Glucose-Modified Proteins Modulate Essential Functions and Apoptosis of Polymorphonuclear Leukocytes

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Abstract. Any modulation of the activity of polymorphonuclear leukocytes (PMNL) is a potential cause of the altered immune response in uremia. Because the level of glycation products is elevated in uremic sera and peritoneal effluents, the effect of glycated proteins on essential functions and on apoptosis of PMNL was investigated. Proteins from sera of healthy donors were incubated with and without glucose. The extent of early glycation was monitored by boronate chromatography and the fructosamine assay. The formation of late glycation products was assessed by fluorescence spectroscopy and Western blotting that used a specific antibody for imidazolone, a late glycation product. With the addition of aminoguanidine, a compound that inhibits the formation of late but not of early glycation products, protein samples with early glycation only were obtained. Glucose-modified proteins increased chemotaxis and activation of the 2-deoxy-D-glucose uptake of PMNL obtained from healthy donors, compared with those of unmodified proteins. PMNL apoptosis, assessed by morphologic changes, by detecting DNA strand breaks, and by measurement of the caspase 3 activity, was increased in the presence of glucose-modified serum proteins. It was found that the formation of late glycation products is necessary for the effect on PMNL chemotaxis. In contrast, early glycation of proteins is responsible for the increase of glucose uptake and apoptosis. It was concluded that the accumulation of glycated proteins in uremic sera and peritoneal fluid may contribute to the diminished immune function observed in uremia, by modulation of essential PMNL functions and acceleration of PMNL apoptosis.

Bacterial infections still represent a main cause for the increased morbidity and mortality among patients with uremia (1,2). This is primarily a consequence of disturbed functions of polymorphonuclear leukocytes (PMNL) in this group of patients (3). PMNL, cells of the first-line unspecific immune defense, migrate to the site of infection along a chemotactic gradient; they ingest the invading microorganisms by phagocytosis and kill them with proteolytic enzymes and toxic oxygen radicals. Disturbances in one of these essential functions will lead to an increased risk for bacterial infections. The importance of apoptotic cell death of PMNL has been recognized within the past few years. PMNL have a short life span and are already programmed to die via apoptosis when they enter circulation (4). Apoptosis is important for the normal resolution of inflammation: apoptotic PMNL, unlike necrotic PMNL, are recognized and taken up by macrophages without the release of proinflammatory cytokines (5,6). In healthy organisms, there is a balance between antiapoptotic and proapoptotic factors. Although a decrease in PMNL apoptosis at the site of inflammation would be harmful for the organism, increased PMNL apoptosis would contribute to the diminished immune function observed in uremia.

Uremic toxins—circulating factors that accumulate in the serum of uremic patients—play a crucial role in the inhibition of PMNL functions. Although a number of factors that modulate essential PMNL functions have already been purified and characterized (reviewed in reference 7), so far no uremic toxins that affect PMNL apoptosis have been identified.

With regard to future diagnosis and therapy, it would be useful to identify such toxins. Because the level of advanced glycation end products (AGE) is elevated in patients with uremia and is even higher than that in patients with diabetes mellitus without renal disease (8), we investigated in the present study the effect of this group of factors on essential PMNL functions and on spontaneous PMNL apoptosis.

Materials and Methods

Patients

The effluents obtained from two patients who were undergoing continuous ambulatory peritoneal dialysis (CAPD) were used for the isolation of glucose-modified proteins by preparative boronate affinity chromatography (see below). Patient 1 received 2.5 L of peritoneal dialysis fluid (lactate buffer) containing 1.5% (wt/vol) glucose three times daily and 2.5 L of peritoneal dialysis fluid (lactate buffer) containing 4.25% (wt/vol) glucose once daily. Patient 2 received 2 L of peritoneal dialysis fluid (lactate buffer) containing 2.3% (wt/vol) glucose twice daily and 2 L of peritoneal dialysis fluid (lactate buffer) containing 1.5% and 2.0% (wt/vol) glucose once daily. Patient 2 (but not patient 1) had diabetes mellitus. Informed consent was obtained

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from the patients. Care was taken that the patients did not have any clinical signs or symptoms of infection that would interfere with our study.

Limulus Amebocyte Lysate Assay

For the semiquantification of endotoxin, we used the limulus amebocyte lysate assay (E-Toxate; Sigma, St. Louis, MO), according to the instructions of the supplier. All collected CAPD effluents and the serum proteins used in this study had <1.25 EU/ml.

Isolation of PMNL

PMNL were isolated by use of discontinuous Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Ten ml of the venous whole blood of healthy donors was anticoagulated by collecting it into a sterile Lithium Heparin Vacutainer tube (Becton Dickinson, Meylan Cedex, France) and then put on top of 12 ml of Ficoll-Hypaque. After centrifugation, erythrocytes were removed from the pellet by hypotonic lysis with ammonium chloride buffer (157 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM ethylenediaminetetraacetate Na_2) on ice. After lysis, the PMNL were collected by centrifugation at 4°C and washed once with ammonium chloride buffer and twice with the buffer used in the functional assay. The cells were counted in a cell counter (Cell Dyn 610; Abbott, Wiesbaden, Germany). The viability of the PMNL obtained by this protocol was >95%, as determined by assessment of the exclusion of ethidium bromide (Gibco-BRL Life Technologies, Gaithersburg, MD) under the fluorescence microscope.

