Differential Modulation of Lymphocyte Proliferative Responses and Lymphokine Secretion in Mice during Development of Immunity to *Chlamydia psittaci*†

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A murine model was utilized to study immune responses occurring during the period of acquisition of immunity to chlamydial infection. C3H (H-2^k) mice were immunized by intramuscular injection of 5×10^3 viable *Chlamydia psittaci* elementary bodies (EBs) by a protocol which permits animals to survive an otherwise lethal intraperitoneal challenge 10 days later with the homologous chlamydial strain. Spleen cells assayed during the 10-day period of development to immunity showed depressed proliferative responses in vitro to the T-cell mitogen, concanavalin A, and also exhibited suppressor cell activity. Spleen cell mitogen responses returned to normal levels by 30 days postimmunization, concomitant with the detectable development in vitro of responses to chlamydia-specific antigen. In marked contrast to the reduced proliferative responses, mitogen-stimulated production of the T-cell-derived lymphokines interleukin-2 and gamma interferon by spleen cells from immunized animals was within the normal range at 10 days postimmunization, and supernatant fluids containing these products had both microbicidal and microbistatic effects on chlamydial organisms in vitro. These results demonstrate that independent regulation of T-cell proliferation and lymphokine production occurs in vivo as part of the development of an antigen-specific protective immune response. These results also suggest that such differential modulation of T-cell responses may contribute to the development of protective immunity to chlamydiae in mice, perhaps through limited T-cell clonal expansion coupled with early or preferential maturation of cytokine-secreting helper T cells.

Chlamydiae are obligate intracellular bacteria responsible for a number of human and animal disease syndromes, including trachoma, sexually transmitted diseases, and psittacosis (8, 22, 23). Chlamydiae have a complex life cycle involving the orderly alternation of an infectious metabolically inactive form (elementary body [EB]) and a noninfectious metabolically active stage (reticulate body) (22). Injection of high concentrations of the infectious EB causes rapid death in mice, whereas inoculation with much lower concentrations results in a latent infection (20).

Despite evidence of both enhanced and diminished cell-mediated and humoral responses to chlamydiae (6, 10, 16–18, 25), the relative importance of various aspects of the immune response in the development of protective immunity to this organism is still unclear. We have adopted a murine model to study acquired immunity to chlamydial infection. In this system, immunity is induced after the injection of low numbers of live organisms. Mice that recover from this primary infection are rendered immune to subsequent challenge with an otherwise lethal high dose of the homologous chlamydial strain but are not protected against infection by unrelated organisms (D. M. Paulnock, R. E. Huebner, L. E. Guagliardi, R. M. Albrecht, and G. I. Byrne, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr no. 618, 1985). The experiments described here were designed to characterize early host responses occurring during the period of development of immunity. Spleen cells from protectively immunized mice exhibited a transient depression in response to the mitogen concanavalin A (ConA)

immediately after immunization. However, although proliferative responses were suppressed, cytokine production by these cells remained intact, as measured by production of both interleukin-2 (IL-2) and gamma interferon (IFN-γ). Lymphokine-containing supernatant fluids from mitogen-stimulated cultures could be shown to inhibit chlamydial growth in vitro and to lyse infected cells. Thus, *Chlamydia* infection in mice appears to specifically sensitize splenic T lymphocytes, producing effective immunity but concomitantly suppressing T-cell proliferative capacity. These results suggest that independent regulation of T-cell proliferative responses and lymphokine production may occur in vivo as part of an antigen-specific immune response. Such differential modulation of T-cell responses may be a key element in the development of resistance to chlamydiae.

MATERIALS AND METHODS

Animals. Adult male C3H mice were obtained from Harlan-Sprague Dawley Co. (Indianapolis, Ind.) and used between 8 and 10 weeks of age. Mice were certified free of murine hepatitis virus at the time of arrival, and the serum was monitored periodically thereafter for the presence of antibodies to the virus. Animals were housed in filter-top cages in AAALAC-approved facilities with restricted access.

Growth of Chlamydia psittaci. *C. psittaci* (6BC strain) was grown in mouse L cells and harvested by sonicating the trypsinized L cells for 15 min at 40 kHz. Lysates were centrifuged for 30 min at 12,000 × g. Supernatant fluids were discarded, and pellets were suspended in 10 ml of phosphate-buffered saline (PBS) containing 0.25 M sucrose and 2% (vol/vol) heat-inactivated fetal bovine serum (*Chlamydia* buffer). Cellular debris was cleared by low-speed centrifu-

gation, and chlamydial suspensions were suspended in *Chlamydia* buffer (6). The infectivity titer of each batch of 6BC was determined in L cells by the 50% infective dose method described by Hatch (10).

Immunization of mice. Mice received 5×10^3 viable EBs serially diluted in PBS from stock suspensions by intramuscular injection in the left flank. Animals immunized in this way survive an otherwise lethal intraperitoneal challenge of 10^6 EBs administered 10 days after the intramuscular injection (Paulnock et al., 25th ICAAC). Control (mock-immunized) mice were similarly injected with PBS.

