Abstract


1. The immunosuppressive effects of drugs such as alcohol or hormones such as cortisol may be age-related. To test this hypothesis, the authors investigated the in vitro effects of ethanol (EtOH) and cortisol on Natural Killer (NK) cell activity of lymphocytes from normal cord blood in comparison with that of lymphocytes from normal adult peripheral blood.
2. K562, an erythroleukemia cell line, was used as a target in a 4 hr $^{51}$Cr release assay.
3. Ethanol at 0.3% (V/V) and cortisol at 0.05, 0.1 and 0.2 μg/ml concentrations, added directly to a mixture of effector and target cells significantly suppressed the NK activity of cord blood lymphocytes in a dose dependent fashion, whereas similar concentrations of either EtOH or cortisol did not manifest significant immunoregulatory effects on NK cell activity of normal adult lymphocytes.
4. Pre-treatment of the target with either EtOH or cortisol for 4 hours did not affect cytotoxicity. Inhibition of cytotoxicity was also not due to direct toxicity of effector cells because lymphocytes treated with either EtOH or cortisol showed normal $^{51}$Cr release and their viability was comparable to that of untreated control cells.
5. This suggests a selective inhibitory effect of EtOH and cortisol on NK activity of neonatal lymphocytes that may be of clinical significance.
Keywords: alcohol, cortisol, natural killer, lymphocytes

Abbreviations: adult peripheral blood lymphocyte (aPBL), antibody dependent cellular cytotoxicity (ADCC), cord blood lymphocytes (CBL), ethanol (EtOH), hypothalamic pituitary adrenal (HPA), Hanks’ balanced salt solution (HBSS), herpes simplex virus (HSV), lymphokine activated killer (LAK), natural killer (NK), Roswell Park Memorial Institute (RPMI)

Introduction

Previous studies suggest that a bidirectional communication network exists between the central nervous system and the immune system which is primarily mediated through shared neuropeptide signals produced in response to the activation of the hypothalamic-pituitary adrenal (HPA) axis (Bateman et al, 1989; Stein et al, 1988; Biondi & Kotzmidis, 1990; Dunn, 1989). It is now becoming clear that lymphocytes not only synthesize and secrete neuropeptides and hormones; they possess receptors for these neuro-endocrine molecules (Solomon 1985). Glucocorticoids are the end products of endocrine-immune interactions. Negative feedback regulation by cortisol on the production of neuropeptides and hormones by activated HPA axis and the immune system has also been reported (Bateman et al, 1989). Natural killer (NK) and antibody dependent cellular cytotoxic (ADCC) activities are considered as the body's early defense mechanisms against viral infection and tumors (Herberman & Holden, 1978; Welch, 1981). Previous studies have demonstrated that glucocorticoids exert significant down-regulation of various immune responses including NK, (Nair & Schwartz, 1984, 1988), polyclonal B cell activation (Yu et al, 1974) and cytokine production (Wahl et al, 1975).

Chronic alcohol consumption is associated with abnormalities of humoral (Drew et al, 1984; Delacroix et al, 1982; Morgan et al, 1980; and Chang et al, 1990) and cellular immunity (Jerrells et al, 1989; Meadows et al, 1992; Ericsson et al, 1980; Watson et al, 1984; Hodgson et al, 1978) including dysfunction of suppressor (Woltjen et al, 1980; Kawanashi et al, 1981), helper (McKeever et al, 1988) and cytotoxic lymphocyte activities (Abdallah et al, 1983; Saxena et al, 1980; Meadows et al, 1989) as well as production of soluble immune mediators (Nair et al, 1993). Considerable evidence suggests an association between alcohol consumption, increased risk of cancer and possible involvement of NK cell activity (Yirmiya et al, 1992). Alcohol consumption is also known to activate the HPA axis resulting in the release of neurohormone or neuropeptides (Redei et al, 1986, 1988; Gottesfeld et al, 1990; Jerrells et al, 1989) which in turn is known to have profound effects on immune functions (Fauci, 1978) and tumor growth (Riely, 1981). The authors have previously reported that lymphocytes from nonalcoholic, healthy donors precultured in vitro with different concentrations of...
alcohol manifest decreased levels of NK, ADCC and lymphokine activated killer (LAK) cell-activities (Nair et al, 1990). The authors have also shown that patients with depression, demonstrating high levels of cortisol, manifested significantly decreased levels of NK activity (Kronfol et al, 1990).

