Cellular Immunity to Mammary Tumor Virus in Normal and Tumor-bearing C3H/HeN Mice

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ABSTRACT

Cellular immunity to mouse mammary tumor virus (MTV) in normal virgin female C3H/HeN-MTV+ (hereafter called C3H+) and C3H/HeN-MTV− (hereafter called C3H−) mice was assessed by the indirect agarose droplet migration inhibition (MI) assay. Spleen cells from C3H+ mice were able to respond to MTV antigens and produced a factor (presumably macrophage MI factor) that inhibited migration of normal mouse peritoneal exudate cells. This reactivity appeared at 14 weeks of age in C3H+ mice but was no longer detectable at 36 weeks. In contrast, reactivity was never displayed by spleen cells of C3H− mice. Injection of 10 μg of MTV in C3H+ or C3H− mice younger than 14 weeks of age induced similar reactivity to MTV antigens in both mouse strains. This pattern of reactivity suggested that normal C3H+ mice are not tolerant to antigens of MTV and that overt infection is required for induction of an immune response. Further investigations were performed to see whether the growth of a syngeneic transplantable mammary tumor (MAT-2) affected the MI response to MTV in both C3H+ and C3H− mice. A response to MTV antigens could be detected in C3H− mice younger than 14 weeks of age, but C3H+ tumor-bearing mice were generally unable to respond to MTV antigens. A similar pattern of results in MI tests with tumor-bearing animals was obtained with a glycoprotein with a molecular weight of 52,000, the major glycoprotein of MTV, as the antigen. The differences in MI reactivity of normal, MTV-immunized, and tumor-bearing C3H+ and C3H− mice that have been observed in these studies indicate that development of cell-mediated immunity to MTV antigens may be dependent on the dose, route, and length of exposure to MTV antigens.

INTRODUCTION

Humoral (5, 12, 13) and cellular (1, 6, 8, 9, 21, 22) immunity against MTV2 antigens have been reported in high spontaneous mammary tumor strains of mice, including C3H/He-MTV+ (8, 9, 22), RIII (9), GR (6, 13), and BALB/cfC3H (1, 6, 21), and in normal BALB/c mice that were presumably infected by horizontally transmitted MTV (2). Thus, the possibility of immunological tolerance to the virus in congenitally or neonatally infected mice, which was suggested in earlier reports (4, 10, 15, 17, 23), has largely been dismissed.

The development of cellular immunity to MTV appeared to be dependent upon the strain and age of the normal virgin MTV-infected mice. Blair et al. (1, 4), using visual MCA's, reported cell-mediated cytotoxic reactivity to MTV-associated antigens in BALB/cfC3H mice at 3 to 32 weeks of age, with the majority of the reactivity developing by 13 to 14 weeks. These authors also reported that some normal BALB/c mice that were not foster nursed also became reactive at around 14 weeks of age (2). Similarly, Stutman (22), using a [3H]proline MCA, found that a majority of C3H/He-MTV+ mice developed reactivity at 6 to 21 weeks of age, with the highest proportion of animals being reactive at 13 to 21 weeks, a time when hyperplastic alveolar nodules are first detected. In contrast to these reports of cellular immunity to MTV in mice neonatally infected with MTV, Lopez et al. (15), using direct capillary tube MI, failed to detect reactivity to MTV in BALB/cfC3H mice up to 12 weeks of age; however, normal BALB/c mice did become reactive by this age. Similarly, Creemers and Bentvelzen (6), using leukocyte adherence inhibition, failed to detect immunity to MTV in BALB/cfC3H and GR mice up to 10 weeks of age.

Some studies have attempted to relate the development of immunity to MTV to the development of spontaneous primary tumors. Blair and Lane (3) have shown that reactivity to MTV is increased in parous BALB/cfC3H mice as compared to virgin females, but decreased reactivity was seen in multiparous mice bearing large tumors. Similarly, Gillette and Lowery (9) detected reactivity to MTV in mice bearing small mammary tumors, whereas mice with advanced primary lesions were somewhat hyporeactive. Creemers and Bentvelzen (6) also reported that MTV-specific reactivity peaked when the primary tumor weight reached about 1 g and then later declined. They further observed that reactivity returned to about one-half the original level when the tumor reached a weight of 3 g. The highest titers of antibodies to MTV have been found in the sera of spontaneous tumor-bearing C3H mice (13). Whether the antibody titers in tumor-bearing mice may directly or indirectly influence cell-mediated responses to MTV is not known.

