IMMUNE DYSFUNCTIONS AND ABROGATION OF THE INFLAMMATORY RESPONSE BY ENVIRONMENTAL CHEMICALS

Richard G. Olsen
Department of Veterinary Pathobiology

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During the past year, the immunotoxic effects of three chemicals were studied: hydrazine (Hz), benzene-α-pyrene (BaP), and 1,1-dimethylhydrazine (UDHR). 1) Hydrazine: mice treated with 5-20 mg/kg Hz showed a suppressed antibody-forming cell response after immunization compared to control mice. This immunosuppression correlated with the Hz-induced in vitro suppression of the lymphocyte blast transformation (LBT) response noted previously.
2) Benzo-α-pyrene: 3(α)P, when added to LBT assays, caused a concentration related suppression of the LBT response which was not due to cytotoxicity.
3) 1,1-dimethylhydrazine: most experiments during the past year involved UDMH. First, mice treated on a short-term basis (9 days) with UDMH were affected similarly to those treated over a 3-month period, i.e., an enhancement of antibody-forming cells and no effect on the LBT response. Secondly, in vitro studies with UDMH in the mixed lymphocyte reaction (MLR) assay showed that UDMH inhibited the MLR of whole splenocyte populations and of non-adherent splenocyte populations. However, when adherent splenocytes were removed, exposed briefly to UDMH, then washed and added back to the non-adherent cells, the MLR of the reconstituted population was enhanced. These experiments indicate that UDMH may be interfering with cellular antigen recognition mechanisms between T-cells (responder) and B-cells (stimulator) in the first two experiments, causing a suppressed MLR, and between suppressor macrophages (adherent cell) and T-cells in the latter experiment, resulting in an enhanced MLR. Thirdly, preliminary studies of the effects of in vitro UDMH on macrophage function showed a slight enhancement of phagocytic activity and a significant enhancement of microbicidal activity of UDMH-exposed macrophages.
Abstract

During the past year, the immunotoxic effects of three chemicals were studied: hydrazine (Hz), benzo-a-pyrene (B(o)P), and 1,1-dimethylhydrazine (UDMH). 1) Hydrazine: mice treated with 5-20 mg/kg Hz showed a suppressed antibody-forming cell response after immunization compared to control mice. This immunosuppression correlated with the Hz-induced in vitro suppression of the lymphocyte blast transformation (LBT) response noted previously. 2) Benzo-a-pyrene: B(o)P, when added to LBT assays, caused a concentration related suppression of the LBT response which was not due to cytotoxicity. 3) 1,1-dimethylhydrazine: most experiments during the past year involved UDMH. First, mice treated on a short-term basis (9 days) with UDMH were affected similarly to those treated over a 3-month period, i.e., an enhancement of antibody-forming cells and no effect on the LBT response. Secondly, in vitro studies with UDMH in the mixed lymphocyte reaction (MLR) assay showed that UDMH inhibited the MLR of whole splenocyte populations and of non-adherent splenocyte populations. However, when adherent splenocytes were removed, exposed briefly to UDMH, then washed and added back to the non-adherent cells, the MLR of the reconstituted population was enhanced. These experiments indicate that UDMH may be interfering with cellular antigen recognition mechanisms between T-cells (responder) and B-cells (stimulator) in the first two experiments, causing a suppressed MLR, and between suppressor macrophages (adherent cell) and T-cells in the latter experiment, resulting in an enhanced MLR. Thirdly, preliminary studies of the effects of in vitro UDMH on macrophage function showed a slight enhancement of phagocytic activity and a significant enhancement of microbicidal activity of UDMH-exposed macrophages.
I) Research Objectives

The major objective of this research initially was to develop rapid, economical immunoassays which would allow accurate evaluation of the potential immunotoxic effects of chemical compounds. It has become apparent from work in this laboratory and others that, because of the complex nature of the immune system, a single assay or battery of assays cannot be universally applied to all chemicals and that simply looking for an effect of a chemical on the immune system is not adequate. Consequently, the focus of our research has shifted slightly to include studying the mechanism and site of action of specific immunotoxicants. Unsymmetrical dimethylhydrazine (UDMH) has been the main compound studied during the past year. Specific objectives have included:

A. Evaluation of cell-mediated immune function
   1. Lymphocyte blast transformation assay (chemically treated animals as well as lymphocytes exposed in vitro to chemical)
   2. Mixed lymphocyte reaction (MLR) (treated mice as well as lymphocytes treated in vitro)
   3. Identification of T-cell subsets (helper and suppressor T-cells) in treated animals and lymphocytes exposed to chemical in vitro
   4. Evaluation of concanavalin A-induced suppressor cell activity

B. Evaluation of humoral immune system

C. Evaluation of macrophage function
   1. Phagocytosis and microbicidal properties (in vitro chemical treatment of murine peritoneal macrophages)
   2. Chemotactic response
   3. Mitogen presentation to lymphocytes
D. Determination of "site of action" of chemical in immune response (preliminary studies)

1. Effects of chemical on adherent (mostly macrophages) and non-adherent (T-cell and B-cell) cells in LBT and MLR reactions.

E. Evaluation of UDMH treatment on development of systemic lupus erythematosus in mice genetically susceptible to autoimmune disease.

II) Status of Research

A. Animals used: All animals used during the past year were young adult mice.

1. CD-1: A variety of Swiss outbred mice (Charles River Laboratories, Wilmington, Mass.) was used for most in vivo and in vitro studies involving the LBT assay, Jerne plaque assay, and macrophage studies.

2. C57Bl/6 and Balb C were used in MLR assays. The C57Bl/6 cells were used as responders and Balb C were used as stimulators.

3. MRL-lpr/lpr and MRL +/- (control) (Jackson Laboratories, Bar Harbor, Maine): These mice (lpr/lpr) are genetically destined to develop autoimmune disease (systemic lupus erythematosus) and lymphoproliferation. They were used to determine the effect of UDMH administration on the development of the autoimmune disease.

B. Chemicals Tested

1. As mentioned, most immunotoxicity experiments were done using UDMH, which was obtained in liquid form from Aldridge Chemical Co., Inc. (Milwaukee, WI). For in vivo experiments, it was diluted in phosphate buffered saline (PBS); for in vitro experiments, it was diluted in culture media.

2. Hydrazine obtained from Aldridge Chemical Co., Inc. was used for an in vivo experiment. It was diluted in PBS before injection.
3. Benzo-α-pyrene (Eastman Kodak Co., Rochester, N.Y.) was dissolved in acetone, then diluted with culture media for in vitro experiments.

C. Results of immunoassays

1. Effects of short-term UDMH treatment of mice on Jerne plaque response: The methodology of the Jerne plaque assay is detailed in preprint no. 1, so will not be repeated here. Since the last reporting period, one experiment was done in which mice were exposed to 0, 10, 25, 50, or 100 mg/kg UDMH daily for nine days. There was no consistent effect on the direct (IgM secreting) Jerne plaque response, but mice in the two higher dose groups showed a slightly increased indirect (IgG-secreting) response (Table 1, first preprint). These results supported our earlier findings that long-term UDMH treatment causes an enhanced direct Jerne plaque response, possibly due to specific abrogation of suppressor activity. It also indicates that regulation of T-cell function (IgG response is T-cell dependent) as well as B-cell function (IgM response) is affected by UDMH.

2. Effects of short-term hydrazine (Hz) exposure on lymphocyte blast transformation (LBT) response and Jerne plaque response of mice: Five mice per dose group were injected intraperitoneally with 0, 1, 5, 20, or 40 mg/kg Hz daily for 9 days. Four days before sacrifice (on the fifth day of Hz treatment) mice were immunized intravenously with sheep red blood cells (0.2% of a 10% suspension). Details of the methodology of each of these techniques are included in preprint no. 1. The Hz treatment had no significant effects on the LBT response, though a slight decrease in response to both concanavalin A (conA) and lipopolysaccharide (LPS) was noted (Table 1).
The Hz treatment induced a significant suppressive effect on the direct Jerne plaque response except for the 5 mg/kg dose (Table 2).

We concluded from these results that Hz does not cause any enhancement of immune function, as does UDMH, but causes suppression of the Jerne plaque response; and it is much more toxic than UDMH. These in vivo results correlate with earlier in vitro experiments in which Hz caused a dose related suppression of the LBT response to both con A and LPS, at concentrations of 5 to 30 \( \mu \text{g/ml} \).

3. Effects of benzo-\( \alpha \)-pyrene on in vitro LBT response to mitogens:

Spleen cells were harvested from normal mice and processed as described for the LBT assay. Benzo-\( \alpha \)-pyrene was dissolved in acetone, then added to the LBT cultures in concentrations ranging from 5-250 \( \mu \text{g/ml} \). The LBT response to con A was decreased at 50-250 \( \mu \text{g/ml} \), and the response to LPS was decreased at 25-250 \( \mu \text{g/ml} \) (Table 3). Viability of cells was similar to control (untreated) cells up to 200 \( \mu \text{g/ml} \), so the depressed LBT response was due to factors other than cytotoxicity.

4. Effects of UDMH exposure on development of autoimmune disease.

A strain of mice (MRL-1pr/lpr) has recently been developed by Jackson Laboratories (Bar Harbor, Maine) which develops systemic lupus erythematosus (SLE) and lymphoid proliferation at 4-7 mo. of age. A pilot experiment was done to determine if injections of UDMH altered the time of onset of signs (Table 4) or life span of these mice.

