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Differential Effects of Two Strains of BCG on Transplantation Immunity to SV40-Induced Tumors in Hamsters[1,2] (38281)

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Mycobacterium bovis, strain BCG, has been shown to augment immunity to malignancy in many experimental immunoprophylactic and immunotherapeutic models. In inbred guinea pigs with transplanted intradermal, syngeneic hepatocarcinomas, BCG has proven highly effective in causing regression of established tumors as well as elimination of regional lymph node metastases (1–3). Immunization with BCG in combination with tumor cells is more effective than either BCG or tumor cells alone for stimulating transplantation immunity (4–6). BCG alone, by various routes of administration, can be protective (7–10), have no effect (9, 10), or on occasion cause enhancement of tumors (9–11) in experimental animals. A similar range of effects has been reported when BCG has been used for treatment of malignancies in man (12–16).

Variables which have been cited in the use of BCG to stimulate immunity against malignancies in experimental models are: source or substrain of bacterium, dose, number of viable organisms, and time and route of administration. A less apparent, but in our experience, critical variable is the ability of the experimental animal to control the infectious process established by the live BCG. This is extremely relevant in developing reliable experimental models for establishing alternatives for therapy in man where BCG infection is limited or can be controlled by drugs.

The induced immune response against tuberculosis after vaccination with BCG is thought to be due to the microorganism being sufficiently virulent to multiply freely until immunization is achieved (17, 18). Morbidity studies in the hamster have shown that BCG preparations differ widely in their virulence (17). The beneficial effect of tumor immunotherapy by intralesional injection of transplanted intradermal tumors in guinea pigs is related to virulence, since the viable organism must grow long enough to establish a granulomatous reaction in the tumor and in regional lymph nodes, with the infection eventually being abolished (19, 20). However, in experimental animals other than guinea pigs (*i.e.*, mice, rats, and hamsters), typical BCG infection is usually progressive (17, 21, 22), and in these systems enhancement of malignancies has been frequently detected (9–11).

In order to gain insight into the basis of protection or enhancement as a function of BCG treatment, we chose the transplantable hamster–SV40-tumor system. This model is advantageous because the tumor immunology has been well-characterized (23) and the hamster has been used to evaluate BCG strains for their virulence in relation to their use as live vaccines for tuberculosis (17). A correlation is made between immunoprophylactic and immunotherapeutic properties and the growth culture kinetics and morphology of a lyophilized and live frozen source of BCG.

Materials and Methods. LVG/LAK male Syrian golden hamsters, 6 weeks old, were used in these studies. The tumor cells were SV40-transformed F5-1 cell line, derived *in vivo* in an LVG/LAK hamster following neonatal in-

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fection. The cells were propagated as previously described (24). The F5-1 line has never been observed to yield infectious virus and contains T, S, TSTA, and fetal antigens. Previous studies in this system have shown that 10^7 irradiated F5-1 cells given intraperitoneally induce complete protection against the tumor challenge of up to 5×10^6 F5-1 cells (24).

One BCG strain selected was the Phipps strain, obtained from the Trudeau Institute, Saranac Lake, New York, and frozen at −70° until just before use. This preparation was compared to the Glaxo preparation of a Danish strain of BCG produced by Glaxo Ltd., which was in lyophilized form and reconstituted with water just before use. Total organism counts were determined by counting serial dilutions in a Coulter Counter set at a 30-μm orifice. The morphologic characteristics and growth kinetics of these two strains were determined by surface growth on Dubos oleic albumin agar in a tenfold dilution series. The plates were examined weekly for 4 weeks.

Transplantation of tumors was achieved by a subcutaneous injection of 5×10^4 F5-1 cells in the upper-left quadrant. From previous experience in our laboratory, this dose of tumor cells was expected to produce tumors in about 60% of the animals. We chose this dose of cells to enable us to detect both enhancement of and protection against tumor development as a result of immunization.

The immunizations were administered intradermally in 0.2-ml volumes on the side contralateral to tumor cell challenge. Animals were immunized with 5×10^6 irradiated F5-1 cells (5,000 R X-ray) and/or 2.5×10^5 viable BCG. The treatment groups of animals (40 animals/group) were immunized as follows: (a) no immunogen, (b) Phipps strain, (c) Glaxo strain, (d) irradiated F5-1 cells, (e) irradiated F5-1 cells admixed with Phipps strain, and (f) irradiated F5-1 cells admixed with Glaxo strain. All immunizations were performed 2 weeks prior to tumor-cell challenge.

