Immunolocalization of *Leishmania* (*Viannia*) *braziliensis* membrane antigens recognized by mAbs SST-2, SST-3, and SST-4

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(*Received 11 December 2002; revised 27 May 2003; accepted 9 June 2003*)

SUMMARY

The immunolocalization of *Leishmania* (*Viannia*) *braziliensis* stage-specific antigens recognized by mAbs was analysed by transmission electron microscopy. The antigen recognized by mAb SST-2 was present at the surface of promastigotes, including the flagellum and flagellar pocket. The reactivity of SST-2 with isolates of different serodemes showed a pronounced microheterogeneity in terms of the number of reactive bands within the low molecular weight range from 24 to 33 kDa. The 180 kDa glycoprotein recognized by mAb SST-3 was present only in the flagellar membrane. SST-3 also recognized multiple discrete bands from 160 to 200 kDa, as observed in several serodemes. In contrast, mAb SST-4, which recognizes a 98 kDa antigen, showed weak labelling on the promastigote surface by transmission electron microscopy and indirect immunofluorescence. Based on Western blotting, indirect immunofluorescence, and solid-phase radioimmunoassay, the antigens recognized by mAbs SST-2, SST-3 and SST-4 were present in all *L*. (*V.*) *braziliensis* analysed, from 7 different serodemes.

Key words: *Leishmania* (*Viannia*) *braziliensis*, monoclonal antibody, specific antigens, flagellum.

INTRODUCTION

During their developmental cycle, parasites of the genus *Leishmania* occur as flagellated promastigotes within the alimentary tract of their insect vector, the phlebotomine sandfly, and as amastigotes in the mononuclear phagocytes of mammalian hosts. Various species and subspecies of *Leishmania* are responsible for a broad spectrum of cutaneous, mucocutaneous, and visceral leishmaniasis, which occur throughout the world and constitute a major public health problem in tropical areas (Modabber, 1993). Members of the *Leishmania mexicana* complex and *Viannia* subgenus are associated with human cutaneous leishmaniasis, and mucocutaneous leishmaniasis, respectively (Lainson & Shaw, 1987; Samady, Janniger & Schwartz, 1996; Herwaldt, 1999). In the New World, mucocutaneous leishmaniasis is caused mainly by *Leishmania* (*Viannia*) *braziliensis*, although some cases have been attributed to other *Leishmania* species (Barral *et al*. 1991; Convit *et al*. 1993; Herwaldt, 1999).

Monoclonal antibodies (mAbs) have been used as tools for immunocharacterization of surface antigens, allowing the identification and classification of New World *Leishmania* species (McMahon-Pratt & David, 1981; McMahon-Pratt, Bennett & David, 1982; Grimaldi, David & McMahon-Pratt, 1987; Shaw, Ishikawa & Lainson, 1989; Grimaldi *et al*. 1991; Hanham, Shaw & Lainson, 1991*a*; Hanham *et al*. 1991*b*; Grimaldi & McManhon-Pratt, 1996). A panel of specific mAbs were used by Grimaldi *et al*. (1987) and Shaw *et al*. (1989) to identify different species of *Leishmania*. Shaw *et al*. (1986) defined 7 different *L*. (*V.*) *braziliensis* serodemes based on mAb reactivities to promastigotes.

We recently produced mAbs against membranes of *L*. (*V.*) *braziliensis*, and characterized the antigens recognized by Western blotting, immunoprecipitation of labelled parasites, and immunofluorescence (Silveira *et al*. 2001). In the present study, we analysed the presence of antigens recognized by mAbs SST-2, SST-3, and SST-4 in several *L*. (*V.*) *braziliensis* serodemes, and their cellular localization by transmission electron microscopy (EM).

