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MACROPHAGES ELICITED WITH HEAT-KILLED BACILLUS CALMETTE-GUÉRIN PROTECT C57BL/6J MICE AGAINST A SYNGENEIC MELANOMA

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There is a close relationship between the response of mononuclear phagocytes to bacterial infections and their capacity to inhibit tumor growth. Macrophages obtained from mice infected with *Listeria monocytogenes* or with Bacillus Calmette-Guérin show enhanced bactericidal activity; in addition, these cells are reported to inhibit the growth of tumors in vivo (1, 2) and destroy tumor cells in vitro (3–5). These findings suggest that mononuclear phagocytes have the capacity to play a pivotal role in defending the host against neoplasms.

To date, however, most of the systems in which macrophage cytotoxicity has been examined have involved incubation of the effector cells with the tumor cell targets in vitro. Cytotoxicity has generally been assessed by release of a radioactive label previously incorporated into the target cell or by a measurement of the number of viable tumor cells remaining at the end of the assay. However, there is no quantitative standard by which cytotoxic effects in vitro can be correlated with inhibition of tumor growth in vivo. Numerous studies have described the destruction in vitro of 60–70% of an inoculum of tumor cells by suitably activated macrophages (6, 7). Yet, if activated macrophages are to make a meaningful contribution to the eradication of neoplasms, the macrophages must be capable of destroying the clonogenic tumor cells within a tumor bed in the tumor-bearing host.

To evaluate the efficacy of mononuclear phagocytes in tumor resistance, we have examined the capacity of these cells to inhibit the formation of tumors by $B_5 59$ cells, a subclone of the B16 melanoma (8, 9), in its syngeneic host, the C57BL/6 mouse. We have chosen this system for several reasons. The B16 melanoma arose spontaneously. Like most spontaneously arising tumors it is not immunogenic (as assayed in challenge-protection experiments) and does not elicit concomitant immunity (Results). The tumor has been extensively studied, grows rapidly to form lethal tumors in vivo, and is easy to propagate and to clone in vitro. The experiments described below examine the tumorigenic potential of $B_5 59$ cells when these cells are injected into syngeneic mice together with various putative cytotoxic cells.

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Materials and Methods

Tumor Cells. $B_5$59 cells, a subclone of the B16 melanoma, have been extensively characterized (8, 9). The cells were maintained in monolayer culture in modified Eagle's medium (MEM)[1] (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 7.5% fetal bovine serum in a humidified atmosphere containing 6% CO_2. Before their use, a semiconfluent monolayer was disaggregated with 0.25% trypsin and EDTA (Grand Island Biological Co.) for 5 min. The cells were then washed twice with phosphate-buffered saline without Ca^{++} or Mg^{++} (PBS), and then resuspended in PBS.

Mice. Female C57BL/6J mice, between 6 and 8 wk of age, were obtained from The Jackson Laboratory (Bar Harbor, Maine). These animals were used either as tumor recipients or as donors of peritoneal cells. For experiments requiring irradiation, mice were exposed to 650 rad from a 2,400-Ci cesium source gamma cell (located at the Sloan-Kettering Institute for Cancer Research, New York). BALB/c nude mice were a generous gift of Dr. Seung-Il Shin (Albert Einstein College of Medicine, Bronx, N. Y.). (C57BL/6 × BALB/c)F_1 mice, 8 wk of age, were purchased from The Jackson Laboratory.

BCG. Mycobacterium bovis, strain Bacillus Calmette-Guérin (BCG) (Pasteur A1011) was obtained frozen from the Trudeau Institute (Saranac Lake, N. Y.); and was used for all experiments. The mice were injected either intravenously or intraperitoneally with 10^7 organisms in 0.1 ml of PBS. 21-24 d later, the mice were injected with an equal number of either live or dead BCG intraperitoneally, and the peritoneal exudate was harvested 3-4 d later. For experiments in which BCG-immunized mice were challenged with the melanoma, the mice were immunized with BCG according to the same protocol. 3-4 d after the second intraperitoneal injection of BCG, the mice were inoculated either subcutaneously or intraperitoneally with $B_5$59 cells. For injection with dead BCG, the vials were autoclaved at a temperature of 120°C at a pressure of 15 lb/in^2 for 25 min, and then diluted to 10^7 organisms/0.1 ml. The peritoneal exudate from mice injected twice with heat-killed BCG contained an average of 35-45% macrophages as determined by Wright-Giemsa staining.

Peritoneal Cells

Peritoneal cells were harvested and maintained in culture by a modification of the method of Cohn and Benson (10) as described by Michl et al. (11).

INFLAMMATORY EXUDATE CELLS. For some experiments, mice were injected intraperitoneally with 1 ml of brewer's thioglycollate medium (Difco Laboratories, Detroit, Mich.), 1 or 4 d before harvest of the peritoneal exudate. Wright-Giemsa staining of cytocentrifuge preparations revealed a differential count of >80% macrophages 4 d after thioglycollate injection, and ~80-90% polymorphonuclear leukocytes when the peritoneal exudate was harvested 1 d after intraperitoneal thioglycollate injection.

