JOURNAL OF CLINICAL MICROBIOLOGY, May 1991, p. 997–1003
0095-1137/91/050997-07$02.00/0

Vol. 29, No. 5

In Vitro Inhibition of Multiplication of *Babesia bigemina* by Using Monoclonal Antibodies

JULIO V. FIGUEROA AND GERALD M. BUENING*

Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211

Received 24 July 1990/Accepted 18 February 1991

A panel of 21 monoclonal antibodies (MAb) against the erythrocytic stages of *Babesia bigemina* was produced. The 21 MAb could be divided into four distinct immunofluorescence groups on the basis of their patterns of binding to *B. bigemina*-infected erythrocytes. Nine MAb positive in the indirect immunofluorescent antibody test with acetone-fixed *B. bigemina*-infected erythrocytes were selected for purification and further characterization. The MAb were species specific, since they did not cross-react with *B. bovis*, but they were not stage specific. The purpose of this study was to evaluate the biological activity of *B. bigemina*-specific MAb in vitro. Three MAb inhibited the multiplication of *B. bigemina* in vitro as assessed by a lowered percentage of parasitized erythrocytes and by a decreased incorporation of [^{3}H]hypoxanthine. These MAb are now being utilized to purify the correspondent parasite polypeptides, either native or recombinant, in order to test them as subunit immunogens.

Babesiosis, a tick-borne disease caused by the erythrocytic phase of protozoan parasites of the genus *Babesia*, prevails in the tropical and subtropical regions of the world in areas where the arthropod vector is found (27). Bovine babesiosis caused by *Babesia bovis* and *Babesia bigemina* is the most important economically, particularly to livestock industries of developing countries (19). Limited use of existing vaccines, primarily due to the inherent constraint of maintaining viability of such vaccines, has prompted the development of nonviable immunogens. Fractions of lysed bovine infected erythrocytes (13), soluble antigens derived from in vitro culture (19), or subunit components of the parasite purified by affinity adsorption (26, 28) are possible sources of such immunogens. Previous work has been aimed at the detection of protective antigens of *B. bovis*, with little characterization of *B. bigemina* antigens as potential immunogens (14).

Monoclonal antibodies (MAb) with defined specificity and having functional inhibitory activity towards parasite development could be used to purify the antigens recognized by those antibodies which might be promising vaccine candidates (3, 7). The present study describes the biological activity of a panel of MAb specific for *B. bigemina* antigens in an in vitro inhibition test.

MATERIALS AND METHODS

Parasites. An original *B. bigemina* Mexican isolate adapted to in vitro culture (23) was used as the source of antigenic material for MAb production. Culture conditions for in vitro parasite multiplication and infected erythrocyte (iRBC) concentration have been previously described (23, 24). In addition, six other *B. bigemina* isolates (6) were adapted to in vitro culture under the same conditions (23). A Mexican isolate of *B. bovis* that has been maintained in culture at this laboratory for several years was used for antigen preparation for the cross-reaction experiment (15).

Production of MAb. BALB/c and RBF/Dn mice were immunized intraperitoneally twice, 4 weeks apart, with 10^8 concentrated *B. bigemina* iRBCs diluted in 0.5 ml of Vega y Martinez solution (24). A final intravenous boosting dose of parasite antigen (~10^7 iRBCs in 100 μl of Vega y Martinez solution) was given a week later, and spleens were removed for cell fusion. Cell fusions were performed essentially as described previously (5, 21, 22). Hybridoma culture supernatant was screened for antibabesial activity by indirect immunofluorescent antibody test (IFAT) on *B. bigemina* Seed iRBC smears. Hybridomas were selected and cloned essentially as described before (21, 22). Class and subclass of immunoglobulins secreted by hybridomas in the culture supernatant were determined with a mouse monoclonal subisotyping kit (Hyclone Laboratories, Logan, Utah) as suggested by the supplier.

IFAT. Immunofluorescence assays were done with air-dried thin films of concentrated *B. bigemina* (24) or *B. bovis* (20) iRBCs. Acetone-fixed slides were reacted for 30 min at 37°C with either undiluted culture supernatant of hybridomas or positive or negative mouse serum included as controls. Fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G (IgG) (Sigma, St. Louis, Mo.) at a 1:60 dilution in phosphate-buffered saline (PBS), pH 7.2, was added to the slides and incubated for 30 min at 37°C. Slides were washed three times for 5 min each with PBS after each reaction. The slides were then mounted in 90% (vol/vol) glycerol in PBS, pH 8.0, containing 1% (wt/vol) *p*-phenylenediamine (12), and examined with an UV epifluorescence microscope. IFAT of smears of the several isolates of *B. bigemina* was done to determine reactivity of the MAb. *B. bovis* iRBC and bovine normal RBC smears were included to test for cross-reactivity and specificity of the MAb.

