A Macrophage Invasion Mechanism of Pathogenic Mycobacteria

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Tuberculosis is the leading cause of death due to an infectious organism, killing an estimated 3 million people annually. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and other pathogenic mycobacteria require entry into host macrophages to initiate infection. An invasion mechanism was defined that was shared among pathogenic mycobacteria including *M. tuberculosis*, *M. leprae*, and *M. avium* but not by nonpathogenic mycobacteria or nonmycobacterial intramacrophage pathogens. This pathway required the association of the complement cleavage product C2a with mycobacteria resulting in the formation of a C3 convertase. The mycobacteria-associated C2a cleaved C3, resulting in C3b opsonization of the mycobacteria and recognition by macrophages.

It is estimated that a third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and an additional 8 million new cases of pulmonary tuberculosis occur each year (*1*). Much of the resurgence of tuberculosis can be attributed to the human immunodeficiency virus epidemic (*2*), which also predisposes infected hosts to other mycobacterial infections, especially *M. avium* (*3*). These mycobacteria are intracellular pathogens that reside almost exclusively within macrophages of infected individuals. In vitro studies have demonstrated a number of mechanisms by which mycobacteria can invade host macrophages including opsonization of mycobacteria with C3 through activation of the alternative pathway of complement, followed by invasion of macrophages by way of the complement receptors CR1, CR3, and CR4 (*4*). Consistent with this mechanism, heat treatment of fresh normal human serum, which inactivates the complement pathways, abolishes its ability to support mycobacteria invasion of macrophages (*4*, *5*). However, heat-treated commercial equine serum or human serum from a mixed lymphocyte reaction retains the ability to markedly enhance *M. avium* uptake by macrophages (*6*). This indicates that the latter serum fractions contain an additional factor, not present in freshly isolated normal human serum, that can facilitate *M. avium* invasion of macrophages.

We have purified the active component from heat-treated equine serum (*7*) and tested its ability to mediate macrophage invasion for different mycobacterial species. The purified component markedly enhanced macrophage invasion by pathogenic mycobacteria [*M. leprae*, bacillus Calmette-Guerin (BCG), and *M. tuberculosis*] but not by fast-growing nonpathogenic mycobacteria (*M. vaccae*, *M. smegmatis*, and *M. phlei*) (Fig. 1A). Other pathogens such as *Leishmania mexicana*, *Listeria monocytogenes*, *Staphlococcus* sp. (Fig. 1B), and *Nocardia asteroides* (*8*) could not use this serum factor to mediate macrophage entry. Thus, this method of invasion appears unique to pathogenic mycobacteria and suggests a virulence mechanism for mycobacterial infection.

The purified active component from equine serum is a 70-kD protein (*7*). The NH_2-terminal sequence of this protein was identical to amino acids 244 to 263 of human complement component C2 in 19 of 20 amino acids (*9*). This sequence corresponds to the NH_2-terminus of the C2a fragment of C2, which is generated during classical complement pathway activation (*10*). This 70-kD protein was of the correct molecular size for C2a and was recognized by a polyclonal antibody to human C2 (*8*). These active fractions had no other detectable proteins on silver staining and no intact C2 by protein immunoblot. Our findings were unexpected because C2a has been shown to function only as part of a complement C3-cleaving enzyme (convertase) and in the absence of C4b has no known activity (*11*).

To confirm the role of C2a in mycobacteria invasion of macrophages, we immunodepleted C2 and its activation fragments C2a and C2b from fractionated heat-treated equine serum and human serum (*12*). Depletion of C2 and its fragments abolished facilitation of *M. avium* uptake by macrophages (Fig. 2A), whereas treatment of the serum samples with antibodies to human fibronectin had no effect (*8*). To demonstrate that C2a was sufficient to stimulate the invasion process, we incubated purified complement components C1 and C4 with antibody-coated erythrocytes (EA) to form a C2 convertase. Upon prolonged incubation with commercially obtained pure human C2, the C4b2a complex forms and then dissociates, leaving C2a free in solution. This C2a enhanced *M. avium* ingestion by macrophages, whereas intact C2 did

