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Collaboration of Bovine T Lymphocytes and Macrophages in T-Lymphocyte Response to *Brucella abortus*

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***Brucella abortus*-induced bovine macrophage-T-lymphocyte collaboration was studied as a prerequisite for the eventual clearance of this infectious organism. Esterase-positive, peripheral blood monocytes functioned as an adherent antigen-presenting cell population. A dual requirement for expression of bacterial antigens in combination with self major histocompatibility complex class II products was required by adherent cells for the activation of T lymphocytes. Comparison of antigen-presenting cell populations that were either trypsinized or nontrypsinized following *B. abortus* ingestion substantiated the need for phagocytosis and antigen processing. A monoclonal antibody (H4) directed against major histocompatibility complex class II determinants was able to block or, with complement, to abrogate T-lymphoctye responses. Measurement of both proliferation and interleukin 2 production via [^{3}H]thymidine incorporation confirmed specific activation of an enriched T-lymphocyte population. These results indicate that in vivo-primed T lymphocytes of peripheral blood origin recognize phagocytized bacterial components of the facultative intracellular bacterium *B. abortus* and may contribute to the removal of the bacteria. Furthermore, bovine peripheral blood-adherent cells function as classic antigen-presenting cells, which suggests that macrophages are capable of processing this bacteria. Therefore, any lymphocyte-mediated dysfunction attributable to *B. abortus* most likely occurs at some point in the cascade of immune events following initial macrophage-T-lymphocyte collaboration.**

Macrophages serve as important resident cells for the facultative intracellular bacterium *Brucella abortus*; however, it is assumed that macrophages phagocytize and kill most ingested species of bacteria within a few hours (4, 7, 35). Resistance to facultative intracellular bacteria depends on successful interaction between specifically sensitized T lymphocytes and macrophages (21). In vivo development of nonspecific resistance to *B. abortus*-infected mice has been described in several studies (5, 13), and these reports have suggested that activated macrophages are responsible for the antibacterial activity. There are also studies in which heightened bactericidal activity has been shown in vitro by macrophages collected from *B. abortus*-infected mice and cattle (3, 11, 29). Most recently, in studies in which *B. abortus*-infected mice have been examined, increased macrophage activation has been reported by several criteria, including spreading, Fc and C3 receptor density, locomotion, and chemotactic responsiveness (3). However, in these in vivo and in vitro studies done by others, interactions between bovine macrophages and T lymphocytes in response to *B. abortus* have not been examined.

Results of studies in mice in which another facultative intracellular bacterium, *Listeria monocytogenes*, was used have indicated that the organisms are phagocytized, killed, and processed (1, 41). Then, bacterial antigenic components are expressed on the macrophage membrane in association with major histocompatibility complex (MHC) class II determinants for recognition by antigen-specific T lymphocytes (10, 43). Proliferation and interleukin-2 (IL-2) production by T lymphocytes occurs following associative recognition of bacterial and MHC components on the macrophage surface (16–18).

Studies of immune response to *B. abortus* by cattle have been limited predominately to antibody production in response to bacterial components (25, 42). However, the principal biologic host for this bacterium is cattle, and few studies have examined the interactions between bovine macrophages and T lymphocytes in the recognition of *B. abortus*. Because bovine macrophages serve as resident cells for *B. abortus*, they might also be central in initiating both positive and negative regulation of immune responses in infected cattle. The manner by which macrophages present *B. abortus* to T lymphocytes may serve to regulate T-lymphocyte recognition and IL-2 production. This cell-cell interaction could be pivotal for the control of this bacterium, as has been demonstrated for other facultative bacteria (21). In this study the interaction between bovine macrophages and T lymphocytes in response to *B. abortus* was examined.

MATERIALS AND METHODS

Cells. Eight Guernsey cattle (age, 3 to 8 years) from the University of Wisconsin-Madison dairy herd that were vaccinated when they were 2 to 3 months old with *Brucella abortus* 19 (smooth strain) (Jensen-Salsbury Lab, Kansas City, Mo.) were the donors of peripheral blood cells. Animals were seronegative for *B. abortus*. Peripheral blood mononulcear (PBM) cells were isolated with Ficoll-Hypaque, and the cells were cultured with acetone-killed *B. abortus* 1119 (smooth strain) (26) for selected days, as indicated below. Eight hours prior to harvesting, the cells were pulsed with 1 μCi of [^{3}H]thymidine, harvested, and counted in a scintillation counter.

