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In vitro study of the potential role of guanidines in leukocyte functions related to atherogenesis and infection

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In vitro study of the potential role of guanidines in leukocyte functions related to atherogenesis and infection.

Background. The blunted immune response upon stimulation in chronic renal failure (CRF) is often coupled to a baseline inflammatory status which has been related to atherogenesis. Uremic biologic fluids and several specific uremic retention solutes alter cell-mediated immune responses, as well as the interaction of calcitriol with the immune system.

Methods. The present study evaluated the influence of different guanidino compounds on DNA synthesis, chemiluminescence production, and CD14 expression of undifferentiated and calcitriol-differentiated HL-60 cells. In a second setup, these guanidino compounds were evaluated for their specific effect on normal human leukocyte oxidative burst activity and tumor necrosis factor-α (TNF-α) expression.

Results. First, several guanidino compounds elicited proinflammatory effects on leukocytes. Methylguanidine and guanidine stimulated the proliferation of undifferentiated HL-60 cells and the antiproliferative effect of calcitriol ($P < 0.05$) was neutralized in the presence of methylguanidine ($P < 0.05$) and guanidinosuccinic acid ($P < 0.05$). The phorbol-myristate-acetate (PMA)-stimulated chemiluminescence production of the calcitriol differentiated HL-60 cells was enhanced in the presence of guanidine ($P < 0.05$). Methylguanidine and guanidinoacetic acid enhanced the lipopolysaccharide (LPS)-stimulated intracellular production of TNF-α by normal human monocytes ($P < 0.05$). Second, several guanidino compounds inhibited the function of leukocytes if they were activated. The PMA-stimulated chemiluminescence production of the calcitriol differentiated HL-60 cells was inhibited by the presence of methylguanidine ($P < 0.05$), guanidinoacetic acid ($P < 0.05$) and guanidinosuccinic acid ($P < 0.05$). After incubation of whole blood in the presence of methylguanidine, the *Escherichia coli* stimulated oxidative burst activity of the granulocyte population was significantly inhibited ($P < 0.05$). In addition, guanidinosuccinic acid had an inhibitory effect on the LPS-stimulated intracellular production of TNF-α by human monocytes ($P < 0.01$).

Conclusion. Guanidino compounds exert proinflammatory as well as anti-inflammatory effects on monocyte/macrophage function. This could contribute to the altered prevalence of cardiovascular disease and propensity to infection in patients with CRF.

Key words: uremia, atherogenesis, guanidines, inflammation, infection, leukocytes.

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Chronic renal failure (CRF) is characterized by functional anomalies of the immune system [1], resulting in an increased incidence of infection and a higher risk for malignancy [2]. In end-stage renal disease (ESRD), the blunted immune response upon stimulation is often associated with an activated baseline inflammatory status which has been related to atherogenesis [3]. There is now extensive evidence that atheromatosis is more frequent and more severe in uremia [4–6]. It has been shown that uremic biologic fluids alter cell-mediated immune responses in vitro [7–9]. The influence of several specific uremic retention solutes such as p-cresol, purines, several peptides (granulocyte inhibitory protein I and II, ubiquitin-like peptide), advanced oxidation protein products, and advanced glycation end products (AGEs) on biologic functions of immune-competent cells has been demonstrated, both in a stimulatory and an inhibitory way [8, 10–15].

In a previous study by our group, the effect of uremic ultrafiltrate (UUF) and fractions of the UUF on leukocyte function was evaluated. Fraction 1 of the UUF elicited an effect that was comparable to that of UUF per se, by enhancing the calcitriol-inhibited HL-60 cell proliferation and by inhibiting its calcitriol-induced differentiation [9]. Guanidino compounds were found to coelute within this fraction 1 of the UUF [16]. For that reason, we thought that as a next step it was worthwhile to evaluate the individual impact of guanidines on elements of immune function.

It is known that plasma and tissue levels of guanidino compounds such as guanidine, guanidinosuccinic acid,

and creatinine, which is the precursor of methylguanidine, and methylguanidine itself are highly increased in uremic biologic fluids and tissues [17–20] and that several guanidino compounds modify key biologic functions [21–23].

Studies on the effect of guanidino compounds on leukocyte function are relatively scarce, however. Hirayama et al [24] described that a mixture of guanidino compounds suppresses the production of superoxide by normal isolated neutrophils. Furthermore, guanidino compounds such as guanidinosuccinic acid, creatinine, and guanidinopropionic acid, have been shown to inhibit HL-60 and K562 cell line proliferation [25]. Finally, Autore et al [26] reported that methylguanidine downregulates the tumor necrosis factor-α (TNF-α) release by activated macrophages, but other guanidino compounds were not evaluated. All these studies evaluated the impact of guanidino compounds on activated leukocytes, pointing to changes in the context of the uremic susceptibility to infection, but the impact on baseline leukocyte function, potentially involved in atherogenesis, was not evaluated.

