Species-specific antigens in salivary glands of phlebotomine sandflies

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SUMMARY

Saliva inoculated by sandfly females during feeding stimulated production of high levels of anti-saliva antibodies. To determine whether 3 species of the genus *Phlebotomus* have species-specific salivary antigens we performed dot-blots and immunoblots using sera from mice, hamsters and rabbits repeatedly bitten by sandflies. Important differences were found in the antigen components of the salivary gland lysates (SGL) of *Phlebotomus papatasi*, *P. perniciosus* and *P. halepensis*. In total 4–9 species-specific antigens were detected in each species by immunoblotting. Cross-reactivity was not detected between *P. papatasi* and the other species tested; in the SGL of *P. papatasi* sera from animals bitten by this species recognized 5–7 major antigens while sera from animals bitten by other species did not react. A weak cross-reaction was observed between *P. perniciosus* and *P. halepensis*; in SGL from *P. perniciosus*, the sera from rabbits and hamsters bitten by this species recognized about 8 intense bands while sera from animals bitten by *P. halepensis* reacted weakly with up to 4 saliva polypeptides.

Key words: *Phlebotomus* spp., *Leishmania* spp., sandfly, saliva, immunoglobulin.

INTRODUCTION

Repeated bites of bloodsucking insects stimulate immune responses to salivary antigens in the vertebrate host. The response is mainly a CD4+ Th1 type induction, which mediates delayed-type reactions, but antibodies are also produced. In addition to homocytotropic antibodies, circulating antibodies reactive with protein components of salivary glands are also induced (for review see Sandeman, 1996). In sandflies (*Diptera: Phlebotominae*), the anti-saliva antibodies were detected both in hamsters repeatedly exposed to bites of *Phlebotomus argentipes* (Ghosh & Mukhopadhyay, 1998) and in mice inoculated by salivary gland lysate (SGL) of *P. papatasi* (Belkaid *et al*. 1998).

In addition to its anti-haemostatic activity the sandfly saliva enhances *Leishmania major* infection by modulating selective macrophage function in the naive host (for reviews see Champagne & Valenzuela, 1996; Wikel, Ramachandra & Bergman, 1996). It was shown recently, however, that mice repeatedly inoculated with SGL of *P. papatasi* made anti-saliva antibodies capable of neutralizing the enhancing activity of SGL on *L. major* infections (Belkaid *et al*. 1998). This important finding suggests that in endemic areas the intensity of previous exposure to bites of uninfected sandflies might influence the susceptibility to *Leishmania* infection and hence the epidemiology of leishmaniases.

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The composition of sandfly saliva varies considerably between species and even between populations of different geographical origin. Thus variability in the immunosuppressive and anti-haemostatic components of saliva was observed in different populations of the New-World vector *Lutzomyia longipalpis* (Warburg *et al*. 1994; Lanzaro *et al*. 1999). Similarly, in Old-World vector (*Phlebotomus* spp.), differences in protein components of the SGL were recently demonstrated between species and even between colonies of the same species (Volf, Tesařová & Nohýnková, 2000). In endemic areas it is likely that individuals at risk of exposure to infected vectors are repeatedly bitten by uninfected sandflies of several species. Therefore, it is important to determine whether there exist species-shared antigens among various sandflies eliciting cross-reactive antibody responses in the bitten host. So far as we are aware, there are no data on whether exposure to salivary components of non-vectorial phlebotomine species has a neutralizing effect on the saliva of a vector species.

In the present study, 3 species belonging to different phlebotomine subgenera were chosen; SGL of *P.* (*Phlebotomus*) *papatasi*, *P.* (*Larroussius*) *perniciosus* and *P.* (*Adlerius*) *halepensis* were tested with sera of laboratory animals repeatedly bitten by these species. *P. papatasi* is a proven vector of *L. major* while *P. perniciosus* and *P. halepensis* are proven and suspected vectors of *L. infantum*, respectively (for review see Lewis & Ward, 1987). To determine whether the 3 sandfly species have species-shared and species-specific antigens we used 2 blotting techniques with sera from animals

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immunized by sandfly bites. We investigated whether antibodies elicited by biting of a particular species cross-react with SGL of another species.

MATERIALS AND METHODS

Sandfly colonies were maintained at 26 °C and 14/10 LD photo-period. Adult flies were given a 50% sucrose solution, and females were fed on sedated mice (*Phlebotomus papatasi* Scopoli), on hamsters (*P. perniciosus* Newstead) or rabbits (*P. halepensis* Theodor). To obtain salivary glands females were dissected at 5–10 days of age as previous experiments showed that protein composition of saliva depends on the age of the fly and the typical electrophoretic pattern is reached 3–5 days after imago emergence (Volf *et al*. 2000). Salivary glands were dissected in cold PBS (pH 7·2) and stored at −70 °C.

