SUPPRESSION OF MACROPHAGE OXIDATIVE METABOLISM BY PRODUCTS OF MALIGNANT AND NONMALIGNANT CELLS*

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Macrophages from tumor-bearing hosts or macrophages incubated with tumor cells, tumor lysates, or tumor cell-conditioned medium (TCM)[1] are often defective in their maturation (1, 2), chemotaxis in vitro or accumulation in vivo (3–14), spreading (15, 16), phagocytosis (17–19), or antimicrobial activity (20) (reviewed in 21). However, few effects of tumor cells on macrophages have been described at the biochemical level. In particular, tumor cells have not been shown to suppress the ability of macrophages to secrete substances cytotoxic for tumors.

Among the macrophage secretory products with cytotoxic potential are the reactive oxygen intermediates (ROI), including superoxide anion (22, 23) and hydrogen peroxide (24). ROI mediate some forms of spontaneous (25) and elicited (26, 27) cytolysis by macrophages. In addition, the production of ROI by macrophages is closely related to their inhibition of a number of intracellular pathogens (reviewed in 28). Herein we report that TCM, as well as medium conditioned by certain nonmalignant cells, selectively suppresses the ability of macrophages to secrete ROI.

Materials and Methods

Tumor Cells. P815 mastocytoma and PU5-1, L1210, EL-4, TLX9, and YAC lymphomas were obtained as described previously (29–31) and passaged as ascites in the mouse strains listed in Table I. GIF was a sarcoma arising spontaneously in the flank of a (BALB/c × DBA/2)F_1 ($CD2F_1$) female mouse, and was studied in its first in vitro passage. MC-6 mammary carcinoma, adenocarcinoma 755, and RC-2 renal adenocarcinoma were from the tumor bank of the Division of Cancer Treatment, National Cancer Institute, and were passaged as ascites in the mouse strains listed in Table I. J774 histiocytoma (32) was maintained in spinner culture in Eagle's minimum essential medium, alpha variant, containing 100 μg/ml streptomycin and 100 U/ml penicillin (Grand Island Biological Company, Grand Island, NY) plus 10% horse serum that had been heat inactivated at 56°C for 30 min (Flow Laboratories, Rockville, MD) (MEM-HS). The other tumors were maintained in stationary cultures in the same medium.

Nonmalignant Cells. Granulosa cells were isolated from proestral rat Graafian follicles as described (33). LLC-PK_1, a pig kidney epithelial cell line (34), was the gift of M. Sudol and E. Reich, The Rockefeller University. Rat fibroblast cultures were established by trypsinization of minced newborn Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington,

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[1] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; Con A, concanavalin A; MAF, macrophage-activating factor; MEM-HS, Eagle's minimum essential medium, alpha variant, containing penicillin, streptomycin, and 10% heat-inactivated horse serum; P-10h, medium conditioned by P815 mastocytoma cells in 10 h; PMA, phorbol myristate acetate; ROI, reactive oxygen intermediates; TCA, trichloroacetic acid; TCM, tumor cell-conditioned medium.

MA) and were used in their second passage. Diploid skin fibroblasts (SK21) from the American Type Culture Collection, Rockville, MD were used in their third passage. Spleen cells from (DBA/2 × BALB/c)F_1 ($D2CF_1$) mice were used after depleting adherent cells during a 2-h incubation in plastic tissue culture flasks. Erythrocytes were obtained from the axillary vessels of $D2CF_1$ mice and collected in heparin (20 U/ml). The above were all cultured in MEM-HS.

Tests for Mycoplasma and Viruses. Cytocentrifuged cells were fixed and stained for 10 min in 1 μg/ml bisbenzimidazol in methanol (Aldrich Chemical Co., Milwaukee, WI) (35). The slides werre examined in a fluorescence microscope with a 366 ± 11 nm excitation filter and an emission filter with a 460-nm cutoff (Carl Zeiss, Inc., New York) to detect cytoplasmic DNA indicative of mycoplasma. In addition, antibody to mycoplasma was sought in the sera of tumor-bearing mice by Microbiological Associates, Walkersville, MD. Before and 4 d after injection of 10^6 P815 ascitic or cultured cells, the sera of four Nelson-Collins Swiss (NCS) mice were tested for lactate dehydrogenase, using a commercial assay kit (Sigma Chemical Co., St. Louis, MO). Elevations in this enzyme activity are characteristic of infection by lactate dehydrogenase-elevating virus (36). Sera of tumor-bearing mice were tested by the Laboratory Animal Research Center, The Rockefeller University, for complement-fixing antibodies to Sendai, ectromelia, mouse hepatitis, lymphocytic choriomeningitis, and mouse adenoviruses, and for hemagglutination-inhibiting antibodies to pneumonia virus, polyoma, minute virus, K-virus (newborn mouse pneumonitis virus), Theiler's encephalomyelitis (GDVII) virus, and reovirus type 3.

Conditioned Media. TCM was collected from 48-h cultures of tumor cells in MEM-HS, with a final cell density of ~1.5 × 10^6/ml and a final viability of >90%. Cells were removed by centrifugation at 500 *g* for 15 min, and the supernatant was dialyzed in Spectra/Por 6 tubing (1,000-dalton cutoff) (Spectrum Medical Industries, Inc., Los Angeles, CA) for 24 h at 4°C against 20–50 vol of MEM. The TCM could be stored for >1 mo at 4°C or >3 mo at −20°C. Alternatively, 5 × 10^6 cells/ml were incubated in MEM-HS for 10 h and the supernatant dialyzed as above. Conditioned medium was also collected from adherent cells in monolayers that were ~75% confluent in 25-cm^2 flasks containing 10 ml MEM-HS. To prepare lymphokines, $D2CF_1$ spleen cells were incubated at 10^8 cells/ml in RMPI 1640 containing 10% fetal bovine serum, pyruvate, glutamine, nonessential amino acids, 2-mercaptoethanol, and antibiotics as described (31). Concanavalin A (Con A) (3 μg/ml) (Sigma Chemical Co.) was added at the beginning or the end of the 48-h incubation, or was omitted.

