Lymphokines Enhance the Capacity of Human Monocytes to Secrete Reactive Oxygen Intermediates

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A B S T R A C T Supernatants from mitogen- or antigen-stimulated human blood mononuclear cells enhanced the capacity of human monocytes or monocyte-derived macrophages (MDM) to release H_2O_2 or O_2^- in response to phorbol myristate acetate or zymosan. The stimulatory effect of lymphokines (LK) lasted ~5 d, regardless of the time of their addition. However, the magnitude of stimulation depended on whether LK were added to freshly explanted monocytes or to MDM. When LK were added on day 0 of culture, they enhanced MDM H_2O_2-releasing capacity ~40% measured on day 3, when H_2O_2-releasing capacity in the controls was maximal. Addition of LK on day 2 retarded the decline in H_2O_2-releasing capacity normally seen by day 5, so that LK-treated cells released about twice as much H_2O_2 as the controls. Addition of LK to MDM that had already lost most of their H_2O_2-releasing capacity (e.g., on day 4–6) restored it to an average of 60% of the values seen with freshly explanted monocytes. In this case, LK-treated cells were about 12 times more active than cells incubated in medium alone. The effects of LK were dose- and time-dependent, with maximal effects requiring 3 d of exposure. The specific activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and myeloperoxidase, and the specific content of glutathione were not diminished in LK-treated MDM, suggesting that increased synthesis of H_2O_2 rather than decreased catabolism probably explained the greater release of H_2O_2 from LK-treated cells. In contrast, release of H_2O_2 was suppressed 93±4% by exposing monocytes for 4 d to hydrocortisone (50%-inhibitory concentration, 1.9±0.3 × 10^{-7} M). Thus, the oxidative metabolism of human mononuclear phagocytes can be markedly modulated in vitro: augmented by mediators released from lymphocytes during an immune response, and suppressed by antiinflammatory corticosteroids.

INTRODUCTION

Studies with mouse peritoneal macrophages have revealed a close correlation between the capacity of the cells to release reactive oxygen intermediates (ROI)[1] such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), and their ability to inhibit the growth of certain intracellular pathogens (1–7). This has been demonstrated not only with macrophages activated in vivo by injection of microbial materials, but also with macrophages exposed in vitro to supernatants of antigen- or mitogen-stimulated lymphocytes (lymphokines [LK]) (1, 2, 5, 6). Incubation in LK enhanced the capacity of murine macrophages both to secrete H_2O_2 and to kill *Trypanosoma cruzi* (1), *Toxoplasma gondii* (2), *Leishmania enrietii* (5), and *Mycobacterium microti* (6).

Human blood monocytes release large amounts of ROI when freshly explanted and challenged with suitable immunologic, pharmacologic, or particulate stimuli (8–12). However, as the cells mature into monocyte-derived macrophages (MDM) over ~5 d in vitro, their capacity to secrete ROI declines sharply, until it comes to resemble that of the resident peritoneal macrophage of the mouse (12). Treatment of human

[1] *Abbreviations used in this paper*: Con A, concanavalin A; hk-BCG, heat-killed BCG; IC_{50}, 50%-inhibitory concentration; LK, lymphokines; LPS, bacterial lipopolysaccharide; MDM, monocyte-derived macrophages; MNL, mononuclear leukocytes; PMA, phorbol myristate acetate; PPD, purified protein derivative (tuberculin); ROI, reactive oxygen intermediates; SOD, superoxide dismutase.

mononuclear phagocytes with LK enhances their capacity to inhibit the intracellular replication of *T. gondii* (13, 14), *T. cruzi* (15), *Legionella pneumophila* (16), *Mycobacterium tuberculosis* (17), *Mycobacterium bovis* (17), and *Staphylococcus aureus* (18). We hypothesized, therefore, that treatment of human monocytes with LK would retard the normal decline in their capacity to secrete ROI, and that treatment of human MDM with LK would elevate their ROI-secreting capacity toward the high levels characteristic of activated rodent macrophages.