Two groups of ten mice each (5 male, 5 female) were injected 3 times weekly with either 25 mg/kg UDMH or phosphate buffered saline (PBS) (diluent control group). This dose of UDMH was chosen because in the previous long-term UDMH exposure experiment it was the dose which had the greatest effect on Jerne plaque response and con A-induced suppressor cell function.
I assay. The results to date indicate that this dose of UDMH did not have any effect on time of onset of initial lesions (lymphadenopathy) (Table 4) or life span (data not shown). A second experiment using these mice is now underway in which antinuclear antibody levels (which correlate with the development of autoimmune disease) are being monitored in mice being injected 3 times weekly with 50 mg/kg UDMH (a control group is being treated with PBS).

3. Effects of UDMH on mixed lymphocyte reaction (MLR)

Methods for the MLR, adherent cell removal, and reconstitution are detailed in preprint no. 2 so will not be repeated here. The results can be briefly recounted as follows:

a. UDMH causes both an in vitro and an in vivo dose related suppression of the MLR. *In vitro* concentrations of 10 to 50 ug/ml of UDMH significantly suppressed the MLR (see Table 1 of preprint no. 2) and daily doses of 10 to 50 mg/kg given to the responder mice suppressed their MLR (see Table 5 of preprint no. 2).

b. Removal of adherent cells from the splenocytes resulted in an increased MLR of the remaining nonadherent cells which agrees with previously published reports. This enhancement occurred regardless of the presence or dose of UDMH (see Table 4, preprint no. 2).

c. The presence of UDMH induced a dose-related suppression of the MLR of the nonadherent cell population, similar to its effects on the MLR of the intact splenocyte population (Table 2, preprint no. 2).

d. Upon reconstitution, i.e., adding back the adherent cells, the MLR returned to a similar value as before removal of the adherent cells (see Table 4, preprint no. 2 under "control"). However, if the adherent cells were incubated with UDMH for 2 hours and then washed prior to
reconstitution, this suppressive effect was abolished and the MLR was actually enhanced compared to the untreated adherent cells at 50 ug/ml (Table 3).

These experiments indicate that UDMH has two different effects in the MLR: a suppressive effect on the cells responsible for recognition of and blastogenic response to major histocompatibility (MHC) antigens (T-cells); and a suppressive effect on the regulatory cell activity found in the adherent cell population resulting in an enhanced MLR after reconstitution (probably macrophages). The first effect on recognition and response apparently overrides the second effect.

6. Effects of in vitro UDMH on macrophage function
   
a. Preliminary experiments have been done to evaluate the effects of UDMH on phagocytosis and microbicidal activity of peritoneal exudate cells (PEC). The PEC (1 x 10^7/ml) were added to live yeast cells (cell to yeast ratio 1:5) and incubated for 2 hours at 37°C in the presence of methylene blue dye. Varying concentrations of UDMH were added to the test cultures. The cell mixture was then pipetted onto a hemocytometer chamber and the percent of PEC containing at least two live (unstained) yeast organisms (percent phagocytosis) as well as the percent of PEC containing at least one killed (dyed blue) yeast organism (% killing) were determined. The results, seen in Table 5, indicate that UDMH slightly enhances phagocytosis and significantly enhances killing.

b. Other experiments either in progress or planned will evaluate the effects of UDMH on response of PEC to chemotactic factors, and on the ability of macrophages (PEC or adherent cells) to present mitogens and antigens to T-cells.
III. Written Publications (see attached preprints)


IV. Professional Personnel Associated with Research Effort

Richard G. Olsen, Ph.D.
Departments of Veterinary Pathobiology, Microbiology (College of Biological Sciences), and Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210

Melinda J. Tarr, D.V.M., Ph.D.
Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210

Debra L. Jacobs, B.S.
Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210

V. Oral Presentations

VI. Significance of Results to Date and Future Direction

Our main focus of interest during the past year has remained on the effects of UDMH on cellular immune function. At first glance, it appears that UDMH has variable immunotoxic effects depending on which assay is used. For instance, it enhances B-cell response to mitogens and enhances antibody forming cells in treated mice, but it suppresses T-cell response to mitogens and suppresses mixed lymphocyte reactions (MLR). However, if one interprets these results in light of some of the recent knowledge gained in the field of immune responder genes and immune recognition, it is quite plausible that UDMH could be affecting certain cell membrane antigens (termed "Ia" antigens) which are necessary for recognition and cooperation among subsets of lymphocytes and macrophages. Different immunoassays evaluate the function of different immunocyte subsets and a pool of immunocytes such as spleen cells contains varying proportions of functional subsets depending on the immune status of the donor animal. So if UDMH was affecting Ia recognition antigens, it could be affecting suppressor cell function as well as helper or effector cell function, and these effects can be manifest as either suppression or augmentation of the immune response, depending on the assay used and the status of the animal. We are hoping that further study with UDMH will help to elucidate the basic mechanism by which immunocytes interact and cooperate with each other. Once these mechanisms are determined at the molecular level, it is possible that the effects of most chemicals on immune function can be predicted, based on their biochemical activity and metabolism.
Table 1. Effects of Short-Term Hz Exposure on LBT Response to Mitogens

<table>
<thead>
<tr>
<th>Dose Hz</th>
<th>Response to ConA (CPM ± SEM)</th>
<th>Response to LPS (CPM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (PBS control)</td>
<td>90190 ± 7840</td>
<td>81594 ± 9336</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>78734 ± 11413</td>
<td>72113 ± 8832</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>85020 ± 12439</td>
<td>71662 ± 8052</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>79163 ± 11157</td>
<td>70494 ± 7095</td>
</tr>
<tr>
<td>40 mg/kg</td>
<td>ND\textsuperscript{a}</td>
<td>ND</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Daily treatment with 40 mg/kg Hz was lethal after 3 to 4 days.
Table 2. Effects of Short Term Hz Exposure on Direct Jerne Plaque Response

<table>
<thead>
<tr>
<th>Dose Hz</th>
<th>No. PFC$^a$/10$^6$ Spleen Cells (+ SEM)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (PBS control)</td>
<td>1440 ± 395</td>
<td>-</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>1030 ± 285</td>
<td>&lt;.025</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>1670 ± 430</td>
<td>NS</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>640 ± 280</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

$^a$PFC = plaque forming cells
Table 3. *In vitro* effects of benzo-α-pyrene on LBT response to mitogens

<table>
<thead>
<tr>
<th>[B(a)P]</th>
<th>Response to con A</th>
<th>Response to LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of Control a</td>
<td>p b</td>
</tr>
<tr>
<td>5 μg/ml</td>
<td>107.4 ± 9.5</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>102.8 ± 13.0</td>
<td>NS</td>
</tr>
<tr>
<td>25</td>
<td>96.4 ± 9.0</td>
<td>NS</td>
</tr>
<tr>
<td>50</td>
<td>91.6 ± 8.9</td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>75</td>
<td>78.8 ± 11.2</td>
<td>p &lt; .001</td>
</tr>
<tr>
<td>100</td>
<td>79.0 ± 12.3</td>
<td>p &lt; .005</td>
</tr>
<tr>
<td>200</td>
<td>34.8 ± 3.1</td>
<td>p &lt; .001</td>
</tr>
<tr>
<td>250</td>
<td>39.0 ± 8.3</td>
<td>p &lt; .005</td>
</tr>
</tbody>
</table>

a Expressed as percent of control (untreated cell) response.
b p value determined by paired "t" test.
Table 4. Effects of 25 mg/kg UDMH on Age of Onset of Lymphadenopathy in MRL-1pr/lpr mice

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Age of onset (days)</th>
<th>Animal No.</th>
<th>Age of onset (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>females</td>
<td></td>
<td>females</td>
</tr>
<tr>
<td>1</td>
<td>81</td>
<td>1</td>
<td>97</td>
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<td>3</td>
<td>99</td>
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<td>5</td>
<td>102</td>
<td>5</td>
<td>97</td>
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<td>mean</td>
<td>93.6</td>
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<td>99.0</td>
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<tr>
<td></td>
<td>males</td>
<td></td>
<td>males</td>
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<tr>
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<td>135</td>
</tr>
<tr>
<td>5</td>
<td>151</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>127.8</td>
<td>mean</td>
<td>123.3</td>
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</table>
Table 5. Effects of *In Vitro* UDMH on PEC Phagocytosis and Killing

<table>
<thead>
<tr>
<th>Concentration UDMH (µg/ml)</th>
<th>% Phagocytosis&lt;sup&gt;a&lt;/sup&gt; (+ SEM)</th>
<th>% Killing&lt;sup&gt;b&lt;/sup&gt; (+ SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>37.1 ± 5.1</td>
<td>6.4 ± 1.0</td>
</tr>
<tr>
<td>25</td>
<td>43.3 ± 4.0</td>
<td>9.9 ± 1.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>41.4 ± 3.7</td>
<td>9.8 ± 2.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>100</td>
<td>41.8 ± 3.8</td>
<td>8.4 ± 1.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent of PEC containing ≥ 2 yeast organisms.<br>
<sup>b</sup>Percent of PEC containing ≥ 1 killed yeast organism.<br>
<sup>c</sup><sup>p</sup>< .005 (student's "t" test)<br>
<sup>d</sup><sup>p</sup>< .025<br>
<sup>e</sup><sup>p</sup>< .05
In Vivo and In Vitro Effects of 1,1-Dimethylhydrazine on Selected Immune Functions