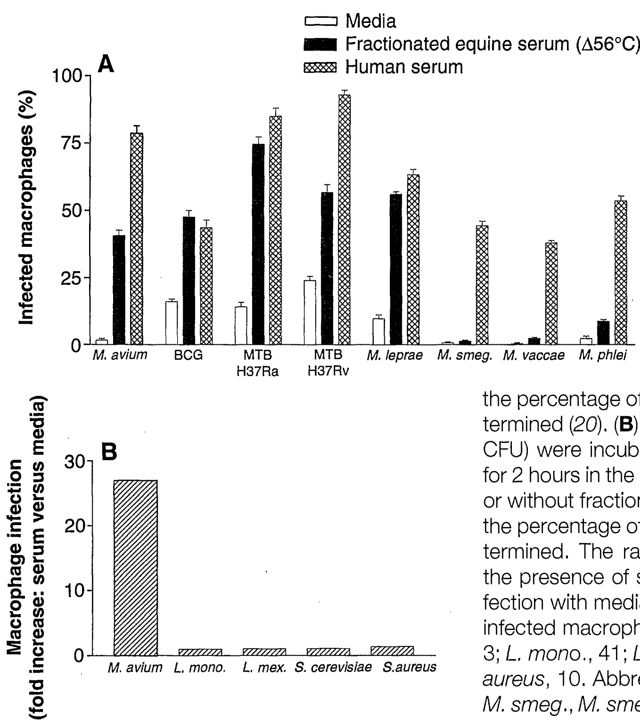

Fig. 1. Use of a heat-stable serum component for macrophage infection by pathogenic mycobacteria. (**A**) The various mycobacterial species [5 × 10^5 colony-forming units (CFU)] were incubated with human macrophages (*19*) in the presence of infection media (RPMI, 0.5% human serum albumin) or infection media with Con A–fractionated Δ56°C equine serum, or 10% fresh normal human serum, and the percentage of infected macrophages was determined (*20*). (**B**) The various organisms (5 × 10^5 CFU) were incubated with human macrophages for 2 hours in the presence of infection media with or without fractionated Δ56°C equine serum, and the percentage of infected macrophages was determined. The ratio of macrophages infected in the presence of serum proteins compared to infection with media alone is shown. Percentage of infected macrophages in media alone: *M. avium*, 3; *L. mono.*, 41; *L. mex.*, 35; *S. cerevisiae.*, 40; *S. aureus*, 10. Abbreviations: MTB, *M. tuberculosis*; *M. smeg.*, *M. smegmatis*; *L. mono*, *Listeria monocytogenes*; *L. mex.*, *Leishmania mexicana*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. aureus*, *Staphylococus aureus*, coagulase negative.

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not enhance *M. avium* invasion (Fig. 2B). These data demonstrate that carry-over of trace amounts of intact C2 to form a classical pathway C3 convertase is not the mechanism for this mycobacterial invasion of macrophages. Therefore, pathogenic mycobacteria have evolved a mechanism to salvage C2a and use it as a means of invading macrophages. C2a does not reassociate with C4b and thus in the absence of these pathogenic mycobacteria, has no ability to cleave C3 (*11*).