Macrophages. Mice >6 wk old of either sex and of the following strains were used with equivalent results: $CD2F_1$ (Simonsen Laboratories, Gilroy, CA), $D2CF_1$, C57BL/6 (Trudeau Institute, Saranac Lake, NY), NCS (The Rockefeller University), and ICR (Taconic Farms, Germantown, NY). Resident cells were collected from the peritoneal cavities of untreated mice, or peritoneal cells were collected 12–20 d after intraperitoneal injection of 7 × 10^6 viable bacilli Calmette-Guérin (BCG) (type 1011, Trudeau Institute) in 0.1 ml 0.9% NaCl; 4–6 d after intraperitoneal injection of 1 ml of 6% sodium caseinate (Lot ABX, Eastman Kodak Co., Rochester, NY) in 0.9% NaCl; or 5 d after intraperitoneal injection of 1 ml of 10% proteose peptone (Difco Laboratories, Detroit, MI) in 0.9% NaCl. Differential counts of these cell populations have been reported previously (24, 25, 29). The cells were suspended in MEM-HS, and 1 ml containing 2 × 10^6 cells was dispensed to each 16-mm Diam well of Cluster trays (Costar, Data Packaging, Cambridge, MA) on which a 13-mm Diam ethanol-cleaned glass coverslip (Clay Adams, Inc., New York) had been placed. 4–6 h later, nonadherent cells were removed, and the medium was replaced with MEM-HS or various dilutions of dialyzed TCM in MEM-HS. Where indicated, peritoneal cells were suspended directly in TCM before plating, and nonadherent cells were left in the cultures.

H_2O_2 Release. After various periods of incubation in test or control media, coverslips with adherent peritoneal cells were rinsed by agitation in three beakers of 0.9% NaCl. Excess fluid was removed with absorbent paper, and the coverslips were placed in Cluster tray wells containing 1.5 ml Krebs-Ringer phosphate buffer with 5.5 mM glucose, pH 7.35–7.40, formulated as described (24). Each well also contained 15 nmol scopoletin, 1.5 purpurogallin units (10 μg) horseradish peroxidase (Sigma Chemical Co.), 1 mM NaN_3 to inhibit catalase, and 150 ng phorbol myristate acetate (PMA) (Consolidated Midlands Co., Brewster, NY). In some experiments, 2 × 10^7 zymosan particles (Sigma Chemical Co.) were exposed for 10 min

to 50% fresh mouse serum at 37°C, washed by centrifugation, and added to the cultures. In this case, the plates were centrifuged at 500 *g* for 3 min at 4°C, and the coverslips were transferred to fresh wells containing the assay mixture described above but with PMA omitted. Assays were initiated by floating the trays in a 37°C water bath. The medium was collected 60 min later, and fluorescence was determined as described (37). Control media were collected from wells lacking cells. In preliminary experiments, the amount of H_2O_2 detected from cells not given PMA or zymosan was <1% of the stimulated values. Separate coverslips were rinsed as above and used to determine adherent cell protein by the method of Lowry et al. (38) using bovine serum albumin as a standard. Results are expressed as the mean ± SEM for nmol H_2O_2 released from triplicate coverslips, divided by the mean mg protein on triplicate coverslips incubated under identical conditions.

Other Assays. Superoxide anion secretion was assayed by the superoxide dismutase-inhibitable reduction of ferricytochrome c (23), using macrophages stimulated with PMA or zymosan as described above. Secretion of arachidonic acid and its metabolites was determined from the radioactivity of media of zymosan-stimulated macrophages that had been labeled to constant specific activity with [^{3}H]-arachidonic acid, as described in detail (39). Lysozyme was measured in the media of 48-h macrophage cultures by recording the decrease in absorbance at 600 nm of a suspension of 100 μg/ml *Micrococcus luteus* (Miles Laboratories, Elkhart, IN) in 0.06 M sodium phosphate buffer in 0.9% NaCl at room temperature, compared with a hen egg white lysozyme standard (Worthington Biochemical Corp., Freehold, NJ) (40).

To measure plasminogen activator, macrophages were incubated for 48 h in TCM or MEM-HS, washed extensively, and incubated an additional 48 h in serum-free MEM. Aliquots of the medium were added to plates coated with ^{125}I-fibrin (41) with and without purified human plasminogen (a kind gift of M. Sudol, The Rockefeller University) and soluble radioactivity was determined 1 h later. The activity of plasminogen activator was calculated by comparison to a urokinase standard.

To compare DNA synthesis in TCM and MEM-HS, 5×10^6 macrophages were incubated in 35-mm plastic petri dishes in 2.5 ml of either medium. At 24 h, 10 μCi [^{3}H]thymidine (2.0 Ci/mmol, New England Nuclear, Boston, MA) was added. At 48 h, the monolayers were washed in phosphate-buffered saline and scraped with a rubber spatula. Cells were sedimented at 8,000 *g*, resuspended in lysis solution (9.5 M urea, 5% 2-mercaptoethanol, 2% NP-40), and frozen and thawed. DNA was precipitated with 10% trichloroacetic acid (TCA) containing 0.1% unlabeled thymidine and washed with the same solution on 0.4-μm pore filters (Millipore Corp., Bedford, MA). The dried filters were used for scintillation counting.

