Opsonization Effects on *Mycobacterium avium* subsp. *paratuberculosis*-Macrophage Interactions

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High antibody titers in ruminants infected with *Mycobacterium avium* subsp. *paratuberculosis* correlates with disease progression. Effects of humoral responses during mycobacterial infection are not completely understood. This study suggests that activation status may be an important factor in determining macrophage ability to limit proliferation of opsonized *M. avium* subsp. *paratuberculosis*.

Generally, the humoral response present during mycobacterial infections has been considered nonprotective; however, the function and influence of the generated antibodies on the course of infection are incompletely understood. Development of high antibody levels in ruminants infected with *Mycobacterium avium* subsp. *paratuberculosis* correlates with deterioration of cell-mediated immunity and development of extensive multibacillary granulomatous lesions (9). The goal of the present study was to test the hypothesis that antibody opsonization of *M. avium* subsp. *paratuberculosis* would not enhance mycobacterial responses in macrophages. We compared the interaction of cultured naive bovine macrophages with *M. avium* subsp. *paratuberculosis* opsonized with serum from naive or infected animals with high anti-*M. avium* subsp. *paratuberculosis* titers.

Immune serum was pooled from five adult bovine cases of *M. avium* subsp. *paratuberculosis* infection that were confirmed by serum enzyme-linked immunosorbent assay for anti-*M. avium* subsp. *paratuberculosis* antibodies and gross and microscopic pathology findings. Anti-*M. avium* subsp. *paratuberculosis* serum levels were determined for each animal by the Iowa State University Veterinary Diagnostic Laboratory using an IDDEX system according to the manufacturer’s instructions. We further titrated the positive serum samples to an endpoint opsonizing activity (indirect immunofluorescence) at >1:500 dilutions for each. Naive serum was pooled from two adult bovines maintained free of infection. Monocytes were generated from peripheral blood mononuclear cells and differentiated into macrophages by 7 days of culture. The 19698 strain of *M. avium* subsp. *paratuberculosis* was obtained from the American Type Culture Collection (Manassas, Va.) and conjugated with fluorescein isothiocyanate (FITC) by a previously described method (1). Bacteria were opsonized for 1 h at 37°C with complete or heat-inactivated serum.

We used fluorescence microscopy to determine bacterial uptake/adherence by scoring 100 macrophages cultured on chambered slides (Nalge Nunc International, Naperville, IL) as either containing or lacking FITC-tagged *M. avium* subsp. *paratuberculosis* at 1 h postinfection. As shown in Fig. 1, opsonization with naive serum (NS), immune serum (IS), and heat-inactivated immune serum (HIS) led to similar uptake, which was increased over nonopsonized bacteria. Opsonization with heat-inactivated NS (HNS) led to similar uptake as nonopsonized bacteria (*P* < 0.05). Based on these results, complete IS and NS were similar in the ability to promote macrophage uptake of *M. avium* subsp. *paratuberculosis*, as were complement and anti-*M. avium* subsp. *paratuberculosis* antibody.

To examine kinetics of uptake/adherence, we determined the phagocytic index (mean fluorescent intensity in R1 × percent gated in M1) of macrophages infected with FITC-tagged bacteria by flow cytometry (Fig. 2). At 30 min postinfection, nonopsonized and HIS- and HNS-opsonized bacteria had the lowest and IS- and NS-opsonized bacteria the highest phagocytic indices (*P* < 0.05). From 60 min on, the phagocytic index of HIS-opsonized bacteria increased to a value similar to IS- and NS-opsonized bacteria, while nonopsonized and HNS-opsonized bacteria remained low (*P* < 0.05). These data indicate that bacterial uptake over time increased regardless of

![FIG. 1. Effect of serum opsonization on uptake/adherence of *M. avium* subsp. *paratuberculosis* by macrophages. The percentage of macrophages containing at least one bacterium was determined by fluorescence microscopy. Values are the means of three replicates of the experiment ± the standard error of the mean. The asterisks indicate significant decreases in the HNS and no-treatment groups below the IS, HIS, and NS treatment groups (*P* < 0.05).](http://cvi.asm.org/Downloaded from on July 19, 2018 by guest)
opsonization treatment. HIS opsonization, however, did not enhance uptake/adherence until 60 min postinfection. Potential mechanisms for this brief delay would include increasing surface expression of Fcγ receptors and/or integrin receptor expression with subsequent potentiation of Fcγ receptors (7, 11).

