NEUTROPHIL ACTIVATION BY MURINE RETROVIRAL INFECTION DURING CHRONIC ETHANOL CONSUMPTION

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Abstract — Aims: Neutrophil adhesion molecule CD11b and reactive oxygen species (ROS) are neutrophil activation markers for evaluating the functional activity of neutrophils. The aim of this study was to determine if neutrophils are activated in murine AIDS and/or chronic ethanol consumption and if neutrophil CD11b expression and ROS production vary when progressive retrovirus infection occurs. Methods: Four groups were studied: control, murine AIDS, ethanol and ethanol plus murine AIDS. Neutrophil activation was assessed by CD11b expression and ROS production using flow cytometry. Results: We found that neutrophils lost their responsiveness to fMLP due to retrovirus or ethanol exposure. In the murine AIDS group, neutrophil CD11b expression was up-regulated along with a significant increase in ROS after 1 month of retroviral infection. After 2 months, neutrophil CD11b and ROS decreased. However, neutrophil CD11b expression further increased after 3 months. In the ethanol consumption group, neutrophil CD11b expression was down-regulated after 2 months, whereas ROS production increased in the first and third months. In the murine AIDS plus ethanol group, there were significant increases in both ROS and CD11b expression during the 3-month observation period. Conclusions: These findings suggest that neutrophil function is impaired by LP-BM5 retrovirus infection and/or chronic ethanol consumption. The pattern of neutrophil CD11b expression and ROS production might help to predict the stage of murine AIDS. Ethanol may further compromise neutrophil function in AIDS.

INTRODUCTION

Neutrophils are the first line of defense against invading foreign microorganisms. Impairment of neutrophil function may contribute to the onset of certain life-threatening bacterial and fungal infections in AIDS individuals (Ellis et al., 1988; Murphy et al., 1988; Vecchietelli et al., 1995). Neutrophil CD11b expression and reactive oxygen species (ROS) production are inflammatory responses to mechanical, bacterial and viral injury. Under physiological conditions, circulating neutrophils are in the resting state. CD11b, a component in the CD11b/CD18 adhesion protein complex, is a sign of neutrophil activation by up-regulation of its expression. The presence of this adhesion complex allows neutrophils to migrate from the blood circulation into target tissue to perform their duty (Cavanagh et al., 1998). ROS is another neutrophil activation marker. Neutrophil-derived ROS may cause an intracellular oxidant stress by retroviral infection. When neutrophils are stimulated, the membrane-bound NADPH oxidase catalyses the production of superoxide anions and hydrogen peroxide. ROS production by neutrophils is also critical for microbial killing. However, neutrophil activation is tightly regulated by many factors. The underlying mechanism remains limited. It may involve cytokine dysregulation, viral products and secondary infection in AIDS. Indeed, many investigators (Morse et al., 1992; Akarid et al., 1995; Liang et al., 1997) described the severity of cytokine dysregulation in murine AIDS. Increased cytokine production by Th2 cells and infected macrophages is a hallmark of murine AIDS. Production of IL-4, IL-6, IL-10 and TNF-α is dramatically elevated (Morse et al., 1992; Akarid et al., 1995; Liang et al., 1997). Retrovirus-infected macrophages can further release abnormally large amounts of IL-1, TNF-α and platelet-activating factor (PAF) (Gelbard et al., 1994; Westmoreland et al., 1996; Serradji et al., 2000). It is well known that IL-1β, TNF-α and PAF are potent neutrophil-activating factors. We therefore hypothesized that neutrophil CD11b expression and ROS production are elevated in murine AIDS.

Because cytokines involved in neutrophil activation are mainly secreted by immune cells, such as T and B cells, and macrophages, the degree of cell destruction by retroviral infection in the different stages of murine AIDS may influence cytokine levels. Eventually, it will reflect on neutrophil activities, such as neutrophil CD11b adhesion molecule expression and ROS production. Therefore, levels of neutrophil CD11b expression and ROS production may become useful prognostic markers to monitor the progressive stages of AIDS.

MacGregor’s studies indicated that ethanol intoxication has profound effects on neutrophil kinetics (Gluckman and MacGregor, 1978; MacGregor et al., 1978, 1988). Of most significance is the inhibition of neutrophil mobilization to sites of inflammation. Most of these effects are likely to be mediated by the mechanism of inhibited neutrophil adhesion molecular expression through cytokine dysregulation. Indeed, Arbabi et al. (1999) found that acute ethanol intoxication inhibits the production of IL-8 and TNF-α. Ethanol consumption could directly increase production of ROS in neutrophils by ethanol dehydrogenase, microsomal oxidation systems and catalase. However, few investigators used both neutrophil CD11b expression and ROS production for neutrophil kinetic and/or functional studies after chronic ethanol consumption. Chronic ethanol consumption in AIDS patients is common. Thus, 14% of HIV-infected patients misuse alcohol (Welch, 2000). Retrovirus and ethanol may interact in a complex manner on neutrophils. Therefore, we investigated both factors on neutrophil CD11b expression and ROS production.

