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Modulation of the Host Immune Response as a Result of *Chlamydia psittaci* Infection

JOYCE K. LAMMERT AND PRISCILLA B. WYRICK*

Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

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After intraperitoneal injection of mice with infectious, inactivated, or envelope preparations of the elementary body of *Chlamydia psittaci*, lymphocyte transformation of spleen cells to the mitogens concanavalin A, phytohemagglutinin, and lipopolysaccharide was significantly reduced 1 and 2 weeks postinjection. Lymphocyte response returned to the control values by 4 weeks. Similarly, transformation of cells by chlamydial antigen was not detected until 4 weeks postinjection. Injection of the noninfectious intracellular reticulate body, in contrast, had little effect on transformation of cells to concanavalin A. When control spleen cells were incubated with infectious or inactivated elementary bodies in vitro, response to all three mitogens was also reduced. The sooner the organisms were added after the addition of mitogen, the greater the reduction in transformation. Incubation with elementary body envelopes and reticulate bodies had no effect on lymphocyte transformation of the spleen cells to concanavalin A. The relationship between the observed ability to reduce the response in the in vitro assay of lymphocyte transformation and the actual in vivo establishment of infection is discussed.

Chlamydiae are obligate intracellular bacteria that are responsible for a number of disease syndromes, including trachoma, sexually transmitted diseases, and psittacosis (9, 27, 31). Cell-mediated and humoral immune responses to the chlamydiae have been recorded after infection in humans and in a variety of animal models (10, 14, 23-25, 36). Despite such responses, chronic or latent infections are the general rule after primary infection. Vaccines and passive transfer of immunity, in general, have not provided solid immunity against challenge (3, 7, 21, 24).

The interactions of the chlamydiae with host cells are very specific. These organisms are able to induce their own uptake into nonprofessional phagocytic cells and subsequently to circumvent phagolysosome fusion and multiply in the cells (6, 37). The multiplication cycle involves two distinct forms of the microorganism: the elementary body (EB), which is the infectious extracellular form, and the reticulate body (RB), which is the metabolically active, highly permeable intracellular form.

It has been demonstrated that many microorganisms modulate the host immune response during or after infection (29, 30). This is one way in which the balance of host and microorganism can be affected. We have examined whether infection with chlamydiae can result in such a modulation of the host immune response. *Chlamydia psittaci* infection in mice provides a suitable model for such studies. Mice that recover from primary infection are resistant to subsequent challenge with a lethal dose of inoculum. However, successful recovery does not denote sterile immunity, and chlamydiae can be isolated from these mice (4, 26). Using lymphocyte transformation as our assay system, we have found that, after in vivo injection of infectious or inactivated *C. psittaci* or in vitro incubation with the microorganisms, [^{3}H]thymidine incorporation in mitogen-stimulated spleen cells is depressed. The contribution of such a modulation of host cell response to the establishment of a latent or persistent infection is discussed.

(A preliminary report was presented at the 80th Annual Meeting of the American Society of Microbiology in Miami Beach, Fla., 11 to 16 May 1980.)

MATERIALS AND METHODS

Animals. Female F_1 mice (C57BL/6 × C3H) were obtained from Cumberland View Farms, Clinton, Tenn., and used at between 8 and 12 weeks of age. Mice were screened for the presence of preexisting chlamydia-neutralizing antibody by testing for the ability of serum to inhibit the formation of inclusions in L cells. Attempts were also made to grow chlamydiae out of the spleens and livers of untreated or hydrocortisone-treated mice. Tests were negative for the presence of chlamydiae or neutralizing antibody before injection. After injection with *C. psittaci*, mice were maintained in an isolation cubicle in the animal facility.

Experimental infection. Intraperitoneal infection of mice with *C. psittaci* has been well characterized (4, 26). Injection of high concentrations of infectious EBs (2×10^8 cells per mouse) causes toxemia-type death in mice within 24 h, whereas injection of a moderate concentration (1×10^8 cells per mouse) results in an infectious syndrome with death occurring at between 2 and 7 days postinjection. Injection of lower concentrations results in a latent form of the infection, and organisms have been grown out for as long as 7 months postinjection. In these studies mice were infected intraperitoneally with 7×10^7 infectious organisms. At 1 month postinfection, small numbers of chlamydiae could be grown out of the spleen when spleen cell suspensions were cocultivated with L cells. Control mice were injected with uninfected L-cell material processed in the same manner as the chlamydia-infected cells.

