Leukemia L1210 Therapy Trials with Antileukemia Serum and Bacillus Calmette-Guérin

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SUMMARY

Further experiments on the therapy of leukemia L1210 with rabbit antiserum prepared against whole leukemia cells have confirmed the mild effectiveness of this agent, which gave no long-term survivors. In contrast, therapy with Mycobacterium bovis (strain Bacillus Calmette-Guérin) gave some long-term survivors, when used under optimal conditions of route and of other still undefined factors, which appear to include the dosage, viability, and strain of BCG.

Mice bearing leukemia L1210 show a strong depression of primary antibody response to sheep erythrocytes. BCG does not increase the primary γG antibody response to sheep erythrocytes.

As a sensitive indicator of leukemia cell damage, it has been possible to use whole-body counting of mice given injections of as few as 100,000 iododeoxyuridine-125I-labeled L1210 cells. This makes it possible to use labeled cells in the standard L1210 assay of the Cancer Chemotherapy National Service Center.

INTRODUCTION

Leukemia L1210 is used in one of the 3 standard chemotherapy screens that are considered very helpful for the selection of substances that subsequently prove to be clinically useful in man (13). This leukemia has an average doubling time of 0.4 to 0.6 day, and there is a 16.6% chance that a single leukemia cell can transfer the disease to a new host (32). However, a weak immune response against this leukemia is present even when it is transplanted in DBA/2 mice (17).

In order to inhibit the i.p. growth of leukemia L1210, it is necessary to inject relatively large doses of an ALKS3 with high cytolytic potency (18, 26, 31). This therapeutic effect is obtained in spite of the immunosuppressive effects of such xenogeneic (heterologous) antisera (7, 33, 38) and in spite of their lack of any strong specificity against leukemia cells (2, 27). In this study, the mild effectiveness of ALKS under various injection regimens is further substantiated.

In direct contrast to ALKS, Mycobacterium bovis (strain BCG) is an effective nonspecific stimulant of immune responses and is active for therapy of transplantable leukemias (14–16). In this report, questions as to the degree of effectiveness of BCG for therapy of leukemia L1210 are answered. Under the conditions of our experiments, BCG did not stimulate the primary antibody responses to sheep erythrocytes.

MATERIALS AND METHODS

Mice and Leukemia. All mice were obtained from the Jackson Laboratory. Lymphocytic leukemia L1210 arose in a DBA/212 mouse painted with methylcholanthrene (12) and was transplanted i.p. in DBA/2 mice.

BCG. We purchased Mycobacterium bovis (strain BCG) as a sterile suspension in 0.9% NaCl solution from the Pasteur Institute, 25 Rue du Dr. Roux, Paris, France (French BCG). Shipments from Paris were by air with refrigeration, and a 2-week outdating period was observed. BCG was also obtained from the Institute for Tuberculosis Research, Chicago, Ill. (U. S. BCG). The latter BCG was supplied either in lyophilized form ("dry") or in suspension ("moist").

ALKS. Seven rabbits were immunized by 2 courses of injections. Each course consisted of 1 s.c. injection of 1 to 2 ml of complete Freund-McDermott adjuvant containing a 20% suspension of leukemia cells in 0.9% NaCl solution, followed by 7 i.v. injections spaced over 3 weeks; each i.v. injection consisted of 10 ml of a 1% suspension of viable leukemia cells in 0.9% NaCl solution (2). Rabbits were bled 7 days after the last immunization, then at weekly intervals just preceding a weekly booster injection. The total volume of pooled antiserum (900 ml) was inactivated at 56° for 30 min and absorbed 3 times with 60 ml of packed DBA/2 erythrocytes and once with 70 ml of DBA/2 plasma. The γ-globulin was separated at 40% saturation of ammonium sulfate, dissolved in 225 ml of distilled water, and dialyzed at 5° against 5 changes of water, then against 0.9% NaCl solution. The cytolytic potency of the resulting preparation was 4520 (mean of 3 determinations; see below).

