A murine monoclonal antibody exhibiting high species specificity for *Histoplasma capsulatum* var. *capsulatum*

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A monoclonal antibody (mAb) exhibiting a high degree of species specificity for the yeast phase of the dimorphic fungus *Histoplasma capsulatum* was produced by a modification of the standard mAb production protocol. The technique for generating mAbs involved the use of the immunosuppressive drug cyclophosphamide to diminish the response in mice to immunodominant cross-reactive epitopes. This mAb exhibited clear specificity and did not react by ELISA with the closely related genera *Blastomyces*, *Paracoccidioides* and *Sporothrix*. In Western blots it recognized a linear determinant on a 70–75 kDa molecule in *H. capsulatum* antigen, with an extremely faint reactivity to antigens of identical molecular mass derived from *Sporothrix* and *Paracoccidioides*, and no reactivity against *Blastomyces* antigen.

Introduction

The dimorphic fungus *Histoplasma capsulatum* var. *capsulatum*, is the causative agent of classical or small-form histoplasmosis, a disease of worldwide distribution, with the exception of Europe. Most commonly this respiratory pathogen causes a primary infection which is asymptomatic in the majority of people, but acute or chronic progressive pulmonary disease or widely disseminated infections can occur, characterized by the presence of small (3–4 µm) intracellular yeasts. A variety of this species, *Histoplasma capsulatum* var. *duboisii*, causes a clinically distinctive infection confined to Central and West Africa, described as large-form or African histoplasmosis, whose principle features are skin or bone lesions. The yeasts found in this form of the disease are large (10–12 µm), but the isolates are morphologically indistinguishable from those seen in classical histoplasmosis. Although there have been a number of attempts to define the antigenic structure of the *Histoplasma* yeast phase, these have tended to concentrate on cross-reactive carbohydrate components (Domer, 1971; Azuma *et al.*, 1974) and it is likely that the antigenic preparations, such as histoplasmin, the culture filtrate of the mycelial form of *H. capsulatum* used in the

serodiagnosis of histoplasmosis, contain such antigens. This has resulted in the generation of false positive results in patients with fungal diseases other than histoplasmosis, notably blastomycosis and paracoccidioidomycosis (Pine *et al.*, 1978; Wheat *et al.*, 1986). It would, therefore, be desirable to generate monoclonal antibodies (mAbs) which may recognize unique antigenic components of *Histoplasma*; two such approaches have been published (Reiss *et al.*, 1986; Kamel *et al.*, 1989). The principal problem in such endeavours has been the immunodominance of cross-reactive carbohydrate epitopes, though there have been attempts to overcome this by, for example, the periodate treatment of antigens prior to usage, which serves to destroy sugar moieties (Reiss *et al.*, 1986). However, recent publications (Thomas, 1987; Matthew & Sandroock, 1987) have demonstrated that the use of the immunosuppressive drug cyclophosphamide makes it possible to depress the immune response against immunodominant epitopes and, in so doing, enhance the production of mAbs recognizing unique epitopes. This is of particular value in mycological studies since it may result in the production of mAbs directed against peptide epitopes which are likely to be much more variable between species than their carbohydrate counterparts.

In this paper we report a successful attempt to raise a

Abbreviations: i.p., intraperitoneal(ly); mAb, monoclonal antibody; OPD, o-phenylenediamine.

species-specific mAb against *H. capsulatum* var. *capsulatum* by firstly challenging mice with its closest antigenic relative, *H. capsulatum* var. *duboisii*, and then treating them with cyclophosphamide in order to destroy proliferating B cells stimulated by this antigen, prior to inoculation with *H. capsulatum* var. *capsulatum*. In this way only B cells capable of recognizing specific *H. capsulatum* var. *capsulatum* antigen remain to be activated.

