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Suppressed Development of Mammary Tumorigenesis in R III Mice Treated Neonatally with BCG (40178)

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Numerous studies during the last few decades utilizing Bacillus Calmette Guerin (BCG) for nonspecific stimulation of antitumor immunity have provided a broad base of information concerning the possibility of enhancement of resistance to a variety of tumors (1–3). Animal studies in particular have given encouraging results concerning nonspecific immunostimulation by BCG in a variety of tumor model systems (4–7). Much less consistent results have been obtained in clinical trials (1, 3), except in certain select tumor systems (8, 9). However, it is apparent that many variables including "host factors" can inherently affect host responsiveness to nonspecific immunostimulation, and this can be best controlled in experimental animal models. In this regard, development of mammary adenocarcinomas in highly inbred strains of mice vertically infected with the mammary tumor virus (MTV) has been widely utilized as a model for various breast cancer studies (10, 11). For example, more than 95% of female R III strain mice develop mammary tumors. A number of factors influencing tumor development in these mice infected in utero by natural passage of MTV has been recently studied. In the present investigation, the effect of infection of R III mice between 3 to 14 weeks of age with BCG on the spontaneous development of virus induced mammary adenocarcinomas was examined. The choice of this animal model system was based on the characteristic that "spontaneity" of mammary tumor development in this mouse strain closely simulates neoplastic development observed in the human situation.

Methods and materials. The albino R III inbred mouse strain in this study has been described in detail (10). These mice are a carrier of MTV propagated vertically from mother to neonate via milk. At least 95% of the females of this strain develop "spontaneous" mammary adenocarcinoma between 90–350 days of age. This strain was originally derived from Bittner's strain and has been maintained for over 40 years by Dr. Dan Moore (Institute for Medical Research, Camden, NJ). The breeding program in this laboratory was initiated from stock kindly supplied by Dr. Moore several years earlier. Female weanling mice ranging in age from 3 to 14 weeks were used for treatment. These animals were injected with viable BCG obtained from the Research Foundation, Chicago, Illinois. One ampule of lyophilized BCG was resuspended into 1 ml sterile saline and diluted with McCoy's 5A medium (Flow Laboratories, Rockville, Maryland) to a final concentration of approximately 1×10^7 infectious units of microorganisms per ml.

One group of 65 experimental mice received a single subcutaneous injection of 0.1 ml BCG (1×10^6 infectious units) by a single time inoculation at selected intervals during the first few months of life. A control group of 60 weanling mice consisted of equal numbers of R III animals most of which were litter mates of the BCG injected mice. The controls were given a placebo inoculation of a similar quantity of McCoy's medium alone at the same time intervals. The BCG treated mice were placed in an isolation room in which conditions of temperature and humidity were the same as that of the non-BCG treated animals housed in a separate room. Animals of both groups were fed Purine mouse pellets and water *ad libitum* over their entire life span.

Visual examinations of each mouse in all groups were made at weekly or semi-weekly intervals for evidence of overt mammary tumor formation. As tumors appeared, date of initial tumor manifestation was determined. Mice which died were autopsied to determine

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the cause of death whenever possible. Tumors, whenever possible, were examined histologically to ascertain that they were indeed mammary adenocarcinomas.

Results. The rate of mammary tumors and death in mice given a single injection of BCG during the first three months of life was markedly lower during the first year of life as compared to the control group of animals (Fig. 1). The first signs of tumor development in the control mice generally became evident during the 12th to 20th week of life. However, tumors in BCG treated animals did not become detectable usually until after the 30th week of life. Thereafter, there was lower incidence of mammary tumor development and death in the BCG treated animals as compared to controls. The average age at death of BCG treated mice was much later than for the control mice, which developed tumors and died at a much earlier age. All of the control untreated animals had developed tumors and/or died at the 54th week of life. At this time, fewer than 30% of the BCG treated animals had died. Nevertheless, by the 100th week of age, all of the BCG treated animals developed mammary adenocarcinomas and succumbed to the tumor or died of other causes.

There was no significant difference between the groups of BCG treated animals which had been injected with the microorganisms at varying times during the first 2 months of life. As can be seen in Table I, 6 of 29 mice injected between 3 and 4 weeks of age with BCG developed mammary tumors at 1 year of age for an incidence of 21%. At this time nearly all controls had died. The average day of tumor development for these six animals was 228 ± 78 days. The other animals in this group did not develop tumors until at least 400 days after birth. Mice injected with BCG at 5 to 8 weeks of age also showed a low incidence of tumors at one year of age (2 out of 26 animals for a tumor incidence rate of 8%). The age at tumor development was 264 and 312 days for the two positive mice. Animals injected with BCG between 11 and 14 weeks of age also showed a low tumor incidence (1 out of 10 for a 10% rate). This tumor appeared at 310 days. However, in all cases, those mice which showed no evidence of tumor by one year of life all developed tumors by the end of the second year.

