BACILLUS CALMETTE-GUÉRIN–ACTIVATED MURINE MACROPHAGES KILL SYNGENEIC MELANOMA CELLS UNDER STRICT ANAEROBIC CONDITIONS

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Murine macrophages activated by such organisms as *Mycobacterium bovis* (strain Bacillus Calmette-Guérin, BCG[1]), *Propionbacterium acnes* (*P. acnes*, formerly known as *Corynebacterium parvum* [1]), or *Listeria monocytogenes* are cytotoxic for tumor cells both in vitro and in vivo (2–7). One mechanism of cytotoxicity involves oxygen-dependent reaction(s) (8, 9). For example, cytolysis of tumor cells is mediated by hydrogen peroxide produced by activated macrophages or granulocytes stimulated with phorbol myristate acetate (PMA) (9–12). Under anaerobic conditions this cytolysis was not observed (11). In addition, lysis of antibody-coated tumor cells by activated murine macrophages (13) or by polymorphonuclear leukocytes is also dependent on oxygen (14). Furthermore, the spontaneous lysis of tumor cells coated with eosinophil peroxidase is mediated by hydrogen peroxide (15).

However, macrophage-mediated killing of tumor cells may occur by other mechanisms, at least some of which appear to be dependent on cytotoxic molecules other than toxic oxygen metabolites (8). The lysis by human monocytes of antibody-coated erythrocytes or tumor cells as well as human tumor cells not coated with antibody, is not inhibited by either inhibitors of the respiratory burst or scavengers of reactive oxygen metabolites (16–18). Similarly, these scavengers of toxic oxygen metabolites did not inhibit the cytotoxic effects of murine macrophages elicited by treatment with *P. acnes* (19), nor did culture of *P. acnes*–elicited macrophages with tumor target cells under reduced oxygen tension prevent tumor cytotoxicity (20). This suggested that *P. acnes*–elicited murine macrophages do not require oxygen to kill tumor cells. However, in these studies, neither the amount of oxygen remaining in the system, nor the capacity of macrophages to utilize this residual oxygen to form toxic oxygen metabolites,

was assessed. Thus, these experiments did not directly exclude a role for oxygen metabolites in the spontaneous killing of tumor cells by activated macrophages.

To resolve this issue we have studied the spontaneous killing of melanoma cells by BCG-elicited macrophages (7) under strictly anaerobic conditions. An assay of cytotoxicity in vitro was developed in which the ability of melanoma cells to form colonies was used as an index of viability. No significant difference in killing by macrophages was observed under aerobic or anaerobic conditions. The results indicate that spontaneous macrophage-mediated cytotoxicity of murine melanoma cells is independent of oxygen.

Materials and Methods

Mice

Female C57BL/6 mice, between 6 and 8 wk of age, were obtained from The Jackson Laboratory (Bar Harbor, ME) and used within 4–8 wk.

BCG

Mycobacterium bovis, strain Bacillus Calmette-Guérin (BCG), Pasteur A1011, was obtained frozen from the Trudeau Institute (Saranac Lake, NY); the BCG was autoclaved (120°C) for 25 min, and then diluted to 10^7 organisms/0.2 ml of phosphate-buffered saline without Ca^{++} or Mg^{++} (PBS).

Peritoneal Cells

Peritoneal cells were harvested and maintained in culture by a modification of the method of Cohn and Benson, as described by Michl et al. (21).

Inflammatory exudate cells. In some experiments, mice were injected intraperitoneally with 0.5 ml of 4.5% Brewer's thioglycollate medium (Difco Laboratories, Detroit, MI), 1 d before harvest of the peritoneal cells. Wright-Giemsa staining of cytocentrifuge preparations revealed the presence of 80–90% polymorphonuclear leukocytes (neutrophils) when the peritoneal exudate was harvested 1 d after intraperitoneal thioglycollate injection.

BCG-elicited peritoneal cells. Mice received two intraperitoneal injections of 0.2 ml of the BCG suspension 21–40 d apart. The peritoneal exudate, harvested 3–4 d after the second injection, contained 35–45% macrophages, 55–65% lymphocytes, and 5% polymorphonuclear leukocytes. In some experiments, adherent peritoneal cells (mostly macrophages) were separated from nonadherent cells by incubating 4–5 × 10^7 peritoneal cells on 100-mm glass petri dishes in minimal essential medium (MEM); (Gibco, Grand Island Biological Co., Grand Island, NY) supplemented with 10% (heat inactivated for 30 min at 57°C) fetal bovine serum (FBS) (Dutchland Laboratories Inc, Denver, PA) for 4 h at 37°C in a humidified CO_2 incubator (12). The plates were then washed three times with 4–5 ml of ice-cold PBS containing 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical Co., St. Louis, MO). To obtain the nonadherent cell fraction, the wash fluids were combined and centrifuged at 150 g for 10 min at 4°C. The cell pellet was resuspended in PBS and kept at 4°C until used. The adherent cell fraction was harvested by adding to each dish 5 ml PBS plus 1 mM EDTA. After incubation for 10 min at 4°C, the cells were gently scraped off the plate with a sterile rubber spatula, centrifuged, and resuspended as described above. ~50% of the cells plated were recovered in the nonadherent cell fraction; the viability of these cells was usually 75–85%. Recovery of adherent cells ranged between 30 and 50% of the cells plated; these were 91–96% macrophages as shown by Wright-Giemsa staining of cytocentrifuge preparations. Viability, as determined by trypan blue exclusion, was usually >70%.

