Immunochemical characterisation of a 29-Kda surface-associated molecule of *Entamoeba histolytica* and its recognition by serum from patients with amoebiasis

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Summary. A 29-Kda cytotoxic molecule of axenically-grown pathogenic *Entamoeba histolytica* (strain HM1) was purified from an amoebic extract by immuno-affinity chromatography with monoclonal antibodies. Immunoreactivity of the purified 29-Kda molecule altered significantly ($p < 0.01$) after exposure to heat or trypsin, but remained unaltered after treatment with sodium metaperiodate. The 29-Kda molecule was recognised by serum from each of 13 patients with amoebic liver abscess. In an ELISA system, the molecule produced significantly higher ($p < 0.01$) OD readings with these serum samples than with samples from asymptomatic cyst passers. No serum from healthy subjects or from patients with idiopathic ulcerative colitis or giardiasis had antibodies that reacted with the 29-Kda molecule. The immune response to the 29-Kda amoebic protein in man may indicate a specific role for this molecule in invasive amoebiasis.

Introduction

Entamoeba histolytica is a protozoan parasite that infects 480 million people with 36 million cases of disabling colitis or extra-intestinal metastasis and 40 000 deaths annually.¹ Cytolysis of the target cells by amoebae is initiated by adhesion of amoebic trophozoites through surface lectins *via* specific receptors;² this is followed by cytolysis of target cells through the action of proteolytic enzymes,^{3–5} cytotoxins⁶ and enterotoxins.^{7,8} The trophozoites of *E. histolytica* cytolys erythrocytes⁹ and other target cells such as Chinese Hamster Ovary cells (CHO), Baby Hamster Kidney cells (BHK), Maiden Derby Canine Kidney cells (MDCK) and Henle-407 cells by direct contact-dependent cytolysis.^{2,6} Thus, the surface-associated adhesive and cytotoxic molecule(s) are important in mediating host-parasite interactions. Several monoclonal antibodies (MAbs) have been developed and used widely to identify surface-associated molecules of amoebic trophozoites. A 220-Kda protein that is inhibited by *N*-acetyl-D-glucosamine,¹⁰ a 170-Kda lectin that is inhibited by *N*-acetyl-D-galactosamine,¹¹ a 112-Kda lectin¹² and a 96-Kda protein¹³ have been implicated in the recognition of target cells by amoebae. Recently, we¹⁴ and others^{15–17} have reported recognition of several amoebic antigens by serum from patients with amoebiasis. An epitope of a 29-Kda surface-associated protein of *E.*

histolytica, identified by MAbs,¹⁸ has been shown to mediate adhesion and cytotoxicity of amoebic trophozoites to the target cells.¹⁹ Furthermore, amoebae from asymptomatic cases showed little or no evidence of expression of a (29–30)-Kda molecule.^{18,20} Thus the (29–30)-Kda protein may have a role in mediating the amoebic disease process. In the present study we have sought to purify the 29-Kda molecule by affinity chromatography with MAbs, to characterise it immunochemically, and to investigate its recognition by serum from patients with amoebiasis.

Materials and methods

Preparation of antigen

E. histolytica (strain HM1) trophozoites grown axenically in Trypticase-Panmede-Serum (TPS-I) medium were harvested in phosphate-buffered saline (PBS; pH 7.2) and disintegrated ultrasonically in a sonicator (MSE Ltd, Crawley, Sussex) with 10 15-s bursts.¹⁹ The sonicated material was designated crude amoebic extract (CAE). The protein content of CAE was determined by the method of Lowry *et al.*²¹

Monoclonal antibodies (MAbs)

A panel of MAbs developed by us was used to identify the 29-Kda molecule of *E. histolytica*. A clone of cells, P4C4F8 (C8), produced MAb C8, of IgG₁ isotype, which recognised the 29-Kda molecule of *E.*

histolytica trophozoites.¹⁹ MAb C8-producing cells were propagated as ascites in BALB/c mice and were precipitated by ammonium sulphate 45% w/v. The precipitated MAb was redissolved in PBS (pH 7.2) and dialysed extensively against distilled water.²²

