Decreased Stimulated Glucose Oxidation and Iodination by Polymorphonuclear Leukocytes from Insulin-Treated Diabetic Subjects

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SUMMARY

To investigate the mechanism of the reported abnormality of polymorphonuclear leukocyte (neutrophil) function in diabetes, the burst of oxidative activity accompanying the killing reaction and the neutrophil iodination reaction were measured in 21 stable, insulin-treated diabetic subjects and 32 nondiabetic subjects of similar age range. Responses to standard killed preparations of *Candida albicans* (*C. albicans*) (glucose oxidation and neutrophil iodination) and *Staphylococcus aureus* (*S. aureus*) (neutrophil iodination) were studied. The iodination reaction was studied using the subjects' neutrophils suspended both in autologous serum and in pooled normal human serum, and the glucose oxidation test was performed using the neutrophils in pooled normal human serum.

There was a significant decrease in the conversion of ^{14}C-1-glucose to $^{14}CO_2$ in the neutrophils from diabetics [0.47 ± 0.04 (SE) cf. 0.64 ± 0.04 nmol/2 × 10^6 neutrophils/60 min, $P < 0.005$] and in neutrophil iodination in response to *S. aureus* in both autologous serum (diabetics .089 ± 0.008 cf. 0.114 ± 0.008 nmol iodine/10^6 neutrophils/90 min, $P < 0.025$) and in pooled human serum (diabetics 0.086 ± 0.009 cf. 0.105 ± 0.006, $P < 0.05$). There was no significant difference in neutrophil iodination in response to *C. albicans*. There was a significant negative correlation between the diabetics' fasting plasma glucose level and neutrophil iodination in response to *S. aureus* in pooled human serum ($r = -0.54$, $P < 0.05$), but not between fasting plasma glucose and glucose oxidation.

It is concluded that there are significant decreases in stimulated glucose oxidation and in the neutrophil iodination reaction (reflecting decreased function of the myeloperoxidase-hydrogen peroxide-halide microbicidal system) in neutrophils from insulin-treated diabetic subjects. These abnormalities may contribute to the defective bactericidal activity of diabetic neutrophils. DIABETES *30*:256–260, March 1981.

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It has long been recognized that diabetic subjects have an increased liability to severe infection. Neutrophil polymorphonuclear leukocytes (hereafter referred to as neutrophils) provide the first cellular defence against invading organisms, and several investigations of the function of neutrophils from diabetic subjects have shown defects in one or more of the steps involved in neutrophil function. In particular, defects in chemotaxis,[1–3] adherence,[4] and phagocytosis[5–9] have been demonstrated. The most recent of these reports dissociated the processes of engulfment and bacterial killing, and found that the killing reaction as well as the engulfment process was abnormal in neutrophils from diabetic subjects.[9] The cause of this defect is unknown.

The steps involved in the intracellular killing process by neutrophils are complex, and include a burst of glucose oxidative activity following engulfment,[10] which is required for optimal bacterial killing,[11] activity of the myeloperoxidase-hydrogen peroxide-halide system,[12] as well as several other oxygen-dependent and oxygen-independent steps. In an attempt to clarify the mechanism of the impaired neutrophil killing process in diabetes, stimulated glucose oxidation and the neutrophil iodination reaction as a marker of the myeloperoxidase-hydrogen peroxide-halide system were studied in a group of stable, insulin-treated diabetic subjects. To determine the contribution of abnormalities in the neutrophils themselves, and effects secondary to defective opsoninization, the neutrophil iodination reaction was performed both in autologous serum (AS) and in pooled normal human serum (PHS).

MATERIALS AND METHODS

Subjects studied. A group of 21 ambulant, nonobese (less than 115% ideal body weight) insulin-treated diabetic subjects (age range 14–74 yr) (mean 41) was studied after an

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overnight fast before receiving their morning insulin. None was suffering from overt infection, and there had been no obvious recent change in their metabolic control or medication. Only three were receiving additional medication (one digoxin and propranolol, one doxepin hydrochloride, and one chlorothiazide). No patient had significantly impaired renal function. The control group consisted of 32 nondiabetic laboratory staff and normal elderly subjects (age range 19–85 yr, mean 42) who were also studied after an overnight fast.