Effect of MAb on *B. bigemina* multiplication in vitro. (i) Experiment 1. Cells from a selected panel of cloned hybridomas secreting antibodies to *B. bigemina*, one hybridoma producing MAb against *B. bovis* (unpublished data), as well as SP2/0 myeloma cells were expanded in culture. Approximately 10^6 cells were injected into BALB/c mice previously primed with pristane. Ten to fourteen days later, ascitic fluids were collected from the peritoneal cavities of the mice and clarified by centrifugation and then filter sterilized.

iRBCs from *B. bigemina* cultures (~3% parasitemia at 48

* Corresponding author.

h) were diluted with normal bovine RBCs to a percentage of parasitized erythrocytes (PPE) of 0.5. The packed-cell volume was adjusted to 20% with complete culture medium (23), and 0.5-ml aliquots were placed in each of the central wells of a 24-well culture plate. The various ascitic fluids were diluted to 40 or 20% (vol/vol) in complete culture medium, and 0.5 ml of each dilution was added and mixed with the previously plated *B. bigemina* cultures. Each ascitic fluid dilution was tested in four culture wells. *B. bigemina* culture plates were then incubated under standard in vitro culture conditions (23). Thus, each well contained 1 ml of culture (four replicates) 10% packed-cell volume, 0.5% *B. bigemina*-infected erythrocytes, and 10 or 20% final concentration of the different ascitic fluids at the beginning of the experiment. After 24 h of incubation, spent culture supernatants (0.8 ml) were removed from the culture wells, and then 0.8 ml of fresh complete culture medium containing the different dilutions of the various ascitic fluids was added. To assess parasite in vitro multiplication (or inhibition), a modified procedure of that described earlier (9) was used. In this experiment, each culture received 0.1 ml of RPMI medium which contained [^{3}H]hypoxanthine (10 μCi/ml; specific activity, >1 Ci/mmol; Amersham, Arlington Heights, Ill.), and the culture plates were incubated for an additional 24 h. At the end of the experiment (48 h), 1.5 μl of culture RBCs from each well was removed to prepare blood smears. The plates were then sealed with masking tape and placed at −70°C until [^{3}H]hypoxanthine incorporation was measured. PPE were calculated at 48 h of culture incubation by counting at least 10^3 RBCs in methanol-fixed smears stained with 5% Giemsa in PBS for 45 min. [^{3}H]hypoxanthine incorporation was evaluated by harvesting the cultures after the 48-h incubation period onto microfiber filters using a manifold system (Amicon, Lexington, Mass.) to which vacuum was applied. The filter paper discs were allowed to dry and then deposited into glass vials containing 15 ml of scintillation fluid (Fisher Scientific, Fair Lawn, N.J.) and counted for 2 min in a liquid scintillation analyzer (Packard, Downers Grove, Ill.). Background counts per minute (CPM) were determined by counting [^{3}H]hypoxanthine incorporation in cultures containing bovine normal RBCs exclusively.

(ii) Experiment 2. Experiment 2 was done to confirm the MAb inhibitory activity to *B. bigemina* by using purified immunoglobulins from ascitic fluids. MAb IgG was purified by procedures described previously (1). The purified IgG fractions of five MAb specific to *B. bigemina*, one MAb specific to *B. bovis*, and IgG from myeloma induced ascitic fluid (IgG protein control) were added to *B. bigemina* complete culture medium to a final concentration of 50 μg of protein per ml of culture medium. The culture media containing the various IgG fractions were added with 10% (vol/vol) packed cell volume of cultured *B. bigemina* iRBCs which had been previously adjusted to contain a PPE of 0.5.

Two hundred microliters of the culture suspensions prepared as described above was delivered to each of four wells of a 96-well culture plate and placed under standard in vitro cultivation conditions. Control cultures to which an equivalent volume of physiological saline solution (10% [vol/vol]) had been added were also included. At 24 h, supernatants were removed, and fresh culture medium was supplemented with the IgG fractions indicated above added to the appropriate wells. Also at this time, 20 μl of RPMI medium containing 0.2 μCi of [^{3}H]hypoxanthine was delivered to each well, and the cultures were uniformly resuspended. The culture plates were then incubated for an additional 24 h. At the end of the experiment, samples (1.5 μl) were taken from the bottoms of the wells and RBC smears were done to assess PPE. The remainder of the procedure was similar to that of experiment 1 except that a semiautomated microplate cell harvester was used to collect the cultures onto microfiber filters.

RESULTS

IFAT reactivity of antibabesial MAb. Four different IFAT reactivity patterns were distinguished during the primary screening on the acetone-fixed preparations of *B. bigemina* Seed strain. Antibodies from group I (seven MAb) stained the iRBCs and two bright fluorescing dots at the blunt end of dividing parasites (paired pyriform organisms) (Fig. 1a). Group II (nine MAb) antibodies reacted with both merozoites and trophozoites and uniformly stained the parasite body (Fig. 1b). Antibodies from group III (three MAb) reacted with a localized area at the anterior, blunt end of mature dividing parasites (Fig. 1c). MAb from group IV (two MAb) reacted weakly with what appears to be the surface of the pyriform stages. However, a stronger reaction was seen at the blunt end of the parasite (Fig. 1d). No fluorescence staining was observed with the heterologous *B. bovis* species. Serum from mice immunized with *B. bigemina* iRBCs had polyclonal antibodies which reacted with both bovine normal RBC and *B. bigemina* iRBC (Fig. 1e). The immunofluorescence pattern produced by nine selected MAb was the same for the seven *B. bigemina* isolates tested.

