Molecular characterization of antigen 24, a specific immunodominant antigen family from *Leishmania infantum*

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SUMMARY

Leishmania infantum immunoelectrophoretic antigen 24 (AG 24), a visceral leishmaniasis associated immunodominant antigen, has been characterized with a monospecific antiserum by combining SDS–PAGE, immunoblotting, metabolic labelling, radio-immunoprecipitation and *in vitro* poly A^+ mRNA translation. AG 24 appeared to correspond to a multi-antigen family of 6–9 members ranging from 20 to 31 kDa and proteinic by nature with no post-translational modifications. A similar banding pattern was recognized by infection sera. AG 24 was not found exposed on the cell surface.

Key words: *Leishmania infantum*, serodiagnosis, immunoelectrophoresis, immunodominant antigen.

INTRODUCTION

Diagnosis of visceral leishmaniasis relies ideally on clinical suspicion based on spleen and liver enlargement and on parasitological confirmation. Due to low sensitivity ($\leqslant 60\%$) of the latter (Pearson & de Queiroz Sousa, 1995) and to high antibody titre resulting from marked B cell stimulation at disease stage, serological testing is often compulsory and efficient. Its value, however, is impeded by a relative lack of specificity inherent to the use of whole cells or crude extracts as antigen. Therefore special efforts have been made to identify defined, specific and sensitive antigens, usually from Western blot patterns with patients' sera or from immunoscreening of recombinant DNA libraries.

Previous work in our laboratory (Le Ray, Afchain & Capron, 1975) has conducted an exhaustive analysis of the antigen make-up of *Leishmania infantum* by immunoelectrophoresis (IEP) with rabbit hyperimmune sera. More than 30 immunoelectrophoretic components were consistently evidenced, 60% of them being genus *Leishmania*-specific. One specific component, positioned as antigen no. 24 (AG 24) on the immunoelectrophoregram, displayed prominent immunogenicity: it was recognized early and strongly by a variety of mammals (man, dog, hamster, mouse) in natural and experimental infections as well as following immunization with promastigotes. Since then, AG 24 has been routinely used in our laboratory as reference standard for serodiagnosis of visceral leishmaniasis (Le Ray *et al*. 1973).

The present paper describes an original methodology developed for the molecular characterization of the immunoelectrophoretic component AG 24, based on polyclonal antisera specific to AG 24 raised by immunizing rabbits with the AG 24 precipitation line excised from the IEP gel. Initial characterization was obtained by SDS–PAGE, immunoblotting and radio-immunoprecipitation. Eventually the AG 24 proteinic nature was assessed by metabolic labelling and by *in vitro* translation mRNA experiments, and was shown to represent a multi-antigen family composed of a set of proteins ranging from 20 to 31 kDa.

MATERIALS AND METHODS

Parasites and cell lines

Leishmania infantum MHOM/67/MA(BE)/ITMAP 263 (clone 7) promastigotes, *Trypanosoma cruzi* Tehuantepec epimastigotes and *Trypanosoma brucei* WA/UG/66/EATRO 1125 procyclics were grown in GLSH medium and maintained as described by Le Ray (1975). Clone 7 was used throughout this study. Its identity was ascertained by isoenzymatic analysis (Pratlong, personal communication).

HL 60 cells (human promyelomonocytic cell line) were obtained from Dr Véronique Kruys at ULB (Brussels Free University). They were grown in supplemented RPMI medium (foetal calf serum

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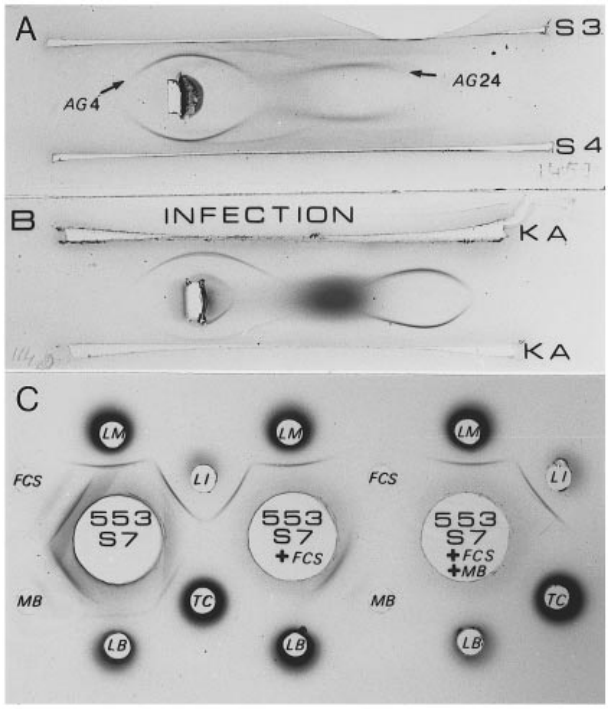

