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Effect on Intraerythrocytic *Anaplasma marginale* of Soluble Factors from Infected Calf Blood Mononuclear Cells

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Blood mononuclear cells (lymphocytes and monocytes) were isolated from infected calves during in vivo control of acute anaplasmosis and cultured with *Anaplasma marginale* organisms. Supernatants from the cultures reduced the proportion of erythrocytes containing viable *A. marginale* in vitro, indicating that an antibody-independent mechanism of rickettsemia control might occur during acute anaplasmosis.

Anaplasmosis is a hemoparasitic disease of cattle that is caused by *Anaplasma marginale* (15), a rickettsial agent that has been placed in the family *Anaplasmataceae* (18). *A. marginale* infects mature erythrocytes, resulting in severe hemolytic anemia that can lead to death (10). Acute rickettsemia is characterized by the presence of parasitized erythrocytes detectable 2 to 3 weeks after infection. The percentage of parasitized erythrocytes peaks at approximately 4 to 5 weeks postinfection before declining to microscopically undetectable levels in surviving cattle. The mechanisms causing reduction in rickettsemia are unknown.

The involvement of antibody in immunity to *A. marginale* has been investigated. High titers of antibody to several of the major surface proteins (MSP) of *A. marginale* develop during infection, and a correlation between antibody titers and protection has been demonstrated (14, 16). However, passive transfer of serum antibody did not protect calves from challenge infection (6), indicating that other mechanisms must be involved as well.

The role and mechanisms of lymphocyte-mediated immunity in anaplasmosis are undefined. Cell-mediated cytotoxicity and leukocyte migration inhibition in *A. marginale*-infected cattle have been demonstrated, and cutaneous hypersensitivity has been correlated with immunity in vaccinated cattle (1, 2). However, the lymphocyte subpopulations mediating protective T-lymphocyte responses and the mechanism of disease control associated with these responses have not been delineated. This study was undertaken to determine whether evidence could be obtained that would suggest that lymphocyte-mediated control of rickettsemia occurs during acute anaplasmosis. The time points examined included those for peak rickettsemia (31 days postinfection) and following control of rickettsemia (45 days postinfection), when few parasitized erythrocytes were detectable by microscopic examination. The data indicate that by 45 days after infection, but not at peak rickettsemia, peripheral blood mononuclear cells can mediate in vitro reduction of viable intraerythrocytic *A. marginale* through elaboration of soluble factors.

Five male Holstein calves from an *A. marginale*-free herd were each inoculated intramuscularly with 875 freshly collected erythrocytes infected with *A. marginale* Florida. Rickettsemia was monitored daily by microscopic examination of Giemsa-stained blood smears, and anemia was monitored daily by determination of packed cell volume as previously described (14). Peak rickettsemia (mean ± 1 standard deviation [SD]) occurred at 31.8 ± 1.3 days postinoculation, at which point the percentage of parasitized erythrocytes was 6.3% ± 1.8% for the four calves (calves 2, 3, 4, and 5) that developed microscopically detectable rickettsemias. Rickettsemia was rare by 60 days postinfection. Packed-cell volumes declined significantly, as determined by Student's *t* test, from 37.6% ± 2.3% before infection to 23.6% ± 3.6% ($P < 0.00009$) approximately 35 days postinfection and had returned to 32.2% ± 1.3% ($P < 0.001$) by 60 days postinfection.

Peripheral blood lymphocytes were phenotyped prior to inoculation of the calves with *A. marginale* and at 14, 31, and 45 days postinoculation. Monoclonal antibodies to the major bovine lymphocyte subpopulation differentiation molecules CD4 (CACT83B; immunoglobulin G1 [IgG1]), CD8 (CACT80C; IgG1), WC1 (BAQ4A; IgG1, expressed on γδ T cells), and surface IgM (BIg73A; IgG1) (3, 8, 9) were used with flow cytometry as previously described (20) to quantitate the lymphocyte subpopulations. In Fig. 1 the means ± 1 SD for the number of lymphocytes in each subpopulation for the four calves that developed microscopically detectable rickettsemias are presented. A statistically significant decrease in the $WC1^+$ subpopulation ($P < 0.002$) occurred by 31 days postinfection. At 45 days postinfection, this subpopulation remained significantly decreased compared with preinfection levels ($P < 0.03$). These data indicate that changes in lymphocyte subpopulations occur during acute infection.