In vitro inhibition test. Addition to cultures of fresh complete culture medium containing the MAb at a 10% concentration showed, as evaluated by PPE, that MAb B1C9A2 and C2F3G3 caused percentages of parasite inhibition of 52 and 62%, respectively (Table 1). Doubling the concentration of ascitic fluids (20%) did not increase the percentages of inhibition of the parasite multiplication (not shown). In fact, by the end of the assay period, cultures exposed to a high concentration (20%) of ascitic fluid from MAb B1C9A2 and C2F3G3 showed only partial inhibition, with percentages of inhibition between 35 and 44%. However, SP2/0 myeloma-induced ascitic fluid or the control MAb anti-*B. bovis* IgG1 never produced inhibition percentages over 10% at either concentration.

Data on incorporation of [^{3}H]hypoxanthine in *B. bigemina* cultures treated with the dilutions of the different ascitic fluids are presented in Table 1. At a final concentration of 10%, all the ascitic fluids containing anti-*B. bigemina* MAb inhibited the incorporation of radiolabel at various degrees. Although SP2/0-induced and anti-*B. bovis* ascitic fluid did not inhibit the incorporation of tritium-labeled hypoxanthine, a substantial decrease in cultures treated with saline solution alone was observed. This decrease resulted in a 21% inhibition. The highest percentages of inhibition were calculated for ascitic fluids containing MAb B1C9A2 and FoxG5, which caused 65 and 53% inhibition, respectively. Increasing the ascitic fluid twofold (20% [vol/vol]) did not, correspondingly, enhance the inhibition percentages of radioactive incorporation (data not shown). In this experiment, however, inhibition of [^{3}H]hypoxanthine incorporation in control cultures treated with saline solution, SP2/0-myeloma ascitic fluid, or control anti-*B. bovis* MAb was not observed.

To exclude the possibility that nonspecific inhibitory components were present in the ascitic fluids, the monoclonal IgGs were purified from the ascites (of those showing maximum inhibition), and their biological activity was further tested for inhibitory activity. At the end of the 48-h assay, it was observed that relevant IgG purified from SP2/0

