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Identification and partial purification of soluble antigens from culture-grown *Besnoitia besnoiti* endozoites

SHKAP (V.), UNGAR-WARON (H.), PIPANO (E.). Identification et purification partielle d'antigènes solubles à partir de culture d'endozoïtes de *Besnoitia besnoiti*. *Revue Élev. Méd. vét. Pays trop.*, 1990, 43 (1) : 63-68.

Des antigènes solubles obtenus à partir des endozoïtes de cultures de *Besnoitia besnoiti* ont été identifiés après leur purification partielle par chromatographie par affinité. Un éluat spécifique obtenu à partir de cette méthode sur une colonne à laquelle avaient été fixés des anticorps provenant du sérum d'une vache naturellement infectée, a montré 7 bandes de polypeptides par électrophorèse (SDS-PAGE). Cinq bandes ont été observées dans l'éluat à partir d'un immuno-adsorbant auquel avaient été couplés des anticorps provenant d'un veau expérimentalement infecté. Les antigènes de l'éluat ont donné une réaction dans le test ELISA. La réactivité des antigènes séparés par électrophorèse avec des sérums bovins inoculés avec les endozoïtes, et des sérums provenant de cas de besnoitiose contractée sur le terrain, a été étudiée par immunotransfert selon la technique de WESTERN. *Mots clés* : *Besnoitia besnoiti* - Endozoïte - Antigène - Isolement - Chromatographie - Technique immunologique.

Humoral antibody formation, of unknown relevance to protective immunity, is characteristic of natural and experimental infection, and is the basis for serological diagnosis (6, 7, 8, 9, 12, 18, 25). However, there is little definitive information on antigens inducing the humoral immune responses in the host.

In the present study we partially purified and identified *B. besnoiti* antigens from culture-grown endozoites by affinity chromatography followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western immunoblotting was used for analysis of the endozoite antigens reacting with sera obtained from *B. besnoiti* naturally or experimentally infected cattle.

INTRODUCTION

Besnoitia besnoiti is a coccidian protozoan causing bovine besnoitiosis (27). The disease is of economic importance because of the losses it causes to the cattle industry due to its high morbidity rate and to hempered reproduction, as affected bulls often become sterile (4, 23). In the acute stage of the disease, fever, inappetance, loss of weight and anasarca are apparent. The acute stage is followed by a chronic stage which is characterized by alopecia, sclerodermia, *seborrhea sicca*, the presence of cysts in the skin and subcutaneous tissues, and orchitis (4). The two developmental stages of the parasite which have been described are endozoites and cystozoites. Endozoites are considered as the fast multiplying organisms which appear intra-or extracellularly in blood smears of experimentally infected cattle and rabbits during the acute stage of besnoitiosis (3, 21, 22). Endozoites can be both propagated in cell cultures and in cells lining the peritoneal cavity of susceptible laboratory animals (2, 17, 19, 25). Cystozoites are the slowly multiplying organisms which develop in cysts during the chronic stage of besnoitiosis (1, 3).

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MATERIALS AND METHODS

Parasites

Besnoitia besnoiti endozoites were obtained and propagated in Vero cell cultures as described by SHKAP, PIPANO and GREENBLATT (25).

Endozoites lysate

Endozoite suspensions free of host cells were obtained by separation on Whatman CF-11 column as described by SHKAP, UNGAR-WARON, PIPANO and GREENBLATT (26). The endozoites were lysed with 0.5% (v/v) Nonidet-40 (NP-40) in the presence of protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.25 M iodoacetamide and 100 KIU/ml of trasylol (Kallikrein Inactivator Units aprotinin, Bayer, Germany), for 18 h at 4 °C. The lysate was then dialysed against PBS, centrifuged for 15 min at 12,000 xg, the precipitate discarded and the clear solution kept frozen at -20 °C until further use.

Sera

Four sera from *B. besnoiti* naturally infected cattle and positive by the indirect fluorescent antibody test

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(IFAT) were used (438, 609, 610, 639). Another six sera (201, 349, 386, 387, 392, 394) were obtained from cattle inoculated with live culture-grown endozoites. Of these, calf 201 received intravenously 10^8 endozoites twice at two months interval and serum was collected at its maximal 1:16,000 IFAT titre. Serum samples from the other 6 calves were collected at titres of 1:4,000 after inoculating them once with 10^9 to 2.5×10^9 live endozoites.

