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Kinetics of Chlamydial Antigen Processing and Presentation to T Cells by Paraformaldehyde-Fixed Murine Bone Marrow-Derived Macrophages

HUA SU AND HARLAN D. CALDWELL*

Immunology Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840-2999

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Macrophages are potential candidates for antigen presentation to chlamydial-specific CD4$^+$ T cells. We have studied the kinetics of chlamydial antigen processing and presentation by using paraformaldehyde-fixed bone marrow-derived macrophages (BMDM) and splenic T cells isolated from chlamydia-infected mice. BMDM were inoculated with different multiplicities of heat-killed chlamydial elementary bodies, and at different times postingestion, the macrophages were fixed with paraformaldehyde and used as antigen-presenting cells in T-cell proliferation assays. T-cell proliferative responses were shown to be dependent on the chlamydial inoculum size, with a multiplicity of 10 chlamydiae per macrophage producing optimum T-cell proliferation. Temporal experiments showed that peak T-cell proliferative responses occurred between 4 and 12 h postingestion of chlamydiae by BMDM. T cells proliferated strongly to antigen when presented by *H-2*-matched BMDM but not when presented by *H-2*-disparate BMDM, demonstrating that T-cell recognition of processed chlamydial antigen was major histocompatibility complex restricted. BMDM inoculated with 10 chlamydiae per cell and fixed at 8 h postinoculation were shown to be as stimulatory to T cells as conventional splenic antigen-presenting cells. Because large numbers of BMDM can be propagated in vitro, and experimental conditions that provide optimum presentation of processed chlamydial antigen to chlamydia-specific CD4$^+$ T cells can be defined, BMDM may be a potentially useful source for the isolation of naturally processed parasite antigen from major histocompatibility complex class II molecules.

Chlamydia trachomatis is an obligate intracellular bacterial pathogen that infects the ocular and urogenital mucosae of humans. Infections caused by *C. trachomatis* are a major cause of preventable blindness and sexually transmitted diseases (13, 20, 32) for which immune intervention strategies are badly needed. Vaccine development for the prevention and control of chlamydial diseases would be greatly facilitated by a better understanding of host immune mechanisms that function in acquired immunity and of those chlamydial antigens that elicit protective immune responses.

The *C. trachomatis* murine biovar, mouse pneumonitis (MoPn), produces infection and disease of the mouse genital tract that closely parallels those in humans caused by human *C. trachomatis* biovars (5, 28, 35) and is therefore a useful model for the study of acquired immunity to chlamydial genital tract infection. Mice infected vaginally with MoPn develop an acute self-limiting infection of the genital tract. Peak shedding of chlamydiae occurs at 7 to 14 days, and the infection lasts 21 to 28 days. Subsequent challenges are characterized by a significant decrease in cervicovaginal shedding of chlamydiae and a rapid resolution of infection. T-cell responses play an important role in naturally acquired immunity in this model. Rank et al. (30) have shown that athymic mice fail to clear chlamydiae from the genital tract, and this inability to resolve infection can be partially restored by adoptive transfer of chlamydia-specific CD4$^+$ T-cell lines (29) or clones (19). Furthermore, in vivo depletion of CD4$^+$ T cells results in persistent chlamydial genital tract infection with a higher incidence of hydrosalpinx (21). Collectively, these findings provide strong evidence that CD4$^+$ major histocompatibility complex (MHC) class II-restricted T-cell responses are critical components of the protective immune response to chlamydial genital tract infection. However, the mechanism(s) by which CD4$^+$ T cells function and the chlamydial antigenic determinants or epitopes that are recognized by protective CD4$^+$ T cells are undefined.

Identification of naturally processed chlamydial peptides recognized by chlamydia-specific protective CD4$^+$ T cells will allow for the identification of new classes or types of chlamydial antigens and epitopes for vaccine development. One approach to achieve this goal is to directly sequence the pathogen-derived T-cell epitopes isolated from MHC molecules. This approach has successfully identified MHC class I-restricted CTL epitopes of *Listeria monocytogenes* (27). Bone marrow-derived macrophages (BMDM) are efficient antigen-presenting cells (APC) that present processed immunogenic peptides complexed to MHC class II molecules to CD4$^+$ T cells (6). Unlike continuous murine macrophage cell lines, murine BMDM are highly phagocytic and can express elevated levels of class II MHC molecules following induction by gamma interferon (IFN-γ). Therefore, BMDM have the potential to be a useful source for the isolation and characterization of naturally processed chlamydial peptide(s) complexed to MHC class II molecules. This report describes the kinetics of natural antigen processing of chlamydiae by murine BMDM and the experimental conditions of processing and presentation which provide optimum responsiveness to chlamydia-specific T cells. These findings may be relevant to future studies aimed at identifying chlamydial T-cell epitopes that elicit protective immune responses.

* Corresponding author. Mailing address: Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840-2999. Phone: (406) 363-9333. Fax: (406) 363-9204.