Fig. 1. Immunoelectrophoretic antigen no. 24 (AG 24) of *Leishmania infantum*, as recognized in water-soluble promastigote extract by early immunization antisera, S3 and S4 (A) and by kala-azar sera, KA, (B). (C) Specificity of AG 24 antiserum tested against homologous *L. infantum* (LI) and heterologous *L. mexicana* (LM), *L. braziliensis* (LB), *Trypanosoma cruzi* (TC), *Mycobacterium butyricum* (MB) and foetal calf serum (FCS). Cross-reactivity was removed by adsorbing the antiserum with heterologous antigens (+FCS, +MB). AG 24 is shared by *L. infantum* and *L. mexicana*.

10 %, penicillin and streptomycin 50 iu/ml, L-glutamine 0·2 mM, sodium pyruvate 1 mM, HEPES 10 mM, non-essential amino-acids 10 %) in 5 % CO_2 atmosphere at 37 °C and passaged every day by diluting 1/2 in fresh medium.

Antigenic extracts

Large-scale preparation of freeze-dried parasite water-soluble antigenic extracts was conducted as described by Le Ray (1975).

For most experiments, small-scale extraction was performed on *L. infantum* clone 7 by freeze–thawing as described next. Promastigotes from 3 or 4-day-old cultures were washed 3 times with Hanks and counted. Each pellet of 10^8 cells was resuspended in 100 μl of hypotonic saline containing the following protease inhibitors: aprotinin 10 μg/ml, antipain 1 μg/ml, benzamidine 17·5 μg/ml (Sigma) and PMSF 10 mM (Boerhinger Mannheim). The suspension was then submitted to 5 cycles of freezing (2 min in liquid nitrogen) and thawing (40 °C waterbath). The lysate was recovered in an Eppendorf tube and centrifuged for 5 min at 14000 ***g***. The supernatant fraction, referred to as soluble *Leishmania* extract, was transferred to a new tube and stored at −20 °C or used immediately.

Both large- and small-scale types of soluble extract were used successfully in precipitation-in-gel as well as in SDS–PAGE and immunoblotting. However, some degree of degradation of high molecular weight proteins in the large-scale soluble extract was observed in immunoblotting experiments. Hence, the use of large-scale type of soluble extracts was confined to precipitation-in-gel assays.

Precipitation-in-gel

Immunodiffusion (ID) and immunoelectrophoresis (IEP) were performed as described by Le Ray (1975).

AG 24-specific antisera

Three categories of antisera were used in this work: (i) oligospecific immune sera from rabbits immunized with *L. infantum* extract, (ii) oligospecific infection sera from proven cases of either kala-azar or canine leishmaniasis, and (iii) monospecific anti-AG 24 rabbit immune sera. Oligospecific infection sera, especially those from kala-azar patients, were carefully chosen so as to show only 1 or 2 precipitation lines beside the AG 24 line upon IEP analysis (Fig. 1, panel B). This is to avoid interpretation problems that might appear when using sera with too many specificities.

Monospecific AG 24 antisera were prepared basically according to Bout, Fruit & Capron (1974). Two farm-bred New Zealand white rabbits were immunized with a pool of AG 24 lines excised from IEP plates prepared with oligospecific antisera. Rabbit 553 received AG 24 lines precipitated with a kala-azar patient serum whereas rabbit 554 was immunized with AG 24 lines from the early serum of a rabbit immunized with total promastigote extract. AG 24 precipitation lines were prepared as follows: 8 × 4 cm IEP plates were run as usual and washed extensively. AG 24 line-containing agarose blocks were excised from IEP gel and finely ground in sterile saline. As a rule, 4–7 lines of AG 24 in 0·5 ml of saline were emulsified in 0·5 ml of Freund's incomplete adjuvant (Difco) plus 0·5 mg desiccated *Mycobacterium butyricum* (Difco). The rabbits were injected weekly in the subscapular cavity on the right and left sides alternatively: rabbit 553 received a total of 20 lines in 5 injections, rabbit 554 received a total of 34 lines in 6 injections. The rabbits were bled weekly from the time of the first injection. All sera were tested in IEP against *L. infantum* lyophilized soluble extract and they were assessed for cross-reactivity with heterologous antigens: foetal calf serum (FCS), rabbit serum (RS), human serum (HS), *T. cruzi* extract (TC), *T. brucei* extract (TB),

and the *M. butyricum* preparation used as adjuvant (MB). Immune cross-adsorption was performed as described by Le Ray (1975). Finally, immunological identity of the putative line 24 from rabbits 553 and 554 was ascertained in IEP with the *bona fide* line 24 reference antiserum by fusion of the precipitation-in-gel lines with one another.

