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Processing and Presentation of an Antigen of *Mycobacterium avium* Require Access to an Acidified Compartment with Active Proteases

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We have generated a murine T-cell hybridoma, 1C9, which recognizes an antigen expressed by a virulent clinical isolate of *Mycobacterium avium*. Both peritoneal exudate macrophages and bone marrow-derived macrophages infected in vitro with *M. avium* process and present the antigen to the T-cell hybridoma. Gel filtration chromatography of a sonicate of *M. avium* followed by T-cell Western blotting (immunoblotting) demonstrated that the antigen recognized by hybridoma 1C9 is approximately 50 kDa. In addition, treatment of macrophages with the lysosomotropic agent chloroquine or with inhibitors of acid proteases inhibits processing and presentation of the antigen. These results indicate that the antigen must encounter an acidic compartment with active proteases for processing and presentation to occur. Our results are discussed in the context of our current understanding of how mycobacterial antigens are processed and presented by infected macrophages to T cells.

Mycobacterium avium is a common cause of disseminated bacterial infection in immunocompromised patients such as those with AIDS and is associated with increased morbidity and mortality (20, 21). A detailed understanding of how *M. avium* infection is controlled by immunocompetent hosts and why fulminant infection develops in immunocompromised patients is essential for the development of new diagnostic methods and treatments. Like other mycobacteria, *M. avium* is taken up by macrophages or monocytes via specific receptors (30) and dwells within a phagocytic vacuole (40). It has been postulated that mycobacterial antigens present in the phagocytic vacuole enter the class II antigen processing pathway, resulting in presentation to $CD4^+$ T cells (3, 15, 22, 32, 38). Cytokines elaborated from the $CD4^+$ cells stimulate macrophage effector functions such as NO production, which leads to eventual destruction of the bacilli (12, 14, 17, 25, 26). T cells have been shown to be essential for the acquisition of protective immunity to *M. avium* in mice (4, 23), but although several antigens of *M. avium* have been cloned (31, 37, 45), nothing is known about which *M. avium* antigens are involved in the activation of protective T cells. Moreover, there have been no studies documenting and characterizing the processing and presentation of a specific *M. avium* antigen by infected macrophages to mouse T cells.

In this report, we demonstrate the processing and presentation of a 50-kDa antigen derived from a virulent clinical isolate of *M. avium*, MAC101. We show that murine macrophages infected in vitro with *M. avium* process and present the antigen to an I-A^d-restricted T-cell hybridoma, 1C9. In addition, we show that the processing and presentation of the 50-kDa antigen requires access of the antigen to an acidified compartment containing active proteases. We relate our results to what is currently known about the maturation of the mycobacterium-containing vacuole down the endosomal-lysosomal pathway, the proteases involved in antigen processing, and the effect mycobacterial infection has on the ability of host macrophages to present antigen to T cells.

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MATERIALS AND METHODS

Media and agar. Tissue culture medium (R10.FCS) consisted of RPMI 1640 (Gibco/BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% GlutaMAX-1 (Gibco/BRL), 5×10^{-5} M 2-mercaptoethanol, and, where indicated, 50 μg of gentamicin per ml. 7H9 medium consisted of 9 parts Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) and 1 part 10× mycobacterial medium supplements consisting of 50 mg of bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo.) per ml, 2% glucose, 0.85% NaCl, 0.06% oleic acid (Fisher Scientific, Pittsburgh, Pa.), and 0.5% Tween 80 (Sigma). 7H9 medium was filtered through a 0.22-μm-pore-size filter before use. 7H10 agar plates were prepared by combining 9 parts autoclaved Middlebrook 7H10 agar (Difco) with 1 part 10× mycobacterial medium supplements and dispensing the mixture into bacteriologic petri dishes (Midwest Scientific, St. Louis, Mo.).

Bacteria. *M. avium* 5-8 (serovar 4) (34) was a kind gift of Timothy Ratliff (Washington University, St. Louis, Mo.). *M. avium* MAC101 (serovar 1) (9) was kindly provided by Lowell S. Young (Kuzell Institute, San Francisco, Calif.) and passaged regularly through mice to maintain virulence. A single colony from an infected spleen was grown at 37°C and in 0% CO_2 to approximately 10^8 CFU/ml in 7H9 medium, and the culture was frozen at −70°C in 7H9 medium containing 20% glycerol. Individual vials were thawed, diluted in 7H9 medium, and cultured to approximately 10^8 CFU/ml before use in experiments. Bacteria were enumerated by plating serial dilutions on 7H10 agar plates and determining the number of colonies after incubation of the plates for 10 days.

Preparation of *M. avium* sonicate and culture filtrate proteins. *M. avium* MAC101 was grown to approximately 10^8 CFU/ml in 2 liters of 7H9 medium in a stir flask. The bacilli were pelleted by centrifugation at $3,000 \times g$ in a Beckman model J221 centrifuge equipped with a JA-10 rotor. The bacilli were washed three times in phosphate-buffered saline (PBS) containing 0.5% Tween 80. They were then transferred to a siliconized tube and sonicated in PBS-Tween 80 with a Fisher Scientific Sonic Dismembrator (Fisher Scientific). Insoluble material was pelleted by centrifugation at $3,000 \times g$ in a Beckman GPR centrifuge. The supernatant was spun in a Beckman Airfuge Ultracentrifuge at 24 lb/in^2 for 10 min to pellet cell wall-derived lipids. Protein content was determined with a Bradford assay (Bio-Rad Laboratories, Hercules, Calif.).