Cytolysis Assay System. A small-scale assay system (25) was used. In outline, each assay tube contained 105 viable lymphocytes, absorbed 10% complement (21), and various dilutions of antiserum. Tubes were incubated for 1 hr at 37° and placed in ice water. For each tube in turn, the supernatant was sucked off the cells that had settled, 0.02 ml of vital dye was added, and 100 cells were classified under the microscope as stained or unstained. Results, in percentage of stained cells, were plotted against final antiserum concentration (%), and
Leukemia L1210 Therapy Trials with ALKS and BCG

the cytolytic titer was read at 50% stained cells, with a correction for stained cells in the control tube (21). The potency of an antiserum was expressed as 100 divided by the cytolytic titer obtained in this assay system. Use of potency (rather than of its reciprocal, the titer) has the advantage that it parallels the strength and is numerically equal to the final dilution of the antiserum.

Radiolabeled Coombs Serum. The myeloma protein M secreted by BALB/c plasma cell tumor MOPC-21A was purified from the serum of tumor-bearing mice by diethylaminoethyl cellulose chromatography and used to immunize rabbits (23). The rabbit antibody was purified by adsorption onto and elution from an insoluble antigen polymer, prepared by cross-linking the myeloma protein with glutaraldehyde (3). This antibody was labeled with $^{131}I$ (22) or with $^{125}I$ and used as the Coombs serum.

Assay of Cell-bound Antibody (24). Mouse anti-sheep erythrocyte serum (0.5 ml), or suitable dilutions thereof (a 1:10 dilution was mostly used), were pipetted into the disposable plastic tubes used eventually for determination of radioactivity. To this were added 1.5 ml of 2% washed sheep erythrocytes containing approximately 900 million cells ($A_{414} = 0.43$ after lysis of 1 ml with 8 ml of H$_2$O in cells of 1-cm light path). Tubes were incubated for 30 min at 37°C, with mixing at 0, 10, 20, and 30 min; then 3.0 ml 0.9% NaCl solution buffered at pH 7.2 with phosphate were added. After mixing, tubes were centrifuged for 6 min at 900 X g, and the supernatant was removed by suction. The sedimentsed cells were resuspended in 0.5 ml of the radiolabeled Coombs antiserum and incubated a second time for 15 min at 37°C, with mixing at 0, 5, 10, and 15 min. Cells were again washed 3 times with 3.0-ml portions of 0.9% NaCl solution. Finally, radioactivity was determined in a Packard Series 5000 γ-ray spectrometer.

Assay of Hemagglutination Activity. Stimpfling's method for hemagglutination of sheep erythrocytes by mouse antiserum was used (34). In outline, 0.1 ml of serial doubling dilutions of antiserum in a buffer containing 1% polyvinylpyrroldione was incubated with 0.05 ml of 2% sheep erythrocytes for 2 hr at room temperature, with mixing at 0.5-hr intervals. Tubes were centrifuged for 30 sec at 1000 X g and read for agglutination by flushing 0.5 ml of 0.9% NaCl solution over the pellicle.

Assay in Vivo. Agents were tested in vivo along the lines in use for cancer chemotherapy screening with leukemia L1210 (4, 30). On Day 0, a stated number (usually 10's) of L1210 cells were injected i.p. into young adult female DBA/2 mice. Therapy injections were begun at least 1 day after implantation of the leukemia. The mean survival time of test and control mice was compared.