Localization of AG 24 in promastigotes

Indirect immunofluorescence on methanol-fixed promastigotes was performed as described by Van Meirvenne, Janssens & Magnus (1975) with AG 24-specific antisera diluted 1/20 and FITC-conjugated anti-rabbit secondary antibodies (Amersham) diluted 1/25.

Direct agglutination was assayed with AG 24-specific antisera on fixed-and-stained promastigotes after the method of El Harith *et al*. (1988) as well as on living parasites kept on ice.

Direct analysis of excised IEP line 24

Rabbit anti-AG 24-specific sera were used to prepare line-24 material in IEP gels. Agarose blocks containing line 24, as well as control neighbouring blocks, were excised and washed in sterile 15-ml Falcon tubes containing 20 volumes of saline (NaCl 9 g/l, NaN_3 0·05 g/l). Tubes were placed in the cold room and slowly rotated. The saline was changed daily for 14 days. Washed blocks were then melted in sample buffer heated at 95 °C for 5 min and loaded onto a 10 % acrylamide gel in the presence of SDS. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250.

In another set of experiments, proteins were transferred from the gel onto a PVDF membrane and reacted with the rabbit anti-AG 24 pre-immune and immune sera.

SDS–PAGE and immunoblotting

Discontinuous polyacrylamide gel electrophoresis in the presence of SDS and immunoblotting was performed essentially as described in Harlow & Lane (1989). Except when stated otherwise, 12 % acrylamide gels were cast into a Miniprotean II apparatus (BioRad). Before loading, samples were denatured by heating (95 °C, 5 min) in 80 mM Tris–HCl, pH 6·8 buffer containing 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0·02 % bromphenol blue. Routinely, extracts from 10^7 promastigotes or 10–20 μg protein were loaded/lane. Molecular weight markers in the 14–200 kDa range (Life Technologies) were used at 100 ng each protein/lane. Gels were run at 80–120 V constant till exiting of the front dye. The gel was then either fixed and stained with Coomassie Brilliant Blue R250 or processed for immunoblotting.

For immunoblotting the proteins were transferred onto PVDF membrane (Immobilon, Millipore) by the semi-dry electrophoretic method using a Transblot SD cell (BioRad). The membrane was lightly stained with Ponceau S to check for the presence of transferred proteins and washed with distilled water. After blocking overnight with 0·5 % Instagel (Tessenderlo Chemie) in phosphate-buffered saline containing 0·1 % Tween (PBS–Tween), the membrane was washed once with PBS–Tween and put in contact with specific antibodies for 2 h at room temperature under mild agitation. The membrane was then washed again 3 times and alkaline phosphatase-conjugated secondary antibodies (anti-human or anti-rabbit IgG, Promega) added for another 1 h. After 3 final washes, alkaline phosphatase was revealed using bromochloro-indolyl phosphate/nitroblue tetrazolium substrate (NBT/BCIP). Routinely, primary antibodies were used at a 1/1000 dilution in PBS–Tween plus 0·25 % Instagel; secondary antibodies were diluted 1/7500 in the same solution.

Ion-exchange chromatography

An attempt was made to separate AG 24 from the bulk of the proteins in the promastigote extract by small-scale batch chromatography on an anion-exchange resin (Q-Sepharose Fast Flow, Pharmacia) and on a cation-exchange resin (CM-Sepharose Fast Flow, Pharmacia). Fifty micrograms of lyophilized promastigote extract were resuspended in 200 μl of Tris buffer (10 mM, pH 8·0) containing 8 M urea, in a sterile Eppendorf tube. Ten microlitres of washed resin microbeads equilibrated in the same buffer were added and the tube was slowly rotated in a cold room for 1 h. The tube was then spun down, the supernatant recovered and the pellet washed 3 times with the urea-containing Tris buffer. Finally, the pellet was resuspended in 30 μl of sample buffer and analysed by SDS–PAGE and immunoblotting as described above.

Metabolic labelling and radio-immunoprecipitation

Metabolic labelling and radio-immunoprecipitation were performed as described by Ouaissi *et al*. (1990) with the following modifications: 10^8 promastigotes from 3-day-old cultures were labelled with [^{35}S]-methionine (Tran-^{35}S-label, ICN) and lysed by freeze–thawing. In each immunoprecipitation, radioactive soluble extract corresponding to 2×10^7 promastigotes was added to 500 μl of precipitation buffer (10 mM Tris–HCl, pH 8·0, NaCl 150 mM, EDTA 2 mM, NP40 0·5 %, SDS 0·3 %) with protease inhibitors and 20 μl of immune or infection serum. Immune complexes were precipitated with Protein A-Sepharose microbeads (Pharmacia). After 4 washes, the immune complexes were eluted and

analysed by SDS–PAGE. Dried gels were autoradiographed on X-OMAT K films (Kodak).