Preparation of neutrophils. Neutrophils were prepared from 15 ml fresh heparinized blood. The lymphocytes and neutrophils were separated on a discontinuous Ficoll-Isopaque density gradient.[13] Five milliliters of Ficoll-Isopaque (specific gravity 1.077, Pharmacia, Uppsala) was layered under 15 ml heparinized blood. This was centrifuged at 1400 × *g* for 20 min. The lymphocytes, monocytes, and platelets that separated out at the Ficoll-Isopaque/plasma interface were discarded. The neutrophils were separated from the red cells by diluting the cells that came through the Ficoll-Isopaque with 15 ml of 0.5 M phosphate-buffered saline (PBS), pH 7.4, and mixing with 5 ml of 6% Dextran T500 (Pharmacia, Uppsala). The red cells were allowed to sediment for 30 min, after which the supernatant was removed and centrifuged at 700 × *g* for 5 min to remove the residual platelets.

The neutrophils were washed three times with PHS, counted, and made up to a concentration of 1 × 10^6 neutrophils/100 μl of PBS. Differential counts showed that the neutrophil suspension routinely contained greater than 90% neutrophils. The small residual red cell contamination was shown not to affect the results for either glucose oxidation or neutrophil iodination.

^{14}C-1-glucose oxidation. The procedure used was modified from that of Keusch et at.[14] The test was performed in a 20-ml container (PMBC tubes, Johns Professional Products, Cheltenham, Victoria,) which had two compartments and was fitted with a top containing a rubber plug through which reagents could be injected.[15] One compartment contained the reaction mixture, which consisted of 2 × 10^6 neutrophils in 700 μl PBS containing 0.06 μCi ^{14}C-1-glucose (3 Ci/mol, Radiochemical Centre, Amersham), 100 μl of *C. albicans*, adjusted to an optical density of 1.3 at 540 nm using a cuvette with a 1-cm light path, and 25 μl of freshly frozen pooled normal human serum. The other compartment contained a miniscintillation vial (Packard Instrument Co. Illinois) with 400 μl Hyamine 10× (Packard, Illinois). The tubes were incubated at 37°C for 1 h, after which the reaction was stopped by the injection of 1 ml of 1-N HCl into the reaction mixture, and the incubation was allowed to proceed for another hour. The minivial was then removed, 5 ml of Instagel (Packard) was added, and the radioactivity was measured in a liquid scintillation spectrometer (Packard). Basal ^{14}C-1-glucose oxidation was measured in the same way, except that *C. albicans* was omitted from the incubation mixture. All tests were performed in duplicate, and at least one nondiabetic control was included on each test day.

Neutrophil iodination. Iodination by neutrophils was measured by conversion of ^{125}I-iodide to a trichloroacetic acid (TCA) precipitable form based on the method described by Pincus and Klebanoff.[16] Five duplicate incubations were done for neutrophils from each subject. One pair measured iodination in response to *S. aureus* using AS as the source of opsonin, one pair in response to *S. aureus* using PHS as the source of opsonin, one pair in response to *C. albicans* with AS, and one pair in response to *C. albicans* with PHS. The final pair was used as a control to measure TCA precipitable radioactivity in the absence of microorganisms. The *S. aureus* and *C. albicans* preparations were each killed suspensions adjusted, after preliminary experiments, to an optical density of 1.0 at 540 nm, using cuvette with a 1-cm light path.

Each incubation mixture consisted of 200 μl of glucose solution (2.2 mmol/L) in Dulbecco phosphate-buffered salt solution.[17] We added 100 μl of the *S. aureus* or *C. albicans* suspension and 25 μl of AS, respectively, to the appropriate tubes. To all the tubes were added 100 μl of ^{125}I Na I (Radiochemical Centre, Amersham) diluted to 0.6 μCi/ml containing 8 nmol of unlabeled sodium iodide and 100 μl of the neutrophil suspension (1 × 10^6 cells). The tubes were gently shaken by hand initially and at 15-min intervals during a 90-min incubation at 37°C.

