ABSTRACT—The ability of thimerosal-killed Blastomyces dermatitidis yeast cells, which greatly enhance the cell-mediated immune response in C57BL/6J mice, to act as an immunopotentiator against EL 4 lymphoma was investigated. Mice treated with yeast cells were protected from as many as 10^6 tumor cells. Complete suppression of tumor growth was observed in treated animals that received ip injections of 10^6 or 10^4 tumor cells. The mice, however, were not immune to further EL 4 lymphoma challenge. The lack of tumor-specific immunity indicated nonspecific suppression of tumor growth probably by macrophages. Ten days after treatment, the peritoneal macrophages from mice that showed complete tumor suppression were tested for their ability to prevent in vitro tumor cell proliferation. These macrophages demonstrated 90% inhibition of [3H]thymidine incorporation by EL 4 tumor cells at a 100:1 effector-to-target cell ratio. Macrophages from B. dermatitidis-treated animals exhibited a twofold increase in specific lysis of EL 4 at 10 and 15 days compared to resident macrophages. Spleen and lymph node cells from protected animals showed no cytotoxic activity against EL 4 in a 51Cr release assay. Treatment of tumor-bearing mice with a single dose of B. dermatitidis was effective only if administered within 24 hours of tumor establishment. These results demonstrated that nonviable B. dermatitidis inhibits the growth of EL 4 under conditions where Corynebacterium parvum fails to do so.—JNCI 1982; 69:1337–1342.

It is a generally accepted theory that macrophages play a major role in resistance against growth of tumor cells within the host tissue. The in vitro cytolytic capacity of activated macrophages is well substantiated (1, 2). Also, macrophages activated by bacterial or protozoal infections are able to inhibit the growth of tumor cells in vivo (3, 4). Microorganisms such as BCG (2), Toxoplasma gondii (3), or Corynebacterium parvum (4) are capable of inducing macrophages that have the capacity to distinguish tumor cells from normal cells. There is a great interest in agents that enhance the ability of macrophages to nonspecifically kill tumor cells. Various agents are also able to activate cultured resident macrophages to a tumoricidal state. These include lymphokines (2), LPS (5), and poly I:C (6). Evidence continues to mount for the importance of macrophages as primary targets of such immunomodulating agents.

Past studies in our laboratory with yeast cells of the dimorphic fungus Blastomyces dermatitidis indicated that this organism elicits a strong cell-mediated immune response (7, 8). We report here results of experiments on the protective effects of B. dermatitidis-induced resistance in C57BL/6J mice against the syngeneic lymphoma EL 4.

MATERIALS AND METHODS

Culture.—The thimerosal-killed whole yeast cell Ag was prepared by the method of Restrepo-Moreno and Schneidau (9) as modified by Spencer and Cozad (7). In all experiments the B. dermatitidis was washed three times in 10 volumes of a sterile PSS to remove the thimerosal. The yeast phase culture of B. dermatitidis 242 was originally isolated from a fatal human case of blastomycosis. The culture was maintained on BHI agar slants at 37°C.

Test animals.—Inbred C57BL/6J mice of both sexes, 2-3 months old were used in this study. The strain was originally obtained from The Jackson Laboratory, Bar Harbor, Maine, and bred in our animal facilities. The mice were separated by sex and given water and Purina Mouse Chow (Ralston Purina Co., St. Louis, Mo.) ad libitum.

Cell line.—The thymus-derived benzene-pyrene-induced lymphoma EL 4 of C57BL mice was used in this study. The cells were maintained as ascites tumors in 8-12-week-old C57BL/6J mice. Transfers were made at 10-12 days by ip injection of 10^6 cells in 0.2 ml HBSS (GIBCO, Grand Island, N.Y.). In vitro cultures of the EL 4 lymphoma were maintained as suspensions in RPMI-1640 medium (GIBCO) supplemented with 10% heat-inactivated FCS (M.A. Bioproducts, Walkersville, Md.). The medium also included 2 mM L-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml (GIBCO). Cells were maintained as suspensions in RPMI-1640 medium (GIBCO) supplemented with 10% heat-inactivated FCS (M.A. Bioproducts, Walkersville, Md.). Cells were carried in logarithmic growth (12- to 14-hr doubling time) within a cell density of 1×10^5 to 2×10^6 cells/ml. EL 4 tumor cell viability under these growth conditions was between 97 and 100% as determined by the trypan blue exclusion test.