Adherent cells were obtained by culturing 2×10^6 PBM cells in microtiter wells containing RPMI 1640 medium supplemented with 100 IU of penicillin per ml and 100 μg of streptomycin per ml, 2 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 10% fetal calf serum. Either 0.1 ml of acetone-killed *B. abortus* at 500 μg/ml or concanavalin A (ConA) at 25 μg/ml was added, and the cultures were incubated overnight at 37°C and with 5% CO_2 in a humidified atmosphere. Follow-

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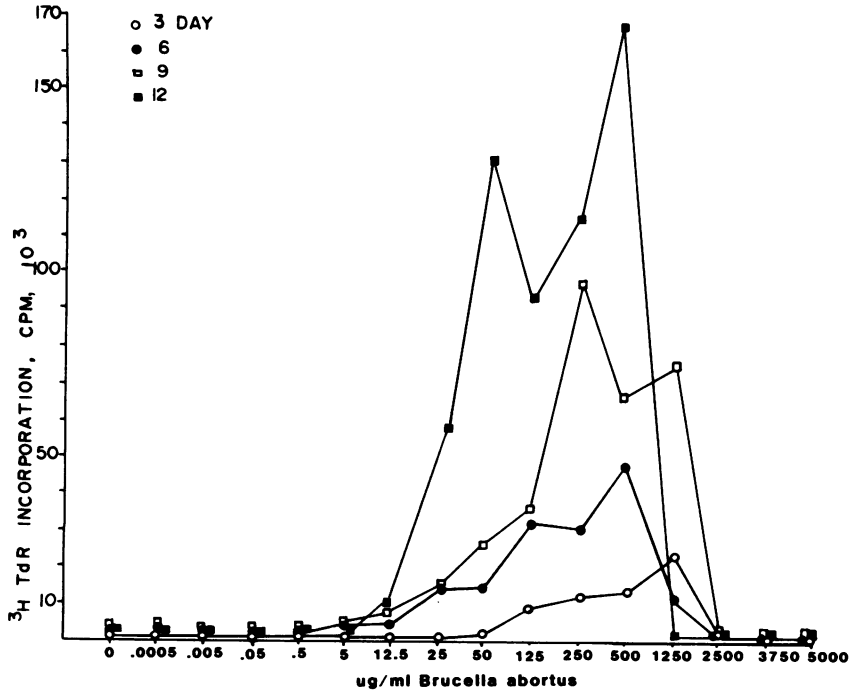

FIG. 1. Dose response of unfractionated PBM cells to *B. abortus* for 3 (○), 6 (●), 9 (□), and 12 days (■). ^{3}H TdR, [^{3}H]thymidine.

ing incubation, the cells were washed three times with medium to remove nonadherent cells, either supplemented with medium or trypsinized twice with 0.5× stock solution of trypsin-EDTA (Grand Island Biological Co., Grand Island, N.Y.), and subsequently washed; fresh medium was then added. Culture wells contained approximately 10^4 to 10^5 adherent cells. Nonadherent cells were obtained from 60 × 10^6 PBM cells cultured overnight in 75-cm^3 flasks containing supplemented medium only. Nonadherent cells were washed and passaged through a Sephadex G10 column by the method of Jerrells et al. (14) and designated as nonadherent cells. Further purification was accomplished by panning on rabbit F(ab')$_2$ anti-bovine immunoglobulin-coated petri dishes by the technique of Mage et al. (22). These enriched cells were designated as T lymphocytes. Nonadherent or T lymphocytes at 4 × 10^6 cells per ml were added to adherent cells. In the figures are shown representative data obtained from at least two animals, and experiments were repeated 3 to 10 times.

Cell characterization. Adherent cells were identified as esterase positive with alpha-naphthyl acetate esterase (32). T lymphocytes bearing D-galactose residues were identified with fluorescein isothiocyanate-labeled peanut agglutinin (E-Y Labs, San Mateo, Calif.), and B lymphocytes were identified with tetramethylrhodamine isothiocyanate-labeled rabbit anti-bovine immunoglobulin with light chain activity as previously described (39).

Monoclonal antibodies B29 and B26 with bovine pan-T-cell specificity were the gifts of W. C. Davis (8, 20). These monoclonal antibodies were added neat in 0.25 ml to 10^6 cells incubated for 45 min on ice with 0.02% NaN_3 and washed three times. Fluoresceinated rabbit anti-murine antibody was added to the cells and incubated for 45 min on ice with NaN_3 followed by three washes prior to examination with a fluorescent microscope. The monoclonal antibody H4 (immunoglobulin G2b [IgG2b]) with specificity for a structural MHC class II framework determinant of human leukocyte antigen-DR (HLA-DR) and cross-reactive with bovine MHC class II antigens (19) was purchased from One Lambda (Los Angeles, Calif.). The monoclonal antibody C5B6 (IgG1) has specificity to bovine monocytes and was produced in our laboratory, and DAS 2 (IgG1) and 9 (IgG1) are monoclonal anti-bovine IgG2 and anti-bovine light chain antibodies, respectively, and were the gift of D. Goldsby (37, 38).

