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# Correction of a Murine Mammary Tumor Virus-associated Immunological Depression by Selective Immunosuppression with Cytosine Arabinoside[1]

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## SUMMARY

Mammary tumor virus (MTV) infection has been shown to be associated with a diminished hypersensitive reaction to methylated bovine serum albumin. Since methylated bovine serum albumin-induced hypersensitivity appears to be a mixed [humoral *versus* cell-mediated immunity (CMI)] reaction, the deficit in reactivity could be caused by, among other things, a direct depression of CMI or an increase in a humoral, blocking component. Assay of oxazolone-induced contact sensitivity and phytohemagglutinin-induced lymphocyte stimulation revealed normal or greater than normal CMI in MTV-positive animals. Treatment of MTV-positive and -negative animals with a regimen of cytosine arabinoside designed to inhibit only humoral immunity and leave CMI intact, corrected the deficit in methylated bovine serum albumin reactivity in MTV-positive mice. Thus, it is suggested that MTV infection may facilitate the production of interfering or blocking humoral immunity.

## INTRODUCTION

The effect of oncogenic virus infection on host immune reactivity has been generally viewed as being a nonspecific depression of both humoral immunity (3, 11-13) and CMI[4] (1, 6). However, it was recently reported by this laboratory that mouse MTV depressed MBSA-induced hypersensitivity, while having little suppressive effect upon humoral immunity. Indeed, in young adult female mice, the 7 S antibody response was stimulated by MTV infection. It was noted that the observed inhibition of the mixed (proposed to be humoral *versus* CMI) MBSA hypersensitivity could have resulted from either a direct depression of CMI or an increase of a humoral blocking component that interfered with the CMI (9).

The present report provides evidence that MTV infection does not suppress CMI directly, but may do so through facilitation of the production of interfering humoral immunity. This has been shown by the use of a drug regimen designed to inhibit humoral immunity without CMI (8). In addition, CMI function has been independently assessed by contact sensitization to oxazolone and by PHA-induced lymphocytic blastogenesis.

## MATERIALS AND METHODS

**Animals.** Strains C3HeB/FeJ, C3H/HeJ, BALB/c, and BALB/cfC3H mice, aged 2 to 4 months, were used for this study. Strains C3HeB/FeJ and BALB/c are MTV negative, whereas strains C3H/HeJ and BALB/cfC3H are MTV positive. The C3H lines were obtained from The Jackson Laboratory, Bar Harbor, Maine. The BALB/c lines originally came from the Cancer Research Laboratory, Berkeley, Calif., and have been maintained by brother-sister mating at Brown University since 1969.

**Assay of MBSA-induced Hypersensitivity.** Hypersensitivity to MBSA was induced by a modification of the method of Crowle *et al.* (4). Mice were given two 1-mg sensitizing doses of MBSA at an interval of 1 week. The challenge dose of 200 $\mu$g was administered into the footpad 14 days after the 1st MBSA injection. The same volume of 0.9% NaCl solution was injected into the opposite footpad. The edema that resulted was measured with a Gilson polygraph-transducer plethysmograph 24 hr after the injection of the challenge antigen, as outlined by Uyeki *et al.* (15).

**Assay of Oxazolone-induced Contact Sensitivity.** Animals were sensitized to oxazolone according to the technique of Zembala and Asherson (16) with slight modification. One-tenth ml of a 3% solution of oxazolone in ethanol was applied to the clipped abdomen of the mice. This was allowed to air dry. On Day 6 after sensitization, the animals were challenged by painting a 3% solution of oxazolone in ethanol on the left hind paw. The right hind paw was untreated and used as a control. Twenty-four hr later, the amount of edema in each paw was measured plethysmographically.