Melanoma Cells

$B_5$59 cells, a subclone of the B16 melanoma (obtained from Dr. S. Silagi, Cornell University Medical College, New York) were maintained in monolayer culture in MEM supplemented with 10% FBS in a humidified atmosphere containing 6% CO_2. At the inception of this project, the $B_5$59 cells were cloned by limiting dilution, and a clone selected that was tumorigenic in vivo and had a plating efficiency of ~90% in vitro. Cells of the same clone were used in all experiments. Before use, a semiconfluent monolayer was disaggregated with 0.25% trypsin and 0.02% EDTA (Gibco) for 1 min. The cells were washed with PBS, counted in a hemocytometer, and then diluted and plated in the growth medium.

Melanoma Cell Susceptibility to H_2O_2

$B_5$59 melanoma cells were plated in 16-mm Costar wells at a density of 10^5 cells/well in 1 ml MEM containing 10% FBS. The medium was removed from the cells, and each well washed three times with PBS. Then 1 ml PBS containing 10^{-1} to 10^{-7} M H_2O_2 (prepared from Superoxol, Mallinkrodt) was added. Cultures were incubated in a 37°C water bath for 4 h. 0.04 ml PBS containing 0.4% trypan blue was added to each well and after 2 min the cells were examined for trypan blue exclusion.

Assay of Spontaneous Killing of Melanoma Cells by BCG-elicited Macrophages

$B_5$59 melanoma cells (10^5) in 1 ml MEM containing 10% FBS were added to each 16-mm well of 24-well plastic tissue culture plates (Costar, Cambridge, MA). The plates were incubated for 16–24 h at 37°C in a humidified CO_2 incubator to allow cells to adhere and spread. Total peritoneal cells or macrophages (from the adherent fraction) were resuspended at 2.5 × 10^6 cells per ml in MEM supplemented with 10% FBS, except where noted. Medium was aspirated from wells containing tumor cells, and the peritoneal cell suspension (2 ml) was added to each well. The plates were incubated in a CO_2 incubator for an additional 24 h. Control wells were treated in exactly the same manner, except that 2 ml of medium supplemented with 10% FBS, without peritoneal cells, was added per well. After the incubation period the medium was aspirated from each well, removed to tubes, and saved so that any tumor cells that detached from the surface during the incubation with macrophages would be retained. After trypsinization the cells from each well were placed in the test tubes and brought to a volume of 10 ml. The samples were diluted 100-fold in two steps with MEM plus 1% FBS. From the final dilution 200 μl was plated into a 60-mm plastic petri dish containing 2 ml MEM plus 10% FBS. The dishes were incubated for 7 d in a humidified CO_2 incubator, and the medium was then removed. Colonies were fixed with 2% vol/vol glutaraldehyde in PBS, stained with 4% methylene blue, and counted using an Artek colony counter (Artek Instruments, Farmingdale, NY). A similar assay has been described (22).

PMA-stimulated Granulocyte Cytotoxicity

As described previously (10), cytotoxicity was assessed using a ^{51}Cr release assay, 1-d thioglycollate-elicited mouse peritoneal polymorphonuclear leukocytes as the cytotoxic effectors, and mouse TLX9 lymphoma cells as the target. TLX9 cells maintained either in syngeneic C57BL/6 mice or in culture in alpha-MEM (Gibco) supplemented with 10% horse serum, were labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA). Mouse granulocytes were suspended at 1.6 × 10^6/ml in MEM plus 1% FBS. PMA (Consolidated Midlands, Brewster, NY) dissolved in dimethyl sulfoxide (DMSO) was added to each 12 × 75 mm glass culture tube to achieve a final concentration of 100 ng/ml. Then 0.5 ml of target cells, and either 0.5 ml of effector cells, or 0.5 ml cell-free medium was added to each tube. Tubes were incubated for 4 h in a humidified CO_2 incubator, then centrifuged at 70 g for 10 min. Each supernatant (0.5 ml) was transferred to another tube and counted in a gamma spectrometer (Packard Instrument Co., Downer's Grove, IL). The percent specific ^{51}Cr release was determined as previously described (10).