Inactivation of *C. psittaci* with heat or UV light. Some EB preparations were treated with UV light (UV inactivated) before immunization (2). Infectivity after treatment was assayed by the inclusion-forming-unit technique (15).

When lymphocyte proliferative responses to chlamydial antigen were tested, the EBs were heat inactivated by incubation in a 56°C water bath for 30 min.

Lymphocyte transformation. Cell suspensions were made from spleens of *Chlamydia*-sensitized or mock-immunized mice at various times after immunization and pooled for assessment of proliferative responses. Erythrocytes in the splenocyte populations were lysed with hemolytic Gey's solution (19) before the initiation of the culture. For all blastogenic assays, cells were suspended in complete medium (RPMI 1640 medium; GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (Hazleton Research Products, Denver, Pa.) at a concentration of 2×10^6 cells per ml and 200-μl aliquots were dispensed into the wells of 96-well flat-bottom microtiter plates (4×10^5 cells per well) (Costar, Cambridge, Mass.). For mitogen-induced blastogenesis, various concentrations of ConA (Sigma Chemical Co., St. Louis, Mo.) in RPMI 1640 medium were added to individual wells in a final volume of 20 μl. In antigen-stimulated blastogenesis assays, cells were plated as described above and heat-inactivated EBs were added at a 10:1 or 1:1 EB/cell ratio. In both cases, cells subsequently were incubated at 37°C in a humidified incubator at 7% CO_2. After 72 h, 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) in 20 μl of RPMI 1640 medium was added to each well, followed by an additional 18-h incubation period. Cultures were harvested onto glass-fiber filter strips with a PHD cell harvester (Cambridge Technology, Inc., Cambridge, Mass.), and the filters were placed in 3 ml of Betafluor (Research Products International, Mount Prospect, Ill.) and counted in a Tri-Carb 300 liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Suppression of normal cell blastogenesis. Spleen cells from mock-immunized mice (control cells) or from mice injected 10 days earlier with 5,000 viable EBs (immune cells) were irradiated with 1,000 rads delivered from a ^{137}Cs source (J. H. Phillips, Glendale, Calif.). The irradiated cells were adjusted to 4×10^6/ml in complete RPMI 1640 medium, and 100-μl aliquots (4×10^5 cells) were added to individual wells of 96-well flat-bottom microtiter plates. An equal number of untreated control or immune cells was added to each well, and the mixed population was stimulated with serial dilutions of ConA for 72 h and processed as described above. Normal cell blastogenic responses to ConA also were measured after stimulation of cells in medium containing 50% (vol/vol) culture supernatant fluids prepared as described below.

Lymphokine preparation. At various times (4, 10, or 30 days) postimmunization (PI), spleens were aseptically removed from infected mice and erythrocyte-free cell suspen-

sions were prepared with hemolytic Gey's solution. Cells were adjusted to 5×10^6/ml in Eagle minimum essential medium supplemented with 10% fetal bovine serum (Hazleton). Cells were cultured either without stimulation or in medium containing either 5 μg of ConA per ml or EBs at a 10:1 EB/cell ratio. Cultures (6 ml) were incubated for 48 h in 25-cm^2 tissue culture flasks (Costar), and cell-free supernatant fluids were obtained by centrifugation. Clarified supernatant fluids then were tested by bioassay for the presence of IL-2 and IFN-γ, as described below. Control supernatant fluids were prepared in exactly the same way, using spleen cells from mock-immunized animals.

Assay for IL-2 in spleen cell supernatant fluids. Supernatant fluids from *Chlamydia*-immune or mock-immunized mice were tested for the presence of IL-2 by bioassay using the IL-2-dependent mouse cytotoxic cell line, CTLL (7). Briefly, 5×10^3 CTLL cells per well were plated in 96-well, flat-bottom microtiter plates (Costar) along with serial dilutions of antigen- or mitogen-stimulated supernatant fluids in a total volume of 200 μl. After 24 h of incubation, the cells were pulsed for an additional 5 h with 1 μCi of [^{3}H]thymidine (Dupont NEN) per well. The samples were then harvested onto glass-fiber filter strips and counted in a scintillation counter as described above. IL-2 concentrations in supernatant preparations were calculated by the method of Robb and Smith (21), using recombinant murine IL-2 (Genzyme, Boston, Mass.) as a standard.

Assay for IFN-γ in spleen cell supernatant. IFN-γ levels in supernatant preparations were assessed both by titration of antiviral activity and by assay of antichlamydial activity.

Measurement of the protective effect of acid-treated cell culture supernatant fluids against cytocidal infection of L929 cells with encephalomyocarditis virus was done by Lee Biomolecular Research Laboratories, Inc. (San Diego, Calif.). Results were expressed as units of IFN antiviral activity per milliliter of culture medium, using a standard mouse IFN reference reagent as the control. Results are expressed as units per milliliter, normalized to the National Institutes of Health mouse interferon reference reagent (1). Levels of IFN-γ detected by this assay in culture supernatant fluids generated in two independent experiments did not vary by more than 10%.