Growth and purification of EBs and RBs. The Cal10 meningopneumonitis strain of *C. psittaci* was grown in 929 L-cell suspension cultures by the method of Tamura and Higashi (32). The L cells were free of mycoplasma contamination (courtesy of Wallace Clyde, Department of Pediatrics, University of North Carolina School of Medicine). The EBs were harvested and purified as previously reported (38). Briefly, cell-free supernatants from infected suspension L-cell cultures were harvested at 48 h postinfection and then subjected to differential centrifugation, followed by centrifugation through 30% sucrose cushions and discontinuous gradients of 38, 44, and 59% Renografin-76 (E. R. Squibb & Sons, Inc., Princeton, N.J.). Infectivity was assayed by the inclusion-forming unit technique (13). The RBs were grown and purified by the technique of Brownridge and Wyrick (5). The RBs are not infectious, so they cannot be titrated by the inclusion-forming unit technique. Protein in the purified RBs was determined by the Lowry assay.

Inactivation of *C. psittaci* with heat, UV light, and immune serum. Purified EBs were treated with UV light by the procedure of Byrne (6). For heat treatment, the EBs were suspended in phosphate-buffered saline (PBS) and incubated in a 56°C water bath for 30 min. Infectivity after treatment was assayed by the inclusion-forming unit technique, and more than 99% of the infectious particles were inactivated.

Antiserum to *C. psittaci* was prepared by repeated intravenous injection of New Zealand white rabbits with purified EBs of the Cal10 strain. Antiserum was ammonium sulfate precipitated, dialyzed, and stored at −70°C. When a 1/10 dilution of antiserum was incubated with EBs (2×10^9) at a 1:1 ratio for 30 min at 37°C, greater than 99% neutralization of infectivity was observed.

EB envelope preparation. EB envelopes were prepared by a modification of the method of Tamura et al. (33). Purified EBs were suspended in PBS without Ca^{2+} and Mg^{2+}, sonicated for 90 s at 40 kc/s (model W140 Sonifier, Heat Systems-Ultrasonics, Inc., Plainview, Long Island, New York), and incubated with 20 μg of trypsin (Worthington Diagnostics, Freehold, N.J.) per ml for 15 min at 37°C, followed by addition of trypsin inhibitor. The EBs were disrupted by use of a Mickle apparatus at 60 cycles per s at 4°C for 10 min. The disrupted material was centrifuged to remove dust and beads, sonicated, and then treated with DNase and RNase (100 μg of each per ml) for 30 min at 37°C. This was followed by a second trypsin treatment. The envelope material was sonicated, layered on a linear sucrose gradient (5 to 45%), and centrifuged for 30 min at 6,000 rpm in the SW25 rotor of a Spinco ultracentrifuge. The envelope band was collected, washed, and suspended in PBS without Mg^{2+} and Ca^{2+}. Envelopes were frozen at −70°C until used. Envelopes were enumerated by direct particle count, and 2×10^9 EB envelopes contained 35 μg of protein.

Lymphocyte transformation. (i) In vivo infection with *C. psittaci*. Mice were infected intraperitoneally with infectious, inactivated, or envelope preparations of *C. psittaci*. The spleens were subsequently harvested, and cells were teased from the spleens, washed several times, and suspended in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (KC Biological, Lenexa, Kans.) at a concentration of 5×10^6 cells per ml. Cells were dispensed in 0.1-ml portions into the wells of 96-well flat-bottomed microtiter plates (Falcon Plastics, Oxnard, Calif.). A 0.1-ml portion of mitogen in medium was added to each well, and plates were incubated at 37°C in a humidified incubator with 5% CO_2. After 48 h, 0.5 μCi of [^{3}H]thymidine (specific activity, 1.9 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y. in 20 μl of RPMI 1640 was added to each well, followed by an additional 10-h incubation period. Cultures were harvested with a Mash II automatic harvester, placed in Econofluor (New England Nuclear Corp., Boston, Mass.), and counted in a Packard liquid scintillation counter. In preliminary studies, the following optimal mitogen concentrations were determined: concanavalin A (ConA), 0.2 μg per well; phytohemagglutinin (PHA), 1 μg per well; lipopolysaccharide (LPS; Sigma Chemical Co., St. Louis, Mo.), 5 μg per well. Optimal incubation times were also determined (see below). All cultures were performed in triplicate, and standard deviations were generally less than 10%. All experiments were repeated a minimum of three times with different batches of purified chlamydiae. Representative experiments are presented because the extent of the decrease varied depending on the individual preparation of chlamydiae used for injection or for in vitro addition. In most studies, spleens from four or more mice were pooled for each injection group.