A decline of various immunological functions with age has been suggested (Miller, 1991). Neonates are known to have increased susceptibility to disseminated and devastating infections (Schaffer and Avery, 1971). The ability of cord blood lymphocytes (CBL) to mediate NK and ADCC activities has been reported, but with conflicting findings (Kohl et al, 1981; Antovelli et al, 1981; Kaplan et al, 1982; Hashimoto et al, 1983; Uksila et al, 1982; Tarkkanen et al, 1982). In a mouse model, age related changes in alcohol induced immunosuppression were also reported (Saad and Jerrells, 1990; Makinoden et al, 1987). These studies suggest that alcohol and age can interact to produce specific changes in the regulation of various immune functions. However, the effect of alcohol, and cortisol on immune functions of lymphocytes has not been clearly elucidated with respect to aging. In the present investigation, the authors examined the direct effect of cortisol and alcohol on NK activity of cord blood lymphocytes in comparison to adult peripheral lymphocytes.

Methods

Preparation of Effector Cells

Neonatal umbilical cord blood samples were collected in heparinized (20 U/ml) tubes under sterile conditions from healthy term infants at vaginal delivery before the expulsion of the placenta. Heparinized (20 U/ml) peripheral blood was obtained simultaneously from healthy unrelated adult volunteers of either sex (20 to 40 years of age). Blood samples were processed immediately after collection. Donors or their parents were apprised of the study and consents were obtained consistent with the policies of the NIH. Mononuclear cells from both adult and cord blood samples were isolated using a modified method of Boyum (Boyum, 1968). Blood was diluted with an equal volume of Ca²⁺ and Mg²⁺-free Hanks' balanced salt solution [HBSS (GIBCO, Grand Island, N.Y.)] and centrifuged at 400 x g for 30 min at 18°C over a cushion of Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.). The mononuclear cell band was harvested, washed three times with HBSS, and resuspended in RPMI 1640 medium containing 25 mM Hepes buffer supplemented with 10% heat-inactivated fetal bovine serum [FBS (GIBCO), 80 ug/ml gentamicin (Schering Corp., Kenilworth, N.J.), and 300 ug fresh glutamine/ml (complete medium).
Depletion of Adherent Cells

Mononuclear cells were depleted of adherent cells as described (Nair and Schwartz, 1983). Briefly, total mononuclear cells were suspended in RPMI 1640 with 10% FBS and passed through a 7 ml column of Sephadex G-10 beads (Pharmacia) equilibrated in the same medium. After 45 min of incubation at 37°C, nonadherent cells were washed through with 1 bed vol of warm (37°C) medium. The cell recovery was >70% of the total input, and monocyte contamination was <2% as indicated by nonspecific esterase staining.

Preparation of Target Cells

Mycoplasma-free human erythroleukemic cell line, K-562, was used as target for NK cells in a 4 hr $^{51}$Cr release assay. Tumor target cells were serially passaged in complete medium and used in cytotoxicity assays no more than 48 hr after the last passage. 200 μCi of $^{51}$Cr as sodium chromate (New England Nuclear, Boston, MA) was added to 0.8ml aliquots of complete medium containing $5 \times 10^6$ washed tumor cells. The cells were incubated at 37°C for 1 hr in a humidified atmosphere of 5% CO₂, with intermittent shaking. After incubation, the cells were washed three times with complete medium and resuspended at a concentration of $1 \times 10^5$ cells/ml.

Assay for NK Cell Activity

NK cell activity was measured in a direct $^{51}$Cr release assay as previously described (Nair et al., 1990). A fixed number of effector cells in complete medium was added to triplicate cultures of $^{51}$Cr-labeled target cells in a V-bottom microtitration plates (Costar, Cambridge, MA). After centrifugation at 40 x g for 10 min, 100μl aliquots were removed from each well and transferred to glass tubes and read in a Packard Model 593 gamma counter. Percentage cytotoxicity was calculated as follows:

\[
\%\text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100
\]

Spontaneous release represents counts released from control wells containing only $1 \times 10^4$ target cells, and total release represents counts obtained from an aliquot of $1 \times 10^4$ target cells.
Data Analysis

The data were analyzed by the student "t" test and the significance of differences in the mean values between control and treated groups were considered significant at P<.05 levels.

