Brief Definitive Report

Co-infection of Macrophages Modulates Interferon γ and Tumor Necrosis Factor–induced Activation Against Intracellular Pathogens

By Carolyn M. Black,* Luiz E. M. Bermudez,‡ Lowell S. Young,‡ and Jack S. Remington*

*From the *Department of Immunology and Infectious Diseases, Research Institute, Palo Alto Medical Foundation, Palo Alto, California 94301, and Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305; and the ‡Kuzell Institute for Arthritis and Infectious Diseases, Medical Research Institute at Pacific Presbyterian Medical Center, San Francisco, California 94115*

Summary

Co-infection of macrophages (Mϕ) with *Toxoplasma gondii* and *Mycobacterium avium–intracellulare* complex (MAC) has been observed in patients with acquired immunodeficiency syndrome (AIDS). In this study we have demonstrated that co-infected murine Mϕ respond differently to cytokine stimulation than Mϕ infected with either of the microorganisms alone. Whereas treatment with interferon γ (IFN-γ) activated both single and co-infected groups of Mϕ to kill *T. gondii*, treatment with TNF did not influence the rate of MAC growth in co-infected Mϕ, in contrast with the inhibition of growth observed in MAC-infected Mϕ. These results suggest that in AIDS patients suffering infection with multiple intracellular pathogens, the ability of cytokines to stimulate microbicidal or static activity in mononuclear phagocytes can be impaired by the presence of more than one of the intracellular organisms.

Toxoplasma gondii and organisms belonging to the *Mycobacterium avium* complex (MAC) are two of the most common pathogens in patients with AIDS (1–2). Both *T. gondii* and MAC block phagosome-lysosome fusion (3, 4) and replicate inside of human monocyte-derived macrophages and murine macrophages (Mϕ) (5, 6). While the IFN-γ–activated Mϕ has been shown to be the key effector cell in host defense mechanisms against *T. gondii* (7), IFN-γ paradoxically induces enhanced intracellular survival of MAC within Mϕ (5, 8). TNF elicits no apparent effect on intracellular replication of *T. gondii* within Mϕ (9). In contrast, TNF-treated Mϕ exhibit static or cidal activity against intracellular MAC (5). Since in vivo production of these cytokines is probably triggered sequentially by infection, the outcome of TNF- and IFN-γ–induced activation of Mϕ during co-infection with these two intracellular pathogens becomes critical in severely immunocompromised patients. Stimulated by the clinical observation of mononuclear phagocytes harboring both *T. gondii* and MAC in the brain of a patient with AIDS, toxoplasmic encephalitis, and mycobacterial meningoencephalitis, we investigated the response of co-infected Mϕ to cytokine activation, and assessed whether successful fusion of lysosomes with phagosomes occurred in co-infected activated Mϕ.

Materials and Methods

Strains and Cytokines. *T. gondii* strain RH and *M. avium* complex strain 101 (serotype 1) were harvested and processed by methods described previously (5, 6). Recombinant murine IFN-γ (2.3×10^7 U/mg protein; <25 pg endotoxin/mg protein) and recombinant murine TNF-α (2.8×10^7 U/mg protein; 12 pg endotoxin/mg protein) were supplied by Genentech, Inc. (South San Francisco, CA). IFN-γ was stored before use at 4°C in a solution of 1×10^7 U/ml PBS containing 1 mg/ml BSA. TNF was stored undiluted at 4°C and never frozen.

Treatment of Macrophages. Unelicited murine peritoneal Mϕ were prepared as described (6) and incubated with 230 U/ml IFN-γ, 1,000 U/ml TNF, or medium alone (IMDM [Gibco Laboratories, Grand Island, NY] containing 10% FCS and 40 μg gentamicin/ml) as a control for 24 h at 37°C. The monolayers were then challenged with *T. gondii* alone (2 tachyzoites per Mϕ); MAC alone (10 bacilli per Mϕ); or with both microorganisms simultaneously at the same multiplicities of infection. After 2 h, all monolayers were thoroughly washed with PBS to remove extracellular microorganisms. At this time, to correspond to the 0 h time point, some of the monolayers on slides were fixed and stained with Diff-Quik stain (American Scientific Products, McGaw Park, IL) with the third step of the procedure omitted. The Kinyoun stain was then performed on the stained monolayers, omitting the counterstain step. This combined staining procedure allowed easy visualization

