Brief Definitive Report

LYMPHOKINE ENHANCES OXYGEN-INDEPENDENT ACTIVITY AGAINST INTRACELLULAR PATHOGENS*

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A number of recent studies have demonstrated that mononuclear phagocytes use an oxygen-dependent mechanism to kill or inhibit the replication of a diverse group of intracellular pathogens (1–10). In the mouse peritoneal macrophage model, this mechanism, which generates superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), appears to be broadly effective since it contributes to the activity of normal and activated macrophages against ingested fungi (6), protozoa (1–5, 8, 9, 11), and mycobacteria (7). Although soluble lymphocyte products (lymphokines) are known to enhance the macrophage's oxidative and antimicrobial activity in parallel (4, 11), the ability of lymphokine-stimulated mouse macrophages to inhibit the growth of *Chlamydia psittaci* does not appear to require respiratory burst activity (12). This report extends the latter observation by demonstrating that once activated by lymphokine, oxidatively deficient human macrophages and mouse fibroblasts can successfully inhibit the intracellular replication of both *Toxoplasma gondii* and *C. psittaci*.

Materials and Methods

Cells. Monolayers of resident peritoneal macrophages from normal BALB/c mice (3), mouse L cells (fibroblasts) (12), and human monocytes derived from the peripheral blood mononuclear cell fraction (10, 13) were prepared and cultivated on glass coverslips in 5% CO_2-95% air at 37°C (3, 10, 12, 13). Peritoneal macrophages and L cells were cultured for 24 h before infection or assay in Eagle's minimum essential medium (Gibco Laboratories, Grand Island, N.Y.) containing 10–20% heat-inactivated fetal bovine serum (Gibco Laboratories), penicillin (100 U/ml), and streptomycin (100 μg/ml) (3, 12). Monocytes from normal donors and three patients with chronic granulomatous disease (CGD)[1] (10) were maintained for 9–16 d before use in RPMI 1640 medium (Flow Laboratories, Inc., Rockville, MD) with 20% heterologous heat-inactivated human serum and antibiotics, and were designated monocyte-derived macrophages (10, 13).

Lymphoyte Products (Lymphokines) and Cell Infection. Cultures of normal BALB/c spleen cells (5×10^6/ml) were stimulated for 48 h with 5 μg/ml of concanavalin A (Con A) (3,

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[1] After triggering with PMA (100 ng/ml), 1 d-old monocytes from these 3 CGD patients released 24 ± 9 nmol/mg per 90 min; ld cells from normal donors released 1,015 ± 58 nmol/mg per 90 min (10).

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11). Human mononuclear cells (3×10^6/ml) were cultivated in 5% CO_2-95% air at 37°C with 15 μg/ml of Con A for 48 h (10). Control (sham) lymphokines, which did not enhance phagocyte oxidative or antimicrobial activity, were obtained from unstimulated cell cultures to which Con A was added at the end of the 48 h incubation period (3, 10, 11). Supernates were centrifuged, sterilized by filtration, and stored at 4°C.

To induce microbistatic activity, (*a*) freshly plated peritoneal macrophages were cultivated for 24 h with 20% lymphokine plus 8 mg/ml of heart infusion broth (HIB) (Difco Laboratories, Detroit, MI) (11); (*b*) L cells did not require HIB and were incubated for 24 h with 20% mouse lymphokine alone; and (*c*) after 9–16 d in culture, monocytes were treated for 3 d with 10% fresh human lymphokine (10, 13). Unstimulated and activated cells were then challenged for 1–2 h with 1×10^6 RH strain *T. gondii* trophozoites (1) or one 50% infectious dose (ID_{50}) of *C. psittaci* (12). Uningested organisms were removed and standard medium was added. 18 h later, the extent of intracellular replication was assessed by enumerating the number of toxoplasmas per vacuole (1) and the percent of cells infected with *C. psittaci* (12). Microbial ingestion by control and activated cells was comparable (3, 10–12, 14).

Oxidative Burst Inhibitors. Lymphokine-stimulated cells were also treated (*a*) 3 h before and during infection with 2 mg/ml catalase or medium free of glucose (1), (*b*) 3 h before infection with 200 ng/ml phorbol myristate acetate (PMA) (15), or (*c*) for 24 h before infection with tumor cell conditioned medium (TCM) (16) provided by Dr. C. Nathan and Dr. A. Szuro-Sudol (The Rockefeller University, New York).