BCG-ELICITED PERITONEAL CELLS. After immunization with heat-killed BCG according to the schedule outlined above, the peritoneal cells were harvested as described. For some experiments, the adherent peritoneal cells were separated from the nonadherent cells by incubating 2×10^7-3×10^7 peritoneal cells on 100-mm glass Petri dishes in MEM that contained 20% fetal bovine serum for 3-4 h at 37°C in a humidified CO_2 incubator. The plates were then washed three times with ~5 ml of ice-cold PBS that contained 1 mM EDTA. To obtain the nonadherent cell fraction, these wash fluids were combined and centrifuged at 150 *g* for 10 min at 4°C. The cell pellet was resuspended in PBS and stored at 4°C until used. The adherent-cell fraction was harvested by incubating the cells remaining on the surface of each glass Petri dish in 5 ml of PBS that contained 1 mM EDTA for 10 min at 4°C. The cells were then gently scraped off with a sterile rubber policeman, centrifuged, and resuspended as described above for the nonadherent cells. Generally, ~50% of the total cells plated were recovered in the nonadherent cell fraction; the viability of these cells was usually ~80%. Recovery of adherent cells averaged between 30 and 50% of the total cells plated; their viability,

[1] *Abbreviations used in this paper:* BCG, *Mycobacterium bovis*, strain Bacillus Calmette-Guérin; BCG-PE, BCG-elicited peritoneal exudate cells; HRP-IgG, horseradish peroxidase-anti horseradish peroxidase immune complexes; IgG-GRBC, IgG-coated, glutaraldehyde-fixed sheep erythrocytes; PBS, phosphate-buffered saline without Ca^{++} or Mg^{++}.

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as determined by trypan blue exclusion, was usually >70%. In all experiments, the cell number reported is the number of viable cells.

Tumor Neutralization Assay. A modification of the tumor neutralization assay developed by Winn (12) was used to assess the effectiveness of various peritoneal exudate cells against B_559 melanoma cells in vivo. Peritoneal cells were harvested as described above, washed, resuspended in PBS, and then mixed at various cell concentrations with B_559 cells. A final vol of 0.2 ml of the cell mixture was then injected either subcutaneously in the flank or intraperitoneally into C57BL/6J mice. Mice were monitored twice-weekly for tumor formation. At the end of each experiment, mice without gross evidence of tumors were killed and dissected. The time of killing of these mice is indicated in the Figure and Table legends. In all experiments, the number of mice injected is the number reported. No mice used in any experiment were excluded from the data reported.

Phagocytosis Assays. Binding and phagocytosis of IgG- or complement-coated sheep erythrocytes was measured as described previously (11). The phagocytic index was determined by multiplying the percentage of macrophages that had ingested erythrocytes by the average number of erythrocytes ingested per macrophage; the binding index was obtained similarly. Uptake of horseradish peroxidase-anti-horseradish peroxidase immune complexes was carried out according to the method of Steinman and Cohn (13). Phagocytosis of IgG-coated glutaraldehyde-fixed sheep erythrocytes was assayed by fluorescence microscopy (J. D. Loike and S. C. Silverstein. Manuscript in preparation.).

Results

Several protocols for eliciting tumoricidal macrophages with BCG have been described (1, 4–6). In most of these reports, living bacteria were injected into the mice. To determine whether an infection with living BCG was necessary for the development of cytotoxic peritoneal cells, we carried out the following experiment. Mice were injected intravenously with live BCG to establish a *Mycobacterium* infection (assayed by a two- to five-fold increase in spleen weight over normal spleen weights at autopsy), and then boosted intraperitoneally 14–21 d later with either live or dead (heat-killed) BCG. Other groups of mice received an initial intraperitoneal injection of dead BCG followed by a boost with dead BCG. The peritoneal exudate cells from animals treated in these ways were harvested, mixed with the melanoma cells, and then injected subcutaneously into the flank of normal C57BL/6 mice. The results of these experiments are presented in Fig. 1. Although the peritoneal cells elicited by live plus live BCG, and live plus dead BCG inhibited tumor engraftment by 60 and 25%, respectively, only the peritoneal cells elicited by two injections of dead BCG were 100% successful in preventing tumorigenesis by the melanoma. Additional experiments showed that peritoneal cells from mice injected intraperitoneally with a single dose of live BCG 24 d before harvest, or with heat-killed BCG 4 d before harvest, failed to inhibit melanoma formation (data not shown). These results indicate that systemic *Mycobacterium* infection is not a prerequisite for the development of tumoricidal peritoneal cells. In fact, systemic infection is relatively inefficient, whereas immunization with two inoculations of dead BCG intraperitoneally is the most efficacious of the six protocols tested. Therefore in all subsequent experiments, mice that had received two injections of dead BCG according to the protocol described in Materials and Methods were used as donors of peritoneal cells.

To determine whether BCG immunization confers systemic immunity to B_559 melanoma cells, mice were immunized with dead BCG, and then injected with the melanoma cells either subcutaneously or intraperitoneally. The results of a representative experiment are presented in Table I. As can be seen from the data, when BCG

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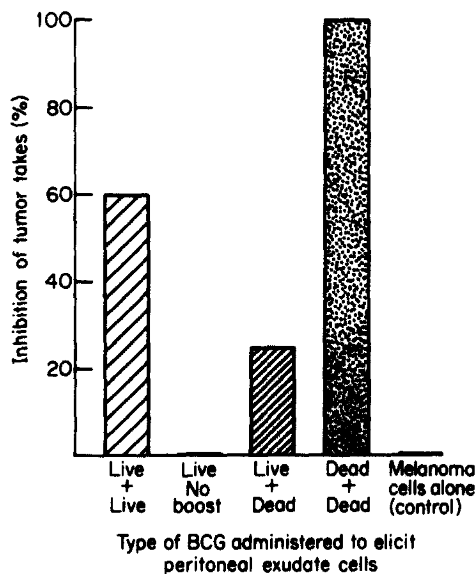

