The Predictive Value of Skin Allograft Survival Times during the Development of Urethan-induced Lung Adenomas in BALB/c Mice

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SUMMARY

The possibility that depression of cell-mediated immunity facilitates the carcinogenic effect of urethan on the mouse lung was studied in the BALB/c strain which is highly susceptible to lung tumor development. Lung adenomas were induced by giving 4-day-old mice an i.p. injection of 1.0 mg urethan. Two months after injection, urethan-treated and 0.85% NaCl solution-treated control mice were test grafted with DBA/2 skin (H-2d → H-2k). In the 1st experiment, urethan caused a modest impairment of graft rejection as indicated by a skewing and flattening of the normal distributions of survival scores in treated mice: 64% of 36 urethan-treated mice had rejection times slower than the control median. Two months after grafting, lung adenomas were found preferentially in these mice rather than in mice with shorter rejection times.

In the 2nd experiment, thymectomy and sham thymectomy were performed 2 to 3 days before urethan treatment. Thymectomized mice of both sexes developed significantly more lung adenomas than did sham-thymectomized controls. Thymectomized males, but not females, had significantly larger adenomas than did controls. Among males, large adenomas occurred in slow allograft rejectors more frequently than expected by chance. Deaths from large, obstructive lung adenomas occurred exclusively among the slowest rejectors whether or not they had intact thymuses. Based on the combined data of the 2 experiments, allograft survival scores were significantly correlated with lung adenoma incidence. We concluded that skin allograft survival scores after urethan treatment provide a useful index of the risk of lung tumor development in BALB/c mice. This relationship can be explained by assuming that cell-mediated immunity normally modifies the expression of the carcinogenic effect of urethan on the mouse lung.

Our findings are interpreted as further evidence for the operation of immunological surveillance during chemical carcinogenesis.

INTRODUCTION

Prehn and Main (15) originally proposed that interference with normal immunological processes is a major part of the tumorigenic action of chemical carcinogens. The validity of this concept has been amply confirmed for the polycyclic hydrocarbon carcinogens: the tumors induced by these chemicals are usually strongly antigenic [see review by Smith (19)], and the hydrocarbon carcinogens are potent immunodepressants (16). The degree of depression produced by individual hydrocarbon carcinogens (as measured by plaque formation by spleen cells) has been shown to correlate highly with their carcinogenic activity (20). More importantly, the kind of immune depression produced by hydrocarbons like 3-methylcholanganthrene has been shown to favor the outgrowth of antigenic tumors (14).

The position of nonhydrocarbon carcinogens within this general framework is unclear. Urethan (ethyl carbamate), a multipotent nonhydrocarbon carcinogen, produces tumors that appear to be only slightly antigenic (13), yet gives the appearance of being a strong immunodepressant (12). This impression is largely based on the potent depressant effect of urethan on humoral immunity (9, 11, 16). The consensus, however, appears to be that carcinogenic doses of urethan are relatively weakly immunodepressive, both for humoral (2, 5) and for cellular immunity (7). This fact is compatible with the antigenic weakness of urethan-induced tumors (13): presumably, highly antigenic tumor variants would be subject to immune elimination or selection in a urethan-treated host. However, Lappe and Steinmuller (7) have predicted that even the weak immunodepressive effect of urethan might contribute to its carcinogenic activity.

Previous studies have shown that the relationship of immune depression to urethan-mediated carcinogenesis varies with the tumor system examined (7). For example, depression of humoral immunity, as measured by the degree of reduction of hemagglutinin or hemolsyn titers against sheep red blood cells, was found to correlate very highly with urethan leukemogenesis in C3Hf and SWR
mice (4, 12). However, another study (21) showed that depression of humoral immunity was unrelated to the risk of urethan lung adenomagenesis in one of these same strains (SWR). In this study, Trainin et al. found that while neonatal thymectomy potentiated lung adenoma formation after urethan treatment there was no correlation in individual mice between the extent of reduction of hemolysin titers and the number of lung adenomas that later developed.