MATERIALS AND METHODS

Parasites and cells

Leishmania (*Viannia*) *braziliensis* promastigotes were cultured at 26 °C by several passages of log-phase parasites in Medium 199 supplemented with 10% (v/v) heat-inactivated foetal calf serum (Cultilab, Campinas, SP, Brasil). Table 1 shows International codes of the *L*. (*V.*) *braziliensis* used. Hybridoma cell

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Parasitology (2003), **127**, 449–456.
DOI: 10.1017/S0031182003003986 Printed in the United Kingdom

Table 1. *Leishmania* (*Viannia*) *braziliensis* International codes

L. (*V.*) *braziliensis**	International code
Serodeme 1 (M11272)	MHOM/BR/1987/M11272
Serodeme 2 (M14039)	MHOM/BR/1992/444MR
Serodeme 3 (M13108)	MHOM/BR/1990/280JM
Serodeme 4 (M11113)	MHOM/BR/1986/008-L1
Serodeme 5 (M11115)	MHOM/BR/1986/024-L1
Serodeme 6 (M11119)	MHOM/BR/1986/030-L1
Serodeme 7 (M14006)	MHOM/BR/1991/389SMPP

* Isolated from patients with cutaneous leishmaniasis at Laboratório de Ensino e Pesquisa em Análises Clínicas of Universidade Estadual de Maringá, Maringá, Paraná, Brazil and identified by Dr J. J. Shaw, Instituto Evandro Chagas, Belém, Pará, Brazil.

lines SST-2 (IgG1), SST-3 (IgG3), and SST-4 (IgG1) were grown in RPMI-1640 medium supplemented with 10% foetal calf serum at 37 °C.

Cellular radio-immunoassay (*RIA*)

Parasites were washed with PBS and resuspended in PBS (4×10^7 parasites/ml). Parasites were adsorbed on 96-well plates pre-coated with 0·1% poly-L-lysine (molecular weight 500 000) for 30 min as described by McMahon-Pratt, Jaffe & Grimaldi (1984*b*). Promastigotes (2×10^6/well) were added, plates were centrifuged for 10 min at 850 ***g***, and parasites were fixed for 15 min with 0·5% glutaraldehyde in cold PBS. Plates were washed with PBS, unbound aldehyde groups were blocked by adding 0·1 M glycine, pH 8·0, for 30 min, and plates were washed again with PBS and used for solid-phase RIA as follows. The plates were blocked with 1% bovine serum albumin (BSA) in PBS (200 μl) for 2 h, incubated with mAb culture supernatant for 2 h. The amount of mAb bound was determined by reaction with 50 μl of rabbit anti-mouse IgG (Cappel, ICN Pharmaceuticals, Aurora, OH) diluted 1 : 500 in 1% BSA/PBS. Plates were washed 3 times with PBS, 50 μl ^{125}I-labelled protein A (Pierce, Rockford, IL) in 1% BSA (10^5 cpm/well) were added, incubated for 1 h, washed 5 times with PBS, and the radioactivity in each well was measured with a gamma-counter (Straus *et al*. 1993). Negative reactivity was defined as well with counts representing less than 0·1% of added ^{125}I-labelled protein A.

Indirect immunofluorescence

Promastigotes (1×10^8) were fixed with 1% formaldehyde in PBS for 10 min. Cells were washed, resuspended in 1 ml of PBS, and 20 μl of the solution were added to each cover-slip. Air-dried preparations were flooded for 1 h with PBS containing 5% BSA, and incubated sequentially with mAb culture supernatant (1 h), and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (DAKO, Carpinteria, CA) diluted 1 : 200 in 1% BSA/PBS (1 h) (Suzuki *et al*. 1997). The cover-slips were washed 5 times with PBS after each incubation, then examined with an epifluorescence microscope. In control experiments in the presence of an irrelevant IgG3 mAb, no fluorescence was observed. The criterion used to define negative reactivity was complete lack of fluorescence on the parasites.

Western blotting

From each serodeme 3×10^8 parasites were solubilized in 1 ml of sample buffer (Tris–HCl 0·123 M, pH 6·8; SDS 0·139 M; glycerol 2·17 M). Samples of 15 μl were applied in 1×5 mm lane, and separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) carried out in 8·0% polyacrylamide slab gels (10×8 cm) (Laemmli, 1970) under non-reducing conditions, and transferred onto nitrocellulose (Towbin, Staehelin & Gordon, 1979). The nitrocellulose was blocked with 5% BSA in PBS for 2 h, incubated with mAbs (culture supernatant) for 2 h, rinsed in PBS, then incubated sequentially with goat anti-mouse IgG conjugated to biotin (DAKO, Carpinteria, CA) diluted 1 : 2000 in 1% BSA/PBS (2 h), avidin conjugated to peroxidase (DAKO, Carpinteria, CA) diluted 1 : 1000 in 1% BSA/PBS (1 h), and 4-chloro-1-naphthol (Straus *et al*. 1996). The reaction was terminated after 5 min by washing with distilled water. Antibody-reactive bands were visualized on nitrocellulose as blue spots.

