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Aminoguanidine and the prevention of leukocyte dysfunction in diabetes mellitus: a direct vital microscopic study

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1 Defective leukocyte-endothelial interactions are observed in experimental diabetes mellitus. The present study investigated the effect of aminoguanidine, an inhibitor of advanced glycation end products formation, on leukocyte-endothelial interactions in alloxan-induced diabetic rats.

2 In rats anaesthetized with sodium pentobarbitone, the internal spermatic fascia was exteriorized and the microcirculation was observed by a closed-circuit TV coupled to a microscope. The number of leukocytes rolling along the venular endothelium and sticking to the vascular wall was determined after topical application of zymosan-activated plasma (1 mg ml^{-1}), as well as the number of adherent and migrated cells after an irritative stimulus (carrageenan 100 μg).

3 The diabetic state decreased the number of rolling, sticking and migrated leukocytes. Pretreatment of diabetic animals with aminoguanidine (250 mg kg^{-1} day^{-1}, for 18 days) normalized these values. To be effective, aminoguanidine had to be administered chronically, starting treatment before induction of the diabetic state.

4 The preventive effect was unrelated to the number of circulating leukocytes, or to the hyperglycaemia or to the hyperosmolality secondary to hyperglycaemia.

5 A non-dialyzed (>12,000-Mr) material in plasma from diabetic, but not normal animals, decreased the number of rolling, sticking and migrated leukocytes in recipient rats. This effect was completely abolished by chronic treatment of diabetic plasma donors with aminoguanidine.

6 The results suggest that a protein modified by glycosylation (>12 kDa) is associated with leukocyte dysfunction in diabetes mellitus and that the ability of aminoguanidine to prevent such dysfunction is related to an inhibitory effect on advanced glycation end products formation.

Keywords: Aminoguanidine; leukocyte-endothelial interactions; diabetes mellitus

Introduction

Alterations of inflammatory events are noticeable from the early stages of poorly controlled diabetes mellitus. Functional changes in the behaviour of microvessels (Altura *et al*., 1979; Bohlen & Hankins, 1982), decreased responsiveness of endothelial cells to vasoactive agents (Fortes *et al*., 1983a,b; 1984), impaired gross inflammatory reactions (Garcia-Leme *et al*., 1973; 1974; Llorach *et al*., 1976), and depressed chemotaxis associated with a plasma factor that interacts with neutrophil receptors for complement-derived chemoattractants (Pereira *et al*., 1987; Sannomiya *et al*., 1990) have been described. These observations and many other aspects of the inflammatory response affected by the diabetic state have been reviewed (Garcia-Leme, 1989; Garcia-Leme & Farsky, 1993).

The mechanisms underlying leukocyte accumulation in a tissue depend on the interaction between the cells and the vascular endothelium. During the development of inflammatory responses leukocytes roll along the lining endothelium of postcapillary venules and eventually become firmly attached to the vascular wall before migrating into tissues. Defective leukocyte-endothelial interactions are observed in experimental diabetes mellitus and are thought to depend on the presence of specific plasma factors (Fortes *et al*., 1991). Plasma and cellular proteins modified by glycosylation in diabetic subjects lead to advanced glycation end products formation that plays a central role in the pathogenesis of diabetic complications (Bucala & Cerami, 1992; Brownlee, 1994). Advanced glycation end products formation is inhibited by aminoguanidine and its analogues (Rudermann *et al*., 1992).

The present study was, therefore, undertaken to investigate whether glycation products influence leukocyte-endothelial interactions in experimental diabetes mellitus.

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Methods

General

Male Wistar rats weighing 180–200 g at the beginning of the experiments were used. The animals were allowed a standard pellet diet and tap water *ad libitum* throughout the observation period. The experimental design included diabetic and control animals either treated or untreated with aminoguanidine (AG). Diabetes mellitus was induced by the intravenous injection of 42 mg kg^{-1} alloxan (ALX) dissolved in physiological saline. Control rats were injected with physiological saline alone. Ten days after ALX injection the presence of diabetes was verified by blood glucose concentrations >11.2 mmol l^{-1} estimated with the aid of a blood glucose monitor. Blood samples were obtained from the cut tip of the tail of the animals. Diabetic animals were always used at the tenth day of ALX injection. AG was given orally as a single daily dose (250 mg kg^{-1}) for 18 days. In the diabetic group, treatment started 8 days before ALX injection. Dosage in controls matched that in diabetic rats. Immediately before use total and differential leukocyte counts were determined in samples of the peripheral blood of the animals. Blood samples were obtained as referred to above. Total leukocyte counts were made in Neubauer chambers. Stained blood films were used for differential leukocyte counts. At the moment of death, blood samples were collected from the abdominal aorta of randomly selected animals in the various experimental groups for assessment of the percentage of glycosylated haemoglobin (HbA_1) by affinity chromatography.