Labeling of L1210 Cells with IUDR-$^{125}I$. Fifty million L1210 cells were injected i.p. into each of several DBA/2 mice. Starting approximately 18 hr later, each mouse received 3 i.p. injections at 3-hr intervals with 5 µCi of IUDR-$^{125}I$. Three hr after the last injection, the peritoneal cavity of each mouse was washed out with Earle's balanced salt solution (Baltimore Biological Laboratory, BioQuest Co., Cockeysville, Md.), and the cells were washed once at 12 X g in the same buffer, resuspended, and standardized in a hemacytometer. Suspensions containing more than 10% erythrocytes were discarded. The radioactivity of $10^6$ leukemia cells usually ranged between 15,000 and 35,000 cpm when determined in a Packard Series 5000 γ-ray spectrometer. Labeled cells were stored at 0°C and injected into mice within 3 hr of harvesting from the peritoneal cavity of the mice used for the labeling. Beginning 1 day before this injection, the experimental mice were given 0.1% sodium iodide in their drinking water to depress accumulation of $^{125}I$ in the thyroid (9).

Whole-Body Counting. Following the suggestions of Dr. Kurt G. Hofer, we supplied specifications for a well scintillation detector for whole-body counting of mice to the Packard Instrument Company, Downers Grove, Ill. The resulting detector consisted of a 3-inch sodium iodide crystal hollowed out to admit a 90-ml plastic test tube (International Equipment Co., Needham, Mass., No. 2806). With a window setting of 12.5 to 87.5 keV for counting $^{125}I$, this counter gave 13,576 cpm/0.01 µCi $^{125}I$ and a background of approximately 52 cpm.

The procedure of Hofer et al. (10) was only slightly modified, to permit longer counting periods. The mouse was placed in a tubular chamber consisting of 2 interlocking 25-ml plastic tubes (International Equipment Co., No. 2839), each with an 8-mm circular opening cut in the bottom. The inner tube was slit from rim to bottom, so that a mouse could be inserted backwards into the tube by pulling its tail along the slit to the bottom. The outer tube was slid over the head of the mouse and over the rim of the inner tube, to form a compact chamber. The mouse's tail was tucked into the inner tube, and the chamber was inserted into the 90-ml tube. A rod was placed on top of the chamber and taped in place to prevent the mouse from escaping. With this arrangement, mice could be counted for 5-min periods or longer without discomfort.

RESULTS

Labeling of Leukemia Cells with IUDR-$^{125}I$. Previously, no less than $10^6$ $^{125}I$-labeled L1210 cells have been used (9). In order to use $10^5$ L1210 cells in the standard in vivo chemotherapeutic assay, it was necessary to label them as strongly as possible without damaging them. Cell damage is certified by an increase in the rate of excretion of $^{125}I$ from mice given injections of labeled cells (10).

Three groups, each of 6 mice, were given injections of $10^5$ L1210 cells that had been labeled with IUDR-$^{125}I$ at 3 different levels of radioactivity (Chart 1) but otherwise as described in "Materials and Methods." The rate of $^{125}I$ excretion from mice given injections of cells labeled at the 2 lower levels was identical, whereas the rate was higher in the 3rd group, indicating cell damage. This damage was too slight to affect the survival time of mice dying from leukemia, which was identical in the 3 groups. It is concluded that 3 injections of 5.0 µCi of IUDR-$^{125}I$ is the highest dose at which there is no apparent cell damage under the conditions of labeling used.

Therapy of Leukemia L1210 with ALKS. Our previous therapy experiments were done with relatively small numbers of mice in each group (26). They showed that only high doses of strongly cytolytic rabbit antiserum to L1210 cells are effective; lower amounts or less potent antiseras are ineffective.
The present experiments with larger groups confirm the increased survival of DBA/2 mice given i.p. injections of 10^6 L1210 cells and treated with ALKS (Table 1). As previously, no permanent survivors were obtained with any of the injection schedules that were tested.

A similar experiment was done with 10^6 L1210 cells labeled with 3 injections of 5.0 µCi of IUDR-125I (see “Materials and Methods”). The excretion of ^125I by mice of the experimental group was substantially more rapid than in mice of the 2 control groups (Chart 2). In addition, there were significant differences in the day of death in the groups given injections of 0.9% NaCl solution, normal serum, or ALKS, which were, respectively, 10.1 ± 0.4, 10.0 ± 0.5, and 11.8 ± 0.9 (mean ± S.D.). Comparison of the 1st and 3rd and the 2nd and 3rd groups by Student’s t test gave p < 0.001 in both cases.

