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Influence of Glucose Levels on the In Vitro Phagocytosis of Bacteria by Human Neutrophils

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A previously developed in vitro method for studying the phagocytosis of bacteria and particles by human neutrophils was used to investigate the influence of different glucose levels on phagocytosis. It was found that high glucose levels (200, 400, and 800 mg of glucose/100 ml) significantly depressed the phagocytosis of *Staphylococcus epidermidis*, *S. aureus*, and *Escherichia coli*. At very low glucose levels, a somewhat decreased phagocytic activity was noted. The strongest phagocytic activity occurred at glucose concentrations of 50 and 100 mg/100 ml. A second effect noted at the higher (200, 400 and 800 mg/100 ml) glucose concentrations was a decreased adhesiveness of the neutrophils to solid surfaces. The mechanism of the decrease in phagocytosis and in neutrophil adhesiveness at higher glucose levels is unknown, but it is not linked to increased osmotic pressures due to the presence of glucose, as ethanol, at the same and even higher osmolal concentrations, had no effect on the phagocytosis. These results show that not only the phagocytic activity of those neutrophils that do adhere to a solid surface is diminished at higher glucose concentrations but also that fewer neutrophils adhere to solid surfaces at higher glucose levels. These two phenomena combined may provide at least part of the explanation for the well-known decrease in resistance to bacterial infections of diabetics.

Although the cause of the decreased resistance of diabetics to bacterial infections is far from being conclusively established (9, 24), it has repeatedly been suspected that impaired phagocytic activity might be implicated (7, 10, 13, 16, 19, 22). As many varying (and sometimes conflicting) results have been reported as different methods were used. Da Costa and Beardsley (4) found a somewhat decreased opsonizing capacity of diabetic patients' serum (in vitro). Horster (7) found a decreased phagocytic index (in vivo) of the leukocytes of depancreatized dogs, as well as a slight decrease in the opsonizing capacity of their serum. Marble et al. (13) found no significant difference between the bactericidal power or phagocytic activity of diabetic as compared to normal (human) blood (in vitro). Kestermann and Vogt (10) and also Rohmann (19) found a somewhat decreased phagocytic index among diabetics (in vitro). Bagiu et al. (1) noted a decrease in phagocytic activity as well as of leukocyte mobility at higher glucose levels, with whole rabbit blood (in vitro). Drachman et al. (5) demonstrated a decreased resistance to pneumococci in alloxan diabetic rats in vivo, whereas their in vitro results showed the principal defect to be due to the high serum glucose levels, rather than to any defect of the neutrophils. Finally, a decrease in the chemotactic activity of the neutrophils of diabetics has recently been described (15).

The measurement of phagocytic activity is of course fraught with variables. The phagocytic power of neutrophils of even normal subjects varies from day to day. Opsonizing serum factors [specific antibodies (20, 24), aspecific immunoglobulins of the immunoglobulin G (IgG) (21, 22) and immunoglobulin M classes (22)] and many of the complement components (6, 8, 14, 16) are too numerous, too dependent on the donor's individual immunological history, and also too variable with time to allow one to attach much importance to slight differences between the opsonizing capacities of sera of different individuals.

One of the only valid methods remaining to test the influence of any given additive on phagocytic activity is to compare the difference in phagocytosis in the additive's absence and in its presence, with neutrophils of a single (normal)

donor, donated at one given moment, in the absence of uncontrolled serum factors. A method developed earlier by the author and his coworkers (21; Stinson and van Oss, J. Reticuloendothel. Soc., *in press*), which fulfills these conditions, was applied to the (in vitro) study of the influence of glucose (and some other additives) on phagocytosis of a number of microorganisms by normal human neutrophils.

MATERIALS AND METHODS

Bacteria. *Staphylococcus epidermidis*, *S. aureus* (haemolyticus), and *Escherichia coli* were grown at 37 C in Tryptose phosphate broth and harvested during exponential growth. They were then washed three times with physiological saline and suspended in Hanks balanced salt solution (*p*H 7.4) to a final concentration of 10^9 bacteria/ml. When "coated" with IgG, they were then placed in a 1% solution of IgG and incubated for 30 min at 37 C (under continuous agitation). The "coated" bacteria were then centrifuged and resuspended in Hanks solution to a final concentration of 2×10^8 bacteria/ml.

Immunoglobulins. Human polyclonal IgG was used in the form of freeze-dried Cohn's fraction II. Immunoelectrophoretic analysis showed this fraction to contain only IgG, 85% of which proved, by analytical ultracentrifugation, to be of the 7*S* variety, whereas 15% consisted of 10*S* dimers.

