MYCOBACTERIUM BOVIS (STRAIN BCG) EFFECTS ON THE GROWTH AND METASTASIS OF A TRANSPLANTABLE HAMSTER LUNG ADENOCARCINOMA[1] [2]

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Abstract. The effect of Bacillus-Calmette-Guerin (BCG) on the growth of an adeno-carcinoma of the lung induced in a Syrian hamster was evaluated. It inhibited intra-dermal growth of the tumor when the tumor cells were mixed with high doses of BCG prior to intradermal injection. The development of lung metastasis after excision of the primary intradermal tumor was inhibited by BCG at doses 100 times lower than those used to inhibit the growth of intradermal tumors. The results suggest that effective immunotherapy may depend on the number of viable BCG cells, the route of administration, and the anatomic location of the tumor.

Syrian golden hamsters when treated intratracheally with benzo(a)pyrene and iron oxide develop respiratory tract malignancies which for the most part resemble the majority of human lung cancers both in location and morphologic characteristics (Saffiotti *et al* 1968). We have been assessing the effects of BCG preparations on the development of primary lung tumors (Zwilling *et al* 1977) and have sought in this study to assess the effect of BCG on the growth and metastasis of a transplantable hamster lung adenocarcinoma induced by the intratracheal application of the carcinogenic agent benzo(a)pyrene.

MATERIALS AND METHODS

Animals. Inbred male Syrian golden hamsters, strain LSH/LAK, were obtained from Charles River Lakeview (Lakeview, N.J.). Animals were housed 5 per cage under standard laboratory conditions and were used when 7–12 weeks of age.

Tumor cells. The tumor cell line used in this study was a bronchogenic adenocarcinoma, designated LG 1002. It was induced in an out-bred Syrian golden hamster by the intra-tracheal instillation of benzo(a)pyrene ad-sorbed to ferric oxide particles and suspended in saline (Saffiotti *et al* 1968). The tumor was maintained by serial passage in the hamster

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cheek pouch. Tumor cell inocula were prepared from the 2nd to 10th transplant generations by digesting small tumor pieces with trypsin and suspending the cells at the desired concentration in Hank's balanced salt solution (HBSS).

Mycobacterial antigen. The Phipps strain of *Mycobacterium bovis*, strain Bacillus-Calmette-Guerin (BCG), TMC#1029, Lot #NC734A was obtained from the Trudeau Institute (Saranac Lake, N.Y.). Vials containing 1.2 x 10^8 colony forming units (cfu) were stored frozen at −70 °C. Dilutions of the BCG for injection were made in Hank's BSS. Concentration of the BCG suspension was accomplished by centrifugation at 200Xg for 20 minutes.

Immunizations. Tumor cell inoculations were given intradermally in the intrascapular region of the back or into the hind footpad of animals anesthetized with Brevitol Sodium. Locally administered BCG was admixed with the tumor cell inoculum prior to injection. Intra-tracheal injections of BCG were performed by the method described by Saffiotti *et al* (1968) for carcinogen instillation.

Monitoring of tumor growth. Growth of the tumor cell inocula given intradermally in the back was monitored by measuring the greatest and the least diameters of the tumor nodule with calipers. Tumor size was expressed as the product of the two diameters. Tumor growth in animals receiving footpad injections was monitored by measuring the footpad thickness with calipers. Footpad tumor growth was expressed as a tumor index which was calculated as the ratio of the thickness of the injected footpad to the thickness of the uninjected footpad.

Intradermal tumors were excised along with sufficient surrounding skin to insure the complete removal of the primary tumor mass. The

wound was inspected for the presence of sub-
cutaneous tumor growth and the edges were
apposed and fastened with sterile skin clips,
which were removed 7–10 days later. Animals
were checked daily for regrowth of the tumor
at the excision site. To enumerate the number
of metastases, the lungs were cleared of blood
by severing the dorsal aorta and injecting 3–5
ml of saline into the right ventricle. They
were then removed, fixed as described by Wil-
liams and Nettersheim (1973) and strained and
cleared according to the method of Yuhas
(1973). Mean tumor sizes and number of
metastases were compared using the Student's
t-test. Tumor incidence was compared using
the Fisher's exact test.

RESULTS

*BCG on the growth of intradermal tu-
mors.* Animals received either 6 x 10⁶,
1 x 10⁷ or 6 x 10⁷ cfu BCG admixed with
either 1 x 10⁴ or 1 x 10⁵ tumor cells. The
growth rate of tumors in animals injected
with 10⁵ tumor cells is shown in figure 1.
The growth of tumors in all 3 BCG-
treated groups was significantly inhibited
after ($P < 0.025$) compared to the control
group. One of 5 animals in the 6 x 10⁷
BCG group and 1 of 3 in the 10⁷ BCG
group failed to develop tumors. Tumors
which did develop in these groups grew

at a slower rate than did the control tu-
mors. All animals receiving 6 x 10⁶ BCG
developed tumors; tumor appearance was
delayed and growth rate decreased.
Similar results were obtained when BCG
was admixed with 1 x 10⁴ tumor cells.