M. avium 5-8 was grown to approximately 10^8 CFU/ml in 7H9 medium. The bacilli were pelleted by centrifugation at $3,000 \times g$. The supernatant containing secreted proteins was concentrated with an Amicon Stir Cell Microconcentrator equipped with a 10,000-molecular-weight cutoff membrane (Amicon, Inc., Beverly, Mass.). Protein content was determined with a Bradford assay (Bio-Rad).

T-cell hybridomas. The T-cell hybridoma 1C9 was developed by use of a standard protocol (2). Briefly, BALB/cAnNCr mice (National Cancer Institute, Bethesda, Md.) were immunized subcutaneously with a preparation of *M. avium* 5-8 culture filtrate proteins emulsified in incomplete Freund adjuvant (Difco). One week later, the draining lymph nodes were removed and the cells were stimulated in vitro for 3 days with the culture filtrate proteins. The cells were then fused with a T-cell receptor $\alpha^-\beta^-$ variant of the BW5147 cell line. Hybridomas were screened for interleukin 2 (IL-2) production in response to *M. avium* culture filtrate proteins with BALB/c macrophages as antigen-presenting cells. Hybridoma 1C9 reacted strongly to the culture filtrate proteins in an I-A^d-restricted manner.

Hybridoma Fl.2 recognizes amino acid residues 409 to 421 of β-galactosidase

in the context of I-A^d (40a). A peptide comprising these residues (VVDEANIETHGMV) was synthesized on a Symphony/Multiplex multiple peptide synthesizer (Protein Technologies, Tucson, Ariz.) and purified by high-performance liquid chromatography on a reverse-phase C-18 column.

Macrophages. Peritoneal exudate macrophages were prepared by injecting BALB/cAnNCr mice with 1 ml of concanavalin A in saline (60 μg/ml intraperitoneally; Sigma), followed 4 days later with 1 ml of peptone (Difco) intraperitoneally. Three days after peptone injection, peritoneal exudate cells were collected in sterile Hanks' balanced salt solution (HBSS; no antibiotics) by peritoneal lavage. The cells were plated at 4×10^5 cells per well in R10.FCS (no antibiotics). Nonadherent cells were removed after 2 h of incubation at 37°C by extensive washing with HBSS. The adherent cells were then used for infection experiments. To prepare bone marrow-derived macrophages, bone marrow cells were isolated from the femurs of BALB/cAnNCr mice. Cells were plated at 2×10^4 per well in 96-well plates and cultured without antibiotics in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% FCS, 1% GlutaMAX-1, 5×10^{-5} M 2-mercaptoethanol, and 10% L-cell-conditioned medium. After 5 days, the cultures were supplemented with fresh medium. Some cultures received mouse recombinant gamma interferon at 100 U/ml (Genentech, Inc., South San Francisco, Calif.) to upregulate class II expression. After 2 days of further incubation, the cultures were used in infection experiments.

Infection experiments. Mycobacteria were suspended in RPMI 1640 supplemented with 10% FCS and 5% heat-inactivated horse serum (HyClone Laboratories, Logan, Utah) and added in various doses to macrophage monolayers. Cultures were incubated at 37°C for up to 2 h. They were then washed extensively with HBSS supplemented with 50 μg of gentamicin per ml to remove free bacilli and fixed with 1% paraformaldehyde. After washing with HBSS to remove the fixative, the cultures were incubated overnight in R10.FCS plus gentamicin to allow regurgitation of any residual internalized paraformaldehyde. 1C9 hybridoma cells (10^5 per well) were added to the fixed monolayers. After 24 h, culture supernatants were collected for the measurement of IL-2 content.

In some experiments, macrophages were pretreated for 20 min with chloroquine (100 μM) or with the protease inhibitor pepstatin A (50 μg/ml; Sigma) or E64 (25 μg/ml; Sigma) before the addition of mycobacteria. The infections proceeded as described above in the continued presence of the drugs.

IL-2 assay. The IL-2 content of media was measured with the IL-2-dependent cell line CTLL-2. Medium conditioned by T hybridoma cells (100 μl) was added to cultures of CTLL-2 cells (5×10^3 per well). After 18 h, the cultures were pulsed with [^{3}H]thymidine (0.4 μCi/well, 2 Ci/mmol; NEN Research Products, DuPont Co., Boston, Mass.) for an additional 24 h. The cultures were then harvested for scintillation counting with a cell harvester (Skatron, Sterling, Va.).

Partial purification of the 1C9 antigen. A column (2.6 by 90 cm; Pharmacia Biotech, Piscataway, N.J.) containing Sephacryl S300HR resin (Sigma) was equilibrated with PBS. MAC101 sonicate (4.5 mg) was passed over the column at a flow rate of 1 ml/min by use of a peristaltic pump (LKB2232 Micro Perpex; Pharmacia Biotech). Fractions (2 ml) were collected with an LKB 2211 Superack fraction collector (Pharmacia Biotech), and protein was detected by monitoring the optical density at 280 nm with a UV monitor (UV-1; Pharmacia Biotech). Individual fractions were tested for the presence of the 1C9 antigen by adding 10 μl of each fraction in duplicate to cultures of 1C9 hybridoma cells (10^5 per well) and the B-cell line TA3 (*H-2*^{k} and *H-2*^{d}; 3×10^4 per well) as antigen-presenting cells and measuring IL-2 production from the T hybridoma cells as described above. Fractions stimulating the hybridoma were pooled and concentrated approximately 40-fold with Centriprep-10 concentrators (Amicon). The concentrated active fractions were subjected to T-cell Western blotting (immunoblotting) as described by Abou-Zeid et al. (1). Briefly, the preparation was separated on a reducing sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (SDS–8% PAGE) gel and transferred to nitrocellulose. Protein bands were visualized with colloidal gold stain (Bio-Rad). Individual bands were excised and dissolved by adding 0.5 ml of dimethyl sulfoxide and incubating for 1 h at 37°C. Nitrocellulose particles were precipitated by adding dropwise a solution of 0.5 M sodium carbonate–0.5 M sodium bicarbonate (pH 9.6) with continual vortexing. The precipitates were washed three times with RPMI 1640 and suspended to a final volume of 200 μl in RPMI 1640. Samples were tested by adding 10 μl to cultures of concanavalin A-peptone-elicited peritoneal exudate cells (PEC) as a source of phagocytic antigen-presenting cells and hybridoma 1C9 (10^5 per well). The IL-2 content of culture supernatants was measured 24 h later.