Anaerobiosis

Preparation of anaerobic cell suspensions and media was carried out using a gas-vacuum manifold in a cold room at 4°C (23). The manifold had a three-way valve connected to a vacuum pump (Air Cadet, Cole Palmer Instrument Co., Chicago, IL) and to a gas tank containing N_2/CO_2 (95:5) (Matheson, East Rutherford, NJ). The gas mixture was passed over prereduced hot copper filings to remove trace amounts of oxygen (23). Glass 100-ml bottles (Gibco) containing 50 ml MEM supplemented with 10% FBS and 10 mM piperazine-N,N'-bis-2-ethanesulfonic acid (Pipes), (Sigma) were tightly sealed with butyl rubber stoppers. Since the tumor cells otherwise render the medium acidic (pH 6–6.2) during the anaerobic incubation, 10 mM Pipes buffer was added to the MEM to maintain the pH in the range 7.2–7.3. Oxygen was removed from the contents of the bottle by five cycles of evacuation and filling with the N_2/CO_2 gas mixture. This medium was then used to resuspend previously centrifuged (60 g) peritoneal and TLX9 cells in 15-ml glass tubes while a constant stream of N_2/CO_2 from the manifold flowed over the cells and the medium. After the cells were resuspended, the tubes were sealed with sterile sleeve type serum stoppers and kept on ice until used.

Adherent tumor cells were plated in Costar multiwell plates as described above. In order to maintain the pH during subsequent anaerobic manipulations, the incubation medium was removed and replaced with PBS containing 1% FBS. The plates were then moved into the anaerobic chamber.

Manipulation of the various cell types was carried out inside an anaerobic glove chamber (Coy Laboratory Products, Ann Arbor, MI) (24). Items were placed into the chamber via an airlock from which air was removed by five cycles of evacuation and filling with a gas mixture of prepurified N_2/H_2 (95:5) (Matheson). Small amounts of oxygen that diffuse into the chamber were removed by the action of palladium-coated asbestos pellets, which catalyze formation of water from H_2 and trace amounts of O_2, and thus maintain an oxygen-free atmosphere.

Gas analysis of the chamber contents was carried out at the end of each of three consecutive experiments. A sample of the gas phase was obtained by pumping it into a 150-ml sampling cylinder and analyzed by mass spectroscopy (Gollub Analytical Service, Berkeley Heights, NJ). In each case no oxygen (the limit of detection is 4 μl of oxygen per liter of gas) was detected in the gas phase of the chamber.

For cytotoxicity experiments the saline covering the adherent cells in the multiwell plates was removed and replaced by either a macrophage suspension in sterile anaerobic culture medium, or this medium alone. Duplicate sets of culture plates were prepared, one for continued anaerobic incubation, and the other for aerobic incubation. When nonadherent tumor cells were used as target cells (e.g. TLX9), cell suspensions were mixed together in glass tubes. Cells to be maintained anaerobically were placed in an anaerobic GAS-PAK jar (25–27) (BBL, Becton-Dickinson, Cockeysville, MD), which was sealed, removed from the chamber, and incubated in a 37°C room for 24 h. The other sets of cells were removed from the chamber and then placed in a 95% air, 5% CO_2 incubator for 24 h. In some experiments, duplicate sets of cells were mixed completely aerobically and incubated in the CO_2 incubator. After the incubation period, all sets of cells were assayed to determine the number of colony-forming units (as described above).

Results

Susceptibility of Melanoma Cells to H_2O_2. BCG-elicited macrophages generate large amounts of H_2O_2 in response to PMA as reported previously (10), but this treatment does not augment their cytotoxic effect on B_559 murine melanoma cells (data not shown). To determine the sensitivity of the B_559 cells to H_2O_2, varying amounts of H_2O_2 were added to cultures of B_559 cells and the cytotoxic effect assessed. Fig. 1 shows that the melanoma cells were relatively resistant to H_2O_2, with an LD_{50} of ~1 × 10^{-4} M, as compared to an LD_{50} of ~4 × 10^{-6} M
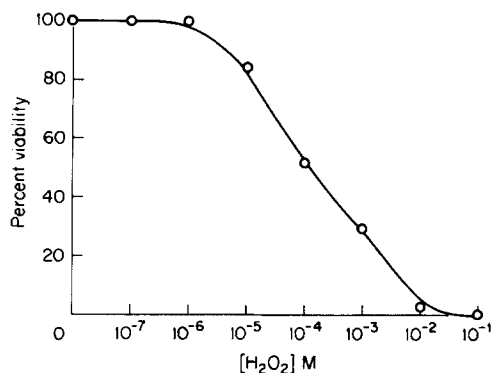
FIGURE 1. Viability of B_559 murine melanoma cells cultured in 16-mm wells in the presence of various concentrations of H_2O_2 (see Materials and Methods). Viability was assessed by trypan blue exclusion.

to 4×10^{-5} M reported for other murine tumor cells (9). This led us to consider whether oxygen was required to effect cytotoxicity in this system.