Preparation of immunizing inoculum and assessment of delayed hypersensitivity.—An Ag emulsion of thimerosal-killed B. der-
matitisid) yeast cells was prepared by the mixing of equal volumes of the yeast suspension and Freund's incomplete adjuvant (Difco Laboratories, Detroit, Mich.). A control suspension was prepared by substitution of PSS for the yeast suspension.

The mice were sensitized according to the method of Cozad and Chang (8). On days 0 and 7, mice were inoculated sc in the inguinal area with 0.1 ml Ag emulsion (containing 2 mg dry wt equivalent of killed B. dermatitisid) yeast cells). A control group was inoculated in the same manner with the PSS emulsion. Some animals were given ip 0.1 or 1.0 mg killed B. dermatitisid in 0.2 ml PBS 5 days before tumor challenge.

To determine the delayed hypersensitivity pattern, we gave 3 mice from each group footpad tests on days 3, 15, and 30 after the initial injection with a killed whole yeast cell suspension. We performed the footpad tests by the procedure of Youmans and Youmans (10), as described by Cozad and Chang (8).

Tumor inoculations.—We conducted preliminary experiments to determine the median survival time of mice that received 102-105 tumor cells without treatment. To test the effect of preimmunization with the Ag emulsion, we challenged groups of 10 mice with a range of 102-106 ip tumor cell injections on days 0, 3, and 15 after the initial Ag emulsion injection. We performed all experiments at least twice.

We also evaluated the protective effects by mixing the yeast cells with tumor cells prior to injection. The mice were given 0.2 ml ip injections of 102-106 EL 4 cells mixed with 0.1 or 1.0 mg killed B. dermatitisid) yeast cells. Survival of the animals was checked daily for 2 months.

To test the therapeutic potential of B. dermatitisid, we gave mice ip inoculations of 102-106 EL 4 cells and treated them with ip injections of 1.0 mg B. dermatitisid in 0.2 ml PBS at various times after tumor challenge. A control group of tumor-bearing mice received ip injections of PBS alone. Treatment times were at 2 hours and at 1, 2, 3, 5, 8, and 11 days.

Collection of peritoneal cells.—We collected peritoneal cells without the use of an exudate-inducing agent by a modification of the method described by Telnai (11). Five milliliters cold RPMI-1640 medium containing 10 U sodium heparin/ml (Fellows Medical Manufacturing Co., Inc., Anaheim, Calif.) and 1% FCS was injected into the peritoneal cavity. The fluid was withdrawn after a short massage.

We pooled the cells from 3 animals and assessed their viability by the trypan blue exclusion test. After centrifugation, we resuspended the cells at the concentration of 5X106 cells/ml in cold RPMI-1640 medium with 1% FCS and placed 0.1 ml of the suspension into wells of a Falcon MicroTest II plate (style 3040; Falcon Plastics, Oxnard, Calif.). After adherence for 60 minutes at 37°C in 5% CO2, we removed nonadherent cells by washing twice with warm PBS and by suction through a Pasteur pipette. Then we added 0.1 ml fresh warm medium containing 10% FCS to the chambers and incubated the plates for 18-24 hours at 37°C in 5% CO2.

