

## EXTRACELLULAR CYTOLYSIS BY ACTIVATED MACROPHAGES AND GRANULOCYTES

### I. Pharmacologic Triggering of Effector Cells and the Release of Hydrogen Peroxide\*

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Until recently, we knew of no biochemical basis for the cytotoxic activity of any mononuclear leukocyte (macrophage or lymphocyte). Thymidine (1) and arginase (2, 3) are now thought to account for some of the *in vitro* cytostatic and cytolytic effects of macrophages. However, those molecules seem unlikely to mediate similar effects of macrophages *in vivo*, except perhaps in areas of minimal perfusion. More is known about the cytotoxic molecules released by granulocytes. When exposed to phagocytic particles (4–6) or lectins (7), granulocytes release hydrogen peroxide. Acting together with myeloperoxidase and a halide (8), granulocyte-derived hydrogen peroxide can participate in the destruction of eukaryotic cells *in vitro* (8, 9). Enzymatically generated hydrogen peroxide has been known for some time to lyse mammalian cells in the presence of a peroxidase and halide (10–12).

Mouse peritoneal macrophages can release hydrogen peroxide almost as copiously as granulocytes, provided the cells are both activated and triggered *in vitro* (13). Similar conditions lead to the release of comparable quantities of superoxide anion (14). These findings led us to investigate the extracellular cytotoxic potential of macrophages after sequential activation and triggering. Suitably activated macrophages, as well as granulocytes, proved to be cytotoxic after exposure to a soluble, membrane-active agent, phorbol myristate acetate (PMA).<sup>1</sup> Study of a variety of effector and target cells provided correlative evidence consistent with a role for hydrogen peroxide as the effector molecule. The rapidity and potency of cytolysis made this model suitable for analysis at the biochemical level, as described in the accompanying report (15).

### Materials and Methods

*Mice.* Female (BALB/c × DBA/2)F<sub>1</sub> (CDF<sub>1</sub>) (H-2<sup>d/d</sup>) (Flow Laboratories, Dublin, Va.) and NCS mice (outbred) (Rockefeller University), aged 6–20 wk, were used, with equivalent results.

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<sup>1</sup> *Abbreviations used in this paper:* BCG, Bacille Calmette-Guérin; DMSO, dimethyl sulfoxide; E:T, effector cell to target cell ratio; FBS, heat-inactivated (56°C, 30 min) fetal bovine serum; KRPG, Krebs-Ringer phosphate buffer with 5.5 mM glucose; LPS, bacterial lipopolysaccharide; MEM, Eagle's minimum essential medium with Earle's salts, penicillin and streptomycin; MEM-S, MEM with 1% FBS; NPAC, nonphagocytic adherent cells; PMA, phorbol myristate acetate.

**Injectibles.** Bacille Calmette-Guérin (BCG), *C. parvum*, casein, and thioglycollate were obtained and administered i.p. as described (13). The BCG was of the Pasteur type (TMC 1011). Peritoneal cells were harvested 10 d–6 wk after the injection of  $10^7$  viable bacilli. Macrophages elicited with a 4% thioglycollate broth obtained from Dr. Richard Johnston (National Jewish Hospital, Denver, Colo.) gave results equivalent to cells elicited with the 10% preparation used in the majority of experiments. Granulocytes were harvested 10–20 h and macrophages 4 d after injection of thioglycollate broth. 1 ml of 1% proteose-peptone (Difco Laboratories, Detroit, Mich.) was given i.p. 4 d before cell collection.

**Preparation of Peritoneal Cells.** Peritoneal cells were collected, subjected to hypotonic lysis of erythrocytes, washed, counted, stained, and scored as described (13). Nonadherent and nonphagocytic adherent cells were prepared from BCG-treated mice as before (16). To remove nonadherent cells, peritoneal cells were added to  $12 \times 75$  mm glass test tubes, centrifuged at 180 *g* for 5 min, and allowed to adhere at 37°C for 1 h in Eagle's minimum essential medium with Earle's salts (MEM) (Grand Island Biological Co., Grand Island, N. Y.) containing 1% heat-inactivated fetal bovine serum (FBS) (Flow Laboratories, Inc., Rockville, Md.), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (MEM-S). The tubes were drained and rinsed three times with Krebs-Ringer phosphate buffer with 5.5 mM glucose (KRPG) (13). To deplete granulocytes,  $2 \times 10^7$  peritoneal cells in 2 ml of MEM-S were layered over 3 ml of Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) in a 15-cubic centimeter conical tube (Corning Glass Works, Science Products Div., Corning, N. Y.) and centrifuged at 300 *g* for 10 min in the cold. The upper cell layer was removed and washed.

**Other Cells.** For mouse erythrocytes, blood was collected from the axillary vessels, anticoagulated with 50 U/ml heparin, and washed twice in KRPG, discarding the buffy coat. For spleen cells, CDF<sub>1</sub> spleens were teased with forceps, the clumps discarded after settling, and the remaining cells subjected to hypotonic lysis (13) and washed twice in MEM. Spinner cultures of the macrophage lines J774 (H-2<sup>d</sup>) and P388D1 (H-2<sup>d</sup>), and the L-cell fibroblast line, were the gift of Dr. Jay Unkeless (Rockefeller University). A monolayer of nontransformed, diploid human foreskin fibroblasts (FS4) was the gift of Dr. P. Sehgal (Rockefeller University). Tumors were maintained by serial passage of ascites: P388 (H-2<sup>d</sup>), P815 (H-2<sup>d</sup>) (from Dr. Unkeless) and SL2 (H-2<sup>d</sup>) (17) in CDF<sub>1</sub> mice, and TLX9 (H-2<sup>b</sup>) (17) in C57BL/6 mice (The Jackson Laboratories, Bar Harbor, Maine).

**Hydrogen peroxide release.** The previously described assay (13) was modified by the use of 5 nmol of scopoletin and 30  $\mu$ g of horseradish peroxidase in 3 ml of KRPG. The assay was performed in an MPF-44 fluorometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Agents tested for their ability to trigger hydrogen peroxide release were: PMA (Consolidated Midland Corp., Brewster, N. Y.), digitonin (Sigma Chemical Co., St. Louis, Mo.), the ionophore A23187 (the gift of Dr. Robert Hamill, Eli Lilly Co., Indianapolis, Ind.), NaF (Fisher Scientific Co., Pittsburgh, Pa.), and the following preparations of bacterial lipopolysaccharide (LPS), all from Sigma Chemical Co.: butanol, phenol, and trichloroacetic acid extracts of *Escherichia coli* 0127:B8, phenol extract of *E. coli* 026:B6, and phenol extract of *Salmonella typhosa*.

