

## EXTRACELLULAR CYTOLYSIS BY ACTIVATED MACROPHAGES AND GRANULOCYTES

### II. Hydrogen Peroxide as a Mediator of Cytotoxicity\*

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In the previous paper (1), we reported that activated macrophages, as well as granulocytes, when pharmacologically triggered, could rapidly lyse a variety of target cells. The cytotoxic potency of effector cells, the susceptibility of target cells to lysis, and the efficacy of triggering agents all conformed closely to the hypothesis that the mediator of cytotoxicity was hydrogen peroxide. The concentration of  $H_2O_2$  which could be generated by effector cells within the period of injury was calculated to be sufficient to lyse target cells.

To obtain more direct evidence regarding the role of hydrogen peroxide, we undertook the studies described below. These included depriving the cultures of oxygen or glucose, adding catalase or superoxide dismutase, and testing scavengers of singlet oxygen or hydroxyl radical. In addition, we measured the cytotoxicity of glucose oxidase-coated particles, which generated  $H_2O_2$  at their surface at the same rate as Bacille Calmette-Guérin (BCG)<sup>1</sup>-activated macrophages after pharmacologic triggering. Finally, we attempted to determine whether extracellular cytotoxicity depended on peroxidase. The results established that hydrogen peroxide mediates extracellular cytolysis by activated macrophages and granulocytes in this system.

### Materials and Methods

*Mice, Activating Agents, Cell Preparation, and Assays for  $H_2O_2$  Release and Cytotoxicity.* These were the same as in the previous paper (1). Where indicated, two additional strains of mice were used. C3H/HeAn1/Cs<sup>b</sup> mice, homozygous for severe acatalasemia (2), were bred in our colony from stock generously provided by Dr. Robert N. Feinstein (Argonne National Laboratories, Argonne, Ill.). As controls, C3H/HeN mice were used (Charles River Breeding Laboratories, Inc., Wilmington, Mass.).

*Oxygen Deprivation.* Krebs-Ringer phosphate buffer with 5.5 mM glucose (3), 1% heat-inactivated fetal bovine serum (FBS) and antibiotics (Krebs-Ringer phosphate buffer with 5.5 mM glucose [KRPGS]) was alternately gassed with nitrogen via a submerged catheter and evacuated, for 10 cycles over 4 h. Traces of  $O_2$  were removed from the  $N_2$  by bubbling it through a solution of 8.2 g of pyrogallol (Sigma Chemical Co., St. Louis, Mo.) in 200 ml of 1 N NaOH. Mineral oil (Fisher Scientific Co., Pittsburgh, Pa.), which had been degassed under vacuum, was layered over the KRPGS. Aliquots of KRPGS were then removed from beneath

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<sup>1</sup> Abbreviations used in this paper: BCG, Bacille Calmette-Guérin; DABCO, diazabicyclooctane; E:T, effector cell to target cell ratio; FBS, fetal bovine serum, heat-inactivated (56°C, 30 min); KRPGS, Krebs-Ringer phosphate buffer with 5.5 mM glucose, 1% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

the oil using a gas-tight nitrogen-flushed syringe and catheter. Effector cells and  $^{51}\text{Cr}$ -labeled target cells, suspended separately in small volumes of KRPGS in conical glass centrifuge tubes, were overlaid with oil and diluted with KRPGS prepared as above. The tubes were centrifuged (180 g, 7 min, 4°C), and the supernate removed from beneath the oil with a nitrogen-flushed syringe and catheter. The cells were washed in this manner three times. Meanwhile, specially designed reaction tubes were evacuated and gassed with  $\text{N}_2$ . These round-bottomed glass test tubes were fitted with sidearms (2-ml capacity). The tubes had an internal diameter of 13.5 mm. Using 1-ml vol in each compartment, the maximum fluid depth was 8 mm, and the surface-to-volume ratio was  $0.14 \text{ mm}^{-1}$ . Opposite the sidearm, each tube was equipped with a stopcock, through which it was gassed and evacuated. The top was sealed with a silicone rubber stopper. The tubes were designed to fit holder 339279 for the TJ6R centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Phorbol myristate acetate (PMA) was added to the target cells under oil. The suspension was immediately taken up into a syringe and dispensed to the sidearms of the reaction tubes under a brisk efflux of  $\text{N}_2$ . PMA was used at a final concentration of 100 ng/ml (10 times the required dose [1]), and the time of its possible contact with oil kept under 60 s, to reduce the possible loss of PMA by partitioning into the oil. A preliminary experiment demonstrated that PMA prepared in this manner triggered a maximal level of cytotoxicity by aerobically cultured granulocytes, both when it was neat and when diluted 1:10, indicating that all the PMA added was recovered in active form (see Fig. 5 in ref. 1). Effector cells were added to the main compartment of the tubes. The stoppers were replaced, and the tubes alternately evacuated for 1-min periods and flushed with  $\text{N}_2$ , for 10 cycles. The tubes were cooled in an ice bath, inverted to mix the tumor cell-PMA suspension with the effector cells, centrifuged (180 g, 5 min, 4°C), and incubated in a 37°C water bath for 4.5 h. The tubes were then cooled on ice and centrifuged (700 g, 10 min, 4°C). 1 ml of supernate was transferred to a sample tube. The remaining fluid was transferred to a residual tube, along with two water rinses of the reaction tube. Sample and residual tubes were counted, and specific release of  $^{51}\text{Cr}$  computed as before (1).

