The stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), is an economically important pest of livestock. Previous studies demonstrated lymphocyte suppression by crude salivary gland extract (SGE) of the stable fly. A dominant 27-kDa protein identified in the SGE was reported to stimulate immunodominant antibody responses in exposed cattle. The purpose of this study was to determine whether this protein, now identified as a homolog of insect proteins named antigen 5 (Ag5), was responsible for the lymphocyte suppression and whether naïve calves can mount an immune response to it. Calves raised in the winter were immunized with recombinant Ag5 (rAg5) expressed in *Drosophila* S2 cells or with “natural” Ag5 protein isolated by preparative gel electrophoresis of SGE. Control calves were immunized with adjuvant alone. Rising antibody concentrations to rAg5 were detected in two of three calves immunized with rAg5 and one of three calves immunized with natural Ag5. Recall lymphocyte responses to rAg5 were detected at 21 and 28 d postimmunization in calves immunized with rAg5 but not in calves immunized with the natural Ag5 or those exposed to adjuvant alone. Mitogen-stimulated bovine lymphocyte responses were not suppressed by rAg5. Further investigation using immunoblotting revealed that rAg5 binds to the Fc and F(ab')2 portions of bovine IgG, but not to an Fab fragment. These findings suggest that Ag5 of the stable fly salivary gland is not immunosuppressive but that it has immunoglobulin binding properties and can invoke specific antibody and memory lymphocyte responses in immunized calves.

KEY WORDS  bovine lymphocytes, lymphocyte suppression, antibody, stable fly, salivary gland protein
biting rate of the tsetse fly potentiates the risk of increased transmission of trypanosomiasis (Torr and Mangwiro 2000).

The best available tool for reducing stable fly populations in confined livestock operations is to reduce larval habitats by sanitation. However, control failures are common. The integration of other control practices, such as the use of residual pesticides and the release of pupal parasites aids in the control of these flies in confined livestock operations. There are no effective means of controlling stable flies attacking range cattle (Campbell and Raun 1971, Campbell and Wright 1976). Typical control strategies (e.g., wet sprays on the legs, dust bags, insecticide impregnated ear tags, and oral larvicides) are either ineffective in lowering stable fly populations below economic levels, promote the development of insecticide resistant populations, or are becoming less desirable because of the increasing public concern about chemical residues in food. Therefore, new strategies and approaches for reducing stable fly populations near cattle are needed. Moreover, recent immunization trials with a salivary protein of the horn fly demonstrated reduced bloodmeal sizes and delayed egg development in flies fed on immunized cattle (Cupp et al. 2004).

In a previous study, we showed that stable fly salivary gland extract (SGE) inhibits mitogen-driven proliferation of bovine lymphocytes, and we identified a dominant protein of 27 kDa in the stable fly salivary gland that seemed to be highly immunoreactive in cattle exposed to flies (Swist et al. 2002). We have subsequently identified the 27-kDa protein as a homolog of antigen 5 (Ag5) from other insect species (X. Wang, J.M.C. Ribeiro, M. J. Wilkerson, A. B. Broce, and M. R. Kanost, in preparation). We have determined the sequence of its cloned cDNA (GenBank accession no. AY190321), and we found that it is expressed specifically in adult salivary glands (X. Wang, A. B. Broce, and M. R. Kanost, in preparation). To further investigate these findings and to determine whether this salivary gland protein would be a good immunogen in cattle, we conducted the current study. The objectives of this study were to 1) determine specificity of bovine antibody response to recombinant Ag5, 2) to determine whether a recombinant form of the protein suppresses bovine lymphocyte proliferation in culture, and 3) to determine whether calves immunized with either recombinant or natural Ag5 preparations produce rising antibody and memory lymphocyte responses.

Materials and Methods

Flies. Stable flies were obtained from a colony maintained in the Department of Entomology (Kansas State University [KSU], Manhattan, KS) and established from wild flies collected in Manhattan in 1990. The larvae were reared in a vermiculite/wheat bran/ fishmeal medium. Adults were fed daily on bovine blood stored in sodium citrate anticoagulant. Twenty-four hours before dissection, male and female flies (4–8 d after eclosion) were fed Gatorade to maintain nutrition without causing ovary development, and then they were anesthetized by lowering body temperature to 4°C. Salivary glands were dissected from the flies according to the method described previously (Swist et al. 2002).

