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A Low Concentration of Ethanol Reduces the Chemiluminescence of Human Granulocytes and Monocytes but Not the Tumor Necrosis Factor Alpha Production by Monocytes after Endotoxin Stimulation

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The ability of polymorphonuclear neutrophils (PMNs) and monocytes (Mϕ) to produce reactive oxygen species (ROS) has been related closely to their potential in the killing of microorganisms. Ethanol has been shown to impair the generation of ROS in these phagocytes after stimulation with some immunogens and to increase the susceptibility of alcohol abusers to infectious diseases. As endotoxemia is common in alcohol abusers, we investigated the effect of ethanol (21.7 mmol/liter) on the luminol-amplified chemiluminescence of PMNs and Mϕ after endotoxin stimulation and the release of tumor necrosis factor alpha (TNF-α) from Mϕ. Further, the efficiency of ethanol to inactivate chemically generated ROS was tested. Significant stimulation of ROS release occurred at endotoxin concentrations of 1 ng/ml or higher in both PMNs and Mϕ. Ethanol significantly suppressed the formation of ROS in both cell types, the decrease being more pronounced in Mϕ (−73.8%) than in PMNs (−45.7%). The correlations between endotoxin concentration and the amount of released ROS showed a dose-dependent, sigmoidal course. Concentrations of endotoxin necessary for half-maximum stimulation were nearly identical (6 to 8 ng/ml) in both PMNs and Mϕ, independent of the presence of ethanol. In contrast to ROS formation, ethanol had no effect on the amount of TNF-α produced by endotoxin-stimulated Mϕ. Ethanol was shown to be unable to decrease the levels of chemically generated ROS under physiological conditions. Therefore, ethanol cannot be assumed to be an "antioxidative" compound but rather seems to modify processes of endotoxin recognition, intracellular signal transduction, or metabolism.

One of the most important functions of polymorphonuclear neutrophils (PMNs) and monocytes (Mϕ) is the phagocytosis and destruction of invasive microbes. This task is, at least in part, fulfilled by the release of reactive oxygen species (ROS) (4). The potential of endotoxin (lipopolysaccharide [LPS]), a constituent of the outer membrane of gram-negative bacteria and a strong immunogen, to induce the release of ROS in both cell types has been demonstrated previously (12, 18, 29). The activation of phagocytes results in the formation of numerous oxidizing agents such as superoxide, hydrogen peroxide, and hypochlorite (36). After stimulation with endotoxin, monocytes produce a number of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), which is a central mediator of the inflammatory response to LPS activation (10). The simultaneous presence of hydrogen peroxide and hypochlorite has been shown to be essential for the enhancement of chemiluminescence by luminol (2, 33). A close correlation between the ability of phagocytes to kill microbes and to induce luminol-amplified chemiluminescence has been demonstrated previously (11, 16).

The immune response of alcohol abusers is suppressed, leading to an increased infection rate and a higher mortality rate due to infectious diseases (1, 31). This suppression in host defense may be due to an impaired formation of reactive oxygen compounds, as ethanol concentrations in blood observed in alcoholics has been shown to decrease PMN-associated chemiluminescence after stimulation with chemotactic peptide (fMLP) (24, 25, 27), phorbol myristate acetate (27), opsonized zymosan (5, 26, 28, 35), and concanavalin A (25) and to suppress the activation of Mϕ with opsonized zymosan (28).

Endotoxin levels have been found to be significantly elevated in patients with alcohol-induced liver diseases (13), implying an effect on the immune system. Hence, we investigated the effect of ethanol at a clinically relevant level (21.7 mmol/liter) on the luminol-enhanced chemiluminescence of granulocytes and Mϕ after stimulation with LPS (*Escherichia coli* O111:B4) at different concentrations.

