

Ethanol inhibits IgE-induced degranulation and cytokine production in cultured mouse and human mast cells

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Abstract

Activated mast cells (MC) can produce a wide variety of potent inflammatory mediators. Excessive alcohol consumption is known to lead to immune deficiency and propensity for pneumonias in particular. As MCs are important in the first line of defence of mucosal membranes we have studied the effect of ethanol (EtOH) on several MC functions. EtOH attenuated dose dependently IgE-induced degranulation of mouse bone marrow derived mast cells (mBMMC) as reflected by the release of granule associated β -hexosaminidase (β -hex). A mean of $26 \pm 7\%$ inhibition of β -hex release was observed in the presence of 5‰ (86 mM) EtOH and nearly complete inhibition in the presence of 20‰ (344 mM) ethanol. The IgE-induced degranulation of mBMMC cultured with EtOH for seven days was inhibited to a similar degree as the degranulation of mBMMC exposed to EtOH for only one hour. Inclusion of 5‰ (86 mM) ethanol in the medium reduced tumour necrosis factor (TNF)- α and interleukin (IL)-8 production in human mast cell line (HMC-1) cells by $55 \pm 7\%$ and $19 \pm 5\%$, respectively, and the presence of 20‰ (344 mM) ethanol inhibited the expression $81 \pm 12\%$ and $59 \pm 14\%$ respectively. These results suggest that, in contrast to previous assumption, ethanol inhibits several critical MC functions at least in vitro. This inhibition of mediator, and cytokine release in particular, could contribute to the immune deficiency associated with chronic alcohol consumption. © 2000 Elsevier Science Inc. All rights reserved.

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Introduction

Mast cells are bone marrow derived effector cells of the immune system which are found abundantly in connective tissue, skin, mucosal membranes and in general in tissues interfacing

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ing with the external environment. Recent findings implicate a role for MC, in addition to immediate type of hypersensitivity reactions and defence against parasitic infections, also in defence against bacterial infections [1,2]. Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases, and several proinflammatory and chemotactic cytokines such as TNF- α , interleukins (IL)-4-IL-6, IL-8, IL-13 and transforming growth factor (TGF)- β [3–5]. In contrast to other cell types of the immune system, MC can store at least TNF- α in its granules and abundant amounts of this cytokine can be released quickly upon MC degranulation. Mast cells are also located in the vicinity of nerves and blood vessels and their activation has been shown to lead into adhesion of leukocytes to endothelium and to leukocyte extravasation [6,7]. A significant role for MC has been implicated in alcohol induced damage of gastric mucosa. Inhibition of mast cell degranulation prior to alcohol administration has been shown to prevent the mucosal damage [8]. Therefore it has been generally assumed that ethanol is a potent activator of MC. However, it is not clear whether ethanol directly activates MC or induces the release of some other MC activating factor, such as substance P or vasoactive intestinal peptide [9].

Alcohol abuse and chronic alcoholism are associated with increased frequency and severity of infectious diseases as well as with elevated incidence of tumours of for example gastrointestinal tract. These effects have been addressed to alcohol induced suppression of immune system and also to increased production of acetaldehyde in the colon by gut bacteria [10]. Ethanol can impair several aspects of immune function, but the precise mechanism is not known. Ethanol has been shown to down regulate cytokine and NO-release in lung alveolar macrophages [11], to inhibit lipopolysaccharide (LPS) induced expression of inducible nitric oxide synthase [12,13] and to suppress LPS stimulated production of TNF- α . Furthermore ethanol suppresses lymphocyte activity [14,15] and up-regulates secretion of corticosteroids [16]. In contrast, an increase of NO production by Kupfer cells has been observed by chronic administration of ethanol in the presence or absence of LPS [17].

MC are important in several aspects of initiation of inflammation, such as recruitment of other immune cells to the site of infection. Therefore, to assess whether the ethanol-induced immune suppression could be related to inhibition of MC activity, we decided to study the effects of ethanol on the functions of MC that are important in initiating of inflammatory reaction i.e. IgE-mediated release of preformed granule associated mediators, as well as the production of inflammatory and chemotactic cytokines, TNF- α and IL-8.