Recombinant Ag5 Preparation. A cDNA for Ag5 obtained from a stable fly salivary gland cDNA library (accession no. AY190321) was expressed from plasmid vector pMT/V5-His A (Invitrogen, Carlsbad, CA) in *Drosophila* Schneider 2 (S2) cells, and the secreted protein was purified by cation exchange chromatography (X. Wang, A. B. Broce, and M. R. Kanost, in preparation). Those fractions containing recombinant rAg5 were pooled, dialyzed against phosphate-buffered saline (PBS) at 4°C, concentrated using Centricon YM-10 centrifugal filtration devices (Millipore, Billerica, MA) to 1 mg/ml, and stored at −80°C. The purity of rAg5 protein was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) analysis. Supernatant harvested from untransfected S2 cells was used as the source of negative control protein (CP).

Preparation of Natural Ag5 for Immunization Studies. Salivary glands dissected from 1- to 7-d-old adult male and female stable flies were used to prepare SGE (Swist et al. 2002). Twenty-five micrograms of SGE protein was separated by SDS-PAGE according to the method of Laemmli (1970) with a 10% separating gel and a 4% stacking gel. After Coomassie blue staining, the band with molecular weight of 27 kDa was excised. The gel slice containing the 27-kDa Ag5 protein was then homogenized and stored at −20°C. A similar gel slice was removed from a lane that did not contain protein to serve as the negative control.

Animals and Immunization Studies. Eight Holstein bull calves (3–4 mo old) were obtained from the Kansas State University-Dairy herd in the winter when stable flies were not present and after the calves had obtained colostrum. The calves were housed in an enclosed barn maintained under the guidelines of the Institutional Animal Care and Use Committee. Four additional adult steers housed at the KSU-Dairy herd were bled for serum samples and initial lymphocyte proliferation studies. Two calves were bled before ingestion of colostrum to obtain a source of antibody negative sera. Maternal antibody was confirmed to be absent in these serum samples by single radial immunodiffusion (VMRD, Pullman, WA) and serum electrophoresis (performed at the clinical pathology laboratory at College of Veterinary Medicine, Kansas State University). All calves were bled before immunization. Calves were immunized at 4 mo of age, twice at 1-wk intervals intramuscularly. Three calves (C22, C23, and C73) were immunized with 100 μg of rAg5 in 200 μl of 0.01 M PBS, pH 7.2, emulsified with equal volumes of TiterMax adjuvant (Sigma-Aldrich, St. Louis, MO). The other three calves (C19, C20, and C21) were immunized with 100 μg of natural Ag5 from the preparative gel emulsified with equal volumes of TiterMax adjuvant (Sigma-Aldrich) as described previously (Yarnall et al. 1988). Two calves were immunized with adjuvant alone as negative controls. One control calf (C17) received adjuvant containing CP (prepared as described previously), whereas the other...
calf (C18) received adjuvant plus a piece of preparative gel that did not contain a protein band. All calves received two injections 1 wk apart. Blood samples were collected at 0, 14, 21, 28, and 42 d postimmunization (DPI) by jugular venipuncture. Whole blood was collected in tubes containing 0.01 M PBS, pH 7.2, and 20% acid-citrate dextrose (ACD, Fisher Scientific, Pittsburgh, PA) from all calves for lymphocyte proliferation studies. Blood was also drawn directly into a serum clot tube by using a vacutainer collection system. The samples were immediately transported to the laboratory in a cooler with ice packs. Processing of the samples was performed within 2 h of blood collection. To collect serum samples for indirect enzyme-linked immunosorbent assay (ELISA) studies, the samples in the clotting tubes were centrifuged at 2000 × g for 20 min at 4°C, and serum was stored at −20°C until analysis.

Isolation of Bovine Lymphocytes from Peripheral Blood. Lymphocytes harvested from venous blood of four adult steers housed at the KSU-Dairy and exposed to stable fly biting was used to test whether rAg5 had immunosuppressive properties. Lymphocyte isolation was performed according to the protocol described previously (Swist et al. 2002) with some modification. Briefly, whole blood (30–60 ml) was placed into tubes containing PBS/ACD (20%). Lymphocytes were separated from other leukocytes by centrifugation on a density gradient (Ficoll-Paque 1.086, Sigma-Aldrich). The mononuclear cell interface was collected and washed three times in PBS/ACD (20%). Ammonium chloride (0.5% NH₄Cl) was used to lyse red blood cells. After washing one more time, lymphocytes were counted using a Neubauer hemocytometer (AO Scientific, Buffalo, NY).