Effects of Anaerobiosis on Melanoma Cells. B_559 cells were incubated under aerobic and anaerobic conditions for 24 h. As shown by phase contrast microscopy, cells incubated under aerobic conditions are flat and have broad cytoplasmic processes, while those incubated under anaerobic conditions are rounded and clumped with long slender processes (Fig. 2). To determine whether anaerobiosis altered the growth or the colony-forming potential of the melanoma cells, 10^5 B_559 cells were plated in each well of a Costar 24-well plate, and incubated for 24 h at 37°C. Some of the plates were sampled immediately and assayed for colony-forming units. Others were incubated for an additional 24 h under either aerobic or anaerobic conditions, and then assayed for colony-forming units. The typical appearance of colonies recovered is shown in Fig. 3.

The results (Table I) show that following aerobic incubation for 24 h, the number of colonies of melanoma cells was approximately equal to the total number of cells plated. Consequently, the cells were viable, indicating colony-forming efficiency of ~90–100%; this does not depend on the initial number of cells in the wells. Therefore, within the first 24-h period, there is apparently little change in the number of cells. Furthermore, when 10^5 melanoma cells are plated and incubated under aerobic conditions for 48 h, 3–4 × 10^5 cells are recovered (by actual count in a hemacytometer) yielding 300–400 colony-forming units as shown in Table I. This indicated that under aerobic conditions tumor cell division occurs every 24–36 h. When 10^5 melanoma cells are incubated for 24 h aerobically and for an additional 24 h anerobically (for a total of 48 h), 1.4–2.2 × 10^5 colony-forming units are recovered (Table II). Thus the melanoma cells remained viable, albeit they did not divide, during the 24-h anaerobic incubation period.

BCG Macrophage-mediated Cytotoxicity Under Aerobic and Anaerobic Conditions. When the melanoma cells are incubated together with BCG-elicited macrophages for 24 h either aerobically or anaerobically, the majority of melanoma cells are destroyed. Direct microscopic observation shows that most of the
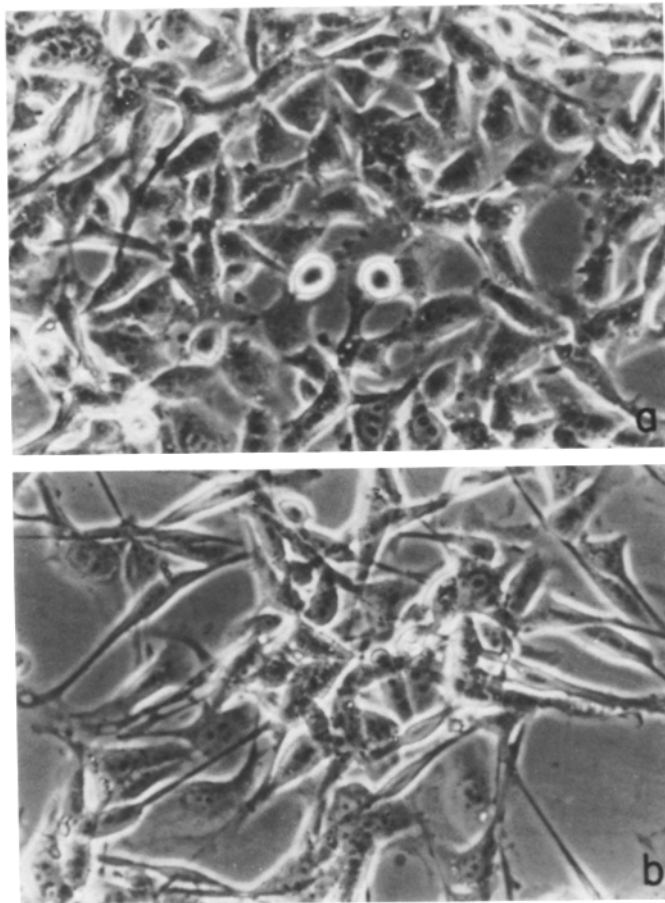
FIGURE 2. Phase contrast micrographs showing B₅59 melanoma cells after culture for 24 h under (a) aerobic conditions or (b) anaerobic conditions (× 100).

melanoma cells are fragmented or stain with trypan blue, in contrast to the control wells, in which the melanoma cells form an intact monolayer and exclude trypan blue. The number of melanoma cells capable of forming colonies decreases by >90% (Fig. 3 and Table II) under aerobic and anaerobic conditions. Fig. 4 shows that the same ratio of effector to target cells (20:1) was required to achieve nearly 90% cytotoxicity, either aerobically or anaerobically. Previous work had established that macrophages and not lymphoid cells, mediate lysis of the melanoma cells (7).

