

POLYPEPTIDES REACTIVE WITH ANTIBODIES ELUTED FROM THE SURFACE OF *BABESIA BOVIS*-INFECTED ERYTHROCYTES

KIM P. AHRENS & DAVID R. ALLRED

Department of Infectious Diseases, P.O. Box 110880, University of Florida Gainesville, FL 32611-0880, U.S.A.

A technique was sought that would enable identification of surface-exposed parasite antigens on Babesia bovis-infected erythrocytes (BbIE) that are not detectable by surface-specific immunoprecipitations. Antibodies which bind to the surface of BbIE were recovered from intact cells using a low pH wash procedure. The eluted antibodies were then used in conventional immunoprecipitation assays to identify parasite-synthesized polypeptides carrying epitopes that are exposed on the surface or are cross-reactive with such epitopes. The results of these experiments support our previous data, obtained using a surface-specific immunoprecipitation technique, in the identification of a repertoire of parasite-derived antigens on the surface of infected erythrocytes (Allred et al., 1991). In addition, two polypeptides of Mr 68,000 and 185,000 were identified which react strongly with the eluted antibodies but which are not detected by surface-immunoprecipitation. These data illustrate the potential of this approach for identification of parasite polypeptides which carry epitopes exposed on, or cross-reactive with exposed epitopes of the infected erythrocyte surface.

Key words: *Babesia bovis* – erythrocyte surface – parasite – derived antigens – antibody elution – antigen identification

Immunity to *Babesia bovis* is widely thought to depend at least in part upon circulating antibody. The mechanisms of antibody-mediated immunity to babesiosis are not yet fully established, but could include antibody-mediated inhibition of erythrocyte invasion by merozoites (Winger et al., 1987; Figueroa & Buening, 1991); antibody-bound complement and lysis of merozoites or infected erythrocytes; and opsonization of infected erythrocytes and/or merozoites via surface-bound antibody (Mahoney et al., 1979). The antigenic profile of the *B. bovis*-infected erythrocyte (BbIE) surface would determine the specificity of the response. The derivation of the BbIE surface antigens could be: materials synthesized by the intracellular parasite and exported to the erythrocyte membrane; materials which become associated with the erythrocyte membrane during invasion or by adsorption from the plasma; or endogenous erythrocyte components which become

modified by parasite activities. The development of immunoprophylactics for immunization of cattle with defined, non-host antigens will require the identification of suitable target antigens and their subsequent cloning and expression in a recombinant system, or delivery via a live vector such as vaccinia virus or *Salmonella* spp.

The surface of the BbIE was previously shown by Curnow (1968), using an agglutination technique, to be antigenically altered; the alterations appeared to be isolate-specific. Using a solid-phase, whole-cell antigen capture assay, we have recently confirmed this result (Allred et al., 1992). Additionally, the use of surface-specific immunoprecipitation procedures enabled us to identify repertoires of parasite-synthesized antigens that are a part of that antigenic modification. In this report, we present the results of a complementary, parallel approach to the identification of polypeptides carrying surface-exposed epitopes. This technique utilizes the adsorption to, and elution from intact BbIEs of antibodies which recognize epitopes on surface-exposed antigens. The surface-eluted antibodies (EAs) can then be used in immunochemical procedures to identify

polypeptide antigens which react with the antibodies. We illustrate this approach by using immunoprecipitation and indirect immunofluorescence assays to identify the antigens recognized by the eluted antibodies, and to demonstrate their subcellular location. Our results provide confirmatory evidence for several of the previously identified surface antigens and suggest that two additional candidate antigens of Mr 185,000 and 68,000 are either surface-exposed or are cross-reactive with antigens present on the BbIE surface.

MATERIALS AND METHODS

Parasite – A United States isolate of *B. bovis*, designated TX for its origin in southern Texas, was used in this study. This isolate was the kind gift of Dr W. Goff, United States Department of Agriculture. The parasites were maintained *in vitro* under microaerophilous stationary phase (MASP) conditions, as described elsewhere (Levy & Ristic, 1980).

