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Comparison of Proteins Synthesized by Two Different Isolates of *Anaplasma marginale*

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We present results on the initial definition of proteins synthesized by two isolates of *Anaplasma marginale*. Bovine erythrocytes infected with *A. marginale* were radioactively labeled with [^{35}S]methionine or a ^{3}H-amino acid mixture during short-term in vitro culture. The labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This technique revealed protein bands of various apparent molecular weights from <14,000 to >200,000. The bands observed represented *A. marginale* proteins because (i) uninfected erythrocytes from the same animal did not incorporate radioisotope during identical culture conditions, and (ii) the incorporation of radioisotope into proteins during culture of infected erythrocytes was inhibited by tetracycline but not by cycloheximide. The radioactive protein profiles of two different isolates of *A. marginale*, from Washington and Florida, were compared by two-dimensional gel electrophoresis. About 200 proteins were resolved in each case. Several proteins differed in position when the two-dimensional gel maps were compared, indicating variations in protein structure between the two *A. marginale* isolates.

Anaplasmosis is a tick-borne disease of cattle and other ruminants caused by *Anaplasma marginale*, a member of the order *Rickettsiales* (23). The disease occurs worldwide and is a particular problem in livestock production in the tropics. After exposure of cattle, anaplasmosis is characterized by a latent period of 3 to 6 weeks, an acute phase (4 to 9 days) of high parasitemia in erythrocytes, and a chronic phase during which low parasitemia may persist indefinitely. Despite many attempts, extra-erythrocytic forms of the parasite have not been identified (9).

Three different methods of immunoprophylactic control of the disease have been used. (i) Premunization with *A. marginale* or *Anaplasma centrale*, followed in some cases by control of the initial infection with tetracyclines (19). This method is not frequently used in the United States because of the variable protection produced against subsequent *A. marginale* infections and the possibility of introducing clinical disease into an area (especially with *A. centrale*, which is not presently found in the United States). (ii) Vaccination with a killed *A. marginale* preparation which still contains erythrocyte stroma (Anaplaz; Fort Dodge Laboratories, Fort Dodge, Iowa). Subsequent field challenge of vaccinated cattle results in the establishment of carriers (2), although the vaccine protects most cattle against clinical disease (19). Occasionally, isoerythrolysis occurs in calves nursing from vaccinated dams (7). (iii) Use of live, attenuated *A. marginale* organisms (24). Reports on the efficacy of this vaccine conflict, varying from complete protection against needle challenge with a heterologous isolate (14) to partial protection against clinical disease in field challenge situations (14, 21). Problems associated with the attenuated vaccine include potential reversion to virulence, clinical illness, diminished milk production, and abortion in lactating dairy cows (19).

Because of these problems, we decided to adopt an alternative approach to immunoprophylaxis against *A. marginale*. The theoretical basis for this approach was to identify and purify the protein antigens synthesized by the parasite which are potential targets for attack by the host immune system. The protein composition of *A. marginale* is not known because the methods available for purifying *A. marginale* organisms from host erythrocytes (3, 8) do not guarantee complete integrity of the parasite and complete absence of host proteins. Analyses of such preparations by electrophoresis and staining methods would not reliably characterize the protein structure of *A. marginale*. However, it has been shown that *A. marginale*-infected bovine erythrocytes, unlike uninfected erythrocytes, will incorporate radiolabeled amino acids into tri-

chloroacetic acid-precipitable material (6, 17). We therefore labeled the proteins of *A. marginale* by metabolic incorporation of radioactive amino acids and analyzed these proteins by one- and two-dimensional gel electrophoresis and fluorography.

MATERIALS AND METHODS

Origin of *A. marginale* organisms. Two isolates of *A. marginale* were used in the experiments. The Washington isolate was obtained from a mature bull with clinical anaplasmosis diagnosed at the Washington State University Veterinary Clinic in 1981. The Florida isolate was a pooled blood sample collected from naturally infected cattle in various sections of Florida in 1955 (22). Infected blood was stored as a stabilate (1 volume of blood to 1 volume of dimethyl sulfoxide in phosphate-buffered saline) in liquid nitrogen before being used to initiate infections in splenectomized calves (15).