Methods

Cell culture

Mouse bone marrow derived mast cells (mBMMC) were generated by culturing bone marrow cells from the tibias and femurs of 6–12 weeks old BALB/c mice (Animal facilities of Helsinki University, Helsinki, Finland) for 2–5 weeks in enriched medium (RPMI-1640 containing 10% FCS (Sigma Chemical Company, St. Louis, USA), 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol) supplemented with 50% WEHI-3 cell (line TIB-68; American Type Culture Collection, Rockville, MD) conditioned medium (WCM) [18] as a source of IL-3. Before isolation of

bone marrow cells the mice were sacrificed by decapitation under CO₂-anesthesia and therefore the animals experienced no pain or suffering. The study was approved by the ethical committee of the Institute of Biomedicine, Helsinki University. Every 7 days the nonadherent cells were transferred into new flasks and suspended into fresh culture medium. Human mast cell line (HMC-1) has been established from the peripheral blood of a patient with mast cell leukemia as described by Butterfiel et al. [19]. The cells were cultured in Iscove's medium supplemented with 10% FCS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1.2 mM monothioglycerol (Sigma).

Recombinant c-kit ligand (rKL) was produced in Cos cells by transiently transfecting the cells by electroporation with artificially truncated KL cDNA (20). The activity of rKL was determined as described previously [20] and was compared with the known amounts of rKL (Immunex Corporation, Seattle). rKL containing cos cell supernatant was used at dilutions giving maximal proliferative activity of mBMMC (1:10–1:6) corresponding to approximately 100–150 ng/ml of KL. In separate experiments the Cos cell supernatant at dilution of 1:5–1:10 was also shown to induce the expression of MMCP-4 gene as previously described [21] (data not shown). Ethanol used in experiments was 99.9% pure.

Activation of MC

For activation studies 3–6 weeks old mBMMC were sensitized for 2 h with anti-dinitrophenyl IgE (10 mg/ml) antibody (Sigma), cells were washed twice with PBS and resuspended at 1×10^6 cells/ml in RPMI 1640 enriched media supplemented with 10% WCM. Thereafter the cells were incubated in the presence of indicated EtOH concentrations for 30 minutes prior to induction of degranulation by challenging them with dinitrophenyl-BSA (Sigma) for 20 min. Cells were pelleted at +4°C and cells and supernatants were analysed for β -hexosaminidase (β -hex) activity as described [22].

IgE-induced activation of mBMMC was assessed also after prolonged (7 days) culture of mBMMC in the presence of varying EtOH concentrations (0–5%). At higher EtOH concentrations ($\geq 20\%$) the viability of the cells on day 7 was compromised and therefore their effect was not studied. Fresh media with or without ethanol was changed daily. On day 7 the number of viable cells was assessed by trypan blue exclusion and equal amounts were taken from ethanol treated and control cells. The cells that had been cultured in the presence of EtOH were divided into two sets. One half of the cells were kept in the same ethanol concentrations and IgE-induced degranulation was assessed. The other half of the cells was washed twice with media and resuspended into media without ethanol. Subsequently IgE-induced degranulation was assessed after 1 h incubation. Furthermore, the same concentrations of EtOH were added, 1 h prior to induction of degranulation, to the cells cultured without ethanol for 7 days.

To exclude the possibility that ethanol could interfere with the enzymatic activity of β -hex, we took supernatants of activated mast cells, containing the released β -hexosaminidase, and measured the enzymatic activity in the presence of different ethanol concentrations. In these experiments EtOH had no significant effect on the enzymatic activity of β -hex (data not shown). The degranulation % was calculated by using following formula: stimulated release/(stimulated release + stimulated residual) – nonstimulated release/(nonstimulated release + nonstimulated residual).

