Antibody Responses of Cattle Immunized with the Tf190 Adhesin of Trichomonas foetus

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The antibody response patterns of cattle after subcutaneous and intranasal immunizations with adhesin Tf190 of Trichomonas foetus were investigated. Reactions of antibody from cattle parenterally immunized with Tf190 revealed antigen specificity and Tf190 sensitization in the majority of the animals, as determined by Western blotting. The results also demonstrated strong preimmune immunoglobulin G2 (IgG2) binding to T. foetus antigens not seen in IgG1 profiles. Subcutaneous injections of Tf190 resulted in significant (P < 0.05) increases in serum IgG1 and IgG2 titers over time, as determined by parasite specific enzyme-linked immunosorbent assay. Immune sera also significantly inhibited parasite adhesion to mammalian cell lines compared to the level of inhibition obtained with preimmune sera (P < 0.05). Intranasal immunization with Tf190 failed to produce measurable parasite-specific antibody in serum; however, this immunization route did result in significant (P < 0.05) increases in parasite-specific IgA titers in cervical mucus secretions from immunized animals that were more resistant to intravaginal challenge with T. foetus than controls. These results suggest that systemic immunization with Tf190 results in serum antibody production and antiparasitic adhesin antibodies. Additionally, the results of challenge experiments with intranasally immunized animals suggests that Tf190 primes protective immune responses that lead to lower rates of infection among these animals.

The antibodies, secreted by the reproductive tract of the female reproductive tract or in the epithelial glands of the penis of the bull (20, 21, 23, 28). There is no approved chemotherapy in the United States, and infected animals are usually culled to control disease in the herd (14). Infected animals can often self-cure, perhaps as a consequence of infection-induced acquired immunity; however, reinfection is common and infected bulls can carry the infection for life, serving as a reservoir for the disease and suggesting that immunity from natural infection rarely protects against challenge. Therefore, the reported herd incidence remains near 10%, despite the availability of a commercial vaccine (15). The present vaccine is also problematic because of its incomplete efficacy, since its use results in only an approximately 30% rate of reduction in the incidence of abortion (15).

Advancements in chemotherapy and vaccine development are complicated by a general lack of knowledge of the immune effector functions that contribute to protection and insufficient understanding of the pathogenesis of trichomoniasis. Parasite-specific antibody responses in cattle to natural T. foetus infection (immunoglobulin G1 [IgG1], IgG2, and IgA isotypes) have been demonstrated by a variety of assays and with a variety of parasite antigens (3, 9, 15, 17, 25) and in experimental infections (2, 27), although antibody effector mechanisms have not been clearly identified.

The mechanisms of pathogenesis of T. foetus are also poorly understood. However, adherence and killing of mammalian cell lines have been demonstrated (5, 6), and recently, the contact-dependent cytotoxicity of T. foetus against bovine vaginal epithelial cells has been documented (26). Monoclonal antibodies (MAbs) specific for parasite adhesin molecules have been shown to inhibit adhesion of the parasite to mammalian tissues (4, 6), and bovine antibodies specific for surface epitopes of T. foetus have been shown to inhibit adherence to and killing of several mammalian cell lines (6, 10). Collectively, these data suggest that adhesion is an important step in the cytopathic mechanism of host cell damage and may be important in the pathogenesis of bovine trichomoniasis as well.

We have identified an adhesin molecule on the surface of T. foetus Tf190 (25) and have now studied the humoral responses in cattle immunized in vivo with Tf190. The purpose of the present study was to investigate the immunogenicity of Tf190 and to define the antibody responses in cattle after immunization with Tf190. We report that parenteral immunizations with Tf190 elicit a strong systemic response in cattle and that immune serum antibodies can significantly inhibit parasite adhesion to mammalian cells. Intranasal immunization decreased the rate of infection in immunized versus unimmunized animals when these animals were challenged by intravaginal inoculation of T. foetus, and parasite-specific antibody was detectable in cervical mucus after intravaginal challenge with live T. foetus in immunized animals that were resistant to infection.