FIG. 1. C57BL/6J mice were injected either intravenously with 10^7 living BCG organisms in 0.1 ml of PBS or intraperitoneally with 10^7 heat-killed BCG organisms in 0.1 ml PBS. 21-24 d later, the mice were injected intraperitoneally with 10^7 live or dead BCG. The peritoneal exudate was harvested 3-4 d later, mixed with the B_659 melanoma cells at a ratio of peritoneal cells:melanoma cells of 20:1, and the mixture was injected subcutaneously into naive C57BL/6J mice. The mice were monitored twice-weekly for tumor formation. 50 d after inoculation of melanoma cells, the mice were killed and dissected. The data are the average of two experiments. At least five mice were used in each group in each experiment.

TABLE I

Effects of Intraperitoneal BCG Immunization on Tumorigenicity of B_559 Melanoma Cells

Route of injection of B_659 cells*	No. mice with tumors/No. mice injected	Latency
		d
Intraperitoneal	0/15	—‡
Subcutaneous	9/9	17-21

* 2 × 10^6 B_659 melanoma cells were injected either intraperitoneally or subcutaneously into syngeneic mice immunized with dead BCG as described in Materials and Methods. Control experiments showed that this tumor cell dose results in tumors in 100% of the naive (non-BCG-immunized) mice injected, with a latent period of 12-16 d.

‡ 35 d after inoculation, the mice were killed and dissected.

immunization was intraperitoneal, and the tumor challenge was also intraperitoneal, 100% of the mice were protected from the tumor. However, when mice were immunized intraperitoneally with BCG and challenged with tumor cells subcutaneously, all of the mice succumbed to the melanoma, despite the presence in their peritoneal cavities of cells capable of eliminating the tumor.

To determine whether the BCG-induced protection was absolute or if it was dependent upon the number of melanoma cells injected, control and BCG-immune

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animals were inoculated intraperitoneally with varying numbers of B_559 cells. At a tumor cell inoculum of 10^5, 2×10^5, or 5×10^5 cells, 100% of the unimmunized animals developed tumors. In contrast, when the same number of cells was inoculated intraperitoneally into the BCG-immune animals, tumor formation was completely inhibited (Fig. 2). The complete absence of tumors was shown by dissection of these mice 60 d after injection with the tumor cells. However, when 10^6 melanoma cells were injected intraperitoneally into the BCG-immunized mice, ~20% of the animals, upon dissection, showed a peppering of the peritoneal cavity with tumor nodules. Furthermore, when 5×10^6 melanoma cells were injected intraperitoneally, 100% of the mice developed tumors at this site. These results indicate that although the BCG-immune mouse is protected against 5- to 10-fold the 100% tumorigenic dose of B_559 cells, a 50-fold-excess number of tumor cells can override this protection.

The results in Table I further suggest that although BCG-immune animals contain tumoridical cells in their peritoneal cavities, these cells are not present and/or cannot be delivered to other sites, such as the subcutaneous tissues, when B_559 cells are inoculated at these distant sites. To examine this issue further, and to determine the minimal number of BCG-immune peritoneal cells required to protect against a subcutaneous challenge with B_559 cells, we mixed varying numbers of peritoneal exudate cells from BCG-immunized mice with a constant number (2×10^5) of B_559 cells and inoculated them subcutaneously into naive C57BL/6J mice. Control experiments showed that 2×10^5 B_559 cells was a 100% tumorigenic dose in these mice.

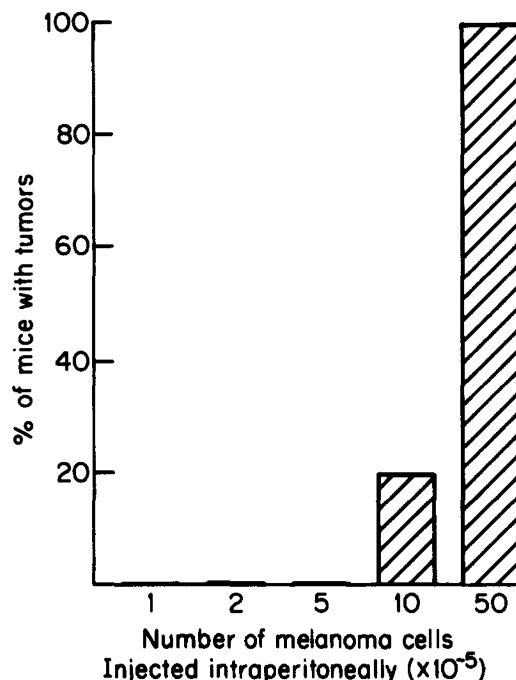

FIG. 2. C57BL/6J mice were primed and boosted with 10^7 heat-killed BCG intraperitoneally as described in Materials and Methods. 4 d after the last injection of BCG, the mice were challenged intraperitoneally with varying numbers of B_559 melanoma cells and then monitored twice weekly for tumor development. Mice were considered tumor free only when no melanoma nodules were observed intraperitoneally upon dissection at 60 d after the melanoma challenge.