Next the effect of EtOH on cytokine production of HMC-1 cells was assessed. Varying concentrations of EtOH were added 1 h prior to the addition of the activators and the cytokine production was assessed after 24 h. As HMC-1 cells do not possess functional IgERI-receptors on their plasma membranes, they were activated with a combination of phorbol 12-myristate 13-acetate (PMA) 50 ng/ml (Sigma) and calcium ionophore A23187 0.5 μ M (Sigma). These concentrations have been shown to result in optimal activation of HMC-1 cells [23]. A23187 was dissolved in EtOH resulting in final EtOH concentration of 0.2‰ and PMA was dissolved in DMSO with a final concentration of 0.05‰ which did not affect the cytokine production of HMC-1 cells (data not shown). Activation was performed in RPMI 1640 medium in the absence of serum. TNF- α and IL-8 were measured from the supernatants using a commercial ELISA-method according to manufacturers recommendations (R&D Systems, London, England). In experiments, where also intracellular TNF- α was measured both cells and supernatants were subjected to three freeze thaw cycles to release the intracellular TNF- α prior to ELISA-measurements. The viability of the cells was assessed by counting the trypan blue excluding cells in the end of each experiment. Viability was not affected by the serum free medium and was >95% at 24 h. The presence of the combination of PMA and ionophore resulted in a decrease of viability of the cells by 20–30% but the presence of EtOH did not have any significant effect on the viability during the 24 h activation.

Cell proliferation

Mast cell proliferation was assessed by assaying the [^{3} H]Thymidine incorporation into cellular DNA. 3–5 weeks old mBMMC were washed twice in enriched medium and incubated for 24 h in the 96-well plates in enriched medium in the presence of different concentrations of EtOH and in the presence of rKL containing Cos cell supernatant at 1:6–1:10 dilutions which gave in preliminary studies the maximum proliferation. EtOH was added 1 hour after the addition of rKL. Cells were pulsed for 4 hours with 1 mCi of [^{3} H]Thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) after which nuclei were harvested onto glass filter papers and washed with water using a cell harvester (Skatron, Norway). All the experiments were done in triplicate. Incorporated radioactivity was determined by using liquid scintillation counting.

Statistical analysis

The effect of increasing ethanol concentration was evaluated in each experimental series by analysis of variance (ANOVA) for repeated measurements. To compare differences between individual ethanol concentrations, two-tailed student's T-test was performed.

Results

The effect of ethanol on IgE-mediated degranulation of mBMMC

As revealed by Fig. 1 a clear dose dependent attenuation of IgE-mediated β -hexosaminidase (β -hex) release from mBMMC by ethanol was evident. The degranulation % (see materials and methods) of mBMMC in the absence of EtOH was 50.9 ± 4.1 and in the presence of 5‰ (86 mM) ethanol 37.6 ± 6.4 . In 20‰ (344 mM) ethanol, virtually no IgE-mediated release of