Immunoprecipitation from in vitro *translated polyA$^+$ RNA*

Total RNA was extracted in parallel from HL60 human cells and *L. infantum* promastigotes by the guanidium/CsCl method. PolyA$^+$ RNA was recovered from total RNA by affinity chromatography on oligo(dT) cellulose. Both types of RNA were quantified by spectrophotometry and aliquots were run on non-denaturing agarose gels using 16S and 23S *E. coli* ribosomal RNAs (Sigma) as markers. Methods were performed as described in Sambrook, Fritsch & Maniatis (1989).

Pellets of washed promastigotes yielded on average $0{\cdot}40 \pm 0{\cdot}11$ μg of total RNA/10^6 promastigotes with a A260/A280 ratio of 1·7. After a single chromatography on oligo(dT) cellulose, 5% of the loaded RNA was recovered as polyA$^+$ RNA which had a A260/A280 ratio close to 2·0. These figures compare favourably with published data on RNA extraction from *Leishmania tropica* and *L. donovani* (Vedel & Robert-Gero, 1987).

In parallel, 419 μg of total RNA were recovered from HL60 cells (A260/A280 ratio = 1·7) Oligo(dT) chromatography yielded 2·4% in weight of polyA+ RNA.

On non-denaturing agarose gel electrophoresis, total promastigote RNA revealed the expected 3 bands corresponding to ribosomal RNA. Occasional higher or lower size bands were also observed: these might correspond to high molecular weight DNA contaminants and 5·8 S or tRNAs respectively. Poly A$^+$ RNA showed a smooth smear contaminated with the 2 larger ribosomal RNA bands.

PolyA$^+$ RNAs were translated *in vitro* by the method of Pelham & Jackson (1976) using commercial nuclease-treated rabbit reticulocyte lysate (Promega) and added 'translation grade' [^{35}S]-methionine (Amersham) as radioactive label. In experiments assessing post-translation modifications, commercial canine pancreatic microsomal membranes (Promega) were added to the reticulocyte lysate after the method of Walter & Blobel (1983). Percentage incorporation of radioactive label and stimulation above background were estimated by TCA precipitation of translation products. All kits and commercial products were used according to the manufacturer's instructions, including prescribed positive and negative control experiments.

AG 24 was searched in translation products by immunoprecipitating with AG 24-specific rabbit immune sera. Experiments were conducted essentially as described above except that translation products and antibodies were dissolved in TNE buffer (50 mM Tris–HCl, pH 7·5, 150 mM NaCl, EDTA 2 mM) plus NP40 2% (v/v). Protein A-Sepharose loaded with immune complexes was washed twice with TNE + 2% NP40, once with TNE + 1% NP40 and once with TNE alone.

Immune complexes eluted from protein A-Sepharose microbeads as well as total translation products were analysed by SDS–PAGE as described above. Dried gels were routinely exposed to X-OMAT K films (Kodak).

RESULTS

Immunoelectrophoretic antigen 24

Immunoelectrophoretic (IEP) antigen no. 24 (AG 24) was shown to be 1 of the first 2 antigens of *L. infantum* to be recognized by the precipitating antibody response during experimental immunization of the rabbit. AG 24 proved to be also the earliest, most frequently recognized antigen during visceral leishmaniasis of man and dog (Fig. 1A and B).

For the analysis of AG 24, the prerequisite was to prepare an antiserum strictly specific to AG 24. Therefore AG 24 precipitation lines were excised from IEP gels and used to immunize 2 rabbits, nos 553 and 554. Their sera were monitored weekly with ID and IEP. Some later bleedings of rabbit 554 showed a faint additional non-AG 24 line: this line had previously been observed in the original rabbit antiserum, i.e. the antiserum used to prepare the AG 24 line for immunizing rabbit 554. When immunization was stopped, AG 24 precipitins attenuated but remained present for over 12 months.

The identity of the putative AG 24 precipitation line from monospecific rabbit antisera 553 and 554 was proven by its fusion in IEP with the *bona fide* AG 24 lines from a reference antiserum (not shown).

AG 24 antisera were assessed for specificity (Fig. 1C). In ID, antisera from both immunized rabbits reacted with heterologous antigenic extracts from *Mycobacterium butyricum*, *Trypanosoma cruzi*, *T. brucei*, *Leishmania mexicana* and *L. braziliensis* as well as to foetal calf serum. Immunoadsorption with non-leishmanial cell extracts and serum totally removed heterologous reactivities while leaving the homologous *L. infantum* reactivity intact.

It is interesting to note that AG 24 monospecific antiserum to *L. infantum* was reacting equally well with *L. mexicana* (Fig. 1C). Following immunoadsorption with *L. braziliensis* or with *L. mexicana* soluble extracts, the homologous *L. infantum* AG 24 line was still present but fainter than before adsorption, indicating partial immunological identity of AG 24 among *Leishmania* species (not shown).

