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In Vivo-Activated Mononuclear Phagocytes and Protective Immunity to Chlamydiae in Mice

ROBIN E. HUEBNER† AND GERALD I. BYRNE*

Department of Medical Microbiology, University of Wisconsin Medical School, Madison, Wisconsin 53706

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Peritoneal macrophages (Mϕs) collected from *Chlamydia psittaci* 6BC-immune mice after intraperitoneal challenge with 10^6 6BC (immune-boosted [IB] Mϕs) were compared by various functional criteria with other in vivo- and in vitro-activated Mϕ populations. While casein-, protease peptone-, and thioglycolate (Thio)-elicited Mϕs were equally susceptible to in vitro infection with 6BC, IB Mϕs did not support chlamydial growth and Mϕs from *Mycobacterium tuberculosis* BCG- or *Listeria monocytogenes*-sensitized mice exhibited intermediate susceptibility to infection. The resistance of IB Mϕs was not due to the ingestion of fewer 6BC organisms, nor were these cells persistently infected, since chlamydiae could not be recovered from infected IB Mϕs after in vitro infection, even after extended incubation times. In contrast, Thio Mϕs stimulated in vitro with gamma interferon (IFN-γ), with or without lipopolysaccharide, resulted in cells that exhibited chlamydiastatic activity which was lost shortly after IFN-γ was removed from the culture medium. Conversely, the antichlamydial activity of IB Mϕs was stable over time but not through the production of autostimulatory cytokines, as evidenced by the lack of stimulation of Thio Mϕs to restrict 6BC replication in coculture experiments. IB Mϕs exhibited enhanced oxidative activity, but anti-IFN-γ antibody did not abrogate this response. IB Mϕs were recovered only from immunized mice that survived an otherwise lethal 6BC intraperitoneal challenge. These cells appear to be important for development of protective immunity to chlamydiae, and evidence suggests that stimulation by cytokines other than IFN-γ (with or without lipopolysaccharide) is required for the observed heightened in vivo activation.

Chlamydiae are obligate intracellular procaryotic pathogens that multiply within membrane-bound vesicles (inclusions) in the cytoplasm of susceptible eucaryotic cells. *Chlamydia trachomatis* is almost exclusively a human pathogen, causing ocular, genital, and systemic infections (16). *Chlamydia psittaci* affects a wide range of avian, mammalian, lower invertebrate, and nonvertebrate hosts. The diseases caused by this organism are as varied as the host range, from enteritis in birds, to abortions and polyarthritis in sheep, to pneumonia and encephalitis in humans.

Several reports have been published on the interactions between chlamydiae and professional phagocytes such as monocytes, macrophages (Mϕs), and polymorphonuclear leukocytes. Yong *et al.* (19) found that after 60 min of incubation with human polymorphonuclear leukocytes, there was a 3-log decrease in the number of viable *C. trachomatis*. Polymorphonuclear leukocytes from patients with chronic granulomatous disease had a chlamydiacidal activity equivalent to that of normal polymorphonuclear leukocytes, suggesting that killing was independent of toxic intermediates of oxygen metabolism. Wyrick et al. (17) evaluated the survival of *C. psittaci* in normal and elicited peritoneal Mϕs in vitro and found that the organisms were internalized within an hour after infection and had differentiated into reticulate bodies that multiplied and matured into infectious elementary bodies capable of initiating a new round of infection upon lysis of the Mϕ. Heat inactivation or opsonization of the chlamydiae with immune sera promoted their destruction through phagolysosome fusion (17).

Gamma interferon (IFN-γ) has been demonstrated to be a potent Mϕ-activating factor, capable of enhancing the antimicrobial activity of Mϕs to a variety of organisms including *Listeria monocytogenes*, *Leishmania donovani*, and *Toxoplasma gondii*. Murine Mϕs and human monocytes stimulated in vitro with IFN-γ also were found to restrict in vitro *C. psittaci* growth (1). This inhibition was not bactericidal, however, as removal of IFN-γ resulted in a resumption of inclusion development.

We previously described a murine model for acquired resistance to chlamydiae in which mice immunized subcutaneously with 5×10^2 viable *C. psittaci* 6BC were protected from an otherwise lethal intraperitoneal challenge with 10^6 homologous organisms. Examination of the cell- and antibody-mediated immune responses revealed that the establishment of protection correlated temporally with the induction of activated Mϕs which presumably restricted in vitro chlamydial growth. The Mϕs are now described in further detail, including comparisons with other in vivo- and in vitro-activated Mϕ populations.

MATERIALS AND METHODS

Mice. A/J mice, 6 to 8 weeks old, were purchased from Jackson Laboratory, Bar Harbor, Maine. The mice were housed under routine conditions in the University of Wisconsin Medical School animal care facilities. Five mice were housed per filter top cage, and the animals were fed and received water ad libitum. For some experiments, C3H/HeN mice were used for comparative purposes. These mice were purchased from Jackson Laboratory and housed as described for A/J mice.

Growth of *C. psittaci*. The 6BC strain of *C. psittaci* was used in all experiments. Confluent monolayers of mouse L929 fibroblasts were infected with 10 ID_{50}s of 6BC (ID_{50}, the volume of a suspension of 6BC required to infect 50% of a given number of L cells) in Hanks balanced salt solution

* Corresponding author.

† Present address: Department of Epidemiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, MD 21205.

