Immunochemical characterisation of antigens and growth inhibition of *Fonsecaea pedrosoi* by species-specific IgG

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Summary. Antigens of *Fonsecaea pedrosoi*, the most common agent of chromomycosis, were characterised by immunoprecipitation with a rabbit antiserum raised against the cell-protein extract and serum from an infected patient. Thirteen antigens were commonly detected and, as some of these antigens could be iodinated, they may be present in the fungal cell wall. Purified IgG from the rabbit antiserum was shown to produce a 50–60% inhibition of fungal growth. Some of the antigens characterised may be important in relation to the stimulation of protective immunity against chromomycosis.

Introduction

Chromomycosis is a chronic mycotic infection caused by seven dimorphic fungi, of which *Fonsecaea pedrosoi* is the commonest, and is characterised clinically by verrucous, crusted and warty lesions that are usually limited to the skin and subcutaneous tissues. Whatever the causative agent, in histological section the fungus appears in the tissues as thick-walled, brown, sclerotic bodies (Carrion and Silva-Hutner, 1971).

The mechanisms by which the saprophytic filamentous phase transforms to the parasitic one in man is not yet clearly understood, although in a previous study we have shown that the thermal stress response contributes to the dimorphism of *F. pedrosoi*. At 37°C the production of certain polypeptides is modified. Antigens associated with growth at 23°C (saprophytic phase) and at 37°C (parasitic phase) were characterised by immunoblotting with sera from immunised rabbits and infected human patients. Some of the antigens that appeared during the parasitic phase in man were thermo-inducible proteins (O. Ibrahim-Granet, C. de Bièvre, M. Jendoubi, unpublished observations).

In the present study we characterised the soluble antigens of *F. pedrosoi* by immunoprecipitation and iodination of the antigenic proteins, and we report the effect of purified immunoglobulins from rabbit antisera on the growth of the fungus.

Materials and methods

F. pedrosoi strain 1335 from the Institut Pasteur Collection was grown in a defined medium containing (g/L): MgSO₄·7H₂O 0.6 g; KCl 1 g; Na₂HPO₄ 2.38 g; KH₂PO₄ 0.91 g and glucose 20 g, supplemented with 1 g of a mixture of 20 amino acids (Drouhet and Mariat, 1952).

Protein labelling

(i) *With ³⁵S.* Cells of *F. pedrosoi* from an 8-day culture were washed and transferred to culture medium without methionine. Cells were then labelled with L-³⁵S-methionine 15 µCi/ml (Amersham; 1100 Ci/mmol) and incubated at 37°C for 24 h. Soluble proteins were obtained after overnight storage of the fungal cells at 4°C in 0.5 M KCl-0.028 M phosphate buffer (pH 7) supplemented with tween 20 0.5%. After centrifugation, the supernate was used for immunoprecipitation.

(ii) *With ¹²⁵I.* Two methods were used—those of Hunter and Greenwood (1962) and Hubbard and Cohn (1972). With the first method, 8-day-old *F. pedrosoi* cells were labelled with 700 µCi of ¹²⁵I in the presence of sodium barbitone buffer pH 7. Iodination was achieved by adding chloramine T 2 mg/ml in the same buffer. Chloramine T is an oxidising agent and iodates cell walls and intracellular proteins. The reaction was stopped by adding sodium metabisulphite at the same concentration as the chloramine T. After labelling, the proteins were dissolved in KCl-phosphate buffer, as above. In the second method, lactoperoxidase was used as a catalyst. This method is the more specific for cell-wall proteins because, with lactoperoxidase only the cell-wall proteins are iodinated. The 8-day-old *F. pedrosoi* cells were suspended in phosphate buffered saline containing 5 mM

glucose. To this mixture, 20 μ l of phenylmethylsulphonyl fluoride 1 mg/ml in methanol, 20 μ l of 5×10^{-5} M potassium iodide in PBS-glucose, 20 μ l of lactoperoxidase 1 mg/ml in PBS-glucose and 300 μ Ci of 125 I were added successively. The reaction was terminated by three additions, at 15-s intervals, of 10 μ l of glucose oxidase.