Transmission EM

Pre-embedding assay. Promastigotes (3×10^8) were fixed with 4% paraformaldehyde and 0·05% glutaraldehyde in 0·1 M Na-cacodylate buffer, pH 7·6 (CB), for 20 min at 4 °C. Cells were washed (3400 ***g*** for 3 min) in 0·1 M pH 7·4 phosphate buffer (PB) and incubated with 0·05% sodium borohydride and 0·1% glycine in PB for 10 min. Parasites were washed with 0·5% BSA and 0·1% gelatin in PB (AGPB) and blocked with 5% goat serum in AGPB for 1 h. Approximately 1×10^7 parasites were incubated with 0·5 ml of mAb (culture supernatant for 1·5 h at 25 °C), washed with AGPB, and incubated with gold (10 nm)-conjugated goat anti-mouse IgG (Electron Microscopy Sciences, Fort Washington, PA) diluted 1 : 20 in 1% BSA/PBS (1·5 h at 25 °C). Parasites were washed with AGPB and PB, fixed with 2·5% glutaraldehyde in PB (10 min at 25 °C), washed in PB, and set in 4% agarose. The agarose was cut and the material was contrasted with 2% osmium tetroxide in CB (1 h at 25 °C), washed with water, and incubated with 0·5% uranyl in 0·3 M sucrose (30 min at 25 °C). The material was then washed with water, gradually dehydrated in ethanol (70%,

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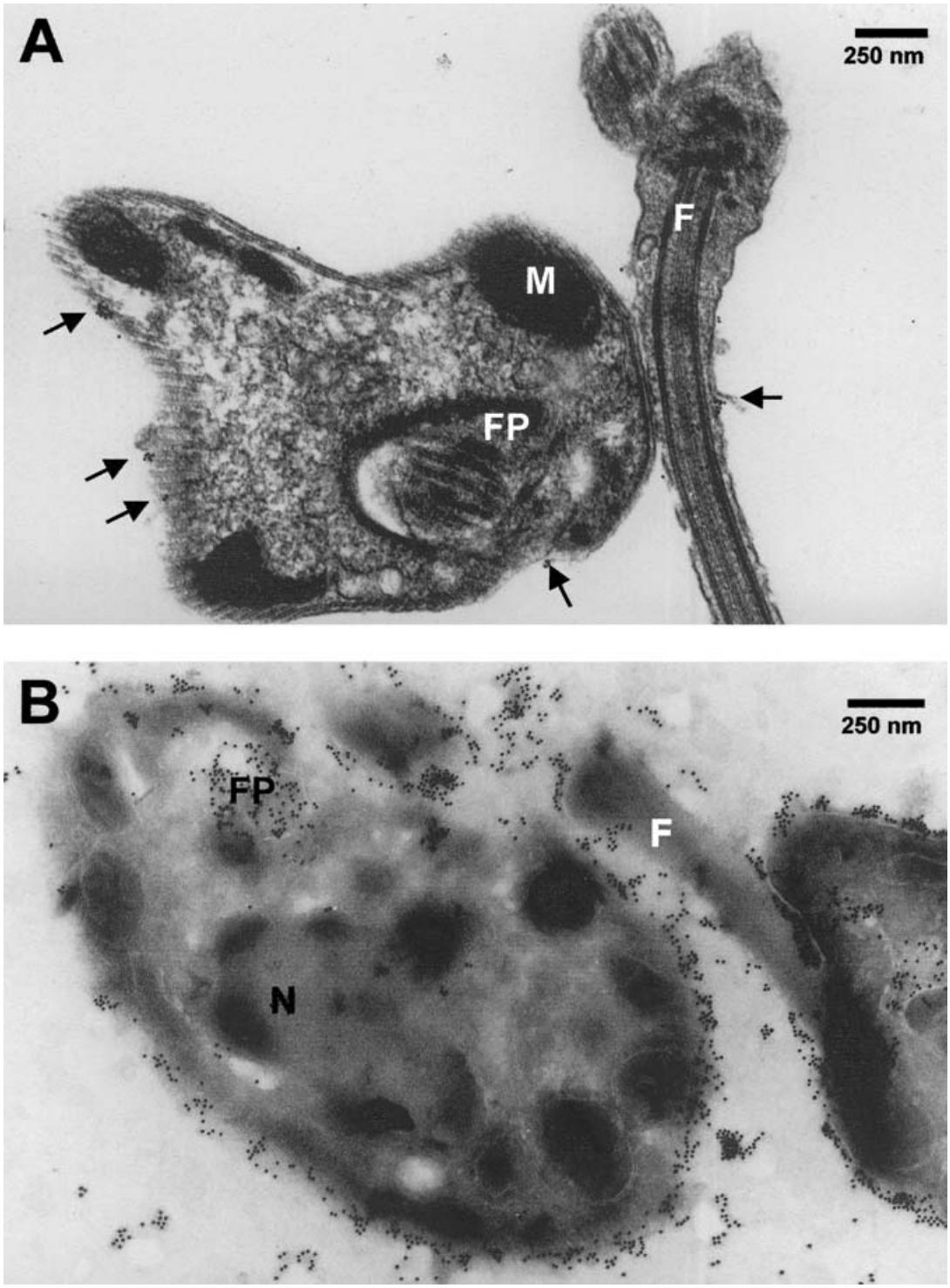