To test whether cell contact is required for cytotoxicity to occur, we used a special chamber (Fig. 5). Macrophages and tumor cells were incubated together on the bottom surface of the well, while tumor cells alone were incubated on a coverslip located above the mixed population at the top of the same well, i.e., both populations of melanoma cells were cultivated in the same medium during the incubation period. At the end of 24 h incubation, either aerobically or anaerobically, the tumor cells plated in both locations were harvested by trypsinization, diluted, and plated to determine the number of colony-forming units
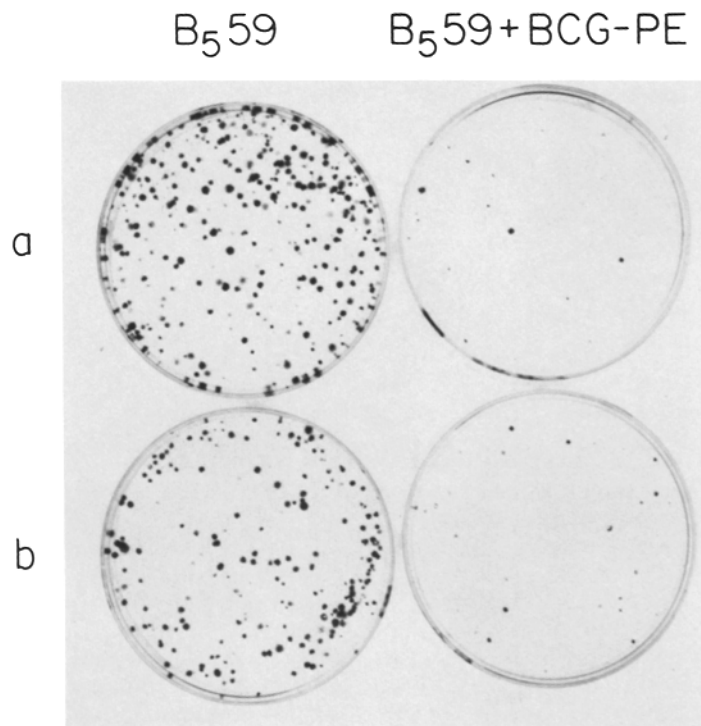
FIGURE 3. Colonies of B$_5$59 melanoma cells after cytotoxicity assay. Melanoma cells were plated in 16-mm wells and then incubated with or without BCG-elicited macrophages for 24 h under aerobic or anaerobic conditions. Melanoma cells were trypsinized and diluted (as described), and then plated in 60-mm petri dishes for 7 d before fixation with 2.5% glutaraldehyde and staining with methylene blue. (a) Colonies recovered after aerobic incubation; (b) Colonies recovered after anaerobic incubation.

remaining. When B$_5$59 melanoma cells and BCG macrophages were plated together on the bottom of the well, cytotoxicity occurred both aerobically and anaerobically, as shown by the low number of colonies recovered (Table III). Little or no decrease in colony-forming activity was observed in melanoma cells maintained on coverslips above the mixed population in the same culture (Table III). These results show that under both aerobic and anaerobic conditions, cytotoxicity is dependent on marophage-tumor cell contact, and is not dependent on a readily diffusible macrophage product, or on an alteration of essential nutrients in the medium.

Amount of Oxygen in the Anaerobic System. To determine whether leukocytes could produce H_2O_2 using any amount of O_2 (remaining in the medium of cultures prepared in the anaerobic chamber) we incubated mouse neutrophils with TLX9 tumor cells under both aerobic and anaerobic conditions. In these experiments, PMA was used to induce H_2O_2 release from mouse neutrophils as described previously (8, 9) during incubation with TLX9 tumor cells. Cytotoxicity occurred only in the presence of both PMA and oxygen; when the cells were anaerobically prepared, treated with PMA, and then incubated anaerobically, killing did not occur (Table IV). Control experiments showed that neutro-

TABLE I

Recovery of Colony-forming Units of Melanoma Cells Cultured in Multiwell Plates for 24 h or 48 h*