The disparity in results obtained by various investigators using different mouse strains and different assay systems makes it difficult to understand the relationship between MTV and host immunity. A better understanding of the interaction of the naturally MTV-infected host with virus-related antigens and the factors leading to development of immunity is required before rational attempts are made to manipulate the host immunologically for prevention of spontaneous tumors or for immunotherapy of established tumors.
To evaluate further the factors influencing development of cell-mediated immunity to MTV, we have measured reactivity to MTV and gp52 by the indirect agarose microdroplet MI assay (14, 16) in a mouse strain with a high incidence of spontaneous mammary tumors. For the elimination of the effects of genetic background on the results, normal mice and tumor-bearing mice of a C3H strain with a high spontaneous mammary tumor incidence due to the neonatal viral infection (C3H+) were compared with genetically identical mice which were not neonatally infected with MTV (C3H-).

MATERIALS AND METHODS

Animals

Female C57BL/6, BALB/c, C3H+, and C3H- mice were obtained from the Frederick Cancer Research Center animal colony, Frederick, Md. The C3H mice were freed of milk-transmitted MTV by cesarean derivation and foster nursing on C57BL/6 mice, a strain with little or no detectable infection with MTV (19). The C3H+ mice were obtained by inoculation of C3H- mice with purified C3H milk-derived MTV. Virgin C3H+ and C3H- female mice develop mammary tumors spontaneously at an average of 9 months, respectively (C. Hansen, personal communication).

Tumor

The third to sixth transplant generations of a mammary tumor (MAT-2), pathologically defined as an adenocarcinoma that spontaneously arose in a C3H+ exbreeder was used. The rate of tumor growth after s.c. inoculation was determined as previously described (7). Tumors were measured by calipers, and the approximate tumor weights were calculated by the formula

\[
\text{Tumor wt (g)} = \frac{\text{Tumor length (mm) \times tumor width (mm)^2}}{2 \times 1000}
\]

Antigens

MTV and gp52 were kindly provided by Dr. W. P. Parks, National Cancer Institute, NIH, Bethesda, Md. These reagents were obtained by density gradient centrifugation of tissue culture supernatants as described previously (20).

Indirect Agarose Microdroplet MI Assay

We used the indirect agarose microdroplet assay as previously described (14, 16).

In Vitro Synthesis of MIF by Spleen Cells from Individual Mice. Spleen cells (5 \times 10^6) from individual mice were suspended in 1 ml of Roswell Park Memorial Institute 1640 medium containing 1% FBS and then were cultured with or without (medium control) antigen for 4 hr at 37°C in a humidified 5% CO2 incubator. The cells were then centrifuged at 150 \times g for 10 min, and the supernatants were discarded to remove most of the antigen. The cells were then resuspended at 5 \times 10^6/ml with fresh medium containing 1% FBS and were placed in a 37°C humidified 5% CO2 incubator. After 48 hr the culture supernatants were collected, supplemented with an additional 9% FBS, and stored at −70°C until tested for MIF activity.

Indirect MI assay. Normal C57BL/6 mouse PEC were induced by i.p. inoculation with 3 ml of light mineral oil and harvested 3 to 4 days later by repeated washing of the peritoneal cavity with a total of 15 ml of cold Hanks’ balanced salt solution with 10% FBS and heparin (100 units/ml). PEC from 5 to 10 mice were pooled and washed 3 times at 200 \times g for 10 min. The PEC were then centrifuged at 200 \times g for 5 min, and the supernatant was removed. The cell pellet was briefly incubated at 37°C in a water bath and was resuspended by gentle agitation in 0.2% agarose (Marine Colloids, Rockland, Maine) at a concentration of 4 \times 10^6 PEC/ml. A 2-μl droplet of 0.2% agarose cell suspension containing 8 \times 10^6 PEC/droplet was placed in the center of each well of Costar plastic culture plates (No. 3596) with a microdispenser (Drummond Scientific Co., Broomall, Pa.). Each droplet was allowed to solidify at room temperature for 5 min, and then 0.1 ml of supernatant from antigen-stimulated or control cultures was added to each well. The plates were incubated for 24 hr at 37°C in a humidified 5% CO2 incubator. The migration area images were then projected onto paper by means of a Reichert microscope fitted with a \times 1 objective and a Zeiss projection tube (projective, f = 63 mm), and the areas were drawn. The areas of the agarose droplet and of the outer cell migration for each well were quantitated by planimetry. The net area of migration of the cells was then obtained by subtracting the inner agarose area from the outer cell area. A migration index was calculated by the formula

\[
\text{Migration index} = \frac{\text{Av. migration area of 4 replicates of supernatants from cultures with antigen}}{\text{Av. migration area of 4 replicates of supernatants from medium control cultures}}
\]

Statistical Analysis

In vitro survival data and in vitro MI values were analyzed by the nonparametric Mann-Whitney U-Test or by the Fisher’s exact test.