Radioisotope labeling of proteins. Uninfected and *A. marginale*-infected erythrocytes (Washington and Florida isolates) were prepared as follows. Blood (30 to 50 ml) was drawn from splenectomized calves with parasitemias of 60 to 88%. The blood was centrifuged at 675 × *g* for 20 min, and the buffy coat was removed four times, with Hanks balanced salts solution without calcium and magnesium used as a wash. From 1×10^9 to 3×10^9 washed erythrocytes were then added to 1 ml of either Eagle minimal essential medium without methionine or Dulbecco modified Eagle medium, each containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. To the minimal medium was added 125 μCi of [^{35}S]methionine (1,021.4 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml, and to the Dulbecco modified minimal medium was added 250 μCi of ^{3}H-labeled mixed amino acids (1 mCi/ml; no. NET-250, New England Nuclear Corp.) per ml. The cells were incubated for 24 or 42 h at 37°C in 5% CO_2 in air and then washed four times with 30 ml of minimal medium or Hanks balanced salts solution without calcium or magnesium. Cells (10^8) were added to individual tubes and pelleted as described above. The pelleted cells were stored as a dry pellet in either lysis buffer (9.5 M urea, 2% [vol/vol] Nonidet P-40 [NP40], 1.6% [wt/vol] ampholytes at pH 5 to 7, 0.4% [wt/vol] ampholytes at pH 3 to 10, and 5% [vol/vol] β-mercaptoethanol) or sodium dodecyl sulfate (SDS)-polyacrylamide gel sample buffer (0.025 M Tris-hydrochloride [pH 6.8], 2% [wt/vol] SDS, 15% [vol/vol] glycerol, 2.5% [vol/vol] β-mercaptoethanol, and a few crystals of bromophenol blue) at −70°C until required for analysis. In certain experiments, 1 or 10 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml or 50 μg of tetracycline (oxytetracycline hydrochloride; Pfizer Inc., New York, N.Y.) per ml was added to the cultures to determine the effect on the incorporation of radioisotope.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in 7.5 to 17.5% (wt/vol) polyacrylamide gradient slab gels under reducing conditions (27). The position of ^{35}S- or ^{3}H-labeled protein bands was revealed by fixing gels in a solution containing 10% (wt/vol) trichloroacetic acid, 10% (vol/vol) acetic acid, and 30% (vol/vol) methanol, followed by processing for fluorography with En^{3}Hance (New England Nuclear Corp.) as described by the manufacturer. Dried gels were exposed to Kodak XAR-2 X-ray film at −70°C. The ^{14}C-labeled proteins used for molecular weight comparison were obtained from Amersham Corp., Arlington Heights, Ill., and included (molecular weight): myosin (200,000), phosphorylase b (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

Processing samples for SDS-PAGE. For the experiments shown in Fig. 1 and 4, 10^8 radiolabeled infected erythrocytes were solubilized directly by being boiled for 3 min in 150 μl of gel sample buffer (see above), and portions were loaded onto the gel. For the experiment shown in Fig. 2, two tubes, each containing a washed pellet of 10^8 radiolabeled cells, were processed as follows. Both pellets were frozen and thawed in 100 μl of buffer A (50 mM Tris-hydrochloride [pH 8.0], 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone) and then ultracentrifuged at 130,000 × *g* for 90 min at 4°C. The supernatant from one tube was removed, diluted with 50 μl of three-times-concentrated gel sample buffer, and boiled for 3 min (water-soluble supernatant). The pellet from the same tube was resuspended in 100 μl of buffer A, 50 μl of three-times-concentrated gel sample buffer was added, and the mixture was boiled for 3 min (water-insoluble pellet). The supernatant from the second tube was removed and discarded, and the pellet was resuspended in 100 μl of buffer A containing 1% (vol/vol) NP40. This mixture was incubated for 30 min on ice before being ultracentrifuged at 130,000 × *g* for 90 min. The supernatant was removed, diluted with 50 μl of three-times-concentrated gel sample buffer, and boiled for 3 min (detergent-soluble supernatant). The pellet was resuspended in 100 μl of buffer A containing 1% (vol/vol) NP40, 50 μl of three-times-concentrated gel sample buffer was added, and the mixture was boiled for 3 min (detergent-insoluble pellet). Equal volumes of each of the four samples (water-soluble and -insoluble fractions, detergent-soluble and -insoluble fractions) were then subjected to SDS-PAGE.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed with isoelectric focusing in the first dimension (ampholyte composition, 1.6% at pH 5 to 7, 0.4% at pH 3 to 10) and gradient SDS-PAGE in the second dimension as described by O'Farrell (20), except that the SDS-polyacrylamide gel had a linear gradient of 7.5 to 17.5% polyacrylamide. The position of ^{35}S- or ^{3}H-labeled protein spots was revealed by fluorography as described above for SDS-PAGE.