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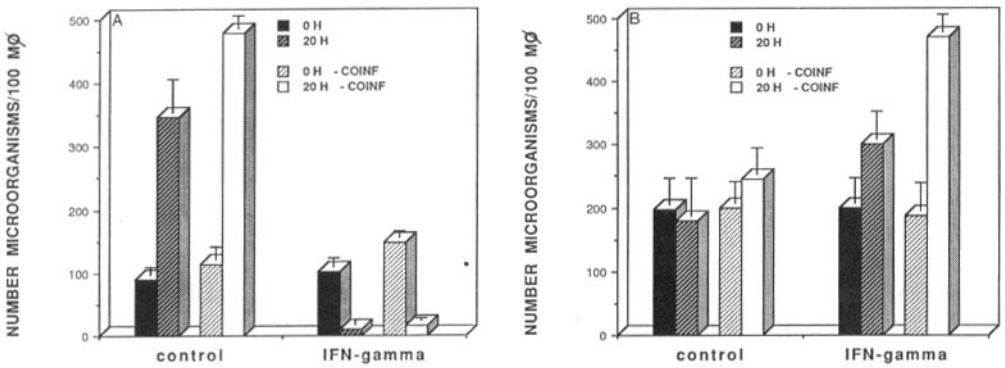

Figure 1. Effect of treatment of Mϕ with IFN-γ on intracellular growth of (*A*) *T. gondii* and (*B*) MAC after single infection (shown as columns marked *h*) and co-infection (shown as columns marked *h-coinf*) with these two agents. Bars represent the standard deviation.

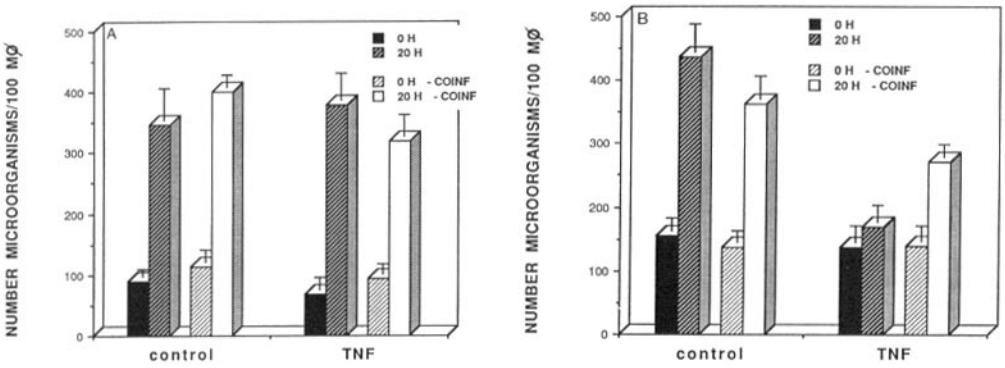

Figure 2. Effect of treatment of Mϕ with tumor necrosis factor (TNF) on intracellular growth of (*A*) *T. gondii* and (*B*) MAC following single infection (shown as columns marked *h*) and co-infection (shown as columns marked *h-coinf*) with these two agents. Bars represent the standard deviation.

and distinction between intracellular *T. gondii* and MAC organisms. The remaining monolayers were incubated an additional 20 h before staining.

Phagosome-Lysosome Fusion. Cytokine-treated and control macrophage monolayers were incubated for 20 min with acridine orange (5 μg/ml) in HBSS (Gibco Laboratories) at 37°C in a humidified atmosphere containing 5% CO_2 and infected as described above. This method by which phagosome-lysosome fusion was quantitated within infected Mϕ has been described and verified by electron microscopy (10, 11). After quantitation of fused phagolysosomes, the coverslips were removed and the slides were soaked briefly in xylene. After staining with modified Diff-Quik as described above, numbers of intracellular microorganisms per 100 Mϕ and percentages of infected cells were determined.

Results

Co-infection in Normal Mϕ. The percentage of infected Mϕ was 56.5 ± 10.2% for monolayers infected with *T. gondii* only, and 70.8 ± 12.7% for monolayers infected only with MAC. The percentages of infected Mϕ in co-infected monolayers were: 6.0 ± 0.9% contained intracellular *T. gondii* only, 21.2 ± 3.7% contained intracellular MAC only, and 50.8 ± 9.9% Mϕ contained both organisms. These percentages were unaffected by treatment of the Mϕ with cytokines. After 20 h of infection, numbers of intracellular *T. gondii* per 100 Mϕ increased nearly fourfold in Mϕ infected with *T. gondii* alone (Fig. 1 *A*). Numbers of intracellular MAC remained approximately the same after 20 h of infection in untreated Mϕ infected with MAC alone (Fig. 1 *B*). In Mϕ co-infected with both pathogens, numbers of intracellular *T. gondii* increased similarly as in Mϕ infected with *T. gondii* alone (Fig. 1 *A*; the apparently higher mean value for co-infected Mϕ was not significant), and numbers of intracellular MAC increased ~21% (Fig. 1 *B*). The results of experiments for which colony-forming units of MAC were counted in parallel by lysing infected and co-infected Mϕ and plating on agar showed percentage increases with time of infection similar to those measured by microscope assay (not shown).