METHODS

Cultivation of human monocytes. Blood monocytes were separated from buffy coat obtained from the Greater New York Blood Center, as reported previously (12). In brief, the cells were diluted twofold in 0.9% NaCl, layered on Ficoll-Hypaque (ρ = 1.077) (Pharmacia Fine Chemicals, Piscataway, NJ), and centrifuged at 700 g for 20 min at 25°C. The mononuclear leukocytes (MNL) were collected, washed as described (12), and suspended in RPMI 1640 (Flow Laboratories, Inc., Rockville, MD) that contained 100 μg/ml streptomycin, 100 U/ml penicillin, and 25% human serum prepared as reported (12). 0.1 ml of a suspension of 1×10^7 MNL/ml was placed on a 13-mm-Diam glass cover slip (Clay-Adams, New York, NY) and incubated for 2 h at 37°C in 5% CO_2:95% humidified air. The nonadherent cells were removed by washing three times with Hanks' balanced salt solution (HBSS) or Eagle's minimum essential medium (Flow Laboratories) at 37°C. The cover slips were then placed in 0.3 ml of RPMI-25% human serum with antibiotics (12). The medium was replaced on days 1, 3, 5, and 7, unless indicated otherwise.

Preparation of LK. MNL were collected from purified protein derivative (tuberculin) (PPD)-positive donors as described above, except that the blood was anticoagulated with 40 U/ml heparin rather than citrate. 5 ml of cells (1×10^7/ml) in RPMI 1640 with 100 μg/ml streptomycin, 100 U/ml penicillin, 5×10^{-7} M 2-mercaptoethanol, 2 mM glutamine, and 25% fresh autologous serum ("enriched RPMI") were incubated for 2 h in 5% CO_2:95% humidified air in an 84-mm-Diam plastic tissue culture dish. Nonadherent cells were collected by replacing the medium three times with warm HBSS while agitating the dish. The recovered cells were centrifuged and suspended in enriched RPMI at 5×10^6/ml. 4 ml of the cell suspension were incubated in 50-mm-Diam plastic tissue culture dishes with $\sim 4 \times 10^4$ BCG (Pasteur type 1011; Trudeau Mycobacterial Collection, Trudeau Institute, Saranac Lake, NY), which had been heat-killed by autoclaving (heat-killed BCG [hk-BCG]). After 5 d at 37°C in 5% CO_2:95% humidified air, the medium was centrifuged to remove cells, dialyzed in Spectra/Por 6 tubing (cutoff, 1,000 daltons; Spectrum Medical Industries, Los Angeles, CA) for 24 h at 4°C against 100 volumes of RPMI 1640, sterilized by filtration (0.22 μm pore size; Millex, Millipore Corporation, Bedford, MA), and stored at -20°C until use. This fluid was designated BCG-LK. Control LK was prepared in the same manner, except that hk-BCG were added at the end of the 5-d incubation. The "medium control" consisted of enriched RPMI that was incubated for 5 d without cells, given hk-BCG, centrifuged, dialyzed, filtered, and stored as above. Concanavalin A (Con A)-induced LK, control LK, and the Con A medium control were prepared as above, except that hk-BCG was replaced in each case by 20

μg/ml Con A (Sigma Chemical Co., St. Louis, MO) and the supernate was collected at 2 d.

Secretion of H_2O_2 or O_2^-. The assays were conducted as described (12), using the fluorometric detection of horseradish peroxidase-catalyzed oxidation of scopoletin to detect H_2O_2, and the spectrophotometric detection of superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c to detect superoxide anion. Phorbol myristate acetate (PMA) (100 ng/ml) (Consolidated Midland, Corp., Brewster, NY) or opsonized zymoson (50 particles per monocyte) were used to trigger secretion of ROI, as described (12). No secretion of ROI was detected in the absence of triggering agents. The data are usually expressed as the mean±SEM for triplicates, divided by the mean protein content of triplicate monolayers that were treated exactly like those used to measure secretion of ROI. Protein was measured by the method of Lowry et al. (19), using bovine serum albumin as a standard.

Assays for O_2^-- or H_2O_2-catabolizing factors. SOD (20), catalase (21), glutathione (22), glutathione peroxidase (23), glutathione reductase (24), and myeloperoxidase (25) were measured by the cited methods, as described earlier (12), except that the concentration of Triton X-100 (Sigma Chemical Co.) in the cell lysate was 0.5%. This concentration of Triton X-100 did not affect the assays.

