Animal Models in Cancer Research Which Could be Useful in Studies of the Effect of Alcohol on Cellular Immunity

Michael A. Chirigos and Richard M. Schultz

Virus and Disease Modification Section, Laboratory of Chemical Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, NIH, Bethesda, Maryland 20205

Abstract

Alcohol appears to exert a depressant effect on host immunity. Animal models useful in studying immune responsiveness in cancer research are discussed, which could be of value in studying the effect of alcoholism.

Allogeneic tumor grafts are poorly rejected in immunosuppressed mice. Of the four major cellular elements of the immune system, the macrophage appears to have a critical role in immune surveillance. Several conditions occur which abrogate or restrict the tumoricidal activity of macrophages. Stress induced by physical restraint results in depressed macrophage activation. The tumoricidal activation induced in macrophages by interferon was markedly depressed in the presence of the corticosteroids, hydrocortisone, prednisone, and dexamethasone. In addition, prostaglandins (PGE1 and PGE2) also were found to decrease interferon activation of macrophages.

Since immune deficiency is a trait of alcoholism and cancer, animal models with defined, measurable, immunological parameters would be useful in studying the effect of alcohol on cellular immunity.

Introduction

An impressive number of publications are appearing in the literature concerning the possible relationship between alcohol and cancer (29). In addition, alcohol has been implicated as a causative agent in altering the immune mechanism of the host (1, 4, 5, 13, 17, 18, 21). Green and Trawbridge (10) report that among the more common and recognizable problems in chronic alcoholism are those related to the cellular elements of the blood. Anemia is the most common hematological complication of alcoholism, with folic acid deficiency being the predominant cause. Thrombocytopenia has been reported to occur with or without anemia as an indirect effect of alcoholism. Granulocytopenia, which is secondary to folic acid deficiency or splenic sequestration, can similarly be seen in the alcoholic, and neutrophil dysfunction (abnormal chemotaxis) in cirrhotic patients has also been described (7).

Of the 4 major cellular types: T-lymphocytes (thymus-derived cells); B-lymphocytes (bone marrow-derived cells); null cells (possibly precursors of T-cells); and the macrophage (histiocyte), the latter has been rigorously examined in only the past few years.

There appears to be a common feature in alcoholism and cancer in that these 2 diseases have an inhibitory effect on the immune mechanism. The effect of trauma to the host-immune mechanism(s) mediated by cancer, physiological perturbation by stress, or alcoholism can be studied in the animal system.

The objective of this report is to describe animal models in cancer research which could be applicable in studying the relationship of alcoholism (which could be a stress to the host immune system) and cancer.

Materials and Methods

Animals. Adult male BALB/c × DBA/2 F1, (hereafter called CD2F1) 6 to 8 weeks old, were supplied by the Mammalian Genetics and Animal Production Section, Drug Research and Development Branch, National Cancer Institute, NIH, Bethesda, Md. The animals were housed in plastic cages and fed Purina laboratory chow with water ad libitum. All animals weighed at least 23 g before they were used for experimentation.

Tumor. The MBL-2 line, a Moloney lymphoid leukemia line originally induced in C57BL/6 mice by inoculation with the Moloney murine leukemia virus has been routinely passaged in our laboratory in the ascites form for >100 passages. The ascites tumor is passed i.p. at weekly intervals.

Allograft Response. MBL-2 ascites cells (1 × 10⁸ cells) were inoculated s.c. in the right inguinal region of CD2F1 mice, and tumor palpations were conducted every third day until regression occurred. In studies to determine abrogation of the allograft response, mice were treated with BCNU (30 mg/kg) s.c. in the scapular area 1, 2, 4, or 6 days before MBL-2 tumor inoculation.

Stress Procedure. Immobilization stress was accomplished by confining individual mice in 30- x 115-mm plastic conical tubes (Falcon Plastics, Oxnard, Calif.) as described by Gisler (9).

Leukemia Cell Cultures. MBL-2 lymphoblastic leukemia cells were maintained as suspension cultures in RPMI-Medium 1640 supplemented with 20% heat-inactivated (56° for 30 min) FCS, gentamicin solution (100 µg/ml), 0.075% NaHCO₃, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (RPMI:FCS). Viability of the cells was determined by the trypan blue exclusion test.