(HBSS; GIBCO Laboratories, Grand Island, N.Y.) for 1 h on a rotary shaker. The medium was aspirated, and the cells were incubated for 2 days at 37°C in 5% CO_2 in medium 199 (Hazelton Research Products, Lenexa, Kans.) supplemented with 1.2% sodium bicarbonate, 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah), 100 μg of streptomycin sulfate, 10 μg of gentamicin sulfate, and 2 μg of cycloheximide per ml. After incubation, the cells were trypsinized, sonicated, and centrifuged at 500 × *g* for 10 min to remove cell debris and then at 10,000 × *g* for 30 min to collect the chlamydiae. Phosphate-buffered saline containing 0.2 M sucrose and 2% heat-inactivated fetal bovine serum was used to resuspend the chlamydia-containing pellet. The number of viable elementary bodies was quantitated by the tissue culture ID_{50} method (6). Purified elementary bodies were prepared by Percoll gradient centrifugation (13).

Preparation of radiolabeled 6BC. ^{3}H-labeled 6BC organisms were prepared by first infecting confluent L929 monolayers as described above. Approximately 12 h after the start of infection, the medium was replaced with leucine-deficient minimum essential medium (GIBCO) supplemented as described previously and containing 10 μCi of [^{3}H]leucine (Amersham, Arlington Heights, Ill.) per ml and a suboptimal concentration of unlabeled leucine. Incubation at 37°C was continued for an additional 54 h, when the chlamydiae were harvested as described above. Purified elementary bodies were prepared by Percoll gradient centrifugation (13).

Mϕ harvest. Immune-boosted (IB) Mϕs were obtained by immunizing mice subcutaneously with 5 × 10^2 viable 6BC and, after 10 days, by challenging intraperitoneally with 10^6 6BC. Peritoneal exudate cells were obtained 4 days postchallenge by lavage of the peritoneal cavity with HBSS containing 5% acid citrate glucose. The cells were pooled in plastic tubes, centrifuged at 1,000 × *g* for 10 min, resuspended in MEM, enumerated with a hemacytometer, and diluted for use at the appropriate concentrations.

In some experiments, mice were injected intraperitoneally with 1 ml of thioglycolate (Thio)–10% Proteose Peptone–6% sodium caseinate–10^6 viable *Mycobacterium tuberculosis* BCG or *L. monocytogenes* 3 or 4 days prior to cell harvest. In other experiments, *L. monocytogenes*-injected mice were restimulated with a live inoculum of *L. monocytogenes* 7 days after the initial sensitization (*Listeria*-boosted mice).

Infection of Mϕs. Mϕs were collected as described above, plated into triplicate, cover slip-containing, 24-well cell culture dishes at a density of 5 × 10^5 cells per well, and allowed to adhere in MEM at 37°C. The nonadherent cells were removed, and 0.1 to 2 ID_{50}s of 6BC, in 1 ml of MEM, was added to each well. In most experiments, the inoculum of 6BC used was chosen to maximally infect 50% of the control cells (Thio Mϕs). Although there was variability in the maximal number of inclusion-containing cells observed from experiment to experiment, the system was internally consistent. After 24, 48, or 72 h of incubation at 37°C, the cells were fixed in methanol and stained with Giemsa, and the fraction of inclusion-containing cells (200 cells counted per cover slip) was determined by light microscopy.

Uptake of 6BC by Mϕs. The relative amount of ^{3}H-labeled 6BC organisms phagocytosed by Mϕs was quantitated by first plating 10^6 cells onto glass scintillation vials in MEM. Nonadherent cells were removed after 3 h of incubation, 3.5 × 10^7 ^{3}H-labeled 6BC organisms were added, and the incubation was continued. At specified times after infection, the cells were washed three times with HBSS, Aquasol was added to each vial, and the cell-associated radioactivity was determined by using a liquid scintillation spectrometer.

Activation of Mϕs with IFN-γ and LPS. Plated Mϕs were incubated in 1 ml of MEM containing 14 ng of recombinant mouse IFN-γ (Genentech, South San Francisco, Calif.) and 100 ng of lipopolysaccharide (LPS) (*Escherichia coli* O127:B8; Difco Laboratories, Detroit, Mich.) per ml for 24 h prior to infection with 6BC. In experiments designed to test whether IFN-γ treatment resulted in microbistatic or microbicidal activity, the IFN-γ-containing medium was aspirated after 24 h and replaced with fresh MEM.

Measurement of Mϕ oxidative activity. Mϕs were obtained and activated in vitro with IFN-γ and LPS as described above. After 24 h of incubation, cover slips were removed, rinsed in HBSS without phenol red, and placed in glass scintillation vials. HBSS (150 μl) then was added to each vial. Some vials also received either 50 μl of phorbol-myristate acetate (250 ng/ml) or 5 × 10^6 6BC to stimulate oxidative activity. Luminol (10 μM) then was added to each vial, and O_2 activity was assessed by chemiluminescence in a Picolite luminometer (United Packard Instruments, Downers Grove, Ill.).

Anti-IFN-γ antibody treatment of Mϕs. Mϕs were activated with IFN-γ and LPS as described above. Anti-IFN-γ antibody (rat anti-murine IFN-γ monoclonal neutralizing immunoglobulin G_1 hybridoma R4-6A2; courtesy of Edward Havell, The Trudeau Institute, Inc., Saranac Lake, N.Y.) was added to replicate wells at a final concentration of 1:40. Cells were incubated in the presence of IFN-γ and antibody for 24 h and then infected with 1.5 ID_{50}s 6BC. Incubation was continued for an additional 24 h, and the number of inclusion-containing cells was determined as described above.