1,1 Dimethylhydrazine Immune Function

Melinda J. Tarr¹
Richard G. Olsen¹,²,³
Debra L. Jacobs¹

¹ Department of Veterinary Pathobiology, The Ohio State University
² Department of Microbiology, College of Biological Science, and
³ Comprehensive Cancer Center of The Ohio State University


Corresponding author:
Melinda J. Tarr
The Ohio State University
Department of Veterinary Pathobiology
1925 Coffey Road
Columbus, Ohio 43210
614-422-5661
Abbreviations Used:

UDMH: 1,1-dimethylhydrazine
PBS: phosphate buffered saline
SRBC: sheep red blood cells
i.p.: intraperitoneally
i.v.: intravenously
HBSS: Hank's balanced salt solution
CCM: cell culture medium
LBT: lymphocyte blast transformation
ConA: concanavalin A
LPS: lipopolysaccharide
cpm: counts per minute
C: complement
sem: standard error of the mean
NS: not significant
ND: not done
lgG: immunoglobulin G
lgM: immunoglobulin M
Abstract

The in vivo phase of the experiments reported here include the evaluation of immune function after short- or long-term treatment of mice with 1,1-dimethylhydrazine (UDMH). Long-term exposure (3 injections/week for 14 weeks) resulted in increased numbers of Jerne plaque forming cells, a trend toward decreased induction of suppressor cell activity by concanavalin A (conA) and no effects on mitogen-induced lymphocyte blast transformation (LBT), compared to saline-treated control mice. These effects were greatest at doses of 10 or 50 mg/kg, while higher doses had less of an effect. In vitro experiments were performed by adding UDMH to normal murine splenocytes in the LBT assay and conA-induced suppressor cell assay. The UDMH induced a significant enhanced response to lipopolysaccharide (LPS) at 10 and 50 μg/ml, and a suppressed response to both conA and LPS at higher concentrations. The UDMH also caused a decrease in suppressor cell activity at 25 μg/ml.

Selective abrogation of suppressor activity or alteration of the suppressor cell-helper cell ratio were suggested as possible mechanisms for the enhancement effect associated with UDMH.

Key words: 1,1-Dimethylhydrazine; Lymphocyte blast transformation; Jerne plaque cells; ConA induced suppressor cells.
Introduction

Numerous environmental pollutants, drugs, or industrial chemicals are now known to suppress or modulate immune functions. Many compounds exert an immunosuppressive effect, often at doses lower than those causing overt toxicity or death. These compounds include polychlorinated biphenyls (Thomas and Hindsdill, 1978; Friend and Trainer, 1970), tetrachlorobenzo-p-dioxin (Faith and Moore, 1977), heavy metals such as lead, cadmium, and mercury (Koller, 1973), and methylNitrosourea (Tarr et al., 1979). Other compounds have been associated with an enhancement of the immune response, or an enhancement at one dose, and suppression at higher or more prolonged doses. For instance, mice exposed to NO or NO2 showed an enhanced antibody response to a T-dependent antigen after 10 weeks of exposure, but a depressed response after 30 weeks (Holt et al., 1979). The same biphasic pattern was seen with graft versus host reactions. Exposure of mice to vinyl chloride also resulted in immunostimulation as measured by enhanced spontaneous lymphocyte blastogenesis and enhanced blastogenic response to phytohemagglutinin and pokeweed stimulation (Sharma and Gehring, 1979). Long-term high dose exposures resulted in less enhancement than short-term high dose exposures. These results correlate with the supposition that the vinyl chloride syndrome experienced by exposed industrial workers may involve an autoimmune phenomenon (Ward et al., 1976).

Numerous other drugs have been associated with autoimmune disease, and some of these induce suppressor-cell malfunctions, such as methyldopa (Kirtland et al., 1980). Exposures to compounds containing the hydrazine moiety (-N-NH2) such as hydralazine (Perry, Jr., 1973) or hydrazine sulfate (Durant and Harris, 1980), have induced systemic lupus erythematosus-like signs. The purpose of the experiments reported here was to examine the in vitro and in vivo effects of a simple hydrazine derivative, 1,1-dimethylhydrazine (UDMH), on immune response and suppressor cell induction in mice.
Materials and Methods

Animals: Male and female Swiss outbred mice weighing 20-35 gm were used for all in vivo or in vitro experiments.

Chemical preparation: The UDMH was obtained in liquid form (Aldridge Chemical Corp.) and further distilled (Dr. Kenneth Back and associates, Wright Patterson Air Force Base, Dayton, OH). It was diluted in phosphate buffered saline (PBS) for in vivo (mouse exposure) experiments, or cell culture media (see below) for in vitro (lymphocyte exposure) experiments.

Animal treatment - in vivo experiments: Two treatment regimes were used in two different experiments. In the short-term exposure experiments, 6 groups of 10 mice each (5 female, 5 male) were injected intraperitoneally (i.p.) daily with PBS (diluent control), 10, 25, 50, 100, or 150 mg/kg UDMH. On the fifth day of injections, they were immunized i.p. with 0.2 ml of a 10% suspension of sheep erythrocytes (SRBC). Five days later, the mice were sacrificed. For the long-term exposure experiment, 5 groups of mice (10 mice per group, 5 male, 5 female) were injected i.p. 3 days per week for 14 weeks with PBS (diluent control), 25, 50, 100, or 150 mg/kg UDMH. Four days before sacrifice, the mice were immunized intravenously (i.v.) with 0.2 ml of a 10% suspension of SRBC.

Spleen cells: Mice were sacrificed by cervical dislocation, and the spleens aseptically removed and placed in L-15 media (Grand Island Biological Co., Grand Island, NY) supplemented with 1% EDTA and 1% antibiotic solution. The spleens were separately teased, strained through #60 wire mesh, and aspirated several times through 18, 25, and 27 g needles. The red cells were lysed with 0.15 M ammonium chloride lysing solution, and the white cells were then washed and suspended in cell culture medium (CCM) consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), supplemented with 2 x 10^{-5} M 2-mercaptoethanol, 10% fetal bovine serum (Sterile Systems, Inc., Logan, UT), and 1% antibiotic solution. The splenocytes were counted and
diluted to $10^6$ lymphocytes/ml. Viability was 97-100%. Percent of lymphocytes ranged from 85-99% (other cells being macrophages or neutrophils).

Lymphocyte blast transformation (LBT) assay: Microtiter plates (CoStar, Cambridge, MA) were filled with 0.1 ml cell suspension ($10^5$ lymphocytes) per well. Concanavalin A (ConA) (Sigma Chemical Co., St. Louis, MO) and lipopolysaccharide (LPS; derived from E. coli 0127:B8; Difco Laboratories, Detroit, MI) were added to murine splenocytes in 10 μl aliquots (0.2 μg/well of ConA and 10 μg/well of LPS). Quadruplicate wells were set up for each mitogen and CCM control. Cultures were incubated at 37°C in a 5% CO₂ humidified incubator for 54 hrs, then pulsed with 0.5 μCi/well of $^3$H-TdR (New England Nuclear, Boston, MA) and harvested 18 hrs later using a multiple automated sample harvester (Otto Hiller Co., Madison, WI). The glass fiber filter disks containing the harvested cells were dried and placed in vials with scintillation cocktail. Counts per minute (cpm) were obtained using a liquid scintillation counter (Packard Tricarb Model 3375, Packard Instrument Co., Downers Grove, IL). "Corrected cpm" were determined by (cpm of mitogen-stimulated cultures) - (background cpm [cultures with CCM]).

UDMH treatment of spleen cells - in vitro LBT experiments: Spleen cells were harvested as described from 24 normal mice. UDMH was added to LBT cultures containing ConA, LPS, or CCM in concentrations of 0 (control), 5, 10, 25, 50, 75, 100, or 150 μg/ml. UDMH was present throughout the incubation period. The LBT assay was carried out as described above.

Jerne plaque assay: The agar-free slide modification described by Cunningham (1965) was used in all assays. Briefly, SRBC were washed and diluted to a 15% suspension in PBS. Spleen cells were harvested as described from SRBC immunized mice (treated with UDMH or PBS), and suspended in RPMI 1640 with 0.5% gelatin (Sigma Chemical Co., St. Louis, MO) at 3 x $10^6$/ml. Guinea pig complement (C) (Grand Island Biological Co., Grand Island, NY) was reconstituted and absorbed with SRBC, if necessary. Rabbit anti-mouse IgG (Miles Laboratories, Elkhart, IN)
was reconstituted with sterile water, absorbed with SRBC, and frozen in 100 μl aliquots. For direct plaque assays (IgM secreting cells), 30 μl of a mixture of 0.3 ml spleen cells, 0.04 ml SRBC suspension, 0.04 ml media, and 0.02 ml C was placed in Cunningham chambers. For indirect plaque assays, 0.04 ml media was replaced with 0.04 ml anti-mouse IgG (1:40 dilution of stock solution). Each test mixture was run in quadruplicate. Controls consisted of cells, SRBC, and media; cells, SRBC and anti-mouse IgG; SRBC and C; and SRBC, anti-mouse Ig and C. Slides were sealed with petroleum jelly, incubated at 37°C for 1 hour, then refrigerated until reading. Plaques were counted on a dissecting microscope using indirect light. Data were expressed as numbers of plaques per 10^6 spleen cells.