Co-infection in IFN-γ- and TNF-treated Mϕ. In Mϕ activated in vitro with IFN-γ and infected with *T. gondii* alone or co-infected, numbers of intracellular *T. gondii* per 100 cells decreased ten-fold by 20 h post infection (Fig. 1 *A*). In marked contrast, numbers of intracellular MAC within IFN-γ-activated Mϕ increased 70% in singly infected cells and 215% in co-infected cells (Fig. 1 *B*). The intracellular growth of *T. gondii* within Mϕ treated with TNF was not significantly different from that within untreated control Mϕ regardless of co-infection (Fig. 2 *A*). However, smaller increases in numbers of intracellular *T. gondii* occurred consistently in co-infected Mϕ treated with TNF as opposed to Mϕ treated with TNF and infected with *T. gondii* alone (Fig. 2 *A*). Whereas the numbers of intracellular MAC increased 182% in singly infected, untreated control Mϕ and 165% in co-infected control Mϕ, this value increased only by 23% in TNF-treated Mϕ that were infected with MAC alone (Fig. 2 *B*). In contrast to this result, TNF-treated Mϕ that were co-infected with MAC and *T. gondii* showed a 144% increase in intracellular mycobacteria per 100 Mϕ. Since the 20-h in-

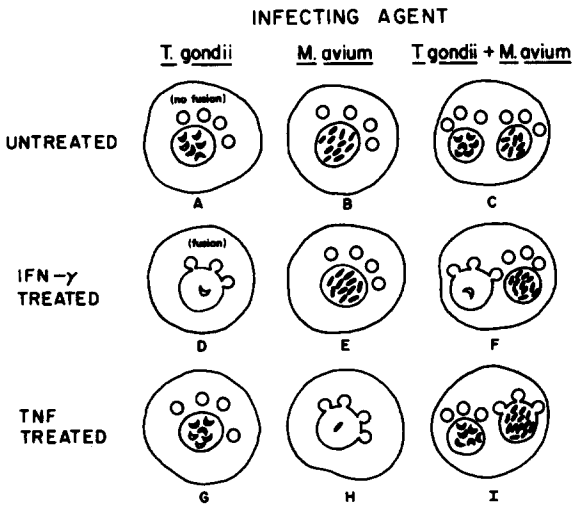

Figure 3. Schematic illustrating the relationship between intracellular fate (replication or inhibition) and fusion of lysosomes with phagosomes containing *T. gondii* or MAC (*M. avium*) in singly infected and co-infected Mϕ that are untreated or treated with cytokines. (*Large circles*) Mϕ; (*small circles*) phagosomes and lysosomes. (*Column on right*) Mϕ co-infected with both agents. (*A* and *B*) Both organisms replicate without fusion of phagosomes with lysosomes within untreated Mϕ; (*C*) these results were the same in co-infected Mϕ; (*D*) Growth of *T. gondii* is inhibited (successful fusion), while (*E*) MAC replicates (no fusion) within IFN-γ-treated Mϕ; (*F*) these fates were not changed within co-infected Mϕ; (*G*) *T. gondii* replicates (no fusion) but (*H*) growth of MAC is inhibited (successful fusion) within TNF-treated Mϕ; (*I*) in contrast, MAC replicated despite successful fusion in Mϕ co-infected with both agents. The fate of *T. gondii* was not altered in co-infected Mϕ.

