Stress and Murine Sarcoma Virus (Moloney)-induced Tumors

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SUMMARY

Stable stress-responsive models have been established in murine (Moloney) virus sarcomas, which lend themselves to further study of intervening immunological mechanisms. Maximum tumor size was increased in sex-segregated male BALB/c mice at 6 and 9 months of age and in females subjected to 3 days of electric shock stress following virus inoculation. Reduction in maximum tumor size was found in females shocked for 3 days prior to virus inoculation. Similarly, females that spontaneously showed fighting behavior developed smaller tumors. Possible endocrine and immunological factors contributing to these varied stress effects are discussed.

INTRODUCTION

Evidence strongly supports the notion that experimental manipulation can influence the induction and course of tumor growth in animals (16). Wide variation in results and differences in direction of influence may be ascribable to the multiplicity of tumor systems and different species and stress procedures used. A significant aspect of the problem has been the internal inconsistency of the course of the given disease. This inconsistency may account for the lack of investigation of underlying mechanisms in terms of metabolic, endocrine, and immunological influences. Thus, it is important to develop an experimental model that would permit focusing on the immune system, currently thought to be fundamental to the control of cancer (15) and to be stress responsive (27). Therefore, we have chosen to study stress effects on a tumor with a highly reproducible course of induction, growth, and regression and in which the role of immune defenses is well established (7). In this communication, we are reporting results concerning stable stress effects, which should lend themselves to study of relevant effects on humoral and cellular immunity.

We chose to explore 3 stresses in mice: sex-segregated housing in males, and electric shock and spontaneous fighting behavior in females. Separation of BALB/c males at weaning into all male groups or into groups of a single male housed with 1 or more females led to development of amyloidosis, autoantibody, and earlier death in the sex-segregated mice, but the course of virus-induced leukemia was not altered (5, 6). Electric shock did not influence the time of onset of Friend virus-induced leukemia in BALB/c mice (21), did not alter the incidence of lymphatic leukemia and methylcholanthrene-induced carcinomas in several strains (14), and did not affect the percentage of “take” or rate of growth of Walker 256 carcinosarcoma (1). Fighting in AKR males led to lower incidence and later onset of leukemia, particularly in the aggressors (17).

Tumor size may be affected directly by adrenocortical hormones or indirectly by their effect on immune mechanisms. Corticosteroids have been reported to render animals more susceptible to viral carcinogenesis (11, 29), and pretreatment with cortisone increased susceptibility to MSV2 tumors in weanling mice and decreased incidence of regression (24). A rise in the level of adrenocorticosteroids is expected as a result of stress (10). We previously demonstrated significant increases in plasma corticosterone levels (>2-fold) under similar electric shock conditions (28).

MATERIALS AND METHODS

MSV, obtained from Dr. A. Fefer, was injected into BALB/c neonates, and extracts of the resulting tumor were prepared as described by Moloney (20), with slight modifications. Extracts were brought to a final concentration of 1 g equivalent/ml. Two such extracts (MSV1 and MSV2) were used in these experiments. Preparations were stored for up to 1 year at −100° in sealed vials. Dilutions of the extracts in Hanks’ balanced salt solution (0.05 ml) were injected i.m. at uniform depth into the medial right hind thigh.

We chose tumor size as chief criterion of the effect of experimental manipulation. Animals were inspected daily until tumors appeared or for 7 days after extract injection. Subsequently, daily caliper measurements of the maximum diameter of right and left thighs were made, and the difference was defined as tumor size. Mann-Whitney ranking statistics3 of maximum tumor size were utilized to determine significance.

Corticosterone was determined by the method of Glick et al. (8). The chloroform extraction of serum was carried out in specially constructed apparatus.

Animals were obtained from Simonsen Laboratories, Gilroy, Calif., and were housed in our facilities for at least 1 week.

3The abbreviations used are: MSV, murine sarcoma virus (Moloney); ACTH, adrenocorticotropic hormone.

3The Mann-Whitney test is a nonparametric analog of the t test applicable when the t test is inappropriate because of distribution problems. In this case, the fact that some animals do not develop tumors makes the t test inapplicable and is also the reason why ranges are not shown on the charts.
prior to beginning of the experiments. Groups of fighting females and age-matched controls were selected by Simonsen Laboratories under our supervision. For purposes of sex-segregated housing, pregnant females were acquired and gave birth in our laboratories.

Shock apparatus consisted of 4 chambers, 8 x 9 inches, with grid flooring electrified alternately to prevent shock avoidance. Timing and randomization of shock were automated. A maximum of 8 animals was placed in a single chamber.

Specific experimental interventions and numbers of subjects are described in “Results.”

RESULTS

Virus Dose. For determining whether any stress effects might mimic virus dose reduction, MSV2 was titered over a 10-fold range of dilution (1:10, 1:30, 1:100). The most noticeable effect of decrease in the amount of virus inoculated was variability in induction time and in rate of tumor growth. A 10-fold decrease in virus dose shifted average maximum peak time from Day 13 to Day 17. Average maximum tumor size induced by the lowest dose was only 10% lower than that caused by the highest dose (Chart 1).