IL-2 assay. Long-term cultures of bovine IL-2-dependent T lymphocytes were derived from a mixed lymphocyte culture as described previously (24) and maintained in 25% (vol/vol) IL-2-containing medium obtained from the cell line MLA-144. Cells were washed in phosphate-buffered saline and suspended in fresh medium, and 10^4 cells per well was added to 96-well round-bottom microtiter plates with dilutions of test supernatants. Plates were incubated for 40 h, pulsed with 1 μCi of [^{3}H]thymidine per well, and harvested 6 h later.

RESULTS

Response to *B. abortus* by bovine lymphocytes. To determine whether *B. abortus* 1119 was stimulatory to sensitized bovine lymphocytes, a dose-response and kinetics profile of adult unfractionated PBM cells was done. Cellular proliferation increased from days 3 to 12 (Fig. 1). The concentration of acetone-killed organisms required to activate lymphocytes varied from 25 to 1,250 μg/ml, with maximal responses occurring between a narrow range (250 to 500 μg/ml). Cells cultured at 6, 9, or 12 days had a biphasic response to the organisms. Nonvaccinated animals failed to respond to *B. abortus* at the concentrations tested (data not shown).

Experiments were performed to examine whether adherent cells could present *B. abortus* 1119 to lymphocytes. Adherent cells were pulsed with selected concentrations of acetone-killed *B. abortus*, and then enriched T cells were added. Figure 2 shows different combinations of adherent

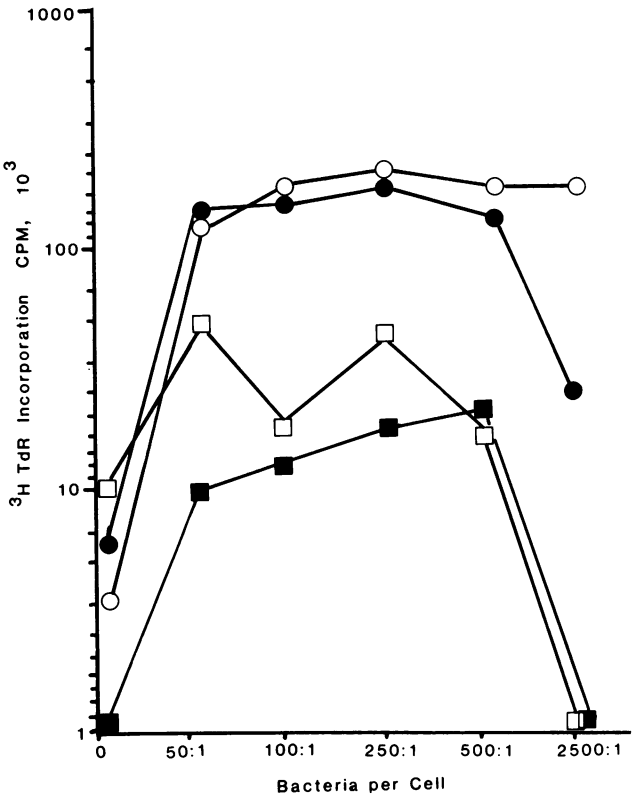

FIG. 2. Response of nonadherent lymphocytes to selected numbers of acetone-killed *B. abortus* presented by adherent cells. The cell combinations were as follows: 4×10^5 nonadherent Sephadex G10-passaged cells alone (■), 2×10^5 adherent cells and 8×10^5 nonadherent Sephadex G10-passaged cells (○), 10^6 adherent cells and 4×10^5 nonadherent Sephadex G10-passaged cells (●), and unfractionated cells (□). ^{3}H TdR, [^{3}H]thymidine.

and nonadherent cells and the response to various numbers of bacteria following 9 days of culture. A broad plateau of response was observed between 50 and 500 bacteria per cell.

Separated cell populations were characterized by esterase staining for macrophages, peanut agglutinin and monoclonal antibodies B29 and B26 for T-lymphocytes, and fluorescent-labeled rabbit anti-bovine immunoglobulin for B-cell identification. Purity and cell composition of the fractionated populations are shown in Table 1. Adherent cells were highly enriched for esterase-positive monocytes, while nonadherent cells panned on anti-immunoglobulin-coated plates were enriched for T lymphocytes.

