Juvenile female *Litomosoides sigmodontis* produce an excretory/secretory antigen (Juv-p120) highly modified with dimethylaminoethanol

M. HINTZ[1], G. SCHARES[2], A. TAUBERT[2], R. GEYER[1], H. ZAHNER[2], S. STIRM[1] *and* F. J. CONRATHS[2]*

[1] *Biochemisches Institut am Klinikum, Justus-Liebig-Universität, Friedrichstrasse 24, D-35392 Gießen, Germany*
[2] *Institut für Parasitologie, Justus-Liebig-Universität, Rudolf-Buchheim-Strasse 2, D-35392 Gießen, Germany*

(*Received 29 December 1997; revised 17 March 1998; accepted 18 March 1998*)

SUMMARY

A 120 kDa antigen produced by juvenile female *Litomosoides sigmodontis* (Juv-p120) was isolated and purified. The amino acid composition of the molecule was determined. Juv-p120 was shown to be highly modified with *N*,*N*-dimethylaminoethanol (28·4 mol%). Treatment of Juv-p120 with potassium hydroxide (*β*-elimination) or with sodium *m*-periodate leads to the destruction of epitopes recognized by antibodies immune affinity-purified with isolated Juv-p120. Juvenile *L. sigmodontis* were shown to release Juv-p120 into the pleural cavity of infected *Mastomys coucha* before the onset of patency.

Key words: *Litomosoides sigmodontis*, filariasis, excretory/secretory products, dimethylaminoethanol.

INTRODUCTION

Human lymphatic filariasis is a spectral disease with a number of clinical manifestations (WHO, 1984). A specific cellular unresponsiveness observed in filariasis patients (Ottesen, Weller & Heck, 1977; Piessens *et al*. 1980) could result from tolerance induced by clonal anergy (King *et al*. 1992) or from a number of other possible mechanisms (for a review, see Maizels *et al*. (1995)).

Certain aspects of the clinical manifestations, e.g. a persisting high microfilaraemia in the presence of an immune response, can be modelled in the *L. sigmodontis*-infection of rodents. In previous experiments, we could show that antibodies specific for 2 dominating microfilarial sheath surface antigens of 40 and 120 kDa (shp3a and shp3) cross-react with an excretory/secretory (E/S) product of 120 kDa (Juv-p120) which is released by juvenile female *L. sigmodontis* 3–4 weeks before they start to liberate microfilariae (Schares *et al*. 1994). The results of this study and the observation that parasitaemic hosts (*Mastomys coucha*; *Sigmodon hispidus*) fail to mount an antibody response to shp3a and shp3 raised the question as to whether Juv-p120 is capable of inducing a hyporesponsiveness. Several E/S products of other parasites have been shown or are suspected of exerting immunomodulating effects. It is of particular interest that a 62 kDa E/S antigen of the filarial nematode *Acanthocheilonema viteae* seems to be capable of inhibiting the proliferation of B cells and can interfere with signal transduction pathways (Harnett & Harnett, 1993). This molecule appears to be modified with carbohydrate moieties and phosphorylcholine in an unusual manner (Harnett *et al*. 1993). Preliminary data suggest that the production of Juv-p120 by juvenile female *L. sigmodontis* coincides with a hyporesponsiveness of T-cells to stimulation with filarial antigens (V. Nurmi, H. Zahner & F. J. Conraths, unpublished observations).

Recently, *N*,*N*-dimethylaminoethanol (DMAE) was described as a component of the microfilarial sheath of *L. sigmodontis* where it could be demonstrated as a constituent of the major sheath surface proteins shp3 and shp3a (Hintz *et al*. 1996). DMAE appears to represent a novel kind of post-translational modification of proteins which seems to be covalently linked to hydroxy-groups of amino acids or carbohydrates via phosphodiester bonds. In the study presented here we report on the biological and biochemical characterization of Juv-p120, the first isolated E/S product, which is known to be modified with DMAE.

* Corresponding author: Institute für Epidemiological Diagnostics, Federal Research Centre for Virus Diseases of Animals, Seestrasse 55, D-16868 Wusterhausen, Germany. Tel: +49 33979 80176. Fax: +49 33979 80222. E-mail: franz.conraths@wus.bfav.de

MATERIALS AND METHODS

Parasites

The life-cycle of *L. sigmodontis* was maintained as described (Lämmler, Saupe & Herzog, 1968). Juvenile female parasites were recovered from the

https://doi.org/10.1017/S0031182098002996 Published online by Cambridge University Press

pleural cavity of infected *Mastomys coucha* on day 38 p.i.; mature adult worms were isolated on day 130 p.i.; microfilariae were recovered from the blood of patently infected *Sigmodon hispidus* by Percoll gradient centrifugation (Chandrashekar *et al*. 1984).

Parasite antigens

Antigen extracts of microfilariae, microfilarial sheaths, juvenile and adult parasites were prepared as described (Schares *et al*. 1994). Serum, pleural exudate and creamy-white lobular masses (Bertram, 1966), frequently present in the pleural cavity of *L. sigmodontis*-infected *M. coucha*, were recovered for antigen detection when the animals were necropsied on day 38 p.i.