The present study evaluates the effect of different specific guanidino compounds on the function of the monocyte/macrophage phenotype, both at baseline and after activation. The monocyte-macrophage is a critical cell type in atherogenesis because the attachment of the activated monocyte to the vascular endothelium is an early event in the genesis of the fatty streak lesion. Our work uses representative parameters such as DNA synthesis by estimation of the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into the DNA, free radical production by the evaluation of the chemiluminescence response and the oxidative burst activity of leukocytes, and the intracellular production of TNF-α. In this way, the study evaluates changes, due to presence of guanidino compounds, with a possible role in atherogenesis, as well as in susceptibility to infection.

METHODS

Studies on HL-60 cells

For the first screening, HL-60 cells were used, with as advantages, unlimited availability of leukocytes with the monocyte/macrophage phenotype after culture in the presence of calcitriol [27] and the possibility to pursue long contact times with lasting cell viability.

Cell line culture

HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained as a continuous culture in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 20% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Merelbeke, Belgium) and 50 μg/mL gentamycin in a humidified atmosphere of 5% CO_2 in air at 37°C. Medium was refreshed every 3 to 4 days. Cell cultures were free of mycoplasma.

Experimental media

In this in vitro setup, HL-60 cells were cultured, for 7 days, in (*1*) medium alone, (*2*) medium containing specific guanidino compounds, (*3*) medium containing 10 nmol/L calcitriol to differentiate HL-60 toward the monocyte/macrophage phenotype, and (*4*) a combination of (2) and (3). The experimental media (1) and (2) were included as controls. Cell viability at the moment of evaluation exceeded 90% as assessed by propidium iodide exclusion on flow cytometry. The guanidino compounds (Sigma Chemical Co., St. Louis, MO, USA) methylguanidine, guanidine, creatinine, guanidinoacetic acid, and guanidinosuccinic acid were dissolved in RPMI 1640, respectively, at 8.4 μg/mL, 2.1 μg/mL, 240 μg/mL, 3.3 μg/mL, and 40 μg/mL, after which the solutions were filtered through a 0.22 μm filter unit (Millipore, Bedford, MA, USA). The applied concentrations are ten times higher than the concentration which had been determined in preliminary experiments in ultrafiltrates from hemodialysis patients (De Deyn et al, unpublished observations).

All guanidino compound solutions used in this study were found to be endotoxin-free (<0.2 IU/mL) (Endosafe® Endochrome-K™) (Charleston, SC, USA).

Evaluation methods for HL-60 cells

BrdU incorporation. After 1 week of incubation, cell proliferation was evaluated by the measurement of the incorporation of BrdU into DNA. Anti-BrdU (Becton Dickinson, San Jose, CA, USA) was used in flow cytometric analysis to identify cells that synthesized DNA during exposure to BrdU (Sigma Chemical Co.).

Cells were incubated for 30 minutes with BrdU (10 μmol/L) in the CO_2 incubator at 37°C. After two washings with 1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS), the pellet was resuspended in 200 μL of PBS on ice and to fix the cells, this suspension was then slowly added to 5 mL of 70% ethanol (−20°C) followed by a 30-minute incubation period on ice. After centrifugation and aspiration of the supernatant, 1 mL 2 N HCl/0.5% Triton X-100 was slowly added to the pellet followed by a 30-minute incubation period at room temperature to denature the DNA. After centrifugation, 1 mL of 0.1 mol/L $Na_2B_4O_4{\cdot}10\ H_2O$, pH 8.5, was added to the pellet to neutralize the acid, followed by a second centrifugation step. Cell concentration was adjusted with 0.5% Tween/1.0% BSA/PBS to achieve 10^6 cells/test. Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated Anti-BrdU for 30 minutes

and washed once in 1 mL 0.5% Tween/1.0% BSA/PBS, and finally resuspended in PBS containing 5 μg/mL propidium iodide before analysis by a FACScan® (Becton Dickinson).