Antisera

A cell-protein extract was prepared from the disrupted cells of a 12-day-old culture grown at 37°C, and a preparation containing 1 mg/ml was used to immunise adult female New Zealand rabbits as previously described (Ibrahim-Granet *et al.*, 1985). A series of ten injections was given subcutaneously at 10-day intervals. The first injection was 4 ml of antigen and 1 ml of 10% potassium alum. Booster injections contained 800 μ g of antigen and 200 μ l of alum. After the last injection, serum was collected and the IgG fraction was prepared by affinity chromatography on protein A-Sepharose (CL-4B; Pharmacia, Uppsala, Sweden) according to the method of Hjelm *et al.* (1972). Human serum was collected from a 50-year old male patient, recently diagnosed as having chromomycosis, and living in Gabon where chromomycosis is endemic. *F. pedrosoi* was isolated from this patient.

Immunoprecipitation

Immunoprecipitation of dissolved 35 S methionine and 125 I labelled proteins was performed with protein A-Sepharose (CL-4B; Pharmacia) according to the method of Kessler (1975). One volume of cell protein extract containing $(1-4) \times 10^5$ cpm was incubated with 5 μ l of rabbit or human antiserum at 4°C overnight. The immunocomplexes were then precipitated for 1 h at room temperature with 50 μ l of protein A-Sepharose. The supernate was removed and the pellet washed six times in KCl (pH 7) buffer in a microfuge (Jouan MR 15) at 4°C. The final pellet was resuspended in 50 μ l of 0.0625 M Tris HCl (pH 6.8) and boiled for 5 min as described by Laemmli (1970).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and auto radiography

One-dimensional electrophoresis was performed according to the method of Laemmli (1970) with 10% polyacrylamide gels. After electrophoresis, the gels were fixed, treated for fluorography (Bonner and Laskey, 1974), dried and autoradiographed.

Inhibition of growth in *F. pedrosoi*

Inhibition of protein synthesis. Eight pellets, 2 mm in diameter, were incubated separately in 2 ml of the synthetic medium described above. IgG purified from

the rabbit antiserum to the protein extract of *F. pedrosoi* was added to four flasks at a final concentration of 1 mg/ml. IgG purified from normal rabbit serum was added at the same concentration to the other four flasks. Each culture was then labelled with 35 S methionine (15 μ Ci/ml) and incubated at 37°C. Cultures were harvested and treated after 24 h, 48 h, 8 days and 10 days.

Protein synthesis was determined by assaying the soluble protein content of the culture extract and cell filtrate. Immunoprecipitation and electrophoresis for each protein extract was undertaken as above.

Inhibition of nucleic-acid synthesis. Six cultures were prepared as in the previous experiments; three were incubated with IgG purified from rabbit antiserum to *F. pedrosoi* and three with IgG from normal rabbit serum. L- 3 H hypoxanthine was added at a concentration of 30 μ Ci/ml. Cultures were harvested after 8, 9 and 12 days. The extent of incorporation of hypoxanthine into nucleic acids was determined after the proteins were extracted in the KCl-phosphate-Tween buffer. This procedure was done to eliminate all traces of protein-bound hypoxanthine. Radiolabelled fungal cells were then counted on filters (GFC-Whatman).

Results

Identification of *F. pedrosoi* antigens

Fig. 1 shows the *F. pedrosoi* polypeptides (lane A) labelled with 35 S methionine and precipitated with normal rabbit serum (lane B), with rabbit serum produced against *F. pedrosoi* cell-protein extract of *F. pedrosoi* cells grown at 37°C (lane C), and with human serum from an infected patient (lane D), as detected by one dimensional 10% SDS-PAGE.

Thirteen antigens of mol. wts (10^3) 115, 98, 94, 84, 80, 75, 70, 67, 55, 51, 49, 35 and 32 were recognised by the rabbit antiserum to *F. pedrosoi*. All these antigens were detected by the human serum but at a lower intensity. The antigens of mol. wts (10^3) 115, 80 and 51 were precipitated by normal rabbit serum.

Fig. 2 shows *F. pedrosoi* polypeptides labelled with 125 I by the chloramine T technique (lane A). Five polypeptides of mol. wts (10^3) 80, 75, 63, 55 and 35 were iodinated. From these polypeptides only that of mol. wt 75×10^3 was detected with lactoperoxidase (lane B). Fig. 3 shows the iodinated *F. pedrosoi* polypeptides (lane A) precipitated by normal rabbit serum (lane B), by rabbit antiserum to *F. pedrosoi* (lane C), and by human serum (lane D). Two antigens of mol. wts $75 (10^3)$ and 55 were recognised by the rabbit anti-*F. pedrosoi* serum and the human serum. The 55×10^3 -mol. wt antigen was recognised by the normal rabbit serum.