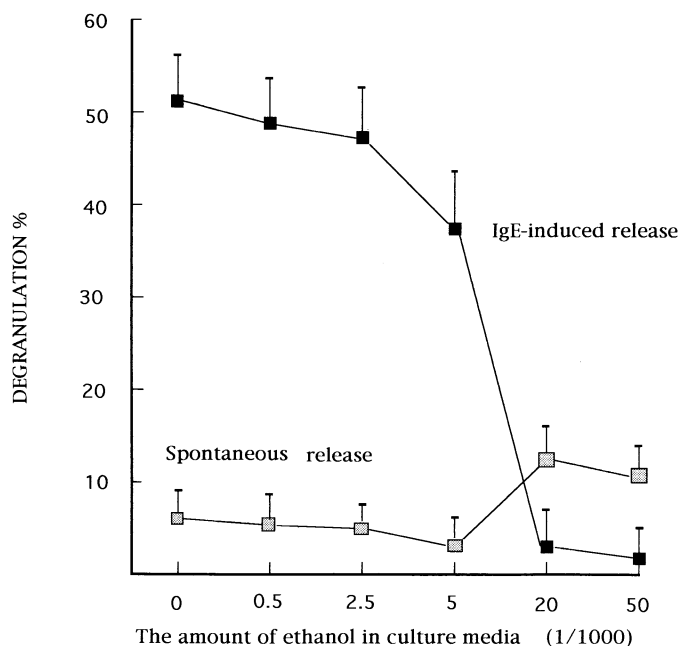


Fig. 1. The IgE-induced and spontaneous release of β -hexosaminidase from mBMMC in varying ethanol concentrations. Before addition of DNB-albumine to induce degranulation, mBMMC were incubated for one hour in the presence of indicated ethanol concentrations. The degranulation % was calculated by using following formula: stimulated release/(stimulated release + stimulated residual) – nonstimulated release/(nonstimulated release + nonstimulated residual). ($n = 4$, mean $\pm$ SEM). In the IgE-induced release $p < 0.05$ control vs. EtOH (5–20%).

β -hex could be observed. The difference in β -hex release between control and ethanol treated cells was statistically significant in the presence of 5‰ and higher ethanol concentrations ($P < 0.05$). No appreciable spontaneous release of β -hex ($< 6.1\%$) could be observed in ethanol concentrations from 0 to 5‰ (0–86 mM). However, at EtOH concentrations of 20‰ and 50‰ (344 and 860 mM) a slight increase of spontaneous β -hex release to 11–13% was evident indicating that some membrane leakage or cell death may have occurred at these high ethanol concentrations (Fig. 1). In two separate experiments the IgE-induced degranulation of mBMMC was assessed also in the presence of EtOH and 4-methylpyrazol (100 mM) which is an inhibitor of alcohol dehydrogenase. Similar inhibition of degranulation by ethanol was revealed (data not shown).

The effect of the presence of EtOH for 7 days in the culture media of mBMMC prior to induction of degranulation was then studied. Fresh media containing 0–5‰ (0–86 mM) EtOH was changed daily and on day 7 the IgE-induced degranulation was assessed. As shown in Fig. 2 there was no difference between the IgE-induced degranulation of those cells that had been cultured in the presence of ethanol for 7 days compared to those mBMMC that were incubated with ethanol only for one hour prior to IgE-induced degranulation. This indicates that chronic exposure to ethanol does not exert any additional inhibitory effect on the degranulation capacity of the cells, nor does it lead into adaptation of the cells to the effects of EtOH. Furthermore, if the cells that had been cultured in the presence of ethanol for seven

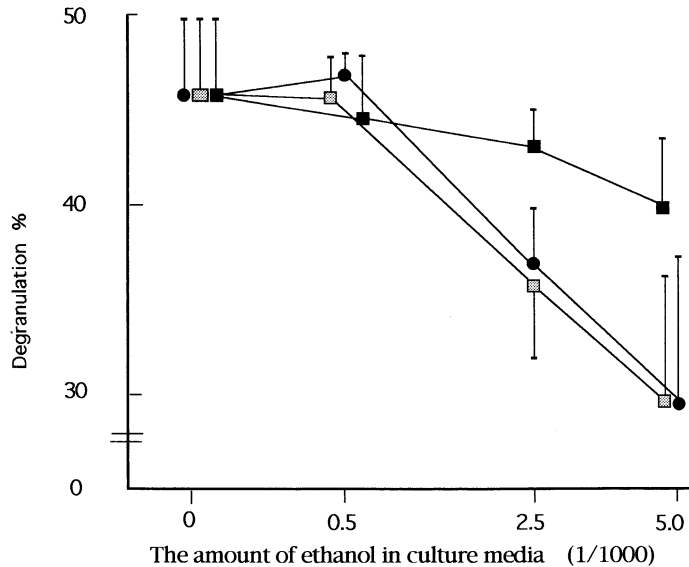


Fig. 2. The IgE-induced degranulation of mBMMC after 7 days of culture in the presence of varying concentrations of ethanol. Media of all cultures was changed daily. On day 7 mBMMC were coated with anti DNB-IgE and challenged with DNB-albumin (see Materials and Methods). Cells cultured in the absence of EtOH for 7 days and EtOH was added 1 h prior to induction of degranulation (open squares). Cells kept in ethanol for 7 days after which degranulation was assessed (filled circles), cells cultured in the presence of EtOH for 7 days but washed twice with PBS 2 h prior to induction of degranulation (filled squares) ($n = 3$, mean $\pm$ SEM).