Localization of AG 24 in promastigotes

In an exploratory attempt to localize antigen no. 24 in promastigote cells, indirect immunofluorescence and direct agglutination were assayed.

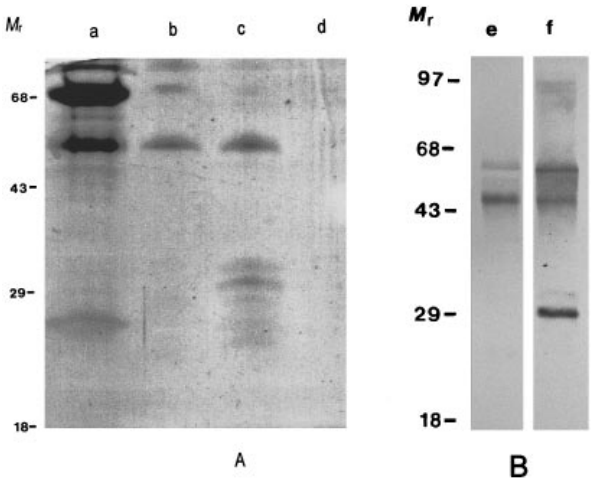

Fig. 2. (A) Direct SDS–PAGE analysis of an AG 24 precipitation line excised from IEP gel (c) in parallel with rabbit serum 553 S7 used to make the IEP (a) and control neighbouring IEP gel areas (b, d). (B) Immunoblotting of AG 24 precipitation line revealed with rabbit pre-immune (e) and AG 24 immune serum (f). Molecular weight markers are given in kDa.

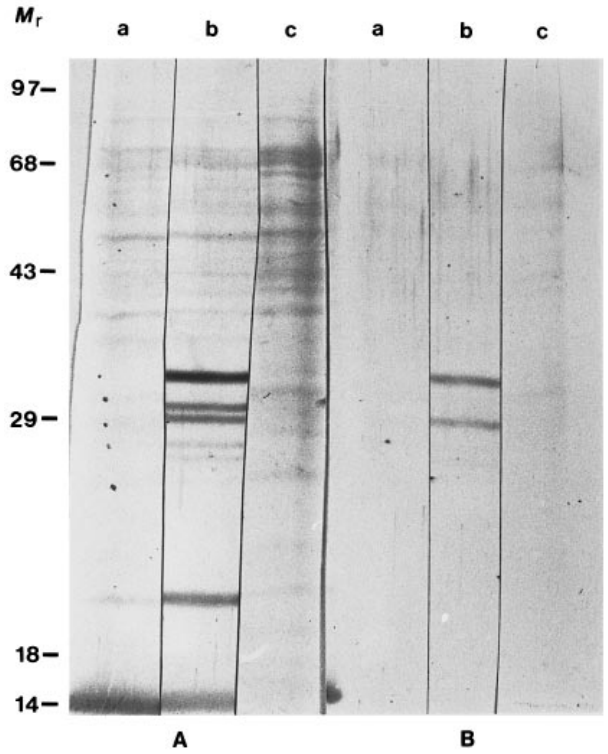

Fig. 3. Immunoblotting of soluble *Leishmania infantum* promastigote total extract (A) and of Q Sepharose fraction (B) revealed with pre-immune rabbit serum (a), AG 24 antiserum (b) or stained with Coomassie Blue (c). Molecular weight markers are given in kDa.

AG 24 antisera did not agglutinate fixed promastigotes in the presence of β-mercaptoethanol. Some agglutinating activity was observed in the absence of β-mercaptoethanol, titres however remained equal to, or slightly higher, than those of pre-immune sera, i.e. $\leqslant 1:640$. The direct agglutination assay was repeated with living promastigotes kept on ice: here again the AG 24 antiserum did not agglutinate more than the pre-immune serum.

Indirect immunofluorescence performed on methanol-fixed promastigotes with AG 24 antisera induced a cytoplasmic fluorescence only.

These results indicate that AG 24 is apparently not exposed externally nor is it a major constituent of the promastigote cell surface.

Direct analysis of the AG 24 precipitation line

When precipitation lines no. 24 excised from IEP gels were submitted to polyacrylamide gel electrophoresis, a set of 5 (or 6) bands was displayed at 31, 30, 28, (27), 26 and 24·5 kDa and a single band at 50 kDa (Fig. 2A). By comparison with pre-immune rabbit serum on the same gel, the 50 kDa band could be attributed to immunoglobulin heavy chains. No obvious correlation could be made between any AG 24 band and a major band in the soluble cell extracts.

Following immunoblotting of the same material, only the 31 and 29·5 kDa bands were recognized specifically by AG24 antiserum (Fig. 2B). In some experiments, however, the 29·5 kDa band presents as a doublet and 2 faint bands at 27·5 and 26·5 kDa appeared.