Processing samples for two-dimensional electrophoresis. Radiolabeled *A. marginale*-infected erythrocytes were prepared for first-dimension isoelectrofocusing by dissolving 10^8 cells in 150 μl of lysis buffer (see above). The lysis buffer was added either to the infected cell pellet immediately after radiolabeling or to a pellet labeled previously and stored at −70°C. The different procedures were used to test the effect of storage on the two-dimensional gel maps obtained. From 4 to 12 μl of lysate was loaded onto the first-dimension isoelectric focusing gels. To confirm whether spots on two different two-dimensional gels over-

lapped, the individual samples were always analyzed separately first and then together on the same gel.

RESULTS

Synthesis of ^{35}S-labeled proteins by *A. marginale*. The radiolabeled-protein profile of *A. marginale*-infected erythrocytes labeled by metabolic incorporation of [^{35}S]methionine is shown in Fig. 1. The polypeptides observed ranged from <14,000 to >200,000 in apparent molecular weight, the majority having an apparent molecular weight of <94,000. To confirm that only *A. marginale* proteins had been labeled, we attempted to incorporate [^{35}S]methionine into the proteins of uninfected erythrocytes taken from the same animal before infection. No radiolabeled bands were detected. The inclusion of penicillin and streptomycin in cultures had no effect on the protein bands observed. The inclusion of tetracycline in the labeling medium completely abolished the incorporation of [^{35}S]methionine into proteins (Fig. 1, lanes 6 and 12). The growth of *A. marginale* is inhibited by this antibiotic, presumably because protein synthesis is inhibited, as is the case in other bacteria (26). Conversely, cycloheximide, an inhibitor of protein synthesis in eucaryotic cells (1), had no effect on the radiolabeled protein bands observed at either 1 (Fig. 1, lanes 7 and 13) or 10 μg/ml (data not shown). These cycloheximide concentrations have been shown to inhibit protein synthesis in intact rabbit reticulocytes (5).

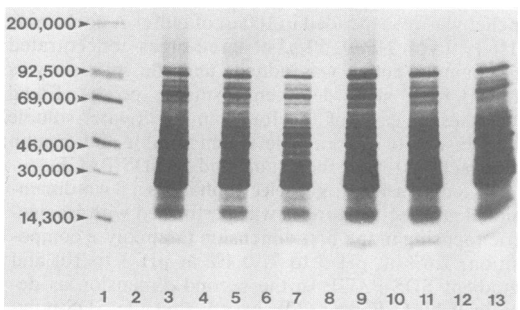

FIG. 1. ^{35}S-labeled proteins synthesized by *A. marginale* during short-term in vitro culture. Uninfected bovine erythrocytes (lanes 2, 4, 8, and 10) or cells from the same animal infected with the Washington isolate of *A. marginale* (lanes 3, 5, 6, 7, 9, 11, 12, and 13) were labeled for 24 (lanes 2 through 7) or 42 (lanes 8 through 13) h with [^{35}S]methionine. Proteins synthesized by 2×10^7 cells were analyzed by SDS-PAGE on 7.5 to 17.5% polyacrylamide gradient gels and by fluorography. Penicillin and streptomycin were omitted from the cultures shown in lanes 2, 3, 8, and 9; tetracycline (50 μg/ml) was added to the cultures shown in lanes 6 and 12; cycloheximide (1 μg/ml) was added to the cultures shown in lanes 7 and 13. ^{14}C-labeled marker proteins of known molecular weights are shown in lane 1.