Chemiluminescence production. Chemiluminescence production by HL-60 cells was determined after 1 week of incubation in the different culture media and after readjustment of the cell count to 1×10^6 cells/mL in every sample. We added 500 μL of luminol solution (56 μmol/L) and 100 μL of a phorbol 12-myristate 13-acetate (PMA) solution (1.5 μg/mL, final concentration) to 50 μL of cell suspension. The test tube was immediately processed into a luminescence analyzer (Lumicon, Hamilton, Switzerland). The photon-counting value, which is indicated arbitrarily in relative light units (RLU), was registered with a counting interval set at 30 seconds and a total recycling time of 30 minutes. Chemiluminescence production was expressed as integrated chemiluminescence emission in counts/30 min, chemiluminescence peak in counts/min and chemiluminescence slope. The integral considers the area under the curve of the chemiluminescence production over a time period of 30 minutes and is the most complete parameter, the peak value is more a snapshot of the upper limit of chemiluminescence production and the slope learns about the kinetics of the initiation of the chemiluminescence production. The three parameters together give a correct and overall image of the chemiluminescence production.

Analysis of membrane bound CD14 expression on HL-60 cells. Expression of CD14, a receptor for lipopolysaccharide (LPS) and differentiation marker, was assessed by direct immunofluorescence after 3 days of culture. Fifty microliters cell suspension was incubated with Simultest™ Leucogate™ (Becton Dickinson) at 4°C in the dark. Simultest™ Leucogate™ contains FITC-conjugated CD45 monoclonal antibodies (Anti-HLe-1) and phycoerythrin-conjugated CD14 monoclonal antibodies (Leu™-M3). After washing procedures, the cells were submitted to flow cytometric analysis (FACScan®) (Becton Dickinson). Fluorescence was standardized by microbeads (Calibrite™ particles) (Becton Dickinson) with amplification and voltage kept constant throughout the procedures. Analysis was performed on 10,000 events (detector threshold FSC-H:200, parameter FSC-H:1.00). The cell population was gated according to forward and right angled light scatter. Background binding was estimated by isotype-matched negative control antibodies (Simultest™ Control) (Becton Dickinson).

Studies on leukocytes in whole blood

During a second analytical phase, the guanidino compounds were tested in whole blood and with application of the maximal concentration (C_{max}) [methylguanidine (C_{uremic} 0.77 μg/mL; C_{max} 1.82 μg/mL); guanidine (C_{uremic} 0.17 μg/mL; C_{max} 0.80 μg/mL); creatinine (C_{uremic} 136.0 μg/mL; C_{max} 240.0 μg/mL); guanidinoacetic acid (C_{uremic} 0.38 μg/mL; C_{max} 0.69 μg/mL); guanidinosuccinic acid (C_{uremic} 6.5 μg/mL; C_{max} 47.0 μg/mL)] as reported in uremic patients in the literature [28–30]. Guanidino compounds were dissolved in a 0.9% sodium chloride solution (Baxter, Lessines, Belgium). The C_{max} was used to compensate for the shorter exposure times in these in vitro experiments in whole blood compared to the chronic exposure in vivo. All guanidino compound solutions used in this study were found to be endotoxin-free (<0.2 IU/mL) (Endosafe® Endochrome-K™) (Charleston, SC, USA).

Sample collection

Heparinized whole blood (sodium heparin Vacutainer® tubes) (Becton Dickinson) was collected from healthy volunteers. The protocol was approved by the local Ethical Committee and an informed consent was obtained from all healthy donors.

Evaluation methods for whole blood

Quantification of oxidative burst activity. After a 10-minute incubation of the heparinized whole blood with saline, methylguanidine, guanidine, creatinine, guanidinoacetic acid, and guanidinosuccinic acid, the Bursttest (Phagoburst®) (Orpegen Pharma, Heidelberg, Germany) was applied for a quantitative determination of the leukocyte oxidative burst activity, according to manufacturer's guidelines. Ice-cooled heparinized whole blood was incubated with unlabeled opsonized *Escherichia coli* (*E. coli*) as a particulate stimulus and without stimulus for negative background control. The generation of reactive oxygen metabolites during the oxidative burst was monitored by the addition and the determination of oxidation of the fluorogenic substrate dihydrorhodamine (DHR) 123. The reaction was stopped by addition of lysing solution (Becton Dickinson), which removes the erythrocytes and results in a fixation of the leukocytes. After a washing step, DNA staining solution was added to exclude aggregation artifacts of bacteria or cells. The percentage of cells having produced reactive oxygen radicals as well as their mean fluorescence intensity (MFI) (enzymatic activity) were analyzed and the different leukocyte populations were identified based on their light scattering properties using a FACScan® (Becton Dickinson).