MATERIALS AND METHODS

Chlamydiae. The murine *C. trachomatis* mouse pneumonitis (MoPn) strain was grown in HeLa 229 cells and elementary bodies (EBs) were purified from infected cells by density gradient centrifugation as previously described (8). Infectious forming units (IFUs) of purified EBs were assayed on HeLa 229 monolayers by indirect fluorescent-antibody staining as previously described (31). For all experiments described below, live chlamydiae were resuspended in Dulbecco's modified Eagle medium (D-MEM; GIBCO Life Technologies, Gaithersburg, Md.) buffered with 10 mM 3-(*N*-morpholino)propanesulfonic acid and heated in a 80°C water bath for 30 min at a density of 3×10^7 IFUs per ml. Confirmation of the heat killing treatment was documented by IFU titrations on HeLa 229 cells. The multiplicity of inoculation (MOI) of heat-killed (HK) chlamydiae used to inoculate macrophages corresponds to the IFU concentration of the viable seed stock preparation prior to heat treatment. HK chlamydiae were used in these experiments to avoid infection of macrophages. UV light-inactivated chlamydiae were not used, because they retain chlamydial cytotoxic activity and therefore, could not be used in those experiments in which higher multiplicities of chlamydial inoculum were required. Last, because class II MHC presentation of chlamydial antigen to T cells involves proteolytic degradation and processing, chlamydial viability and native conformation of chlamydial proteins are not critical issues in these experiments.

Mouse strains. C57BL/10SnJ (*H-2*^{b}), B10.A/SgSnJ (*H-2*^{a}), B10.D2/oSnJ (*H-2*^{d}), B10.BR/SgSnJ (*H-2*^{k}), B10.Q/SgJ (*H-2*^{q}), and B10.PL(73NS)/Sn (*H-2*^{u}) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice of both sexes at 2 to 4 months of age were used for experimentation.

Cultures of BMDM. Macrophage cultures were prepared by the method of Brunt et al. (6). Briefly, macrophages were obtained by culturing bone marrow plugs from mouse femurs in D-MEM supplemented with 20% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), gentamicin (50 μg/ml), 2-mercaptoethanol (50 μM), and 20% L-929 cell conditioned medium (33). Cultures were maintained in 5% CO_2 at 37°C in plates (150 by 25 mm) (Corning Incorporated, Corning, N.Y.) until they formed confluent monolayers (4 to 5 days). Three femoral plugs consistently produced a confluent macrophage monolayer which yielded 2×10^7 to 4×10^7 macrophages per plate. The macrophage monolayer was washed three times with 20 ml of Dulbecco's PBS (D-PBS) to remove nonadherent cells. Twenty milliliters of Ca^{2+}- and Mg^{2+}-free D-PBS containing 2 mg of glucose per ml was then added to the macrophage monolayer, and the plate was incubated for 20 min at 37°C. The D-PBS solution was then pipetted over the surface of the monolayer until the majority of macrophages were detached. Macrophages were collected by centrifugation and then plated in either 24- or 96-well flat-bottom tissue culture plates (Linbro; Flow Laboratories, McLean, Va.) in D-MEM containing 10% FCS (D-MEM-10) with 20 ng of recombinant murine IFN-γ (rIFN-γ) per ml (PharMingen, San Diego, Calif.). Medium was supplemented with rIFN-γ to increase levels of class II MHC expression by BMDM (6).

Chlamydial ingestion and proteolytic degradation by BMDM. Macrophages were plated onto 12-mm-diameter glass coverslips in 24-well tissue culture plates in 1 ml of D-MEM-10 containing rIFN-γ at a density of 7.5×10^5 cells per well. After incubation at 37°C for 40 h, the confluent monolayers were washed with D-MEM and inoculated with HK chlamydiae at an MOI of 10 in 250 μl of D-MEM for 30 min at 37°C ($T = 0$). The inocula were removed, and the monolayers were washed twice with 500 μl of D-MEM to remove uningested chlamydiae. The monolayers were then refed with 1 ml of D-MEM-10, and the plates were incubated for 30 min, 2 h, and 4 h at 37°C in 5% CO_2. After each incubation period, the media were removed, and the monolayers were washed twice with 1 ml of D-PBS and fixed for 15 min at room temperature with 0.5 ml of absolute methanol. The monolayers were stained with either monoclonal antibody (MAb) MP-33b which is specific to the MoPn major outer membrane protein (MOMP) or MAb EVI-H1 which is specific to chlamydial lipopolysaccharide (LPS) at 37°C for 30 min. Both MAbs recognize heat-stable epitopes that are surface exposed on MoPn EBs. After the macrophages were washed, they were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G at 37°C for 30 min. The coverslips with macrophage monolayers were washed in D-PBS, mounted in buffered glycerol, and examined for immunofluorescence. The kinetics of proteolytic degradation of chlamydiae by macrophages was assessed by comparing the antigenic signals obtained with each of the MAbs at different times post-chlamydial ingestion.

BMDM as chlamydial APC. Macrophages were plated into 96-well plates at densities of 5×10^3, 1.5×10^4, 5×10^4, and 1.5×10^5 in 100 μl of D-MEM-10 containing 20 ng of rIFN-γ per ml at 37°C for 40 h. The media were removed, and the monolayers were washed three times with 150 μl of D-MEM. The monolayers were then inoculated with 50 μl of HK chlamydiae suspended in D-MEM at a MOI of 0.1, 1, or 10 chlamydiae per macrophage and incubated for 30 min ($T = 0$) at 37°C. The inocula were removed, and the monolayers were washed with D-MEM. The macrophages were then used as viable APC in T-cell proliferation assays or were refed with D-MEM-10, incubated at 37°C for 30 min and 2, 4, 8, 12, 24, 34, 48, and 72 h, washed, and lightly fixed with 1% paraformaldehyde for 15 min at room temperature (6). The macrophages were washed and then incubated with 100 μl of 0.1 M lysine for 30 min at 37°C. The plates were kept at 4°C in D-PBS and washed once with D-MEM before the T cells were added.