RESULTS

The T-cell hybridoma 1C9 produces IL-2 in response to an antigen expressed by *M. avium* MAC101. To study the processing and presentation of *M. avium* antigens by infected macrophages, we generated T-cell hybridomas from lymph node cells of BALB/c mice which had been immunized with a preparation of *M. avium* 5-8 (serovar 4) (34) proteins. One hybridoma, 1C9, which reacted to the immunizing preparation in the context of I-A^d (data not shown), was selected for further study. We first tested whether the antigen recognized by hybridoma 1C9 was expressed by *M. avium* MAC101 (serovar 1) (9), a virulent strain isolated from an AIDS patient. 1C9 cells were incubated with the B-cell lymphoma TA3 as a source of antigen-presenting cells and increasing doses of a sonicate of MAC101. Culture supernatants conditioned by the hybridoma were collected and measured for IL-2 content (see Materials and Methods). As shown in Fig. 1, we observed a dose-dependent production of IL-2 from the hybridoma in response to the sonicate, thus demonstrating that the antigen recognized by hybridoma 1C9 is expressed by this virulent clinical isolate of *M. avium*. We therefore chose strain MAC101 for our subsequent studies.

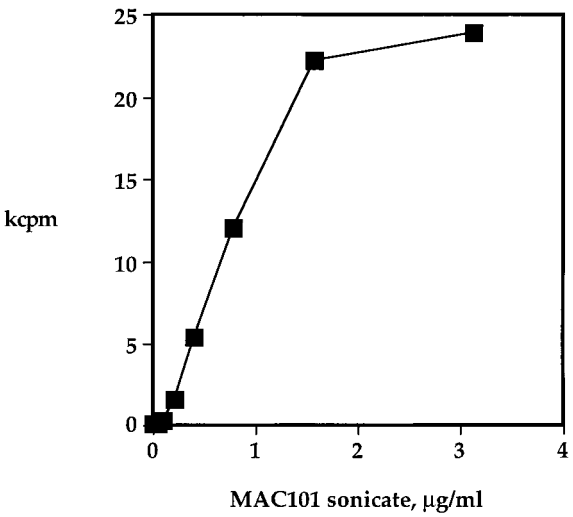

FIG. 1. T-cell hybridoma 1C9 responds to an antigen present in a sonicate of *M. avium* MAC101. T hybridoma cells (10^5 per well) were incubated with the B-cell line TA3 (3×10^4 per well) and increasing doses of a sonicate of MAC101. After 24 h, culture supernatants were collected and tested for IL-2 content as described in Materials and Methods. The experiment shown is representative of more than 10 similar experiments performed independently. The values are the means of duplicate cultures. Ranges were less than 15% of the mean values. Error bars were omitted for clarity.

The antigen recognized by hybridoma 1C9 is processed and presented in vitro by MAC101-infected mouse macrophages. We next determined whether the antigen recognized by hybridoma 1C9 is presented by MAC101-infected mouse macrophages in vitro. Adherent PEC elicited by concanavalin A and peptone treatment were employed as an enriched source of activated macrophages high in class II expression. PEC monolayers were incubated with various doses of a log-phase culture of MAC101, ranging from 4 to 40 viable mycobacteria per macrophage. At various time points, the macrophage monolayers were washed extensively to remove extracellular mycobacteria. To stop further antigen processing, and to preclude the effects of macrophage cytokines (such as transforming growth factor β and IL-10) and mycobacterial components (such as lipoarabinomannan) which could inhibit T-cell activation (7, 8, 28), the infected macrophages were fixed with paraformaldehyde. Hybridoma 1C9 was added to the fixed monolayers, and IL-2 production was measured. As shown in Fig. 2a, MAC101-infected PEC stimulated a high level of IL-2 production by hybridoma 1C9. When a high dose of MAC101 was employed (40 mycobacteria per macrophage), 1C9-antigen-major histocompatibility complexes (MHCs) were detectable 1 h after the initiation of infection. When a 10-fold-lower dose of MAC101 was employed, the kinetics of presentation were slower: 1C9 antigen-MHCs were detected 90 min after the initiation of infection and were still increasing at 2 h. Figure 2b