Anaesthetized animals were repeatedly exposed to sandfly bites (20–80 sandfly females per animal, 4–6 times over a period 2 months). One week after the last exposure the animals were bled, and the sera were used for blotting experiments.

It would have been desirable for the antibody responses to SGL from different species of sandflies to be compared in sera from the same host species. Unfortunately the feeding preferences of the different species of sandflies do not allow this. Thus, although the 3 species of sandflies all feed readily on man they have different feeding preferences for laboratory animals. *P. papatasi* feed well on mice. In contrast *P. perniciosus* and *P. halepensis* will not feed on mice and although they will both feed on hamsters and rabbits the latter thrives better on rabbits, as described above.

Dot-blot

Salivary gland lysate (SGL) was obtained by repeated freezing/thawing and centrifugation (2500 ***g*** for 5 min) of the sample (40 glands in 80 μl of PBS) just before use. SGL was dotted on nitrocellulose membrane (Serva, pore size 0·2 μm) in 2 μl volumes (corresponds to 1 gland per dot) and the antigen was stabilized on the discs by drying at room temperature. The membrane with the antigen was blocked with 5% skim milk in Tris–Tw buffer (20 mM Tris, 150 mM NaCl, pH 7·6, with 0·1% Tween 20) overnight at 4 °C and stored at −20 °C until use. The membrane was subsequently incubated for 1 h with sera diluted 1:500 and 1:1500 in Tris–Tw followed by 4 washings in Tris–Tw and incubation with appropriate secondary antibodies conjugated with horseradish peroxidase (SEVAC, Prague) diluted 1:1000 in Tris–Tw. Swine anti-mouse (SwAM/Px), anti-hamster (SwAHa/Px) or anti-rabbit immunoglobulins (SwAR/Px) were used, respectively. The peroxidase reaction product was visualized in the substrate solution with diaminobenzidine.

Immunoblotting

Electrophoresis (SDS–PAGE) was carried out using the discontinuous system according to Laemmli (1970) under reducing conditions on 12% slab gels (thickness 0·75 mm, constant voltage 200 V) using the Mini-Protean II apparatus (BioRad). Lysate was boiled with sample buffer for 3 min and the supernatant obtained (2500 ***g*** for 5 min) was used for further work. The equivalent of 5 glands (4–5 μg of protein) was loaded in 1 lane. The first part of the gel carrying the separated proteins was stained by Coomassie blue or silver according to the method of Bollag & Edelstein (1991) and the second part was electrotransferred to nitrocellulose membrane using Semi-Phor apparatus (Hoefer Scientific Instruments, San Francisco). The Western blots were blocked in 5% skim mild in Tris–Tw buffer overnight at 4 °C and cut into strips. Strips were incubated for 1 h with sera diluted 1:500 and 1:1500 in Tris–Tw, and 1 h with peroxidase conjugate (SEVAC, Prague) diluted as described above. Anti-*P. papatasi* sera originated from bitten mice, anti-*P. perniciosus* and anti-*P. halepensis* sera from rabbits and hamsters. Swine anti-mouse (SwAM/Px), anti-hamster (SwAHa/Px) or anti-rabbit immunoglobulins (SwAR/Px) were used, respectively. The whole reaction was terminated as described above.

RESULTS

Dot blot

Extracts of *P. perniciosus*, *P. papatasi* and *P. halepensis* glands were tested with sera of laboratory animals repeatedly bitten by these species. In all cases sera strongly reacted with homologous antigen (Fig. 1). At the dilution 1:500, a weak cross-reaction was observed in some, but not all pairs: sera from mice bitten by *P. papatasi* gave a weak reaction with SGL of *P. halepensis*, but did not react with *P. perniciosus*. On the other hand, sera against *P. halepensis* cross-reacted with SGL of *P. perniciosus*, and vice versa. Using a dilution 1:1500 the specificity of the reaction was even more obvious. Sera recognized almost exclusively the homologous SGL, the only exception was a serum from a rabbit bitten by *P. halepensis* which gave a very weak reaction with *P. perniciosus* (Fig. 1).

Immunoblotting

The electrophoretic profiles of SGL differed significantly between species. From 5 to 8 prominent proteins bands with molecular masses ranging from 28 to 50 kDa were visualized by silver staining in each SGL (Fig. 2).

