Modulation of Bovine Lymphocyte Response by Salivary Gland Extracts of the Stable Fly, *Stomoxys calcitrans* (Diptera: Muscidae)

S. L. SWIST,[1] M. J. WILKERSON,[1] C. R. WYATT,[1] A. B. BROCE,[2] AND M. R. KANOST[3]

Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS 66506

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ABSTRACT The effect of salivary gland extract of the stable fly, *Stomoxys calcitrans* (L.), on bovine lymphocyte proliferation was determined, and antibody reactivity to salivary gland proteins was characterized in cattle exposed to stable flies. Salivary glands were dissected from male and female flies (4–8 d after eclosion), and protein extracts were made by freeze–thaw cycles. Salivary gland extract (SGE, 1 and 5 μg) significantly inhibited mitogen-driven proliferation of bovine lymphocytes, compared with 1 and 5 μg of identically prepared midgut extract (ANOVA, $P < 0.05$). Phytohemagglutinin A (PHA) stimulated lymphocyte responses were suppressed by 61.7 and 79.5% (mean values) with 1 and 5 μg of SGE, whereas concanvalin A (Con A) stimulated responses were suppressed by 62.9 and 77.1% (1 and 5 μg). In contrast, midgut extract (1 and 5 μg) minimally suppressed PHA (12.7% $\pm$ 12.6 and 18.7% $\pm$ 15.5) and Con A-driven responses (13.8% $\pm$ 20.5 and 24.6% $\pm$ 14.9), respectively. Viability studies using propidium iodide and flow cytometry demonstrated that SGE was not cytotoxic. Two-color immunofluorescence studies identified T and B lymphocytes as the nonviable cells in the cultures. Western blot analysis of serum collected from five dairy cows during periods of low and high fly exposure identified an immunodominant 27 kDa protein among the salivary gland proteins. These results indicate that exposure of cattle to stable fly saliva during blood feeding results in an antibody response to salivary proteins and that the saliva has a potential to modulate T lymphocyte function.

KEY WORDS Bovine lymphocytes, lymphocyte suppression, antibody, mitogen stimulation

BLOOD-SUCKING ARTHROPODS produce many pharmacologically active molecules in their saliva that counter host defenses, including pain–itch responses, hemostasis, and immune responses (Ribeiro 1987, 1995; Wikel 1999). Some of the arthropod salivary proteins act as vasodilators (Lerner et al. 1991, Champagne et al. 1995, Beerntsen et al. 1999), interfere with thrombin and factor Xa clotting factors (Valenzuela et al. 1996, Cappello et al. 1998, Stark and James 1998, Waidhet-Kouadio et al. 1998), and affect platelet aggregation (Ribeiro 1995). Salivary gland extracts from black flies (*Simulium vittatum* Zetterstedt) and sand flies inhibit host lymphocyte responses, including antibody production, cytokine expression, and in vitro mitogen-driven lymphocyte proliferation (Cross et al. 1993, Wikel 1999). A peptide, maxadilan, isolated from the Old World sand fly (*Phlebotomus papatasi* Scopoli) saliva inhibits tumor necrosis factor alpha production by macrophages, a cytokine important for killing of intracellular organisms (Soares et al. 1998), which in turn enhances survival of the obligate intracellular parasite *Leishmania major* Friedlin (Wikel 1999, Morris et al. 2001, Wikel and Alarcon-Chaidez 2001). Similar to tickborne pathogens (Wikel 1996, Zeidner et al. 1996, Wikel and Bergman 1997), the immunomodulatory effect of fly saliva plays a significant role in the establishment and persistence of vector-borne pathogens (Wikel 1999).

The stable fly, *Stomoxys calcitrans* (L.), is an economically important pest that affects the health of livestock, for example cattle in feedlots (Campbell et al. 1987), dairies (Stork 1979) or on free range (Hall et al. 1982). Stable fly exposure in cattle has been associated with increased body temperature, urinary nitrogen output, and cortisol levels (Schwinghammer et al. 1986). Behavioral responses, including bunching of the herd, foot stomping, and head throwing, lead to reduced feed consumption and weight gains (Wieman et al. 1992). However, little research has been done on the effects of the stable fly on bovine immune responses. The purpose of this study was to determine whether or not stable fly salivary gland extracts had immunomodulatory effects on bovine lymphocytes. We demonstrated inhibition of mitogen-driven proliferation of bovine lymphocytes by stable fly salivary gland extract, and identified an immunodominant protein of 27 kDa in the stable fly salivary gland. These

[1] Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506 (e-mail: wilkersn@vet.ksu.edu).