ConA-induced suppressor cell assay: Spleen cells were harvested as described. Cells from UDMH-treated mice were incubated with conA (1 μg/ml) or CCM for 48 hours, then washed, incubated with mitomycin C (30 μg/10^7 cells) (Sigma Chemical Co., St. Louis, MO) for 30 min. at 37°C and washed three times in Hank’s balanced salt solution (Grand Island Biological Co., Grand Island, NY) with 0.3 M α-methyl-d-mannoside. The number and percent of live cells was determined, and the cells were suspended in CCM at 3 x 10^6 live cells/ml. One tenth ml of this conA-induced suppressor cell population or the control cell population (incubated in CCM) was added to 0.1 ml of fresh spleen cells (3 x 10^6/ml) collected from a normal (untreated) donor mouse as previously described. ConA, LPS, or CCM was then added to the cell cultures (quadruplicate wells for each mitogen and cell combination), and they were incubated and harvested as described for the LBT assay. The percent suppression was determined by:

\[
\text{Percent Suppression} = \frac{100 - (\text{cpm of cultures with suppressor (conA-induced) cells})}{\text{(cpm of cultures with control (media-incubated) cells})} \times 100
\]

For the in vitro UDMH experiments, suppressor cells from normal mice were induced by conA as described, but in the presence of 25 μg/ml UDMH or CCM. The rest of the
procedure was carried out as described. The suppressor cell activity generated in the presence of UDMH was compared to that generated by conA alone.

Cell viability: Cell viability was determined by the trypan blue dye exclusion method. Percent viability was determined by:

\[
100 \times \frac{\text{no. live cells}}{\text{no. total cells}}
\]

Statistics: For all in vivo experiments, values from UDMH-treated groups were compared to those of the control (PBS-treated) group, and evaluated for significant differences by the student T test. For the in vitro tests, values from UDMH-treated cells were expressed as percent of the control (untreated cell) values and significant differences were evaluated using the paired T test.

Results:

In vivo experiments - UDMH-treated mice:

Jerne plaque formation: Direct and indirect plaque responses were determined 5 days after i.p. immunization in the short-term exposure experiment. The 50 and 100 mg/kg dose groups formed more indirect plaques than the control group (1557 ± 516 and 1762 ± 648, respectively, compared to 1011 ± 433 for the control group), although these increases were not statistically significant (table 1). No significant or consistent change was seen in the direct plaque response. In the long-term exposure experiment, direct (IgM) plaques were measured 4 days after i.v. SRBC immunization. Mice in the 10 and 50 mg/kg dose groups showed a significantly higher response than controls (p < .001) (table 1). Responses of the higher dose groups (100, 150 mg/kg) were the same as those of the control group.

ConA-induced suppressor cells: Both short-term and long-term UDMH exposure caused a decreased induction of suppressor cell activity as seen in table 2, although the decreases were not statistically significant. The 50, 100, and 150 mg/kg dose groups were most affected in both experiments, manifest by less suppression of the fresh cell response to conA, and actual enhancement of the response to LPS.
LBT assay: None of the UDMH-treated groups showed either suppressed or elevated LBT response to mitogens compared to the controls. All groups showed good responses to both conA and LPS (data not shown).

Toxicity: None of the mice in the short-term study died or showed any signs of toxicity. In the long-term study, 7 out of 10 mice in the highest dose group (150 mg/ml) died by the end of the study; the other three appeared healthy. All mice surviving at the end of the experiment were necropsied and selected tissues were examined histologically, and no significant gross or histologic abnormalities were present.

Effect of in vitro exposure of cells to UDMH:

LBT assay: The LBT response of normal cells to conA was suppressed by UDMH at concentrations of 25 to 150 μg/ml (figure 1). The response to LPS was significantly enhanced at 10 and 25 μg/ml, and depressed at 100 to 150 μg/ml. The suppression of the LPS response was not due to decreased cell viability as a result of UDMH exposure (table 3), however, the viability of the cultures stimulated with conA was somewhat decreased at 50 to 150 μg/ml UDMH.

Effect of UDMH on in vitro conA-induced suppressor cell activity: The UDMH inhibited conA-induced suppressor cell activity in the LBT assay. The suppression of the fresh cell LBT response to conA in 3 separate experiments was decreased by an average of 17.2% (p < .05), and to LPS by 29% (NS), when UDMH (25 μg/ml) was present during suppressor cell induction (Table 4).

Discussion

Both the in vivo and in vitro experiments suggest that UDMH inhibits suppressor cell function. Direct and indirect Jerne plaque formation were increased in UDMH-treated mice, and conA-induced suppressor cell activity was diminished in treated mice. The in vitro studies indicated that UDMH caused an enhanced response to the mitogen LPS, and also abrogated suppressor cell induction in normal spleen cells. In the treated mice as well as in culture, it took relatively low doses or concentrations of UDMH to
exert these effects, while higher amounts were similar to controls. This indicates that
the suppressor cell population is preferentially inhibited at low doses or that the
suppressor-helper ratio is altered in favor of helper cell activity (increased helper
activity or decreased suppressor activity). Higher in vivo doses apparently do not affect
this ratio, nor are they immunosuppressive, however, in vitro exposure of lymphocytes
to higher concentrations of UDMH causes inhibition of the LBT response, and toxicity
at even higher concentrations.

Inhibition of suppressor cell function would correlate with the observed SLE-like
syndrome induced by hydralazine and hydrazine sulfate. It is well established that
natural cases of SLE (Bresnihan and Jasin, 1977) as well as other autoimmune diseases
such as Graves disease (Aoki et al., 1979) or multiple sclerosis (Reinherz et al., 1980)
are associated with a decrease in suppressor cell function. More recently, drug-induced
autoimmune disorders have been associated with decreased suppressor T-cell function
such as that reported for methyldopa-induced autoimmune hemolytic anemia (Kirtland et
al., 1980). In patients taking methyldopa, polyclonal antibody production was increased
compared to normal subjects, and suppressor T-cell activity of cells from these patients
on normal cell polyclonal antibody production was diminished. Cyclophosphamide at
certain dose levels will also abrogate suppressor T-cell function (Schwartz et al., 1978).

Certainly, other mechanisms may explain the enhancement effects of UDMH. Being
a small and unstable molecule, it may alter membrane permeability and ion flux,
enhancing the mitogenic stimulus. It is known to act as a growth promotor for
fibroblasts at low concentrations (Blakeslee, unpublished data) and it caused somewhat
higher background counts in our lymphocyte cultures, although a dose effect was not
seen.

The immunoenhancement effects of UDMH could also be related to the interaction
of the hydrazine moiety with pyrimidine and/or purine bases of DNA. Dubroff and Reid
(1980) have demonstrated several "altered nucleoside products" as a result of incubation
of thymidine or deoxycytidine with hydralazine; and Shank (1980) reported that rats treated with hydrazine showed increased methylation of guanine DNA in the liver. These interactions could result in either acquired immunogenicity of host DNA or, if lymphocyte DNA is affected, abrogation of immune tolerance.

The effects of UDMH, at least partially, may also be genetically determined. Hydralazine-induced SLE occurs nearly exclusively in patients with the "slow acetylator" phenotype (Perry et al., 1970), and the reported case of hydrazine sulfate-induced SLE (Durant and Harris, 1980) was also in a person with the slow acetylator phenotype. It would be of interest to determine the relationship, if any, between acetylase activity and lymphocyte function and interactions.

Regardless of the mechanism, it is apparent that subtoxic doses of hydrazine compounds may enhance the immune response and cause autoimmune disease. This is an important observation, since the hydrazine moiety is prevalent in the environment, found in mycotoxins in common edible mushrooms (Toth et al., 1978), herbicides (maleic hydrazine) (Zurkel, 1957/1963), and missile propellants (such as UDMH) (Beck and Thomas, 1970). It is also present in such commonly used drugs as hydralazine, isoniazid, and hydrazine sulfate. Many hydrazine compounds are known to possess overt toxic or carcinogenic properties, but immunotoxic effects may occur earlier and at lower exposure levels.
References


Table 1. Effects of Short- and Long-Term UDMH Exposure on Jerne Plaque formation.

**Short Term Exposure**

<table>
<thead>
<tr>
<th>Dose UDMH</th>
<th>No. PFC/10^6 Spleen Cells</th>
<th>Direct (± SEM)</th>
<th>Indirect (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-Term Exposure^a</td>
<td>Control (PBS)</td>
<td>296 ± 91</td>
<td>1011 ± 433</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>179 ± 66</td>
<td>865 ± 317</td>
</tr>
<tr>
<td></td>
<td>25 mg/kg</td>
<td>214 ± 60</td>
<td>1126 ± 385</td>
</tr>
<tr>
<td></td>
<td>50 mg/kg</td>
<td>179 ± 43</td>
<td>1537 ± 516</td>
</tr>
<tr>
<td></td>
<td>100 mg/kg</td>
<td>262 ± 74</td>
<td>1762 ± 648</td>
</tr>
</tbody>
</table>

**Long-Term Exposure^b**

<table>
<thead>
<tr>
<th>Dose UDMH</th>
<th>No. PFC/10^6 Spleen Cells</th>
<th>Direct (± SEM)</th>
<th>Indirect (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-Term Exposure^b</td>
<td>Control (PBS)</td>
<td>1110 ± 340</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>1880 ± 430^c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mg/kg</td>
<td>1860 ± 430^c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mg/kg</td>
<td>1380 ± 320</td>
<td></td>
</tr>
<tr>
<td></td>
<td>150 mg/kg</td>
<td>1190 ± 120</td>
<td></td>
</tr>
</tbody>
</table>

a) Mice treated daily for nine days and immunized with SRBC 5 days before sacrifice.

b) Mice treated 3X weekly for 14 weeks and immunized with SRBC 4 days before sacrifice.

c) p < .001
Table 2. Effects of Short-Term and Long-Term UDMH Exposure of Mice on Suppressor Cell Induction by Con A.