**Cytotoxicity Assay.** Target cells, usually numbering  $3 \times 10^7$ , were suspended in 2 ml of MEM with 100–200  $\mu$ Ci of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (1 mCi/ml in saline, New England Nuclear, Boston, Mass.), incubated at 37°C in 7% CO<sub>2</sub> in air for 45 min, and washed four times by centrifugation in MEM-S. Cell concentration was determined by hemocytometer counting and viability by trypan blue exclusion. The desired number of target cells (usually  $2 \times 10^4$ – $6 \times 10^4$ ) in 1 ml of MEM-S was dispensed to  $12 \times 75$  mm borosilicate glass culture tubes (Fisher Scientific Co.) in an ice bath. The tubes already contained 0.02 ml of KRPG in which was dissolved the pharmacologic agent to be tested. Unless otherwise stated, the latter was PMA (maintained as a stock solution of 0.3 mg/ml in dimethylsulfoxide at –70°C). Control tubes contained an equal volume of dimethyl sulfoxide (DMSO) (Sigma Chemical Co.) in KRPG. The activity of PMA in KRPG was lost if it was passed through a Millipore filter (Millipore Corp., Bedford, Mass.). Next, 1 ml of MEM-S containing various numbers of effector cells (usually  $2 \times 10^5$ – $1 \times 10^6$ ) was added, giving a final concentration of PMA of 10 ng/ml (16.7 nM) and of DMSO of 0.0033% (vol/vol). The tubes were transferred to precooled carriers and centrifuged at 180 *g* for 5 min (TJ6R, Beckman Instruments, Inc., Fullerton, Calif.). The carriers were placed in a 37°C water bath for 60 s, then transferred to a humidified incubator (7% CO<sub>2</sub>) at 37°C for 4.5 h. They were then centrifuged at 700 *g* for 10 min, and the upper 1 ml transferred to a

second tube (sample tube). The original tube was designated the residual tube. Both tubes were counted in a gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). To measure the cytotoxicity of reagent hydrogen peroxide, dilutions of Superoxol (J. T. Baker Chemical Co., Phillipsburg, N. J.) in 0.02 ml KRPG were added to the tubes instead of PMA, and effector cells were omitted. Assays were done in triplicate.

*Computations.* Percent release (R) of  $^{51}\text{Cr}$  was taken to be:

$$R = \frac{2.02 (\text{sample} - \text{Bgd})}{\text{sample} + \text{residual} - 2\text{Bgd}} \times 100,$$

where Bgd indicated machine background of the gamma spectrometer, and 2.02 = total assay volume/volume of sample. Specific release (SR) was taken as:

$$\text{SR} = \frac{\bar{R}_{\text{experimental}} - \bar{R}_{\text{spont.}}}{\bar{R}_{\text{max.}} - \bar{R}_{\text{spont.}}} \times 100,$$

where  $\bar{R}_{\text{max.}}$  represented the mean percent of  $^{51}\text{Cr}$  released from an aliquot of target cells diluted fivefold in distilled water and frozen four times in an acetone-dry ice bath, and  $\bar{R}_{\text{spont.}}$  was the mean percent of  $^{51}\text{Cr}$  released by target cells cultured without effector cells. The SEM for triplicate R values was adjusted by dividing it by the factor,  $(\bar{R}_{\text{max.}} - \bar{R}_{\text{spont.}})/100$ . Partway through these studies, the availability of a computer made it practical to estimate the SEM more accurately as:

$$\text{SEM} = \left[ \{n_{\text{exp.}} [(\bar{R}_{\text{max.}} - \bar{R}_{\text{spont.}})\text{SEM}_{\text{exp.}}]^2 + n_{\text{spont.}} [(\bar{R}_{\text{max.}} - \bar{R}_{\text{exp.}})\text{SEM}_{\text{spont.}}]^2 + n_{\text{max.}} [(\bar{R}_{\text{exp.}} - \bar{R}_{\text{spont.}})\text{SEM}_{\text{max.}}]^2 \} / n_{\text{exp.}} (\bar{R}_{\text{max.}} - \bar{R}_{\text{spont.}})^4 \right]^{1/2} \times 100.$$

For 100 consecutive triplicates, the SEM as calculated above averaged 2.16% release of  $^{51}\text{Cr}$ , whereas the simple proportional SEM for the same triplicates averaged 2.04%. For consistency, when data are presented below for individual triplicates, the simple proportional SEM is reported.

Effector cell to target cell (E:T) ratios were calculated on the basis of the number of macrophages and monocytes added, except for cells harvested 10–20 h after injection of thioglycollate broth, in which case the ratio was based on the number of granulocytes (neutrophils plus eosinophils). The number of cells of each type was calculated from their percentage on the differential count and the total number of peritoneal cells added.

*Scanning Electron Microscopy.* As performed by Dr. Gilla Kaplan, cultures in 1 ml of MEM-S on glass coverslips were fixed by the addition of an equal volume of 5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, dehydrated through graded alcohols, transferred to amyl acetate, subjected to critical point drying in liquid  $\text{CO}_2$  in a Sorval CPD system, coated with gold (Edwards 5150 sputter coater (Edwards High Vacuum, Inc., Grand Island, N.Y.)), and examined in an ETEC Autoscan (ETEC, Inc., Hayward, Calif.)

## Results

*Extracellular Lysis of P388 Lymphoma Cells by BCG-Induced Peritoneal Cells and by Granulocytes in the Presence of PMA.* Table I summarizes the results of all 91 experiments conducted in the standard manner using the lymphoma P388 as a target. Peritoneal cells taken from BCG-treated mice (BCG cells) and granulocyte-rich peritoneal cells taken from mice 10–20 h after injection of thioglycollate broth, readily lysed P388, but only when triggered by PMA. Peritoneal cells from untreated mice, or from mice given thioglycollate broth 4 d previously, were ineffective. The compositions of the effector cell populations are shown in Table II.

The validity of the assay was investigated further. BCG-induced macrophages were cultured with P388 cells with or without PMA and examined by scanning electron microscopy, kindly performed by Dr. Gilla Kaplan. In two experiments, there were 76 and 91% reductions, respectively, in the total number of visible P388 cells in the

TABLE I  
Lysis of P388 Lymphoma Cells by Peritoneal Cells Triggered with PMA

Treatment of mice	No. exp.	E:T ratio* mean $\pm$ SEM (range)	Percent specific release of $^{51}\text{Cr}\ddagger$ mean $\pm$ SEM (range)	
			DMSO vehicle§	PMA
BCG	38	19 $\pm$ 1.2 (7-30)	1.5 $\pm$ 1.0	78.1 $\pm$ 2.1 (39.6-104)
Thioglycollate, 10-20 h (granulocytes)	20	13 $\pm$ 2.1 (3-33)	1.0 $\pm$ 0.7	87.3 $\pm$ 3.4 (50.5-113)
None	26	18 $\pm$ 1.3 (7-27)	-6.2 $\pm$ 1.9	-4.4 $\pm$ 1.6 (-18.1-11.4)
Thioglycollate, 4 d**	7	36 $\pm$ 3.9 (22-45)	-8.2 $\pm$ 6.0	-0.4 $\pm$ 2.6 (-15-4.9)

\* Effector to target ratio, calculated from the number of peritoneal cells added and the percentage of effector cells on the differential count (see Materials and Methods).

