HYDROGEN PEROXIDE RELEASE FROM MOUSE PERITONEAL MACROPHAGES
Dependence on Sequential Activation and Triggering*

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Hydrogen peroxide is one of the most important antimicrobial weapons of polymorphonuclear leukocytes (PMN)1 (1), and is also involved in some of their antitumor effects (2). Our understanding of hydrogen peroxide metabolism in mononuclear phagocytes and its possible contribution to their antimicrobial and antitumor mechanisms is much less advanced.

There are striking similarities between PMN and many preparations of mononuclear phagocytes in their metabolic responses to stimuli such as phagocytosis (3-5) or membrane-active agents (6, 7), including the respiratory burst, enhanced hexose monophosphate shunt activity, and superoxide production (3-7). In PMN, these responses are closely linked to hydrogen peroxide production (8-13).

It would be logical to expect, therefore, that mononuclear phagocytes also could be stimulated to bursts of H2O2 production, although their H2O2 metabolism might differ in some respects from that in PMN. In particular, the remarkable plasticity of structure and function of mononuclear phagocytes, whereby many of their attributes are susceptible to sustained "activation," might lead one to anticipate inducible changes in their metabolism of H2O2.

In this study, use of a sensitive and specific assay for H2O2 permitted the demonstration that mouse peritoneal macrophages could be triggered to release H2O2 into the extracellular medium almost as copiously as PMN. The ability to trigger such release depended on the state of activation of the macrophages. Potential activating agents differed markedly in their ability to prime macrophages for H2O2 release.

Materials and Methods

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Mice. Mice of either sex, aged 2-8 mo, were obtained as follows: (BALB/c x DBA/2)F1 (CDF), from Flow Laboratories, Inc., Rockville, Md.; C3H/HeN, from the colony of Dr. Malcolm

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1 Abbreviations used in this paper: BCG, Bacille Calmette-Guérin; DMSO, dimethyl sulfoxide;
FCS, fetal calf serum; HBSS, Hank's balanced salt solution; HPO, horseradish peroxidase;
KRPG, Krebs-Ringer phosphate buffer with glucose; MEMS, Eagle's minimal essential medium
with 20% FCS, NPAC, nonphagocytic adherent cells; PMA, phorbol myristate acetate; PMN,
polymorphonuclear leukocytes; SEM, standard error of the mean.

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Mitchell, Yale University; and CDI, from Charles River Breeding Laboratories, Wilmington, Mass. No differences in results were noted using different strains.

**Activating Agents.** Bacille Calmette-Guérin (BCG), Phipps strain, TMC 1029, lot 9, was from the Trudeau Institute, Saranac Lake, N. Y., and was stored at -70°C. 0.1 cc containing approximately 10⁶ viable organisms, was injected intraperitoneally (i.p.). Formalin-killed Corynebacterium parvum (Coparvax), lot CA-449, was the generous gift of Dr. Richard Tuttle, Burroughs Wellcome Co., Research Triangle Park, N.C. 0.1 cc containing 0.7 mg in saline was injected i.p. Higher doses were associated with excessive numbers of PMN. Thioglycollate (Difco Laboratories, Detroit, Mich.) was given i.p. as 1 cc of a 6% solution in saline. Sodium caseinate, practical grade, from Eastman Kodak Co., Rochester, N. Y., was given i.p. as 1 cc of a 6% solution in saline. Heat-inactivated (56°C, 30 min) fetal calf serum (FCS; Flow Laboratories) was given i.p. in 1 cc.

**Cell Preparation.** Peritoneal cells were lavaged from normal or previously injected mice as described (14), except that phenol red was omitted from the Hanks' balanced salt solution (HBSS). After centrifugation at 200 g, the cell pellet was resuspended in 2 cc of 0.2% saline for 30 s to lyse erythrocytes. This was important to eliminate exogenous catalase (12). Isotonicity was restored with 2 cc of 1.6% saline, additional HBSS was added, centrifugation repeated, and the cells resuspended in cold modified Krebs-Ringer phosphate buffer with glucose (KRPG; 120 mM NaCl, 4.8 mM KCl, 0.54 mM CaCl₂, 1.2 mM MgSO₄, 15.6 mM sodium phosphate buffer, pH 7.4, and 11 mM dextrose). Cell number was determined by counting in a hemocytometer. Aliquots were taken for the H₂O₂ release assay to provide a wide range of cell concentrations. Another aliquot was air-dried on a glass slide and stained (Diff-Quik, Harleco, Philadelphia, Pa.) for differential counts of 300 cells. The percentage of PMN detected was the same if smears were prepared in a cytocentrifuge. Neutrophils and eosinophils were recorded together as "PMN." Another aliquot, containing about 2 x 10⁶ cells, was added to 2 cc of Eagle's minimal essential medium (GIBCO, Grand Island, N.Y.) containing 100 U penicillin, 100 μg streptomycin and 0.2 cc FCS per ml (MEMS) in a 35-mm plastic dish (no. 3001, Falcon Plastics, Div. BioQuest, Oxnard, Calif.) and allowed to adhere at 37°C under 5% CO₂/95% air for 3-4 h. The dishes were then vigorously rinsed in four beakers of normal saline and used to assay the amount of adherent cell protein, as described (15). In some experiments, about 2 x 10⁷ cells were plated in 5 cc of MEMS in 60-mm plastic dishes for 3 h, the nonadherent cells decanted and plated again for 1 h, and the remaining nonadherent cells washed four times in KRPG and assayed. Adherent cells from the first plating were then rinsed in 12 mM lidocaine hydrochloride as reported (16, 17). After washing four times in KRPG, and used for the H₂O₂ assay or assay of protein content. In some experiments, peritoneal cells were plated on 11.5 x 22-mm flying coverslips in Leighton tubes in MEMS for 3 h; the slips were rinsed in three beakers of normal saline and added directly to cuvettes containing KRPG for H₂O₂ assay. They were positioned in the cuvette in a standard manner so as not to affect the initial fluorescence. After assay, the slips were rinsed again, along with matched slips not exposed to the assay, and the adherent cell protein determined. Finally, some cells were plated in 35-mm dishes in MEMS for 3 h, rinsed in four beakers of saline, and the medium replaced with KRPG (containing scopoletin and horseradish peroxidase—see below), of which samples were taken at intervals for assay.