When transformation to chlamydial antigen was tested, erythrocytes were removed from cell suspensions by treatment with 0.83% NH_4Cl and then cells and antigen were incubated together for 5 days in RPMI 1640 containing 10^{-5} M 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y.). Cells were subsequently pulsed with [^{3}H]thymidine for 24 h and harvested as described above. Chlamydial antigen consisted of purified EBs that had been UV inactivated and stored at −70°C until used. At a protein concentration of 0.3 μg per well (1.5 μg/ml), transformation of responding spleen cells to chlamydial antigen was optimal.

(ii) In vitro incubation of chlamydiae with spleen cells. Spleens were removed from untreated mice, and cell suspensions were prepared as described above except that erythrocytes were first removed by treating the cells with 0.83% NH_4Cl. Infectious, inactivated, or envelope preparations of chlamydiae were added to spleen cells at a ratio of 100:1 or 10:1 and incubated at 37°C for 1.5 h with rotation. The preparations were then dispensed into microtiter plates, and mitogen was

added. Preliminary studies indicated no difference in the degree of reduction of lymphocyte transformation if the remaining chlamydiae were removed by washing after the 1.5-h incubation period or allowed to remain for the entire 58 h. The ability of the chlamydiae to bind mitogen was tested by incubating ConA with chlamydiae and subsequently removing the chlamydiae. The treated ConA was able to induce transformation of spleen cells to the same extent as the untreated ConA. To eliminate the possibility that any decrease in mitogen response was caused by simple disruption of normal cell-cell interaction due to the presence of the chlamydiae, control spleen cell cultures (no chlamydiae added) contained an equivalent amount of protein from sonicated L cells.

(iii) Mouse serum. Infected and control mice were bled by cardiac puncture. The serum was removed from clotted blood, heat inactivated, and sterilized by passage through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.). Fresh serum or serum stored at −20°C was diluted 1/10 and used instead of fetal bovine serum in the lymphocyte transformation assay with spleen cells from uninjected mice.

(iv) Macrocultures. For supernatant exchange and delayed in vitro addition of chlamydiae, spleen cells were prepared as described above, and 1 ml of 2×10^6 cells per ml was added to plastic tubes (12 by 75 mm; no. 2001; Falcon Plastics). ConA was added to the cultures at a concentration of 1.5 μg per culture. For harvesting, 3 ml of PBS was added to each culture, and the cultures were blended in a Vortex mixer and collected on glass fiber filter disks (Reeve-Angel, Clifton, N.J.) with suction. Filters were then washed once with 5% trichloroacetic acid and three times with PBS, dried, and counted in the scintillation counter.

Immunofluorescent staining of spleen cells. Mouse spleen cells were characterized by their surface markers. Spleen cell suspensions were washed, pelleted, and then resuspended with 0.5 ml of a 1/20 dilution of rabbit anti-mouse thymocyte serum (Cappel Laboratories, Downingtown, Pa.), rabbit anti-mouse immunoglobulin serum (Cappel), or normal rabbit serum. Cells were incubated for 30 min at room temperature, then washed three times, resuspended in the fluorescein-conjugated immunoglobulin gG fraction of goat anti-rabbit serum (Cappel), and incubated for 30 min at room temperature. Cells were washed again three times and examined with a Leitz fluorescent microscope. A total of 300 cells were counted per slide, and the numbers represent the mean of three experiments. The specificity of the commercial antisera was tested and confirmed before use.

T-cell enrichment. Spleen cell preparations were enriched for the presence of T cells by using the column adherence method of Julius et al. (12). Nylon wool was washed to remove toxic products and dried, and 0.6 g was added to each 10-ml plastic syringe. Autoclaved syringes were preincubated with RPMI 1640 for 45 min at 37°C before the addition of cells. Cells were prepared as follows. A total of 10^8 spleen cells were incubated in a 100-mm plastic petri plate for 1 h at 37°C. Nonadherent cells were then removed by gentle agitation, added to a second petri plate, and incubated for an additional 1 h. Nonadherent cells were then added to nylon wool columns and incubated for 45 min at 37°C, and the first 20 ml of effluent was collected. Recovery after nylon wool passage ranged from 20 to 30%. Adherent cells were removed from the first petri plate with a rubber policeman and used as the source of adherent cells in additional experiments.

Statistics. In all experiments, numbers represent the mean of triplicate cultures. Standard deviations were generally less than 10%. Differences in lymphocyte responses were analyzed by Student's t test. Results for which $P \geq 0.05$ were defined as not significant.