Splenocytes as chlamydial APC. Spleens were harvested from normal C57BL/10 mice, single-cell suspensions were prepared, and erythrocytes were lysed with 0.17 M Tris-buffered ammonium chloride. Splenocytes (10^7/ml) in D-MEM were incubated with HK chlamydiae at MOIs of 0.1, 1, and 10 per cell in a 37°C shaking water bath for 1 h. The cells were washed twice with phosphate-buffered balanced salt solution (PBBS), pH 7.2, containing 5% FCS and resuspended in D-MEM-10 at a concentration of 10^7 cells per ml. Splenocytes were plated at 100 μl per well in 96-well flat-bottom plates and then irradiated (3,000 rads) in a ^{137}Cs gamma irradiator.

Isolation of T cells. C57BL/10 female mice that had resolved a primary genital tract infection with MoPn and had been rechallenged vaginally 30 to 40 days following resolution of primary infection were the sources of chlamydia-specific (immune) T cells. Secondary challenge resulted in infections of reduced duration and with significantly less chlamydial shedding (data not shown). Mice were sacrificed 10 days following the secondary chlamydial challenge, and their spleens were harvested. Single-cell suspensions were prepared from control (uninfected C57BL/10 mice) and immune spleens in PBBS containing 5% FCS. T cells and $CD4^+$ T cells were isolated from splenocyte suspensions by immunoaffinity chromatography using IsoCell Mouse T-cell and IsoCell mouse CD4 isolation kits (Pierce, Rockford, Ill.). $CD8^+$ T cells were isolated from splenocyte suspensions by immunoaffinity chromatography using Cellect plus mouse CD8 isolation kit (Biotex Laboratories, Inc., Edmonton, Alberta, Canada). The immunoaffinity isolation of T cells was performed as described by the

manufacturers. Surface antigens of enriched T-cell, $CD4^+$, and $CD8^+$ cell populations were determined by flow cytometry. T-cell suspensions contained 90% or greater T cells and less than 1% B cells. $CD4^+$ and $CD8^+$ T cells isolated by immunoaffinity chromatography were equal to or greater than 75% of the total cell population. Enriched $CD4^+$ and $CD8^+$ populations contained 0.2% or less of contaminating $CD8^+$ or $CD4^+$ cells, respectively. Control (nonimmune) T cells were similarly prepared from naive, sex- and age-matched mice by identical procedures.

T-cell proliferation assays. Immune or control T cells suspended in D-MEM-10 were added to the 96-well plates (2.5×10^5 cells per well) containing viable or fixed macrophages or splenocyte APC, and the plates were incubated at 37°C in 5% CO_2 for 5 days. A 20-μl volume of D-MEM-10 containing 1.0 μCi of [^{3}H]thymidine (New England Nuclear, Du Pont, Wilmington, Del.) was added to each well, and the plates were incubated for an additional 18 h. The cells were then harvested, and radioactive incorporation was measured with a liquid scintillation counter (LS6000LL; Beckman Instruments, Palo, Alto, Calif.). The results are expressed as the mean ± standard deviation of triplicate cultures.

RESULTS

Ingestion and degradation of HK chlamydiae by BMDM. To define conditions of chlamydial uptake, ingestion, and proteolytic degradation by professional APCs, monolayers of BMDM were pulsed with HK MoPn EBs at an MOI of 10 and the cells were incubated for various periods of time postinoculation. The macrophage cultures for determining the efficiency of chlamydial uptake and proteolytic degradation were fixed with methanol and then stained by indirect immunofluorescence with a MAb specific to either the MOMP or LPS. Both these MAbs recognize heat-stable epitopes located at the surface of intact chlamydiae. These analyses were done to determine the efficiency of uptake of chlamydiae by BMDM and to monitor the antigenic signals of protein and nonprotein antigens at various time periods following chlamydial ingestion. Strong immunofluorescence staining of MoPn by both anti-MOMP and anti-LPS MAbs was observed at $T = 0$ and 30 min postinoculation (Fig. 1). Staining with the anti-MOMP and anti-LPS antibodies showed individual EBs associated with macrophages at each of these time periods. The numbers of EBs ingested by macrophages varied from cell to cell, with the majority of macrophages containing multiple organisms. The staining patterns of EB surfaces by the two MAbs differed. Anti-MOMP antibodies produced discrete staining of the EB periphery, whereas anti-LPS staining was more diffuse and made individual EB particles appear larger. By 2 h postinoculation, distinct differences in the staining patterns were observed with the anti-MOMP and anti-LPS MAbs. The numbers of individual EBs stained by the anti-MOMP MAb diminished significantly at this time period. Antigenic signals were commonly associated with what appeared to be aggregates of EBs that mostly appeared to be localized within large intracellular vesicles. At 4 h postinoculation, there was no detectable staining of chlamydial EBs by the anti-MOMP MAb. In contrast, macrophages stained with the anti-LPS antibody exhibited intense fluorescence staining of chlamydiae at 2 and 4 h postinoculation. Two distinct patterns of immunofluorescence were observed: (i) staining of EB aggregates in large vesicles similar to that observed with the anti-MOMP stained macrophages at 2 h postinoculation, and (ii) diffuse punctate fluorescence throughout the macrophage cytoplasm. Interestingly, this same staining pattern was observed in macrophages up to 7 days postinoculation (data not shown). These findings show that HK EBs are efficiently taken up by BMDM which effectively degrade the chlamydial MOMP within 2 h of EB ingestion.