‡ The viability of P388 immediately before adding effector cells averaged 96.1  $\pm$  0.4%. The  $^{51}\text{Cr}$  release after 4 cycles of freezing and thawing averaged 85.0  $\pm$  0.7%. The spontaneous release without PMA averaged 14.7  $\pm$  0.9%, and the spontaneous release with PMA averaged 15.6  $\pm$  0.9% (48 preparations of tumor cells). Assay time, 4.5 h, with  $2 \times 10^4$ - $8 \times 10^4$  P388 cells per tube.

§ DMSO, 0.0033% (vol/vol).

|| PMA, 10 ng/ml (16.7 nM).

¶ Cells harvested 10-20 h after i.p. injection of thioglycollate broth.

\*\* Cells harvested 4 days after i.p. injection of thioglycollate broth.

TABLE II  
Characterization of Peritoneal Cell Suspensions

Treatment of mice	No. exp.	No. cells per mouse $\times 10^{-6}$ mean $\pm$ SEM	Percent of peritoneal cells, mean $\pm$ SEM				
			Macrophages + monocytes	Lymphocytes	Mast cells	Eosinophils	Neutrophils
BCG	53	5.7 $\pm$ 0.3	40.9 $\pm$ 1.2	52.0 $\pm$ 1.2	0.14 $\pm$ 0.04	0.13 $\pm$ 0.03	6.8 $\pm$ 0.3
Thioglycollate, 10-20 h	23	13.7 $\pm$ 0.8	25.9 $\pm$ 1.2	8.2 $\pm$ 1.5	0.06 $\pm$ 0.04	2.9 $\pm$ 0.60	63.0 $\pm$ 2.1
None	45	4.7 $\pm$ 0.2	45.1 $\pm$ 1.5	53.3 $\pm$ 1.6	0.57 $\pm$ 0.12	0.32 $\pm$ 0.06	0.67 $\pm$ 0.10
Thioglycollate, 4 d	8	26.0 $\pm$ 4.1	78.5 $\pm$ 3.8	10.4 $\pm$ 2.5	0.23 $\pm$ 0.10	8.0 $\pm$ 1.6	3.3 $\pm$ 1.3

cultures containing BCG-induced macrophages and PMA, with 89 and 96% reductions in the number of P388 cells which appeared viable by morphologic criteria. The cytotoxicity assay was performed at a somewhat lower effector cell density, and resulted in 71 and 76% specific release of  $^{51}\text{Cr}$  in the same experiments.

The BCG-induced macrophages were well spread, but PMA induced an even greater degree of spreading. As seen in Fig. 1, the zone of macrophage-tumor cell contact was disk-like, without extensive areas of membrane apposition. Lysis was extracellular (Fig. 1 c, d), and no phagocytosis of tumor cells was observed. Lymphocytes adjacent to dead tumor cells appeared undamaged (Fig. 1 d).

Release of  $^{51}\text{Cr}$  also correlated closely with reduction in the number of trypan blue-excluding tumor cells. For these experiments, adherent granulocytes were used as effector cells, to minimize the possibility that any effector cells would be mistaken for target cells in the hemocytometer. A mean specific release of  $^{51}\text{Cr}$  of 87% was associated with a mean reduction in trypan blue-excluding lymphoma cells of 89%.

The cytotoxicity assay was not affected by retention of  $^{51}\text{Cr}$  by adherent effector cells. All the  $^{51}\text{Cr}$  added could be recovered after rinsing the reaction tubes with

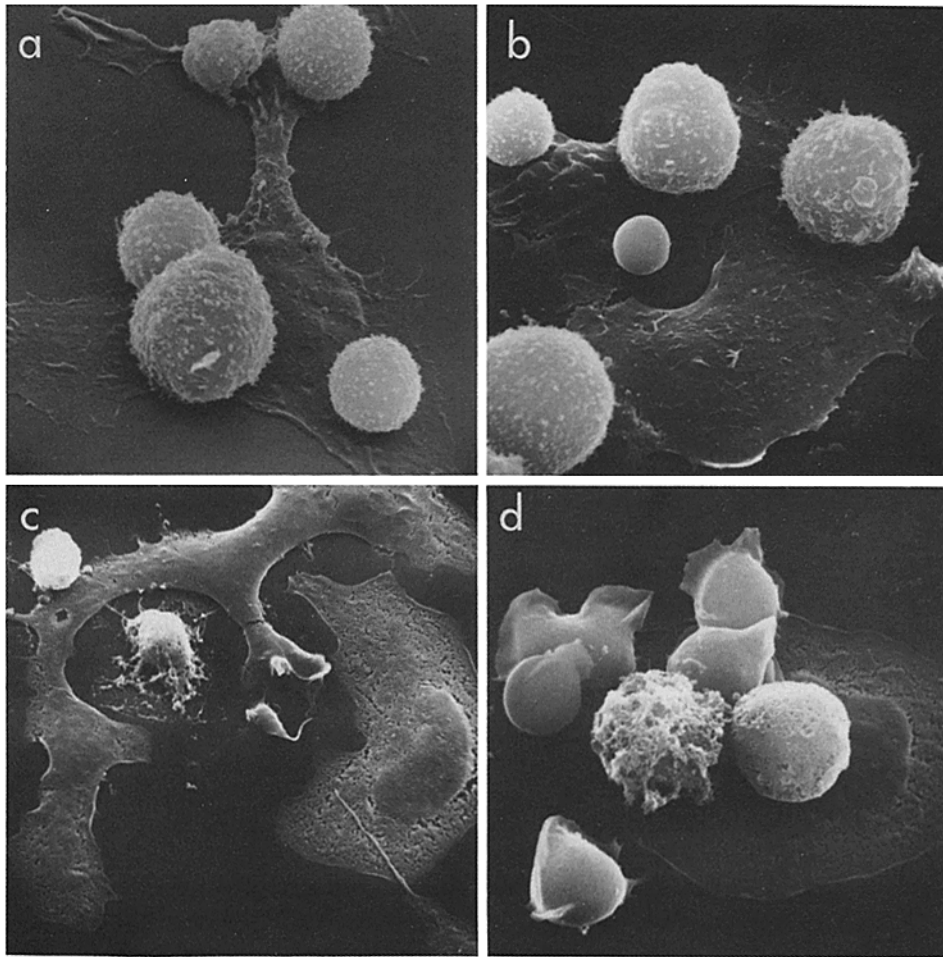


FIG. 1. Scanning electron micrographs of peritoneal cells from BCG-treated mice cultured for 4.5 h with P388 lymphoma cells. Cultures in (a) and (b) received DMSO vehicle (0.0033%). Those in (c) and (d) received PMA (10 ng/ml). Magnifications: (a)  $\times 3,280$ , (b)  $\times 1,804$ , (c)  $\times 1,640$ , (d)  $\times 1,488$ .