To compare protein synthesis by macrophages in TCM and MEM-HS, 48-h cultures like those above were pulsed for the last 6 h with 100 μCi [^{35}S]methionine (450 Ci/mmol, New England Nuclear), and the monolayers were washed, scraped, and lysed as above. Aliquots of the lysate were applied to filters and boiled in 10% TCA with 0.1% unlabeled methionine for 5 min, soaked in cold 5% TCA, and washed in cold 98% ethanol. The dried filters were used for scintillation counting. The content of glutathione (42) and the specific activity of glutathione reductase (43), glutathione peroxidase (44), catalase (45), and guaiacol peroxidase (46) were measured as described. Phagocytosis was measured using ^{14}C-acetyl starch granules in 20-min uptake periods, as described (47).

Results

Suppression of Macrophage H_2O_2-releasing Capacity by Conditioned Media. In preliminary experiments, co-culture of BCG- or caseinate-activated macrophages for 24–48 h in a 2:1 ratio with P815 mastocytoma, P388 lymphoma, or TLX9 lymphoma cells ablated detectable H_2O_2 release when the macrophage monolayers were subsequently washed to remove tumor cells and then challenged with the secretagogue, PMA (48). To eliminate the possibility that tumor cells exerted this effect by exhausting the medium, subsequent experiments used TCM dialyzed against fresh MEM. As shown in Table I, conditioned medium from 11 of 11 tumors tested markedly suppressed the H_2O_2-releasing capacity of caseinate-activated macrophages. Many of the TCM were tested

TABLE I

Effect of Conditioned Media on H_2O_2 Release by Caseinate-activated Macrophages

Medium* conditioned by	Species and strain	Negative tests for contamination	Percent inhibition‡
P815 mastocytoma	Mouse, $D2CF_1$ or $CD2F_1$	§, ‖, ¶, **	97.9
PU5-1 lymphoma	Mouse, BALB/c		100.0
L1210 lymphoma	Mouse, $CD2F_1$		92.4
EL-4 lymphoma	Mouse, C57BL/6		96.2
TLX9 lymphoma	Mouse, C57BL/6	§, ‡‡	68.2
YAC lymphoma	Mouse, A/Sn	§	99.0
GIF sarcoma	Mouse, $CD2F_1$		89.6
J774 histiocytoma	Mouse, BALB/c		67.5
MC-6 mammary carcinoma	Mouse, BALB/c		60.5
Adenocarcinoma 755	Mouse, C57BL/6		94.9
Renal adenocarcinoma 2	Mouse, $CD2F_1$		98.5
Erythrocytes	Mouse, $D2CF_1$		9.0
Peritoneal macrophages	Mouse, $D2CF_1$		25.3
Splenic lymphocytes	Mouse, $D2CF_1$		27.2
Diploid skin fibroblasts	Human	§	64.8
Fibroblasts (from newborns)	Rat		75.1
LLC-PK_1 cells, epithelial kidney	Pig		99.1
Graafian follicle granulosa cells	Rat		100.0

* Medium was conditioned for 48 h as described in Materials and Methods and dialyzed against 20–50 vol of MEM before use.

‡ Macrophages from 2×10^6 caseinate-elicited peritoneal cells were incubated in conditioned media for 48 h, washed, and challenged with PMA to measure H_2O_2 release over 60 min in comparison to macrophages incubated for 48 h in MEM-HS alone. Data are from five experiments, in which values for the control macrophages averaged 463 nmol H_2O_2/mg cell protein/60 min.

§ Tested for mycoplasma contamination by bisbenzimidazol staining.

‖ Tested for lactate dehydrogenase-elevating virus.

¶ Tested for Sendai virus.

** Tested for pneumonia virus, reovirus 3, Theiler's encephalomyelitis virus, polyoma virus, ectromelia virus, murine hepatitis virus, murine adenovirus, lymphocytic choriomeningitis virus, newborn mouse pneumonitis virus, minute virus, and mycoplasma by serologic techniques (see Materials and Methods).

‡‡ Negative for all organisms listed above (**), except positive for minute virus of mice.

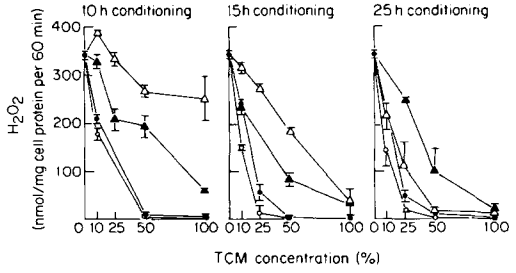

FIG. 1. Influence of tumor cell number and incubation time on potency of TCM. Medium was conditioned for 10, 15, or 25 h by P815 cells initially cultured at 5×10^5/ml (△), 1×10^6/ml (▲), 5×10^6/ml (●), or 1×10^7/ml (○). The media were dialyzed and added at the indicated concentrations (%, vol/vol) to adherent cells from 2×10^6 caseinate-elicited peritoneal cells. 48 h later, TCM was washed off and H_2O_2 released measured in response to PMA. Means and SEM of triplicates are indicated.

also with BCG-activated macrophages, and gave the same results as with the caseinate-elicited cells (data not shown). The tumors were syngeneic or allogeneic to the macrophages, adherent or nonadherent, lymphocytic, histiocytic, or fibroblastic, carcinogen-induced, x-ray induced, or spontaneously arising, and were tested after in vivo or in vitro passages numbering from one to many hundreds. Testing for mycoplasma, lactate dehydrogenase-elevating virus, and other murine viruses was negative with P815 mastocytoma, the tumor chosen for the most of the further experiments.