Immunoblot analysis revealed 4–9 major antigenic bands in each species, most of them appeared to be

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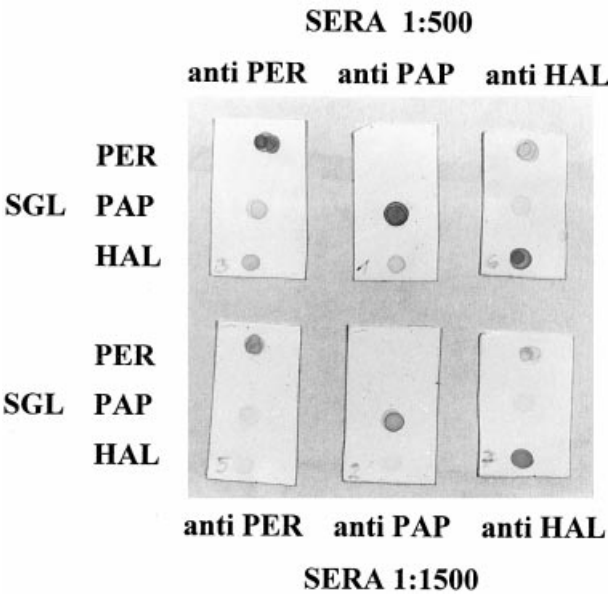

Fig. 1. Dot blot of sandfly salivary gland lysates with sera of mice repeatedly exposed to bites of *Phlebotomus papatasi* (anti-PAP), and hamsters repeatedly exposed to *P. perniciosus* (anti-PER), or *P. halepensis* (anti-HAL). Two dilutions of sera (1:500, 1:1500) are shown.

species specific. No reaction was observed between *P. papatasi* and other species tested. In SGL of *P. papatasi* homologous sera recognized from 5 to 7 major antigens. Prominent and consistently present were 2 bands of 30 and 33 kDa, the 42–44 kDa doublet and the 70 kDa doublet. On the other hand, sera from hamsters and rabbits bitten by *P. perniciosus* or *P. halepensis* did not react with *P. papatasi* SGL (Fig. 2).

A weak cross-reaction was observed between *P. perniciosus* and *P. halepensis*. In SGL from *P. perniciosus*, the sera of animals bitten by this species recognized about 8 major bands while the sera from animals bitten by *P. halepensis* gave a weak reaction with 1–4 polypeptides. Anti-*P. perniciosus* sera originating from a different source (hamster or rabbit) reacted similarly: in *P. perniciosus* SGL they recognized the same bands and differed only in the intensity of binding to polypeptides of 36–39 kDa (Fig. 2).

Similar specificity of antibody reaction was observed using SGL of *P. halepensis*: sera of rabbits and hamsters bitten by this species recognized 4–8 antigens, the most prominent ranging from 31 to 42 kDa; while anti-*P. perniciosus* sera cross-reacted very weakly with 2 or 4 bands (Fig. 2).

No reaction was found in the control strips where pre-immune sera were used or the serum step was omitted (data not shown).

DISCUSSION

Salivary antigens inoculated by sandfly females during feeding stimulated production of high levels of anti-saliva antibodies. In both methods used, the antibodies of repeatedly bitten animals detected specific salivary antigens in dilutions of sera 1:500–1500. Contrary to the findings of Ghosh &