Effects of hydrocortisone. Where indicated, hydrocortisone phosphate (Sigma Chemical Co.) was added to the culture medium on day 0, and fresh medium containing the drug was replaced on day 2.

RESULTS

Dose-dependent effect of LK on H_2O_2-releasing capacity of human MDM. Monocytes were incubated for 4 d in RPMI 1640 with 25% human serum. The MDM were then exposed for an additional 2 d to LK and control media. On day 6, the cells were washed and tested for their ability to release H_2O_2 in response to PMA. As shown in Fig. 1, supernatant from lym-
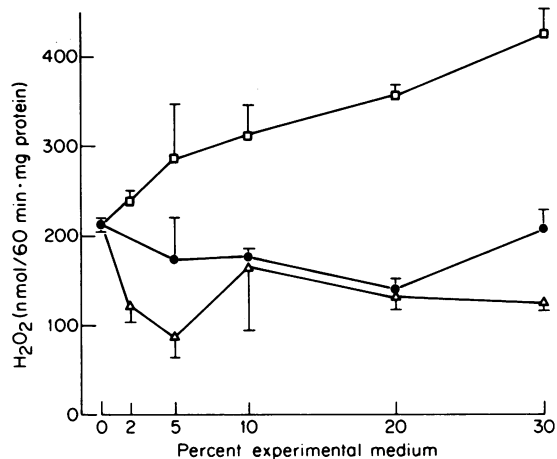

FIGURE 1 Effect of concentration of BCG-LK on H_2O_2 release from MDM. Monocytes were cultured for 4 d before LK and control media were added. Assays were performed 2 d later using PMA as a stimulus. Data are means±SEM for triplicates in a representative experiment. □, BCG-LK; ●, control medium; △, control LK.

phocytes of a PPD-positive donor cultured with hk-BCG (BCG-LK) doubled the H_2O_2-releasing capacity of the MDM, compared with MDM that had been cultured for the previous 2 d in RPMI 1640-human serum alone. This effect increased linearly with the concentration of BCG-LK over the range tested (2-30%). In contrast, no augmentation of H_2O_2-releasing capacity was seen after incubation in supernatant from the same lymphocytes to which hk-BCG was added just before removal of the lymphocytes (control LK). Likewise, RPMI 1640-human serum, which had been incubated for 5 d in parallel with the lymphocytes and exposed to BCG (medium control), did not increase the H_2O_2-releasing capacity of the MDM (Fig. 1).

The above LK were obtained from nonadherent MNL and were dialyzed against RPMI 1640 before use (14). When BCG-LK was prepared from unseparated MNL and used without dialysis, the dose-response curve usually peaked at lower concentrations than those shown in Fig. 1 (e.g., 2-5%), but showed suppressive effects at higher concentrations (e.g., 10-30%) (data not shown).

Dialyzed supernatants collected 2 d after stimulating nonadherent MNL with Con A (Con A-LK) gave results like those with BCG (Fig. 2). However, controls containing Con A also stimulated H_2O_2-releasing capacity (Fig. 2) (26).

H_2O_2-releasing capacity was elevated by LK whether expressed per cover slip or per milligram cell protein on concurrently measured, identically treated cover slips. LK had no consistent effect on the amount of cell protein per cover slip (data not shown). However, LK-treated cells were usually more spread out than controls.
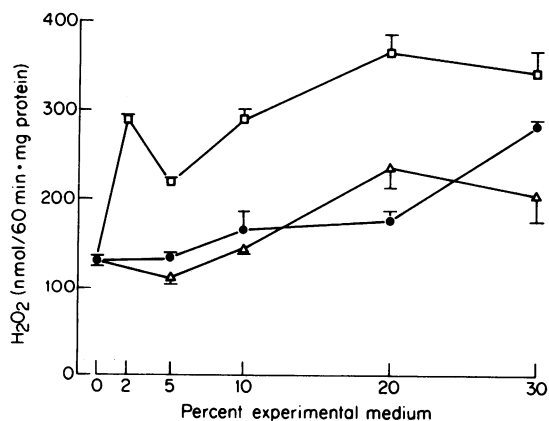