Sera

Rabbit hyperimmune sera were raised against adult female parasites or against constituents of the microfilarial sheath soluble in the presence of 2-mercaptoethanol and sodium dodecyl sulfate (SDS). Antibodies from the anti-sheath serum were immune affinity-purified either on formaldehyde-fixed microfilariae (thus directed against microfilarial sheath antigens and cross-reacting with Juv-p120) or on rpHPLC-purified Juv-p120. These antibodies were used as probes on immunoblots and histological sections as described elsewhere (Schares *et al*. 1994).

Purification of Juv-p120

Juvenile female *L. sigmodontis* were recovered from the pleural cavity on day 38 p.i., briefly dried on filter paper (Schleicher & Schüll, Dassel; Ref.-No. 311609), filled into cryo-tubes (Nunc) and frozen in liquid nitrogen until further use. Approximately 400 parasites (400 ± 50 mg) were dissected using microscissors, once gently frozen and thawed, and 4 times extracted by suspending in 1·6 ml of buffer (0·1 M Tris–HCl, 1 mM EDTA, pH 8) and centrifugation (10000 ***g***, 15 min, room temperature). The pooled supernatants were centrifuged again (10000 ***g***, 30 min, room temperature) resulting in a raw-homogenate which was desalted using a PD-10 Sephadex®G-25M column (Pharmacia, Freiburg, Germany) and 0·1 M Tris–HCl, pH 8. The homogenate (approximately 20 mg protein in a total of 6 ml) was fractionated on a strong anion-exchange matrix (SAX; Resource™ Q, 1 ml column, Pharmacia, Freiburg, Germany) using increasing salt concentration (5 min 100 % eluent A, followed by a gradient reaching 100 % eluent B after 20 min; eluent A: 20 mM Tris–HCl, pH 8; eluent B: eluent A supplemented with 1 M NaCl; flow rate: 1 ml/min; 37 °C; Fig. 1A). Aliquots (250 μl) of the Juv-p120 containing the SAX fraction were further separated by size-exclusion chromatography (SEC) on a GF-450 column (9·4 mm × 250 mm, 15 nm pore size, 10 μm particle size, Zorbax, Newport, Delaware, USA) which was eluted with 160 mM sodium phosphate buffer, pH 7·0, supplemented with 20 % (v/v) acetonitrile; flow rate: 1 ml/min at 37 °C (Fig. 1B). A final purification step consisted of the separation of the Juv-p120-containing SEC fractions by reversed-phase HPLC (rpHPLC) on a C4 column (2·1 mm × 250 mm, 30 nm pore size, 5 μm particle size; Vydac, Hesperia, CA, USA) eluted with a gradient of trifluoroacetic acid (TFA) and acetonitrile (buffer D: 0·11 % TFA in water; buffer E: 0·08 % TFA in acetonitrile; gradient starting with 100 % buffer D and reaching 100 % buffer E after 40 min at a flow rate of 200 μl/min at room temperature; Fig. 1C).

Amino acid analysis

Approximately 0·1 μg Juv-p120 was hydrolysed (Tous *et al*. 1989) and investigated by 9-fluorenylmethoxycarbonyl amino acid analysis (Miller, Narkates & Niemann, 1990; Hensel *et al*. 1995).

Carbohydrate constituent analysis

Starting from 11·3 μg of rpHPLC–purified Juv-p120, carbohydrate constituents were identified as alditol acetates as described previously (Geyer *et al*. 1982).

Treatment with O*-glycanase, PNGase F, endoglycosidase H, phospholipase C, proteinase K, potassium hydroxide or sodium* m*-periodate*

Aliquots of 5 μg Juv-p120 were reacted with 2·5 mU *O*-glycanse (Boehringer, Mannheim, Germany), 20 U phospholipase C (Boehringer Mannheim, Germany), or 1 μg proteinase K (from *Tritirachium album*, Boehringer, Mannheim, Germany), respectively, in a total volume of 50 μl of phosphate-buffered saline for 1 h at 37 °C. Equal amounts of Juv-p120 were treated with peptide:*N*-glycosidase F (PNGase F) or endoglycosidase H (New England Biolabs, Schwalbach, Germany) according to the manufacturer's instructions, or were incubated with variable concentrations of potassium hydroxide ranging from 10 to 150 mM in a volume of 20 μl under nitrogen at 37 °C overnight (Maizels *et al*. 1991). The reaction with potassium hydroxide was stopped by adding 2 μl of 1 M Tris–HCl, pH 7·5. To destroy carbohydrate epitopes by oxidation, aliquots of 5 μg Juv-p120 were reacted with 2·5 mM sodium *m*-periodate in 0·1 M sodium acetate buffer, pH 5·5, for 20 min at room temperature in the dark, or left untreated. Abundant periodate was removed by addition of sodium disulfite to a final concentration of 75 mM and incubation for 5 min at room temperature. All samples were analysed by SDS–PAGE

https://doi.org/10.1017/S0031182098002996 Published online by Cambridge University Press