additional medium. Cytotoxicity was only slightly affected by varying the concentration of FBS over a wide range. For example, BCG-induced cells incubated with  $^{51}\text{Cr}$ -labeled P388 cells at an E:T ratio of 18 in the presence of PMA caused 79% specific release of  $^{51}\text{Cr}$  at 0.1% FBS, 83% at 1.0% FBS (the standard amount), and 91% at 5% FBS. Results were similar when tumor cells were allowed to settle at unit gravity onto monolayers of BCG-induced macrophages on coverslips, compared to centrifuging target cells and effector cells together in round-bottomed tubes in the standard manner (data not shown).

In the absence of effector cells, PMA had no deleterious effect on P388 cells, as judged by scanning electron microscopy. Likewise, no toxicity of PMA for P388 cells was discernible by trypan blue exclusion,  $^{51}\text{Cr}$  release (Table I), or the survival time of mice injected i.p. with the P388 cells remaining after 4.5 h of culture with DMSO

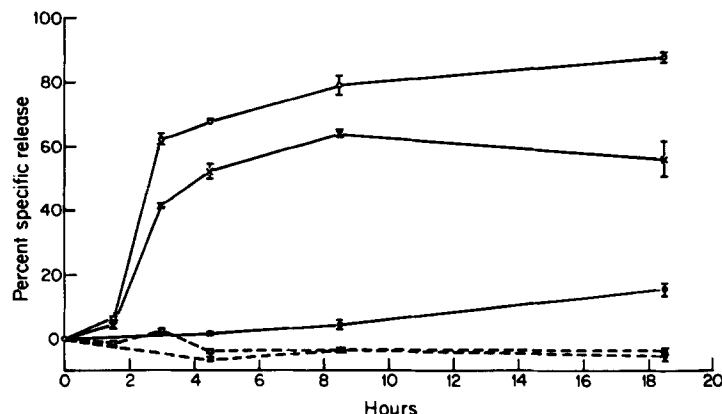


FIG. 2. Time-course of  $^{51}\text{Cr}$  release from P388 lymphoma cells;  $\times$ — $\times$ , with BCG-induced peritoneal cells and PMA (10 ng/ml), not centrifuged. The remaining groups were centrifuged at the start of the assay:  $\circ$ — $\circ$ , with BCG-induced peritoneal cells and PMA;  $\circ$ — $\circ$ , with BCG-induced peritoneal cells and DMSO vehicle;  $\bullet$ — $\bullet$ , with normal peritoneal cells and PMA;  $\bullet$ — $\bullet$ , with normal peritoneal cells and DMSO vehicle. E:T ratio, 9. Means and SEM for triplicates.

( $12.3 \pm 0.4$  d) or PMA ( $12.6 \pm 0.5$  d) (initial cell number,  $6 \times 10^4$ , with six mice per group).

*Time-Course.* The above experiments were terminated at 4.5 h. To learn how fast lysis occurred, and whether the superiority of BCG-induced cells over normal cells, as well as their requirement for PMA, was still evident at later times, samples were harvested at intervals from 1 to 18.5 h. As shown in Fig. 2, little lysis was evident until 3 h. Thereafter,  $^{51}\text{Cr}$  was rapidly released from P388 cells in the presence of BCG cells and PMA, with only a slight increment between 4.5 and 18.5 h. Even at 18.5 h, BCG cells cultured without PMA failed to induce lysis. Cytolysis by BCG-induced peritoneal cells was augmented if the tubes were centrifuged at the start of the assay (Fig. 2). Normal peritoneal cells, cultured with PMA, were minimally cytotoxic only at 18.5 h.

We next investigated whether the period of injury to the tumor cells might be shorter than the time required for them to release  $^{51}\text{Cr}$ . BCG-induced peritoneal cells were allowed to adhere in test tubes, and nonadherent cells were removed. The tumor cells were added and centrifuged. At intervals, the pellets were disrupted, and one-half the medium transferred to a second tube. Thus, the second tube contained one-half the target cells, but few or no effector cells. All tubes were harvested at 4.5 h. As shown in Fig. 3, those target cells which were removed from adherent effector cells at 0 time or at 30 min exhibited no or only partial  $^{51}\text{Cr}$  release at 4.5 h. However, if P388 cells were removed from the adherent BCG cells after 1 h or more, they were fully lysed when tested at 4.5 h. It appeared that BCG cells inflicted an injury during the 1st h, which resulted in near-maximal release of  $^{51}\text{Cr}$  at 4.5 h.

*Influence of Effector Cell Number.* To compare the efficacy of eight different cell populations, effector cells were added to cultures of P388 cells at a variety of doses. As shown in Fig. 4, peritoneal cells from mice which had received BCG, *C. parvum*, or short-term thioglycollate treatment (granulocytes) were extremely effective, with similar dose-response curves, showing maximal or near maximal lysis at E:T ratios of

## I. CYTOLYSIS BY MACROPHAGES AND GRANULOCYTES

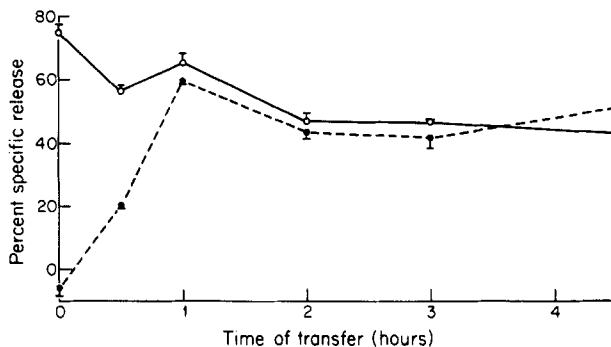


FIG. 3. Effect of separating target cells from effector cells at various times after starting the assay.  $^{51}\text{Cr}$ -labeled P388 lymphoma cells were centrifuged onto preadherent BCG-induced macrophages in the presence of PMA. At the times indicated, the pellets were disrupted. One-half the tumor cells, but few or no effector cells, were transferred to a second tube (●-●). The transferred cells as well as the target cells remaining behind with the effectors (O-O) were all harvested at 4.5 h. E:T ratio, 9. Means and SEM of triplicates.