RESULTS

In vivo injection of *C. psittaci*. Initial studies were performed to determine whether infection of mice with chlamydiae had any effect on the ability of host cells to respond to mitogen. Mice were injected intraperitoneally with 7×10^7 infectious EBs per mouse. The infected mice showed no outward signs or symptoms of infection; however, splenomegaly of varying duration was observed for up to 2 months postinfection. Spleen cells from infected mice sacrificed 1 and 2 weeks postinfection exhibited reduced [^{3}H]thymidine uptake relative to control cells when they were incubated with the T-cell mitogens ConA and PHA and the B-cell mitogen LPS (Fig. 1). At 4 weeks postinfection, the uptake had returned to control values. In repeated studies it was observed that although the pattern remained consistent, the extent of the decrease and the length of time before the response returned to normal varied depending on the

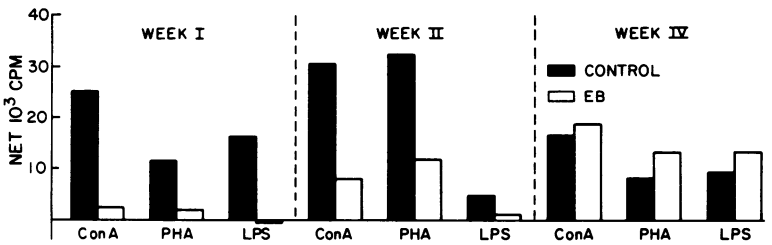

FIG. 1. Mitogen responsiveness of spleen cells from mice infected with *C. psittaci*. Mice were injected intraperitoneally with 7×10^7 infectious EBs and sacrificed at the indicated intervals. Net 10^3 cpm = 10^3 cpm for cells with mitogen − 10^3 cpm for cells with medium. At 1 and 2 weeks postinfection, the difference in response between control and test animals was significant at $P \leq 0.001$ to 0.01.

TABLE 1. Spleen cells assayed by indirect immunofluorescence for theta and immunoglobulin[a]

Spleen cells	% Theta positive	% Immunoglobulin positive	% Null
Control[b]	35.0 ± 1.0	35.9 ± 1.7	29.2 ± 2.7
EB	39.6 ± 0.4	36.1 ± 2.5	24.3 ± 2.7

[a] A total of 300 cells were counted per slide, and the numbers represent the mean of three experiments ± standard deviation. Null cells are cells that did not stain with either the anti-theta antisera or the anti-immunoglobulin antisera.

[b] Mice were injected intraperitoneally with 7×10^7 infectious EBs (EB) or with L-cell material (control), and spleens were harvested at 2 weeks postinfection.

individual preparation of chlamydiae used for injection. The decrease in [^{3}H]thymidine uptake was not due to a decrease in cell viability during culture or during exposure to [^{3}H]thymidine, as monitored by the trypan blue exclusion technique. The mean cell count, as measured by [^{3}H]thymidine uptake for the unstimulated cultures, also showed little variation in cells from infected and control animals. The difference was not caused by a shift in the sensitivity of the cells from infected mice to the optimal mitogen concentration or a shift in the time of maximal response of the cells to the mitogen. The presence of splenomegaly did not correlate with the decrease in mitogen response, and the spleens remained enlarged after the mitogen response had returned to control levels. It was possible that the population of cell types within the spleen was altered during infection, resulting in a depletion in the number of transformable cells. However, no difference was observed in the percentage of T and B cells found in control and infected spleens as determined by indirect immunofluorescence (Table 1).

The ability of spleen cells from infected mice to respond to chlamydial antigen was also tested (Table 2). With the exception of day 3, postinfection transformation to the specific antigen was not observed, in spleen cells from infected mice during the first 2 weeks, which correlates with the observed decrease in transformation when cells were incubated with mitogen. At 4 weeks postinfection, there was a significant response of infected cells to chlamydial antigen.

In some infections (20, 35) it has been found that mitogen or antigen responses, or both, can be modulated by serum factors. Fresh or frozen serum from infected mice sacrificed at the stated time intervals did not have a suppressive effect on mitogen-induced transformation when added to spleen cells from untreated mice.

It was of interest to determine whether the reduction in uptake of [^{3}H]thymidine after incubation with mitogen was a function of the infectious process or caused by the mere presence of the chlamydiae. Three preparations of nonviable chlamydiae were used for injections: UV- or heat-treated EBs or EB envelopes. UV-treated EBs retain the ability to induce their own uptake and evade phagolysosome fusion in nonprofessional phagocytic cells, whereas heat-treated organisms lose these functions (6, 37, 38). It is not known whether envelopes can inhibit phagolysosome fusion or be taken up by nonprofessional phagocytes such as L cells. As observed at 2 weeks postinjection with infectious EBs, transformation of spleen cells by the test mitogen was reduced after injection of the threee nonviable preparations (Fig. 2). The degree of reduction did not seem to be strongly dependent on the concentration of chlamydiae injected, as 10^3 infectious EBs were as effective as 10^8 were. Incubation of chlamydiae with neutralizing antibody in vitro leads to rapid uptake and degradation of the microorganisms when incubated with macrophages (37, 38). When infectious chlamydiae were incubated with neutralizing antibody in vitro and subsequently injected into mice, a reduction in lymphocyte transformation was still observed (Table 3).

