larities probably arose independently.

Note added in proof: After this report went to press, P. G. Haneline and S. M. Case undertook an electrophoretic comparison of enzymes in the specimens whose albumins we had compared immunologically. There is approximate agreement between the electrophoretic and immunological results. In particular, the comparison of enzymes encoded by ten genes indicates that whereas the major alleles at most loci in "*wrightorum*" are identical electrophoretically with those of *H. eximia*, the major alleles at most loci in *H. regilla* are different. We are grateful to Haneline and Case for permission to refer to the electrophoretic evidence, which they intend to publish in full later. These workers are affiliated with the California Academy of Sciences, San Francisco, and the Department of Zoology, University of California, Berkeley, repectively. Their electrophoretic studies are being carried out in S. Y. Yang's laboratory, Museum of Vertebrate Zoology, University of California, Berkeley.

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29. A referee has suggested that the albumins of "*wrightorum*" and *H. regilla* could have undergone rapid divergent evolution despite a recent common ancestry of these frogs. If this were true, the "*wrightorum*" and *H. regilla* albumins should differ more from *Litoria* albumin than do the albumins of the other hylids in Fig. 1. This is not so.
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Malignant Melanoma: Specific Immunity Induced by Bacillus Calmette-Guérin in BALB/c Mice

Abstract. *Previous treatment of BALB/c mice with bacillus Calmette-Guérin (BCG) will significantly protect them against intramuscular challenge of S-91 melanoma but not against a mammary carcinoma or a methylcholanthrene-induced sarcoma. Lymphocyte-mediated cytotoxicity studies correlate with in vivo data in showing that BCG-immune lymphocytes are specifically cytotoxic to S-91 melanoma target cells but not to the carcinoma or sarcoma target cells.*

Injection of bacillus Calmette-Guérin (BCG) into animals produces not only specific immunity to tubercle bacilli but also leads to development of nonspecific immunity to a variety of foreign antigens (*1*), among which are a number of malignant tumors. In humans, a nonspecific imune stimulation by BCG has been employed in the treatment of sarcomas (*2*), leukemia (*3*), and, most notably, malignant melanoma (*4*). Although animal investigations have shown nonspecific BCG-induced regressions of sarcomas (*5*), mammary carcinoma (*6*), and leukemia (*7*), there is an absence of animal studies demonstrating the effectiveness of BCG in the prevention and treatment of melanoma. We now document BCG-induced protection against a murine malignant melanoma and demonstrate preliminary evidence that suggests the possibility of a specific BCG-induced immunity to this malignant melanoma.

Three different malignant tumors—a melanoma (S-91), a mammary carcinoma, and a methylcholanthrene-induced sacroma—were used in syngeneic BALB/c mice. Test animals received 0.1 ml of BCG (Research Foundation,

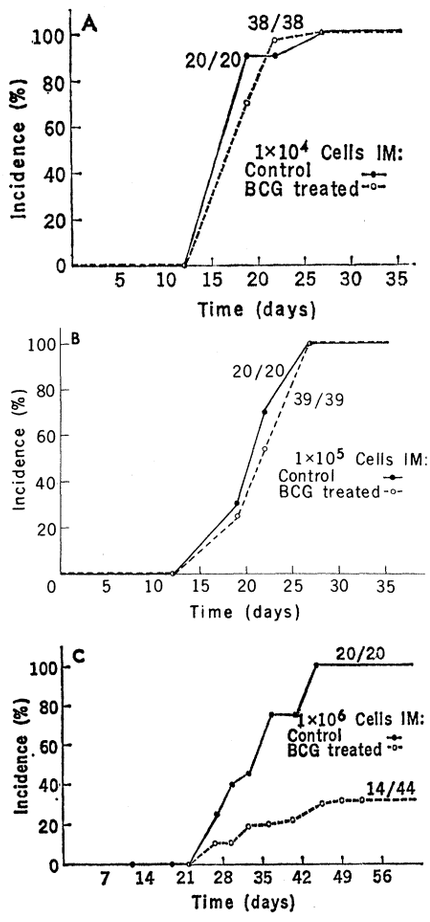

Fig. 1. (A) Incidence of sarcoma in control mice and those treated with BCG. (B) Incidence of mammary carcinoma in control mice and those treated with BCG. (C) Incidence of malignant melanoma in control mice and those treated with BCG. *IM*, intramuscular.