Medium. Hanks balanced salt solution containing 7 g of NaCl, 350 mg of $NaHCO_3$, 350 mg of KCl, 200 mg of $MgSO_4 \cdot 7H_2O$, 55 mg of $Na_2HPO_4 \cdot 2H_2O$, and 55 mg of KH_2PO_4 per liter, at *p*H 7.4, was used without addition of the usual amount (0.09%) of glucose. Instead, where necessary, glucose was added to the desired concentration (0.05, 0.1, 0.2, or 0.8%). For the ethanol experiments, ethanol was added to Hanks solution containing 0.09% glucose. For the sucrose experiments, sucrose was added to glucose-free Hanks solution.

Phagocytosis. Phagocytosis of the bacteria by human neutrophils was studied in Mackaness coverslip chambers (12). Monolayers of peripheral leukocytes from a few drops of finger-prick blood given by a single, healthy donor were prepared on round glass cover slips by the method given by Newsome (17). These cell sheets, generally consisting of about 200 to 400 neutrophils per mm^3 (this number varies with the donor and with the day of donation but is quite constant for any given single donation), were placed into the Mackaness chambers and incubated for 25 min at 37 C in a Hanks balanced salt solution containing the ultimate concentration of glucose, ethanol, or sucrose plus 1% human serum albumin. After that first incubation period, the Mackaness chambers were emptied and a suspension of bacteria (at a final concentration of 2×10^8/ml) in Hanks solution with the final appropriate concentration of glucose, ethanol, or sucrose was put into the chamber and incubated at 37 C. After 15 min, the chambers were tapped on their sides to dislodge any free particles adhering to the surface of the monolayers. The cover slips were then removed, washed gently in saline, air-dried at room temperature, and stained.

Staining and counting of phagocytized bacteria. Gram stain was used for the detection of phagocytized staphylococci, and simple crystal violet was used for *E. coli*. The total number of polymorphonuclear cells and the total number of active phagocytic cells were counted on two traverses of the monolayer taken at right angles to each other through the approximate center of the coverslip. A total of 40 cells were counted. The percentage of polymorphonuclear cells that had engulfed bacteria was designated the phagocytic index. The mean number of bacteria in those cells that had accomplished phagocytosis was recorded as the avidity index. The phagocytic activity was then expressed as the average number of bacteria phagocytized per neutrophil (the product of the above two indexes), and the standard deviations of the average were calculated for all phagocytic activities thus obtained.

Neutrophil adhesiveness. The degree of adhesiveness of neutrophils to the glass surfaces of the cover slips was found by going through the same procedures as above, but without the addition of bacteria to the Hanks solutions. After drying and Gram staining of the cover slip, the number of neutrophils per microscopic field was counted on two traverses of the monolayer taken at right angles to each other through the approximate center of the cover slip and averaged from 20 observations. The standard deviations of these averages were then calculated. The neutrophil adhesiveness was then expressed as the average number of adhering neutrophils per microscopic field.

RESULTS

Influence of glucose on phagocytosis. The phagocytic activities of normal human neutrophils with respect to *S. epidermidis*, *S. aureus*, and *Escherichia coli* are represented in Fig. 1 and 2. In Fig. 1 the results are given for unopsonized bacteria and in Fig. 2 the bacteria had been treated with 1% human IgG, which acts as a rather powerful, largely aspecific (21; M. W. Stinson and C. J. van Oss, J. Reticuloendothel. Soc., *in press*), heat-stable opsonin, which can, as is obvious from a comparison of Fig. 1 and 2, as much as double the phagocytic activity. It is clear from both sets of data that the phagocytic activity of peripheral neutrophils is strongest at low and high normal glucose levels (0.05 and 0.1%) and that that activity is significantly decreased at hyperglycemic glucose levels (0.2, 0.4, and 0.8%). It also becomes clear that in the absence of glucose phagocytic activity is also somewhat depressed, although to a lesser degree than at the higher hyperglycemic levels.