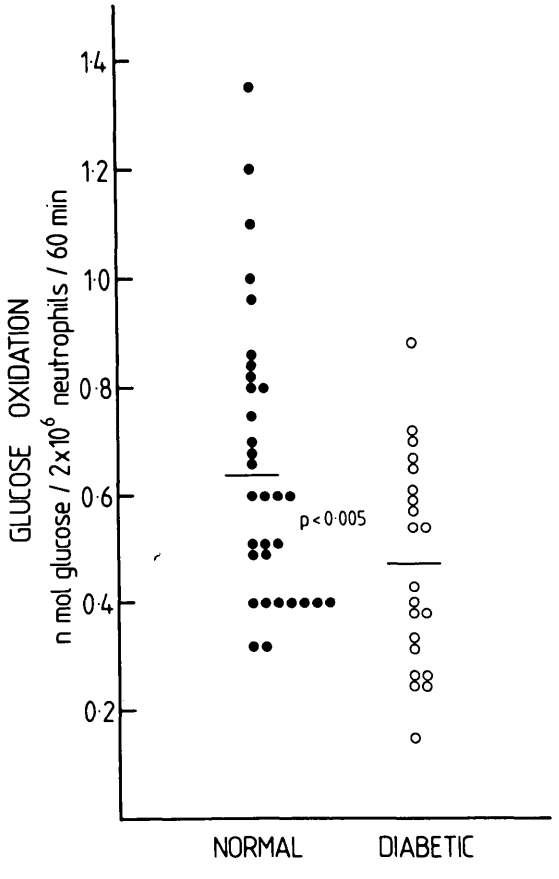
The reaction was terminated by the addition of 2 ml of 10% TCA. The precipitate was washed three times with PBS and the precipitated ^{125}I counted in an autogamma spectrometer (Packard Instrument Co. Illinois).

Statistical evaluation. Statistical evaluation was performed by Student's *t* test for unpaired data.

RESULTS

^{14}C-1-glucose oxidation. Neutrophils from diabetic subjects showed decreased ^{14}C-1-glucose oxidation to $^{14}CO_2$ in

FIGURE 1. Glucose oxidation in neutrophils from the normal and diabetic subjects. The mean value is indicated.



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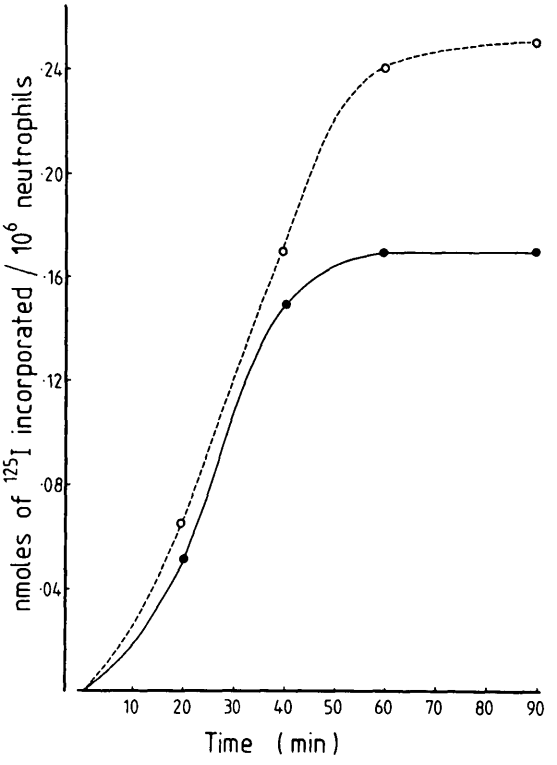


FIGURE 2. Time course of the neutrophil iodination reaction in neutrophils from normal subjects in response to killed preparations (O.D. 1.0) of *S. aureus* ●——● and *C. albicans* ○—○. Each point represents the mean of duplicate determinations.

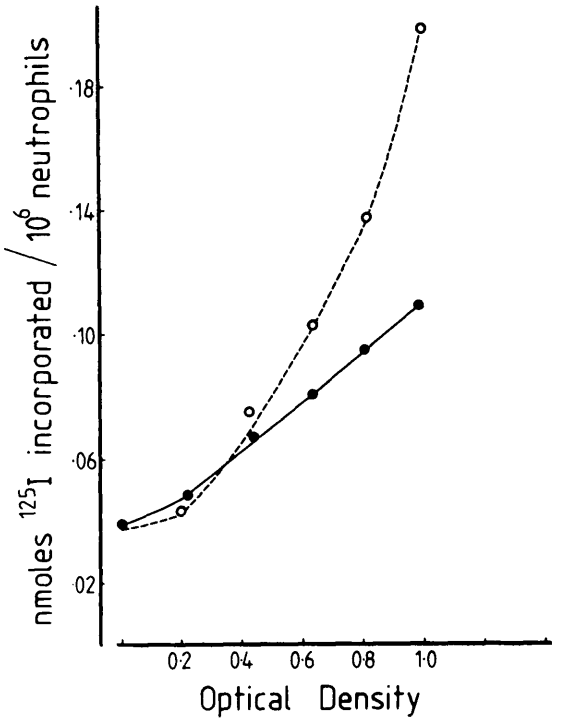


FIGURE 3. Relationship between the concentration of the microbial preparation and the neutrophil iodination reaction. Symbols as in Figure 2. Optical density was measured at 540 nm.

response to *C. albicans* (Figure 1). However, nonstimulated glucose oxidation was identical in neutrophils from nondiabetic and diabetic subjects [0.11 ± 0.01 (SE) nmol glucose/2 × 10^6 neutrophils/60 min in both groups]. There was no correlation between ^{14}C-1-glucose oxidation and the fasting plasma glucose at the time of the test (data not shown).