[2] Department of Entomology, College of Agriculture, Kansas State University, Manhattan, KS 66506.

[3] Department of Biochemistry, College of Arts and Sciences, Kansas State University, Manhattan, KS 66506.

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findings have important implications in cattle, because stable flies can be mechanical carriers of pathogens (Knapp et al. 1992).

Materials and Methods

Flies. Stable flies were obtained from a colony maintained in the Department of Entomology, Kansas State University, established from wild flies collected in Manhattan, KS, 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 and then anesthetized by lowering body temperature to 4°C.

Salivary Gland/Gastrointestinal Tract Extract. Salivary glands and midgut were dissected from the flies and placed in phosphate-buffered saline (PBS; 150 mM NaCl, pH 7.5), transferred to 100 μl of 150 mM NaCl, and stored at −80°C. Salivary glands and gastrointestinal tracts were disrupted by four rapid freeze–thaw cycles. The tissue lysates were cleared of debris by vortexing (30 s), followed by centrifugation (16,600 × *g*, 30 s) (Sorvall, Newton, CT), and filter sterilization (0.45 μm; Fisher, Pittsburgh, PA). Protein concentrations were determined using the BCA protein assay (Pierce Co., Rockford, IL).

Animals. Holstein cows were housed at the Kansas State University Dairy Barn and maintained under the guidelines of the Institutional Animal Care and Use Committee. Whole blood was collected from six Holstein cows for lymphocyte proliferation studies, and serum samples were obtained from five of the same cows during periods of peak fly exposure (May–August) and low fly exposure (November–February). During the time frame of this study, the cows were kept in pens at the dairy unit, where horn flies, *Haematobia irritans* (L.), have not been found because of the lack of intact cowpats for their larval development (ABB, unpublished).

Isolation of Bovine Lymphocytes from Peripheral Blood. Whole blood (30–60 ml) was collected by venipuncture and placed into glass tubes containing preservative-free heparin. Lymphocytes were separated from granulocytes by centrifugation on a density gradient (Ficoll-Paque 1.077, Sigma, St. Louis, MO). The mononuclear cell interface was collected and washed three times in PBS to remove the density gradient. Red blood cells were lysed with ammonium chloride (0.8% NH_4Cl), and lymphocytes were counted using a Neubauer hemocytometer (AO Scientific, Buffalo, NY).

Lymphocyte Proliferation Assay. To determine whether stable fly saliva has immunosuppressive effects, bovine lymphocytes were stimulated in vitro with T cell-specific mitogens phytohemagglutinin A (PHA) or concanavalin A (Con A) in the presence of various concentrations of salivary gland extracts (SGE) or midgut extracts (GE). Initial experiments compared the effects of adding SGE simultaneously with Con A or 2 h after stimulation with Con A. For the remainder of experiments, the SGE was added simultaneously with each mitogen.

Lymphocytes at 1×10^6 cells/ml in RPMI-1640 medium (Gibco/BRL, Rockville, MD) containing 10% heat-inactivated fetal bovine serum (HyClone Lab, Logan, UT), L-glutamine (0.3 mg/ml), penicillin (10,000 U/ml), streptomycin (10 mg/ml), and gentamycin (12.5 mg/ml) were plated out in flat bottom, 96-well plates (Corning, Corning, NY). Triplicate wells were stimulated with PHA or Con A (1 μg/ml, Sigma, St. Louis, MO) with or without addition of SGE or GE at 1 and 5 μg/ml. Lymphocyte cultures were incubated for 72 h at 37°C with 5% CO_2. Unstimulated lymphocytes were used as the background control for the endogenous effects of the tissue extracts. To measure cell proliferation, 0.2 μCi of [^{3}H]thymidine was added to cells 18 h before harvest. The cells were harvested after 72 h of culture using an automated multiwell cell harvester (PHD cell harvester; Brandel, Gaithersburg, MD). The amounts of tritiated thymidine incorporated into DNA of proliferating lymphocytes were expressed as the radioactive counts per minute (cpm) determined by liquid scintillation counting (Beckman Instruments, Fullerton, CA). Proliferation was expressed as a stimulation index (SI), a ratio of the cpm for mitogen-stimulated cells divided by the cpm of unstimulated cells. The percentage of inhibition (% I) of mitogen-driven lymphocyte proliferation imparted by the presence of the stable fly tissue extract (SGE or GE) was determined by the following formula:

$$\frac{SI\,(mitogen) \;-\; SI\,(mitogen \;+\; tissue\ extract)}{SI\,(mitogen)}$$

Two-Color Immunofluorescence for Identifying Lymphocyte Subsets and Viability. To determine the viability of lymphocytes following exposure to SGE or GE, treated cells were assessed by flow cytometry using propidium iodide, a vital dye that intercalates into DNA when cellular membranes are damaged. To determine which lymphocyte subsets (T or B) contributed to the nonviable and viable populations at 72 h of mitogen stimulation, aliquots of cells (2×10^6) were harvested and incubated with mouse monoclonal antibodies (10 μg/ml) specific for a B cell determinant (BAQ44A, IgM; VMRD, Pullman, WA) and to a T cell determinant CD3 (MM1A, IgG_1; VMRD). Incubations were for 30 min at 4°C, and followed by three wash cycles in PBS plus 2% gamma globulin-free horse serum (Sigma). Fluorescein-conjugated anti-mouse IgM or IgG (10 μg/ml; Caltag, San Francisco) was added to tubes and incubated for 30 min at 4°C, followed by three washes with PBS. For viability analysis, 10 μl of propidium iodide (10 μg/ml) was added to each of the antibody-labeled tubes. Data were collected and analyzed on 10,000 cells using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA).

Western Immunoblotting. Western blot assays were used to identify the presence of antibodies to stable fly salivary gland proteins in serum samples

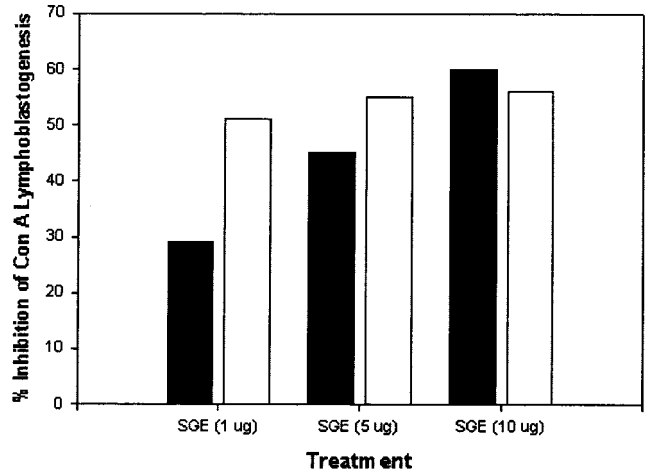

Fig. 1. The percent inhibition of concanavalin A (Con A) stimulated lymphocyte proliferation was determined on lymphocytes from one cow after addition of salivary gland extract (SGE) simultaneously with Con A (1 μg/ml) (black bars) or after 2 h of preincubation with Con A (1 μg/ml) (open bars).

from the same cows in which lymphocyte function was suppressed by SGE. To determine whether antibody responses to the SGE proteins differed between fly seasons, serum samples were collected from each cow during high fly exposure (May–August) and low exposure (November–February). A total of five cows were sampled because one cow had been culled before serum collection.