Protein determination

The protein content of the antigenic preparations was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDAL (15) with bovine serum albumin as standard.

Preparation of immunoabsorbents

Crude immunoglobulin fractions (Ig's) from serum of a naturally infected cow (438) and from an experimentally infected calf (201) were precipitated with ammonium sulfate at 40% saturation. After dialysis against PBS, these preparations served for the further coupling to Sepharose 4B (Pharmacia, Uppsala) activated with cyanogen bromide as described by PORATH, AXEN, and ERNBACK (24). Twenty gram of CNBr-activated Sepharose were added to a mg/ml solution of the crude Ig's in 0.1 M NaHCO_3 (50 ml). The mixture was kept overnight at 4°C with gentle stirring, filtered and washed with 0.1 M NaHCO_3 . The immunoabsorbent was then blocked with 0.1 N ethanolamine, rinsed with water, PBS, 0.1 M acetic acid, again with PBS, 0.1 M NH_4OH . The immunoabsorbents were stored in PBS containing 0.02% sodium azide at 4°C. Under these conditions 2-2.5 mg protein were bound per gram of wet weight Sepharose.

Fractionation of endozoite lysates on immunoabsorbents

Crude lysates of endozoites in PBS were passed on column-packed immunoabsorbents (Sepharose-Ig's-438 and Seph-Ig's-201). Absorbance at 280 nm of 1 ml fractions was followed until all unbound proteins were removed. Column-bound antigens were eluted with 0.1 M NH_4OH , pH 9.6, and the protein-containing fractions pooled. The solution was immediately brought to a neutral pH and dialysed against PBS containing protease inhibitors at a concentration 10 times lower than mentioned above for endozoite lysis. Aliquots of affinity-purified antigens were freeze-dried and kept at 4°C until further used.

ELISA tests

Polystyrene plates (Nunc, Denmark) were coated either with the crude endozoite lysate or with affinity-purified antigens. Coating was performed at 5 µg/ml protein in 0.1 M carbonate-bicarbonate buffer, pH 9.6, and incubated for 18 h at 4°C. All steps of the ELISA reactions were performed in volumes of 0.1 ml per well. After the plates had been rinsed 3 times with PBS containing 0.05% Tween 20 (PBST), serum samples diluted 1:100 in PBST and 0.02% BSA were introduced into antigen-coated wells and further incubated at 4°C for 18 h. After 3 more washings, alkaline phosphatase-conjugated rabbit anti-bovine IgG (Bio-Makor, Rehovot, Israel) was introduced into the wells at a 1:2 000 dilution and incubated at 37°C for 2 h. An additional washing step identical to the prior one was carried out after incubation. The substrate disodium-4-nitrophenyl phosphate hexahydrate (Fluka) made up to a mg/ml solution in 0.05 M sodium carbonate buffer containing 1 mM MgCl_2 , pH 9.6 was added to the wells and incubation proceeded for 30 min at room temperature. Reaction was stopped by addition of 50 µl 3N NaOH per well and absorbance measured at 405 nm in an automatic MR 580 Dynatech ELISA reader.

Western immunoblotting

Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulphate (SDS) and 10% polyacrylamide (SDS-PAGE) was performed according to LAEMMLI (14). Samples were reduced with 2-mercaptoethanol in 1% SDS before being applied to the gel. After electrophoresis at 160 V for 5 h, gels were either stained with Coomassie brilliant blue (Schwartz, Mann.), or transferred to 0.45 µm nitrocellulose paper in a Hoefer TE-42 Transfor apparatus. Electrotransfer was carried out as described by TOWBIN, STAHELIN and GORDON (28) in Tris-glycine buffer (25 mM Tris, 192 mM glycine), pH 8.3 and 20% (v/v) methanol at 45V, for 18 h at 4°C. The nitrocellulose was further blocked with a 3% casein solution by incubation for 3 h at room temperature and washed 3 times with PBST. This was followed by incubation with bovine sera diluted 1:50 in PBS containing 1% BSA, for 18 h at 4°C. After 3 additional washings with PBST, the nitrocellulose was incubated with a peroxidase-conjugated affinity purified rabbit anti-bovine IgG (Kirkegaard and Perry, Maryland) diluted 1:1 000 in PBS containing 1% BSA, for 2 h at room temperature. The membrane was washed as above, and bands were visualized by immersing the membrane in a fresh solution of 0.005% 3,3'-diaminobenzidine (Sigma, Mass.) and 0.03% cobalt chloride supplemented with 0.4% H_2O_2 . Reference molecular weight markers run in parallel with samples included heavy and light chains of bovine IgG and IgM, BSA and ovalbumin.