Preparation of Glucose-Modified Proteins

Sera obtained from five healthy donors were incubated in the presence or absence of 1 M D-glucose (Merck, Darmstadt, Germany) and in the presence or absence of aminoguanidine (Sigma) at 37°C for 10 d. We investigated which aminoguanidine concentration was necessary to inhibit the formation of late glycation products, determined by measurement of fluorescence. Human serum albumin (final concentration 40 mg/ml) was incubated for 10 d at 37°C in buffer only or with 1 M D-glucose in the presence of various concentrations of aminoguanidine. Afterward, the extinction and emission fluorescence spectra were taken. The results obtained showed that 10 mM aminoguanidine is sufficient to block the formation of fluorescence material. We therefore used this concentration in the following experiments. All samples contained penicillin-streptomycin (Gibco-BRL) at a final concentration of 100 U/ml. The sera were dialyzed against phosphate-buffered saline (PBS) by use of a membrane with a molecular weight cutoff of 1000 d (Spectra/Por, Laguna Hills, CA), sterilized by filtration, and frozen in aliquots at −80°C.

Molecular Characterization of Glucose-Modified Proteins

Early Glycation Products. We used two different methods to measure the success of our *in vitro* modifications. (*1*) Boronate gel affinity chromatography was routinely used in clinical laboratories to quantify the concentration of glycated hemoglobin or albumin (9). The percentage of glycated proteins was determined by application of 50 μl of protein solution onto a 1-ml boronate affinity gel column (GLYCO GEL II; Pierce, Rockford, IL) with the use of binding buffer (250 mM ammonium acetate, 50 mM $MgCl_2$ [pH 8.05]). The bound material was eluted with elution buffer (100 mM Tris-HCl, 200 mM sorbitol [pH 8.5]). Afterward, the protein concentration of the unbound and bound material was determined by the bicinchoninic acid protein assay (Pierce). The percentage of protein bound to the boronate column is shown in Figure 1A. (*2*) Using the fructosamine assay (10) (Roche Diagnostics GmbH, Mannheim, Germany), according to the instructions of the supplier, we confirmed the results obtained by boronate gel affinity chromatography. The results of the fructosamine assay that chemically measures early glycation are shown in Figure 1B. We obtained the same results with both assays, *i.e.,* proteins incubated in the presence of glucose but in the absence of aminoguanidine (S+) or presence of glucose and aminoguanidine (S+AG). We found an increased degree of modification, compared with that of proteins incubated with buffer alone (S−). The presence of aminoguanidine alone (S−AG) did not increase the percentage of proteins bound to the boronate column (Figure 1A) and did not increase the signal in the fructosamine assay (Figure 1B).

Late Glycation Products. We used two different methods to assess the extent of late glycation. (*1*) The results from fluorescence spectroscopy (Luminescence Spectrometer LS B 50; Perkin Elmer, Überlingen, Germany) are shown in Figure 2A. Excitation and emission spectra were taken at wavelengths of 340 and 415 nm, respectively. (*2*) By Western blotting, we detected the formation of imidazolone, a late glycation product. After electrotransfer of the proteins from the sodium dodecyl sulfate polyacrylamide gels to a nitrocellulose membrane, imidazolone was detected by a specific monoclonal antibody (final concentration, 5 μg/ml) (11), horseradish peroxidase–labeled goat–anti-mouse antibodies, and

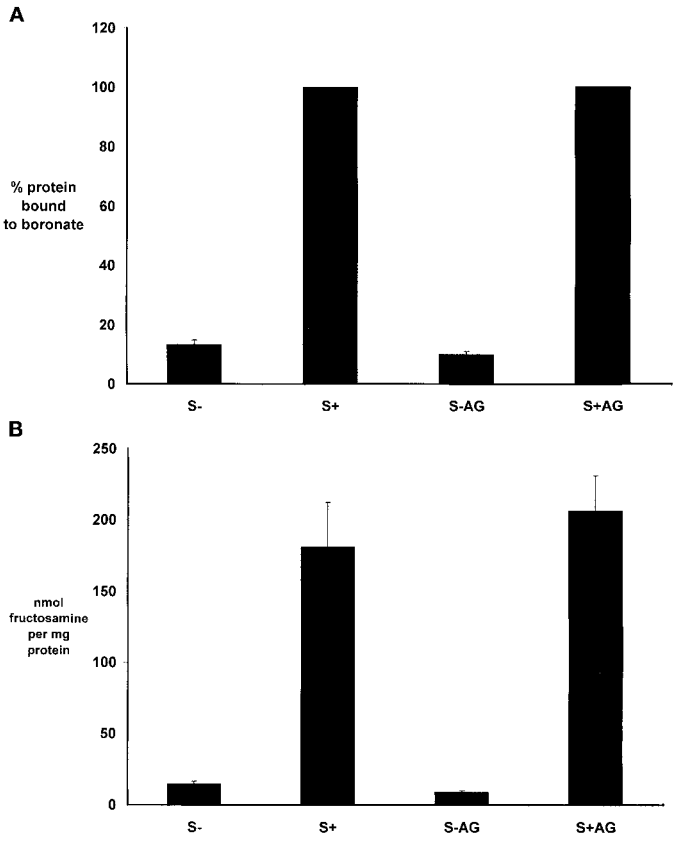

Figure 1. Measurement of early glycation products in human serum protein samples ($n = 5$) by boronate gel affinity chromatography (A) or fructosamine assay (B). The serum protein samples were incubated with buffer alone (S−), with glucose (S+), with buffer and aminoguanidine (S−AG), and with glucose and aminoguanidine (S+AG), as described in the Materials and Methods section. (A) Percentage of protein bound to a boronate affinity gel column. (B) Fructosamine (nmol) per mg protein. Mean values ± SEM.

the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). The results of the Western blotting experiments are shown in Figure 2B. In agreement with the results of the fluorescence spectroscopy (Figure 2A), the highest signal that was specific for imidazolone was obtained with proteins that were incubated with glucose alone (S+), whereas the signal of proteins that were incubated with glucose and aminoguanidine (S+AG) stayed in the same range as that obtained with proteins that were incubated with buffer only (S−).