No. of cells plated per well	Final dilution	Colony-forming units		48-h culture
		24-h culture		
		Estimated	Counted	Counted
10^5	1:500	200	227	350
			229	
5×10^4	1:500	100	120	225
			96	
10^4	1:500	20	27	66
			25	
	1:50	200	204	ND[‡]
			164	
5×10^3	1:500	10	11	32
	1:50	100	92	ND
10^3	1:500	2	4	6
			2	
	1:50	20	22	ND
10^2	1:10	10	7	ND
			11	

* $B_5$59 melanoma cells were enumerated in a hemocytometer and plated at various concentrations in 16-mm wells and incubated aerobically for either 24 h or 48 h. The cells were then trypsinized, diluted as indicated, and plated in 60-mm plates. Colonies were counted after 7 d. The number obtained represents the colony forming units. Each value is the average of triplicate wells.
‡ ND, not determined.

TABLE II

Macrophage-mediated Cytotoxicity Under Aerobic and Anaerobic Conditions*

Exp.	Aerobic			Anaerobic		
	$B_5$59[‡]	$B_5$59 + BCG-PE[§]		$B_5$59	$B_5$59 + BCG-PE	
	Colonies per plate	Colonies per plate	% Cytotoxicity	Colonies per plate	Colonies per plate	% Cytotoxicity
1	338	34	90.0	147	18	87.8
2	489	12	98.5	225	9	96.5
3	437	64	86.1	225	17	92.1
Mean (n = 18 exp.)		91.9			91.9	
		(Range 77–98.9)			(Range 80–100)	
SD		± 6.68			± 6.73	

* Cell populations were mixed in the anaerobic chamber and the mixtures were incubated either aerobically or anaerobically. Cytotoxicity (%) was expressed as the number of melanoma colonies recovered after incubation with BCG-PE divided by the number of colonies recovered after incubation without the effector cells, × 100. (The cytotoxicity observed was the same as for $B_5$59 cells and BCG-PE prepared aerobically and incubated aerobically).
‡ $B_5$59 cells were plated 24 h before the addition of BCG-PE at an effector-target ratio of 25:1.
§ BCG induced peritoneal exudate (BCG-PE) harvested 4 d after the second of two intraperitoneal injections of heat-killed BCG.
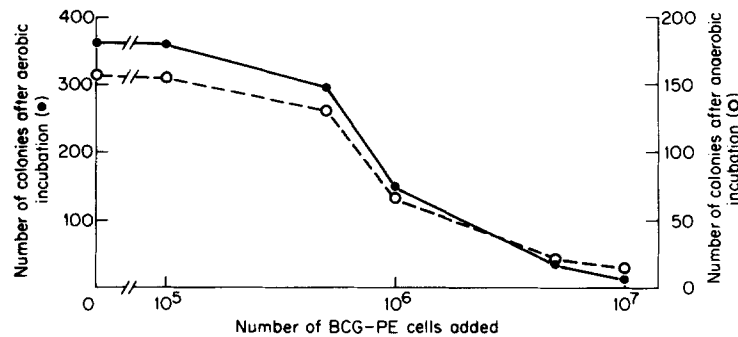
FIGURE 4.   Influence of different numbers of BCG-induced peritoneal cells on the colony forming ability of melanoma cells after 24 h incubation either aerobically (●) or anaerobically (○). B_559 melanoma cells were prepared, and then various amounts of freshly harvested BCG-elicited peritoneal cells were added to the cultures.
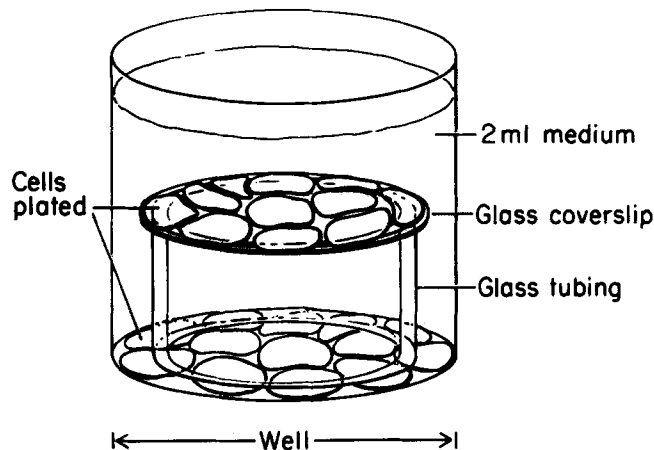

FIGURE 5.   Diagram of chamber for simultaneous incubation of tumor cells with a mixed population of tumor and effector cells. Chamber is a 16-mm well (plastic) in which a 12-mm glass tube acts as a support for a 13-mm glass coverslip. B_559 melanoma cells were plated on the glass coverslip or on the bottom surface of the well 24 h before the addition of BCG-elicited macrophages to the bottom of the well.

phils prepared in the anaerobic chamber and then incubated aerobically, were still capable of mediating tumor cell lysis (Table IV). These experiments confirm that the anaerobic system used contains insufficient O_2 to support oxygen-mediated cytotoxicity to TLX9 tumor cells by neutrophils.