**Assay for H₂O₂ Release.** As described fully (12), the 480-nm emission from reduced scopoletin, when excited by light at 350 nm, is extinguished when scopoletin is oxidized by H₂O₂ in the presence of horseradish peroxidase (HPO). Under assay conditions, the loss of fluorescence was proportional to the concentration of H₂O₂, and was recorded continuously (12). The assay was modified slightly as follows: buffer, KRPG; total volume per cuvette, 3.0 cc; scopoletin, 10 nmol per cuvette; HPO, 0.06 mg (about 30 U) per cuvette. The above reagents and the cells were mixed by inversion and left in the fluorometer for 5 min to reach 37°C. A base line reading was made for 2 min, a "triggering agent" added, the contents mixed, and fluorescence recorded for at least 5 min. With flying coverslips, fluorescence was recorded intermittently immediately after mixing, with care to reposition the slip in the standard manner. H₂O₂ release was determined from a standard curve prepared under the above conditions with monoethylperoxide (Polysciences, Inc., Warrington, Pa.; 12). At the same cell concentration, replicates agreed within a few percent. The greatest source of variability within a given experiment was the use of a wide range of cell concentrations; at the lower cell concentrations, H₂O₂ release per cell tended to be greater than at the higher cell concentrations. Variability among different experiments might be related in part to the use of cells from single mice.
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Stimuli for \( \text{H}_2\text{O}_2 \) Release. PMA (12-O-tetradecanoyl-phorbol-13-acetate; Consolidated Midland Corp., Brewster, N.Y.) dissolved in dimethylsulfoxide (DMSO, Sigma Chemical Co., St. Louis, Mo.) was added to give a final concentration of 0.1 \( \mu \text{g} \) PMA/ml, 0.04% DMSO (vol/vol). Concanavalin A (grade III, Sigma Chemical) was added to give 42-420 \( \mu \text{g} \)/ml. Cytochalasin B (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in DMSO was added to give 4.2 \( \mu \text{g} \) cytochalasin/ml, 0.17% DMSO. The ionophore A23187 was the kind gift of Dr. Robert Hamill, Lilly Research Laboratories, Indianapolis, Ind. and was added in DMSO to give 0.33 \( \mu \text{g} \)/ml, 0.03% DMSO. Starch granules prepared as described (18) were opsonized by mixing for 30 min at 37°C with 0.005 cc of pooled normal human serum per milligram starch, and washing three times in KRPG by centrifugation. 1 mg of opsonized starch was added per cuvette, a dose selected to give no detectable quenching or light-scattering.

Other Reagents. Catalase (Sigma Chemical) was added to give 2,000-4,000 Sigma U/ml. Superoxide dismutase (Truett Laboratories, Dallas, Tex.) was added to give 42 \( \mu \text{g} \)/ml (125 + U/ml). Ferricytochrome-c (type VI, Sigma Chemical) was added to give 83-138 nmol/ml.

Use of Adherence to Estimate Macrophage Content of Cell Suspensions. Protein determinations on known numbers (by hemocytometer count) of thoroughly washed preparations of adherent peritoneal cells which had been brought back into suspension with the aid of lidocaine (16, 17) gave the following results per 10^6 cells: cells from untreated mice, 0.0796 ± 0.0029 mg; cells from mice given thioglycollate 4-5 days previously, 0.1073 ± 0.0076 mg (mean ± SEM from four and three experiments, respectively). These values were greater than those previously reported (14, 19) for whole peritoneal cell suspensions, perhaps because the earlier values reflected the contribution of lymphocytes. However, the results compare well with those reported for adherent normal (0.080 mg) and thioglycollate-elicited peritoneal cells (0.130 mg) after overnight cultivation (20). An accurate factor could not be obtained in this manner for adherent peritoneal cells from BCG-treated mice because of the high proportion of adherent lymphocytes and the difficulty in removing intact macrophages (17). The above values were used to convert adherent cell protein values determined in each experiment to estimated numbers of adherent cells. Because a known number of peritoneal cells had been plated, the percent of peritoneal cells that were adherent could be determined. With cells from mice given BCG, C. parvum, or FCS, the same conversion factor was used as for cells from untreated mice, because the stained cells appeared to be nearly the same size; for cells from casein-treated mice, which appeared to be intermediate in size between normal cells and thioglycollate-elicited cells, the factor used was the mean of the factors for those cell types. The percentage of macrophages estimated in the above way was compared to the percentage estimated from stained smears.