Antisera – The antisera used in this study were hyper-immune, post-infection sera collected from Holstein steers, # 326 (spleen-intact) and # 2106 (splenectomized), after repeated challenge with virulent parasites of the TX isolate. The specificities of these antisera for this isolate have been previously described (Allred et al., 1992).

Elution of antibodies – Intact infected bovine erythrocytes were enriched from *in vitro* cultures on Percoll cushions as described (Allred et al., 1992). The infected erythrocytes were washed two times at 2000 xg for 5 min at 4°C, using Vega y Martinez (VYM) buffer (Vega et al., 1985), and were resuspended to a 50% packed cell volume in additional VYM buffer. "Free parasites" were collected by incubating culture at 37°C under ambient gas conditions for 4 h to reduce reinvasion. Parasites were then harvested by differential centrifugation at 200 xg for 5 min, followed by centrifugation at 3000 xg for 10 min. The parasites were washed with VYM buffer as above and resuspended to a 10% suspension. This parasite preparation, while primarily intact parasites, also was contaminated with erythrocyte stroma and other particulate debris. No attempt was made at this point to prevent such contamination. A quantity of antiserum equal to 1/4 the volume of the cell suspensions was added to each. The cells were incubated with the antiserum for 1 h at 4°C on a rotating mixer, then were washed four

times with VYM buffer as before. The antibodies were then eluted with 0.1 M glycine HCl, 0.1 M sodium chloride, pH 3.0, as described by Rekvig & Hannestad (1977). After incubation in the low pH buffer, the cells were sedimented at 15,000 xg for 30 seconds. The supernatant was collected and immediately neutralized with 0.5 M tris, pH 10.5. The antibodies were concentrated by centrifugation through Centricon (Amicon, Danvers) ultrafiltration units with a molecular weight cutoff of 30,000 and were washed by repeated dilution and re-concentration from 0.01 M tris, 0.15 M sodium chloride, pH 7.5. Ovalbumin (Sigma Chemicals, St. Louis) was added to a final concentration of 1 mg/ml to help stabilize the antibodies.

Immunoassays – Indirect immunofluorescence assays, conventional immunoprecipitations, pronase treatment of intact erythrocytes, and the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of samples were performed as described previously (Allred et al., 1992).

RESULTS

Recovery of antibodies – Recovery of antibody from the surface of the infected cells was detected by the use of an indirect immunofluorescence assay (IFA). Eluted antibodies (EAs) were incubated on acetone-fixed smears of *B. bovis* cultures at a dilution of 1/10, then were visualized using affinity-purified rabbit anti-bovine immunoglobulin G antibody conjugated with fluorescein isothiocyanate (FITC-R α BIgG). Figure 1 demonstrates the reactivity of the EAs with infected erythrocytes. Both the erythrocyte and the intracellular parasite fluoresce when incubated with EAs adsorbed from hyper-immune post-infection serum on BbIE. No fluorescence was observed when antibodies adsorbed from pre-immune serum were used as the primary antibody, when primary antibody was omitted, or when using EAs that had been adsorbed from hyper-immune serum onto uninfected erythrocytes. The pattern of fluorescence observed with BbIE - eluted hyper-immune EAs matched that seen when hyper-immune serum was used as the primary antibody. Membrane-associated fluorescence detected with the BbIE-eluted hyper-immune EAs was observed to increase progressively with maturation of the intracellular parasite (Fig. 2). Treatment of intact BbIE with pronase prior to their use in IFAs had little or no obvious effect on