These findings suggest that a degradation product of the cell envelope may be responsible for the reduced lymphocyte transformation. It was therefore of interest to test whether injection of the RB, which has lost some of the rigid components found in the envelope of the EB and lacks a number of virulence determinants (5, 18, 33), would have a similar effect on lymphocyte transformation. Injection of RBs, at concentrations up to 10 times that of EBs, did not have an effect on the ability of spleen cells from the

TABLE 2. Transformation of mouse spleen cells by chlamydial antigen[a]

Spleen cells	Net 10^3 cpm ± SD at day postinjection:							
	1	3	5	7	9	11	14	28
EB	1.2 ± 0.16	9.5 ± 1.3	1.9 ± 0.57	0.8 ± 0.16	3.5 ± 0.63	1.6 ± 0.58	1.3 ± 0.29	43 ± 3.5
Control	2.9 ± 0.4	0.9 ± 0.19	3.3 ± 0.30	1.1 ± 0.53	2.9 ± 0.52	1.2 ± 0.53	1.9 ± 0.4	3.4 ± 0.79

[a] Mice were infected with 7×10^7 infectious EBs (EB) or L-cell material (control), spleen cells were harvested at the indicated days postinjection, and the response to EB antigen or medium was recorded. Net 10^3 cpm = 10^3 cpm with ConA − 10^3 cpm with medium.

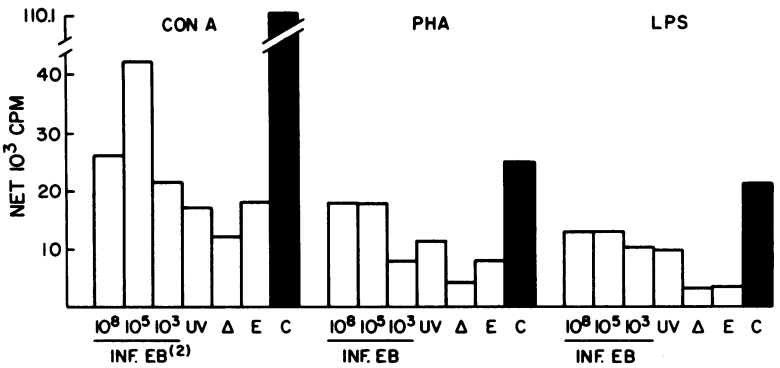

FIG. 2. Mitogen responsiveness of spleen cells from mice injected with treated or infectious *C. psittaci*. Mice were injected with 10^8, 10^5, or 10^3 infectious EBs, 10^8 UV-treated (UV) or heat-treated (Δ) EBs, 10 μg EB envelopes (E), or with L-cell material (C). Spleens were harvested at 2 weeks postinjection. $P \leq 0.001$ to 0.01.

injected mice to be transformed by ConA (Table 4). The component responsible for the decrease in lymphocyte transformation is apparently found on the infectious extracellular EB but not on the noninfectious intracellular RB.

To determine whether the decrease in lymphocyte transformation was due to the production of suppressor substances during in vitro incubation or conversely to the inability to produce the soluble signals necessary for transformation, medium exchange was performed between cultures of control and EB spleen cells at various times after the addition of mitogen (Table 5). It is clear that the medium exchange had no effect on the ability of either cell type to respond to the mitogen ConA. The role that macrophages might play in this decrease in response was tested by depleting spleen cells of macrophages via adherence and subsequently adding back these cells. It was observed (Table 6) that depletion of the macrophages decreased the ability of the subsequent T-enriched fraction from both control and EB spleen cells to respond to ConA. The addition of EB macrophages to T-enriched control cells returned the response of those cells to the level of undepleted cells, suggesting that macrophages from EB-injected mice are not impaired in their ability to participate in the transformation response. The addition of control macrophages to T-enriched EB cells also returned the response to ConA to the undepleted level, but it did not bring the response to the level of the undepleted control cells.