As shown in Table I, there was little suppression of H_2O_2-releasing capacity after 48 h incubation of macrophages in medium conditioned by erythrocytes or macrophages. Spleen cell-conditioned medium was variably suppressive. Diploid fibroblasts and ovarian follicular cells in early passage and a long-passaged porcine epithelial cell line had clear-cut suppressive effects.

Kinetics of Conditioning of Medium by P815 Cells and Dose Dependence of the TCM. To quantify the conditioning of medium by P815 cells, different numbers of tumor cells ranging from 5×10^5/ml to 1×10^7/ml were cultured in MEM-HS for periods varying from 10 to 25 h. The TCM were then dialyzed against fresh MEM and added in dilutions to caseinate-activated macrophages. 48 h later, the monolayers were washed to remove TCM, and their H_2O_2-releasing capacity was tested upon challenge with PMA. As shown in Fig. 1, the suppressive potency of TCM depended both on the number of tumor cells per volume of medium and the duration of the tumor cell culture. For example, 100% suppression of macrophage H_2O_2-releasing capacity followed a 48 h exposure to 50% (vol/vol) of dialyzed medium conditioned by 5×10^6 P815 cells/ml for 10 h, or by 5×10^5 P815 cells/ml for 25 h. Dialyzed medium conditioned by 5×10^6 P815 cells/ml for 10 h was designated P-10h and used in most of the subsequent experiments. In the experiment indicated in Fig. 1, 50% suppression of H_2O_2 release followed incubation of macrophages for 48 h in 13% of P-10h.

Kinetics of Suppression of H_2O_2 Release by TCM. 100% P-10h was added to unseparated BCG-elicited peritoneal cells (Fig. 2 A), adherent peritoneal cells from BCG-elicited exudates (Fig. 2 B), or adherent, caseinate-elicited peritoneal cells (Fig. 2 C). In each case, H_2O_2 release was suppressed nearly completely. However, BCG-elicited macrophages lost H_2O_2-releasing capacity spontaneously over the same time period,

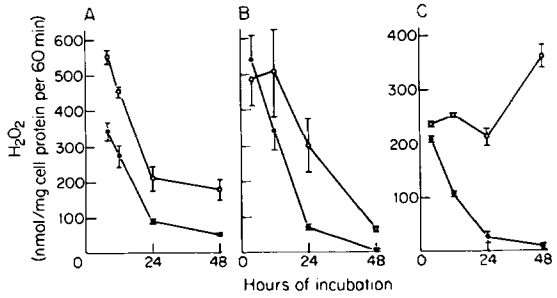

FIG. 2. Time course of suppression of H_2O_2 release by TCM. TCM was prepared by incubating 5×10^6 P815 cells/ml for 10 h and dialyzing the supernatant. Cells cultured in TCM (●) or MEM-HS alone (○) were (A) from BCG-elicited peritoneal exudates; (B) as in (A) with nonadherent cells removed before addition of test media; or (C) from caseinate-elicited peritoneal exudates, with nonadherent cells removed. H_2O_2 release in response to PMA was tested after the indicated periods of incubation. Means and SEM of triplicates are shown.

as reported earlier with this (49) and other (50) microbial vaccines. TCM accelerated this decline. In contrast, caseinate-activated macrophages retained or even increased their H_2O_2-releasing capacity during 48 h in vitro, so that the suppressive effect of TCM was much more evident. With the latter macrophages, 50% suppression of H_2O_2-releasing capacity was obtained by 18 h of exposure to P-10h.

In an earlier report, caseinate-elicited guinea pig macrophages in monolayer cultures required 2–3 d to respond to the lymphokine, macrophage-activating factor (MAF). However, the period of exposure to MAF could be shortened to 1–2 d if the macrophages had been in culture for 1–2 d before MAF was added (51). Similarly, the 48-h period required for complete suppression of H_2O_2 release by TCM could be caused by prolonged interaction of TCM with macrophages or the gradual acquisition by macrophages of the capacity to respond quickly to TCM. To investigate the latter possibility, macrophages were incubated with P-10h throughout the 48-h culture period, or only for the final 24, 6 or 1 h. Only macrophages exposed to TCM for 48 h showed complete suppression (data not shown).

Dose Dependence and Reversibility of Effect of Media Conditioned by Nonmalignant Cells. The suppressive activity in medium conditioned by newborn rat fibroblasts, human skin fibroblasts, and mouse spleen cells appeared to be present in 3.5- to 7-fold lower concentrations than that in P815 TCM, judging by the concentration of conditioned medium required to cause 50% inhibition of H_2O_2 release (Fig. 3 A). Moreover, within 48 h of its removal, the effect of nonmalignant cell-conditioned medium was completely reversed, whereas the effect of TCM persisted (Fig. 3 B).