Intracellular TNF-α production. After a 10-minute incubation of the heparinized whole blood with saline alone as a control or with saline containing methylguanidine, guanidine, creatinine, guanidinoacetic acid, and guanidinosuccinic acid at C_{max}, the blood was stimulated with 1 μg/mL LPS (Sigma Chemical Co.), and without LPS for negative background control for 2 hours in a humidified atmosphere of 5% CO_2 in air at 37°C. After

Table 1. Effect of guanidino compounds on DNA synthesis of HL-60 cells

% BrdU-positive cells	Medium	Guanidino compounds	Calcitriol	Calcitriol + guanidino compounds
Methylguanidine	30.9 ± 5.4	34.7 ± 12.5[a]	23.4 ± 5.0[b]	27.6 ± 1.8[c]
Guanidine	28.9 ± 9.3	40.8 ± 13.6[a]	20.6 ± 9.6[b]	20.6 ± 7.3
Creatinine	30.5 ± 5.5	29.6 ± 10.2	23.3 ± 4.6[b]	22.8 ± 2.3
Guanidinoacetic acid	25.4 ± 1.5	24.7 ± 1.9	20.7 ± 3.6[b]	22.1 ± 5.5
Guanidinosuccinic acid	19.4 ± 1.9	19.5 ± 5.0	14.7 ± 3.2[b]	19.6 ± 2.5[c]

BruU is 5-bromo-2′-deoxyuridine. Mean ± SD ($N = 6$).
[a] $P < 0.05$ versus medium; [b] $P < 0.01$ versus medium; [c] $P < 0.05$ versus calcitriol.

this incubation period, 10 μg/mL Brefeldin A (BFA) (Sigma Chemical Co.) was added to inhibit intracellular transport and to retain the cytokines inside the cell; subsequently the incubation was continued for another 2 hours, after which FACS lysing solution was added to lyse the erythrocytes and to fix the external epitopes. Then the cells were permeabilized using FACS permeabilizing solution, after which they were stained for intracellular TNF-α (Fastimmune™ Anti-Hu-TNF-α) (Becton Dickinson) and with mouse Ig controls for estimation of the aspecific binding. Samples were analyzed using the FACScan® (Becton Dickinson).

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed using one-way repeated measures analysis of variance (ANOVA) followed by a paired Wicoxon signed rank test in case of significant ANOVA. A P value of less than 0.05 was considered significant.

RESULTS

Effect of different guanidines on HL-60 cell function

BrdU incorporation (DNA synthesis). The addition of methylguanidine and guanidine (Table 1) in the culture medium significantly enhanced the DNA synthesis (a marker of cell proliferation) of HL-60 ($P < 0.05$ versus medium). The presence of creatinine, guanidinoacetic acid, and guanidinosuccinic acid in the culture medium, on the other hand, had no effect on HL-60 cell DNA synthesis.

When HL-60 cells were cultured in the presence of calcitriol to differentiate the cells toward the monocyte/macrophage phenotype, the percentage of cells incorporating BrdU (synthesizing DNA) was significantly decreased, confirming the known antiproliferative effect of calcitriol ($P < 0.01$ versus medium). The presence of methylguanidine and guanidinosuccinic acid in the calcitriol containing culture medium significantly counteracted this antiproliferative effect of calcitriol ($P <$ 0.05 versus calcitriol) and induced again more DNA synthesis. No effect of creatinine, guanidine, and guanidinoacetic acid in the presence of calcitriol was observed (Table 1).

Table 2. Effect of guanidino compounds on the characteristics of the luminol-amplified chemiluminescence production of phorbol-myristate-acetate (PMA)-stimulated HL-60 cells

	Medium	Guanidino compounds	Calcitriol	Calcitriol + guanidino compounds
Methylguanidine				
Integral	5.3 ± 2.2	10.0 ± 7.0[a]	82.3 ± 79.0[a]	48.4 ± 49.0[a,b]
Peak	5.1 ± 3.4	8.7 ± 3.5	43.1 ± 10.5[a]	25.17 ± 9.3[a,b]
Slope	0.4 ± 0.5	0.6 ± 0.5	5.2 ± 1.9[a]	1.6 ± 0.6[a,b]
Guanidine				
Integral	11.7 ± 11.4	26.1 ± 19.0[a]	154.3 ± 72.2[a]	208.5 ± 89.4[a]
Peak	13.5 ± 10.5	13.3 ± 5.5	92.7 ± 35.9[a]	132.3 ± 81.9[a]
Slope	2.1 ± 2.8	1.5 ± 1.4	14.2 ± 11.2[a]	27.0 ± 25.9[a]
Creatinine				
Integral	4.9 ± 2.4	10.0 ± 6.3[a]	28.4 ± 15.6[a]	22.8 ± 11.6[a]
Peak	3.9 ± 1.8	5.5 ± 3.0	15.9 ± 10.4[a]	18.7 ± 8.5[a]
Slope	0.2 ± 2.1	0.4 ± 0.4	1.7 ± 1.6[a]	1.8 ± 1.4[a]
Guanidinoacetic acid				
Integral	5.3 ± 3.2	8.1 ± 2.6	65.9 ± 13.1[a]	55.3 ± 8.1[a,b]
Peak	5.2 ± 2.3	7.2 ± 5.7	29.2 ± 6.3[a]	23.0 ± 3.7[a]
Slope	0.6 ± 0.6	0.8 ± 0.7	5.1 ± 1.9[a]	3.6 ± 0.8[a]
Guanidinosuccinic acid				
Integral	3.8 ± 2.5	4.9 ± 4.5	14.1 ± 5.3[a]	16.1 ± 7.9[a]
Peak	6.8 ± 2.3	6.8 ± 3.8	24.1 ± 2.8[a]	19.4 ± 0.8[a,b]
Slope	0.7 ± 0.3	1.0 ± 0.4	4.9 ± 0.8[a]	2.8 ± 0.8[a,b]

