Selective Thymus-derived Cell Enrichment in the Rat Spleen as a Result of Immunodepression by Urethan

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SUMMARY

The early effect of urethan on cell-mediated immunity was studied in Fischer adult rats by means of skin homografts, graft-versus-host assays, and in vitro lymphocyte stimulation by phytohemagglutinin.

A weak but significant survival prolongation of Lewis rat skin grafted in urethan-treated Fischer rats 1 day after the end of the treatment was observed. Contrary to expectation, the Fischer spleen cells taken 1 day after the end of urethan treatment showed a graft-versus-host reactivity about 4 times that of the controls when injected into Fischer X Bf F1, hybrid newborn rats; this reactivity receded to values slightly below the normal levels if the donor cells were taken 7 days after the end of urethan treatment. In vitro assays demonstrated that cells with heightened in vivo capacity to mount a graft-versus-host reaction showed also an increased proliferative response to phytohemagglutinin. The hypothesis is suggested that urethan can distinguish between two mononucleated spleen cell populations. The more numerous would be destroyed (1 day after treatment, the number of mononucleated spleen cells is reduced to less than one-third); the other, i.e., that involved in cell-mediated immune responses, would be much less affected by the carcinogen. It would result in a relative increase of thymus-dependent cells in the spleen of treated animals.

INTRODUCTION

Immunodepression seems to be a widespread condition during chemical carcinogenesis (1, 9). Since it is a very early phenomenon after carcinogen administration, it could act by allowing tumor neoantigens to escape from the host immunological surveillance. One important mechanism by which this appears to be possible involves the depression of cell-mediated immunity of the primary host.

Previous studies indicate that such a depression occurs during hydrocarbon carcinogenesis (4). The same effect was also observed in mice neonatally exposed to urethan (5, 7); however, this condition gives information more on the impairment of the immunological development than on the depression of a fully immunocompetent system.

The present study was undertaken to investigate the early effect of urethan, given to young adult rats, on cell-mediated immunity by means of skin homografts across a weak histocompatibility barrier, GVH2 assays, and in vitro lymphocyte stimulation by PHA.

The results of our experiments presented us with an unexpected phenomenon not observed before with chemical carcinogens, i.e., a greatly heightened capacity of the spleen cells surviving the strong lymphocytolytic action of urethan to mount a GVH reaction and to react in vitro to PHA stimulation. We interpret this finding as a relative increase of T-cells produced by the selective pressure of urethan on the whole spleen population.

MATERIALS AND METHODS

Skin Graft Experiments. Grafts were performed by use of Fischer (Arsal; Pomezia, Roma, Italy) and Lewis (Microbiological Associates, Inc., Bethesda, Md.) female inbred rats, 4 to 5 months old, as hosts and donors, respectively. This combination shares the same strong histocompatibility allele (AgB-1), the donor-host incompatibility being due to differences at multiple non-AgB loci.

Ethyl carbamate (urethan; Carlo Erba, Milano, Italy) was dissolved in sterile, distilled water at a concentration of 100 mg/ml and injected i.p. into hosts in 5 doses of 0.5 mg/g body weight on Days 0, 1, 2, 3, and 4 (at a total dosage of 2.5 mg/g body weight) in order to avoid the irreversible coma that we observed when the total amount of urethan was given in single doses of 1 mg/g body weight. All controls received injections of sterile, distilled water. The Fischer rats were grafted on the 5th day after treatment had begun. The procedure used was essentially that of Billingham (2). Casts were removed 6 days later, and the grafts were examined daily from the appearance of the 1st lesions until complete rejection. The significance of the differences between treated animals and controls has been calculated for both MST and MFLT by means of Student's t test.

GVH Assays. The assays were performed according to Simonsen's spleen assay (8): 4- to 5-month-old Fischer rats were given injections of urethan or distilled water as above, the last injection being given 24 hr or 7 days before the assay. The rats were grafted on the 5th day after treatment had begun. The procedure used was essentially that of Billingham (2). Casts were removed 6 days later, and the grafts were examined daily from the appearance of the 1st lesions until complete rejection. The significance of the differences between treated animals and controls has been calculated for both MST and MFLT by means of Student's t test.

The abbreviations used are: GVH, graft-versus-host; PHA, phytohemagglutinin; T-cells, thymus-derived cells; MST, mean survival time; MFLT, mean 1st lesion time.