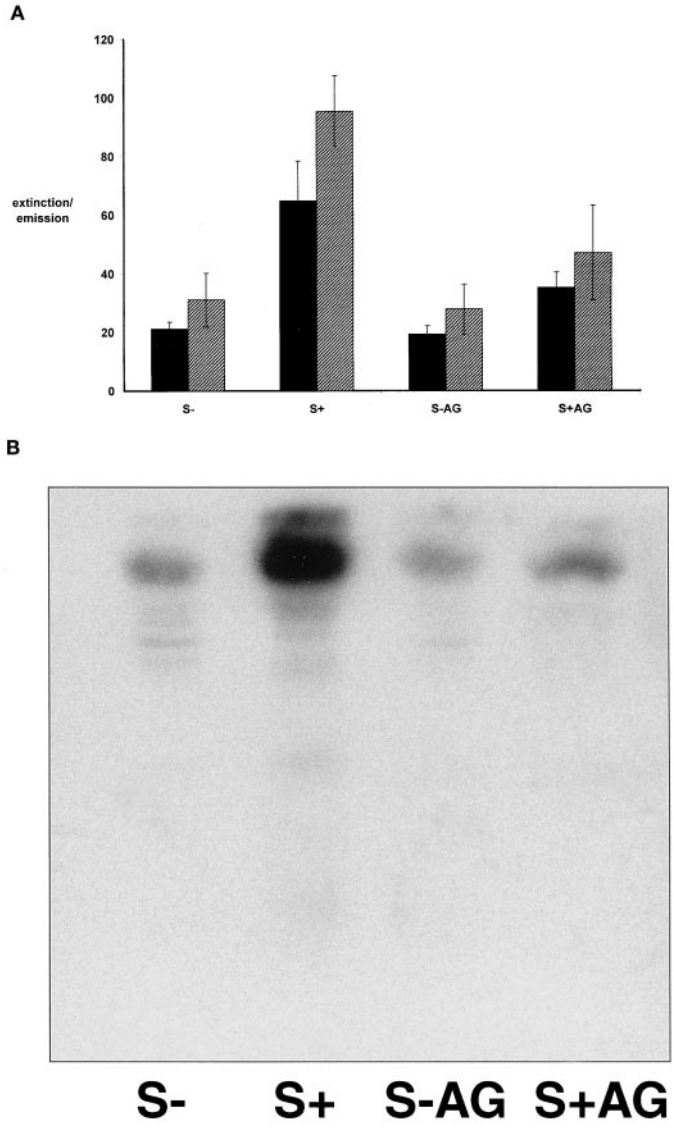

Figure 2. Measurement of late glycation products in human serum protein samples ($n = 5$) by fluorescence spectroscopy (A) or Western blotting (B). The serum protein samples were incubated with buffer alone (S−), with glucose (S+), with buffer and aminoguanidine (S−AG), and with glucose and aminoguanidine (S+AG), as described in the Materials and Methods section. (A) The fluorescence maxima of the extinction (■) and emission (▨) spectra in arbitrary units. Mean values ± SEM. (B) Western blot with use of a specific monoclonal antibody that detects imidazolone.

Isolation of Glycated Proteins by Preparative Boronate Affinity Chromatography

Peritoneal effluents of patients who were undergoing CAPD were concentrated approximately 20-fold with Cuprophan membranes, with a cutoff of approximately 5 kD (Hemoflow E4; Fresenius, Oberursel, Germany), and applied onto a 10-ml boronate affinity gel column (GLYCO GEL II; Pierce). The bound material was eluted as described above and dialyzed against PBS by use of membrane with a molecular weight cutoff of 1 kD (Spectra/Por).

PMNL Function Tests

Chemotaxis. PMNL chemotaxis was determined by the under-agarose method (12). PMNL were resuspended at a concentration of $0.5 \times 10^6/10$ μl in Dulbecco's PBS (pH 7.2; Gibco-BRL Life Technologies) or in PBS containing the proteins under investigation and incubated for 15 min at 37°C before the start of the chemotaxis assay. N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma), dissolved in Hanks' balanced salt solution (Gibco-BRL Life Technologies), was used as a chemoattractant at a concentration of 4.2×10^{-7} M. During the migration step, the agarose plates were incubated for 2 h at 37°C. Then the cells were fixed with methanol and paraformaldehyde and stained with Giemsa (Merck, Darmstadt, Germany). The distance that the cells migrated under the agarose was measured under the microscope.

