Further Characterization of the Impaired Bactericidal Function of Granulocytes in Patients with Poorly Controlled Diabetes

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SUMMARY

Granulocytes of patients with diabetes mellitus have an impaired capability to engulf bacteria, but it is not clear whether subsequent intracellular killing, which has separate energy sources, is also defective.

We separately assayed engulfment and intracellular killing of *Staphylococcus aureus* 502a by granulocytes of 17 diabetic patients with fasting hyperglycemia to better characterize the phagocytic defect. Diabetic granulocytes engulfed a smaller proportion than controls of a 10^6 inoculum of bacteria after 20 minutes of incubation in vitro (56.8 ± 9.4 per cent versus 72.4 ± 3.6 per cent, mean ± S.E. of 10 patients and paired controls, $p < 0.05$), but after 60 minutes of incubation this defect had disappeared. Intracellular killing of staphylococci by granulocytes from seven diabetics (68.6 ± 8.9 per cent of a 10^6 inoculum) was less ($p < 0.01$) than that of paired controls (80.3 ± 4.5 per cent) after two hours of incubation. Seven patients were retested during a period of improved diabetes' control; intracellular killing of staphylococci by granulocytes of six of the seven increased considerably and either exceeded the paired control value or approached it to within 75 per cent.

These data suggest that a primary defect exists in intracellular killing of staphylococci by granulocytes from poorly controlled diabetics in addition to that previously shown in engulfment. This bactericidal activity becomes more efficient when the diabetes is brought under better control. DIABETES *27*:889-94, September, 1978.

Polymorphonuclear leukocytes provide man's first line of defense against bacterial infection. These cells accumulate at sites of bacterial entry, where they efficiently phagocytose invading microorganisms. The complex processes that lead to engulfment and killing of bacteria by granulocytes have been the object of intensive investigation in recent years. As a consequence of these efforts, several conditions have been identified in which a tendency toward recurrent bacterial infections is associated with defective granulocyte function.[1-3]

Diabetes mellitus is one such disorder shown to be associated with altered granulocyte function.[4-9] Moreover, patients with diabetes often have bacterial infections that are difficult to eradicate, and these complicate the clinical management of the diabetic state. Since evidence is increasing that rigorous antidiabetic treatment may decrease the frequency and severity of many complications of diabetes mellitus,[10-12] it is important to establish whether the disturbances in granulocyte function also are favorably influenced by assiduous control of the diabetes.

Experimental techniques employed in past studies of granulocyte function in diabetes mellitus have not adequately differentiated between the two sequential, independent, phagocytic processes of bacterial engulfment and intracellular killing. Consequently, it is not firmly established that the observed defect in bacterial killing by diabetic granulocytes[6] is primary or secondary to impaired engulfment. This study was designed to characterize more precisely the granulocyte functions of bacterial engulfment and intracellu-

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lar killing in diabetic patients as well as to determine the effects of improved diabetes' control on these parameters of granulocyte function.

MATERIALS AND METHODS

Definitions. In this study the term phagocytosis refers to the entire process of granulocyte interaction with bacteria. The term engulfment describes the phase of the phagocytic process in which bacteria are internalized by the granulocyte, and intracellular killing defines the process that results in the death of engulfed bacteria.

Patients. Seventeen ambulant, unhospitalized, diabetic patients (nine men and eight women—median age, 50 years; range, 23 to 60 years) with fasting hyperglycemia (fasting glucose concentration in serum: mean, 288.2 mg. per deciliter; range, 188 to 452 mg. per deciliter) and no evidence of ketoacidosis, infection, renal disease, or any other diabetic complication were studied after giving their informed consent. Nine patients were receiving oral hypoglycemic drugs while eight were taking insulin. On a day of study, granulocytes from one patient and a normoglycemic control subject were tested simultaneously for phagocytic activity against a strain of *Staphylococcus aureus* 502a. Patients and controls were matched for sex and race but not for age.

Experimental design. In order to obtain maximum information about the phagocytic activities of granulocytes, two separate assay systems were utilized. Granulocytes from seven diabetics and their controls (group 1) were incubated with the test bacteria for two hours to determine their maximum capacity for engulfment and intracellular killing. Engulfment was also assessed by microscopic examination of stained smears of incubating mixtures of leukocytes and bacteria from five of these seven patients and controls.

Granulocytes from the remaining 10 patients and their controls (group 2) were tested at earlier intervals to compare the kinetics of the engulfment phase. According to this protocol, mixtures of granulocytes and bacteria were sampled at intervals during one hour of incubation, and engulfment was quantitated as described below.