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newborn rats, obtained from Fischer female rats and Bf male rats (Simonsen Laboratories, Inc., Gilroy, Calif.), as recipients. Spleens were removed aseptically and were weighed, and a cell suspension from 5 pooled spleens was prepared in Hanks' balanced salt solution. Doses varying from $2.5 \times 10^6$ to $10 \times 10^6$ mononucleated cells suspended in 0.1 ml Hanks' solution were injected i.p. into newborn rats. Litters were killed 9 days later, and the index of spleen enlargement was evaluated by dividing the spleen weight:body weight ratio of the inoculated animals by the average ratio of their untreated littermates. The mean spleen index for each cell dose was computed by averaging the indices obtained from each litter. The significance of the differences in reactivity between different cell preparations was calculated by means of the analysis of variance, and cell dose-spleen index regression lines for a $3 \times 3$ factorial experiment were computed.

**Conditions of Cell Culture.** Spleen cell suspensions were prepared by techniques fully described elsewhere (3) and were washed and suspended in culture medium (Eagle's minimal essential medium; Schwartz BioResearch, Inc., Orangeburg, N. Y.) supplemented with calf serum (15%) or fresh BN rat serum (5%), penicillin (100 i.u./ml), and streptomycin (100 µg/ml). Each culture consisted of 1,000,000 lymphocytes suspended in a total volume of 1 ml medium, with or without PHA-P (0.003 ml) (Difco Laboratories Inc., Detroit, Mich.). All cultures were conducted in triplicate in 13-× 100-mm disposable flint glass tubes (Belco Glass, Inc., Vineland, N. J.). The tubes were closed with Morton stainless steel caps and maintained upright in a 95% air:5% CO$_2$ humid atmosphere at 37°. At appropriate times after the cultures were begun, 0.1 ml of medium containing 0.25 µCi tritiated thymidine (thymidine-methyl$^3$H; specific activity, 6.5 Ci/mmole; The Radiochemical Centre, Amersham, England) was added, the pellets were dissolved in formic acid (0.4 ml) and 6 ml of medium containing 0.25 µCi tritiated thymidine (thymidine-methyl$^3$H; specific activity, 6.5 Ci/mmole; The Radiochemical Centre, Amersham, England) was added, the tubes were returned to the incubator for 12 hr, and the radioactivity of the DNA fraction was assessed according to a procedure that improved the sensitivity of the usual assay (10). The pellets were dissolved in formic acid (0.4 ml) and 6 ml methyl Cellosolve. Ten ml scintillation liquid (0.4% PPO and 0.005% POPP in toluene) were added, and radioactivity was determined in a Nuclear Chicago Mark I spectrometer with 133 Ba external standardization (with an efficiency of approximately 20%). Counts among replicate cultures rarely varied more than 20%.

**RESULTS**

**Skin Grafts.** The results, including the MFLT and MST of the L→F combination, are shown in Table 1. In this system, urethan treatment significantly prolonged the MST of grafts performed 5 days following the 1st urethan exposure and also delayed the appearance of the earliest signs of rejection.

**GVH Assays.** These experiments were devised to investigate the reactivity of mononucleated spleen cells from control and urethan-treated Fischer rats against a strong (AgB) histocompatibility barrier. Doses of $2.5 \times 10^6$, $5 \times 10^6$, and $10 \times 10^6$ mononucleated cells taken from control or treated donors 1 or 7 days after the last injection were given i.p. to FFB1 newborn rats.

Chart 1 shows data referring to spleen indices obtained from inoculating 140 newborns during 2 successive experiments. The response of cells taken from treated donors is completely different if the GVH assay is made 24 hr or 7 days after the end of urethan treatment.

The GVH reactivity of spleen cells taken 24 hr after the end of treatment is about 4 times that of controls, whereas after 7 days, the capacity of the cells to mount a GVH reaction falls to slightly below the normal levels. The slopes of the regression lines are not significantly different (Table 2); therefore, the horizontal distance between lines indicates the difference in potency between the different cell populations.

**Lymphocyte Cultures.** These experiments were devised to investigate PHA reactivity of Fischer rat spleen cells from control and urethan-treated donors 1 day after the end of the treatment. Thymidine$^3$H was added 12 hr before culture termination.