Expression of Data. The data are presented in the following ways, as specified in the tables. (a) as mean \( \text{H}_2\text{O}_2 \) release per 10^6 peritoneal cells (macrophages, lymphocytes, and PMN) over 5 min after the addition of the triggering agent, including the latency period; (b) as in a, but correcting for PMN. The contribution of PMN was determined from the product of the number of cells in the cuvette, the percentage of PMN in the suspension, and a standard value for the activity of PMN (see Results). This product was subtracted from the observed \( \text{H}_2\text{O}_2 \) release, and the remainder was expressed per 10^6 cells exclusive of PMN (macrophages and lymphocytes); (c) as corrected for PMN, and expressed per 10^6 macrophages. The latter were estimated from the number of cells in the cuvette and the percentage of cells scored as macrophages on stained smears.

To correct base-line values for spontaneous loss of fluorescence, 5-min recordings were made from 14 cell-free preparations over the course of these studies, and the mean rate plus 2 SD (8.9 ± 43 pmol scopoletin/min) was subtracted from the resting values observed with cells. This correction was not applied to data obtained after addition of a triggering agent. Values for triggered cells that would have been reduced to zero by such a correction have been so indicated.

Results

Composition of Peritoneal Cell Suspensions. Two methods were compared to estimate the proportion of peritoneal cells that were macrophages (Table I). Differential counts on stained smears had the disadvantage that there appeared to exist a continuous spectrum of morphologic features, from typical macro-


TABLE I

Composition of Peritoneal Cell Suspensions

| Injected i.p. | Macrophages by stained smears* | Macrophages by % adherent cells‡ | PMN§ |
|--------------|-------------------------------|---------------------------------|------|
| Nothing      | 44.56 ± 6.23 (8)              | 40.72 ± 3.72 (3)                | 0.64 ± 0.24 (6) |
| BCG          | 47.16 ± 3.72 (15)             | 45.03 ± 4.53 (13)               | 5.76 ± 1.12 (16) |
| Thioglycollate| 84.23 ± 2.81 (11)             | 54.96 ± 5.92 (8)                | 1.31 ± 0.45 (11) |
| Casein       | 48.10 ± 6.58 (7)              | 55.85 ± 8.11 (5)                | 3.24 ± 0.90 (7)   |
| FCS          | 27.11 ± 4.30 (5)              | 25.24 ± 4.09 (5)                | 2.53 ± 0.81 (5)   |
| C. parvum    | 38.24 ± 3.07 (14)             | 46.28 ± 9.79 (10)               | 16.33 ± 3.52 (14) |

* Mononuclear cells with reniform nuclei, and/or intermediate to large size, and/or intermediate to low nuclear: cytoplasmic ratio on unspun, air-dried, stained glass slides, as percentages of 300 cells scored. Number of experiments in parentheses.
‡ Adherent cellular protein in washed monolayers, converted to number of adherent cells, and divided by number of peritoneal cells initially plated, as described in Materials and Methods.
§ Neutrophils plus eosinophils in stained smears, as percentage of 300 cells scored.

phages to typical lymphocytes. Determination of the proportion of adherent cells by protein measurement had the disadvantage that not all adherent cells were macrophages, and not all macrophages adhered. Nonetheless, on the average, as shown in Table I, the two methods agreed closely when applied to peritoneal cells from untreated mice, or mice given BCG, casein, FCS, or C. parvum. With thioglycollate-treated mice, however, a substantial proportion of morphologic macrophages failed to adhere (Table I, and observation of the nonadherent cells). Differential counts on stained smears were selected as an estimate of the macrophage content of peritoneal cell suspensions.

Because human peripheral blood PMN release copious amounts of H₂O₂ after appropriate stimulation (12), it was important to take into account the effect of the PMN that contaminated the peritoneal cell suspensions. Their representation, which is summarized in Table I, ranged from 0.64 ± 0.24% of peritoneal cells from untreated mice, to 16.33 ± 3.52% of those from mice given C. parvum i.p. 4-25 days before. To determine their contribution to H₂O₂ release, peritoneal cell suspensions intentionally enriched for PMN were prepared.

H₂O₂ Release by PMN. Peritoneal cells were harvested from mice given thioglycollate, casein, or BCG 18-24 h previously, and from mice with hemoperitoneum. In seven such experiments, the proportion of PMN in the peritoneal cell suspension was 46.57 ± 4.37%. Peritoneal macrophages were not expected to release substantial amounts of H₂O₂ when harvested this soon after such injections, as shown below. Spontaneous H₂O₂ release was 0.096 ± 0.029 nmol/10⁶ PMN per 5 min; PMA-induced H₂O₂ release was 9.460 ± 0.994 nmol/10⁶ per 5 min. These figures were used to estimate the H₂O₂ released by PMN in peritoneal cell suspensions containing low numbers of PMN.