In vitro addition of *C. psittaci* to mouse spleen cells. Infectious, UV- or heat-treated EBs were added to spleen cells from untreated mice at EB-to-spleen cell ratios of 100:1 and 10:1. Lymphocyte transformation with the three mitogens was significantly reduced after the addition of all three chlamydial preparations (Fig. 3). The degree of reduction was dependent on the amount of chlamydiae added, with the 100:1 ratio reduc-

TABLE 3. ConA response of spleen cells from mice injected with antibody-neutralized *C. psittaci*[a]

Injected with:	ConA response (net 10^3 cpm[b] ± SD)	P[c]
Preimmune + EB	56.1 ± 6.0	<0.001
Immune A + EB	44.5 ± 1.4	<0.001
Immune B + EB	53.7 ± 6.3	<0.001
Preimmune + L cell	108.8 ± 14.7	

[a] Infectious EBs were incubated for 30 min at 37°C with preimmune serum or immune serum (batch A or B) before injection in mice. This resulted in greater than 99% neutralization of infectivity when tested for the appearance of inclusions in L cells. Mice were injected with 7×10^7 neutralized EBs or L-cell material, and spleens were harvested at 2 weeks postinjection.

[b] Net 10^3 cpm = 10^3 cpm with ConA − 10^3 cpm with media.

[c] Level of significance of response relative to preimmune plus L-cell-injected mice, by Student's *t* test.

TABLE 4. ConA response of spleen cells from mice injected with the RB or EB of *C. psittaci*[a]

Injected with (μg):	ConA response (net 10^3 cpm[b] ± SD)
RB (250)	24.5 ± 2.1
RB (50)	19.6 ± 1.5
RB (25)	23.2 ± 3.0
EB (20)	7.5 ± 0.76
L cell (50)	20.9 ± 0.54

[a] Mice were injected intraperitoneally with 8×10^7 infectious EBs, 20 μg of protein per mouse, RBs, or L-cell material at the stated concentrations. Spleens were harvested at 2 weeks postinfection.

[b] Net 10^3 cpm = 10^3 cpm with ConA − 10^3 cpm with media.

TABLE 5. Effect of medium exchange on the ability of spleen cells from control and EB-infected mice to respond to ConA[a]

Spleen cells	Medium	ConA response (net 10^3 cpm ± SD) after:		
		4 h[b]	24 h	48 h
EB	Control	14.3 ± 1.8	11.6 ± 1.7	14.3 ± 1.5
EB	EB	16.8 ± 0.35	14.1 ± 0.86	14.4 ± 1.3
Control	EB	47.1 ± 3.3	41.8 ± 4.3	45.2 ± 1.3
Control	Control	40.8 ± 4.0	37.5 ± 5.4	43.2 ± 2.3

[a] Control and EB spleen cell culture tubes were spun at 150 × *g* for 10 min. Media were then exchanged between EB and control tubes or added back to the same tube. Cultures were then gently blended in a Vortex mixer and returned to the incubator for the remaining incubation period. Spleens were harvested at 2 weeks postinfection.

[b] Time at which medium exchange occurred after the cells were put into culture.

ing transformation more effectively than the 10:1 ratio did. When the chlamydiae were removed from the spleen cells by washing after the initial incubation period and before the addition of mitogen, lymphocyte transformation was reduced as effectively as when chlamydiae were allowed to remain for the entire 58-h culture and labeling period. Cell viability during culture or during exposure to [^{3}H]thymidine, as determined by trypan blue uptake, was the same in cultures with and without added microorganisms. The effect of time on the ability of *C. psittaci* to reduce lymphocyte transformation was examined by adding the EB to the cultures at various times after the spleen cells had been incubated with ConA. As the EB was added at later times (Fig. 4), the response to ConA began to approach that of the control response. Incubation of cells with EB envelopes or the RB did not cause a reduction in [^{3}H]thymidine uptake in response to ConA (Table 7).

DISCUSSION

The outcome of an infection ultimately depends on the interaction between the infectious agent and the immune response of the host. The initial effect of the microorganism on the initiation of the immune response may be an important factor in the outcome, in the case of chlamydiae in the establishment of a latent or persistent infection.

A generalized depression in the ability of host cells to respond, as evidenced by the low level of transformation to T- and B-cell mitogens and specific chlamydial antigen, was observed after the in vivo injection of chlamydiae. Unlike infection with such parasites as *Trypanosoma congolense* or *T. bruzei* (19, 22), extensive changes in the relative number of B, T, and null cells were not observed. Because adherent cells from EB spleens are capable of restoring the response of adherent cell-depleted control cells to ConA, the

TABLE 6. Restoration of ConA response by the addition of adherent cells[a]

Spleen cells	Adherent cells added from:		ConA response (net 10^3 cpm[b] ± SD)
	EB	Control	
EB spleen	—[c]	—	12.5 ± 1.3
EB T	—	—	1.7 ± 0.25
EB T	1×10^5	—	10.0 ± 0.09
EB T	2.5×10^5	—	5.4 ± 0.85
EB T	—	1×10^5	12.8 ± 1.5
EB T	—	2.5×10^5	14.2 ± 1.4
Control spleen	—	—	40.4 ± 0.44
Control T	—	—	1.8 ± 0.14
Control T	1×10^5	—	53.0 ± 1.3
Control T	2.5×10^5	—	34.0 ± 4.1
Control T	—	1×10^5	41.8 ± 3.5
Control T	—	2.5×10^5	32.3 ± 2.4

[a] T-enriched spleen cells (T) were tested for the ability to respond to ConA with or without the addition of plastic adherent cells from EB or control spleens. Each experiment used 5×10^5 spleen cells. Spleens were harvested at 2 weeks postinfection.