Fig. 1. Transmission EM of *Leishmania* (*V*.) *braziliensis* promastigotes using mAb SST-2. (A) Promastigotes were fixed, incubated sequentially with SST-2 and gold-conjugated anti-mouse IgG, and set in Araldite. (B) Promastigotes processed for cryosection. Grids were incubated sequentially with SST-2 and with gold-conjugated anti-mouse IgG. FP, flagellar pocket; F, flagellum; M, mitochondria; N, nucleus. Arrows indicate gold particle labelling.

90%, and absolute), and infiltrated sequentially with propylene oxide (2 times for 15 min), Araldite in propylene oxide (1 : 2; v/v; 1 h); Araldite in propylene oxide (1 : 1; v/v; overnight; agitation); and Araldite (5 h; desiccator). After polymerization, the material was cut (70–90 nm thickness). The grids were contrasted with uranyl acetate (8 min) and with lead citrate (4 min) (Straus *et al*. 1996), and then examined by JEOL1200 EX-II transmission EM under 80 kV.

Post-embedding assay in cryosections. In some experiments 1×10^8 promastigotes were washed with PBS, fixed with 4% paraformaldehyde and 0·1%

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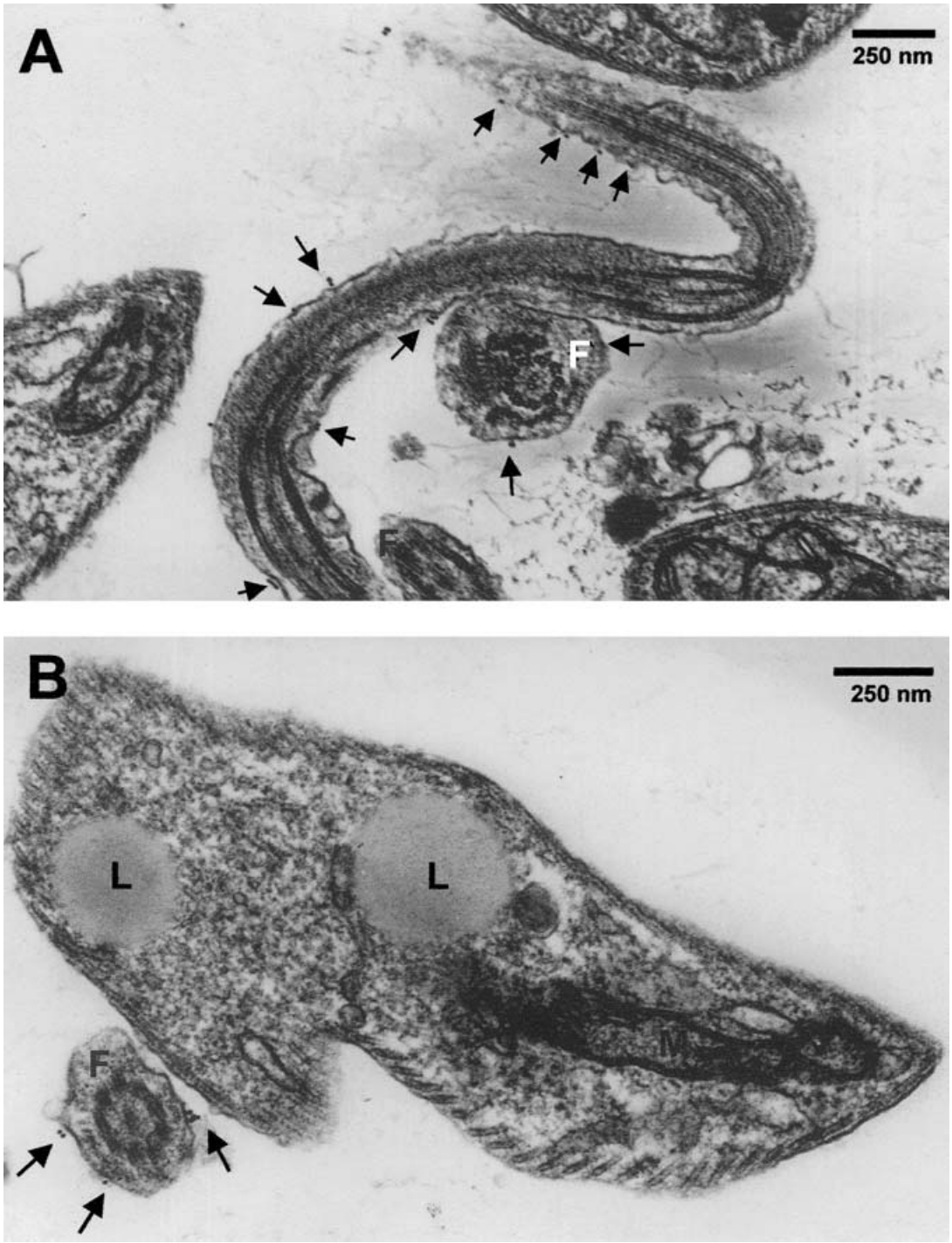

Fig. 2. Transmission EM of *Leishmania* (*V.*) *braziliensis* promastigotes using mAb SST-3. Promastigotes were fixed, incubated sequentially with SST-3 and gold-conjugated anti-mouse IgG, and set in Araldite. F, flagellum; M, mitochondria; L, lipidic vesicles. Arrows indicate gold particle labelling.