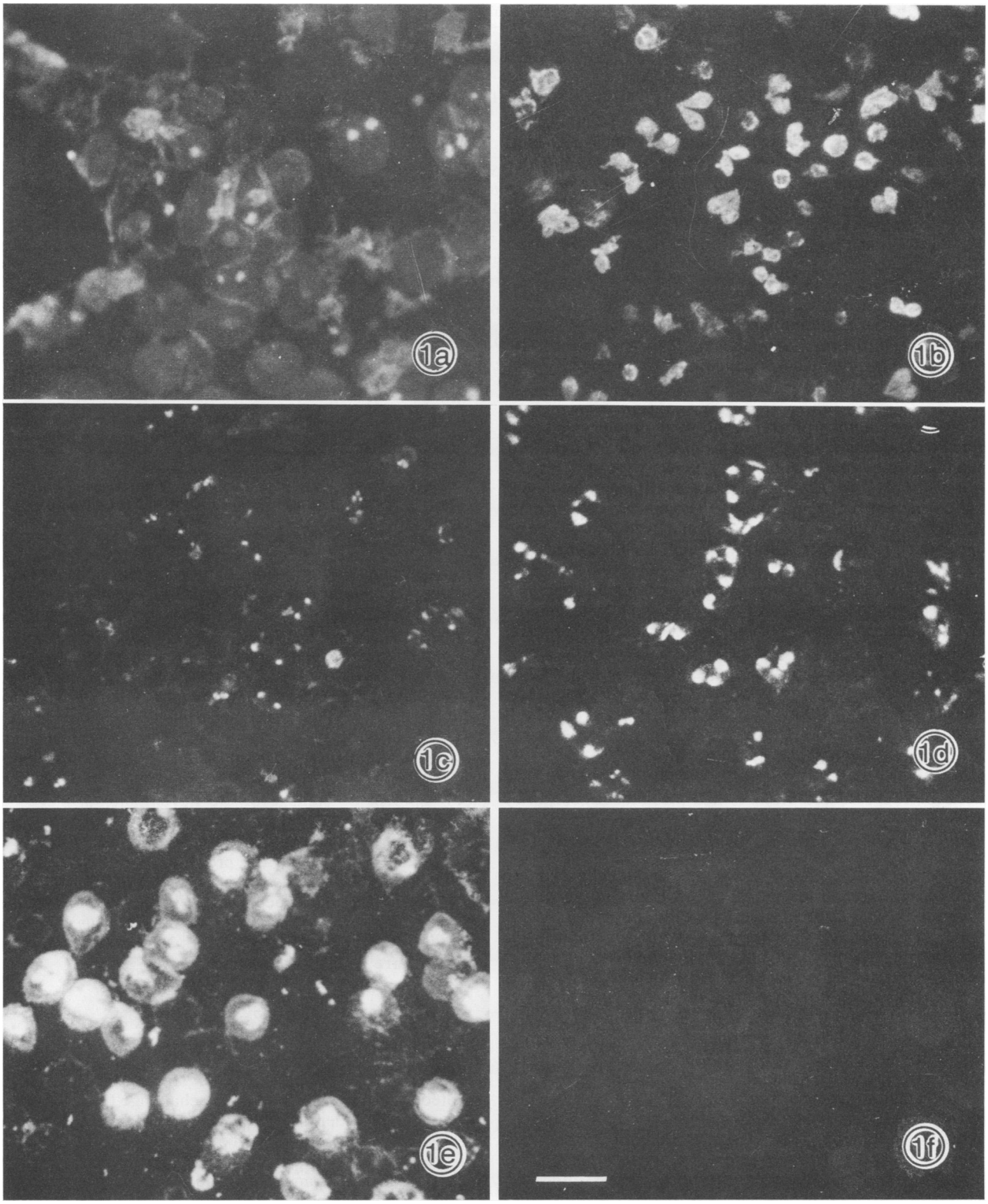

FIG. 1. Photomicrographs of IFAT reaction patterns produced by MAb directed against the erythrocytic stages of *B. bigemina*. Acetone-fixed slides of Seed-infected erythrocytes were reacted with: (a) group I MAb J1B4; (b) group II MAb 12C2; (c) group III MAb C2F3G3; and (d) group IV MAb B1B3C4. (e) Reaction pattern produced by serum from mice immunized with the Seed strain and reacted with *B. bigemina* Seed-infected RBCs. (f) Reaction pattern produced by normal mouse serum. Bar = 10 μm.

TABLE 1. Inhibition[a] of the multiplication in vitro of *B. bigemina* by ascitic fluid (10%) containing MAb

MAb	Isotype	PPE[b]	% Inhibition[c]	[^{3}H]hypoxanthine incorporation[d]	% Inhibition[e]
Normal		4.00 ± 0.5		2,986.25 ± 675.3	
Saline		4.31 ± 0.49	0	2,372.75 ± 262.08	21
SP2/0		3.82 ± 0.33	5	2,808.00 ± 211.49	6
J1B4	IgG1	3.46 ± 0.67	15	1,926.25 ± 244.42	35
IF9	IgG2a	3.82 ± 0.33	5	1,920.25 ± 287.12	36
FC/M	IgM	ND[f]		ND[f]	
12C2	IgG2a	3.14 ± 0.62	25	1,780.00 ± 218.5	40
B1C9A2	IgG1	2.19 ± 0.11*	52	1,057.75 ± 47.73*	65
C2F3G3	IgG2b	1.84 ± 0.27*	62	1,811.00 ± 956.93	39
B1B3C4	IgG2a	3.49 ± 0.75	15	2,271.25 ± 875.06	24
B1E2E8	IgM	3.47 ± 0.46	15	2,143.00 ± 584.34	28
FoxG5	IgG2a	3.06 ± 0.28*	27	1,410.75 ± 368.45*	53
Control	IgG1	3.65 ± 0.70	10	3,087.50 ± 575.55	0

[a] By 48-h cultivation assay.
[b] Mean ± standard error; $n = 4$. *, Significantly different from control SP2/0. One-tailed Student's t test ($P < 0.05$).
[c] Percentage of inhibition = PPE normal − PPE MAb/PPE normal − PPE initial (0.5) × 100.
[d] CPM ± standard error; $n = 4$. *, Significantly different from control SP2/0. One-tailed Student's t test ($P < 0.05$).
[e] Percentage of inhibition = CPM normal − CPM MAb/CPM normal × 100.
[f] ND, Not done. Addition to culture caused clot formation.

myeloma-induced ascitic fluid apparently caused a substantial inhibition of *B. bigemina* multiplication as determined by the decreased PPE (Table 2), which resulted in an inhibition percentage of 50% compared with the PPE in normal cultures. This enhanced inhibition caused by normal mouse IgG, however, could not be documented as calculated by the incorporation of [^{3}H]hypoxanthine (Table 2). The incorporation rate was essentially the same in cultures treated with SP2/0, saline solution, or untreated normal culture.