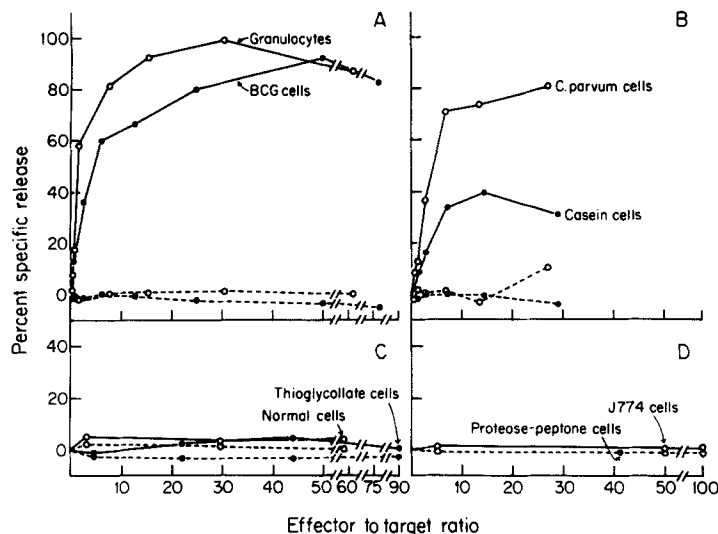


FIG. 4. Influence of different numbers of effector cells, incubated with  $2 \times 10^4$   $^{51}\text{Cr}$ -labeled P388 lymphoma cells for 4.5 h. Solid lines, with PMA (10 ng/ml). Dotted lines, with DMSO vehicle. Cells elicited with proteose-peptone (panel D) were tested at a single cell number, and gave the same value with or without PMA. Means of triplicates. SEM ranged from 0.1 to 4.0%.

25, 50% lysis at E:T ratios from 1.4 to 4.5, and detectable lysis at ratios as low as 0.5. When PMA was omitted, however, no cytotoxicity resulted with these cells even at E:T ratios as high as 77 (Fig. 4 a, b). In contrast, normal peritoneal cells, cells taken 4 d after thioglycollate or proteose-peptone, and J774 cells (a transformed, macrophage-like line [18]) failed to lyse P388 cells at any dose tested, up to E:T ratios of 100 (Fig. 4 c, d). Casein-induced peritoneal cells had an intermediate efficacy (Fig. 4 b).

The relative potency of the cell populations tested above correlated precisely with their ability to release hydrogen peroxide in response to PMA. The ability of BCG-, *C. parvum*-, and casein-induced cells, as well as granulocyte-rich populations, to release

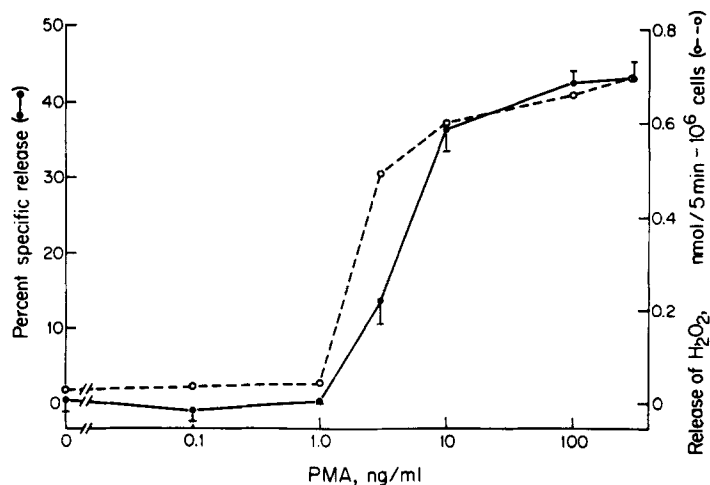


FIG. 5. Effect of concentration of PMA on lysis of  $^{51}\text{Cr}$ -labeled P388 lymphoma cells (●—●) by BCG-induced peritoneal cells, and on release of  $\text{H}_2\text{O}_2$  (○---○) by the latter. For the cytotoxicity assay, the E:T ratio was 23. Spontaneous release of  $^{51}\text{Cr}$  ranged from  $8.1\% \pm 0.4$  to  $9.7\% \pm 0.1$  for the different doses of PMA. Means and SEM of triplicates.

detectable  $\text{H}_2\text{O}_2$ , and the relative inability of thioglycollate-induced macrophages and normal peritoneal cells to do so, after exposure to PMA, were reported earlier (13). We found that proteose-peptone induced macrophages released  $<0.10$  nmol  $\text{H}_2\text{O}_2/10^6$  macrophages/5 min after PMA (four experiments), and  $\text{H}_2\text{O}_2$  release from J774 cells with or without PMA was undetectable (two experiments).

**Effect of PMA Dose.** To pursue further the correlation between lysis of P388 and secretion of  $\text{H}_2\text{O}_2$ , the effect of PMA dose on both processes was determined, using BCG-induced peritoneal cells. The dose-response curves were almost identical, showing a nearly optimal effect at 10 ng/ml (16.7 nM) (Fig. 5). As much as 100 ng/ml PMA failed to trigger  $\text{H}_2\text{O}_2$  release or cytotoxic activity using peritoneal cells from untreated mice (not shown).

**Nature of the Effector Cell from BCG-Treated Mice.** The adherent cells in the BCG-induced peritoneal cell population displayed full lytic activity toward P388, whereas the nonadherent cells were inactive. One of three such experiments is shown in Table III. Nonphagocytic adherent cells, isolated from the adherent population, were inactive as well (Table III). This strongly implied that the effector cell was both adherent and phagocytic. These findings correlated with the ability of the same populations to release  $\text{H}_2\text{O}_2$  in response to PMA (13).

The remaining possibility, that granulocytes could account for all of the activity seen with adherent peritoneal cells from BCG-treated mice, seemed remote, after considering the data in Table II and Figs. 1 and 4. From these, it seemed that BCG-induced peritoneal cells, especially when tested at lower E:T ratios, contained too few granulocytes to account for anything but a small percentage of their activity. Moreover, when BCG- or *C. parvum*-induced peritoneal cells were centrifuged on Ficoll-Hypaque, their content of granulocytes could be reduced markedly, yet cytotoxic activity was reduced only slightly (Table IV).