Closer scrutiny of the components of the phagocytic activity (= phagocytic index × avidity index; *see* Tables 1 and 2) shows that the decrease in phagocytic activity at increased glucose concentrations is for the greater part due to a decrease in the mean avidity index and,

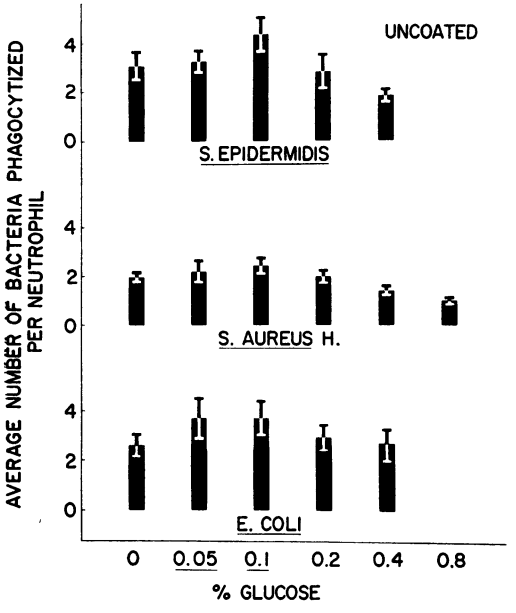

FIG. 1. *Phagocytic activities of human neutrophils towards three different microorganisms (uncoated) as a function of glucose levels. ("Normal" glucose concentrations are underlined.) The standard deviations from the averages are indicated below and above the tops of the solid bars. At the highest glucose concentrations (0.8%), there not always were enough adhering neutrophils to obtain significant counts. For the statistical significance, see Results.*

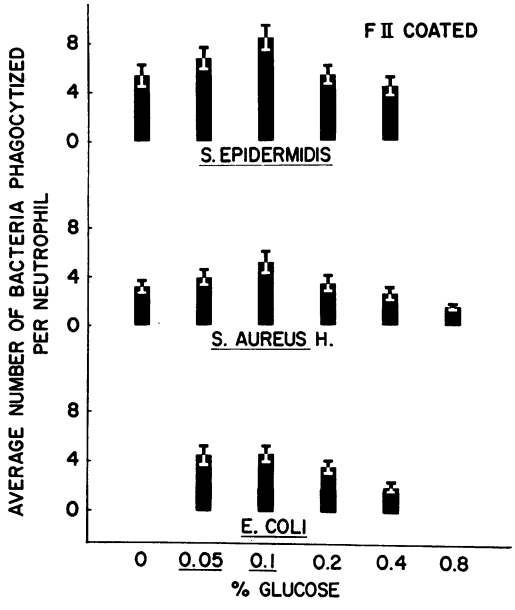

FIG. 2. *Phagocytic activities of human neutrophils towards three different microorganisms opsonized with human IgG (Cohn's fraction II-coated) as a function of glucose levels. Conditions otherwise as described for Fig. 1. At 0% glucose, no phagocytic counts were obtained with E. coli because of accidental breakage of the cover slip with the leukocyte monolayer. For the statistical significance, see Results.*

much more slightly and mainly at extremes of glucose excess, to a decrease in the mean phagocytic index. This means that, under hyperglycemic conditions, those neutrophils that accomplish phagocytosis engulf fewer bacteria than at "normal" glucose concentrations. The fraction of the total number of neutrophils that engages in phagocytosis decreases much more slightly when the glucose concentration increases.

Influence of glucose on neutrophil adhesiveness. In the course of this work, it often proved difficult to find sufficient numbers of neutrophils adhering to the cover slip at the highest (0.8%) glucose level studied (Fig. 1 and 2) to obtain significant data on their phagocytic activity. This prompted an investigation into the adhesiveness of neutrophils to glass surfaces as a function of the concentration of glucose (Fig. 3).

The adhesiveness (in Fig. 3 expressed as the average number of neutrophils adhering per microscopic field of approximately 0.01 mm²) was clearly decreased under hyperglycemic glucose concentrations and was most reduced at the highest concentration (of 0.8% glucose).

Influence of ethanol on phagocytosis and on neutrophil adhesiveness. To investigate the question of whether the influence of glucose on phagocytosis and on neutrophil adhesiveness could be due to simple osmotic forces, it became desirable to study the influence of another carbohydrate of, if possible, even smaller molecular weight than glucose. Ethanol was chosen. It can be seen from Fig. 4 and 5 that ethanol, in addition to and at the same concentrations as (and thus at even higher osmolal concentrations than) the glucose studied, had no influence whatever on either phagocytosis or neutrophil adhesiveness.