The control MAb anti-*B. bovis* IgG1 inhibited parasite multiplication at relatively low percentages (39%) as calculated by the two procedures. MAb FoxG5 did not have statistically significant inhibitory activity as calculated by both techniques and at the same IgG concentration as the normal mouse IgG. As determined by the PPE count, MAb C2F3G3 did not have a statistically significant inhibitory activity compared with that in the SP2/0 IgG treated cultures. However, treated cultures had a reduced incorporation of [^{3}H]hypoxanthine, which resulted in an significantly increased percentage of inhibition which was calculated as high as 70% more than that caused by the control IgG. The two other purified MAb IgGs, B1C9A2 and B1B3C4, caused relatively high percentages of inhibition as calculated by the iRBC counting method but also substantially elevated inhibitory activity as calculated by the radionuclide incorporation technique (68 and 88% inhibition, respectively). The reduced PPE and [^{3}H]hypoxanthine incorporation caused by both MAb were statistically significant as compared with that caused by purified IgG from SP2/0 myeloma-induced ascitic fluid. Observation with the light microscope of smears from the culture wells treated with the different ascitic fluids or purified IgGs revealed that the majority of the MAb with inhibitory activity did not cause agglutination of iRBCs or parasites and did not alter the parasites' morphology (Fig. 2). However, MAb C2F3G3 and particularly MAb B1C9A2 had inhibitory activity that was associated with a retardation of development or damage of the developmental stages that give rise to the paired mature stages (Fig. 3). Up to 20% of the counted iRBCs contained babesial stages that were apparently damaged by the presence of specific antibody. The affected organisms were observed to be larger than normal and generally vacuolated (Fig. 3a), but on occasion, dense giant bodies were found which contained dispersed, heavily stained chromatin (Fig. 3b). Multiple-infected RBCs with bizarre forms were also seen.

TABLE 2. Inhibition[a] of the multiplication in vitro of *B. bigemina* by purified MAb IgG

MAb	PPE[b]	% Inhibition[c]	[^{3}H]hypoxanthine incorporation[d]	% Inhibition[e]
Normal	3.71 ± 0.75		849.00 ± 198.0	
Saline	3.24 ± 0.30	15	816.87 ± 133.28	4
SP2/0	2.08 ± 0.20	51	849.50 ± 102	0
12C2	1.63 ± 0.16*	65	570.00 ± 63.18*	33
B1C9A2	0.86 ± 0.08*	89	271.12 ± 123.38*	68
C2F3G3	1.61 ± 0.21	65	25.87 ± 30*	73
B1B3C4	1.26 ± 0.02*	76	96.38 ± 25*	89
FoxG5	2.30 ± 0.50	44	671.50 ± 94.74	21
Control MAb	2.47 ± 0.19	39	522.00 ± 61.25*	39

[a] By 48-h cultivation assay.
[b] Mean ± standard error; $n = 4$. *, Significantly different from control IgG (SP2/0-induced ascitic fluid). One-tailed Student's t test ($P < 0.05$).
[c] Percentage of inhibition = PPE normal − PPE MAb/PPE normal − PPE initial (0.5) × 100.
[d] 24-h pulse, added during exchange of culture medium (0.2 μCi). CPM (mean ± standard error), $n = 4$. *, Significantly different from control IgG (SP2/0-induced ascitic fluid). One-tailed Student's t test ($P < 0.05$).
[e] Percentage of inhibition = CPM normal − CPM MAb/CPM normal × 100.

DISCUSSION

In recent years, there has been a renewed interest in improving antibabesial vaccines. Major constraints are that cattle immune to one *B. bigemina* strain are partially or not protected against a heterologous challenge (2), and serological tests have demonstrated that antigenic differences between isolates of *B. bigemina* exist (4). In this study, a panel of MAb was produced against *B. bigemina* erythrocytic stages. The MAb panel was used to identify cross-reacting antigens in a group of *B. bigemina* isolates from geographically different regions (6).

MAb have been used to define grouping of antigenic determinants on the basis of stage and species specificities of hemoprotozoan parasites by using immunofluorescence staining patterns (10, 11, 17, 18). In this study, MAb from