Integral, cpm/30min/10^3; peak, cmp 10^3; slope, 10^3. Means ± SD ($N = 6$).
[a] $P < 0.05$ versus medium; [b] $P < 0.05$ versus calcitriol.

Because cell proliferation can be considered as a cellular change enabling a more pronounced inflammatory response, these data point out that at least some of the guanidino compounds might contribute to the enhanced inflammatory status in uremia.

Chemiluminescence-production. The effect of guanidino compounds on PMA stimulated chemiluminescence production of undifferentiated HL-60 was evaluated as a control. Table 1 illustrates that the presence of methylguanidine, guanidine, and creatinine in the culture medium was found to enhance this response for the integral ($P < 0.05$ versus medium), but not for the peak or slope. Addition of calcitriol to the culture medium induced cell differentiation toward the monocyte/macrophage phenotype, resulting in an enhanced chemiluminescence production. In this condition, the PMA stimulated chemiluminescence production was significantly inhibited when cells were cultured in the presence of methylguanidine (integral, peak, and slope values), guanidinoacetic acid (only the integral value), and guanidinosuccinic acid (peak and slope values) ($P < 0.05$ versus calcitriol) (Table 2) (representative experiments in Fig. 1). In contrast, guanidine enhanced the PMA-stimulated chemiluminescence production in a significant way for the slope and peak values. The presence of creatinine in the calcitriol containing culture medium