Method of antigen presentation of *B. abortus* by adherent cells. Although the data shown in Fig. 2 indicate that adherent cells present *B. abortus* to nonadherent cells, the mechanisms for antigen presentation have not been examined. To determine whether presentation by adherent cells involves active bacterial processing or merely passive adherence to antigen-presenting cells, trypsinization was used to remove passively adhered bacteria from the cell surface. The data in Fig. 3A suggest that presentation of bacteria by adherent cells is required for the response of Sephadex G10 nonadherent cells, as well as cells further enriched by passage over anti-immunoglobulin-coated dishes. Adherent or nonadherent cells alone failed to respond. Adherent cells pulsed with bacteria and subsequently trypsinized (Fig. 3B) could likewise present bacterial antigens to Sephadex G10 nonadherent cells and enriched T lymphocytes for a response comparable to that of the nontrypsinized system (Fig. 3A). Removal of whole bacteria from the surface of adherent cells by trypsin was confirmed by scanning electron microscopy (data not shown).

Results of previous work in our laboratory indicate that adherent cells pulsed with high doses (25 μg/ml) of ConA failed to initiate T-lymphocyte proliferation because of suppressor cell induction. This system might represent a model for the induction of T-lymphocyte suppression (34). Because macrophages could internalize and present *B. abortus* to T lymphocytes, the ability of macrophages to present ConA to T lymphocytes was determined. Plastic adherent cells cultured with 25 μg of ConA per ml overnight were washed with α-methyl-D-mannoside at a concentration of 20 mg/ml in phosphate-buffered saline and trypsinized or nontrypsinized. Nonadherent cells were added to the ConA-pulsed adherent cells and cultured for 8 days. As shown above, nonadherent lymphocytes obtained by Sephadex G10 selection or Sephadex G10 selection followed by nonadherence to anti-immunoglobulin-coated plates responded to adherent cells presenting *B. abortus* (Fig. 4). In contrast, nonadherent lymphocytes usually failed to respond to ConA when presented by adherent cells. This decrease in T-lymphocyte response to ConA was not due to an inability of ConA to bind cells because in the presence of adherent cells ConA induces suppressive T cells (30).

Animal variation in nonadherent lymphocyte response to *B. abortus* or ConA presentation by adherent cells. Because cattle represent an outbred population, we were concerned that the observations should be reproducible in a number of animals. Figure 5 examines the response of eight animals. Although variation occurred among animals, nonadherent lymphocytes responded to adherent cells presenting *B. abortus* in seven of the eight animals, with animal 3 being the exception. Similarly, the ConA response was below control levels in five of the eight animals. In only one animal (4) was the ConA response greater than the *B. abortus* response.

TABLE 1. Percentage of cell types in adherent and nonadherent populations

Cell population	Percent positive[a]				
	Esterase	PNA	SIg	B29	B26
Nonadherent pre-G10	15 (6–24)	99	ND[b]	ND	ND
Nonadherent post-G10	0.2 (0–1.1)	98	9 (6–14)	87 (80–94)	75 (70–81)
Anti-immunoglobulin panned	0	95 (86–100)	5 (0–13)	84 (82–86)	75 (71–79)
Plastic adherent	98 (97–98)	ND	ND	ND	ND
Unfractionated	19 (18–20)	63	20 (17–25)	70 (66–73)	58 (44–71)

[a] Abbreviations: PNA, Peanut agglutinin; SIg, surface immunoglobulin; B29 and B26, monoclonal antibodies with pan-T-lymphocyte specificity. Values in parentheses represent the range.
[b] ND, Not determined.