*Preparation of Particle-Bound Glucose Oxidase.* In preliminary experiments, starch granules (2.6  $\mu$  diameter) from seeds of *Amaranthus caudatus* (4) caused no  $^{51}\text{Cr}$  release from P388 lymphoma cells when they were centrifuged together. 10 mg of starch granules were suspended in 1 ml of 0.1 M sodium phosphate buffer, pH 8.2. 0.2 ml of ethanol was added containing 0.12 mg *p*-benzoquinone (J. T. Baker Co., Phillipsburg, N.J.). After 1.5 h in the dark, the particles were washed three times by centrifugation, and resuspended in 2 ml of 0.1 M sodium bicarbonate containing 10 mM  $\epsilon$ -amino-*n*-caproic acid (Sigma). After 1.5 h, the particles were washed three times, resuspended in 2 ml of tris-(hydroxymethyl)aminomethane buffer, pH 7.4, mixed with 86 mg of 1-ethyl-3-(3-diaminoethylamino)propylcarbodiimide hydrochloride (Pierce Chemical Co., Rockford, Ill.) for 2 h, washed three times, and resuspended in 2 ml of bicarbonate buffer with 139 Sigma U of glucose oxidase (type V) for 16 h at room temperature. Glycylglycine (Sigma), 2 ml of a 0.2-M solution in phosphate buffer, was added for 1 h. The particles were washed until the supernate contained no detectable glucose oxidase activity as assayed by the generation of  $\text{H}_2\text{O}_2$  in KRPG (1). The particles themselves were then assayed for  $\text{H}_2\text{O}_2$ -generating activity in the same manner, and their concentration adjusted to give rates within the range produced by  $4 \times 10^5$ – $1 \times 10^6$  BCG-activated macrophages triggered by PMA (3).

*Other Assays.* Peroxidase cytochemistry was performed according to Kaplow (5). Glucose concentration was measured by the glucose-6-phosphate dehydrogenase-catalyzed reduction of NADP (6).

*Other Reagents.* Superoxide dismutase (SOD) was from Truett Laboratories, Dallas, Tex., or from Sigma. Catalase (from bovine liver, twice recrystallized), ferricytochrome *C* (type VI), sodium benzoate, bilirubin, *D*-alpha-tocopherol (type IV), histidine hydrochloride, butylated hydroxytoluene, arginine hydrochloride, arginase, galactose, and thymidine were from Sigma. Potassium cyanide and sodium azide were from Fisher. Mannitol and sodium iodide were from Mathieson, Coleman, and Bell, Norwood, O. Diazabicyclooctane (DABCO) and diphenylfuran were from Eastman Kodak Co., Rochester, N.Y. Lactoperoxidase was from Calbiochem, San Diego, Calif.

## Results

*Oxygen Deprivation.* Table I records the results of four experiments in which P388

TABLE I  
Inhibition of Cytotoxicity by Anaerobiosis

| Cultures with BCG-induced cells*              | Percent specific release of $^{51}\text{Cr}\ddagger$ |             |              |             |
|---|--|-------------|--------------|-------------|
|   | Exp. 1   | Exp. 2      | Exp. 3       | Exp. 4      |
| <b>Aerobic</b>                                |  |             |              |             |
| I. In MEM-S§                                  | 79.8 ± 2.6   | —           | 85.3 ± 0.9   | 98.3 ± 0.8  |
| II. In KRPGS                                  | 78.7 ± 5.6   | 54.9 ± 2.7  | —            | —           |
| III. In KRPGS, anaerobic → aerobic¶           | —  | 55.8 ± 2.9  | 64.5 ± 1.1   | 75.6 ± 2.8  |
| IV. BCG cells, anaerobic → aerobic** in KRPGS | —  | 49.5        | —            | 73.0 ± 4.6  |
| <b>Anaerobic</b>                              |  |             |              |             |
| V. In KRPGS‡‡                                 | -15.4 ± 0.1  | -26.4 ± 6.6 | -17.0 ± 14.2 | -13.2 ± 3.7 |

\* Effector to target cell ratios in the four experiments were 11, 17, 29, and 22.

‡ Means ± SEM for triplicates with  $2 \times 10^4$  to  $4 \times 10^4$   $^{51}\text{Cr}$ -labeled P388 lymphoma cells and 100 ng/ml PMA. Spontaneous release averaged: Set I, 19.1%; Set II, 30.3%; Set III, 31.5%; Set V, 36.9%.

§ Eagle's minimum essential medium with Earle's salts, antibiotics, and 1% FBS.

|| KRPG, antibiotics, and 1% FBS.

¶ KRPGS was alternately evacuated and flushed with  $\text{N}_2$  for 10 cycles over 4 h, then overlaid with oil. A portion was removed from beneath the oil and exposed to air. P388 and BCG cells were suspended in this medium and assayed.

\*\* BCG cells were overlaid with oil and washed three times into previously deoxygenated KRPGS. A portion was removed and cultured aerobically in the KRPGS used for Set III.

‡‡ P388 and BCG cells were both washed under oil in deoxygenated KRPGS. They were dispensed into reaction tubes, into the sidearm or main compartment, respectively, and alternately evacuated and flushed with  $\text{N}_2$  for 10 cycles before being mixed, centrifuged, and cultured. See Materials and Methods.

lymphoma cells were cultured with BCG-activated macrophages and PMA, aerobically and anaerobically. Anaerobiosis abrogated the ability of BCG-activated macrophages to lyse P388 cells. Anaerobic preparation of the cells, followed by admission of air, resulted in full expression of cytotoxicity, indicating viability of the effector cells under these conditions. In separate experiments, scopoletin and horseradish peroxidase were added to the medium, and tumor cells and serum were omitted. After 1 h under hypoxic conditions, there was no change in the fluorescence of scopoletin, indicating that the release of  $\text{H}_2\text{O}_2$  from BCG-activated macrophages in the presence of PMA had been reduced to an undetectable level.