Therapy of Leukemia L1210 with BCG. When 10^2 cells of leukemia L1210 were injected i.p., and therapy injections were also given i.p., starting 1 day later, survival was not significantly prolonged (p = 0.23 for Groups A and B (Table 2)), nor was prolongation obtained at 10^3 cells (Groups D and E). Results for 2 additional extensive experiments at 10^4 and 10^5 cells were also negative and are therefore not presented. When 10^2 cells were injected i.p. and therapy injections were also given i.p. but starting 5 days later, 1 permanent survivor was obtained; however, the increase in survival time of mice that succumbed to leukemia was not significantly changed (p = 0.21 for Groups A and C).

Because of the above poor results in the therapy of i.p.-injected leukemia cells, all subsequent experiments were done with s.c.-injected leukemia cells, which kill mice a little more slowly and thereby give the therapeutic agent more time to act. For 10^4 s.c.-injected L1210 cells, i.p. therapy was not effective (p > 0.05 for Groups G, H, and I compared to Group F), except in the case of United States moist BCG (p < 0.01 for Group J compared to Group F). When therapy was given i.v., it was effective in Experiment 1 (Table 2), since permanent survivors were obtained in all experimental (L to N) but not in control groups (F and K). In 2 later experiments (Experiments 2 and 3, Table 2), statistically significant therapy could not be reproduced with fresh lots of French BCG. However, only 3 therapy injections were given in Experiment 2. Positive results were again obtained in the next experiment with a different lot of BCG (Table 3). Thus, of 4 separate experiments in which therapy injections with BCG were given i.v., 2 experiments were positive and 2 negative.

French BCG given i.v. at 1 mg/20 g of body weight was nontoxic (Group B, Table 3), while reconstituted United States dry BCG was toxic (Group C). The quantities injected were based on the weight figures quoted by the respective manufacturers. With the absorbance at 600 mètre used as a turbidimetric estimate of relative concentration, we found it to be 4.2 times higher per mg of United States BCG than per mg of French BCG. In the case of United States BCG, the weights quoted by the manufacturer were nominal values per mg of BCG, while French BCG was toxic (Group E). The quantities injected were based on the weight figures quoted by the respective manufacturers. With the absorbance at 600 mètre used as a turbidimetric estimate of relative concentration, we found it to be 4.2 times higher per mg of United States BCG than per mg of French BCG. In the case of United States BCG, the weights quoted by the manufacturer were nominal values per mg of BCG, while French BCG was toxic (Group E).
chosen to reflect the viability of the vaccine. Serial autopsies suggested that the deaths of mice given injections of United States BCG were due to miliary tuberculosis; small lesions typical of this disease were widely disseminated in livers, kidneys, and spleens of mice autopsied on Day 16 of an experiment (Table 4). In addition, mechanical blockage of vascular channels may have been a factor in some of the deaths from United States BCG.

In order to test whether mice carrying a transplantable leukemia or given injections of BCG experienced a change in humoral immune response, we investigated the antibody response to sheep red blood cells (Table 3). The radiolabeled Coombs serum assay (see "Materials and Methods") was previously found to be specific for γG-globulin antibody, to give direct proportionality between quantity of antibody present and radioactivity bound to the cell surface, and to have a standard deviation of 5.5%. In contrast, the hemagglutination assay (see "Materials and Methods") is not specific for γG antibody and had a much larger standard deviation (50.9%) in our hands.

Mice given injections of leukemia cells had a much lower γG antibody response to sheep RBC (p = 0.01); this response was even lower in mice given therapy with BCG and bled on Day 16. In mice not given injections of leukemia cells, even French BCG appeared to depress the γG antibody response. The French BCG was active therapeutically in this experiment, since there were 2 100-day survivors (Group E, Table 3).