Indirect ELISA. The solid phase was prepared by coating the wells of ELISA plates with rAg5 at 1 μg/ml (100 μl per well) in 0.1 M PBS, pH 7.2, at 4°C overnight. After removal of unbound rAg5 by washing the plate with PBS containing 0.05% of Tween 20 (PBS-T, vol/vol) three times, calf sera at 0, 14, 21, 28, and 42 DPI (1/1000 dilution in PBS-T) were added to the wells vol:vol) three times, calf sera at 0, 14, 21, 28, and 42 DPI plate with PBS containing 0.05% of Tween 20 (PBS-T, vol/vol) three times, calf sera at 0, 14, 21, 28, and 42 DPI. After removal of unbound rAg5 by washing the wells with PBS-T, the substrate PA) at 1:2000 dilution in PBS-T to each well, and the temperature. The wells were then washed three times with PBS-T. The bovine immunoglobulin that bound to the stable fly protein was detected by adding horse-radish peroxidase (HRP)-goat anti-bovine Fab (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:2000 dilution in PBS-T to each well, and the plate was incubated for 45 min at room temperature. After washing the wells with PBS-T, the substrate 3,3,5,5-tetramethylbenzidine (100 μl/well; Sigma-Aldrich) was used to develop the colorimetric reaction. Finally, stop solution (0.5 M H₂SO₄) was added to each well at 30 μl per well. The optical density (OD) at 450 nm of each well was measured on an automatic ELISA plate reader (Universal Microplate Reader, EL800, Bio-Tek Instrument, Inc., Winooski, VT). The assay was performed in duplicate.

Lymphocyte Proliferation Assay. Bovine lymphocytes collected from the calves immunized with either rAg5, natural Ag5, or adjuvant alone (control calves) were stimulated in vitro with a T-cell–specific mitogen concanavalin A (Con A, Sigma-Aldrich) in the presence of various concentrations of rAg5 or natural Ag5. Lymphocytes were plated in flat bottom, 96-well plates (Corning Inc., Corning, NY) at a concentration of 1 × 10⁶ cells per ml in RPMI 1640 medium (Gibco/BRL, Rockville, MD) containing 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 1-glutamine (0.3 mg/ml), penicillin (10,000 U/ml), streptomycin (10 mg/ml), and gentamycin (12.5 mg/ml). Assays were performed in triplicate. Wells were stimulated with Con A (1 μg/ml) with or without addition of rAg5 or natural Ag5 at 1 and 5 μg/ml. Lymphocyte cultures were incubated for 72 h at 37°C with 5% CO₂. Unstimulated lymphocytes were used as the background control for the nonspecific proliferation by medium alone. To measure cell proliferation, 0.2 μCi of [³H]thymidine was added to cells 18 h before harvest. The cells were harvested using an automated multilwell cell harvester (Inotech Biosystems, Rockville, MD). The amounts of ³H incorporated into DNA of proliferating lymphocytes were expressed as the radioactive counts per minute (cpm) determined by liquid scintillation counting (Beckman Coulter Inc., Fullerton, CA). Proliferation was expressed as a stimulation index (SI), a ratio of the cpm for mitogen-stimulated cells divided by the cpm of nonstimulated cells. To determine the suppressive effects of the stable fly protein preparations, a range of 1–100 μg/ml of either rAg5, CP, or SGE was added with Con A to the lymphocyte cultures. The percentage of inhibition (% I) of mitogen-driven lymphocyte proliferation imparted by the presence of recombinant or native protein was determined by the formula SI (Con A) − SI (Con A + protein)/SI (Con A).

Immunoblotting. Four micrograms of protein per lane of protein (rAg5 or SGE) was treated with the reducing sample buffer and separated by SDS-PAGE according to the method of Laemmli (1970) with a 10% separating gel and a 4% stacking gel. The proteins were electrophoretically transferred from gels to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) by using Trans-Blot SD semi-dry transfer cell system (Bio-Rad). The immunoblots were developed under the following conditions at room temperature. The PVDF membranes were blocked with 1% (wt/vol) BSA (99%, γ-globulin free, Sigma-Aldrich) in PBS-T at room temperature for 1 h and cut into strips. After washing three times with PBS-T, two strips from each membrane were incubated for 1 h with serum (diluted 1:100 in PBS-T) from two different steers that had been exposed to stable fly bites. Two additional strips from each membrane were incubated for 1 h with serum (diluted 1:100 in PBS-T) from two newborn calves collected before ingestion of colostrum. One strip from each membrane was not incubated with a primary antibody. After washing three times in PBS-T, all five strips were incubated with an HRP-conjugated sheep anti-bovine IgG (H + L) diluted 1:1000 in PBS-T. Three additional strips from each membrane were incubated with HRP-bovine IgG Fab, HRP-bovine IgG F(ab’)₂ (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:2000 dilution in PBS-T to each well, and the plate was incubated for 45 min at room temperature. After washing the wells with PBS-T, the substrate 3,3,5,5-tetramethylbenzidine (100 μl/well; Sigma-Aldrich) was used to develop the colorimetric reaction. Finally, stop solution (0.5 M H₂SO₄) was added to each well at 30 μl per well. The optical density (OD) at 450 nm of each well was measured on an automatic ELISA plate reader (Universal Microplate Reader, EL800, Bio-Tek Instrument, Inc., Winooski, VT). The assay was performed in duplicate.
ImmunoResearch Laboratories, Inc.) diluted 1:1000 in PBS-T for another hour. For detection, all membranes were washed twice for 5 min each with PBS, pH 7.2. The 3,3′-diaminobenzidine tetra hydrochloride liquid substrate system (Sigma-Aldrich) was added to develop the color reaction.