**Glucose Deprivation.**  $\text{H}_2\text{O}_2$  released from BCG-activated macrophages appears to arise by dismutation of superoxide (3). The latter is formed by the reduction of molecular oxygen by NADPH (7), which in turn arises from the hexose monophosphate shunt pathway of glucose oxidation (7). Deprivation of exogenous glucose might therefore retard the formation of  $\text{H}_2\text{O}_2$ . We found that 2 h of culture in glucose-free medium was sufficient to abolish detectable  $\text{H}_2\text{O}_2$  release from BCG-induced macrophages or from granulocytes (not shown). Using freshly harvested cells, the minimal glucose concentration required to support full  $\text{H}_2\text{O}_2$  release was 0.3 mM (Fig. 1). Glucose could not be replaced by 5.5 mM galactose (Fig. 1).

BCG-induced macrophages, as well as granulocytes, were unable to lyse P388 cells when glucose was withheld from the medium (MEM-1% dialyzed FBS) or when only galactose was added (Fig. 1). Cytotoxic activity was supported by glucose concentrations of 0.3 mM glucose or more for macrophages, and 0.1 mM or more for granulocytes (Fig. 1). The effect of glucose deprivation was reversible. When the cultures were made 5.5 mM in glucose after 4 h without hexose, full cytotoxic activity

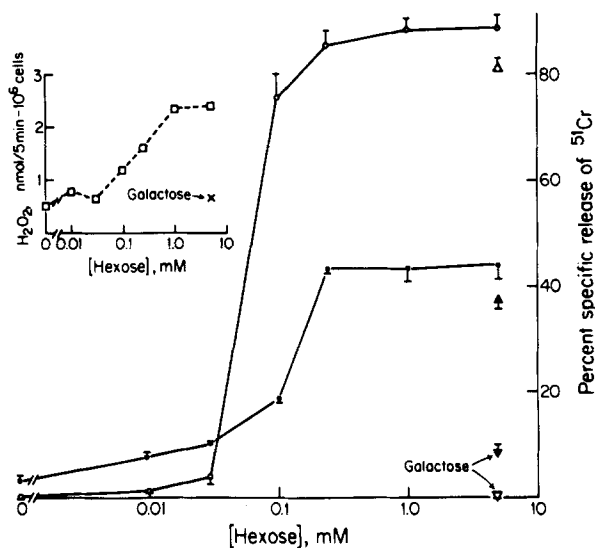


FIG. 1. Effect of hexose concentration on cytotoxicity and  $\text{H}_2\text{O}_2$  release. Solid lines indicate percent specific release of  $^{51}\text{Cr}$  from  $2 \times 10^4$   $^{51}\text{Cr}$ -labeled P388 cells incubated with 10 ng/ml PMA and BCG cells (●) at an E:T ratio of 25, or granulocytes (○) at an E:T ratio of 13, as a function of glucose concentration. (▼) indicates percent  $^{51}\text{Cr}$  release when P388 cells and BCG cells were cultured in 5.5 mM galactose. (▽) indicates results for P388 and granulocytes in 5.5 mM galactose. Effector cells were precultured for 2 h at the indicated hexose concentrations before adding P388 and PMA. (▲) indicates results when BCG cells were cultured for 4 h with no hexose, then made 5.5 mM in glucose and cultured another 4.5 h with P388 and PMA. (Δ) indicates analogous results for granulocytes cultured 4 h without hexose, then made 5.5 mM in glucose. Spontaneous release ranged from 6.7% to 9.1%. The medium was MEM-1% FBS (FBS dialyzed 18 h against a 60-fold excess of normal saline). Inset (dotted line),  $\text{H}_2\text{O}_2$  release from the same preparation of BCG cells for 5 min after adding PMA (expressed per  $10^6$  leukocytes) as a function of glucose concentration. (X) indicates results in 5.5 mM galactose. The  $\text{H}_2\text{O}_2$  assay was performed with fresh cells in Krebs-Ringer phosphate buffer, omitting the 2-h preculture.

was restored (Fig. 1). The galactose used in these experiments contained  $<0.14\%$  glucose by enzymatic assay.

**Effects of Catalase, Ferricytochrome C, and Superoxide Dismutase.** Three of five similar experiments are shown in Table II. Catalase, in doses which abolished detectable  $\text{H}_2\text{O}_2$  release from the effector cells, abrogated their cytotoxic effect (mean reduction in cytotoxicity of 99.8% in the five experiments). Ferricytochrome C, which oxidizes superoxide, diminishing its dismutation to  $\text{H}_2\text{O}_2$ , markedly reduced the cytotoxicity of BCG-induced macrophages (Table II, exp. 1). At low effector cell to target cell (E:T) ratios, ferricytochrome C also inhibited the cytotoxicity of granulocytes (exp. 2), but it had less of an effect at higher E:T ratios (exp. 3). Superoxide dismutase, which promotes the dismutation of superoxide to  $\text{H}_2\text{O}_2$ , overcame the inhibitory effect of ferricytochrome C. When cytotoxicity had been blocked by ferricytochrome C and restored by superoxide dismutase (SOD), it could again be abolished by catalase (Table II, exp. 2). By itself, SOD had no substantial effect on cytotoxicity (mean reduction of 9.3% in the five experiments). In addition, SOD did not lead to cytotoxicity when added to normal peritoneal cells or thioglycollate-induced macrophages, with or without PMA (not shown). In the above studies, SOD was used in an amount sufficient to restore the full rate of  $\text{H}_2\text{O}_2$  release from  $1.4 \times 10^6$  PMA-triggered granulocytes in the presence of  $65 \mu\text{M}$  ferricytochrome C, the latter being