Methods

Antigen preparation. Mycelial isolates of *H. capsulatum* var. *capsulatum* (two), *H. capsulatum* var. *duboisii* (two), *Blastomyces dermatitidis* (one), *Paracoccidioides brasiliensis* (three) and *Sporothrix schenckii* (two) were obtained from the National Collection of Pathological Fungi, Mycological Reference Laboratory, Colindale, London, UK (NCPF nos 4100 and 4088; 4094 and 4077; 4076; 3285, 4115 and 4095; 3181 and 3268, respectively), and transformed to the yeast phase on slopes of brain/heart infusion (BHI) medium, supplemented with 0.2 mM-L-cysteine, at 37 °C. Cultures of each fungus were then subcultured in liquid BHI broth at 37 °C, collected by filtration (Whatman paper no. 2) and washed twice in phosphate-buffered saline (PBS; 0.01 M, pH 7.4). Yeast samples were then divided into two subsamples, to one of which a cocktail of protease inhibitors was added (Birk, 1976; Bergmeyer, 1984) in order to prepare antigen specifically for use in Western blotting. All samples were then homogenized in turn using a bead beater (Biospec Products). The soluble cell extract was collected, centrifuged at 2000 g for 7 min and the supernatant collected to yield a cytoplasmic antigen, divided into portions and frozen at -70 °C. Protein was estimated in each sample using the Coomassie Blue method (Read & Northcote, 1981). Antigens from the original mycelial isolates of NCPF nos 4088, 4094 and 3285 were prepared in a similar way. Histoplasmin was obtained from Dr Leo Kaufman, Centres for Disease Control, Atlanta, Georgia, USA.

Immunization protocol. Five male Balb/c mice were injected intraperitoneally (i.p.) with *H. capsulatum* var. *duboisii* antigen (50 µg protein per mouse) in Freund's complete adjuvant. An i.p. inoculation of cyclophosphamide (Sigma) in PBS (40 mg per kg body weight) was given to each mouse after 3 d, and after a further 12 d the mice received an i.p. inoculation of *H. capsulatum* var. *capsulatum* (50 µg protein per mouse) in Freund's incomplete adjuvant. A final i.p. inoculation of the var. *capsulatum* antigen in PBS was given 6 d later, and 2 d after this mice were test bled and their sera screened by ELISA to determine the animal with the greatest differential polyclonal response to the two *Histoplasma* antigens. This mouse was given an intravenous inoculation of the *H. capsulatum* var. *capsulatum* antigen (50 µg protein total) in PBS and used 3 d later for the fusion.

Fusion protocol. Cells of the myeloma line sp2/O were fused with spleen cells from the chosen mouse in a ratio of 1:10, according to a modification of the protocol of Zona & Brooks (1982) using polyethylene glycol 4000. Hybridomas were plated on to 96-well microtitre plates and 10 d post fusion, colonies were screened by ELISA. The respective *H. capsulatum* variant antigens in 0.06 M-sodium carbonate buffer (pH 9.6) were used to coat 96-well microtitre plates at a protein concentration of 1 µg/per well (100 µl per well), overnight at 4 °C. The wells were then washed in PBS/Tween (0.05%), blocked for 1 h at 37 °C with 1% (w/v) bovine serum albumin in PBS/Tween and then incubated for 1 h at 37 °C in culture supernatants. Immunoglobulin (Ig) peroxidase-linked conjugate goat anti-mouse IgG

P₀ (G&M IgG P₀) (Jackson), at a dilution of 1:5000 in PBS/Tween, was then added. The substrate *o*-phenylenediamine (OPD: 0.2 mg ml⁻¹, with 0.005% H₂O₂ in 0.01 M-sodium citrate buffer, pH 5.0) was used to visualize positive reactions, after washes in PBS/Tween and PBS. Clones showing strain specificity were then expanded and subcloned twice before a final expansion into a 25 ml culture flask. mAbs were then collected after i.p. inoculations of 10⁴ hybridoma cells into Balb/c mice previously primed with pristane. These mice were tapped over a period of a week after the development of ascites. Ascitic fluids, diluted 1:100, were used to assess the species specificity of the mAbs produced, by ELISA. The yeast and mycelial antigens described previously, along with histoplasmin, were used at 1 µg per well as above.