Discussion

Many studies have implicated toxic oxygen metabolites in the microbicidal and tumoricidal activities of phagocytes. Products of the respiratory burst have been shown to be involved in the killing of tumor cells by macrophages and granulocytes (8–11). Several recent reports suggest, however, that cytotoxic activity is not necessarily dependent on the generation of reactive oxygen intermediates. These studies have not investigated the role of oxygen in cytotoxicity directly by

TABLE III

Coincubation of Melanoma Cells with Mixed Population of BCG-elicited Macrophages and Melanoma Cells Under Aerobic and Anaerobic Conditions*

Location of cells[‡] in wells		% Cytotoxicity Condition of incubation			
		Aerobic		Anaerobic	
Bottom	Top (coverslip)	Bottom	Top	Bottom	Top
B$_5$59 + BCG$_m$	B$_5$59	95.4	16.3[§]	92.8	2
B$_5$59 + BCG$_m$	None	98.1	—[∥]	98.1	—
B$_5$59	B$_5$59	1	1	1	1
BCG$_m$[§]	B$_5$59	—	—	—	1
None	B$_5$59	—	1	—	1
B$_5$59	None	1	—	1	—

* This is a representative experiment of three carried out.
‡ B$_5$59 cells were plated as described in the legend for Fig. 5. Tumor and effector cells were incubated together for 24 h either aerobically or anaerobically and then the cytotoxicity was assayed as in Table I. BCG-induced peritoneal exudate was obtained as in Table I, and macrophages prepared by adherence as described (Materials and Methods).
§ In other experiments there was no reduction in the number of melanoma cells on the coverslips above BCG-elicited cells and B$_5$59 melanoma cells on the bottom of the well.
∥ —indicates no cells plated in this location.

TABLE IV

Lysis of TLX9 Cells by PMA*-stimulated Neutrophils[‡] Under Aerobic and Anaerobic Conditions

Preparation conditions	Incubation conditions	Specific release (%)[§]	
		− PMA	+ PMA
Aerobic	Aerobic	0.7	16.8
Anaerobic	Aerobic	−2.1	21.2
Anaerobic	N$_2$/H$_2$/CO$_2$	3.5	−1.6

* PMA (phorbol myristate acetate) 10 ng/ml.
‡ Cells harvested 17 h after intraperitoneal injection of thioglycollate broth.
§ From 2 × 10^4 ^{51}Cr-labeled TLX9 cells (Means of triplicates).

excluding oxygen from the reaction systems, but have utilized two indirect approaches.

Klassen and Sagone (17) showed that monocytes from patients with chronic granulomatous disease, although genetically incapable of the phagocyte respiratory burst, can still lyse antibody-coated lymphoblastoid cells. Furthermore, scavengers of superoxide, hydrogen peroxide, hydroxyl radicals, singlet oxygen, and inhibitors of myeloperoxidase, failed to inhibit lysis of human tumor cells by normal human monocytes (16, 17). Bryant and Hill (19) have shown that scavengers of toxic oxygen metabolites such as catalase, superoxide dismutase, and cytochrome c did not inhibit the cytotoxic effects of P. acnes–elicited macrophages of murine tumor cells (19); Weinberg and Haney showed that these

same scavengers did not inhibit the killing of human tumor cells by human monocytes or macrophages (18). The conclusion drawn from these studies is that, since the use of scavengers did not eliminate cytotoxic effects, the cytotoxicity observed was independent of oxygen. Although these scavengers inactivate reactive oxygen metabolites in solution, it is not clear that they could penetrate the limited space between the macrophage and its target tumor cell (28). When monocytes are bound to ligand (antibody or complement)-coated surfaces, molecules with molecular weight of 50,000 or greater are excluded from the zone of contact (29). This suggests that a molecule like catalase, of molecular weight 250,000, might not enter the cleft between macrophage and tumor cell and therefore would be unavailable to inactivate any peroxide secreted into this space.

In further studies, hypoxia did not affect lysis of antibody-coated target tumor cells by normal human monocytes (17), nor did anaerobic culture of *P. acnes*–elicited murine macrophages with mouse tumor cells inhibit tumor cell death (20). However, in these studies, the cell populations were mixed aerobically and neither the amount of residual O_2 or ambient O_2 present initially was measured nor was its role in cytotoxicity directly assessed.