2-Deoxy-D-Glucose Uptake. The hexose uptake was assessed by a method used routinely in our laboratory (13–15) and described elsewhere (16). Isolated PMNL were resuspended in PBS at a concentration of 1×10^6 cells/ml and preincubated for 5 min at 37°C in a shaking water bath. Then up to 20 μl of PBS containing the tested proteins or the same volume PBS was added to 200-μl of cell suspension and incubated for 15 min. Ten μl of PBS or fMLP methyl-ester (fMLP-M; Sigma), at a final concentration of 10^{-7} M, were added, and the incubation was continued for 15 min at 37°C. Then 100 μl 2-deoxy-D-[1-^{3}H]glucose (Amersham Pharmacia Biotech) stock solution (5 μCi/ml 100 μM D-glucose in PBS) were added and incubated for 60 min. To confirm the linearity of the glucose transport over this period of time, we stopped the reaction at various time points. We found a linear time dependence of the unstimulated as well as stimulated glucose uptake for incubation time points up to 60 min (data not shown). This is in agreement with previous reports on PMNL glucose transport that used the deoxy-glucose tracer technique (17). The uptake was stopped by addition of 1 ml of ice-cold PBS. The samples were centrifuged at 20,000 × *g* for 20 s, and the pellet was washed once with ice-cold PBS. Afterward, the supernatant was removed, and the pellet, together with the 1.5-ml reaction tube, was put into a scintillation vial containing 10 ml of Optifluor (Packard Instrument B.V.-Chemical Operations, Groningen, The Netherlands). The incorporated radioactivity was determined with a liquid scintillation counter.

We determined the incorporated activity in unstimulated ("basal") and "stimulated" cells both in the absence and presence of the substance under investigation. We obtained our results in cpm. Because we used freshly isolated PMNL (with their normal biologic variability) in the individual experiments and because we were interested only in the relative effect caused by the substance under investigation, we normalized our results by setting the value for the unstimulated cells in the absence of substance as 100% and calculate the percentage of the other samples.

Spontaneous Apoptosis of PMNL

PMNL were isolated under sterile conditions as described above. The cell suspension (6×10^6 cells/ml) was incubated at 37°C for 20 h. The spontaneous apoptotic cell death was assessed by three different methods.

Morphologic Features. The PMNL suspension was mixed with fluorescence DNA-binding dyes and examined under the fluorescence microscope. Acridine orange (Merck, Darmstadt, Germany) and ethidium bromide (Life Technologies-BRL) were added, to a final concentration of 5 μg/ml each. Acridine orange is taken up by the cells, binds to DNA by intercalation between stacked base pairs, and appears green. Ethidium bromide is taken up only by dead cells without intact cell membrane and also binds to DNA but stains it orange to a stronger extent than does acridine orange. Because the DNA in nonapoptotic cells is structured within the nucleus and the DNA in apoptotic cells is condensed, the following four cell populations can be observed and counted under the microscope: (*1*) viable, nonapoptotic with green, structured nucleus; (*2*) apoptotic with green, condensed nucleus; (*3*) late apoptotic with orange, condensed nucleus; and (*4*) necrotic with orange structured nucleus (usually not seen in this experimental setup).

DNA Strand Breaks. DNA strand breaks, characteristic for the apoptotic cell death, were detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end–labeling staining (TUNEL), with use of the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH), according to the instructions of the supplier. Fluorescein labels incorporated in nucleotide polymers were detected and quantified by flow cytometry (Epics XL-MCL; Coulter, Hialeah, FL).

Caspase 3 Activity. Caspase 3 activity was determined by use of a colorimetric protease assay kit (Chemicon International, Inc., Temecula, CA), according to the instructions from the supplier.

Statistical Analysis

The results were statistically evaluated by paired analysis that used the Wilcoxon test.

Results

Glucose Modification Modulates the Effect of Serum Proteins on PMNL

Chemotaxis. We used the under-agarose method (12) to test the influence of protein modification with glucose on the chemotactic response of PMNL. PMNL exerted an increased movement toward fMLP in the presence of glucose-modified proteins (S+) but not in the presence of unmodified serum proteins (S−) or in the presence of buffer alone (Figure 3). The increased movement toward fMLP was not observed when we used serum proteins modified in the presence of both glucose and aminoguanidine (S+AG), which suggests that late glycation products are responsible for the chemotaxis-enhancing effect. We used 5 mg/ml as the final protein concentration in the chemotaxis assay. As can been seen in Figure 4, 5 and 10 mg/ml glucose-modified (S+) proteins stimulate chemotaxis more than comparable concentrations of unmodified (S−) serum proteins.

2-Deoxy-D-Glucose Uptake. We investigated the effect of glucose-modified serum proteins on the uptake of ^{3}H-labeled 2-deoxy-D-glucose, considered as a measurement of the metabolic activity of PMNL. We found that the relative activation of the glucose uptake by fMLP-M was higher in the presence of glucose-modified proteins (S+), compared with that of unmodified proteins (S−) or buffer. As shown in Figure 5, serum proteins modified by early glycation seem to be responsible for this effect, because the increase in activation of glucose uptake is highly significant in the presence of both serum proteins incubated with glucose and aminoguanidine (S+AG) and proteins incubated with glucose alone (S+).