Chart 2 shows the progress of thymidine$^3$H incorporation by lymphocytes from individual control or urethan-treated donors in culture medium supplemented with calf serum. Six normal and 8 urethan-treated cell cultures were performed in 2 successive assays. Normal cells react to PHA stimulation, with a maximum reaction occurring between the 30th and 50th hr, after which thymidine incorporation by PHA cultures is similar to that of nonstimulated controls, in which incorporation is always very low. Cells from urethan-treated donors do not show a clear response to PHA stimulation; at the 54th hr the incorporation is like that of non-PHA-stimulated cultures.

**Table 1**  
*Lewis rat homograft survival time in Fischer rats after urethan exposure*

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Strain combination</th>
<th>Treatment</th>
<th>Grafting time$^a$ (days)</th>
<th>MFLT (days)</th>
<th>MST (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>18</td>
<td>♀ Lewis→♀ Fischer</td>
<td>Urethan</td>
<td>5</td>
<td>10.61 ± 0.49$^b$</td>
<td>13.55 ± 0.45</td>
</tr>
<tr>
<td>II</td>
<td>14</td>
<td>♀ Lewis→♀ Fischer</td>
<td>Distilled water</td>
<td>5</td>
<td>8.58 ± 0.49</td>
<td>11.08 ± 0.55</td>
</tr>
</tbody>
</table>

*Statistical significance of differences*

1 vs. II, $p < 0.01$ both for MFLT and MST

$^a$ After the beginning of the treatment.

$^b$ Mean ± S.E.
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Chart 1. GVH reactivity across a strong histoincompatibility. Mean spleen indices obtained in FBF litters given doses of $2.5 \times 10^6$ to $10 \times 10^6$ Fischer rat spleen cells taken from control (*) or urethan-treated (2.5 mg/g body weight) donors, 24 hr (●) or 7 days (○) after the end of the treatment. Newborn rats were given injections when 6 to 7 days old and were killed 9 days later. Each point represents the mean of about 15 recipients. Slopes of the 3 regression lines are not significantly different (Table 2). The horizontal distance between lines indicates approximately the difference in potency between treated and standard preparations. A statistical evaluation of these data is given in Table 2.

Table 2
Statistical evaluation of data from the GVH assays in Chart 1

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Freedom degrees</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>Fischer rats</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>2</td>
<td>35.5488</td>
<td>17.7744</td>
<td>28.7054</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Doses</td>
<td>2</td>
<td>19.3264</td>
<td>9.6632</td>
<td>15.6059</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Linear</td>
<td>1</td>
<td>19.2098</td>
<td>19.2098</td>
<td>31.0236</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Residual</td>
<td>1</td>
<td>0.11660</td>
<td>0.11660</td>
<td>0.1883</td>
<td>N.S.°</td>
</tr>
<tr>
<td>Doses X treatments</td>
<td>4</td>
<td>1.1824</td>
<td>0.2956</td>
<td>0.4774</td>
<td>N.S.</td>
</tr>
<tr>
<td>Error</td>
<td>135</td>
<td>83.6020</td>
<td>0.6192</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

° N.S., not significant.

Chart 2. Proliferative response of $1 \times 10^6$ spleen lymphocytes taken from normal (*) or urethan-treated (●) (2.5 mg/g body weight) Fischer rats, 24 hr after the end of the treatment, and cultured in 1 ml Eagle's minimal essential medium supplemented with calf serum (15%) and with (—) or without (—) Difco P PHA (0.003 ml/ml). Cultures were performed in 13- x 100-mm disposable flint glass tubes closed with Morton stainless steel caps and maintained upright in a 95% air:5% CO₂ humid atmosphere at 37°C. Each point represents the mean of 6 or 8 cultures, each performed in triplicate with cells from individual control or urethan-treated donors, respectively, ± S.E. Thymidine-3H (0.25 μCi in 0.1 ml medium) was added to each culture 12 hr before culture termination.

Chart 3 shows the proliferative response to PHA of pooled spleen cells from 8 normal or urethan-treated donors cultured in medium supplemented with fresh BN rat serum. In this condition, the peak of thymidine-3H incorporation by urethan-treated cells rises to values 3 times that of normal cells.

Spleen Weight and Spleen Cell Number. As shown in Table 3, a significant decrease in spleen weight of urethan-treated...
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Fischer rats was observed 5 days after treatment had begun. The decrease in the number of mononucleated spleen cells was even more marked, so that the mononucleated cells:spleen weight ratio was also lowered.

