ROLE OF ACTIVATED MACROPHAGES IN ANTIBODY-DEPENDENT LYSIS OF TUMOR CELLS*

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Mononuclear leukocytes are able to destroy tumor cells under a variety of circumstances. In few such instances is the biochemical basis of cytotoxicity understood. Recently, we demonstrated that activated macrophages, stimulated by phorbol myristate acetate (PMA),[1] secreted substantial amounts of hydrogen peroxide (1). The release of this oxidant from macrophages correlated with the extracellular lysis of tumor cells (2). In fact, under the conditions tested, hydrogen peroxide was both necessary and sufficient for cytolysis by activated macrophages (3). The secretion by phagocytic leukocytes of toxic species of reduced oxygen can be triggered not only pharmacologically, but also immunologically, such as by contact with antibody-coated particles or aggregated immunoglobulin (4–9). Therefore, we attempted to learn whether activated macrophages would lyse tumor cells when the tumor cells were coated with homologous antibody, and whether reactive metabolites of oxygen would play a role in this form of cytolysis. Tests of these hypotheses are reported below and in the accompanying paper (10).

Materials and Methods

Mice. Mice of either sex were obtained at 6–8 wk of age. CD_2F_1 ([BALB/c × DBA/2]F_1) mice were from Flow Laboratories, Inc. (Rockville, Md.), Charles River Breeding Laboratories, Inc. (Wilmington, Mass.), or Simonson Laboratories (San Francisco, Calif.). C57BL/6 and C57BL/10 mice were from The Jackson Laboratory, Bar Harbor, Maine, Trudeau Institute, Saranac Lake, N. Y., and Charles River Breeding Laboratories, Inc. A/Sn mice were from The Jackson Laboratory.

Injection of Eliciting Agents and Harvest of Peritoneal Cells. Bacille Calmette-Guérin (BCG) (7×10^6 viable organisms, Pasteur type 1011, Trudeau Institute), heat-killed *Corynebacterium parvum* (0.7 mg, Burroughs-Wellcome, Research Triangle Park, N. C.), and thioglycollate broth (1 ml of a 10% solution, Difco Laboratories, Detroit, Mich.) were injected intraperitoneally as described (1). Peritoneal cells were collected from untreated mice (resident cells); 12–20 h after injection of thioglycollate broth (granulocytes); 3–5 d after thioglycollate broth; 5–7 d after *C. parvum*; or 10 d–5 wk after BCG (BCG cells). The collection, hypotonic lysis, washing, enumeration, and differential counting of peritoneal cells were as before (1). Differential counts during this study were the same as those given elsewhere (1, 2).

Tumor Cells. Tumors were passaged as ascites. P388 was maintained in CD_2F_1 mice, and TLX9 in C57BL/6 mice (2). YAC-1 was the kind gift of R. Keller, Immunobiology Research

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[1] *Abbreviations used in this paper:* BCG, Bacille Calmette-Guérin; E:T, effector:target; FBS, fetal bovine serum; FMS, fresh mouse serum; KRPG, Krebs-Ringer phosphate buffer with 5.5 mM glucose; KRPGS, KRPG that contained 1% FBS; MEM, Eagle's minimum essential medium; MEMS, MEM that contained 10% FBS; NK, natural killer; PMA, phorbol myristate acetate.

Group, Zürich, and also of E. Lattime and O. Stutman, Memorial Sloan-Kettering Cancer Center, New York, and was maintained in A/Sn mice. EL4-BU and Russian Leukemia (C57BL/6 mice), and L1210/CTX and P388/adria (CD_2F_1 mice), were from the Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). Erythrocytes were lysed by hypotonic shock (1) if they represented >1% of the cells. Equivalent results were obtained when tumors were maintained as stationary suspension cultures in Eagle's minimum essential medium (MEM) alpha modification with 100 U/ml penicillin and 100 μl/ml streptomycin (Grand Island Biological Co., Grand Island, N. Y.) that contained 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) (Flow Laboratories, Inc.) (MEMS) at 37° in 5% CO_2 in air.

Sera. Spleens were removed aseptically from CD_2F_1 mice (H-2^d), or from C57BL/6 or C57BL/10 mice (H-2^b), placed in MEM, and crushed with a sterile glass stopper. One-seventh of a spleen was injected intraperitoneally into mice of the opposite H-2 type every other week for three to six injections. Results were equivalent, but titers were higher, if the spleens were taken from mice that had received BCG intraperitoneally. 1 wk after the last injection, blood was collected, and the serum was frozen at −80°C until use. Results were unaffected by heating the serum at 56°C for 30 min. Mouse serum used as a complement source was collected from the donors of the peritoneal cells in the same experiment.

Labeling and Sensitization of Tumor Cells. Tumor cells were labeled with sodium ^{51}Cr and washed four times as described (2). Up to 1×10^6 cells were then suspended in 0.1 ml of Krebs-Ringer phosphate with glucose (1) that contained 1% FBS (KRPGS) and variable amounts (Results) of alloantiserum. After 30 min on ice, 0.1 ml of 40% fresh mouse serum in KRPGS was added, giving a final concentration of 20%, and the cells were incubated for 10 min in a 37°C water bath. The cells were then washed one to three times by centrifugation, or diluted directly in MEMS. As controls, alloantiserum was replaced with nonimmune mouse serum from the same strain of mice (sham sensitization), or mouse serum was omitted (unsensitized cells). The final concentration of mouse serum in the assay with sensitized or sham-sensitized cells ranged from 0 to 3% (usually <0.3%) without affecting the results.