Macrophages as a Source of H₂O₂. As shown in Table II, H₂O₂ release was readily demonstrated when PMA was added to peritoneal cell suspensions from BCG-treated mice. In 6 experiments, the change in fluorescence of scopoletin was reduced to 2.19 ± 1.41% of control when catalase was added (2,000-4,000 U/ml), and in 10 experiments, it was reduced to 0.00 ± 0.00% of control when
### Table II

| Exp. | Initial suspension | Cells adherent to coverslip | Postadherent suspension | Nonadherent cells |
|------|-------------------|-----------------------------|------------------------|------------------|
| 19   | 19.51 ± 2.80      | -                           | -                      | 0.23**           |
| 31   | 9.13 ± 0.77       | -                           | 3.03                   | 0.04**           |
| 93   | 5.90 ± 0.60       | 4.81                        | 1.79 ± 0.32            | 0.09             |
| 98   | 5.12 ± 0.16       | 6.29 ± 1.26                 | 2.10 ± 0.13            | 0.00 ± 0.00      |

* Mean ±SEM for replicates (single value where no SEM shown). Corrected for estimated contribution of PMN (see text).
† Peritoneal cells from C3H/HeN mice (experiments 19, 31) or CDF1 mice (experiments 93, 98) given BCG from 10 to 25 days previously. Data expressed per 10⁶ macrophages, as estimated from stained smears (see text). No detectable H₂O₂ release before adding PMA.
§ Coverslips with adherent cells washed and placed in fluorometer for assay. Number of adherent cells estimated from protein measurements on matched coverslips not exposed to PMA. No correction made for nonphagocytic adherent cells (NPAC).
¶ Washed monolayers of adherent cells brought back into suspension with the aid of lidocaine. Viability by trypan blue exclusion, 98.28 ± 0.61%. Data expressed per 10⁶ viable cells. No correction made for NPAC.
** Derived from raw data within 2 SD of the mean for rate of spontaneous loss of fluorescence.

H₂O₂ was omitted. Given the specificity of these enzymes, these data indicate that the recorded fall in fluorescence of scopoletin was the result of release of H₂O₂ (12).

The observed H₂O₂ release could be accounted for by the activity of adherent peritoneal cells when they were studied in situ on well-washed coverslips, or to a lesser extent, when they were recovered from a well-washed monolayer (Table II). Almost none of the activity could be attributed to nonadherent cells (Table III). In one experiment, nonphagocytic adherent cells (NPAC) were isolated and assayed, but released no detectable H₂O₂. We concluded, therefore, that when the contribution of PMN had been subtracted, H₂O₂ released by the peritoneal cell suspensions arose from macrophages, and not from lymphocytes.

**Effect of Various Treatments of the Mice.** Fresh peritoneal cell suspensions from untreated mice did not release significant amounts of H₂O₂ in the standard assay (Table III). They were not devoid of H₂O₂-releasing capacity, however, for when large numbers of them were cultured in plastic dishes for 4-5 h including 1-1.5 h in the presence of PMA, A23187, concanavalin A with cytochalasin B, or opsonized starch granules, there was a substantial fall in scopoletin fluorescence in the supernate, which could be abolished by adding catalase or omitting HPO (data not shown). When expressed per 10⁶ cells/5 min, however, this H₂O₂ release was small (about 1 nmol).

Peritoneal cells from mice given BCG 1 min before sacrifice resembled untreated peritoneal cells in their lack of rapid PMA-induced H₂O₂ release (Table III). This indicated that the presence of BCG organisms did not in itself augment H₂O₂ release. There was a gradual increase in PMA-induced H₂O₂...
Table III

PMA-Induced $H_2O_2$ Release by Peritoneal Cells from Mice Treated With Various Agents