Prior treatment of C2a with the irreversible serine protease inhibitor diisopropylfluorophosphate (DFP) markedly decreased C2a enhancement of *M. avium* entry into macrophages (Fig. 3A), suggesting a role for C3, the only known C2a substrate, in C2a-dependent macrophage invasion. Addition of DFP to *M. avium* preopsonized with C3 by the alternative pathway did not block its invasion of macrophages, confirming that DFP acted on C2a and not on the macrophages (*8*). Prior incubation of *M. avium* with both C2a and C3, but not with either component individually, markedly increased its ingestion by human macrophages (Fig. 3B). Furthermore, the combination of C2a- and C3-treated mycobacteria stained positively with an antibody to human C3 (anti-C3) (*8*), indicating that incubation of mycobacteria with C2a resulted in deposition of C3 on the mycobacteria. C3 cleavage was measured directly by enzyme-linked immunosorbent assay (ELISA) with antibodies specific for C3a, a cleavage product of C3. Incubation of pathogenic mycobacteria with C2a and C3, but not C2 and C3, resulted in C3 cleavage (Fig. 3C). Thus, pathogenic mycobacteria can use C2a but not intact hemolytically active C2 to form a C3 convertase.

The initial experiments showed that C2a could enhance mycobacteria invasion of macrophages without an exogenous source of C3, suggesting that macrophages could be a source of C3. To address this possibility, we incubated macrophages from $C3^{-/-}$ mice (*13*) or control mice with heat-treated serum from the $C3^{-/-}$ mice as a source of murine C2a. Under these conditions, *M. avium* was not ingested by the $C3^{-/-}$ macrophages but was able to invade wild-type macrophages (Fig. 3D). Addition of heat-treated equine serum as a source of C2a and C3 restored *M. avium* invasion of $C3^{-/-}$ macrophages, demonstrating that these cells were not abnormally resistant to infection (Fig. 3E). Additional in vitro experiments indicated that the predominant *M. avium* opsonin is C3b, the ligand for CR1, and not C3bi, the ligand for CR3 (Fig. 3F).

Macrophage invasion by mycobacteria can proceed by opsonization with C3 by the alternative complement pathway or by interactions of mannose-containing cell wall glycolipids with macrophage lectins (*14*).

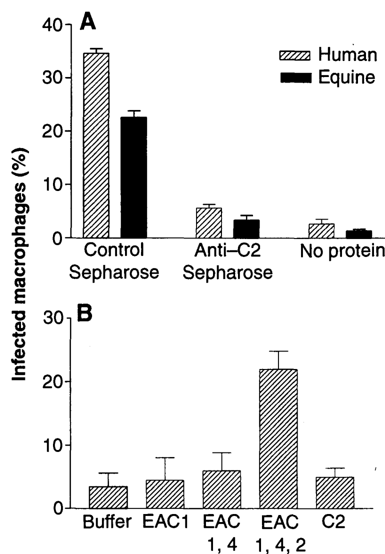

Fig. 2. C2a is the active component from heat-treated serum that enhances *M. avium* entry into macrophages. (**A**) Partially purified C2a from heat-treated human and equine serum was tested for its ability to support *M. avium* invasion of human monocyte-derived macrophages after incubation with anti-C2 sepharose or control sepharose (*12*). (**B**) Human macrophages and *M. avium* were incubated with supernatant from immunoglobulin M–coated sheep erythrocytes (EA) treated sequentially with complement proteins C1, C4, and C2 or with C2 alone (*21*). The conditions that generate C2a (that is, EAC1, 4, 2) produce invasion-promoting activity.