Polyacrylamide gel electrophoresis (PAGE), electroblotting and immunoenzyme development. A modification of the method of Tsang *et al.* (1983) was employed. Each of the *Histoplasma* variant antigens along with the other dimorphic fungal antigens containing protease inhibitors (total 100 µg protein per gel) were electrophoresed in a Tris/glycine/sodium dodecyl sulphate (SDS) running buffer using a mini Protean II cell (Bio-Rad) on a 10% (w/v) polyacrylamide gel at 200 V for 1 h. Prior to use all samples were boiled for 2–5 min and treated with 2-mercaptoethanol and SDS. Gibco BRCD molecular mass markers were run simultaneously. A semi-dry multi-gel electroblotter (Ancos) was then used to transfer separated antigens to nitrocellulose paper, in a Tris-glycine buffer, for 30 min at 200 mA.

Immunoenzyme development of nitrocellulose strips was performed by first blocking them with PBS containing casein (Marvel skimmed milk powder, 30 g l⁻¹) for 1 h at 37 °C, then washing in PBS/Tween, followed by incubation with mAbs made up 1:1 in PBS/Tween containing casein. After three washes in PBS/Tween, strips were probed with G&M IgG P₀ diluted 1:250 in PBS/Tween (1 h incubation at 37 °C), washed in PBS/Tween and PBS, and a colour reaction was then generated by the addition of the substrates 3,3'-diaminobenzidine tetrahydrochloride and 4-chloro-1-naphthol in PBS. A final tap water wash was used to terminate the reaction.

Determination of subclasses of mAbs. The Serotec subclassing kit was used. mAbs were first bound to *H. capsulatum* var. *capsulatum* antigen on ELISA plates, and then identified with the appropriate peroxidase linked anti-mouse IgG subclass probe, which was then visualized with OPD.

Results

The ELISA performed on sera from the five test mice used in the inoculation regime identified three individuals showing clear differential activity against the two varieties of *H. capsulatum*, but with considerably greater reactivity to *H. capsulatum* var. *capsulatum*. Fig. 1 shows ELISA absorbance values plotted against the serum titre of the individual used in the fusion. Control mice, inoculated with both variants of *H. capsulatum* without being treated with cyclophosphamide, showed no difference in serum reactivity to either variant antigen (data not shown).

One mAb obtained (designated C69) exhibited clear differential activity between the variants of *H. capsulatum* by ELISA, as shown in Fig. 2. In addition C69 showed no appreciable reaction to any of the yeast antigens of *P. brasiliensis*, *B. dermatitidis* and *S. schenckii*, nor to the mycelial antigens of *H. capsulatum* var. *duboisii*

Fig. 1. Polyclonal response of the mouse chosen for fusion against var. *capsulatum* antigen and var. *duboisii* antigens, as measured by absorbance values in ELISA. △, Reactivity to var. *capsulatum*; ▲, reactivity to var. *duboisii*.

Fig. 2. Reactivities of four mAbs (ascitic fluids at 1:100) to a representative range of isolate antigens, as defined by absorbance values in ELISA. Reactivity to: ○, var. *capsulatum* (4100); ●, var. *duboisii* (4094); ◆, *P. brasiliensis* (3285); ■, *B. dermatitidis* (4076); ▲, *S. schenckii* (3181); ◇, histoplasmin. Other yeast and mycelial isolates of each species exhibited very similar reactivities (data not shown).

and *P. brasiliensis*. ELISA absorbance values of 0.2 and below are considered negative by comparison with negative controls. Three other mAbs were produced, designated E12, E18 and D4. E12 showed partial cross-reactivity with the other fungal antigens, whereas E18 and D4 showed complete cross-reactivity. C69, along with the other mAbs, showed high reactivity to histoplasmin in this ELISA.

Western blot analysis showed that the species-specific mAb C69 recognized a linear determinant on a molecule with an apparent molecular mass of 70–75 kDa on nitrocellulose strips containing antigen of *H. capsulatum* var. *capsulatum* while there was no reaction on strips containing *H. capsulatum* var. *duboisii* (Fig. 3). mAb C69

Fig. 3. Western blot analysis of species-specific mAb C69 (A tracks) and cross-reactive mAb D4 (B tracks). Tracks A1 and B1 are var. *capsulatum* antigen; A2 and B2 are var. *duboisii* antigen; A3 and B3 are *B. dermatitidis* antigen; A4 and B4 are *P. brasiliensis*; A5 and B5 are *S. schenckii*.

did not react with *B. dermatitidis* antigen, though it showed an extremely faint reactivity with a 70–75 kDa molecule in *P. brasiliensis*. There was also a very faint reaction to *S. schenckii*. The partially cross-reactive mAb E12 (by ELISA) and the fully cross-reactive mAbs E18 and D4 showed a variation in binding intensity between species, although it was much greater than that seen using mAb C69. Once again they all appeared to recognize determinants on a 70–57 kDa molecule (the binding of mAb D4 to these antigens is shown in Fig. 3). Subclassing of these four mAbs revealed that all of them belonged to the IgG1 subclass.