Affinity purification of 29-Kda antigen

The 29-Kda amoebic antigen was affinity purified as described earlier.²³ Briefly, 5 mg of MAb C8 directed at the 29-Kda antigen was covalently coupled to 1 ml of cyanogen bromide-activated sepharose-4 B gel in 0.1 M borate buffer saline, pH 8.3. A portion containing 5 mg of CAE protein was applied to an immuno-adsorbent column previously equilibrated with PBS (pH 7.2) at 4°C for 3 h. The bound antigen was eluted at 4°C with 0.1 M glycine-HCl buffer, pH 2.5. Several identical elutions of CAE protein were made through affinity gel. The fractions containing protein were pooled, dialysed extensively against distilled water and concentrated by lyophilisation (Kontron Instruments, Zurich, Switzerland).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting

The purity of affinity-purified antigen was confirmed by SDS-PAGE.²⁴ The specificity was investigated by Western immunoblotting.²⁵ Briefly, 100 µg of CAE protein or 40 µg of affinity-purified antigen was subjected to electrophoresis in a 5% stacking gel and a 10% separating gel under reducing conditions in an electrophoretic cell (BioRad Laboratories, Richmond, CA, USA) at 25 mA for 4 h. Resolved proteins from gels were either stained or transferred on to nitrocellulose paper (0.2-µm pore diameter) at 200 mA for 3 h. The strips were blocked with bovine serum albumin (BSA) 3% and incubated with MAb C8 or hyperimmune serum obtained from mice immunised with CAE, followed by incubation with anti-mouse-HRP conjugate (Dakopatts a/s Glostrup, Denmark). The colour reaction was developed with 3 mg of 4-chloro-1-naphthol in 6 ml of PBS, pH 7.2, and 5 µl of H₂O₂ as substrate.

Physico-immunochemical characterisation of the 29-Kda antigen

Immunoreactivity of the 29-Kda antigen after heating and exposure to trypsin or sodium metaperiodate was assessed in an ELISA system.²⁶ The sensitivity to heat was determined by exposing 29-Kda antigen to 60°C or 100°C (boiling) for 10 min before coating on to the ELISA plate. The effect of proteolytic digestion was determined by treating wells coated with 29-Kda antigen with trypsin (10, 100 or 1000 µg/ml) at 37°C for 2 h. Wells containing untreated antigen served as controls. The periodate oxidation was accomplished by treating wells coated with the 29-

Kda antigen with 0.025 M, 0.05 M or 0.1 M sodium metaperiodate in 20 mM sodium acetate buffer, pH 4.5, in the dark at 4°C for 24 h. Antigen-coated wells treated with acetate buffer alone were included as controls. Altered immunoreactivity of treated antigen was assessed by incubating with MAb C8 (1 in 1000) and then with anti-mouse-HRP conjugate. The reaction was developed with *o*-phenylene-diamine (OPD) as substrate (5 mg of OPD in 10 ml of citrate buffer, pH 5.0, and 5 µl of H₂O₂). The effect of heat, trypsin and periodate treatments was determined by comparing optical density (OD) values of treated wells with untreated control wells. Each experiment was set up in triplicate and repeated at least twice.

Recognition of 29-Kda protein by human serum

A total of 33 serum samples was collected from confirmed cases of amoebic liver abscess (13 patients), asymptomatic amoebic cyst passers (five patients), idiopathic ulcerative colitis (five patients) and giardiasis (five patients) and from five healthy subjects. The criteria for diagnosis of these patients have been reported elsewhere.²⁷ Each serum sample was tested against the 29-Kda affinity-purified amoebic protein in an ELISA system.²⁸ Briefly, the ELISA was performed in microtitration plates (M/S Costar Corporation, Broadway, Cambridge, MA, USA) by coating wells with a 100-µl portion containing affinity-purified 29-Kda protein, 1 µg/ml in 0.05 M carbonate buffer, pH 9.6. Wells were blocked with BSA 3% and incubated with each serum diluted 1 in 4000. After treatment with anti-human-HRP conjugate (The Binding Site Ltd, Edgbaston, Birmingham) diluted 1 in 4000, the colour reaction was developed with OPD as substrate. The reaction was stopped by adding 50 µl of 6N H₂SO₄ and the OD was measured at 492 nm in an ELISA reader (Uniskan-1; Lab Systems, Helsinki, Finland).