<table>
<thead>
<tr>
<th>Dose UDMH</th>
<th>Percent Suppression of Fresh Cell Response To:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con A (+ SEM)</td>
<td>LPS (+ SEM)</td>
</tr>
<tr>
<td>Short-Term</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposure&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Control (PBS)</td>
<td>49.2 ± 12.9</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>59.6 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>25 mg/kg</td>
<td>37.7 ± 20.0</td>
</tr>
<tr>
<td></td>
<td>50 mg/kg</td>
<td>36.5 ± 18.4</td>
</tr>
<tr>
<td></td>
<td>100 mg/kg</td>
<td>30.5 ± 9.7</td>
</tr>
<tr>
<td></td>
<td>150 mg/kg</td>
<td>30.7 ± 24.9</td>
</tr>
<tr>
<td>Long-Term</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposure&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Control (PBS)</td>
<td>48.4 ± 11.2</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>42.1 ± 14.4</td>
</tr>
<tr>
<td></td>
<td>50 mg/kg</td>
<td>24.9 ± 15.7</td>
</tr>
<tr>
<td></td>
<td>100 mg/kg</td>
<td>47.3 ± 13.3</td>
</tr>
<tr>
<td></td>
<td>150 mg/kg&lt;sup&gt;c&lt;/sup&gt;</td>
<td>55.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice treated daily for nine days and immunized with SRBC 5 days before sacrifice.

<sup>b</sup> Mice treated 3X weekly for 14 weeks and immunized with SRBC 4 days before sacrifice.

<sup>c</sup> Only one animal tested.
Table 3. Viability of Splenocytes Incubated with UDMH and Con A or LPS.

<table>
<thead>
<tr>
<th>Concentration UDMH</th>
<th>Con A</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% viability (± SEM)</td>
<td>p</td>
</tr>
<tr>
<td>0 (control)</td>
<td>86.5 ± 2.2</td>
<td>91.5 ± 1.5</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>80.5 ± 4.6</td>
<td>NSb</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>67.0 ± 6.5</td>
<td>p &lt; .025</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>68.3 ± 8.0</td>
<td>p &lt; .05</td>
</tr>
<tr>
<td>150 µg/ml</td>
<td>68.5 ± 3.9</td>
<td>p &lt; .005</td>
</tr>
<tr>
<td></td>
<td>95.5 ± 0.5</td>
<td>p &lt; .025</td>
</tr>
<tr>
<td></td>
<td>92.0 ± 2.5</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>89.0 ± 1.9</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>78.0 ± 3.5</td>
<td>p &lt; .01</td>
</tr>
</tbody>
</table>

a P value determined by Student t test
b NS = not significant
Table 4. Effects of UDMH in Cultures of In Vitro Suppressor Cell Induction by Con A.  

<table>
<thead>
<tr>
<th></th>
<th>Con A</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>Suppressor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>induced in presence</td>
<td>7.2</td>
<td>-5.1</td>
</tr>
<tr>
<td>of UDMH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suppressor cells</td>
<td>19.9</td>
<td>21.4</td>
</tr>
<tr>
<td>induced alone (control)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a p value from paired "t" test
Figure 1. Effects of UDMH on the LBT response of normal splenocytes to ConA and LPS (vertical bars represent standard error of the mean).

● = ConA    ■ = LPS

a $p < .001$;  b $p < .005$;  c $p < .01$ (paired t test).
CONCENTRATION OF UDMH \((\text{CH}_2\text{NNH}_2)\) (µg/ml)
Effects of 1,1-Dimethylhydrazine on Murine
Mixed Lymphocyte Reaction

Debra L. Jacobs¹
Melinda J. Tarr¹
Richard G. Olsen¹,²,³

¹ Department of Veterinary Pathobiology
² Department of Microbiology, College of Biological Science
³ Comprehensive Cancer Center, The Ohio State University

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Scientific Research.

Corresponding author: Debra L. Jacobs, Department of Veterinary Pathobiology,
The Ohio State University, 1925 Coffey Road, Columbus, Ohio 43210. (614)422-5661.
INTRODUCTION

Current research in the field of immunotoxicology has provided information regarding the adverse effects of different chemicals on the immune system. Immunosuppressive effects have been demonstrated using subtoxic and/or subcarcinogenic levels of chemicals such as hexachlorobenzene(1), polychlorinated biphenyls(1,2,3), polybrominated biphenyls(4), tetrachlorobenzo-p-dioxin (TCDD)(5), lead(6), cadmium(6), mercury(6), and methylnitrosourea(7). These compounds may affect either the cellular mediated arm of the immune system, as is the case of TCDD, the humoral arm(5), or both.

Experimental evidence suggests that simple hydrazine (Hz) compounds, which are used as rocket fuels, may inhibit or inactivate immunoregulatory function. Lymphoblast transformation assays (LBT) using murine splenocytes treated in vitro with the Hz compounds result in a dose related suppression of concanavalin A (Con A) stimulation, a T cell mitogenic response. However, low dosages of unsymmetrical dimethylhydrazine (UDMH) and symmetrical dimethylhydrazine (SDMH) enhance the LBT response to lipopolysaccharide (LPS) a B cell mitogen, while the higher doses of these compounds suppress this response. Jerne plaque assays performed with splenocytes from mice treated in vivo with Hz compounds result in an increased number of direct plaque forming cells (PFC) over that of untreated controls(8). The enhancement of LBT responses and of PFC could be related to a dysfunction of regulatory activity brought about by the Hz compounds. Clinical evidence obtained from patients treated for hypertension with hydrazine, a Hz analogue, correlates "lupus erythematosus-like syndrome" (an autoimmune disease associated with suppressor cell dysfunction) with this drug therapy.(9)
The concept of a specific cell, i.e. "suppressor cell", responsible for regulation of immune functions has been widely discussed throughout the scientific literature. Macrophages, adherent T cells, and Con A induced T cells have been identified as "suppressor cells" capable of suppressing LBT, Jerne plaque, and mixed lymphocyte reaction (MLR) responses.\textsuperscript{(10,11)} It is currently thought that the adherent cell fraction of the leukocyte cell population is responsible for regulating the MLR.\textsuperscript{(10,11)} Since UDMH possibly induces an alteration of immunoregulatory activity, the effects of UDMH on the MLR and its immunoregulatory system were examined.
Materials and Methods

Animals:

The mice used in the MLR experiments, BalbC and C57Bl/6, aged 6 weeks to 6 months, were obtained from Lab Supply through The Ohio State University Animal Procurement Service, Columbus, Ohio.

Unsymmetrical Dimethylhydrazine (UDMH)

The UDMH was obtained from Aldridge Chemical Corporation in a liquid form and purified by distillation by Dr. Kenneth Back and Associates, Wright Patterson Air Force Base, Dayton, Ohio. For *in vitro* cell culture, the UDMH was diluted with culture media (see below). For *in vivo* studies, the UDMH was diluted with phosphate buffered saline (PBS).

Culture Media

Culture media used throughout these experiments consisted of RPMI 1640 with 25 mM hepes buffer (Grand Island Biological Co., Grand Island, N.Y. (GIBCO) supplemented by $2 \times 10^{-5}$ 2-mercaptoethanol, 2.5% pooled human sera (Type AB O, GIBCO), 1% L-glutamine (GIBCO), and 1% antibiotic solution.

Lymphocyte Isolation:

Mice were sacrificed by cervical dislocation and their spleens aseptically removed. Single cell suspensions were obtained by straining the spleens through a 60 mesh brass screen into L-15 media (GIBCO) supplemented with 1% EDTA and 1% antibiotic solution. They were then passed through a series of 18, 25 and 27 gauge needles. A hemolyzing agent of .15 M ammonium chloride was used to lyse erythrocytes. The cell suspension was then washed with L-15 and
suspended in the culture media. Lymphocytes were counted with a hemocytometer and viabilities determined by use of trypan blue stain.