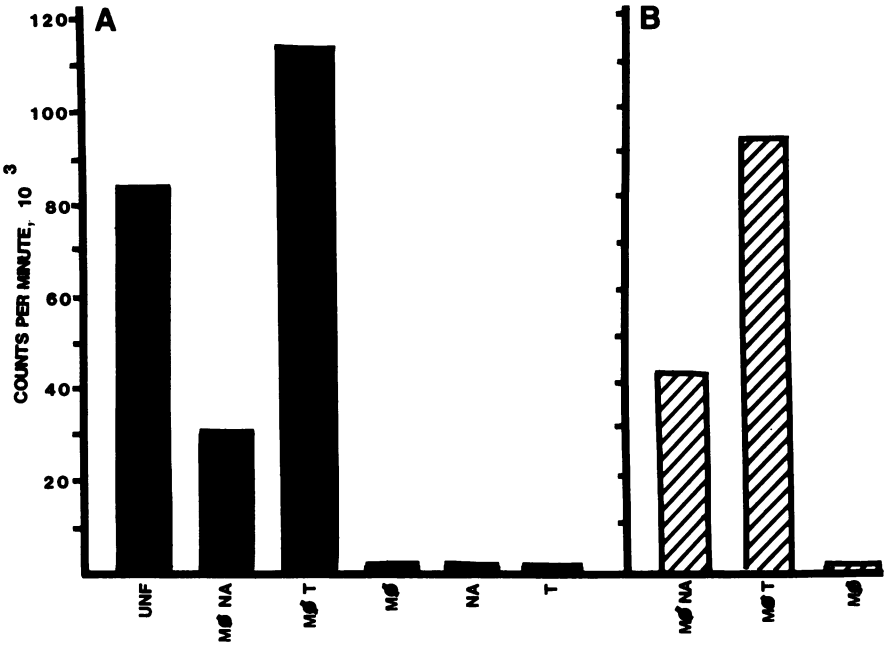

FIG. 3. Requirement of adherent cells for the response of T lymphocytes to *B. abortus*. Combinations of macrophages, Sephadex G10-passaged nonadherent cells, Sephadex G10- and anti-immunoglobulin-passaged cells, or unfractionated cells were cultured with *B. abortus*. (A) Nontrypsinized adherent cells cultured overnight with *B. abortus*, washed, and cultured for 8 days with T lymphocytes. UNF, Unfractionated; Mϕ NA, macrophage-nonadherent cell; Mϕ T, macrophage-T lymphocyte; Mϕ, macrophage; NA, nonadherent cell; T, T lymphocyte. (B) Adherent cells cultured with *B. abortus* overnight, trypsinized, and cultured for 8 days with T lymphocytes. Abbreviations are as described for panel A.

Therefore, in seven of eight animals adherent cells pulsed with *B. abortus* could present bacterial antigens to nonadherent lymphocytes.

IL-2 production by *B. abortus*-activated T lymphocytes. Supernatants from lymphocytes cultured with *B. abortus* 1119-pulsed macrophages that were trypsinized or nontrypsinized were collected and assayed on bovine IL-2-dependent T lymphocytes. Figure 6 illustrates that IL-2 was produced in both trypsinized and nontrypsinized systems. However, in cultures in which macrophages were trypsinized, IL-2 production was delayed by 24 h.

MHC class II involvement in T-cell proliferation. Antigen presentation by macrophages involves associative recognition of foreign antigen with MHC class II molecules by T lymphocytes of mice responding to *L. monocytogenes* (10) and T lymphocytes of humans responding to *Yersinia* spp.

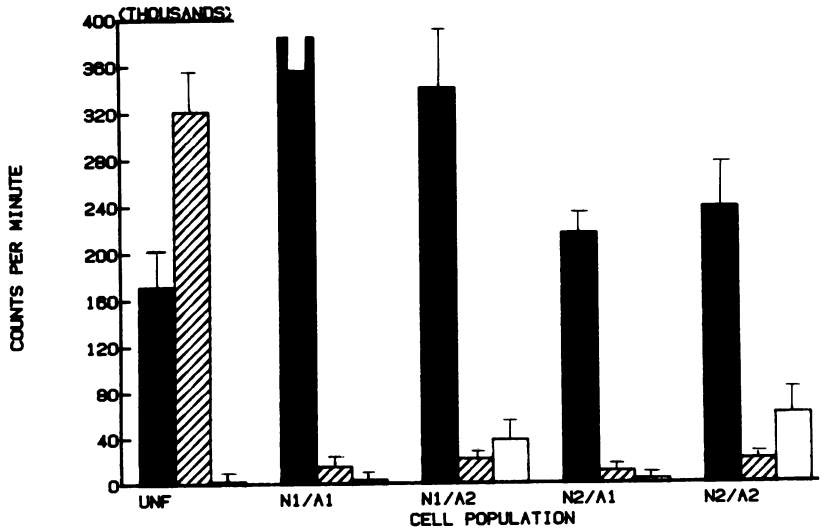

FIG. 4. Comparison of adherent cells presenting *B. abortus* and ConA. *B. abortus*, 100 μg/ml (■); ConA, 25 μg/ml (▨); or control medium (□) were cultured with adherent cells overnight. Adherent cells were nontrypsinized (A1) or trypsinized (A2) and cultured for 8 days with T lymphocytes enriched by Sephadex G10 selection (N1) or Sephadex G10 selection followed by nonadherence to anti-immunoglobulin-coated plates (N2). UNF, Unfractionated.