**Assay of Spleen Cell PHA Responsiveness.** Spleens were homogenized in 15-ml Ten Broeck homogenizers in 5 ml of culture medium. The clumps were allowed to settle out, and the rest of the suspension was centrifuged at 500 $\times$ *g* for 5 min at 4°. The pellet was washed twice in medium. The cells were then resuspended in medium at a concentration of 3.0 $\times$ $10^6$ nucleated cells per ml. Cell counts were done on a Model B Coulter counter. PHA-M (Difco, Detroit, Mich.) of the same lot number was used throughout. Stock solutions were prepared by dissolving the contents of each

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[4] The abbreviations used are: CMI, cell-mediated immunity; MTV, mammary tumor virus; MBSA, methylated bovine serum albumin; PHA, phytohemagglutinin; ara-C, cytosine arabinoside.

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vial in 5 ml medium, from which 2-fold dilutions were then prepared. Five-tenth ml of PHA was added to 1 ml of cell suspension in 12- x 75-mm Falcon plastic tubes. The tubes were incubated at 37° in a 5% $CO_2$ in air atmosphere. Triplicate cultures were prepared for each determination. The cultures were labeled after 2 days of incubation with 0.5 $\mu$Ci of [$^3$H]thymidine with a specific activity of 6.0 mCi/mmole (Schwarz/Mann, Orangeburg, N. Y.) and harvested 18 hr later. The cell suspension was filtered on a 0.45 $\mu$m Millipore filter (Millipore Co., Bedford, Mass.) and washed twice with 5% cold trichloroacetic acid for 3 min. The filter was placed in 20 ml of scintillation fluid (5 g PPO: 0.25 g POPOP:100 ml toluene) and radioactivity was counted in a Packard Model 3373 Tri-Carb liquid scintillation counter.

**ara-C Treatment.** ara-C was given on Days 1 to 5 of the 14-day sensitization period for MBSA hypersensitivity in a dose (20 mg/kg/day) which has previously been shown to inhibit production of humoral immunity while leaving CMI intact (8).

## RESULTS

**Response of MTV-negative and MTV-positive Mice to Oxazolone.** Our previous experiments had shown that C3HeB/FeJ (MTV-negative) animals respond well to MBSA, whereas C3H/HeJ (MTV-positive) mice, and C3HeB/FeJ mice foster fed on C3H/HeJ mothers, respond poorly to MBSA. It was felt that this might have been due to a compromised CMI response; therefore, since the response elicited 5 days after induction of oxazolone contact sensitivity is mediated solely by CMI (5), it was of interest to test the relative responsiveness of the MTV-positive and -negative animals to this antigen. As shown in Table 1, both strains responded well to oxazolone. In fact, although the difference is not significant, the MTV-positive (C3HeB/FeJ mice) had a greater response than did the MTV-negative (C3HeB/FeJ) mice. Similar results were obtained in the next series of experiments in which PHA responsiveness was assayed.

Table 1

*Oxazolone contact sensitivity in C3HeB/FeJ and C3H/HeJ mice*

| Strain | No. of animals | $\mu$l of edema |
|---|---|---|
| C3HeB/FeJ | 5 | 43.1 ± 6.8[a] |
| C3H/HeJ | 5 | 52.3 ± 8.9 |

[a] Mean ± S.E.

**Responsiveness of Spleen Cells to PHA of MTV-negative and MTV-positive Mice.** Spleen cells from BALB/c (MTV-negative), BALB/cfC3H (MTV-positive) and from C3HeB/FeJ (MTV-negative) and C3H/HeJ (MTV-positive) female mice were tested for their responsiveness to PHA by their ability to incorporate tritiated thymidine. Peak incorporation for all strains was found at a 1:32 dilution from stock of PHA. The data at this concentration are shown in Table 2. The complete curves of responsiveness for dilutions ranging from undiluted (1), in 2-fold steps, to 1:512 (10) are shown in Chart 1.

Responsiveness to PHA was greater with spleen cells from both strains of MTV-infected mice than with cells from their MTV-free costrains. Thus, the ratio of cpm in stimulated to nonstimulated cultures at the peak stimulation was 5.9 for the BALB/c, and 24.9 for the BALB/cfC3H ($p < 0.001$). Likewise, a ratio of 1.9 was found for the C3HeB/FeJ cells, compared with 10.4 for the C3H/HeJ cells ($p < 0.005$). The difference in reactivity was found throughout the PHA dilution curve, being significant at the 5% level, or less, at dilutions of 1:4 to 1:128 with cells of the BALB/c genotype and 1:2 to 1:512 with the cells of the C3H genotype.