The chlamydiastatic activity of spleen cell supernatant preparations was tested by the method of Byrne and Kreuger (6). Macrophage target cells for chlamydial infection were harvested by peritoneal lavage from C3H mice injected intraperitoneally 5 days previously with 1 ml of 3% (wt/vol) thioglycolate broth (Difco Laboratories, Detroit, Mich.). Peritoneal cells were collected by centrifugation and suspended in minimal essential medium supplemented with 10% fetal bovine serum (Hazelton). The cells then were plated onto glass cover slips (12-mm diameter, VWR Scientific, Inc., San Francisco, Calif.) in the wells of 24-well plates (Costar). Plated cells were incubated for 30 min at 37°C in a humidified atmosphere of 7% CO_2, washed free of nonadherent cells, and returned to the incubator with or without the addition of supernatant fluids from ConA- or EB-stimulated spleen cells at various dilutions. After treatment with spleen cell supernatant fluids for 6 h, macrophages were washed extensively and infected with one 50% infective dose of strain 6BC in Hanks balanced salt solution (GIBCO) as previously described (3). Fresh minimal essential medium was added (1 ml per well) after a 90- to 120-min adsorption period. Evidence of parasite replication was measured 18 to 20 h after infection by determining the fraction of cells containing Giemsa-stained inclusions. Previous studies have

TABLE 1. Responses of spleen cells from *C. psittaci*-immunized mice to the mitogen ConA

Spleen cell source[a]	cpm[b] at the following concn (μg/ml) of ConA:					
	0	0.5	1.25	2.5	5	10
Control mice (mock immunized)	185 ± 4	122,357 ± 4,636	134,041 ± 2,458	170,510 ± 20,554	179,566 ± 18,147	172,249 ± 26,246
Immunized mice						
4 days PI	605 ± 89	121,120 ± 5,893	298,875 ± 25,923	397,253 ± 16,482	98,408 ± 10,880	4,093 ± 165
10 days PI	164 ± 75	123[c] ± 24	206[c] ± 20	434[c] ± 68	355[c] ± 30	301[c] ± 17
30 days PI	343 ± 80	72,787 ± 3,688	84,313 ± 2,321	136,115 ± 6,036	152,976 ± 10,342	85,965 ± 15,497

[a] Four mice per group. Spleen cells were collected from mice 4, 10, or 30 days PI with 5,000 EBs (immunized mice) or 10 days after a mock immunization with PBS (mock-immunized mice). For each group, pooled spleen cells were cultured at 4×10^5 cells per well with various concentrations of ConA for 72 h.

[b] Values represent the cpm (mean ± standard deviation) of [³H]thymidine incorporation in triplicate samples. Results shown are from a single experiment representative of greater than 20 separate experiments.

[c] $P < 0.05$ by two-tailed Student's t test.

shown that the activity of the chlamydiastatic factor present in such supernatant preparations is neutralized by antibodies to IFN-γ (4).

Cytotoxic factor activity in immune and control spleen cell supernatant fluids was determined by a [³H]thymidine release assay using L-cell targets, as previously described (6). Briefly, L-cell monolayers in 75-cm² flasks were incubated for 16 to 20 h in the presence of 2 μCi of [³H]thymidine (Dupont NEN) (specific activity, 40 to 60 Ci/mmol) per ml. After incubation, cells were washed extensively in Hanks balanced salt solution and then infected with 10 50% infective doses of *C. psittaci* or mock infected with Hanks balanced salt solution alone. After adsorption and uptake of the inoculum, infected L cells were trypsinized, counted, and distributed as 100-μl portions (10^4 cells) into individual wells of 96-well flat-bottom microtiter plates (Costar). The total medium in each microtiter well was brought to a final volume of 200 μl by the addition of various dilutions of supernatant fluids prepared as described above. Plates were incubated for 30 h at 37°C in a CO_2 incubator and subsequently processed to measure the amount of [³H]thymidine released into the culture medium. For the measurement of radiolabel release from each sample (experimental counts per minute [cpm]), plates were centrifuged for 5 min at 400 × g, and 100-μl aliquots were then drawn off and added to 3 ml of water-soluble scintillant (Aquasol; Dupont NEN). Triton X-100 (Sigma) at a concentration of 0.05% was added to quadruplicate samples of labeled cells to determine the maximal release of [³H]thymidine (maximum cpm). The cpm released by infected L cells also was measured in wells containing cells and medium alone (spontaneous cpm). All samples were tested in quadruplicate assays, and a mean value of label released was obtained. The percent specific [³H]thymidine released was determined according to the following formula: percent specific [³H]thymidine release = [(experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100.

Previous studies have shown that the cytotoxic activity of these supernatant preparations is neutralized by antibodies to IFN-γ (5).