Cytotoxicity Assay. Variable numbers of peritoneal cells (usually 1×10^6) and tumor cells (usually 2×10^4 to 4×10^4) were dispensed at 4°C to 12- × 75-mm glass tubes in 2 ml of MEMS (the FBS concentration was usually 1 or 5%, but ranged from 0.1 to 10% with equivalent results). The tubes were centrifuged (180 *g* for 5 min), warmed for 60 s in a 37°C water bath, and incubated for 4.5–8 h at 37°C in 5% CO_2 in air. The tubes were centrifuged at 700 *g* for 10 min, and the upper 1 ml removed to another tube. Controls received 10–100 ng/ml of PMA as before (2). In separate experiments, 4.8 ± 1.4% of the radioactivity remained associated with the tubes after gentle washing with warm KRPG at the end of the assay. This retention of ^{51}Cr was not affected by the presence or absence of effector cells, nor by the use of sensitized, sham-sensitized, or unsensitized target cells. Thus, effector cells did not retain detectable radiolabel from the target cells. Equivalent results were obtained with microtest plates (Costar, Data Packaging, Cambridge, Mass.). In this case, 8×10^5 to 9×10^5 peritoneal cells and 2×10^4 to 4×10^4 tumor cells were incubated in 0.2 ml of MEMS/6.4-mm well. The percent specific release of ^{51}Cr was calculated as before (2), except that in the plate assay, 0.1 ml of supernate was removed for counting, and the total radioactivity per well was estimated by counting portions of the initial tumor cell suspension. The simple proportional standard error of the mean (2) is reported for triplicates.

Depletion of Macrophages with Monoclonal Antimacrophage Antibody. Dr. Ralph Steinman (The Rockefeller University, New York) kindly provided the IgG fraction of a DEAE column eluate of ascites from a hybridoma (1.1). 14 μg of 1.1 protein in 1 ml of Tris buffer, pH 9, was added to 35-mm plastic culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at room temperature for 1–6 h. The dishes were rinsed six times with KRPG and then given 2×10^6 BCG peritoneal cells that were nonadherent to untreated plastic tissue culture dishes after 2 h of incubation in MEM-5% FBS. The 1.1-treated dishes were centrifuged (150 *g* for 2 min) and incubated at 37°C for 60–90 min. The medium was collected, and the monolayer was jetted gently with warm KRPG from a pipette. The nonadherent cells were pooled and washed by centrifugation.

Other Monoclonal Antimacrophage Antibodies. 2.4G2 and its Fab were prepared as described (11). 2.6 and its Fab were prepared by the same techniques (I. Mellman, R. Steinman, J.

Unkeless, and Z. Cohn. Manuscript in preparation.). Their specificities are shown in the legend to Fig. 6.

Scanning Electron Microscopy. Samples were processed as described (2).

Results

Lysis of Sensitized Lymphoma Cells by Various Populations of Peritoneal Cells. In an earlier study, when mice were injected with BCG or *C. parvum,* their peritoneal macrophages acquired the capacity to release copious amounts of hydrogen peroxide in the presence of PMA (1), and thereby to lyse lymphoma cells (2, 3). In the present study, peritoneal cells from such mice lysed TLX9 or P388 lymphoma cells, not only in the presence of PMA (Table I), but also when the tumor cells had been sensitized with alloantiserum and fresh mouse serum (FMS) (Table I). The results in Table I were obtained with three different alloantisera for each tumor. Similar alloantiserum-dependent cytolysis by BCG peritoneal cells was observed in smaller numbers of experiments with four additional lymphoma targets (EL4-BU, Russian Leukemia, L1210/CTX, and P388/adria) (not shown).

In contrast to the results with activated peritoneal cell populations, peritoneal cells from untreated mice, or those elicited with thioglycollate broth, were unable to lyse sensitized lymphoma cells (Table I). Such peritoneal cells were previously found to release little hydrogen peroxide in response to PMA (1). As before (2), they lysed lymphoma cells poorly in the presence of PMA (Table I).

Granulocyte-rich peritoneal cell populations were highly effective in lysing lymphoma cells in the presence of PMA (Table I; [2]). However, the same cell populations failed to lyse sensitized TLX9 cells (Table I).

Dependence of Cytolysis on Concentration of Alloantiserum and FMS. The extent of lysis of

TABLE I

Alloantiserum-dependent Lysis of Lymphoma Cells by Activated Peritoneal Cells

Tumor	Peritoneal cells	E:T ratio*	Mean percent specific release of ^{51}Cr ± SEM (number of experiments)‡			
			Sensitized§	Sham sensitized‖	Unsensitized¶	Sham or unsensitized plus PMA**
TLX9	BCG‡‡	24 ± 5	65.1 ± 2.7 (30)	12.9 ± 2.0 (22)	8.3 ± 2.5 (13)	57.9 ± 4.1 (23)
	C. parvum§§	40 ± 18	38.4 ± 15.1 (3)	10.3 ± 6.2 (2)	8.9 ± 1.8 (2)	51.0 ± 24.3 (3)
	Thioglycollate‖‖	63 ± 27	6.7 ± 1.2 (3)	1.7 ± 5.1 (2)	−3.9 ± 1.7 (2)	3.7 ± 1.3 (3)
	Resident¶¶	28 ± 7	1.8 ± 3.0 (8)	−4.6 ± 2.3 (6)	−4.3 ± 1.0 (6)	1.1 ± 1.1 (7)
	Granulocytes***	54 ± 16	3.8 ± 1.3 (4)	0.5 ± 1.6 (3)	−0.7 ± 0.3 (3)	73.2 ± 19.7 (4)
P388	BCG	26 ± 3	41.6 ± 5.1 (10)	7.0 ± 2.2 (10)	9.7 ± 7.6 (2)	69.8 ± 7.5 (9)
	Resident	26 ± 4	4.9 ± 2.3 (3)	1.3 (1)	2.4 ± 3.0 (2)	2.8 ± 2.0 (2)

* The E:T ratio is calculated from the number of macrophages and monocytes added, except where indicated for granulocytes. The target cells were 2×10^4–4×10^4/culture.