Cytostasis assay.—We used a modified version of the procedure described by Goldman and Bar-Shavit (12). After the 24-hour incubation, we added to the wells 0.1 ml RPMI-1640 with 10% FCS containing the desired concentration of EL 4 cells. We set up controls of 0.2 ml RPMI-1640 medium with tumor cells alone, reincubated the plates for 7 hours, and added 0.5 μCi [3H]dThd (20 Ci/mmol; New England Nuclear Corp., Boston, Mass.). Controls containing macrophages alone with [3H]dThd were included. We incubated the plates at 37°C and 5% CO2 for an additional 24 hours and then harvested the samples with a MASH unit (M.A. Bioproducts). We dried the filter strips, placed the disks in toluene scintillation fluid, and counted them in a Beckman LS 100C liquid scintillation counter.

Cytotoxicity assay.—We labeled the EL 4 cells by incubating 5X106 cells for 3 hours in 1.0 ml RPMI-1640 medium with 10% FCS containing 10 μCi [3H]dThd (5 Ci/mg; Amersham Corp., Arlington Heights, Ill.) (13). After incubation we washed the cells three times with medium and resuspended them at 5X106 cells/ml.

We added the desired concentration of labeled EL 4 cells in 0.1 ml medium to wells containing 5X105 macrophages that had been incubated for 24 hours. Wells were set up for spontaneous and maximum release for each tumor cell concentration used. We ran all samples in triplicate. We incubated the plates for an additional 27-30 hours at 37°C and 5% CO2, added 0.1 ml of a 2 N HCl solution to the maximum-release wells to obtain total counts, and centrifuged the plates for 15 minutes at 2000 x g. We then removed 0.1-ml samples and counted the radioactive label in a Packard Tri-Carb gamma counter. The percentage of specific lysis was obtained with the use of the formula: Percent specific lysis = [(cpm test sample - cpm spontaneous release)/maximum cpm of target cells]x100.

SEM of EL 4 lymphoma cells and macrophages.—After the harvest of the peritoneal cells, a 0.5-ml suspension of 1X106 cells/ml was plated in petri dishes that contained three glass cover slips. After 60 minutes at 37°C and 5% CO2, all nonadhering cells were washed away with warm PBS. We added fresh warm medium containing 10% FCS and incubated the cells 18-24 hours at 37°C in 5% CO2.

After 24 hours, we replaced the medium with fresh medium containing 5X106 EL 4 cells/ml and incubated it for various time intervals up to 48 hours at 37°C and 5% CO2. The medium was then aspirated, and the cover slips were fixed with 0.5% glutaraldehyde in PBS at room temperature for 18-24 hours.

We further prepared the samples for SEM by submitting them to a series of ethyl alcohol dehydrations. The cover slips were critical point dried in a Pelco Critical Point Dryer (No. 99 model H; Ted Pella Co., Trustin, Calif.) with the use of liquid CO2 at a critical pressure of 1,072 pounds per square inch. The samples were then gold coated in a Technics Hummer sputter coater and examined in an International Scientific Instruments Super II scanning electron microscope at the noted magnifications with an accelerating voltage of 25 kV.

Chromium release assay.—We evaluated the cytotoxic activity of lymphocytes with the use of an established procedure (14). EL 4 cells, 1X106 in 1.0 ml, were incubated in serum-free RPMI-1640 medium containing 100 μCi ⁵¹Cr-sodium chromate (500 mCi/mg; Amersham Corp.) for 1 hour at
37°C in 5% CO₂. After incubation, the cells were washed twice and adjusted to 2×10⁷ cells/ml (target cells) in RPMI-1640 with 10% FCS. Spleen or lymph node cells (effector cells) from mice showing tumor suppression at 10 or 15 days were adjusted to 1×10⁶ cells/ml. Then 0.1 ml of the target cells were placed in a round-bottomed microtiter plate (Linbro Scientific Co., Inc., Hamden, Conn.). The plate was incubated at 37°C in 5% CO₂ for 4 hours. At the end of this time, we centrifuged the plate at 200×g for 15–20 minutes, removed carefully 0.1 ml supernatant, transferred it to a 7.5×100-mm disposable glass tube, and counted the radioactivity for 10 minutes in a Packard Tri-Carb gamma counter. The percent cytotoxicity was obtained by the formula: Percent specific lysis = [(counts of test group − spontaneous release)/maximum cpm of target cells]×100.