Sera were collected from the five calves prior to inoculation and at 34, 41, and 48 days postinoculation. They were evaluated by competition inhibition enzyme-linked immunosorbent assay (ELISA) as previously described (13) for reactivity with recombinant MSP-5. MSP-5 is a surface protein of *A. marginale* that is recognized by sera from all persistently infected cattle (11). Competition inhibition ELISA results were analyzed by comparing the average of two optical density (OD) readings per test serum with the means and SDs for five known positive and five known negative sera. A test serum was considered positive if the average of the OD readings was 3 SDs below the mean OD of the known negative samples. Sera from the four calves that developed detectable rickettsemias contained anti-MSP-5 antibody at multiple time points (Table 1), indicating that these calves had become persistently infected with the organism. The results were confirmed by Western blot (immunoblot) analyses using whole organisms as antigens (data not shown).

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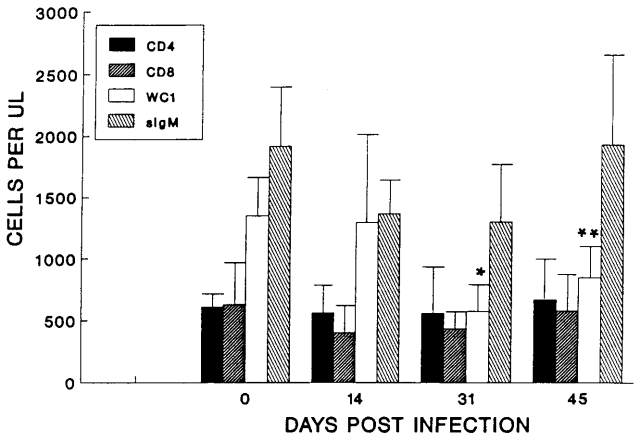

FIG. 1. Comparison of peripheral blood lymphocytes in each major subpopulation during acute *A. marginale* infection for calves which developed detectable rickettsemias. Bars represent mean numbers of lymphocytes per microliter of blood for each subpopulation at 0, 14, 31, and 45 days postinfection. Error bars represent 1 SD ($n = 4$). Significance is designated with one star ($P < 0.002$) or two stars ($P < 0.03$).

Peripheral blood lymphoid cells were collected from the calves at 31 and 45 days postinoculation by centrifugation of blood on a solution of 1.086 g of Ficoll-Hypaque per ml (20) and were cultured with or without sonicated *A. marginale* organisms prepared from infected erythrocytes frozen at 44% rickettsemia. The sonicated organisms were suspended in 3 ml of dimethyl sulfoxide per 40 ml of starting erythrocyte suspension and were diluted 1:4 into Dulbecco's modified Eagle's medium containing antibiotics and 13% calf serum (DME). The diluted suspension was used at 5 μl/2 × 10^6 lymphocytes. After incubation for 5 days at 37°C in 5% CO_2, the cultures were centrifuged to remove the cells, and the supernatants were collected and frozen at −20°C.

To assay for the effect of supernatants on in vitro parasite viability, the supernatants were thawed and serially diluted in RPMI 1640 containing 10% fetal bovine serum (RPMI-FBS), and 100 μl of each dilution was added to the wells of a flat-bottom 96-well culture plate. Washed, infected erythrocytes (15 to 30% rickettsemia) at a final concentration of 2.5% erythrocytes in RPMI-FBS were added at 100 μl per well. Medium controls of serially diluted DME were included, as were positive controls of infected erythrocytes in RPMI-FBS and negative controls (uninfected erythrocytes). After incubation at 37°C and 5% CO_2 for 3 days, the erythrocytes were washed in phosphate-buffered saline and incubated in a 50-μg/ml final concentration of the vital dye hydroethidine for 20 min at 37°C. Hydroethidine is converted into ethidium within cells only in the presence of NADP-dependent dehydrogenase activity (7). The ethidium is intercalated into nucleic acids, producing the characteristic red fluorescence detectable by flow cytometry. We have previously demonstrated that only viable hemoparasites convert hydroethidine into ethidium (19). A minimum of 10,000 erythrocytes per sample was analyzed by flow cytometry to determine the viability of intraerythrocytic *A. marginale*. After background fluorescence in negative control cells (less than 3%) was subtracted, the percent reduction in infected erythrocytes (termed "% killing" in Fig. 2) was calculated by subtracting the percentage of fluorescent erythrocytes from supernatant-treated samples from the percentage of fluorescent erythrocytes from the medium control and then dividing by the percentage of fluorescent erythrocytes from the medium control and multiplying by 100. *A. marginale* viability in the medium controls was equal to that in the positive controls, indicating that the supernatant medium was not toxic to the rickettsiae.