Salivary gland and midgut extracts containing 5.0 μg of protein were fractionated on a 4–20% gradient polyacrylamide gel (ISC Bioexpress, Kaysville, UT) (Laemmli 1970). Proteins were electrophoretically transferred from SDS–PAGE (polyacrylamide gel electrophoresis) gels to nitrocellulose paper (Bio-Rad Laboratories, Hercules, CA; Hoeffer Apparatus, Bio-Rad Laboratories). Nitrocellulose sheets were incubated with 10% hydrogen peroxide for 30 min to inhibit endogenous peroxidases and then blocked for 30 min in a solution containing 5% rabbit serum and 1% nonfat milk. The nitrocellulose sheets were incubated with the test bovine serum (1:100 in PBS) for 1 h at room temperature. After three washes in PBS-Tween 20, the sheets were incubated with either mouse anti-bovine IgG_1 or mouse anti-bovine IgG_2 (1:1000; Serotec, Raleigh, NC) for 1 h at room temperature. The membranes were then incubated with $F(ab')_2$ rabbit anti-mouse IgG coupled with horseradish peroxidase (1:5000; Serotec). Antibody bound to protein bands was detected using an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). As a negative control, the nitrocellulose membrane containing SGE proteins was incubated with secondary antibodies in the absence of cow sera.

Silver Stain of PAGE. SGE and GE proteins fractionated by SDS–PAGE were defined by silver staining the proteins separated in the gel (Bio-Rad Laboratories) (Merril et al. 1984). Relative molecular weights of the proteins in the SGE and GE were estimated by comparison with known molecular weight standards (Amersham Pharmacia Biotech).

Statistical Analysis. Differences in the mean stimulation indices among the treatment groups were determined by one-way ANOVA using SIGMASTAT (Jandel Software, San Rafael, CA). Dunnett's method was used to determine differences in the means among the treatment groups exposed to stable fly tissue compared with the control (mitogen-stimulated lymphocytes without fly tissues). Tukey's test was used to compare the percentage of inhibition of mitogen stimulation by fly tissue extracts among the treatment groups. Kruskal–Wallis one way ANOVA on ranks was performed on the viability data to determine differences in viability among treatment groups. Statistical significance was established at $P < 0.05$.

Results

Effect of Salivary Gland Extract on Mitogen-Induced Lymphocyte Proliferation. Based on the BCA protein assay, a pair of salivary glands removed from a single stable fly was equivalent to 1 μg of protein, a protein yield per salivary gland within the range reported for salivary gland extracts of black flies and sand flies (Cross et al. 1993, Valenzuela et al. 2001). In an initial experiment using lymphocytes from a single cow, SGE inhibited Con A-stimulated lymphocyte proliferation (Fig. 1). The degree of inhibition increased as the concentration of SGE increased and occurred when SGE was added simultaneously with Con A (Fig. 1, black bars). A possible explanation for this inhibition was that the SGE inhibited mitogen stimulation of lymphocytes by competing with the mitogen for binding sites on the T cell receptor. In fact, SGE not only inhibited lymphocytes prestimulated with Con A for 2 h (Fig. 1, open bars), but the percent inhibition of the lymphocyte responses increased

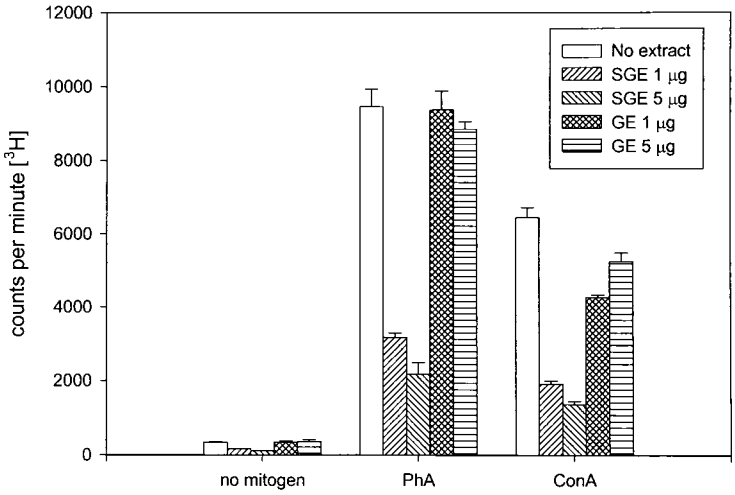

Fig. 2. Lymphocyte proliferation assay determined by incorporation of tritiated thymidine defined as counts per minute of radioactivity. Lymphocytes from one cow were nonstimulated (no mitogen), or stimulated with either PHA or Con A. Error bars represent one standard error of the mean of triplicate replicates.

from 30 to 50% with 1 μg of SGE and from 45 to 55% with 5 μg of SGE, when SGE was added after mitogen. The time of addition of the highest concentration of SGE (10 μg) did not significantly change the degree of inhibition. Because of the difficulties in obtaining large quantities of protein for the experimental assays, only 1 and 5 μg of tissue extract were used in subsequent experiments.