RESULTS

Endozoite lysates containing 5 mg protein were applied to the immunoabsorbents. Affinity chromatography was performed as described in Materials and Methods. Recovery of the bound protein was 10 % of the total amount of the endozoite lysate loaded onto either column as measured by absorbance at 280 nm. The band pattern of the antigenic preparations before and after affinity chromatography on Sepharose Ig's-438 followed by SDS-PAGE and stained with Coomassie brilliant blue is shown in Figure 1. The crude endozoite lysate exhibited at least 22 polypeptide bands, of which 3 main and 4 faint bands of apparent molecular weight of 125 000, 97 000, 90 000, 71 000, 38 000, 32 000 and 26 000 were seen in the specific eluate dissociated from the column-bound antibodies. A similar electrophoretic pattern of eluted antigens was observed when an endozoite lysate was loaded on the Sepharose-Ig's-201 immunoabsorbent (Fig. 2), except for the 125 000, 38 000 and 32 000 proteins which did not appear in the specific eluate from the latter immunoabsorbent. An evident but faint band of about 60 000 observed in the eluate obtained from the Sepharose- Ig's- 201 column was not discernible in the eluate from 438-Ig's bound Sepharose.

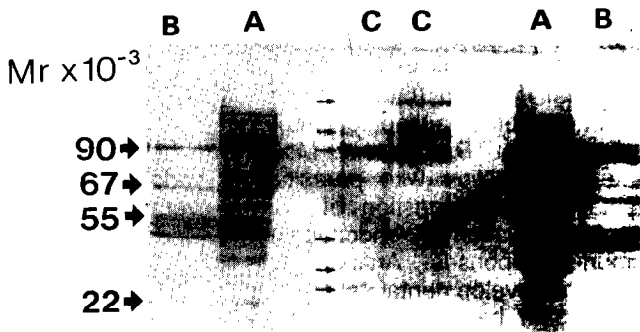


Fig. 1 : Polypeptide bands resolved on 10 % SDS-PAGE under reduced conditions and stained with Coomassie brilliant blue : crude *B. besnoiti* endozoite extract applied to a column of Sepharose-Ig's from a naturally infected cow (A) ; unbound eluate from the column (B) and specific eluate (C). The position of MW markers is indicated on the left side.

ELISA microtitre plates were coated with either crude *B. besnoiti* endozoite antigen or with specific eluate and further reacted with different serum samples (438, 609, 610, 639, 201, 387 IFAT-positive, and 6 IFAT-

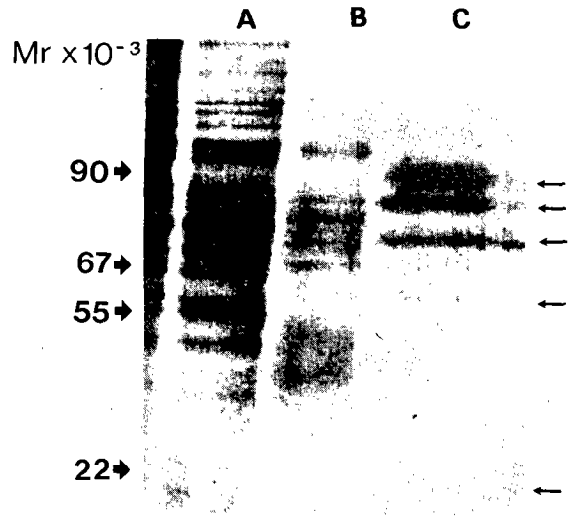


Fig. 2 : Polypeptide bands resolved and stained as above : crude *B. besnoiti* endozoite extract applied to a column of Sepharose-Ig's from a hyperimmune bovine serum (A) ; unbound eluate (B) and specific eluate (C).

negative). As shown in Figure 3, lower ELISA absorbance readings were recorded with the affinity purified antigens as compared to reactivity of the crude lysate using the same serum samples. On the other hand, the background was lower with the purified antigen than with the crude lysate. Eight additional serum samples exhibiting positive IFAT titers up to 1:256 to *Toxoplasma gondii*, to *Anaplasma centrale* and to *Babesia bovis* were also examined (data not shown). The results obtained were in the range of ELISA readings