DISCUSSION

A prolongation of MST of Lewis rat skin homografts performed in Fischer rats 5 days after the beginning of urethan treatment was observed. The result is to be evaluated taking into account that (a) it refers to a period of time that does not overlay tumor appearance; (b) the challenged antigenic difference approaches more closely that resulting from the malignant transformation than does the male-to-female incompatibility thus far successfully tested (7); and (c) it has been obtained by giving urethan to adult rats and not to newborns as in previous studies (5).

The GVH assays, originally performed to better quantify this depressive effect by relating the immunological reactivity to a definite cell number, have actually given paradoxical results. GVH reactivity of donor spleen cells, taken 1 day after the end of urethan treatment, rose to values about 4 times that of control cells. One week after the end of urethan treatment, this GVH reactivity receded to values slightly below the normal levels. The phenomenon of increased GVH reactivity can support at least 2 different hypotheses: (a) it could represent an actual heightening of the capacities of single cells to mount a cell-mediated immune response; and (b) it may be that, underlying the apparent phenomenon of an increased GVH reactivity of single cells, a selection by urethan is performed on a complex cell population.

The 1st hypothesis seems at present to be the less probable, since it would imply that urethan stimulates the "immunological program" of the cells implicated in the cell-mediated response, increasing their individual efficiency. On the other hand, this hypothesis does not account for the slight but significant prolongation of MST of skin homografts performed on urethan-treated hosts.

According to the selective hypothesis, urethan would distinguish between 2 mononucleated spleen cell populations. The more numerous, probably related to the humoral response, would be drastically impaired by the carcinogen. The degree of urethan sensitivity of the other spleen cell population, i.e., that involved in the cell-mediated immune response.

Table 3

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Treatment</th>
<th>Timea</th>
<th>Spleen weight (mg)</th>
<th>Nucleated cells x 10^6</th>
<th>Mean ratio of nucleated cells x 10^6 spleen weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Urethan</td>
<td>5</td>
<td>308.23 ± 34b</td>
<td>102.22 ± 12</td>
<td>0.331</td>
</tr>
<tr>
<td>10</td>
<td>Urethan</td>
<td>12</td>
<td>447.77 ± 118</td>
<td>122.38 ± 40</td>
<td>0.273</td>
</tr>
<tr>
<td>30</td>
<td>Distilled water</td>
<td>5, 12</td>
<td>542.00 ± 44</td>
<td>361.15 ± 30</td>
<td>0.666</td>
</tr>
</tbody>
</table>

a Days after treatment had begun.
b Mean ± S.E.
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responses, would be much smaller. The result would be a relative enrichment of the spleen population in cells involved in the cell-mediated immune responses. A slight diminution and/or impairment of these cells on the whole would explain the slight prolongation of the skin homograft survival observed.

When this selected cell population is tested in a GVH assay, which measures the relative potency for a given number of immunocompetent cells, urethan-selected cells would show a highly specific cell-mediated reactivity to hide any concomitant immunodepression. The drastic diminution (to less than one-third) of mononucleated spleen cells in urethan-treated donors at the time of GVH assay supports this hypothesis.

Moreover, when GVH assay is performed by using spleen cells taken 7 days after the end of urethan treatment, GVH reactivity recedes to values slightly below the normal levels, thus showing the weak and persistent depression of cellular immunity just suggested by the prolongation of skin homograft survival.

It is known that cellular immunity comprehends various immune responses, such as GVH reaction, homograft rejection, delayed hypersensitivity, and antacrier helper activity in humoral response (6). The T-cells involved in these phenomena are known to respond in vitro to PHA stimulation. However, in the 1st PHA in vitro experiment using calf serum, and according to previous studies (10), we were not able to provoke an evident response of urethan-selected cells to PHA, in comparison to the responsiveness of control cells. This finding, which was difficult to explain, was reversed by a 2nd experiment, in which we used fresh BN rat serum. In this case, the response to PHA of spleen cells from urethan-treated donors was about 3 times that of controls. This finding is in agreement with the hypothesis that we are dealing with a selected spleen population greatly enriched in T-cells. We are not yet able to give a satisfactory explanation for the lack of responsiveness to PHA when calf serum, instead of BN rat serum, is added to the culture medium.

In conclusion, this study seems to have overcome the problem of immunodepression in carcinogenesis and to offer a method that enables one to distinguish and separate the T-cells in a heterogenous population like that of the spleen.

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