To demonstrate that the inhibition of SGE was specific to this tissue extract and not due to endogenous substances such as proteases present in any tissue, the effects of GE on lymphocyte responses of one cow were compared with SGE. As illustrated for lymphocytes of one cow (Fig. 2), SGE caused a 3–4-fold reduction in the amount of tritiated thymidine incorporated by lymphocytes stimulated with PHA or Con A, whereas GE caused little to no reduction in the incorporation compared with cells stimulated with mitogen alone. Pooled data generated from all six dairy cows were consistent with these findings. Stimulation indices were lower for mitogen-stimulated cultures exposed to SGE compared with GE or mitogen stimulation alone (Table 1). The data demonstrate that a similar degree of inhibition of PHA and Con A-stimulated cells occurred with 1 μg of SGE (61.7 ± 12.7% for PHA; 62.9 ± 12.1% for Con A) as with 5 μg of SGE (79.5 ± 4.6% for PHA; 77.1 + 8.9% for Con A). Inhibition by 1 and 5 μg concentrations of control tissue (GE) did not vary between concentrations of protein and was significantly less compared with SGE (ANOVA $P < 0.05$) (Table 1).

Lymphocyte Viability and Composition of Viable versus Nonviable Lymphocytes. The proportion of total lymphocytes that were dead by 72 h of culture (30–50%) were similar regardless of whether mitogen-stimulated cells were exposed to stable fly tissue extract or not (Table 2). This finding suggested the fly tissue extracts were not directly cytotoxic. Therefore, cell death was attributable to culture conditions. Using flow cytometry and two-color immunofluorescence, the lymphocyte subsets that contributed to the live

Table 1. Pooled data for phytohemagglutinin A (PHA) and concanavalin A (Con A) stimulated lymphocyte responses in the presence or absence of stable fly tissue extracts GE: midgut extracts; SGE: salivary gland extracts

Pooled data (n = 6)	Stimulation index Mean (± SD)	% Inhibition Mean (± SD)
PHA + no extract	29.8 (17.0)	—
PHA + 1 μg GE	25.1 (12.3)	12.7 (12.6)
PHA + 5 μg GE	22.6 (8.9)	18.7 (15.5)
PHA + 1 μg SGE	10.2 (3.8)[a]	61.7 (12.7)[b]
PHA + 5 μg SGE	5.69 (2.3)[a]	79.5 (4.6)[b]
Con A + no extract	21.7 (13.9)	—
Con A + 1 μg GE	16.9 (12.5)	13.8 (20.5)
Con A + 5 μg GE	15.1 (13.3)	24.6 (14.9)
Con A + 1 μg SGE	8.4 (5.6)	62.9 (12.1)[b]
Con A + 5 μg SGE	4.1 (3.6)[a]	77.1 (8.9)[b]

[a] $P < 0.05$ (Dunnett's method for multiple comparisons versus control group without extract).
[b] $P < 0.05$ (Tukey's test for multiple comparisons).

Table 2. Effects of mitogen (PHA: Phytohemagglutinin, Con A: concanavalin A) stimulation and fly extracts (GE: midgut; SGE: salivary gland) on the viability of bovine lymphocytes in culture

Fly extract	% PI + Unstimulated Mean (± SD)	% PI + PHA Stimulated Mean (± SD)	% PI + Con A Stimulated cells Mean (± SD)
No extract	40.2 (5.1)	39.6 (0.3)	37.4 (2.4)
SGE (1 μg)	31.8 (4.8)	40.1 (6.5)	37.2 (0)*
SGE (5 μg)	29.9 (10.5)	50.2 (1.7)	30.1 (13.2)
GE (1 μg)	37.7 (3.2)	46.4 (6.3)	39.6 (2.3)
GE (5 μg)	39.1 (7.5)	43.1 (10.2)	43.2 (2.8)

Data were pooled from lymphocytes of two cows. * n = 1. Each condition was performed in triplicate.
The percentage of nonviable cells was determined by propidium iodide (PI) staining.
$P = 0.77$ (Kruskal-Wallis one-way ANOVA on ranks).