[b] Net 10^3 cpm = 10^3 cpm with ConA − 10^3 cpm with media.

[c] —, Cells not added.

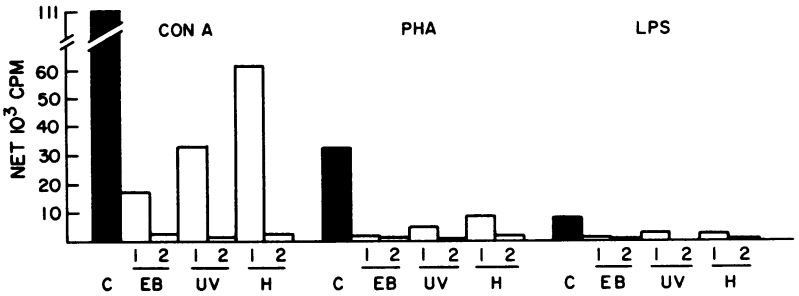

FIG. 3. Mitogen responsiveness of spleen cells after the in vitro addition of *C. psittaci*. Additions were as follows: L-cell material (C), infectious EBs (EB), UV-treated EBs (UV), or heat-treated EBs (H). Chlamydiae or L-cell material was added to spleen cells at ratios of 10:1 (labeled 1) and 100:1 (labeled 2). $P \leq 0.01$.

ability of the macrophages to perform the necessary functions required for transformation, such as the production of conditioned medium, is not impaired (1, 2, 17). Medium exchange up to 48 h postincubation did not affect the uptake of [^{3}H]thymidine by control or EB cells in response to ConA. This eliminated the role of soluble suppressor substances such as cold thymidine, which is produced by the degradation of DNA from other cells in culture by activated macrophages. Cocultivation experiments done with various ratios of spleen cells from EB-injected and control mice (data not shown) did not reveal the presence of active suppressor cells in the EB spleen cell suspensions.

It does not seem likely that the reduction in lymphocyte transformation was due to the ability of the chlamydiae to block mitogen-binding sites on the surface of the cells. Levy (15) found that the addition of the lectin wheat germ agglutinin blocks the attachment of chlamydiae to the surface of L cells, whereas the same concentration of ConA or *Ricinus communis* lectin causes little inhibition of attachment. She concluded that the attachment of chlamydiae is fairly specific for an *N*-acetyl-D-glucosamine-containing receptor on the host cell. This suggests that the binding of the chlamydiae does not block the binding of the three mitogens tested in this study. This observation is further supported by the observation that increasing the concentration of ConA did not improve the response of cells to the mitogen.

Significantly, the reduction was not a function of the infectious process; rather, it appeared to be due to a heat-stable component of the EB envelope. It has been demonstrated by in vitro studies that phagolysosome fusion does occur when heat-treated and antibody-neutralized chlamydiae are taken up by macrophages (37, 38). Therefore, it is possible that incomplete degradation of the chlamydiae occurs and that a persistent bacterial component is responsible for the initiation of signals leading to reduced lymphocyte function. The persistence of cell wall components in macrophages has been reported for other bacteria such as group A streptococci

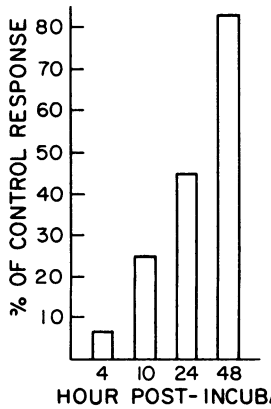

FIG. 4. Addition of *C. psittaci* to cultures at 4, 10, 24, and 48 h after the addition of ConA. *C. psittaci* was added at an EB-to-spleen cell ratio of 100:1. Percentage of control response to ConA = 100 × (net counts per minute with chlamydiae added/net counts per minute with fresh medium added).

