Ascorbic Acid Metabolism in Diabetes Mellitus[a]

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Levels of ascorbic acid (AA) are decreased in various tissues of animals with experimental diabetes.[1,2] Altered plasma levels and turnover of AA have been reported in diabetic patients,[3,4] but these observations and their biologic importance have not been explained. Noting the structural similarity between glucose and AA (FIG. 1), Mann suggested in 1974 that glucose and vitamin C might occupy the same membrane transport system.[5] He subsequently reported with Newton that elevated glucose levels interfered with cellular AA transport in erythrocytes.[6] Others have observed inhibition by glucose of AA transport *in vitro* by human lymphocytes[7] and bovine endothelial cells.[8] Bigley *et al.* described competitive inhibition between the *in vitro* uptake of dehydroascorbic acid and glucose analogues by human polymorphonuclear leukocytes (PMN) and fibroblasts, and concluded on the basis of kinetic data that the competing ligands utilized the same membrane carrier.[9]

We recently subjected normal human volunteers to intravenous glucose tolerance testing in order to study the effect of acute hyperglycemia *in vivo* on the concentration of AA in mononuclear leukocytes (MNL).[10] Significant decreases in intracellular AA levels promptly followed the rapid glucose infusions.

The concentrations of AA in polymorphonuclear leukocytes and mononuclear leukocytes reportedly exceed plasma levels 25-fold and 80-fold, respectively, in normal individuals,[11] although the physiologic significance of this concentration gradient is not understood. Several published observations suggest a possible association between intracellular ascorbate, abnormal PMN function, and an increased frequency of infection in subgroups of patients with congenital leukocyte disorders.[12–23] Furthermore, ascorbate may affect neutrophil and monocyte chemotaxis.[15,16,23–25] Leukocyte dysfunction occurs in diabetes mellitus,[26–31] and is particularly associated with hyperglycemia and poor control.[32–35]

In this study we compared the effects of an acute intravenous glucose load on the ascorbic acid levels of polymorphonuclear leukocytes and mononuclear leukocytes in

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normal subjects. We examined concurrently whether leukocyte chemotaxis changed in association with alterations in intracellular AA. Finally, we investigated the effects of protracted *in vivo* hyperglycemia, maintained for up to four hours by a glucose clamp technique, on leukocyte ascorbic acid concentrations and chemotactic function.

MATERIALS AND METHODS

Normal male volunteers (ages 27-68) were studied according to a protocol approved by the Human Subjects Review Committee of the University of Washington, and after obtaining written informed consent. Subjects were asked to withhold all over-the-counter medications, including vitamins, for at least 24 hours before the study and to maintain a 12-hour overnight fast. Ten subjects received glucose as a bolus infusion, followed by examination of its effects on leukocyte AA concentrations.

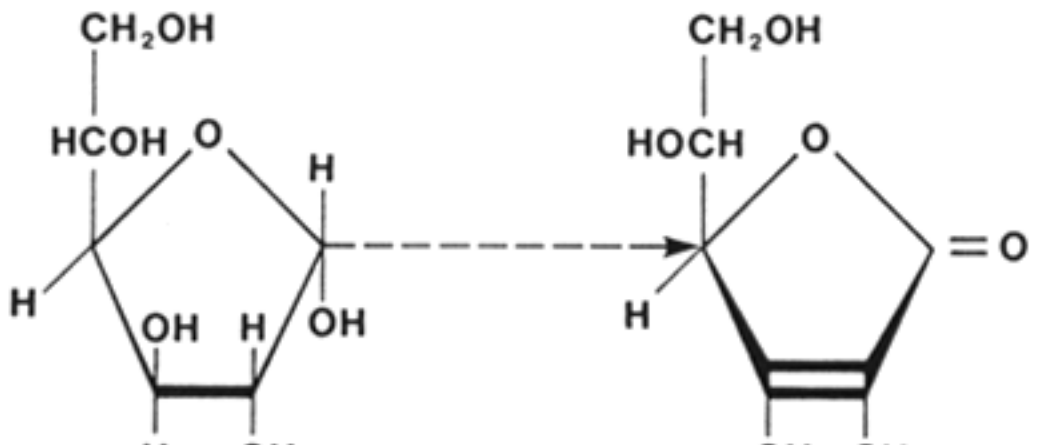

FIGURE 1. Molecular structures of D-glucose and L-ascorbic acid.