MATERIALS AND METHODS

Parasites and parasite antigens. Two strains of parasites, a high-passage-number clone, clone MT85–330.1 (strain Tf330.1), originally isolated in 1985 and a low-passage-number isolate, isolate TFC-5–1, obtained from a 1997 outbreak in Montana were maintained in vitro at 37°C with Diamond’s medium (12)
without agar containing 5% donor calf serum (Atlanta Biologicals, Atlanta, Ga.) and 10 μg of gentamicin sulfate per ml. Strain TF330.1 was used for the following TF190 preparations, Western blots, all immunizations, and intravaginal challenge. Strain TFC-5-1 was used for enzyme-linked immunosorbent assay (ELISA), inhibition ELISA, and comparison to TF330.1 in the adhesion assays. Whole parasite extract was obtained as described previously (25). Briefly, the parasites were washed in phosphate-buffered saline (PBS; pH 7.2) by centrifugation (400 × g, 5 min) and were extracted on ice at 10° parasites/ml of extraction buffer (50 mM Tris (pH 8), 100 mM NaCl, 5 mM EDTA, 1% s-octyl-β-D-glucopyranoside, 100 μM leupeptin, 100 μM trans-epoysuccinyl-l-leucylamido-(4-guanidino)butane). TF190 antigen was then prepared by affinity chromatography with MAb 32.3B3.5, as described previously (25), and was hophorized prior to use for intranal immunizations.

Cattle and immunizations. (i) Subcutaneous immunizations (experiment 1). Ten virgin adult cows were immunized subcutaneously at multiple injection sites with 100 μg of TF190 in alum on days 0, 20, and 125 (300 μg total). Ten control animals received only alum in the same manner.

(ii) Intranasal immunizations (experiment 2). All animals tested negative for T. foetus infection, as determined by standard sampling of cervical mucus followed by culture for parasite detection (1) prior to the experiment. Six adult cows were given an initial subcutaneous injection of 100 μg of TF190 in alum followed by three intranasal doses of 20 μg of cholera toxin subunit B (CT-B; Sigma, St. Louis, Mo.) on days 21 and 58 (300 μg total). TF190 plus CT-B was dissolved in PBS and was placed on small (1/16-in.) absorbent disks (Whatman no. 1 filters; Fisher Scientific, Pittsburgh, Pa.). The disks were inserted into each animal intranasally with a plastic calf balling gun. Six control animals received alum and CT-B only at the same times. Serum was taken from all animals on day 0, prior to immunization, and was designated the preimmunization serum.

Challenge with T. foetus. Six animals that received TF190 intranasally and six control animals that received cholera toxin only were infected intravaginally with 10° live T. foetus organisms (TF330.1), each in buffered saline with glucose, on day 77. The challenged animals were monitored for 30 days by weekly sampling of cervical mucus with artificial insemination pipettes, followed by culture in Diamond's medium and examination by phase-contrast microscopy for the presence of parasites. Cultured cervical mucus samples contained no significant bacterial contamination.

Assessment of antibody responses. (i) Western blotting. Whole extracts of T. foetus (TF330.1) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide), followed by Western blotting. Blots were probed with bovine sera, cervical mucus samples, or nasal secretions. Cervical mucus and nasal secretions were treated immediately after removal from the cow with 2 ml of Hanks balanced salt solution (Atlanta Biologicals, Norcross, Ga.) containing 0.15% dithiothreitol (Sigma) and were physically sheared by multiple passages through a 20-gauge needle. Antibody subtypes were characterized by incubation with sheep anti-IgG1, anti-IgG2, and anti-IgA (all subtypes were anti-bovine heavy chain specific; Bethesda Laboratories, Montgomery, Tex.). Some blots were also probed with mouse anti-bovine IgG1 (heavy chain specific; VMRD, Pullman, Wash.). Subtypes antibody were incubated at 2 μg/ml (3 h), followed by incubation with anti-mouse horseradish peroxidase (HRP)-labeled secondary antibody (1 μg/ml, 1 h; ICN Pharmaceticals, Costa Mesa, Calif.) or anti-sheep HRP-labeled secondary antibody (1 μg/ml, 1 h; Bethyl Laboratories). Blots were developed with tetramethylbenzidine membrane peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

(ii) ELISA. ELISAs were performed as follows: 100 μl of whole T. foetus extract (obtained by using strains TFC-5-1 and TF 330.1 and prepared as described above) containing 137.5 μg of extract/ml (as determined by the bichromeric acid protein assay; Pierce Chemicals, Rockford, Ill.) was plated in 96-well plates (in PBS), and the plates were incubated overnight at room temperature. Afterward, the plate was blocked in 1% fish gelatin-PBS for 1 h and rinsed three times with 0.05% Tween 20 in PBS, and dilutions of bovine sera or cervical mucus in 0.05% Tween 20 plus 0.01% fish gelatin were added. The plates were washed and incubated at room temperature with sheep anti-bovine IgG1 or anti-IgG2 heavy-chain-specific antibody (1 μg/ml, 1 h). The plates were then washed and HRP-labeled anti-sheep antibody (1 μg/ml) was added. 2.2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) peroxidase substrate solution (Kirkegaard & Perry Laboratories) was used for detection, and the mean absorbances of duplicate samples at 405 nm were recorded (THERMOMAX; Molecular Devices, Sunnyvale, Calif.). Statistically significant differences (P < 0.05) were detected between treatments by analysis of variance and Tukey's comparison of means (Prism, version 3; Graph Pad, San Diego, Calif.).

