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Isolation and Partial Characterization of Culture-Derived Soluble *Babesia bovis* Antigens

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Immunochemical analyses of soluble antigens derived from microaerophilous stationary phase cultures of *Babesia bovis* demonstrated that at least three parasite antigens were released in vitro. These antigens have molecular weights within the range of 37,000 to 40,000, fast electrophoretic mobility in the albumin and alpha$_1$ regions, and are proteinaceous in nature as determined by the sensitivity to proteolytic enzymes trypsin and papain. Purification of these antigens should allow complete characterization of their respective physicochemical and immunogenic properties.

Babesia bovis is an intraerythrocytic protozoan parasite and a causative agent of the tick-borne blood disease of cattle, bovine babesiosis. The analysis of blood-derived babesial antigens has been difficult because of the relatively low parasitemias and various immunopathological processes occurring in infected animals.

Recently, two culture systems have been developed for the continuous propagation of *B. bovis* organisms. Erp et al. (7) devised the Spinner flask culture system which allowed relatively short-term growth with concomitant production of soluble antigenic material. Subsequently, continuous cultivation of *B. bovis* was obtained; however, parasite numbers remained low (8). Immunization of cattle with cell-free soluble antigens prepared from culture supernatants has been shown to provide protection against a homologous tick-borne challenge with virulent *B. bovis* organisms (21).

Considerable improvement in cultivation of *B. bovis* has been achieved with the development of the microaerophilous stationary phase (MASP) system (16). The maintenance of high parasite yields offers an abundant source of soluble antigens which are released into the culture medium. Recent experiments have shown that these parasite products are effective in inducing protective immunity to bovine babesiosis (K. L. Kuttler et al., unpublished data).

The present study was concerned with the identification of soluble babesial antigens derived from the high-yield MASP culture system. Electrophoretic analyses of these antigens provided valuable information as to their physicochemical properties.

MATERIALS AND METHODS

Antigen preparation. *B. bovis* organisms were cultured by the method of Levy and Ristic (16). When cultures reached parasitemias of ca. 15%, supernatants (consisting of 40% normal bovine serum, 60% medium 199 supplemented with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and antibiotics, hemoglobin, and soluble parasite antigens) from several flasks were pooled, centrifuged at 12,100 × *g* for 30 min at 4°C, and then filtered through a 0.45-μm membrane filter (Millipore Corp.) to remove all erythrocytic stroma and corpuscular parasite material. Supernatants were finally lyophilized and stored at 4°C until use. In addition, normal control supernatants from uninfected cultures were similarly prepared.

Antiserum production. Rabbits were utilized as a source of polyspecific anti-*B. bovis* antibodies because of the suboptimal immunoreactivity of bovine antisera in gels. Coincident with the need for purified *B. bovis* antigens for rabbit antiserum production (due to the relatively high bovine serum content of the MASP culture medium), the culture of *B. bovis* in rabbit erythrocytes and serum was established in our laboratory (Esparza et al., unpublished data).

Briefly, cultures were initiated with parasitized bovine erythrocytes and with various amounts of bovine and rabbit serum. With each subsequent passage, only rabbit erythrocytes were added. Likewise, with each change of medium only rabbit serum (40% concentration) was used. Culture supernatant containing potential immunogens was collected only after the third subculture in which only rabbit blood elements were used to minimize the risk of collecting bovine serum contaminants. At that time, 30 ml of supernatant was collected over an 8-day period from cultures with an average parasitemia of 2.8%. The supernatants were then pooled, centrifuged (12,100 × *g*, 30 min, 4°C), filtered (0.45-μm membrane filter), and lyophilized. A 125-mg amount of lyophilized culture supernatant (ca. 95 mg total protein, of which greater than 90% derived from rabbit serum) was reconstituted with 0.6 ml of sterile distilled water (5× concentration) and then mixed with an equal volume of Freund adjuvant for immunization into each of three rabbits. The rabbits were given three weekly subcutaneous injections: the

first with Freund complete adjuvant and the last two with Freund incomplete adjuvant. By day 9 after the third inoculation, the rabbits had responded with a mean anti-*B. bovis* indirect fluorescent antibody titer of 1:427. These specific antisera were collected, pooled, and frozen at −20°C until use in immunoelectrophoretic studies.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lyophilized MASP *B. bovis*-infected and uninfected culture supernatants were mixed with SDS (SDS to dry weight ratio, 3:1), reconstituted with sterile distilled water to affect a 2× antigen concentration, and then dialyzed against 0.01 M phosphate buffer (pH 7.1) containing 0.1% SDS for 48 h at 4°C. Next, supernatants were incubated at 100°C for 2 min or 37°C for 3 h and chilled, and 10 μl of bromophenol blue and 10 μl of 2-mercaptoethanol were added to 250 μl of each sample solution. The samples were then overloaded (ca. 700 μg of protein per 10 μl of sample application volume) onto an SDS-containing polyacrylamide gel (7.5% acryladmide, 0.1 M phosphate buffer [pH 7.1], 0.1% SDS) and electrophoresed at 190 mA for 4.5 h at 8°C. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue R-250 for 2 h at 25°C and then destained for 24 h at 25°C in a solution containing 30% ethanol and 10% acetic acid.