Immunoblotting

When soluble cell extract from *L. infantum* promastigotes was blotted onto PVDF membrane after polyacrylamide gel electrophoresis (SDS–PAGE), a pattern of 5 bands (Fig. 3) was revealed specifically by AG 24 rabbit antisera. This pattern consisted of a strong band at 31 kDa, 2 bands at 29·5 and 28·5 kDa and 2 fainter ones at 27·5 and 26·5 kDa. An additional strong band at 21 kDa was noticed that was also recognized faintly by pre-immune sera.

An attempt was made to purify AG 24 from the soluble extract by ion-exchange chromatography on Q and CM Sepharose. At pH 8·0, in the presence of 8 M urea, 5 out of the 6 candidate AG 24 components were retained on Q Sepharose whereas the 29·5 kDa component could not be seen neither on Q (Fig. 3) nor on CM. This observation suggests biochemical heterogeneity in addition to size heterogeneity among the putative components of antigen no. 24.

Metabolic labelling and immunoprecipitation

By SDS–PAGE analysis of immune complexes precipitated with specific AG 24 antisera from soluble extract of [^{35}S]methionine-labelled promastigotes, 9 bands were evidenced ranging from 21 to 31 kDa (i.e. 21, 22·5, 24, 25, 26, 27, 28, 29 and 30·5 kDa; Fig. 4). Although the intensity of some bands (e.g. the band at 28 kDa) varied from

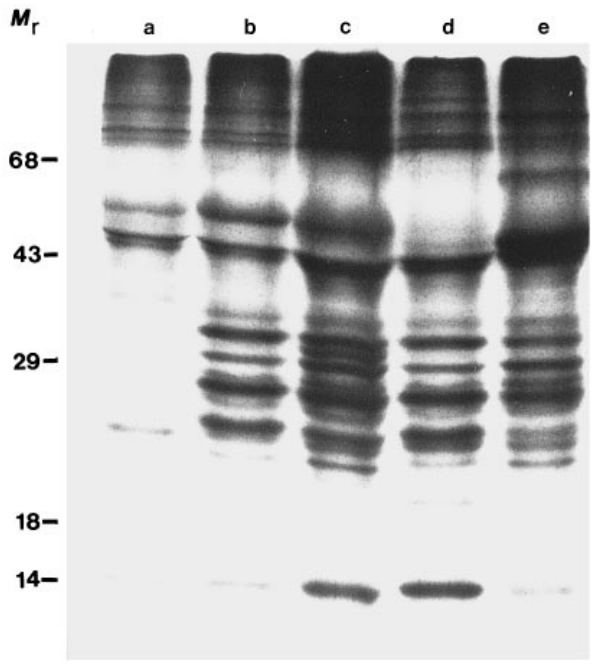

Fig. 4. Metabolic labelling and radio-immunoprecipitation of AG 24 from soluble extract of [^{35}S]methionine-labelled promastigotes with AG 24-specific rabbit antisera: 553 S0, pre-immune serum (a); 553 S3, first positive immune serum (b); 553 S7, peak immune serum (c); 553 S20, terminal antiserum (d) and rabbit 554 S2b, first positive immune serum (e). Molecular weight markers are given in kDa.

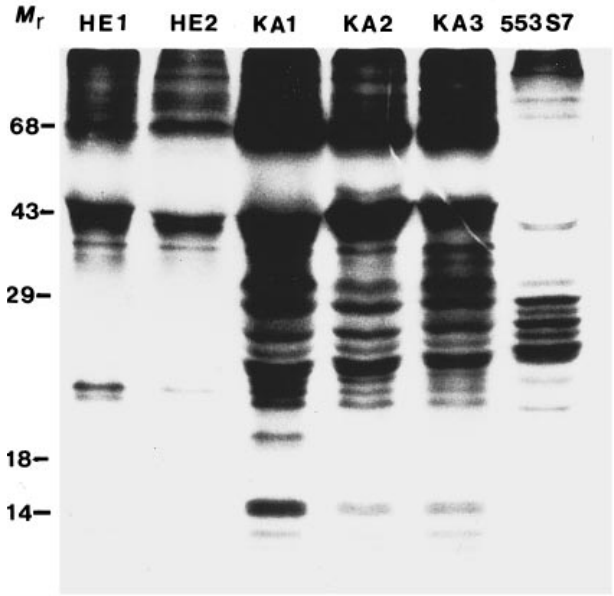

Fig. 5. Radio-immunoprecipitation of AG 24 with sera from patients with visceral leishmaniasis (HE, healthy European; KA, kala-azar; 553 S7, rabbit AG 24 antiserum). Molecular weight markers are given in kDa.

antiserum to antiserum, the overall profile was specifically (i.e. not seen with pre-immune sera) and consistently associated with AG 24 line reactivity.