(iii) Adhesion inhibition assays. Adhesion of T. foetus was evaluated as described previously by coculture of 3H-labeled T. foetus with unlabeled target cells (6). Briefly, parasites were labeled overnight with 3HJharacil and added to either HeLa cells or bovine macrophage cell line M617 in 24-well plates. Preimmunization sera or immune sera (as determined by ELISA and Western blotting) from animals immunized with TF190 (experiment 1) were then added (final dilution, 1:100), and the cultures were incubated for 30 min at 37°C. The monolayers were gently washed twice with warm medium; after the last rinse the liquid was aspirated out of each well, 250 μl of 1% SDS in water was added to each well, and the number of counts per minute was determined with a liquid scintillation analyzer (1500 Tri-Carb; Packard, Meriden, Conn.). Percent inhibition was calculated by the following formula: [(counts per minute for the control – counts per minute determined experimentally)counts per minute for the control] × 100. Data were analyzed by analysis of variance and Tukey's comparison of means, with significance indicated by a P value of <0.05.

RESULTS

In order to determine if TF190 could elicit antibody responses, cattle were immunized with TF190 by subcutaneous injection and their sera were assayed for TF190-specific antibodies. Results from experiments in which gels from Western blots of whole parasite extract were reacted with sera showed that for 7 of 10 animals immunized with TF190, as described for experiment 1, antigen-specific IgG1 antibody responses were detectable in serum at day 125 of immunization. Preimmunization sera from these same animals did not recognize the TF190 antigen (Fig. 1, lanes 1, 3, 5, and 7). Sera from animals immunized with alum only also did not recognize the TF190 antigen (data not shown). The reaction pattern of polyclonal sera from immunized animals was consistent with that of the TF190-specific MAb 32.3B3.5 (compare lane 9 with lanes 2, 4, 6, and 8 in Fig. 1). Sera from all TF190-immunized animals recognized both the 140- and 60-kDa subunits of TF190 (Fig. 1,
lanes 2, 6, and 8), only the 140-kDa subunit (Fig. 1, lane 4), or only the 60-kDa subunit (data not shown).

The results of Western blotting with sera from control animals with whole parasite antigen demonstrated the presence of IgG2 that reacted with several *T. foetus* antigens (Fig. 2, lanes 3 and 7). A complex pattern was seen in the IgG2 reactions of immune sera, whereas a restricted pattern was observed in the IgG1 reactions (Fig. 1). However, immune sera from the same animals also contained Tf190 antigen-specific IgG2 antibody that recognized the 60-kDa subunit of Tf190 (e.g., Fig. 2, lanes 4 and 8), as well as Tf190 antigen-specific IgG1 (Fig. 1, lanes 2, 6, and 8, and Fig. 2, lanes 2 and 6). Collectively, the results of Western blot analysis with serum antibodies indicate that subcutaneous immunization with Tf190 results in antigen-specific IgG1 and IgG2 responses. However, the results of similar experiments in which Western blots were probed with anti-*T. foetus* sera from animals immunized intranasally (experiment 2) showed no IgG1 or IgG2 reactions with Tf190 and, therefore, no evidence of systemic sensitization to Tf190 (data not shown).

Results of ELISA of serum antibodies demonstrated increased levels of parasite-specific antibodies after parenteral immunization (experiment 1). Immune sera (day 125) contained levels of parasite-specific antibody responses that were higher (*P < 0.05*) than those in preimmunization sera (Fig. 3A and B). Parasite-specific IgG1 levels in immune sera were significantly different from those in preimmunization sera at all dilutions from 1:250 to 1:3,200 (Fig. 3A). Significant increases in parasite-specific IgG2 levels in immune sera versus preimmunization sera were also evident at day 125 (Fig. 3B). However, this difference was not significant at higher dilutions, suggesting that the relative strength of the IgG1 response was greater than that of the IgG2 response. In addition, Tf190-specific antibody was demonstrated in immune sera by inhibition ELISA, in which immune sera (but not preimmunization sera) inhibited the binding of Tf190-specific MAb 32.3B3.5 to *T. foetus* antigen (data not shown).