RESULTS

Preimmunization effects on survival of mice.—We then preimmunized the mice with B. dermatitidis in Freund’s incomplete adjuvant. Footpad tests with representative mice demonstrated a delayed hypersensitivity pattern that is consistent for B. dermatitidis (8), with a peak response at 15–18 days post primary injection. We then challenged the remaining mice with various tumor inocula at different times post primary immunizing dose. The results are presented in text-figure 1. Mice challenged 3 days after the initial B. dermatitidis injection showed a significant increase in mean survival time. The lower the initial tumor inoculum, the more significant was the increase in mean survival time compared to that of controls.

In some experiments, mice were given ip injections of 0.1 or 1.0 mg killed B. dermatitidis in PBS without adjuvant 5 days prior to tumor challenge. Both doses of yeast cells caused significant (and similar) increases in mean time of survival of treated mice compared to that of control mice (text-fig. 2). The increase in survival was very similar to that seen in mice immunized 3 days before tumor challenge with B. dermatitidis in Freund’s incomplete adjuvant (text-fig. 1).

Mixing of EL 4 cells with B. dermatitidis.—We mixed a dry equivalent weight of 0.1 or 1.0 mg killed B. dermatitidis with 10²–10⁶ EL 4 cells and immediately injected the mixture ip into mice to determine if B. dermatitidis could induce a host response capable of suppressing tumor growth. EL 4 cells in these mixtures showed no significant decrease in viability even after 4 hours as determined by trypan blue exclusion test. Within several days, all mice that had received the mixture developed an inflammatory response at the site of injection. Mice that received EL 4 alone showed no such response. At 30 days, the inflammation had receded in a majority of the animals that received B. dermatitidis. By 60 days, all mice in the control groups were dead as the result of tumor growth, but a significant number of animals that received the mixture were alive and free of tumors (table 1). At 65 days, the living mice were challenged with 1×10⁴ EL 4 cells given by ip injection. The mice all died as a result of tumor development.

Treatment of EL 4 cells with B. dermatitidis.—To determine if B. dermatitidis could cause regression of established tumors,
TABLE 1.—Suppression of tumor growth by B. dermatitidis

<table>
<thead>
<tr>
<th>No. of EL 4 tumor cells injected</th>
<th>Tumor suppression 60 days post treatment with B. dermatitidis&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None, control</td>
<td>0.1 mg</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Expt 1</td>
<td>Expt 2</td>
<td>Expt 1</td>
<td>Expt 2</td>
</tr>
<tr>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0/10 0/10</td>
<td>0/10 0/10</td>
<td>0/10 0/10</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0/10 1/10</td>
<td>0/10 6/10</td>
<td>2/10 1/10</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0/10 10/10</td>
<td>10/10 10/10</td>
<td>10/10 10/10</td>
</tr>
<tr>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0/10 10/10</td>
<td>10/10 10/10</td>
<td>10/10 10/10</td>
</tr>
</tbody>
</table>

<sup>a</sup>No. of animals tumor-free/No. given EL 4 tumor cell injections.

In vitro cytotoxicity of peritoneal macrophages.—We tested peritoneal macrophages taken from mice 10–15 days after receiving 0.1 or 1.0 mg B. dermatitidis mixed with 10<sup>6</sup> EL 4 cells for their ability to release [<sup>3</sup>H]dUrd-prelabeled EL 4 cells. The data in text-figure 4 show that there was at least a twofold difference in the amount of lysis caused by macrophages from treated mice compared to the amount from normal resident macrophages. There was a consistent drop in the amount of lysis when the E:T was increased from 10:1 to 100:1.