Chemotaxis by leukocytes from eight of the subjects was examined as well. Six of the eight returned at a later date for similar studies during continuous glucose infusions to maintain the blood glucose above 250 mg/dl for 4 hours.

Two baseline blood samples (−5, 0 minutes) were initially obtained from an antecubital vein prior to all glucose infusions. During studies with a single acute challenge of i.v. glucose, baseline blood sampling was followed by administration of 0.3 g per kg of 50% glucose given as a bolus to an indwelling butterfly needle in the contralateral antecubital vein. Blood samples were drawn at 5, 15, 20, 30, 60, 90, and 120 minutes after the glucose bolus. Glucose clamp studies were performed on another day several weeks later. Baseline blood samples were obtained, followed by the infusion of a loading dose of 25 g of 50% glucose over 12 minutes into an antecubital vein. In order to maintain the plasma glucose concentration above 250 mg/dl, the loading dose was followed by a continuous infusion of 10% glucose at a rate of 1 g per minute or, if necessary, 20% glucose at a rate of 2 g per minute.[36] Blood samples were subsequently drawn from the contralateral antecubital vein at 5-15 minute intervals to monitor the plasma glucose levels, and at 60, 120, 210, and 240 minutes for plasma and intracellular leukocyte AA levels. Tests of leukocyte chemotaxis were done at baseline and at 210 and 240 minutes.

Plasma was separated from cellular components by centrifuging 20 ml heparin anticoagulated venous blood. An aliquot of plasma was frozen at −20°C for subsequent

glucose determination. The remainder of the plasma and aliquots of the MNL and PMN fractions (described below) were processed on the same day of sample collection for ascorbic acid determination as described previously.[37]

After dilution with PBS, the cells were separated by centrifugation over a Ficoll-Hypaque solution with a density of 1.095 g/ml.[38] Two distinct fractions representing predominantly PMN and MNL cells were separately washed and counted using a hemocytometer. Smears were prepared and stained by Diff-Quick (Scientific Products) for differential cell counting. The PMN fraction contained 95-98% PMNs, with mononuclear cells composing the non-PMN cell contaminants. The MNL fraction represented 85 ± 5% lymphocytes, 8 ± 4% monocytes, with the remainder composed of contaminating PMNs. After aliquots of the PMN and MNL fractions were removed for determinations of ascorbic acid, the remaining cells were reserved for tests of chemotactic function.

Plasma glucose was determined by the glucose oxidase method using the Technicon (Tarrytown, N.Y.) Autoanalyzer II. Plasma and cellular ascorbic acid levels were determined by HPLC.[37] The intra-assay coefficient of variation (CV) is 2.4%. The inter-assay CV is 4.3%.

Chemotaxis of PMN and MNL cells was measured in microchemotaxis chambers (Neuroprobe Co., Bethesda, Md.) using the modified Boyden technique.[39,40] PMNs were diluted with Gey's basic salt solution (with 2% bovine serum albumin fraction V, without phenol red, pH 7.2 ± 0.1; Gibco) to contain 1×10^6 /ml, and chemoattractants used were 5% and 10% human zymosan activated serum (ZAS). For MNL, the cells were diluted to contain 1×10^6 /ml myeloperoxidase positive cells; 10% and 20% ZAS were used as chemoattractants. Additionally, chemotaxis for both cell fractions was mesured to the chemoattractant, 10^{-8} M *N*-formylmethionyl-leucyl-phenylalanine-methylester (FMLP). PBS was used for the control. Chemoattractant (25 μl) was added to each chamber's bottom compartment, with 50 μl of cell suspension introduced to the top compartment. For PMN, chemotaxis was quantified as the total number of cells that migrated completely through a 3μ pore size filter and were visible in five adjacent light microscopic fields (450×), after incubation for three hours at 37°C (5% CO_2, 100% humidity). For MNL, the filter pore size was 5 μ; incubation time was 90 minutes at 37°C (5% CO_2, 100% humidity), and chemotaxis was quantified as the total number of cells that migrated completely through the filter and were visible in five adjacent light microscopic fields (970×). Chemotaxis was determined in triplicate for each chemoattractant and the replicates averaged.

Changes from baseline for values of leukocyte intracellular AA concentrations and leukocyte chemotaxis were evaluated using Student's *t*-test. Correlation between leukocyte AA and plasma AA concentrations was tested by least-squares linear regression analysis. The relationship between mean fasting intracellular AA concentrations and the nadir of mean intracellular AA after glucose infusions was examined by one-way analysis of variance. Associations between changes in chemotaxis and decreases in leukocyte AA concentration were evaluated by the Kendall rank order correlation test.