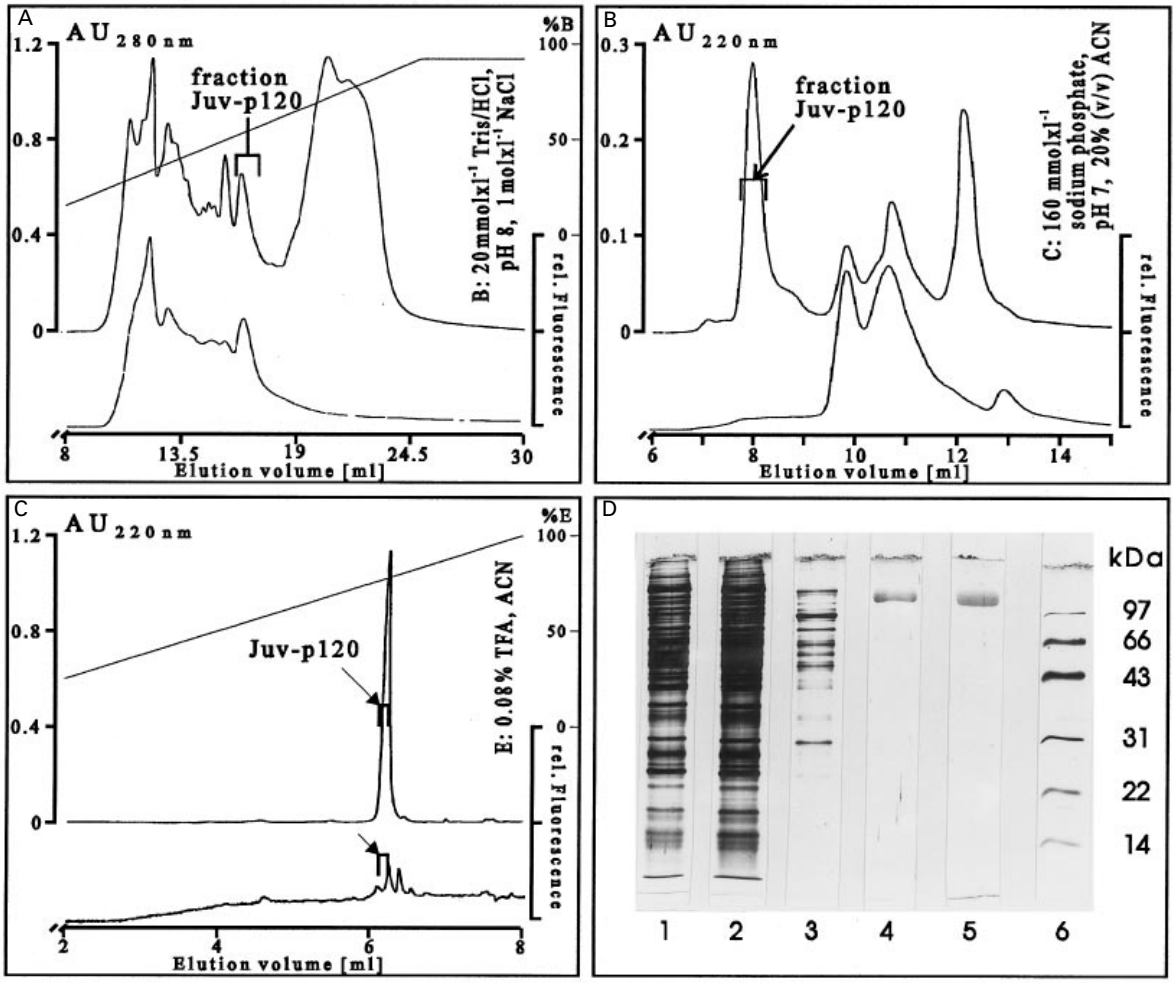

Fig. 1. Approximately 24 μg Juv-p120 were purified in 3 consecutive HPLC-purification steps comprising anion-exchange chromatography (A), SEC (B) and rpHPLC (C) from a raw homogenate prepared from 400 juvenile female *Litomosoides sigmodontis*. The positions of Juv-p120-containing fractions are indicated by arrows. The relative purity of HPLC-isolated Juv-p120 is demonstrated by SDS–PAGE (D, lane 5) in comparison with material after different purification steps (lane: 1, raw homogenate; 2, desalting; 3, strong anion-exchange; 4, SEC; 6, molecular mass marker). Detection was carried out by successive silver and Coomassie staining, absorption (AU) at 280 or 220 nm and fluorescence emission at 354 nm, excitation at 295 nm). It should be noted that there is no detectable fluorescence of tryptophan in Juv-p120 (see (C)).

and silver staining (Oakley, Kirsch & Morris, 1980), or by immunoblotting using antibodies immune affinity-purified on rpHPLC-purified Juv-p120.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Protein samples were separated on 12·5 % sodium dodecylsulfate-polyacrylamide gels and were stained with Coomassie Brilliant Blue or silver salts, or transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore Inc., Eschborn, Germany) and probed with antibodies as described (Bardehle *et al*. 1991). Bound immunoglobulins were visualized with peroxidase-labelled goat anti-rabbit IgG (Dianova, Hamburg, Germany) and 4-chloro-1-naphthol in combination with hydrogen peroxide as a substrate.