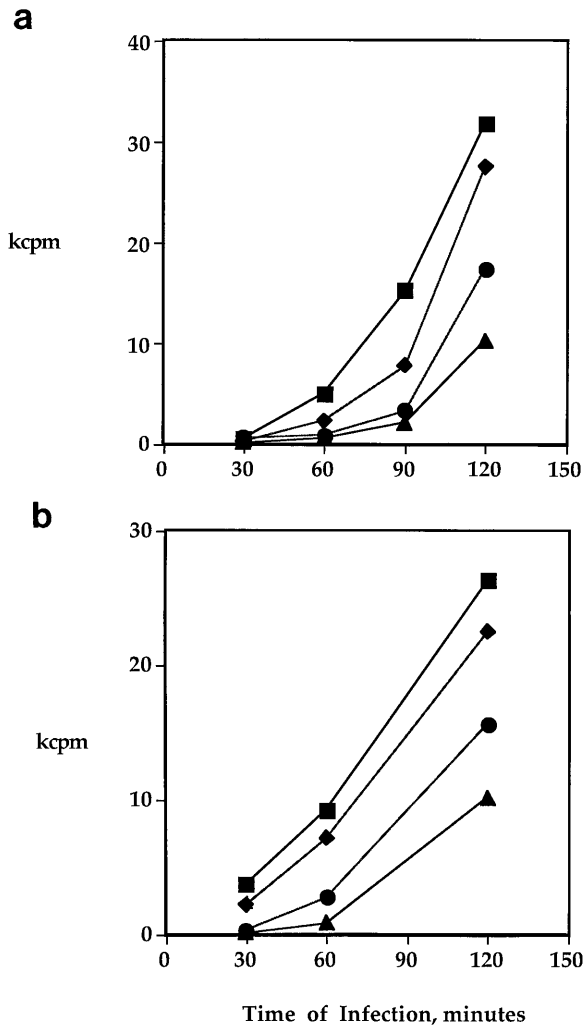

FIG. 2. The 1C9 antigen is processed and presented by macrophages infected in vitro with MAC101. (a) Concanavalin A-peptone-elicited macrophages were infected for 30, 60, 90, or 120 min with different doses of MAC101 bacilli and then fixed with paraformaldehyde. Hybridoma 1C9 (10^5 cells per well) was added, and culture supernatants were collected and tested for IL-2 content after 24 h. The experiment shown is representative of five similar experiments performed independently. (b) Bone marrow-derived macrophages were infected for 30, 60, or 120 min, fixed with paraformaldehyde, and tested for their ability to stimulate IL-2 production from hybridoma 1C9 as described for panel a. The experiment shown is representative of three similar experiments performed independently. In both experiments, the values are the means of duplicate cultures. Ranges were less than 20% of the mean values. Error bars were omitted for clarity. Infection levels for both panels were 40:1 (■), 20:1 (◆), 8:1 (●), and 4:1 (▲) (mycobacteria per macrophage).

shows that bone marrow-derived macrophages infected with MAC101 also presented the antigen recognized by hybridoma 1C9; the kinetics of presentation were very similar to those observed with PEC. Pretreatment of the macrophages with 100 U of gamma interferon per ml to upregulate MHC class II expression was required for presentation to occur (data not shown).

The antigen recognized by hybridoma 1C9 is a 50-kDa protein. To begin to purify the antigen recognized by hybridoma 1C9, we first employed gel filtration chromatography. A sonicate of MAC101 was passed over a Sephacryl S300 gel filtration column, and individual fractions were collected and tested for their ability to stimulate IL-2 production from hybridoma 1C9. Figure 3 shows that the antigen eluted as a broad peak in the molecular mass range of 66 to 200 kDa. Next, the active gel filtration fractions were pooled and concentrated approximately 40-fold to maximize the amount of antigenic material for the next purification step. SDS-PAGE analysis of the starting MAC101 sonicate and the concentrated, active gel filtration fractions indicated that the gel filtration chromatography was particularly effective at removing high-molecular-weight aggregates from the starting preparation (data not shown). The antigen was then identified by T-cell Western blotting, a technique which relies on the ability of macrophages to take up antigen-bearing particles derived from nitrocellulose strips of Western blots (1, 24). The concentrated, active gel filtration fractions were applied to two lanes of a reducing SDS–8% PAGE gel. After separation by PAGE, the proteins were transferred electrophoretically to nitrocellulose, and individual bands were visualized by colloidal gold staining. One lane was used as a reference (Fig. 4a). Bands with bound protein were cut out of the second lane, and each strip was dissolved and precipitated as described in Materials and Methods. Individual precipitates were tested for their ability to stimulate IL-2 production from hybridoma 1C9 with PEC as antigen-presenting cells. Figure 4b shows that a precipitated nitrocellulose fraction containing a protein with a molecular mass of approximately 50 kDa specifically stimulated IL-2 production by hybridoma 1C9. The fact that the 1C9 antigen elutes from the gel filtration column at a higher apparent molecular weight than is evident in the T-cell Western blot suggests that under the nonreducing conditions of the column, the antigen is dimerized or otherwise aggregated. The 50-kDa antigen stimulated higher levels of IL-2 production by hybridoma 1C9 in two T-cell Western blot experiments in which the nitrocellulose strips dissolved and precipitated for the assay were not stained with colloidal gold (data not shown). Direct staining of the proteins with colloidal gold was necessary for precise removal of individual protein bands from the blot and unambiguous identification of the antigen. Thus, with T-cell Western blotting, we were able to identify efficiently the antigenic material within a complex mixture of proteins.