TABLE II  
Effect of Catalase, Superoxide Dismutase, and Ferricytochrome C on Cytotoxicity

| Additions                            | Percent specific release of $^{51}\text{Cr}^*$ |                                  |                                  |
|--------------------------------------|--|----------------------------------|----------------------------------|
|                                      | Exp. 1<br>BCG cells‡<br>E:T 22                 | Exp. 2<br>Granulocytes§<br>E:T 7 | Exp. 3<br>Granulocytes<br>E:T 13 |
| DMSO vehicle                         | -0.5 ± 2.6                                     | -1.1 ± 0.2                       | 1.0 ± 0.2                        |
| PMA¶                                 | 88.6 ± 3.9                                     | 50.5 ± 1.4                       | 90.5 ± 2.7                       |
| Catalase** + DMSO                    | 4.0 ± 5.4                                      | 0.1 ± 0.8                        | -6.4 ± 2.7                       |
| Catalase + PMA                       | 2.3 ± 0.9                                      | -0.7 ± 1.1                       | -5.7 ± 2.0                       |
| SOD‡‡ + DMSO                         | -15. ± 1.7                                     | 1.9 ± 0.5                        | -0.3 ± 1.4                       |
| SOD + PMA                            | 93.9 ± 3.7                                     | 31.4 ± 0.8                       | 95.5 ± 3.4                       |
| Catalase + SOD + DMSO                | -3.7 ± 1.5                                     | —                                | -2.4 ± 1.0                       |
| Catalase + SOD + PMA                 | -2.8 ± 2.9                                     | —                                | -4.3 ± 2.0                       |
| Cytochrome C§§ + DMSO                | 8.4 ± 2.3                                      | -2.9 ± 0.6                       | 6.3 ± 2.2                        |
| Cytochrome C + PMA                   | 20.2 ± 3.2                                     | 7.7 ± 3.8                        | 76.7 ± 6.6                       |
| Cytochrome C§§ + SOD + DMSO          | 2.5 ± 0.7                                      | -0.4 ± 0.9                       | 2.6 ± 2.9                        |
| Cytochrome C + SOD + PMA             | 47.9 ± 1.8                                     | 47.7 ± 1.8                       | 91.1 ± 1.7                       |
| Cytochrome C + SOD + catalase + DMSO | —  | -1.3 ± 0.6                       | —                                |
| Cytochrome C + SOD + catalase + PMA  | —  | -2.2 ± 0.3                       | —                                |

\* Mean ± SEM for triplicates with  $2 \times 10^4$   $^{51}\text{Cr}$ -labeled P388 cells. In the absence of effector cells, the percent spontaneous release in each group in the order listed above averaged: 12.8, 14.2, 21.1, 19.1, 15.2, 16.6, 26.9, 22.2, 11.9, 13.3, 12.7, 14.4, 9.7, 9.9.

‡ Peritoneal cells collected 2 wk after injection of BCG.

§ Peritoneal cells collected 10–20 h after injection of thioglycollate broth.

|| DMSO, 0.0033% (vol/vol).

¶ PMA, 10 ng/ml.

\*\* Catalase, 2,000 U/ml.

‡‡ Superoxide dismutase, 300 U/ml.

§§ Ferricytochrome C, 130  $\mu\text{M}$  in Exps. 1 and 2, 65  $\mu\text{M}$  in Exp. 3.

sufficient to abolish detectable  $\text{H}_2\text{O}_2$  release from the cells over 5 min after the addition of PMA. Thus, the SOD was enzymatically active.

These data indicated that  $\text{H}_2\text{O}_2$  was necessary for the cytotoxic effect of BCG-activated macrophages and granulocytes when triggered by PMA. Superoxide appeared to be important as a precursor of  $\text{H}_2\text{O}_2$ , but by itself, did not appear to be cytotoxic.

*Effect of Scavengers of Singlet Oxygen and Hydroxyl Radical.* As shown in Table III, the singlet oxygen quenchers DABCO (8–10), diphenylfuran (10, 11), bilirubin (9, 12), histidine (9), and tocopherol (9), and the hydroxyl radical scavengers mannitol (13–16), ethanol (11, 13, 16–18), benzoate (13, 16–20), and histidine (11, 17, 21) had no effect on the PMA-triggered cytolysis of P388 by granulocytes at all nontoxic concentrations tested. The antioxidants tocopherol (22, 23) and butylated hydroxytoluene (22) were without effect (Table III), but their limited solubility makes it difficult to interpret the negative results.

*Effect of Particle-Bound Glucose Oxidase.* The above studies indicated that  $\text{H}_2\text{O}_2$  was necessary for the cytotoxic effect of BCG-activated macrophages in this system, but they did not indicate whether it was sufficient. To investigate this question, we constructed particles which resembled macrophages in their spatial relation to the target cells and in their generation of a flux of  $\text{H}_2\text{O}_2$ , but which were otherwise inert. The effect of starch particles with covalently coupled glucose oxidase is shown in

TABLE III  
Effect of Scavengers of Singlet Oxygen and Hydroxyl Radical on Cytotoxicity

| Experiment               | Agent added        | Highest non-toxic concentration tested* | Percent specific release of $^{51}\text{Cr}\ddagger$ |
|--------------------------|--------------------|---|--|
| A                        | None               |   | 97.6 $\pm$ 2.2                                       |
|                          | Diazabicyclooctane | 10 mM                                   | 79.9 $\pm$ 8.7                                       |
|                          | Bilirubin          | 10 $\mu\text{M}$                        | 95.9 $\pm$ 4.7                                       |
|                          | Histidine          | 10 mM                                   | 86.3 $\pm$ 1.6                                       |
|                          | Mannitol           | 50 mM                                   | 97.4 $\pm$ 1.0                                       |
|                          | Benzoate           | 20 mM                                   | 81.3 $\pm$ 2.7                                       |
|                          | Ethanol            | 100 mM                                  | 95.2 $\pm$ 7.7                                       |
|                          | B                  | None                                    |  |
| Diphenylfuran            |                    | 3.3 $\mu\text{M}$                       | 106.5 $\pm$ 1.2                                      |
| Tocopherol               |                    | 450 $\mu\text{M}$                       | 96.0 $\pm$ 3.3                                       |
| Butylated hydroxytoluene |                    | 4.3 $\mu\text{M}$                       | 107.0 $\pm$ 3.8                                      |

\* Each agent was tested at four concentrations over  $\log_{10}$  increments. An agent was considered nontoxic at a given concentration if it caused no significant increase in spontaneous release above that when no agent was added.