Statistical analysis. The proliferative responses of normal and experimental animals were compared by two-tailed t test at the mitogen or antigen concentrations routinely yielding the maximum proliferative response (ConA, 1.25 and 2.5 μg/ml; chlamydial antigen, 10:1 EB/cell ratio) and were considered significantly different at the level of $P < 0.05$. In experiments measuring the production of soluble factors, activities in the supernatant preparations were compared with the activities seen by treatment with medium alone.

RESULTS

Immunization with *C. psittaci* is characterized by an early, transient suppression of splenic T-cell proliferative responses. Mice immunized intramuscularly with 5,000 *Chlamydia* EBs exhibited splenomegaly and a pronounced increase in the total number of splenocytes per spleen. The increase in spleen size peaked by 10 days PI (at approximately 15×10^8 cells per spleen), but the size returned to normal within 30 days (data not shown). Spleen cells also were tested for their ability to respond to the T-cell mitogen ConA and to specific chlamydial antigen at several time points after infection. Spleen cells taken from immunized mice 4 days PI displayed heightened proliferative responses to ConA (Table 1) compared with the response of mock-immunized controls. However, by 10 days PI, when splenomegaly was maximal, there was a pronounced reduction in splenic T-cell responses at all concentrations of ConA tested (Table 1). Lymphocyte responses to ConA were within the normal range by 30 days PI, concomitant with the return of normal spleen size (Table 1).

A similar analysis of proliferative responses to chlamydial antigen revealed that proliferative responses to heat-inactivated EBs were not detectable at 4 or 10 days PI, but stimulation with EBs at a 10:1 EB/cell ratio induced a strong proliferative response by 30 days PI (Table 2).

Addition of *Chlamydia*-sensitized spleen cells suppressed the response of normal cells to mitogen. To determine if the

TABLE 2. Responses of spleen cells from *C. psittaci*-immunized mice to specific antigen

Spleen cell source[a]	cpm[b] at the following EB/cell ratio[c]:	
	10:1	1:1
Control mice (mock immunized)	3,031 ± 287	2,147 ± 219
Immunized mice		
4 days PI	2,201 ± 109	1,062 ± 75
10 days PI	4,421 ± 203	4,066 ± 221
30 days PI	85,547[d] ± 2,173	9,777 ± 1,073

[a] Four mice per group. Mice were immunized with 5,000 viable EBs or with PBS. AT 4, 10, or 30 days PI, spleens from immunized mice were pooled and cultured with chlamydial antigen (EB) for 72 h. Spleen cells from mock-immunized mice were harvested 10 days after immunization with PBS.

[b] Values represent the cpm (mean ± standard deviation) of [³H]thymidine incorporation in triplicate cultures. Results shown are from a single experiment which was representative of 10 separate experiments.

[c] Chlamydia EBS were heat inactivated at 56°C for 30 min and added at a 10:1 or 1:1 EB/cell ratio.

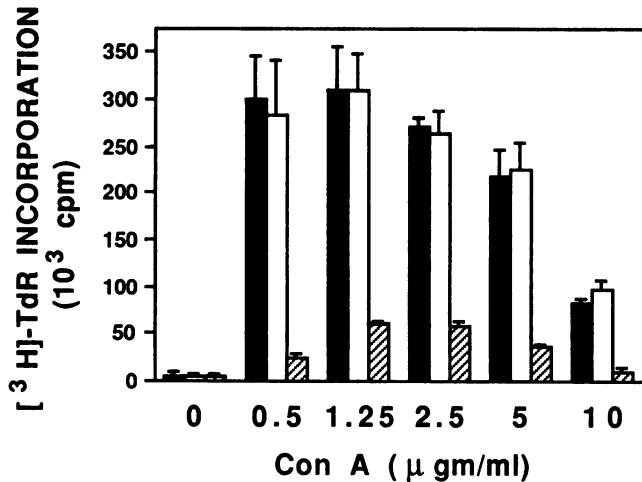[d] $P < 0.05$ by two-tailed Student's t test.

FIG. 1. Irradiated cells from *Chlamydia*-sensitized mice suppress normal cell blastogenic responses. Pooled spleen cells from either four normal mice or four mice infected for 10 days with 5,000 EBs were irradiated with 1,000 rads delivered from a ^{137}Cs source. Irradiated cells (normal, ■; immune, ▨) (5×10^5) were added to an equal number of untreated control splenocytes, and the mixed population was stimulated in vitro with the indicated dose (in micrograms per milliliter) of ConA for 72 h. Control splenocytes also were cultured with 50% (vol/vol) supernatant fluid obtained from immune spleen cells after culture for 48 h at 37°C (□). The data are represented as the mean cpm of triplicate samples ± standard deviation from a single experiment which was representative of 20 separate experiments from 20 separate immunizations. [^{3}H]-TdR, [^{3}H]thymidine.