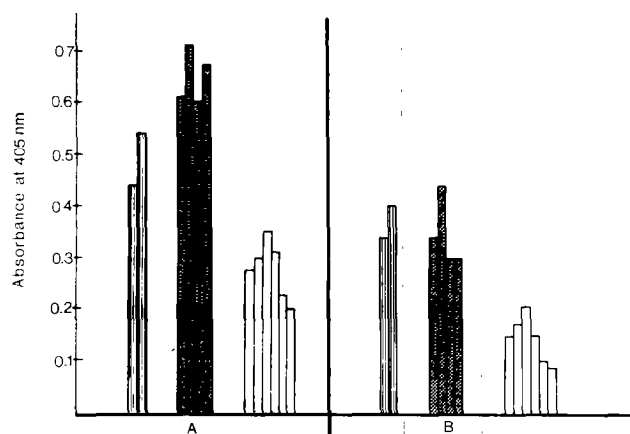


Fig. 3 : ELISA readings obtained by coating plates with crude *B. besnoiti* endozoite lysate (A) and the affinity purified antigen (B). The tested sera came from experimentally infected cattle, naturally infected cattle, and *Besnoitia* IFAT-negative cattle. Bars represent individual serum samples.

observed with *Besnoitia*-IFAT-negative cattle as in Figure 3. Reactive antigens of electrophoretically resolved *B. besnoiti* endozoite lysates were identified using the Western immunoblotting technique. Bovine sera from either endozoite inoculated or naturally infected cattle reacted with *B. besnoiti* antigens and exhibited multiple banding patterns (Fig. 4). The homologous sera from endozoite inoculated cattle revealed about 12 polypeptide bands, 7 of which were intensely stained and were of relative molecular weight ranging from 97 000 to 26 000. The binding pattern observed with antisera from a naturally infected cattle (lines 1-4) was similar to that seen in the homologous reaction, except for at least 2 bands of 125 000 and 123 000. These bands were clearly seen in the heterologous reactions, but did not appear in reactions with the anti-endozoite sera (lines 5-10). No immunostaining was observed with two sera samples from *Besnoitia*-IFAT-negative cattle or bovine sera with an antibody titre of 1:256 to *Toxoplasma gondii* (data not shown).

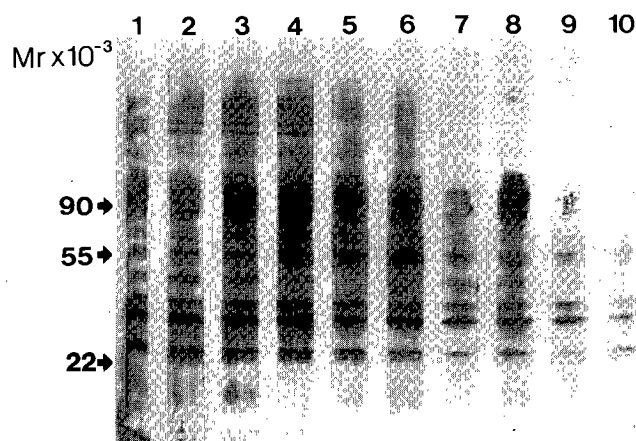


Fig. 4 : Western immunoblots of the endozoite antigens reacting with four sera from naturally infected cattle (lanes 1-4) and six sera from endozoite-inoculated animals (lanes 5-10).

DISCUSSION

The results presented above showed the feasibility of applying the affinity chromatography technique for purification and isolation of serologically active *Besnoitia besnoiti* antigens from lysates of culture-grown endozoites. Although only 10 % of the total protein lysate were bound and eluted from the columns, the process of desorption of antigens from immunoadsorbent did not affect the functional activity of the eluted