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Mice injected with 2×10^5 B_559 cells and an equal number of BCG-immune peritoneal cells all developed tumors. However, mice inoculated with the same number of B_559 cells and a 20-fold or greater excess of BCG immune peritoneal cells did not develop tumors (Fig. 3), whereas mice inoculated with B_559 cells and a 10-fold excess of BCG-immune peritoneal cells exhibited a delay in tumor formation. Nevertheless, 80% of the mice in this last group ultimately succumbed to the melanoma. Because the number of cells in the BCG-immune peritoneal cavity is ~5 $\times 10^6$, these results suggest that roughly the same number of BCG-immune cells is required to protect subcutaneously as is required to protect intraperitoneally.

To determine whether inhibition of tumor engraftment could be produced by leukocytes induced by inflammatory stimuli other than BCG, we mixed 2×10^5 B_559 cells with 5×10^6 peritoneal cells from mice injected 4 d previously with thioglycollate broth and injected this mixture subcutaneously into C57BL/6J mice. In this case, the peritoneal exudate consisted of 80–90% macrophages by Wright-Giemsa staining. These mice all developed melanomas at the site of injection (Table II). We had previously noted that peritoneal cells from BCG-immunized mice contain 6–15% polymorphonuclear leukocytes (by Wright-Giemsa staining). To determine whether polymorphonuclear leukocytes could inhibit melanoma formation, peritoneal exudate cells (>90% of which were polymorphonuclear leukocytes) were obtained from mice injected 1 d previously with thioglycollate medium. Mice inoculated with a mixture of these exudate cells and B_559 cells all developed tumors (Table II). Similar results were obtained when resident peritoneal cells (which are ~30–40% macrophages) were injected together with B_559 cells (Table II). These findings demonstrate that resident macrophages, thioglycollate-elicited macrophages, and polymorphonuclear leukocytes are incapable of inhibiting tumor formation when mixed with B_559 cells. The failure of polymorphonuclear leukocytes to inhibit tumor formation is especially noteworthy, because Nathan et al. (14) have shown that when these cells are stimulated pharma-

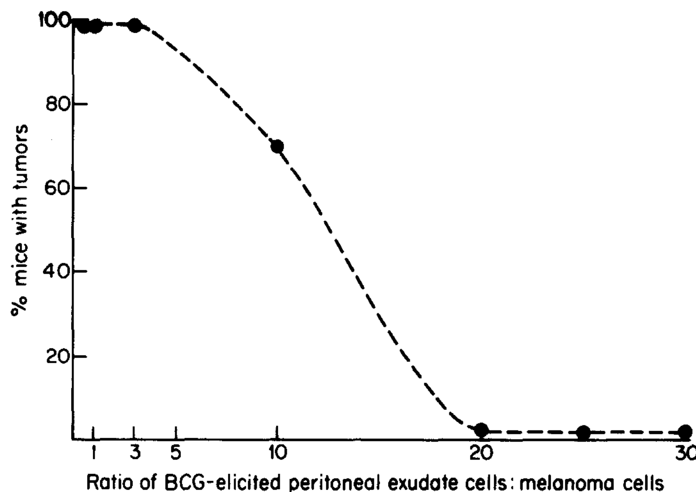

FIG. 3. B_559 melanoma cells (2×10^5) were mixed with varying numbers of BCG-induced peritoneal cells and injected subcutaneously into syngeneic mice. The mice were monitored twice weekly for tumor formation. 35 d after tumor inoculation, all mice were killed and dissected for tumors that might have been too small to detect by palpation. None were found.

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TABLE II
Resident and Thioglycollate-Broth-elicited Peritoneal Cells Do Not Suppress Tumorigenicity of B_559 Melanoma Cells

Source of peritoneal cells*	No. mice with tumors/No. mice injected	Latency‡
		d
BCG-immunized mice	1/30	40
Thioglycollate-injected mice		
1 d (80-90% polymorphonuclear leukocytes)	10/10	8-10
4 d (80% macrophages)	25/25	12-16
Resident (uninjected)	18/19	16-20
Control: B_559 cells alone	20/20	10-14

* The peritoneal cells were elicited from C57BL/6J mice as described in Materials and Methods. Heat-killed BCG was used for immunization. In each case, naive C57BL/6J mice were inoculated subcutaneously with the cell mixture that consisted of 2×10^6 B_559 cells and 5×10^6 peritoneal cells.

‡ Latency = the number of days before the tumor nodule is palpable. Mice without gross evidence of tumors 60 d after inoculation were killed and dissected.

cologically, they do have the capacity to secret copious amounts of H_2O_2 and, thereby, destroy some tumor cells.

The data presented above, therefore, demonstrate that only the peritoneal exudate induced by BCG has tumoricidal activity when tested in vivo. Because the peritoneal cells from BCG-immunized mice were harvested 3-4 d after the second intraperitoneal injection of dead bacteria (Materials and Methods), it was possible that some dead bacteria remained in the peritoneal exudate. There have been many reports of the efficacy of live BCG introduced intralesionally to induce regression of established tumors (15, 16). Therefore, to determine whether dead bacteria were responsible for the abrogation of tumorigenicity in these experiments, the BCG-containing peritoneal cells were sonicated in a Heat Systems sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) to release their content of BCG before admixture with the B_559 cells, and the entire mixture was then injected subcutaneously into normal C57BL/6J mice. As Table III shows, only viable BCG-elicited cells are capable of preventing the engraftment of the melanoma. In other experiments (data not shown), injection of dead BCG into B_559 melanomas growing in unimmunized animals did not cause either rejection or regression of the tumor, further strengthening the conclusion that dead bacilli in the peritoneal cells do not themselves inhibit tumor engraftment.