observed reduction in proliferative responses was the result of active suppression, immune splenocytes were added to normal mouse spleen cell cultures at a 1:1 ratio and the [^{3}H]thymidine incorporation was measured after stimulation of the mixture with various concentrations of ConA. Splenocytes from immunized mice received a dose of 1,000 rads to eliminate their contribution to the thymidine incorporation measured. Control cultures contained an identical number of irradiated spleen cells obtained from mock-immunized mice. The addition of 50% irradiated immune cells to normal splenocyte cultures resulted in the reduction of responsiveness of the normal cells to ConA (Fig. 1, $P < 0.05$ for all concentrations of mitogen tested compared with control cells). Similar tests of the suppressive capacity of various numbers of immune spleen cells demonstrated that this reduction of responsiveness was cell concentration dependent and could be manifested by the addition of immune cells up to a 0.25:1 immune cell/normal cell ratio (data not shown). In contrast to the results obtained with immune

spleen cells, addition of cell-free supernatant preparations obtained from cultures of cells with suppressor activity did not reduce the mitogen response of normal cells (Fig. 1).

Immunization with nonviable *C. psittaci* failed to evoke protection and suppression. Splenic proliferative responses of immunized mice were compared with the responses of groups injected with nonviable *C. psittaci* to determine if the development of suppression was associated with the induction of active immunity or was the result of exposure to chlamydial constituents per se. Mice injected intramuscularly with 10^3 or 10^6 UV-inactivated EBs were not protected from a lethal intraperitoneal challenge (Table 3). Spleen cells taken from these animals also showed ConA-induced blastogenic responses within the normal range, although slightly decreased responses (relative to control cell responses) were observed at higher mitogen concentrations (Table 4). In contrast, mice rendered immune to challenge by an initial injection of 5,000 viable EBs again exhibited significantly decreased spleen blastogenic responses to ConA 10 days PI (Table 4).

Mitogen-stimulated immune spleen cell cultures produced IL-2. The ability of spleen cells from immunized animals to produce various cytokines in response to mitogen or antigen stimulation was also monitored. For these experiments, spleen cells obtained from mock-immunized mice or mice 4, 10, or 30 days PI were stimulated with 5 μg of ConA per ml for 48 h in vitro. Cell-free culture fluids were obtained by centrifugation and tested by biological assay for the presence of IL-2 and IFN-γ.

The presence of IL-2 in immune and control spleen cell supernatants was assessed in a proliferative assay by using the IL-2-dependent murine CTLL cell line as responder cells. Substantial levels of [^{3}H]thymidine incorporation were seen in CTLL cells stimulated for 24 h with various dilutions of supernatant fluids obtained after mitogen stimulation of

TABLE 3. Assessment or protective immunity in mice after immunization with *C. psittaci*[a]

Form and no. of organism used for immunization	% Survival[b]
None (mock immunized with PBS)	0
5.0 viable EBs	100
5,000 viable EBs	100
10^3 UV-inactivated EBs	0
10^6 UV-inactivated EBs	0

[a] Mice (four mice per group) were immunized with 5 or 5,000 viable EBs or with 10^3 or 10^6 UV-inactivated EBs. (Chlamydia EBs were inactivated by 15 min of exposure to UV light. UV-inactivated EBs are not able to form detectable inclusions in L cells or macrophages.) Mice were challenged with 10^6 viable EBs 10 days PI.

[b] Animals were observed for a period of 2 months; at the end of this observation period, the percentage of the animals surviving was determined.

TABLE 4. Comparison of spleen cell mitogen responses in mice immunized with *C. psittaci*[a]

Form and no. of organisms used for immunization	cpm[b] at the following concn (μg/ml) of ConA:					
	0	0.5	1.25	2.5	5	10
None (mock immunized)	6,983 ± 895	325,380 ± 31,394	365,680 ± 46,195	432,225 ± 31,779	397,975 ± 44,462	349,153 ± 83,091
5,000 viable EBs	1,355 ± 104	1,105[c] ± 98	1,289[c] ± 256	674[c] ± 122	456[c] ± 195	491[c] ± 93
10^6 UV-inactivated EBs	1,871 ± 172	281,434 ± 30,980	320,188 ± 13,903	185,922 ± 29,747	170,614 ± 24,082	145,478 ± 4,989

[a] Mice were immunized intramuscularly with either 5,000 viable EBs or 10^6 UV-inactivated (by 15-min exposure to light) EBs. At 10 days PI, spleen cell responses to ConA were assessed by using pooled spleen cells (four mice per group).