An explanation for this paradoxical finding is that depression of cellular rather than humoral immunity is responsible for the facilitating effect of neonatal thymectomy on urethan adenoma formation. As a hypothesis, we propose that possession of cellular immune competence is a requisite for control of lung adenoma formation. Since a skin allograft model has shown that urethan per se interferes with cellular immunity (7), it should be possible to test this hypothesis by establishing a relationship between individual allograft survival time and lung adenoma formation in urethan-treated mice. A corollary of this hypothesis is that neonatal thymectomy facilitates urethan adenoma formation to the degree that it depresses cellular immunity.

These predictions were examined in newborn BALB/c mice which are highly susceptible to the immunodepressive (7) and lung adenomagenic (22) effects of urethan. An added advantage of using this strain is that neonatal thymectomy has a very small depressive effect on cellular immunity (8), giving an overlapping spectrum of graft survival scores between intact and thymectomized mice (cf. Ref. 7). We predicted that the extent of prolongation of allograft survival would be an index of subsequent tumorigenesis whether or not the thymus was present.

MATERIALS AND METHODS

The procedures used here are essentially the same as those reported previously (7). In the 1st experiment, 1 mg urethan was injected i.p. as 0.1 ml 1% solution of urethan in 0.85% NaCl solution to 4-day-old BALB/cCrgl mice of both sexes. A comparable number of 4-day-old controls were given injections of 0.85% NaCl solution. Two months later, all mice were grafted with skin from female DBA/2HeIcr mice as described above. Female recipients were excluded because previous experience had suggested that with this test system immune depression would only be detectable in males (7). All mice were examined monthly for evidence of tumors and those in respiratory distress were killed. All of the surviving mice were autopsied 17 months after urethan treatment and the number and size of lung adenomas were recorded. In both of the experiments, experimental and control mice were read “blind” in that neither their previous graft score nor their experimental group were known at the time of autopsy.

RESULTS

Experiment 1. The results of the first experiment are summarized in Chart 1. The skewed distribution of rejections for the urethan-treated mice (cross-hatched) as compared to the controls (white) suggests that urethan treatment impaired skin graft rejection in some males and females. MST's of urethan-treated mice averaged 1 to 2 days longer than those of controls. In accord with previous findings (7), this effect appeared to be more dramatic in the males (MST, urethan-treated = 15.6 ± 1.4 versus MST, controls = 13.8 ± 1.1) than in the females (MST, urethan-treated = 13.2 ± 1.4 versus MST, controls = 12.3 ± 1.2). However, group differences were not significant because of the wide spread of rejection scores in urethan-treated animals.

The total number of lung adenomas recorded for the mice rejecting on any given day is shown in Chart 1 below the histograms for each experimental group. There was a tendency for mice exhibiting the longest rejection times to have the largest number of lung adenomas. This trend is most evident in the males, although too few tumors were actually counted to permit analysis based on individual datum. Consequently, a statistical test of these data was performed by dividing the urethan treated mice into 2 groups: those with allograft rejections below the control MST, and those with allograft rejection above the control MST.

This compilation is presented in Table 1 which shows the incidence of adenomas in urethan-treated males and females partitioned according to allograft survival time. There was a tendency for more urethan-treated mice to
have allograft survival times above the control median (23 out of 36 or 64%), although this distribution could have arisen by chance ($\chi^2 = 2.78, 0.10 > p > 0.05$).

Lung adenomas, however, were partitioned nonuniformly between the 2 groups. Twice as many mice developed lung tumors in the group with rejection scores above the control median ("slow rejectors") than in the group with scores below the control median ("fast rejectors") ($17/23$ versus $5/13$, respectively; $\chi^2 = 4.30, p < 0.05$). Multiple tumors tended to be more frequent among slow rejectors ($10/23$) than among fast rejectors ($3/13$). This tendency was reinforced by the significant difference in the average number of tumors per mouse between the 2 groups. Slow rejectors averaged 1.39 tumors/mouse as compared to 0.62 tumor/mouse for fast rejectors ($t = 2.13, df/38, p < 0.05$).

**Experiment 2.** Eight mice in the 2nd experiment were killed prematurely because of respiratory distress. On autopsy, these mice were found to have large adenomas which had obstructed their breathing. There were previous allograft records for 6 of the mice. These mice had the longest allograft survival times (17 to 20 days) of all the mice grafted. An additional male with the next to longest graft survival (19 days) was killed at 14 months with a large skin papilloma on the dorsum. This male had developed another skin papilloma within a few weeks of test allografting at the site of the graft bed. No papillomas were recorded in urethan-treated mice with shorter survival times.