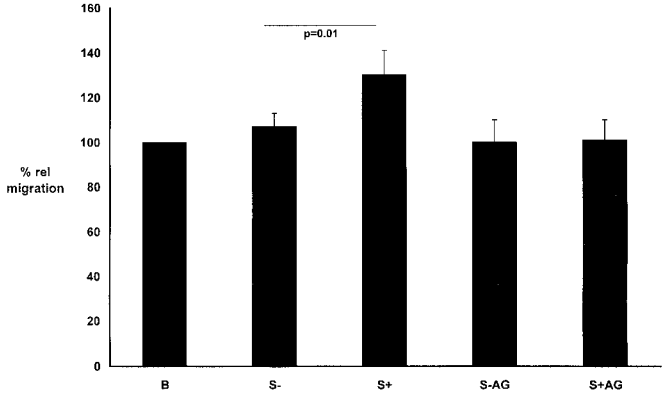

Figure 3. Influence of human serum protein samples incubated with buffer (S−), with glucose (S+), with aminoguanidine (S−AG), and with glucose and aminoguanidine (S+AG) on the chemotactic movement of polymorphonuclear leukocytes (PMNL) toward N-formyl-methionyl-leucyl-phenylalanine (fMLP), compared with PMNL chemotaxis in the presence of buffer alone (B) ($n = 8$). Mean values ± SEM.

In our 2-deoxy-D-glucose uptake assay, we used 10^{-7} M fMLP-M, a concentration that has been shown in pilot experiments to cause maximal stimulation of PMNL suspended in buffer, while being aware that there is a slight batch-to-batch variation for the optimal concentration for activation. 2-Deoxy-D-glucose uptake was higher in the presence of glucose-modified proteins (S+), compared with that of unmodified proteins (S−) over a concentration range from 10^{-5} to 10^{-8} M. A maximum activation for both S− and S+ occurred at 10^{-7} M. FMLP is 10-fold more potent than fMLP-M to "chemoattract" PMNL. We did not, however, observe a significant difference between fMLP and fMLP-M in the stimulation of PMNL 2-deoxy-D-glucose uptake (data not shown).

Apoptosis. We tested the influence of protein modification with glucose on spontaneous PMNL apoptosis. A much

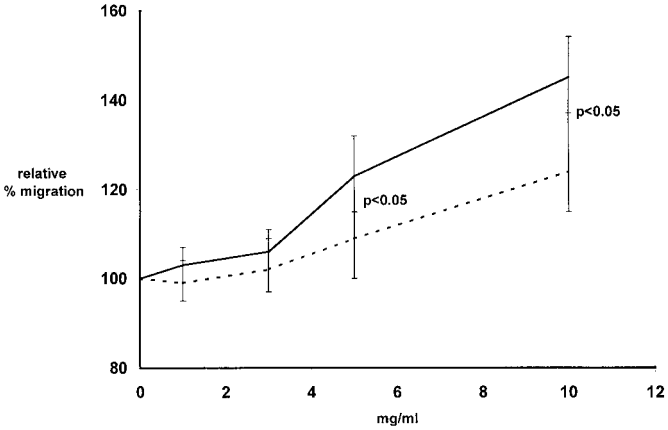

Figure 4. Influence of human serum protein samples incubated with buffer (S−, dotted line) or with glucose (S+, solid line) at different final concentrations on the chemotactic movement of PMNL toward fMLP ($n = 6$). Mean values ± SEM.

lower percentage of PMNL survived in the presence of buffer, compared with the presence of unmodified serum proteins (S−), indicating that serum proteins *per se* are able to decrease PMNL apoptosis. In contrast, modified serum proteins (S+) were able to increase the spontaneous apoptotic cell death of PMNL and thereby decrease the number of viable PMNL. Serum proteins that are modified in the presence of both glucose and aminoguanidine (S+AG)—and therefore contain only early glycation modifications—decrease the number of viable PMNL by increasing PMNL apoptosis as well (Figure 6A). By detecting DNA-strand breaks using the TUNEL method (see Material and Methods section), we obtained similar results (Figure 6B). In agreement with these data, Figure 6C shows that the activity of caspase 3, a central executioner molecule in the apoptotic process, is increased in the presence of glucose-modified (S+ and S+AG), compared with the presence of unmodified (S− and S−AG) proteins. We used 5 mg/ml as the final protein concentration in the apoptosis assays. As can been seen in Figure 7, 5 and 10 mg/ml glucose-modified (S+), compared with unmodified serum proteins (S−), increase the spontaneous apoptotic cell death of PMNL and thereby decrease the number of viable PMNL.

Effects of Glycated Proteins Isolated from Uremic Patients on PMNL

We tested the influence of proteins present in the effluent of patients who were undergoing CAPD on the uptake of 2-deoxy-D-glucose by PMNL. We compared proteins binding to boronate (BBP) and proteins not binding to boronate (PD−). At a protein concentration of 1 mg/ml, BBP significantly increased the uptake of 2-deoxy-D-glucose by PMNL (Figure 8). However, PD− also show a significant effect on 2-deoxy-D-glucose uptake, although insignificantly less so than BBP at this protein concentration. Therefore, there are other factors/proteins in addition to BBP present in CAPD effluents that modulate chemotaxis, 2-deoxy-D-glucose uptake, and apoptosis. Obviously, our *in vitro* findings with modified serum proteins may not by applicable directly to the effect on PMNL in the CAPD environment.