Solubility of ^{35}S-labeled proteins. We next investigated the solubility of the different proteins synthesized by *A. marginale* by analyzing their relative distribution when extracted into aqueous or aqueous plus detergent phases (Fig. 2). The major labeled proteins of apparent molecular weights 94,000, 29,000, and 16,000 were predominantly water soluble and were released into the supernatant by freeze-thawing the infected erythrocytes. The majority of radiolabel was not solubilized by freeze-thawing infected cells in an aqueous buffer and remained associated with the pellet fraction. This does not mean that all these proteins are not water soluble, but merely that freeze-thawing in aqueous buffer does not extract them. Some of these pellet-associated proteins were solubilized by 1% (vol/vol) NP40 detergent, e.g., the major labeled proteins of 35,000 and 24,000 apparent molecular weight. Other proteins were not solubilized by either freeze-thawing in an aqueous buffer or by incubation in 1% (vol/vol) NP40. These results indicate that for the optimal extraction and purification of *A. marginale* proteins, a number of different aqueous and detergent solubilization methods will be needed.

Comparison of ^{35}S-labeled proteins synthesized by two isolates of *A. marginale*. After the initial characterization of *A. marginale* proteins synthesized in vitro, we compared the protein profiles of two different isolates of the organism from geographically separated areas (Florida and Washington states). Preliminary experiments revealed no difference in the one-dimen-

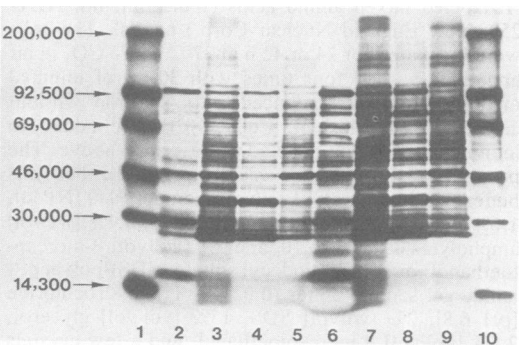

FIG. 2. Solubility of *A. marginale* proteins. Erythrocytes infected with the Washington isolate of *A. marginale* were labeled with [^{35}S]methionine for 42 h (as shown in Fig. 1, lane 11). Proteins were then extracted, first with buffer A and then with buffer A plus 1% NP40, and analyzed by SDS-PAGE on 7.5 to 17.5% polyacrylamide gradient gels and by fluorography. Lanes: 1 and 10, ^{14}C-labeled marker proteins; 2 and 6, water-soluble fraction; 3 and 7, water-insoluble fraction; 4 and 8, detergent-soluble fraction; 5 and 9, detergent-insoluble fraction. Either 15 (lanes 2 through 5) or 40 (lanes 6 through 9) μl of each fraction was loaded on the gel.

sional SDS-PAGE patterns obtained from the two isolates; we therefore used two-dimensional gel electrophoresis to improve the protein resolution. This technique should be more sensitive in detecting amino acid sequence variations between proteins, but correct storage conditions for radiolabeled samples are important to avoid the generation of artifactual spots (20). We observed that artifactual spots can result from storing radiolabeled, *A. marginale*-infected erythrocytes as a dry pellet at −70°C (Fig. 3C). These artifactual spots were eliminated when lysis buffer was used to store the samples. When samples were processed in lysis buffer, about 200 spots were resolved (on the original fluorographs), and three major labeled spots appeared to be unique to either the Washington or Florida isolate (Fig. 3A and B, arrows). These spots all had similar apparent molecular weights but different isoelectric points. Otherwise the majority of spots from the Washington and Florida isolates were found in the same position on the two two-dimensional gel maps.