‡ Spontaneous release for TLX9 averaged: sensitized, 15.9 ± 0.8; sham sensitized, 15.1 ± 0.8; unsensitized, 15.6 ± 1.4; with PMA, 11.4 ± 0.5. Maximum release after freezing and thawing averaged 86.7 ± 0.7. The corresponding values for P388 were: 18.6 ± 1.5, 15.0 ± 1.0, 16.2 ± 1.7, 17.2 ± 1.4, and 89.3 ± 0.8.

§ Target cells incubated with one of three alloantisera directed against either TLX9 or P388, followed by exposure to 20% FMS, before dilution and addition to effector cells.

‖ Target cells prepared as in §, but with nonimmune mouse serum in place of alloantiserum.

¶ Target cells not exposed to mouse serum.

** PMA, 10–100 ng/ml.

‡‡ From mice given 7×10^6 viable BCG i.p. from 10 d–5 wk before collection.

§§ From mice given 0.7 mg heat-killed *C. parvum* 5–11 d before collection.

‖‖ From mice given 1 ml of 10% thioglycollate broth 3–5 d before collection.

¶¶ From untreated mice.

*** From mice given thioglycollate broth 12–20 h before collection.

TLX9 by BCG peritoneal cells depended upon the concentration of alloantiserum used for sensitization (Fig. 1). Lysis by BCG peritoneal cells was augmented slightly by including 10% or more FMS in the sensitization step (Fig. 1). Up to 20% FMS did not cause antibody-dependent lysis in the absence of effector cells, although 50% FMS did so to a minor degree (Fig. 1). With rabbit serum as a complement source, the alloantisera typically lysed 100% of lymphoma cells at dilutions of 1:60–1:200, and gave 50% lysis at 1:500–1:1,200. Sensitization was routinely performed with 1:5–1:20 alloantiserum, followed by 1:5 FMS, before dilution and mixture with effector cells. Results were the same if the target cells were washed repeatedly after the sensitization step.

Specificity and Contact Dependence of Cytolysis. Five experiments with similar results were performed to determine whether the lysis of sensitized lymphoma cells by BCG peritoneal cells was immunologically specific. As shown in Table II for one such experiment, BCG peritoneal cells lysed TLX9 when the alloantiserum was directed against TLX9, and lysed P388 when the alloantiserum was directed against P388. However, no lysis of TLX9 above background occurred with anti-P388 alloantiserum, and no lysis of P388 above background occurred with anti-TLX9 alloantiserum. In addition, no lysis above background of either lymphoma occurred in the presence of unlabeled, sensitized lymphoma cells of the other type. This was not a result of interference by the increased number of target cells present, because cytolysis proceeded normally in the presence of P388 cells, if TLX9 cells were labeled and sensitized, and vice versa (Table II). Furthermore, PMA-induced cytolysis of each

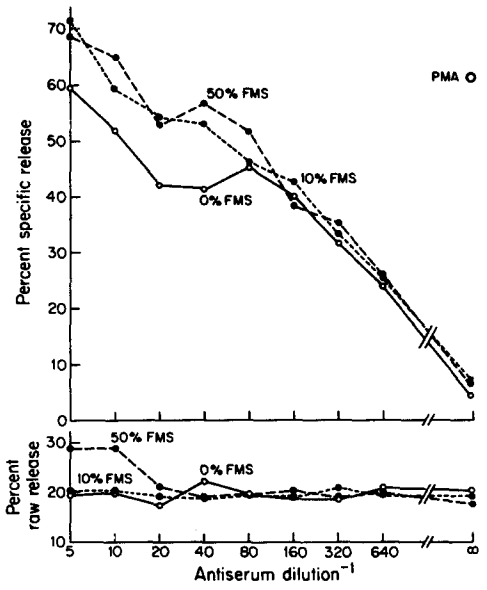

FIG. 1. (Upper panel) Effect of concentration of alloantiserum on lysis of 2×10^4 TLX9 cells by a 75-fold excess of BCG peritoneal cells (containing 44% macrophages and monocytes) over 8 h. O——O, no FMS added as a complement source during the sensitization step; ●- - -●, 10% FMS added; ⊘- - -⊘, 50% FMS added; O, cytolysis in the absence of antiserum but in the presence of PMA. Means of triplicates. SEM ranged from 0.1 to 4.8% (mean: 1.8%). (Lower panel) Spontaneous release of ^{51}Cr by TLX9 cells in the absence of effector cells, as influenced by the concentration of alloantiserum and FMS during the sensitization step.

TABLE II

Specificity of Alloantiserum-dependent Lysis by BCG Peritoneal Cells

Tumor*		Alloantiserum directed against	PMA	Percent specific release of ^{51}Cr‡
^{51}Cr-labeled	Unlabeled			
TLX9	—	Neither§	—	27.6 ± 1.6
TLX9	—	TLX9	—	90.5 ± 1.7
TLX9	—	P388	—	24.0 ± 1.3
P388	—	Neither	—	13.6 ± 1.2
P388	—	P388	—	71.8 ± 1.4
P388	—	TLX9	—	13.7 ± 1.3
TLX9	P388	TLX9	—	77.2 ± 7.5
P388	TLX9	TLX9	—	10.5 ± 1.3
P388	TLX9	P388	—	67.1 ± 0.9
TLX9	P388	P388	—	32.1 ± 1.5
TLX9	—	Neither	+	88.2 ± 2.1
TLX9	P388	Neither	+	87.7 ± 0.7
P388	—	Neither	+	90.1 ± 1.0
P388	TLX9	Neither	+	88.2 ± 8.7

* 2×10^4 cells incubated with a 54-fold excess of macrophages from BCG-treated mice. Spontaneous release was 17.4 ± 0.4% for TLX9 and 16.1 ± 1.2% for P388 in 7 h.