| Injected i.p. | Time from injection to sacrifice | Before PMA: $H_2O_2$ cells/min*¹ | After PMA: $H_2O_2$ cells/10⁶ cells per 5 min* | Number of experiments |
|--------------|---------------------------------|----------------------------------|-----------------------------------------------|----------------------|
| Nothing      | 0.00 ± 0.00                      | 0.14 ± 0.06                      | 0.11 ± 0.06                                   | 7                    |
| BCG          | 0 min                            | 0.00 ± 0.00                      | 0.47 ± 0.19                                   | 1                    |
|              | 1 min                            | 0.00 ± 0.00                      | 0.36 ± 0.07                                   | 1.05 ± 0.47          | 1                  |
|              | 40 min                           | 0.00 ± 0.00                      | 0.27 ± 0.11                                   | 2.34 ± 0.90          | 1                  |
|              | 1 day                            | 0.00 ± 0.00                      | 1.80 ± 0.20                                   | 0.00 ± 0.00          | 1                  |
|              | 2 days                           | 0.02 ± 0.02                      | 1.15 ± 0.19                                   | 0.27 ± 0.15          | 1.14 ± 0.63        | 1                  |
|              | 3 days                           | 0.00 ± 0.00                      | 1.31 ± 0.05                                   | 0.54 ± 0.21          | 1.65 ± 0.40        | 2                  |
|              | 4 days                           | 0.01 ± 0.01                      | 0.98 ± 0.11                                   | 0.41 ± 0.12          | 2.30 ± 0.67        | 1                  |
|              | 5 days                           | 0.00 ± 0.00                      | 1.72 ± 0.17                                   | 1.37 ± 0.18          | 6.51 ± 0.85        | 1                  |
|              | 7 days                           | 0.00 ± 0.00                      | 1.75 ± 0.66                                   | 1.40 ± 0.11          | 3.15 ± 0.60        | 2                  |
| 10-73 days   | 0.02 ± 0.02                      | 3.91 ± 0.65                      | 3.62 ± 0.65                                   | 7.90 ± 1.65          | 10                 |
| 101 days     | 0.00 ± 0.00                      | 0.52 ± 0.12                      | 0.43 ± 0.12                                   | 0.81 ± 0.23          | 1                  |
| Thioglycollate| 4-14 days**                      | 0.00 ± 0.00                      | 0.35 ± 0.08                                   | 0.23 ± 0.05          | 0.28 ± 0.06        | 10                 |
|              | multiple injections***           | 0.00 ± 0.00                      | 1.13 ± 0.46                                   | 0.90 ± 0.37          | 2.84 ± 0.83        | 2                  |
| Casein       | 4-7 days                         | 0.02 ± 0.02                      | 2.04 ± 0.62                                   | 1.90 ± 0.63          | 5.27 ± 1.02        | 3                  |
|              | 14 days                          | 0.00 ± 0.00                      | 0.52 ± 0.06                                   | 0.43 ± 0.07          | 1.17 ± 0.18        | 1                  |
|              | multiple injections†             | 0.00 ± 0.00                      | 2.31 ± 0.60                                   | 1.89 ± 0.61          | 3.08 ± 1.07        | 4                  |
| C. parvum    | 4-25 days                        | 0.01 ± 0.01                      | 1.30 ± 0.49                                   | 0.93 ± 0.36          | 2.76 ± 0.95        | 6                  |
| FCS          | 1-11 days§§                      | 0.00 ± 0.00                      | 0.34 ± 0.06                                   | 0.19 ± 0.05          | 0.67 ± 0.28        | 5                  |

* Means ± SEM. For single experiments, these values were derived from the results for different cell concentrations. For pooled experiments, the values were derived from the means for each experiment.

† Corrected for the contribution of PMN and for spontaneous loss of fluorescence (see text).

‡ See text.

§ Proportion of macrophages in cell suspensions estimated from stained smears (see text).

¶ Value based on low cell numbers and/or low activity per 10⁶ cells such that raw data were within 2 SD from the mean for rates of spontaneous loss of fluorescence.

** Injections were given 13 and 5 days before sacrifice in the first experiment, and 17 and 9 days in the second.

†† In two experiments, double injections were given, at 12 and 4 days, and at 14 and 6 days before sacrifice. In two experiments, triple injections were given, at 21, 13, and 5 days, and at 26, 18, and 4 days before sacrifice.

§§ In one experiment, injections were given 7 and 4 days before sacrifice. In one experiment, injections were given 7 and 4 days before sacrifice. A value of more than 2 SD greater than the mean for rates of spontaneous loss of fluorescence was found only in the experiment performed 11 days after injection (1.71 nmol/10⁶ macrophages).

release with time after injection of BCG, which was first evident at 49 h and continued to mount over about 10 days. Thereafter, PMA-induced $H_2O_2$ release, nearly as copious as that seen with mouse peritoneal PMN, was seen for 10 wk after BCG injection (Table III). The only exceptions were two experiments (not shown) using BCG that had thawed during shipment; here, $H_2O_2$ release was only one-fifth that shown for equivalent times after injection of BCG shipped frozen. The viability of the thawed shipment of BCG was about one-quarter that of the frozen shipment, as determined by colony counts.

Macrophages from mice given thioglycollate 4–5 days previously were more spread out and vacuolated, and had a higher protein content, than those from BCG-treated or untreated mice. Surprisingly, however, they released very little detectable $H_2O_2$ in response to PMA. This was so whether the cells were studied 4 (six experiments), 5 (two experiments), 7 (one experiment), or 14
days (one experiment) after injection of thioglycollate (data pooled in Table III). The rate of $H_2O_2$ release did not increase when the cells were observed for over an hour after adding PMA (data not shown).

When $1 \times 10^6$ peritoneal cells from a BCG-treated mouse, releasing 4.36 nmol $H_2O_2/5$ min after PMA, were mixed with $1 \times 10^6$ peritoneal cells from a thioglycollate-treated mouse, themselves releasing 0.004 nmol, the mixture released only 1.54 nmol when exposed to the same amount of PMA. This was consistent with catabolism of $H_2O_2$ by thioglycollate-induced peritoneal cells, or with the oxidation by $H_2O_2$ of moieties associated with the cells, in competition with scopoletin.

When mice were given a second injection of thioglycollate approximately a week after the first, and their peritoneal cells collected after another 5-9 days, much more substantial $H_2O_2$ release could be detected in response to PMA (Table III).