$\ddagger$  Means  $\pm$  SEM for triplicates for  $4 \times 10^4$   $^{51}\text{Cr}$ -labeled P388 lymphoma cells with granulocytes at an effector:target ratio of 7, and with PMA at 10 ng/ml. Spontaneous release with PMA alone was 8.9% in Exp. A and 11.8% in Exp. B.

Table IV. Such particles, sedimenting with the tumor cells and producing  $\text{H}_2\text{O}_2$  at a rate similar to that of BCG-activated, PMA-triggered macrophages, imitated the cytotoxic effect of the latter. Starch particles from which the glucose oxidase had been omitted during preparation, or from which the coupling agent had been omitted, were inactive, indicating that the cytotoxic activity was dependent on covalently bound glucose oxidase. Catalase abolished the cytotoxicity of particle-bound glucose oxidase, indicating that cytolysis was mediated by  $\text{H}_2\text{O}_2$ , and not by the utilization of glucose or accumulation of hydrogen ion which accompany catalysis by this enzyme.

*Role of Peroxidase.* The peroxidase content of the cells used in these studies was examined cytochemically with the light microscope. The percentage of peroxidase-positive cells was the same as the percentage of granulocytes, using peritoneal cells from BCG-treated mice (14 experiments), untreated mice (9 experiments), or mice injected with thioglycollate broth 10–20 h before (3 experiments). Thus, BCG-induced macrophages were almost uniformly negative for peroxidase by this technique, but were accompanied by a small percentage of peroxidase-positive granulocytes. P388 cells were peroxidase-negative (two experiments).

To examine the role of heme-containing peroxidases, we studied the effects of the inhibitors, cyanide and azide. As shown in Table V, these compounds did not decrease cytotoxicity by BCG-activated macrophages or by granulocytes. In fact, when cytotoxicity was low, azide and cyanide increased it. However, azide did not lead to the expression of cytotoxicity by normal macrophages (Table V).

We attempted to augment cytotoxicity by adding lactoperoxidase and iodide. These experiments were conducted in 0.1% serum, the minimum concentration supporting viability of the target cells. As shown in Table VI, lactoperoxidase and iodide strongly inhibited cytotoxicity.

We speculated that P388 lymphoma cells may have become coated with granulocyte myeloperoxidase in ascites, and that such a surface coat of myeloperoxidase could be

TABLE IV  
Cytotoxic Effect of Glucose Oxidase Covalently Coupled to Starch Particles

| Experiment | Addition   | Percent specific release* |            |
|------------|--|---------------------------|------------|
|            |  | No catalase               | Catalase‡  |
| A          | Particles, treated with benzoquinone and glucose oxidase, releasing 4.0 nmol H <sub>2</sub> O <sub>2</sub> /5 min§ | 104.3 ± 2.8               | 15.5 ± 2.4 |
|            | Untreated particles  | -1.1 ± 1.6                | 12.6 ± 2.6 |
| B          | Particles, treated with benzoquinone and glucose oxidase, releasing 2.0 nmol H <sub>2</sub> O <sub>2</sub> /5 min  | 83.8 ± 3.0                | -5.6 ± 1.6 |
|            | Particles, treated with glucose oxidase alone ¶  | 1.3 ± 0.0                 | 0.5 ± 1.0  |
|            | Particles, treated with benzoquinone alone**   | 3.6 ± 0.8                 | 4.9 ± 1.2  |

\* From  $2 \times 10^4$  <sup>51</sup>Cr-labeled P388 lymphoma cells. Means ± SEM for triplicates. Spontaneous release, 14.3% in Exp. A, 11.0% in Exp. B.

‡ Catalase, 2,000 U/ml.

§ Starch particles activated with benzoquinone, coupled to glucose oxidase, and washed, as in Materials and Methods.  $3 \times 10^7$  particles/tube.

|| As for note §, but  $1 \times 10^6$  particles/tube.

¶ Particles not activated with benzoquinone before exposure to glucose oxidase and subsequent washing.

\*\* Particles treated as in note §, but glucose oxidase omitted.

TABLE V  
Effect of Azide and Cyanide on Cytotoxicity

| Experiment | Effector cell | E:T ratio | Percent specific release from P388* |                         |            |
|------------|---------------|-----------|-------------------------------------|-------------------------|------------|
|            |               |           | No additions                        | NaN <sub>3</sub> , 1 mM | KCN, 1 mM  |
| A          | BCG cells‡    | 9         | 75.7 ± 2.9                          | 77.5 ± 2.5              |            |
|            |               | 2         | 43.5 ± 3.9                          | 70.3 ± 0.9              |            |
|            | Granulocytes§ | 18        | 85.9 ± 2.7                          | 89.6 ± 2.7              |            |
|            |               | 2         | 55.1 ± 1.6                          | 73.7 ± 1.9              |            |
|            | Normal cells  | 11        | -3.1 ± 0.8                          | 0.6 ± 0.9               |            |
| B          | BCG cells     | 30        | 64.3 ± 2.4                          | 90.8 ± 3.3              | 94.4 ± 4.2 |
| C          | BCG cells     | 26        | 86.3 ± 1.3                          | 89.0 ± 2.1              | 91.9 ± 2.9 |
|            | Granulocytes  | 13        | 98.0 ± 2.0                          | 96.8 ± 1.9              | 96.5 ± 5.7 |

\* Means ± SEM for triplicates with  $2 \times 10^4$  <sup>51</sup>Cr-labeled P388 cells and 10 ng/ml PMA. With PMA, but without effector cells, spontaneous release ranged from 10.4 to 23.1%. With effector cells, but without PMA, specific release averaged 1.4%, except for granulocytes in Exp. C. In that case, specific release was  $-0.8 \pm 0.1\%$  without additions,  $38.1 \pm 3.1\%$  with azide, and  $44.3 \pm 7.6\%$  with cyanide.