‡ Mean ± SEM for triplicates.

§ Nonimmune mouse serum used in place of alloantiserum.

lymphoma was unaffected by the presence of the other (Table II). Thus, cytolysis of sensitized lymphoma cells was immunologically specific. Moreover, it appeared to be effective only against target cells in antibody-dependent contact with the effector cells because bystander cells were completely spared.

Characterization of Cytolysis by Scanning Electron Microscopy. Sensitized TLX9 cells appeared morphologically normal, with numerous surface microvilli, when observed within 10 min of their addition to BCG peritoneal cells (Fig. 2a). By 30 min, however, some of the lymphoma cells had lost microvilli, and displayed surface pitting (Fig. 2b). By 1 h, most of the lymphoma cells exhibited pitting, and some were replaced by collections of cellular debris (Fig. 2c). At 3 h (Fig. 2d) and 7 h (Fig. 2e), almost no intact TLX9 cells remained, whereas collections of rubble-like material were evident on many of the macrophages. In contrast, sham-sensitized TLX9 cells incubated with BCG peritoneal cells were still morphologically normal at 7 h (Fig. 2f), and sensitized lymphoma cells were unaffected by 7 h incubation with resident peritoneal cells (Fig. 2g). All damaged TLX9 cells seen were in contact with macrophages. A minority of damaged TLX9 cells were also adjacent to lymphocytes. No freestanding TLX9 cells were observed to be damaged. The number of damaged TLX9 cells in contact with the same macrophage ranged from one to three. No phagocytosis of lymphoma cells was detected.

Time-Course of Cytolysis. The rapidity of cytolysis observed by scanning electron microscopy was confirmed by ^{51}Cr release. In the presence of BCG peritoneal cells, half-maximal specific release of ^{51}Cr from sensitized TLX9 cells occurred by 1 h, and maximal release by 5 h (Fig. 3). With less potent alloantisera, maximal release was sometimes delayed to 8 h (not shown). In contrast, peritoneal cells from untreated mice lysed sensitized TLX9 cells poorly even after 20 h of incubation (Fig. 3).

Effector:Target Ratios. Lysis of sensitized TLX9 cells by BCG peritoneal cells was