Hamsters from all groups were randomly chosen and killed at several intervals (3–5 animals per timepoint) for histology and gross pathology. Paraffin-embedded, 5-μ tissue sections were stained with hematoxylin and eosin for histopathology and with the Ziehl–Neelson procedure for acid-fast organisms.

For intralesional injection of BCG, 5 or 10 tumor-bearing hamsters were randomly chosen from groups 1, 2, and 3. The animals were intralesionally injected with 2.5×10^5 viable BCG in 0.2 ml volume. The unimmunized and Phipps-strain-immunized tumor-bearing hamsters received intralesional Phipps-strain injection, and the Glaxo-strain-immunized tumor-bearing hamsters received intralesional Glaxo-strain injection. The average diameter of tumors on the day of injection was between 7 and 17 mm. Measurements were made on tumors twice weekly.

Results. The total counts/ml and the growth kinetics of BCG are shown in Table I. With respect to BCG colony count on solid media, the Phipps strain grew faster than the Glaxo strain. Peak growth was obtained in 2 weeks by the Phipps strain, whereas the Glaxo strain required 3 weeks to each maximum growth. The Phipps strain formed large, spreading colonies on a solid media (Fig. 1a). They had dense, opaque centers surrounded by more transparent, irregular edges. The colony surface was wrinkled, consisting of closely folded serpentine cords. The colonies spread until they coalesced into large masses. The Glaxo strain formed smaller colonies of two sizes, both of which were smaller than the Phipps strain (Fig. 1b). The colony surface had a beady appearance, but formed no serpentine cords.

The effect of BCG on the development of SV40-induced tumor transplantation immunity in the hamster can be seen in Fig. 2. Immunization with Phipps strain delayed tumor appearance, although final tumor incidence was only

TABLE I. BCG Growth and Particle Counts.[a]

Strain	Particle count	2nd week colony count	3rd week colony count
		counts/ml	
Glaxo	3.0×10^7	1.7×10^6	2.0×10^6
Phipps	1.7×10^7	3.9×10^6	5.2×10^6

[a] Coulter counter settings: Orifice—30 μm; 1/aperture current—0.707; amplification—4; lower threshold setting—7; and sample volume—5 μl.

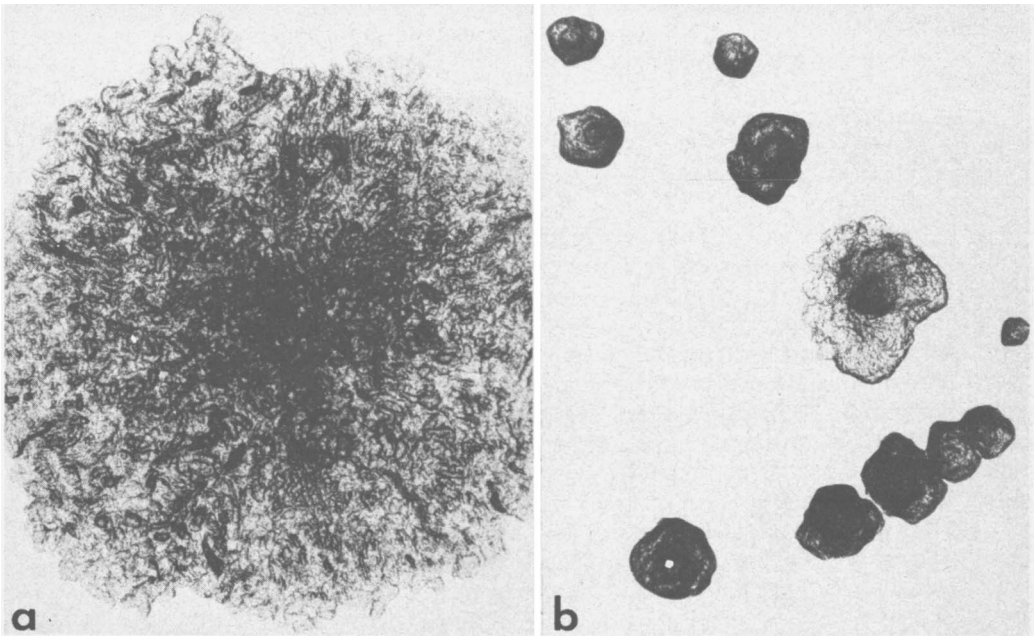

FIG. 1. Colony morphology of BCG strains brown on Dubos Oleic–Albumin agar for 4 weeks. (a) Phipps strain (× 8). (b) Glaxo strain (× 8).