Discussion

The presence of cross-reactive epitopes in pathogenic dimorphic fungi belonging to the genera *Histoplasma*, *Blastomyces*, *Sporothrix* and *Paracoccidioides* (Reyes-Montes *et al.*, 1982; Kashkin *et al.*, 1978) has held back the development of highly sensitive serodiagnostic tests, and although complement fixation and immunodiffusion tests are effective in the diagnosis of up to 90% of

patients with histoplasmosis (Wheat *et al.*, 1982, 1983), this cross-reactivity leads to the generation of an unacceptable number of false positives. Lack of sensitivity has also been a problem in radioimmunoassays for *Histoplasma* antigen (Wheat *et al.*, 1987) since such tests are only effective in disseminated cases of histoplasmosis. Such problems could be greatly lessened by the production of species-specific mAbs which could be used not only for the detection of antigen in body fluids but also in the isolation of epitopes specific for *Histoplasma* which could then be used to improve serological testing. Thus far only Reiss *et al.* (1986) and Kamel *et al.* (1989) have reported partially successful techniques for generating species-specific mAbs. The latter raised four mAbs, two of which, judging by their reactivity on Western blots, were almost certainly directed against carbohydrate epitopes. None of them were reactive to either the H or M antigens (Pine *et al.*, 1978) of histoplasmin, the antigens principally used in serodiagnosis. However, Reiss *et al.* (1986) raised one mAb, CB4, which recognized the M antigen of *Histoplasma* and defined this antigen as having a molecular mass of between 70 and 75 kDa. None of their mAbs showed high species specificity. In contrast, it would appear that mAb C69 recognized an epitope of the same molecular mass (70–75 kDa), and also recognized histoplasmin; it is thus likely to be directed against a presumptive M antigen, whilst exhibiting high species specificity across a range of isolates, particularly by ELISA. The mAbs raised by Reiss *et al.* (1986) were only weakly reactive by ELISA using microdilution wells coated with the purified M antigen, whilst mAb C69 showed good reactivity against presumptive M antigen.

The use of cyclophosphamide to abrogate the murine antibody response to selected antigens when attempting to raise species-specific mAbs obviously has great potential in the mycological field. It is clear even from the results of the differential activity of polyclonal serum from mice treated with the immunization protocol used that the drug is at least partially suppressing the total humoral response to the first antigen used, in this case *H. capsulatum* var. *duboisii*. In circumstances such as these cyclophosphamide is thought to have two modes of action, firstly by killing proliferating B cells and secondly by effecting long-term T cell suppression (Asherson, 1984). It would seem likely that the former effect is of more importance in the protocol described, so that B cells proliferating in response to the inoculation with *H. capsulatum* var. *duboisii* are killed, leaving stem cells with the ability to recognize antigens specific to *H. capsulatum* var. *capsulatum* intact. These are subsequently stimulated, resulting in the production of a mAb that not only distinguishes between the two *H. capsulatum* varieties, but also shows a high degree of species specificity.

Another approach to obtain species-specific mAbs using this drug, and one which is currently under evaluation in our laboratory, would be to challenge mice initially with a cocktail of fungal antigens from a number of cross-reactive species, then treat with cyclophosphamide before inoculating with the species antigen of interest.

The 70–75 kDa molecule to which these mAbs bind evidently contains both epitopes that are conserved and regions which vary both between variants and between species. Using the specific mAb C69 we now hope to elucidate the nature of the variable epitope on this molecule, and if it is a pure peptide, to sequence and synthesize it. If patients with histoplasmosis also recognize this epitope it could then form the basis of a more specific and sensitive means of serodiagnosis.

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