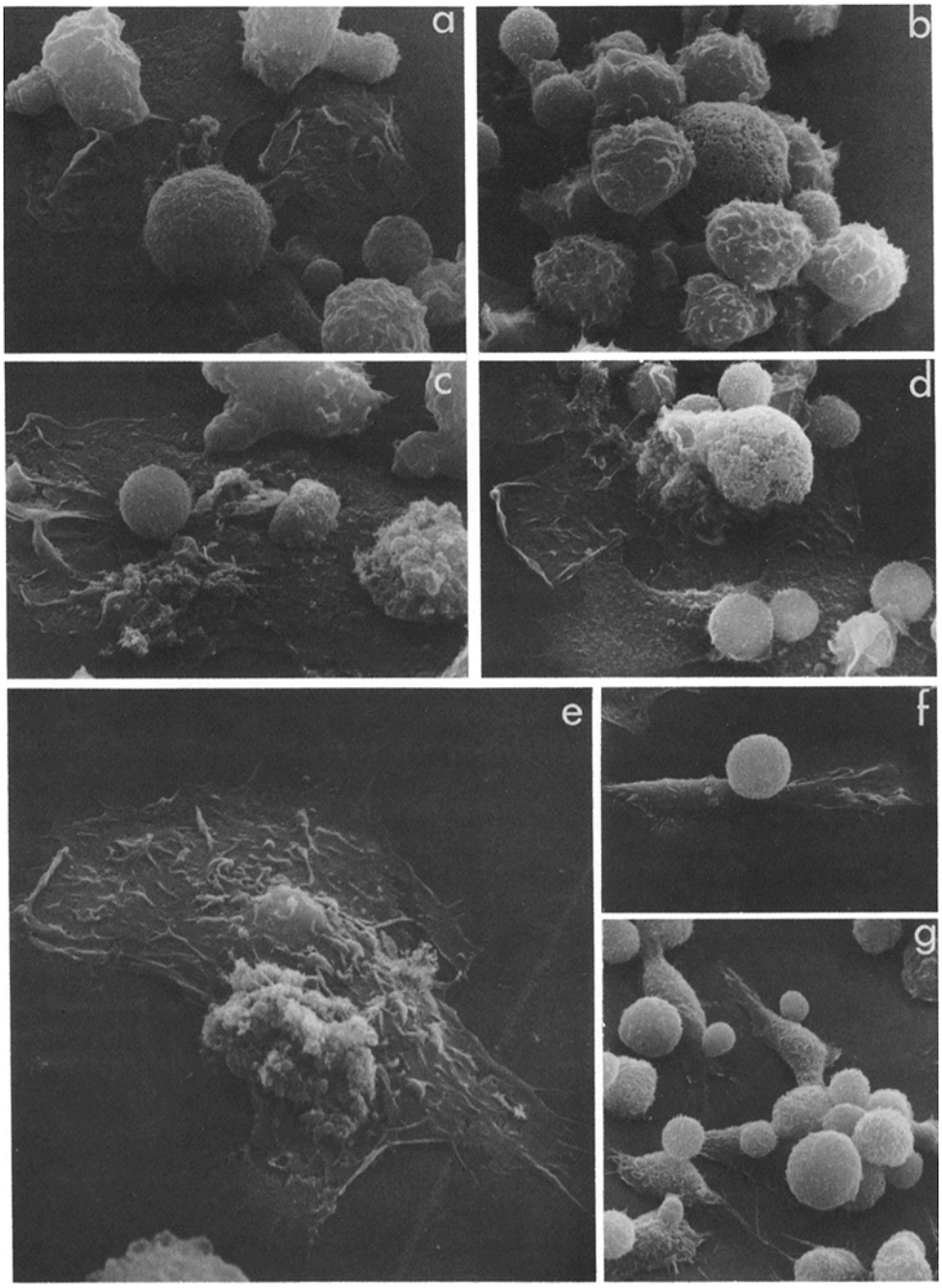

FIG. 2. Scanning electron micrographs of sensitized (a–e, g) or sham-sensitized (f) TLX9 cells, incubated with BCG peritoneal cells (a–f) or resident cells (g), for the following times: (a) 10 min, × 2,350; (b) 30 min, × 2,270; (c) 1 h, × 2,800; (d) 3 h, × 2,000; (e) 7 h, × 3,700; (f) 7 h, × 1,200; (g) 7 h, × 1,200.

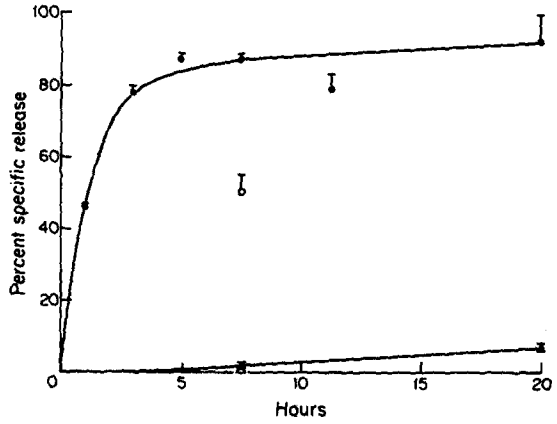

FIG. 3. Time-course of cytolysis of 4 × 10^4 sensitized TLX9 cells by 1.9 × 10^6 BCG cells (●) (containing 45% macrophages and monocytes) or 1.5 × 10^6 resident cells (▲) (containing 44% macrophages and monocytes) in 2 ml of MEM-5% FBS. (○ and △) are corresponding values with sham-sensitized TLX9 cells in the presence of PMA. At the indicated time points, beginning with 1 h, spontaneous release averaged 5.7, 9.2, 11.0, 14.3, 17.5, and 25.8%. Means ± SEM of triplicates.

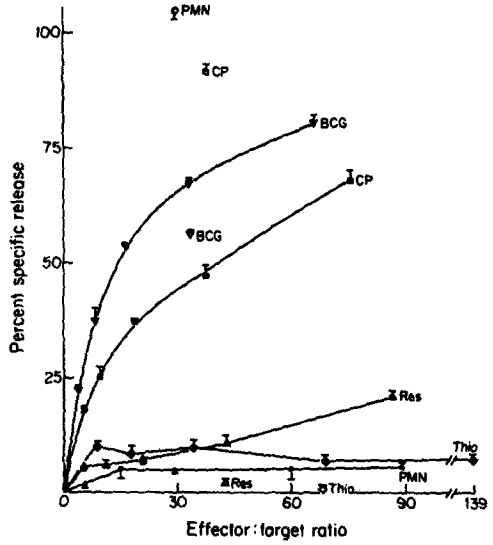

FIG. 4. Effect of peritoneal cell number on lysis of 2 × 10^4 sensitized TLX9 cells (closed symbols) over 8 h in 12- × 75-mm tubes. Resident cells (Res) contained 58% macrophages and monocytes; BCG cells, 44%; *C. parvum* cells (CP), 50%; and thioglycollate broth-elicited cells (Thio), 92%. Polymorphonuclear leukocyte-rich exudates (PMN) contained 60% granulocytes. For comparison, the E:T ratios are expressed in terms of the presumptive effector cells indicated above. Open symbols, unsensitized TLX9 cells plus PMA. Means ± SEM of triplicates.

half-maximal at effector:target (E:T) ratios of ~9 (expressed in terms of the macrophages and monocytes present) when tested in 12- × 75-mm glass tubes (Fig. 4). When tested in microtest wells, half-maximal lysis required E:T ratios of ~3, although maximal lysis tended to be lower (Fig. 5). Results were the same whether the effector or the target cell number was varied, or whether the microtest wells had flat, round, or conical bottoms (not shown). The relative inability of resident, thioglycollate-

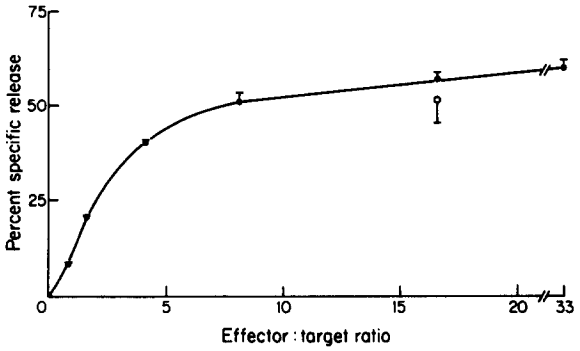

FIG. 5. Effect of 3.3×10^5 BCG-activated macrophages on ^{51}Cr release from sensitized TLS9 cells (●) ranging from 1×10^4 to 4×10^5/6.4-mm well. (○) represents unsensitized TLX9 cells plus PMA. Specific release from unsensitized TLX9 cells in the presence of BCG cells without PMA was $-1.6 \pm 1.3\%$. Means ± SEM of triplicates.