13% lower than the control (Fig. 2(a)). In combination with irradiated tumor cells, the Phipps strain had a synergistic effect on transplantation immunity resulting in a significant protection against tumor challenge. Immunization with the Glaxo strain resulted in a significant enhancement of tumor incidence (Fig. 2(b)). The time of tumor appearance paralleled the controls until after the seventh week, when enhancement led to 100% tumors. This coincided with the time when systemic BCG infections became histologically apparent. The Glaxo strain mixed with irradiated tumor cells afforded some protection by delaying tumor appearance and slightly decreasing tumor incidence, but these results were not statistically significant.

Histologically, the BCG injection in hamsters was progressive. Both strains grew well in hamsters with evidence of granulomas in the draining lymph nodes (superficial distal axillary) at 10–14 days; thus, granulomas were present in the lymph nodes contralateral to the challenge site at the time of tumor-cell challenge. The first evidence of progressive infection for both BCG preparations in hamsters was characterized by the appearance of histiocytosis in the spleen and liver at 4 weeks, and in the lungs and distant lymph nodes at 10 weeks. At 26 weeks, the BCG infection was more pronounced in the Phipps-strain-infected groups, presenting macroscopic tuberculous lesions resulting in splenic enlargement, lung and liver nodules, and intestinal adhesions.

In the Glaxo-strain-infected groups, the disease process was not yet macroscopically visible at 26 weeks; however, the disease could be seen histologically in the lymph nodes as well as in the lung and liver. Progressive systemic infection was further exemplified by the presence of numerous acid-fast organisms in tissue sections of spleen and lymph nodes of BCG-immunized hamsters 16 weeks after immunization. At 26 weeks, there were no BCG-related deaths among the groups. This is contrary to reports of hamster sensitivity to BCG and is probably related to the low dose of organisms and the fact that the inoculations were given by the intradermal route rather than the intraperitoneal or intravenous route as used by others (17, 22). The systemic infections in our experimental animals progressed at such a slow rate that the studies in transplantation immunity were completed before any detrimental effects of BCG infection were found. In the present experiment, the first deaths were seen by 28 weeks when four hamsters of the Phipps-strain-immunized group were dead, compared

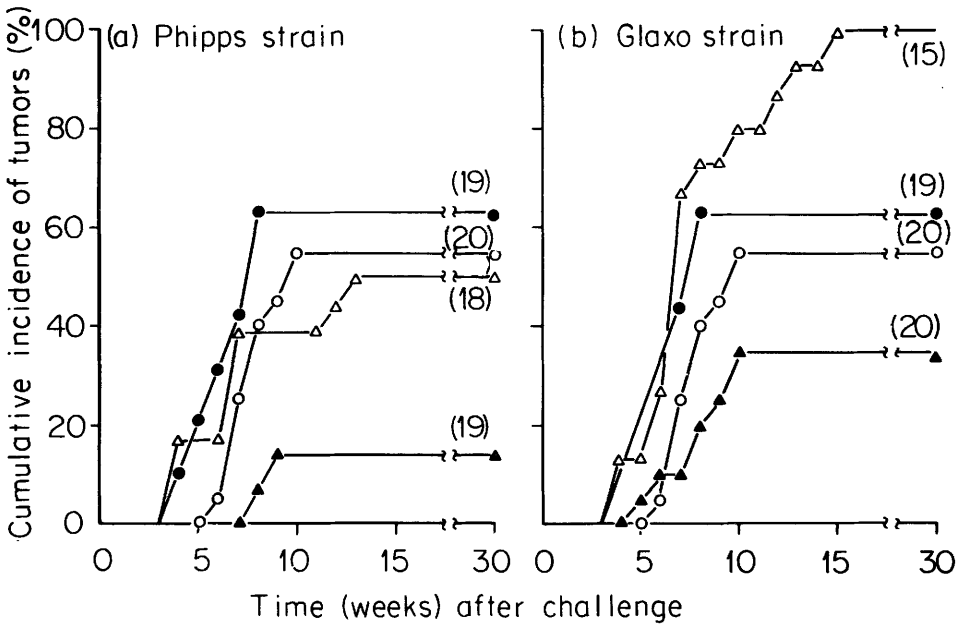

FIG. 2. Cumulative tumor incidence in hamsters challenged with 5×10^4 F5-1 cells after immunization with BCG alone (Δ—Δ), BCG admixed with irradiated tumor cells (▲—▲), irradiated tumor cells alone (○—○), or unimmunized controls (●—●). The number of animals in each group is noted in parentheses.

with one of the Glaxo-strain-immunized group and none of the controls. This pattern continued when eight of the Phipps group, compared with three Glaxo and no control hamsters, were dead by 33 weeks. Eleven Phipps-strain-immunized hamsters, 10 Glaxo, and 10 control animals remained alive at 32 weeks.