Two series of experiments were performed. In one, the effect of AG treatment on the defective leukocyte-endothelial interactions of diabetic rats was assessed. In the other, the effect of plasma factors, obtained from either AG-treated or untreated diabetic animals, was determined by intravenous injections of the dialyzed material into normal controls.

Direct vital microscopy of the microcirculation

The animals were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.) and the internal spermatic fascia of the wall of the scrotal chamber was exteriorized for microscopic examination *in situ* (Fortes *et al*., 1991). This was done through a longitudinal incision of the skin and dartos muscle in the midline over the ventral aspect of the scrotum and opening of the cremaster muscle to expose the internal fascia. The procedure does not require extensive surgical manipulation for the observation of the vascular network and provides a valuable means for transilluminating a tissue for quantitative studies of the microcirculation. The animals were maintained on a special board thermostatically controlled at 37°C, which included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with warmed (37°C) Ringer-Locke solution, pH 7.2–7.4, containing 1% gelatin. The composition of the solution was (mmol l^{-1}): NaCl 154, KCl 5.6, $CaCl_2.2H_2O$ 2, $NaHCO_3$ 6 and glucose 5. The rate of outflow of the solution onto the exposed tissue was controlled to maintain the preparation in continuous contact with a film of the liquid. A 500-line television camera was incorporated to a triocular Zeiss microscope to facilitate the observation of the enlarged image ($\times 3400$) on the video screen. Images were recorded on a video recorder by use of a $\times 40$ long distance objective with a 0.65 numerical aperture. An image-splitting micrometer was adjusted to the phototube of the microscope (Baez, 1973). The image-splitter sheared the optical image into two separate images and displaced one with respect to the other. By rotating the image-splitter in the phototube the shearing was maintained in a direction at right angles to the axis of the vessel. The displacement of one image from the other allowed measurement of the vessel diameter. Vessels selected for study were third-order venules, defined according to their branch order location within the microvascular network (Gore & Bohlen, 1977; Rhodin, 1980). These vessels corresponded to postcapillary venules; their diameters ranged from 12 to 16 μm.

Interaction of leukocytes with the luminal surface of the venular endothelium was studied in a 100 μm segment of the vessels by use of recorded images (Farsky *et al*., 1995). Leukocytes moving in the periphery of the axial stream, in contact with the endothelium, are considered to be rollers (Atherton & Born, 1972; Dahlén *et al*., 1981). These leukocytes move sufficiently slowly to be individually visible and can be counted as they roll past a selected point on one side of the vessel. Leukocytes adhering to the endothelium (stickers) and migrating into the adjacent perivascular tissue are seen following the application of irritative stimuli to the preparation. The number of sticking and migrated leukocytes was evaluated by means of the recorded images. Counts were independently scored by two observers. A given section of the vascular bed was tested only once for the determination of the number of rollers or stickers and no more than two determinations were performed on a single animal. These were averaged for each animal. The number of leukocytes accumulating at the connective tissue adjacent to a postcapillary venule was determined in a standard area of 1,000 μm^2. This area was defined on the video screen, 10 μm in tissue corresponding to 3.4 cm on the screen (magnification $\times 3400$). Five different fields were evaluated on a single animal to avoid variability based upon sampling. Data were then averaged for each animal.