The relative capacities for $H_2O_2$ release by macrophages from BCG-treated, thioglycollate-treated, or untreated mice were preserved over a wide range of cell concentrations (Fig. 1).

Peritoneal cells from mice injected with casein released substantial amounts of $H_2O_2$ in response to PMA (Table III). $H_2O_2$ release was detectable earlier than with BCG (4 days), but appeared to decline faster (by 14 days) and tended to be of lower magnitude. Multiple injections of casein did not augment the response (Table III).

Treatment with a formalin-killed preparation of $C. parvum$ yielded peritoneal cells with a modest degree of PMA-induced $H_2O_2$ release, which appeared to peak on the 11th day after injecting 0.7 mg ($7.42 \pm 1.06$ nmol/10$^6$ macrophages per 5 min on day 11). Prodigious degrees of $H_2O_2$ release, well over 10 nmol/10$^6$ macrophages per 5 min, were seen in three experiments 5 days after injecting 0.7, 1.4, or 2.1 mg of $C. parvum$, but contamination by PMN was high, and the results have been excluded from analysis.

Macrophages from mice given FCS from 1 to 11 days before sacrifice were mostly inactive in PMA-induced $H_2O_2$ release (Table III).

Role of "Triggering" Agent. No matter how the mice had been treated, their peritoneal cells usually released no detectable $H_2O_2$ in vitro until a triggering agent was added (Table III). Four materials were found to serve well as triggering agents: PMA, the ionophore A23187, concanavalin A in combination with cytochalasin B, and opsonized phagocytic particles. Responses to PMA were of the greatest magnitude and shortest latency, but no attempt was made in this study to optimize conditions for the use of the other agents, and the results are therefore not presented.

Dose-response curves for PMA over the range 0.005-0.83 $\mu$g/ml were determined for peritoneal cells from mice given $C. parvum$, thioglycollate, or no treatment, and for peritoneal exudates rich in PMN. All showed a plateau including the standard dose of 0.1 $\mu$g/ml, indicating that differences in $H_2O_2$ release among various cell preparations could not be attributed to either suboptimal or suppressive doses of PMA.

As shown in Table IV, the onset of $H_2O_2$ release was apparent about 40 s after PMA was added to each type of cell preparation used in this study, with two exceptions. Peritoneal cells from untreated mice began to release a small
FIG. 1. PMA-induced \( \text{H}_2\text{O}_2 \) release as a function of the number of macrophages in the cuvette. C3H/HeN mice were untreated, or given thioglycollate 4 days or BCG 14 days before collection of peritoneal cells. Values corrected for the contribution of PMN and the estimated proportion of macrophages, based on stained smears (untreated mice, 47.5% macrophages and 0% PMN; mice given thioglycollate, 77.5% macrophages and 0.5% PMN; mice given BCG, 38.5% macrophages and 3% PMN). All assays performed in the same experiment. Lines fitted by the method of least squares.

amount of \( \text{H}_2\text{O}_2 \) after about 14 min (Table IV), whereas the latency for BCG-elicited peritoneal cells, when studied adherent to coverslips, was about 2 min. The significance of these differences is unclear.

*Derivation of \( \text{H}_2\text{O}_2 \) from Superoxide.* In human peripheral blood PMN, \( \text{H}_2\text{O}_2 \) appears to arise during the respiratory burst mainly from the dismutation of superoxide anion (13, 21, 22). This pathway was examined indirectly in peritoneal cells from BCG-treated mice. Ferricytochrome c, which is reduced by superoxide and therefore diverts it from the dismutation reaction (23), abolished \( \text{H}_2\text{O}_2 \) release in response to PMA (Table V). Superoxide dismutase, which catalyzes the dismutation of superoxide to \( \text{H}_2\text{O}_2 \), overcame the depressive effect of ferricytochrome c. By itself, superoxide dismutase either had little effect or enhanced the detectable \( \text{H}_2\text{O}_2 \) release (Table V). These observations suggest that the \( \text{H}_2\text{O}_2 \) released by macrophages from BCG-treated mice was derived from superoxide anion.

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Table IV

Latency of Response to PMA

| Injected i.p.          | Onset of H₂O₂ release after addition of PMA |
|------------------------|---------------------------------------------|
| Nothing                | None apparent in 10 min (12)*               |
| BCG                    | 39 ± 1 (61)†                                |
| Thioglycollate         | 43 ± 2 (20)                                 |
| Casein                 | 40 ± 3 (15)                                 |
| FCS                    | 34 ± 2 (2)§                                |
| C. parvum              | 40 ± 2 (28)                                 |
| PMN-rich preparations  |                                             |
| BCG                    | 42 ± 4 (7)                                  |
| Thioglycollate¶        | 46 ± 3 (14)                                 |
| Casein¶                | 37 ± 1 (4)                                  |

* In three experiments with prolonged observation, of which two employed cells adherent to coverslips, H₂O₂ release was observed after 14 ± 1 min. Number of observations in parentheses.
† Mean seconds ± SEM. With cells adherent to coverslips, latency was about 2 min.
§ In seven other observations, no onset of H₂O₂ release was discernible.
¶ Either 1 day after BCG injection, or from a group of mice all members of which had peritonitis, whether or not injected.
¶¶ 1 day after injection.