MATERIALS AND METHODS

Blood donors and cell preparation. All procedures with blood and leukocytes were performed either in gamma-irradiated or depyrogenized (ethylene oxide-treated) plasticware. Blood (40 ml) was taken from healthy, overnight-fasted male volunteers ($n = 6$; age, 32 ± 1.2 years; nonsmokers; mean daily alcohol intake, <10 g/day) and collected into heparinized syringes. Further, 10 ml of blood was collected in a syringe for blood serum preparation. PMNs and peripheral blood mononuclear cells (PBMCs) were isolated as described by Patel et al., with slight modifications (27). Five-milliliter portions of blood were layered over 3.5 ml of Polymorphprep (Nycomed Pharma AS, Oslo, Norway) at room temperature in a pyrogen-free plasticware vial. The vial was centrifuged for 30 min at 450 × *g* without brake activation of the centrifuge. The resulting layer with PMNs and the layer containing PBMCs were isolated and washed with 15 ml of isotonic, pyrogen-free sodium chloride solution (10 min, 450 × *g*). In previous experiments ($n = 28$), the percentage of Mϕ in the PBMC layer was determined to be 24.6% ± 2.9% (Sysmex cell counter K-1000; Sysmex GmbH, Norderstedt, Germany) (unpublished results). As Mϕ in the fraction containing PBMCs are generally accepted to be the only cells capable of phagocytosis (36), adequate numbers of Mϕ and PMNs were applied in the experiments. After cell counting, the cell concentration was adjusted with RPMI 1640 medium (Biochrom, Berlin, Germany) to give 20.0×10^5 PBMCs or 5.0×10^5 PMNs per ml, resulting in comparable cell concentrations of Mϕ and PMNs in both solutions.

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Chemiluminescence measurement. Microtiter plates (Maxisorp; Nunc, Wiesbaden Germany) additionally depyrogenized by ethylene oxide treatment were used for chemiluminescence measurements. Cavities of the plates were filled with RPMI medium and blood serum (final concentration, 10%). In every second cavity, ethanol (ad injectabile; Braun, Melsungen, Germany) was added to a final concentration of 0.1% (21.7 mmol/liter). One blank (LPS-free) and five final endotoxin (*E. coli* B4:O111 [Sigma, Deisenhofen, Germany]) concentrations from 0.1 ng/ml to 1.0 μg/ml, each concentration 10-fold higher than the previous one, were used for stimulation. Finally, 2.0×10^4 PMNs or 8.0×10^4 PBMCs in RPMI medium were added to each cavity. After computer-controlled addition of 50 μl of 10 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) solution in 0.2 M borate buffer (pH 9.0), the chemiluminescence measurement was started immediately in a luminescence microplate reader (BMG Labtechnologies, Offenburg, Germany). To avoid any effects of the luminol or the associated change in pH on the luminescence, the light intensity measurement started with luminol addition and lasted for 1 s. The luminescence intensity was measured every 8 min (final measurement after 56 min).

Direct interactions of ethanol with ROS. To determine the direct (chemical) potential of ethanol to destroy highly reactive oxygen species under comparable conditions, we mixed RPMI medium, luminol in borate buffer, endotoxin, and serum to provide identical concentrations, as described above. Hydrogen peroxide (0.1%, 25 μl) and potassium ferricyanide (1.0×10^{-5} M, 25 μl) were added in parallel from two computer-controlled syringes, and chemiluminescence was measured during the same time. This experiment was repeated 10 times with and without 0.1% ethanol (21.7 mmol/liter).

TNF-α assay. For measurement of TNF-α release, 20.0×10^4 PBMCs were kept for 1.5 h (37°C, 5% CO_2) until Mϕ became adherent. The nonadherent cells were discarded, and the adherent Mϕ were incubated for 4 h (37°C, 5% CO_2) with increasing LPS concentrations, with and without ethanol. The concentrations of blood serum, ethanol, and LPS were identical to those used in the chemiluminescence assays. After 4 h of incubation, the supernatant was taken and kept frozen (−20°C) until TNF-α quantification by enzyme-linked immunosorbent assay (ELISA). After endotoxin challenge, the cells that were adherent to the plastic bottoms of the cavities were counted under the microscope.