‡ Peritoneal cells from BCG-treated mice.

§ Peritoneal cells from mice 10-20 h after injection of thioglycollate broth.

|| Peritoneal cells from untreated mice.

essential for the cytotoxic effect of BCG-activated macrophages. To test this, we grew P388 in vitro for five passages over 16 days, so that the original cell surface area was diluted more than 200,000-fold by division and growth. Cultured target cells were fully as susceptible to lysis as were freshly harvested ascites cells (Table VI).

From these experiments, we concluded that while myeloperoxidase was present, at least within granulocytes, there was no evidence that it promoted cytotoxicity in this assay.

*Effect of Erythrocytes.* Based on the results obtained so far, erythrocytes might be expected to inhibit the cytolysis of lymphoma cells by one or more of four mechanisms. First, they would be expected to serve as alternate (cold) targets (1). Second, they could release catalase when lysed. Third, their heme iron might promote the decom-

TABLE VI  
Effect of Lactoperoxidase and Iodide on Cytotoxicity of BCG-Induced Peritoneal Cells

| Target cells                             | Additions | Percent specific release of $^{51}\text{Cr}^*$ |                |
|--|-----------|--|----------------|
|  |           | DMSO vehicle                                   | PMA            |
| P388 ascites, $2 \times 10^4$ (E:T 30)   | None      | $-0.1 \pm 2.2$                                 | $64.4 \pm 2.4$ |
|  | LPO, NaI‡ | $0.1 \pm 0.8$                                  | $11.9 \pm 1.8$ |
| P388 culture, § $2 \times 10^4$ (E:T 30) | None      | $4.1 \pm 1.2$                                  | $96.4 \pm 0.4$ |
|  | LPO, NaI  | $-3.5 \pm 0.8$                                 | $7.7 \pm 2.1$  |

\* Means  $\pm$  SEM for triplicates. Experiments conducted in MEM made 0.1% in FBS. PMA and DMSO as in Table II.

‡ Lactoperoxidase, 100 mU/ml plus NaI, 0.1 mM.

§ P388 ascites cells were cultured for 16d (5 passages), with an estimated dilution of the original cell surface area by more than  $2 \times 10^5$ .

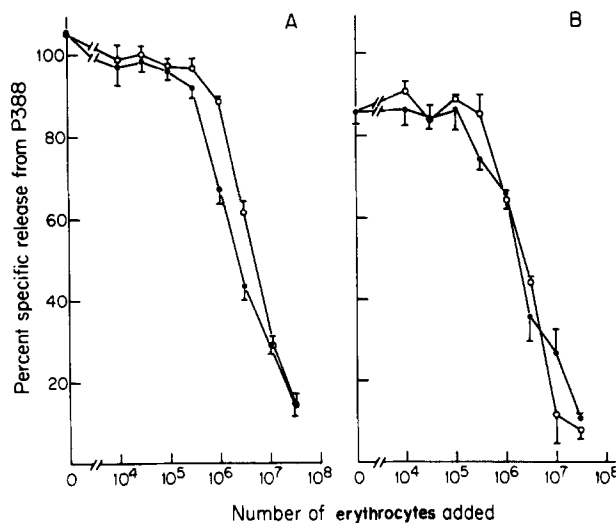


FIG. 2. Inhibition of cytotoxicity by erythrocytes.  $4 \times 10^4$   $^{51}\text{Cr}$ -labeled P388 lymphoma cells were incubated with 10 ng/ml PMA and granulocytes at an E:T ratio of 7 (A) or BCG-induced macrophages at an E:T ratio of 12 (B). Varying numbers of unlabeled erythrocytes were added, either from C3H/HeN mice (closed circles) or C3H/HeAn1/Cs<sup>b</sup> mice (open circles). The latter are acatalasemic (2). Means and SEM for triplicates.

position of  $\text{H}_2\text{O}_2$  (24, 25). Fourth, they might release other substances, such as glutathione and glutathione peroxidase, which could consume  $\text{H}_2\text{O}_2$ . Indeed, mouse erythrocytes inhibited the cytolysis of lymphoma cells by granulocytes (Fig. 2A) and by BCG-activated macrophages (Fig. 2B). Erythrocytes from normal and acatalasemic mice were equally effective (Fig. 2). Thus, catalase was unlikely to be responsible for the inhibitory effect.

*Lack of Effect of Arginine, Arginase, and Thymidine.* One chemically defined mechanism of cytotoxicity by activated macrophages in vitro is secretion of arginase, with depletion of arginine from the medium (26, 27). In addition, some lymphoma cells are susceptible to growth inhibition in vitro by the amounts of thymidine secreted by macrophages (28). However, as shown in Table VII, addition of arginase or thymidine to cultures of P388 cells resulted in no cytolysis under the standard assay conditions. The cytolysis effected by PMA-triggered, BCG-activated macrophages was not diminished by addition to the medium of large amounts of arginine (Table VII).