Paraformaldehyde-fixed BMDM as chlamydial APC. We examined both viable and paraformaldehyde-fixed BMDM as chlamydial APC following ingestion of HK EBs. Initially, splenic T cells from chlamydia-challenged and control mice were tested in proliferation assays with viable or fixed BMDM following ingestion of chlamydiae. For these experiments, viable macrophages were plated in 96-well plates at 1.5×10^5 cells per well, inoculated with different MOIs of HK EBs, and then incubated with T cells. In those experiments in which fixed BMDM were used, macrophages were plated at the same density and inoculated with the same MOI of HK EBs. The macrophages were incubated for 8 h at 37°C and then lightly fixed with paraformaldehyde. Following fixation, T cells were added to the plates at the same concentrations as those used with viable macrophages. The T-cell proliferative responses obtained with viable and paraformaldehyde-fixed BMDM as chlamydial APC are shown in Fig. 2. Paraformaldehyde-fixed BMDM inoculated with chlamydial MOIs of 0.1 and 1 did not generate significant proliferative responses from immune T cells. In contrast, fixed macrophages that were inoculated with chlamydial MOIs of 10 and 100 were highly stimulatory to immune T cells. Optimum T-cell proliferation occurred when BMDM inoculated with a chlamydial MOI of 10 were used. There was a slight but insignificant reduction in the proliferative response of immune T cells incubated with fixed BMDM inoculated with an MOI of 100. In contrast to fixed BMDM, immune T cells incubated with viable BMDM failed to proliferate, irrespective of the number of HK EBs ingested by the macrophages. Examination of the viable macrophage cultures inoculated with a chlamydial MOI of 10 by phase microscopy revealed very few intact T cells, indicating that the negative proliferative responses at the higher chlamydial MOIs were the result of significantly depleted T-cell numbers in these cultures. A likely explanation for the inhibitory activity of viable macrophages on T-cell proliferation is enhanced macrophage activation with secretion of products that are inhibitory or toxic to T cells, as described by others (37). It is apparent from these results that unlike viable BMDM, fixed BMDM are potent in vitro chlamydial APC.

We next compared the proliferative responses of T cells and T-cell populations enriched for $CD4^+$ and $CD8^+$ cells by using paraformaldehyde-fixed BMDM as chlamydial APC (Fig. 3). Total T cells and T cells enriched for $CD4^+$ cells exhibited strong proliferative responses to fixed BMDM. In contrast to $CD4^+$ cells, immune $CD8^+$ cells exhibited a very marginal proliferative response with BMDM as chlamydial APC. These findings support a classical exogenous antigen processing and presentation pathway of chlamydial peptides by class II molecules to $CD4^+$ T cells following ingestion of HK EBs by BMDM. This result was not particularly unexpected, since nonviable EBs were used as antigen; however, it was important to define, since a major focus of this work was to develop an in vitro system for optimizing experimental conditions for the presentation of processed chlamydial peptides complexed to class II molecules to chlamydia-specific $CD4^+$ T cells. Because $CD4^+$ T cells were the predominant responding phenotype in the total population of immune T cells, all subsequent experiments described below were done with unfractionated populations of purified splenic T cells.

Effect of macrophage density on T-cell proliferative response. We next examined the effect of macrophage density on their ability to function as chlamydial APC. BMDM were plated in 96-well plates at different densities and inoculated