proteins as observed in the ELISA tests. It has to be noted that although higher background ELISA readings were observed with the crude lysate than with the immunopurified antigens, the specificity of either antigenic preparation was ascertained by the negative results obtained with sera to *Toxoplasma gondii*, *Anaplasma centrale* and *Babesia bovis*. Reduced ELISA background readings were obtained when higher dilutions of sera were used (data not shown), however a 1:100 dilution was chosen as being adequate for the detection of antibodies in weakly positive sera. Upon affinity chromatography seven polypeptide bands were resolved after SDS-PAGE of the purified endozoite antigen on Sepharose-Ig's-438 (Fig. 1), while only five bands appeared after elution of the specific antigen on Sepharose-Ig's-201 (Fig. 2). Indeed, the immunoblot analysis of electrophoretically resolved antigens from the crude lysate revealed differences in the binding pattern of sera from naturally infected cattle when compared to reactivity of sera from experimentally-induced infection by endozoites. The 125 000 and 123 000 Coomassie-stained proteins of the specific eluate obtained from Sepharose-Ig's-438 were exhibited in the Western blots with serum 438 and with three more sera obtained from field cases of bovine besnoitiosis (Fig. 4). These proteins were not observed in the affinity purified eluate from Sepharose-Ig's-201 column run on SDS-PAGE and further stained with Coomassie blue, nor in the blots. On the other hand, the 60 000 band which was clearly stained in only one of the specific eluates (Fig. 2) was detected in all blots (Fig. 4). These results indicated the presence of some unique antigens due to natural infection (by oocysts ?) or the stage-specific diversity in the antigenic profile of cystozoites and endozoites of *B. besnoiti*. Moreover, the inoculation route, or infection with parasites at various developmental stages (ingestion of oocysts versus subcutaneous inoculation of endozoites) may induce different immune responses. Antigenic dissimilarities between different developmental stages in the life cycle of *Toxoplasma* have been reported (5, 20, 11, 16, 20). LUNDE and JACOBS (16) showed that fluorescent-labelled antibodies reacted with bradyzoites only, while anti-tachyzoite serum reacted with both tachyzoites and bradyzoites. A unique immunogenic protein distinguishing between acute and chronic infection has been described (5). IgM antibodies directed against a 6kD antigen were detected in sera from acute, but not chronic toxoplasmosis (5). Using monoclonal antibodies to tachyzoites and sporozoites of *T. gondii*, unique stage-specific proteins were demonstrated by one- and two- dimensional SDS-PAGE and Western immunoblotting (10). Similar findings were reported for merozoites and sporozoites of another coccidian protozoan *Eimeria faeciformis* (13). Protection studies with *Besnoitia* antigens described here need to be performed to define the target proteins of the protective immune response.

SHKAP (V.), UNGAR-WARON (H.), PIPANO (E.). Identification and partial purification of soluble antigens from culture-grown *Besnoitia besnoiti* endozoites. *Revue Élev. Méd. vét. Pays trop.*, 1990, 43 (1) : 63-68.

Soluble antigens from culture-grown *Besnoitia besnoiti* endozoites were identified following their partial purification by affinity chromatography. A specific eluate obtained after affinity chromatography on a column to which antibodies from serum of a naturally infected cow were bound exhibited seven polypeptide bands on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Five bands were observed in the eluate from an immunoadsorbent to which antibodies from an experimentally infected calf were coupled. Eluted antigens were reactive in the enzyme-linked immunosorbent assay (ELISA). Reactivity of electrophoretically resolved antigens with sera of endozoite-inoculated cattle and sera from field cases of besnoitiosis were studied using the Western immunoblotting technique. *Key words* : *Besnoitia besnoiti* - Endozoite - Antigen - Isolation - Chromatography - Immunological test.

SHKAP (V.), UNGAR-WARON (H.), PIPANO (E.). Identificación y purificación parcial de antígenos solubles a partir de cultivos de endozoitos de *Besnoitia besnoiti*. *Revue Élev. Méd. vét. Pays trop.*, 1990, 43 (1) : 63-68.

A partir de endozoitos de cultivos de *Besnoitia besnoiti* se obtuvieron e identificaron antígenos solubles, previa purificación parcial mediante cromatografía de afinidad. Un eluato específico, obtenido mediante este método, sobre una columna a la cual se fijaron anticuerpos provenientes del suero de una vaca infectada en forma natural, mostró 7 bandas de polipéptidos por electroforesis en gelatina de poliacrilamida dodecilsulfato sódica (SDS-PAGE). Mediante un adsorbente inmunológico, al cual fueron acoplados anticuerpos provenientes de un ternero infectado experimentalmente, se observaron 5 bandas en el eluato. Los antígenos del eluato reaccionaron ante el test ELISA. La reactividad de los antígenos separados por electroforesis, con los sueros bovinos inoculados con los endozoitos y con los sueros provenientes de besnoitiosis contraída en el campo, se estudiaron mediante inmunotransferencia, siguiendo la técnica de WESTERN. *Palabras claves* : *Besnoitia besnoiti* - Endozoito - Antígeno - Aislamiento - Cromatografía - Técnica inmunológica.

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