TABLE III

Lysis of Sensitized TLX9 Cells by Peritoneal Cell Subpopulations

Experiment	Assay	BCG peritoneal cells				
		Unseparated*	Adherent ‡	Nonadherent to plastic§	Nonadherent to plastic coated with 1.1 antibody‖	Nonphagocytic adherent cells¶
A	Percent ^{51}Cr release**	32.5	21.9	13.3	ND‡‡	−0.4
	Percent macrophages§§	32.8	ND	21.0	ND	1.7
B	Percent ^{51}Cr release	24.8	22.4	28.2	5.9	ND
	Percent macrophages	42.9	ND	22.5	5.6	ND
C	Percent ^{51}Cr release	54.2	30.8	19.8	5.8	ND
	Percent macrophages	45.2	ND	17.7	5.8	ND

* 9×10^5 BCG peritoneal cells were incubated with 2×10^4 TLX9 cells in 6.4-mm microtest wells in MEM-5% FBS.

‡ 9×10^5 cells were allowed to adhere to the wells for 3 h, or 3×10^5 fresh cells were allowed to adhere successively three times for 1 h each, with repeated rinsing of the monolayers.

§ Cells were decanted after gently agitating a tissue culture plastic dish in which they had been incubated for 3 h. 5×10^5 cells/well were used as effectors.

‖ Cells nonadherent after 2 h as in § were centrifuged onto dishes coated with the monoclonal antimacrophage antibody, 1.1. Cells nonadherent to 1.1-treated dishes were used at 5×10^5/well.

¶ Prepared as elsewhere (12), and used at 5×10^5/well.

** Specific release for sensitized TLX9 less specific release for sham-sensitized TLX9.

‡‡ ND, not done.

§§ Differential counts of macrophages and monocytes were performed on cytocentrifuge slides as elsewhere (1).

induced, and granulocyte-rich peritoneal cells to lyse sensitized TLX9 cells was preserved over a wide range of E:T ratios (Fig. 4).

Nature of the Effector Cell. When BCG peritoneal cells were allowed to adhere to plastic in the presence of FBS, both the adherent and the nonadherent populations lysed sensitized TLX9 cells (Table III). However, under such conditions, the nonadherent population contained a substantial proportion of morphologically identifiable macrophages and monocytes (22.2 ± 1.3% in 23 experiments) (morphologic criteria in: [1]). Mononuclear phagocytes were not reduced below 14% of the nonadherent

cells by using tissue culture plastic or glass from six different manufacturers, eight lots of FBS, or up to three cycles of adherence. Therefore, we tested a monoclonal antimacrophage antibody, 1.1. This reagent gave strongly positive indirect immunofluorescence with mouse peritoneal macrophages, but only weak or negative reactions with lymphocytes from mouse peritoneum or spleen (R. Steinman. Personal communication.). In three experiments, BCG peritoneal cells nonadherent to 1.1-coated dishes contained from 0 to 5.8% (mean: 3.8%) of morphologically identifiable mononuclear phagocytes. 96% of the cells were lymphocytes by morphology. The ability of such populations to lyse sensitized TLX9 cells was markedly reduced (Table III). However, some cytolytic capacity remained, which appeared to correspond to the residual proportion of mononuclear phagocytes (Table III). Supernates from the incubation of peritoneal cells in 1.1-treated dishes did not reduce cytolysis when used to resuspend unseparated or conventionally prepared nonadherent cells, thus suggesting that immune complexes were not released from such dishes to exert a blocking effect (not shown). The adherent population of BCG peritoneal cells has been characterized in detail (12). In addition to macrophages, it contains up to 18% of nonphagocytic cells bearing Fc receptors, which are probably B lymphoblasts (12). When these nonphagocytic adherent cells were isolated, they were unable to lyse sensitized TLX9 cells (Table III).

Role of the Trypsin-resistant Fc Receptor The results to this point suggested that specific interaction of antibody-coated lymphoma cells with BCG-activated macrophages could elicit an extracellular cytotoxic response similar to that elicited with the pharmacologic agent, PMA. To obtain more direct evidence regarding a ligand-receptor interaction triggering cytotoxicity, we pretreated BCG cells with a monoclonal antibody, 2.4G2, which specifically recognizes the trypsin-resistant Fc receptor (FcR II) on mouse peritoneal macrophages (11). Because antimacrophage antibodies may block macrophage Fc receptors nonspecifically, presumably by interactions involving the Fc of the antibodies (13), we included two controls. First, we used Fab of the anti-Fc receptor antibody. Second, we examined the effect of another monoclonal antimacrophage antibody, 2.6, which recognizes a 21,000-dalton surface moiety distinct from, and at least five times as abundant as FcR II. (I. Mellman, R. Steinman, J. Unkeless, and Z. Cohn. Manuscript in preparation.). Like 2.4G2, 2.6 was used both intact and as Fab. As shown in Fig. 6A, 2.4G2 markedly inhibited the lysis of sensitized TLX9 cells by BCG peritoneal cells. In four experiments, inhibition averaged 82 and 74% for optimal doses of the intact antibody and its Fab. In individual experiments, this inhibition reached 100 and 97%, respectively. Inhibition was specific for antibody-dependent cytolysis, because there was no inhibition of PMA-dependent cytotoxicity against sham-sensitized TLX9 cells (Fig. 6B). In addition, 2.6 and its Fab had no inhibitory effect (Fig. 6).