Assays for Oxidative Activity and Phagolysosomal Fusion. Cellular oxidative activity was assessed by the scopoletin method for H_2O_2 release (1, 4, 10, 13), qualitative nitroblue tetrozolium (NBT) reduction (10, 11), and luminol-enhanced chemiluminescence (17). The latter was inhibited >90% by superoxide dismutase (100 μg/ml) plus catalase (200 μg/ml). Phagosome-lysosome fusion was assessed by the acridine orange technique (1).

Results

Oxidative and Antimicrobial Activity of Unstimulated and Lymphokine-treated Cells. Resident peritoneal macrophages, L cells, and normal human macrophages displayed comparably low levels of oxidative activity, and supported the intracellular replication of both *T. gondii* and *C. psittaci* (Table I and Fig. 1). These unstimulated cells generated small amounts of H_2O_2 after PMA triggering, and showed relatively little or no NBT reduction or chemiluminescence in response to toxoplasma or chlamydial ingestion. The addition of lymphokine, however, induced all three cell populations to exert appreciable microbistatic activity. Consistent with previous observations (10, 11, 13), this acquired antimicrobial activity for human and mouse macrophages was paralleled by a clearly enhanced capacity to generate oxygen intermediates in response to either PMA or microbial ingestion (Table I). Lymphokine-stimulated L cells also achieved virtually identical toxoplasmastatic and chlamydiastatic effects, but displayed this activity in the absence of an augmented oxidative burst response.

Monocytes from patients with CGD, cells which generate scant amounts of O_2^- and H_2O_2[1] (5, 8, 10), were also cultivated for 9–16 d and then treated with lymphokine before infection. Like oxidatively deficient L cells, activated CGD macrophages readily inhibited the replication of both *T. gondii* and *C. psittaci* (Table I). Not unexpectedly, lymphokine stimulation did not enhance the CGD macrophage's minimal level of oxidative activity.

Effect of Oxidative Burst Inhibitors. To further document that the microbistatic effects of in vitro activated, oxidatively deficient cells did not require an intact respiratory burst, lymphokine-treated L cells were subjected to four conditions

TABLE I

Cellular Oxidative and Antimicrobial Activities

Cell population and treatment*	H_2O_2 Release‡	Percent cells NBT-positive§			Chemiluminescence‖		Antimicrobial activity¶	
	PMA	PMA	Zymosan	Toxoplasma	PMA	CP	Toxoplasmas per vacuole	Percent cells CP-infected
Resident mouse macrophages								
Control	78 ± 12	91 ± 2	82 ± 4	17 ± 4	29.2 ± 7.8	11.3 ± 4.2	4.6 ± 0.2	40 ± 5
+ LK + HIB	122 ± 10	89 ± 4	75 ± 2	59 ± 7	148.0 ± 91.2	33.9 ± 18.2	1.2 ± 0.2	10 ± 2
Mouse L cells								
Control	42 ± 7	0	—**	0	0.134	—	5.8 ± 0.3	46 ± 8
+ LK	35 ± 9	0	—	0	0.167	—	1.8 ± 0.1	5 ± 1
Normal human macrophages								
Control	129 ± 17	83 ± 4	82 ± 8	22 ± 4	—	—	4.8 ± 0.3	43 ± 6
+ LK	702 ± 68	93 ± 5	87 ± 3	69 ± 6	—	—	1.9 ± 0.4	7 ± 2
CGD macrophages								
Control	36 ± 11	<1	0	0	—	—	4.6 ± 0.4	40 ± 13
+ LK	49 ± 10	<1	<1	0	—	—	2.0 ± 0.3	6 ± 2

* Cells were cultivated in medium alone (control) or treated with lymphokine (LK) as described in Materials and Methods.

‡ nmol of H_2O_2 released per mg of adherent cell protein in 90 min after triggering with PMA (100 ng/ml) (1, 10). Results are the mean ± SEM of 3–19 experiments for each cell type performed in triplicate.

§ Percent of cells showing blue-black formazan precipitation 1 h after ingestion of opsonized zymosan particles (5×10^6/ml), viable toxoplasmas (1×10^6/ml), or stimulation with PMA (100 ng/ml) (1–3, 10). Mean ± SEM of three to four experiments.

‖ Maximum Δcpm/min $\times 10^{-3}$ during 15 min of exposure to PMA (100 ng/ml) or 50 ID_{50} of *C. psittaci* (CP) (17). Background Δcpm/min $\times 10^{-3}$ for unstimulated cells: mouse macrophages (control) 3.6 ± 0.2, (LK + HIB) 13.0 ± 8.2; L cells (control) 0.125, (+ LK) 0.287. Results are the mean ± SEM of 5–10 experiments for mouse macrophages and the mean of 2 similar experiments for L cells, each performed in duplicate.