TNF-α sandwich ELISA was performed according to standard ELISA procedures by incubating the supernatants in microtiter plates coated with a monoclonal mouse anti-human TNF-α antibody (IC Chemikalien GmbH, Ismaning, Germany). After a wash, adherent TNF-α was double labeled with a polyclonal rabbit anti-human TNF-α antibody (Genzyme Corp., Cambridge, Mass.) and a peroxidase-conjugated monoclonal goat immunoglobulin G Fab anti-rabbit antibody (Medac GmbH, Hamburg, Germany). TNF-α was quantified by incubating the antibody complex with an *o*-phenylene diamine–hydrogen peroxide solution in citrate buffer (pH 5.0) in parallel with a recombinant human TNF-α standard (Genzyme Corp.). TNF-α concentrations were related both to the number of applied cells (PBMCs) and to the number of adherent cells, which were assumed to be Mϕ, as only these cells are capable of adherence (see Table 2). The viability of the cells was tested at the end of the experiments with the trypan blue test.

Data evaluation. Values (relative luminescence units [RLU]) are given as means ± standard deviations (SD), if not otherwise indicated. Significance was tested with the Mann-Whitney U test (CSS software; StatSoft Inc.). Mathematical approximation of the luminescence-time *f*(RLU) course (see Fig. 1) was performed by a biphasic function (equation 1), with *e* standing for Euler's number:

$$f(\mathrm{RLU}) = A_0 + A_1 \cdot t^{A_2} \cdot e^{(-A_3 \cdot t)} \quad (1)$$

The area under the curve (AUC) of this approximation was assumed to correspond to the total amount of released ROS of the applied cells in one cavity. Sigmoidal correlations between the AUC of equation 1 and the applied endotoxin concentration were fitted by equation 2 (see Fig. 2 and 3)

$$F(\mathrm{RLU} \cdot t) = \frac{A_O}{1 + e^{[A_1 - A_2 c(\mathrm{LPS})]}} \quad (2)$$

A_0, A_1, A_2, and A_3 were assigned the approximating variables of equations 1 and 2; c(LPS) is the LPS concentration; and t is the time (in minutes).

RESULTS

Luminol-amplified luminescence values and, therefore, increased production of ROS was already detectable at 8 min after endotoxin (LPS) stimulation in both PMNs and PBMCs. The highest values were reached after 24 min, and, approximating the last measurement at 56 min, the chemiluminescence intensity declined against the background level (Fig. 1). All time courses of chemiluminescence intensity were fitted by equation 1 with regression coefficients higher than 0.95.

The total amount of released ROS (calculated as the AUC below function 1 and approximated by equation 2) was significantly increased at an endotoxin concentration of 1.0 ng/ml or higher without alcohol ($P < 0.05$). In the presence of 0.1% ethanol (21.7 mmol/liter), the AUC was significantly elevated at LPS concentrations of 10 ng/ml or higher in comparison to the unstimulated cells, for both PMNs (Fig. 2) ($P < 0.05$) and Mϕ (Fig. 3) ($P < 0.05$).

For PMNs stimulated with an endotoxin concentration of 1.0 ng/ml or more, ethanol depressed significantly the amount of ROS produced ($P < 0.05$) (Fig. 2). ROS formation by Mϕ after being challenged with endotoxin was also significantly depressed ($P < 0.05$) in the presence of ethanol (21.7 mmol/liter) at LPS concentrations of 0.1 and 1 ng/ml. This depression was more pronounced ($P < 0.01$) at endotoxin concentrations of 10 ng/ml or higher (Fig. 3).

Points of inflection of the sigmoidal regression (corresponding to half-maximum stimulation of the cells) were 6.0 ng of LPS per ml without ethanol and 8.0 ng of LPS per ml with ethanol in PMNs (Fig. 2). Monocytes reached half-maximum