days were washed to remove EtOH and placed in media without alcohol, there was a tendency (n.s.) for the degranulation ability of the mBMMC to return closer to that of the control cells indicating that ethanol's inhibitory effect on degranulation was at least to a certain extent reversible.

The effect of ethanol on cytokine production of HMC-1 cells

The results on the effect of ethanol on the production of two important cytokines, the proinflammatory TNF- α and chemotactic IL-8, by activated HMC-1 cells are shown in Fig. 3. Unactivated HMC-1 cells produced 4.3–10.9 pg/ml TNF- α per 1×10^6 cells but no detectable amounts of IL-8 during a 24 h activation period. No increase of the production of TNF- α or IL-8 could be observed with either PMA (50 ng/ml) or ionophore A23187 (0.5 μ M) alone. However, upon addition of both PMA (50 ng/ml) and A23187 (0.5 μ M), a clear increase of TNF- α (98.3–475 pg/ml) and IL-8 (2610–4140 pg/ml) production was evident. In the presence of EtOH together with PMA and ionophore, a dose dependent suppression of TNF- α and to a lesser degree of IL-8 production could be observed. A statistically significant inhibition of TNF- α production was observed in the presence of 1% and higher EtOH concentrations as compared to the control cells ($p < 0.05$, $n = 4$). Significant inhibition of IL-8 production was observed at 10% and higher ethanol concentrations ($p < 0.05$, $n = 3$). In the presence of 20% (344 mM) EtOH TNF- α and IL-8 production were inhibited by $81 \pm 12\%$ and $59 \pm 14\%$, respectively.

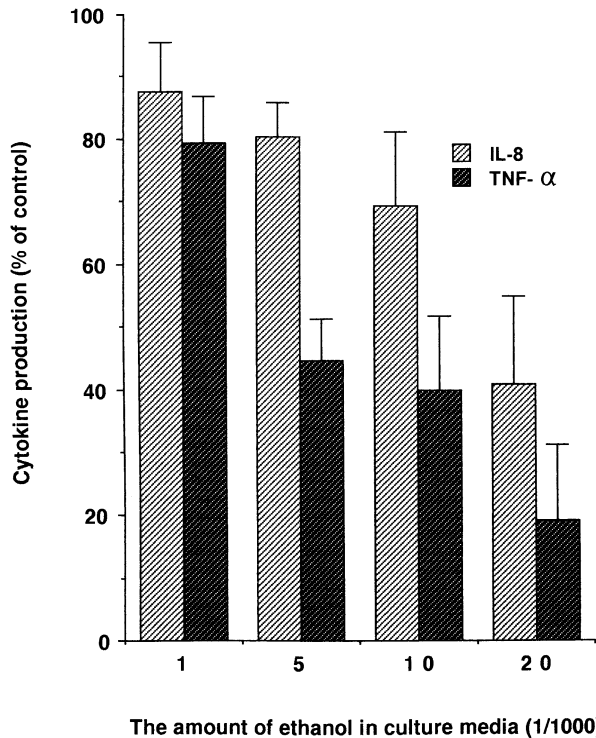


Fig. 3. The effect of ethanol on TNF- α and IL-8 protein production in HMC-1 cells. The cells were stimulated with the combination of phorbolmyristylacetate (50 ng/ml) and calcium ionophore A23187 (0.5 μ M). Ethanol was added 1 h before the addition of the activators and 24 h after the activation of the cells TNF- α and IL-8 were measured from the culture media supernatants by using a commercial ELISA assay. Mean of four (TNF- α) and three (IL-8) separate experiments is shown ($\pm$ SEM). For TNF- α experiment, $p < 0.05$ control vs. EtOH ($\geq 1\%$), for IL-8 experiment $p < 0.05$ control vs. EtOH ($\geq 10\%$).