Reversibility of Suppression by TCM. To determine whether the suppressive effect of TCM was reversible, caseinate-activated macrophages were incubated in various dilutions of P-10h for 48 h. As usual, H_2O_2-releasing capacity was suppressed at that time in a dose-dependent manner. The remaining cultures were then placed in fresh MEM-HS and tested at intervals over the next 6 d. As shown in Fig. 4, the suppressive effect of TCM persisted for 4 d after its removal. By the 8th d of culture, H_2O_2-releasing capacity of macrophages which were last exposed to TCM 6 d previously,

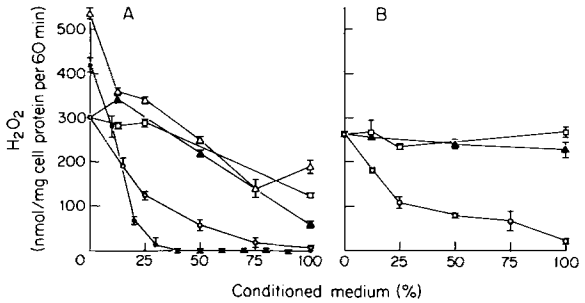

FIG. 3. (A) Dose-dependent effects of media conditioned by different cells. H_2O_2 release was measured 48 h after incubation of caseinate-activated macrophages in the indicated concentrations of dialyzed media conditioned by P815 cells at 5×10^6/ml for 10 h (○) or 1×10^6 for 48 h (●); by newborn rat fibroblasts (▲) or mouse spleen cells (□) at 5×10^6/ml for 10 h; or by a 75% confluent monolayer of human skin fibroblasts at 0.4 ml/cm^2 for 48 h (△). Means and SEM for triplicates are indicated for data from three experiments. (B) Rapid reversal of suppressive effect of media conditioned by spleen cells (□) and newborn rat fibroblasts (▲) but not by P815 cells (○). After 48 h incubation in the conditioned media at the indicated concentrations, the coverslips were washed and placed in MEM-HS for an additional 48 h before H_2O_2 release was measured in response to PMA.

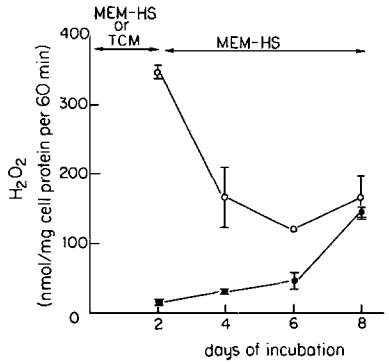

FIG. 4. Delayed reversal of suppression after removing TCM. Caseinate-activated macrophages were incubated for 48 h in TCM (●) or MEM-HS (○), washed, and placed in fresh MEM-HS alone. H_2O_2 release was measured at 2-d intervals.

increased to equal that of cells never exposed to TCM. Thus, the effect of TCM was reversible, but only after a long delay.

Effect of Lymphokines on Suppression of H_2O_2 Release by TCM. Resident or inflammatory mouse peritoneal macrophages can be induced by lymphokines to exhibit an enhanced capacity to release H_2O_2 (50, 52–54). It was of interest to learn whether lymphokines would prevent the suppressive effect of TCM on H_2O_2 release or hasten the reversal of this suppression after removal of TCM. As shown in Table II, a 2-d incubation in 10% Con A-induced lymphocyte supernatant plus TCM resulted in suppression. A 2-d incubation in lymphokines in the absence of TCM did not reverse the suppression seen with macrophages exposed to TCM during the previous 2 d. This was the case whether caseinate-activated or proteose peptone-elicited macrophages were used. The potency of the lymphokine preparation was confirmed by its ability to triple the H_2O_2-releasing capacity of the proteose peptone-elicited macrophages (Table II).

Effect of TCM on Secretion of ROI Triggered by PMA or Zymosan. TCM was unlikely to suppress apparent PMA-induced H_2O_2 release by interference with the binding of PMA to the macrophages or the reaction of H_2O_2 with the fluorescent indicator, because the macrophages were removed from TCM and washed before the assay. Nonetheless, to exclude these possibilities, we used a different stimulus for secretion and a different assay for detection of ROI. As shown in Table III, TCM suppressed O_2^- release from macrophages, as markedly as it suppressed H_2O_2 release. Moreover, the release of both substances was suppressed by TCM when zymosan was used as the stimulus instead of PMA. However, the degree of suppression using zymosan was usually less than with PMA. The reason for this difference is not known.

H_2O_2-Catabolizing Pathways in TCM-treated Macrophages. Suppression of H_2O_2 release from TCM-treated macrophages could result either from decreased synthesis of H_2O_2 or from its increased catabolism. To investigate the latter possibility, we measured the specific activity or specific content of the known major H_2O_2-catabolizing moieties in mononuclear phagocytes, namely, catalase, glutathione peroxidase, glutathione reductase, glutathione, and myeloperoxidase. As shown in Table IV, 48 h of exposure to P-10h resulted in decreases in the activity of catalase, glutathione peroxidase, and the content of glutathione, and no changes in the other factors. Thus, no increase in

TABLE II

*Effect of Lymphokines on Suppression of H_2O_2 Release by TCM**

Lymphocyte supernatant	Caseinate-elicited macrophages incubated days 0–2 with:		Proteose peptone-elicited macrophages incubated days 0–2 with:	
	MEM-HS	P-10h	MEM-HS	P-10h
Added on days 0–2:				
Con A stimulated‡	379 ± 32§	147 ± 41	ND‖	
Con A control¶	398 ± 81	144 ± 66		
No Con A added**	415 ± 36	112 ± 47		
None‡‡	381 ± 15	91 ± 30		
Added on days 2–4:				
Con A stimulated	368 ± 24§§	47 ± 3	126 ± 29§§	8.2 ± 0
Con A control	245 ± 5	48 ± 7	51 ± 20	8.2 ± 0
No Con A added	ND		38 ± 4	7.5 ± 1
None	416 ± 20	23 ± 3	45 ± 5	7.4 ± 1

* Macrophages from 2×10^6 peritoneal cells were incubated for 48 h in 90% MEM-HS or 90% dialyzed P-10h, plus 10% of the indicated lymphocyte supernatant, washed, and tested for H_2O_2 release on day 2 (upper half of table). Alternatively, 10% additional MEM-HS was added on day 0 instead of lymphocyte supernatant, and on day 2, all media were replaced with 90% MEM-HS plus 10% of the indicated lymphocyte supernatant, until testing for H_2O_2 release on day 4 (lower half of table).