Chicago) containing 1×10^8 viable organisms in the footpad on day 1, intramuscularly on day 7, and intraperitoneally on days 21 and 35. Test animals were then randomly divided into three groups, and each group was injected on day 42 with a single suspension of cells from one of the tumors. The number of tumor cells injected was the lowest dose of each tumor that had been found to grow in 100 percent of control animals after intramuscular injection. Injections of 1×10^6 melanoma cells, 1×10^5 carcinoma cells, and 1×10^4 sarcoma cells were utilized. Animals were examined for tumor incidence twice weekly. The results are shown in Fig. 1. When compared to controls, growth of the carcinoma and the sarcoma was not influenced by prior treatment with BCG. However, mice pretreated with BCG and then challenged with 1×10^6 melanoma cells, given intramuscularly, showed a definite delay in the onset of tumor development, as well as a significantly lower tumor incidence in comparison with controls ($P < .001$).

Lack of immunogenicity of the carcinoma and the sarcoma was not responsible for the absence of a response to previous treatment with the BCG. We have demonstrated that these tumors are highly immunogenic on the basis of tumor rejection after amputation of growing tumors and rechallenge with suspensions of tumor cells.

Lymphocyte-mediated cytotoxicity tests were performed to determine whether similar results could be demonstrated in vitro. Lymphocytes immune to BCG were obtained from the spleens of BALB/c mice immunized with BCG in the same manner as that described for the in vivo experiment. One week following the fourth injection of BCG, spleen homogenates from ten mice treated with BCG were passed through nylon-wool columns, which resulted in 95 to 99 percent pure lymphocyte preparations. Tumor-specific immune lymphocytes were obtained from animals made immune to each respective tumor by amputation of growing tumors followed by challenge with suspensions of live tumor cells. One week after the challenge with tumor cells, spleens were removed from ten animals immune to each tumor, and lymphocyte preparations were made by means of the nylon-wool method described above. Control lymphocytes were obtained in a similar fashion from the spleens of normal BALB/c mice.

The cytotoxicity tests were performed as follows. Target cells from each of the three tumor cell lines were plated out in Microtest (Falcon Plastics) plates (approximately 150 cells per well), after which control lymphocytes and lymphocytes from tumor-immune and BCG-immune mice were added at a ratio of 50 : 1 to target cells. Thirty-six replicate wells were included for each set of lymphocytes to be tested, and the entire experiment was conducted twice. After 48 hours of incubation at 37°C in an atmosphere containing 5 percent CO_2, lymphocytes were removed, the plates were stained, and the remaining viable target cells were counted. Cytotoxic indices (CI) for each well were calculated as follows:

$$CI = \frac{\text{No. VCC} - \text{No. VTC}}{\text{No. VCC}}$$

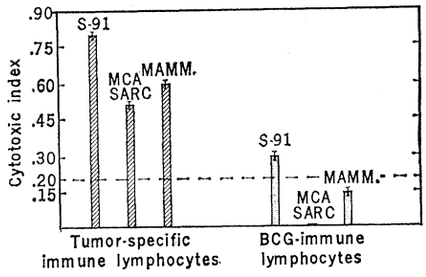

Fig. 2. Results of lymphocyte-mediated cytotoxicity utilizing specific immune lymphocytes and BCG-immune lymphocytes against tumor target cells. Cytotoxic indices against methylcholanthrene-induced sarcoma (*MCA SARC*), mammary carcinoma (*MAMM.*), and S-91 melanoma for tumor-specific immune lymphocytes and BCG-immune lymphocytes.

where VCC is viable control cells and VTC is viable test cells. A CI of > 0.20 was statistically significant ($P < .05$).

From the data (Fig. 2), it can be seen that lymphocytes immune to each of the tumors demonstrated significant cytotoxicity against target cells of their respective tumors ($CI > 0.20$). However, BCG-immune lymphocytes showed significant cytotoxicity only against S-91 target cells ($CI = 0.30$), and, furthermore, this cytotoxicity was significantly different from that seen against the mammary carcinoma ($P < .05$) and the sarcoma ($P < .01$). These results are identical to the in vivo experiment and they suggest the possibility of a specific BCG-induced protection against malignant melanoma in an inbred strain of laboratory mice.

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