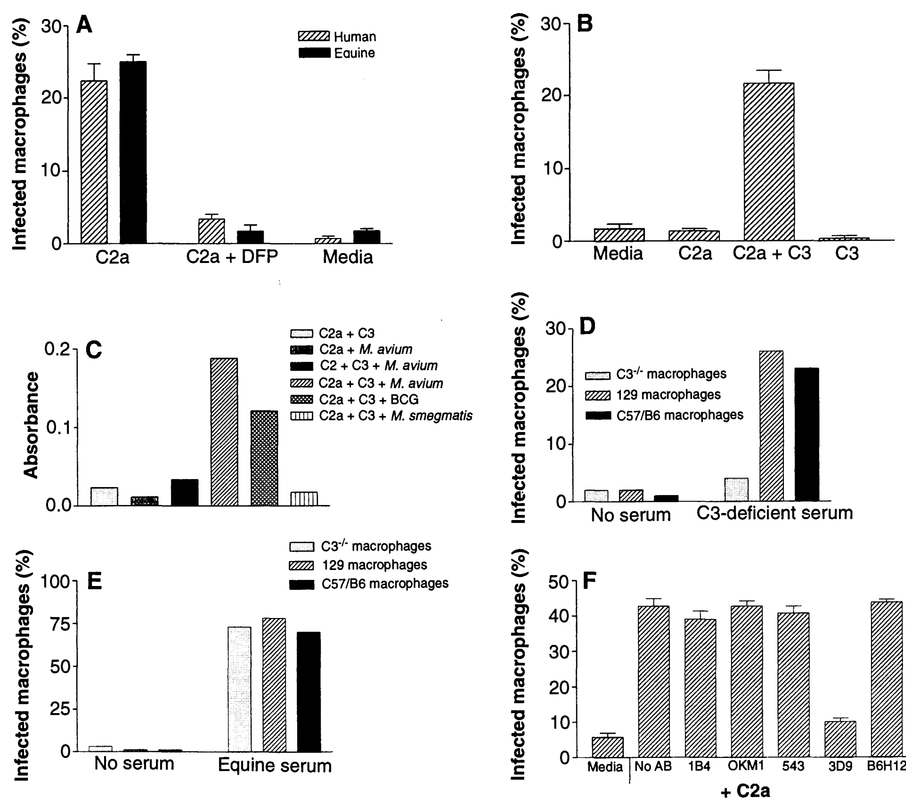

Fig. 3. *Mycobacterium avium* and C2a function as a C3 convertase. (**A**) Partially purified C2a from heat-treated human and equine serum was incubated for 1 hour at 25°C with or without 5 mM DFP, a serine protease irreversible inhibitor. The C2a samples were dialyzed at 4°C against PBS to remove free DFP and then incubated with *M. avium* and human monocyte-derived macrophages to test the C2a fractions for invasion-enhancing activity. (**B**) After preincubation of *M. avium* with either partially purified C2a or 50 U of C3 or both, the organisms were washed and resuspended in infection media and tested for their ability to invade human macrophages. (**C**) *Mycobacterium avium* BCG, and *M. smegmatis* (2×10^7), were incubated with intact C2, C2a, and C3 as indicated for 1.5 hours at 37°C. Cleavage of human C3) was assessed by enzyme-linked immunosorbent assay for C3a (Quidel, San Diego, California). (**D**) *Mycobacterium avium* invasion of mouse bone marrow macrophages (*22*) was tested in the presence of infection media only or infection media containing 10% immune complex–treated serum (Δ56°C) from $C3^{-/-}$ mice (*23*). (**E**) Same as (C) except that 10% heat-treated equine serum was used. (**F**) *Mycobacterium avium* invasion of human macrophages in the presence of partially purified C2a was assessed after preincubation of the macrophages with monoclonal antibodies (30 μg/ml): 1B4 (*24*) and OKM1 (*25*) blocking antibodies to CR3; 543 (*26*) nonblocking and 3D9 (*27*) blocking antibody to CR1; B6H12 irrelevant binding control antibody against integrin-associated protein (*28*). Media: *M. avium* added to macrophages in the absence of C2a.

However, neither of these mechanisms is specific to pathogenic mycobacteria, which have evolved as extremely successful intracellular pathogens. Here we describe a mycobacterial macrophage invasion mechanism that is not shared by other organisms including other nonmycobacterial intracellular pathogens and other mycobacteria that are not intracellular pathogens. This C2a-dependent entry pathway thus has the characteristics of a virulence mechanism for pathogenic intracellular mycobacteria. The invasion mechanism is very sensitive to C2a since 1 to 10 nM C2a is sufficient to mediate a maximum rate of invasion in vitro (*8*). This requires cleavage of <1% of the serum concentration of C2, suggesting that local concentrations of C2a in a biologically significant range could be obtained at the site of a mycobacterial infection. Subsequent steps after this mycobacteria-dependent mechanism for C2a cleavage of C3 are identical to what has already been shown for mycobacteria opsonized with C3b by the alternative complement pathway, including invasion of the macrophage by way of complement receptors and survival and replication within macrophage phagosomes (*14*).