Synthesis of ^{3}H-labeled proteins. To confirm the observation of similar gel maps by an independent procedure, erythrocytes infected with either of the two *A. marginale* isolates were radiolabeled with a mixture of 15 ^{3}H-labeled amino acids (not including methionine). A large number of polypeptides were labeled, the majority ranging in apparent molecular weight from 14,000 to 94,000; no polypeptides were labeled in erythrocytes taken from the same animal before infection (Fig. 4). The relative densities of the ^{3}H-labeled bands appeared to be similar to the densities of the same bands in the ^{35}S fluorographs. One exception was a band of 74,000 apparent molecular weight, which was heavily labeled in ^{35}S but not in ^{3}H fluorographs. Otherwise the similarity of the ^{3}H and ^{35}S gel profiles suggested that the density of a radioactive band was primarily an indication of the amount of protein being synthesized rather than of its methionine content.

Comparison of the two-dimensional maps obtained from the Washington and Florida isolates by ^{3}H-amino acid labeling (Fig. 5) revealed differences between the isolates similar to those seen on the ^{35}S maps. The three major spots unique to either the Washington or Florida isolate are shown (Fig. 5A and B, arrows). The reduction in the number of spots detected on the ^{3}H-labeled compared with the ^{35}S-labeled two-dimensional fluorographs was due to the lower specific activity of the ^{3}H-amino acids as compared with the [^{35}S]methionine. Even with an exposure time of 3 months for the ^{3}H gels (Fig. 5), fewer spots were detected on them than on ^{35}S gels exposed for 1 month (Fig. 3).

DISCUSSION

Short-term in vitro cultures of *A. marginale*-infected bovine erythrocytes have been shown to maintain infectivity, with microbial replica-

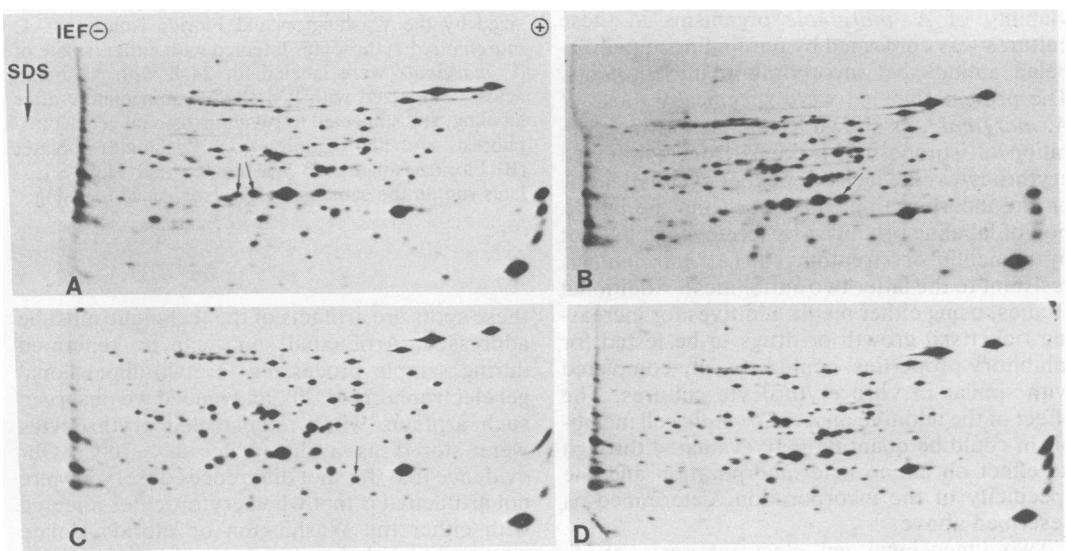

FIG. 3. Comparison of ^{35}S-labeled proteins synthesized by the Washington and Florida isolates of *A. marginale*. Infected erythrocytes were labeled for 24 h with [^{35}S]methionine, solubilized with lysis buffer, and subjected to two-dimensional gel electrophoresis and fluorography. (A and C) Washington isolate; (B) Florida isolate; (D) Washington and Florida isolates run on the same gel. (A, B, and D) Infected erythrocytes were solubilized in lysis buffer immediately after being labeled and washed. (C) Infected erythrocytes were labeled and then stored as a dry pellet at −70°C for 2 months before the addition of lysis buffer and electrophoresis. IEF, Isoelectric focusing.