MLR treated with UDM+ (Control MLR)

Stimulator cells were obtained by incubating splenocytes from Balb C mice with 50 μg/1x10^7 cells of Mitomycin C (Sigma Chemical Company, St. Louis, MO) for 1/2 hour at 37°C and then washing 3 times with Hank’s Balanced Sal Solution (HBSS)(GIBCO). The cells were resuspended in the culture media at a concentration of 1x10^7 cells/ml. Fifty μl of stimulator cells (5x10^5/well) and 50 μl of untreated responder cells from C57Bl/6 mice (cell concentration 5x10^6/ml or 2.5x10^5/well) were plated in quadruplicate in round bottomed microtiter plates. One hundred μl/well of each dose of UDMH was then added (in quadruplicate) to the plate as well as a media control. Other controls for this assay were responder cells with UDMH, stimulator cells with UDMH, and responder cells and stimulator cells from C57Bl/6 mice with UDMH (autologous controls). The test and control cultures were incubated for seven days at 37°C in a 5% CO₂ humidified atmosphere. Eighteen hours prior to termination, the cells were labeled with .5 μCi/well ^3H-TdR (New England Nuclear, Boston, MA.) The tests and controls were harvested with a multiple automated sample harvester (Otto Hiller Company, Madison, WI) and after 1 hour drying time the glass fiber disks containing the harvested cells were placed in vials with scintillation cocktail and counted with a liquid scintillation counter (Packard Tricarb Model 3375, Packard Instruments Company, Downers Grove, IL) to determine ^3H-TdR incorporation by counts per minute (cpm). Results were recorded as corrected cpm which are obtained by subtracting the autologous control cpm from the MLR test cpm.
MLR of non-adherent responder cell population

Stimulator cells were treated as mentioned above. Responder cells at a concentration of $5 \times 10^6$ cells/ml, were adhered for 2 hours to 60x15 mm plastic petri dishes (Falcon Labware, Oxnard, CA). After this incubation period, the non-adherent cells were removed by washing the plates 3 times with media. The non-adherent cells were counted and resuspended to a concentration of $5 \times 10^6$ cells/ml. Responder and stimulator (intact spleen cell population) cells were plated with UDMH and the assay completed as described above.

MLR with UDMH treated adherent cells

Stimulator cells were prepared as described above. Responder cells were prepared as follows: After removal of the non-adherent cell population, the adherent cells were treated for 2 hours with differing dosages of UDMH. Removal of the adherent cells from the petri plates was accomplished by incubating them for 1/2 hour with PBS lacking calcium and magnesium, scraping them gently with a rubber policeman, and then vigorously washing them five times with media. The adherent and non-adherent cells were reconstituted, brought to a cell concentration of $5 \times 10^6$ cells/ml, and the assay was performed as described above, except that UDMH was not added to the cultures during the seven day incubation period. The volume in the wells was brought to .2 ml with media.

Preliminary MLR testing with mice exposed to UDMH

C57Bl/6 mice were injected interperitoneally with 10, 25, or 50 mg/kg doses of UDMH or PBS for one week. The mice were sacrificed, the spleens removed, and the cells used as responder cells in the MLR assay.
Results

Effects of UDMH on MLR

UDMH caused a significant dose related suppression on the MLR at concentrations of 10, 25, and 50 μg/ml (Table 1).

Effects of adherent cell removal on MLR

The removal of adherent cells resulted in a significantly increased MLR as compared to the control MLR at 0, 10, 25 and 50 μg/ml UDMH. (Table 4). In addition, the UDMH caused a dose related suppression of MLR of the non-adherent cell population at concentrations of 25 and 50 μg/ml, similar to that of the control MLR (Table 2).

MLR of reconstituted non-adherent and UDMH-treated adherent cells

Adherent cells incubated for 2 hours with media and then reconstituted with non-adherent cells and incorporated into the MLR resulted in a response (1628 ± 499 cpm) similar to that of control MLR (1271 ± 351 cpm). Treatment of adherent cells with 10, 25, and 50 μg/ml UDMH for 2 hours prior to reconstitution resulted in a significantly greater response when compared to the control MLR at the same concentrations (Table 4). In addition, the preincubation of adherent cells with 50 μg/ml UDMH resulted in a significant increased MLR of the reconstituted population compared to preincubation with media (Table 3), and the lower UDMH concentrations also resulted in a slight increase.

Preliminary MLR testing with mice exposed to UDMH

Preliminary results from MLRs performed with responding cells from mice injected with PBS, 10, 25 and 50 mg of UDMH/kg mouse indicate that UDMH exerts
a suppressive effect on the MLR when it is administered in vivo. There is a significant decrease in MLR of mice injected with UDMH at doses of 10, 25, and 50 μg/UDMH/kg mouse as compared to MLR done with cells from untreated mice and mice treated with PBS (Table 5).

Discussion

These studies have shown that in vitro UDMH clearly caused a dose related suppression of the mixed lymphocyte response. This data indicates that a mechanism which is necessary for the MLR responder-stimulator relationship is being altered without affecting autologous controls.

There are several possibilities which can explain this phenomenon. UDMH may be causing a change in Ia antigen recognition. Experimentation shows that Ia a-antisera specifically inhibits MLR stimulation without affecting autologous controls (12). It is possible that UDMH also acts in this manner. Another possibility is that UDMH may alter cellular membrane structure or function and therefore inhibit recognition.

Classically, the MLR has been described as a responder T-cell population recognizing and proliferating in response to a heterologous stimulator B-cell population (15). However more emphasis is being placed on the importance of the monocyte in this reaction. It is essential in an MLR for the monocyte to be present and functional in either the stimulating cell population, responding cell population, or both (13). Since the MLR decreases in the presence of UDMH, UDMH may selectively alter the function of the monocyte or its products, as well as of the responder T-cell or stimulator B-cell population.
The same UDMH dose-related suppression of the MLR is also seen upon removal of adherent cells from the responder cell population. However the response stimulation in this assay is higher than in the control assay, regardless of the presence of UDMH, indicating that UDMH is exerting its suppressive effects on the non-adherent (T-cell) subset of the responder population and/or the stimulator cell population.

It was also noted that the MLR of the non-adherent cell population was higher than that of the control (intact) cell population, regardless of the presence or absence of UDMH. This indicates a suppressor or regulator function present in the non-adherent cell population which is independent of the effects of UDMH. This suppressor property of adherent cells on the MLR has been noted by other investigators (10,11,14). The cell responsible for this effect is most likely a macrophage, but could also be an adherent T-cell or B-cell.

Further experiments demonstrated that brief exposure to UDMH incubation of adherent cells alone prior to reconstitution with non-adherent cells and MLR assay abolished the suppressor activity of the adherent cell population, and actually tended to enhance the response. At least two explanations for this effect exist. First, transient exposure to UDMH could result in activation of the adherent cell population. To correlate with this theory, preliminary studies in our laboratory have indicated that UDMH enhances phagocytic and microbicidal properties of peritoneal macrophages during a 2 hour incubation period, indicating a non-specific activation of macrophages.

Another explanation for the UDMH-induced loss of adherent cell suppressor activity would be that UDMH specifically abrogates the suppressor cell component of the adherent cell population. Incubation of the adherent cell component of the
responder cells with UDMH had a similar enhancing effect on the MLR as removal of the adherent cell population. Other experiments in our lab have shown that low-concentration UDMH caused an enhanced lymphoblast transformation response to lipopolysaccharide²; and mice treated with UDMH responded to sheep erythrocyte immunization with increased numbers of plaque-forming cells compared to control mice. Furthermore, in vivo and in vitro UDMH treatment inhibited concanavalin A-induced suppressor activity.

Other possible explanations for the effects of UDMH undoubtedly exist. Experiments are now underway to examine the effects on the MLR of exposure of different components of the stimulator cell population to UDMH and on brief exposure to UDMH of the non-adherent responder cell population. Additionally, the effects of UDMH on mitogen presentation of macrophages, and of mitogen and antigen receptor mobility on lymphocytes will be examined.
References


Table 1. Effects of UDMH on control MLR

<table>
<thead>
<tr>
<th>Concentration of UDMH (µg/ml)</th>
<th>MLR (^a)</th>
<th>p value (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>1271 ± 351</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>1507 ± 607</td>
<td>n.s.</td>
</tr>
<tr>
<td>10</td>
<td>822 ± 315</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>25</td>
<td>121 ± 216</td>
<td>&lt; .005</td>
</tr>
<tr>
<td>50</td>
<td>412 ± 233</td>
<td>&lt; .025</td>
</tr>
</tbody>
</table>

\(^a\) - Stimulation expressed as corrected counts per minute (cpm) ± standard error (see text).

\(^b\) - Determined by student's T-test.

Table 2. Effects of UDMH on MLR of non-adherent cell population.

<table>
<thead>
<tr>
<th>Concentration of UDMH (µg/ml)</th>
<th>MLR of non-adherent cell (^a)</th>
<th>p value (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>2679 ± 741</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>2185 ± 695</td>
<td>n.s.</td>
</tr>
<tr>
<td>10</td>
<td>2032 ± 643</td>
<td>n.s.</td>
</tr>
<tr>
<td>25</td>
<td>1327 ± 210</td>
<td>&lt; .025</td>
</tr>
<tr>
<td>50</td>
<td>1223 ± 643</td>
<td>&lt; .050</td>
</tr>
</tbody>
</table>
Table 3. Effects of UDMH pre-treatment of adherent cells on the MLR of the reconstituted cell population.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Concentration of UDMH (μg/ml)</th>
<th>MLR of reconstituted and adherent cells\textsuperscript{b}</th>
<th>p value\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>1628 ± 499</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>2287 ± 628</td>
<td>n.s.</td>
</tr>
<tr>
<td>10</td>
<td>1944 ± 628</td>
<td>n.s.</td>
</tr>
<tr>
<td>25</td>
<td>2244 ± 583</td>
<td>n.s.</td>
</tr>
<tr>
<td>50</td>
<td>2658 ± 657</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Adherent cells were incubated with UDMH for 2 hours, washed, then added back to the non-adherent cell population.

\textsuperscript{b} Expressed as corrected cpm (see text) \pm standard error of the mean.