Results

Data presented in Table 1 demonstrate the in vitro effects of alcohol on NK activity of cord blood lymphocyte (CBL) compared to adult peripheral blood lymphocyte (a-PBL). CBL depleted of adherent cells demonstrated significantly (p<0.001) lower level of NK activity (14.8%) compared to a-PBL (26.7%) at 25:1 effector to target (E:T) cell ratio. CBL also demonstrated significant suppression of NK activity at other E:T cell ratios of 100:1, 50:1 and 10:1 compared to a-PBL (data not presented). Direct addition of alcohol at concentrations of 0.1, 0.2 and 0.3% to the reaction mixture of a-PBL and target cells did not produce any effect on NK activity; the percent cytotoxicities being 24.5, 23.8 and 21.4% respectively compared to 26.7% manifested by control a-PBL. Alcohol at 0.1, 0.2 and 0.3% manifested a dose dependent inhibition of NK activity of CBL; the percent cytotoxicities being 10.3, 8.4 and 7.2 respectively compared to 14.8% produced by control CBL. Alcohol at higher concentration (0.3%) produced statistically significant (p<.02) suppression of NK activity of CBL.

Since cortisol is known to affect the immune responses of adult lymphocytes, in general, and infants born by vaginal delivery also demonstrate higher blood cortisol levels (Gaspasoni et al, 1991); we examined the direct effect of cortisol on NK activity of CBL. The data presented in Table 2 show that cortisol at concentrations, 0.05, 0.1 and 0.2μg/ml added directly to the reaction mixture of a-PBL and target cells did not produce any significant inhibitory effects; the cytotoxicities being 24.3, 21.4 and 22.7% respectively compared to 25% cytotoxicity produced by untreated control cultures. However, cortisol at similar concentrations produced significant inhibitory effect on the NK activity of CBL; the cytotoxicities being 8.5 (p<0.05), 6.6 (p<0.01) and 6.5% (p<0.005) respectively at 0.05, 0.1 and 0.2 μg/ml, compared to 14.8% manifested by untreated control CBL. These results suggest that cortisol produces a selective inhibitory effect on NK activity of CBL.
### Table 1

**Effect of Direct Addition of Alcohol on NK Activity of Lymphocytes**

<table>
<thead>
<tr>
<th>Effector Source</th>
<th>Concentration of Alcohol (%)</th>
<th>% Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPBL</td>
<td>0</td>
<td>26.7±2.5</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>24.5±2.2 (NS)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>23.8±0.8 (NS)</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>21.4±2.5 (NS)</td>
</tr>
<tr>
<td>CBL</td>
<td>0</td>
<td>14.8±2.0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>10.3±2.9 (P&lt;.7)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>8.4±2.7 (P&lt;.2)</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>7.3±2.2 (P&lt;.02)</td>
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</tbody>
</table>

EtOH (v/v) was added directly to a mixture of effector cells plus target cells and the NK activity was measured against prelabeled K562 target cells. Values represent mean % cytotoxicity ± SD of triplicate determinations from 13 experiments using 13 different cord blood and 13 different adult peripheral blood lymphocyte samples. The NK activity was measured at 25:1 E:T cell ratio. Statistical significance of differences in the mean values was determined by two tailed student "t" test.

### Table 2

**Effect of Direct Addition of Cortisol on NK Activity of Lymphocytes**

<table>
<thead>
<tr>
<th>Effector Cell Source</th>
<th>Concentration of Cortisol (µg/ml)</th>
<th>% Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPBL</td>
<td>0</td>
<td>25.7±4.8</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>24.3±2.9 (NS)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>21.4±3.7 (NS)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>22.7±2.9 (NS)</td>
</tr>
<tr>
<td>CBL</td>
<td>0</td>
<td>14.8±2.0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>8.5±2.5 (P&lt;.05)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>6.6±2.5 (P&lt;.01)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>6.5±2.0 (P&lt;.005)</td>
</tr>
</tbody>
</table>