To determine whether oxygen was involved as a substrate for generation of cytotoxic molecules involved in the spontaneous killing of melanoma cells by BCG-elicited macrophages, we examined the ability of these effector cells to kill B_559 cells under strictly anaerobic conditions. These studies demonstrate that macrophage-mediated cytotoxicity of murine melanoma cells can occur anaerobically, i.e., in an atmosphere containing undetectable levels of oxygen.

That the methods used for removal of the oxygen from the anaerobic system were effective is supported by two findings. First, the amount of O_2 was <4 ppm O_2 (the detectable limit) in the gas phase in the anaerobic chamber in which all the manipulations were carried out. In air-saturated water, the concentration of O_2 at $25°C$ is $260 \mu M$ (30, 31). Since the amount of oxygen in the gas phase of the chamber was <4 μl per liter of gas, we calculated that the maximum concentration of oxygen in the medium in the chamber was 5 nM. The respiratory burst of human neutrophils has been measured under hypoxic conditions (32). O_2^- production was half maximal when the gas phase contained 3.5 ml of O_2 per liter of gas (equivalent to ~10 μM oxygen in the medium) and 1,000-fold greater than the amount present in our experiments. Second, PMA-stimulated neutrophils were unable to kill TLX-9 target tumor cells under similar anaerobic conditions. Thus, any trace amount of O_2 remaining in the medium was insufficient to permit generation by the neutrophils of tumoricidal quantities of H_2O_2.

It should be noted that anaerobic culture for 24 h is an unphysiologic condition and did impose a stress on the B_559 tumor cells. The melanoma cells remain alive but are altered in appearance (Fig. 2), and fail to divide during the anaerobic incubation period. While it is unclear whether the cells cultured anaerobically are rendered more susceptible to macrophage-mediated cytotoxicity, our experiments show that cytotoxicity occurs at the same effector/target ratio under aerobic and anaerobic conditions (Fig. 4 and Table II). Furthermore, the experiments in which tumor cells are cocultivated with a mixture of tumor cells and effector cells, show that macrophage tumor cell contact is required for

cytotoxicity to occur either aerobically or anaerobically (Table III). Therefore, despite the stress of anaerobic culture on the tumor cells, cytotoxicity is mediated by contact with BCG-macrophages, requires the same number of effector cells, and is not due to a nonspecific alteration of the medium (33, 34).

The mechanism of cytotoxicity in this system has not been determined. Several mechanisms of tumor cell killing by secretory products of activated macrophages have been proposed (for review, see reference 8). It has been assumed that oxygen is not involved in those lytic mechanisms in which a cytotoxic molecule, e.g. a cytotoxic protease (35), is secreted. However, the ability of activated macrophages to synthesize or release any of these cytolytic molecules in the absence of O_2, has not yet been investigated. Thus, we do not know whether any of these existing mechanisms account for anaerobic cytotoxicity in this system.

The sensitivity to oxidant injury of most tumor cells has not been determined. Our experiments showed that $B_5$59 melanoma cells are extremely resistant to exogenously added H_2O_2 (see Fig. 1). This resistance is 50–100-fold greater than that reported for tumor cells such as TLX9 (10, 11), a target cell regarded as susceptible to oxygen-dependent cytotoxicity. Oxygen-independent mechanisms of macrophage-mediated cytotoxicity are likely to be required for the elimination of tumor cells that are resistant to oxygen metabolites.

One implication of our studies is that the macrophage can play an important role in host defense under hypoxic conditions. That murine melanoma cells can be killed by activated macrophages during a 24-h period of anaerobic incubation does not, however, exclude a role for oxygen during aerobic killing. In fact, both mechanisms may be operative, or even synergistic, in the hypoxic environment in the center of tumor masses.

Summary

We have studied the spontaneous killing of $B_5$59 melanoma cells by Bacillus Calmette-Guérin (BCG)-elicited macrophages under strictly anaerobic conditions to investigate the role of oxygen in macrophage-mediated cytotoxicity. The number of melanoma cells capable of forming colonies after aerobic or anaerobic incubation with BCG-macrophages was used as the index of cytotoxicity. The BCG-macrophages killed melanoma cells regardless of the amount of oxygen present. The killing observed was proportional to the ratio of effector cells added; a ratio of 25:1 effector to target cells was required to achieve nearly 90% cytotoxicity both aerobically and anaerobically. This cytotoxicity was not dependent on a diffusible macrophage product nor on alteration of the medium by macrophages, since tumor cells incubated in the same culture medium, but not in contact with a mixed population of tumor cells and macrophages, were not killed. These results also indicated that macrophage-mediated cytotoxicity was dependent on macrophage–tumor cell contact. The mechanism responsible for the oxygen-independent cytotoxicity is unknown at present.

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