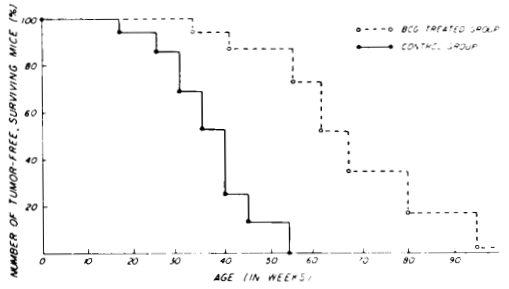

FIG. 1. Incidence of mammary adenocarcinomas in high tumor incidence R III strain female mice treated within the first to third month of life with a single dose of BCG (1×10^6 infectious units) subcutaneously as compared to control untreated mice (60–65 mice per group were used at the start of the experiment). Differences between mean survival time of treated vs control mice statistically significant ($P = <0.05$ by Student's t test).

TABLE I. INCIDENCE OF MAMMARY TUMORS DURING THE FIRST YEAR OF LIFE OF R III MICE INJECTED BETWEEN 1 TO 3 MONTHS OF AGE WITH BCG.

Age at BCG inoculation (wks)[a]	Tumor Incidence[b]	Average at first detection[c]
3–4	6/29 (21%)	228 ± 78
5–8	2/26 (8%)	288 ± 24
11–14	1/10 (10%)	310
None (controls)	52/53 (98%)	261 ± 67

[a] Mice infected subcutaneously as indicated with 1×10^6 live BCG (infectious units).

[b] Number of mice showing evidence of tumors on overt examinations by 365 days of age in each group. Parenthesis is percent with tumors.

[c] Average ± standard error at which mice first showed overt tumors per group (age in days).

[d] Control mice given placebo only. (Eight of sixty control mice died of nontumor causes during first year of life.)

Discussion. These results indicate that a single injection of BCG during the first two months of life may alter the average age at which mammary tumors appear in R III mice and the survival rate of the animals during the first 1–2 years of life. Although at the end of the second year animals from both groups had developed tumors and succumbed to the disease, there was a consistent difference in the rate of tumor development between the BCG treated and control groups. The group of animals injected with BCG at about 3–4 weeks of life showed a higher rate of tumor development during the first year of life, as compared to animals injected between 5 to 8

weeks of life, although due to small numbers it is difficult to assess the significance of these data. The smaller group of mice injected during the 3rd month of life also developed a much smaller percentage of tumors, suggesting that if BCG is indeed altering the incidence of spontaneous MTV induced mammary adenocarcinomas in these animals by an immunologic mechanism, that animals treated during the first month of life, when the immune system may be weakest, may have the least response. It has been well documented that immunologic maturity, both of the B and T lymphocyte system, is more pronounced after the first month of life (12). Thus administration of BCG during the 2nd and 3rd months of life, prior to any overt development of adenocarcinoma, may be more optimal in stimulating a nonspecific host defense based on immunologic mechanisms.

It seems noteworthy that earlier studies by Old *et al.* (6) in a similar spontaneous mammary tumor system studying BCG and mammary tumors (but BCG was not given until 7–8 weeks after birth) gave results which are much like those seen here. However, that study lasted less than one year and mortality within the control group was considerably lower (i.e. 50%) at the time of termination of the experiment. When transplanted mammary tumor was studied by Old *et al.* (6) BCG still enhanced survival rates and resulted in decreased tumor diameter. Conversely, Weiss showed that specific immunotherapy for spontaneous mouse mammary tumors resulted in increased tumor susceptibility, as measured by tumor size (7).

Studies are in progress to determine whether the BCG treated R III animals show increased specific or nonspecific reactivity against MTV *in vitro* and *in vivo* and whether or not there are immunologically mediated mechanisms which can be related to the slower rate of development of adenocarcinoma in the animals. Furthermore, the effect of BCG on R III mice which have established mammary tumor adenocarcinomas is being investigated in the model system described here.

Summary. The effects of Bacillus Calmette Guerin on the development of spontaneous virus induced mammary adenocarcinomas in the mammary tumor prone R III strain of mice was investigated. A single injection of 1×10^6 infectious units of living BCG administered to mice between 3 to 4 weeks of age was found to significantly inhibit the rate of development of mammary adenocarcinoma as compared to control mice receiving a placebo injection of medium only. The incidence of spontaneous mammary tumors was essentially identical in both groups (96 vs 98%). However, mammary tumors developed at a consistently later time in the BCG treated animals as compared to the appearance of tumors in the control mice. Mice injected with BCG during the 2nd and 3rd month of life showed a moderately slower rate of development of tumors as compared to animals injected during the 3rd and 4th weeks of life. The possible role of BCG in nonspecific stimulation of resistance to the tumor virus mediated by immunologic factors seems likely.

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