\textsuperscript{c} p value determined by student's T test.
Table 4. Effect of adherent cell removal, UDMH treatment and reconstitution compared to control MLR.

<table>
<thead>
<tr>
<th>Concentration of UDMH(μg/ml)</th>
<th>Control MLR(a)</th>
<th>MLR of non-adherent cells</th>
<th>p value</th>
<th>MLR with UDMH(b) treated adherent cells</th>
<th>p value(c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>media</td>
<td>1271 ± 351</td>
<td>2679 ± 741</td>
<td>&lt;.05</td>
<td>1628 ± 499</td>
<td>n.s.</td>
</tr>
<tr>
<td>5</td>
<td>1507 ± 607</td>
<td>2185 ± 695</td>
<td>n.s.</td>
<td>2287 ± 628</td>
<td>n.s.</td>
</tr>
<tr>
<td>10</td>
<td>822 ± 315</td>
<td>2032 ± 643</td>
<td>&lt;.05</td>
<td>1944 ± 628</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>25</td>
<td>121 ± 216</td>
<td>1327 ± 210</td>
<td>&lt;.001</td>
<td>2244 ± 583</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>50</td>
<td>412 ± 233</td>
<td>1223 ± 643</td>
<td>&lt;.05</td>
<td>2658 ± 657</td>
<td>&lt;.005</td>
</tr>
</tbody>
</table>

\(a\) MLR using intact splenocyte population as responder cells (cmp ± S.E.)

\(b\) MLR using non-adherent cells reconstituted with UDMH treated and washed adherent cells as responder cell population.

\(c\) Determined by student's T-test.
Table 5. Effects of UDMH on MLR when administered in vivo.

<table>
<thead>
<tr>
<th>Dose (mg UDMH/kg mouse)</th>
<th>MLR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>4577 ± 791</td>
<td>--</td>
</tr>
<tr>
<td>PBS</td>
<td>4340 ± 929</td>
<td>n.s.</td>
</tr>
<tr>
<td>10</td>
<td>2733 ± 1344</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>25</td>
<td>2115 ± 503</td>
<td>&lt;.025</td>
</tr>
<tr>
<td>50</td>
<td>505 ± 42</td>
<td>&lt;.005</td>
</tr>
</tbody>
</table>

<sup>a</sup> cpn

<sup>b</sup> Determined by student's T-test.
Species Variation in Susceptibility to Methylnitrosourea-Induced Immunosuppression

Melinda J. Tarr¹
Richard G. Olsen¹,²,³

¹ Department of Veterinary Pathobiology, The Ohio State University
² Department of Microbiology, College of Biological Science, and
³ Comprehensive Cancer Center of the Ohio State University

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Corresponding author:

Melinda J. Tarr
The Ohio State University
Department of Veterinary Pathobiology
1925 Coffey Road
Columbus, Ohio 43210
614-292-3661
Abstract:

Methylnitrosourea (MNU) was shown to suppress the cell-mediated immune system of mice. Cutaneous allografts were retained longer than controls in mice treated with 25 or 50 mg/kg MNU. The lymphocyte blast transformation (LBT) responses to the mitogens concanavalin A (con A) and lipopolysaccharide (LPS) of splenocytes from treated mice were also suppressed three days following treatment. By 7 and 42 days, however, the LBT responses to con A were higher than the controls for the 10 and 25 mg/kg dose groups. The rebound in the response to LPS was not nearly so pronounced and at 7 and 42 days was still suppressed for the 50 mg/kg dose group.

In additional experiments, the LBT response of normal murine splenocytes to con A and LPS were significantly suppressed after exposure to 25 μg/ml MNU, and the LBT response of human lymphocytes to con A was suppressed after exposure to 60 μg/ml MNU.

The results of these studies were compared to previous experiments in cats. It was concluded that cats are more immunosuppressed by MNU than mice, and suppression of the LBT response following exposure of normal lymphocytes to MNU is in the order cats > mice > humans.

Species Immunosuppression Methylnitrosourea
Introduction:

There is increasing concern by private and governmental agencies over the potential adverse effects of industrial chemicals and food additives on the immune system of exposed animals and man (Vos and Van Genderen, 1973). Generalized immunosuppression, for example, is induced by numerous chemicals such as polyhalogenated biphenyls (Friend and Trainer, 1970; Loose et al., 1973; Street and Sharma, 1975), heavy metals such as lead, cadmium, and mercury (Koller, 1973), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Faith and Moore, 1977; Thigpen et al., 1975), both in controlled experimental animal studies, and, in the case of polybrominated biphenyls, after natural exposure (Bekesi et al., 1978). Methylnitrosourea (MNU), a potent resorptive carcinogen derived from the nitrosamine family (Druckery et al., 1967) was recently demonstrated to be immunosuppressive in cats (Tarr et al., 1979). There was suppression of cell-mediated immunity in MNU-treated cats as evidenced by prolonged allograft retention and suppressed lymphocyte blast transformation (LBT) response to mitogens (Tarr et al., 1979). In addition, MNU-treatment of normal feline lymphocytes resulted in suppressed LBT responses to mitogens (Tarr and Olsen, 1980). We then attempted to show the same correlation of immunosuppression between in vivo and in vitro experiments in mice, as well as determine the in vitro effects of MNU on human lymphocytes. The studies reported here compare the in vivo effects of MNU in cats and mice, and the in vitro effects in cats, mice, and humans.

Materials and Methods:

Animals: Mice: Young adult Swiss outbred mice and C57BL/6 (for skin graft donors) were purchased from Lab Supply, through The Ohio State University Animal Procurement Service, Columbus, Ohio.
Human: Four healthy volunteers from our technical staff served as human blood donors.

Methylnitrosourea: The MNU (kindly supplied by Dr. Ming Chang of our department) was dissolved in a citrate-phosphate buffer (pH 6.8) and diluted in media (see below) immediately prior to animal injection or incubation with lymphocytes.

Animal inoculation - in vivo experiments: Swiss outbred mice were inoculated intraperitoneally with MNU in citrate phosphate buffer, in doses of 0, 10, 25, 50, or 75 mg/kg. Each dose group consisted of 6 to 16 mice, with equal numbers of males and females.

Lymphocyte isolation: Mouse spleens were harvested aseptically in Hanks balanced salt solution, and pressed through a no. 60 grid mesh. The splenic pulp was aspirated several times through 18g, 20g, 22g, and 25g needles, and then washed in phosphate buffered saline. Red blood cells were lysed in 0.155 M NH4Cl solution and the splenocytes were then washed in L-15, and resuspended in culture medium consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), 1% antibiotics (2 x 10^2 U/ml penicillin and 0.1/mg/ml streptomycin), 5 x 10^{-5} M 2-mercaptoethanol, and 10% heat inactivated fetal bovine serum (Sterile Systems, Inc., Logan, Utah).

Human lymphocytes were isolated from heparinized peripheral blood by centrifugation through a Ficoll-hypaque gradient as previously described for cats (10). The lymphocytes were washed, counted and resuspended in medium consisting of minimum essential media with Spiner's Salts (MEM-S) (Grand Island Biological Co., Grand Island, NY), 1% antibiotics, NaHCO3 and 5% normal pooled heat inactivated human serum.
Cutaneous allografts: On the day of MNU injection, cutaneous allografts (CA) were performed according to the method of Manning and Kreuger (1974). Briefly, 1 cm round biopsies were removed from the skin of a single sacrificed donor C57BL/6 mouse. A graft bed was prepared on each recipient mouse (8 mice per dose group) and the biopsies were fixed in place with cyanoacrylate glue (Krazy Glue, Inc., Chicago, IL). The grafts were kept bandaged for six days, then checked daily for signs of rejection (devitalization, scaling, necrosis). The grafts were scored as rejected when 90% or more of the graft was completely devitalized. Grafts that did not become vascularized or were chewed off were not included in the results.

LBT assays: 1) MNU-treated mice: Lymphocytes were harvested from the spleens of MNU-treated or control, (untreated) mice at 3, 7 or 42 days following treatment (6 to 16 mice per dose group), and suspended in culture medium at 1x10^6 live mononuclear cells/ml. One-tenth ml aliquots of cell suspension (10^5 cells) were placed in 96-well microtiter plates (Costar, Cambridge, Mass.). Cell cultures from each mouse were stimulated in quadruplicate with con A (Sigma Chemical Co., St. Louis, Mo.) (final concentration 1.0 µg/ml), lipopolysaccharide (LPS; from Serratia marcescens) (DIFCO Laboratories, Detroit, MI) (final concentration 50 µg/ml), or medium (for control background counts). The cultures were incubated 48 hrs, pulsed with tritiated thymidine (H^3T) (0.5 µCi/well) (New England Nuclear, Boston, MA), and then harvested 24 hrs later with a multiple automated sample harvester (Otto Hiller Co., Madison, WI). The incorporated H^3T was measured as counts per minute (CPM) using a liquid scintillation counter (Packard Tricard Model 3375, Packard Instrument Co., Downers Grove, IL). The results are expressed as "corrected" CPM for MNU-treated mice ((CPM of mitogen-stimulated cultures) - (CPM of media control cultures)), or as percent of control response ((CPM of treated animals or cells) + (CPM of untreated control animals or cells)) X 100 for each mitogen.
2) MNU treatment of lymphocytes from normal mice and humans: Lymphocytes were collected from 20 normal mice and 4 human volunteers, as described. The cells were suspended in medium without serum, and incubated in freshly dissolved MNU at concentrations of 0, 1, 5, 10, 25, 50, 75 and 100 μg/ml for 1/2 hr at room temperature. They were then washed in medium, resuspended in medium with serum and tested in the LBT assay as already described. Murine cells were stimulated with con A and LPS as described above, and human cells with 50 μg/ml con A and 6.125 μg/ml phytohemagglutinin (PHA) (DIFCO Laboratories, Detroit, MI).