glutaraldehyde at 4 °C for 30 min, washed with PBS, and incubated with 50 mM ammonium chloride for 30 min in order to block free aldehyde groups. The parasites were washed with PBS and set into 10% gelatin to provide a supporting matrix. Fragments (2–3 mm) were incubated in 2·3 M sucrose, overnight at 4 °C. The specimens were placed onto a metal carrier and frozen in liquid nitrogen. Cryosections (40–50 nm) were mounted on nickel grids, washed with PBS, blocked with PBS containing 1% BSA and 5% goat serum for 30 min, incubated with hybridoma culture supernatant for 90 min, and with colloidal gold (10 nm) conjugated to goat anti-mouse IgG diluted 1 : 20 in 1% BSA/PBS for 90 min. After each incubation the grids were washed 3 times with 1% BSA in PBS. After the last wash with 1% BSA, the sections were washed with distilled water, dried, and stained with a mixture of 3% uranyl acetate and 1·5% methyl-cellulose (1 : 9; v/v) in 2 cycles of 10 min. Excess solution was removed with filter paper, and

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Table 2. Reactivity of mAbs SST-2, SST-3, and SST-4 with various *Leishmania braziliensis* serodemes, by indirect immunofluorescence (IIF) and solid-phase radioimmunoassay (RIA)

	SST-2		SST-3		SST-4	
	IIF*	RIA† (cpm)	IIF*	RIA† (cpm)	IIF*	RIA† (cpm)
Serodeme 1	+	8675	+	4040	−	1150
Serodeme 2	+	8635	+	2490	−	900
Serodeme 3	+	10155	+	4970	−	3765
Serodeme 4	+	9285	+	3755	−	3955
Serodeme 5	+	10870	+	3750	−	1725
Serodeme 6	+	8175	+	3980	−	905
Serodeme 7	+	9910	+	4730	−	2620

* IIF, immunofluorescence: (−), negative reactivity; (+), positive reactivity.
† mAb reactivity with 2×10^6 parasites/well.