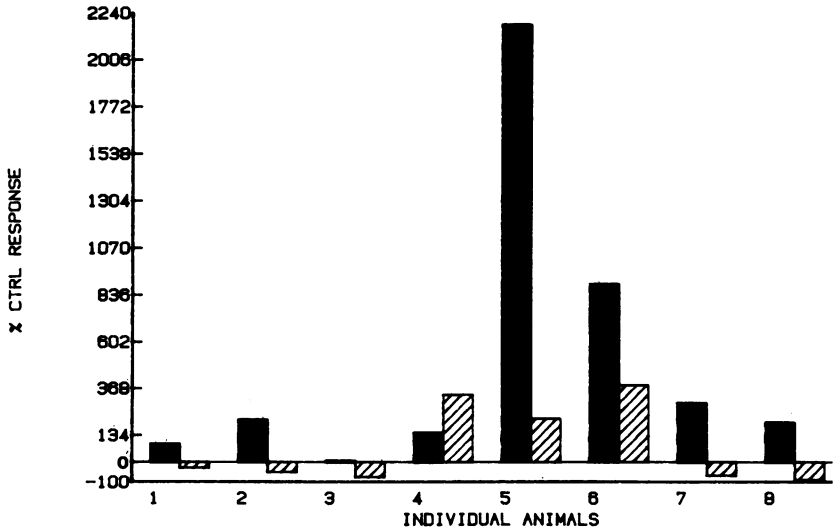

FIG. 5. Response of T lymphocytes from eight animals to adherent cells presenting *B. abortus* (■) or ConA (▨). Percent control (CTRL) response was calculated as follows: (T-lymphocyte response in cultures containing adherent cells presenting antigen or mitogen/the T-lymphocyte response in cultures containing adherent cells presenting medium only) × 100.

(40) and *Escherichia coli* (9, 23). The role of MHC class II molecules in antigen presentation by bovine macrophages of *B. abortus* was evaluated. Macrophages pulsed with *B. abortus* were treated with the monoclonal antibody H4 or C5B6 and complement or complement only (Fig. 7A). Treatment with the monoclonal antibody H4 and complement significantly ($P < 0.05$) reduced the proliferation of T lymphocytes. In contrast, trypsinization of macrophages prior to monoclonal antibody H4 and complement treatment failed to alter the subsequent T-lymphocyte response (Fig. 7B). Treatment with the monoclonal antibody C5B6 and complement or complement alone failed to alter the T-lymphocyte response.

When the anti-MHC class II monoclonal antibody H4 was present throughout the 8-day culture period (Fig. 8), a significant reduction in proliferation was observed with unfractionated PBM cells or T lymphocytes enriched by two methodologies.

DISCUSSION

Numerous workers have contended that a facultative relationship is advantageous for intracellular bacteria, such as *B. abortus*, to evade the immune system (6, 7, 33). In the experiments reported here, the mechanisms of collaboration between bovine macrophages and T lymphocytes in response to killed *B. abortus* 1119 have been investigated. Results of our experiments established that (i) unfractionated lymphocytes respond to *B. abortus*; (ii) adherent cells pulsed with *B. abortus* stimulated T-lymphocyte proliferation, and removal of surface-bound bacteria by trypsiniza-