Leishmania specificity of this AG 24-associated profile was checked by immune cross-adsorption of

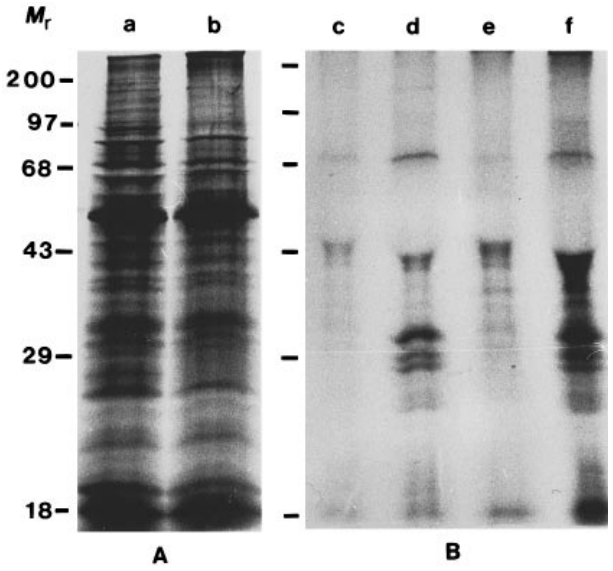

Fig. 6. *In vitro* translation and immunoprecipitation. (A) *Leishmania infantum* polyA+ RNA was translated in rabbit reticulocyte lysate alone (a) or with added microsomes (b). (B) Immunoprecipitation with pre-immune (c, e) or peak (d, f) AG 24 antiserum was performed on the product of RNA translated alone (c, d) or with added microsomes (e, f). Molecular weight markers are given in kDa.

AG 24 antiserum with heterologous cell extracts. Adsorption with *Mycobacterium butyricum* prior to immunoprecipitation did not affect the AG 24-associated profile. Two bands at 21·5 and 61 kDa respectively were removed by *T. cruzi*. In contrast, homologous *L. infantum* soluble promastigote extract efficiently removed the whole AG 24-associated immunoprecipitation profile (not shown).

The diagnostic value of the observed profile was confirmed with AG-24 mono- and oligospecific sera from kala-azar patients (Fig. 5): both immunoprecipitated specifically the same 20–31 kDa set of bands. The curved aspect of some of these bands is due to bowing under the high load of immunoglobulin light chains in the same region of the gel (not shown). A similar banding pattern was also obtained with canine leishmaniasis sera and with mouse *L. infantum* experimental infection sera. Sera from oriental sore patients, which do not recognize AG 24 but another antigen in IEP, did not display the AG 24-associated profile (not shown).

Immunoprecipitation from in vitro *translated polyA$^+$RNA*

PolyA$^+$RNA from *L. infantum* promastigotes was efficiently translated *in vitro* in rabbit reticulocyte lysate. Samples of 400 ng poly A$^+$RNA/25 μl volume of reaction reproducibly gave $> 3{\cdot}3\,\%$ incorporation of [^{35}S]methionine, with a 20-fold stimulation above background. Upon SDS–PAGE analysis (Fig. 6A) a profile of strongly labelled bands extending from < 14 kDa to > 200 kDa was repro-

ducibly observed. One prominent band at 50 kDa was tentatively identified as tubulin. Addition of canine pancreatic microsomal membranes decreased [^{35}S]methionine incorporation by 50 % but the total protein profile was found to be unchanged.

The AG 24 peak antiserum from rabbit 553 was used to immunoprecipitate promastigote mRNA translation products. As compared to pre-immune serum, it revealed a specific profile of 5 bands at 30·5, 29, 28, 26·5 and 25 kDa (Fig. 6 B). Another 6 bands (71, 22·5, 22, 21, 20 and 18 kDa) were found to increase in intensity from pre-immune to immune serum, and accordingly they were not considered as being specific. Addition of canine pancreatic microsomal membranes to the translation mixture did not alter the profile of immunoprecipitated material. The same profile was seen with the peak antiserum from rabbit 554 (not shown).

No such specific bands were seen when using polyA$^+$RNA from a non-*Leishmania* source (HL60 cells, synthetic luciferase messenger) or water in *in vitro* translation experiments (not shown).

DISCUSSION

Polyclonal AG 24-specific antiserum was raised by immunizing rabbits with immunoelectrophoretic AG 24 precipitation lines excised from preparative immunoelectrophoresis (IEP) gels. AG 24 antiserum-associated reactivity to culture medium (foetal calf serum) and to immunoadjuvant (Freund's mycobacterial additive) was easily removed by immunoadsorption. Subsequent monospecificity of antiserum to AG 24 was shown by precipitation-in-gel and AG 24 cross-reactivity of *L. infantum* with other leishmanias (*L. mexicana*, *L. braziliensis*) was confirmed, with no cross-reaction with other trypanosomatids (*Trypanosoma cruzi*, *T. brucei*).