Coculture of Thio and IB Mϕs. To determine whether IB Mϕs released autocrine factors, Mϕs were collected and plated into 24-well dishes at concentrations of 0:1, 1:1, 1:2,

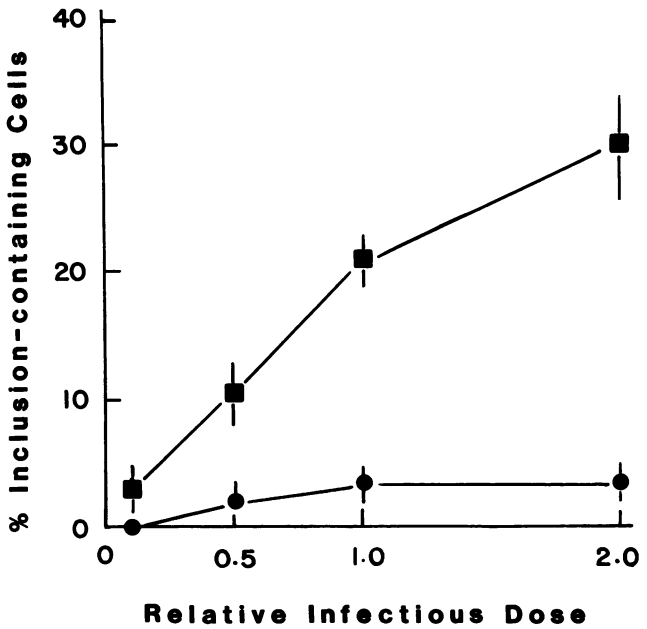


FIG. 1. Dose-response curve of in vitro susceptibility of Thio (■) and IB (●) Mϕs to *C. psittaci*. Mϕs were infected with 0.1, 0.5, 1.0, or 2.0 ID_{50}s of 6BC, and after 24 h they were fixed in methanol and stained with Giemsa. The percentages of cells showing inclusions were then recorded. Each data point represents the mean of triplicate determinations. Standard deviations are indicated by the vertical lines.

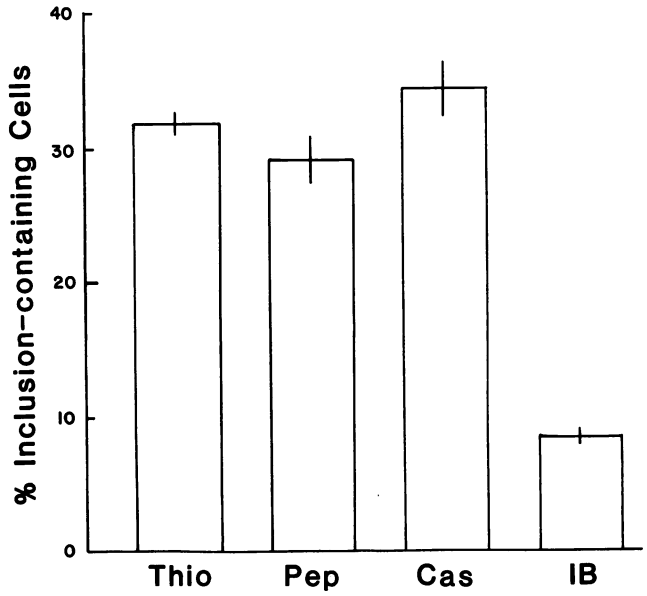


FIG. 2. Comparison of Mϕs elicited with a variety of inflammatory mediators to support chlamydial growth in cell culture. Thio, Proteose Peptone (Pro), sodium caseinate (Cas), and IB Mϕs were obtained as described in Materials and Methods and infected with 1.5 ID_{50}s of 6BC. The percentages of inclusion-containing cells were measured after 24 h of in vitro incubation. Each bar represents the mean of triplicate determinations. Standard deviations are indicated by the vertical lines.

and 1:0 ratios of Thio:IB. The final concentration in each well was 5×10^5 cells. Cocultured Mϕs were then infected with 1.5 ID_{50}s of 6BC, and the amount of chlamydial growth was determined as described above.

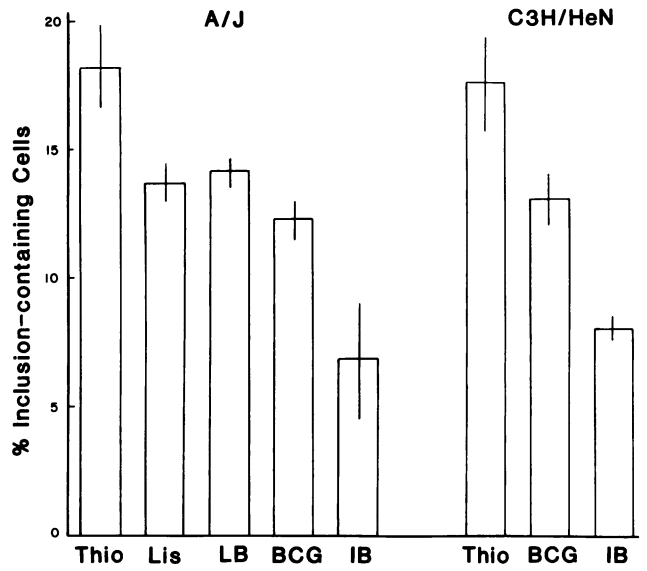


FIG. 3. Comparisons of in vitro susceptibility to 6BC growth for Mϕs activated in vivo with *M. tuberculosis* BCG or *L. monocytogenes* (Lis) and IB Mϕs from 6BC-immune mice. Thio-elicited Mϕs were included as a positive control. *Listeria*-boosted (LB) Mϕs also were included. Cells were taken from both A/J and C3H/HeN mice. Each bar represents the mean of triplicate determinations. Standard deviations are indicated by the vertical lines.

Statistics. Statistical comparisons were performed by using the Student *t* test for paired means.