**Effect of ara-C Treatment of MBSA-induced Hypersensitivity in MTV-positive and MTV-negative Mice.** Since ara-C treatment in a low-dose regimen seems capable of selectively depressing antibody production and leaving cell-mediated immunity intact, it was utilized in this experiment in order to attempt to investigate the participation of a humoral-blocking factor in the MBSA response. Shown in Table 3 and Chart 2 are the results of 2 experiments in which the difference between the response of the MTV-positive and that of the MTV-negative animals to MBSA is negated. In Table 3, doses of 10, 20, and 40 mg ara-C per kg had no significant effect in the MTV-negative (C3HeB/FeJ) mice; whereas, in the MTV-positive (C3H/HeJ) mice, ara-C at a dose of 10 mg/kg significantly stimulated MBSA-induced hypersensitivity. Chart 2 illustrates the same type of result, only in this case, 20 mg/kg significantly stimulated MBSA-induced hypersensitivity in both strains.

Table 2

*PHA response of spleen cells from MTV-infected and MTV-free mice*

| Mouse strain | MTV status | [$^3$H]Thymidine incorporation (cpm/culture) | | |
|---|---|---|---|---|
| | | Unstimulated | PHA-stimulated[a] | Ratio[b] |
| BALB/c | Free | 536.4 ± 58.1[c] | 3,148.3 ± 342 | 5.9 |
| BALB/cfC3H | Infected | 413.6 ± 68.1 | 10,307.7 ± 1,680 | 24.9[d] |
| C3HeB/FeJ | Free | 1,541.9 ± 303 | 3,071.2 ± 892 | 1.9 |
| C3H/HeJ | Infected | 1,224.0 ± 183 | 12,823.0 ± 3,249 | 10.4[e] |

[a] Dilution of stock PHA, 1:32.
[b] Mean cpm in stimulated cultures divided by mean cpm in control cultures.
[c] Mean ± S.E.
[d] $p < 0.001$ from BALB/c value.
[e] $p < 0.005$ from C3HeB/FeJ value.

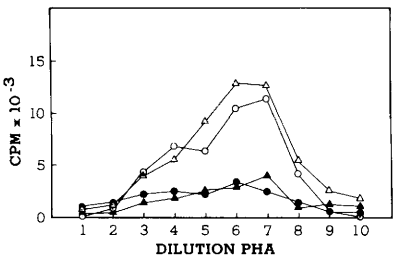


Chart 1. Mean cpm/culture of [$^3$H]thymidine incorporated by spleen cells from MTV-free and MTV-infected mice in 2-fold dilutions of PHA beginning with stock PHA as Dilution *1*. ●, MTV-free BALB/c; ▲, MTV-free C3HeB/FeJ; ○, MTV-infected BALB/cfC3H; △, MTV-infected C3H/HeJ.

Table 3

*Effect of ara-C on MBSA-induced hypersensitivity in MTV-positive and MTV-negative mice*

| Treatment | No. of animals | Strain | μl of edema |
|---|---|---|---|
| 0.9% NaCl solution | 5 | C3HeB/FeJ | 82.0 ± 14.2[a] |
| ara-C, 10 mg/kg/day[b] | 6 | C3HeB/FeJ | 66.4 ± 14.6 |
| ara-C, 20 mg/kg/day | 6 | C3HeB/FeJ | 55.6 ± 11.1 |
| ara-C, 40 mg/kg/day | 6 | C3HeB/FeJ | 43.9 ± 9.3 |
| 0.9% NaCl solution | 7 | C3H/HeJ | 31.9 ± 5.6[c] |
| ara-C, 10 mg/kg/day | 6 | C3H/HeJ | 57.2 ± 4.6[d] |
| ara-C, 20 mg/kg/day | 6 | C3H/HeJ | 48.9 ± 10.6 |
| ara-C, 40 mg/kg/day | 6 | C3H/HeJ | 46.3 ± 9.0 |

[a] Mean ± S.E.