When granulocytes were added back in different numbers to Ficoll-Hypaque-passaged BCG cells, the resulting  $^{51}\text{Cr}$  release from P388 cells was equal to the sum of



TABLE III  
Cytotoxicity of Subpopulations of BCG-Induced Peritoneal Cells

Peritoneal cells	No. added	Percent specific release of $^{51}\text{Cr}^*$	
		DMSO vehicle $\ddagger$	PMA $\S$
Unseparated	$1 \times 10^6$	$-14.8 \pm 2.6$	$81.9 \pm 5.1$
Adherent	From $1 \times 10^6$ $\parallel$	$-17.2 \pm 3.0$	$89.7 \pm 6.4$
Nonadherent	$2 \times 10^6$	$-11.2 \pm 4.1$	$7.6 \pm 2.4$
	$1 \times 10^6$	$-13.1 \pm 0.9$	$5.3 \pm 2.5$
	$4 \times 10^5$	$-11.7 \pm 0.8$	$1.4 \pm 1.5$
NPAC $\parallel\parallel$	$9.3 \times 10^5$	$-13.9 \pm 2.0$	$-3.3 \pm 5.5$
	$8 \times 10^4$	$-3.0 \pm 1.3$	$-2.7 \pm 1.7$

\* From  $2 \times 10^4$   $^{51}\text{Cr}$ -labeled P388 cells. Means  $\pm$  SEM of triplicates.

$\ddagger$  DMSO, 0.0033% (vol/vol).

$\S$  PMA, 10 ng/ml.

$\parallel$   $1 \times 10^6$  peritoneal cells were added to the tubes, allowed to adhere for 1 h, and nonadherent cells rinsed out (see Materials and Methods).

$\parallel\parallel$  Nonphagocytic adherent cells, isolated as described (16). NPAC were 94.5% viable, with 0.84% contamination by phagocytic cells.  $1 \times 10^6$  unseparated peritoneal cells were estimated to contain  $8.6 \times 10^4$  NPAC, based on finding 41% macrophages-monocytes in the differential count, and 21% nonphagocytic cells by observation of well-washed, postphagocytic adherent cell monolayers.

TABLE IV  
Effect of Reduction of Granulocyte Content on Cytotoxic Activity of Peritoneal Cells

Experiment	Treatment of mice	Peritoneal cells	Number added	Percent granulocytes	Percent specific release of $^{51}\text{Cr}^*$
A	<i>C. parvum</i>	Unseparated	$5 \times 10^5$	24.0	$63.8 \pm 1.2$
		After FH $\ddagger$	$5 \times 10^5$	4.6	$65.8 \pm 4.0$
B	BCG	Unseparated	$1 \times 10^6$	12.2	$86.8 \pm 1.5$
		After FH	$1 \times 10^6$	5.6	$70.1 \pm 0.8$
C	BCG	Unseparated	$4 \times 10^5$	9.5	$63.4 \pm 2.0$
		After FH	$4 \times 10^5$	3.7	$50.1 \pm 2.2$
		Unseparated	$2 \times 10^5$	9.5	$39.3 \pm 1.5$
		After FH	$2 \times 10^5$	3.7	$32.9 \pm 1.1$

\* From  $2 \times 10^4$   $^{51}\text{Cr}$ -labeled P388 cells with PMA (10 ng/ml). Means  $\pm$  SEM for triplicates.

$\ddagger$  Cells taken from the upper layer after centrifugation on Ficoll-Hypaque (see Materials and Methods).

the lysis caused by the same number of each effector population tested separately (not shown). Thus there was no evidence that granulocytes synergized with BCG cells; they were simply additive. We concluded that both granulocytes and BCG-activated macrophages were capable of lysing P388 cells when triggered with PMA.

*Lysis of Other Targets.* The sensitivity to lysis of a variety of targets was then tested, and compared with their sensitivity to lysis by reagent  $\text{H}_2\text{O}_2$ . As shown in Figs. 6 and 7, various cells differed in their susceptibility to lysis in both tests. For example, the concentration of  $\text{H}_2\text{O}_2$  causing 50% specific release of  $^{51}\text{Cr}$  ranged from  $3.63 \times 10^{-6}$  M in the case of P388 to  $3.85 \times 10^{-5}$  M for J774. The correlation between susceptibility to lysis by PMA-triggered granulocytes and by reagent  $\text{H}_2\text{O}_2$  was extremely close ( $r = 0.98$ ) (Fig. 8).

In less extensive tests, BCG-induced peritoneal cells demonstrated similar results with many of the same target cells. For example, PMA-triggered BCG cells caused 86% specific release of  $^{51}\text{Cr}$  from syngeneic erythrocytes at E:T ratios as low as 0.047

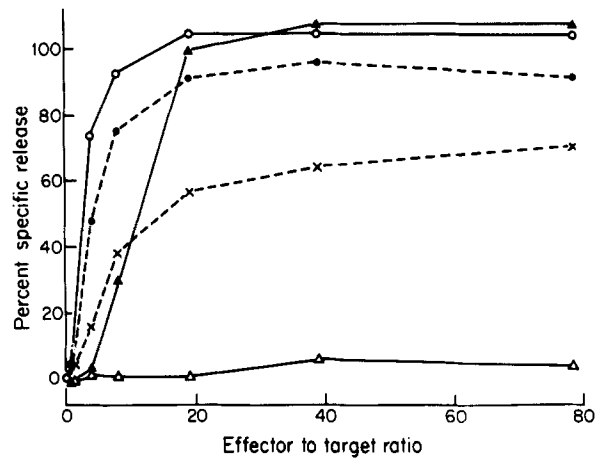


FIG. 6. Susceptibility of various target cells to lysis by PMA-triggered peritoneal granulocytes.  $2 \times 10^4$   $^{51}\text{Cr}$ -labeled target cells were cultured for 4.5 h with 10 ng/ml PMA plus varying numbers of peritoneal exudate cells collected 17 h after injection of thioglycollate broth (78.6% granulocytes). The target cells and their spontaneous release of  $^{51}\text{Cr}$  with PMA, followed by their specific release when incubated with a 40-fold excess of granulocytes but without PMA, were:  $\circ$ — $\circ$ , P388 (14.7%, -1.6%);  $\bullet$ — $\bullet$ , TLX9 (12.4%, -4.2%);  $\blacktriangle$ — $\blacktriangle$ , L cells (33.9%, 2.7%);  $\times$ — $\times$ , spleen cells (19.8%, 5.7%);  $\triangle$ — $\triangle$ , J774 (17.2%, 1.1%). Means of triplicates. SEM ranged from 1.2 to 3.0%.