Neutrophil iodination reaction. The time course of the neutrophil iodination reaction in response to *S. aureus* and *C. albicans* in neutrophils from normal subjects is shown in Figure 2. It can be seen that a plateau is reached by 60 min. The relationship between the optical density of the microbial preparation and the neutrophil iodination in neutrophils from normal subjects is shown in Figure 3. It can be seen to increase approximately linearly between optical densities of 0.2 and 1.0 for both microorganisms, with a steeper gradient for the *C. albicans*. There was no difference in the basal rate of neutrophil iodination. However, neutrophils from the diabetic subjects showed significantly impaired neutrophil iodination in response to *S. aureus* in both AS and PHS (Figure 4). There was no significant difference in neutrophil iodination in response to *C. albicans* between neutrophils from normal and diabetic subjects in either AS or PHS (Table 1).

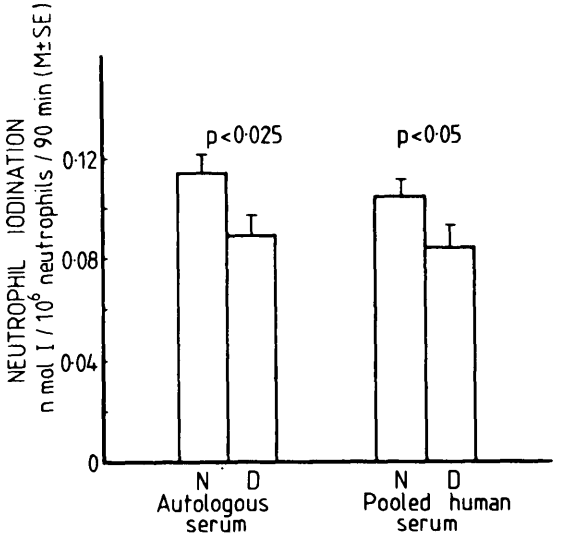
There was a significant negative correlation between neutrophil iodination in response to *S. aureus* in PHS and the fasting plasma glucose at the time of the test in the 16 diabetic subjects in whom simultaneous readings were available (Figure 5).

TABLE 1
Neutrophil iodination in response to *C. albicans* in neutrophils from nondiabetic and diabetic subjects in autologous serum or pooled human serum

Source of neutrophils	Source of opsonin	Neutrophil iodination (mean ± SE) nmol I/10^6 neutrophils/90 min	Signif. cf. control
Nondiabetic	AS	0.23 ± 0.01	
Diabetic	AS	0.21 ± 0.01	N.S.
Nondiabetic	PHS	0.23 ± 0.01	
Diabetic	PHS	0.20 ± 0.01	N.S.

N.S. $P > 0.05$.

FIGURE 4. Neutrophil iodination in neutrophils from normal (N) and diabetic (D) subjects in AS and PHS. Mean and SEM are shown for 32 normal and 21 diabetic subjects.



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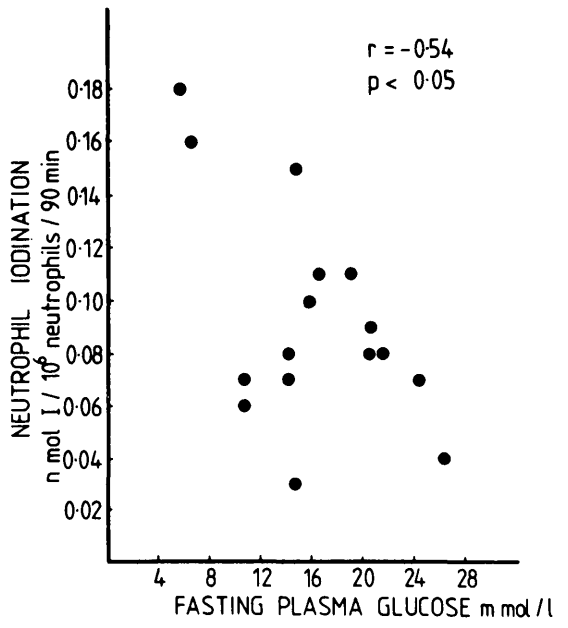

FIGURE 5. Relationship between neutrophil iodination and the fasting plasma glucose at the time of the test in neutrophils from diabetic subjects. PHS was used as the source of the opsonins.