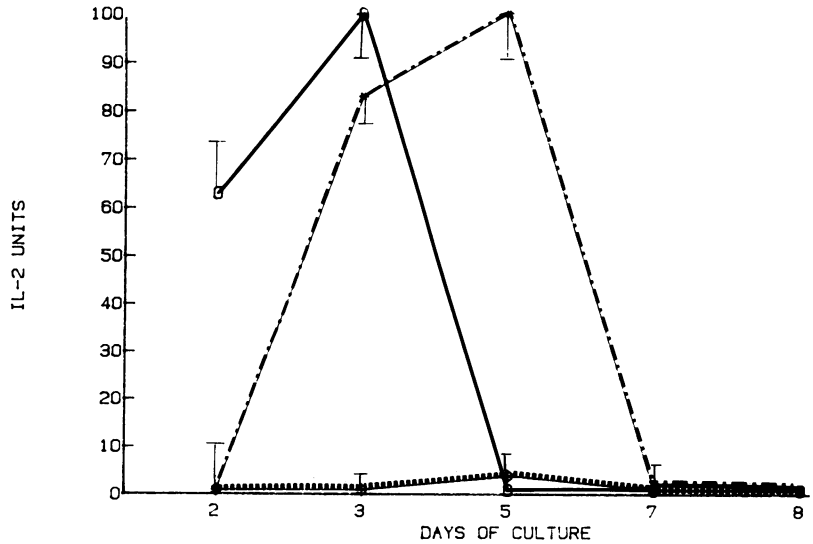

FIG. 6. IL-2 synthesis by T lymphocytes cultured with *B. abortus*-presenting adherent cells. Nontrypsinized *B. abortus*-presenting adherent cells and T lymphocytes (□——), trypsinized *B. abortus* presenting adherent cells and T lymphocytes (★-----), or adherent cells only and T lymphocytes (S. . .). Supernatants were collected on the indicated days of culture and assayed on an IL-2 dependent bovine cell line. Bars represent standard deviations.

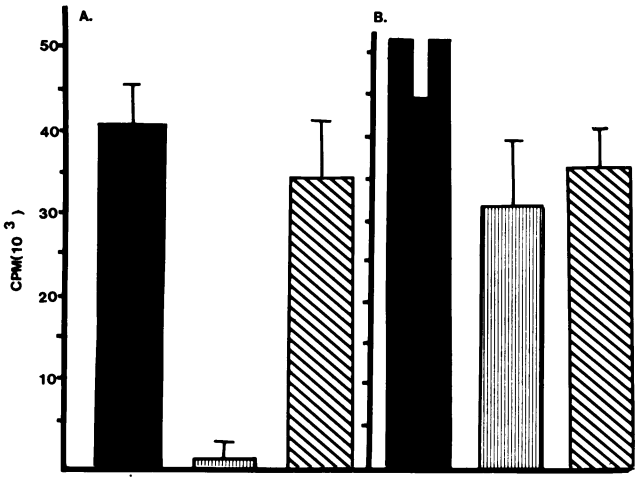

FIG. 7. Effect of anti-MHC class II monoclonal antibody (H4) and complement on *B. abortus*-presenting macrophages (A) or trypsinized *B. abortus*-presenting macrophages (B). *B. abortus*-presenting macrophages were incubated for 1 h with complement (■), H4 and complement (▥), or C5B6 and complement (▧) and then washed, followed by the addition of T lymphoctyes. Bars represent standard deviations.

tion of adherent cells did not alter the T-lymphocyte response; (iii) T lymphocytes proliferated to *B. abortus*-pulsed adherent cells but not to ConA- (25 μg/ml) pulsed adherent cells; (iv) IL-2 was produced by T-lymphocyte cultures in response to either trypsinized or nontrypsinized *B. abortus*-pulsed adherent cells; and (v) MHC class II products were required for recognition or response by T lymphocytes because a monoclonal antibody against MHC class II products could prevent the T-lymphocyte response.

Few studies have examined bovine lymphocyte responses to *B. abortus* (2, 36). Results of the present study indicate a biphasic response at various bacterial concentrations and times. Whether the biphasic response represents different cell populations, the effects of different bacterial antigens, or the response of the same cell population influenced by maturation and growth factors requires further investigation. Development of monoclonal antibodies to bovine lymphocyte subpopulations and isolated bacterial components may clarify this observation. Excess antigen in culture had a suppressive effect; this is an observation of other investigators, who used mouse lymphocytes cultured with *E. coli* (23). Suppression could result from suppressor T cells (27, 28) or, more likely, from the toxic effects of the bacterial lipopolysaccharide. Other workers have reported that excess free antigen (2,4-dinitrophenol-keyhole limpet hemocyanin) in vitro suppressed the T-lymphocyte response to macrophage-bound antigen (15).

Interestingly, trypsinizaton of adherent antigen-presenting cells did not prevent T lymphocytes from responding. This suggests that adherent cells phagocytized sufficient numbers of *B. abortus* and that bacterial antigens were later expressed on the cell surface after removal of surface-bound bacteria by trypsin. Similar results have been reported recently by others with *L. monocytogenes* (1). In contrast, macrophages pulsed with ConA failed to present this lectin in a stimulatory manner to T lymphocytes. The difference in the results of these two systems could be explained by lectin-induced suppressive cells as observed in bovine (34) and human (30) lymphocytes that turn off de novo production of IL-2, as described previously (12). Macrophages pulsed with a known stimulatory concentration of phytohemagglutinin (10 μg/ml) activate guinea pig T lymphocytes (31); therefore, lectins bound to adherent cells can lead to activation or suppression of T-lymphocyte proliferation. It would be of interest with *B. abortus* to determine whether