FIGURE 2 Effect of concentration of Con A-LK on H_2O_2 release from MDM. Monocytes were cultured for 4 d before LK and control media were added. Assays were performed 2 d later, using PMA as a stimulus. Data are means±SEM for triplicates in a representative experiment. □, Con A-LK; ●, control medium; △ control LK.
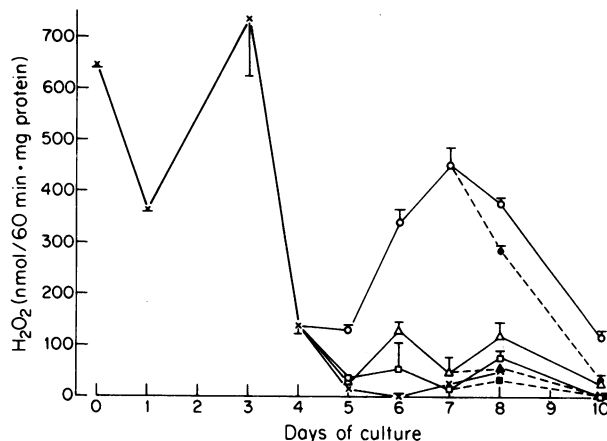

FIGURE 3 Kinetics of enhancement of H_2O_2 release by 10% BCG-LK (open circles), control LK (open triangles), or control medium (open squares) added to MDM on day 4 of culture. Values for cells incubated in medium alone are indicated by crosses. On day 7, some of the MDM were removed from LK and control media, rinsed, and placed in medium alone for 1-3 additional days (closed symbols). Data are means±SEM for triplicates in a representative experiment, using PMA as a stimulus.

Kinetics of LK effect. Monocytes were tested for H_2O_2 release on days 0, 1, 3, and 4 of culture (Fig. 3). H_2O_2-releasing capacity declined to 21% of the initial level by 4 d. At that point, the remaining MDM were incubated in 10% BCG-LK, control LK, or medium alone, for intervals ranging up to an additional 6 d. As shown in Fig. 3, 1 d of exposure to BCG-LK maintained H_2O_2 release at the same level as on day 4. In contrast, H_2O_2 release from MDM incubated from day 4 to 5 in control LK, control medium, or fresh medium alone continued to decline, reaching ∼4% of the day 0 value. After 2-3 d incubation in BCG-LK, H_2O_2-releasing capacity rose to 70% of the level seen on day 0. Thus, by the 7th d of culture, LK-treated MDM released from 9 to 30 times more H_2O_2 per milligram cell protein than the controls. Thereafter, H_2O_2-releasing capacity of LK-treated MDM fell sharply, although it remained higher than concurrent controls at all times tested. This decline was hastened when LK was replaced with fresh medium (Fig. 3).

Next, we examined the effect of adding BCG-LK for 3-d periods beginning on each of the first 7 d of culture. As shown in Table I, the rise in H_2O_2-releasing capacity usually seen on days 2-4 was higher when BCG-LK was present. The decline in H_2O_2-releasing capacity usually seen on days 5-6 was delayed by BCG-LK. Thus, monocytes treated with LK from days 2-5 released 96% as much H_2O_2 as those tested on day 0, whereas the controls released only 42% as much. After day 5, H_2O_2-releasing capacity declined in all

TABLE I

Effect of the Time of Addition of BCG-LK on H_2O_2 Release from Cultured Human Monocytes[*]

Incubation period	H_2O_2		
	12% BCG-LK	12% Control LK	12% Control medium
	nmol/60 min per mg protein		
Day 0	—	—	425±6‡
Day 0–3	827±107	455±114	609±46
Day 1–4	984±52	603±16	602±20
Day 2–5	410±22	198±24	180±24
Day 3–6	342±89	237±47	73±13
Day 4–7	122±9	51±15	73±11
Day 5–8	64±6	25±4	17±4
Day 6–9	38±3	0±0	0±0

* After the indicated periods of incubation, cover slips with adherent cells were rinsed and exposed to PMA to trigger the release of H_2O_2.
‡ Means±SEM from triplicate or quadruplicate cultures, except duplicate values for the controls assayed on day 3.

cultures, remaining relatively higher in those exposed to LK.