RESULTS

Resistance of IB Mϕs to in vitro infection with 6BC. Figure 1 demonstrates the results of infecting IB and Thio Mϕs with various amounts of 6BC. Thio cells showed a linear increase in the numbers of inclusion-containing cells with increasing multiplicities of infection. IB cells were refractile to chlamydial growth, with only 3% exhibiting demonstrable inclusions regardless of the initial inoculum of 6BC, thus demonstrating that the vast majority of IB Mϕs were activated in vivo such that they did not support chlamydial growth within the range of inocula tested. The small fraction of cells that supported chlamydial growth may represent resident cells that responded in a manner similar to that of Thio Mϕs.

Susceptibility of elicited and inflammatory Mϕs to in vitro infection with 6BC. Sterile inflammatory cells other than Thio Mϕs were tested for in vitro susceptibility to chlamydiae (Fig. 2). Sodium caseinate- and Proteose Peptone-elicited Mϕs were as susceptible to infection with chlamydiae in vitro as those elicited with Thio broth. Therefore, Mϕs activated in vivo in response to a challenge with 6BC were functionally distinct from other Mϕ populations elicited in response to sterile inflammatory agents. Mϕs from BCG- and *L. monocytogenes*-sensitized mice showed some inhibition of chlamydial growth, but even these in vivo-activated cells were not nearly as effective as were IB Mϕs in resisting intracellular development of chlamydiae subsequent to in vitro infection (Fig. 3).

Phagocytosis of radiolabeled 6BC by Mϕs. Activated Mϕs reportedly ingest fewer *Legionella* or *Rickettsia* species than

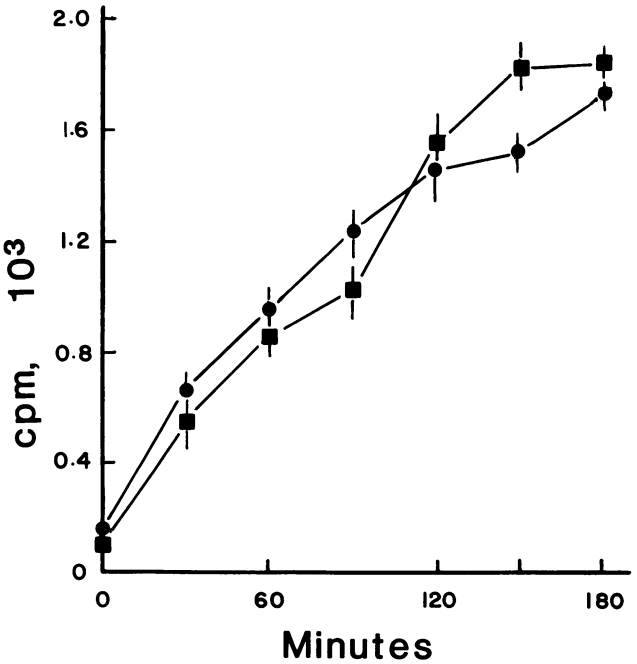


FIG. 4. Phagocytosis of ^3H-labeled 6BC by Thio and IB Mϕs. Thio (■) and IB (●) Mϕs were obtained and plated into glass scintillation vials as described in Materials and Methods. The cells were infected with 3.5×10^7 ^3H-labeled 6BC, and cell-associated counts per minute were determined after the indicated times. Each data point represents the mean of triplicate determinations. Standard deviations are indicated by the vertical lines.

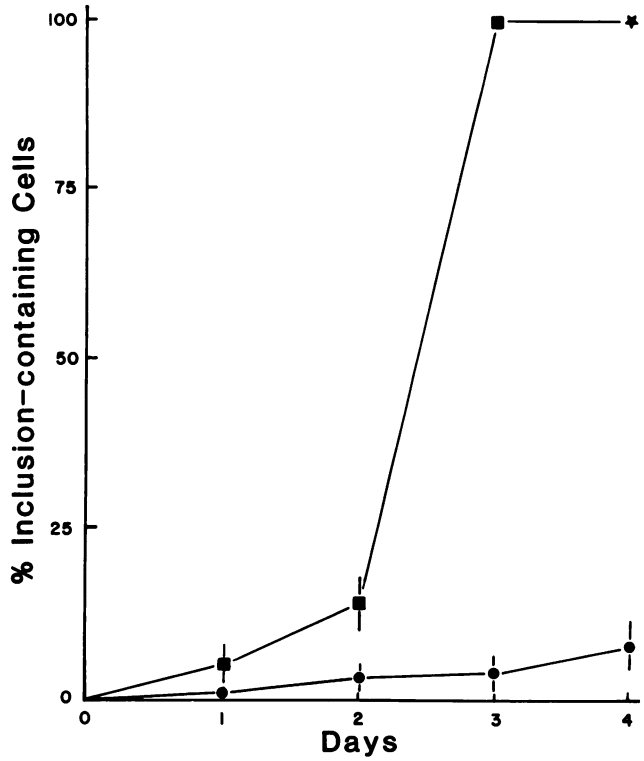


FIG. 5. Effect of prolonged in vitro incubation of Thio and IB Mϕs infected with a low multiplicity of infection of *C. psittaci*. Thio (■) and IB (●) Mϕs were obtained as described in Materials and Methods and infected with 0.1 ID_{50} of 6BC. At 1, 2, 3, and 4 days postinfection, the Mϕs were fixed in methanol and stained with Giemsa, and the numbers of inclusion-containing cells were determined. There were no intact Thio cells by 4 days (★). Each data point represents the mean of triplicate determinations. Standard deviations are indicated by the vertical lines.

unactivated cells, thereby inhibiting growth by excluding these bacteria from the intracellular environment that they require for replication (14). To test this possibility for the resistance of IB Mϕs to 6BC infection, both IB and Thio Mϕs were infected in vitro with ^3H-labeled 6BC, and the uptake of organisms was monitored over time. The radiolabeled chlamydiae were observed to associate with the IB Mϕs at the same rate and to the same extent as with the Thio Mϕs (Fig. 4). Therefore, the lack of inclusion development seen in the IB Mϕs could not be attributed to the uptake of fewer organisms.