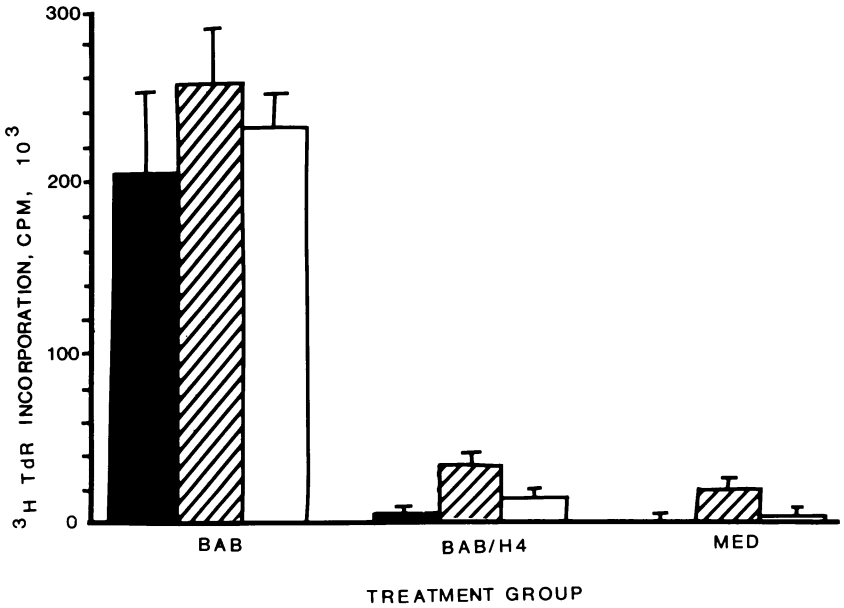

FIG. 8. Effect of anti-MHC class II monoclonal antibody (H4) present continuously in culture. Adherent cells were pulsed with *B. abortus* (BAB) or medium only (MED) and cultured with unfractionated PBM cells (■); T lymphocytes enriched by Sephadex G10 selection (▨); or Sephadex G10 selection followed by monoclonal antibodies H4, DAS2 and DAS9, and complement (□). Monoclonal antibody H4 was added to selected cultures (BAB/H4) for 8 days. 220204, a monoclonal antibody of the same isotype with specificity for bovine herpesvirus failed to inhibit the response (data not shown). Bars represent standard deviations. ^{3}H TdR, [^{3}H]thymidine.

the suppressive effect observed with greater than 1,250 μg of bacteria per ml was due to a similar mechanism.

IL-2 and gamma interferon from antigen-activated T lymphocytes serve to regulate the functions of other T lymphocytes and macrophages, respectively. Evidence for IL-2 production in this study substantiates the role of T lymphocytes in this system. Cultures containing trypsinized adherent cells had a delay in IL-2 synthesis, suggesting the removal of MHC class II antigens, bacterial antigens, or both and the subsequent reexpression of these components. The requirement for the association of bacterial antigens with MHC class II products probably explains this delay, with cell surface expresson of both molecules being necessary.

In addition to lymphokines, the requirement for cell surface MHC class II products on antigen-presenting cells is fundamental for T-lymphocyte responses to foreign antigens. Bovine T-lymphocyte proliferation was inhibited nearly 100% when a monoclonal antibody specific for an MHC class II framework determinant and complement was used to treat the adherent cell population prior to antigen processing, indicating the requirement for viable macrophages in antigen processing. In contrast, if adherent cells were trypsinized just subsequent to treatment with the monoclonal antibody, a competent T-lymphoctye response ensued. This suggests that the cell surface determinant to which the monoclonal antibody binds was removed by trypsinization. If the monoclonal antibody was present continuously in the culture in the absence of complement, T-lymphocyte responses were inhibited (Fig. 8), indicating the recognition requirement of MHC class II products by T lymphocytes. The requirement of MHC class II products and bacterial antigens denotes that *B. abortus* does not function in a manner analogous to that of a mitogen for T lymphocytes.

The interrelationship between T lymphocytes and macrophages has developed as a protective mechanism for the control of intracellular pathogens like *B. abortus*. Phagocytosis of *B. abortus* is not sufficient to prevent infection (7). Results of the present experiments substantiate that T lymphocytes recognize *B. abortus* on antigen-presenting cells of cattle. However, host mechanisms that contribute to elimination or persistence of sequestered *B. abortus* remain to be fully identified. Characterization of T-lymphocyte populations in cattle and the assessment of soluble mediators, in addition to IL-2, may contribute to the understanding of cellular immune responses to facultative intracellular bacteria resident in macrophages.

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