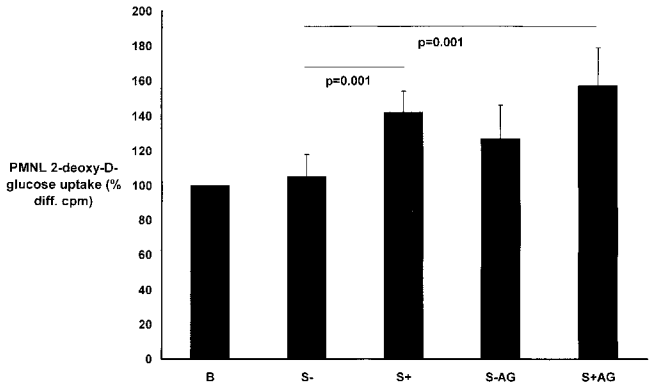

Figure 5. Influence of human serum protein samples incubated with buffer (S−), with glucose (S+), with aminoguanidine (S−AG), and with 2-deoxy-D-glucose and aminoguanidine (S+AG) on the relative activation of PMNL 2-deoxy-D-glucose uptake by fMLP methyl-ester (fMLP-M), compared with PMNL 2-deoxy-D-glucose uptake in the presence of buffer alone (B) (n = 10). Mean values ± SEM.

Discussion

The aim of the present study was to identify a class of uremic toxins that is able to modulate PMNL apoptosis and essential PMNL functions. In previous studies, we identified several proteins that inhibit 2-deoxy-D-glucose uptake (13–15) and chemotaxis (18). Unlike for essential PMNL functions, no

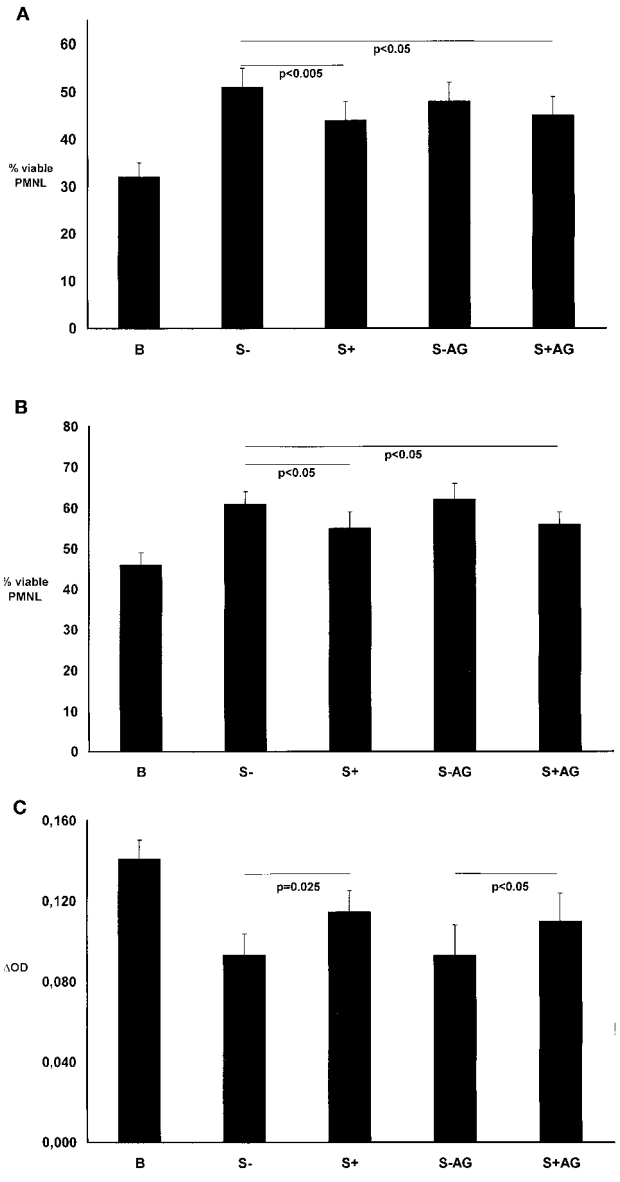

Figure 6. Influence of human serum protein samples incubated with buffer (S−), with glucose (S+), with aminoguanidine (S−AG), and with glucose and aminoguanidine (S+AG) on the spontaneous PMNL apoptosis. The percentage of viable cells was determined by morphologic criteria (n = 15; A) and by detection of DNA strand breaks with the use of the terminal deoxynucleotidyl transferase–mediated dUTP nick end–labeling staining method (n = 13; B). The activity of caspase 3 was determined by a colorimetric assay (n = 8; C). Mean values ± SEM.

uremic toxin with the potential to modulate PMNL apoptosis has been described so far. Recently, it was shown that uremic PMNL undergo accelerated apoptosis *in vitro* (19) and that constitutive cellular factors are partly responsible for this observation. However, uremic plasma also has been shown to accelerate PMNL apoptosis (19).

We chose to investigate the effect of glucose-modified proteins on PMNL. In the presence of glucose, proteins are nonenzymatically modified at their lysyl side chains or at the amino-terminal amino groups. At the beginning of the glycation reaction, the Amadori product is formed via a Schiff base intermediate. At later stages and after a variety of complex chemical rearrangements, AGE are formed. This modification is responsible for physical changes in proteins and tissues (20) and finally leads to protein cross-linking. Glycation is an unavoidable, minor feature of the physiologic metabolism: 6 to 15% of human serum albumin is glycated in normal human serum (21). However, in some pathophysiologic states, such as renal insufficiency and diabetes mellitus, AGE levels are elevated as a result of decreased renal clearance and/or increased rate of glycation. Interestingly, AGE levels are twice as high in patients with end-stage renal disease, compared with those in patients with diabetes mellitus without renal disease (8). The AGE level remained 3.5- to 6.0-fold above normal, regardless of the kind of treatment (high-flux dialysis, conventional hemodialysis, or peritoneal dialysis). Even though a high-flux hemodialysis session can reduce AGE to approximately 50%, the AGE concentration goes back to the pretreatment range within 3 h (22). The clinical relevance of AGE is underlined by reports (reviewed in reference 21) that show that high levels of AGE are involved in cell activation such as increased expression of extracellular matrix proteins, of vascular adhesion molecules, and of cytokines or growth factors. The AGE-related cell activation is associated with monocyte chemotaxis (23), angiogenesis, oxidative stress, and cell proliferation (24).