[b] Values represent the cpm (mean ± standard deviation) of [^{3}H]thymidine incorporation of triplicate cultures. Results from one experiment, which was representative of three separate experiments, are shown.
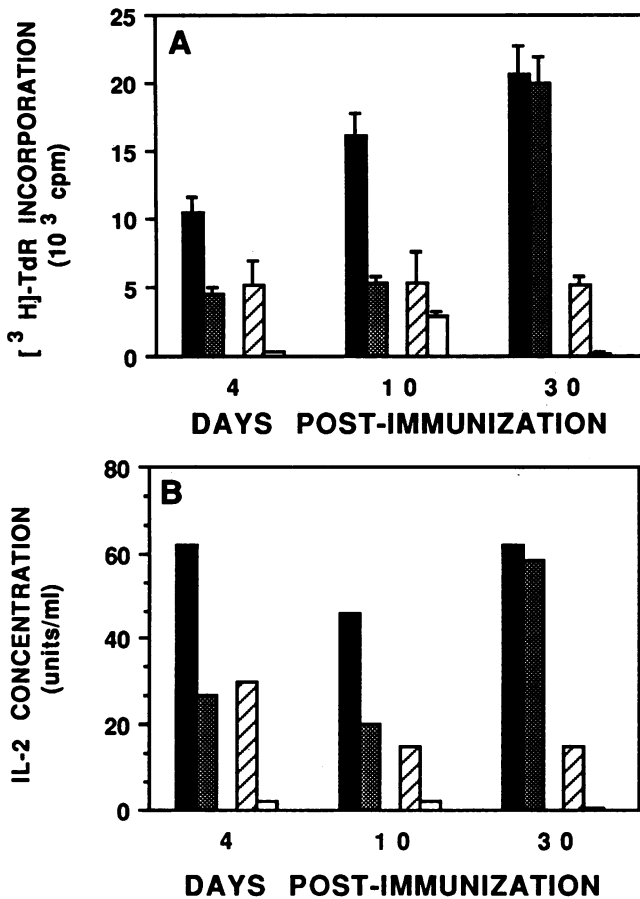
[c] $P < 0.05$ by two-tailed Student's t test.

FIG. 2. Spleen cell supernatants from immunized mice contain IL-2. (A) Spleens were harvested from mock-immunized or *Chlamydia*-sensitized mice 4, 10, or 30 days PI. Pooled splenocytes were cultured at 5×10^6 cells per ml in medium containing 5 μg of ConA per ml (normal, ■; immune, ▨) or a 10:1 EB/cell ratio (normal, □; immune, ▨) for 48 h. Cell-free supernatant fluids were collected by centrifugation of the splenocyte cultures and added to CTLL cells at a final dilution of 1:4 in medium. Thymidine incorporation by CTLL cells was measured 24 h later. Values represent the mean cpm of [³H]thymidine ([³H]-TdR) incorporation (± standard deviation) of quadruplicate cultures from a single experiment which was representative of three separate experiments. (B) IL-2 concentrations in each preparation were calculated by using recombinant murine IL-2 as a standard, as described in Materials and Methods.

cells from either mock-immunized or *Chlamydia*-immunized mice at 4, 10, and 30 days PI (Fig. 2A). Calculation of the IL-2 concentrations present at each time point revealed that the levels of IL-2 present in supernatant preparations made using spleen cells from immunized animals were slightly lower than those detectable in similar preparations made from normal spleen cells (Fig. 2B).

Similar analysis of cell-free supernatant preparations collected from splenocyte cultures derived from spleen cells of immunized or mock-immunized mice stimulated with chlamydial antigen (at a 10:1 EB/cell ratio) demonstrated that spleen cells of immunized mice, but not normal cells, produced low levels of IL-2 at all time points after immunization (Fig. 2B). Although the overall level of IL-2 activity was lower than that observed after mitogen stimulation, the presence of significant levels of IL-2 was detectable before the appearance of a proliferative response to antigen, which was first demonstrable at 30 days PI (Fig. 2B).
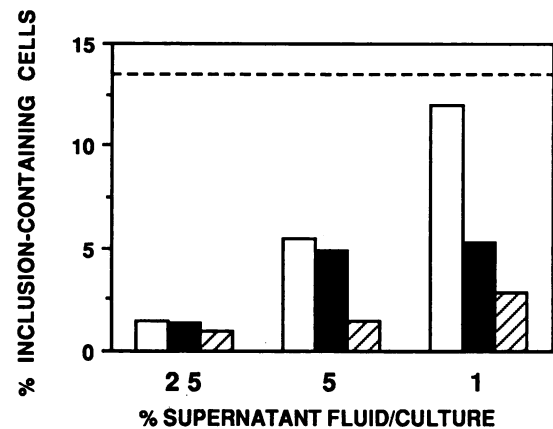

FIG. 3. Soluble spleen cell-derived mediators can activate macrophages to restrict chlamydial replication. Thioglycolate-elicited macrophages were incubated in medium containing the indicated amounts of supernatant preparations for 24 h before infection with *C. psittaci*. Supernatant fluids were collected from ConA-stimulated spleen cells of control mice (□) or mice 10 days after immunization with 5,000 viable EBs (■) and from EB-stimulated spleen cells of mice 10 days after immunization (▨) as described in Materials and Methods. After medium removal, the cells were infected with one 50% infective dose of *Chlamydia* EBs and examined for evidence of chlamydial replication (inclusion formation) 18 to 20 h later. Values represent the percentage of inclusion-containing cells. The percentage of cells containing inclusions in cultures treated with medium alone (13.4%) (– – – – – –) is shown. Results are shown from one experiment, which was representative of three separate experiments done using supernatant fluids prepared from a pool of cells from four mice per group. Levels (in units per milliliter) of IFN-γ detected in the same supernatant preparations by assessment of antiviral activity were as follows: 7.4 for ConA-stimulated control cells, 51 for ConA-stimulated immune cells, <1 for EB-stimulated control cells, and 17 for EB-stimulated immune cells.