Experimental protocols

The number of rolling, sticking and migrated cells was determined to evaluate, first, the influence of AG treatment in diabetic animals relative to controls; and, second, the effect of diabetic plasma factors injected into control animals. The number of rollers and stickers was estimated in 10 min periods. Adhesion was investigated in two circumstances: (i) following exposure of the preparation to zymosan-activated homologous plasma that is a source of components of the complement system; (ii) following the injection of an irritant (100 μg carrageenan) into the scrotum to induce a local inflammatory response. In the first circumstance, the internal spermatic fascia, after a suitable control period of normal circulation, was exposed to 0.1 ml of a solution containing 10% zymosan-activated homologous plasma in physiological saline. To obtain activated plasma, zymosan was incubated (1 mg ml^{-1}) with plasma of normal animals for 1 h at 37°C. Following centrifugation at 1,600 *g* for 10 min, the zymosan-activated plasma was diluted 1:10 with physiological saline and topically added to the preparation. Adhesion of leukocytes was assessed 1, 5 and 10 min thereafter. Plasma treated identically, except for the addition of zymosan, was tested as control. In the other circumstance, animals were submitted to a local inflammatory response evoked by the injection of 0.1 ml of a solution containing 1 mg ml^{-1} carrageenan sodium salt in physiological saline into the scrotal chamber of the animals. Adhesion was evaluated 1 h after the injection of the irritant. Migration was investigated in the carrageenan-injected groups, 1 h after the administration of the irritant.

To investigate the influence of plasma factors, blood was collected from the abdominal aorta of anaesthetized animals in the presence of heparin. Untreated control and diabetic rats or control and diabetic rats treated with AG were used as blood donors. After centrifugation of blood, pooled plasma samples obtained from each group of animals, were dialyzed to equilibrium during 20 h at 4°C against 2×100 vol of 0.01 M ammonium carbonate buffer, pH 7.30–7.35, with 12,000-M_r retention dialysis tubing. Protein concentration in dialyzed plasma samples was spectrophotometrically determined at 280 nm. Mean ± s.e.mean plasma protein values were 82.67 ± 2.03 and 77.00 ± 3.51 mg ml^{-1} ($n = 3$) in diabetic and control plasma samples, and 80.33 ± 0.66 and 79.00 ± 2.08 mg ml^{-1} ($n = 3$) in samples obtained from diabetic and control rats treated with AG, respectively. Contact with glass was avoided throughout the extraction procedure. After lyophilization, the resulting material was stored at −20°C. At the moment of use, 100 mg of the lyophilized material was dissolved in 0.5 ml of sterile physiological saline and injected intravenously into control, non-diabetic rats. Tests were performed 1 h later.

Materials

Aminoguanidine bicarbonate salt, alloxan monohydrate, zymosan A from *Saccharomices cerevisae*, heparin sodium salt were obtained from Sigma; carrageenan sodium salt, a 60,000–100,000 M_r polysaccharide composed of sulphated galactose units was from Marine Colloids and affinity chromatographic columns from Bio Rad Laboratories.

Statistical analysis

All data are presented as means ± s.e.mean. One-way analysis of variance was performed followed by the Tukey-Kramer multiple comparisons test. $P < 0.05$ was considered to be statistically significant.

Results

Characteristics of study group

Relative to controls, animals rendered diabetic by the injection of ALX 10 days before exhibited a significant reduction in body weight gain during this period (59 ± 3 and 17 ± 4 g in control and diabetic animals, respectively, $P < 0.001$). Blood glucose levels had significantly increased from control values of 5.6 ± 0.3 to 28.6 ± 1.3 mmol l^{-1} ($P < 0.001$) as had glycosylated haemoglobin percentage (3.4 ± 0.2 and 7.1 ± 0.3 in control and diabetic animals, respectively, $P < 0.001$). Pretreatment with AG did not significantly alter these parameters in either control or diabetic animals (data not shown).

Leukocyte-endothelial interactions and the effect of aminoguanidine administration

Quantitation of the rolling cell flux in the different groups of animals showed that the ALX-induced diabetic state resulted in a marked decrease in the number of rolling leukocytes, relative to controls. Treatment of diabetic rats with AG, for 18 days, resulted in an increase in the number of rolling leukocytes. The values attained matched those observed in control animals. In contrast, treatment of control rats with AG was without effect. Results are presented in Figure 1. The observed changes were not dependent on the number of circulating leukocytes because total and differential counts in the peripheral blood were equivalent in normal and diabetic animals, either treated or untreated with AG (data not shown). However, AG was ineffective when given after the installation of the diabetic state, i.e. after ALX administration. A single dose of the drug (250 mg kg^{-1}) given by the oral route 3 h before testing or equal daily doses (250 mg kg^{-1}) administered for up to 7 days before testing did not ameliorate the leukocyte rolling behaviour in diabetic animals. Values estimated were 37.6 ± 2.4 ($n=5$) and 32.1 ± 2.7 ($n=7$) rolling leukocytes 10 min^{-1}, in AG-treated and untreated diabetic rats, respectively.