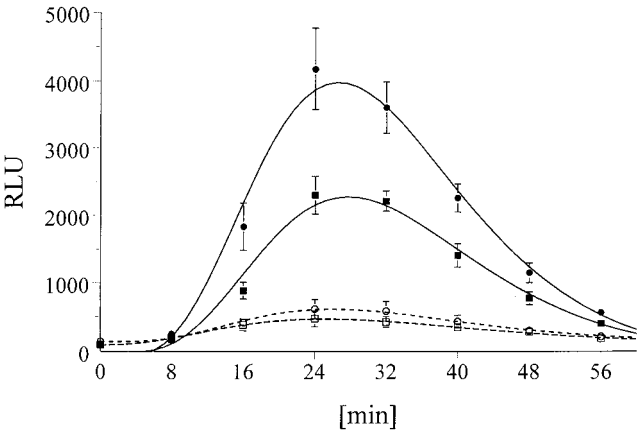

FIG. 1. Time course of chemiluminescence (expressed as mean RLU ± standard error of the mean) of granulocytes with (solid lines) or without (dashed lines) 10 ng of endotoxin (*E. coli* O111:B4) per ml in the presence (■, □) or absence (●, ○) of ethanol (21.7 mmol/liter). The AUC for comparative statistics was calculated from the function given in equation 1.

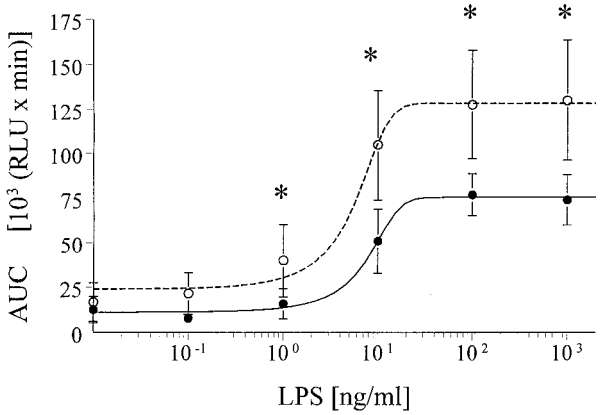

FIG. 2. Correlation between endotoxin concentration and release of ROS from PMNs in the presence (solid line) or absence (dashed line) of 21.7 mmol of ethanol per liter. ROS release was calculated as the AUC (mean ± SD) from the function given in equation 1. The course of AUC dependence on endotoxin concentration was approximated by equation 2 for calculation of the LPS concentration necessary for half-maximum stimulation (point of inflection, as given in the text). *, $P < 0.05$.

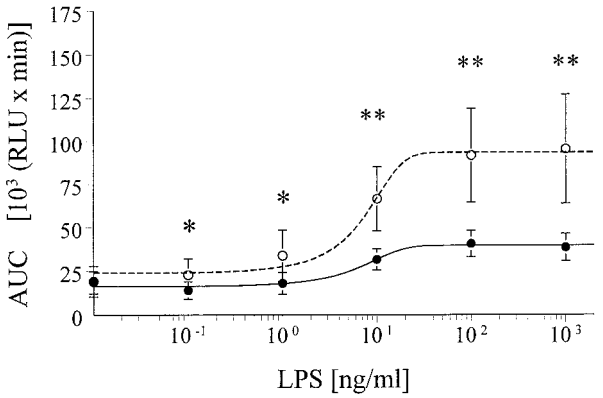

FIG. 3. Correlation between endotoxin concentration and release of ROS from PBMCs in the presence (solid line) or absence (dashed line) of 21.7 mmol of ethanol per liter. ROS release (AUC [mean ± SD]) and the point of inflection were calculated as described for PMNs. *, $P < 0.05$; **, $P < 0.01$.

release of ROS (point of inflection) at 7.8 ng of LPS per ml without alcohol and 6.9 ng of LPS per ml with alcohol (Fig. 3).

Maximum LPS-induced chemiluminescence was weaker in monocytes within the PBMC fraction (68.7% of the value reached by polymorphonuclear neutrophils, calculated for identical cell numbers [$P < 0.01$]). At endotoxin concentrations of maximum ROS stimulation (100 and 1,000 ng/ml), Mϕ in the presence of other PBMCs were more sensitive to ethanol in ROS production (−73.8%) than were PMNs (−45.7%), if only endotoxin-induced stimulation was taken into account and background signals were neglected.