Prolonged in vitro cultivation of 6BC-infected Mϕs. Persistence of chlamydiae is a feature of many naturally occurring chlamydial diseases. To determine whether IB Mϕs were persistently infected and thus a potential factor contributing to this phenomenon, cells were plated, infected with a low inoculum of 6BC, and examined after extended incubation times. Thio Mϕs were similarly infected. Thio Mϕ controls allowed multiple rounds of bacterial replication such that by day 4, the monolayer was completely destroyed (Fig. 5). IB Mϕs, however, restricted chlamydial growth such that only 8% of the cells showed inclusions after 4 days of cultivation. Previous studies (data not shown) revealed the presence of two populations of adherent cells in the peritoneal cavities of IB mice. The major population of IB Mϕs, probably newly recruited into the peritoneum in response to the chlamydial challenge, resisted in vitro infection with 6BC. A minor population, comprising 5 to 20% of the IB cells, continued to support chlamydial growth. These cells are probably resident Mϕs, and their presence accounts for the slight increase in the number of inclusion-containing cells seen during prolonged incubation.

Comparative susceptibility of in vivo- and in vitro-activated Mϕs to 6BC infection. IFN-γ has been shown to activate Mϕs in vitro to exhibit both bactericidal and chlamydiastatic activity (1). Therefore, a potential role for IFN-γ in the in vivo activation of IB Mϕs was examined by comparing IFN-γ in vitro-activated Thio Mϕs with respect to susceptibility to in vitro chlamydial infections. Incubation of Thio Mϕs with 14 ng of recombinant murine IFN-γ and 100 ng of LPS per ml for 18 h prior to infection resulted in cells that restricted chlamydial replication (Fig. 6). The antichlamydial activity of IB Mϕs was clearly evident in the absence of IFN-γ-LPS treatment, although inhibition was somewhat enhanced (*P* values ranged from less than 0.1 at a relative infectious dose of 1.5 to less than 0.01 at a relative infectious dose of 2.0) by treatment with IFN-LPS. Differences between in vitro- and in vivo-activated cells were seen upon removal of IFN-γ from the medium and continued culture in vitro (Fig. 7). This resulted in reversal of the inhibition of inclusion development in IFN-γ-treated Thio Mϕs. The number of inclusion-containing cells increased fivefold after 48 h of incubation without IFN-γ. In contrast, no real increase in the fraction of infected IB Mϕs was observed in groups of cells incubated either with or without IFN-γ. The

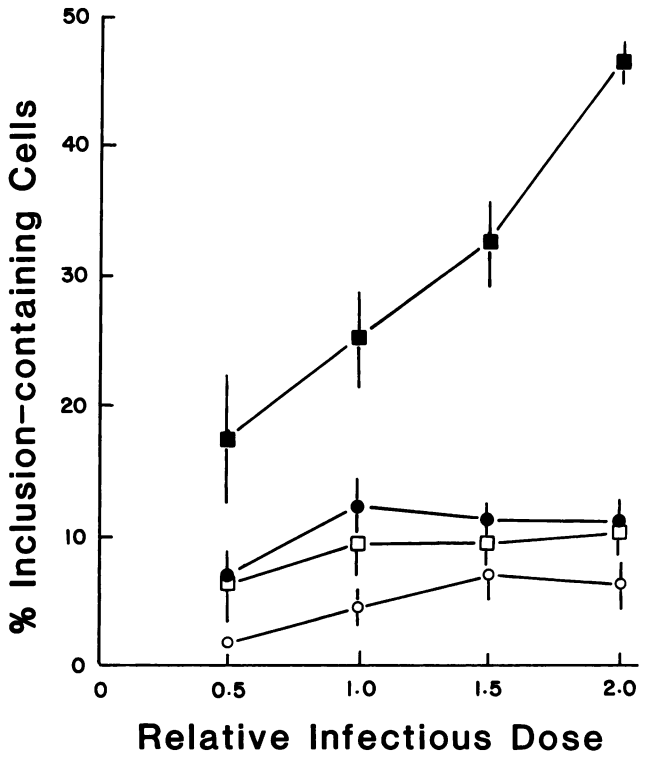


FIG. 6. Susceptibility of in vitro- and in vivo-activated Mϕs to infection with *C. psittaci*. Thio (■) and IB (●) Mϕs were obtained and plated. Some cells were pretreated with 14 ng of recombinant murine IFN-γ and 100 ng of LPS per ml (□, Thio Mϕs; ○, IB Mϕs) for 24 h prior to infection with 0.5, 1.0, 1.5, or 2.0 ID_{50}s of 6BC. After 24 h of in vitro incubation, the percentages of inclusion-containing cells were quantitated and reported as described in the legend to Fig. 1.