In this experimental tumor model of hamsters, attempts at immunotherapy by intralesional BCG injections proved to be generally ineffective. Our results agree with previous experiments in mice (25, 26). Progressive tumor growth occurred in all 10 Glaxo-strain-immunized hamsters intralesionally injected with Glaxo strain BCG. Also, progressive tumor growth occurred in all eight unimmunized animals intralesionally injected with Phipps strain BCG. Three of five Phipps-strain-immunized hamsters injected intralesionally with Phipps-strain BCG grew progressively, while one animal from this group showed partial regression and another complete regression after intralesional injection of Phipps strain. Other experiments in the laboratory (A. K. Szakal and M. G. Hanna, Jr., unpublished) involving the intralesional injection of Phipps strain into primary and transplanted DMBA-induced skin carcinomas of hamster were also ineffective in decreasing tumor growth rate or inducing regression.

Discussion. The present studies of immunoprophylaxis with BCG show protective effects, as measured by delay of appearance and reduction of tumor incidence, with the Phipps strain and actual enhancement of tumor incidence with the Glaxo strain. In combination with irradiated tumor cells, both had synergistic effects on the development of transplantation immunity in the hamster. However, the effect of the Glaxo strain was significantly lower than the Phipps strain.

Further, our results indicate that there are differences in BCG strains and although our results are limited to only two strains, it is possible that these differences may have influenced the efficacy of the BCG strains tested as immunoprophylactic or immunotherapeutic agents. These differences cannot strictly be correlated with total organism count but may possibly be associated with the kinetics of growth, virulence, and the colonial morphology on solid media. Our results on differences between Phipps and Glaxo BCG/strain colony morphology confirms the results of Mackaness *et al.* (27). Bunch-Christensen *et al.* have attributed these differences to genetic variations between strains of the

microorganism (17). We suggest that lyophilization of the Glaxo strain is probably not the single factor in its failure to stimulate immunity in this system since other lyophilized strains have been shown to be useful for immunotherapy in man (14, 28). There is ample other evidence that strain variability may be important in the effectiveness of BCG treatment. A recent comparison of different BCG preparations in mice disclosed a broad range of immunostimulant potential and therapeutic effectiveness (29). In a recent clinical trial in Britain using the Glaxo strain, the conclusion was that it had no effect on the treatment of lymphoblastic leukemia (13). In contrast, a French study of immunotherapy with the Pasteur strain showed significant maintenance of remission in leukemia (30). A study in Uganda compared the scarification and intradermal vaccination modes of BCG administration in malignant melanoma (14). All patients intradermally treated developed recurrent tumors within 30 weeks, while the scarified patients held in remission up through 59 weeks. The Ugandan study relates to strain and dose differences because the scarified patients received lyophilized Pasteur strain in a dose 10,000 times higher than the intradermally injected patients, who received the Glaxo strain.

The precise infection conditions in experimental animals are extremely important. Enhancement of tumors may be a consequence of antigenic competition developed by the systemic infection. An induced systemic BCG infection in adult guinea pigs abrogated the ability to develop delayed sensitivity to PPD and rendered the animals unable to suppress tumor growth at sites of BCG infection (31). Another point to be considered is that an adjuvant effect elicited by the number of nonviable organisms in a preparation may result in "blocking" factors. Which of the conditions was responsible for enhancement of tumors in the present experiments in hamsters cannot be defined at the present time. However, the results emphasize the variety of factors that must be considered in interpreting BCG-mediated immunoprophylaxis or therapy in experimental animals where systemic or progressive infection is a rule, and cautions against extrapolations of efficacy-screening experiments in these models with immunoprophylaxis or therapy in man.

Summary. Using a transplantable SV40 tumor system in hamsters, we evaluated the immunoprophylactic and immunotherapeutic properties of lyophilized Glaxo-strain and fresh frozen Phipps-strain BCG. These results were correlated with growth-culture kinetics, virulence, and colony morphology of the two preparations. The results demonstrate an immunoprophylactic effect with Phipps, as measured by delay of appearance and reduction of total tumor incidence. An actual enhancement of tumor incidence was obtained with the Glaxo strain. In combination with irradiated tumor cells, both had synergistic effects on the development of transplantation immunity in the hamster. However, the effect of the Glaxo strain was significantly lower than that of the Phipps strain.

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