In the modified Winn-type assays described above, two distinct cell populations were mixed together and inoculated into normal hosts. Although the recipient mice were always syngeneic with both the B_559 melanoma cells and the BCG-elicited peritoneal cells, there was the possibility that the BCG-elicited peritoneal cells stimulated the production of cytotoxic effector cells of recipient origin and that the melanoma cells were destroyed as bystanders to a host versus graft reaction. That the cytotoxicity and prevention of tumorigenicity is not a result of a bystander effect is shown by experiments in which 5×10^6 resident peritoneal cells from unimmunized (C57BL/6J [$H\text{-}2^b$] $\times$ BALB/c [$H\text{-}2^d$])F_1 mice were mixed together with the melanoma cells before their subcutaneous injection into C57BL/6J mice. 9 of the 10 mice thus

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TABLE III

Requirement for Viable Peritoneal Cells in the Modified Winn Assay

BCG-elicited peritoneal cells*	Cell viability‡	No. mice with tumors/ No. mice injected
	%	
Untreated	90	0/20‖
Sonicated§	0	19/20

* Peritoneal cells were obtained from mice immunized twice with heat-killed BCG as described in Materials and Methods. 5×10^6 viable BCG-elicited peritoneal cells or the sonicated debris from 5×10^6 BCG-peritoneal cell equivalents (determined by protein assay) were mixed with 2×10^5 B_559 cells and injected subcutaneously into naive C57BL/6J mice.

‡ Cell viability was determined microscopically by trypan blue exclusion. The sonicates were used only when no whole cells were observed on microscopic examination.

§ Peritoneal cells were suspended in PBS at a concentration of 5×10^7/ml, kept on ice, and subjected to three 10-s bursts in a Heat Systems sonicator.

‖ Mice without gross evidence of tumors 60 d after inoculation were killed and dissected.

TABLE IV

Effect of Adherent and Nonadherent BCG-elicited Peritoneal Cells on Tumor Formation by B_559 Cells

BCG-elicited peritoneal cells*	No. mice with tumors/No. mice injected
Total peritoneal cells + B_559 cells	1/30
B_559 cells alone	20/20
NON-AD peritoneal cells + B_559 cells	8/9
AD peritoneal cells + B_559 cells	3/15
NON-AD + AD + B_559	0/4

* BCG-elicited peritoneal cells were obtained as described in Tables II and III. The cells were separated into adherent (AD) and nonadherent (NON-AD) fractions by plating them on glass Petri dishes, as described in Materials and Methods. The cells were recovered and 5×10^6 viable cells of each type was mixed with 2×10^5 B_559 cells and injected subcutaneously into C57BL/6J mice. Mice without evidence of tumors 60 d after inoculation were killed and dissected.

inoculated developed melanomas at the site of injection, despite the presence of allogeneic cells in the nascent tumor bed.

To identify the cell type(s) that inhibit melanoma engraftment, BCG-elicited peritoneal cells were separated into adherent and nonadherent subsets by plating them for 4 h on glass Petri dishes (Materials and Methods). Approximately 40% of these cells adhere to the dishes. The glass-adherent and nonadherent cell populations were collected separately, mixed with the melanoma cells, and injected subcutaneously into C57BL/6J mice. 80% of the mice that received adherent BCG-elicited cells together with melanoma cells were protected from tumor formation, whereas 88% of the mice inoculated with a mixture of nonadherent BCG-elicited cells and the melanoma cells developed tumors (Table IV), and died. The latent period of tumor appearance in the latter group was slightly longer than in animals inoculated with melanoma cells alone. In addition, in a reconstitution experiment, mice injected with

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a mixture of melanoma cells and 2.5×10^6 cells each of the adherent and nonadherent cell fractions were completely protected against tumor formation.

Examination of this BCG-adherent cell fraction by phase-contrast microscopy indicated that the majority of these cells were macrophage-like in appearance. To further characterize these cells, we examined their expression of Fc and complement receptors and their capacity to phagocytize a variety of particles. As indicated in Table V, glass-adherent cells from the peritoneal cavities of normal or BCG-immunized C57BL/6J mice were roughly equivalent with respect to both the percentage of mononuclear leukocytes that bound IgG- or complement-coated erythrocytes and the total number of immune ligand-coated erythrocytes attached to each leukocyte. It is important to note, however, that a significantly smaller percentage of the BCG-elicited adherent mononuclear cells ingested IgG or complement-coated erythrocytes than did the adherent cells from normal mice, and each of these BCG-elicited adherent cells ingested fewer immune ligand-coated erythrocytes than did its unstimulated counterpart.

Resident macrophages from outbred Swiss mice (from The Rockefeller University,

TABLE V
Phagocytosis by C57BL/6J Adherent Cells

	Ingestion of E(IgG)*			Binding of E(IgG)		
	Percentage of macrophages with E(IgG) ingested	Phagocytic index‡	Phagocytic index: control	Percentage of macrophages with E(IgG) attached	Attachment index‡	Attachment index: percentage of control
Resident	88	630	100	74	1,192	100
BCG elicited	57	242	38	79	1,192	100
	Ingestion of E(IgM)C'§			Binding of E(IgM)C'		
Resident	79	362	100	81	1,481	100
BCG elicited	58	149	41	80	1,193	81
	Ingestion of IgG-GRBC					
Resident	95	2,184	100			
BCG elicited	96	1,378	64			
	Ingestion of HRP-IgG					
Resident	92	NA‖				
BCG elicited	90	NA				

C57BL/6J peritoneal cells, either BCG elicited or resident (unelicited), were plated on 13-mm glass coverslips at a density of 2×10^5–2.5×10^5 cells per coverslip in MEM + 10% fetal bovine serum. The cells were allowed to adhere for 4 h at 37°C, and then binding and phagocytosis of IgG- or complement-coated erythrocytes or immune complexes was carried out as described in Materials and Methods.