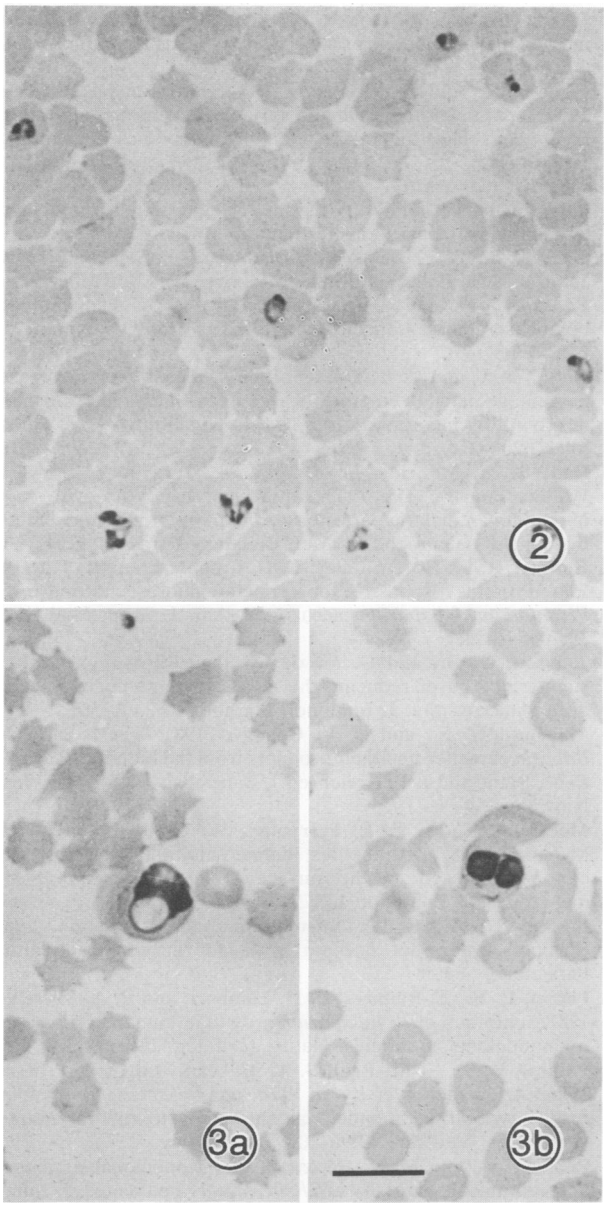

FIG. 2. Photomicrograph of *B. bigemina*-infected erythrocytes after addition of anti-*B. bovis* MAb. Bar = 10 μm.

FIG. 3. Photomicrographs of *B. bigemina*-infected erythrocytes after addition of inhibitory MAb B1C9A2. Bar = 10 μm.

group I stained iRBCs when hybridoma supernatant was used as the source of antibody. However, antibodies in ascitic fluid reacted also with normal RBCs. Parasite antigens recognized by MAb to *B. bigemina* of the other three groups were parasite specific (as evidenced by the lack of reaction with bovine normal RBC antigens) but not stage specific, since all developmental forms seen in culture were routinely reactive in the IFAT. Moreover, the parasite antigens were demonstrated to be species specific as evidenced by the negative reaction observed with *B. bovis* parasites.

Phillips et al. (17) showed that three MAb produced to the rat-adapted *Babesia divergens* did not cross-react with *Babesia microti* or *B. bovis* iRBCs. In contrast, Wright and Riddles (27) reported that a MAb reactive with a low-molecular-weight antigen of *B. bovis* also reacted with a similar antigen of *B. bigemina* and *B. divergens*. Antigenic diversity among *B. divergens* isolates or *Theileria parva* isolates has been reported to occur, as detected by MAb in immunofluorescence assays (17, 18). However, MAb produced to *B. bigemina* (14) and *B. bovis* (8, 27, 28) were shown to recognize cross-reactive epitopes in babesial isolates from different areas. Consequently, the presence of conserved epitopes may not be uncommon for *Babesia* species.

For the identification of potential protective *B. bigemina* antigens, MAb which are capable of interrupting the multiplication of the parasite in vitro could provide valuable functional information. Initially, by using neat ascitic fluids, five MAb to *B. bigemina* which had a functional inhibitory activity in vitro were identified. However, after purification of the IgG fraction from the MAb-containing ascitic fluids, only three MAb were found to inhibit the parasite growth or multiplication at significant levels. Further experiments have clearly defined the localization and biochemical characteristics of the parasitic components recognized by the inhibitory MAb analyzed in this study (6). Immunoelectron microscopy studies demonstrated that MAb B1C9A2, B1B3C4, and C2F3G3 recognized surface-exposed babesial epitopes. MAb B1C9A2 immunoprecipitated a parasite antigen having a relative mobility of 22 kDa, whereas both MAb C2F3G3 and B1B3C4 immunoprecipitated parasite components banding at relative sizes of 62, 60, and 58 kDa (6). Two of the three inhibitory MAb were associated with direct damage to the parasite, although the mechanism of such effect cannot be immediately explained. Perrin et al. (16) reported the production of MAb to the protozoan parasite *Plasmodium falciparum*, which had inhibitory activity in vitro. They hypothesized that several mechanisms could take place to explain the functional activity of the MAb. (i) The MAb binds to the surface coat of the infective merozoite, blocking the invasion process of an RBC by interfering with the parasite ligand-RBC receptor. (ii) MAb bind to parasite antigen in the surface of an iRBC containing mature stages (schizonts) and somehow modify the parasite's metabolism. (iii) MAb could reach the intracellular parasite since schizont-infected human RBCs have an increased permeability. Which of these, or any other mechanism, is occurring in the *B. bigemina* system is to be tested in the future by using highly synchronized culture of *B. bigemina* and inhibitory MAb at various IgG concentrations added at different times. Winger et al. (25) reported that a MAb directed against a merozoite surface coat antigen of *B. divergens* significantly inhibited the merozoite invasion process of bovine erythrocytes. The MAb to *B. bigemina* showing the highest inhibitory activity reported in this study recognize an epitope present on the surface of the parasite (6). However, unlike those directed to *B. divergens*, it is unlikely that the MAb are blocking the invasion of RBCs by the parasite under the in vitro conditions reported here. Evidence accumulated so far indicates that B1C9A2, C2F3G3, and B1B3C4 to lesser extent retard or inhibit parasite division with a subsequent drop in PPE as evidenced by the presence of relatively high numbers of damaged parasites and by the presence of a very low number of free merozoites in the culture medium as well as by the decreased incorporation of [^{3}H]hypoxanthine. [^{3}H]hypoxanthine incorporation by *B. bovis* in culture has been considered a suitable procedure to measure the in vitro multiplication of the parasite (9). The discrepancy observed