Presentation of the 1C9 antigen requires an acidified compartment with active proteases. It is well established that soluble proteins entering antigen-presenting cells via endocytosis require processing for presentation to $CD4^+$ T cells to occur. Antigens are first cleaved by lysosomal proteases in acidified compartments; the resulting peptides associate with nascent class II molecules which are then transported to the antigen-presenting cell surface (19, 46). It has been postulated that mycobacterial antigens in the phagocytic vacuole are processed in an analogous fashion (3, 15, 22, 32, 38), but this has not been confirmed with a specific *M. avium* antigen. To demonstrate that the 1C9 antigen must be processed for presentation to occur, we pretreated PEC with the lysosomotropic agent chloroquine, which inhibits acidification of phagocytic and endocytic vacuoles. The macrophages were then infected for 2 h with MAC101 in the continued presence of the drug, fixed with paraformaldehyde, and tested for their ability to stimulate 1C9 T cells. The data in Fig. 5 show that chloroquine treatment of the PEC completely inhibited presentation of the 1C9 antigen. This result strongly suggests that the antigen must encounter an acidified vacuole for presentation to occur.

We next investigated the role of lysosomal proteases in the processing of the 1C9 antigen. Recent studies have shown that the acid proteases cathepsin B, cathepsin D, and cathepsin L are present in *M. avium*-containing vacuoles (39a). We therefore tested the effect of pepstatin A, which inhibits cathepsin D, and E64, which inhibits cathepsins B and L, on the presen-

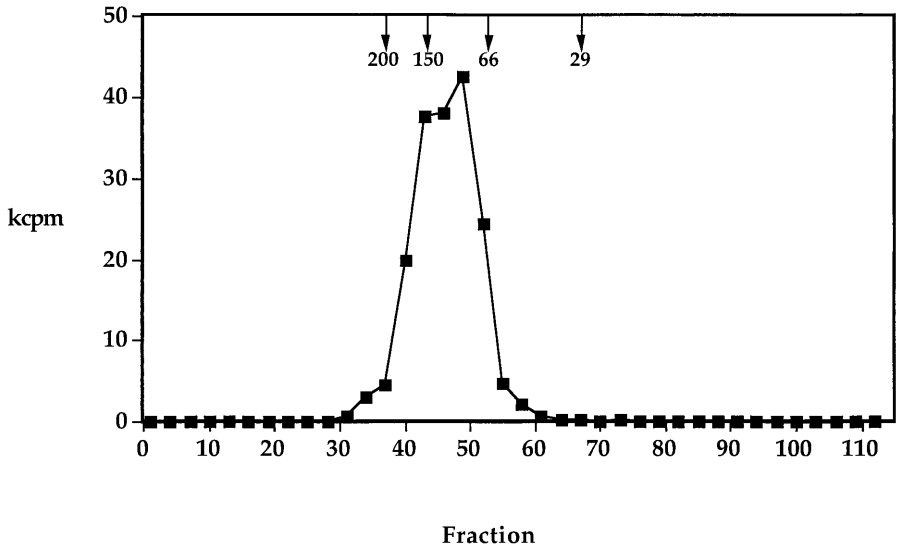

FIG. 3. The 1C9 antigen elutes as a broad peak upon gel filtration of the MAC101 sonicate. Four and one-half milligrams of MAC101 sonicate was fractionated by passing the sonicate over a Sephacryl S300 gel filtration column. Fractions were tested for their ability to stimulate hybridoma 1C9 (1×10^5 cells per well) by using TA3 B cells (3×10^4 cells per well) as antigen-presenting cells. The IL-2 content of culture supernatants was assayed after 24 h. Arrows indicate the fractions at which molecular weight standards eluted from the column. The numbers under the arrows indicate the molecular mass in kilodaltons.

tation of the 1C9 antigen. PEC monolayers were preincubated with pepstatin A, E64, or the two drugs combined. The PEC were then infected for 2 h with various doses of MAC101 in the continued presence of the inhibitors, fixed with paraformaldehyde, and tested for their ability to stimulate hybridoma 1C9. As shown in Fig. 6a, treatment of the PEC with pepstatin A or E64 alone significantly inhibited presentation of the 1C9 antigen. A combination of the two protease inhibitors had an additive effect and almost completely inhibited presentation of the 1C9 antigen when lower doses of MAC101 were employed for infection.

A possible explanation for the observed inhibition is that the protease inhibitors block class II invariant chain (Ii) cleavage, thereby inhibiting maturation of functional class II molecules and association with antigenic peptides (10). To address this, in the same experiment, PEC which had been treated with the protease inhibitors and infected with MAC101 were tested for their ability to present an antigenic peptide to a different T-cell hybridoma, Fl.2, which is specific for amino acids 409 to 421 of β-galactosidase in the context of I-A^d (40a). As shown in Fig. 6b, treatment of infected PEC with the protease inhibitors had no effect on their ability to present the β-galactosidase peptide to hybridoma Fl.2, thus indicating that treatment with the protease inhibitors does not block the function of surface class II molecules. Taken together, these results suggest that the protease inhibitors specifically block proteolysis of the 1C9 antigen and are not affecting the integrity of the class II antigen presentation pathway as a whole in the time frame examined.

DISCUSSION

In this communication, we have described a T-cell hybridoma, 1C9, which reacts with an antigen expressed by a virulent clinical isolate of *M. avium*, MAC101. The antigen is present in a sonicate of MAC101 and strongly stimulates the hybridoma to produce IL-2 when processed and presented by a B-cell line. Fractionation of the MAC101 sonicate by gel filtration chromatography followed by T-cell Western blotting revealed that the antigen is approximately 50 kDa. More importantly, we have shown that the 1C9 antigen is processed and presented by macrophages which have been infected by live MAC101. Experiments in which macrophages were pretreated with chloroquine or protease inhibitors demonstrated that internalization of the mycobacteria and encounter of the antigen with an acidified vacuole containing active proteases are necessary for presentation of the antigen to occur.