Mitogen-stimulated immune spleen cell cultures produced IFN-γ. Levels of IFN-γ present in supernatant preparations were assessed both by titration of antiviral activity and by bioassay.

The levels of chlamydiastatic activity in ConA-stimulated supernatant preparations from spleen cells from immune and mock-immunized mice were assessed by activating peritoneal macrophages to restrict chlamydial replication in vitro, as described in Materials and Methods. When *C. psittaci* was added to thioglycolate-elicited macrophages pretreated with medium alone, evidence of parasite replication was detected 20 h after infection. Examination of Giemsa-stained cell populations (triplicate samples, 200 cells per sample) revealed that an average of 13.4% of the adherent cells contained demonstrable cytoplasmic inclusions (Fig. 3). Supernatant fluids prepared by ConA stimulation of cells from immunized animals 10 days PI displayed marked chlamydiastatic activity at all concentrations tested ($P < 0.05$ compared with medium alone [Fig. 3]). Pretreatment of macrophages with medium containing as little as 1% (vol/vol) supernatant fluid from cells obtained from mice 10 days PI, for example, resulted in only 5.3% of the cells containing demonstrable inclusions (Fig. 3). Supernatant preparations from antigen-stimulated cultures of cells from immunized animals also inhibited chlamydial growth at all dilutions tested, including substantial inhibition at a 1% (vol/vol) concentration in the medium (2.8% inclusion-containing cells [Fig. 3]). In contrast, supernatant fluids from antigen-stimulated normal cells allowed maximal parasite replication

at all concentrations tested (data not shown). Chlamydial replication also was reduced when the elicited macrophages were pretreated with high concentrations of supernatant fluid from ConA-stimulated cells from mock-immunized mice, indicating the presence of IFN-γ in these preparations. Further dilution of this preparation, however, allowed near-maximal chlamydial growth (Fig. 3, 12% inclusion-containing cells with 1% mock-immune supernatant), confirming by bioassay the differing levels of IFN-γ present in medium from the immune and nonimmune spleen cell cultures.

Analysis of the acid-labile antiviral activity present in the supernatant preparations used in the above experiment revealed that cultures of immune spleen cells stimulated with ConA contained higher levels of IFN-γ than did similarly stimulated normal spleen cells. Levels of IFN-γ detected by this method in supernatant preparations were measured at 51 U/ml for immune cells and 7.4 U/ml for control cells. Immune spleen cells stimulated with EBs (10:1 EB/cell ratio) also produced detectable levels of IFN-γ (17 U/ml), while similarly stimulated control cells produced <1 U of IFN-γ per ml.

Mitogen-stimulated immune spleen cell supernatant preparations exhibited cytotoxic activity. The presence of cytotoxic activity in supernatant fluids from ConA- or EB-stimulated cells was determined by a [^{3}H]thymidine release assay, as described in Materials and Methods. In this assay, uninfected, labeled L cells displayed <3% specific [^{3}H]thymidine release after 30 h of incubation at 37°C. Chlamydia-infected L cells were lysed in a dose-dependent manner after incubation of the cells in mitogen-stimulated supernatant fluids obtained from either Chlamydia-immunized or mock-immunized mice (Fig. 4). However, the levels of L-cell lysis after treatment with the ConA supernatant preparations from immunized mice were significantly higher ($P < 0.05$ at all dilutions) than those observed with the control ConA supernatants at all dilutions tested (Fig. 4). Cell-free supernatant preparations from immune splenocytes stimulated with heat-inactivated chlamydial antigen (EB) also contained detectable levels of cytotoxic activity for Chlamydia-infected L cells (Fig. 4). No cytotoxic activity was detectable in supernatant preparations from antigen-stimulated normal cells (data not shown).