Statistical Analysis. Differences in the lymphocyte proliferation among the treatment groups were determined by one-way analysis of variance (ANOVA) and Holm-Sidak method for pairwise comparison procedures SigmaStat (SPSS Inc., Chicago, IL). A one-way repeated measures ANOVA and multiple comparisons versus the control animal was performed to determine differences among the lymphocyte recall responses (performed in triplicate) between individual calves. Differences in antibody concentration between the calf treatment groups and days post immunization were determined using a two-way ANOVA with repeated measures model and multiple comparisons with computations from PROC GLIMMIX of the SAS system (version 9.1.3, SAS Institute, Cary, NC). Statistical significance was established at P values <0.05.

Results

SDS-PAGE Analysis. The rAg5 protein was detected as a 27-kDa single band by SDS-PAGE followed by Coomassie blue staining (Fig. 1A). The SDS-PAGE analysis of the SGE stained with silver stain revealed a major band running at 27 kDa as shown in Fig. 1B. 

Effect of rAg5 on Mitogen-Induced Lymphocyte Proliferation. Lymphocytes from four steers were stimulated in culture with the T-cell mitogen Con A.

![Fig. 1. SDS-PAGE analysis of rAg5 stained with Coomassie blue (A) and SGE stained with silver stain (B) depicting the dominant 27-kDa protein (native Ag5) of the extract.](https://academic.oup.com/jme/article-abstract/45/1/94/874205)

![Fig. 2. Lymphocyte proliferation assay determined by incorporation of tritiated thymidine defined as counts per minute of radioactivity. Lymphocytes from four steers were treated with five separate conditions: no stimulation (no mitogen), stimulated with Con A alone, Con A plus 3.0 µg/ml of rAg5, CP, or crude SGE. Error bars represent one standard error of the mean of three replicate experiments.](https://academic.oup.com/jme/article-abstract/45/1/94/874205)
The lymphocytes from all steers proliferated in response to the mitogen (Fig. 2), with incorporation of 
$[^{3}H]$ thymidine significantly higher than unstimulated cells ($P < 0.01$). SGE suppressed Con A-stimulated
lymphocyte proliferation in all steers, ranging from 55% (steer 2) to 90% (steer 4) inhibition. However, rAg5 at 3.0 $\mu g/ml$ did not suppress Con A-stimulated lymphocyte proliferation; the mean cpm for Con A + rAg5 (25,165) was no different than Con A-stimulated proliferation when control protein was added (27,125 ± 2218) (Fig. 2). Moreover, the addition of lower and higher protein concentrations of SGE or rAg5 ranging between 0.1 and 10 $\mu g/ml$ did not significantly change the results (data not shown).

Antibody Responses to Ag5 in Immunized Calves. Antibody concentration measured by ELISA for control calves (C18 and C17) exposed to adjuvant alone did not significantly change over time. Two of three calves immunized with rAg5 produced antibody responses to rAg5, detected beginning at 21 DPI peaking at 28 DPI for one calf (C22) and 42 DPI for the other calf (C23). There was a significant increase in the overall group mean antibody concentrations at 28 and 42 DPI compared with 0 DPI for calves immunized with rAg5 protein ($P < 0.01$), consistent with a rising antibody response after vaccination. One calf immunized with Ag5 protein isolated by preparative gel electrophoresis of SGE produced a slight increase in antibody concentration, peaking at 21 DPI (C19), whereas the other two calves (C73 and C20) did not respond to Ag5. There were no significant differences at 21 DPI compared with 0 DPI for the Ag5 group (Fig. 3).