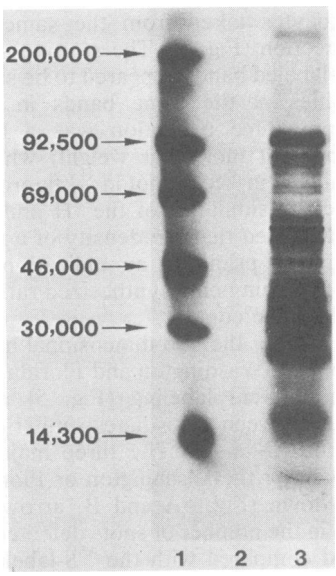

FIG. 4. ^{3}H-labeled proteins synthesized by *A. marginale* during short-term in vitro culture. Uninfected bovine erythrocytes (lane 2) and erythrocytes from the same animal infected with the Washington isolate of *A. marginale* (lane 3) were labeled for 24 h with ^{3}H-amino acids. The proteins synthesized by 2.7×10^{7} cells were analyzed by SDS-PAGE on 7.5 to 17.5% polyacrylamide gradient gels and by fluorography. ^{14}C-labeled marker proteins are shown in lane 1.

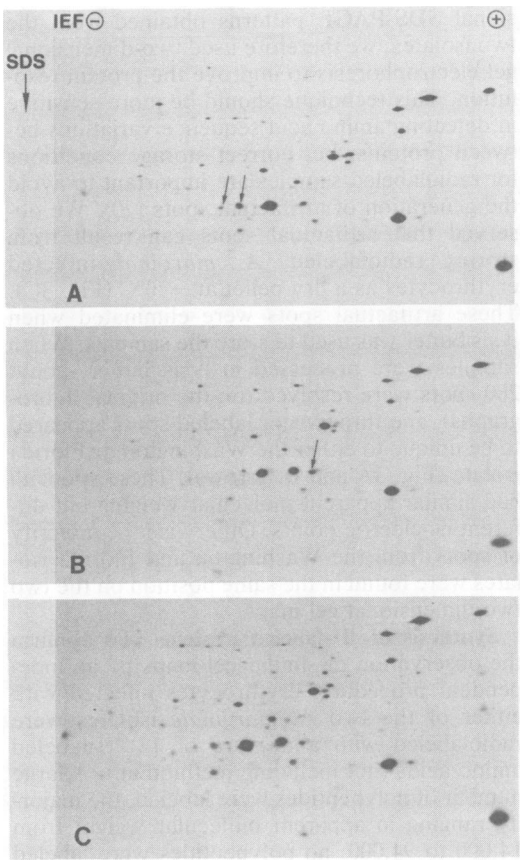

FIG. 5. Comparison of ^{3}H-labeled proteins synthesized by the Washington and Florida isolates of *A. marginale*. Erythrocytes infected with either isolate of *A. marginale* were labeled for 24 h with ^{3}H-amino acids, solubilized with lysis buffer immediately after labeling, and subjected to two-dimensional gel electrophoresis and fluorography. (A) Washington isolate; (B) Florida isolate; (C) Washington and Florida isolates run on the same gel. IEF, Isoelectric focusing.

tion (10, 18) and metabolic activity (6, 17). The viability of *A. marginale* organisms in these cultures was confirmed by our finding of radiolabeled amino acid incorporation into proteins. The proteins labeled were specifically those of *A. marginale*, as shown by the lack of incorporation of isotope into proteins from uninfected erythrocytes, the lack of effect of cycloheximide on the incorporation of isotope, and the inhibition of labeling produced by tetracycline but not by penicillin or streptomycin (*A. marginale* is resistant to the latter two antibiotics). Additional studies, using either media additives for increasing rickettsial growth or drugs to be tested for inhibitory properties, could be easily completed with similar in vitro erythrocyte cultures. The effect of the additive on *A. marginale* cell metabolism could be quantitatively evaluated through its effect on amino acid incorporation and the specificity of the incorporation, determined as described above.