This C2a-mediated invasion mechanism may be involved in both the establishment of an infection and in its propagation. Previous work has shown that lung exposure to *M. tuberculosis* results in an inflammatory response that leads to complement activation and C2a production (*15*). Additional studies have shown that patients with active pulmonary tuberculosis have high levels of circulating immune complexes (*16*) with activation of the classical complement pathway resulting in increased serum levels of C2a (*17*). The presence of C2a during several stages of a mycobacterial infection, along with conservation of this C2a-dependent uptake pathway among pathogenic mycobacteria, suggests that this invasion mechanism has a crucial role in mycobacterial pathogenesis. The mycobacterial cell wall component required for this invasion process could provide a new target for therapeutic intervention.

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7. Equine serum (250 ml) was heat treated by incubation at 56°C for 30 min. The serum was diluted to 1 liter with Hanks' buffered saline, 1 mM $CaCl_2$, $MnCl_2$, and $MgCl_2$ ($HBSS^{3+}$) and run through a column containing 4 ml of packed concanavalin A (Con A)–agarose equilibrated in $HBSS^{3+}$ at a flow rate of 0.35 ml/min. The column was washed with $HBSS^{3+}$ and the bound proteins eluted with 14 ml of HBSS, 0.1 M α-methyl mannoside. The sample (13.5 ml) was diluted to 140 ml with 20 mM tris (pH 7.6) and loaded onto a heparin-Sepharose column containing 8 ml of heparin-Sepharose resin equilibrated in 20 mM tris (pH 7.6). The bound protein was eluted with 14 ml of 20 mM tris, 1.2 M NaCl (pH 7.6). The heparin-Sepharose elution (13.5 ml) was diluted to 140 ml with 20 mM tris (pH 7.6) (final NaCl concentration = 116 mM). This material was loaded onto a Mono Q fast protein liquid chromatography (FPLC) HR 5/5 anion-exchange column equilibrated in 20 mM tris, 100 mM NaCl (pH 7.6) with a Waters FPLC at a flow rate of 1.0 ml/min. Flow-through was collected and used in the subsequent purification step. The column flow-through was concentrated and the buffer exchanged into 25 mM triethylamine, 100 mM NaCl (pH 11.0). Final volumes of sample equaled 3.5 ml. A portion of this sample (3.4 ml) was loaded onto a Mono P FPLC 5/5 chromatofocusing column equilibrated in 25 mM triethylamine, 100 mM NaCl (pH 11.0) buffer. Bound proteins were eluted with Pharmalyte (pH 8.0 to 10.5), 100 mM NaCl (pH 8.0). Fractions (1 ml) were collected and the pH adjusted to neutrality. Fractions were analyzed for protein purity by SDS–polyacrylamide gel electrophoresis (PAGE), silver stained, and examined for activity in the mycobacteria-ingestion assay. Fraction 22, which showed the highest activity, gave only a single 70-kD band on SDS-PAGE, indicating that the protein was purified to apparent homogeneity. A sample (0.5 ml) of this fraction was sent to Midwest Analytical (St. Louis, MO) for analysis of NH_2-terminal sequence. Partially purified C2a used in some experiments was obtained by Con A fractionation of heat-treated (56°C) human serum (mixed lymphocyte reaction–C^+serum, North American Biologicals, Miami, FL) or equine serum as described above or by batch mode. Protein eluted with HBSS, 0.1 M α-methyl mannoside was exchanged into phosphate-buffered saline (PBS) and used in the experiments at ~0.15 mg/ml.
8. J. S. Schorey *et al.*, data not shown.
9. Seventy-kilodalton protein (NH_2-terminus), KIQIQRSGHLNLYLLLD**A**SQ; human C2a (NH_2-terminus), KIQIQRSGHLNLYLLLD**C**SQ. The single–amino acid difference between the purified equine protein and the NH_2-terminus of human C2a is shown in bold. Abbreviations for the amino acid residues: A, Ala; C, Cys; D, Asp; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; Q, Gln; R, Arg; S, Ser; and Y, Tyr.