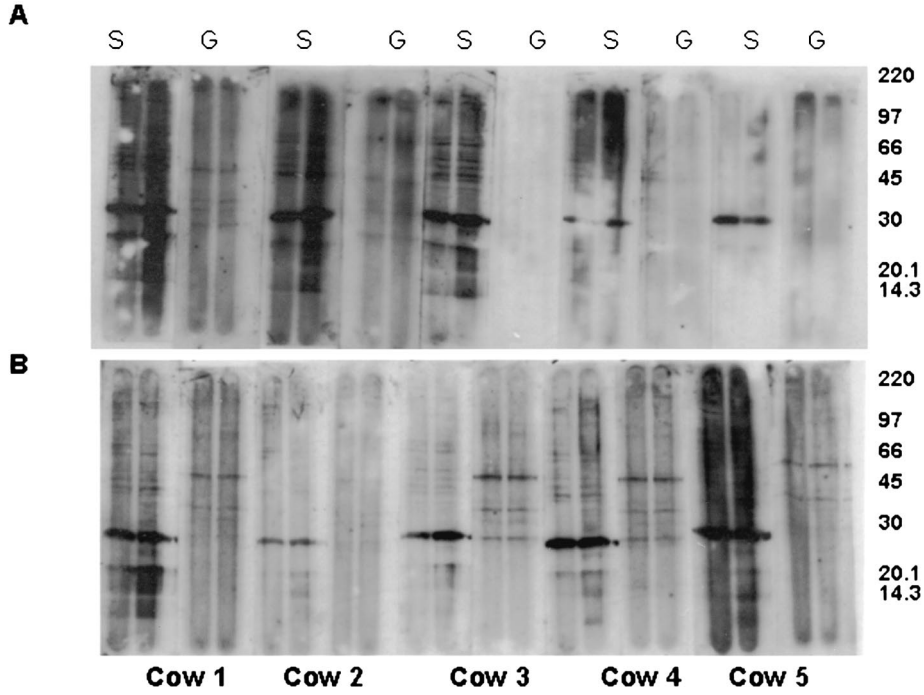

Fig. 3. Western blot analysis of bovine serum against SGE (S lanes) and GE (G lanes). For each pair of lanes, the left lane was immunoblotted with a winter serum sample and the right lane was immunoblotted with a spring serum sample. (A) Anti-bovine IgG_1; (B) Anti-bovine IgG_2.

versus dead populations was determined. At 72 h of culture, the live cells consisted of B lymphocytes (36%), T lymphocytes (28%), and mononuclear cells that were negative for the B and T cell markers (36%). Nonviable lymphocytes identified by propidium iodide staining and lymphocyte determinants were CD3+ T lymphocytes (39%), BAQ44A+ B lymphocytes (44%), and nonT/B cells (16%).

Detection of Immunodominant Salivary Gland Proteins by Western Blot. Western immunoblot assays were used to determine if antibodies to stable fly salivary gland proteins could be present in serum samples from the same cows in which lymphocyte function was suppressed by SGE. IgG subclasses were analyzed for a predominance of one subclass over the other. Both IgG_1 and IgG_2 subclass antibodies in cow serum reacted with several salivary gland proteins. For all cows, the signal was strongest against a low-molecular-weight protein of 27 kDa (Fig. 3). A different, but weak, serum antibody profile was observed with GE (Fig. 3). In some cows, (i.e., Cow 2), the intensity of the band for the 27 kDa protein appeared greater for IgG_1 subclass compared with IgG_2. Comparison of antibody responses between fly seasons demonstrated no apparent difference in antibody subclass reactivity or strength of reactivity between sera collected from high and low fly exposure times.