Recall Lymphocyte Responses to Ag5. All calves immunized with rAg5 had recall lymphocyte responses to rAg5 at 21 or 28 DPI. The lymphocyte response of calf 22 was significantly higher than the control calf at 28 DPI ($P < 0.05$). Lymphocyte responses to rAg5 of calf 23 were significantly higher than the control calf at both 21 and 28 DPI ($P < 0.05$), whereas calf 73 had significantly higher lymphocyte responses than the control calf only at 21 DPI ($P < 0.05$). Lymphocytes from calves immunized with natural Ag5 did not respond to rAg5 protein in vitro and were not significantly different than the control calf (Fig. 4).

Immunoglobulin Binding Properties of rAg5. Serum from cattle exposed to stable fly bites reacted with a prominent band at 27 kDa in the SGE (Fig. 5A, lanes 1 and 2). However, we noted that serum from newborn calves never exposed to stable flies and known to be deficient in antibodies (determined by serum protein electrophoresis and radial immunodiffusion assays to be
below detectable limits, < 0.2 g/dl of IgG) also reacted with the 27-kDa protein (Fig. 5A, lanes 3 and 4). This reaction, albeit less intense, was found to be due to the binding of the secondary antibody to the 27-kDa protein (Fig. 5A, lane 5). Furthermore, fragments of bovine IgG including Fc and F(ab’)2 reacted with the 27-kDa Ag5 protein in SGE (Fig. 5A, lanes 7 and 8), but the Fab fragment did not (Fig. 5A, lane 6).

The recombinant Ag5 reacted with all sources of antibody tested in a similar manner (Fig. 5B). The secondary sheep anti-bovine IgG antibody reacted with the recombinant protein with or without the addition of steer serum samples or newborn calf serum samples (Fig. 5B, lanes 1–5). The Fc fragment of purified HRP-labeled bovine IgG (Fig. 5B, lane 7), but not the HRP-labeled Fab fragment of purified bovine IgG (Fig. 5B, lane 6) reacted with the recombinant protein. In addition, the F(ab’)2 fragment of HRP-labeled purified bovine IgG reacted weakly to rAg5 (Fig. 5B, lane 8).

**Discussion**

Our findings demonstrated that rAg5 does not suppress mitogen-driven lymphocyte proliferation in culture, indicating either the recombinant form of the protein lacks immunosuppressive properties or that other salivary gland substances may be responsible for the immunosuppressive activity present in SGE of the stable fly. The immunization trial demonstrated that naïve calves raised in the winter (when stable flies were not present) can produce rising antibody responses to rAg5 and in one calf to the natural Ag5 from SGE. This finding indicates that specific antibody responses can be produced to Ag5 in the bovine even though the protein binds bovine immunoglobulin nonspecifically via the Fc or F(ab’)2 portions. However, the ELISA that was used in this study incorporated anti-bovine Fab, which was shown by Western blot not to react to Ag5. Although Ag5 binds to antibodies nonspecifically, the presence of rising antibody concentrations over time after immunization indicates induction of specific antibody responses to the protein instead of nonspecific binding. In contrast, calves immunized with adjuvant alone produced no change in the signal for the indirect ELISA throughout the 42-d investigation period. Similarly, in other systems, specific antibody responses to Fc receptor-like binding proteins of bacteria have been reported in immunized cattle (Yarnall and Corbeil 1989).

Furthermore, calves immunized with rAg5 develop memory lymphocyte responses when lymphocytes were restimulated in culture with rAg5. Because only the calves immunized with rAg5 developed recall lymphocyte responses, memory epitopes may have been altered in the natural Ag5 preparations isolated by gel electrophoresis.

The immunization trial demonstrated that rAg5 evokes bovine immune responses (antibody and lymphocyte) and is potentially a good immunogen to pursue further challenge studies to determine if prior immu-
Leishmania major, has proven to be protective against infection by stable flies. Although vaccination of the host may be a means for the tick to evade the host immune system.

Studies involving the ixodid tick R. appendiculatus revealed that host IgG found in the tick was excreted via salivation at feeding, and the immunoglobulin binding proteins in tick hemolymph and salivary glands are thought to be responsible for such excretion.

The discovery of an immunoglobulin excretion system in ticks indicates that they have a highly developed mechanism to protect themselves from their host’s antibody attack (Wang and Nuttall 1994, Wang and Nuttall 1999). Salivary glands of R. appendiculatus male ticks contain a number of IgB proteins. It has been postulated that males secrete a cocktail of IgB proteins into the feeding lesion where they help protect adjacent females from the host’s anti-tick immune response (Wang and Nuttall 1995b).

Collectively, these findings show promise that the very abundant Ag5 protein in salivary glands of the stable fly may be a good candidate immunogen to pursue future challenge studies to determine whether bovine immune responses generated toward this unique protein could decrease feeding and reproductive efficiency of the stable fly.

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