RESULTS

Intracellular AA Leukocyte Levels

The concentration of AA in polymorphonuclear leukocytes in the fasting state was about half that in mononuclear leukocytes, as shown in TABLE 1. In order to

permit comparison with plasma AA levels, the intracellular AA concentration was expressed as mg/dl, converted from $\mu g/5 \times 10^6$ cells, using cell volume data from the literature.[41]

We found the average intracellular AA level in PMN was 17-fold concentrated compared with plasma, and MNL levels 43-fold increased. The MNL AA and plasma AA levels were correlated ($r = 0.65$, $p = 0.04$, $n = 10$), but no significant correlation was found between PMN AA and plasma AA ($r = 0.35$, $p = 0.32$).

FIGURE 2 demonstrates the effect of acute hyperglycemia on leukocyte AA levels. Ascorbic acid levels were expressed as the percentage of the basal concentration to facilitate analysis, since we observed a significant correlation between the fasting intracellular AA levels and the nadir of intracellular AA after glucose infusion for both MNL ($r = 0.79$, $p < 0.01$, $n = 10$) and PMN ($r = 0.75$, $p < 0.05$, $n = 10$). The decline in intracellular AA induced by hyperglycemia was not as dramatic in PMN (nadir $-26 \pm 12\%$ [mean $\pm$ SD] at 30 minutes, $n = 8$, $F = 6.29$, $p < 0.001$) compared to that in MNL ($-38 \pm 21\%$ at 20 minutes, $n = 8$, $F = 4.64$, $p < 0.01$). Similar results were obtained when statistical significance was calculated for the absolute decreases in PMN and MNL mean intracellular AA levels. The effect persisted for 30 minutes in MNL compared with 120 minutes in PMN. Plasma AA levels did not change.

TABLE 1. The Comparative Ascorbic Acid Concentration in Leukocytes and Plasma in Ten Normal Subjects in the Fasting State

	Measured			Calculated	
	MNL ($\mu g/5 \times 10^6$ Cells)	PMN ($\mu g/5 \times 10^6$ Cells)	Plasma (mg/dl)	MNL (mg/dl)	PMN (mg/dl)
Range	0.34-2.55	0.16-1.1	0.12-3.12	18.9-141.7	7.1-48.9
Mean	1.12	0.56	1.43	62.2	24.8
SD	0.66	0.34	0.89	36.8	15.2
Mean cell/plasma ratio				43×	17×
Cell/plasma ratio (range), Evans *et al.*[11]				26-84×	14-33×

Leukocyte Chemotaxis

We initially tested leukocyte chemotaxis before and after the declines in intracellular AA concentrations induced by acute hyperglycemia. Chemotaxis was measured for MNL at -5, 0, 15, and 30 minutes, since the decline in the intracellular AA concentration reached a nadir between 15-20 minutes and persisted for 30 minutes. Chemotactic function was evaluated in PMN at 0, 60, 90, and 120 minutes, since the decline in intracellular AA persisted for 120 minutes. We were not able to demonstrate any acute change in chemotactic function in either MNL or PMN following the single glucose pulse.

Unlike the observations following the single bolus infusion of glucose, chemotaxis by both PMN and MNL was significantly suppressed to all chemoattractants tested after exposure to hyperglycemia *in vivo* for 210 or 240 minutes (TABLES 2 and 3; FIG. 3). During these glucose clamp studies, plasma glucose was successfully maintained throughout the duration of the infusion above 250 mg/dl. We observed a decrease in the plasma AA concentration from a mean baseline concentration of 0.82 mg per dl to 0.56 mg per dl at 240 minutes (FIG. 3). Total urinary AA output was

measured during the infusion in four subjects, and varied from 1-20 mg/4 h, which appeared to be sufficient to account for the decrease in plasma AA. Mean intracellular concentrations of AA decreased from the baseline in both MNL and PMN, and remained depressed throughout the period of the hyperglycemic clamp. However, this decrease was not statistically significant at all points due to small sample size.