TABLE 1. *Phagocytosis of Staphylococcus aureus H. (uncoated) as a function of glucose levels*

Per cent glucose	Mean phagocytic index	Mean avidity index	Mean phagocytic activity
0	0.65	2.92	1.90 ± 0.30
0.05	0.65	3.36	2.18 ± 0.49
0.1	0.68	3.59	2.43 ± 0.39
0.2	0.68	2.96	2.00 ± 0.32
0.4	0.63	2.32	1.48 ± 0.25
0.8	0.55	1.77	0.98 ± 0.19

TABLE 2. *Phagocytosis of Staphylococcus aureus H. (Cohn's fraction II-coated) as a function of glucose levels*

Per cent glucose	Mean phagocytic index	Mean avidity index	Mean phagocytic activity
0	0.68	4.67	3.15 ± 0.61
0.05	0.73	5.31	3.85 ± 0.69
0.1	0.75	6.80	5.10 ± 0.89
0.2	0.70	4.86	3.40 ± 0.75
0.4	0.65	3.92	2.55 ± 0.57
0.8	0.58	2.65	1.53 ± 0.30

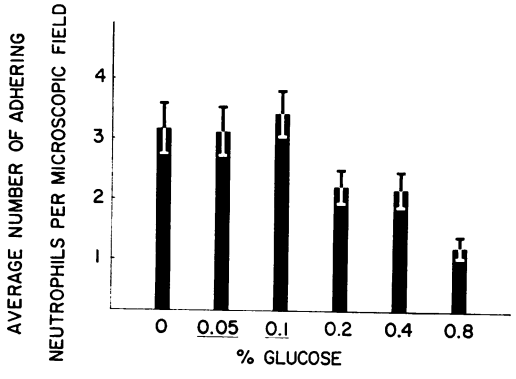

FIG. 3. *Neutrophil adhesiveness to the glass surface of the cover slips as a function of glucose levels. ("Normal" glucose concentations are underlined.) The standard deviations from the averages are indicated below and above the tops of the solid bars. For the statistical significance, see Results.*

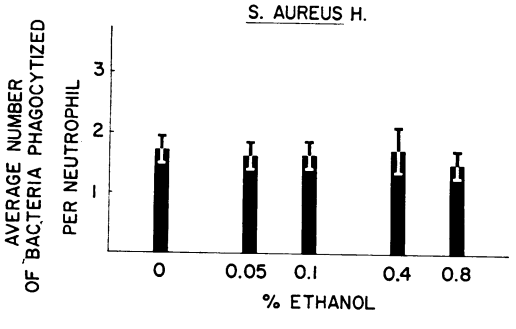

FIG. 4. *Phagocytic activity of Staphylococcus aureus H. as a function of ethanol levels. The standard deviations from the averages are indicated below and above the tops of the solid bars.*

Influence of sucrose on phagocytosis and on neutrophil adhesiveness. A preliminary experiment (with "uncoated" *S. epidermidis*) showed (Table 3) that with sucrose there was also a certain depression of phagocytic activity at the higher

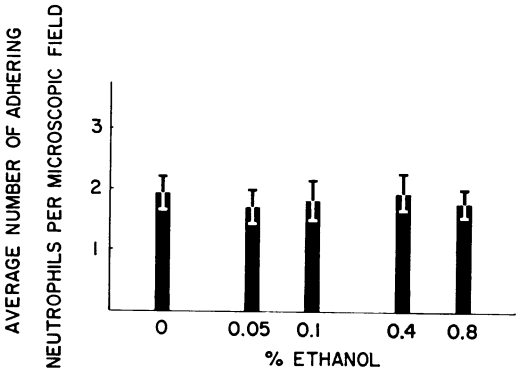

FIG. 5. *Neutrophil adhesiveness to the glass surface of the cover slips as a function of ethanol levels. The standard deviations from the averages are indicated below and above the tops of the solid bars.*

TABLE 3. *Phagocytosis of Staphylococcus epidermidis (uncoated) and neutrophil adhesiveness as a function of sucrose concentration*

Per cent sucrose	Mean phagocytic index	Mean avidity index	Mean phagocytic activity	Avg no. of neutrophils adhering per microscopic field
0	0.63	2.16	1.30 ± 0.24	0.87 ± 0.17
0.05	0.63	2.37	1.50 ± 0.28	0.90 ± 0.18
0.1	0.70	2.43	1.70 ± 0.31	0.90 ± 0.18
0.2	0.63	2.26	1.42 ± 0.23	0.73 ± 0.15
0.4	0.63	1.89	1.20 ± 0.23	0.63 ± 0.15
0.8	0.57	1.59	0.91 ± 0.20	0.67 ± 0.13

concentrations though not as pronounced as with glucose. As in the case of glucose, this depression was mainly (but again not entirely) due to a decrease in the avidity index at the higher concentrations. Although in this experiment the total amount of normally adhering neutrophils was rather on the low side (Table 3), it still is clear that at the higher sucrose concentrations the neutrophil adhesiveness to glass was also diminished.