Cortisol (µg/ml) was added directly to a mixture of effector cells plus target cells and the NK activity was measured against prelabeled K562 target cells. Values represent mean % cytotoxicity ± SD of triplicate determinations from 13 experiments using 13 different cord blood and 13 different adult peripheral blood lymphocyte samples. The NK activity was measured at 25:1 E:T cell ratio. Statistical significance of differences in the mean values was determined by two tailed student "t" test.
Previously the authors have reported that CBL show significantly lower NK, ADCC and target binding activities compared to adult PBL (Nair et al, 1985). We also showed that patients with a history of intravenous drug abuse, often associated with excessive alcohol consumption, demonstrated decreased levels of NK, ADCC, interferon and interleukin-2 induced NK activities. (Nair et al, 1986) Further, we showed that lymphocytes from adult healthy subjects, pre-cultured \textit{in vivo} with varying concentrations of alcohol corresponding to \textit{in vivo} intoxicating levels manifested decreased levels of NK and ADCC activities; whereas direct addition of alcohol did not produce any significant suppression of NK activity (Nair et al 1986). The authors (Nair and Schwartz, 1984) and others (Callewaert et al, 1991) have shown that corticosteroids manifested significant inhibitory effects on NK activity of normal adult lymphocytes. However, the effect of alcohol and cortisol on NK activity of neonatal lymphocytes has not been clearly elucidated. The present investigation shows that alcohol and cortisol demonstrate a selective inhibitory effect on NK activity of CBL as compared to a-PBL. Direct addition of cortisol (equivalent to plasma levels in depressed patients) or alcohol (similar to in vivo intoxicating levels in human) to the mixture of a-PBL and target cells did not produce any significant effect on the NK activity. However similar concentrations of alcohol and cortisol manifested significant inhibition of NK activity of CBL. The suppression caused by either cortisol or alcohol is not due to toxicity because the viability of treated CBL was comparable to that of control cultures (data not presented). Suppression caused by cortisol or alcohol on NK activity of CBL is also not due to selective effect on target cells because in control experiment K562 target cells preincubated with cortisol or alcohol for 4 hr demonstrated similar levels of susceptibility to lysis (13.9% cytotoxicity at 25:1 E:T cell ratio) by fresh effector cells as untreated target cells (14.2%). Further, the spontaneous release of $^{51}$Cr from the target cells treated with alcohol or cortisol was less than 5% of total labeling and was comparable to the spontaneous release from untreated target cells. The inhibitory effect of cortisol on NK activity of CBL was demonstrable at concentration as low as 0.05 and 0.1 ug/ml, levels lower than the blood concentrations seen in depressed patients. In human, reports on the ontogeny of NK cells are conflicting (Rosenberg et al, 1972; Saksela et al, 1979; Abo et al, 1982). Defects in the ability of neonates to mediate NK activity against tumor and virus infected targets have been described by several investigators (Nair et al, 1985; Slukin and Chernishov, 1992).

Recently, Gasparoni et al (1991) showed a significantly high level of blood cortisol in infants born by vaginal delivery. The presence of high levels of cortisol in the blood of neonates born by vaginal delivery (Gasparoni et al, 1991) may corroborate with our finding of lower level of NK activity seen in neonates born by vaginal delivery and possible role for cortisol on NK suppression. However a previous study (Frazier et al, 1982) reported that lymphocytes from babies delivered by C-section without labor
demonstrated significantly lower levels of NK and ADCC activities against Herpes Simplex Virus (HSV) infected Chang liver cells than that of babies delivered vaginally. This study did not (Frazier et al 1982) suggest a negative effect of cortisol on NK activity, since cortisol levels were reported to be lower in neonates delivered by C-section. It is possible that the depressed levels of NK activity as reported in their studies (Frazier et al, 1982) could have been due to the general anesthesia given for the C-section. However, the results presented herein provides direct evidence of selective inhibitory effects of cortisol on NK activity of CBL compared to no such effect observed on a-PBL with similar levels of cortisol. The elucidation of molecular mechanisms underlying alcohol or cortisol induced NK suppression may subsequently yield information applicable for the prevention or control of infections in neonates. Further, the synergistic role of alcohol and cortisol on NK activity remains to be studied.

Conclusion

This study shows a selective suppressive effect of alcohol (similar to in vivo intoxicating levels in humans) and cortisol (equivalent to plasma levels in depressed patients) on NK activities of lymphocytes from cord blood compared to that of adult peripheral blood lymphocytes, that may be of clinical significance.

Acknowledgements

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Alcohol and cortisol effects on cord blood NK cells


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