**DISCUSSION**

These data show that ALKS produces statistically significant prolongation in the life-span of mice given leukemia injections. However, no permanent survivors were obtained, nor was ALKS “active” by the criteria set for the Cancer Chemotherapy National Service Center primary screen with L1210 cells, which require an increased survival of at least 25% in repeated trials.

What can be done to make ALKS more effective? An increase in its present low specificity against leukemia cells (2, 27) seems essential. This objective might be achieved with an anti-L1210 serum prepared by repeated immunization of DBA/2 mice with X-ray-sterilized L1210 cells (17). This reagent could be used to identify leukemia-specific antigens.

### Table 2

*Therapy of leukemia L1210 with BCG*

<table>
<thead>
<tr>
<th>Experiment no. and group</th>
<th>L1210 cells</th>
<th>Therapy injections</th>
<th>Results of therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose</td>
<td>Route</td>
<td>Agent&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>-------------------------</td>
<td>------</td>
<td>-------</td>
<td>-------------------</td>
</tr>
<tr>
<td>1-A</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>i.p.</td>
<td>0.9% NaCl Solution</td>
</tr>
<tr>
<td>1-B</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>i.p.</td>
<td>French BCG – A</td>
</tr>
<tr>
<td>1-C</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>i.p.</td>
<td>French BCG – B</td>
</tr>
<tr>
<td>1-D</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>i.p.</td>
<td>0.9% NaCl solution</td>
</tr>
<tr>
<td>1-E</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>i.p.</td>
<td>French BCG – A</td>
</tr>
<tr>
<td>1-F</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>0.9% NaCl solution</td>
</tr>
<tr>
<td>1-G</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>French BCG – A</td>
</tr>
<tr>
<td>1-H</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>French BCG – B</td>
</tr>
<tr>
<td>1-I</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>French BCG – B</td>
</tr>
<tr>
<td>1-J</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>United States moist BCG</td>
</tr>
<tr>
<td>1-K</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>United States moist BCG</td>
</tr>
<tr>
<td>1-L</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>French BCG – A</td>
</tr>
<tr>
<td>1-M</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>French BCG – B</td>
</tr>
<tr>
<td>1-N</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>United States moist BCG</td>
</tr>
<tr>
<td>2-O</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>United States moist BCG</td>
</tr>
<tr>
<td>2-P</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>French BCG – C</td>
</tr>
<tr>
<td>3-Q</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>French BCG – C</td>
</tr>
<tr>
<td>3-R</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>s.c.</td>
<td>French BCG – D</td>
</tr>
</tbody>
</table>

<sup>a</sup> French BCG – A was kept at +10° for 1 week longer before use than French BCG – B. All injections were in a 0.5-ml volume. French BCG – C and BCG – D were different lots, used soon after arrival. Neither French BCG nor United States moist BCG were toxic when given i.v. at a dose of 1 mg/injection.
Table 3

<table>
<thead>
<tr>
<th>Therapy injections (i.v.)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sera of Day 10</th>
<th>Sera of Day 16</th>
<th>Results of therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Dose/20 g body weight (mg)</td>
<td>% counts bound</td>
<td>Hemagglutination potency</td>
</tr>
<tr>
<td>A</td>
<td>0.9% NaCl solution 500</td>
<td>11.7</td>
<td>288</td>
</tr>
<tr>
<td>B</td>
<td>French BCG 1</td>
<td>10.7</td>
<td>192</td>
</tr>
<tr>
<td>C</td>
<td>United States dry BCG 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.8</td>
<td>96</td>
</tr>
<tr>
<td>D</td>
<td>0.9% NaCl solution 500</td>
<td>5.8</td>
<td>120</td>
</tr>
<tr>
<td>E</td>
<td>French BCG 1</td>
<td>6.0</td>
<td>72</td>
</tr>
<tr>
<td>F</td>
<td>United States dry BCG 1</td>
<td>4.7</td>
<td>72</td>
</tr>
</tbody>
</table>

<sup>a</sup> Therapy injections were given i.v. on Days 1, 5, 9, 13, 17, and 23 after injection of L1210 on Day 0. All injections were in a total volume of 0.5 ml.