Ethanol did not decrease the chemiluminescence when a chemical system (hydrogen peroxide-potassium ferricyanide) was used for the generation of ROS (Table 1). Also, different endotoxin concentrations had no influence on the concentration of active, luminescence-inducing ROS.

Endotoxin addition resulted, as expected, in increased expression of TNF-α protein in Mϕ. The TNF-α release was dose dependent and significantly increased at LPS concentrations of 10 ng/ml or higher (Table 2). In the present system, ethanol did not have any influence on the production of TNF-α from monocytes, either the applied (PBMCs) or adherent (Mϕ) cells (Table 2).

For each experiment, viability was found to be higher than 97% at the end of the experiment.

DISCUSSION

The results presented above confirm findings showing a release of ROS in PMNs (19) and activation of Mϕ by LPS (18). They are not consistent with investigations postulating the failure of endotoxin in the stimulation of ROS production of phagocytes (38). According to the results of this study, endotoxin is a potent activator of ROS release from Mϕ and PMNs if blood serum factors are present.

Furthermore, a clear inhibition of ROS formation in PMNs and Mϕ by ethanol at low concentrations after LPS elicitation in vitro is evident. Ethanol also inhibits zymosan-induced chemiluminescence of phagocytes in whole blood (5), indicating a similar effect of ethanol on immunogen-stimulated chemiluminescence in complex cell systems and isolated cell types. The course of chemiluminescence impairment in PMNs and Mϕ indicates a classical, noncompetitive inhibition (Fig. 2 and 3). From the present experiments, the exact type of impairment in receptor binding or enzyme activity cannot be deduced, and further experiments are necessary to clarify this result. Simple noncompetitive inhibition of an enzyme, which is responsible for the formation of one of the ROS after endotoxin stimulation by ethanol, could be a possible explanation for the reduced ROS generation.

A direct reaction of ethanol with the formed ROS and a direct chemical inactivation of these compounds is not consistent with the fact that ethanol is not able to reduce the concentration of chemically generated ROS. This is evident from the failure of ethanol to reduce the chemiluminescence within the hydrogen peroxide-potassium ferricyanide system (Table 1). Based on chemical theory, ethanol is unable to provide mesomerically stabilized forms after splitting off of an unpaired electron and therefore does not possess the ability to form stabilized radicals itself, lacking essential structural demands. Therefore, a direct "antioxidative" effect of ethanol (7, 15, 35) is unlikely.

A reduced phagocytosis in PMNs and Mϕ has been shown to occur after incubation with ethanol, and the impaired phagocytosis has been discussed as a possible reason for reduced ROS release (6, 21). In the experiments of the present study, no particles for phagocytosis were presented to the phagocytes. Nevertheless, a reduction in ROS formation was evident. Therefore, phagocytosis does not seem to be an indispensable trigger for ROS production in this case.

Ethanol or its first metabolite, acetaldehyde, has been assumed to be cytotoxic (22); such a cytotoxic effect could be responsible for reduced ROS formation. However, the high percentage of viable cells at the end of all experiments is inconsistent with this hypothesis.

An increase of the cytosolic calcium concentration is assumed to serve as an activating signal of the physiological response during the respiratory burst of leukocytes (23), but low ethanol concentrations were not found to change basal levels of calcium after stimulation (25, 27). A short-term increase in the intracellular concentration of cyclic AMP (cAMP) occurs after phagocyte stimulation (30), whereas a long-term elevated cytosolic cAMP concentration in phagocytes is associated with a decline in phagocytic functions (9, 14, 20). Ethanol has been demonstrated to increase intracellular cAMP levels (3, 17, 35). Hence, the ethanol-associated elevation of cytosolic cAMP concentration has to be taken into

TABLE 1. Influence of endotoxin and ethanol (21.7 mmol/liter) on the inactivation of chemically generated ROS from a hydrogen peroxide-potassium ferricyanide system

Exptl condition	Chemiluminescence (AUC [10^3 RLU · t])/cavity[a] at endotoxin concn (ng/ml) of:					
	0	0.1	1.0	10	100	1,000
Without ethanol	29.4 ± 3.2	29.8 ± 2.3	29.8 ± 2.4	30.4 ± 3.0	30.5 ± 2.6	30.4 ± 1.9
With ethanol	31.0 ± 1.8	30.9 ± 1.4	31.2 ± 1.6	31.1 ± 1.8	31.3 ± 2.1	31.1 ± 2.2

[a] Values are means ± SD. There are no significant differences between any of the values.