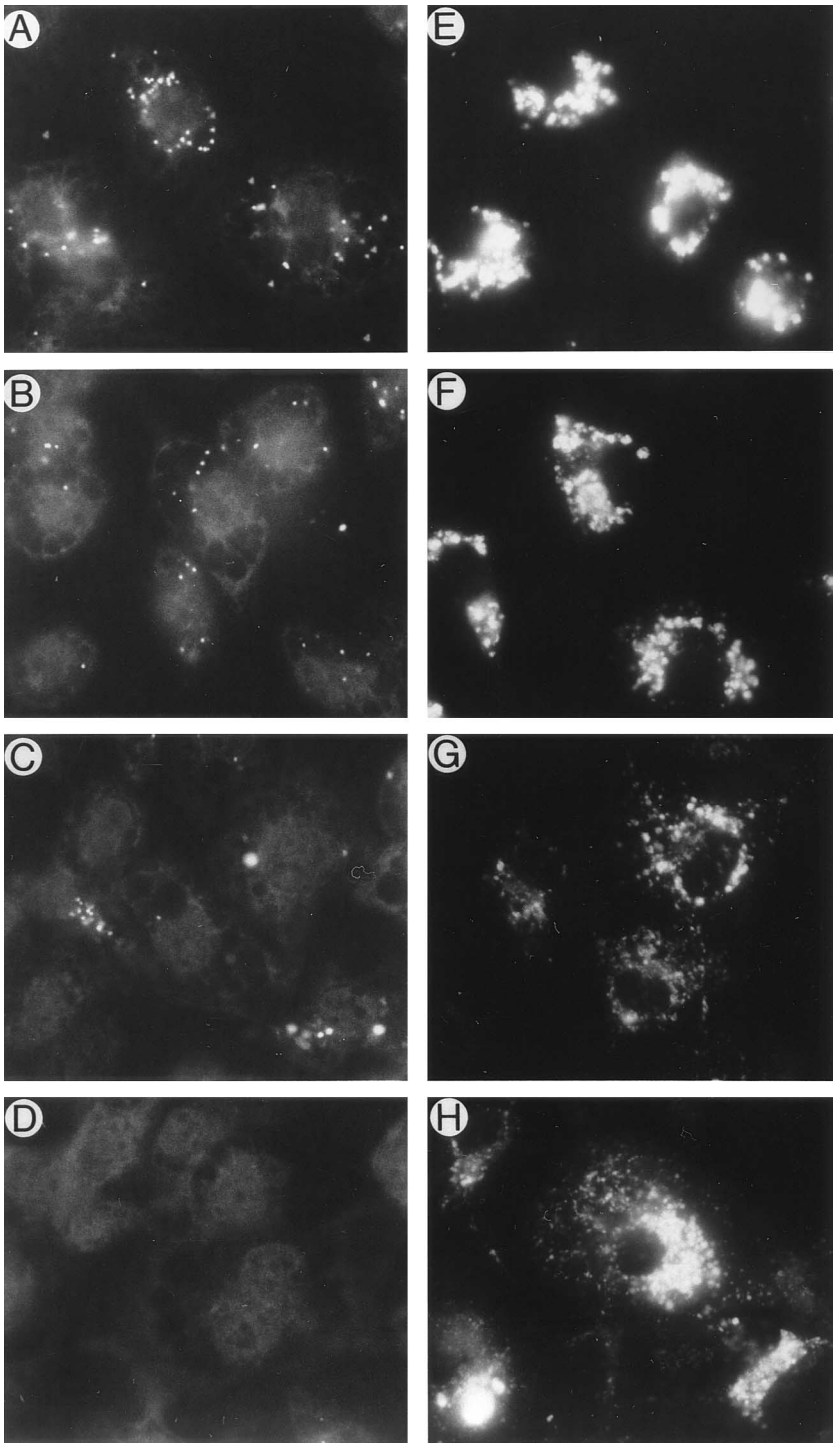

FIG. 1. Ingestion and proteolytic degradation of HK chlamydiae by BMDM. Immunofluorescence staining of BMDM following ingestion of HK chlamydiae with anti-MOMP MAb MP-33b and anti-LPS MAb EVI-H1. (A to D) Anti-MOMP at $T = 0$ (A) and 30 min (B), and 2 h (C) and 4 h (D) postinoculation of MoPn. (E to H) Anti-LPS at $T = 0$ (E) and 30 min (F), 2 h (G) and 4 h (H) postinoculation of MoPn. Magnification, ×600.

with HK MoPn at an MOI of 10. The macrophages were incubated for 8 hrs and fixed with paraformaldehyde. The fixed cells were then used in proliferation assays with both immune and control T cells (Fig. 4). Optimum proliferation of chlamydia-specific T cells was observed with macrophage densities of 5×10^4 and 1.5×10^5 cells per well. Significant proliferative responses occurred with lower macrophage densities (5×10^3 and 1.5×10^4 cells per well); however, T-cell responses obtained from these cultures were three to four times less than those with higher densities of macrophages.

Kinetics of chlamydial antigen processing and presentation by paraformaldehyde-fixed BMDM. BMDM were inoculated

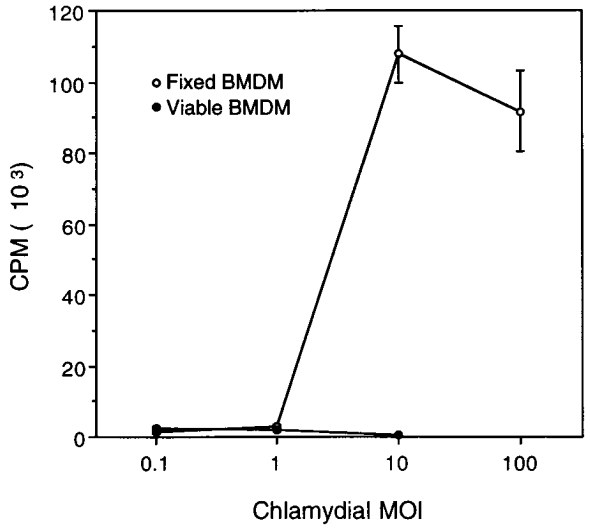

FIG. 2. Comparison of viable and fixed BMDM as chlamydial APC. Macrophages were seeded in 96-well plates at 1.5×10^5 cells per well and inoculated with different MOIs of HK MoPn EBs. Macrophages were incubated at 37°C for 30 min, washed, and then used as viable APC or incubated for an additional 8 h at 37°C, fixed with paraformaldehyde, and used as APC. The plates were then incubated at 37°C for 5 days with T cells. Cultures were pulsed with [^{3}H]thymidine 18 h prior to harvesting. The proliferative responses of T cells isolated from noninfected control mice were less than 1,000 cpm.

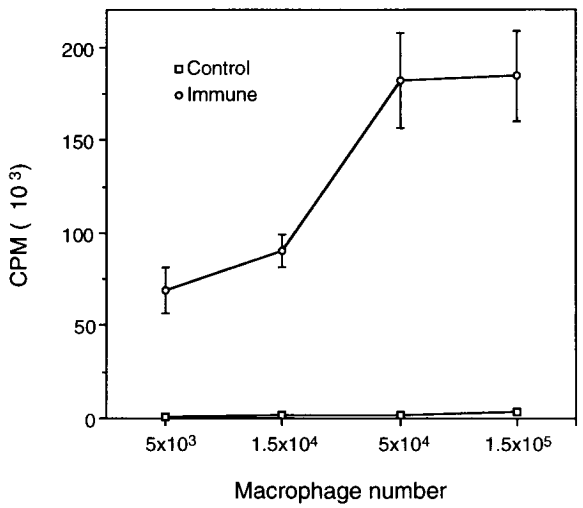

FIG. 4. Effect of macrophage density on T-cell proliferation. BMDM were plated in 96-well plates at the densities shown. They were then inoculated with HK MoPn EBs at an MOI of 10, washed, incubated at 37°C for 8 h, and fixed with paraformaldehyde. Control and immune T cells were added to the fixed macrophages, and the cultures were incubated for 5 days at 37°C.