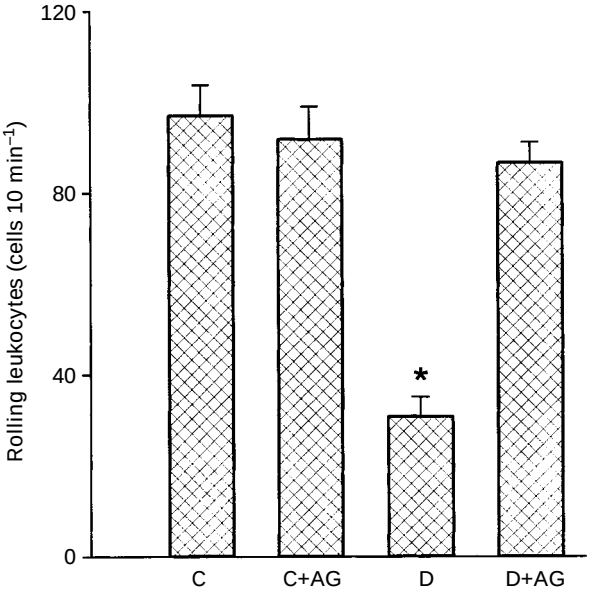

Figure 1 Rolling leukocytes in postcapillary venules of untreated control (C, $n=10$) and diabetic (D, $n=8$) rats and in control and diabetic rats treated with aminoguanidine (C+AG, $n=8$; D+AG, $n=13$, respectively). Values are means±s.e.mean. $*P<0.001$ vs values in other groups.

Table 1 Number of leukocytes adhered to postcapillary venules of diabetic rats and matching controls after contact with zymosan-activated plasma (ZAP): effect of aminoguanidine (AG) administration

		Adhered leukocytes (100 μm venule length) at			
Animals	*Treatment*	*1 min*	*5 min*	*10 min*	n
Diabetic	None	1.5±0.3*	4.8±0.6**	7.0±0.8**	6
Control	None	4.0±0.8	13.2±0.7	18.8±1.2	5
Diabetic	AG	5.2±1.4	13.8±2.2	16.8±2.4	5
Control	AG	5.6±2.3	14.7±2.3	19.0±4.0	5

For testing, the preparations were exposed to 0.1 ml of a solution containing 10% ZAP in physiological saline. Values are means±s.e.mean for n animals in each group. $*P<0.05$, $**P<0.01$ vs values in other groups.

Tests performed at varying intervals (1 to 10 min) after the topical application of zymosan-activated plasma to the exposed tissue showed a reduction in the number of cells adhering to the vessel wall in diabetic animals as compared to controls. Treatment with AG prevented the impaired response in diabetic rats and produced no effect in matching controls (Table 1). No adhesion was observed following the application of non-activated plasma to the preparations (data not shown). Impaired adhesion and migration were observed after carrageenan injection in diabetic animals. Whereas in matching controls firmly adhered leukocytes were seen 1 h after the injection of carrageenan, and this was accompanied by cell migration into tissue, in diabetic rats the number of adhered and migrated leukocytes were comparatively reduced. AG prevented the impaired responses of diabetic animals. Results are shown in Figure 2.