Seven patients, three from group 1 and four from group 2, agreed to more intensive management of their diabetes for a period of two to three weeks after the test of granulocyte function. Improved control was achieved by increasing the dose of insulin or hypoglycemic agent; no attempt was made to alter the diets of these ambulatory patients. The response to manipulation of therapy during this period was monitored by determination of the glucose concentration in 24-hour urine specimens collected in fractionated portions.

Granulocyte function studies were repeated during this period. Conditions for the repeated assays were identical to those used initially for each patient; sampling times remained the same, and in every case the individual who donated granulocytes for control of the initial study also served as the control for the repeated assay.

Preparation of granulocytes. Leukocytes were separated from heparinized fasting venous blood by dextran sedimentation,[1] washed twice in calcium-free and magnesium-free Hank's balanced salt solution (from BBL, Cockeysville, Md.), and suspended in Hank's balanced salt solution (BBL), pH 7.4, supplemented with glucose and gelatin (100 mg. per deciliter each) (supplemented HBSS). The concentration of viable granulocytes in these suspensions was adjusted to 5×10^6 per milliliter; granulocytes regularly comprised about 80 per cent of all suspended leukocytes.

Preparation of serum. Human serum was used in the assay system as a source of opsonin. Aliquots (2 ml.) of serum from a single donor of blood type AB were stored for no more than three weeks at $-20°$ C. On a day of study, an aliquot was thawed and diluted with 6 ml. of supplemented HBSS.

Preparation of bacteria. An inoculum of the test strain of bacteria (*S. aureus* 502a, courtesy of Dr. S. J. Klebanoff, Seattle) was cultured in trypticase soy broth at 37° C. for four hours, washed twice in 0.9 per cent NaCl, and suspended in supplemented HBSS. A concentration of about 10^7 viable staphylococci per milliliter was achieved by diluting five times a suspension of bacteria equal in turbidity to that of a standard suspension of barium sulfate.[13]

Incubation of leukocytes and bacteria. Mixtures of leukocytes and bacteria were prepared by adding 0.8 ml. of the leukocyte suspension from a patient or control, 0.2 ml. of the bacterial suspension, and 1 ml. of diluted serum to disposable plastic tubes (12 × 75 mm.). These mixtures, thus, consisted of about 2.5×10^6 granulocytes and 1×10^6 bacteria per milliliter and 10 per cent serum. They were incubated in duplicate at 37° C. and rotated slowly end over end. Additional tubes were incubated without granulocytes to control for serum bactericidal activity against the test organism.

Quantitation of engulfment and intracellular killing. At intervals prescribed by the experimental design,

aliquots (0.5 ml.) were removed from incubating mixtures of leukocytes and bacteria for determination of engulfment, or intracellular killing, or both. Intracellular killing during a stipulated interval was equal to the difference between the initial bacterial inoculum (determined by pour plating[1] of serial dilutions obtained immediately after mixing leukocytes, bacteria, and serum) and the total number of bacteria that remained viable (also determined by pour-plate counting) in the aliquot sampled at the proper time.

To determine engulfment, it was necessary to count the number of viable bacteria within the granulocytes. This was achieved by a previously published technique[14] that included separation of the granulocytes from extracellular bacteria by sedimentation; brief incubation of the separated granulocytes in a solution containing penicillin and streptomycin to kill adherent, extracellular bacteria; and, finally, lysis of granulocytes in distilled water and pour-plate counting of the liberated bacteria. Engulfment was equal to the number of viable bacteria that remained within the granulocytes added to the number that had been killed by the granulocytes.[14]

Engulfment was observed microscopically on additional preparations of granulocytes and bacteria from five patients and controls from group 1. For each of these subjects, three such mixtures were prepared: each contained 1×10^6 granulocytes and sufficient bacteria to yield ratios of granulocytes to bacteria of 1:1, 1:2, and 1:10. These mixtures, in supplemented HBSS with 10 per cent serum, were incubated for 30 minutes with slow tumbling. After that time, cover slip smears were made and stained with Wright's reagents. One hundred consecutive, unclumped granulocytes were identified on each smear; the numbers containing no bacteria, one or two bacteria, and more than two bacteria were recorded.