Adrenocortical Hormones. We tested the effect of ACTH on tumor growth to evaluate the influence of high levels of endogenous hormone concurrent with tumor development. Two groups of ten 8-week-old male BALB/c mice were given s.c. injections of ACTH gel (Armour Pharmaceutical Co., Kankakee, Ill.), 1 unit/animal/day, starting either 2 days prior to virus inoculation or 8 days afterwards, the day on which the tumor is expected to appear, and continuing to Day 16 after virus inoculation. ACTH-treated animals, bled on Days 3 and 10 after initiation of ACTH treatment at 2 hr after morning injection, showed average corticosterone levels of 420 (range, 200 to 660) and 400 ng/ml (range, 170 to 550), compared with pooled control levels of 40 and 80 ng/ml, respectively. Both ACTH-injected groups showed a statistically nonsignificant increase in maximum tumor size. ACTH treatment did significantly reduce the variability in maximal tumor size (\( p < 0.001 \)).

Pooled plasma corticosterone levels of fighting females (230 ng/ml) and their nonfighting controls (190 ng/ml) and of 8-month-old segregated males (90 ng/ml) and their mixed-sex housed controls (130 ng/ml) did not differ significantly.

Sex-segregated Housing. Sex-segregated housing was instituted after the pattern of Ebbesen (5, 6). Male littermates were either housed singly with 2 or 3 females or they were segregated into groups of 4 to 8 males at weaning. Litters born during the experiment were permitted to remain in the cage (11.5 x 7.5 inches) for 3 weeks; thus, cages occasionally contained as many as 30 animals. (In a previous experiment, no effect of crowding on maximum tumor size could be demonstrated.) Sex-segregated and control animals were inoculated with MSV from the same batch of virus at 3, 6, and 9 months of age. There was no difference between the 3-month-old groups (Chart 2A); but at 6 months, maximum tumor size was significantly increased (0.05 < \( p < 0.10 \)) in sex-segregated animals over controls (Chart 2B). The increase is much more marked (\( p < 0.001 \)) after 9 months (Chart 2C). In a 2nd experiment, a more significant difference (\( p < 0.001 \)) was found at 6 months (Table 1).

Electric Shock. For evaluation of effects of acute intermittent stress, female 7-week-old mice were shocked for 4 hr daily between 10 p.m. and 2 a.m., randomly, 10 times/hr. For a 10-sec period, 2.0 to 4.0 ma at constant current were administered. One experimental group (15 animals) was treated for 3 days prior to virus injection and then removed from the shock box. Chart 3 shows the results in comparison with littermate controls. Shocked animals showed a decrease in maximum tumor size as well as a lower incidence (\( p < 0.005 \)). These findings have been replicated in our laboratory

4 Ratios in this and subsequent charts represent proportion of animals with measurable tumors.

5 Data on maximum tumor size of all reported experiments are summarized in Table 1. Specific reference to the table is made only for experiments for which there are no charts.

Chart 1. Virus dose dependence of average tumor size of 6-week-old males. •, inoculated with MSV, 1:10 dilution, 10 out of 10; ○, inoculated with MSV, 1:30 dilution, 9 out of 10; ●, inoculated with MSV, 1:100 dilution, 9 out of 10.
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Chart 2. Average tumor size of animals injected with MSV, 1:10 dilution. Controls housed with 2 to 3 females after weaning. A, sex-segregated males, 3 months old. o, sex-segregated, 13 out of 16; •, controls, 12 out of 15. B, sex-segregated males, 6 months old. o, sex-segregated, 11 out of 15; •, controls, 12 out of 15. C, sex-segregated males, 9 months old. o, sex-segregated, 18 out of 18; •, controls, 10 out of 15.

with an even greater degree of significance (Table 1). A 2nd experimental group (10 animals) of 5-month-old female mice received the same schedule of electric shock administered for 3 days beginning on day of virus inoculation. Again, controls were littermates. Postinoculation shocked animals showed a significant increase in maximum tumor size ($p < 0.05$) as well as a higher incidence of tumors (Chart 4). These findings have now been replicated in a younger group of animals (Table 1). Maximum tumor size in older animals is considerably less than in younger females.

**Fighting Females.** Our animal supplier routinely destroys groups of female BALB/c mice that, for undetermined reasons, spontaneously manifest fighting behavior when housed in groups following weaning. Such groups represent a small and variable fraction of their production. Female mice housed in groups of 15 to 20 following weaning that spontaneously developed fighting behavior were inoculated with MSV at 5 to 7 weeks of age, as were comparable nonfighting control animals. Chart 5 shows that the maximum tumor size is significantly decreased ($p < 0.05$) in the fighting group.