RESULTS

Isolation and purification of Juv-p120

Starting from a raw homogenate of approximately 400 juvenile female *L. sigmodontis*, approximately 24 μg Juv-p120 were purified in 3 consecutive HPLC-purification steps comprising anion-exchange chromatography (Fig. 1 A), size exclusion chromatography (Fig. 1 B) and rpHPLC (Fig. 1 C). Thus, juvenile parasites contain Juv-p120 in relatively large amounts of at least 60 ng/worm. By contrast, juvenile male *L. sigmodontis* lack this moleculae (data not shown). The relative purity of rpHPLC-isolated Juv-p120 is demonstrated by SDS–PAGE and silver Coomassie staining (Fig. 1 D), lane 5) showing a single band of 120 kDa.

Co-migration of purified Juv-p120 with a compound of the same molecular mass present in a crude

https://doi.org/10.1017/S0031182098002996 Published online by Cambridge University Press

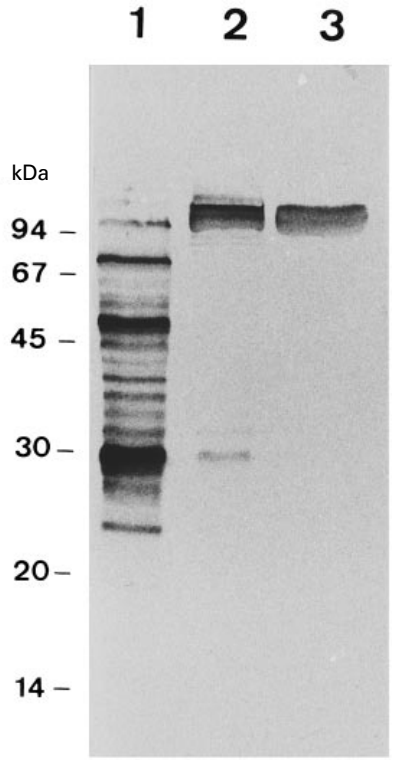

Fig. 2. Extracts of adult female (lane 1), juvenile female *Litomosoides sigmodontis* (lane 2) or HPLC-purified Juv-p120 (lane 3) were subjected to SDS–PAGE, electroblotted onto a PVDF membrane and probed with rabbit anti-microfilarial sheath antibodies immune affinity-purified on intact formaldehyde-fixed microfilariae. Bound antibodies were visualized with goat anti-rabbit IgG peroxidase conjugate and 4-chloro-1-naphthol in combination with hydrogen peroxidase as a substrate.

extract of juvenile parasite was demonstrated by immunoblotting (Fig. 2). Anti-sheath antibodies immune affinity-purified on intact, formaldehyde-fixed microfilariae recognize Juv-p120 strongly in a crude extract of juvenile parasites recovered on day 35 post-infection (lane 2) and after purification (lane 3). The antigen appears to be less abundant in older female parasites recovered around day 130 post-infection (lane 1), which in turn contain several additional constituents recognized by the antibodies.

Composition of Juv-p120

HPLC-purified Juv-p120 protein was hydrolysed and its amino acid and DMAE composition determined (Table 1). The analysis revealed that the polypeptide contains relatively large amounts of glutamine/glutamic acid (18·3 mol%), followed by leucine (14·6 mol%) and proline (13·8 mol%). The most interesting finding is, however, that DMAE represents 28·4 mol% of Juv-p120. Monosaccharide constituent analysis further revealed the presence of fucose, mannose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine. Neutral carbohydrates

Table 1. Amino acid (aa) and dimethylaminoethanol (DMAE) composition of Juv-p120

	Mol%*
Glycine	3·2 ± 0·6
Alanine	6·0 ± 1·3
Valine	2·4 ± 0·5
Leucine	14·6 ± 0·9
Isoleucine	3·5 ± 0·5
Serine	8·2 ± 0·6
Threonine	7·7 ± 0·5
Cysteine	N.D.
Methionine	0·8 ± 0·3
Arginine	3·4 ± 0·5
Lysine	2·8 ± 0·4
Histidine	4·3 ± 0·7
Phenylalanine	6·9 ± 1·0
Tyrosine	2·9 ± 1·1
Tryptophan	N.D.
Proline	13·8 ± 1·3
Asparagine/aspartic acid	1·1 ± 0·4
Glutamine/glutamic acid	18·3 ± 1·9†
Dimethylaminoethanol	28·4 ± 4·4‡

* ±, Standard deviation, $n = 11$.
† Sum of aa = 100 Mol%.
‡ Sum of aa + DMAE = 100 Mol%.

represent approximately 2% of the mass of the protein (calculated as the total mass of the amino acid constituents).