TABLE 1. Presence of antibody to MSP-5 in sera of calves inoculated with *A. marginale*

Calf no.	Detectable rickettsemia	MSP-5 antibody[a] at:		
		34 DPI[b]	41 DPI	48 DPI
1	No	Negative	Negative	Negative
2	Yes	Positive	Positive	ND[c]
3	Yes	Positive	Positive	Positive
4	Yes	Positive	Positive	Positive
5	Yes	Positive	Positive	Positive

[a] Scored positive when OD was ≥3 SDs below the mean OD of the known negative samples.
[b] DPI, days postinoculation.
[c] ND, not determined.

There was no reduction in *A. marginale* viability in erythrocytes reacted with supernatants after culture with lymphocytes collected at 31 days postinoculation (data not shown). At 45 days postinoculation, however, all five calves had blood mononuclear cells which produced soluble factors capable of reducing rickettsemia in a concentration-dependent pattern, especially when stimulated with the organisms (Fig. 2). Blood mononuclear cells taken from an uninfected control animal did not produce such factors. There was no apparent relationship between lymphocyte phenotypes for any of the calves at culture initiation and the percent reduction in viability observed. Examination of the culture supernatants by using the Griess reaction to detect nitrites (4), the stable byproducts of the respiratory burst which would implicate macrophages (12), failed to detect these products (data not shown). Thus, the soluble factors are made by blood mononuclear cells; however, the specific cell type(s) that produces these factors remains unidentified.

Calf 1 differed from calves 2 to 5. Although it was inoculated with *A. marginale*, calf 1 did not develop detectable rickettsemia and did not become persistently infected, suggesting early control followed by termination of infection. Knowles et al. recently showed that of 35 *A. marginale*-inoculated calves, one did not develop detectable rickettsemia and did not become persistently infected, while a second calf did develop detectable rickettsemia but did not become persistently infected (11). If a small proportion of calves inoculated with *A. marginale* can terminate infection, this fact would suggest that heterogeneity in the immune response to the parasite exists. Mononuclear cells of calf 1 had rickettsicidal activity at 45 days postinoculation but not at 31 days postinoculation, like those of the other calves; however, the activity could not be enhanced by in vitro incubation with *A. marginale*. This lack of inducibility could indicate that the rickettsicidal activity was the result of vigorous innate immunity or of an ongoing in vivo immune response.

T lymphocytes have been implicated in protection in hemoprotozoal diseases. In mouse malaria models, gamma interferon produced by $CD4^+$ T lymphocytes early in acute infection mediates killing of intraerythrocytic protozoa in vitro through activation of macrophages (17). In human malaria, both $CD4^+$ and $CD8^+$ T lymphocytes, in the presence of autologous monocytes, can inhibit growth of intraerythrocytic protozoa in vitro, via soluble factors (5). Our data are consistent with a change in immune responsiveness over time in calves with acute anaplasmosis. In particular, we demonstrate the presence of soluble factors made in vitro by lymphoid cells