Influence of plasma factors

To investigate the influence of plasma factors on leukocyte-endothelial interactions, plasma samples obtained from untreated and AG-treated rats were dialyzed, lyophilized and injected intravenously into normal animals. Tests performed 1 h later showed that whereas the final product obtained from control rats failed to interfere with the number of leukocytes normally rolling along the venular endothelium, the lyophilized material obtained from diabetic rats produced a marked decrease in the number of rolling leukocytes. The inhibitory activity was completely abolished by pretreatment of diabetic plasma donors with AG. Results are presented in Table 2. Similar results were observed when leukocyte adhesion and migration were investigated during the course of the local inflammatory reaction evoked by carrageenan. In animals receiving plasma factors from control donors, firmly adhered leukocytes were seen 1 h after the injection of carrageenan and cells accumulated in the connective tissue adjacent to the venule in a pattern characteristic of the inflammatory reaction. In

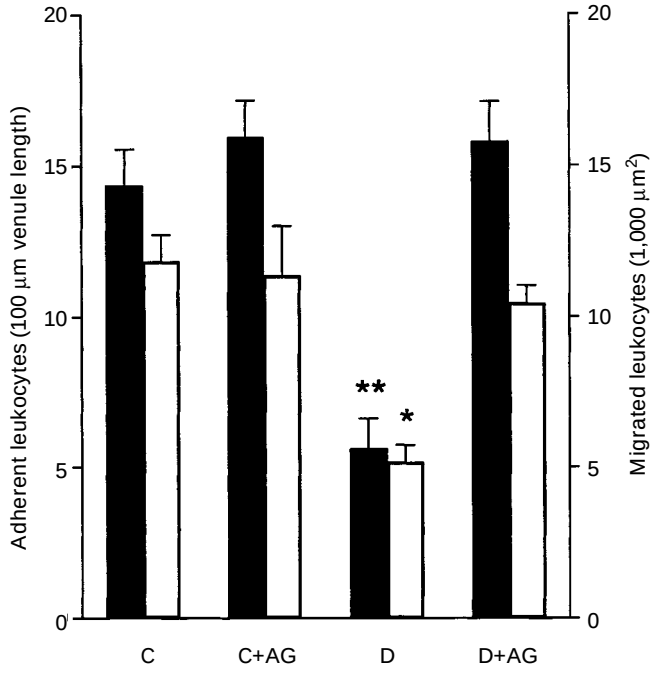

Figure 2 Cells sticking to the endothelium of a 100 μm segment of postcapillary venules (solid columns) and accumulation of leukocytes in a standard area (1,000 μm^2) of the perivascular tissue (open columns) in untreated control (C, $n=5$) and diabetic (D, $n=6$) rats and in control and diabetic rats treated with aminoguanidine (C+AG, $n=5$; D+AG, $n=7$, respectively). Values are means±s.e.mean. $*P<0.01$ and $**P<0.001$ vs corresponding values in other groups.

Table 2 Number of rolling leukocytes in postcapillary venules of normal rats following the intravenous injection of dialyzed and lyophilized plasma samples

Plasma donors	*Treatment*	*Rolling leukocytes (number 10 min^{-1} per venule)*	n
Diabetic	None	38.0 ± 4.5*	8
Control	None	100.1 ± 4.8	5
Diabetic	AG	102.5 ± 3.8	6
Control	AG	106.3 ± 4.5	5

Tests were performed 1 h after the intravenous injection of dialyzed and lyophilized material (100 mg) dissolved in sterile physiological saline. Values are means ± s.e.mean for *n* animals in each group. *$P < 0.001$ vs values in other groups.

Table 3 Number of adhered and migrated leukocytes during the course of an inflammatory response in normal rats following the intravenous injection of dialyzed and lyophilized plasma samples

Plasma donors	*Treatment*	*Adhered leukocytes (100 μm venule length)*	*Migrated leukocytes (1,000 μm^2)*	n
Diabetic	None	4.1 ± 0.6*	5.8 ± 0.5*	7
Control	None	15.7 ± 1.3	13.5 ± 0.5	5
Diabetic	AG	16.9 ± 0.8	12.9 ± 0.9	7
Control	AG	13.8 ± 0.5	14.1 ± 0.7	5

The dialyzed and lyophilized material (100 mg dissolved in sterile physiological saline) was injected by the intravenous route, immediately before the injection of carrageenan (100 μg in physiological saline) into the scrotal chamber of the animals. Tests were performed 1 h later. Values are means ± s.e.mean for *n* animals in each group. *$P < 0.001$ vs values in other groups.

contrast, animals receiving diabetic plasma factors exhibited a marked reduction in the number of adhered and migrated leukocytes. This effect was prevented by treatment of diabetic donors with AG (Table 3).