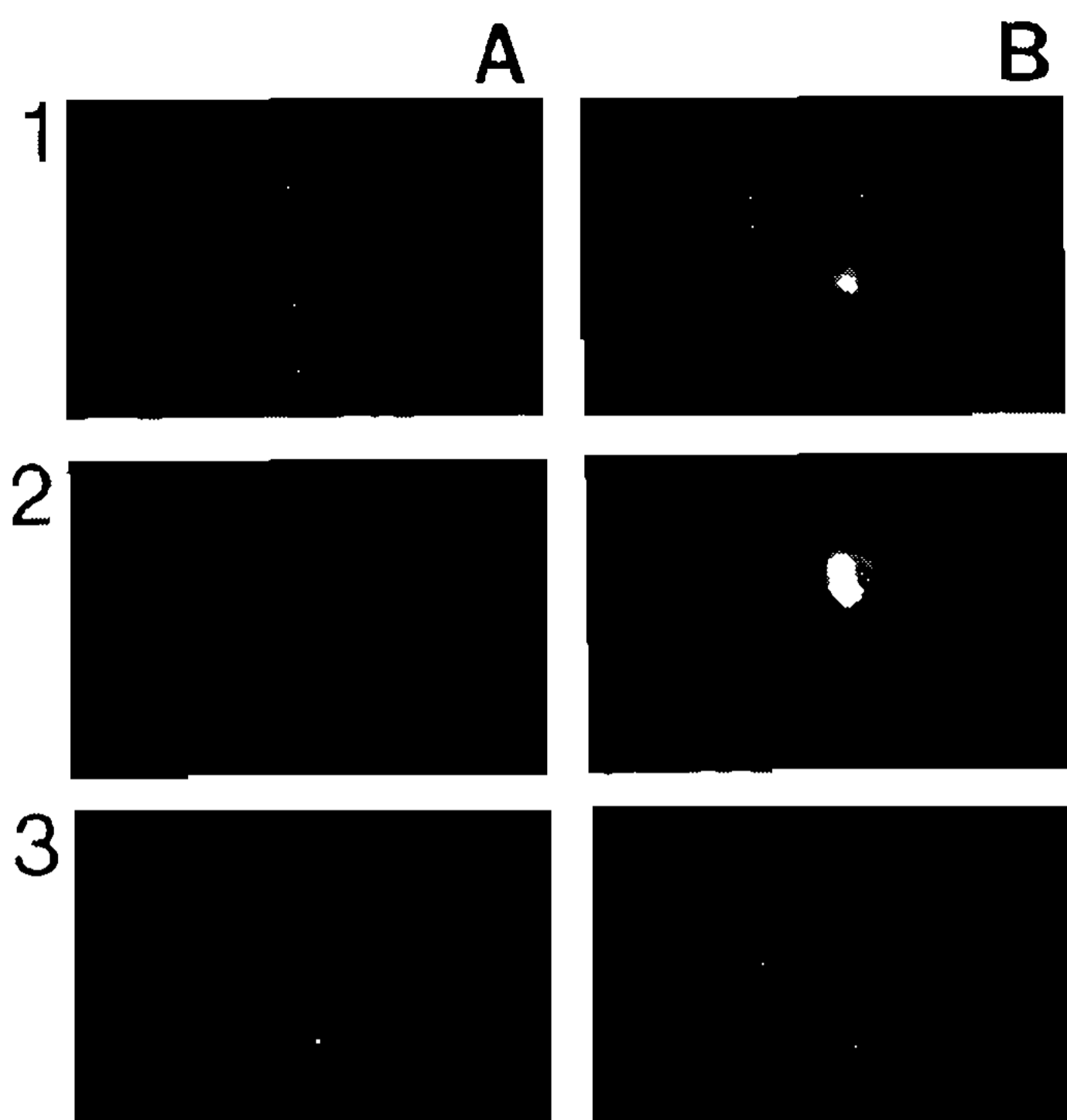


Fig. 1: indirect immunofluorescence assay for antigens on TX isolate *Babesia bovis*-infected erythrocytes. Panels: A, pre-immune serum or antibodies; B, hyper-immune serum or antibodies; 1, infection sera from calf B326 (anti-TX isolate); 2, B326 antibodies eluted from the surface of TX-isolate *B. bovis*-infected erythrocytes; 3, B2106 antibodies eluted from the surface of uninfected erythrocytes held in culture for three days.