*BCG on the growth of tumors inoculated
into the footpad.* Since the skin of most
rodents does not lend itself to a vigorous
delayed-type inflammatory response, the
footpad was studied as an alternate site
of inoculation. In this experiment, 10⁴
tumor cells were admixed with 10⁵ to 10⁷
cfu BCG and injected into the hind foot-
pad of the hamsters. Injection of 10⁴
tumor cells alone and 10⁷ cfu BCG alone
served as controls. Tumor growth was
measured as the ratio of the thickness of
the tumor injected footpad to the unin-
jected footpad. The results, expressed
as a tumor index, are shown in figure 2.
At days 30 and 37, the mean tumor indices
for the groups receiving 10⁴ and 10⁷ cfu
BCG were significantly smaller ($P < 0.05$)
when compared to the tumor control
group. By day 42, all of the BCG-
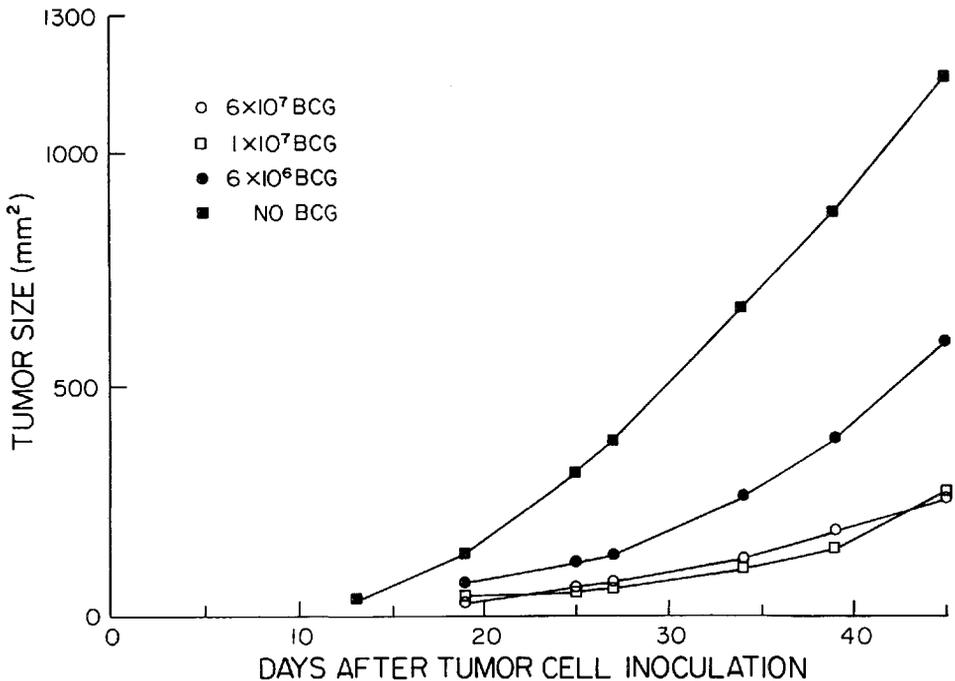treated groups had significantly smaller

FIGURE 1. Effect of BCG on the growth of LG1002 tumors. Varying numbers of BCG
were admixed with 1 x 10⁵ LG1002 tumor cells and inoculated intradermally and tumor growth
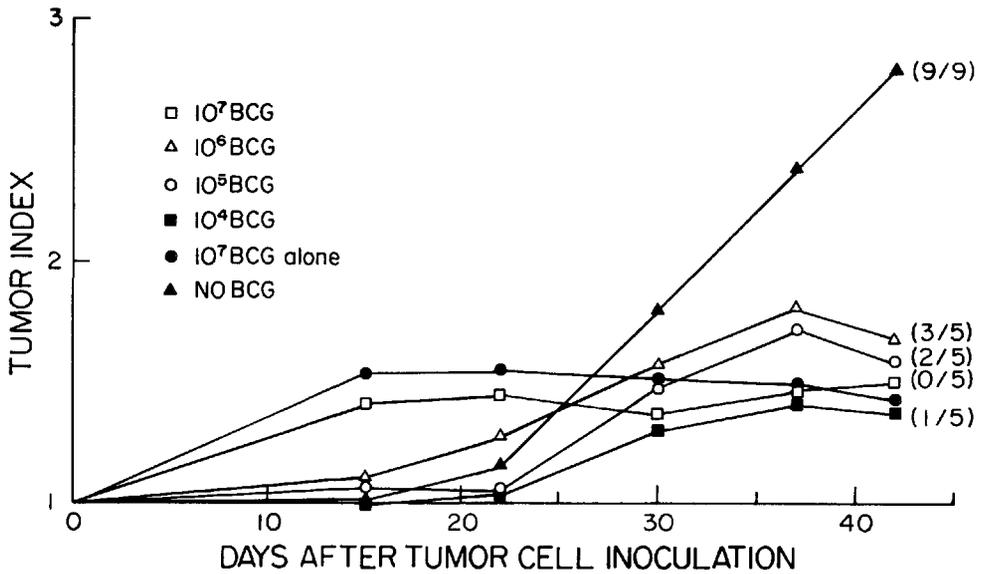was monitored for 45 days.