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MATERIALS AND METHODS

Reagents

The reagents and sources were as follows: LDS-751 and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR, USA); mouse fluorescein isothiocyanate (FITC)-conjugated anti-CD11b mAb was purchased from Pharmingen (San Diego, CA, USA); f-Met-Leu-Phe (fMLP) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Phosphate-buffered saline (PBS) was filtered using a 0.45 μm pore filter prior to all experiments.

Animals

Female C57BL/6N mice (National Cancer Institute) at 8–12 weeks of age and weighing about 20–22.5 g were randomly assigned to four different groups: control, murine AIDS, ethanol and murine AIDS plus ethanol. Mice were housed in transparent plastic cages with a stainless wire lid in a room at 20–22 °C with constant humidity and a 12 h:12 h light:dark cycle (lights on at 07.00). Murine AIDS was induced by LP-BM5 murine leukaemia retrovirus infection, as done previously in our laboratory (Wang et al., 1995; Liang et al., 1997). Infection leads to the rapid induction of clinical symptomatology that is similar to human AIDS. In the first week, 10% (v/v) ethanol in autoclaved tap water was made available to the chronic ethanol-fed mice in a 300 ml plastic bottle with a stopper. The ethanol concentration was increased in increments of 10% at 1-week intervals, from an initial 10% to a final concentration of 20% (v/v) and kept at 20% (v/v) for the rest of the treatment period. Mice were active at night; therefore, we took blood samples at night (after lights were turned off for 3.5 h) to estimate blood-ethanol concentration (Sigma Diagnostics Alcohol Kit).

The average blood-ethanol concentration was 66.9 mg/dl (0.0669%). The non-ethanol fed mice were given the same diet, except that the water bottles contained only water. No weight loss was found at the end of the experimental period between the non-ethanol and ethanol-fed mice.

Neutrophil CD11b expression and ROS production

Flow cytometry was used to identify neutrophils and their activity in whole blood. Freshly drawn, citrated whole blood was mixed with the vital nucleic acid stain, LDS-751 (1 μg/ml). Labelling leucocytes with LDS-751 allowed for the investigation of leucocyte characteristics in whole blood and thus avoided leucocyte isolation procedures, which are known to cause artificial leucocyte activation (Macey et al., 1992). This mixture of whole blood and LDS-751 was used for all subsequent leucocyte experiments. Neutrophil CD11b and ROS measurements were performed by incubating saturating concentrations of FITC-labelled mouse CD11b antibody, and 80 mM DCFH-DA with the whole blood/LDS-751 mixture. Samples were incubated in a 37°C water bath for 15 min, and then diluted with 0.5 ml of cold PBS. During FACS (fluorescence-activated cell sorter) scanning (Becton Dickinson, FACSPlan Clinical Flow Cytometer), a 488 nm argon laser light was used for excitation, and fluorescence emission was detected as forward scatter (FSC), which is a measure of cell size, and side scatter (SSC), which is a measure of cell granularity. In addition, a threshold fluorescence was set on the LDS-751 signal that allowed list-mode data collection on leucocytes in whole blood without interference from erythrocytes. Thus, neutrophil subpopulations can be separated on the basis of their dot plots pattern on FSC, SSC and the fluorochrome intensity of LDS-751 (red) in the FL3 channel (Fig. 1) (detector FL3 is...
for red fluochrome). The green fluorescence intensity due to bound FITC-labelled CD11b antibody was monitored in the FL1 channel (detector FL1 is for green fluochrome). For measurement of ROS, this method (Himelfarb et al., 1992) used the properties of DCFH-DA, which rapidly diffused across the cell membrane and was then trapped within the cell by a deacetylation reaction. In the presence of hydrogen peroxide, this compound was oxidized to DCF, which is highly fluorescent in the FL1 channel. To investigate the capacity of neutrophils to up-regulate the CD11b adhesion molecule expression and ROS generation in response to bacterial infection, we used fMLP (10⁻⁶ M final concentration), a bacterial peptide, to stimulate neutrophils for 15 min ex vivo before analysis by flow cytometry. The data from the FACS processing was further analysed using WinMDI 2.8. Data were expressed as total fluorescence intensity (TFI = mean channel of fluorescence × % of positive events).

Haematology parameter

Blood cell counts were determined by an automated cell counter (Serono Diagnostics, Allentown, PA, USA; Model 9018 CP). Leucocyte differential counts were verified manually on Wright stained blood smears.