¶ 18 h after infection. Results are the mean ± SEM of 5–9 duplicate experiments for each cell type for *T. gondii* (toxoplasma) and of 5–14 experiments for *C. psittaci* (CP). The mean percent inhibition of *C. psittaci* replication induced by lymphokine stimulation [(percent control cells infected − percent treated cells infected)/percent control cells infected] × 100 (12) was as follows: mouse macrophages, 75%; L cells, 89%; normal human macrophages, 84%; and CGD macrophages, 80%.

** Not done.

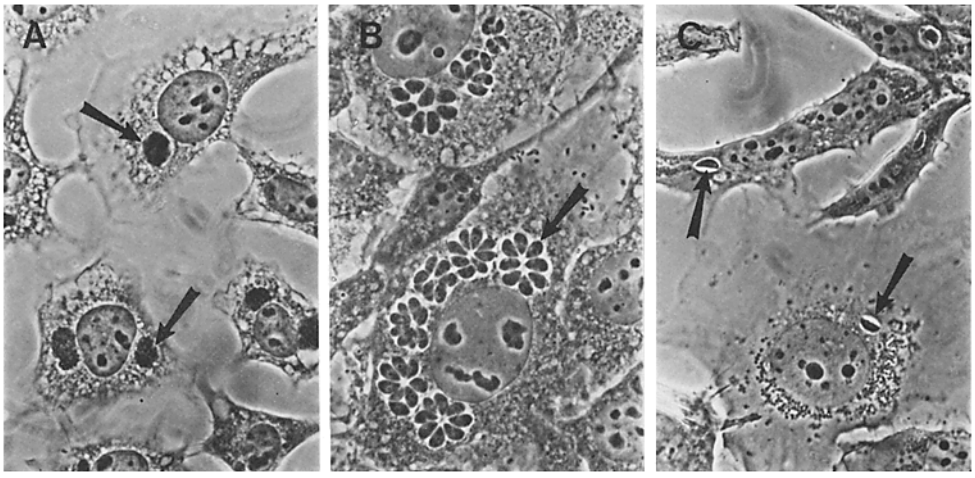

FIGURE 1. Phase-contrast micrographs showing mouse L cells 18 h after infection supporting the intracellular replication of *C. psittaci* (*A*) and *T. gondii* (*B*). In *A*, each of the large inclusions contains numerous chlamydiae. In *C*, L cells pretreated with 20% Con A lymphokine effectively inhibit *T. gondii* replication 18 h after infection. × 800.

TABLE II

*Effect of Oxidative Burst Inhibitors on Lymphokine-stimulated Antimicrobial Activity**

Pretreatment	Toxoplasmas per vacuole‡		Percent inhibition of C. psittaci replication‡	
	Mouse macrophage	L cell	Mouse macrophage	L cell
None	1.2 ± 0.1	1.9 ± 0.2	85 ± 4	83 ± 6
No glucose	3.6 ± 0.4	2.0 ± 0.2	80 ± 5	76 ± 3
PMA	3.4 ± 0.3	2.1 ± 0.3	75 ± 6	74 ± 4
Catalase	3.2 ± 0.3	1.8 ± 0.2	83 ± 4	85 ± 5
TCM	3.5 ± 0.4	2.2 ± 0.3	80 ± 3	81 ± 3

* Cells were activated overnight, and before infection were treated with the indicated agents as described in Materials and Methods. Results are the mean ± SEM of three to five experiments, 18 h after infection.

‡ For unstimulated control cells cultivated in medium alone, the number of toxoplasmas per vacuole was 4.7 ± 0.3 (mouse macrophages) and 5.8 ± 0.4 (L cells). Percent inhibition of *C. psittaci* replication was calculated as indicated in Table I legend. For control cells, 42 ± 6% (mouse macrophages) and 48 ± 5% (L cells) were infected with *C. psittaci*.

TABLE III

*Phagolysosomal Fusion within Microbistatic Cells**

Lymphokine-treated cells	Percent vacuoles stained at 2 h‡		
	Zymosan	Viable toxoplasmas	Dead toxoplasmas
Peritoneal macrophages	48 ± 6	10 ± 4	59 ± 7
L cells	ND§	4 ± 2	71 ± 5
CGD macrophages	62 ± 6	12 ± 5	69 ± 7

* Cells were activated for 1–3 d (see Materials and Methods), pulsed for 20 min with acridine orange, 5 μg/ml, and 30 min later were challenged for 1 h with 5×10^6 opsonized zymosan particles or 1×10^6 viable or heat-killed toxoplasmas (1). After 2 h of additional incubation, cells were examined using simultaneous phase-contrast and fluorescent microscopy for the transfer of acridine orange to phagocytic vacuoles (1).