Peritoneal Macrophages. Noninduced peritoneal macrophages were harvested from mice as previously described (23) and washed in RPMI Medium 1640, and approximately 4 × 10⁶ macrophages were seeded into 16-mm wells on tissue culture cluster plates (Costar, Cambridge, Mass.) in 1.0 ml of RPMI:FCS. The cultures were incubated for 90 min, and macrophage monolayers were washed thoroughly with jets of medium before use in experiments.

Macrophage Activation Assay. Our technique for measuring the ability of interferon to produce growth inhibitory macro-

---

1 Presented at the Alcohol and Cancer Workshop, October 23 and 24, 1978, Bethesda, Md.
2 To whom requests for reprints should be addressed.
phages in vitro has previously been described (22). Monolayers of macrophages in 16-mm wells were treated with partially purified interferon (Lot 1835) at a final concentration of 1000 units/ml culture medium. Agents tested for inhibitory activity on macrophage function were added in 100-μl aliquots to the macrophage cultures for the duration of exposure to interferon (16 hr at 37°). Following the incubation period, the macrophage cultures were washed 3 times with medium to remove interferon globulin-sepharose (20). Prostaglandins E1, E2, and F2α (PGE1, PGE2, and PGF2α) were partially dissolved in absolute ethyl alcohol, and dilutions were concentrated solutions of corticosteroids or prostaglandins were made in Dulbecco's phosphate-buffered saline (pH 7.2) in 10-fold concentrations prior to addition to macrophage cultures. Concentrations of in vivo glucocorticoid treatment to regulate macrophage cytotoxic function, and the ability of stress to increase circulating levels of cortisol in mice (19), we investigated whether macrophages collected from stressed mice would have a similar depressed response to in vitro exposure with interferon or Escherichia coli LPS. The cytotoxic activity of macrophages, harvested immediately following 20 hr of physical restraint of CD2F1 mice, was inhibited by 81 and 28% as compared to normal, unstressed mice in macrophages activated by LPS (10 μg/ml) or interferon (100 units/ml), respectively (Table 2). However, the stress-induced suppression of macrophage function could be overcome by increasing the concentration of interferon to 1000 units/ml.

Using the same test system, we evaluated the ability of acute stress to inhibit interferon activation of macrophages in vivo. Mice were physically restrained for 20 hr either one day prior to or the same day as interferon treatment (1 × 10⁴ units i.p.), and peritoneal macrophages were harvested from all groups 20 hr after interferon treatment. Macrophages from unstressed mice treated with interferon inhibited MBL-2 tumor cell proliferation by 77%. Stress applied either one day prior to or the

Results

Suppressive Effect of Drug on Allograft Tumor Systems. To evaluate whether BCNU is immunosuppressive and to what extent, suppression of the allograft response to the MBL-2 leukemic cells in drug-pretreated CD2F1 mice was tested. Any potential antitumor effect was avoided by withholding tumor inoculation until BCNU treatment was completed. No residual antitumor effect was anticipated since it has been reported that the duration of an effective concentration of BCNU in mice is 1 hr or less (6).

Results in Table 1 show that the allograft response was abrogated as a result of pretreatment with BCNU (30 mg/kg). All nontreated control animals rejected the allograft within 18 days. In contrast, when BCNU was administered up to 4 days prior to tumor inoculation, all animals developed progressively growing tumors without any rejection occurring. Treatment 6 days prior to tumor inoculation resulted in only 40% death with tumor and 60% regression. These results show the immunosuppressive effect of BCNU and the length of duration of suppressive effect. Of particular interest is the observation that the host under immune stress does demonstrate an altered response to a graft rejection.

Role of Stress on Macrophage Tumoricidal Activity. The vital role of mononuclear phagocytes in host defense against neoplasia, graft rejection, and infectious organisms has been amply demonstrated. An assay developed for measuring the ability of agents to induce macrophage-mediated tumor cell cytotoxicity (22, 23) can be useful in evaluating what stress conditions have on macrophage activity. Due to both the ability of in vivo glucocorticoid treatment to regulate macrophage cytotoxic function, and the ability of stress to increase circulating levels of cortisol in mice (19), we investigated whether macrophages collected from stressed mice would have a similar depressed response to in vitro exposure with interferon or Escherichia coli LPS. The cytotoxic activity of macrophages, harvested immediately following 20 hr of physical restraint of CD2F1 mice, was inhibited by 81 and 28% as compared to normal, unstressed mice in macrophages activated by LPS (10 μg/ml) or interferon (100 units/ml), respectively (Table 2). However, the stress-induced suppression of macrophage function could be overcome by increasing the concentration of interferon to 1000 units/ml.