Molecular weights of soluble *B. bovis* antigens were estimated as each antigen preparation was run simultaneously with known standards. The respective relative mobilities were calculated and plotted against the log molecular weights of the protein markers.

CIE. Immunoelectrophoretic analysis of soluble *B. bovis* antigens was conducted by crossed immunoelectrophoresis (CIE) (4). MASP infected and normal control culture supernatants were reconstituted in sterile distilled water to a 10× concentration and dialyzed against 0.02 M phosphate buffer (pH 8.0) for 48 h at 4°C. Final antigen concentration after dialysis was ca. 5×. Ten microliters of antigen sample was applied to a 1% agarose gel containing 4% polyethylene glycol (15) and electrophoresed for 2 h at 10 V/cm in a 0.025 M tris(hydroxymethyl)aminomethane-barbituate buffer, pH 8.6. Second-dimension electrophoresis was then conducted at 2 V/cm for 16 h across a gel containing 12.7 μl of rabbit anti-*B. bovis* serum per cm^2 gel. All gels were finally stained with Crowle double stain (5).

Enzyme sensitivity. The enzymes alpha-amylase (EC 3.2.1.1, type II-A, Sigma Chemical Co., St. Louis, Mo.), triacylglycerol lipase (EC 3.1.1.3, type VII, Sigma), papain (EC 3.4.22.2, type IV, Sigma), pepsin (EC 3.4.23.1, Sigma), and trypsin (EC 3.4.21.4, GIBCO Laboratories, Grand Island, N.Y.) were prepared as previously described (22) and added to *B. bovis* culture supernatants at a ratio of 4 mg of enzyme to 400 mg of lyophilized culture supernatant. Reaction mixtures were reconstituted in 2 ml of the appropriate phosphate buffer to affect a final 3× concentration and then incubated for 16 h at 37°C. Enzyme sensitivity was analyzed by CIE as described above.

RESULTS

Study of the physical properties of culture-derived soluble *B. bovis* antigens demonstrated the presence of at least two antigens with molecular weights between 37,000 and 40,000 daltons. Identical results were obtained whether culture supernatants were heat-treated at 37°C (Fig. 1) or 100°C.

Immunoelectrophoretic analysis of MASP *B. bovis* culture supernatant showed that at least three soluble antigens are produced in vitro (labeled antigens 1, 2, and 3 of Fig. 2A). Antigen 1 has an electrophoretic mobility in the albumin range, whereas antigens 2 and 3 migrate to the $alpha_1$ region.

Supernatants from infected cultures were subjected to enzyme treatment for an assay of their biochemical properties. Crossed immunoelectrophoretograms indicated that at the enzyme-to-antigen ratio utilized, soluble *B. bovis* antigen was completely degraded by papain and partially degraded by trypsin and lipase. The effect of alpha-amylase and pepsin upon subsequent antigenic reactivity was negligible. It should be noted that at the antigen concentration required for optimal enzyme-antigen reactivity (3×) only antigen 1 could be adequately detected.

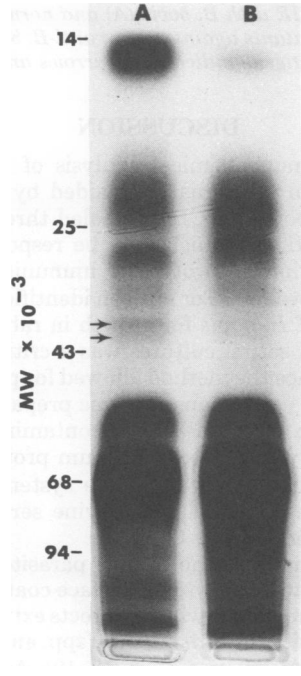

FIG. 1. *SDS-PAGE of MASP-derived B. bovis (A) and normal (B) culture supernatants after heat treatment at 37°C for 3 h. Arrows indicate location of parasite antigens along log molecular weight scale.*

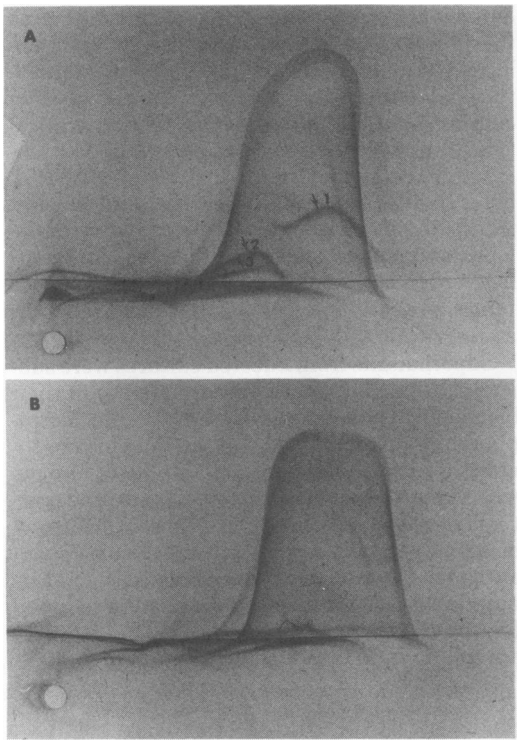

FIG. 2. *CIE with B. bovis (A) and normal (B) culture supernatants against rabbit anti-B. bovis serum. Parasite antigens indicated by arrows and numbers 1, 2, and 3.*