TABLE 7. ConA response of spleen cells incubated in vitro with infectious EB, EB envelopes, or RB[a]

Addition to spleen cells	ConA response (net 10^3 cpm[b] ± SD)
EB	0.8 ± 0.7
RB	29.6 ± 1.4
EB envelopes	27.4 ± 3.0
L-cell material	23.0 ± 2.5

[a] Concentrations of chlamydial components added: infectious EBs = 100:1 EB-to-spleen cell ratio, 29 μg of protein per well; EB envelopes = 200:1, 35 μg of protein per well; RBs = 30 μg of protein per well; L-cell material = 30 μg of protein per well.

[b] Net 10^3 cpm = 10^3 cpm with ConA − 10^3 cpm with media.

and has been associated with both the modulation of the host immune response and the pathogenesis of chronic disease (29, 30). Although chlamydiae possess an outer membrane similar to that of other gram-negative bacteria, they do not have a typical peptidoglycan structure, and the existence of a functional lipid A has not been unequivocally demonstrated (8, 11, 16, 28); therefore, it is possible that the responsible cell envelope component is unique to these obligate intracellular bacteria. The significance of reduced lymphocyte function during infection in vivo is that this component is present in the extracellular infectious form of the organism, and more specifically in the cell envelope, but not in the intracellular noninfectious form.

Transformation to the test mitogens was also reduced when EBs were added directly to spleen cells from untreated mice in vitro. However, in contrast to the in vivo injection of EB, the reduction observed after the in vitro addition of EB depended on the concentration of chlamydiae added and required an intact, if not viable, organism. As the organisms were added to the spleen cell cultures at various times after the addition of ConA, the degree of transformation increased, reaching near-normal levels when added after 48 h. This indicated that the presence of the chlamydiae did not simply inhibit the uptake of the [^{3}H]thymidine, which was added for the final 10 h of incubation. When unbound chlamydiae were removed by washing after the initial 1.5-h incubation period, lymphocyte transformation was reduced to the same degree as it was when the organisms were allowed to remain for the entire culture period. Among the possibilities suggested by these findings are that the chlamydiae directly blocked an early step in the transformation process and that the chlamydiae acted indirectly by inducing the production of suppressor cells. Wainberg and Israel (34) have observed a similar decrease in transformation when live or UV-inactivated viruses are coincubated with lymphocytes. Uptake is not required because cells that lack receptors for entry and penetration of the virus are also affected.

In their study, inhibition is nonspecific in that membrane vesicles and pelleted material from the supernatant fluids of normal and virus-transformed cells are both effective. In contrast, we found inhibition to be specific for the extracellular form of the organism because neither L-cell material nor RBs produced an inhibitory effect. The micrograms of protein added in both studies were similar, so the difference might be due to the preparation or source of the vesicles and supernatants that they used.

The variability observed in the percent inhibition of lymphocyte transformation after the in vivo or in vitro addition of different batches of chlamydiae was not unexpected. In most of the studies, the concentration of chlamydiae added was based on the number of inclusion-forming units of EB that were present. However, the percent infectivity in general varied from one purified batch to another and was generally less than 50%. As a result, an unknown number of noninfectious organisms were present in addition to the infectious organisms in any given experiment. In these studies, it was found that inactivated organisms were also capable of inhibiting lymphocyte transformation; therefore, the exact number of effective organisms added in any given experiment probably varied. The identification and isolation of the chlamydial envelope components responsible for the inhibition of lymphocyte transformation might help to eliminate this variability.

The decrease observed in mitogen responsiveness after the in vivo injection or in vitro addition of EB is not a unique phenomenon; it has been reported with a number of different disease syndromes, most significantly in those that result in chronic or latent infections such as tuberculosis, fungal infections, and parasitic infestations. No doubt the ability of the host to respond effectively early in the infection could be an important factor in the establishment of a chronic or latent infection. Based on our data obtained from both in vivo and in vitro studies, we might predict that the chlamydiae can reduce the host response after infection in two ways. Early in the infection, when large numbers of chlamydiae are being released in a small area, high EB-to-macrophage ratios exist. Cells that are not destroyed by outright infection with chlamydiae and their subsequent multiplication might be damaged in such a way that they cannot perform the functions necessary to initiate a specific immune response. This would correlate with the inhibition observed in lymphocyte transformation after in vitro incubation of EB and cells. Later, when the host begins to respond with antibody and activated macrophages, the persistence of a partially degraded chlamydial cell wall component may result in an additional reduction in host immune function. The intracellular multiplication of chlamydiae under idealized in vitro conditions takes from 48 to 72 h. In vivo, where nutritional conditions are likely to be less optimal, multiplication might be expected to take even longer. The ability to reduce the host immune response early in the infection may allow the slow-growing chlamydiae a chance to multiply and establish themselves intracellularly, leading to a persistent infection.

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