**Effect of macrophages on tumor proliferation.**—To determine if peritoneal macrophages could halt the growth of EL 4 cells in vitro, we examined [<sup>3</sup>H]dThd incorporation by EL 4 cells in the presence of various macrophage preparations (text-fig. 3). At a 100:1 E:T, resident macrophages reduced [<sup>3</sup>H]dThd incorporation into EL 4 by 44%. A significant difference in the amount of incorporation was not seen in the presence of macrophages from mice given 0.1 or 1.0 mg B. dermatitidis within 2 hours of tumor cell injection. The amount of survival decreased at least threefold if the treatment was given 1 day after tumor cell injection. No animal treated between 3 and 11 days with a single dose of B. dermatitidis showed significant increase in survival (text-fig. 2).

**Table 2.—Regression of established tumors after treatment with B. dermatitidis**

<table>
<thead>
<tr>
<th>No. of EL 4 tumor cells injected</th>
<th>Treatment time</th>
<th>Tumor regression 60 days post treatment with:&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1.0 mg B. dermatitidis</th>
<th>0.2 ml PBS</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2 hr</td>
<td>6/10 0/10</td>
<td>0/10</td>
<td>&lt;0.02</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>2/10 0/10</td>
<td>0/10</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 days</td>
<td>0/10 0/10</td>
<td>0/10</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2 hr</td>
<td>9/10 0/10</td>
<td>0/10</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>2/10 0/10</td>
<td>0/10</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 days</td>
<td>0/10 0/10</td>
<td>0/10</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2 hr</td>
<td>10/10 0/10</td>
<td>0/10</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>6/10 0/10</td>
<td>0/10</td>
<td>&lt;0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 days</td>
<td>4/10 0/10</td>
<td>0/10</td>
<td>&lt;0.08</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>No. of animals tumor-free/No. given injections of tumor.

<sup>b</sup>Chi-square test with Yates' correction. NS=not significant; P≥0.10.

dThd incorporation at a 100:1 E:T. This was greater than a 45% difference when compared to resident macrophages at the same concentration. The difference between these 2 groups was even greater at 1:1 and 10:1 E:T.

**Interpretation of the data**

**Text-figure 3.—Macrophage-mediated cytostasis of EL 4 assessed by inhibition of [<sup>3</sup>H]dThd incorporation.**

- **Control** (PBS): Normal resident macrophages. There was a consistent drop in the amount of lysis when the E:T was increased from 10:1 to 100:1.

- **Macrophages from mice showing complete suppression of tumor growth at 10 days after having received 10<sup>6</sup> EL 4 + 1.0 mg B. dermatitidis.** Data are means ± SE.
or 1.0 mg *B. dermatitidis* mixed with $10^4$ EL 4 cells. The tests were done at 10 and 15 days after injection of the mixture. In one experiment, lymph node and spleen cells were taken at 10 days from mice that had received the mixture on days 0, 3, and 5. No cytotoxic lymphocytes were detected in spleen and lymph node preparations from mice that showed suppression of tumor growth.

**DISCUSSION**

The results presented here demonstrate that thimerosal-killed *B. dermatitidis* yeast cells can cause suppression of EL 4 lymphoma in C57BL/6J mice. Other studies on the mechanisms of tumor regression by immunopotentiating agents have shown that macrophages are important effector cells (1, 3). Previous studies in this laboratory have shown that *B. dermatitidis* can activate macrophages to become fungicidal (McDaniel LS, Cozad GC: Submitted for publication). It is possible that *B. dermatitidis*-dependent tumor suppression is mediated by activated macrophages. Although the possible involvement of other mechanisms in the tumor suppression cannot be ruled out, our experiments show that peritoneal macrophages from mice treated with nonviable *B. dermatitidis* yeast cells plus EL 4 lymphoma cells have an enhanced potential for in vitro tumor cell stasis and killing.