Fig. 1. SDS-PAGE fluorogram of *F. pedrosoi* polypeptides labelled with ^{35}S methionine (lane A), and precipitated by normal rabbit serum (lane B), by rabbit anti-serum to *F. pedrosoi* grown at 37°C (lane C) and by the human serum (lane D).

Inhibition of growth

Inhibition of protein synthesis. The growth of eight ^{35}S -labelled cultures growing in a defined medium in the presence of (i) IgG purified from rabbit antiserum to *F. pedrosoi* cell extracts, or (ii) IgG purified from normal rabbit serum was observed for 10 days. Macroscopically, better growth of the cultures incubated with normal IgG occurred. This observation was confirmed by counting (i) the amount of the unused ^{35}S methionine in the culture medium at 24 h, 48 h, 8 days and 10 days, (ii) the protein content of the cell extract, (iii) the immunoprecipitation of the cell extract, (iv) the protein content of the culture filtrate, and (v) the immunoprecipitation of the culture filtrate. All these variations are illustrated in fig. 4.

The respective curves B (protein content of the cell extract), C (immunoprecipitation of the cell extract), D (protein content of the cell filtrate) and E (immunoprecipitation of the cell filtrate) indicate greater protein synthesis in the cells growing with normal IgG.

Calculation of the mean variance of the ratio of protein synthesis in the presence of IgG to protein

Fig. 2. SDS-PAGE of *F. pedrosoi* polypeptides labelled with ^{125}I and with chloramine T (lane A) and lactoperoxidase (lane B). Mol. wt markers are given in the left-hand lane. From top to bottom mol. wts (10^3): 92, 69, 46 and 30.

synthesis in the presence of anti-*F. pedrosoi* IgG, calculated from the cpms corresponding to each flask for the same age, gave values of:

- 1.99 for protein synthesis of the cell extract;
- 1.84 for immunoprecipitation of the cell extract;
- 1.015 for protein synthesis of the cell filtrate;
- 1.80 for immunoprecipitation of the cell filtrate.

With the exception of protein synthesis in the culture filtrate, these values indicate a growth inhibition of approximately 50% by the anti-*F. pedrosoi* IgG.

Inhibition of nucleic-acid synthesis. Nucleic acid synthesis was evaluated by labelling the fungal cultures with ^3H hypoxanthine. Cells growing with normal or anti-*F. pedrosoi* IgG were counted after dissolving the protein. Results are illustrated graphically in fig. 5. Both curves indicate maximum DNA and RNA synthesis at 9 days and a diminution at 12 days. The comparison between

Fig. 3. SDS-PAGE of iodinated *F. pedrosoi* polypeptides (lane A), immunoprecipitated with normal rabbit serum (lane B), with the rabbit anti-serum to cells grown at 37°C (lane C) and by the human serum (lane D). Mol. wt markers are as given in fig. 2.

the cpm values of cells growing in the presence of normal IgG and of cells growing in the presence of anti-*F. pedrosoi* IgG shows a mean inhibition of approximately 60% for the 8 and 9 day cultures.

Electrophoresis of proteins and immunoprecipitation

The cell extract and culture filtrate proteins determined by 10% SDS-PAGE are shown in figs 6 and 7 respectively. For cell extracts, the comparison of the protein patterns of cells grown in the presence of anti-*F. pedrosoi* IgG for 8 and 10 days (lanes A and C) and the patterns of cells grown in the presence of normal IgG (lanes B and D), show that polypeptides of mol. wt (10^3) 172.5, 136 and 20 appear to be specific to the cells grown in the presence of anti-*F. pedrosoi* IgG. In parallel, polypeptides of mol. wt (10^3) 72, 58, 43 and 40 were specific to cells grown in the presence of normal IgG.

For culture filtrates (fig. 7), a polypeptide of mol. wt 63×10^3 was specific to cells grown in the presence of anti-*F. pedrosoi* IgG (lane A) and a polypeptide of mol. wt 68.5×10^3 was specific to cells grown in the presence of normal IgG (lane B).