Table V

Evidence for Origin of H₂O₂ from Superoxide

| Experiment | Peritoneal cells alone | With superoxide dismutase§ | With ferricytochrome c|| | With SOD + ferricytochrome c¶ |
|------------|------------------------|----------------------------|------------------------|--------------------------|
| 17         | 8.44                   | 14.41                      | 0                      | 9.61                     |
| 23         | 5.09                   | 4.56                       | 0                      | 4.39                     |
| 31B        | 8.26                   | 9.49                       | 0                      | 5.28                     |

* PMA-induced H₂O₂ release corrected for contribution of PMN, and for the proportion of macrophages in the peritoneal cell suspensions, as estimated from stained smears.
† C3H/HeN mice injected with BCG 10, 12, and 22 days before cell collection.
§ Superoxide dismutase (SOD), final minimum activity per milliliter: 125 U in experiments 17 and 23; 250 U in experiment 31B.
|| Ferricytochrome c, final amount per milliliter: 83 nmol in experiments 17 and 31B; 138 nmol in experiment 23.
¶ Same amounts as in § and ||, except 250 U SOD in experiment 23.

Discussion

Mouse peritoneal macrophages were capable of releasing up to about 8 nmol of H₂O₂/10⁶ cells per 5 min, a capacity essentially the same as that of mouse peritoneal PMN (9 nmol) and similar to that reported for human peripheral blood PMN (5 nmol) when assayed by the same fluorometric technique.¹

¹ R. K. Root and J. Metcalf. 1977. The role of extracellular Ca++ in oxidase activation of human granulocytes. PMA-induced H₂O₂ release greater than 5 nmol/10⁶ cells per 5 min was attainable with human PMN under somewhat different assay conditions, including continuous agitation and the presence of azide. Manuscript in preparation.
The data reported here should be regarded as lower limits for rates of $H_2O_2$ production. The assay measured only $H_2O_2$ that was released from cells and that was not expended in the oxidation of reactants other than scopoletin, including the cells or materials associated with them. Differences in the amount of $H_2O_2$ detected by this assay using different cell preparations could have been due to differences in the formation, catabolism, or distribution of $H_2O_2$, or combinations of these. Studies of oxygen consumption, specific metabolic pathways, and further cell mixing experiments may help to distinguish among these possibilities.

If the contribution of small numbers of PMN were taken into account, the remaining $H_2O_2$ release from suspensions of peritoneal cells from BCG-treated mice appeared to arise from macrophages. Nonadherent peritoneal cells were inactive. When adherent peritoneal cells were studied on coverslips, their PMA-induced $H_2O_2$ release could account for all the $H_2O_2$ release seen with the initial cell suspension. However, the 5-min assay period included a 2-min latency for adherent cells, but only a 39-s latency for the parent cell suspensions. Thus, after the latency, the rate of $H_2O_2$ release from adherent cells on coverslips was about 1.5 times that of cells in suspension. This raises the possibility that adherence itself may augment the effect of PMA in inducing $H_2O_2$ release. When adherent peritoneal cells from BCG-treated mice were brought back into suspension with lidocaine and jets of saline, their PMA-induced $H_2O_2$ release was still considerable, but was somewhat less than predicted from the activity of the initial cell suspension. Two factors may account for this discrepancy. First, BCG-elicited peritoneal cells appeared to adhere to plastic more tightly than those from untreated mice (17). Consequently, attempts to dislodge them may have inflicted more cell damage than previously described for cells from untreated mice (17). Second, the tight adherence of BCG-elicited macrophages may have resulted in enrichment of the recovered population for NPAC, which appeared to be inactive in $H_2O_2$ release. Although the proportion of NPAC in the lidocaine-treated suspensions was not measured in the present study, it was noted that only 37% of the recovered cells appeared to be large macrophages; the remainder could have been either macrophages or NPAC by morphologic criteria.

Elicitation of the burst of $H_2O_2$ release required two sequential stimuli. First, the macrophages had to be "activated" by appropriate treatment of their donors, a process requiring 4 or more days with the regimens used. Second, these primed macrophages had to be "triggered" by any of a variety of stimuli thought to act at the cell membrane. Triggering agents included PMA, a fatty acid ester of a polyfunctional diterpene alcohol; A23187, an ionophorous antibiotic from fermentation of *Streptomyces*; concanavalin A, a phytoglycoprotein thought to interact with macrophage surface receptors (24); and opsonized particles, which were phagocytized. Omission of either the activating or the triggering stimulus made $H_2O_2$ release almost undetectable. This two-stage process for inducing $H_2O_2$ release is very similar to the requirement for sequential "priming" and triggering in induction of plasminogen activator release from mouse peritoneal macrophages (25).