DISCUSSION

The results presented here demonstrate an unusual pattern of lymphocyte responses occurring during the in vivo immune response to C. psittaci in mice. T-cell responses exhibited a transient suppression early in the course of infection but returned to normal levels approximately 30 days PI. Spleen cells taken from immunized mice at 10 days PI, but not their cell-free products, also suppressed the responses of normal cells to ConA, suggesting the presence of an active cellular suppressor mechanism in immunized animals. Although spleen cell proliferative responses were depressed in the period after immunization, the production of several lymphokines by immune spleen cell cultures, including IL-2 and IFN-γ, was readily detectable during the course of infection. Thus, during the time when protective immunity was acquired (10 days PI), spleen cell proliferative responses to mitogen were suppressed while lymphokine production in response to mitogen remained at or near normal levels. Stimulation of immune spleen cells with the homologous chlamydial antigen in vitro resulted in a lower overall level of lymphokine production. However, lymphokine activity was detectable before the appearance of a
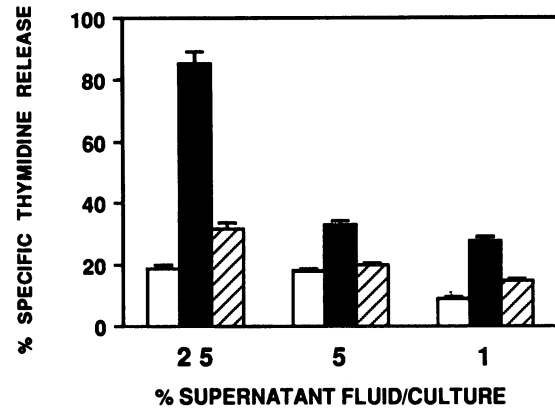

FIG. 4. Mitogen-stimulated spleen cell supernatants contain factor(s) which lyse Chlamydia-infected cells. Culture supernatant fluids were collected from ConA-stimulated spleen cells of mock-immunized mice (□) or from ConA-stimulated (■) or EB-stimulated (▨) spleen cells from immunized mice 10 days PI. Cells from four mice were pooled for each spleen cell preparation. [^{3}H]thymidine-labeled L cells were infected with ten 50% infectious doses of viable EBs and then incubated with the indicated concentration of spleen cell supernatants for 30 h. Cell lysis was measured by quantitation of specific [^{3}H]thymidine by the formula given in Materials and Methods. Labeled, mock-infected cells that were similarly treated consistently demonstrated a specific [^{3}H]thymidine release of <3%. Maximum release (100%), quantitated by detergent lysis of labeled cells, was routinely 500 to 550 cpm/10^4 cells. Values represent the mean of quadruplicate samples ± standard deviation from one experiment that was representative of three separate experiments. Levels (in units per milliliter) of IFN-γ detected in the same supernatant preparations by assessment of antiviral activity were as follows: 7.4 for ConA-stimulated control cells, 51 for ConA-stimulated immune cells, <1 for EB-stimulated control cells, and 17 for EB-stimulated immune cells.

proliferative response to antigen, suggesting that the cells were specifically stimulated by antigen during the period of development of immunity.

These data suggest that a complex pattern of immune responses is stimulated after the administration of viable Chlamydia organisms to competent hosts. At least part of this process appears to involve the selective modulation of T-cell responses such that certain cellular activities (proliferation) are suppressed while others (lymphokine production) remain intact. The suppression of proliferative responses is dependent on an active cellular component found in spleens of immunized animals. We do not yet know what aspects of the host response contribute to the down regulation of mitogen responsiveness observed during the period after infection with C. psittaci. One possibility is that the infection of macrophages and the subsequent presentation of chlamydial antigen by these cells either fails to provide or interferes with the signal(s) necessary to induce proliferation of immune splenocytes but can serve as a sufficient and effective signal for initiation of lymphokine production by stimulated cells. This possibility is strengthened by our recent observation of a temporal relationship between the establishment of protective immunity and the induction of activated macrophages in vivo (12). Thus, it may be that antigen-responsive helper T cells receive an early and preferential maturational signal, forcing them to undergo limited clonal expansion to lymphokine-secreting effector cells. The limited clonal expansion, reflected in the lack of proliferation by lymphocytes in vitro in response to activation signals provided by a mitogen or antigen, may result from the action

of a third-party suppressor cell that is simultaneously activated during the response to *C. psittaci*, either by antigen-presenting cells or by microbial products directly. Results from recent in vitro studies of T-cell activation have suggested that distinct intracellular signals can control the stimulation of proliferation versus lymphokine production in antigen-stimulated cells (9, 11), an observation that mirrors the experimental results presented here. The molecular mechanisms of the selective modulation observed in our system are currently being determined.

In summary, we have demonstrated the presence of marked suppression of spleen cell blastogenic responses in animals immunized with viable *Chlamydia* organisms. However, the capability for production of a variety of soluble factors by these cells was at or near normal levels in these animals even at the time when spleen cell proliferation was reduced. In our system, this uncoupling of lymphokine production and proliferation does not appear to be solely a pathogen-associated event but rather is associated with the administration of a dose and form of the organism that routinely leads to development of immunity. These results suggest that, in contrast to a number of human and animal bacterial infections in which detectable suppression of the host immune response has correlated with increased pathogen virulence (13, 14, 24), immunosuppression after immunization with *C. psittaci* may be indicative of immune response modulation which functions in the development of protective immunity. Our current experiments are aimed at determining whether these events are linked functionally and genetically.

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