There was considerable experiment-to-experiment variation in the effect of LK added on different days of culture (compare Table I and Fig. 3); however, the observations emphasized above were repeatedly observed. Thus, in three experiments in which LK was added from day 0 to day 3, LK-treated cells released 1.4±0.3 (mean±SEM) times as much H_2O_2 per milligram cell protein on day 3 as did untreated cells on day 0, and 1.9±0.1 times as much as cells incubated in control media from day 0 to day 3. In five experiments in which H_2O_2 release on days 6–8 was compared to that of the same cell preparations on day 0, exposure to LK for 2–3 d resulted in 0.6±0.2 times as much H_2O_2 per milligram cell protein as for the freshly explanted cells, whereas samples of the same cells incubated 6–8 d in medium alone released only 0.06±0.03 times as much as on day 0. When MDM were exposed to LK for 2–3 d beginning on days 3–8 of culture, they released 4.0±0.9 times as much H_2O_2 per milligram cell protein as cells incubated concurrently in control LK (17 experiments), 6.3±2.0 times as much as cells in control medium (14 experiments), and 11.8±5.5 times as much as in medium alone (11 experiments).

Effects of LK assayed with another stimulus for H_2O_2 release and another assay for ROI. Either BCG-LK or Con A-LK augmented the release of ROI from MDM, whether PMA or opsonized zymosan was used as a stimulus, and whether H_2O_2 or O_2^- was measured (Table II). However, the amounts of O_2^- detected were much lower than the amounts of H_2O_2 measured si-

multaneously in matched cultures. The reason for the differential detection of H_2O_2 and O_2^- is not known. It was unaffected by including 1,000–2,000 Sigma units of catalase per milliliter in the assay for O_2^- to prevent possible reoxidation of ferrocytochrome c by H_2O_2 (27) (ratio of ferrocytochrome c detected with catalase:without catalase = 0.9±0.1 in four experiments).

Effects of LK on the activity of intracellular scavengers of ROI. LK might increase ROI-secreting capacity either by augmenting synthesis of ROI, or by decreasing intracellular catabolism of ROI. To investigate the latter possibility, we cultured MDM in BCG-LK, Con A-LK, or control media, and measured the specific activity of SOD, myeloperoxidase, catalase, glutathione peroxidase, and glutathione reductase, and the specific content of glutathione. As shown in Table III, all media containing Con A tended to increase the specific activity of SOD. None of the experimental media affected any of the other intracellular scavengers of ROI.

Effect of hydrocortisone on H_2O_2-releasing capacity. The experiments described above suggested that H_2O_2-releasing capacity of monocytes and MDM could be augmented by exposure to LK. We were interested to learn whether H_2O_2-releasing capacity could also be modulated downward. To test this, we exposed monocytes to hydrocortisone, which in pharmacologic doses can decrease the ability of the host to handle infections by intracellular pathogens (28). As shown in Fig. 4, H_2O_2 release from monocytes was nearly abolished by 4 d of exposure to 3×10^{-7} M hydrocortisone. 2 d of exposure to concentrations as much as 33-fold higher were without effect (Fig. 4), and 3 d of exposure gave an intermediate effect (data not shown). In three separate experiments like that shown in Fig. 4, 4 d of exposure to hydrocortisone inhibited

TABLE II

Effect of LK on H_2O_2 and O_2^- Release Triggered by PMA or Opsonized Zymosan[*]

Treatment	PMA		Opsonized zymosan	
	H_2O_2	O_2^-	H_2O_2	O_2^-
	nmol/60 min per mg protein			
12% Control LK	74±6	54±2	98±13	43±0.3
12% BCG-LK	303±19	71±3	216±28	65±0.6
10% Control LK	104±17	24±9	54±29	43±1.8
10% Con A-LK	267±25	64±13	164±17	90±21

Data are means±SEM from three experiments.
* LK was added on day 4 and replaced on day 6. The assays were conducted on day 8.