Two-dimensional gel electrophoresis of *A. marginale* proteins resolved about 200 different spots. Of these, three major labeled proteins appeared to be unique to either the Washington or Florida *A. marginale* isolate. These three proteins had similar molecular weights but different isoelectric points. The possibility that these spots are artifacts of the technique must be addressed. Artifactual spots can be generated during sample processing for two-dimensional gel electrophoresis (20), and indeed we observed such artifacts when radiolabeled erythrocytes were stored as a dry pellet at −70°C. Our evidence that the spot differences observed were not artifactual is that when erythrocytes infected with either the Washington or Florida isolate were radiolabeled under identical culture conditions and then immediately lysed, and the two samples were electrophoresed simultaneously, the same unique spots were apparent. Even more convincing was the observation that identical unique spots were obtained when infected erythrocytes were labeled with either [^{35}S]me-

thionine or a ^{3}H-amino acid mixture not containing methionine.

There are various possible explanations for the origin of the protein differences observed between the Washington and Florida *A. marginale* isolates. One possibility is that there were amino acid sequence changes caused by mutation. An alternate explanation is that different proteins were expressed at different times during the infection and caused the spot changes observed in the gels. We consider this explanation less likely because *A. marginale*-infected erythrocytes were harvested for labeling at similar parasitemia levels in calves infected with either the Florida or Washington isolate. To completely eliminate the possibility, however, it would be necessary to radiolabel infected erythrocytes from both isolates at different times throughout the infection and compare the two-dimensional gel maps obtained. A third explanation is that the *A. marginale* organisms from each isolate had different morphologies and therefore synthesized some different structural proteins. This explanation is based on data showing that the structure of *A. marginale* organisms is variable. Some isolates appeared to have filamentous appendages (or "tails"), whereas others did not (11, 16). At least in part, these differences may result from different fixation techniques (25). However, using identical conditions of fixation and fluorescence microscopy, we observed appendages on the Washington isolate of *A. marginale*, whereas we did not find them on the Florida isolate. It was also observed previously by others that the Florida isolate lacks such appendages (11).

Whatever the origin of the unique spots observed on the two-dimensional gels, these proteins would assume greater significance if they were located on the surface of the *A. marginale* organism. There are some studies which suggest that antigenic differences occur among various isolates of *A. marginale* (12, 14, 21). For example, premunization of cattle with an attenuated *A. marginale* vaccine afforded complete protection against needle challenge with a virulent Texas isolate, but incomplete protection against a field strain in Colombia (14). However, in another series of experiments, premunization with an attenuated *A. marginale* strain isolated in the United States did protect against challenge with a virulent East African isolate (13).

In work with partially purified antigens, no differences were detected among the water-soluble antigens of three *A. marginale* isolates (4). In interpreting these data, one must consider the results shown in Fig. 2 of the present study, which show that many *A. marginale* proteins are not readily extracted into aqueous buffers. The water-insoluble proteins may be the important ones to consider in analyzing surface antigen changes. Clearly, further experiments need to be performed to define the extent of antigenic changes occurring among *A. marginale* organisms from various geographical locations. It should be possible to use the radiolabeling and gel techniques described here, in conjunction with immunoprecipitation and surface-reactive monoclonal antibodies, to positively identify the surface proteins of *A. marginale* and detect structural changes in these proteins in different isolates of the parasite. Proteins which are located on the surface of the organism and which are unchanged in many *A. marginale* isolates would be candidates for use in immune protection studies.

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