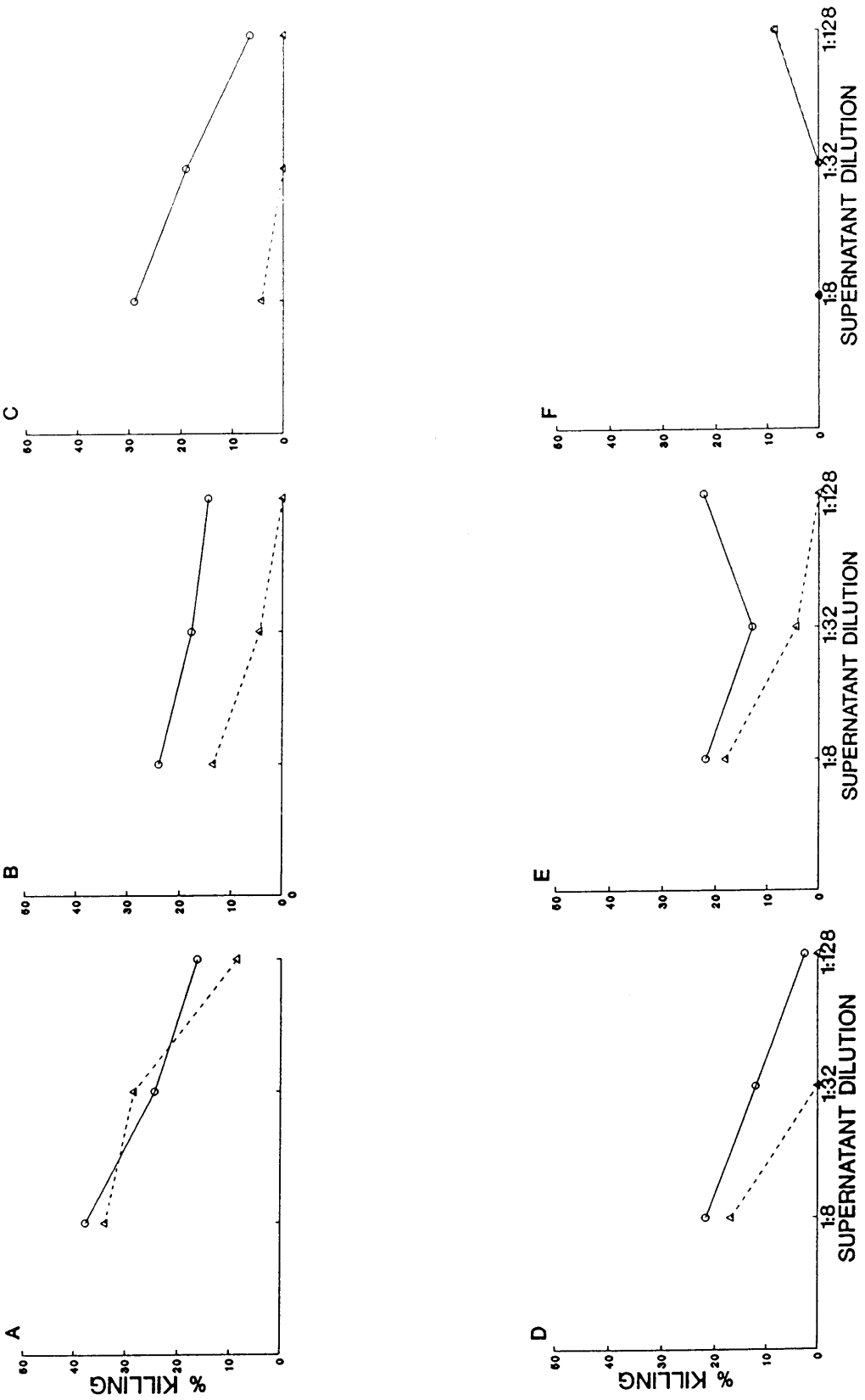

FIG. 2. Reduction of viability of intraerythrocytic rickettsiae by supernatants from cultured lymphoid cells collected 45 days postinfection. (A through E) Percent killing of serially diluted supernatants from stimulated (solid line) or unstimulated (dotted line) cells of infected calves 1 through 5, respectively. (F) Percent killing of supernatants from lymphoid cells from an uninfected animal.

collected during the control phase of acute disease that reduce the proportion of erythrocytes containing viable *A. marginale*. This observation suggests the presence of a mechanism independent of antibody that might be protective in vivo. If this activity is important in vivo, identification of both the cells and the factor(s) that mediate this activity might lead to the development of new strategies to protect calves against anaplasmosis.

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