Characterization of Salivary Gland Extract Proteins. Because the serum of all cows showed strong reactivity to the 27 kDa protein, the SGE and GE were further characterized by SDS–PAGE and silver stain. Bands of varying intensity between 14 and 97 kDa, with a prominent protein band localized at 27 kDa, were detected in the SGE (Fig. 4, Lane A.). Multiple faint bands were detected in the GE (Fig. 4, Lane B).

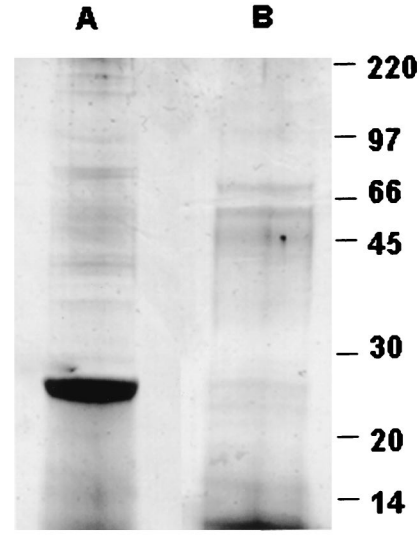

Fig. 4. Silver-stained SDS–PAGE gel of *Stomoxys calcitrans*' (A) SGE, 5 μg; and (B) GE, 5 μg.

Discussion

Stable flies bite mammalian hosts to obtain blood meals necessary for reproduction and nutrition; however, hemostatic defenses, pain–itch responses, and immune responses can interrupt successful feeding (Wikel 1982). It is not surprising, therefore, that short-term and long-term blood-feeding arthropods have mechanisms to modulate these host responses (Wikel 1999, Gillespie et al. 2000, Wikel and Alarcon-Chaidez 2001). The effect of stable fly salivary extract on bovine lymphocytes has not previously been investigated. In this report, SGE from *S. calcitrans* inhibited mitogen-stimulated proliferation of bovine lymphocytes. Because the plant mitogens (Con A and PHA) induce cellular division through T cell receptor clustering by binding to mannose and *N*-acetylgalactosamine-modified glycans residues on the T cell receptor (Hubbard et al. 1986; Demetriou et al. 2001), we tested the hypothesis that the SGE may be inhibiting responses by competing for lectin-binding sites on the lymphocyte. Prior stimulation with Con A did not change the ability of the SGE to inhibit lymphocyte proliferation. In fact, the reduction in lymphocyte responses was greater with lower concentrations of SGE when the extract was added after Con A stimulation. Suppression of T lymphocyte proliferation was not the result of direct cytotoxicity of the salivary gland extract because viability was similar when cells were treated with mitogen without the fly extracts.

The observation that cell death is high after 72 h of mitogen stimulation is not a novel finding, because early studies using trypan blue dye to estimate viability demonstrated decreased viability of mitogen-stimulated lymphocytes over time (Rossier and Landry-Pigeon 1972). In this study, nearly equal proportions of T and B lymphocytes contributed to the viable and nonviable cells at 72 h of stimulation, indicating that even though B lymphocytes were not directly stimulated by the T cell-specific mitogens, their proliferation or survival is likely dependent on signals (i.e., cytokines) provided by activated T cells. The cells that were negative for B and T cell determinants could include macrophages, dendritic cells, or natural killer lymphocytes. The level of suppression of Con A-driven lymphocyte blastogenesis induced by stable fly SGE described in our study was similar to that (68.4%) reported for tick SGE (*Dermacentor andersoni* Stiles) on lymphocytes collected from purebred *Bos indicus* and *Bos taurus* (Ramachandra and Wikel 1995), even though the lymphocytes were pretreated with SGE 2 h before the addition of Con A in that report.