In both PMN and MNL, the decrements in chemotaxis compared to the mean baseline were similar ($p > 0.05$) for all the experimental chemoattractants. Changes

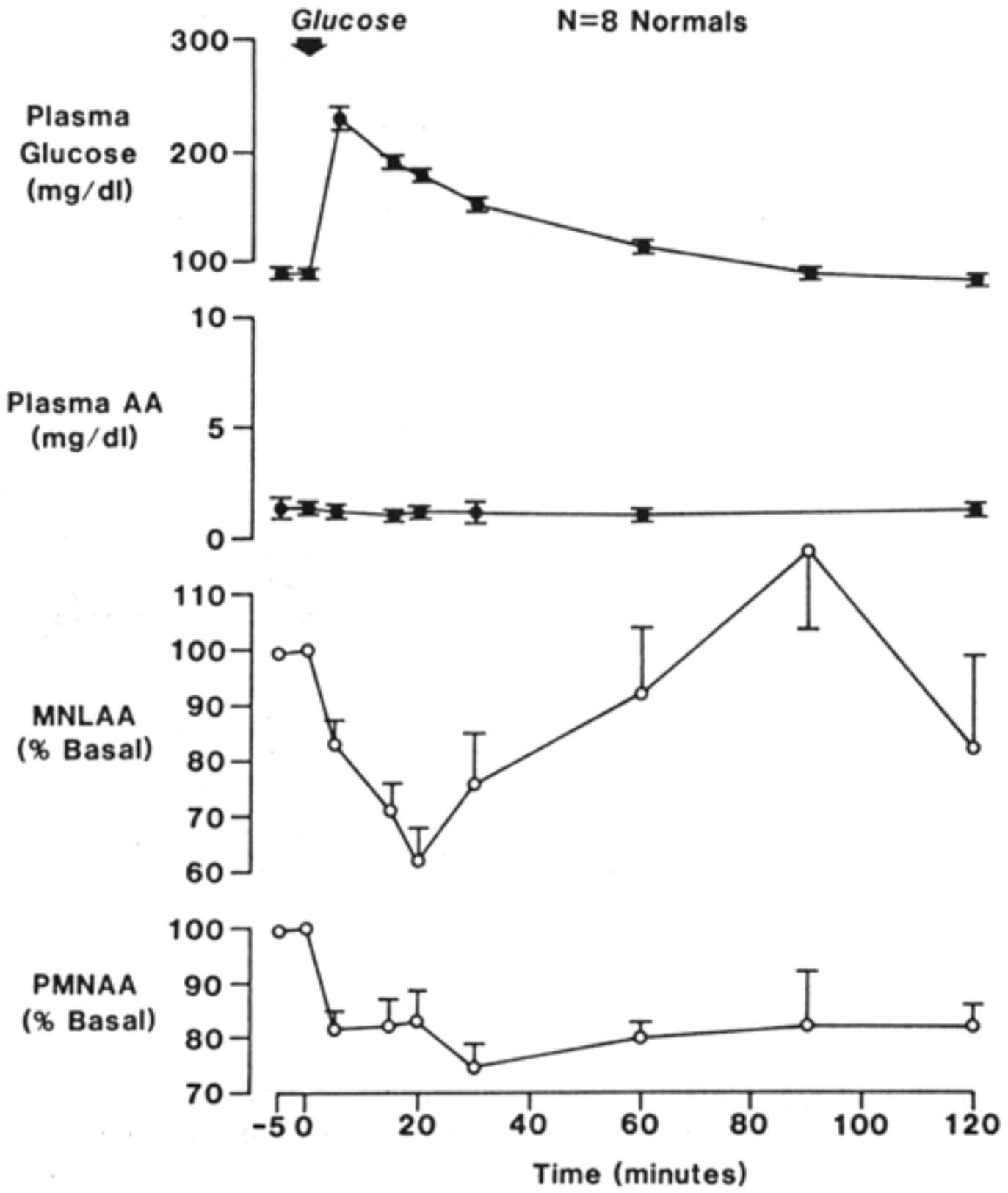

FIGURE 2. The effect of acute hyperglycemia on plasma, mononuclear (MNL), and polymorphonuclear (PMN) leukocyte ascorbic acid (AA) concentrations ($n = 8$, mean ± SEM).

in chemotaxis to all chemoattractants were combined for an analysis of possible association between changes in chemotaxis and the corresponding decreases in intracellular AA concentration. The changes in the various tests of chemotaxis by PMN correlated significantly with decreases in intracellular AA at 210 min ($p = 0.03$) and 240 min ($p < 0.01$). Changes in tests of chemotaxis by MNL correlated with decreases in intracellular AA at 240 min ($p = 0.03$), but the correlation was not significant at 210 min ($p = 0.1$).