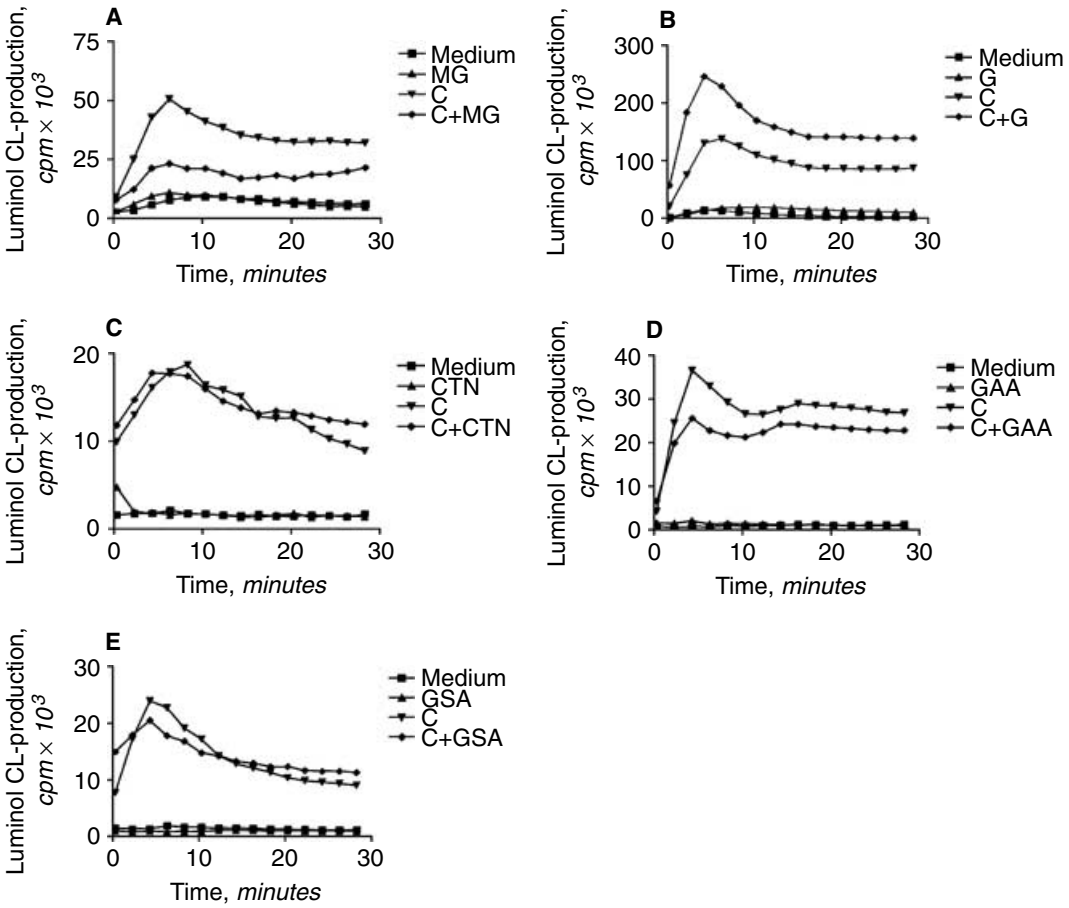

Fig. 1. Representative time curves of luminal-dependent chemiluminescence (CL) assay from phorbol-myristate-acetate (PMA)-stimulated HL-60 cells. The cells were cultured in medium, in medium supplemented with guanidino compounds [methylguanidine (MG), guanidine (G), creatinine (CTN), guanidinoacetic acid (GAA), and guanidinosuccinic acid (GSA)] both in the absence and the presence of calcitriol (C). Calcitriol-treated cells are to be considered as the equivalent of monocyte/macrophage cells. In parallel to the average results illustrated in Table 2, there is a marked decrease in response of the calcitriol-treated cells in the presence of methylguanidine and a less marked inhibition in the presence of guanidino acetic acid and guanidinosuccinic acid. Guanidine, in contrast, has a stimulatory effect. The impact of creatinine is neutral.

did not influence the PMA stimulated chemiluminescence production values in either way (Fig. 1) (Table 2).

Even if no significant changes were found for some indices of chemiluminescence production, the same trend was maintained as for the significant findings under the same conditions.

These data suggest that activation of chemiluminescence production (by guanidine) might be related to the progression of enhanced inflammatory response and atherogenesis, while be suppression of chemiluminescence production (by methylguanidine, guanidinoacetic acid, and guanidinosuccinic acid) might be related to the susceptibility to infection of the uremic patient.

CD14 expression. The expression of CD14 on HL-60 cells was induced by the presence of calcitriol in the culture medium. The presence of guanidino compounds in the culture medium, on the other hand, had no effect on the calcitriol-induced expression of membrane-bound CD14 (data not shown).

Effect of the guanidines on normal human leukocytes in whole blood

Oxidative burst activity. Guanidino compounds did not alter the baseline oxidative burst activity of leukocytes. None of the tested guanidino compounds had an effect on the *E. coli*–stimulated production of reactive oxygen species by normal monocytes. The *E. coli* stimulated production of reactive oxygen species by granulocytes, on the other hand, was inhibited when whole blood was incubated in the presence of methylguanidine, with a mean fluorescence of 427.0 ± 140.1 versus 464.5 ± 142.1 ($P < 0.05$). Guanidinoacetic acid, guanidine, creatinine, and guanidinosuccinic acid had no effect on the *E. coli*–stimulated oxidative burst activity of granulocytes.

These data further point out the potential role of methylguanidine in the enhanced susceptibility to infection of the uremic patient.

Intracellular TNF-α production by LPS-stimulated monocytes. Baseline TNF-α production by monocytes

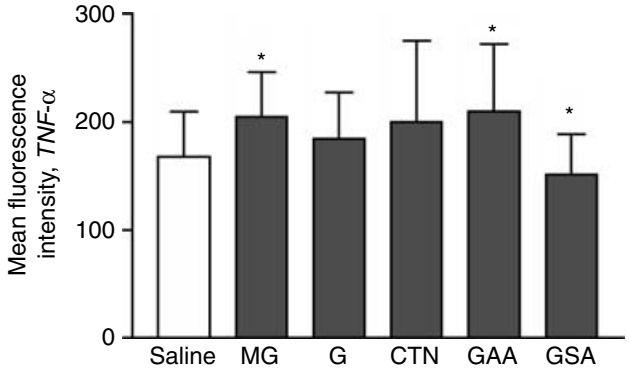

Fig. 2. Evaluation of the lipopolysaccharide (LPS)-stimulated intracellular tumor necrosis factor-α (TNF-α) production by monocytes after incubation of whole blood. The evaluation was performed n the presence of methylguanidine (MG), guanidine (G), creatinine (CTN), guanidinoacetic acid (GAA), and guanidinosuccinic acid (GSA) compared to saline. $^*P < 0.05$ versus saline ($N = 8$).