Statistical analysis

All statistics were calculated using Prism Statistical software (version 3.0). Comparisons between groups were made using ANOVA with Newman–Keuls post hoc testing. Data are presented as means ± SEM.

RESULTS

Total white blood cell (WBC) and neutrophil counts

The results of WBC and neutrophil counts are summarized in Table 1. Although total WBC counts were not significantly different among the groups, the per cent of neutrophil [neutrophil (%)] and absolute neutrophil counts (ANC) were significantly increased after 3 months of murine AIDS and murine AIDS plus ethanol consumption compared to the control group (P < 0.05). In the murine AIDS plus ethanol consumption group, there was a significant decrease of neutrophil (%) and ANC at 2 months compared to the control group (P < 0.05). However, after 3 months, neutrophil (%) and ANC were dramatically increased.

Neutrophil CD11b expression and ROS production

In LP-BM5-induced murine AIDS, circulating neutrophil CD11b (Fig. 2) increased, even in the first month of infection (P < 0.05). In the following month of murine AIDS, neutrophil CD11b expression went back to the control level. Three months post retrovirus infection, CD11b expression reached a new high point (P < 0.001). Neutrophil-derived ROS, another marker for neutrophil activation and killing activity, increased after 1 month of LP-BM5 infection (P < 0.001) (Fig. 3). Thereafter, ROS production decreased. Increased neutrophil ROS production was coupled with CD11b up-regulation in 1-month murine AIDS mice. After 2 months of LP-BM5 infection, neutrophil CD11b expression and ROS production both fell back to unstimulated control levels. After 3 months of infection, murine AIDS mice exhibited general malaise. At this time, neutrophil CD11b expression dramatically increased, but ROS production remained consistent.

In the chronic ethanol consumption group, neutrophil CD11b expression was down-regulated, especially after 2 months of ethanol consumption (P < 0.05) (Fig. 2). Neutrophil ROS production significantly increased after 1 month (P < 0.05), returned to the control level after 2 months, and then increased again after 3 months of ethanol consumption (P < 0.05) (Fig. 3). After 1 month of ethanol consumption, an increased neutrophil ROS production was not accompanied by CD11b up-regulation. After 2 months, neutrophil CD11b was down-regulated, but neutrophil ROS production did not change. After 3 months, neutrophil ROS production was highly induced by chronic ethanol consumption, but neutrophil CD11b expression was not affected.

In the murine AIDS with chronic ethanol consumption group, no significant difference in neutrophil CD11b expression (Fig. 2) was observed in the first or second month. However, a significant increase in neutrophil CD11b expression occurred (P < 0.01) during the 3-month observation period. The neutrophil ROS production (Fig. 3) had a complex pattern. It increased in the first month (P < 0.001), then tended to decrease in the second month and then increased (P < 0.001) again after

<table>
<thead>
<tr>
<th>Group</th>
<th>Month</th>
<th>Total WBC count (10⁹/µl)</th>
<th>Neutrophils (%)</th>
<th>Neutrophil count (10⁹/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>3.10 ± 0.34</td>
<td>6.17 ± 1.50</td>
<td>0.19 ± 0.10</td>
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<tr>
<td></td>
<td>1</td>
<td>1.73 ± 0.26</td>
<td>11.98 ± 3.92</td>
<td>0.21 ± 0.11</td>
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<td></td>
<td>2</td>
<td>2.68 ± 0.23</td>
<td>16.50 ± 2.19</td>
<td>0.44 ± 0.07</td>
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<tr>
<td></td>
<td>3</td>
<td>5.06 ± 1.18</td>
<td>26.83 ± 4.97*</td>
<td>1.36 ± 0.54*</td>
</tr>
<tr>
<td>Murine AIDS</td>
<td>1</td>
<td>2.95 ± 0.33</td>
<td>5.98 ± 1.23</td>
<td>0.17 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.45 ± 0.44</td>
<td>2.00 ± 0.40**</td>
<td>0.09 ± 0.02**</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.59 ± 0.70</td>
<td>22.00 ± 6.44**</td>
<td>1.23 ± 0.50**</td>
</tr>
<tr>
<td>Murine AIDS plus ethanol</td>
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<td>2.89 ± 0.25</td>
<td>5.73 ± 1.33</td>
<td>0.17 ± 0.13</td>
</tr>
<tr>
<td></td>
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<td>1.97 ± 0.22</td>
<td>4.83 ± 0.87</td>
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<tr>
<td></td>
<td>3</td>
<td>3.67 ± 0.41</td>
<td>11.76 ± 1.64</td>
<td>0.43 ± 0.09</td>
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</table>

Data are expressed as means ± SEM in eight mice for each group. The values at month zero represented the control level of white blood cell (WBC) and neutrophil counts.