Statistical analyses. Values obtained for engulfment and intracellular killing by diabetic granulocytes were compared with those of paired controls at each time interval by use of the one-tailed signed-ranks test.[15] The correlation coefficient for the relation between intracellular killing and serum glucose concentration was derived by linear regression analysis using the method of least squares.[16]

RESULTS

The progressive intracellular killing of *S. aureus* 502a by granulocytes from the seven diabetic patients in group 1 is compared with that of controls in figure 1. The figure shows that diabetic granulocytes killed fewer bacteria after 60 and 120 minutes of incubation than did control granulocytes. In addition, it was found that, among individual diabetics in group 1, there was a trend toward a negative relationship between the serum glucose concentration at the time of study and intracellular killing of *S. aureus* at 120 minutes ($y = 149.3 - 0.29x$, $r = -0.649$, $0.15 > p > 0.10$). This was an interesting observation in view of the fact that the patients' granulocytes were separated from their serum and were tested in vitro in the presence of a physiologic glucose concentration (100 mg. per deciliter).

Engulfment was also determined on these mixtures of bacteria and granulocytes from the patients in group 1 after 120 minutes of incubation. Granulocytes from the diabetic patients engulfed 89.9 ± 2.9 per cent (mean ± S.E.) of the initial inoculum; the corresponding control value was 87.7 ± 3.1 per cent. Thus the impairment in intracellular killing manifested by diabetic granulocytes at 120 minutes was not secondary to decreased engulfment at that time, since the latter function was equal to that of controls.

On the other hand, assessment of engulfment in stained smears prepared after 30 minutes of incubation suggested that, during this interval, engulfment by diabetic granulocytes was defective (figure 2). At bacteria-granulocyte ratios of 2:1 and 1:1, staphylococci were engulfed by fewer diabetic granulocytes than by those from controls ($p < 0.05$, signed-ranks test). In preparations containing 10 bacteria per granulocyte, virtually all granulocytes from both patients and controls contained numerous bacteria.

The bacterial engulfment process of diabetic granulocytes proceeded to completion somewhat differently from that in controls (table 1). Evaluation of preparations incubated for 10 minutes was unsatisfactory by this technique, as there was wide variability in engulfment among the control group (range, 0 to 79 per cent). However, after 20 minutes of incubation, engulfment of *S. aureus* by control granulocytes was reliably measured, and a significant reduction was observed in the proportion of bacteria engulfed by diabetic granulocytes. These values confirmed the microscopic assessment of engulfment (figure 2), which demonstrated a similar impairment during the early stages of incubation of bacteria and diabetic granulocytes. It is notable, however, that, after 60 minutes, engulfment by patient's cells was equal to that of controls.

More intensive diabetic treatment was associated with a significant ($p < 0.01$) reduction in fasting glucose concentrations (table 2). During this period,

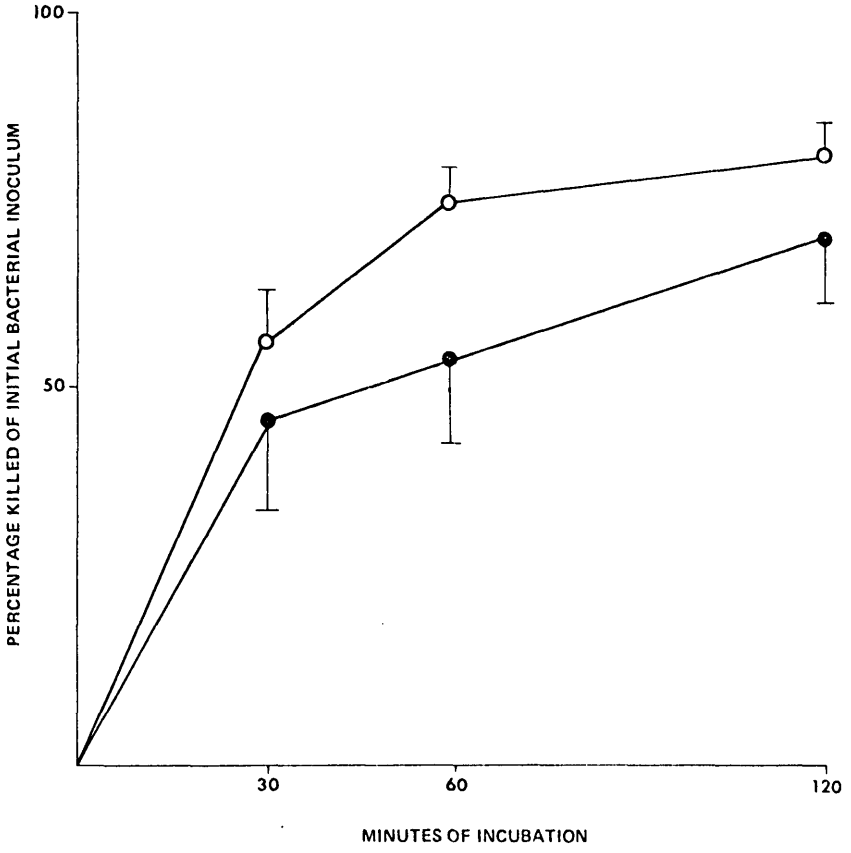

FIGURE 1

Intracellular killing of *S. aureus* 502a by granulocytes of diabetic patients. The semibracketed points are means ± S.E. for the seven patients in group 1 (closed circles) and their controls (open circles). The differences between diabetic and control responses are significant at 60 minutes ($p < 0.03$) and 120 minutes ($p < 0.01$). The initial bacterial inoculum was roughly 1×10^6 bacteria per milliliter, and the ratio of granulocytes to bacteria was 2.5 to 1.