between percentages of inhibition estimated by measuring PPE and [^{3}H]hypoxanthine incorporation in *B. bigemina* cultures added with normal mouse IgG cannot be explained immediately. Part of the difference could be accounted for by the fact that free, exoerythrocytic merozoites (whose presence in the slides from cultures treated with normal mouse IgG was more evident than in cultures treated with the MAb) were not included in the PPE estimate. Thus, a decreased PPE value, particularly in normal IgG-treated cultures, could actually represent an underestimated calculation; whereas, the [^{3}H]hypoxanthine incorporation by the parasites in those cultures could reflect a more accurate estimate since all metabolically active parasites, whether intraerythrocytic or extraerythrocytic, were harvested for the radioisotope counting technique. As for the reason for merozoite exoerythrocytic accumulation caused by normal mouse IgG, it cannot be explained at this time. In addition, *B. bigemina* has different in vitro growth characteristics than *B. bovis*; it tends to grow more slowly, and high PPE are not usually reached as for *B. bovis* cultivated in vitro (23, personal observations). Alternatively, a single pulse labeling of [^{3}H]hypoxanthine was used in this study, as opposed to the labeling every 12 h of *B. bovis* cultures (9). Whether any of these events accounts for the lack of correlation between both tests is not known. Nevertheless, measuring the incorporation of [^{3}H]hypoxanthine by the parasite seemed to be a better indicator of the effect produced by the MAb in the in vitro culture system, since estimation of PPE by light microscopic examination took into account all iRBCs whether they contained damaged parasites or not. To discern at the light-microscope level a live parasite from a damaged one is a difficult task unless an obvious morphological change has occurred. Studies to test whether the parasite growth inhibition is dose and time dependent and whether it requires accessory elements (complements) to be carried out will require further experimentation using growth-synchronized cultures.

In conclusion, a set of specific MAb to *B. bigemina* which inhibit its multiplication in vitro has been produced. Such MAb can now be utilized to purify the correspondent parasite epitopes which could be important in the development of a subunit immunogen against cattle babesiosis caused by *B. bigemina*.

ACKNOWLEDGMENTS

This work was supported in part by USAID grant DPE-5542-G-SS-4026-00 and COR grant C-2-02294, College of Veterinary Medicine, UMC. Julio V. Figueroa was partially supported by INIFAP-SARH, Mexico City, Mexico.

We acknowledge the technical assistance of Karen K. McLaughlin and the secretarial assistance of Ellen Swanson.