‡ Percent of vacuoles housing ingested zymosan or toxoplasmas that stained bright red-orange. Unstained intravacuolar particles and parasites fluoresced pale green (1). Results are the mean ± SEM of three to four experiments performed in duplicate.

§ Not done.

known to inhibit the macrophage's capacity to secrete O_2^- and H_2O_2 by up to 90% (1, 15, 16). As previously reported, pretreatment with PMA (15), catalase (1), TCM (16), and medium free of glucose (1) all readily reversed the toxoplasmastatic activity of in vitro activated peritoneal macrophages (Table II). These same procedures, however, had no appreciable effect on either the inhibition of *T. gondii* replication by activated L cells or the chlamydiastatic capacity of lymphokine-stimulated L cells or macrophages (12).

Phagolysosomal Fusion within Microbistatic Cells. Since lysosomal agents may be important in oxygen-independent antimicrobial activity, and the ability to inhibit phagosome-lysosome fusion may aid *T. gondii* and *C. psittaci* in parasitizing normal cells (12, 18), we also explored whether fusion takes place in oxidatively deficient microbistatic cells. As judged by the transfer of acridine orange from prelabeled lysosomes (1), fusion readily occurred with vacuoles that housed ingested zymosan particles and heat-killed *T. gondii* (Table III). Within the same activated cell populations, however, <12% of viable intravacuolar toxoplasmas showed flu-

orescent staining, which suggested that lymphokine-induced toxoplasmastatic activity can occur in the absence of lysosomal fusion.

Discussion

These results suggest that in addition to their well established capacity to enhance the oxidative activity of both human and murine macrophages (4, 10, 11, 13), soluble lymphocyte products can also effectively stimulate and thus regulate an apparent oxygen-independent intracellular antimicrobial mechanism as well. Since the latter's toxoplasmastatic effects do not appear to depend upon phagolysosomal fusion, it would seem unlikely that the delivery of lysosomal enzymatic constituents directly mediate this microbistatic activity. Other investigators (5, 18) have similarly concluded that lysosomal fusion is not a prerequisite for the inhibition of *T. gondii* replication. It should be noted, however, that acidification of macrophage phagosomes may occur in the absence of fusion (19), and it is possible that some small molecular weight cytoplasmic proteins may not require lysosomal fusion for vacuolar entry. Thus, the effects of either or both of these two potential antimicrobial mechanisms might explain the activity we have observed. In addition, since the assays we used to designate cells as oxidatively deficient detect only the O_2^- and H_2O_2 secreted extracellularly, it is also still possible that lymphokine may have enhanced the intracellular production of O_2^- or H_2O_2, which then could have played some role.

Other studies with human monocytes have also provided data supporting the presence of this apparent oxygen-independent mechanism and its activity against intracellular pathogens. Although less active than normal cells, fresh CGD monocytes have been reported to kill 45–50% of ingested toxoplasmas (5, 8), and effectively inhibit *C. psittaci* replication (14). In addition, despite displaying little activity against the promastigote form of *Leishmania donovani* during the first 24 postphagocytic hours (whereas normal cells kill 80–90%), CGD monocytes can kill 40% of ingested promastigotes by 48 h, and this activity can be strikingly enhanced by lymphokine (10). However, lymphokine-activated CGD monocytes inhibit but do not kill the amastigote form of *L. donovani* (10), suggesting that intracellular pathogens display differential sensitivity to not only oxygen-dependent mechanisms (1–4), but to oxygen-independent agents as well. These studies indicate, therefore, that in addition to the effects of toxic oxygen intermediates, the presence, activity, and susceptibility to oxygen-independent mechanisms should also be considered when examining those factors which determine the fate of intracellular pathogens.

Summary

To determine if mechanisms other than the generation of toxic oxygen intermediates are active against intracellular pathogens, oxidatively deficient mouse L cells and monocyte-derived macrophages from patients with chronic granulomatous disease were stimulated with soluble lymphocyte products. Despite no enhancement in oxidative activity, these cells displayed effective microbistatic activity against both *T. gondii* and *C. psittaci*. These results suggest a potential role for nonoxidative mechanisms in the mononuclear phagocyte's activity against intracellular pathogens, and indicate that lymphokines can regulate both oxygen-dependent and oxygen-independent antimicrobial responses.

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