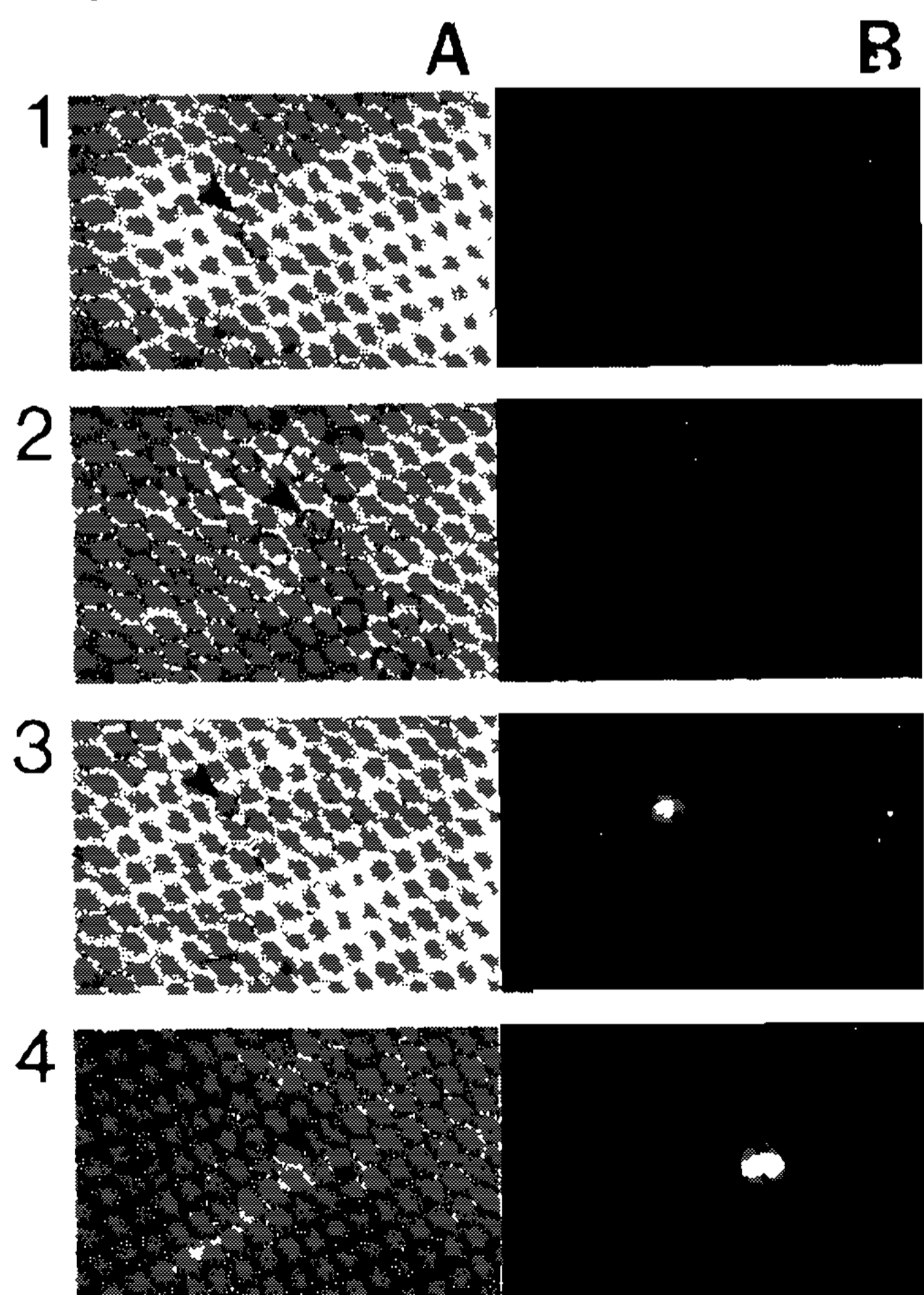


Fig. 2: correlation of parasite maturation with parasite- and membrane-associated fluorescence, as detected by IFA with surface-eluted antibodies. Panels: A, phase-contrast images of erythrocyte fields; B, fluorescence images of the same fields; 1, BbIE-eluted pre-immune B326 EAs; 2-4, BbIE-eluted hyper-immune B326 EAs. Panels 2-4 show erythrocytes containing parasites of progressively greater maturation. The infected erythrocytes are indicated with arrowheads.