TABLE VII  
*Lack of Effect of Arginine, Arginase, and Thymidine on Cytotoxicity*

| Cells                   | Additions* | Dosage            | Percent specific release of $^{51}\text{Cr}\ddagger$ |
|-------------------------|------------|-------------------|--|
| P388                    | arginase   | 10 mU/ml          | 1.6 $\pm$ 1.1  |
|                         |            | 100 mU/ml         | 1.7 $\pm$ 0.7  |
|                         |            | 1 U/ml            | 1.5 $\pm$ 0.1  |
| P388                    | thymidine  | 10 $\mu\text{M}$  | 1.6 $\pm$ 1.6  |
|                         |            | 100 $\mu\text{M}$ | 0.5 $\pm$ 1.0  |
|                         |            | 1 mM              | 0.6 $\pm$ 0.8  |
| P388 cells + BCG cells§ | arginine   | 0                 | 32.3 $\pm$ 1.9                                       |
|                         |            | 0.3 mg/ml         | 48.3 $\pm$ 1.4                                       |
|                         |            | 1.0 mg/ml         | 38.1 $\pm$ 3.1                                       |
|                         |            | 3.0 mg/ml         | 38.4 $\pm$ 0.7                                       |

\* All cultures contained PMA, 10 ng/ml. Medium contained 0.1 mg/ml arginine by formulation.

‡ Means  $\pm$  SEM for triplicates with  $2 \times 10^4$   $^{51}\text{Cr}$ -labeled P388 lymphoma cells. Spontaneous release from P388 with PMA was 8.1%  $\pm$  0.4.

§ E:T ratio, 12.

### Discussion

These studies demonstrated that the release of hydrogen peroxide by activated macrophages and by granulocytes after pharmacologic triggering was both necessary and sufficient for the lysis of lymphoma cells under the conditions used. Our findings do not define the manner in which  $\text{H}_2\text{O}_2$  led to target cell death, they do not rule out the participation of factors supplied by the medium or by the target cells themselves, and they do not necessarily bear on the biochemical basis of cytotoxicity in other assay systems. Nonetheless, the results described above and in the previous report (1) help to define the considerable potential of leukocytes for peroxide-mediated extracellular cytotoxicity, and in the case of mononuclear leukocytes, establish a biochemical basis for cell-mediated cytotoxicity.

Oxygen and glucose may be viewed as the precursors of hydrogen peroxide. Depletion of oxygen abolished both  $\text{H}_2\text{O}_2$  release and cytolysis of lymphoma cells by BCG-activated macrophages in response to PMA. Reduction of the ambient glucose concentration to 0.03 mM or less markedly impaired both  $\text{H}_2\text{O}_2$  release and cytotoxicity by BCG-activated macrophages and by granulocytes. Galactose could not substitute for glucose. These findings suggest that when exogenous glucose is severely restricted, leukocytes are unable to augment their production of  $\text{H}_2\text{O}_2$  after pharmacologic triggering, despite the possibilities of deriving glucose from glycogenolysis, gluconeogenesis, or epimerization of galactose. Alternatively, a low glucose concentration might selectively inhibit the extracellular release of  $\text{H}_2\text{O}_2$  in response to a stimulus such as PMA. In any case, deprivation of oxygen and glucose may be useful techniques for investigating the role of  $\text{H}_2\text{O}_2$  in situations involving leukocytes in which there might be limitations to the access of extracellular reagents such as catalase. In the present system, catalase abolished cytotoxicity.

The cytotoxic effect of macrophages could be mimicked by starch particles of roughly the size of the cell, which generated fluxes of  $\text{H}_2\text{O}_2$  at their surface similar to those of PMA-triggered leukocytes. Such particles would not be expected to release singlet oxygen, superoxide anion, hydroxyl radical, peroxidase, lysozyme, plasminogen activator, other neutral proteases, lysosomal acid hydrolases, prostaglandins, thymi-

dine, arginase, complement components, or any of the other potentially cytotoxic substances reported to be secreted by macrophages. The cytotoxic effect of these glucose oxidase-coated particles could be abolished by catalase.

Superoxide dismutase did not inhibit cytotoxicity. Thioglycollate-elicited macrophages and J774 cells, which were reported to release superoxide in response to PMA (29), were not cytotoxic (1). These results suggest that superoxide anion was not cytotoxic in the present system. However, the inhibition of cytotoxicity with ferricytochrome *C* and reversal of the inhibition with superoxide dismutase implied that superoxide was important as a precursor of  $H_2O_2$ . Cytotoxicity was unaffected by agents used to quench singlet oxygen, namely DABCO (8–10), diphenylfuran (10, 11), bilirubin (9, 12), histidine (9), tocopherol (9), and azide (9, 30), or by agents used to scavenge hydroxyl radical, namely histidine (11, 17, 21), ethanol (11, 13, 16–18), mannitol (13–16), benzoate (13, 16–20), tocopherol (22, 23), and butylated hydroxytoluene (22).

Cytotoxicity did not appear to depend on a heme-containing peroxidase. Myeloperoxidase was carried into the assay system within granulocytes, although it was not detected in BCG-activated macrophages by light microscopic cytochemistry. Azide and cyanide, inhibitors of myeloperoxidase, did not reduce cytotoxicity. In fact, when cytotoxicity was low, azide and cyanide augmented it. The augmentation might have been due to enhanced production of reactive metabolites of oxygen (reviewed in 31); inhibition of catalase in the effector cells, target cells, or serum-containing culture medium; interference with tumor cell repair mechanisms; or perhaps even to inhibition of a peroxidase, because exogenous lactoperoxidase and iodide markedly inhibited cytotoxicity. The latter finding appears to contradict earlier studies (32). Previous experiments on the role of peroxidases in extracellular killing, however, were performed in serum-free medium (32–34). We observed an anticytotoxic effect of lactoperoxidase when the serum concentration ranged from 0.1 to 20%. Peroxidase may catalyze the oxidation of substances in serum by  $H_2O_2$  (35, 36), thereby expending  $H_2O_2$  before it can interact with target cells. In one report, for example, when zymosan, iodide, myeloperoxidase, and glucose oxidase were incubated in medium containing 10% serum, 80% of the resulting iodination was of serum proteins and only 20% was of zymosan (35). Further study is necessary to define the role of peroxidase in extracellular cytotoxic mechanisms at the concentrations of plasma found at inflammatory sites. The possible involvement of a peroxidase not inhibited by cyanide or azide also deserves consideration.