‡ Spleen cells were incubated 48 h in enriched RPMI-fetal bovine serum. Con A (3 μg/ml) was added at the start of the incubation. The supernatant was dialyzed against MEM and added to the macrophage cultures at a final concentration of 10%.

§ H_2O_2, nmol/mg cell protein per 60 min, assayed on day 2. Mean ± SEM for three experiments, each in duplicate or triplicate.

‖ Not done.

¶ Prepared as in (‡), except that Con A was added at the end of the 48 h incubation of lymphocytes.

** Prepared as in (‡), except that no Con A was added.

‡‡ Macrophage cultures supplemented with an additional 10% MEM-HS instead of lymphocyte supernatant.

§§ H_2O_2, nmol/mg cell protein per 60 min, assayed on day 4. Mean ± SEM from a single experiment.

TABLE III

*Effect of TCM on Release of Hydrogen Peroxide and Superoxide from Caseinate-activated Macrophages**

Experiment	Medium	Hydrogen peroxide‡		Superoxide‡	
		PMA§	Zymosan‖	PMA	Zymosan
A	MEM-HS	267	426	69	22
	TCM¶	4.7 (98)**	112 (74)	8.8 (87)	12 (45)
B	MEM-HS	374	485	138	36
	TCM	15 (96)	169 (65)	19 (86)	28 (22)
C	MEM-HS	355	405	37	54
	TCM	28 (92)	239 (41)	8.7 (76)	17 (69)
D	MEM-HS	518	447	ND‡‡	ND
	TCM	38 (93)	280 (37)		

* Adherent cells from 2×10^6 peritoneal cells tested after 48 h incubation.

‡ nmol/mg cell protein per 60 min (mean of duplicates for H_2O_2; single determinations for O_2^-).

§ PMA 100 ng/ml.

‖ Opsonized with fresh mouse serum (see Materials and Methods). 2×10^7 particles per coverslip.

¶ Medium conditioned for 48 h by P815 cells and dialyzed before use.

** Percent inhibition.

‡‡ Not done.

TABLE IV

*Effect of TCM on H_2O_2-Catabolizing Activities of Macrophages**

Assay	Incubation medium	
	MEM-HS	P-10h
Catalase‡	2.00×10^{-3}	1.66×10^{-3}
Glutathione§	13.2	6.8
Glutathione reductase‖	55.6	50.7
Glutathione peroxidase‖	354	162
Myeloperoxidase¶	0	0

* Caseinate-activated macrophages were incubated for 48 h in the indicated media before assay. Data from one of two similar experiments.

‡ Baudhuin U/mg cell protein.

§ nmol of the tripeptide/mg cell protein.

‖ nmol NADPH oxidized/mg cell protein/min.

¶ Guaiacol and H_2O_2 as substrates.

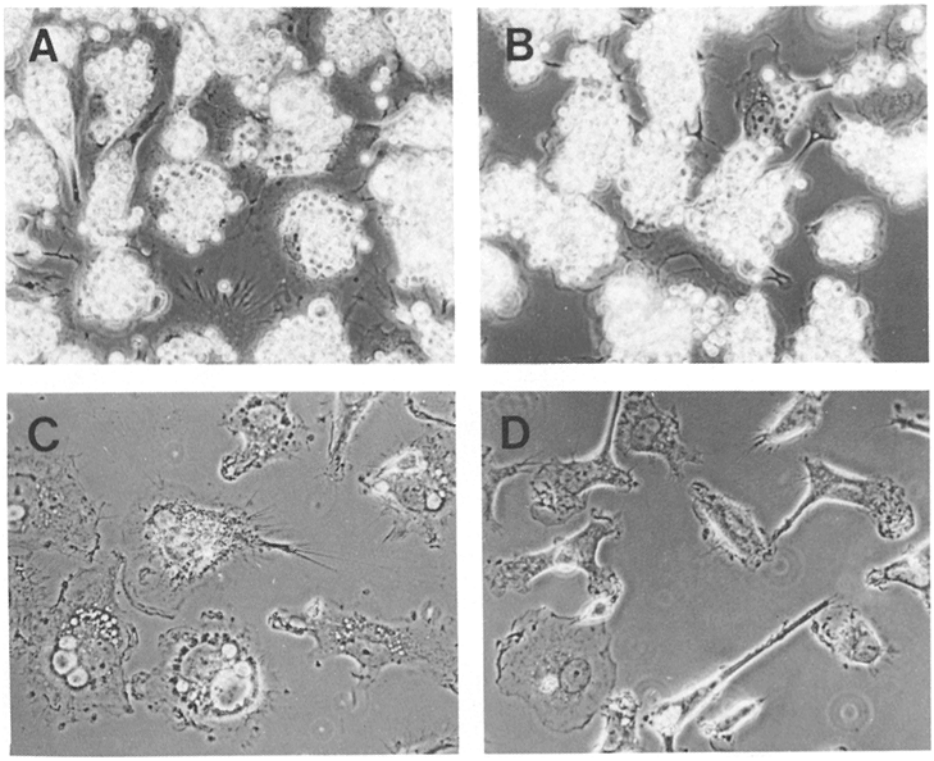

FIG. 5. Phase-contrast photomicrographs (× 1,340) of caseinate-activated macrophages incubated for 48 h in TCM (A, C) or MEM-HS (B, D), with (A, B) or without (C, D) 20-min exposure to starch granules from the seeds of *Amaranthus caudatus*.

H_2O_2-catabolizing pathways could be found to account for diminished H_2O_2 release after exposure to TCM.