The ability of the anti-Tf190 immune sera to inhibit parasite adhesion was investigated. Immune sera significantly reduced the level of parasite adhesion to the HeLa cell line and the bovine cell line compared to the levels of adhesion of the preimmunization sera (Fig. 4A to C). Treatment with immune serum reduced the level of parasite adhesion to mammalian cells by 40 to 56% compared to the level of adhesion observed in controls treated with preimmunization serum from the same animal. This reduction in adhesion was observed with both a high-passage-number parasite strain, strain Tf330.1 (Fig. 4A), and a low-passage-number strain, strain TFC-5–1 (Fig. 4B).

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**FIG. 2.** Comparison of bovine antibody reactions reveals strong preimmunization IgG2 binding to *T. foetus* antigens not seen in IgG1 profiles. Whole extracts of *T. foetus* subjected to SDS-PAGE followed by Western blotting were probed with Tf190 immune sera (day 147) or preimmunization sera (day 0) from animals in experiment 1, followed by probing with anti-IgG1 or anti-IgG2 antibodies and secondary sheep anti-bovine. Lanes 1 and 5, preimmunization sera; lanes 2 and 6, immune sera probed with anti-IgG1; lanes 3 and 7, preimmunization sera; lanes 4 and 8, immune sera with probed with anti-IgG2. The numbers on the right indicate the major bands of Tf190 established by the reactivity pattern of Tf190-specific MAb 32.3B3.5.

**FIG. 3.** Subcutaneous immunization with Tf190 influences levels of parasite-specific IgG1 (A) and IgG2 (B) antibodies. ELISA was performed as described in Materials and Methods with preimmunization sera (day 0) and immune sera (day 125) from animals in experiment 1. The data shown are for two representative animals (animals 4 and 9), with statistically significant differences between preimmune (P) and immune (I) sera detected at the indicated dilutions (*, *P < 0.05*). OD and Od, optical density.
Thus, immunization with Tf190 induced production of a functional antibody capable of inhibiting parasite adhesion.

Evidence of systemic immunization was absent in animals immunized intranasally with Tf190, and antigen-specific antibody responses in cervical mucus samples from these animals were undetectable 75 days after the initial immunization. However, 30 days after intravaginal challenge antigen-specific IgA antibody (but not IgG1 or IgG2 antibody) was detected in secretions of immunized animals by Western blotting analysis with parasite antigen (data not shown). However, only the 60-kDa subunit of Tf190 was recognized, and a high level of background binding to non-Tf190 epitopes was evident. Further evaluation of cervical mucus by ELISA revealed that the animals that were protected from infection had statistically significant increases in parasite-specific IgA 30 days after challenge (Fig. 5), although anti-Tf190 antibodies were not detected in nasal secretions. Nasal immunization of cattle resulted in a 50% decrease in the cumulative infection rate in Tf190-immunized animals (33% infection rate) compared to that in controls (66% infection rate), although this difference was not statistically significant ($P = 0.25$), probably due to the small numbers of animals in each group ($n = 6$, respectively).

These results suggested that intranasal immunization with Tf190 primed the reproductive tract for subsequent increases in parasite-specific antibody responses after the antigen challenge and that this priming led to partial protection against parasite challenge.

**DISCUSSION**

Trichomoniasis in cattle continues to be an important problem, despite control efforts and the availability of a commercial whole-cell vaccine. In order to determine the immunogenicity of a biochemically characterized adhesin of *T. foetus*, Tf190, in cattle, we examined the antibody responses obtained by two routes of immunization with Tf190 (25). The results of these experiments suggested that subcutaneous immunization with Tf190 induced a systemic response characterized by the production of antigen-specific IgG1 antibody that recognized both the 140- and 60-kDa subunits of Tf190. Additionally, the data presented here reveal that although in preimmunization sera there were complex patterns of IgG2 binding to several parasite antigens, IgG2 antibodies specific for Tf190 were present only after immunization. Thus, both IgG1 and IgG2 serum antibody responses to Tf190 resulted from subcutaneous immunization with Tf190.

The strong binding of IgG2 in preimmunization sera to Western blots of *T. foetus* is an important observation to consider in the development of new serological assays since antibody-binding assays such as ELISA may present false-positive results. The reason for the presence in preimmunization sera there were complex patterns of IgG2 binding to several parasite antigens, IgG2 antibodies specific for Tf190 were present only after immunization. Thus, both IgG1 and IgG2 serum antibody responses to Tf190 resulted from subcutaneous immunization with Tf190.

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FIG. 4. Tf190 immune antibodies in the sera of animals inhibit parasite adhesion to mammalian target cell lines. (A) Parasite strain Tf 330.1 and HeLa cells; (B) parasite strain TFC-5-1 and HeLa cells; (C) parasite strain Tf 330.1 and bovine macrophage cell line M617. Parasites were treated with immune sera (day 125; black bars) or preimmunization sera (day 0; shaded bars) of animals from experiment 1. Numbers above the solid bars indicate percent inhibition, and statistically significant differences were detected as indicated ($*, P < 0.05$).

