch [13] was unable to demonstrate a neutrophil-bactericidal defect in burn patients, but it is likely that his choice of technics for examining the variable was responsible for his inability to uncover significant abnormalities.

The experimental data demonstrating that a phagocytic defect is unusual in burn or transplant patients are conclusive. These data include the PIK indices, lysostaphin studies, electron microscopic studies, sequential myeloperoxidase studies,[10] and the observation that bacteria are phagocytized and killed by burn neutrophils at a normal rate for the first hour without further killing. The host abnormality obviously has resulted from a defective intracellular bactericidal activity. The reasons for this abnormality are not clear, but the rates of degranulation in burn neutrophils estimated from a previous study [10] and in the electron microscopic study reported herein and elsewhere [28] appeared to approximate the rate in normal persons, suggesting that the defect was related to an abnormality of lysosomal enzymes.

Several bactericidal mechanisms have been found to be of importance in the neutrophils of man.[23] Chronic granulomatous disease of childhood has been the most completely studied of the several hereditary conditions associated with abnormalities of neutrophilic function.[36] However, the precise enzymatic basis for the defect is still controversial although it seems to be related to a deficiency of the generation of hydrogen peroxide within the leukocyte.[23] Strauss and co-workers [33] have demonstrated that a myeloperoxidase-hydrogen peroxide-chloride system is an important antibacterial mechanism, but Klebanoff and Pincus [24] have suggested the possibility of a microbicibal control mechanism in which a deficiency of myeloperoxidase activity is offset by an increase in non-enzymatic activity of intraleukocyte hydrogen peroxide. If the MPO system is involved in the bactericidal defect of the burn neutrophil, it must be related to some other component of this pathway, since intraleukocyte MPO levels in the burn neutrophils were found to be identical to controls.

Lysozyme has been felt to provide another potential

mechanism of intraleukocyte antibacterial activity.[23] Since neutrophil lysozyme levels had previously been found to be low in burn patients,[1] they were studied more critically in conjunction with tests of neutrophil function and with the exception of one patient, no correlation between intraleukocyte lysozyme levels and neutrophil function could be established. Two patients had normal or greater than normal mean lysozyme levels despite periodically poor neutrophil function, yet their post-burn courses were smooth and not complicated by septic episodes. One patient who had lysozyme levels done during two periods separated by 3 weeks, developed serious Pseudomonas sepsis (Fig. 11), and lost most of his skin grafts in the period when his intraleukocyte lysozyme levels were significantly lower than normal. During the second period, as his burn healed, the levels were strikingly higher and above normal. Leukocyte lysozyme levels were significantly lower in the patients who died with sepsis than in those who did not. From these few patients, it is difficult to draw definitive conclusions, but it would appear that intraleukocyte lysozyme levels have predictive value for the outcome of the burn patient's clinical course.

Zeya and Spitznagel fractionated polymorphonuclear leukocytes from guinea pig[37] and rabbit[38] peritoneal exudates, and a series of lysosomal components could be eluted after electrophoresis on cellulose acetate paper which showed species specific antibacterial activity distinct from lysosomal enzymes. Welsh and Spitznagel[35] more recently separated homogenates of human neutrophils into different fractions, demonstrating that the lysosomes of human neutrophils comprise at least three and possibly four distinct cytoplasmic particles. Cationic proteins were associated with lysozyme and beta glucuronidase containing granules, but antibacterial activity was present in four of the five cell fractions examined. Unfortunately, quantitative methods for measurement of the specific cationic antibacterial proteins have not been described, and these data for burn neutrophils are not available. When this methodology becomes available, resolution of the specific antibacterial deficiencies will be possible and thereby insight into their ultimate control facilitated.

The cyclic pattern of neutrophil function described earlier[4] has been confirmed in this study. The discovery of this cyclic variation in neutrophil function not only explains the unexpected occurrence of infection, but also periods of unexpected resistance. The rhythmic nature of the observed cycle suggested that it may have endocrinological control. A series of experiments in laboratory animals have demonstrated that the cyclic variation is not regulated by the pituitary gland, adrenal glands, gonads, or pineal body,[3] but other endocrinological control mechanisms have not been excluded.

The observation that the neutrophils showed cyclic deficiencies in their ability to kill different types of organisms with abnormalities which did not coincide was at first both surprising and disconcerting. The inoculum reduction of each species by burn and control neutrophils had a consistent range, but there were variations between bacterial species of as much as a full $\log_{10}$ when tested against the same neutrophils. Burn neutrophils showed a consistent bactericidal defect against *Staphylococcus* and *Serratia*, demonstrating cyclic patterns of dysfunction which were in phase in four patients. Burn neutrophil-bactericidal activity was less consistent against *Streptococcus* and *Pseudomonas* and was occasionally normal, although the normal intervals were interrupted by cycles of startling bactericidal inefficiency, which were in phase in three patients. It is the lack of correlation between the different organisms which should be emphasized since it may be important in delineating mechanisms responsible for the leukocyte abnormality. These observations add to the evidence that neutrophils have several bactericidal pathways which may be specific for different bacteria.[23,35,37,38]

Bell and co-workers[14] have studied the antibacterial activity of leukocytes which were derived from inflammatory exudates following dermal abrasion. They demonstrated that phagocytosis and killing of staphylococci and the intracellular contents of lysozyme and cathepsin were not significantly different from neutrophils obtained from peripheral blood. Therefore, they suggested that normal leukocytes did not undergo functional alteration during tissue mobilization. Their findings help to explain the 2–3 day lag period before quantitative cultures of the burn wound reflected the changes in neutrophil function since neutrophils are end cells with a life span of about 3 days. Thus, neutrophils entering the burn eschar or other inflammatory focus could be expected to exert functional characteristics of that population for an additional 2–3 days when they would be replaced by a new population of neutrophils.