Spontaneous Lysis of Unsensitized Cells. In the absence of alloantiserum or PMA, BCG cells rarely caused substantial lysis of TLX9. Thus, in 36 experiments in which TLX9 cells were treated with nonimmune alloserum before incubation with BCG cells, specific release of ^{51}Cr averaged $5.7 \pm 0.7\%$. However, there were eight additional experiments, performed in the same manner, in which cytolysis averaged $26.7 \pm 2.9\%$. This was suggestive of natural killer (NK) activity (14). Therefore, experiments were conducted with YAC lymphoma, which is highly sensitive to lysis by NK cells (14). When 2×10^4 unsensitized YAC cells were incubated with 9×10^5 BCG peritoneal

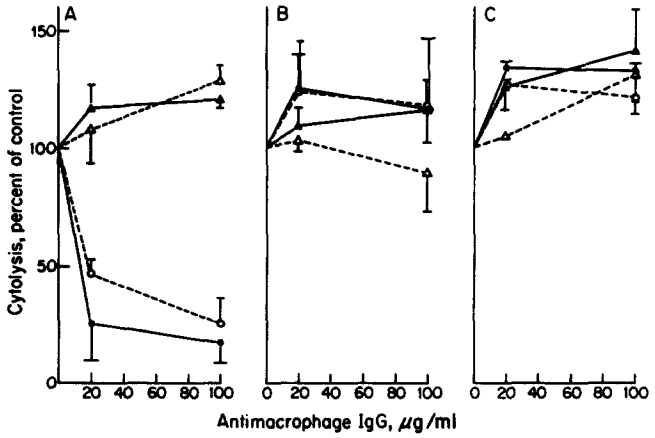

FIG. 6. Effect of two monoclonal antimacrophage antibodies and their Fab on cytolysis of 2×10^4 lymphoma cells by 8×10^5 BCG peritoneal cells (51 ± 3% macrophages and monocytes) per well over 7 h in microtest plates. (A) Antibody-dependent cytolysis of TLX9. (B) PMA-induced cytolysis of TLX9. (C) Spontaneous cytolysis of YAC. ●, intact 2.4G2 IgG; ○, Fab of 2.4G2; 2.4G2 recognizes the trypsin-resistant receptor on mouse macrophages for the Fc of aggregates of IgG_1 or IgG_{2b} (11). ▲, intact 2.6 IgG; △, Fab of 2.6; 2.6 recognizes a 21,000-dalton surface antigen that is at least five times more abundant than the FcR (I. Mellman, R. Steinman, J. Unkeless, and Z. Cohn. Manuscript in preparation.). Means and SEM are shown for four experiments, of which two included YAC, and are expressed as percentage of the values obtained without antimacrophage antibodies. The latter values were: TLX9, sensitized, 45.1 ± 8.0%; TLX9, sham sensitized, plus PMA, 65.7 ± 5.9%; YAC, unsensitized, 42.8 ± 11.7%. Specific release from sham-sensitized TLX9 calls in the absence of PMA but in the presence of BCG cells and antimacrophage antibodies averaged 9.6%, compared with 8.4% without antimacrophage antibodies. Effector cells were incubated for 30 min at 4°C with the indicated concentrations of antimacrophage antibodies before tumor cells were added.

cells, there was 50.0 ± 8.1% specific release of ^{51}Cr (eight experiments). Lysis of YAC was not observed after incubation with resident peritoneal cells or thioglycollate broth-elicited mononuclear cells or granulocytes (not shown). In contrast to antibody-dependent cytotoxicity, lysis of YAC was not inhibited by antibody against FcR II (Fig. 6C). Thus, peritoneal cells from mice given BCG could mediate both antibody-dependent and spontaneous cytolysis in short-term assays, the most prominent form depending upon the target cell.

Discussion

We conclude that tumor cells coated with specific, homologous immunoglobulin interact with at least one class of Fc receptors (FcR II) (11) on activated mouse peritoneal macrophages. The interaction triggers rapid and potent extracellular cytolysis, limited to the tumor cells in antibody-dependent contact with the macrophages.

Two lines of evidence support the interpretation that mononuclear phagocytes were responsible for most of the lysis of sensitized lymphoma cells. By scanning electron microscopy, damaged tumor cells were always observed in contact with macrophages. Damaged tumor cells were sometimes seen in contact with lymphocytes at the same time as with macrophages, but were not found associated with lymphocytes alone, nor standing free. Morphologic injury was evident by 30 min and was extensive by 1 h. The simplest explanation for these observations is that macrophages were the

predominant effector cells. Alternative explanations would have to be complicated; for example, that lymphocytes injured only those target cells in contact with macrophages and then quickly withdrew. Second, cytolytic activity was present in the adherent population, where it could not be attributed either to nonphagocytic adherent cells (lymphoid cells with Fc receptors [12]) or to granulocytes. Cytolytic activity was present also in the nonadherent population, where it correlated with the number of nonadherent mononuclear phagocytes identified by morphologic criteria. Depletion of the latter cells, by incubation on dishes coated with antimacrophage antibody, led to a corresponding depletion of cytolytic activity, down to a level of ~6%. However, as with macrophage-depletion techniques based on the use of silica, carrageenan, iron, glass, plastic, Sephadex, or nylon, it is possible that a minor population of cells other than macrophages may also have been selectively affected. Thus, the data are most consistent with the mediation of alloantiserum-dependent cytolysis by mononuclear phagocytes under the conditions tested. The possible participation of other cell types is not excluded.

The ability of macrophages to lyse antibody-coated tumor cells has been controversial. According to the present results, many of the earlier negative reports (e.g., [15–20]) may have been influenced by the use of resident or inflammatory rather than activated macrophages. For example, Ojo and Wigzell (19) studied mouse peritoneal cells 3 d after injection of *C. parvum,* an interval that in our experience was too brief to result in macrophage activation (1). The only cells in such populations capable of lysing alloantiserum-coated P815 cells appeared to be NK cells (19).

In contrast, there are many reports that testify to the ability of various mononuclear phagocyte populations to lyse antibody-coated tumor cells or cell lines. Effector cells in such studies have come from the peritoneal cavity (21–28), bone marrow cultures (29, 30), tumor tissue (31, 32), peripheral blood (33, 34), or macrophage cell lines (35, 36). Only a few studies, however, have described a rapid, potent, lytic process mediated by primary tissue macrophages against tumor cells coated with antibody from the same species (24–26). Likewise, few studies have considered the role of macrophage activation in antibody-dependent lysis of tumor cells (e.g., [24, 28, 35, 36]).