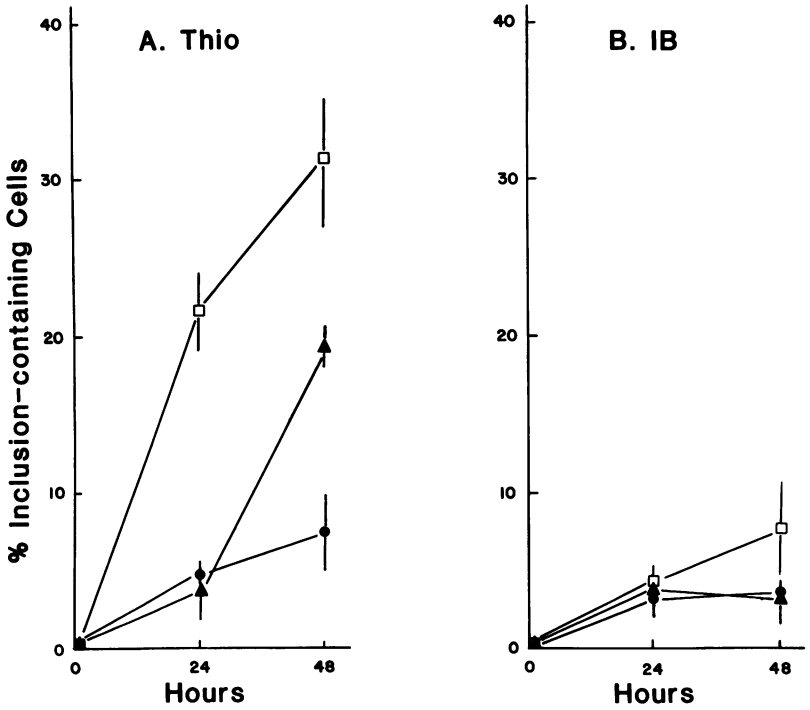
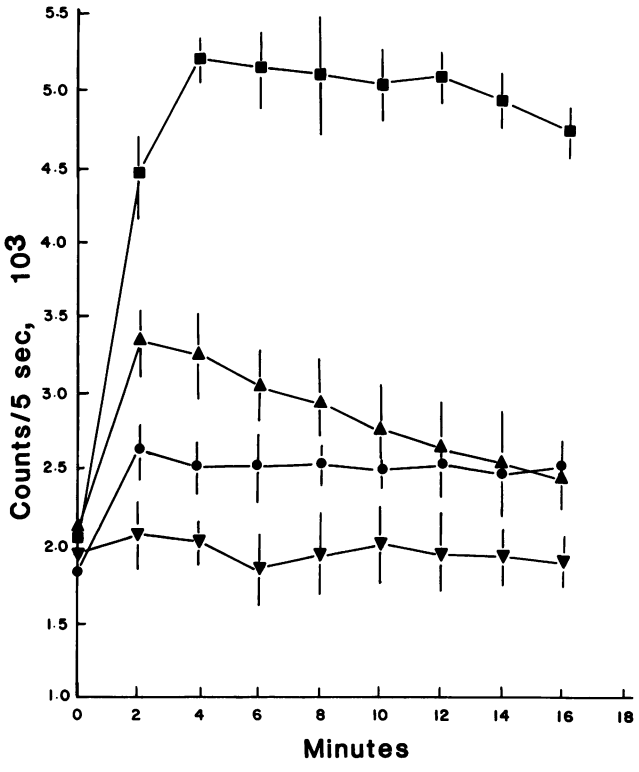


FIG. 7. Stability of in vitro and in vivo activation of Mϕs to restrict *C. psittaci* in culture. Thio-elicited (A) and IB (B) Mϕs were obtained and treated with 14 ng of IFN-γ and 100 ng of LPS per ml for 24 h prior to infection with 1 ID_{50} of 6BC. After 24 h, the IFN-LPS was removed from some cells (▲), while others were infected in the continuous presence of IFN-LPS (●). Untreated cells served as controls (□). Measurements of inclusion-containing cells were made after 24 and 48 h of in vitro incubation as described in the legend to Fig. 1.

modest additional inhibitory effect observed in Fig. 6 and the increase in the inclusion-containing cells seen after 48 h of incubation were consistent observations that probably reflected outgrowth of chlamydia in resident cells which always made up a portion of the IB population. The activation of the IB Mϕ fraction, however, appeared to be unique in that this phenotype was stable with time.

Chemiluminescence response of in vivo- and in vitro-activated Mϕs. Activated Mϕs exhibit enhanced oxidative activity (9), and therefore the chemiluminscent response of IB Mϕs was measured (Fig. 8) as an indicator for O_2 metabolite production. When phorbol-myristate acetate was used as the stimulant, Thio Mϕs pretreated with IFN-γ-LPS exhibited a positive response, with unstimulated controls showing no evidence of respiratory activity. IB Mϕs, however, reacted differently. Even without additional in vitro stimulation, these cells showed an oxidative activity equivalent to that of Thio Mϕs activated for 24 h with IFN-γ-LPS. Treatment of these cells with IFN-γ caused a further increase in their activity, suggesting that perhaps the in vivo signal might be different from the response induced by IFN-γ. 6BC did not stimulate a respiratory burst in any of the cell populations examined (Fig. 9). This is consistent with other reports indicating that phagocytosis of chlamydiae did not induce enhanced oxidative metabolism (2) unless the organisms were opsonized (5). Since 6BC organisms were apparently irreversibly inhibited in IB Mϕs, these results provide further support for the O_2-independent nature of the anti-chlamydial activity induced in mononuclear phagocytes.