To our knowledge, this is the first characterization of the processing and presentation of an *M. avium* antigen to a specific T cell. We do not propose that all mycobacterial antigens are processed and presented to T cells in this way. Other antigen processing pathways, such as the class I presentation pathway and the recently described presentation by CD1 molecules, are important for immunity to mycobacteria (5, 18, 27, 39); moreover, some mycobacterial antigens do not require processing by antigen-presenting cells at all (29). Nevertheless, the fact that numerous soluble antigens have been shown to be processed and presented by a similar mechanism by the class II presentation pathway strongly suggests that our results with the 1C9 antigen are broadly applicable to class II-restricted mycobacterial antigens.

A search through the most recently updated version (April 1996) of the MycDB database (6) did not reveal any *M. avium* proteins of the same apparent molecular weight as the 1C9 antigen. Thus, it is likely that the 1C9 antigen is novel. Further characterization of this antigen will permit the determination of whether it plays a role in stimulating protective immune responses to *M. avium.*

It is important to relate our results to what is known about the maturation of the mycobacterium-containing vacuole down the endosomal-lysosomal pathway, the proteases involved in antigen processing and presentation, and the effect mycobacterial infection has on the ability of host macrophages to stimulate T cells. Like other mycobacteria, *M. avium* is taken up into macrophages via specific receptors and dwells within a phagocytic vacuole. Several recent studies have demonstrated that live mycobacteria evade destruction in host macrophages by inhibiting the maturation of the phagocytic vacuole along the endosomal-lysosomal pathway (13, 30, 40, 44). Sturgill-

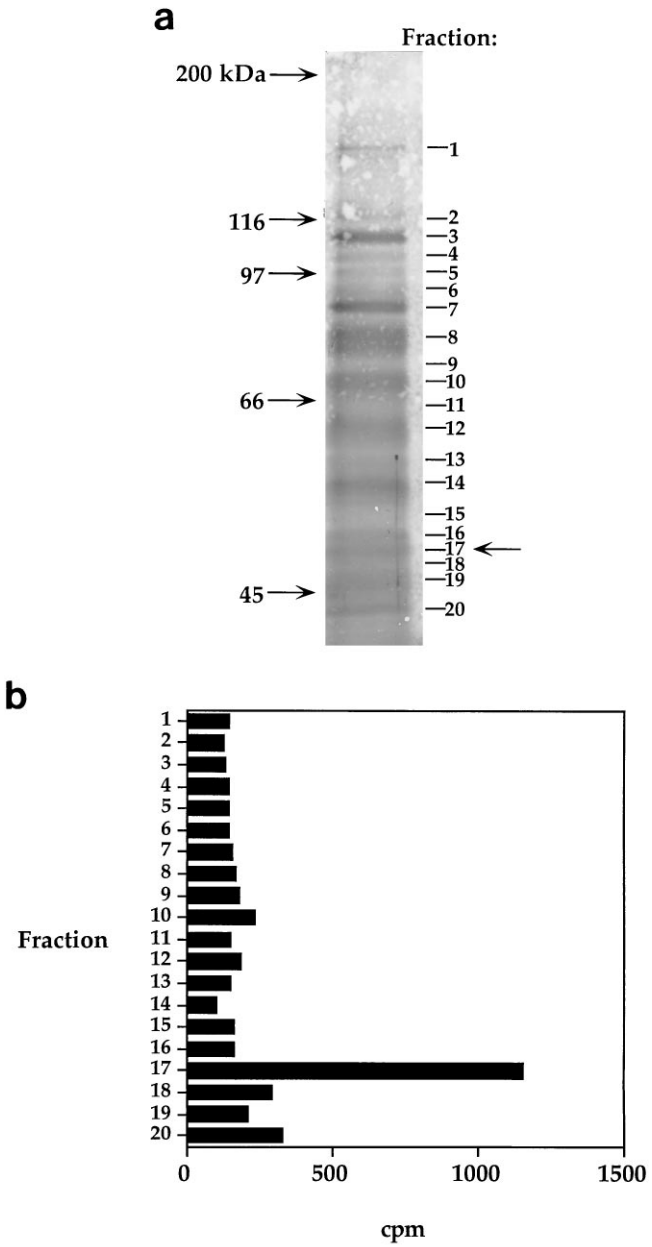

FIG. 4. The 1C9 antigen is a 50-kDa protein. (a) Fractions from the Sephacryl S300 column containing antigenic activity were concentrated, run on a reducing SDS–8% PAGE gel, and transferred to nitrocellulose. Proteins were detected by staining with colloidal gold. The numbers to the left denote the positions of migration of molecular mass markers. The numbers to the right denote individual strips of nitrocellulose which were tested in the T-cell Western blot assay. The arrow on the right indicates the band with antigenic activity. (b) Peritoneal exudate macrophages were incubated with the precipitated nitrocellulose fractions indicated in panel a. Hybridoma 1C9 (10^5 cells per well) was added, and the IL-2 content of culture supernatants was measured 24 h later. The values indicate the means of duplicate cultures. Ranges were less than 10% of the mean values. Error bars were omitted for clarity. The experiment shown is representative of three similar experiments performed independently.