Using the same test system, we evaluated the ability of acute stress to inhibit interferon activation of macrophages in vivo. Mice were physically restrained for 20 hr either one day prior to or the same day as interferon treatment (1 × 10⁴ units i.p.), and peritoneal macrophages were harvested from all groups 20 hr after interferon treatment. Macrophages from unstressed mice treated with interferon inhibited MBL-2 tumor cell proliferation by 77%. Stress applied either one day prior to or the

Table 1

<table>
<thead>
<tr>
<th>Time of observation (day)</th>
<th>None</th>
<th>Day −6</th>
<th>Day −4</th>
<th>Day −2</th>
<th>Day −1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T/T</td>
<td>R</td>
<td>T/T</td>
<td>R</td>
<td>T/T</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>100</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>90</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>100</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>60</td>
<td>40</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>33</td>
<td>40</td>
<td>60</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

% D/T: percentage of mice dying with tumor/

\* All mice were inoculated s.c. with 1 × 10⁴ MBL-2 ascites cells on Day 0. There were 10 mice/group. BCNU was injected i.p. on indicated days prior to MBL-2 ascites cell inoculation.

\* T/T, percentage of mice with tumor/total number of mice; R, percentage of regression of tumors; % D/T, percentage of mice dying with tumor.
same day as interferon treatment resulted in a decrease of macrophage-mediated cytostasis. Stress applied one day prior to interferon treatment resulted in a 75% reduction of interferon activity; when stress was applied on the same day as interferon treatment, a 54% reduction of interferon activity occurred (Table 3). Stress in the absence of interferon treatment did not induce any significant effect on the cytotoxic activity of resting macrophages.

**Role of Corticosteroids on Macrophage Cytotoxic Function.** Hydrocortisone has been reported to block nonspecific, macrophage-mediated cytotoxicity. Using the macrophage assay, we tested whether corticosteroids would produce an abrogation of macrophage function in vivo. Peritoneal macrophages harvested 24 hr after interferon treatment (1 x 10^4 units/animal) inhibited MBL-2 proliferation by 94% (Chart 1). The corticosteroids hydrocortisone, prednisone, and dexamethasone when administered i.p. at 1 to 100 mg/kg simultaneously with interferon suppressed macrophage tumor killing at increasing doses tested. Dexamethasone almost completely nullified the ability of interferon-treated macrophages to inhibit MBL-2 cell growth at the 10 and 100 mg/kg doses.

**Role of Prostaglandins on Macrophage Tumoricidal Function.** When PGE_1 or PGE_2 was added to macrophage cultures simultaneously with interferon, the interferon induction of tumoricidal macrophages was markedly reduced (Table 4), although macrophages were observed to be activated as defined by morphological criteria and remained fully viable. Both PGE_1 and PGE_2 showed similar kinetics of inhibiting macrophage function. PGE_1 and PGE_2 at the 10^{-5} M concentrations caused 97 and 94% inhibition of tumoricidal activity, respectively, compared to the interferon control value (62% tumoricidal activity). In contrast, PGE_2 was ineffective at all concentrations tested. Indomethacin, the prostaglandin synthetase inhibitor, was without effect on resting macrophages.

**Discussion**

The results reported here suggest that the allograft response to MBL-2 leukemia may be useful as a model system for testing agents, particularly chronic alcohol intake conditions, to evaluate whether these agents or conditions interfere with the cellular immune response.

Our results show that within a 20-hr stress period macrophages from stressed mice are markedly less responsive to stimulation by adjuvants capable of invoking a strong macrophage tumoricidal activity. This was demonstrated to occur whether the immunostimulator was added to macrophages from stressed mice in vitro or injected into the stressed mice. The correlation between stress and decreased macrophage responsiveness is of particular interest in that several investigators have implied that stress-induced immune suppression

---

**Table 2**

<table>
<thead>
<tr>
<th>Macrophage source</th>
<th>Macrophage-mediated cytostasis following exposure to Interferon (1000 units/ml)</th>
<th>Interferon (100 units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal CD2F</td>
<td>89 ± 5^a, b</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>Stressed CD2F, c</td>
<td>17 ± 6</td>
<td>95 ± 2</td>
</tr>
</tbody>
</table>

a MBL-2 leukemia growth inhibition by treated macrophages was determined after 48 hr of incubation from triplicate cultures.

b Macrophages were harvested immediately from mice which had been physically restrained for 20 hr.

c Macrophages were harvested from stressed mice 20 hr after interferon treatment and tested for cytotoxicity against MBL-2 leukemia cells.