The mechanism of suppression of lymphocyte proliferation by SGE is not known. It could, however, result from a direct effect by pharmacological components of the SGE that are not present in GE. For example, serine proteases (hypodermins A and B) have been identified in the larvae of common cattle grubs [*Hypoderma lineatum* (Villers)] that affect innate and adaptive immune responses by depleting serum complement and inhibiting T lymphocyte responses (Boulard and Bencharif 1984, Nicolas-Gaulard et al. 1995). Cross et al. (1993) demonstrated that the salivary gland extract of the black fly suppressed murine B and T lymphocyte responses to mitogens, 50%, reduced expression of class II major histocompatability antigens on antigen-presenting cells, and decreased cytokine production by lymphocytes. Salivary gland lysates of the Old World and the New World (*Lutzomyia longipalpis* (Lutz and Neiva)) sand flies secrete vasodilatory substances, such as adenosine and maxadilan that suppress macrophage function (Hasko et al. 1996, 2000) and inhibit T lymphocyte activation (Qureshi et al. 1996). Alternatively, antigens from the stable fly SGE might induce suppression in the host indirectly by activating antigen specific clonal expansion of suppressive lymphocytes, such as CD3+ gamma delta T cell receptor bearing cells, which constitute 10–30% of the T lymphocytes in adult cattle (Tizard 1996) and release immunosuppressive cytokines, that is, transforming growth factor-beta (Rhodes et al. 2001).

Sera from dairy cows exposed to stable flies demonstrated strong IgG reactivity to a 27 kDa protein in SGE. This antibody response to stable fly salivary proteins is likely due to inoculation of cows with saliva during blood-feeding by the flies under natural conditions. We evaluated IgG because it is the most abundant class in serum, comprising ≈80% of the total serum immunoglobulins, and IgG_1 constitutes the major subclass secreted in colostrum (Tizard 1996). Individual animals showed no difference in IgG_1 and IgG_2 reactivity to salivary gland antigens collected during high and low fly exposure times. Quantitative methods are needed to determine whether or not there are differences in antibody titers among the IgG subclasses against the immunodominant SGE protein.

The observation that the dairy cows had developed serum antibodies reactive to stable fly salivary gland antigens is an interesting finding in light of the immunosuppressive properties of the SGE observed in the in vitro assays. Although it is possible that the antibody responses represent cross reactive antibodies that recognize salivary protein epitopes in common with stable flies and horn flies [*Haematobia irritans* (L.)], we argue that concurrent exposure to horn flies is not very likely because the cows were kept in the dairy facility where horn flies are not found. A clear explanation of the dichotomous observations between the effects of SGE on lymphocytes in vitro and the in vivo generation of antibody responses secondary to fly exposure is not available at this time. However, others have reported simultaneous immunosuppression and immunoenhancement with SGE from a single species of arthropods. For example, tick SGE caused enhanced lymphocyte responses to a polyclonal B cell activator, LPS, even though it suppressed T cell mitogen responses (Ramachandra and Wikel 1995). Moreover, Cross et al. 1993 noted that in vivo inoculation with black fly SGE did not affect the mitogenic potential of splenic lymphocytes, whereas in vitro exposure to the extract suppressed mitogen-induced proliferation of lymphocytes. Because the 27 kDa pro-

tein in stable fly salivary glands appears to be immunodominant in cattle, future studies will be directed at further characterizing this protein and identifying its direct immunomodulatory function.

Recently, a 15 kDa protein was isolated from the saliva of the Old World sand fly, the vector that transmits *Leishmania major* to humans. This salivary gland protein from the vector was a novel immunogen that conferred protection in mice subsequently challenged with *L. major* (Valenzuela et al. 2001). Antibody responses in cattle reported for other Diptera include the buffalo fly (*Haematobia irritans exigua* De Meijere) (Kerlin and Allingham 1992) and the common cattle grub (Chabaudie and Boulard 1992). Vaccination of cattle and other domestic animals against components of stable fly saliva is a realistic goal. In fact, Schlein et al. (1976) reported that rabbits immunized with different types of tissues from *S. calcitrans* can produce antibodies against each fly tissue, resulting in alterations in growth and function of the tissues (Schlein and Lewis 1976).

This report identifies immunosuppressive properties of stable fly SGE in bovine immune cells. This property of stable flies may contribute to increasing the susceptibility of cattle to vectorborne pathogens, such as anthrax, brucellosis, or salmonella (Knapp et al. 1992). Development of vaccines against immunodominant proteins of arthropod tissues could become a reasonable tool in reducing the success of bloodmeal acquisition by the vector and reducing the potential for immune compromise and susceptibility to pathogens during fly feeding.

Acknowledgments

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