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

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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.

T. 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, toxoplasmodic 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 × 10^6 U/mg protein; <25 pg endotoxin/mg protein) and recombinant murine TNF-α (2.8 × 10^6 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 × 10^6 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.
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% CO₂ 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 215% 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-
UNTREATED

IFN-γ TREATED

TNF TREATED

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 circle) 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.

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 Macrophages. 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 increase 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.

We thank Martin Wu for his excellent technical assistance during this study.

This work was supported by Public Health Service grant AI-04717 from the National Institutes of Health and from a MacArthur Foundation grant in molecular parasitology.

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Received for publication 29 January 1990 and in revised form 17 May 1990.

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