<sup>b</sup> All mice were given i.p. injections of 30 million washed sheep RBC on Day 0. The percentage counts bound was corrected for controls with sera from mice not given injections. All results were the mean of duplicate determinations on 2 separate days.

<sup>c</sup> 100-day survivors were excluded from this mean.

<sup>d</sup> The dosage was reduced to 0.5 mg/20 g of body weight after Day 9.

Table 4

<table>
<thead>
<tr>
<th>Evidence of tuberculosis infection in mice given BCG injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG injections (1 mg/20 g of body weight) were given i.v. on Days 1, 5, 9, and 13, and the mice were autopsied on Days 10 and 16. There were at least 4 DBA/2 mice per group. Liver, spleen, and kidneys were fixed in 10% formalin, sectioned, and stained either with hematoxylin and eosin or with Kinyoun’s carbofuchsin acid-fast stain.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Day of autopsy</th>
<th>Spleen weights, mean ± S.D. (mg)</th>
<th>p</th>
<th>Miliary-type granulomas in liver sections, approximate % area</th>
<th>Number of acid-fast bacilli per section of liver or spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>10, 16</td>
<td>95 ± 16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>French BCG</td>
<td>10</td>
<td>140 ± 16</td>
<td>&lt;0.02</td>
<td>0</td>
<td>1–15</td>
</tr>
<tr>
<td>French BCG</td>
<td>16</td>
<td>137 ± 25</td>
<td>&lt;0.05</td>
<td>1</td>
<td>1–6</td>
</tr>
<tr>
<td>United States dry BCG</td>
<td>10</td>
<td>211 ± 32</td>
<td>&lt;0.01</td>
<td>2</td>
<td>300–900</td>
</tr>
<tr>
<td>United States dry BCG</td>
<td>16</td>
<td>317 ± 26</td>
<td>&lt;0.001</td>
<td>45</td>
<td>500–200</td>
</tr>
</tbody>
</table>

Is enhancement in the growth rate of leukemia cells a likely result of treatment with ALKS? Three conditions must generally be fulfilled to make enhancement likely. First, the tumor cells must have a low concentration of antigens recognized as foreign by the host in which they are growing (20, 37). Second, the enhancing antibodies must block the strongest of these incompatible histocompatibility antigens (19, 20, 37). Third, the enhancing antibodies must be present in moderate concentrations, since high concentrations can cause immune cytolysis of the tumor cells and growth inhibition, not enhancement (19, 20, 37). With regard to the concentration of relevant cell surface antigens, the lowest concentrations are found in long-transplanted leukemias such as EL4 (11) or in those few murine leukemias derived from marrow rather than thymus precursor cells; such leukemias tend to be susceptible to enhancement with antiserum prepared against them. As a class, however, murine leukemias possess a concentration of cell surface antigens higher than carcinomas and much higher than sarcomas. Conversely, susceptibility to enhancement tends to be lowest for leukemias and highest for sarcomas (19, 20, 37).

An interesting parallel exists between use of BCG in Europe and in the United States, whether BCG is used for mass inoculation as a protection against human tuberculosis or for therapy of mouse leukemias. Continental work on use of BCG for immunization against tuberculosis has been generally positive (8), whereas both positive (1, 29) and negative (5, 6) reports have appeared in the United States. Similarly, the work of Mathé et al. (14–16) on the use of BCG against mouse leukemias has been uniformly positive, while we must report 2 negative as well as 2 positive experiments with French BCG, and all but 1 of our experiments with United States BCG were negative. Thus, this study serves notice that BCG may prove to be a controversial agent also when used for therapy of leukemia.