* E(IgG), IgG-coated sheep erythrocytes.

‡ Phagocytic and attachment indices were determined as described in Materials and Methods.

§ E(IgM)C', complement-coated sheep erythrocytes.

‖ NA, not applicable.

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New York) do not ingest complement-coated erythrocytes via their complement receptors (17, 18). The finding that complement receptors of resident macrophages from C57BL/6J mice promote phagocytosis of these particles was therefore unexpected and requires additional investigation.

To further explore the phagocytic capacity of BCG-elicited adherent cells, we measured their ability to ingest IgG-coated glutaraldehyde-fixed sheep erythrocytes (IgG-GRBC) or horseradish peroxidase-anti-horseradish peroxidase immune complexes (HRP-IgG). In these experiments, 96% of the BCG-adherent cells ingested IgG-GRBC; similarly, 90% of these cells ingested the HRP-IgG immune complexes (Table V). These results confirm that between 90 and 95% of the BCG-elicited adherent mononuclear cells bear Fc receptors and are phagocytic. We conclude, therefore, that nearly all of them are macrophages.

The experiments described thus far show that BCG-elicited macrophages are required to inhibit the grafting of the B_559 melanoma; however, they do not indicate whether these macrophages act as cytotoxic effector cells or require, in addition, the ingress and participation of host leukocytes. To examine these questions, x-irradiated C57BL/6J or nude BALB/c mice were used as recipients of a mixture of BCG-elicited peritoneal cells and melanoma cells. Mice were irradiated with 650 rad (Materials and Methods) 24–36 h before inoculation with the cell mixture.

Mice irradiated with 650 rad have markedly decreased numbers of leukocytes in their blood, beginning 24–48 h after irradiation; this depression in circulating leukocytes lasts for 15–20 d (19). In studies to be reported in detail elsewhere (T. A. Calvelli, V. H. Freedman, S. Silagi, and S. C. Silverstein. Manuscript in preparation.) we have observed that a variant melanoma cell line that elicits a pronounced inflammatory response when inoculated subcutaneously into normal C57BL/6J mice, fails to evoke any infiltrations of either polymorphonuclear or mononuclear leukocytes when injected into C57BL/6J mice previously irradiated with 650 rad. For this reason, we assume that C57BL/6J mice given 650 rad of total-body irradiation are incapable of mounting an inflammatory response to the tumor inoculum in the subcutaneous tissue.

When mice irradiated 24–36 h before were injected with BCG-elicited peritoneal cells admixed with B_559 cells, 14 out of 16 animals had no evidence of tumor 51 d later (Table VI). Similarly, when the same experiments were carried out using nude mice as recipients, 11 out of 13 nude mice had no tumors 60 d after inoculation. In all cases, control animals given only the melanoma cells succumbed to the tumors (Table VI). These experiments show that immunologically impaired mice are protected from developing a melanoma by the simultaneous administration of BCG-elicited peritoneal cells with the tumor cells. We conclude therefore that the BCG-elicited peritoneal cells themselves are capable of exerting a cytotoxic effect against the tumor cells, and that participation of host leukocytes is not required for the prevention of melanoma formation.

The B_559 cells used in these experiments do not elicit concomitant immunity. This was shown in experiments in which a total of 20 C57BL/6J mice were inoculated with 2×10^5 B_559 cells in the left flank. All of these mice developed tumors with a latent period of 11.5 ± 2.8 d. 13–19 d after the first inoculation (a time when tumors were already manifest) these mice were injected with 2×10^5 B_559 cells in the opposite

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TABLE VI

Effect of BCG-induced Peritoneal Exudate Cells on Tumorigenicity of B_559 Cells in x-irradiated or Nude Mice

Cells injected	No. mice with tumors/No. mice injected					
	Irradiated*		Nude‡		Normal§	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
B_559	8/8	5/5	3/4	3/3	4/4	6/6
B_559 +BCG-PE‖	1/8	1/8	0/5	2/8	0/5	0/5

* 6- to 8-wk-old C57BL/6J female mice were exposed to 650 rad from a cesium gamma source. 24-36 h later, the mice were inoculated subcutaneously with either 2×10^5 B_559 cells or a mixture of 2×10^5 B_559 cells and 5×10^6 BCG-elicited peritoneal exudate cells (BCG-PE). Mice without gross evidence of tumors were killed and dissected 48 d after inoculation.

‡ 8- to 12-wk-old BALB/c nude mice were used. The experimental protocol was as described above except that the surviving mice were killed and dissected 54 d after inoculation.

§ 6- to 8-wk-old C57BL/6J female mice were used. The experimental protocol was as described above except that the surviving mice were killed and dissected 60 d after inoculation.

‖ Peritoneal exudate cells were elicited by immunizing C57BL/6J mice with heat-killed BCG as described in Materials and Methods and in Tables II-V.