was negligible with a percentage of 2.26 ± 0.40 TNF-α–positive cells. The presence of methylguanidine, guanidine, creatinine, guanidinoacetic acid, and guanidinosuccinic acid did not influence baseline TNF-α production. The LPS-stimulated intracellular production of TNF-α by monocytes, on the other hand, was significantly enhanced when whole blood was incubated in the presence of methylguanidine and guanidinoacetic acid ($P < 0.05$) (Fig. 2). In contrast, incubation of whole blood in the presence of guanidinosuccinic acid had a slight but significant inhibitory effect on the LPS-stimulated monocytic intracellular production of TNF-α ($P < 0.01$) (Fig. 2). Incubation of whole blood in the presence of methylguanidine, guanidine, creatinine, guanidinoacetic acid, and guanidinosuccinic acid did not influence the percentage of TNF-α–positive monocytes (data not shown). Incubation of whole blood in the presence of guanidine and creatinine, however, had no significant effect on the LPS-stimulated monocytic intracellular production of TNF-α, although there was a trend for increase. More production of TNF-α for the majority of studied guanidino compounds points to a proinflammatory and proatherogenic impact.

DISCUSSION

In this in vitro study, the effect of different guanidino compounds on the function of leukocytes was evaluated. In an initial experiment, using the HL-60 cell line, the effect of methylguanidine, guanidine, creatinine, guanidinoacetic acid, and guanidinosuccinic acid on specific parameters such as BrdU incorporation (DNA synthesis), chemiluminescence response (free radical production) and CD14 expression (differentiation marker and receptor for LPS) was studied in the absence (as control) and the presence of calcitriol (summarized in Table 3). Calcitriol is known to differentiate HL-60 cells toward the monocyte/macrophage phenotype. The second experimental set up, using whole blood, tested the effect of guanidino compounds on the oxidative burst activity and intracellular TNF-α production of leukocytes in whole blood (summarized in Table 4).

Some of the tested guanidino compounds were found to elicit proinflammatory responses. Methylguanidine and guanidine enhanced the basal cell proliferation of undifferentiated HL-60 cells. The proliferation of the calcitriol-differentiated HL-60 cells increased when cultured in the presence of methylguanidine and guanidinosuccinic acid. In addition, guanidine enhanced the PMA-stimulated chemiluminescence production of calcitriol-differentiated HL-60 cells and the LPS-stimulated TNF-α production by monocytes was increased after incubation with methylguanidine and guanidinoacetic acid.

Conversely, under certain conditions, guanidino compounds also elicited an anti-inflammatory response. Methylguanidine and guanidinoacetic acid suppressed the PMA-stimulated chemiluminescence production of calcitriol-differentiated HL-60 cells. Methylguanidine also suppressed the *E. coli*–stimulated oxidative burst activity of granulocytes and guanidinosuccinic acid decreased the LPS-stimulated TNF-α production by monocytes.

These results illustrate, specifically for the guanidines, the dual response of the immune system in uremia. This dual response has been suggested by other studies as well. On one hand, baseline immune function is considered to be activated which might be related to atherogenesis [5, 31]; on the other hand, stimulated immune function is often suppressed [7], which may be related to the susceptibility for infection. According to our data, several guanidines play a role in this process. It is of note that infectious disease in its turn induces inflammation and that specific infections such as *Chlamydia pneumoniae* have been suggested to be related to atherogenesis both in the normal [32] and the uremic population [33].

Because cell proliferation can be considered as a cellular change reflecting an inflammatory response, our data on the enhanced cell proliferation of HL-60 cells cultured in the presence of methylguanidine and guanidine and on the neutralization of the antiproliferative effect of calcitriol by methylguanidne and guanidinosuccinic acid could point in the direction of the involvement of guanidino compounds in the enhanced inflammatory status in uremia. In contrast, Nathan et al [25] reported that creatinine, guanidinopropionic acid, and guanidinosuccinic acid inhibit the growth of HL-60 cells and of the erythroleukemia cell line K562. It is of note, however, that these authors applied concentrations up to 200 times higher than those used in the present study; in addition, proliferation was determined by counting the colony formation on tissue culture dishes, which

Table 3. Effect of different guanidines on HL-60 cell function

	DNA synthesis	DNA synthesis in the presence of calcitriol	Chemiluminescence production	Chemiluminescence production in the presence of calcitriol	CD14 expression
Methylguanidine	$P < 0.05$ ↑	$P < 0.05$ ↑	$P < 0.05$ ↑	$P < 0.05$ ↓	NS
Guanidine	$P < 0.05$ ↑	NS	$P < 0.05$ ↑	$P < 0.05$ ↑	NS
Creatinine	NS	NS	$P < 0.05$ ↑	NS	NS
Guanidinoacetic acid	NS	NS	NS	$P < 0.05$ ↓	NS
Guanidinosuccinic acid	NS	$P < 0.05$ ↑	NS	$P < 0.05$ ↓	NS