Effect of TCM on Macrophage Morphology and Phagocytosis. The reversibility of its effects suggested that TCM was not toxic to macrophages. However, to assess the

possibility of toxicity more directly, we compared the ability of TCM-treated and control macrophages to phagocytize radiolabeled starch granules under conditions shown to maximize the detection of differences in phagocytic rates among various macrophage populations (47). TCM-treated macrophages were avidly phagocytic (Fig. 5 A), ingesting 4.5 ± 0.0 mg starch/mg cell protein in 20 min, compared with 5.4 ± 0.2 mg starch/mg cell protein in 20 min for control macrophages (Fig. 5 B). TCM-treated macrophages appeared morphologically normal by phase-contrast microscopy (Fig. 5 C). They tended to be more spread out on glass than the controls (Fig. 5 D), an effect noted by others culturing macrophages with tumor cells (55) or tumor-induced ascites (56).

Effect of TCM on Macrophage DNA and Protein Synthesis. We next addressed the question of whether TCM had a selective effect on secretion of ROI, or whether it suppressed macrophage metabolism in general. We focused first on DNA and protein synthesis because of the observation that the adherent cell protein content of macrophage monolayers incubated for 48 h in TCM averaged 30.5 ± 2.7 μg, compared with 20.4 ± 1.5 μg for cells incubated in MEM-HS alone (mean ± SEM for 25 experiments). This could be due to increased adherence of the TCM-treated cells. Alternatively, TCM might enhance cell replication or net protein synthesis per cell. As shown in Table V, TCM-treated, caseinate-activated macrophages incorporated

TABLE V

Effect of TCM on DNA and Protein Synthesis

Precursor	Incubation medium	
	MEM-HS	TCM*
	cpm/mg protein	
[^{3}H]Thymidine‡	(31 ± 19) × 10^3	(12 ± 7) × 10^3
[^{35}S]Methionine§	2.9 × 10^7	3.9 × 10^7

* Medium conditioned for 48 h by P815 cells and dialyzed before use.
‡ Mean ± SEM from three experiments.
§ Data from one of two similar experiments.

TABLE VI

Effect of TCM on Macrophage Secretory Products Other Than ROI

Secretory product	Macrophages	Incubation medium*	
		MEM-HS	TCM‡
Lysozyme§	Activated‖	137	106
Plasminogen activator¶	Activated	10.3	11.2
Arachidonate**	Activated	11.8 ± 2.8	7.9 ± 2.8
	Resident‡‡	38.3	34.6

* Macrophages were incubated for 48 h. Lysozyme was measured at that time. Plasminogen activator was measured after a further 48-h incubation in serum-free MEM. Arachidonate was measured after a further 24-h incubation in MEM-HS containing [^{3}H]arachidonic acid.
‡ Medium conditioned for 48 h by P815 cells and dialyzed.
§ μg/mg cell protein. Means from three experiments.
‖ Elicited with sodium caseinate.
¶ mU/mg cell protein (data from one of two similar experiments).
** pmol/μg cell protein, including both arachidonic acid and its oxidized metabolites (means ± SEM from four experiments).
‡‡ From untreated mice (means from two experiments).

small amounts of thymidine at a rate ~40% that of macrophages incubated in MEM-HS. Incorporation of methionine into protein was similar in both populations.

Other Macrophage Secretory Products. To determine whether TCM suppressed macrophage secretion nonspecifically, we compared the release of lysozyme, plasminogen activator, and arachidonic acid products from macrophages incubated for 48 h in TCM or MEM-HS. As shown in Table VI, each substance was secreted in similar amounts from both cell populations. It is of interest that caseinate-activated macrophages released less arachidonic acid than resident cells, as has been observed with macrophages elicited by agents other than casein (57, 58).

Discussion

Mononuclear phagocytes undergo dramatic shifts in their capacity to secrete ROI as they differentiate. Thus, human blood monocytes secrete abundant H_2O_2 in response to membrane stimulation. As monocytes mature in vitro into larger, more actively phagocytic cells, their ability to synthesize and secrete H_2O_2 wanes (59, 60). H_2O_2-secretory capacity of human macrophages can be reinduced by factors in the culture medium of stimulated lymphocytes.[2] Similarly, resident peritoneal macrophages in the mouse resemble human monocyte-derived macrophages with regard to their low levels of H_2O_2 secretion (24). Under the influence of lymphokines in vitro (50, 52–54) or the probable influence of antigen and antigen-specific T lymphocytes in vivo (61), such tissue macrophages gradually acquire a capacity for H_2O_2 secretion similar to that of the blood monocytes from which they were derived (59).

To this sequence can now be added another stage, the ability of certain cell-conditioned media, especially TCM, to induce a gradual, profound, relatively selective, nontoxic, and slowly reversible deactivation of macrophage oxidative metabolism. Thus, a secretory product of tumor cells appears to influence the macrophage in a manner opposite to certain secretory products of lymphocytes, with nearly the same time course. In our experiments, the suppressive effect of TCM predominated over the stimulatory effect of lymphokines. However, only a limited range of conditions was tested.

A principle suppressing macrophage H_2O_2-releasing capacity was detected in the culture medium of all 11 diverse types of tumors tested, as well as in medium conditioned by diploid fibroblasts, ovarian follicular cells, or an epithelial cell line. Little or no suppressive activity was provided by the medium of erythrocytes or macrophages, and variable amounts with spleen cells. The suppressive activity in the medium from nonmalignant cells could be diluted out much more readily than that in TCM. In addition, suppression induced by nonmalignant cell-conditioned medium was reversible within 2 d, whereas that induced by TCM did not reverse within 4 d and began to reverse by the 6th d after its removal. Whether the suppressive activities of malignant and nonmalignant cells represent different amounts of the same substance, or different substances, must await further characterization of the active materials.