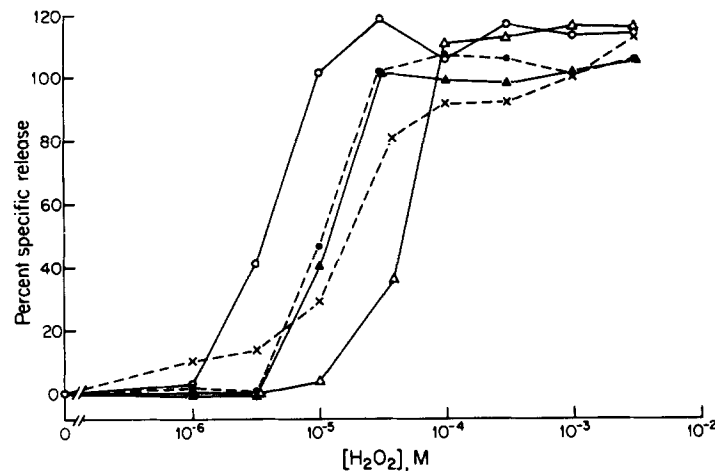


FIG. 7. Susceptibility of various target cells to lysis by reagent  $\text{H}_2\text{O}_2$ . Cells cultured as in Fig. 6, but with reagent  $\text{H}_2\text{O}_2$  instead of effector cells and PMA. Notation of cell types as in legend of Fig. 6. Means of triplicates. SEM ranged from 1.5 to 3.6%.

(1:21), 50% of maximum release at an E:T ratio of 0.025 (1:40), and significant lysis ( $10.8\% \pm 0.5$ ) at an E:T ratio of 0.016 (1:63). One-half-maximal lysis of erythrocytes by syngeneic granulocytes, in comparison, occurred at an E:T ratio of 0.017 (1:60).

*Use of Agents Other than PMA.* It was of interest to test other soluble substances which, like PMA, are membrane-active, but which would not trigger  $\text{H}_2\text{O}_2$  release. LPS and digitonin have both been reported to stimulate hexose monophosphate shunt activity (19) and superoxide release (14, 20, 21) from leukocytes. Yet, we have been

## I. CYTOLYSIS BY MACROPHAGES AND GRANULOCYTES

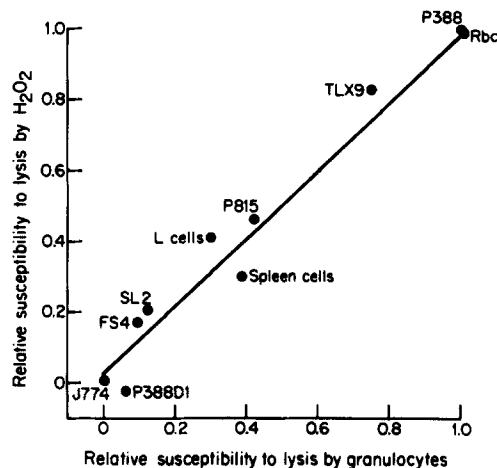


FIG. 8. Comparison of susceptibility to lysis by reagent  $H_2O_2$  and by PMA-triggered granulocytes for 10 types of  $^{51}Cr$ -labeled target cells. From titrations like those shown in Figs. 6 and 7, the percent specific release of  $^{51}Cr$  was determined for each target cell when incubated with  $1 \times 10^{-5} M H_2O_2$  or with  $1.5 \times 10^6$  granulocytes plus 10 ng/ml PMA. The results are plotted relative to the equivalent values for P388 cells in the same experiment. All target cells were tested at  $2 \times 10^4$ , except for erythrocytes at  $3 \times 10^6$  per tube. The line (least squares fit) has a slope of 0.98 and a correlation coefficient of 0.98.

TABLE V

*Effect of Membrane-Active Agents on Cytotoxicity*

Agent added*	Percent specific release‡
DMSO vehicle§	$-3.3 \pm 1.3$
LPS	$3.0 \pm 0.6$
Digitonin¶	$3.9 \pm 1.0$
PMA**	$71.2 \pm 3.8$

\* Effector cells were BCG-induced peritoneal cells at E:T ratio of 18.

‡ Means  $\pm$  SEM for triplicates with  $2 \times 10^4$   $^{51}Cr$ -labeled P388 cells. Spontaneous release for each set, in the order listed, was 12.2, 9.9, 9.9, and 12.6%.

§ DMSO, 0.0033% (vol/vol).

|| LPS, lipopolysaccharide from *E. coli* 027:B8, butanol extracted, 30  $\mu g/ml$ .

¶ Digitonin, 0.3  $\mu g/ml$ .

\*\* PMA, 10 ng/ml.

unable to detect  $H_2O_2$  release from BCG-induced macrophages using five preparations of LPS (see Materials and Methods) over the range from 333  $\mu g/ml$  to 33  $\mu g/ml$  for up to 1.5 h after addition, nor from mouse peritoneal granulocytes using digitonin in the range from 10 ng/ml to 10  $\mu g/ml$ , which was toxic. When added to cultures of BCG-induced peritoneal cells and P388 cells, neither LPS nor digitonin caused  $^{51}Cr$  release, although PMA did (Table V).

Suitable concentrations of NaF (20 mM) (22) and A23187 (0.33  $\mu g/ml$ ) (23) triggered  $H_2O_2$  release, but were toxic to P388 cells at the same concentrations.

## Discussion

These results establish a close correlation between hydrogen peroxide release and the extracellular cytotoxicity of activated macrophages and granulocytes when pharmacologically triggered.

The present findings differ from most reports of immunologically nonspecific macrophage-mediated cytotoxicity in several respects. The cytotoxic effects in the present model were extremely rapid, with nearly maximal release of  $^{51}\text{Cr}$  within 4.5 h, and with evidence that injury to the target cells was complete within 1 h, in contrast to systems in which injury was apparent between about 18 h and 5 d (24–30). The cytotoxicity was associated with target cell death, rather than reversible cytoostasis (31). The cytotoxic activity of effector cells was potent, with 50% lysis of P388 lymphoma cells occurring at E:T ratios of 1.4–4.5, and 50% lysis of mouse erythrocytes at E:T ratios of 0.017–0.025.

Most likely, the rapidity and potency of effects were due to pharmacologic triggering of the macrophages and granulocytes. We felt such a system deserved study, both because it facilitated analysis at the biochemical level, and because it might be a model for other forms of macrophage-mediated cytotoxicity whose induction can be resolved into the same two steps, that is, activation and triggering.

Russell, Doe, and McIntosh (32), and Doe and Henson (33), demonstrated  $^{51}\text{Cr}$  release from P815 mastocytoma cells when the latter were cultured with macrophages from tumors (32) or thioglycollate-induced exudates (33) plus LPS. We considered an agent to trigger a response when the response was absent or very small until the agent was added, and appeared very rapidly thereafter. The requirement for 16 h of exposure to LPS in the studies cited (33) makes it likely that LPS acted as an activating agent, rather than as a triggering agent. It is not clear whether LPS might thereafter have also acted as a triggering agent, or in some other capacity, such as through activation of complement components released by the macrophages. Thus, our inability to trigger cytotoxicity with LPS may be due to fundamental differences in kinetics of the assays, or less likely, to differences in LPS preparations, effector cells, or target cells. The same points arise in comparing the present findings with those of Weinberg, Chapman and Hibbs (34), concerning LPS enhancement of macrophage anti-tumor activity in a 60-h assay (34).