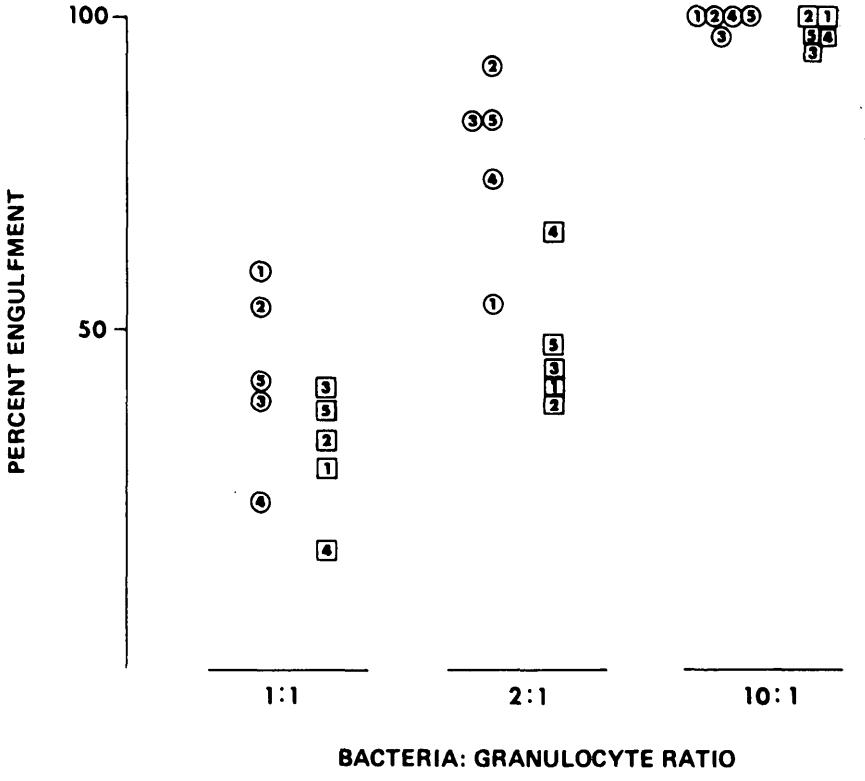

FIGURE 2

Phagocytosis of *S. aureus* 502a by granulocytes of diabetic patients. The boxed numbers identify values for five diabetic patients from group 1; the paired control of each is identified by the corresponding circled number. Per cent engulfment refers to the percentage of granulocytes that contained more than two bacteria upon microscopic examination of stained films prepared after 30 minutes of incubation at the stipulated bacterium-granulocyte ratios. The responses of the two groups are different ($p < 0.05$) at 1:1 and 2:1 bacteria-granulocyte ratios.

TABLE 1

Engulfment of *S. aureus* 502a by granulocytes of diabetic patients in group 2*

Time of incubation (min.)	Percentage engulfed of initial bacterial inoculum† Diabetic granulocytes	Control granulocytes	P
20	56.8 ± 9.4	72.4 ± 3.6	0.05
60	88.1 ± 4.4	86.8 ± 4.2	N.S.‡

*Initial bacterial inoculum was 1×10^6 bacteria per milliliter, and the ratio of granulocytes to bacteria was 2.5 to 1.

†Mean ± S.E.

‡Not significant.

granulocyte function improved in six of seven diabetic patients ($p < 0.03$, one-tailed signed-ranks test). The four patients (patients 5, 6, 11, and 14) who had initial results that were considerably poorer than their controls showed striking increases in granulocyte intracellular killing during the period of improved control. Of the four patients (those from group 2) who also had engulfment assessed before and during the period of more intensive therapy, three improved during the latter period. Patient 10, who sustained a modest decline in intracellular killing, also had a decrease in engulfment upon retesting.