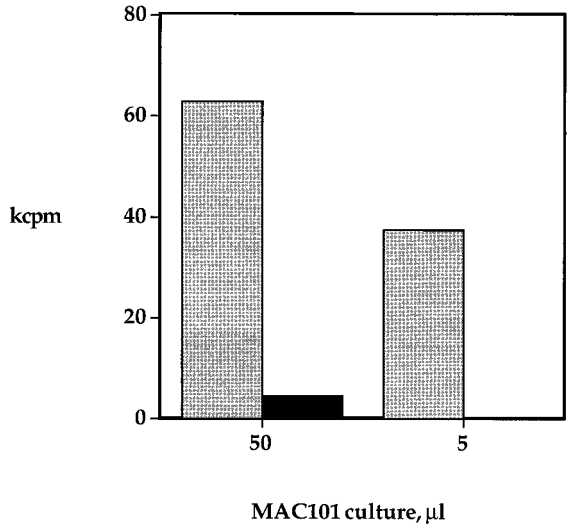

FIG. 5. Chloroquine blocks the presentation of the 1C9 antigen by infected macrophages. Peritoneal exudate macrophages were pretreated for 20 min with 100 μM chloroquine (■) or no inhibitor (▩) and infected with the indicated doses of a log-phase culture of MAC101 for 2 h in continued presence of the drug. The macrophages were then fixed with paraformaldehyde. Hybridoma 1C9 was added, and culture supernatants were collected for the measurement of IL-2 content 24 h later. The values indicate the means of duplicate cultures. The ranges were less than 10% of the mean values.

Koszycki et al. have shown that phagocytic vacuoles isolated from bone marrow-derived mouse macrophages infected with *M. avium* MAC101 fail to acidify below pH 6.0; this incomplete acidification correlates with the failure of these vacuoles to acquire the vesicular proton-ATPase (40). These results were extended by the demonstration that vacuoles containing live *M. avium* or *Mycobacterium tuberculosis* do not contain the mannose-6-phosphate receptor, a marker for late endosomes, and do not acquire biotinylated dextran, which accumulates in lysosomes (44). In addition, in a detailed study of *M. tuberculosis* phagosomes from human monocytes, Clemens and Horwitz observed that phagosomes containing live bacilli contained little gold-labeled BSA, which collects in lysosomes, and acquire little cathepsin D, a lysosomal protease (13). Heat-killed bacilli, in contrast, were processed completely to lysosomes. We have shown that pretreatment of macrophages with chloroquine or inhibitors of the lysosomal proteases cathepsin D, B, and L significantly inhibited presentation of the 1C9 antigen. These results suggest that the mycobacteria must access lysosomes for presentation of the 1C9 antigen to occur. Since we employ cultures of MAC101 in log-phase growth for our experiments, few dead bacilli are expected to be present. Moreover, our preliminary experiments indicate that macrophages incubated with heat-killed bacilli present the 1C9 antigen poorly (data not shown). Interpreted in light of the studies mentioned above, then, our data taken together suggest that the 1C9 antigen is derived from metabolically active bacilli which have been killed during the process of infection and which are processed to lysosomes. An alternative explanation for our results is that the 1C9 antigen is secreted by metabolically active mycobacteria which remain at an earlier stage of the endosomal-lysosomal pathway and that the secreted antigen travels on to lysosomes. We think this explanation is less likely, however, because the 1C9 antigen is not detectable in culture filtrates of MAC101, indicating that it is not constitutively secreted by MAC101 (data not shown).

It is well established that antigens must be processed into peptides for presentation to T cells to occur (19). This processing involves the action of proteases in the acidified endosomal-lysosomal compartment. A number of groups have employed specific inhibitors to identify proteases involved in the processing of soluble antigens and to define the sites of peptidase cleavage (11, 16, 35, 36, 41–43). Collectively, these stud-