Nonviable *B. dermatitidis* yeast cells appear to be more effective than *C. parvum* at stopping the growth of EL 4 tumor in vivo. As evidence, O'Neil and Stebbing (15) recently reported that preimmunization of mice with *C. parvum* failed to inhibit the growth of EL 4 in C57BL/6J mice. Conversely, preimmunization with *B. dermatitidis* significantly increased the mean survival time of mice challenged with EL 4. This protective effect was seen at 3–6 days after sc or ip injection of *B. dermatitidis*. It is curious that the route of injection had little effect on the time sequence of protective effects.

It was originally thought that the highest degree of enhanced survival would coincide with the peak in phagocytic activity of peritoneal macrophages (i.e., 15–18 days post primary *B. dermatitidis* injection). However, our experiments showed that this was not the case. The protective effects dropped off, and the mean survival time returned to that of the controls by day 15, even though peritoneal macrophages remained activated. An interpretation of these results may include the possible involvement of natural killer cells. Alternatively, the lack of correlation between antitumor and phagocytic activities could be the result of induction of functionally different macrophage populations as has been reported in other systems (16).

*B. dermatitidis* produces a granulomatous reaction in tissue (17). Such reactions have been reported for other agents currently used in immunotherapy (18). *B. dermatitidis* yeast cells are known to contain cell wall-associated toxin (19). Although the *B. dermatitidis* endotoxin has not been extensively characterized, it is doubtful that this toxin is identical to bacterial endotoxin that is capable of activating macrophages in vitro to a tumoricidal state (5). As evidence, it has been reported that *C. albicans* possesses an endotoxin that shares some of the properties of bacterial LPS but is not exactly comparable to it (20). Nevertheless, such a substance could mediate the protective effects we observed.

We have considered the possibility of direct interaction between nonviable *B. dermatitidis* and EL 4 tumor cells. Our data suggest that some interaction may be important since the highest degree of protection was obtained by the mixing of yeast cells with tumor cells prior to injection. However, we do not believe that the protective effects result solely from an adverse action of yeast cells against tumor cells. For example, significant protection was obtained by preimmunization of mice with *B. dermatitidis* alone. There were no adverse effects on EL 4 observed by the light microscope. Also, at a 10:1 ratio of nonviable yeast cells to tumor cells in vitro, the EL 4 cells continued to incorporate $[^3H]$dThd at near normal levels (data not shown). In addition, the protective effects were not limited to mixing yeast cells with tumor cells in vitro; the same results could be obtained by the injection of yeast cells and tumor cells separately on opposite sides of the peritoneum.

The preliminary results we obtained on the therapeutic potential of *B. dermatitidis* indicate that the yeast cells had a therapeutic effect only if administered within 24 hours after tumor transplantation. It has been reported that immunoadjuvants can generate cells that are capable of suppressing cell-mediated immune responses (21). Possibly, *B. dermatitidis* suppresses cellular immunity in tumor-bearing mice, thereby enhancing tumor growth. However, no conclusion can be drawn until further data have been gathered on the effects of *B. dermatitidis* on tumor-bearing mice. A regimen that involves several doses of *B. dermatitidis* over a
period of time probably would be more therapeutically effective than a single dose.

The SEM revealed that there was strong interaction between macrophages from mice that showed complete suppression of tumor growth and EL 4 cells in vitro. A lack of such interaction between EL 4 and resident macrophages was also noted. Also, it has been reported that intimate contact between target cells and macrophages is required for tumor cell lysis in vitro (22, 23). Our observations of EL 4 with the SEM agree with these findings.

We were unable to detect cytotoxic lymphocytes in the spleen or lymph nodes of mice that showed suppression of tumor cell growth. Local immunity could have generated cytotoxic lymphocytes within the peritoneum, but we did not test for such cells. However, the fact that mice showing tumor suppression were not resistant to further EL 4 challenge indicates a lack of specific immunity. This observation supports the idea of nonspecific suppression of tumor growth by macrophages.

Our experiments indicate that nonviable B. dermatitidis yeast cells can significantly effect the outcome of tumor growth in the mouse system. This preparation may offer a viable, and when applied in human beings, the possibility of infection in relatively immunodeficient cancer patients would be eliminated.

REFERENCES