To assess whether EtOH inhibits the production and/or secretion of cytokines we next studied simultaneously the effect of EtOH on intracellular as well as secreted TNF- α levels in HMC-1 cells. The HMC-1 cells were stimulated with the combination of Ca-ionophore and PMA in the absence and presence of 10 and 20% ethanol and both intracellular and secreted TNF- α levels were measured 24 h later. As shown in Fig. 4 the unstimulated HMC-1 cells stored very small amounts of TNF- α into their intracellular granules. Upon stimulation the amount of intracellular TNF- α increases to certain extent but the great majority of TNF- α is secreted outside the cell. Importantly, the activation of the cells in the presence of ethanol did not result into intracellular accumulation of TNF- α . Instead a decrease of the levels of intracellular TNF- α was seen concomitantly with the decreased levels of secreted TNF- α indicating that the inhibitory effect of EtOH is not caused by inhibition of the secretory machinery of the cells.

The effect of ethanol on proliferation of MC

To assess the effect of ethanol on proliferation, we studied the recombinant kit ligand (rKL)-induced [^{3} H]Thymidine uptake of mBMMC. In previous studies rKL has been shown

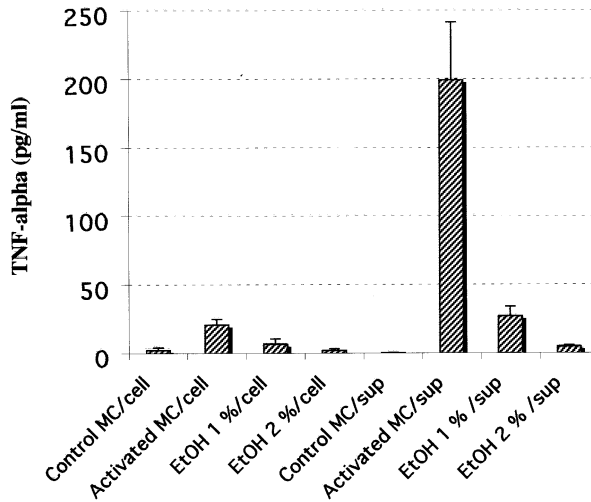


Fig. 4. The effect of ethanol on intracellular vs. secreted TNF- α levels in HMC-1 cells. The cells were stimulated with the combination of phorbolmyristoylacetate (50 ng/ml) and calcium ionophore A23187 (0.5 μ M). Ethanol was added 1 h before the addition of the activators. TNF- α was measured 24 h later from both the cells (MC/cell) and supernatants (MC/sup) in the presence and absence of ethanol. Mean of two separate experiments is shown ($\pm$ SEM).

to be a potent proliferation and maturation factor for mBMMC [21,24]. In the absence of rKL no appreciable [^{3} H]Thymidine uptake by the mBMMC could be observed but in the presence of rKL a pronounced uptake of [^{3} H]Thymidine was evident (Fig. 5). The presence of 0.5–5‰ (8.6–86 mM) ethanol had no clear effect on the [^{3} H]Thymidine uptake. In higher ethanol concentrations of 20‰ and 50‰ (344–860 mM) an attenuation of 13.1% and 44.5% respectively, of [^{3} H]Thymidine uptake by mBMMC could be observed. In two other separate experiments, EtOH was added 2–5 hours prior to addition of rKL but similar results were obtained. In that case in the presence of 20 and 50‰ EtOH the [^{3} H]Thymidine uptake of mBMMC was inhibited by 4.8% (4.5–5.2%) and 25.5% (24.1–27%) respectively. This indicates that EtOH does not interfere with the binding of rKL to its receptor c-kit.