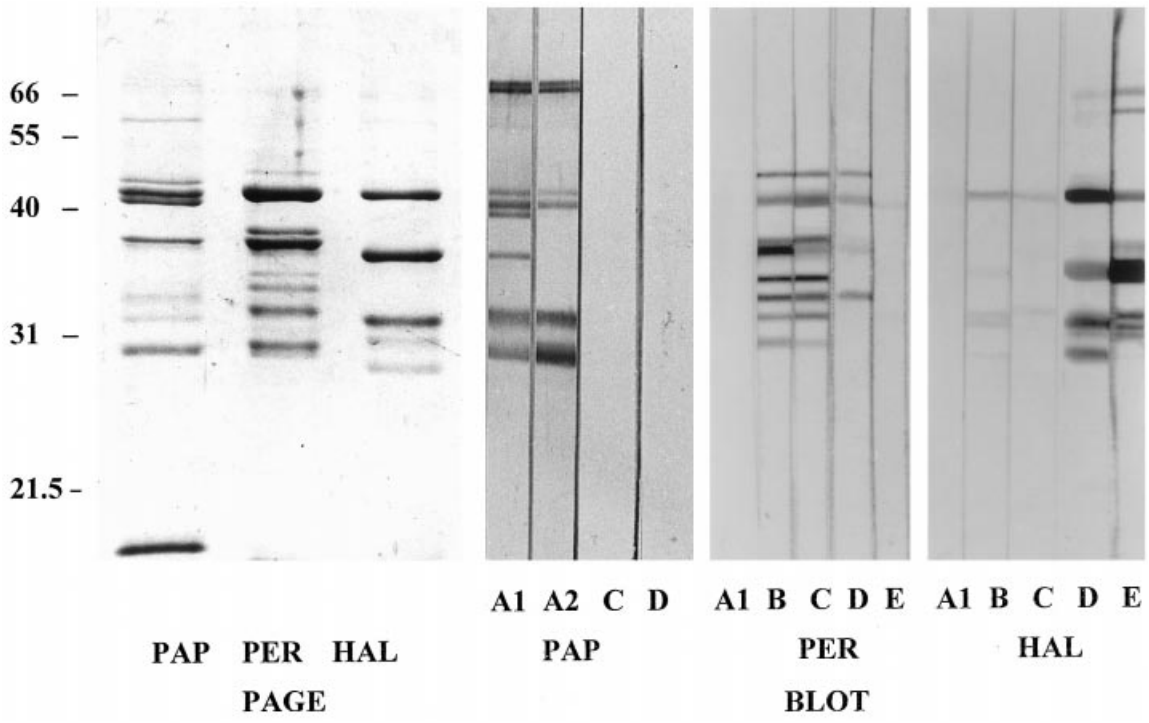

Fig. 2. SDS–PAGE and immunoblotting of *Phlebotomus papatasi* (PAP), *P. perniciosus* (PER) and *P. halepensis* (HAL) salivary gland lysate with sera of animals repeatedly exposed to sandfly bites: 2 mice bitten by *P. papatasi* (A1, A2); rabbit (B) and hamster (C) bitten by *P. perniciosus*; rabbit (D) and hamster (E) bitten by *P. halepensis*.

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Mukhopadhyay (1998), we have not observed increased mortality of females fed on previously sensitized animals (data not shown).

Our experiments showed the antibodies specifically recognizing distinct antigenic bands in SGL of 3 sandfly species. In most cases, antigens in *Phlebotomus* saliva were species-specific and antibodies elicited by biting of a particular species did not cross-react with SGL components of another species. Both the dot-blot and immunoblot revealed that there is no cross-reactivity of *P. papatasi* with other species tested. Partial cross-reactivity was observed between *P. halepensis* and *P. perniciosus* only.

Antigenic components reacting with immunoglobulins of bitten animals have been characterized by blotting techniques in various groups of blood-sucking insects like tsetse flies (Mařha *et al*. 1986), fleas (Greene *et al*. 1993; McKeon & Opdebeeck, 1994) and triatomine bugs (Volf, Grubhoffer & Hošek, 1993). In mosquitoes, a number of antigens reacting with IgG and IgE antibodies from bitten individuals have been identified. Both species-specific and species-shared antigens have been found in various mosquito species (Penneys *et al*. 1989; Peng, Li & Simons, 1996, 1998; Brummer-Korvenkontio *et al*. 1997). Peng *et al*. (1996), who studied the reaction of human sera with thoracic extracts of *Aedes vexans*, *Culex tarsalis* and *Culiseta inornata*, reported 9 antigens shared by all 3 species, 6 antigens shared by 2 species and only 3 antigens being species-specific. On the other hand, most salivary antigens appeared to be species-specific when pure saliva and sera from bitten mice were used (Brummer-Korvenkontio *et al*. 1997). In their study, distinct cross-reactivity was observed only between saliva proteins of 2 taxonomically closely related species *Aedes communis* and *A. punctor*.

Similarly, in sandflies the shared antigens seem to be restricted to related taxons. Cross-reactive antigenic bands were observed in *P. perniciosus* and *P. halepensis* belonging to closely related subgenera *Larroussius* and *Adlerius* but not in *P. papatasi*, a member of subgenus *Phlebotomus* which, according to morphological characters and rDNA sequences, creates the separate branch (Rispail & Léger, 1998; Depaquit *et al*. 1998).

Results presented in this work showed striking differences between species. In addition, preliminary experiments revealed antigenic variability between colonies of different geographical origin: 3 *P. papatasi* colonies tested shared most, but not all antigens (Rohoušová and Volf, unpublished observations). Therefore, we presume that the neutralizing effect of antibodies in sensitized subjects may decrease with the phylogenetic distance of the sandfly taxons. This might have important implications regarding the development of a transmission blocking vaccine based on salivary antigens.

In summary, most components of sandfly saliva are highly immunogenic. Immunoblot analysis has revealed striking specificity of antigen components in the 3 sandfly species studied. These data are important for characterization of salivary proteins and the understanding of the interplay between vectors, parasites, and hosts.

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