**Table 3**

<table>
<thead>
<tr>
<th>Interferon treatment (10^4 units/mouse i.p.)</th>
<th>Stress</th>
<th>Macrophage-mediated cytoactivity (MBL-2 target cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Stress</td>
<td>Macrophage-mediated cytoactivity (MBL-2 target cells)</td>
</tr>
<tr>
<td>Day 0</td>
<td>Day 0</td>
<td>77 ± 2^c, d</td>
</tr>
<tr>
<td>Day 0</td>
<td>Day 1</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Day 0</td>
<td>Day 2</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>Day 0</td>
<td>Day 1</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>Day 0</td>
<td>Day 2</td>
<td>2 ± 5</td>
</tr>
<tr>
<td>Day 0</td>
<td>Day 1</td>
<td>1 ± 3</td>
</tr>
</tbody>
</table>

^ Mice were stressed by physical restraint for 20 hr.

^ Peritoneal macrophages were harvested 20 hr after interferon treatment and tested for cytotoxicity against MBL-2 leukemia cells.

^ MBL-2 growth inhibition by treated macrophages was determined after 48 hr of incubation from triplicate cultures.

^ Mean ± S.E.
may be an etiologic factor in the appearance of several immunological diseases (2, 24–26).

Glucocorticoids have been shown to inhibit a number of macrophage functions. Werb et al. (28) reported that monocytes and macrophages contain saturable glucocorticoid-binding proteins with specificity for binding cortisol, corticosterone, and related synthetic steroids such as dexamethasone and that they have dissociation constants for binding within physiological ranges. In the present report, corticosteroids suppressed the cytotoxicity of interferon-treated macrophages and that they have dissociation constants for binding within physiological ranges. In the present report, corticosteroids suppressed the cytotoxicity of interferon-treated macrophages at what can be considered physiological concentrations. Although the mechanism by which they act on inhibiting macrophage tumoricidal activity is unknown, their activity may be mediated by either stabilization of cell membranes or stimulation of production of cyclic AMP (16).

Prostaglandins of the E series have been previously reported to exert a number of inhibitory effects on macrophages besides cytotoxicity, including random locomotion (8), lessened production of plasminogen activator (27), and colony-stimulating factors for macrophage stem cells (14).

The process of macrophage activation appears to be self-limiting, since it has been reported that activated macrophages release high levels of PGE₂ (3). Our present results indicate that PGE₁ and PGE₂, but not PGE₃, abrogate the ability of interferon to activate macrophages to their tumoricidal capacity. Such prostaglandins could act in a negative feedback inhibition to limit cell activities. This is illustrated in Chart 2. The tumoricidal activity of macrophages can be stimulated by several agents both in vitro and in vivo conditions. The tumoricidal activity of the macrophage can be abrogated by inhibitory levels of prostaglandin (PGE₁ or PGE₂).

Preliminary evidence suggests that the ability of PGE₁’s to inhibit macrophage cytotoxic function is related to elevated levels of intracellular cyclic 3’-5’-adenosine monophosphate. In addition, cells transformed by viruses or chemicals in vitro have been shown to produce several-fold more prostaglandins than do their normal counterparts (12). These tumor cell products could subvert local macrophages and allow the tumor to progress in the presence of these potentially tumoricidal cells.

In conclusion, a more comprehensive knowledge of the stimuli and conditions under which the cytotoxic activity of macrophages is maintained in vivo may clarify the role of the activated macrophage in immunity. In the past, much emphasis has been placed on factors that enhance the functional activity of the macrophage system. It appears that there are opposing factors that may subvert the functional activity of the activated macrophage.

The model test systems for cancer research presented in this report could lend themselves to studies in determining whether a corollary exists between alcohol and cancer, particularly with reference to the role of the macrophage in defense mechanisms. In a recent report, peripheral blood monocytes from patients with liver cirrhosis, several of alcoholic origin, were found to be impaired in their ability to phagocytose or kill 2 strains of Candida (11). These findings led the authors to conclude that the function of the macrophage-macrophage system is impaired in patients with alcoholic postnecrotic and cryptogenic cirrhosis.

References
M. A. Chirigos and R. M. Schultz


Animal Models in Cancer Research Which Could be Useful in Studies of the Effect of Alcohol on Cellular Immunity

Michael A. Chirigos and Richard M. Schultz


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/39/7_Part_2/2894

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.