Not all agents commonly used to activate macrophages sufficed to activate them for $H_2O_2$ release. BCG, which establishes a chronic intracellular infection,
gave the most profound, prolonged, and consistent activation in this regard. Low doses of a nonviable preparation of *C. parvum* gave activation of much briefer duration. Peritoneal macrophages were also effective over a relatively brief period after injection of casein. Macrophages from mice treated with BCG, *C. parvum, or casein were less spread out and less vacuolated than macrophages from thioglycollate-treated mice. The latter have been reported to be highly activated not only morphologically but also with regard to pinocytosis (20), complement receptor-mediated phagocytosis (26), ATP levels (27), and secretion of collagenase (28), elastase (29), and plasminogen activator (30). Yet thioglycollate-elicited macrophages were only slightly more active than macrophages from untreated or FCS-injected mice in detectable H$_2$O$_2$ release.

It has become increasingly evident that many features of macrophage activation can be dissociated from each other (14, 18, 31, 32). Activation is therefore not a stereotypic response, and different regimens for inducing activation cannot be regarded as equivalent. Antitumor activity has been ascribed to macrophages from mice treated with BCG (e.g. 33) and with *C. parvum* (e.g. 34), but has repeatedly been absent with macrophages from mice treated with thioglycollate (31, 32). The antitumor activity of casein-elicited and FCS-elicited mouse peritoneal macrophages has not to our knowledge been tested. Thus, in a rough way, based on the evidence available so far, activation for H$_2$O$_2$ release and activation for antitumor activity may be correlated.

Recent studies with human PMN have shown that their PMA-induced H$_2$O$_2$ release accounts for at least 70% of their oxygen consumption during the PMA-induced respiratory burst (see footnote 3). If PMA-induced H$_2$O$_2$ release by macrophages from BCG-treated mice also proves to account for a substantial fraction of their increment in oxygen consumption, this would suggest that formation of H$_2$O$_2$ is of considerable importance in the physiology of the macrophage, and would give weight to the hypothesis that H$_2$O$_2$ formation may play an important role in the enhanced antimicrobial and antitumor capacities ascribed to macrophages activated in certain ways.

Previous measurements of H$_2$O$_2$ formation or release have been reported for mononuclear phagocytes from several species and anatomic compartments, using various methods of elicitation of the cells and a number of assays for H$_2$O$_2$. Data from most of these studies have been included in Table VI. The limitations of several of the indirect methods for assaying H$_2$O$_2$ have been discussed by Klebanoff (35). The importance of taking into account the degree of contamination by PMN deserves emphasis. With macrophages active in H$_2$O$_2$ release, such as those from BCG-treated mice, the relative contribution by PMN to total H$_2$O$_2$ release (mean, 7.42%) tended to be about the same as their relative number in the cellular preparation (mean, 5.76%). With poorly active macrophages, however, a substantial proportion of total H$_2$O$_2$ release by peritoneal cells could be attributed to small numbers of contaminating PMN. Judging from Table VI, the main differences between the present study and previous studies appeared to be (a) an assay that is probably more sensitive; (b) a triggering agent that may result in greater extracellular release (although this point has not been thoroughly explored); and (c) the recognition that the peritoneal cells must be activated or elicited in an appropriate way.
With a latency of about 40 s after addition of a triggering agent, \( \text{H}_2\text{O}_2 \) release appears to be one of the fastest macrophage responses to any stimulus that has been susceptible to quantitation. As such, it may be suited to the analysis of the interaction of various agents with the cell membrane and its receptors. It is also of interest that the ratio of \( \text{H}_2\text{O}_2 \) release obtainable with BCG-activated macrophages compared to untreated macrophages is one of the largest quantitative changes reported to be associated with macrophage activation. The fluorometric assay for \( \text{H}_2\text{O}_2 \) release is sensitive to the \( \text{H}_2\text{O}_2 \) output from as few as \( 2 \times 10^5 \) macrophages, when appropriately activated and triggered, and thus permits studies to be made upon cells from individual mice. It is hoped that this assay will facilitate the experimental approach to a variety of questions concerning the interaction of macrophages with lymphocyte mediators, cell surface ligands, microbes, other particulates, and nucleated cells.
Summary

Using a specific and sensitive fluorometric assay, the H_2O_2 release from as few as 2 \times 10^5 mouse peritoneal macrophages could be detected continuously and quantitated. It is emphasized that the assay measured H_2O_2 release, not production. Induction of H_2O_2 release required sequential application of two stimuli: the administration of an activating agent to the mice from 4 days to 10 wk before cell harvest, and the exposure of the cells in vitro to a triggering agent. BCG was most effective as an activating agent, resulting in peritoneal macrophages which could be triggered to release H_2O_2 almost as copiously (8 nmol/10^8 macrophages per 5 min) as mouse peritoneal PMN (9 nmol/10^6 PMN per 5 min). Casein and C. parvum could also serve as activators, but thioglycolate and FCS were ineffective after single injections. PMA was a potent triggering agent, resulting in a maximal rate of H_2O_2 release after a latency of about 40 s for cells in suspension. Other triggering agents included the ionophore A23187, concanavalin A in the presence of cytochalasin B, and phagocytosis. H_2O_2 release could be attributed to macrophages and PMN in peritoneal cell suspensions or in preparations of adherent peritoneal cells, but not to lymphocytes. Indirect evidence suggested that the H_2O_2 detected was formed from superoxide anion. These observations appear to justify renewed interest in the idea that H_2O_2 may be important in macrophage antimicrobial and antitumor mechanisms.

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