DISCUSSION

The immunochemical analysis of MASP *B. bovis* culture supernatants, aided by the high-resolving power of CIE, revealed three distinct soluble antigens which may be responsible for the induction of protective immunity against bovine babesiosis. For antigen identification, adaptation of *B. bovis* for growth in rabbit erythrocyte and serum cultures was a critical development since the method allowed for production of antisera against an antigenic preparation relatively free of bovine serum contaminants. Approximately 8 or 9 bovine serum proteins were detected in the rabbit culture system as compared with 40 proteins in bovine serum which may be identified by CIE (2).

One or more of the soluble parasite antigens is thought to be merozoite surface coat, a loosely adherent substance which protects extraerythrocytic parasites of *Plasmodium* spp. and *Babesia* spp. from destruction (1, 3, 18, 19). Apparently, the surface coat is shed into the culture medium at the time merozoites enter erythrocytes. Growth of these parasites in vitro can then provide a ready source of soluble surface coat material and other parasite metabolic products for antigenic characterization. In contrast to antigens obtained from the blood of infected animals, culture-derived antigens would not be altered by host biochemical and immunological processes. Antigens may be easily collected from culture supernatants or extracted from merozoites agitated in cold saline (20).

The isolation of soluble *B. bovis* antigens released in vitro confirms recent data concerning the *B. bovis* antigens obtained from a semipurified lytic extract of infected erythrocytes (13). Goodger and co-workers reported that protamine sulfate precipitates of their antigen preparation contained one major and two minor babesial antigens (13). These most probably are identical to the respective antigens designated 1, 2, and 3 in the present study. Goodger (9) previously demonstrated two specific intraerythrocytic antigens by fluorescent-antibody techniques. One of these antigens was found in or on the internal rim of the erythrocyte membrane. The other antigen resided in granules on the internal stromata of the infected erythrocyte. Gravely et al. (14) observed that antibodies to soluble *B. bovis* antigens derived from Spinner flask cultures were more specific for antigens found on erythrocyte membranes than on or within the parasites themselves. Recent preliminary data from immunofluorescence studies conducted in our laboratory, utilizing rabbit monospecific antibodies to specific culture-derived *B. bovis* antigens, would suggest that so-called antigen 1 is the same as that reported to reside on the internal erythrocyte membrane, antigen 2 is identical to that found in granules, and antigen 3 appears to be associated directly with the parasite (S. Montenegro, M. A. James, and M. Ristic, manuscript in preparation). In addition, similar electrophoretic findings in the present study and in that of Goodger (9) lend further evidence to the identity of these babesial antigens.

B. bovis antigens, in particular the one associated with infected erythrocyte stromata (presumably antigen 2), have been known to complex with fibrinogen and other related proteins, a phenomenon which may affect coagulation, fibrinolysis, and blood agglutination in infected animals (10–12). Besides the pathological consequences of complex formation, it also seems that binding of antigenic determinants to host serum proteins may act to conceal parasites and their antigens, thereby preventing immunological recognition by host lymphocytes.

The estimated molecular weights for at least two soluble MASP-derived *B. bovis* antigens as determined by SDS-PAGE approximate 37,000

to 40,000. These figures correlate well with those reported (ranging from 44,000 to 55,000 daltons) for certain soluble antigens of *Plasmodium* and *Babesia* species (6, 13, 17, 23). Necessary confirmatory autoradiographic experiments are currently in progress.

The biochemical nature of *B. bovis* antigens as determined by reactivity in gels after enzyme treatment would indicate they are highly proteinaceous. Data concerning the relative contribution of lipid or polysaccharide moieties remain equivocal, however. Although CIE analyses seemed to show at least a partial degradation by lipase, no staining of antigen immunoprecipitates was evident when the lipid stain Sudan Black was used. Likewise, Goodger (9) found that specific precipitin bands failed to stain for lipid, peroxidase, or polynucleotides. In the present study, no antigen susceptibility to alpha-amylase was apparent; however, this enzyme has been shown to affect the antigenicity of *B. canis* antigens (N. Atyabi, M.S. thesis, University of Illinois, Urbana, 1979).

The protective nature of each soluble *B. bovis* antigen has yet to be determined. Previously, crude culture supernatants derived from Spinner flask cultures have been shown to protect against a tick-borne *Babesia* challenge exposure (21). More recently, preliminary experiments indicated that the MASP culture system produces protective immunogens which are capable of stimulating even higher antibody levels with a strong anamnestic response (K. L. Kuttler, unpublished data). The purification of soluble *B. bovis* antigens and the subsequent production of large quantities of monospecific antibodies will enable assessment of their protective capabilities. Furthermore, availability of purified antigens should also facilitate the study of antigenic variation and strain cross-immunity in future investigations.

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