We modified with glucose *in vitro* serum proteins that were obtained from healthy donors. The degree of early glycation was determined by analytical boronate chromatography (Figure 1A) and the fructosamine assay (Figure 1B). Although the fructosamine assay reacts with glycated proteins but not AGE, it was described by Wu *et al.* (25) that boronate gel affinity chromatography also reacts with AGE. One could speculate that proteins that already contain AGE structures still expose early glycation structures on their surfaces that react with boronate. However, our results show that proteins that are modified by early—and not by late—glycation are responsible for the effects on PMNL glucose uptake and apoptosis. Therefore, the presence or absence of AGE in the boronate binding material does not influence our conclusions. The degree of AGE formation was determined by fluorescence spectroscopy (Figure 2A) and by detection of imidazolone, a known AGE structure, by use of a specific monoclonal antibody that recognizes this epitope (11) in a Western blotting experiment (Figure 2B).

We tested the effect of unmodified and glucose-modified serum proteins on the chemotactic movement of PMNL using the under-agarose method. PMNL chemotaxis is increased in the presence of glucose-modified (S+) serum proteins, compared with the control samples (S−, Figure 3). We also wanted to answer the question of whether early or late glycation products are responsible for the observed effect. The compound aminoguanidine inhibits the AGE formation, possibly by scavenging fragmentation products formed from the Amadori products (26), but does not prevent the formation of the early glycation products (27). In the present study, we could show that these effects of aminoguanidine were observed in our experimental setup as well: aminoguanidine efficiently abolished the formation of late glycation (Figure 2) but did not influence the content of early glycation products when proteins were modified with glucose (Figure 1). Although preparation S+ contains early and late glycation products, preparation

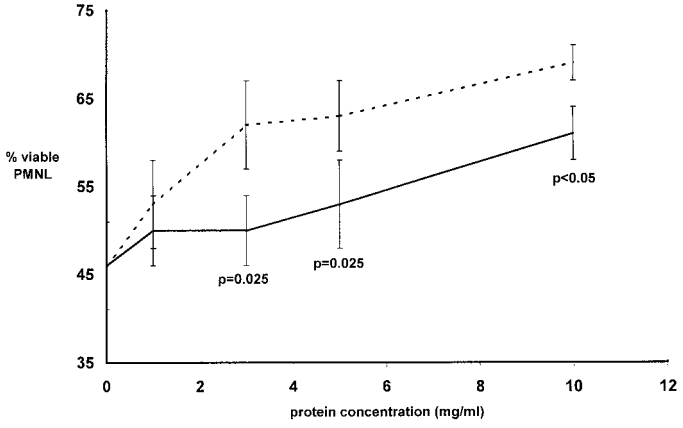

Figure 7. Influence of human serum protein samples incubated with buffer (S−, dotted line) or with glucose (S+, solid line) at different final concentrations on the spontaneous PMNL apoptosis, as determined by morphologic criteria ($n = 7$). Mean values ± SEM.

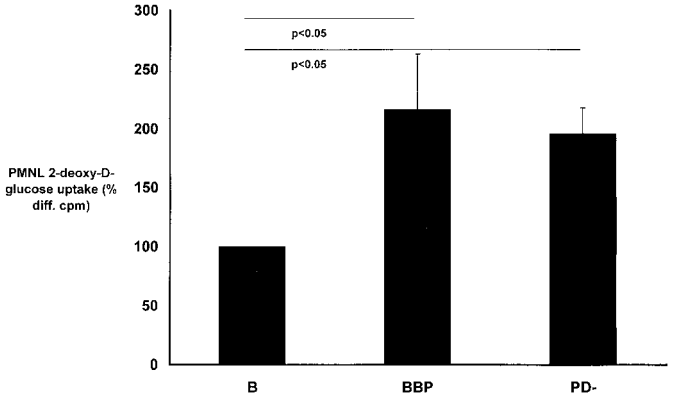

Figure 8. Influence of proteins binding to boronate (BBP) isolated from the effluent of patients who were undergoing continuous ambulatory peritoneal dialysis and of proteins not binding to boronate (PD−), both in a concentration of 1 mg/ml on the relative activation of PMNL 2-deoxy-D-glucose uptake (defined as the difference between the basal and the stimulated values normalized by setting the difference in the absence of substance as 100%) by fMLP-M, compared with PMNL 2-deoxy-D-glucose uptake in the presence of buffer alone (B) ($n = 6$). Mean values ± SEM.

S+AG contains early glycation products only. As shown in Figure 3, the presence of late glycation products seems to be necessary for the stimulatory effect on PMNL chemotaxis. This finding is in agreement with the stimulatory role of AGE-modified proteins found for other cells.