RESULTS

Immunity to MTV in Normal C3H/HeN Mice. Chart 1A summarizes the migration inhibitory reactivity of spleen cells from normal C57BL/6, C3H+, and C3H- mice younger than 12 weeks of age when triggered with MTV (1 μg protein per ml). We had determined earlier from dose-response studies with this antigen that 1 μg was optimal for inducing MIF. No statistical difference was observed among these 3 strains of mice by Mann-Whitney nonparametric analysis. A migration index cutoff value for this antigen of 0.85 was based on the mean migration index minus 2 S.D.’s, and all migration index values below 0.85 were considered positive. When C3H+ mice more than 14 weeks old were tested against MTV, all of 13 mice gave positive inhibition reactions, whereas only 1 of 13 C3H- mice was positive (Chart 1B). Reactivity of C3H+ mice was no longer detectable at 36 weeks of age, and the C3H- mice remained negative (Chart 1C).

Tumor-induced Immunity to MTV. For the determination of the effects of tumor growth on induction of cellular
immunity to MTV at a time prior to spontaneous appearance of reactivity, 8-week-old C3H+ and C3H− mice were inoculated with MAT-2. Table 1 shows that, when trocar fragments of tumor were injected s.c., progressive tumor growth was induced in all mice of both strains, and MdST's of both groups of mice were similar. In contrast, when single-cell suspensions of MAT-2 were injected s.c., some differences in tumor growth patterns between C3H+ and C3H− mice were noted. With 5 × 10⁵ tumor cells, MdST was significantly longer in C3H− mice (Table 1). Moreover, when a lower cell concentration (5 × 10⁴) was inoculated, C3H− mice developed a lower incidence of tumors compared to C3H+ mice. These data suggest that C3H− mice are more reactive immunologically against antigens of MAT-2 tumor than are C3H+ mice. To avoid major differences in tumor burden that could affect the in vitro analysis, we gave C3H+ and C3H− mice injections s.c. by trocar of fragments of MAT-2. Chart 2 shows that the tumor-bearing C3H− mice produced MIF in response to MTV. C3H− reacted significantly more (p < 0.01) than C3H+ mice on Days 12, 19, and 26. In addition, the amount of tumor present appeared to influence the degree of MI reactivity. A total of 3 of 28 (11%) C3H+ mice were positive on Day 19 when the tumors were medium sized (0.5 to 1.0 g), and C3H+ mice were negative at all other test times. A total of 6 of 12 (50%) C3H− mice bearing small tumors (0.05 to 0.2 g) produced MIF to MTV, and 23 of 27 (85%) animals were reactive as the tumor progressed to 0.5 to 1.0 g. The incidence of reactive C3H− mice decreased as the tumor burden increased thereafter, and when the tumors reached 4.0 g, only 2 of 7 (28%) mice were reactive. Thus, there appeared to be an eclipse (loss) in reactivity in C3H− mice with increased tumor burden.

Similar results were obtained when gp52, the major glycoprotein of MTV, was used as the antigen in the assay (Chart 3) at a concentration of 0.05 µg protein per ml (a dose that did not induce MIF production in normal C3H+ and C3H− mice younger than 14 weeks). Tumor-bearing C3H+ mice were again largely unreactive (2 of 10 positive mice when the tumors weighed 0.5 to 1.0 g), whereas tumor-bearing C3H− mice had significantly more reactivity,
and their reactivity varied with the extent of tumor. This reactivity of C3H- mice seems to be specific for MTV antigens since purified glycoproteins with molecular weights of 69,000 or 71,000, antigens obtained from Rauscher murine leukemia virus, did not induce production of MIF in the same mice (A. Tagliabue, unpublished observation).

Chart 4 shows the MI reactivity of C3H- tumor-bearing mice when tested simultaneously with MTV and gp52 antigens. The chart is broken into quadrants indicating the cutoff values for each antigen. The majority of mice either reacted to both antigens (21) or failed to react to either antigen (10). Linear regression analysis of the data revealed an R value of +0.563 and a significant correlation in reactivity to the 2 antigens (p < 0.01). Thus, the reactivity of the C3H- tumor-bearing mice against MTV was probably directed largely against its major glycoprotein, gp52.

Active Immunity to MTV. In an attempt to determine whether the lack of reactivity of tumor-bearing 8- to 12-week-old C3H+ mice was due to the inability of these young mice to respond to antigens of MTV, we gave i.p. injections of 10 μg of MTV to normal 8-week-old C3H+ and C3H- mice, and their ability to make MIF in response to MTV was assessed. Table 2 shows that all C3H+ and C3H- mice tested were reactive to MTV on Day 7 after immunization, whereas on Day 14 only 1 of 4 C3H+ and 1 of 4 C3H- mice were reactive.