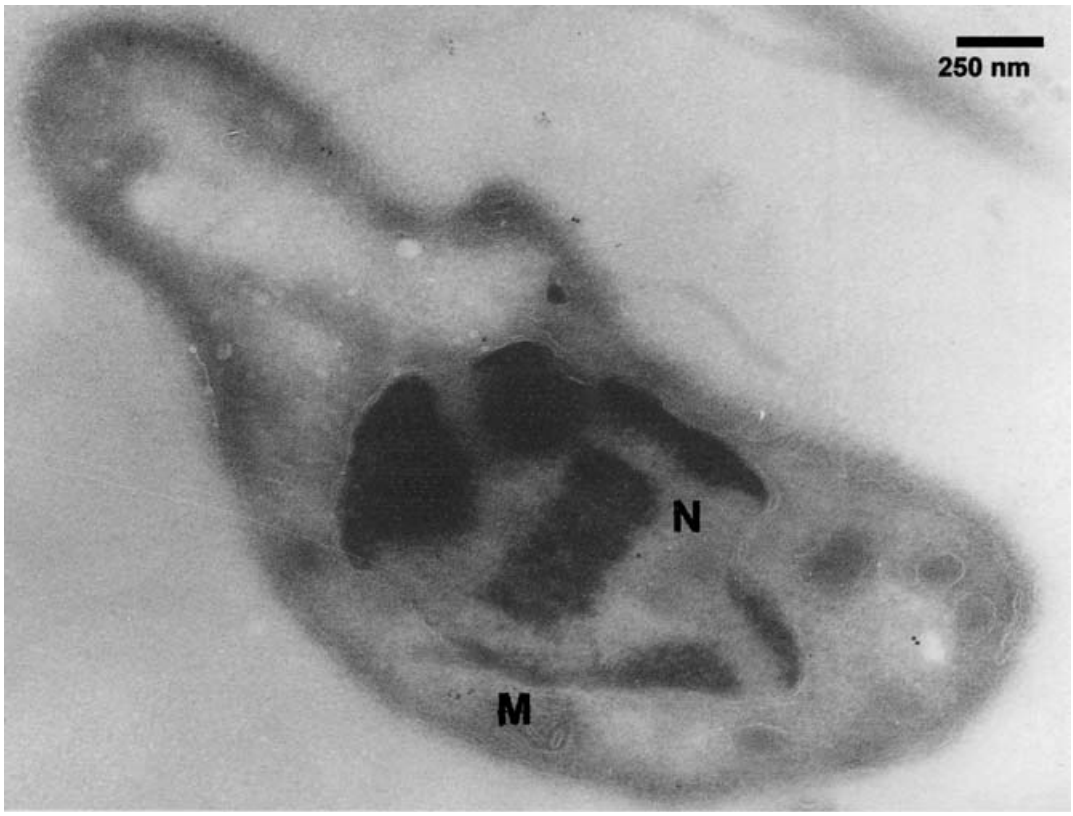

Fig. 3. Transmission EM of *Leishmania* (*V.*) *braziliensis* promastigote using mAb SST-4. Grids were incubated sequentially with SST-4 and gold-conjugated anti-mouse IgG. M, mitochondria; N, nucleus. Arrows indicate gold particle labelling.

the sections were examined by JEOL 1200 EX-II transmission EM.