Discussion

In this study we show that, in contrast to previous assumptions, ethanol clearly attenuates several important functions of mast cells. It suppresses the IgE-induced degranulation of mBMMC and at relatively low EtOH concentrations (1–5‰), which had no effect on cell viability, inhibits the production of TNF- α by HMC-1 cells. The inhibition of degranulation is most likely due to ethanol and not to its metabolite acetaldehyde, as similar results were observed also in the presence of 4-methylpyrazol, an alcohol dehydrogenase inhibitor (data not shown). However, it should be noted that also other pathways for the oxidation of ethanol exist in addition to the alcohol dehydrogenase. The inhibitory effect of EtOH on MC degranulation and cytokine production is unlikely to be caused by its effect on cell viability as up to 20‰ of EtOH had no clear effect on the number of viable cells at 24 h nor did it have any

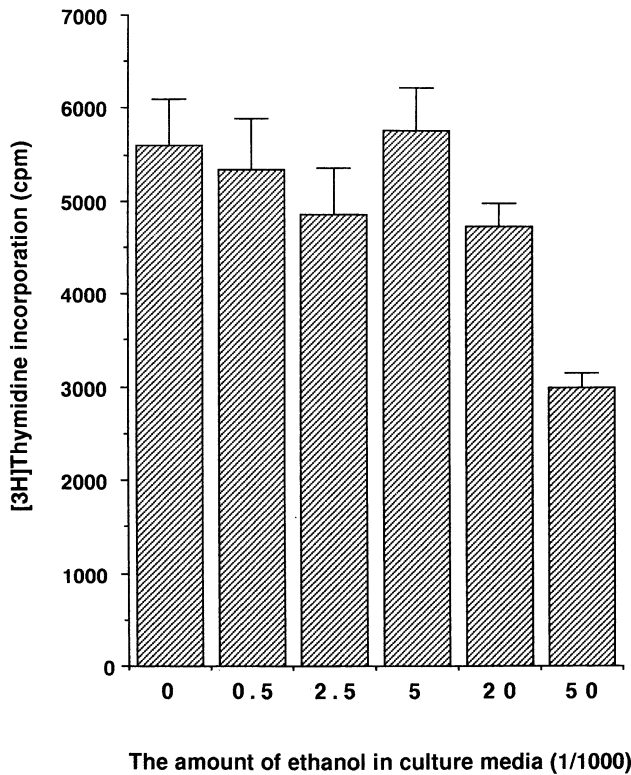


Fig. 5. The rKL-induced [³H]Thymidine uptake of mBMMC in the presence of varying ethanol concentrations. EtOH was added 1 hour after the addition of rKL containing Cos cell supernatant (see materials and methods for details). Cells were pulsed for 4 hours with 1 mCi of [³H]Thymidine and the [³H]Thymidine labeled nuclei were harvested 24 h after addition of EtOH. Experiments were done in triplicate ($n = 2 \pm \text{SEM}$).

significant effect on the incorporation of [³H]Thymidine. No significant intracellular accumulation of TNF- α (Fig. 4) in the presence of EtOH could be observed indicating that EtOH does not significantly inhibit the secretion of TNF- α outside the cell. Therefore it seems most likely that EtOH interferes with the signal transduction system of MC. However, also a plasma membrane perturbing effect by EtOH has been suggested [25].

The strong inhibition of IgE-induced degranulation in the presence of EtOH is somewhat surprising as it has been generally assumed that EtOH induces histamine release from mast cells. For example the damage to gastric mucosa after alcohol ingestion has been shown to be MC dependent and it could be prevented by stabilization of mast cells prior to ethanol ingestion [8,9]. However, inhibition of concanavalin A stimulated histamine release from rat peritoneal mast cells by EtOH has been reported and this inhibition was attributed to ethanol's effects on plasma membrane [25]. Modest increase in the unstimulated histamine release at high ethanol concentration was observed in the present study and it is possible that mast cells residing in gastric mucosa are exposed to even higher EtOH concentrations, which could result into spontaneous histamine release. However, two alternative explanations are more likely. First, it is possible that the activation of MC in gastric mucosa is not directly caused by