Anti-IFN-γ antibody treatment of Mϕs. The role of IFN-γ also was assessed by the addition of anti-IFN-γ antibody to IB and Thio Mϕs stimulated in vitro for 24 h with IFN-γ–LPS. The number of inclusion-containing IB cells was somewhat (but not significantly) increased upon addition of



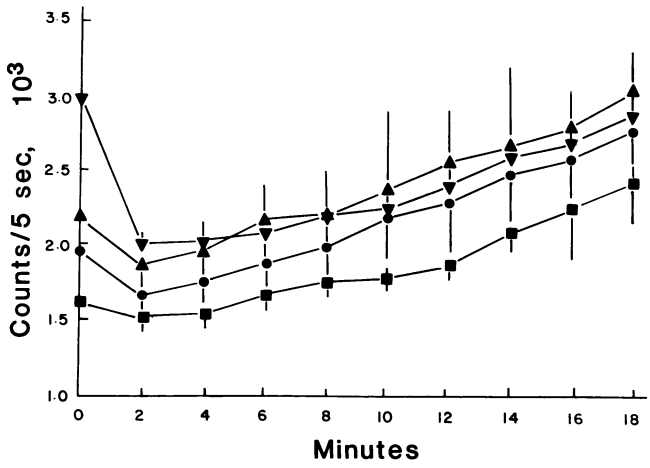


FIG. 9. Chemiluminescence response of Mϕs to *C. psittaci*. Thio (▼) and IB (▲) Mϕs were obtained and stimulated with 5×10^6 6BC. Luminol (10 μM) was added, and the chemiluminescence was measured in a Picolite luminometer. Some cells were also activated with IFN-γ (14 ng/ml) and LPS (100 ng/ml) 24 h prior to stimulation with 6BC (●, Thio Mϕs with IFN-LPS; ■, IB Mϕs with IFN-LPS). Each data point is the mean of triplicate determinations. Standard deviations are indicated by the vertical lines.

IFN-γ-specific antibody in the absence of exogenous IFN-γ (Table 1),and 50% of the IFN-mediated inhibition of 6BC growth in Thio Mϕs was abrogated upon similar treatment. Addition of anti-IFN-γ antibody also moderately influenced the fraction of inclusion-containing cells in IFN-γ–LPS-treated IB Mϕs.

Coculture of IB and Thio Mϕs. These experiments were done to determine whether the stable activation of IB Mϕs was the result of autostimulatory factors secreted by IB cells. Therefore, the ability of the IB Mϕs to influence the susceptibility of Thio Mϕs to chlamydiae was tested by coculturing the two populations at various ratios of IB:Thio Mϕs for 24 h prior to infection with 6BC. The data demonstrate a linear relationship between the proportion of Thio Mϕs present and the fraction of inclusion-containing cells (Fig. 10). Just the opposite would have been expected if autostimulatory substances were produced by IB Mϕs. Thus, IB Mϕs were incapable of activating Thio Mϕs to restrict chlamydial replication under the conditions of the experiment.

DISCUSSION

The Mϕs generated in IB mice clearly were a population of cells activated such that they dramatically restricted intracellular growth of chlamydiae. Functionally, IB Mϕs were distinct from Mϕs elicited with sterile inflammatory agents such as Thio, Proteose Peptone, and sodium caseinate. The former were resistant to in vitro infection with *C. psittaci*, while the latter supported chlamydial growth. IB Mϕs also exhibited an inhibitory capacity that exceeded that of IB populations similarly produced by sensitization with either BCG or *L. monocytogenes*.

The in vitro resistance of IB Mϕs to chlamydial infection was neither the result of altered phagocytosis of the organism nor the result of a persistent infection. Inhibition of inclusion development in the IB Mϕs was the result of an event that occurred subsequent to ingestion of 6BC. Enhanced respiratory activity and the subsequent generation of toxic O_2 intermediates such as O_2^- and H_2O_2 has been found to be important for the intracellular killing of a variety of intracellular microbes, including *Trypanasoma cruzi* (11) and *Leishmania* species (10). The chemiluminescent response of IB Mϕs provided evidence that IB Mϕs were indeed oxida-

TABLE 1. Effect of anti-IFN-γ antibody on the growth of *C. psittaci* in Mϕs

Mϕ source	% Inclusion-containing cells[a]			
	Growth medium		Medium + IFN-γ-LPS[b]	
	−Ab	+Ab	−Ab	+Ab
Thio elicited	24.0 ± 3.3	ND[c]	7.7 ± 1.4	15.8 ± 2.9[d]
IB	10.5 ± 3.6	16.0 ± 1.3	7.7 ± 1.7	10.0 ± 1.3[e]

[a] Cells were infected with 1.5 ID_{50}s of 6BC, and measurements were made as described in the legend to Fig. 3. −Ab, Without antibody; +Ab, with anti-IFN-γ antibody (1:40 dilution) added to cells prior to infection. Values are expressed as means ± the standard deviations.

[b] Mϕs were pretreated with IFN-γ (14 ng/ml) and LPS (100 ng/ml) 24 h prior to infection.

[c] Not done.

[d] Significantly different from IFN-γ–LPS-treated cells in the absence of antibody (P, <0.005).

[e] Significantly different from IFN-γ–LPS-treated cells in the absence of antibody (P, <0.05).

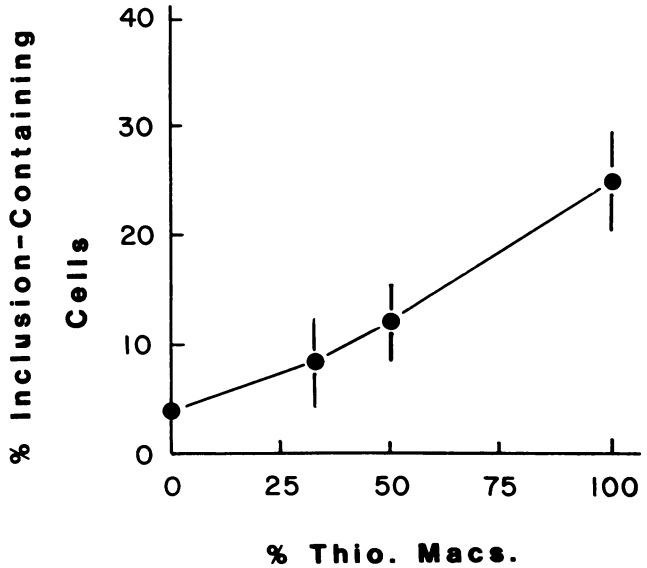


FIG. 10. Effect of coculture of Thio and IB Mϕs on the growth of *C. psittaci* in Thio Mϕs. Cells were obtained and cocultured at ratios of 1:0, 1:1, 1:2, and 0:1 of IB:Thio Mϕs (0, 33, 50, and 100% Thio Mϕs respectively) for 24 h prior to infection with 1.5 ID_{50}s of 6BC. The numbers of inclusion-containing cells were determined and recorded after 24 h of in vitro incubation as described in the legend to Fig. 1.