TABLE III
Effect of LK on Scavengers of O_2^- and H_2O_2 within MDM*

Treatment‡	Superoxide dismutase	Myeloperoxidase	Catalase	Glutathione peroxidase	Glutathione reductase	Glutathione (GSH + GSSG)
	U/mg protein	$\times 10^{-2}$ U/mg protein	$\times 10^{-3}$ U/mg protein	nmol/min/mg protein	nmol/min/mg protein	nmol/mg protein
Con A system						
Medium alone	38±5 (4)	0.38±0.07 (2)	11.4±2.5 (3)	154±48 (3)	70±15 (3)	35.0±3.3 (4)
Medium control	57±9 (4)	0.44±0.0 (2)	13.4±0.7 (3)	156±22 (3)	74±8 (3)	41.9±2.1 (4)
Control LK	62±7 (4)	0.45±0.07 (2)	12.1±1.1 (3)	141±20 (3)	70±10 (3)	39.5±3.5 (4)
Con A-LK	69±10 (4)	0.49±0.04 (2)	12.1±1.2 (3)	145±7 (3)	68±4 (3)	40.6±2.5 (4)
BCG system						
Medium alone	35	0.45	8.8	203	49	34.3
Medium control	36	0.51	8.1	177	38	38.6
Control LK	32	0.49	10.1	247	55	43.7
BCG-LK	38	0.47	10.4	249	49	45.2

* Mean±SEM for the number of experiments indicated in parentheses. Data for BCG system are from one experiment. LK were added on day 4 and assays conducted on days 7, 8, or 9.
‡ LK and medium control added at 10% (vol/vol).

H_2O_2 release from MDM by 93±4% (mean±SEM), with a 50%-inhibitory concentration (IC_{50}) of 1.9±0.3 $\times 10^{-7}$ M. Concentrations of hydrocortisone that suppressed H_2O_2 release were not toxic to MDM, as judged by both the morphology and the amounts of adherent cell protein of treated monolayers as compared with untreated controls.

DISCUSSION

Over the last 10 years, many changes in human monocytes or MDM have been observed after incubation in LK. These include enhanced adherence (29), oxidation of the first carbon of glucose (29), synthesis of nuclear RNA (30), phagocytosis of latex particles (30), activity of plasminogen activator (31), expression of Fc receptors (32), and cytotoxicity toward tumor cells (33, 34). Except for the last two features, most of these changes have been small. Moreover, none of them is likely to be directly related to the enhanced antimicrobial activity of LK-treated human mononuclear phagocytes observed with certain protozoal (13–15) and bacterial (16–18) pathogens. In contrast, the LK-induced enhancement of ROI-secreting capacity described in the present study may represent a close biochemical correlate of macrophage activation in man, as it does in the mouse (1–6). To establish such a correlation firmly, further studies will be needed in which oxidative metabolism and antimicrobial activity are measured in the same cell populations (18) and altered in parallel by a variety of interventions.

Antigen- or mitogen-induced lymphocyte products had different effects on the H_2O_2-secreting capacity of human blood-derived mononuclear phagocytes, depending on the time of addition of LK to the cultures. These differences appeared to reflect the time-dependent changes in ROI-secreting capacity that occurred spontaneously in untreated cultures. Thus, if LK were added to early cultures that could still secrete copious H_2O_2, LK elevated this capacity slightly during the first few days of incubation, and then delayed the decline usually observed by the fifth day. On the other hand, if LK were added to MDM that had already lost most of their capacity to secrete ROI, then H_2O_2 release was restored much of the way toward the high levels characteristic of freshly explanted monocytes.
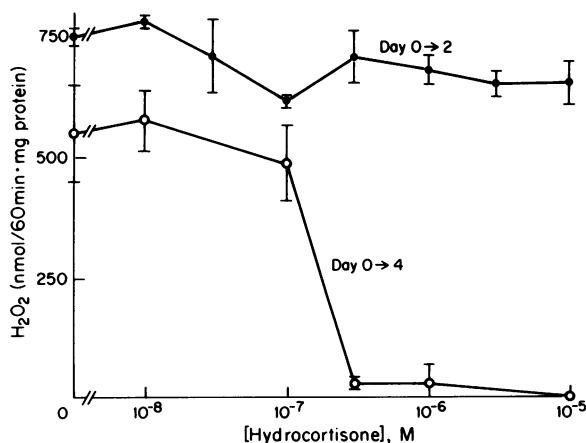

FIGURE 4 Effect of hydrocortisone on H_2O_2 release in response to PMA. Hydrocortisone phosphate was added at the indicated concentrations on day 0 and assays conducted on day 2 or on day 4. In the latter case, fresh medium containing hydrocortisone was added on day 2. Data are means±SEM of triplicates in a representative experiment.