All the surviving mice autopsied at 17 months of age except 1 female had lung adenomas. The tumor incidence was comparable in both sexes so that the data are pooled for comparison. The results of the 2nd experiment are summarized in Table 2. By 17 months after urethan treatment there were significantly more tumors in the thymectomized mice than in the sham-thymectomized mice ($5.25$ adenomas/mouse versus $3.45$ adenomas/mouse, respectively; $t = 2.63, df/43, p < 0.02$). Calculation of an exact $p$ value by the Mann-Whitney $U$ test (17) gave $p = 0.0068$.

The average size of adenomas in thymectomized as compared to that in sham-thymectomized mice did not differ significantly for the groups as a whole (average tumor diameter was $3.02$ mm versus $2.72$ mm, respectively; $t = 0.93, df/182, p > 0.2$). However, the average lung adenoma size was significantly greater in thymectomized males than in sham-thymectomized males ($3.80$ mm versus $3.45$ mm, respectively; $t = 2.14, df/81, p < 0.05$). Seventeen urethan-treated males (10 thymectomized and 7 sham-thymectomized$^3$), which had been allografted when 2 months old, were assayed for lung adenomas. The incidence data for these mice have been pooled with the incidence data obtained in Experiment 1. Chart 2 shows the relationship between tumor incidence and skin allo-

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**Table 1**

*Predictive Value of Skin Allografts*

<table>
<thead>
<tr>
<th>Allograft survival times</th>
<th>Group</th>
<th>Below control median, $N = 13$</th>
<th>Above control median, $N = 23$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proportion with adenomas</td>
<td>No. of adenomas</td>
<td>Proportion with adenomas</td>
</tr>
<tr>
<td>Males</td>
<td>14</td>
<td>2/5</td>
<td>2</td>
</tr>
<tr>
<td>Females</td>
<td>13</td>
<td>3/8</td>
<td>6</td>
</tr>
<tr>
<td>Totals</td>
<td>5/13</td>
<td>8</td>
<td>17/23°</td>
</tr>
<tr>
<td>Average</td>
<td>38%</td>
<td>0.62/mouse</td>
<td>74% 1.39/mouse$^4$</td>
</tr>
</tbody>
</table>

$^a$ Differences between proportions significant at $p < 0.05$.

$^b$ Differences between means significant at $p < 0.05$. 

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$^3$ In accord with previous data (7), the MST was longer for the thymectomized males ($15.7 \pm 1.0$ days) than for the sham-thymectomized males ($14.2 \pm 1.2$ days), but the numbers are too small for significance.
Table 2

Lung adenoma incidence in sham-thymectomized and thymectomized BALB/c mice 17 months after a perinatal injection of 1 mg urethan

Fractions indicate number of tumors over the total number of mice in each category.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham thymectomy</th>
<th>Thymectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males (N = 19)</td>
<td>44/12</td>
<td>39/7</td>
</tr>
<tr>
<td>Females (N = 26)</td>
<td>56/17</td>
<td>45/9</td>
</tr>
<tr>
<td>No. tumors/no. mice</td>
<td>100/29</td>
<td>84/16</td>
</tr>
<tr>
<td>Average no. tumors</td>
<td>3.45/mouse</td>
<td>5.25/mouse*</td>
</tr>
<tr>
<td>Average tumor size</td>
<td>2.72 mm</td>
<td>3.02 mm*</td>
</tr>
</tbody>
</table>

* Difference between means significant at p < 0.02.
* Difference between means not significant.

Chart 2. Scatter diagram showing the plot of lung adenomas as a function of skin allograft rejection time for 57 mice in Experiment 1 and 2. Mice in the 1st experiment were autopsied 4 months and those in the 2nd experiment 17 months after urethan treatment.

Graph survival time in the 2 experiments as a plot of the number of lung adenomas against rejection time for 57 individual mice. The correlation coefficient for these 2 variables was calculated by performing a regression analysis on an IBM 6400 computer. There was a significant correlation between the number of lung tumors counted at autopsy in an individual mouse and its previous allograft survival score (r = 0.3055, df 55, p < 0.02).