Statistical significance. It will be noted that in a number of cases a certain overlap occurs between the phagocytic activities (Fig. 1 and 2) or neutrophil adhesiveness (Fig. 3) at neighboring glucose concentrations when the average deviations are taken into account. It nevertheless is obvious from all of the data that without exception the phagocytic acidity (as well as the neutrophil adhesiveness) always tended to be lower under conditions of diabetic than at normal glucose levels. For technical reasons, it was not feasible to study the phagocytic activities of

more than 40 neutrophils for any one glucose (or ethanol) dilution for each microorganism used. Nor was it practicable to undertake neutrophil adhesiveness studies involving more than 20 counts for any one glucose (or ethanol) dilution used on any given day. But it is possible and permissible to group together a number of data to obtain quantitative insight into the statistical validity of that tendency. The levels of statistical significance (expressed as the *P* values) of the differences in phagocytic activity obtained with a number of the grouped data are as follows. For the data of Fig. 1, at 0.1% glucose the phagocytic activity is significantly higher than at (100 observations each): 0% glucose, $P = 0.024$; 0.2% glucose, $P = 0.044$; 0.4% glucose, $P = 0.0004$. For the data of Fig. 2, at 0.1% glucose the phagocytic activity is significantly higher than at (150 observations each): 0% glucose, $P = 0.0012$; 0.2% glucose, $P = 0.0006$; 0.4% glucose, $P = <0.0001$. The levels of significance of the differences in neutrophil adhesiveness to glass are as follows (for data of Fig. 3 together with data not illustrated, a total of 200 observations). At 0.1% glucose, the neutrophil adhesiveness is: not significantly different from that at 1% glucose, $P = 0.952$; only slightly higher than at 0.2% glucose, $P = 0.496$; significantly higher than at 0.4% glucose, $P = 0.0001$; significantly higher than at 0.8% glucose, $P = 0.0001$.

The standard deviations of the phagocytic activities were obtained in the classical way (2), and the probabilities (*P* values) of the differences in phagocytic activities among groups of results at the various glucose levels studied being due to chance occurrences were obtained by means of the normal error curve (2).

DISCUSSION

From these results, it is clear that when intrinsically variable parameters such as opsonic powers of patients' or normal sera and properties of (or residual glucose levels in) patients' neutrophils can be avoided, there still remains the fact that normal neutrophils phagocytize less well at hyperglycemic glucose concentrations. At high glucose concentrations, the adhesive properties of the neutrophils are also significantly decreased. As it is likely that neutrophil adhesiveness is an important attribute of the phagocyte in surface phagocytosis (26), it follows that both direct phagocytosis and surface phagocytosis by normal neutrophils are significantly impaired at hyperglycemic glucose levels, which suggests that the sole increase in blood glucose levels may by itself be enough to depress the resistance to bacterial infections quite seriously in all cases of insufficiently corrected diabetes.

The possibility that *p*H changes (possibly indirectly caused by metabolic changes in the neutrophils at high glucose levels) may play a role could be rejected. It was verified that the addition of various amounts of glucose did not materially influence the *p*H (= 7.4) of the surrounding medium, either with neutrophils alone or after the addition of bacteria to the chambers. (It was found that *p*H values below 7.0 could cause a slight depression of phagocytosis as well as of the adhesiveness of neutrophils to glass, whereas *p*H values between 7.0 and 8.0 had no influence on these phenomena.)

It is not yet possible at this stage to speculate about the mechanism by which increased glucose levels depress phagocytosis. But the lack of influence of ethanol on the phagocytic activity of normal neutrophils would at least eliminate any suspicion (5) that the phenomenon is caused by undue osmotic exertions of extracellular glucose on the neutrophils. [Although excessive alcohol levels have been linked to increased susceptibility to infections in man, its main influence seems to be on diapedesis (3).]

The slight but definite decrease in phagocytic activity at hypoglycemic glucose levels (Fig. 1 and 2) accords quite well with the concept that neutrophils need to metabolize a certain amount of glucose to function optimally. This would also be in agreement with recent results obtained with human subjects who were subjected to a very low caloric diet, which decreased their phagocytic index (11).

Finally, it is interesting to note (Table 3) that higher sucrose levels also tend to depress phagocytic activity [see also Rode and Gordon (18)] as well as neutrophil adhesiveness, and a systematic study of the influence of other carbohydrates on phagocytosis might aid in discovering the mechanism by which these sugars depress phagocytosis.

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