RESULTS

Immunolocalization of antigens recognized by mAbs SST-2, SST-3, and SST-4 was carried out by transmission EM. Antigens recognized by SST-2 were visualized only on the surface of *L.* (*V.*) *braziliensis* promastigotes, including the flagella (Fig. 1A). Post-embedding labelling of cryosections (Fig. 1B) showed stronger labelling on all parasite surfaces and the flagellar pocket, in comparison with pre-embedding labelling. By pre-embedding assay mAb SST-3 labelled only the flagellar membrane (Fig. 2A, B), the recognized antigens were also probed by post-embedding labelling on cryosections, but only very weak labelling was observed, suggesting that these flagellar antigens are better preserved by the pre-embedding method. mAb SST-4 did not show any labelling by the pre-embedding method, and a minimal labelling on the surface of promastigotes was detected by post-embedding labelling on cryosections by transmission EM (Fig. 3).

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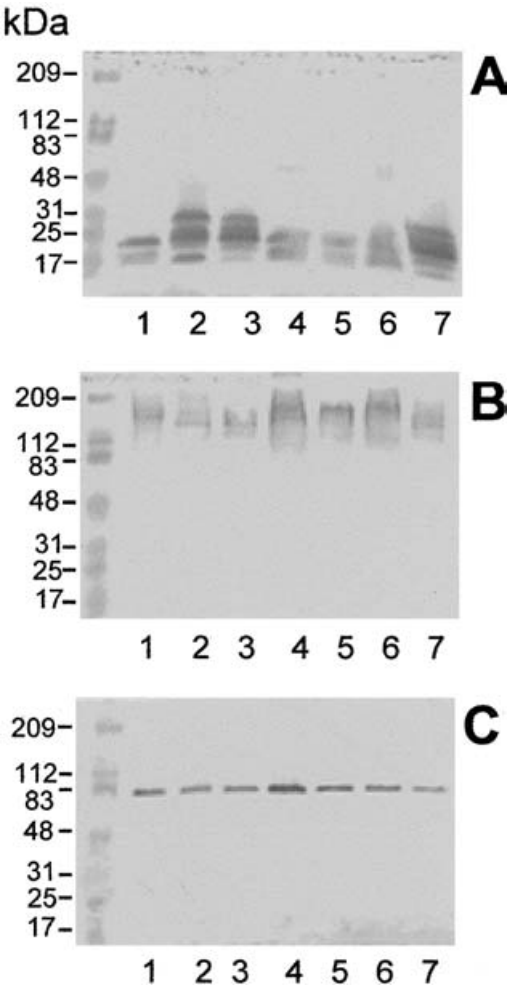

Fig. 4. Western blot of *Leishmania* (*V.*) *braziliensis* promastigotes with mAbs SST-2 (A), SST-3 (B), and SST-4 (C) after separation of whole cell lysates by SDS–PAGE in the absence of β-mercaptoethanol. Samples equivalent to $4{\cdot}5 \times 10^6$ promastigotes were loaded in each lane. Lanes 1 through 7 correspond to serodemes 1 through 7, respectively.

In order to confirm that mAbs SST-2, SST-3, and SST-4 were reactive with all *L.* (*V.*) *braziliensis* serodemes, we performed an indirect immunofluorescence assay. Strong fluorescence was observed for SST-2 and SST-3 with all *L.* (*V.*) *braziliensis* stocks tested. SST-2 labelled the entire surface of the promastigote, including the flagellum, whereas SST-3 showed strong reactivity only with the flagellum. mAbs SST-2 and SST-3 were also reactive with all serodemes tested by solid-phase RIA. SST-4 showed positive reactivity only by solid-phase RIA; no labelling was detected by indirect immunofluorescence assay. Table 2 summarizes reactivity of the mAbs with different serodemes by indirect immunofluorescence and solid-phase RIA.

The antigens recognized by mAbs SST-2, SST-3, and SST-4 in *L.* (*V.*) *braziliensis* were analysed by Western blotting. mAb SST-2 strongly recognized a 28 kDa component in all serodemes (Fig. 4A). Multiple low molecular weight components (24–32 kDa) were observed, and some serodemes shared common bands. SST-2 also showed weak reactivity with a 72 kDa component of some stocks. SST-3 recognized several bands of ~180 kDa, and showed distinct reactivity patterns for the different serodemes tested, varying from 160 to 200 kDa (Fig. 4B). mAb SST-4 recognized a ~98 kDa antigen present in all serodemes (Fig. 4C).