DISCUSSION

This study shows that, in addition to the previously described abnormalities of neutrophil function in diabetic subjects, stimulated glucose oxidation and the neutrophil iodination reaction are also decreased. Before it is assumed that the observed decreases are important primary components of impaired neutrophil function in vivo, other possible explanations must be considered. First, since it has been shown that the engulfment of bacteria by neutrophils from diabetic subjects is impaired,[5-9] the possibility that the decreased stimulated glucose oxidation and neutrophil iodination were secondary to impaired engulfment must be examined. Since engulfment is a rapid process, normally complete within 20 min of exposure of neutrophils to bacteria,[18] and since the impaired engulfment observed in diabetic neutrophils at early time phases becomes normal after less than 60 min,[9] it would appear unlikely that the reduced stimulated glucose oxidation (studied for 60 min) and neutrophil iodination in response to *S. aureus* (studied for 90 min) could be accounted for by the rather modest early decrease in engulfment found in diabetic neutrophils. The time course of the neutrophil iodination reaction, with maximal iodination reached by 60 min (Figure 2), would support this.

In the case of the glucose oxidation experiments, the possibility that the apparent rate of stimulated glucose oxidation depends on the extent of dilution of the specific activity of the added glucose by endogenous glucose produced by glycogenolysis within the neutrophils must be considered. As it is quite conceivable that the rate of glycogenolysis differs in the neutrophils from the diabetic compared with the nondiabetic subjects, this could account for differences in the apparent rates of stimulated glucose oxidation between the two groups of neutrophils. The fact that the basal rate of glucose oxidation did not differ between the groups goes against this possibility, but does not totally exclude it. The neutrophil iodination experiments are not susceptible to this artifact.

The neutrophil iodination reaction measures the capacity of neutrophils to generate H_2O_2 and utilize available halide for microbial killing.[19] Together with the generation of superoxide and hydroxyl radicals, the myeloperoxidase-H_2O_2-halide system comprises the oxygen-dependent antimicrobial system of the neutrophil, which has a major role in the killing reaction.[20] The fact that the reaction was normal with *C. albicans* probably reflected the strong stimulus provided by this preparation at the concentration used (Figure 3), and may demonstrate that the neutrophils from diabetic subjects can react normally if the stimulus is great enough. Alternatively, it may have reflected different mechanisms by which *C. albicans* might activate the myeloperoxidase-H_2O_2-halide system. These possibilities require further clarification. The decreased stimulated glucose oxidation in the neutrophils from the diabetics shows that the neutrophil response to *C. albicans* is not normal in all respects.

The cause of the decreased neutrophil iodination in the neutrophils from the diabetic subjects is unclear. There is obviously considerable overlap with normal, commensurate with the fact that the subjects were studied when ambulant, stable, and free from infection. A similar overlap was found when the overall effectiveness of bacterial killing was studied.[9] The negative correlation between neutrophil iodination and fasting plasma glucose suggests that the defect in the neutrophil iodination reaction may depend on the degree of metabolic abnormality. This is compatible with the previous observation that intracellular killing in neutrophils from diabetics improved with improved metabolic control.[9] The experiments in the PHS showed that the abnormality was associated with the neutrophils themselves rather than with a circulating serum factor. The role of insulin deficiency (relative or absolute) is not clear. Mowat and Baum found that the defect in chemotaxis of the diabetic leukocyte was corrected by incubation of the cells with insulin.[1] Insulin in high concentrations has been shown to have effects on glucose metabolism in diabetic, but not in normal, neutrophils,[21,22] but no studies have been performed to examine a possible role of insulin in the myeloperoxidase-H_2O_2-halide system. The demonstration of direct actions of insulin on intracellular H_2O_2 production in adipocytes[23] raises the possibility that insulin may have a direct effect on this system, but this requires further investigation.

In conclusion, the present study has shown abnormalities at two important steps involved in the killing reaction in neutrophils from diabetic subjects. The biologic significance of these abnormalities and further delineation of the pathogenetic mechanisms involved in the abnormal function of neutrophils from diabetics and the consequent increased liability to infection awaits further investigation.

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