We used a standard CFU assay to evaluate proliferation of opsonized and nonopsonized *M. avium* subsp. *paratuberculosis* recovered from lysates of infected resting and activated macrophages. Colonies were counted after 4 weeks of incubation in a 37°C incubator. To account for differences in macrophage uptake of opsonized and nonopsonized bacteria, we determined the percent change in CFU between 4 and 48 h postinfection. To prevent antibody-mediated agglutination interference with CFU data, macrophages were washed thoroughly with medium prior to lysis to remove any agglutinating antibody. A significant opsonin effect on *M. avium* subsp. *paratuberculosis* growth was not identified in resting macrophages, where variation in CFU recovery was high (data not shown). There was a trend for reduced survival of IS-, HIS-, and NS-opsonized bacteria compared to nonopsonized bacteria in resting cells, again which did not reach statistical significance. CFU variation was reduced in lysates from activated macrophages, which is potentially due to synchronization of macrophage responses following gamma interferon/lipopolysaccharide treatment.

As shown in Fig. 3A, opsonization with IS, which is rich in complement and anti-*M. avium* subsp. *paratuberculosis*, appears to favor bacterial growth in activated macrophages. This may be due to predominate complement receptor-mediated uptake, or alternatively interaction between Fc and complement receptors. Use of multiple receptor types for entry of *M. tuberculosis* into macrophages has been described previously, and it is hypothesized that in vivo this may be the most relevant mechanism of uptake (2, 6a). In contrast, HIS-opsonized bacteria had restricted growth in activated macrophages, comparable to nonopsonized bacteria. This likely was mediated by Fc

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**FIG. 2.** Kinetics of uptake/adherence of opsonized *M. avium* subsp. *paratuberculosis*. (A) Dot blot assay demonstrating macrophage gate R1. (B) Histogram showing uninfected macrophages. (C) Histogram demonstrating macrophages containing fluorescently tagged bacteria defined as falling within the M1 region, which was determined by comparison of infected macrophages with uninfected macrophages. (D) Kinetics of adherence/uptake of opsonized and nonopsonized bacteria at 30, 60, and 120 min postinfection by flow cytometry. The phagocytic (Ph.) index was calculated by multiplying the mean fluorescence intensity by the percentage gated in M1. The value reported at each time point is the mean of three replicates of the experiment ± the standard error of the mean. SSC, side scatter; FSC, forward scatter; Gm, geometric mean; CV, coefficient of variance.
receptor uptake and initiation of bactericidal mechanisms. Unexpectedly, growth of HNS-opsonized bacteria did not parallel the nonopsonized bacterial data. This is potentially through the influence of additional opsonic factors in the absence of specific antibody and complement, which would include natural antibodies and collectins (5, 8, 10).

To further examine the ability of Fc receptor-mediated control of bacterial proliferation, we incubated activated macrophages with anti-CD18/CD11b (VMRD, Pullman, WA) to block bovine complement receptor 3. We infected the macrophages with anti-CD18/CD11b (VMRD, Pullman, WA) to control of bacterial proliferation, we incubated activated macrophages and collectins (5, 8, 10).

specific antibody and complement, which would include natural antibodies and collectins (5, 8, 10). To further examine the ability of Fc receptor-mediated uptake while opsonization with both complement and material killing in activated macrophages via Fc receptor-mediated uptake while opsonization with both complement and antibody favors survival.

We next asked whether complement or antibody opsonization would influence intracellular trafficking of M. avium subsp. paratuberculosis. To address this, we examined phagosome acidification by measuring colocalization of FITC-tagged bacteria with Lysotracker Red using laser confocal microscopy as previously described (4). As shown in Table 1, the percent acidified phagosomes containing HIS-opsonized bacteria started high and then decreased over time, while nonopsonized bacteria had the opposite pattern. NS induced a steady level of phagosome acidification. At the last time point, HIS- and NS-opsonized bacteria had lower levels of phagosome acidification than nonopsonized bacteria (P < 0.05). These data demonstrate that opsonins altered phagosome acidification patterns. However, increasing phagosome acidification does not coincide with increased mycobacterial killing, which has also been demonstrated for macrophages infected with M. avium subsp. avium (3). Differences in survival also do not appear to be directly mediated by nitric oxide production, as these values were similar among opsonization groups in activated and resting macrophages (data not shown).

Taken together, these data suggest that macrophage activation status is a factor in determining the ability of macrophages to control proliferation of antibody-opsonized mycobacteria. This conclusion does not support our initial hypothesis that humoral responses would not promote macrophage mycobacterial functions. It seems, therefore, that given the appropriate environment (successful macrophage activation and predominate antibody opsonization) humoral responses to M. avium subsp. paratuberculosis may have protective capacity.

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