Post-translational modifications

In order to detect and further characterize post-translational modifications of Juv-p120, the molecule was reacted with *O*-glycanase, phospholipase C, and proteinase K, PNGase F and endoglycosidase H, potassium hydroxide, and sodium *m*-periodate, and the effects on the electrophoretic mobility and on the antigenicity of the protein were measured. Treatment with *O*-glycanase, PNGase F and endoglycosidase H had no effect on Juv-p120, whereas proteinase K digested it completely and abrogated any reactivity with Juv-p120-specific antibodies (data not shown). After incubation with phospholipase C, most of the Juv-p120 was still present, but an additional band with slightly higher electrophoretic mobility appeared which was still detected by Juv-p120-specific antibodies. Under mild alkaline conditions, *O*-glycosidic linkages between glycans and serine or threonine residues can easily be split by β-elimination. It could be shown that Juv-p120 loses most of its antigenicity for an affinity-purified antibody when treated with potassium hydroxide (Fig. 3B). Potassium hydroxide treatment of the Juv-p120 with 10 or 20 mM left the recognition by the antibody unaffected whereas treatment with 50 mM reduced it. After incubation of Juv-p120 with 100 mM potassium hydroxide, the antibodies failed to detect the antigen (Fig. 3B). A silver-stained gel

https://doi.org/10.1017/S0031182098002996 Published online by Cambridge University Press

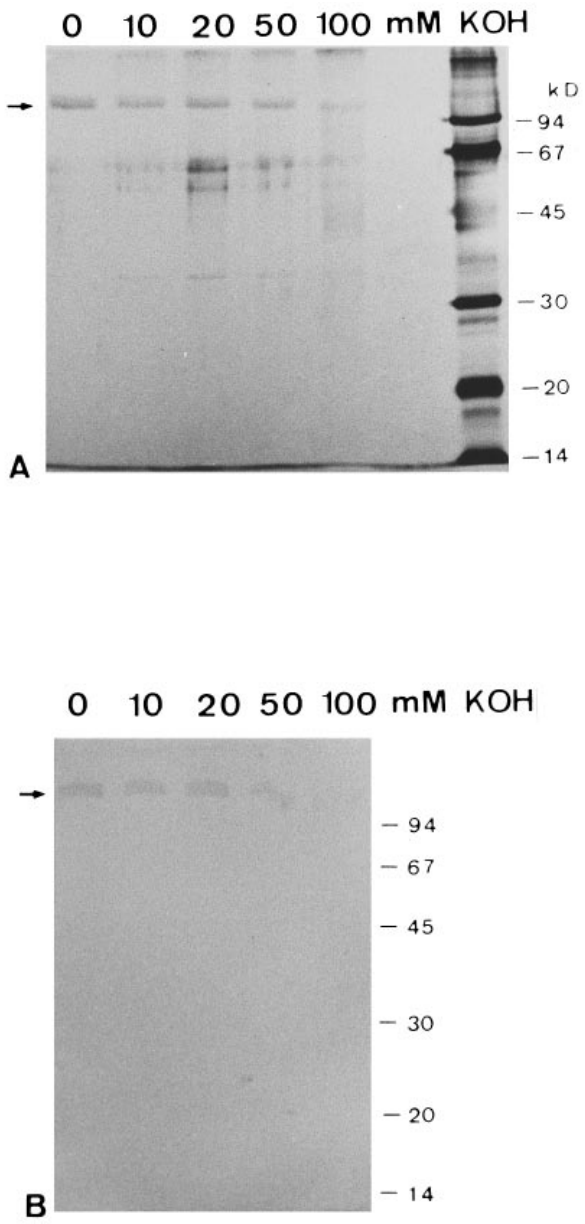

Fig. 3. Aliquots of 10 μg HPLC-purified Juv-p120 were treated with increasing concentrations of potassium hydroxide as detailed in the Materials and Methods section. The samples were split in half and separated in 12·5 % SDS–polyacrylamide gels. One gel was silver stained (A). Proteins from the second gel were electroblotted onto a PVDF membrane and probed with rabbit anti-microfilarial sheath antibodies immune affinity-purified on HPLC-purified Juv-p120 (B). Bound antibodies were visualized with goat anti-rabbit IgG peroxidase conjugate and 4-chloro-1-naphthol in combination with hydrogen peroxide as a substrate.

loaded with equal amounts of the samples shows, however, that the 120 kDa molecule is still present, although moderate degradation may have occurred as indicated by a smear visible between approximately 38 and 50 kDa (Fig. 3A). The epitopes recognized by the anti-Juv-p120 antibody were also destroyed by treating Juv-p120 with 2·5 mM sodium *m*-periodate (data not shown).