The results of the immunoprecipitation reactions of cell extracts and culture filtrates are shown in figs 8 and 9, respectively. Comparison of the antigen

Fig. 4. Growth kinetics of *F. pedrosoi* cultures labelled with ^{35}S methionine and incubated at 37°C for up to 10 days with normal (N) and specific (Sp) immunoglobulins. A—amount of methionine remaining in medium; B—protein synthesis in cell extract; C—immunoprecipitation of cell extract; D—protein synthesis in culture filtrate; E—immunoprecipitation of culture filtrate.

patterns of cells grown either in the presence of anti-*F. pedrosoi* IgG (fig. 8—lanes A, C and E) or in the presence of normal IgG (lanes B and D) shows that the antigens of mol. wt (10^3) 63 and 35 were preferentially recognised by the anti-*F. pedrosoi* IgG. In parallel, antigens of the culture filtrate of mol. wt (10^3) 63, 27 and 17 were preferentially detected by the anti-*F. pedrosoi* IgG (fig. 9—lanes A and C) and an antigen of mol. wt 20×10^3 was preferentially detected by the normal IgG (lane D).

Discussion

In this study of the antigens involved in chromomycosis, with rabbit anti-serum to *F. pedrosoi* grown at 37°C and serum from an infected person, 13 soluble antigens of mol. wts (10^3) between 115 and 32 were identified by immunoprecipitation.

Fig. 5. Growth kinetics of *F. pedrosoi* cultures labelled with ³H hypoxanthine incubated with normal (curve 1), and specific (curve 2) immunoglobulins.

Fig. 7. SDS-PAGE fluorograms of *F. pedrosoi* polypeptides (culture filtrate) labelled with ³⁵S methionine and incubated with anti-*F. pedrosoi* IgG for 8 and 10 days (lanes A and C) and with normal IgG for 8 and 10 days (lanes B and D).

Fig. 6. SDS-PAGE fluorograms of *F. pedrosoi* polypeptides (cell extract) labelled with ³⁵S methionine and incubated with anti-*F. pedrosoi* IgG for 8 and 10 days (lanes A and C) and with normal IgG for 8 and 10 days (lanes B and D).

Fig. 8. Immunoprecipitation of *F. pedrosoi* polypeptides (cell extract) grown in the presence of anti-*F. pedrosoi* IgG for 8 and 10 days (lane A and C) and with normal IgG (lane B and D). Lane E represents the immunoprecipitation of the extract of cells grown with anti-*F. pedrosoi* IgG to which 5 μ l of the total anti-*F. pedrosoi* (37°C) rabbit serum was added.

Fig. 9. Immunoprecipitation of *F. pedrosoi* polypeptides (culture filtrate) grown in the presence of anti-*F. pedrosoi* IgG for 8 and 10 days (lanes A and C) and with normal IgG (lanes B and D).

Polypeptides of mol. wts (10^3) 80, 75, 63, 55 and 35 were iodinated with the chloramine T and polypeptides of mol. wts (10^3) 75 and 55 were immunoprecipitated by both rabbit and human serum. The iodination of the 75×10^3 -mol. wt polypeptide obtained after labelling with lactoperoxidase indicates its presence in the fungal cell wall. We have

shown that the growth of *F. pedrosoi* is inhibited by anti-*F. pedrosoi* immunoglobulins. Protein and 50–60% of the nucleic acids synthesis can be inhibited by adding IgG, 1 mg/ml, to the growth medium.

By immunoprecipitation of the cell extracts, antigens of mol. wts (10^3) 63 and 35 were preferentially recognised by the anti-*F. pedrosoi* IgG. In parallel, antigens of the cell filtrate of mol. wts (10^3) 63, 27 and 17 were also preferentially recognised by the anti-*F. pedrosoi* IgG.

In a study of the response of *F. pedrosoi* to thermal stress (O. Ibrahim-Granet, C. de Bièvre, M. Jendoubi, unpublished observations), cultures incubated at 37°C showed fungal differentiation into thick-walled cells resembling the sclerotic bodies observed *in vivo*. At this temperature, the production of polypeptides of mol. wts (10^3) 95, 75, 70, 62, 60, 45, 42, 35 and 23 were either induced or enhanced, suggesting that they may be related to cell differentiation. The results described show that the polypeptides of mol. wts (10^3) 75, 70, 65 and 35 were immunogenic in man, indicating their involvement in the humoral response. The 63×10^3 and 35×10^3 -mol. wt polypeptides were shown to be implicated in fungal growth inhibition. These antigens—mol. wts (10^3) 75, 70, 63 and 35, may be important in stimulating protective immunity against chromomycosis and our results may be of value in the future development of a vaccine.

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