TABLE 2. Chemotaxis by MNL from 6 Normals to Various Chemoattractants before and during Hyperglycemic Clamp (Mean ± SD)

	Time		
	Mean Baseline	210 min	240 min
Buffer	78 ± 3	75 ± 5	73 ± 4[a]
FMLP[d]	64 ± 3	59 ± 3[b]	58 ± 3[c]
10% ZAS[e]	104 ± 2	96 ± 3[c]	95 ± 3[c]
20% ZAS[e]	140 ± 4	134 ± 3[b]	128 ± 5[c]

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.
[d] Formyl-methionyl-leucyl-phenylalanine-methylester.
[e] Zymosan activated serum.

DISCUSSION

In these studies using the sensitive HPLC method to measure AA, we have confirmed that both polymorphonuclear leukocytes and mononuclear leukocytes concentrate ascorbic acid *in vivo*. The intracellular AA concentration averaged 17-fold in PMN compared with fasting plasma levels and 43-fold in MNL, comparable to concentrtions calculated by Evans *et al.*[11] Our data revealed a correlation between MNL AA and plasma AA, but a similar relationship could not be shown for PMN.

In order to assess a possible physiologic correlation of the acute decreases in leukocyte intracellular AA observed after i.v. glucose infusion, we examined whether changes occurred simultaneously in leukocyte chemotaxis. A central role for AA in leukocyte function has been implied by previous observations, but has not been clearly defined. Addition of exogenous AA to PMN has been reported to stimulate oxidative metabolism [12] and glycolysis,[13] to enhance microtubule assembly,[14] to stimulate random and directional movement,[15] and to prevent chemotactic deactivation.[16] In one study the AA effect on neutrophil mobility was related to the inhibition of the activity of

TABLE 3. Chemotaxis by PMN from 6 Normals to Various Chemoattractants before and during Hyperglycemic Clamp (Mean ± SD)

	Time		
	Mean Baseline	210 min	240 min
Buffer	25 ± 3	19 ± 1[a]	19 ± 1[a]
FMLP[c]	29 ± 3	23 ± 4[a]	23 ± 4[a]
5% ZAS[d]	64 ± 3	57 ± 3[a]	55 ± 6[a]
10% ZAS[d]	105 ± 3	97 ± 3[b]	95 ± 5[b]

[a] $p < 0.01$.
[b] $p < 0.001$.
[c] Formyl-methionyl-leucyl-phenylalanine-methylester.
[d] Zymosan activated serum.

the myeloperoxidase (MPO) H_2O_2/halide system.[17] Ascorbic acid has been shown to improve abnormal bactericidal activity in several conditions.[18] Ascorbic acid given to patients with Chediak-Higashi syndrome [19,20] and chronic granulomatous disease [12,21] has been reported to improve neutrophil function and clinically to decrease the frequency of infection.