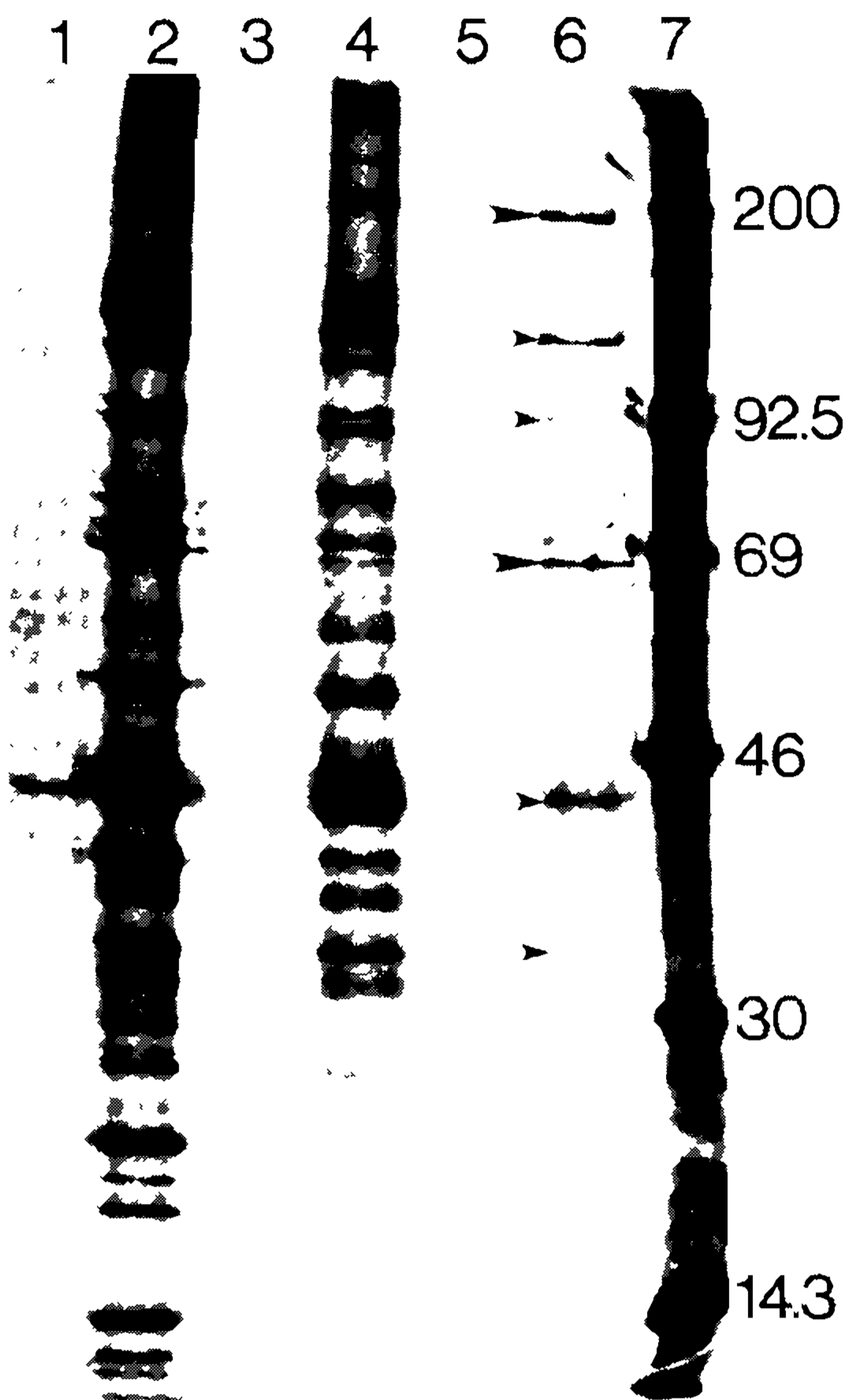


Fig. 3. immunoprecipitation of *Babesia bovis*-synthesized polypeptides with antibodies eluted from the cell surface. Immunoprecipitated antigens were analysed by SDS-PAGE on 7.5-16% gradient gels. Lanes: 1,2 B326 serum; 3,4, antibodies eluted from a *B. bovis* "free parasite" preparation; 5,6, antibodies eluted from the surface of intact *B. bovis*-infected erythrocytes; 7, [¹⁴C]-molecular mass markers. Lanes 1, 3 and 5 involved pre-immune serum or antibodies, whereas lanes 2, 4 and 6 involved hyper-immune serum or antibodies. The relative mass of each molecular weight standard is indicated in kilodaltons.

fluorescence of the intracellular parasite, or the BbIE membrane or cytoplasm (not shown).