TABLE VII

Injection of B_559 Cells or B_559 Cells Mixed with BCG-PE Cells Does Not Induce Immunity against the B_559 Melanoma

First inoculation*	Amputation‡	Second inoculation§	Mice with tumors	Latent period after second inoculation
				d
B_559	Yes	None	0/8‖	—
B_559	Yes	B_559	8/8	12-14
B_559 + BCG-PE	No	None	0/6‖	—
B_559 + BCG-PE	No	B_559	6/6	12-14

* C57BL/6J mice were injected subcutaneously in the thigh with 2×10^5 B_559 cells or subcutaneously in the flank with 2×10^5 B_559 cells and 5×10^6 BCG-PE cells.

‡ After the tumors were palpable (8 d), the tumor-bearing leg was removed surgically.

§ 2×10^5 B_559 cells were injected subcutaneously in the flank on the contralateral side 29 d after the first inoculation.

‖ Control animals were followed for 60 d.

flank. Tumors developed at the second inoculation site in all mice at least as rapidly (mean latent period 9.6 ± 2 d) as at the site of the initial tumor challenge.

To confirm that B_559 cells are not immunogenic and cannot be employed to immunize mice against this melanoma, mice were rechallenged with B_559 cells after surgical removal of a well-developed melanoma. These mice developed tumors at the second inoculation site at approximately the same rate as naive mice given a primary injection of tumor cells (Table VII).

It was possible that the BCG-elicited peritoneal cells exerted an adjuvant effect and promoted immunity to B_559 cells. Were this to occur, the immunity evoked could play an important role in inhibiting melanoma formation in mice inoculated with BCG-elicited peritoneal cells and B_559 cells. The experiments described in Table VI with irradiated or nude mice as recipients suggested that this does not occur. Nevertheless, we performed the following experiment to exclude this possibility. Mice

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were inoculated with a mixture of BCG-elicited peritoneal cells and B_559 cells. As expected, none of these mice developed tumors. 30 d after the first inoculation, these mice were rechallenged with B_559 cells on the contralateral side. All of these animals developed melanomas with the same rapidity as the control mice (Table VII). Thus, the prevention of tumor formation by the concomitant administration of BCG-peritoneal cells and the B_559 melanoma cells does not stimulate immunity to a second challenge with the B_559 cells.

Discussion

The potential importance of activated macrophages in tumor immunity has been recognized for nearly a decade. Scores of reports document the capacity of appropriately stimulated adherent cells to inhibit tumor cell growth, or to promote tumor lysis in cell culture systems (reviewed in 20, 21). However, comparatively few investigators have examined the effects of co-injection of immunologically activated peritoneal macrophages with syngeneic tumor cells on tumorigenesis in a syngeneic host animal (22–24). Our study was undertaken to define optimal conditions for eliciting such activated cells, to assess the capacity of these cells to inhibit tumor formation by a well-characterized syngeneic tumor in vivo, and to evaluate the relative roles of naive host-derived leukocytes and of stimulated donor-derived leukocytes in inhibiting tumor formation in vivo.

Systemic infection with a living microorganism, as described in several other investigations (1–7), is not necessary to elicit protection against B16 melanoma. In fact, systemic infection may be counter-productive (Fig. 1). Systemic infection with BCG induces natural suppressor cells in the bone marrow and spleen (25, 26). These suppressor cells have been shown to inhibit cell-mediated immunity in vitro (26). If a similar mechanism exists in vivo, systemic infection with live BCG may promote inhibition of immune reactions that are essential for tumor cell eradication. Our experiments indicate that local immunization with dead BCG, rather than systemic infection with live BCG, is a more successful adjuvant for immunotherapy. This suggestion is supported by reports describing potent anti-tumor effects after local administration of cell walls from either BCG or from *Nocardia rubra*, a *Mycobacterium*-related organism (22).

Immunological cross-reactivity between BCG and a human melanoma has been suggested as a mechanism for the tumor-inhibitory effects of BCG (27–29). Two lines of evidence indicate that such cross-reactivity is not responsible for the effects of BCG immunization reported here. First, peritoneal lymphocytes (nonadherent cells) from BCG-immunized mice do not inhibit tumorigenesis by B16 melanoma cells (Table IV). These lymphocytes do respond to BCG antigens, however. This is indicated by our preliminary findings that peritoneal lymphocytes from BCG-immunized mice confer the capacity to resist challenge with B16 melanoma cells upon naive mice when they are administered together with heat-killed BCG. Heat-killed BCG alone has no effect (V. H. Freedman and S. C. Silverstein. Unpublished observations.). Second, intraperitoneal BCG immunization does not confer systemic immunity to B16 melanoma cells. Mice challenged subcutaneously, that is, at a site distant from the original immunization(s), develop melanomas (Table II). In addition, mice inoculated with mixtures of BCG-elicited peritoneal cells (containing BCG) and B_559 tumor cells, are not immune to subsequent challenge with this tumor (Table VII).

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Systemic immunity to B16 melanoma cells can be elicited by immunizing C57BL/6J mice with a nontumorigenic variant of this tumor (30). Thus when a suitable cross-reacting antigen is used for immunization, systemic immunity to B16 melanoma is observed. We conclude that immunological cross-reactivity between BCG and B16 melanoma cells is not responsible for the inhibitory effects of BCG-elicited peritoneal cells or for tumorigenesis by B16 melanoma.