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12. To make the protein A–anti-C2, we incubated 0.05 ml of the rabbit polyclonal serum against human C2, which cross-reacts with equine C2, with 0.05 ml of packed protein A–Sepharose (Sigma) in 0.9 ml of PBS for 2 hours at 4°C. The protein A–Sepharose with coupled antibody was washed twice with PBS and resuspended in 0.1 ml of PBS. Approximately 0.2 mg of Con A–fractionated proteins from heat-treated equine serum or human serum (obtained from a mixed lymphocyte reaction) in PBS was incubated with protein A–Sepharose or protein A–Sepharose with bound anti-C2 for 3 hours at 4°C. The protein A was subsequently removed and the treated samples tested for the ability to support *M. avium* invasion of human monocyte-derived macrophages. No detectable C2 or C2 fragments were observed in the anti-C2–treated samples by protein immunoblot analysis with goat anti-C2 (human).
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20. The human or mouse macrophages were plated onto eight-well LabTek chamber Permanox slides 2 to 14 hours before the infection experiments at 5×10^4 cells per well. A mycobacteria to macrophage ratio of 10:1 was used in all experiments. This ratio was also used when other organisms were tested. The macrophages, mycobacteria, and C2a-containing material were incubated for 2 hours at 37°C, 5% CO_2 in 0.2 ml of RPMI, 0.5% human serum albumin (infection media). The cells were washed with PBS, fixed with acetone/methanol (1:1), and stained for pathogenic mycobacteria with a TB Fluorescent Stain Kit O (Becton Dickinson, Cockney, MD). Macrophages infected with *M. smegmatis*, *M. vaccae*, and *M. phlei* were fixed in 2.5% glutaraldehyde and stained with acridine orange. The number of macrophages containing attached and internalized mycobacteria was scored visually with a Nikon Microphot-FX fluorescent microscope. The number of macrophages with associated mycobacteria per 100 cells is given as percent infected. For some experiments, the data are presented as the mean ± SD for 300 macrophages counted and represent variability within a given experimental chamber well. Defining the number of macrophages infected with organisms other then mycobacteria was done by visual inspection with a Zeiss Axioskop microscope.
21. A C1-containing fraction was isolated from human serum as described (*18*). Antibody-coated erythrocytes (EA) (Sigma) were incubated in 0.9 ml of Isogevers Veronal-buffered saline, 0.15 mM $CaCl_2$, 1 mM $MgCl_2$ ($GVBS^{2+}$) sequentially with 0.1 ml of C1 fraction, purified commercial C4 (50,000 U), and C2 (25,000 U) (Advanced Research Technologies, San Diego, CA). Each incubation was for 30 min at 30°C, except for the last incubation after addition of C2, which was for 2 hours. EA were washed with $GVBS^{2+}$ after incubation of $GVBS^{2+}$ with C1 and C4. After the final 2-hour incubation, EA were removed by centrifugation, and 0.1 ml of the supernatant was tested for the ability to support *M. avium* invasion of human monocyte-derived macrophages.
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23. Blood from $C3^{-/-}$ mice were obtained through the eye orbital. The blood was allowed to clot for 30 min at 25°C and then 30 min at 4°C. The serum was cleared of clotted blood by centrifugation and immediately treated with a bovine serum albumin (BSA)–anti-BSA immune complex for 1 hour at 37°C. The immune complex was removed by centrifugation and the serum incubated at 56°C for 30 min to inactivate the complement pathways and stored at −70°C until needed.
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