FIGURE 2.   Growth of LG1002 tumor cells following footpad inoculation of BCG-tumor cell mixtures.   Numbers in parenthesis denote number of animals with tumor/number inoculated. 1 x10⁴ LG1002 cells were admixed with varying numbers of BCG and tumor growth was monitored for 42 days.

$(P<0.01)$ tumors compared to the controls.   Footpad swelling in animals receiving 10^7 BCG mixed with 10^4 tumor cells was observed to be comparable in size to that seen in animals receiving 10^7 BCG alone.   All animals receiving 10^7 BCG failed to develop tumors and the tumor incidence in animals receiving other doses of BCG varied from 20% to 60%.

BCG on the development of pulmonary metastasis.   Studies of the effect of BCG on lung metastasis were initiated because of our observation that intradermal injection of LG1002 tumor cells resulted in numerous metastases to the lung.   In preliminary experiments we determined that lower doses of BCG were more effective at reducing the number of metastases than higher doses and that BCG treatment was more effective when administered after excision of the primary tumor rather than at the same time or prior to excision.

Table 1 shows the results of an experiment in which 1 x 10⁴ cfu BCG was injected intratracheally on the same day as tumor excision or 1 or 2 weeks following excision.   Three weeks after excision

TABLE 1

*The effect of intratracheal BCG on the development of lung metastasis.**

Week of BCG Admin.	No. Animals	No. Lung Metastasis
0	11	85.2 ± 25
1	11	86.7 ± 17
2	6	39.3 ± 9**
Untreated	13	106.7 ± 20

*Groups of hamsters were injected intradermally with 5 x 10⁴ LG1002 tumor cells. Four weeks later the primary tumor was excised and 1 x 10⁴ cfu BCG injected intratracheally on the same day as excision or 1 or 2 weeks following excision. On the third week following tumor excision, the animals were sacrificed and the number of metastases per lung enumerated.
**$P<0.05$.

animals were sacrificed and the mean number of metastases per lung in animals not receiving BCG was 106.7.   When animals were treated with BCG at the time of excision or one week following tumor excision, the number of metastases per lung was reduced to 85.2 and 86.7 respectively.   When treatment was delayed until 2 weeks following excision of the primary tumor, the mean number of

metastases per lung was significantly reduced to 39.3   ($P<0.05$).

DISCUSSION

Suppression of tumor growth by BCG was observed to be dose related, higher doses of BCG being more effective.   The minimum dose of BCG that significantly suppressed intradermal tumors was 6 x 10^6 cfu.   This finding is consistent with those of others (Baldwin and Pimm 1973, Harmel *et al* 1973, Kreider *et al* 1976, Zbar *et al* 1971).   Barlett *et al* (1976) have shown that the ratio of BCG to tumor cells may be as important as the absolute number of BCG; high BCG to tumor cell ratios were inhibitory while lower ratios gave little protection.

An increase in the effectiveness of BCG-mediated inhibition of tumor growth was noted when the BCG-tumor cell mixture was inoculated into the footpad of the hamster.   Significant tumor suppression was observed in the footpad at doses of BCG less than that required for suppression of intradermal growth.   An intense inflammatory reaction to the injection of BCG alone was also observed.

BCG was generally more effective at inhibiting the development of lung metastases when given after excision of the primary tumor.   These findings are in agreement with those of Sparks et al (1973) in which postoperative immunotherapy with subcutaneous injections of BCG or BCG tumor cell vaccine resulted in a significant increase in the median survival time.   Proctor *et al* (1976), however, have reported that BCG given 10 days prior to subcutaneous tumor inoculation was generally more effective in inhibiting the development of lung metastases than BCG given on the same day or 10 days after tumor.   In our studies the dose of BCG found to be most effective in inhibiting metastases was at least 100 fold less than the effective dose found to inhibit the growth of intradermal tumors.

The mechanism by which BCG affects tumor growth appears to be complex in nature.   It has been suggested that lymphocytes reacting to BCG antigens may nonspecifically destory tumor cells (Meltzer *et al* 1976).   During the course of this reaction, lymphocytes may also produce a variety of lymphokines that result in activation of macrophages (Fidler *et al* 1976, Kripke *et al* 1977).   It has been reported by Zwilling and Campolito (1977) and Hibbs *et al* (1972) that macrophages activated as a result of BCG infection acquire the capacity to destroy a variety of tumor cells.   The results of our investigation indicate that the inflammatory response induced by BCG may differ depending on anatomical location.   Effective immunotherapy using agents such as BCG may depend on the number of viable organisms and the route of administration.

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