ethanol, but instead by some other MC activating factor, which is increased by ethanol. This factor could be for example the neuropeptide substance P or vasointestinal peptide, which are both potent activators of MC and have been shown to be increased in gastric mucosa after alcohol ingestion [8,9]. Secondly, it is possible that acetaldehyde, the metabolite of EtOH, could be responsible for activation of MC. This possibility is supported by the recent reports on Japanese patients with alcohol-induced asthma. These studies indicate that acetaldehyde, but not ethanol, induces bronchoconstriction which is associated with histamine liberation [26]. In addition, very recently acetaldehyde was shown to induce histamine release from rat peritoneal mast cells [27]. Further work on the effect of acetaldehyde on MC activation is being conducted in our laboratory.

Compared to the 1 h exposure to EtOH, the presence of 0.5–5‰ ethanol in the MC culture media for seven days prior to IgE-induced degranulation, did not result into any additional inhibition of mBMMC degranulation, nor did it result into adaptation of the cells to the inhibitory effect of EtOH. Removal of ethanol prior to induction of degranulation resulted in increased capacity of MC to degranulate, indicating that the effect of EtOH is at least to a certain extent reversible and that the effect is not due to toxicity of EtOH. During the culture of mBMMC, ethanol containing, as well as the control medium was changed once a day and the evaporation of ethanol was not prevented. Therefore the EtOH concentrations in the culture media varied somewhat. However, this type of variation of EtOH levels perhaps mimicks better the blood levels of alcohol in persons consuming alcohol on a daily basis.

Relatively low EtOH concentrations (1–5‰) suppressed clearly TNF- α and to a lesser degree IL-8 production in HMC-1 cells. With regard to the immunosuppressive effect of EtOH the inhibition of cytokine release is clearly more relevant than the inhibition of histamine release which was observed only at higher EtOH concentrations, rarely observed even in chronic alcoholics. Several studies have been published on the effects of ethanol on cytokine production of various cell types [see 28]. An increase in the lipopolysaccharide induced expression of IL-1 and TNF- α by EtOH in hepatic cells has been observed [29] and increased secretion of IFN- γ , TGF- α and IL-6 was detected in psoriatic keratinocytes. On the other hand, decreased production of IL-10 and TGF- β together with increased production of TNF- α and IL-1- β , was found in monocytes treated with EtOH [30]. Inhibition of LPS induced TNF- α production in Kupffer cells by EtOH was shown to depend on the decreased NF- κ B transcription factor activation [31].

Alcoholic persons are known to be prone to bacterial pulmonary infections, tuberculosis, bacteremia, peritonitis, endocarditis, and meningitis [32]. Granulocytopenia and thrombocytopenia are known complications of alcoholism and are attributed to bone marrow suppression [33,34]. Ethanol has been shown to inhibit human T-cell proliferation in vitro [35]. Brayton et al., [36] reported a slowed mobilization of leukocytes into traumatized skin, but the ability of polymorphonuclear leucocytes to ingest or to kill bacteria remained intact. Macrophages from peritoneum, alveoli and spleen of alcoholic rats expressed increased density of Fc receptors but phagocytic function was clearly depressed [37]. Alcoholic rats were shown to have lower TNF- α activity in serum and bronchoalveolar fluid in response to LPS injection [38], which is also in agreement with our present results.

To conclude, the anatomic location of mast cells in the interface between inner and outer surfaces in skin and in mucosal membranes suggests a role for MC in the first line of immune

defence. Indeed, recently mast cells have been shown to be important in addition to defence against parasitic infections also in host defence against bacterial infections [1,2]. Therefore the clear attenuation of MC mediator release, TNF- α and IL-8 in particular, could result in attenuated recruitment of neutrophils in the presence of already relatively low ethanol concentrations and therefore in part explain the immune deficiency known to be associated with chronic alcohol consumption. However, further studies are needed to establish the *in vivo* significance of these findings.

Acknowledgments

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