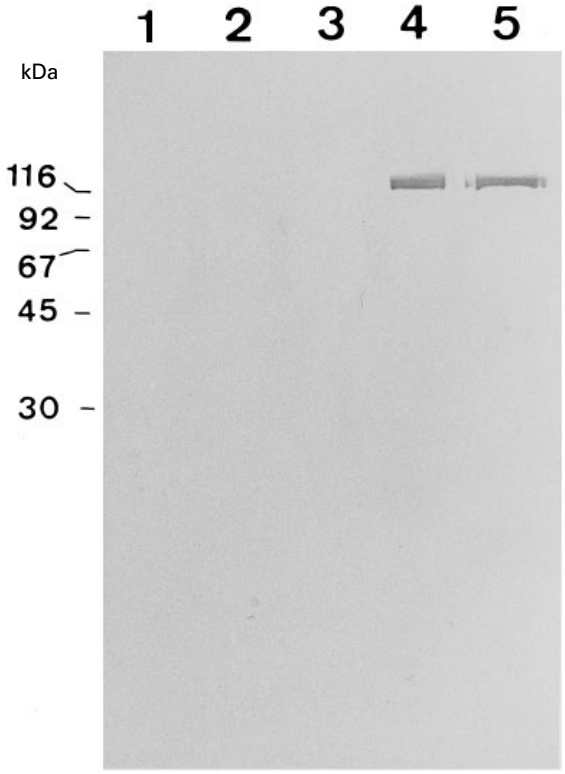

Fig. 4. Fifty μg normal *Mastomys coucha* serum (lane 1), 25 μg (lane 2) and 50 μm (lane 3) *Litomosoides sigmodontis*-infected *M. coucha* serum (day 38 p.i.), 10 μg (lane 4) and 20 μg (lane 5) pleural exudate from *L. sigmodontis*-infected *M. coucha* (day 38 p.i.) were separated by SDS–PAGE, transferred to a membrane and probed with rabbit anti-microfilarial sheath antibodies immune affinity-purified on HPLC-purified Juv-p120. Bound antibodies were visualized with goat anti-rabbit IgG peroxidase conjugate and 4-chloro-1-naphthol in combination with hydrogen peroxide as a substrate.

Presence in pleural exudate

When serum samples and pleural exudate from the pleural cavity of *L. sigmodontis*-infected *M. coucha* were probed with antibodies affinity-purified on HPLC-purified Juv-p120, Juv-p120 could be detected in pleural exudate but not in serum (Fig. 4).

DISCUSSION

Juv-p120 is a female-specific molecule soluble in aqueous solutions. Other gender-specific E/S products have been reported for adult *B. malayi* (Kwan-Lim *et al*. 1989). Antibodies directed against microfilarial sheath surface antigens cross-react with Juv-p120 (Schares *et al*. 1994). The molecule could be extracted from juvenile female parasites and purified almost to homogeneity employing a 3-step HPLC protocol. The identity of purified Juv-p120 with the original molecule present in juvenile female worms was shown by the binding of antibodies which recognize only Juv-p120 and a minor band of 30 kDa in a crude extract of juvenile *L. sigmodontis*.

https://doi.org/10.1017/S0031182098002996 Published online by Cambridge University Press

Juv-p120 appears to be a protein modified with DMAE, phospholipids, and carbohydrates. Like the microfilarial sheath proteins shp3/3a of *L. sigmodontis* (Hintz *et al*. 1994), Juv-p120 is highly DMAE-modified. The amino acid profile of Juv-p120, however, reveals only approximately 16 mol% serine/threonine, whereas 50–60 mol% S/T were found in shp3/3a. Nevertheless, the S/T content of Juv-p120 is sufficient to allow a high degree of modification by phosphate-bound DMAE. Further studies including fragmentation and analysis of individual, small DMAE-containing peptides are required to localize the DMAE-containing regions in Juv-p120. It is of considerable interest that a circulating parasite antigen produced by adult *B. pahangi* has been described which has a similar size (105–110 kDa) as Juv-p120 and contains phosphorylcholine epitopes (Weil *et al*. 1990). Juv-p120, however, is not recognized by a monoclonal antibody (TEPC15) directed against phosphorylcholine (F. J. Conraths, unpublished observations). The finding that only some of the Juv-p120 migrated with higher electrophoretic mobility after treatment with phospholipase C may indicate that the addition of phospholipids to the molecule is optional. It cannot be ruled out completely, however, that cleavage with phospholipase C remained incomplete.

The epitopes recognized by an immune affinity-purified antibody directed against Juv-p120 can be destroyed by treating Juv-p120 with sodium *m*-periodate or potassium hydroxide. Both results suggest that carbohydrates may contribute to the formation of these epitopes. Since potassium hydroxide and *O*-glycanase both attack *O*-glycosidically linked carbohydrates, the results with the former (destruction of the epitopes) and the latter (intact epitopes) treatment are conflicting at first sight, but may be explained by the limited specificity of *O*-glycanase. From the presence of mannose revealed by monosaccharide constituent analysis, one might speculate that Juv-p120 carries also *N*-linked glycans, which, however, could not be split off by treatment with PNGase F and endoglycosidase H. More detailed analyses are required to decide as to whether Juv-p120 contains in fact both *N*-, and *O*-glycosidically bound carbohydrates. After treatment with potassium hydroxide, hardly any shift in the electrophoretic mobility could be observed, which may indicate that *O*-linked carbohydrates do not contribute to the mass of the molecule in a significant fashion.