Specificity of recovered antibodies – The specificity of the EAs for parasite-derived antigens was determined by immunoprecipitation of biosynthetically-labeled antigens from BbIE of the TX strain that were prepared as NP-40-soluble antigen, as previously described (Allred et al., 1992). Antibodies adsorbed from pre-immune or hyper-immune sera then eluted from the surface of infected or uninfected cells were used, along with samples in which the primary antibody was omitted. Unfractionated pre-immune and hyper-immune sera were used as controls on the total

immunoprecipitation patterns. The results of one such experiment are shown in Fig. 3. Four of the bands precipitated by surface-immunoprecipitation (Allred et al., 1991), namely the Mr 125,000, 93,000, 42,000 and 33,500 bands (marked with small arrowheads), are also immunoprecipitated with hyper-immune eluted antibodies. In addition, major bands of Mr 185,000 and 68,000 were precipitated using the hyper-immune EAs (large arrowheads; Fig. 3). No significant precipitations occurred when pre-immune EAs, pre-immune serum, or hyper-immune EAs eluted from uninfected erythrocytes were used as primary antibody (Fig. 3), or upon omission of the primary antibody (not shown). In contrast, hyper-immune serum precipitates a large number of immunoreactive-synthesized polypeptides, as expected (Fig. 3).

DISCUSSION

Immunity to bovine babesiosis is thought to be mediated in part by a humoral response against the infected erythrocyte. Immunity following a single exposure to *B. bovis* may be solid against homologous challenge, but is only partially cross-protective against heterologous isolates (Mahoney et al., 1979). The antibody response to the infected erythrocyte surface is similarly isolate-specific (Curnow, 1968; Mahoney et al., 1979; Allred et al., 1992). Therefore, antibodies directed against the infected erythrocyte surface may play a role in determining the severity of disease. Often, in areas of endemic stability, it would be advantageous to ameliorate the severity of the disease without necessarily preventing it altogether. It would therefore be useful to identify the parasite-derived antigens on the infected erythrocyte surface and to determine the nature of the antigenic diversity displayed among isolates. We previously described the identification of repertoires of isolate-specific surface antigens using surface-specific immunoprecipitations of *B. bovis*-infected erythrocytes that had been metabolically labeled with [³⁵S]-methionine. This method involved the use of isolate-specific polyclonal hyper-immune infection sera (Allred et al., 1992). We identified on the TX isolate a repertoire of seven surface antigens of Mr 135,000, 125,000, 107,000, 93,000, 42,000, 33,500 and 16,000. However, drawbacks to that approach include the necessity for the antigen/antibody complexes to be solubilized by non-ionic detergents and to remain stably associated during subsequent handling. Surface antigens which were not easily solubilized in non-ionic detergents because of asso-

ciation with certain erythrocyte components, such as cytoskeletal elements, would then be undetected. To circumvent this problem, we bound antibodies to the surface of intact infected erythrocytes, then eluted the antibodies and used them in immunoprecipitation and IFA assays to identify the antigens recognized and their subcellular locations.

The low pH method of antibody isolation described in this study was originally developed for use in blood banking to allow the identification of offending antibodies in human transfusion reactions (Rekving & Hannestad, 1977). In our experience, little hemolysis of infected or uninfected cells occurs during stripping of the antibodies from the cell surface. The bovine antibodies that were recovered by this technique functioned well as the primary antibody in IFA reactions and in immunoprecipitation reactions. Background from non-specifically bound or trapped antibodies was negligible.

The EAs that are recovered from the surface of the TX isolate, when used in IFAs, reacted with the parasite at all stages of development, although reactivity was least with young trophozoites. On the other hand, fluorescence of the erythrocyte cytoplasm and membrane was absent in cells infected with very young parasites, but appeared and increased progressively with parasite maturation, indicating the accumulation of antigen in each compartment (Fig. 2). This result is consistent with, although it does not constitute proof for, the export of these antigens from the parasite to the erythrocyte surface. The testing of this hypothesis will await immunoelectron microscopic localization of the antigens with monospecific antibodies. Cloning of the encoded genes and observations of their development expression will further substantiate the nature of these antigens.