Discussion

The present results suggest a positive association between glycosylation reactions and leukocyte dysfunction in experimental diabetes mellitus and the potential for AG to prevent defective leukocyte-endothelial interactions in this condition. The suggestion is supported by the following observations. First, the number of rollers, stickers and migrated cells which was reduced after the induction of the diabetic state, again normalized following pretreatment of the animals with AG. Second, no quantitative changes were observed in control animals under the same dose regime of the drug. Third, the effect of AG was unrelated to the number of circulating leukocytes, because total and differential counts in the peripheral blood were equivalent in animals either treated or untreated with the drug; and was not related to hyperglycaemia *per se* or hyperosmolality secondary to hyperglycaemia, because blood glucose levels and the percentage of glycosylated haemoglobin (HbA_1) remained higher in diabetic rats treated with AG. To be effective, AG had to be chronically administered, dosage starting before the installation of diabetes. When given three days after the onset of the diabetic state, when leukocyte functions are already impaired (Sannomiya *et al*., 1990) AG proved ineffective in ameliorating leukocyte-endothelial interactions in diabetic animals.

Defective leukocyte-endothelial interactions and leukocyte chemotaxis in diabetes mellitus are ascribed to the interference of plasma constituents. The substance responsible was shown to be heat labile (56°C), destroyed by incubation with trypsin and retained after dialysis with 12,000-M_r retention dialysis tubing. These characteristics provide good evidence that the defective functions are associated with a circulating protein (Pereira *et al*., 1987; Sannomiya *et al*., 1990; Fortes *et al*., 1991). Several plasma and cellular proteins are modified by glycosylation, a non-oxidative reaction between glucose and amino groups, in diabetes mellitus, as a function of sugar concentration and time (Higgins & Bunn, 1981; Means & Chang, 1982; Kennedy *et al*., 1982). AG inhibits the formation of advanced glycation end products by reacting with Amadori-derived fragmentation products such as 3-deoxyglucosone (Edelstein & Brownlee, 1992). That a >12 kDa glycosylated protein might be responsible for leukocyte dysfunction in diabetes mellitus is supported by the following observations: (i) control rats injected intravenously with material obtained after dialysis (12,000-M_r) of diabetic rat plasma behaved as diabetic animals in that they exhibited a reduced number of rollers, stickers and migrated leukocytes; (ii) the inhibitory activity was completely abolished by chronic treatment of diabetic plasma donors with AG.

The initial phase of inflammation, a transient slowing of neutrophils in postcapillary venules (rolling behaviour) is mediated by the selectin family of adhesion molecules (Lawrence & Springer, 1991; Ley *et al*., 1991). Subsequently, firm adhesion of neutrophils to the vessel wall occurs via interaction of the CD11/CD18 complex (β2 integrins) to endothelial ligands as intercellular adhesion molecule-1 (ICAM-1) (von Adrian *et al*., 1991; 1992). Therefore, blockade of cell adhesion molecules, either on leukocytes or endothelial cells or both, can effectively inhibit inflammation. Recent observations on the behaviour of leukocytes in the microcirculation of the internal spermatic fascia, by intravital microscopy, suggested that plasma proteins from alloxan-induced diabetic rats might regulate the expression of ICAM-1 (Sannomiya *et al*., 1996). Furthermore, a >10 kDa, heat labile (56°C) glycosylated protein, separated from the serum of diabetic rats, is capable of decreasing membrane fluidity of control leukocytes, as shown by flow cytometry studies (Masuda *et al*., 1990).

Any dysfunction in the sequence of events leading to the ability of leukocytes to migrate efficiently in response to inflammatory stimuli could result in an increased susceptibility of the host to infectious diseases (Ward, 1974; Gallin *et al*., 1980). It is well established that certain infections occur almost exclusively in diabetic patients and that many diabetic patients have a worse prognosis once infection is established (Wilson, 1986; Garcia-Leme, 1989; Garcia-Leme & Farsky, 1993).

It is plausible, therefore, to suggest that a >12 kDa glycosylated protein is responsible for the defects in leukocyte-endothelial interactions and chemotaxis in diabetes mellitus, which represents an aggravating factor for host defense in the first stages of infection. Finally, the results presented here indicate that the ability of AG to prevent leukocyte dysfunction is related to an inhibitory effect on advanced glycation end products formation.

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