FIG. 5. Parasite-specific, mucosal IgA antibody responses increase in animals intranasally immunized with Tf190. Animals in experiment 2 were given Tf190 (black bars) or control immunizations (alum, CT-B) (shaded bars) and were then challenged intravaginally with *T. foetus*. Four of six Tf190-immunized animals included in this study (mean of Tf190) cleared the infection, while four of six control animals (mean of control) maintained the infection. Day 0 indicates the day of intravaginal challenge; day 30 indicates 30 days after challenge. Statistically significant differences were detected between the indicated experimental groups ($*, P < 0.05$) compared to the results obtained for the control group at day 30 (black bars). OD, optical density.
as cross-reactivity between antigens of *T. foetus* and the cattle’s natural protozoal fauna. It is unlikely to be an anamnestic response since these animals were culture negative for *T. foetus* prior to parenteral immunization. Similarly, sera from uninfected exposed patients contain antibodies to heat shock proteins of *T. vaginalis*, which were explained to be natural antibodies (11). However, a 38-kDa antigen was recognized only by antibodies present in sera of infected women, suggesting that active infection is required to elicit a response to this antigen.

Immune sera from immunized cattle significantly inhibited parasite adhesion to mammalian cells, indicating that immunization with TT190 elicited functional antibodies, as was demonstrated previously for murine antibodies to this antigen (6). Inhibition of adhesion of a high-passage-number line of *T. foetus* (which has continuously been in culture since 1985) and a low-passage-number isolate (which has been passaged less than five times; isolate TFC–5–1) by these antibodies also suggests that the target epitopes are stable over a time span of at least 12 years. Since parasitic adherence has been shown to lead to cytotoxicity toward mammalian cell lines (5), including bovine vaginal epithelium (26), this antiadhesion antibody function could prevent direct parasite damage of host cells and help control infection.

After intranasal immunization there was an absence of detectable anti-TT190 antibodies in serum. The absence of a systemic response after immunization by the nasal route is not surprising since immunization by such a route targets mucosal tissues including those of the reproductive tract (24). Therefore, we expected to see evidence of antigen sensitization in the form of antibodies in nasal and cervical secretions after intranasal immunization and prior to challenge with live *T. foetus*. However, immunization did not result in detectable parasitesspecific antibodies in nasal or cervical secretions prior to intravaginal challenge with *T. foetus*, as initially hypothesized. Instead, the parasite-specific IgA level was significantly higher 30 days after challenge in TT190-immunized animals that were partially protected from infection (33% cumulative infection rate) than in control animals (66% cumulative infection rate). This observation suggests that the animals receiving TT190 were primed for a protective immune response upon challenge with live *T. foetus*, even though sensitization to TT190 did not result in detectable IgA prior to challenge. However, challenge appears to have triggered a protective mucosal immune response in these animals. This result is similar to previous observations that systemic priming with *T. foetus* antigens followed by vaginal boosting enhances production of genital, parasite-specific IgA (9). If subcutaneous immunization with TT190, which resulted in a strong systemic response and production of antiadhesion antibodies, was followed by an intranasal booster prior to challenge, a combined systemic and mucosal immune response might occur and might enhance overall immunity to *T. foetus*.

Several studies have demonstrated various forms of increased resistance to infection with *T. foetus* resulting from parenteral immunization (3, 8, 13, 15, 16, 18, 19). One study observed an initial infection rate in cattle immunized with killed *T. foetus* that was equal to the infection rate in controls; however, the immunized animals cleared the infection more rapidly than the controls did (13). Other studies have shown increases in pregnancy rates in vaccinated animals compared to those in controls (an 82.6% successful pregnancy rate in vaccinated animals compared to a 60.53% successful pregnancy rate in controls [15]). Corbeil and colleagues showed that immunization with a subcellular antigen of *T. foetus* resulted in a 20 to 40% reduction in infection rates in immunized animals compared to the rate in controls (9). In the present study we have shown that immunization with the purified adhesin, TT190, decreased the infection rate after challenge (66% in controls versus 33% in immunized animals).

How these subcellular antigens of *T. foetus*, including TT190, provide partial protection from challenge is not known, and further investigations aimed at increasing the efficacy of immunization with TT190 are needed. A better understanding of the mechanisms of the effector functions operating in immunized and protected animals will be required to elucidate the function of antibody in protective immunity.

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