Perhaps the only previous study to combine these features was that of Yamazaki et al. (24). Those workers observed that resident peritoneal macrophages failed to lyse syngeneic, ^{51}Cr-labeled mammary adenocarcinoma cells sensitized with syngeneic anti-tumor IgG_{2a}. However, adherent peritoneal cells from mice given BCG, bacterial lipopolysaccharide, or glycogen were able to mediate cytolysis (24). Yamazaki et al. (24) proposed that the cytotoxicity observed by many workers using macrophages from specifically immunized mice might reflect a two-stage process: lymphocyte-mediated macrophage activation, followed by antibody-dependent cytotoxicity by activated macrophages.

Jolley et al. (25) observed [^{125}I]iododeoxyuridine release from alloantiserum-coated mouse lymphoma cells incubated for 20 h with resident peritoneal cells. The same peritoneal cells inhibited DNA synthesis by unsensitized tumor cells (25), raising the question of whether the macrophages were activated, perhaps by intercurrent infection. Indeed, strong evidence supporting that possibility was provided elsewhere by the same workers (37). Several investigators have observed lysis of sensitized, virus-infected cell lines by resident peritoneal cells (23, 26, 27). Such populations of

peritoneal cells failed to lyse sensitized tumor cells in the present study. The reasons for the discrepant results are not clear, but the possibility should be considered that cell lines may become more susceptible to cell-mediated cytotoxicity after viral infection.

Monoclonal anti-FcR II (11) and its Fab markedly inhibited antibody-dependent, cell-mediated cytolysis. Inhibition was specific, in that monoclonal anti-FcR II did not affect PMA-induced cytolysis by the same effector cells, nor was inhibition of antibody-dependent cytolysis observed with a monoclonal antibody directed against a more abundant macrophage surface antigen that was distinct from FcR II.

Based on their lysis of unsensitized YAC cells, the peritoneal cells from BCG-treated mice used in this study may have contained NK cells (38). The role of the Fc receptor in the cytolytic function of NK cells is disputed. Aggregated IgG (39), immune complexes (40), and anti-immunoglobulin (39) have been reported to block NK cell function in man, but aggregated IgG did not do so in the mouse (41). Our results with monoclonal antibody to FcR II suggest that this receptor was not required for spontaneous cytolysis of YAC, whatever effector cell was involved.

We found that populations of mononuclear phagocytes that were previously shown to release substantial amounts of hydrogen peroxide in response to PMA (1) were capable of lysing antibody-coated lymphoma cells, whereas populations that released very little H_2O_2 in response to PMA (1) mediated little or no antibody-dependent cytotoxicity. Peritoneal granulocytes represented an exception to this relationship. Granulocytes both secreted H_2O_2 (1) and lysed lymphoma cells (2) in the presence of PMA, but were ineffective in antibody-induced cytolysis under the conditions tested. In man, granulocytes can participate in antibody-dependent cytotoxicity (8, 34, 42, 43), but appear to require a higher density of antibody molecules on the target cells than do mononuclear effectors (34).

E:T ratios are commonly used to compare the potency of cytotoxic processes. At low E:T ratios, PMA-induced cytolysis was more potent than antibody-dependent cytolysis (not shown). However, the latter comparison may be misleading. Antibody-dependent cytolysis, unlike PMA-induced cytolysis, required cell-cell contact. Scanning electron microscopy showed that one antibody-coated tumor cell was usually associated with only one macrophage. With TLX9 at higher E:T ratios, antibody-dependent and PMA-induced cytotoxicity gave essentially the same results (Table I).

Under some conditions, sensitized tumor cells are phagocytized by macrophages (44, 45). We did not observe phagocytosis of sensitized tumor cells, as assessed either by retention of ^{51}Cr or by scanning electron microscopy. A particle must be opsonized globally to be engulfed by a macrophage (46). Perhaps redistribution of immune complexes on the tumor cell surface is one factor which determines whether extracellular lysis will predominate over endocytosis.

We found earlier that activated macrophages, when pharmacologically triggered, killed extracellular tumor cells by oxidative means (2, 3). In the present study, an extracellular lytic response of similar potency was elicited from the same mononuclear effector cell populations without pharmacologic agents. Cytolysis was triggered, instead, by a physiologic substance—that is, antibody. Whether the latter form of cytolysis may also have an oxidative basis is the subject of the accompanying report (10).

Summary

Treatment of mice with Bacille Calmette-Guérin (BCG) or *C. parvum* activates their peritoneal macrophages to release increased amounts of H_2O_2, and thereby to lyse extracellular tumor cells, in response to a pharmacologic agent, phorbol myristate acetate (PMA) (1–3). In the present study, the same bacterial vaccines activated peritoneal cells to become cytolytic to lymphoma cells sensitized with alloantiserum, in the absence of PMA. Resident peritoneal cells, or those elicited with thioglycollate broth, were ineffective, not only in PMA-induced lysis, but also in antibody-dependent lysis of tumor cells. The cytolytic effect of BCG peritoneal cells toward sensitized tumor cells appeared to be mediated mostly by macrophages. Cytotoxicity was immunologically specific, contact dependent, rapid, and efficient. Phagocytosis of intact tumor cells was not involved. Alloantiserum-dependent cytolysis was specifically blocked by the Fab fragment of a monoclonal antibody directed against the trypsin-resistant macrophage Fc receptor (FcR II). Thus, tumor cells coated with homologous immunoglobulin interact with FcR II on activated macrophages to trigger an extracellular cytolytic response.

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