**DISCUSSION**

We have demonstrated stable stress effects on the MSV tumor system, one in which cellular and humoral components of immunological rejection have been convincingly demonstrated (12). Our observations include both enhancement and suppression of tumor growth by different stress systems. It is known that stress-responsive hormones may have paradoxical effects on biological systems, which may be adaptive or maladaptive, depending on hormone level (24). Regarding immunological response, it has been shown that either too low or too high levels of adrenocorticosteroid fail to support lymph node growth in tissue culture (2). However, our results in ACTH-treated animals indicate that increased
Table 1

Cumulative distribution of maximum tumor size in all experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>No. of animals</th>
<th>0–50 (%)</th>
<th>0–100 (%)</th>
<th>0–150 (%)</th>
<th>0–200 (%)</th>
<th>0–250 (%)</th>
<th>0–300 (%)</th>
<th>0–350 (%)</th>
<th>0–&gt;350 (%)</th>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>15</td>
<td>13</td>
<td>20</td>
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<td>40</td>
<td>53</td>
<td>80</td>
<td>100</td>
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<tr>
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<td>Control</td>
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<td>27</td>
<td>33</td>
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<td>13</td>
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<td>53</td>
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</tr>
<tr>
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<td>36</td>
<td>36</td>
<td>36</td>
<td>73</td>
<td>80</td>
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<td>100</td>
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<tr>
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<td>18</td>
<td>5</td>
<td>22</td>
<td>28</td>
<td>67</td>
<td>94</td>
<td>100</td>
<td></td>
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<td>14</td>
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<td>47</td>
<td>60</td>
<td>73</td>
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<td>Control</td>
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<td>13</td>
<td>20</td>
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<td>33</td>
<td>53</td>
<td>67</td>
<td>100</td>
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<td>38</td>
<td>44</td>
<td>56</td>
<td>63</td>
<td>88</td>
<td>94</td>
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</tr>
</tbody>
</table>

| Tumor-reducing treatments           |               |                |          |           |           |           |           |           |           |           |         |
| Preinjection shock, 7 wk, female,   | Control       | 15             | 20       | 20        | 20        | 27        | 27        | 40        | 80        | 100       | <0.005  |
| Experiment I                        | Preinjection shock | 15         | 33       | 33        | 40        | 40        | 60        | 67        | 100       |           |         |
| Preinjection shock, 7 wk, female,   | Control       | 15             | 0        | 0         | 0         | 0         | 0         | 13        | 27        | 100       | <0.0002 |
| Experiment II                       | Preinjection shock | 15         | 7        | 7         | 7         | 7         | 40        | 53        | 88        | 100       |         |
| Fighting females                     | Control       | 15             | 13       | 20        | 27        | 33        | 33        | 53        | 67        | 100       | <0.05   |
| Fighting                            | Fighting      | 16             | 25       | 38        | 44        | 56        | 63        | 88        | 94        | 100       |         |

*a NS, not significant.

**corticosteroid production does not cause either increase or decrease in maximum tumor size. The absence of large changes in corticosterone levels due to sex segregation of males or to spontaneous fighting in females provides additional proof that this hormone, the most important corticosteroid in mice (9), does not per se cause the reported changes. Suppression of virus-induced tumor growth might be mediated by interferon, which we have previously shown to be**

Chart 3. Average tumor size of 7-week-old females. Shock treatment was administered for 3 days prior to injection of MSV, 1:100 dilution. ○, shock, 14 out of 15; ●, controls, 15 out of 15.
increased by acute stress (28). However, while interferon induced by polyinosinic-polycytidylic acid inhibited induction of MSV tumors in 4- to 6-day-old mice, it enhanced induction in 20-day-old animals (4). Thus, any stress-responsive increase of interferon in the age groups of our animals is not likely to account for tumor suppression.

Direct metabolic effects on viral infectivity or tumor growth cannot be excluded. However, no significant shifts in peak time of tumor comparable to effects of lowering virus dose were found. In the experiment on sex-segregated rearing, a proper control is difficult to establish. Factors present in the cages containing females and infants, such as different microbial flora, might contribute to the reported findings. Ebbesen (5, 6), however, has shown that castration or reserpine treatment of sex-segregated males reverses the effects of the housing condition, which are therefore dependent on hormonal or behavioral factors rather than on fortuitous differences in the environment. Nonsteroid hormone influences, particularly somatotropin and thyroxin, both of which are stress responsive (18, 19), could have direct effects on tumor growth but also are known to affect immune response (22, 23). We feel that immunological approaches offer the most promise in elucidating underlying mechanisms.

Immunologically based enhancement of tumor growth may be due to increase of early production of “blocking” antibody, to depression of cellular immunity, or to cytotoxic antibody levels (3, 13). These factors are now under investigation. In stressed rats, we have previously shown a decrease in antiflagellin antibody (26) and in cellular immune responses (A. A. Amkraut, G. F. Solomon, and A. Purdue, Stress-induced Suppression of Graft-versus-Host Reaction, in preparation). We have recently also demonstrated stress-induced enhancement of reaginic antibody production (unpublished data).

We observed a progressive decrease in maximum tumor size with age in mixed-sex housed males, which could be the result of sex hormone influence. We noted, however, a similar age-dependent decrease in females (Table 1). It is possible that repeated fortuitous exposure to tumor virus or a cross-reacting antigen might induce a low level of tumor immunity that is abolished by effects of sex segregation in males. If repeated infection by virus is a factor in maximum tumor size, the kinetics of formation of virus-neutralizing antibody must also be taken into account. These immunological variables are currently under investigation.

ACKNOWLEDGMENTS

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