Table 1. *Effect of Coinfection on Fusion of Lysosomes with Phagosomes Containing T. gondii or MAC or Both in Normal and Cytokine-treated Macrophages*

Infection	Percent cells showing fused phagolysosomes*		
	Control	IFN-γ–treated	TNF-treated
T. gondii only	8.0‡	25.2	8.1
MAC only	6.0	7.5	30.9
Coinfected: *T. gondii*§	4.0	14.5	0.0
MAC§	4.5	0.0	20.4
Both¶	0.0	2.5	5.5

* Percentages of infected cells were verified by staining the same monolayers post-acridine orange stain with a modified Giemsa/Kinyoun stain.
‡ Data are the means of duplicate monolayers.
§ Phagosomes within coinfected cells that contained only the indicated species.
¶ Phagosomes within coinfected cells that contained both infecting agents.

cubation period represents a relatively short time for the slow-growing MAC, a day-to-day experimental variation was observed in the multiplication of MAC in untreated Mϕ (Figs. 1 *B* vs. 2 *B*). Although inocula were constant for all experiments, small variations between experiments in number of MAC per Mϕ at time zero were observed and may have affected the rate of intracellular growth. In all experiments, however, the treated and control groups shown together were infected in parallel and treated in an identical fashion except for the presence of the indicated cytokine.

Fusion of Lysosomes with Phagosomes Containing T. gondii or MAC or both in Normal and Cytokine-treated Mϕ. The acridine orange stain made it possible to easily visualize the fused phagolysosomes and to distinguish intraphagosomal *T. gondii* from MAC. At 2 h post infection, an average of 6% of infected, untreated control Mϕ contained fused phagosomes regardless of whether co-infected or infected singly with *T. gondii* or MAC (range 4.0–8.5%; Table 1). In Mϕ activated with IFN-γ, however, the percentage of cells exhibiting fusion of parasitophorous phagosomes with lysosomes increased to over 25% in Mϕ infected with *T. gondii* alone and 14% in co-infected Mϕ (Table 1). In contrast, IFN-γ-activated Mϕ that were infected with MAC showed no significant increases in fusion of phagosomes with lysosomes. A reverse trend was observed in Mϕ treated with TNF: fusion of phagosomes with lysosomes was significantly increased in Mϕ that were infected singly or co-infected and contained only MAC within the phagolysosome, whereas, in contrast, little fusion was observed within Mϕ infected with *T. gondii* alone and within phagosomes of co-infected Mϕ that contained only *T. gondii*. In the case of phagosomes of co-infected Mϕ that contained both *T. gondii* and MAC, fusion was inhibited regardless of whether Mϕ were treated with cytokines (Table 1). These co-infected phagosomes were only observed occasionally, however. Most phagosomes observed contained only one species of infecting agent (not shown).

Discussion

The results presented in this report (summarized in Fig. 3) demonstrate that when Mϕ are co-infected with both *T. gondii* and MAC, the inhibition of intracellular growth of MAC that is usually observed within TNF-treated Mϕ is abrogated by the presence of the co-infecting *T. gondii*, in spite of successful fusion of phagosomes containing MAC with lysosomes. This result suggests that the clinical outcome of co-infections may be complicated due to exacerbation of one infection by the other, and that resolution of the infections may be prolonged regardless of therapeutic intervention.

For most intracellular pathogens, the ability to inhibit phagosome-lysosome fusion correlates with intracellular replication. This was true for *T. gondii* in all cases, and was true for MAC in normal and IFN-γ–treated Mϕ, in which the mycobacteria inhibited phagosome-lysosome fusion and replicated intracellularly. However, agents such as *Mycobacterium*

lepraemurium (12), *Nocardia asteroides* (10), and *Leishmania donovani* (13) are capable of multiplying within the fused phagolysosome. Our finding that MAC was capable of intracellular replication in co-infected cells in the face of fusion of phagosomes with lysosomes in TNF-treated Mϕ is therefore not wholly unexpected. It is surprising, however, that coinfection of TNF-treated Mϕ with both organisms did not alter the successful fusion of lysosomes with phagosomes containing MAC, but did alter the ability of the Mϕ to inhibit replication of MAC. This strongly suggests that, within TNF-treated Mϕ infected only with MAC, some antimycobacterial mechanism other than phagosome-lysosome fusion plays a critical role in the inhibition of intracellular growth. It is of interest that, in phagosomes of co-infected Mϕ that contained both pathogens, a strong inhibition of phagosome-lysosome fusion was observed, indicating that the ability of *T. gondii* to inhibit phagosome-lysosome fusion overcomes the ability of the TNF-treated macrophage to successfully fuse these vesicles as they do for phagosomes that contain only MAC. It should be noted, however, that phagosomes containing both agents were not common, and the data strongly suggest that *T. gondii* and MAC probably do not commonly reside in the same phagosomes within co-infected Mϕ. The fates of individual phagosomes within a co-infected Mϕ regarding fusion with lysosomes are therefore independent from each other.

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Address correspondence to Dr. Carolyn M. Black, Centers for Disease Control, Mail Stop GO5, Atlanta, GA 30333

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