Because of differences in assay conditions, it is difficult to compare this work with previous studies of the biochemical basis of macrophage-mediated cytotoxicity (37–42). Weinberg and Hibbs (41) observed no inhibition of macrophage anti-tumor activity by catalase in a 60-h assay. The anti-tumor activity of proteose-peptone-elicited rat macrophages was also undiminished by catalase in a 36-h assay (R. Keller, personal communication). Perhaps macrophage secretion of  $H_2O_2$  is not involved in the above assays. On the other hand, it is conceivable that  $H_2O_2$  could be released by macrophages from segments of membrane triggered by close contact with target cells. Exogenous catalase might not gain access to such sites. Moreover, the stimuli, if any, presented by malignant cells to macrophages might trigger a lesser degree of  $H_2O_2$  release than seen with PMA. At low fluxes of  $H_2O_2$ , the predominant action of catalase is peroxidatic, so that catalase might augment cytotoxicity, as well as inhibit

it (43, 44). In another study, the lack of effect of catalase might be related to the dose (42).

Sorrell et al. (42) reported that hypoxia reduced only slightly the degree to which *C. parvum*-elicited peritoneal cells inhibited thymidine uptake by target cells. One interpretation is that the mechanism of inhibition of thymidine uptake in that study was independent of  $H_2O_2$  secretion by macrophages. Indeed, the cytotoxic index was substantially reduced by replacing the culture medium during the assay, an effect compatible with the participation of macrophage-derived thymidine (28) or arginase (26, 27). The time required for target cells to recover from the effects of excess thymidine or insufficient arginine is unknown, so it is not clear to what degree such effects can be discounted by changing the medium during the terminal hour of culture. It is also possible that the inhibition of cytotoxicity observed in that study might have been greater if the medium had been further deoxygenated.

Spontaneous cytolysis of erythrocytes by cultured macrophages was abolished by anaerobiosis in the experiments of Melsom (37). In that study, cyanide also inhibited cytotoxicity, in contrast to the present report.

Weinberg and Hibbs observed marked inhibition of macrophage anti-tumor activity by erythrophagocytosis (41). This was attributed to possible alterations in lysosomal function. We also noted a profound anticytotoxic effect when unlabeled erythrocytes were added to cultures of macrophages or granulocytes and  $^{51}Cr$ -labeled lymphoma cells. This effect did not appear to be due to erythrocytic catalase. Erythrocytes may simply have consumed  $H_2O_2$  as cold target cells, or catalyzed its decomposition enzymatically or by means of iron chelates (24, 25).

It is interesting to compare the present findings with those of MacLennan and Golstein (45), who demonstrated that the lethal-hit stage of T cell-mediated cytolysis was dependent on glucose (45). Glucose subserved some function other than provision of energy (45). We know of no evidence that lymphocytes can release  $H_2O_2$ . Nonetheless, it is worth considering whether  $H_2O_2$  may be involved in lymphocyte-mediated cytolysis. To investigate such a possibility, our experience with macrophages suggests that it would be necessary to measure  $H_2O_2$  release from a purified population of cytotoxic T cells during exposure to the stimuli which induce cytolysis, that is, after activation and triggering. MacDonald and Koch (Table II in reference 46) found a mean reduction of only 32% in T cell-mediated cytolysis under hypoxic conditions. However, this leaves open the possibility that further deoxygenation of the medium might have had a more pronounced effect.

Immunologic triggering of granulocytes and mononuclear phagocytes leads to many of the same changes in oxidative metabolism as does exposure to PMA (47-51). Thus it would be of considerable interest to learn whether activated macrophages, when immunologically triggered, may become cytotoxic by virtue of releasing hydrogen peroxide. If true, such a finding could provide a link between the cytotoxic mechanism investigated here and the response of the host to neoplasia, infection, and other inflammatory states.

### Summary

When deprived of oxygen, Bacille Calmette-Guérin (BCG)-activated macrophages no longer lysed P388 lymphoma cells. Both  $H_2O_2$  release and cytotoxicity by BCG-activated macrophages and by granulocytes triggered with phorbol myristate acetate

(PMA) were markedly inhibited when the glucose concentration in the medium was reduced to 0.03 mM or less, or if glucose were replaced with galactose. Catalase abolished PMA-triggered cytotoxicity by both types of effector cells, whereas superoxide dismutase had no effect. Ferricytochrome *C* reduced the cytotoxicity of BCG-activated macrophages, an effect which was largely reversed by superoxide dismutase. 10 drugs, thought to quench singlet oxygen and/or scavenge hydroxyl radical, did not affect cytotoxicity in this system. Neither azide nor cyanide reduced cytolysis, but there was marked inhibition by lactoperoxidase and iodide. This suggested that cytotoxicity was not dependent upon myeloperoxidase, and that lactoperoxidase may have diverted  $H_2O_2$  from the oxidation of target cells to oxidation of substances in serum. Mouse erythrocytes, although sensitive targets, interfered with the cytolysis of lymphoma cells, probably by competition for  $H_2O_2$ . Starch particles with covalently bound glucose oxidase resembled macrophages in their spatial relation to the target cells and in the flux of  $H_2O_2$  they generated from their surface, but were not expected to produce any other potentially toxic products. Such particles lysed lymphoma cells, and the lysis was prevented by catalase. Neither arginase nor thymidine appeared to be involved in cytolysis by BCG-activated macrophages under the conditions used. These findings demonstrated that release of  $H_2O_2$  was both necessary and sufficient for cytolysis by BCG-activated macrophages and by granulocytes when pharmacologically triggered.

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