An additional feature of Experiment 2 was that the mice which had had prolonged survival times when tested at 2 months after urethan treatment tended to have larger tumors 13 to 15 months later than did the mice which had had shorter survival times. Tumor sizes were indistinguishable between thymectomized and sham-thymectomized mice except as they pertained to allograft rejection time. Consequently, the data are pooled to facilitate comparison.

These data are presented in Chart 3. There are 3 or 4 mice in each graft survival category. The apparent relationship between increasing tumor size and allograft survival time was again tested by calculating a correlation coefficient for average tumor sizes and graft survival time. The value of the correlation coefficient was highly significant (r = 0.6014, df 15, p < 0.01), indicating that, in urethan-treated mice, lung tumor size as well as incidence is highly correlated with allograft survival.

DISCUSSION

The possibility that perinatally administered urethan depresses allograft immunity is reinforced by the previous study of Lappe and Steinmuller (7) who found that urethan treatment prolonged the survival of weakly incompatible allografts. We incorporated this possibility in analyzing the results of the 1st experiment. In Experiment 1, we assumed that urethan-treated mice with allograft survival times above the control median (slow rejectors) included those mice which had impaired immune competence. The fact that lung tumors were twice as common among such slow rejectors than among the remainder of the urethan-treated mice is interpreted as an indication that diminished immune competence was causally related to tumor development. This idea was rigorously tested by combining the tumor incidence of the first 2 experiments and calculating a correlation coefficient between skin allograft survival score and number of lung adenomas for each mouse. A similar test was done between allograft scores and lung adenoma size. The fact that there was a highly significant correlation between allograft survival time and both of the pairs of parameters examined confirmed our initial prediction.

The results of the 2nd experiment are consistent with this finding. As had been found in 2 other mouse strains, C3H and SWR (21), neonatal thymectomy facilitated urethan lung adenoma development in BALB/c mice.
Both thymectomized males and females developed significantly more tumors than did their corresponding sham-thymectomized controls. These facts fit the general pattern of potentiating of chemical carcinogenesis by neonatal thymectomy reported by most other investigators (see review by Miller (10)).

The most likely explanation is that, even in BALB/c mice, neonatal thymectomy impairs the normal development of the immune response (8). While it was not possible to estimate the extent of immune impairment accomplished by thymectomy in our experiment, it was evident that mice with longer allograft survival times generally developed larger adenomas. This occurred whether or not their thymuses were intact; for example, the first tumor-caused deaths in Experiment 2 occurred in the slowest allograft rejectors independently of thymic status.

The fact that thymectomized males but not females had larger adenomas than did intact controls is compatible with the greater susceptibility of male BALB/c mice to urethan-mediated immune depression (7). It may be that a considerable portion of the depressive effect of thymectomy in urethan-treated BALB/c's was obscured by the depressive effect of urethan per se. It was our impression that thymectomy was effective in potentiating lung tumorigenesis only to the extent that it contributed to the prolongation of skin allograft survival.

The previously cited study (21), in which no correlation was found between hemolysin titer and lung adenoma incidence in neonatally thymectomized mice, can now be explained in the light of our findings. It appears that immunological competence of the cellular type is more important than competence of the humoral type in modifying urethan lung adenoma development.

It is tempting to speculate that the poorer control of tumorigenesis in slow skin allograft rejectors is a reflection of an impairment of their ability to perform immunological surveillance against emerging neoplastic clones (3). The graded increase of tumor size and number with decreasing allograft reactivity (corroborated by the correlation tests) strongly supports this idea. Such results are in keeping with the findings of other tumor systems in which there was a strong inverse correlation between allograft reactivity and tumor incidence (1, 6).

We conclude that impairment of cell-mediated immunity facilitates urethan carcinogenesis in the mouse lung and that an allograft survival score soon after urethan treatment is of predictive value for determining the extent of such facilitation. Since urethan itself appears responsible for some impairment of allograft immunity (cf. Ref. 7), we suggest that urethan indirectly facilitates adenoma development by interfering with immunological surveillance. To the extent that this proves true, urethan behaves like the hydrocarbon carcinogens in favoring tumorigenesis through immune depression. We further suggest that, in some tumor systems, urethan may appear to be an incomplete carcinogen ("initiator") simply because it is a quantitatively weaker immune depressant than the hydrocarbon carcinogens.

REFERENCES

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