Antibodies eluted from the surface of infected erythrocytes taken from *in vitro* cultures of *B. bovis* captured four of the seven surface antigens previously identified on the TX isolate by surface-immunoprecipitation (Allred et al., 1992); namely, those of Mr 125,000, 93,000, 42,000, and 33,500. Antigens of Mr 135,000, 107,000 and 16,000, also a part of the surface antigen repertoire, were captured poorly with this antibody preparation. Similar preparations made from the same hyper-immune serum have effectively captured the Mr 135,000 and

107,000 antigens in other experiment (not shown), whereas the 16,000 Mr antigen has not been. Each of these is a quantitatively minor antigen relative to the others detected by surface-immunoprecipitation and may represent low abundance, specifically processed forms of the more abundant antigens. On the other hand, the EAs identified two additional antigens which carry epitopes that are surface exposed or are cross-reactive with epitopes exposed on the surface of the infected erythrocyte. In the TX isolate these antigens are approximately Mr 185,000 and 68,000 as determined by SDS-PAGE.

One anomaly that must be addressed is that surface-immunoprecipitations, which are performed by binding antibodies to intact erythrocytes prior to washing and solubilizing the cells, gave no indication that these two antigens might be present on the infected erythrocyte surface. Although strong reactivity of the BbIE-eluted EAs occurred with the 185,000 and 68,000 polypeptides in conventional immunoprecipitation, this does not constitute proof that these two polypeptides are themselves exposed on the infected erythrocyte surface. Indeed, the results of IFA analyses with BbIE-eluted EAs indicated that the antigens recognized were associated not only with the erythrocyte membrane, but also with the erythrocyte cytoplasm and the intracellular parasite (Fig. 1). Removal of surface antigens from the infected erythrocyte with pronase treatment did not abrogate immunofluorescence. This is not surprising as antigens that are present beneath the membrane cannot be distinguished from those on the surface by IFA because of the small size of the cells and the limited resolution of light microscopy. Additional scenarios besides surface exposure include the possibilities that these polypeptides are precursors to the other surface antigens which have not been processed, or that they are "unrelated" polypeptides which coincidentally share cross-reactive epitopes with surface-exposed antigens. Alternatively, these polypeptides may be present on the erythrocyte surface but are not solubilized by NP-40 when in this location. A strong precedent for this exists with the surface antigen, PfEMP-1, of *Plasmodium falciparum* (Leech et al., 1984) and the SICA⁺ antigen of *Plasmodium knowlesi* (Howard et al., 1983). PfEMP1 and SICA⁺ are not solubilized from infected erythrocytes with Triton X-100; however, those antigens can be solubilized with 2% sodium dodecylsulfate (SDS) and immunoprecipitated after dilution of the SDS. Association with erythrocyte com-

ponents, perhaps cytoskeletal in nature, prevents the solubilization of the PfEMP-1 and SICA⁺ antigens with non-ionic detergents. In contrast, the metabolically-labeled 185,000 and 68,000 polypeptides of *B. bovis*-infected erythrocytes are solubilized with non-ionic detergent during conventional immunoprecipitation (Fig. 3). However, those copies of each polypeptide that are solubilized with NP-40 could represent nascent polypeptides that have not yet been matured to a form, or transported to a site that is insoluble in non-ionic detergents. These possibilities await further clarification.

The approach described in this study does not directly identify the antigens that are exposed on the surface of the infected cell. However, it does allow the rapid identification of polypeptides carrying epitopes that are exposed on the surface or which cross-react with such epitopes, and it is easily performed with polyvalent antisera. Therefore, with refinement this approach may yield a rapid assay for the identification of putative surface antigens on intact parasites or parasitized host cells. Similarly, cross-reactive surface antigens could be identified among antigenically diverse isolates by the adsorption and elution of antibodies from heterologous antisera, and their subsequent use in analytical techniques. These possibilities are being explored.

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