TABLE 2. TNF-α production by Mϕ after 4 h of incubation with increasing concentrations of endotoxin with or without 0.1% ethanol

Exptl condition[a]	TNF-α production (pg)/1,000 PBMCs or Mϕ (mean ± SD)[b] at endotoxin concn (ng/ml) of:						
	0	0.01	0.1	1.0	10	100	1,000
Applied PBMCs without ethanol	0.4 ± 0.9	0.2 ± 1.1	0.5 ± 0.9	1.1 ± 1.3	3.7 ± 2.2*	5.7 ± 1.1**	8.0 ± 1.9**
Applied PBMCs with ethanol	0.4 ± 0.9	0.4 ± 1.2	0.7 ± 0.8	1.6 ± 1.5	4.1 ± 2.1*	6.3 ± 1.1**	8.3 ± 2.7**
Adherent Mϕ without ethanol	17 ± 51	7 ± 66	24 ± 47	58 ± 62	182 ± 122*	291 ± 158**	377 ± 132**
Adherent Mϕ with ethanol	23 ± 41	13 ± 72	34 ± 40	90 ± 82	218 ± 176*	311 ± 150**	399 ± 218**

[a] After incubation with LPS.
[b] No significant differences were found between results with and without ethanol at a single endotoxin concentration. Asterisks (*, $P < 0.05$; **, $P < 0.01$) indicate significantly higher yields of TNF-α compared to zero stimulation (endotoxin [LPS] concentration of 0 ng/ml). Compared to zero stimulation, Mϕ produced significantly higher amounts of TNF-α at LPS concentrations of 10 ng/ml or higher, independently of the presence of ethanol.

account as a major mechanism in the depression of ROS generation.

Endotoxemia is common in alcoholics (8), but the LPS concentration in the plasma of these patients is still nearly 3 orders of magnitude lower (15 to 40 pg/ml [13]) than the measured concentration necessary to reach half-maximum stimulation (6 to 8 ng/ml). This effect may be due to isolation from the complex environment in whole blood and the modified interaction between the investigated and the endothelial cells, resulting in reduced sensitivity of the cells to endotoxin challenge. On the other hand, the response to the endotoxin stimulus is very fast, indicating a short-term intracellular response which is unlikely to be due to complex cell interactions or cytokine network-associated reactions.

Furthermore, as LPS-binding protein, a plasma factor important for LPS recognition, is present at milligram-per-milliliter concentrations in human blood (34) and a 10% autologous serum concentration was used during stimulation, an impairment of LPS recognition by PMNs and Mϕ is unlikely. It may be possible that ethanol-accompanied impairment starts to play a role in cases of local bacterial infections, in which the LPS concentration determined for half-maximum stimulation can be easily reached.

The influence of physiologically relevant ethanol concentrations on LPS-stimulated ROS formation of phagocytes parallels its effect on these cells when other stimuli are used (5, 25–28, 35). Therefore, ethanol-induced suppression of ROS released from these cells does not seem to depend on the kind of applied stimulus but rather is related to changes in intracellular metabolism or signal transduction.

TNF-α production has been reported by Szabo et al. and Verma et al. to be suppressed after acute ethanol exposure (32, 37). Although we showed a significant increase of TNF-α release at an LPS concentration as low as 10 ng/ml in comparison to unstimulated controls, we were not able to confirm the findings of those authors, as no suppression of TNF-α production by 0.1% ethanol over the applied LPS concentration range was evident. The inconsistency of this result may be due to different experimental conditions, as the suppression of TNF-α release from LPS-activated Mϕ by ethanol was reported after preincubation with alcohol, without the presence of ethanol during LPS challenge, and with the application of fetal calf serum instead of autologous human serum.

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