From these studies, it is obvious that burn and transplant patients were more apt to acquire life-threatening infections when their neutrophilic function was abnormal than when it was normal, and the risk of infection was directly related to the degree of abnormality of neutrophilic function. The observations are of particular significance to burn patients because of their probable relationship to the pathophysiological mechanisms responsible for systemic invasion by flora in the burn wound. The neutrophil has the primary responsibility for localization of infection in the acute inflammatory process of the burn wound. The periodic defects in neutrophilic-bactericidal function which allow prolonged intracellular survival, the clear ability of bacteria to multiply intracellularly where they are protected from

antimicrobial drugs and other phagocytes, and the ultimate release of viable bacteria upon death of the neutrophil, help to explain the sudden or unexpected development of sepsis in these patients.

Since the cyclic variation of neutrophilic function has been found to be a universal phenomenon, and resistance to infection is clearly related to the efficiency of bacterial killing by neutrophils, it is likely that most opportunistic infections in man are associated with physiologic or acquired abnormalities of neutrophilic antibacterial function. It is evident that further investigation into the biochemical pathways and regulatory mechanisms of this important process will be necessary before its manipulation can be brought to therapeutic advantage.

Summary

Physiologic variations in neutrophilic function occur normally in man, having a cyclic rhythmicity with a period approximating 14–24 days. Acquired abnormalities of neutrophilic antibacterial function, such as those which have been extensively documented in burn and transplant patients, are superimposed upon the physiologic abnormalities associated with the cycle causing periods when neutrophilic function, as expressed by the NBI, is extremely poor. Regardless of the basis for the defects, increasing abnormalities of neutrophil function have been significantly associated with increasing susceptibility to the development of life-threatening sepsis. The control mechanism for the periodic function has not yet been elucidated. In the acquired abnormality, however, leukocytes phagocytize normally and degranulation appears to be intact, thus implicating one or more intraleukocyte bactericidal mechanisms, notably the enzymes contained in lysosomal granules. Within neutrophils, there are a multiplicity of bactericidal mechanisms with different specificities, and bactericidal efficiency for four separate organisms were observed to vary at different times. This observation suggests that the mechanisms are extremely complex, and an abnormality of any single intraleukocyte bactericidal mechanism can not be expected to explain the spectrum of acquired abnormalities which have been observed. Bacteria have been noted to multiply within burn neutrophils, associated with an arrest of the bactericidal mechanisms after one hour, to produce an intracellular infection. While inside leukocytes, the bacteria are protected from the lethal or inhibitory effects of antibiotics.

From our clinical and laboratory observations during the past five years and from the supporting evidence provided by other investigators, we advance the hypothesis that abnormalities of neutrophilic function appear to be the most important variable of immunological defense relating to the development of opportunistic infections in man.

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Discussion

Dr. Basil A. Pruitt, Jr. (San Antonio): We at the Army Burn Unit have been interested in these same changes, because sepsis continues to be the most common cause of death in our burn patients. We have followed the changes in circulating granulocytes in a laboratory rat burn model, both infected and uninfected; and following early demargination in the uninfected burn, total circulating granulocytes return to approximately normal levels. However, with the infected model there is a persistent granulocytic defect.

If one further looks, as shown on the following slide (slide), at both the dividing and the nondividing compartments, one can see that in the uninfected animal the subsequent restitution of granulocytes is due to a marked increase in the dividing compartment, whereas in the infected animals there is a persistent decrease in both the dividing and the nondividing compartments.

I ask Dr. Alexander: Can you separate out a specific granulocytic defect from the effect of a progressively older average aged leukocyte?

President Moore: I would like to ask Dr. Alexander a question. Has he examined the effect of antibiotics themselves on this particular leukocytic activity?

Dr. J. Wesley Alexander (Closing): To answer Dr. Pruitt's question first, we have not been able to relate the apparent age of neutrophils to their function, although we do not feel that we have been able to study this with sufficient clarity to really define whether or not abnormalities may be associated with immature granulocytes.

There have been some studies reported in the past in patients with myelocytic leukemias in which there is evidence that stages before the metamyelocyte are inefficient in bacterial killing, but once the stab form is reached, they can kill efficiently.

In our studies on either burn patients or controls we have not been able to establish a relationship between segmented and nonsegmented ratios and their efficiency of bacterial killing.

To Dr. Moore's question about the effect of antibiotics on this important mechanism, we have done several studies in experimental animals which would indicate that antibiotics, in themselves, do not cause an inefficiency of killing by the neutrophils. However, they do cause many other problems, and we feel that the use of prophylactic antibiotics in patients who are susceptible to opportunistic infection should be rigidly avoided.