REFERENCES

1. **Bruck, C., J. A. Drebin, C. Glineur, and D. Portetelle.** 1986. Purification of mouse monoclonal antibodies from ascitic fluid by DEAE Affi-gel blue chromatography. Methods Enzymol. **121**:587–596.
2. **Callow, L. L.** 1967. Sterile immunity, coinfectious immunity and strain differences in *Babesia bigemina* infections. Parasitology **57**:455–465.
3. **Cohen, S.** 1984. Monoclonal antibodies in parasitic infectious diseases. Br. Med. Bull. **40**:291–296.
4. **Curnow, J. A.** 1973. The use of a slide agglutination test to demonstrate antigenic differences between *Babesia bigemina* parasites. Aust. Vet. J. **49**:290–293.
5. **De St. Groth, S. F., and D. Scheidegger.** 1980. Production of monoclonal antibodies: strategy and tactics. J. Immunol. Methods **35**:1–21.
6. **Figueroa, J. V., G. M. Buening, D. A. Kinden, and T. J. Green.** 1990. Identification of common surface antigens among *Babesia bigemina* isolates by using monoclonal antibodies. Parasitology **100**:161–175.
7. **Fine, E., J. Inselburg, J. Obeing, and A. Hanson.** 1987. *Plasmodium falciparum*-inhibitory monoclonal antibodies produced by human hybridomas. Parasite Immunol. **9**:305–320.
8. **Goff, W. L., W. C. Davis, G. H. Palmer, T. F. McElwain, W. C. Johnson, J. F. Bailey, and T. C. McGuire.** 1988. Identification of *Babesia bovis* merozoite surface antigens by using immune bovine sera and monoclonal antibodies. Infect. Immun. **56**: 2363–2368.
9. **Goff, W. L., and C. E. Yunker.** 1986. *Babesia bovis*: increased percentage parasitized erythrocytes in cultures and assessment of growth by incorporation of [^{3}H]-hypoxanthine. Exp. Parasitol. **62**:202–210.
10. **Hall, R., J. McBride, G. Morgan, A. Tait, J. W. Zolg, D. Walliker, and J. Scaife.** 1983. Antigens of the erythrocytic stages of the human malaria parasite *Plasmodium falciparum* detected by monoclonal antibodies. Mol. Biochem. Parasitol. **7**:247–265.
11. **Howard, R. F., H. A. Stanley, G. H. Campbell, and R. T. Reese.** 1984. Proteins responsible for a punctate fluorescence pattern in *Plasmodium falciparum* merozoites. Am. J. Trop. Med. Hyg. **33**:1055–1059.
12. **Johnson, G. D., and G. M. de C. Nogueira-Araujo.** 1981. A simple method of reducing the fading of immunofluorescence during microscopy. J. Immunol. Methods **43**:349–350.
13. **Mahoney, D. F., and B. V. Goodger.** 1981. The isolation of *Babesia* parasites and their products from the blood, p. 323–335. *In* M. Ristic and J. P. Kreier (ed.), Babesiosis. Academic Press, New York.
14. **McElwain, T. F., L. E. Perryman, W. C. Davis, and T. C. McGuire.** 1987. Antibodies define multiple proteins with epitopes exposed on the surface of live *Babesia bigemina* merozoites. J. Immunol. **138**:2298–2304.
15. **Palmer, D. A., G. M. Buening, and C. A. Carson.** 1982. Cryopreservation of *Babesia bovis* for in vitro cultivation. Parasitology **84**:567–572.
16. **Perrin, L. H., E. Ramirez, P. H. Lambert, and P. A. Miescher.** 1981. Inhibition of *P. falciparum* growth in human erythrocytes by monoclonal antibodies. Nature (London) **289**:301–303.
17. **Phillips, R. S., G. M. Reid, S. A. McLean, and C. D. Pearson.** 1986. Antigenic diversity in *Babesia divergens*: preliminary results with three monoclonal antibodies to the rat-adapted strain. Res. Vet. Sci. **42**:96–100.
18. **Pinder, M., and R. S. Hewett.** 1980. Monoclonal antibodies detect antigenic diversity in *Theileria parva* parasites. J. Immunol. **124**:1000–1001.
19. **Ristic, M., and S. Montenegro-James.** 1988. Immunization against *Babesia*, p. 164–189. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla.
20. **Rodriguez, S. D., G. M. Buening, and C. A. Carson.** 1986. *Babesia bovis*: purification and concentration of merozoites and infected bovine erythrocytes. Exp. Parasitol. **61**:236–243.
21. **Taggart, R. T., and I. M. Samloff.** 1983. Stable antibody-producing murine hybridomas. Science **219**:1228–1230.
22. **Van Deusen, R. S.** 1984. Making hybridomas, p. 16–25. *In* N. J. Stern and H. R. Gamble (ed.), Hybridoma technology in agricultural and veterinary research. Rowman & Allanheld Publishers, Lanham, Md.
23. **Vega, C. A., G. M. Buening, T. J. Green, and C. A. Carson.** 1985. In vitro cultivation of *Babesia bigemina*. Am. J. Vet. Res. **46**:416–420.
24. **Vega, C. A., G. M. Buening, S. D. Rodriguez, and C. A. Carson.** 1986. Concentration and enzyme content of in vitro-cultured *Babesia bigemina*-infected erythrocytes. J. Protozool. **33**:514–518.
25. **Winger, C. M., E. U. Canning, and J. D. Culverhouse.** 1987. A monoclonal antibody to *Babesia divergens* which inhibits merozoite invasion. Parasitology **94**:17–27.

26. **Wright, I. G., G. B. Mirre, K. Rode-Bramanis, M. Chamberlain, B. V. Goodger, and D. J. Waltisbuhl.** 1985. Protective vaccination against virulent *Babesia bovis* with a low-molecular-weight antigen. Infect. Immun. **48**:109–113.
27. **Wright, I. G., and P. W. Riddles.** 1989. Biotechnology in tick-borne diseases: present status, future perspectives, p. 325–340. *In* FAO-UN. (ed.), Biotechnology for livestock production. Plenum Press, New York.
28. **Wright, I. G., M. White, P. D. Tracey-Patte, R. A. Donaldson, B. V. Goodger, D. J. Waltisbuhl, and D. F. Mahoney.** 1983. *Babesia bovis*: isolation of a protective antigen by using monoclonal antibodies. Infect. Immun. **41**:244–250.