It is tempting to speculate that excreted/secreted Juv-p120, which can be detected in large amounts in the pleural exudate of *L. sigmodontis*-infected *M. coucha*, has an impact on the immune system of the host. Epitopes of Juv-p120 which cross-react with microfilarial surface antigens may lead to a clonal anergy of the respective lymphocytes specific for these determinants, thus allowing microfilariae bearing cross-reacting epitopes on their sheath surface to remain untouched by the immune system. This hypothesis is consistent with the finding that no infected *M. coucha* could be found which responded to Juv-p120 or the cross-reacting sheath surface antigens shp3a and shp3 by producing antibodies (Bardehle *et al*. 1992; Schares *et al*. 1994). In human filariasis it has been observed that *in utero* and/or neonatal exposure to maternal filarial infections may alter the immune responses of the progeny (Ottesen, 1980, 1984, 1989; Piessens, Wadee & Kurniawan, 1987). Weil and colleagues (1983) postulated that the low frequency of parasite-specific lymphocytes observed among highly microfilaraemic individuals may result from *in utero* or prenatal sensitization. In the *B. pahangi*–jird model, a greater number of infected male offspring from infected female jirds became microfilaraemic (92%) as compared to infected male progeny from uninfected mothers (50%) (Klei, Blanchard & Coleman, 1986). *B. pahangi*-infected jirds exhibited significantly reduced titres of serum IgG antibodies to *Brugia* antigens as compared to the offspring of uninfected mothers (Bosshardt *et al*. 1992). Our own results lead to the hypothesis that Juv-p120 could be a molecule which may help to establish tolerance to microfilariae in *L. sigmodontis*-infected animals. A clarification of this question can now be addressed with characterized and HPLC-purified Juv-p120. Furthermore, it will be necessary to investigate a potential impact of DMAE and DMAE-modified antigens on the immune system.

The authors wish to thank Brigitte Hofmann, Markus Schwinn and Horst Thiele for excellent technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 272, Teilprojekt A4.

REFERENCES

BARDEHLE, G., CONRATHS, F. J., FAHRENHOLZ, F., HINTZ, M., LINDER, D., SCHARES, G., SCHOTT, H.-H., SCHÜTZLE, B., STIRM, S., STÜBER, W. & ZAHNER, H. (1991). A major *Litomosoides carinii* microfilarial sheath protein (gp22): amino terminal sequence and immunological studies with corresponding synthetic peptides. *Parasitology* **103**, 387–394.

BARDEHLE, G., HINTZ, M., LINDER, D., SCHARES, G., SCHOTT, H.-H., STIRM, S. & ZAHNER, H. (1992). *Litomosoides carinii*: extraction of the microfilarial sheath components and antigenicity of the sheath fractions. *Parasitology Research* **78**, 501–508.

BERTRAM, D. S. (1966). Dynamics of parasitic equilibrium in cotton rat filariasis. *Advances in Parasitology* **4**, 255–319.

BOSSHARDT, S. C., MCVAY, C. S., COLEMAN, S. U. & KLEI, T. R. (1992). *Brugia pahangi*: effects of maternal

filariasis on the responses of their progeny to homologous challenge infection. *Experimental Parasitology* **74**, 271–282.

CHANDRASHEKAR, R., RAO, U. R., RAJASEKARIAH, G. R. & SUBRAHMANYAM, D. (1984). Separation of viable microfilariae free of blood cells on Percoll gradients. *Journal of Helminthology* **58**, 69–70.

GEYER, R., GEYER, H., KÜHNHARDT, S., MINK, W. & STIRM, S. (1982). Capillary gas chromatography of methylhexitol acetates obtained upon methylation of *N*-glycosidically linked glycoprotein oligosaccharides. *Analytical Biochemistry* **121**, 263–274.

HARNETT, W. & HARNETT, M. M. (1993). Inhibition of murine B cell proliferation and down-regulation of protein kinase C levels by a phosphorylcholine-containing filarial excretory-secretory product. *Journal of Immunology* **151**, 4829–4837.

HARNETT, W., HOUSTON, K. M., AMESS, R. & WORMS, M. J. (1993). *Acanthocheilonema viteae*: phosphorylcholine is attached to the major excretory-secretory product via a N-linked glycan. *Experimental Parasitology* **77**, 498–502.

HENSEL, J., HINTZ, M., KARAS, M. LINDER, D., STAHL, B. & GEYER, R. (1995). Localization of the palmitoylation site in the transmembrane protein p12E of Friend murine leukaemia virus. *European Journal of Biochemistry* **232**, 373–380.

HINTZ, M., HIRZMANN, J., HOBOM, G., LINDER, D., LOTTSPEICH, F., SCHOTT, H.-H., CONRATHS, F. J., ZAHNER, H. & STIRM, S. (1994). *Litomosoides carinii* microfilarial sheaths: partial amino acid sequences of several major polypeptide constituents. *Molecular and Biochemical Parasitology* **67**, 69–78.

HINTZ, M., KASPER, M., STAHL, B., GEYER, R., KALINOWSKI, H.-O., KARAS, M., KÜHNHARDT, S., SCHOTT, H.-H., CONRATHS, F. J., ZAHNER, H. & STIRM, S. (1996). Dimethylaminoethanol is a major component of the *Litomosoides carinii* microfilarial sheath. *Molecular and Biochemical Parasitology* **76**, 325–328.