**Cell viability:** Viability of MNU-treated lymphocytes was determined using the Trypan blue dye exclusion test, and was expressed as (no. live cells/total no. cells) × 100. One hundred cells were counted for each determination, at the end of the 3 day LBT incubation period.

**Statistics:** The Students t test was used to determine significant differences between control (untreated) and MNU-treated cell cultures or animal groups.

**Results:**

**Skin grafts:** The MNU-treated mice showed a dose-related increase in skin graft retention time, which was significant at 25 and 50 mg/kg MNU (p<.001). This is compared to previous data obtained for cats (table 1). (No results were obtained for mice treated with 75 mg/kg MNU since most of these animals died 2-3 weeks following treatment).

**LBT assay - MNU-treated mice:** The results of these experiments are summarized in figure 1. At 3 days post-inoculation (dpi) of MNU, the response to both con A and LPS was significantly decreased for the 25 and 50 mg/kg dose groups (the
only dose groups tested at this time). The corrected CPM responses to con A were 41.8 ± 21.3 (standard error of the mean) percent (p<.001) and 74.3 ± 15.8% (p<.05) of the control group for the 25 and 50 mg/kg dose groups, respectively. The LPS responses were 51.7 ± 19.0% (p<.005) and 61.9± 13.3% (p<.005) of the control group for the 25 and 50 mg/kg dose groups.

At 7 dpi, the CPM responses to both con A and LPS were significantly decreased (p<.001) from the control group for mice in the highest dose group (75 mg/kg), (21.9% of control for con A and 11.3% of control for LPS). For the 50 mg/kg dose group, the CPM response to LPS was significantly lower (p<.005) (58.4 ± 31.7% of control), however, the CPM response to con A was similar to control values. Mice treated with 25mg/kg MNU showed a significantly higher (p<.005) CPM response to con A (224.6 ± 45.3% of control), but no significant difference in CPM response to LPS. The 10 mg/kg dose group show no significant differences from the control group.

At 42 dpi, mice in the 10 mg/kg dose group demonstrated a significantly increased CPM response to con A (264.3 ± 22.9% of control) (p<.005) and to LPS (165.3 ± 17.2% of control) (p<.01). There was also a decrease in response to LPS in the 50 mg/kg dose group (69.4 ± 4.3% of control) (p<.01). (No results were obtained for the response to con A). Mice in the 75 mg/kg dose group had all died by 42 dpi.

**LBT assay:** In vitro exposure to MNU of lymphocytes from normal (untreated) animals. The LBT responses to con A of lymphocytes from normal mice, humans, and cats (from a previous study) (Tarr and Olsen, 1980) after a 1/2 hour exposure to MNU are compared in figure 2. All three species demonstrated a dose-related suppression, but there were differences in species susceptibility. Cat lymphocytes were the most susceptible, being significantly suppressed at 10 μg/ml MNU (82.7 ± 3.6% of the control response; p<.001). Murine spleen cells were suppressed at 25 μg/ml (76.6 ± 11.7%;
p<.05), and human lymphocytes were the most resistant, showing significant suppression at 60 μg/ml (49.9 ± 8.8%; p<.001).

The responses of MNU-exposed murine lymphocytes to the T-cell mitogen con A and the B-cell mitogen LPS are compared in figure 3. There were no significant differences in the degree of suppression between the responses to con A and LPS.

Cell viability: There was no significant decrease in viability of MNU-treated lymphocytes at any dose compared to untreated lymphocytes (table 2).

Discussion:

The results of these experiments demonstrate that mice exposed to MNU were immunosuppressed. Treated mice showed significant prolongation of skin graft retention after a single dose of 25 or 50 mg/kg, and also showed a suppressed LBT response to both con A and LPS 3 days after MNU treatment with 25 or 50 mg/kg MNU. However, by 7 dpi, the responses had returned to normal (LPS) or enhanced (con A) levels in the two lower dose groups.

Comparing the effects of MNU in mice to those in outbred cats (Tarr et al., 1979), it appears that cats were markedly more immunosuppressed by a lower dose of MNU (15 mg/kg). Treated cats retained their skin grafts for an average of 74 days (compared to the control group value of 16.3 days), and demonstrated a suppressed LBT response of peripheral blood lymphocytes to both con A and pokeweed mitogen for at least 12 weeks after MNU injection.

It might be argued that the reason for the increased susceptibility of the cat to MNU-induced immunosuppression is that the route of administration of MNU in the cat was intravenous (i.v.), whereas it was intraperitoneal (i.p.) in the mouse. However, the MNU molecule is very small, has the same permeability as urea, and is rapidly
absorbed across epithelial barriers. It is a resorptive carcinogen, that is, it induces tumors in target organs regardless of its route of administration (Swenberg et al., 1975). Other experiments by us have indicated that oral administration of 15 mg/kg MNU to cats induced a degree of immunosuppression similar to that resulting from i.v. administration (unpublished data). Thus, the difference in susceptibility of the cat and mouse to MNU-induced immunosuppression must be primarily a species-related phenomenon.

A species difference was again seen with the LBT response to mitogens of in vitro MNU-treated lymphocytes. Feline lymphocytes were most susceptible, showing significant suppression at 10 μg/ml MNU. Murine lymphocytes were first significantly suppressed at 25 μg/ml; however, at higher concentrations of MNU, feline and murine lymphocytes were suppressed to the same degree. Human lymphocytes were relatively resistant, being suppressed at 60 μg/ml MNU. So it appears that the species difference in immunosuppression seen in in vivo MNU-treated cats and mice is reflected by the difference in suppression of the LBT response of feline and murine MNU-treated lymphocytes, at least at the lower concentrations.

The in vitro experiments also indicate that MNU suppresses murine T-cells and B-cells equally, since the LBT responses to con A and LPS were equally affected. The in vivo experiments showed similar results soon after treatment, i.e. at 3 dpi, the LBT responses to con A and LPS of splenocytes from MNU-treated mice were equally suppressed. At 7 and 42 dpi, however, the LBT response to con A was greater than the response to LPS in the three lower dose groups, suggesting that the splenic T-cell population was less affected or had a greater rebound capacity than the splenic B-cell population after MNU treatment.

In conclusion, it appears that the immunosuppressive effects of MNU seen in MNU-treated animals can be demonstrated by the simple in vitro LBT assay using
MNU-treated lymphocytes. In addition, it reflects species differences in susceptibility to MNU-induced immunosuppression. If further experiments uphold the correlation between in vivo and in vitro immunosuppression, this assay may represent one rapid, simple and economical method of screening environmental pollutants or industrial chemicals for potential immunotoxic effects.
References


Table 1. Comparison of Cutaneous Allograft Retention Times in MNU-treated Cats and Mice

<table>
<thead>
<tr>
<th>Animal</th>
<th>Dose MNU</th>
<th>Skin graft-retention (days ± s.e.m.)</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cats</td>
<td>Control</td>
<td>16.25 ± 0.48</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(untreated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 mg/kg</td>
<td>74.25 ± 9.75</td>
<td>p&lt;.005</td>
</tr>
<tr>
<td>Mice</td>
<td>Control</td>
<td>10.70 ± 0.66</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(untreated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>9.33 ± 1.77</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>25 mg/kg</td>
<td>14.80 ± 0.91</td>
<td>p&lt;.001</td>
</tr>
<tr>
<td></td>
<td>50 mg/kg</td>
<td>18.66 ± 1.42</td>
<td>p&lt;.001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values for control and treated groups for each species were compared using the student T-test.
Table 2. Percent Viability of Con A-Stimulated Cells After Incubation with MNU.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Feline</th>
<th>Murine</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>98.4 ± 1.6³</td>
<td>63.5 ± 7.9</td>
<td>97.7 ± 0.9</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>100.0 ± 0</td>
<td>64.5 ± 6.2</td>
<td>98.8 ± 0.8</td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>100.0 ± 0</td>
<td>ND</td>
<td>99.0 ± 0.6</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>100.0 ± 0</td>
<td>ND</td>
<td>94.0 ± 1.6</td>
</tr>
<tr>
<td>75 µg/ml</td>
<td>96.3 ± 3.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>86.1 ± 6.6</td>
<td>60.0 ± 16.1</td>
<td>91.0 ± 3.3</td>
</tr>
</tbody>
</table>

³ Expressed as percent viability ± standard error of the mean.
Figure 1. LBT responses to con A and LPS of splenocytes from MNU-treated mice. Expressed as % of the response of untreated control mice: (corrected CPM of treated mice/corrected CPM of control mice) x 100. Bars represent standard error of the mean. P values represent significant differences from control responses. Control response = 100%.
Figure 2. Effects of 1/2 hr preincubation with MNU on the LBT response of feline, murine and human lymphocytes to con
A. — = human; —— = feline; ---- = murine.
Figure 3. Effects of 1/2 hr preincubation with MNU on the LBT response of murine splenic lymphocytes to con A and LPS.

● = con A; □ = LPS.
DAT
ILM