*P < 0.05, control vs 3 months of murine AIDS.

**P < 0.001, control vs 2 (decrease) or 3 months (increase) of murine AIDS plus ethanol consumption.
In the first month, neutrophils induced a higher level of ROS without CD11b up-regulation. In the second month, no significant difference of neutrophil CD11b expression and ROS production was observed. Thereafter, an increased neutrophil CD11b expression was parallel to ROS production in the third month of retroviral infection plus ethanol consumption.

**fMLP stimulated neutrophil CD11b expression and ROS production**

To determine the capability of the neutrophil response to bacterial infection, we used fMLP, a bacterial peptide, to stimulate neutrophils for 15 min *ex vivo*. For neutrophil CD11b expression (Fig. 4A–C), fMLP stimulation caused a significant increase (*P < 0.05) that was exhibited in the control group only. No significant differences were found in all other groups in the presence or absence of fMLP. For neutrophil ROS production (Fig. 5A–C), similar results were found for...
neutrophil CD11b expression. There was a significant increase in neutrophil ROS production ($P < 0.05$) after being stimulated by fMLP in the control group and the early first month of ethanol consumption. However, no significant difference in neutrophil ROS production was found after stimulation by fMLP in the different stages of murine AIDS, murine AIDS plus ethanol consumption, and chronic ethanol consumption alone.

**DISCUSSION**

Neutrophil CD11b expression plays a key role in mediating firm adhesion of neutrophils to vascular endothelial cells prior to transmigration into their target sites. Thereafter, activated neutrophils release ROS that is toxic to microorganisms. In this study, we applied fMLP *ex vivo* stimulation to determine the ability of the neutrophil response to invading organisms. We found that neutrophil CD11b expression and ROS production were impaired in response to fMLP stimulation during the different stages of LP-BM5 infection and/or chronic ethanol consumption. This result suggests that neutrophil function is diminished by AIDS and/or chronic ethanol consumption. The neutrophil functional abnormalities may be due to several factors, such as: (1) ethanol or its metabolites; (2) viral particles from LB-BM5 infection; (3) cytokine dysregulation; and/or (4) neutrophil receptor desensitization.

Ethanol intoxication has been observed to decrease the adherence of neutrophils to endothelial cells (Gluckman and MacGregor, 1978; MacGregor *et al*., 1978). Our results further support this observation because neutrophil CD11b adhesion molecules decrease after moderate ethanol consumption. The impairment of neutrophil CD11b expression may be related to decreased proinflammatory cytokine production, such as TNF-$\alpha$ and IL-8 (Arbabi *et al*., 1999), which are potent neutrophil stimulators. It is well known that ethanol induces oxidative stress in many cell types (Brown *et al*., 2001; Sun and Sun, 2001). Neutrophil ROS may be induced directly by the microsomal ethanol-oxidizing systems or indirectly by inflammatory cytokines. In our study, an initial peak of neutrophil ROS production may be dominated by ethanol-oxidizing systems. After 2 months of ethanol consumption, a reduced level of neutrophil ROS with down-regulated neutrophil CD11b suggests that decreased proinflammatory cytokines override the effect of ethanol-oxidizing systems. As mice continually drink, an increase of neutrophil ROS production with normal neutrophil CD11b expression suggests that neutrophils are tolerant to the response of proinflammatory cytokines; however, ethanol-oxidizing systems in neutrophils may be not affected. Although ROS is a necessary agent for bacterial killing, at this point ROS may trigger neutrophil apoptosis before neutrophils reach the inflammatory site. This phenomenon suggests that chronic ethanol consumption increases susceptibility to infection (Jareo *et al*., 1995).

Cytokines released from infected cells are a hallmark of LP-BM5 infection. Proinflammatory cytokines, such as TNF-$\alpha$, IL-1, IL-6, and PAF, increase in AIDS (Gelbard *et al*., 1994; Akarid *et al*., 1995; Liang *et al*., 1997). They are all potent neutrophil activators. In our study, we found that increased neutrophil ROS production was accompanied by neutrophil CD11b up-regulation in the first month of LP-BM5 infection, even though the neutrophil response to fMLP was diminished. At this time, up-regulation of neutrophil CD11b and ROS may be due to cytokine stimulation. After 2 months of LP-BM5 infection, both neutrophil CD11b expression and ROS production returned to the baseline level. This decrease may be due to: (1) a decrease in cytokine production; and/or (2) neutrophil receptor desensitization. A decreased cytokine level may be related to a massive destruction of cytokine-producing cells due to rapid viral replication. Neutrophil desensitization could be also related to cytokine dysregulation.