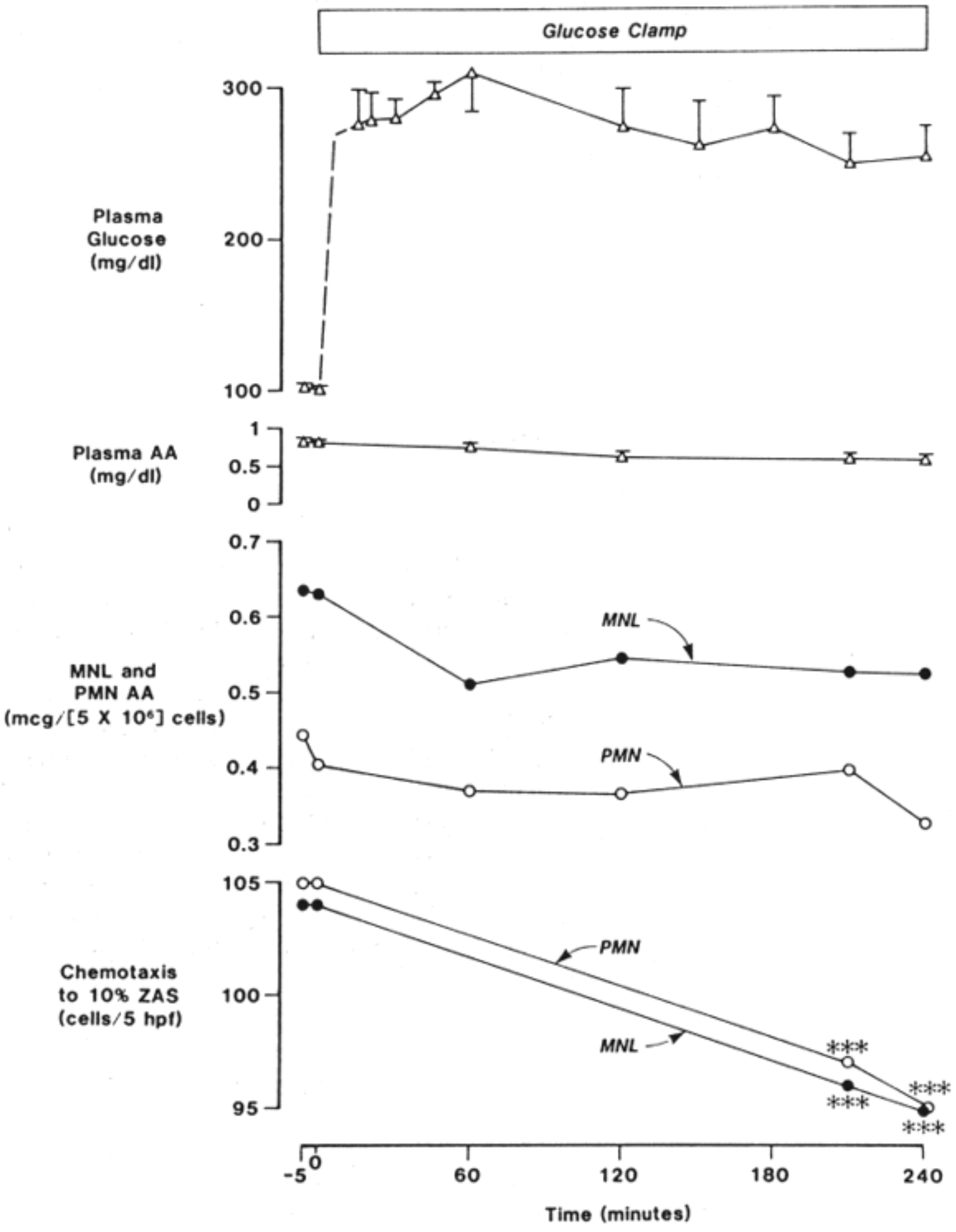

FIGURE 3. The effect of prolonged hyperglycemia maintained by a glucose clamp on plasma, mononuclear (MNL), and polymorphonuclear (PMN) leukocyte AA levels, and chemotaxis by PMN and MNL to 10% zymosan activated serum (ZAS) ($n = 6$; ***$p < 0.001$).

Abnormal PMN chemotaxis has been associated with hyperglycemia,[30,31] as have impaired intracellular killing and impaired phagocytosis.[32,33] PMNs from patients with poorly controlled diabetes reportedly failed to kill bacteria as effectively as neutrophils from normal subjects or patients with controlled diabetes.[34,35] The mechanisms responsible for these effects have not been demonstrated.

We elected to measure chemotaxis as a screening test for leukocyte function since it represents an important initial step in the acute inflammatory response, a function known to be affected *in vitro* by ascorbic acid [15,16,24] and reported to be depressed in diabetes mellitus.[23,25–31] We were unable to demonstrate acute changes in chemotactic function of either PMN or MNL from normal men following acute, transient hyperglycemia. However, following prolonged hyperglycemia, maintained *in vivo* by an intravenous glucose clamp for 3½ hours or longer, chemotaxis by both PMN and MNL directed at several experimental chemoattractants was significantly inhibited. Of particular interest, the changes in chemotaxis by both cell types correlated with decreases in the intracellular concentrations of ascorbic acid after 4 hours of continous hyperglycemia. These observations support the hypothesis that chronic hyperglycemia, as in the case of diabetes mellitus, may impair leukocyte chemotactic function by chronically depressing intracellular ascorbate. Continuous increased urinary losses of AA via osmotic diuresis in parallel with chronic glycosuria are likely to deplete the plasma compartment of ascorbic acid. These hypothetical mechanisms could result in a defective acute inflammatory response in diabetes, which in turn might contribute to increased susceptibility to bacterial infections and perhaps to delayed wound healing, which depends on prompt early infiltration by PMNs and macrophages to initiate the normal cellular sequence of wound repair.[42–44]