The uptake and accumulation of 2-deoxy-D-[1-^{3}H]-glucose serves as a quantitative measurement of the state of activation of phagocytic cells. We reevaluated our assay and found, in agreement with reports elsewhere in which the deoxy-glucose tracer technique was used (28), a linear time dependence of the unstimulated as well as stimulated 2-deoxy-D-glucose uptake for incubation time points up to 60 min. The uptake of 2-deoxy-D-glucose reflects the glucose transport only at low substrate concentration ($<$0.1 mM), whereas the following phosphorylation step becomes rate limiting at higher concentrations (29). Because we used very low concentrations in our assay (32 μM glucose and 0.2 μM deoxy-glucose as tracer), we are confident that our data represent glucose transport. Furthermore, McCall *et al.* (30) found as well, in a similar experimental setup, that hexokinase is not rate limiting.

We measured the uptake of 2-deoxy-D-glucose in the presence of glucose-modified (S+) proteins and control sera (S−) and found that the relative activation by fMLP-M was significantly higher in the presence of the modified samples. Serum proteins that are incubated in the presence of aminoguanidine (S+AG) show the same effect on the activation of glucose uptake (Figure 5) as that of proteins that are incubated in the presence of glucose alone (S+). Therefore, early and not late glycation products are responsible for the effect observed.

We investigated the effect of the glucose-modified proteins on spontaneous PMNL apoptosis by detecting the characteristic morphologic changes such as condensation of the nucleus under the fluorescence microscope (Figure 6A). These results were confirmed by detection of DNA-strand breaks by use of the incorporation of fluorescein-labeled nucleotides and quantification by flow cytometry (Figure 6B), as well as by measurement of the activity of caspase 3 (Figure 6C). Because apoptotic PMNL are removed by macrophages under *in vivo* conditions, we did not distinguish between apoptotic and late apoptotic cells and show in the results of our apoptosis assays the percentage of viable cells. We showed that PMNL die faster in the presence of glucose-modified (S+) proteins, compared with the controls (S−). These results are consistent with the finding of Cendoroglo *et al.* (19) that apoptotic cell death of PMNL is increased in uremia. Recently, it also was shown that an increase in cell death by apoptosis of mononuclear cells is induced by uremia and, independently, by dialysis treatment (31). Figure 7 shows that the presence of human serum *per se* is able to decrease PMNL apoptosis in a concentration-dependent manner. This effect has been described elsewhere (32) and has been confirmed in our system.

That diminished functions of apoptotic PMNL have been described elsewhere (19) is not contradictory to our results, because we investigated the immediate effect of protein samples on normal PMNL, whereas Cendoroglo *et al.* (19) determined the functions of PMNL that are already undergoing apoptotic cell death. In agreement with these data, we also found, in experiments that used apoptotic PMNL, greatly decreased chemotactic movement and 2-deoxy-D-glucose uptake, compared with freshly isolated PMNL (data not shown). Serum proteins that are incubated in the presence of aminoguanidine (S+AG) show the same effect on spontaneous PMNL apoptosis (Figure 6) as proteins that are incubated in the presence of glucose alone (S+). Therefore, early and not late glycation products are responsible for the effect observed.

It is beyond the scope of the present study to investigate the molecular mechanism of the effects described in this work. This issue should be addressed in future experiments. However, in a first set of experiments, we could show that the presence of glucose-modified proteins leads to an increased, concentration-dependent raise of intracellular calcium levels in PMNL after stimulation with fMLP (33).

That the effect of unmodified serum proteins is different from the effect of buffer alone is not surprising, considering the wide variety of protein factors present. It could be argued that the maximum activation possible is affected by the increased basal levels in the presence of proteins. However, this should apply for the *in vivo* situation as well, regardless of the underlying mechanism. Studies to investigate the mechanism are ongoing in our laboratory.

That PMNL die faster in the presence of modified serum proteins could be the result of the abolished functions of survival factors contained in human serum. However, we modified two individual proteins that are not considered to be survival factors, albumin and β2-microglobulin, in the same way as the serum proteins studied in this article and found basically the same effect on PMNL apoptosis (data not shown). This result is in favor of the possibility that our data could be explained by a more specific yet unidentified mechanism, *e.g.,* the existence of specific receptors. Even though we did not identify individual chemical structures that are responsible for the PMNL modulating effects found in this study, our experimental setup—the incubation of the same protein samples in the absence and presence of glucose—makes us confident that it is the modification by glucose *per se* that changes the functional properties of the proteins in the ways described above. Furthermore, we isolated glycated BBP from the effluent of patients who were undergoing CAPD and showed that they exhibit a similar effect on PMNL 2-deoxy-D-glucose uptake (Figure 8). However, as in the case of chemotaxis and apoptosis, the effect of BBP was not significantly different from that of PD−, which suggests that other factors in addition to boronate binding proteins modulate PMNL functions. This assumption is supported by our recent finding that the presence of carbamoylated proteins, another group of modified proteins enriched in uremic plasma, also increases PMNL apoptosis (34).

In conclusion, we demonstrate in this article that glycated proteins that accumulate in uremic sera are able to modulate essential PMNL functions such as chemotaxis and glucose uptake. Furthermore, we showed that glycated proteins accelerate the apoptotic cell death of PMNL and may contribute to the diminished immune function observed in uremia. This is the first time that a group of uremic toxins has been shown to modulate PMNL survival.

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