with different MOIs of HK chlamydiae, and at different time periods following ingestion of chlamydiae, the macrophages were fixed with paraformaldehyde and used as APC in T-cell proliferation assays. The results of these kinetic experiments are shown in Fig. 5. Distinct differences were observed in T-cell proliferative responses with respect to the chlamydial MOI used to inoculate macrophages and the time period that macrophages were incubated following ingestion of chlamydiae and fixation with paraformaldehyde. Optimum T-cell responsiveness occurred when BMDM inoculated with a chlamydial MOI of 10 were used as APC. Immune T cells incubated with macrophages inoculated with an MOI of 10 produced a 40-fold increase in [^{3}H]thymidine uptake over that of T cells incubated with BMDM inoculated with an MOI of 1. Kinetic experiments showed that optimal T-cell proliferation occurred with macrophages that were fixed at 4, 8, and 12 h post-chlamydial inoculation. This temporal relationship between T-cell responsiveness and time postingestion of chlamydiae by macrophages was evident at all three MOIs. These findings show that optimum T-cell stimulatory activity is dependent on the number of chlamydiae ingested and the time of phagocytic processing by BMDM.

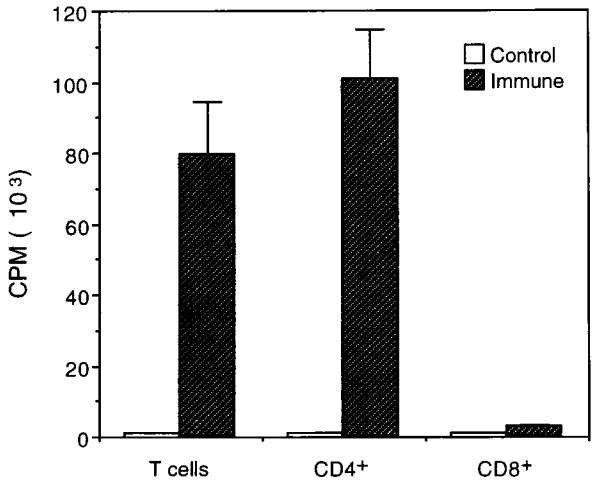

FIG. 3. Proliferative responses of chlamydia-specific T cells and CD4$^+$ and CD8$^+$ cells to fixed BMDM. Macrophages were seeded in 96-well plates at 1.5×10^5 cells per well and inoculated with an MOI of 10 of HK MoPn EBs. Following incubation at 37°C for 8 h, macrophages were fixed and used as chlamydial APC. T cells or CD4$^+$ or CD8$^+$ cells (2.5×10^5 cells per well) were added to the plates, the plates were incubated for 5 days, and the wells were pulsed with [^{3}H]thymidine for the last 18 h of culture.

MHC restriction of chlamydia-responsive T-cell proliferation. To determine if T-cell responsiveness to fixed BMDM was MHC restricted, macrophages were prepared from five B10 congenic mouse strains differing at *H-2* and then used as APC in T-cell proliferation assays. For these experiments, macrophages were inoculated with a chlamydial MOI of 10 and fixed with paraformaldehyde at 8 h postinoculation because these conditions had produced optimum T-cell responsiveness to fixed C57BL/10 macrophages as APC. As shown in Fig. 6, immune T cells responded strongly to chlamydial antigen presented by *H-2*-matched BMDM from C57BL/10 (*H-2^b*) mice, but not by BMDM from congenic strains disparate at *H-2*. Thus, chlamydia-specific T-cell responsiveness to fixed BMDM was MHC restricted.

Comparison of fixed BMDM and splenocytes as chlamydial APC. We compared the antigen-presenting capabilities of fixed BMDM to splenocytes to determine if differences in the ability to stimulate chlamydia-specific T-cell responsiveness existed between the two populations. For these experiments, splenocytes were inoculated with chlamydial MOIs of 0.1, 1, and 10, plated at 10^6 cells per well, irradiated, and incubated for 5 days with immune or control T cells. For comparison, paraformaldehyde-fixed BMDM inoculated with the same MOIs and then fixed 8 h postinoculation were assayed with the same pool of immune and control T cells (Fig. 7). Stronger proliferative responses were observed with immune T cells incubated with splenic APC inoculated with chlamydiae at MOIs of 0.1 and 1 than those induced by fixed BMDM inoculated with the same