These levels came within the range of values seen with mouse peritoneal macrophages activated in vivo or in vitro (1–6).

With time, ROI-secreting capacity of human MDM always declined to levels characteristic of mature macrophages from the uninflamed peritoneal cavity of the mouse. That is, the enhancement by LK of H_2O_2-releasing capacity was limited to a total period of $\sim$5 d, regardless of the concentration of LK or the time or frequency of its addition. It is not known if the transient nature of the action of LK reflects a physiologic response to the initial stimulus; a sequence of responses to a mixture of mediators, some of which may have a suppressive effect; an eventual response of the macrophage to some of its own secretory products, perhaps those induced by LK, such as prostaglandins (35); or a deficiency in some aspect of the in vitro environment.

The factors in the lymphoid supernatants responsible for augmenting ROI-secreting capacity have not been characterized. Thus, we do not know their relation to previously described LK that affect macrophages, such as migration inhibitory factor (36) or macrophage-activating factor (37, 38). Pabst et al. recently demonstrated an effect of bacterial lipopolysaccharide (LPS) on the O_2^--secreting capacity of human monocytes (39). Traces of LPS may have been present in our media. However, all components added to LK-rich media were also added to control media. The control media differed only in the time of addition of antigen or mitogen. Thus, any effects of LPS should have been present also in the controls. However, this leaves open the possibility of a synergistic interaction between LPS and LK (40).

LK did not alter the activity in human MDM of a wide variety of intracellular scavengers of ROI. Con A led to an increase in SOD activity, but this was not attributable to LK. Thus, it is most likely that increased detection of H_2O_2, released by MDM after exposure to LK, reflected increased synthesis of H_2O_2. As yet we have no information as to whether this results from induction of an oxidase or one of its cofactors or activators, or decreased activity of a regulator.

The H_2O_2-releasing capacity of human monocytes could be modulated downward as well as upward. This was shown with hydrocortisone, which nearly ablated detectable H_2O_2-releasing capacity in a dose- and time-dependent manner. The effective concentrations of hydrocortisone (IC_{50}, 2×10^{-7} M) are within the physiologic range. However, in the normal host, diurnal declines in cortisol levels may forestall the suppression of H_2O_2 release seen here with sustained exposures lasting 3–4 d. The need for such prolonged exposure may explain why much smaller suppressive effects were noted by Lehmeyer and Johnston, who added 10^{-3} M prednisolone to human monocytes only at the

time of assay for ROI (41). Likewise, Masur et al. did not observe decreased release of ROI from activated mouse peritoneal macrophages exposed to 2×10^{-4} M hydrocortisone for 24 h (42). The suppression of H_2O_2-releasing capacity of mononuclear phagocytes by prolonged exposure to hydrocortisone might be one factor in the tendency of pharmacologic doses of corticosteroids to reduce host resistance to a number of microbial pathogens (28).

It seems likely that there is a stage in the development of mononuclear phagocytes when the marrow precursor cannot mount a respiratory burst. By the time the monocyte circulates, it is endowed with the remarkable ability to respond to membrane stimulation by rapidly metabolizing large amounts of molecular oxygen to incompletely reduced and potentially toxic intermediates. However, the monocyte appears destined to lose much of this capacity as it matures into a macrophage, at least in vitro. At this point, its oxidative metabolism closely resembles that of the resident macrophage in the uninflamed mouse peritoneal cavity. The present findings suggest that during an immune response, lymphocytes release products, under the influence of which the oxidative metabolism of human mononuclear phagocytes can be markedly and reversibly enhanced.

ACKNOWLEDGMENTS

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