KING, C. L., KUMARASWAMI, V., POINTDEXTER, R. W., KUMARI, S., JAYRAMAN, K., ALLING, D. W., OTTESEN, E. A. & NUTMAN, T. B. (1992). Immunologic tolerance in lymphatic filariasis. Diminished parasite-specific T and B lymphocyte precursor frequency in the microfilaraemic state. *Journal of Clinical Investigation* **89**, 1403–1410.

KLEI, T. R., BLANCHARD, D. P. & COLEMAN, S. U. (1986). Development of *Brugia pahangi* infections and lymphatic lesions in male offspring of female jirds with homologous infections. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **80**, 214–216.

KWAN-LIM, G. E., GREGORY, W. F., SELKIRK, M. E., PARTONO, F. & MAIZELS, R. M. (1989). Secreted antigens of filarial nematodes: a survey and characterization of in vitro excreted/secreted products of adult *Brugia malayi*. *Parasite Immunology* **11**, 629–654.

LÄMMLER, G., SAUPE, E. & HERZOG, H. (1968). Infektionsversuche mit der Baumwollrattenfilarie *Litomosoides carinii* bei *Mastomys natalensis* (Smith, 1834). *Zeitschrift für Parasitenkunde* **30**, 281–290.

MAIZELS, R. M., BLAXTER, M. L., ROBERTSON, B. D. & SELKIRK, M. E. (1991). *Parasite Antigens, Parasite Genes. A Laboratory Manual for Molecular Parasitology*. Cambridge University Press, Cambridge.

MAIZELS, R. M., SARTONO, E., KURNIAWAN, A., PARTONO, F., SELKIRK, M. E. & YAZDANBAKHSH, M. (1995). T-cell activation and the balance of antibody isotypes in human lymphatic filariasis. *Parasitology Today* **11**, 50–56.

MILLER, E. J., NARKATES, A. J. & NIEMANN, M. A. (1990). Amino acid analysis of collagen hydrolysates by reverse-phase high-performance liquid chromatography of 9-fluorenylmethyl chloroformate derivatives. *Analytical Biochemistry* **190**, 92–97.

OAKLEY, B. R., KIRSCH, D. R. & MORRIS, N. R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Analytical Biochemistry* **105**, 361–363.

OTTESEN, E. A. (1980). Immunopathology of lymphatic filariasis. *Springer Seminars in Immunopathology* **2**, 373–385.

OTTESEN, E. A. (1984). Immunological aspects of lymphatic filariasis and onchocerciasis in man. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **78** (Suppl.) S9–S18.

OTTESEN, E. A. (1989). Filariasis now. *American Journal of Tropical Medicine and Hygiene* **41** (Suppl.) S9–S17.

OTTESEN, E. A., WELLER, P. F., HECK, L. (1977). Specific cellular immune unresponsiveness in human filariasis. *Immunology* **33**, 413–421.

PIESSENS, W. F., MCGREEVY, P. B., PIESSENS, P. W., MCGREEVY, M., KOIMAN, J., SAROSO, H. S. & DENNIS, D. T. (1980). Immune responses in human infections with *Brugia malayi*. Specific cellular unresponsiveness to filarial antigens. *Journal of Clinical Investigation* **65**, 172–179.

PIESSENS, W. F., WADEE, A. A. & KURNIAWAN, L. (1987). Regulation of immune responses in lymphatic filariasis. In *Filariasis – Ciba Foundation Symposium* **127** (ed. Evered, D. & Clark, S.), pp. 164–173. John Wiley & Sons Limited, Chichester.

SCHARES, G., SCHÜTZLE, B., ZAHNER, H. & CONRATHS, F. J. (1994). Surface antigens of *Litomosoides carinii* microfilariae: agglutinating antibodies react with sheath components of 40 and 120 kilodalton. *Parasitology* **109**, 73–82.

TOUS, G. I., FAUSNAUGH, J. L., AKINYOSOYE, O., LACKLAND, H., WINTER-CASH, P., VITORICA, F. J. & STEIN, S. (1989). Amino acid analysis on polyvinylidene difluoride membranes. *Analytical Biochemistry* **179**, 50–55.

WEIL, G. J., HUSSAIN, R., KUMARISWAMI, V., TRIPATHY, S. P., PHILLIPS, K. S. & OTTESEN, E. A. (1983). Prenatal allergic sensitization to helminth antigens in offspring of parasite-infected mothers. *Journal of Clinical Investigation* **71**, 1124–1129.

WEIL, G. J., CHANDRASHEKAR, R., LIFTIS, F., MCVAY, C. S., BOSSHARDT, S. C. & KLEI, T. R. (1990). Circulating parasite antigen in *Brugia pahangi*-infected jirds. *Journal of Parasitology* **76**, 78–84.

WORLD HEALTH ORGANIZATION (1984). *Filariasis – Fourth Report. WHO Technical Report Series* No. 702. WHO, Geneva.