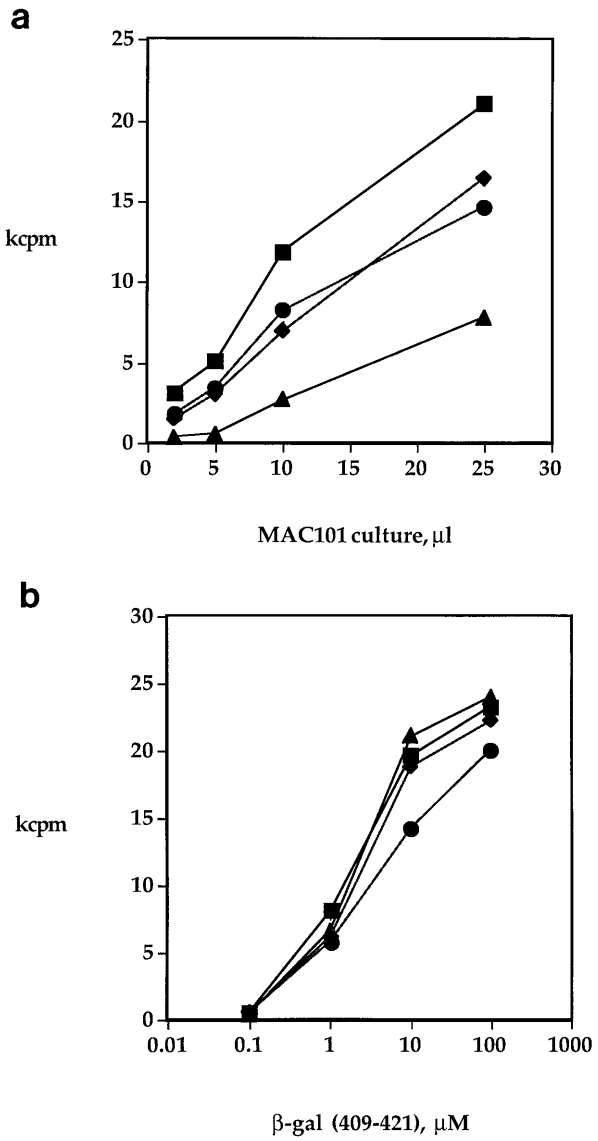

FIG. 6. (a) Processing of the 1C9 antigen by infected macrophages is blocked by inhibitors of acid proteases. Peritoneal exudate macrophages were either left untreated or pretreated for 20 min with E64, pepstatin A, or the two inhibitors combined. The macrophages were then infected for 2 h in the continued presence of the drugs. The macrophages were then fixed. 1C9 T cells were added, and culture supernatants were collected 24 h later for the measurement of IL-2 content. Treatment of the macrophages with vehicle alone (dimethyl sulfoxide) had no effect on 1C9 antigen presentation (data not shown). (b) Treatment of macrophages with acid protease inhibitors does not affect their ability to present antigenic peptides. PEC were either left untreated or pretreated for 20 min with E64, pepstatin A, or the two drugs combined. They were then infected for 2 h with MAC101 (10 μl of the same MAC101 culture used in the experiment described for panel a) in the continued presence of the drugs and fixed. The β-galactosidase-specific T-cell hybridoma Fl.2 was added along with the indicated doses of the peptide β-galactosidase(409–421). IL-2 production in the culture supernatants was measured 24 h later. The values indicate the means of duplicate cultures. Ranges were less than 15% of the mean values. Error bars were omitted for clarity. Symbols: ■, no inhibitor; ◆, E64; ●, pepstatin A; ▲, E64 plus pepstatin A.

ies have shown that two lysosomal proteases, the aspartyl protease cathepsin D and the cysteine protease cathepsin B, play an important role in the proteolysis of some antigens. Puri and Factorovich demonstrated that pepstatin A, an inhibitor of cathepsin D, blocks the presentation of three different antigens to their cognate T-cell clones (35), and Rodriguez and Diment have shown that processing and presentation of an ovalbumin epitope to an I-A^d-restricted T-cell hybridoma requires the action of cathepsin D (36). In addition, Takahashi et al. showed that digestion of sperm whale myoglobin with cathepsin B is sufficient to generate three different epitopes to specific T-cell clones (41). Moreover, a thorough study of the processing of myoglobin by macrophages strongly suggested that the combined activity of cathepsin D and cathepsin B leads to the generation of immunodominant epitopes to myoglobin-primed T cells (42). We have now extended these findings to a mycobacterial antigen within the context of an infected cell. Pretreatment of peritoneal exudate macrophages with pepstatin A or E64, an inhibitor of cathepsins B and L, significantly inhibited the presentation of the 1C9 antigen, and combined, the two inhibitors almost completely blocked presentation when the macrophages were infected with lower doses of *M. avium*. These results demonstrate that an antigen derived from an intact pathogen requires the action of these aspartyl and cysteine proteases for presentation to T cells to occur.

Studies on the processing of Ii suggested a role for cysteine proteases in the cleavage of Ii, which is required for the dissociation of Ii from nascent class II molecules and the association of antigenic peptides (10). Thus, an alternative interpretation to the observed inhibition of 1C9 antigen presentation by E64 is that Ii processing is blocked. We have shown that E64-treated, fixed macrophages are not deficient in their ability to present peptide to an I-A^d-restricted T-cell hybridoma specific for β-galactosidase. Thus, E64 treatment has no observable effect on functional cell surface class II molecules. However, we cannot rule out the possibility that inhibition of Ii cleavage significantly blocks the association of the 1C9 epitope with nascent class II molecules before they reach the cell surface. Taken together, then, our data strongly suggest that the activity of cathepsin D is required for proteolytic cleavage of the 1C9 antigen; cathepsins B and L may be required for further proteolysis of the antigen or may play an essential role in its ability to associate with nascent class II molecules.

It should be noted that our data do not exclude the possibility that other proteases are essential for processing of the 1C9 antigen. Several other proteases have been implicated in antigen processing and could very well be important for generation of the 1C9 epitope (11, 35, 43). In addition, while some proteases are required for the generation of specific epitopes, some may cleave at sites which destroy epitopes (43). It is therefore reasonable to expect that peptides derived from different mycobacterial antigens which associate with specific MHC class II molecules will require the action of different combinations of proteases.

It is likely that the processing and presentation of a given mycobacterial antigen are affected by the activation state of the macrophage and both the duration and level of infection of the macrophage. Pancholi et al. have recently reported that human monocytes infected for 8 days with *Mycobacterium bovis* BCG have a markedly reduced ability to present *M. bovis* antigens to primed T cells or to specific T-cell clones. In contrast, acutely infected (2 days) macrophages presented such antigens efficiently (33). The chronically infected monocytes could present tuberculin purified protein derivative, anti-CD3, and bacterial superantigens perfectly well and showed no apparent down-regulation of MHC class II expression. It was concluded that once they have established an infection, mycobacteria are able selectively to block the presentation of their own antigens to T cells. Our preliminary experiments indicate that bone marrow-derived macrophages continue to present the 1C9 antigen as many as 7 days after the initiation of infection (19a); however,

consistent with the findings of Pancholi et al., the level of presentation is reduced. Importantly, presentation of the 1C9 antigen at such late time points requires a high level of infection, approximately 50 mycobacteria per macrophage, which is 10-fold higher than the dose employed by Pancholi et al. Thus, mycobacteria may be capable of sequestering their antigens only when the host macrophage is infected with a relatively small number of bacilli. This might occur during the acute stages of infection; increased presentation of mycobacterial antigens might occur upon bacillary growth within the macrophage or upon activation of macrophage effector functions.

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