The most obvious difference between the work of Mathé and our work has been the need to ship French BCG to us from Paris. While the air shipment is refrigerated, loss of
viability due to a delay of approximately 3 days is a possibility. Before we were aware that the outdating of BCG might be a problem, we asked Mr. I. Wodinsky of A. D. Little, Inc., Cambridge, Mass., to check our inconsistent results by running a precisely parallel therapy experiment with 10^4 L1210 cells injected s.c. Initially, we provided Mr. Wodinsky with fresh samples of both French and United States BCG. When the next shipment of BCG arrived from Paris, we switched to it in our laboratory, but we did not supply Mr. Wodinsky with it. Our experiment gave positive results with French BCG but not with United States BCG, whereas his gave negative results with both preparations. It seems questionable that technical difficulties due to several consecutive i.v. injections of BCG or else variations in the technique for injection of the leukemia cells are responsible for the inconsistency between the 2 laboratories. A more likely explanation is that the viability of the BCG preparation is an important parameter for successful therapy.

The reason for our lack of success with United States BCG when given i.v. appears to be its higher infectivity in DBA/2 mice, as compared to the French BCG. Higher infectivity could be due to higher viability or to lower antigenicity due to strain differences. In addition, physical blockage of vascular channels could be partly responsible for the greater toxicity of the more concentrated United States BCG preparation. In any case, this study confirms the strict requirements (14–16) regarding the route of administration of BCG. Similarly, differences in the strain of BCG and its method of inoculation could well be important factors in the lower effectiveness of BCG when used for mass inoculation against tuberculosis in the United States (1, 5, 6, 8, 29).

In retrospect, the need for documentation of the number of viable BCG organisms used in the above experiments is apparent. Unfortunately, the assay for viability is not precise, since it requires 4 to 6-week period of incubation of culture tubes containing 10-fold serial dilutions of BCG suspensions (R. G. Crispen, private communication). The precision of the results is therefore relatively low. Further, in the case of Freund-McDermott adjuvant, a killed tubercle bacillus of different strain is used for nonspecific stimulation of immune response. Hence, viability can only be one of several factors that contribute to the effectiveness of BCG.

Failure of BCG to stimulate γG antibody production confirms previous suggestions (14–16) that BCG is a stimulator of cellular rather than of humoral responses. Such stimulation requires time not granted by the rapid growth of leukemia L1210. For less rapidly growing leukemias and other tumors, BCG may well prove to be a valuable nonspecific stimulator of host defenses (39).

In our experiments, i.v. therapy with BCG could be effective if begun after s.c. injection of 10^4 L1210 cells. If no therapy was given, such inoculations were uniformly fatal (Table 2), as are much smaller inocula (32). Therefore, nonspecific immunological stimulation with BCG raised the threshold of the number of L1210 cells that can be eliminated, rather than growing progressively. Different threshold ranges exist for different mouse leukemias and solid tumors (13–17) and by analogy may also exist for different types of human tumors. For each individual, this tumor-characteristic threshold of the number of L1210 cells that can be eliminated, and hormonal and nutritional status, as well as upon the growth potential and antigenic structure of his own particular tumor. If the number (or mass) of the tumor cells can be decreased sufficiently, and the threshold for their elimination raised up to or beyond this level by nonspecific (or better, by specific) immune stimulation, then successful therapy is achieved. Classes of tumors such as choriocarcinoma, Burkitt's lymphoma, and Wilms' tumor, which are exceptional in that chemotherapy is sometimes successful, may possess characteristically high threshold levels, and these may in turn be due to an autoantigenicity that (for a tumor) is relatively high.

ACKNOWLEDGMENTS

Best thanks are due to Mrs. Cynthia M. Robinson for her excellent assistance in the later stages of this work and to Dr. G. Mathé and Dr. R. G. Crispen for supply of BCG and helpful discussions. The help given by Dr. K. G. Hofer, Dr. W. Prensky, and Dr. W. L. Hughes in labeling L1210 cells was instrumental in success with that portion of this study.

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