No evidence for cell surface location of AG 24 could be found by direct agglutination assay on promastigotes while immunofluorescence with AG 24 antiserum indicated AG 24 cytoplasmic presence only.

AG 24 displayed a striking size heterogeneity: a specific set of 5–9 bands, depending on the sensitivity of the technique used, was observed in the 21–31 kDa range of molecular weight.

The discrepancies between the results of the different approaches employed may be attributed in part to technical causes such as the difference in resolving power of 12 % versus 10 % acrylamide (as we are dealing with a number of bands in a close range of molecular weights) or [^{35}S]methionine labelling versus Coomassie Blue staining. The observation that, when analysing the AG 24 precipitation line directly, only 2 of the 6 bands are clearly recognized in immunoblotting, suggests that antibodies have been preferentially elicited against conformational epitopes which are lost during the SDS and heat denaturation steps in SDS–PAGE. This may also explain the discrepancy between immunoprecipitation and immunoblotting on total promastigote lysate.

Besides size heterogeneity, AG 24 also displayed biochemical heterogeneity: the 29·5 kDa component appeared not to interact with a cation-exchange resin whereas the other components did.

Size heterogeneity of AG 24 could be artefactual *pro parte*. Size heterogeneity in protein studies is commonly attributed to protein degradation: this possibility seems remote as we used a cocktail of protease inhibitors during extraction and processing of the material. Repeated immunization with IEP gels and Freund's complete adjuvant could induce an antibody response to antigens other than the genuine AG 24 but this interference cannot take place during *in vitro* mRNA translation and the same AG 24 complex banding pattern was evidenced also with sera from VL patients. Accordingly, the artefactual origin of AG 24 heterogeneity seems to be ruled out.

Heterogeneity might originate at the genome level, with the components of the AG 24 complex being the products of various genes within a polymorphic gene family. Alternatively, these components may be coded by totally unrelated genes and have only a few antigenic determinants in common. In the former case, extensive sequence homologies are expected; in the latter, homologies would be limited to a few short stretches of amino acids. In both situations, different mRNA species would be produced. Examples of similar situations can be found in the recent literature. In *Leishmania*, the PSA-2 gene family codes for surface proteins of 90, 80 and 46 kDa in promastigotes and for a 50 kDa protein in amastigotes; they all are recognized by the same monoclonal antibodies (Symons *et al.* 1994) and have similar charges.

The source of heterogeneity might also be found at the level of the mRNA. A single mRNA species could be translated from different start codons into proteins of different sizes. Alternatively, a single precursor RNA could be differentially spliced into mRNAs of different sizes. In both cases a perfect or near-perfect sequence homology is expected between the various gene products.

At the present time it is not possible to document these two sources of heterogeneity. The matter, however, should be resolved soon as we are now in the process of cloning the DNA sequences coding for AG 24.

A third source of heterogeneity could come from the AG 24 components having a precursor-to-product relationship and/or differential post-translational modifications. This seems unlikely in the case of AG 24, in view of the results of mRNA *in vitro* translation. As *in vitro* translation systems are unable to perform post-translational modifications

such as glycosylation or peptide cleavage, and as the profile of the *in vitro*-translated AG 24 antigen was identical to the one from the promastigote, we conclude that the size heterogeneity is not due to post-translational modifications.

Altogether it appears that the major immunoelectrophoretic antigen no. 24 of *Leishmania infantum* is actually a molecular complex of antigens, and most probably a multi-antigen family. A similar situation has been observed with *T. cruzi*: each of 3 monoclonal antibodies recognized its major IEP antigen no. 5 and all 3 immunoprecipitated 4 components of molecular weight 72, 51, 43 and 24 kDa (Orozco *et al*. 1984) while each component was shown to have particular biochemical properties (Lemesre, 1985). Similarly, the *Fasciola hepatica* major antigen has been shown to correspond actually to an antigen family with diverse properties of its members (Tailliez & Korach, 1970). Our results thus document the general concept of antigenic personality (Capron, 1970). The fact is of basic biological interest for the forthcoming functional identification of AG 24 components in terms of biochemical properties and genetic relatedness.

Further work will concentrate on the cloning of corresponding genes from an expression library screened with the specific AG 24 antiserum described herein. This will make it possible to evaluate the diagnostic value of each individual component of AG 24 as well as to compare them to other published candidates such as the 18–31 kDa set of 4 antigens described by Marty *et al*. (1995) and the 18–33 kDa antigens identified by Correia *et al*. (1996).

The methodology developed here for the characterization of AG 24 can be applied to any antigen identified as a precipitation line in immunoelectrophoresis. Also, direct analysis of precipitation lines on SDS–PAGE opens the way to direct amino-terminal sequencing of the protein or proteins involved.

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