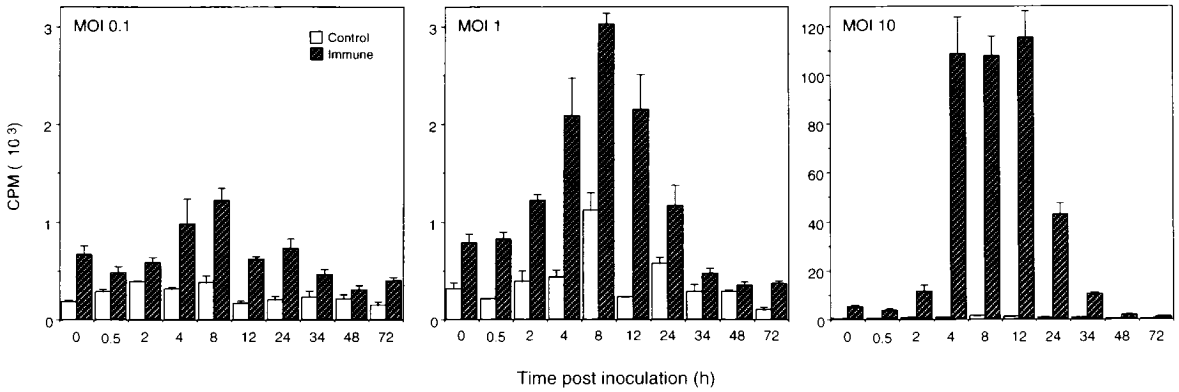

FIG. 5. Kinetics of chlamydial antigen processing and presentation by paraformaldehyde-fixed BMDM. Macrophages were plated at a density of 1.5×10^5 cells in 96-well plates and then inoculated with chlamydial MOIs of 0.1, 1, and 10. After the macrophages were washed, they were refed with culture media and incubated at 37°C. At the individual times shown, macrophages were fixed with paraformaldehyde and then used as APC in T-cell proliferation assays. Optimum T-cell proliferation was produced with BMDM inoculated with a chlamydial MOI of 10. Kinetic studies showed that optimum T-cell responsiveness occurred between 4 and 12 h post-chlamydial inoculation. This temporal relationship is most evident in macrophage cultures inoculated with MOIs of 1 and 10.

MOIs. However, at an MOI of 10, the proliferative responses of immune T cells were similar for splenic APC and fixed BMDM.

DISCUSSION

The importance of $CD4^+$ T cells in the development of protective immunity to chlamydial genital tract infection in the mouse is well established (19, 21, 29). Further investigations will likely focus on defining the mechanisms by which $CD4^+$ cells mediate protection and identification of chlamydial peptides or epitopes complexed to class II MHC molecules that are recognized by protective $CD4^+$ cells. The development and characterization of an APC system that would provide sufficient quantities of chlamydial peptide antigen complexed to class II molecules could allow for the direct sequence determination of naturally processed immunodominant chlamydia-derived T-cell epitopes. Identification of chlamydial peptides recognized by protective $CD4^+$ T cells provides the prospect of using these antigens in a subunit vaccine. B cells and macrophages are potent APCs for $CD4^+$ T cells (14, 22, 36); however, B cells do not effectively ingest chlamydiae (unpublished observations). Continuous murine macrophage cell lines express class II molecules poorly, even after induction with IFN-γ, so they are unattractive as chlamydial APC because they would yield insufficient amounts of complexed chlamydial peptide antigen(s) for subsequent isolation and characterization. We find murine BMDM are potent APC, following mild fixation with paraformaldehyde, and our kinetic experiments define experimental conditions that optimize presentation of processed chlamydial antigen to chlamydia-specific T cells. Large numbers of BMDM can be grown in vitro, making them useful as APC for isolation of class II MHC molecules and sequencing of chlamydial peptides complexed to them.

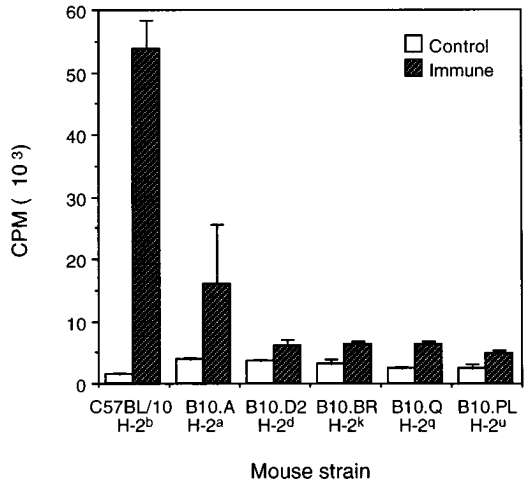

FIG. 6. *H-2* restriction of chlamydia-specific T-cell proliferation. BMDM were prepared from each of the different B10 congenic mouse strains shown. The macrophages were inoculated with a chlamydial MOI of 10, incubated for 8 h postinoculation, fixed with paraformaldehyde, and then used in T-cell proliferation assays using immune or control splenic T cells from C57BL/10 mice. Strong T-cell responses were observed with syngeneic $H\text{-}2^b$ macrophages and T cells. In contrast, T cells from C57BL/10 mice failed to proliferate or proliferated weakly when BMDM derived from mice disparate at *H-2* were used as chlamydial APC.

Presentation of foreign peptides by APC takes place when many of the class II molecules are complexed to autologous peptides (3, 7, 22) with as few as 0.1% of the class II molecules being occupied by foreign peptides (15). This may make identification of naturally processed chlamydial peptides complexed to class II molecules a formidable task. However, with the generation of chlamydia-specific protective $CD4^+$ clones and their T-cell hybridomas, it should be possible to identify chlamydia-specific peptides eluted from class II molecules. With the advent of methodologies which provide peptide sequence information at the pico- to subpicomole level (17, 18), it may be both feasible and practical to accomplish these objectives.