[b] Drug given on Days 1 to 5 of the sensitization period.

[c] C3H/HeJ control is significantly different from the C3HeB/FeJ control at $p < 0.01$.

[d] C3H/HeJ, given 10 mg/kg/day of ara-C, is significantly different from the C3H/HeJ control.

These results are suggestive of a normal CMI response compromised by a strong humoral-blocking component in the C3H/HeJ (MTV-positive) mice.

## DISCUSSION

This report presents evidence that the observed diminished response to MBSA in MTV-infected animals is not due to direct depression of CMI reactivity.

Blair *et al.* (1) have shown that MTV infection is associated with depressed immune function in aging mice. That young adult MTV-infected animals, however, have an essentially normal (with respect to the non-MTV-infected costrains) CMI is supported by several observations. First, oxazolone-induced contact sensitivity, in its early stages, has a strong T-cell proliferation (5), and this response is stronger (or at least not diminished) in the MTV-infected animals. In addition, PHA responsiveness, a T-cell assay (7, 10, 11) was superior in spleen cells from MTV-infected animals. Finally, the pharmacological removal of the proposed blocking, humoral immune component of MBSA revealed equally strong CMI components in the 2 strains. We have observed that C3HeB/FeJ and C3H/HeJ mice do not differ in their acute inflammatory response to serotonin, as measured by paw edema (unpublished observation). It

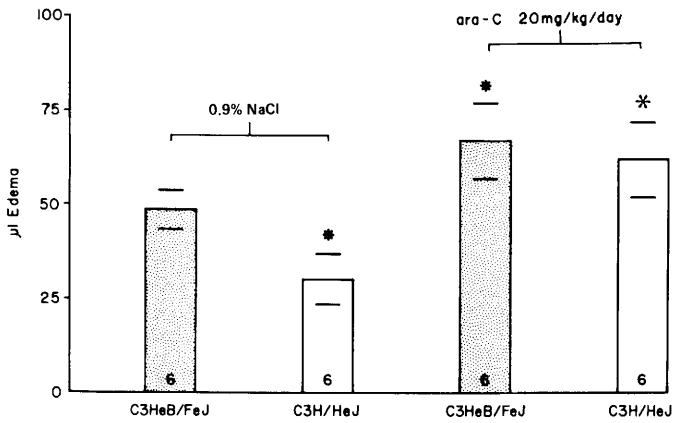


Chart 2. Effect of ara-C (Days 1 to 5, 20 mg/kg/day) on MBSA-induced hypersensitivity in C3HeB/FeJ (MTV-negative) and C3H/HeJ (MTV-positive) mice. The *numbers* in the *bars* are the number of animals used. *, Significantly different from C3HeB/FeJ control; *, significantly different from C3H/HeJ control.

seems reasonable, therefore, to speculate that MTV infection may facilitate the production of interfering humoral immunity. This is supported to some degree by the observation that C3HeB/FeJ female mice fostered from birth to weaning on C3H/HeJ mothers, and therefore MTV infected, show a greater response of 7 S hemolysin plaque-forming cells to sheep red blood cells than do their C3HeB/FeJ counterparts (9).

Since neither the cellular mediation of MBSA-induced hypersensitivity nor the exact mode of action of ara-C is fully appreciated, several possible explanations of these data must be entertained. That the mechanism of ara-C action is due to a selective depression of a B-cell population seems likely, but it is equally possible that specific T helper cell inhibition, specific T suppressor cell stimulation, or specific T effector cell stimulation could all explain its selective immunosuppressive action. However, low doses of ara-C (early) do not compromise or stimulate oxalone-induced contact sensitivity, the skin allograft response, or CMI to tumor isografts (2) suggesting that T effector cell stimulation does not occur.

Regardless of the mechanism involved, "selective" immunosuppression with ara-C stimulated a strong response in an animal which, without such treatment, responds poorly. Elucidation of the mechanism of this effect should further our knowledge of pharmacological manipulation of immune reactivity.

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