SUMMARY

Competition for membrane transport between glucose and ascorbic acid (AA) has been shown *in vitro* in human lymphocytes, granulocytes, and fibroblasts. Therefore, we examined the effects of acute administration of i.v. glucose on AA levels in mononuclear (MNL) and polymorphonuclear leukocytes (PMN) and on leukocyte chemotaxis. Plasma glucose and AA, MNL AA, PMN AA, and chemotaxis by MNL and PMN were measured before and after i.v. glucose in fasted normal male volunteers. A decline in AA occurred in PMN as well as MNL, but decreases in AA induced acutely by transient hyperglycemia were not associated with changes in chemotaxis. However, under conditions of prolonged hyperglycemia maintained by a glucose clamp technique, significant changes ($p < 0.01$) in chemotaxis by both PMN and MNL were observed after 210 and 240 min, with changes in chemotaxis to several chemoattractants significantly correlated with decreases in intracellular AA after 240 min ($p < 0.05$). These results are consistent with the hypothesis that chronic hyperglycemia may be associated with intracellular deficits of leukocyte AA, an impaired acute inflammatory response, and altered susceptibility to infection and faulty wound repair in patients with diabetes.

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DISCUSSION OF THE PAPER

B. LANE (*Columbia University School of Public Health, New York, N.Y.*): It's well known that diabetics are at greater risk for glaucoma and ocular hypertension than normals. My 1980 case-control study showed that ascorbic acid and the glucose tolerance factor, chromium, appeared to collaborate in lowering intraocular pressure. One explanation was a putative role of ascorbic acid as an insulin receptor potentiator.

Are you suggesting that your explanation of competition for transport is complementary to this previous explanation? Or do you feel that your explanation displaces the older explanation?

R. E. PECORARO (*Seattle VA Medical Center, Seattle, Wash.*): No, I don't think it's relevant to your observations. The data suggest strongly that transport occurs by the same transport mechanism in these cells. It's very clear from other data that's been discussed today and yesterday that in other cellular systems the transport mechanism appears to be quite different, so I would be very hesitant to extrapolate this information to other tissues.

B. LANE: Are you saying that there may be more a factor here of transport than potentiation of insulin receptors?

R. E. PECORARO: Well the insulin induces the glucose transporter at the cell membrane and this has been shown in fibroblasts both for ascorbic acid and for glucose. There's increased uptake of both of those after induction by insulin for a matter of hours.

E. J. DILIBERTO (*Wellcome Research Labs, Research Triangle Park, N. C.*): Even though you didn't show any fall in the ascorbate levels in plasma, did you by chance measure the dehydroascorbic acid levels as well?

R. E. PECORARO: No, we didn't. I think that would clarify a lot of these questions about uptake if we were able to do that. Our method measures both the dehydroascorbate and ascorbic acid as ascorbic acid.

J. HATHCOCK (*Food and Drug Administration, Washington, D.C.*): Do you think that ascorbic acid supplementation would help remedy the impaired leukocyte status in the hyperglycemic diabetics?

R. E. PECORARO: We have not done the appropriate work to answer that question.

S. TANNENBAUM (*Massachusetts Institute of Technology, Cambridge, Mass.*): Why did you lump monocytes and lymphocytes when they have such different function and origin? You essentially obliterated any interesting information that you might have derived from the monocytes by having lymphocytes there.

R. E. PECORARO: With regard to the chemotaxis?

S. TANNENBAUM: With regard to levels of ascorbic acid or chemotaxis.

R. E. PECORARO: It's a technical problem. Separations of cell types are difficult, and they have to be processed quickly in order to do studies such as chemotaxis without major artifacts. Based on limited data, monocyte levels appear to have much higher intracellular levels than lymphocytes. I would point out though that the chemotaxis results on the mononuclear cell fraction represented only monocyte chemotaxis in that peroxidase positive cells were aliquotted for those studies.

UNIDENTIFIED SPEAKER: When using the Ficoll gradient technique, do you have aggregates of white leukocytes close to the red cell layer and have difficulty in separating them? Do you lose any of your leukocytes in that way?

R. E. PECORARO: You lose some. We've examined carefully the various cellular makeups of these fractions, and red cell contamination is not a problem. It's less than 5%.