Our findings confirm and extend those of others demonstrating that paraformaldehyde fixation of BMDM does not significantly affect their ability to function as APC (6). Fixed BMDM generated potent chlamydia-specific T-cell stimulatory activity that is MHC restricted, indicating that paraformaldehyde-fixed BMDM express both functional MHC class II and other T-cell costimulatory molecules (38). Optimum APC stimulatory activity for chlamydia-specific T cells was achieved with high densities of BMDM that were fixed after their ingestion of HK chlamydiae. In contrast, high densities of viable BMDM clearly suppressed or completely inhibited T-cell proliferation. This inhibitory activity likely reflects an enhanced state of

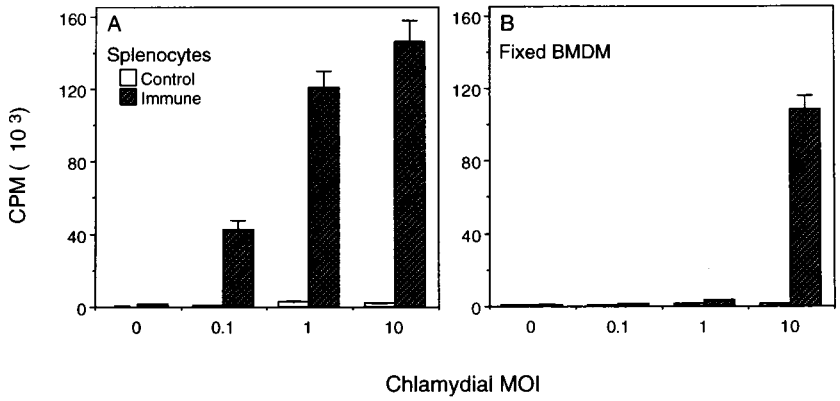

FIG. 7. Comparison of splenocytes and fixed BMDM as chlamydial APC. Splenocytes from C57BL/10 mice were inoculated with chlamydial MOIs of 0.1, 1, and 10. The cells were washed, irradiated, and used as chlamydial APC in T-cell proliferation assays. BMDM were inoculated with the same chlamydial MOIs, incubated for 8 h postinoculation, fixed with paraformaldehyde, and used in T-cell proliferation assays. A distinct dose-response relationship was observed with splenocytes as the APC and the multiplicity of chlamydial inoculum. This dose-response relationship was not as evident with fixed BMDM as the APC; however, at an MOI of 10, the fixed BMDM were nearly as stimulatory to immune T cells as splenocytes inoculated with the same MOI.

macrophage activation following ingestion of chlamydiae, presumably by promoting secretion of T-cell inhibitory cytokines, prostoglandins, or hydrogen peroxide, which suppress lymphocyte proliferation (23, 24). In addition, our findings also demonstrate that fixed BMDM are as effective as splenocytes as chlamydial APC which provide further support for the utility and suitability of fixed BMDM as chlamydial APC.

Another potential use for BMDM as chlamydial APC would be to study natural chlamydial antigen processing, with the goal of understanding the relationship of chlamydial outer membrane structure as it relates to the immunodominance of outer membrane proteins. Chlamydial EBs are unique in that their cell wall outer membrane contains three cysteine-rich proteins, MOMP, 60-, and 15-kDa proteins (1, 2, 9, 11) that form a disulfide cross-linked supramacromolecular structure (16, 25, 26). This structure is thought to be the functional equivalent of peptidoglycan which is not detectable in cell wall preparations of chlamydiae (4, 12). The topological arrangement of these proteins is not known exactly, but only MOMP is accessible to antibody and proteolytic cleavage in situ (34). MOMP is immunodominant and elicits a consistent high-titer antibody response following chlamydial infection or immunization. In contrast, the antibody responses to the 60- and 15-kDa proteins are more variable and are often undetectable. We find that BMDM with ingested HK chlamydiae exhibit increased intracytoplasmic staining with antisera prepared against the 60-kDa cysteine-rich protein following incubation at 37°C and strong anti-60-kDa fluorescent signals persist for as long as 7 days following ingestion of MoPn (unpublished observations). As shown here, antibodies against chlamydial LPS yield similar staining patterns. It is not clear whether this finding represents free LPS molecules or LPS molecules complexed to outer membranes. The persistent antigenic signals with anti-60-kDa and LPS antibodies clearly differ from those observed for surface epitopes of MOMP which disappear completely by 4 h postinoculation of macrophages with MoPn. These data, although preliminary, suggest that the EB outer membranes are inefficiently proteolyzed and persist within macrophage lysosomes. It is known that reduction of disulfide bonds is a key step in antigen processing. The unfolding of protein antigens increases the accessibility of the nascent polypeptide chain to proteolytic processing enzymes. For example, Collins et al. (10) have shown reduction and alkylation of hen lysozyme and ribonuclease A vastly increase their proteolysis and the generation of specific immunogenic peptides bound to class II MHC molecules recognized by T-cell hybridomas. Thus, the immunogenicity or lack thereof of the chlamydial cysteine-rich 60- and 15-kDa outer membrane proteins may be related to their high cysteine content and extensive intra- and inter-disulfide bond cross-linking. Theoretically, such highly disulfide-bonded proteins might resist unfolding, making them inaccessible to proteolytic processing enzymes within lysosomes. Conversely, the immunodominance of MOMP might relate to its more efficient degradation by lysosomal proteases and subsequent association of MOMP-derived peptides with class II molecules for presentation to $CD4^+$ T cells. The BMDM system described here could be very useful for experiments to study natural processing of chlamydial antigens. Specifically, this system should allow definition of how chlamydial outer membrane structure influences antigen processing by macrophages and to what extent the unique structure of the EB outer membrane contributes to immunogenic characteristics of EB outer membrane protein antigens.

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