We compared eight types of effector cells. They varied from strongly cytotoxic to completely noncytotoxic. Their activity in the cytotoxicity assay correlated with their ability to release  $\text{H}_2\text{O}_2$  in response to the same triggering agent, PMA. Specifically, macrophages activated by treatment of the mice with BCG or *C. parvum* were highly effective, as were mouse peritoneal granulocytes. All three cell populations released abundant  $\text{H}_2\text{O}_2$  in response to PMA (13). Casein-induced peritoneal cells were intermediate in efficacy in both assays. Normal peritoneal macrophages, and macrophages elicited with thioglycollate broth or proteose-peptone, which released very little  $\text{H}_2\text{O}_2$  in response to PMA, were inactive. Also inactive was the macrophage-like tumor line, J774 (18), which released no detectable  $\text{H}_2\text{O}_2$ .

On the other hand, there was a discrepancy between cytotoxic potency and previous reports of superoxide release from various preparations of macrophages. For example, PMA was found to trigger as much superoxide release from thioglycollate-elicited macrophages as from BCG-activated macrophages (14). J774 also released substantial amounts of superoxide (14). Because extracellular superoxide anion would be expected to dismutate spontaneously to hydrogen peroxide, our inability to detect either cytotoxicity or substantial amounts of extracellular hydrogen peroxide using thioglycollate-elicited macrophages and J774 cells was unexpected. Possible explanations for this apparent paradox are under study.

Granulocytes were roughly twice as potent as BCG-activated macrophages in

cytotoxicity and in  $H_2O_2$  release. In part, this may be due to inclusion of some nonphagocytic adherent cells in the tally of macrophages, because the former are inactive in this system. Apart from nonphagocytic adherent cells (NPAC), there was extreme heterogeneity in size and cytologic maturity among the macrophages and monocytes in the BCG-induced population. It is possible that a subpopulation accounted for much of the activity, and was correspondingly more potent on a per cell basis.

10 types of target cells were compared for susceptibility to lysis, both by pharmacologically triggered macrophages and granulocytes, and by reagent  $H_2O_2$ . Susceptibility to lysis, although highly variable, was the same in both tests for a given type of target cell. The target cells used were malignant or normal, syngeneic, allogeneic, or xenogeneic, adherent or nonadherent, lymphocytic, erythrocytic, fibroblastic, or macrophage like, murine or human. Too few cells were tested to generalize about their susceptibility to lysis in terms of any of these categories.

The  $LD_{50}$  for various target cells ranged from  $3.63 \times 10^{-6}$  M to  $3.85 \times 10^{-5}$  M  $H_2O_2$ . BCG-activated macrophages continued to release  $H_2O_2$  at a nearly constant rate for at least 1 h after the addition of PMA. In a previous study, the mean rate of release of  $H_2O_2$  after addition of PMA was 7.9 nmol/ $10^6$  cells per 5 min for BCG-induced macrophages, and 9.5 nmol for granulocytes (13). For unknown reasons, the corresponding values during the present study were lower (2.5 and 5.8, respectively). Using the lower values, and assuming there are  $4 \times 10^5$  macrophages in the typical assay to which  $10^6$  BCG-induced peritoneal cells are added in a final volume of 2 ml, the  $H_2O_2$  concentration would be expected to reach  $6 \times 10^{-6}$  M in the 1st h, which corresponds to the period of injury. For granulocytes,  $6.6 \times 10^5$  polymorphonuclear leukocytes in the standard assay using  $10^6$  peritoneal cells would be expected to generate  $2.3 \times 10^{-5}$  M  $H_2O_2$  in the same period. Although these calculations are useful, caution is needed in their interpretation. First, reagent  $H_2O_2$  was added as a bolus, although the cells released  $H_2O_2$  as a flux. Catabolism may have prevented cell-derived  $H_2O_2$  from reaching the projected final concentrations. On the other hand, the concentration of  $H_2O_2$  in the vicinity of the target cells may have been higher than calculated, because of their proximity to the effector cells.

Of the three membrane-active agents tested, only the one triggering  $H_2O_2$  release from activated macrophages and granulocytes led to cytotoxicity when those cells were cultured with tumor cells. The dose-response curve for PMA was the same in both assays.

The above findings point strongly to a role for hydrogen peroxide in the extracellular cytotoxic mechanisms of activated macrophages, and tend to confirm the findings of others using granulocytes (8, 9). However, the evidence presented here is circumstantial. Further experiments were designed to learn whether hydrogen peroxide release from activated macrophages was both necessary and sufficient for cytotoxicity. The results are described in the accompanying report (15).

### Summary

Lymphoma cells were rapidly lysed by activated macrophages and granulocytes in the presence of PMA. Release of  $^{51}Cr$  from lymphoma cells correlated closely with their destruction as viewed by scanning electron microscopy, and with reduction in the number of trypan blue-excluding cells. The standard assay involved  $^{51}Cr$  release

measured at 4.5 h, but injury appeared to be complete in 1 h. Of eight different types of effector cells tested, only those releasing abundant  $H_2O_2$  in response to PMA were effective, that is, BCG-, *C. parvum*-, or casein-activated macrophages, or thioglycollate-elicited granulocytes. Normal macrophages, J774 cells, or macrophages elicited with thioglycollate broth or proteose-peptone were ineffective. BCG-activated macrophages and granulocytes caused 50% specific release of  $^{51}Cr$  from P388 lymphoma cells at E:T ratios between 1.4 and 4.5, and from mouse erythrocytes at E:T ratios of 0.017 to 0.025. 10 types of target cells varied widely in their susceptibility to lysis by reagent  $H_2O_2$ , with one-half-maximal lysis occurring at  $H_2O_2$  concentrations ranging from  $3.63 \times 10^{-6}$  M to  $3.85 \times 10^{-5}$  M. Effector cells were expected to generate approximately that much  $H_2O_2$  during the period of injury. Susceptibility of the target cells to lysis by PMA-triggered granulocytes correlated closely with their sensitivity to  $H_2O_2$  ( $r = 0.98$ ). The membrane-active agents LPS and digitonin, which did not trigger  $H_2O_2$  release, did not trigger cytotoxicity. The dose-response curve for triggering of  $H_2O_2$  release by PMA was identical to that for triggering cytotoxicity. These results provided strong circumstantial evidence for the importance of  $H_2O_2$  in extracellular cytolysis by activated macrophages and granulocytes when pharmacologically triggered.

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