DISCUSSION

Results of this study show that granulocytes from ambulant, unhospitalized diabetics with fasting hyperglycemia have an impaired ability to both engulf and kill opsonized staphylococci. These defects are modest; nevertheless they appear to improve after more intensive diabetes' management and reduction of fasting glucose concentrations. Techniques employed in previous studies showing impaired function of granulocytes from diabetic patients[5,6] have failed to distinguish whether the defect observed in intracellular killing was primary or was caused by delayed engulfment of the test organism. One other study found primary defects in both engulfment and intracellular killing in diabetic granulocytes,[9] but many of the patients studied had clinical evidence of infection and it is known that infection itself may impair granulocyte function.[17] This problem was circumvented in the present experiments because granulocytes were obtained from diabetic outpatients who were otherwise healthy and free of infection.

TABLE 2

The effect of improved control of serum glucose concentration on intracellular killing of *S. aureus* 502a by granulocytes of diabetic patients

Patient	Serum glucose concentration (mg./dl.) A*	B†	Intracellular killing A	B
2	315	245	78‡	90‡
5	316	242	0	76
6	350	240	35	107
8	226	190	103	142
10	270	215	98	86
11	284	230	44	89
14	288	186	53	106

*A = Initial study.

†B = Study performed during period of more intensive management (see text).

‡Number derived by comparing the proportion of initial bacterial inoculum killed by diabetic granulocytes with that proportion killed by the paired control during a 60-minute (patients 8, 10, 11, 14 from group 2) or 120-minute (patients 2, 5, 6 from group 1) incubation, i.e.:

$$\text{Intracellular killing} = \frac{\%\text{ initial inoculum killed by diabetic granulocytes}}{\%\text{ initial inoculum killed by paired control granulocytes}} \times 100$$

The engulfment of foreign material by granulocytes in vitro normally progresses rapidly; experiments using bacteria[2] and inert particles[18] suggest that, under conditions similar to those employed in the present study, the engulfment phase of phagocytosis is completed within 20 minutes after exposure of granulocytes to the foreign particles. Consequently, even though the present data show that engulfment of staphylococci by diabetic granulocytes ultimately became comparable to that of controls, the significant reductions observed at 20 minutes by colony counting and at 30 minutes by microscopic observations confirm previous reports of impaired phagocytosis in diabetic patients.[5,6,9]

It could be argued that the lagging rate of intracellular killing of staphylococci by diabetic granulocytes after 60 minutes of incubation (figure 1) is an artifact resulting from impaired engulfment of bacteria at an earlier interval. However, the reduction in intracellular killing persisted for 120 minutes. Since engulfment had become normal 60 minutes before that time (table 1), a reduction secondary to a defect in engulfment at that interval seems unlikely.

Recent advances in our understanding of granulocyte function now require that perturbations in bactericidal activity be linked as closely as possible to intracellular biochemical events. Studies of chronic granulomatous disease,[1,19,20] an inherited disorder of granulocyte function in which patients have repeated infections owing to an impaired ability to kill certain types of bacteria following normal engulfment, have led to the delineation of a number of steps required for effective killing of microorganisms by granulocytes. Although granulocytes derive much of their energy

from glycolysis and conversion of glucose to lactate and pyruvate,[21] they also take up oxygen from their environment, especially during phagocytosis.[1] This oxygen is utilized by cellular oxidases to generate substances, such as the superoxide radical[19] and hydrogen peroxide,[20] that have potent bactericidal activity. Techniques recently developed to quantitate these important steps in the normal bactericidal pathway need to be applied to characterize more precisely the defect in granulocyte function in the diabetic. Specifically, the role of insulin in these reactions must be clarified. Insulin is necessary neither for glucose entry into the granulocyte[21] nor, apparently, for the respiratory burst that accompanies phagocytosis.[22] However, adequate availability of the hormone does facilitate metabolism of glucose to glycogen and lactate in the diabetic granulocyte;[21] it is possible that the lack of insulin might also hamper the other metabolic processes of phagocytosis, such as phospholipid synthesis.[23]

The abnormalities in granulocyte function of these diabetic patients were modest, a fact that correlates with the relatively mild, clinical expression of their diabetes. Previous measurements of granulocyte function were made on diabetic patients with either marked hyperglycemia[5,6] or frank ketoacidosis;[4] our patients were ambulant, unhospitalized, and free of infection. Although the determinants of granulocyte dysfunction in diabetes are not known, this apparent correlation between granulocyte function and diabetes' control should not be overlooked. For this reason, the controversial issue of diabetes' control and its relation to the complications of the disease should again be raised. This and previous studies[5-7] suggest that disturbances in host defense mechanisms of diabetic patients are ameliorated by better availability of insulin. Therefore, good control of diabetes appears to be an important means of minimizing the likelihood of infection in patients with diabetes mellitus.

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