Reactivity to MTV in Normal BALB/c Mice. We were interested in determining whether we could detect horizontal transmission of MTV reactivity by housing MTV- mice of a different strain with C3H+ mice for 8 weeks. For this purpose some 8-week-old BALB/c mice were put in isolation after arrival from the breeding farm. Other 8-week-old BALB/c mice were housed in a room where normal or MAT-2 tumor-bearing C3H+ mice were housed. After 8 weeks (i.e., at 16 weeks of age), both groups of BALB/c mice were tested for their ability to produce MIF in response to MTV. Only 1 of 10 of the isolated BALB/c mice was found to be reactive to MTV, whereas all 6 mice housed for 8 weeks in the same room with MTV-infected C3H+ mice were reactive to MTV (Chart 5).

DISCUSSION

The indirect agarose microdroplet MI assay used here measures the release of a soluble factor, MIF, upon recognition of antigen by previously sensitized lymphocytes. Using this assay we have demonstrated the development of cellular immunity to MTV in C3H+ mice, which have a high mammary tumor incidence. These data support the conten-
tion that C3H+ animals are not tolerant to the virus (1, 5, 6, 8, 9, 12, 13, 21, 22). The development of this immunity was not detectable until the animals were at least 14 weeks of age, and reactivity preceded visible primary tumor development. Immunity, however, disappeared by 36 weeks of age in these mice, at times when they presumably had subclinical tumors. These results confirm earlier studies that described cellular immunity in strains with a high incidence of spontaneous mammary tumors. Reactivity against mammary target cells or MTV has been detected in visual MCA’s (1), [14]proline MCA (22), direct and indirect capillary tube MI (9, 18, 21), lymphocyte proliferation (6, 9, 21, 22), and leukocyte adherence inhibition (6). Our data with C3H+ mice further suggest that discrepancies among some of the above-mentioned studies might be attributed to the different ages of the mice used in those assays.

Reactivity in MI against MTV was not detected in normal C3H– mice at any age, but it could be induced in these animals by inoculation with MTV (Table 2). Similarly, Ihle et al. (13) described a lack of development of antibodies to MTV in C3H– mice. Thus, it appears that spontaneous development in C3H mice of cellular as well as humoral immunity against MTV is dependent on neonatal infection with virus.

In contrast to the findings with C3H– mice, normal female BALB/c mice developed MI reactivity to MTV if they were housed for several weeks within the same room as MTV-bearing mice. This was presumably due to horizontal transmission of MTV, as previously reported by Blair and Lane (2). Others (1, 21) have also shown development of cellular immunity to MTV in normal BALB/c mice and have suggested that these animals are exposed to antigens later found on MTV-induced tumors (15) or that reactivity may be due to activation of an endogenous gene coding for MTV antigens (15, 21). Thus, the unreactivity of C3H– mice to horizontal transmission of MTV, in contrast to the reactivity found in BALB/c mice, suggests that the conditions for sensitization to environmental MTV infections may vary among different strains, and this is a further factor to be considered in studies of spontaneous immunity to MTV in normal mice.

In initial attempts to determine the effect of tumor growth on MI reactivity to MTV, we studied C3H+ and C3H– mice bearing the transplantable mammary tumor MAT-2 and found that reactivity appeared to be dependent on the virus status of the animal and also on tumor burden. Only a small proportion of tumor-bearing C3H+ mice reacted to MTV or gp52. This finding may be analogous to the loss of spontaneous MTV reactivity during mammary tumor growth, with peak activity when tumors weigh 0.5 to 1.5 g.

In contrast to the poor reactivity of C3H+ tumor-bearing mice, a large proportion of C3H– mice were capable of responding to MTV or gp52 when tumor burdens were up to 2 g in size. These data indicate that when MTV antigens, apparently mainly gp52, are presented to C3H+ mice on growing tumor cells, the C3H– mice are able to react more vigorously than do C3H+ mice. This may be 1 of the factors responsible for the greater resistance of C3H– mice to progressive growth of a small inoculum of MAT-2. In both strains of animals, with increased tumor burden the MI reactivity largely or totally disappeared.

The reasons for the major differences in MI in normal and tumor-bearing C3H+ and C3H– mice remain to be determined. Since these strains are genetically identical and differ only in their MTV status, the presence of virus from birth must in some way account for the later patterns of reactivity upon exposure to exogenous MTV via environmental exposure or growth of MTV-positive tumor cells.

REFERENCES


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