FIG. 8. Chemiluminescence response of Mϕs to phorbol-myristate acetate. Thio (▼) and IB (▲) Mϕs were obtained and stimulated with phorbol-myristate acetate (250 ng/ml). Luminol (10 μM) was added, and the chemiluminescence was measured in a Picolite luminometer. Some cells also were activated with both IFN-γ (14 ng/ml) and LPS (100 ng/ml) 24 h prior to phorbol-myristate acetate stimulation (●, Thio Mϕs with IFN-LPS; ■, IB Mϕs with IFN-LPS). Each data point represents the mean of triplicate determinations. Standard deviations are represented by the vertical lines.

tively active, but treatment of Mϕs with superoxide dismutase to abrogate O_2^- activity had no effect on the number of inclusion-containing cells (data not shown). Therefore, the lack of inclusion development in IB Mϕs did not appear to be the result of O_2-dependent killing of chlamydiae. This result was consistent with previous reports of the oxygen-independent nature of chlamydia inhibition, even in cells with enhanced oxidative activities (2). Oxygen-independent systems also play a role in killing some intracellular pathogens. Uptake of both *Rickettsia* (12) and *Legionella* (9) species was shown to be decreased in IFN-γ-treated Mϕs activated with IFN-γ. Other proposed bactericidal mechanisms include the production of cytoplasmic cationic proteins (17), limitation of essential intracellular nutrients (3), and acidification of the phagosome either with or without lysosomal fusion (4). The induction of antichlamydial activity in IB Mϕs apparently was more than merely growth inhibitory. Attempts at reversing the activation of IB Mϕs were unsuccessful. Treatment of the cells with cycloheximide or tryptophan had no effect on chlamydial growth, suggesting that the resistance of IB Mϕs to infection did not require continued protein synthesis in vitro, nor was it the result of increased intracellular tryptophan catabolism (data not shown), as demonstrated previously by the inhibition of chlamydial replication in human uroepithelial (T24) cells (3).

Activated IB Mϕs also were compared with Mϕs stimulated in vitro with IFN-γ. Although both IB and Thio Mϕs stimulated with IFN-γ and LPS restricted chlamydial growth, in vitro activation of Thio Mϕs was transient and required the continued presence of IFN-γ, whereas activation of IB Mϕs was stable over time. The inability to fully abrogate the in vivo activation of IB Mϕs with anti-IFN-γ antibody or mimic it in vitro with IFN-γ–LPS suggested that factors other than or in addition to IFN-γ were required to achieve fully activated cells. The observation that the addition of anti-IFN-γ antibody resulted in an increase in the fraction of inclusion-containing cells in IB Mϕs not stimulated in vitro with IFN-γ is not easy to explain. If IFN-γ contributed to in vivo activation, then the protein must somehow have been carried over to the in vitro culture system. The most likely source for this possible carryover would have been the small proportion of nonadherent cells not removed by washing procedures. If this were in fact the case, then the action of IFN-γ in vivo must be different from that observed in vitro for the elicited Mϕs populations tested. The stability and apparent bactericidal activity of IB Mϕs, with or without the direct participation of IFN-γ, still make this population of cells a unique phenotype with respect to antichlamydial activity. Clearly, IFN-γ is an important Mϕs activation factor, but other non-IFN-γ factors also have been demonstrated to be important, although non-IFN factors have not as yet been well characterized. Supernatant fluids from concanavalin A-stimulated human lymphocytes were shown to contain a factor distinct from IFN-γ that activated Mϕs to kill *Leishmania donovani* in vitro (7). Crawford et al. (R. Crawford, M. Meltzer, D. Finbloom, J. Ohara, and W. Paul, Fed. Proc. **46**:1028, 1987) demonstrated that interleukin-4-activated murine peritoneal Mϕs killed fibrosarcoma cells. Coculture of IB and Thio Mϕs suggested that in the system investigated here, autostimulatory cytokines were not produced by IB Mϕs. Thus, if a non-IFN-γ cytokine played a role in the in vivo activation, it was produced by a separate inducer population. Evidence for in vivo cooperation between IFN-γ and other lymphokines has been provided by Belosevic and Nacy (M. Belosevic and C. Nacy, Fed. Proc. **46**:1028, 1987), who found that in vitro stimulation of Mϕs with IFN-γ in conjunction with B-cell-stimulating factor 1, granulocyte-monocyte colony-stimulating factor, or interleukin 2 resulted in Mϕs resistant to *Leishmania major* infection in vitro, whereas treatment of Mϕs with IFN-γ alone did not affect the susceptibility of the cells to *Leishmania major*. A similar phenomenon may be responsible for the results reported here.

IB Mϕs were present only after intraperitoneal challenge of previously immunized mice, consistent with the concept of prior sensitization as a requirement for heightened cell-mediated reactivity. The induction of IB Mϕs correlated temporally with protective immunity and therefore provides impetus to further characterize these cells. Understanding the mechanism of in vivo activation not only will be important in identifying the nature of protective immunity to chlamydiae and how this immunity might be put to practical use but also will shed light on the phenomenon of Mϕ activation in general.

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