Suppression of macrophage H_2O_2 release by TCM was recently described by Nelson et al. (62). In that study, macrophages were only exposed overnight to conditioned media, which might explain why suppression was less consistently observed than in

[2] A. Nakagawara, N. M. DeSantis, N. Nogueira, and C. F. Nathan. Lymphokines enhance the capacity of human monocytes to secrete reactive oxygen intermediates. *J. Clin. Invest.* In press.

the present work.

Thomas and Fishman (63) reported that tumor cells can interfere with the detection of H_2O_2 release from macrophages by competing for O_2 during the assay. Interference by tumor cells in the H_2O_2 assay did not appear to be involved in our experiments, because the effects were seen with TCM in the absence of tumor cells; the TCM itself was removed before the assay; and similar results were observed using a different assay for superoxide anion.

Some of the previously described effects of tumor cells on macrophages have been attributed to lactate dehydrogenase-elevating virus (13, 20). The P815 mastocytoma used for the majority of our experiments was shown to be free of this activity. Evidence of contamination by other viruses or by mycoplasma was not found. The factor suppressing macrophage oxidative metabolism passed through an ultrafiltration membrane with a cutoff of 300,000 daltons, suggesting that the active factor is not a virus. However, further work is needed to compare the activity studied in the present report with tumor-associated inhibitors of macrophage morphologic polarization in response to chemotactic stimuli (14). These inhibitors appear to be antigenically related to murine retroviruses (14). Tumor cells often secrete prostaglandins (64), and prostaglandins can impede macrophage activation as measured by H_2O_2-releasing capacity (65). However, the active factor in TCM is not likely to be a prostaglandin, because it was nondialyzable and was inactivated by trypsin and boiling (unpublished observations). Further characterization of the suppressive factor is underway.

Macrophages incubated in TCM appeared healthy and exhibited enhanced spreading often associated with "activation" (55, 56). They phagocytized starch granules and synthesized protein at about the same rates as controls. Moreover, TCM-treated macrophages secreted three products at rates similar to control cells: lysozyme, whose release from macrophages is constitutive and not much affected by their state of activation (40); plasminogen activator, whose secretion is inducible and much greater from activated or inflammatory than from resident macrophages (41); and arachidonic acid and its oxygenated metabolites, which are also secreted in response to triggering stimuli, although less extensively from activated and inflammatory macrophages than from resident cells (57, 58). We have not yet identified the individual arachidonate products secreted by TCM-treated macrophages, and therefore do not know what proportion of the released arachidonate is oxygenated. Nonetheless, it is noteworthy that arachidonic acid oxygenation constitutes a respiratory burst similar in its elicitation and kinetics to that which produces ROI. The minimal effect of TCM on the secretion of lysozyme, plasminogen activator, and arachidonic acid and its metabolites emphasizes the lack of toxicity of TCM and the relative specificity of its suppression of secretion of ROI.

In preliminary experiments, TCM-treated macrophages were markedly suppressed in their ability to kill certain intracellular protozoal pathogens, which normally succumb to oxygen-dependent microbicidal processes within macrophages (H. Murray, A. Szuro-Sudol, and C. Nathan, manuscript in preparation). This suggests that levels of ROI were diminished not only extracellularly but also intracellularly in macrophages exposed to TCM. These experiments indicate further that the impaired respiratory burst of TCM-treated macrophages is manifest not only upon challenge with PMA or zymosan, but also in response to physiologically relevant stimuli.

Transient suppression of macrophage oxidative metabolism by products of prolif-

erating nonmalignant cells, such as fibroblasts, might benefit the host during the healing stages of inflammation. Macrophages could then phagocytize antibody-coated (66) or other (67) debris without releasing ROI, which might otherwise damage nascent fibroblasts (68), parenchymal cells (69), endothelial cells (70), or ground substance (71). Perhaps tumor cells exaggerate and exploit such a regulatory mechanism. Profound and long-lasting suppression of macrophage oxidative metabolism by tumor cells may help them escape cytotoxicity, while setting the stage for the growth of opportunistic microbial pathogens.

Summary

Each of 11 tumors tested produced a factor that markedly suppressed the ability of macrophages to release H_2O_2 or O_2^- in response to phorbol myristate acetate or zymosan. Four of seven normal cell types produced a similar activity, which was 3.5–7 times lower in titer than that in tumor cell-conditioned medium (TCM), and which was much more rapidly reversible in its effects. TCM caused 50% inhibition of H_2O_2 release when it was present in the medium for 48 h at a concentration of 13%, or when 100% TCM was present in the medium for 18 h. The H_2O_2-releasing capacity of macrophages incubated in TCM only returned to control levels by 6 d after its removal. TCM prevented augmentation of H_2O_2-releasing capacity by lymphokines. The titer of suppressive activity in TCM depended on both the concentration of tumor cells and the duration of their incubation. TCM did not augment the activity of catalase, myeloperoxidase, glutathione peroxidase, or glutathione reductase or the content of glutathione within macrophages, suggesting that decreased synthesis rather than increased catabolism was responsible for reduced secretion of H_2O_2. Suppression of the release of H_2O_2 or O_2^- by TCM appeared to be a relatively specific effect, in that TCM increased macrophage spreading and adherence to glass while exerting little influence on rates of phagocytosis, synthesis of protein, or secretion of lysozyme, plasminogen activator, or arachidonic acid and its metabolites. Thus, tumor cells and some normal cells can secrete a factor that selectively deactivates macrophage oxidative metabolism.

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