Table 4. Effect of guanidines on normal human leukocytes

	Oxidative burst *E. coli*-stimulated granulocytes	Oxidative burst *E. coli*-stimulated monocytes monocytes	Lipopolysaccharide-stimulated intracellular tumor necrosis factor-α (TNF-α) production by monocytes
Methylguanidine	$P < 0.05$ ↓	NS	$P < 0.05$ ↑
Guanidine	NS	NS	NS
Creatinine	NS	NS	NS
Guanidinoacetic acid	NS	NS	$P < 0.05$ ↑
Guanidinosuccinic acid	NS	NS	$P < 0.05$ ↓

is a less accurate method compared to the flow cytometric method used in this study. Our data also demonstrate the pro-inflammatory effect of guanidine on the PMA-stimulated chemiluminescence production of calcitriol differentiated HL-60 cells and on the LPS-stimulated intracellular production of TNF-α by normal monocytes. It is known that the monocyte respiratory burst leads to activation of nuclear factor-kappaB (NF-κB) [34], a transcription factor directly involved in the activation of genes responsible for inflammation [35] and present in atherosclerotic lesions [36]. In addition, TNF-α elicits its diverse biologic responses involved in atherosclerosis, such as secretion of proinflammatory cytokines and expression of adhesion molecules [37]. It serves as an endogenous mediator of inflammatory, immune, and host defense functions [38] and is mainly synthesized by cells of the monocyte/macrophage phenotype.

Serum TNF-α concentration has been found to be elevated in patients with CRF [39, 40]. The reason for this evolution has yet not been elucidated but is probably multifactorial. Retention of guanidines could be a possible trigger for enhanced TNF-α production by monocyte/macrophage. In contrast, in a study by Autore et al [26], methylguanidine inhibited the TNF-α release by activated macrophages, but again these results were obtained at concentrations 100 times higher than the ones used in the present study.

Simultaneously, anti-inflammatory responses in the presence of some of the guanidino compounds were observed, as demonstrated by the inhibition of the calcitriol-induced and PMA-stimulated chemiluminescence response by HL-60 cells cultured in the presence of guanidinoacetic acid and methylguanidine and by the inhibitory effect of guanidinosuccinic acid on the LPS-stimulated intracellular TNF-α production by normal monocytes. An inhibition of the zymosan-stimulated superoxide production in the presence of guanidinosuccinic acid, guanidinopropionic acid, and guanidinobutyric acid and by a guanidino compound mix (guanidinosuccinic acid, guanidinopropionic acid, guanidinobutyric acid, methylguanidine, and guanidinoacetic acid) had already been demonstrated for the neutrophilic cell population [24]. The data demonstrating the anti-inflammatory effect of guanidino compounds could implicate the impaired function of monocyte/macrophages in host defense and the enhanced propensity to infection in uremia.

The question might be raised why different guanidines have an opposite impact on different aspects of leukocyte functional response. A possible explanation is that these functions are not interrelated, and that guanidino compounds, in part due to the heterogeneity of their chemical structure, might exert distinct effects on distinct mechanisms of leukocyte response. The discrepancies might in part be attributable as well to methodologic factors. When the HL-60 cell line was studied using chemiluminescence, we evaluated cells uniquely of the monocyte/macrophage phenotype, while the oxidative burst activity was studied using whole blood which allows cell to cell (erythrocyte-thrombocyte-monocyte-lymphocyte-granulocyte) interaction during incubation. In the latter case, the activity of the different cell types is made only afterwards by flow cytometric analysis. Hence, cells of the monocytic phenotype have been submitted to different stimuli if they were isolated (as in the HL-60 model) or submitted to the cross-talk with other cells (as in whole blood).

It is of note that the present data match the data from our previous studies where an increase in the proliferation of calcitriol-differentiated HL-60 cells and inhibition of the chemiluminescence production was observed in the presence of UUF and fraction 1 of this UUF [9]. Guanidines were identified to elute within this fraction 1 [16].

Finally, since virtually every cell type composing the vascular wall has been shown to produce reactive oxygen species, it would be interesting, in the context of the pathogenesis of atherosclerotic lesions, to study the effect of guanidino compounds on the reactive oxygen species production by endothelial and/or vascular smooth muscle cells [41]. The question should be raised in how far the present data regarding reactive oxygen species production by leukocytes can be extrapolated to cells of the vessel wall.

CONCLUSION

Guanidino compounds were shown to exert proinflammatory as well as anti-inflammatory effects on monocyte/macrophage function. These effects could contribute to the altered prevalence of cardiovascular disease and the propensity to infection in patients with ESRD.

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