The nature of the cytotoxic cells in the BCG-elicited peritoneal exudate has been explored. The effector cells are concentrated exclusively in the adherent cell fraction (Table IV). At least 90% of these adherent cells possess Fc receptors (as measured by their uptake of HRP-IgG), and >95% of them are phagocytic (as measured by their injection of IgG-GRBC). Their phagocytic capacity distinguishes the vast majority of these cells from the nonphagocytic adherent cell described by Nathan et al. (31) and identifies them as mononuclear phagocytes. However, mononuclear phagocytes elicited by immunization with heat-killed BCG, and maintained in vitro for 4 h differ in several respects from resident macrophages or from macrophages induced by other inflammatory stimuli (18, 32). They ingest fewer erythrocytes than their resident or inflammatory counterparts (Table V; and [31]), and they exhibit a lower pinocytic rate[2] than either resident or inflammatory macrophages (V. H. Freedman and S. C. Silverstein. Unpublished observations.).

BCG elicits natural killer cells (34) and it has been suggested that this cell may be responsible for the in vitro tumoricidal activity of BCG-elicited mononuclear cell preparations. Most investigators report that both murine and human natural killer cells are neither adherent nor phagocytic (35-37); the effector cells used in our experiments are adherent and >90% of them are phagocytic. Thus is seems unlikely that natural killer cells make a significant contribution to the tumor-inhibitory properties of mononuclear phagocytes elicited with heat-killed BCG.

However, there may be a relationship between natural killer cells and mononuclear phagocytes. Reinherz et al. (38) have shown that human mononuclear cell populations that express natural killer activity contain a surface antigen that is present on monocytes and granulocytes. They suggest that human natural killer cells are members of the monocyte-myeloid series.

Our experiments show that mononuclear phagocytes elicited by two immunizations with heat-killed BCG prevent tumorigenesis by B16 melanoma cells in syngeneic C57BL/6J mice. They suggest that these cells eradicate B16 melanoma cells in the absence of additional participation by host leukocytes. This was tested by inoculating nude or sublethally irradiated (650 rad) recipient mice with mixtures of BCG-immune peritoneal cells and melanoma cells (Table VI). 84% of the nude mice and 87% of the irradiated mice were protected from tumor formation by the BCG-immune peritoneal cells. However, 100% of the control mice were protected from tumor formation by the BCG-immune peritoneal cells. Thus it is possible that host-derived leukocytes may play some role in the permanent eradication of the melanoma graft.

Despite the presence in their peritoneal cavities of cells capable of destroying the tumor inoculum, BCG-immunized B57BL/6J mice are not protected from melanoma formation when injected with this tumor subcutaneously. This could occur as a

[2] Edelson and Erbs (33) reported that BCG-elicited macrophages have an enhanced pinocytic rate. The cells used in their study differed from those used here because the cells used by Edelson and Erbs were maintained in culture for 24 h before measurement of their pinocytic rate.

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consequence of at least two separate processes. First, blood monocytes from BCG-immunized mice may not be capable of inhibiting tumorigenesis when co-injected with B16 melanoma cells into naive C57BL/6J mice. Monocytes may develop tumoricidal capacity only after their emigration from the vascular compartment. Second, Synderman and Pike (39) have documented the formation by tumors of inhibitors of monocyte chemotaxis in vitro. Histological studies of the early stages of melanoma development in naive or BCG-immunized mice show that there are very few host leukocytes in the melanoma before its vascularization (T. A. Calvelli, V. H. Freedman, S. Silagi, and S. C. Silverstein. Manuscript in preparation.). Thus melanoma cells may fail to promote inhibit leukocyte emigration from the vascular compartment into the tumor bed.

However, within the peritoneal cavity, the interaction of BCG with BCG-sensitive T-lymphocytes promotes the formation of lymphokines. These lymphokines act as chemoattractants for the influx of blood monocytes (40) and as signals for monocyte differentiation (41, 42). Monocytes may therefore differentiate into cytotoxic effector cells only after they have emigrated from the blood into the tissues, and only when they are in proximity with antigen driven T-lymphocytes within the same tissue compartment. These cytotoxic macrophages lack the capacity to migrate to other tissues, but once implanted within the substance of a tumor, they destroy the tumor cells with which they are in contact. Hibbs (43, 44) has come to a similar conclusion from his studies of mice immunized with live toxoplasma or BCG.

Summary

We have demonstrated that a murine cytotoxic peritoneal cell can be elicited by intraperitoneal immunization with heat-killed *Mycobacterium bovis*, strain Bacillus Calmette-Guérin (BCG). When these cells are injected together with cells of clone $B_5 59$ of B16 melanoma in a Winn-type transfer assay into syngeneic C57BL/6J mice, the tumorigenic potential of the melanoma is completely abrogated. Similarly, mice immunized intraperitoneally with dead BCG are protected against intraperitoneal challenge with a number of B16 melanoma cells sufficient to cause tumors in 100% of control mice. However, mice immunized intraperitoneally with dead BCG are not protected against tumor formation when B16 melanoma cells are injected subcutaneously. Co-injection of BCG-elicited peritoneal cells with B16 melanoma cells into nude or sublethally irradiated (650 rad) mice inhibits tumor formation in >85% of the mice, indicating that additional participation of host bone marrow- or thymus-derived leukocytes is not required to eradicate the tumor implant. The effector cell in the BCG-induced peritoneal exudate is adherent and phagocytic and is a mononuclear phagocyte. Nonadherent lymphoid cells from the same BCG-induced peritoneal exudate and from thioglycollate-broth-elicited granulocytes and macrophages neither prevent nor delay B16 tumor formation.

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