DISCUSSION

Few specific *L.* (*V.*) *braziliensis* antigens are described in the literature: a 72 kDa protease in the promastigote surface (Legrand *et al.* 1987; Kutner *et al.* 1988; Kutner, Pellerin & Brénière,1990); a 47–54 kDa glycoprotein (Rodríguez, Hernández & Merino, 1983; Misle, Márquez & Hernández, 1985; Nagakura *et al.* 1986); a 25 kDa antigen by (Misle *et al.* 1985) and 17 and 24 kDa promastigote stage-specific antigens (McMahon-Pratt, Bennett & Jaffe, 1984*a*). We recently described 3 mAbs, SST-2, SST-3, and SST-4, reactive with promastigotes of this species (Silveira *et al.* 2001). mAb SST-2 is specific for *L.* (*V.*) *braziliensis*, and recognizes a conformational epitope in a doublet peptide of low molecular weight (24 and 28 kDa) on the parasite surface. mAb SST-3 is specific for the *L.* (*V.*) *braziliensis* flagellum and recognizes a carbohydrate epitope with ~180 kDa surface glycoprotein. mAb SST-4 recognizes a conformational epitope in a 98 kDa protein of *L* (*V.*) *braziliensis*, and cross-reacts weakly with a protein of the same molecular weight in *L.* (*V.*) *panamensis*. In the present study we analysed immunolocalization of the antigens recognized by mAbs SST-2, SST-3, and SST-4 in *L.* (*V.*) *braziliensis* promastigotes, and their presence in several *L.* (*V.*) *braziliensis* serodemes.

Transmission EM showed strong SST-2 labelling at the promastigote surface and the flagellar pocket. Labelling was best observed in cryosections. The presence of these antigens in all serodemes was demonstrated by indirect immunofluorescence and solid-phase RIA, and confirmed by Western blotting, whereby the 28 kDa antigen recognized by SST-2 was present in all *L.* (*V.*) *braziliensis* tested. There was microheterogeneity in terms of small molecular weight components recognized by this mAb (1–5 bands from 18 to 32 kDa).

Indirect immunofluorescence showed that mAb SST-3 labelled exclusively the flagellum, in all serodemes analysed. Transmission EM demonstrated that the recognized antigen is present at the flagellar surface. Under Western blotting, SST-3 recognized a family of high molecular weight isoforms (160–200 kDa), varying according to the *Leishmania* stock used. In most cases the stocks shared 2 or 3 common bands; strongest reactivity was generally observed for the 160 and 180 kDa components.

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Inhibition of mAb SST-2 and SST-3 binding by periodate oxidation, as demonstrated by Silveira *et al*. (2001), suggests that the recognized antigens are glycosylated, and that this glycosylation may be responsible for the microheterogeneity of the *L*. (*V*.) *braziliensis* glycoproteins.

Microheterogeneity in parasite antigens recognized by mAbs was also described by Jaffe, Rachamin & Sarfstein (1990) in various isolates of *L*. (*L*.) *donovani*.

The 98 kDa protein recognized by SST-4 is present in all serodemes of *L*. (*V*.) *braziliensis* tested, as shown by Western blotting and solid-phase RIA. By transmission EM, SST-4 showed little labelling in promastigotes, in agreement with the lack of reactivity observed by indirect immunofluorescence. However, the small amount of 98 kDa protein present on the parasite surface may play an important role in parasite–macrophage interaction, as suggested by our previous study (Silveira *et al*. 2001).

In conclusion it has been demonstrated that the ~18–32 kDa antigens, 160–180 kDa glycoproteins, and 98 kDa protein, recognized respectively by mAbs SST-2, SST-3, and SST-4, are specific antigens present in all serodemes of *L*. (*V*.) *braziliensis* tested. These mAbs provide useful tools for identification of this parasite species, and for elucidating roles of the respective antigens in promastigote interaction with mammalian host cells or insect vector intestinal epithelium.

The authors thank Maria Vieira Seles for technical assistance, Drs Jeffrey Shaw and Edna Ishikawa from Instituto Evandro Chagas, Belém, Pará, Brazil, for the identification of *Leishmania* and Dr Stephen Anderson for editing of the manuscript. This work was supported by FAPESP, CNPq, PRONEX and CAPES.

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https://doi.org/10.1017/S0031182003003986 Published online by Cambridge University Press