Results

Affinity purification of 29-Kda antigen

The 29-Kda amoebic protein was successfully eluted from the affinity column by elution under acidic conditions (fig. 1). SDS-PAGE analysis of purified antigen revealed a single band at the 29-Kda position (fig. 2). Immunoblotting of affinity-purified 29-Kda antigen with hyperimmune serum or MAb C8 revealed a discrete single band at a position corresponding to a molecular mass of 29-Kda (fig. 3).

Physico-immunochemical characterisation

The exposure of affinity-purified 29-Kda antigen at 60°C significantly reduced its reactivity to MAb C8 and exposure at 100°C almost abolished its immunoreactivity (fig. 4a). Similarly, proteolytic digestion of

Fig. 1. Affinity gel elution profile of the 29-Kda molecule of *E. histolytica*.

Fig. 2. SDS-PAGE of *E. histolytica* antigens. Lane (A) crude amoebic extract (CAE) antigen; (B) affinity-purified 29-Kda antigen.

the 29-Kda antigen with trypsin reduced its immuno-reactivity to MAb C8 progressively as the concentration was raised (fig. 4b). However, the immuno-reactivity of the 29-Kda antigen was unaffected by sodium metaperiodate oxidation (fig. 4c).

Fig. 3. SDS-PAGE immunoblots of *E. histolytica* antigens: (A) CAE antigen with anti-amoebic hyperimmune serum; (B) affinity-purified 29-Kda protein with hyperimmune serum; and (C) 29-Kda protein with MAb C8.

Recognition of 29-Kda protein by human serum

The mean OD value of antibodies to the 29-Kda protein in serum from healthy subjects was 0.022 (SD 0.015) (fig. 5). A cut-off OD value for significance was calculated to be 0.052—the mean plus two SD of the OD values observed in healthy subjects. The mean OD values of serum from patients with giardiasis and ulcerative colitis were below the cut-off value. Serum from 13 cases of amoebic liver abscess recognised the 29-Kda antigen in the ELISA system; the mean OD value of anti-29-Kda antibodies in these patients was 0.777 (SD 0.023). OD values obtained with serum from asymptomatic cyst passers was 0.220 (SD 0.046). Although the 29-Kda amoebic protein could be recognised by serum samples from asymptomatic cyst passers, their reactivity was significantly lower (mean OD 0.22, SD 0.046) than the reactivity (mean OD 0.777, SD 0.023) of serum from patients with hepatic amoebiasis.

Discussion

The involvement of surface-associated molecules in modulation of the disease process requires recognition and development of specific immune responses by the host's immune system. Often, the proteins expressed on the cell surface of microbes are important in mediating interactions with target cells, but the integral membrane proteins have extensive hydropho-

Fig. 4. Immunoreactivity of affinity-purified 29-Kda molecule after (a) heat treatment, (b) proteolytic digestion with trypsin, (c) sodium metaperiodate oxidation (mean OD of two sets tested in triplicate); * $p < 0.01$.

bic regions making them difficult to purify and characterise. In this study, we have successfully purified a protein that we had identified earlier as an epitope of a membrane-associated antigen expressed

Fig. 5. Recognition of 29-Kda protein of *E. histolytica* in ELISA by serum from (A) patients with amoebic liver abscess, (B) asymptomatic cyst passers, (C) patients with idiopathic ulcerative colitis, (D) patients with giardiasis, and (E) healthy subjects. The broken line represents the cut-off OD value; bars represent SD.

on the surface of *E. histolytica* trophozoites.¹⁹ This antigen was found to be free from other amoebic proteins and has a molecular mass of 29-Kda. Immunochemical characterisation of the epitope revealed it to be protein in nature—it was heat-sensitive and proteolytic digestion of purified antigen reduced its immunoreactivity, whereas sodium metaperiodate oxidation did not. The 29-Kda molecule was recognised by the human host and specific antibodies were generated in response to amoebic infection. However, the antibody response to the 29-Kda molecule in patients with amoebic liver abscess was significantly higher ($p < 0.01$) than that observed in asymptomatic amoebic cyst passers. This suggests a variable degree of recognition of this molecule in man. Since the 29-Kda molecule is a component of the amoebic plasma membrane, a significant immune response may be generated only if the amoebic trophozoite expresses this molecule. The low level of anti-29-Kda antibodies in cyst passers may indicate that the 29-Kda molecule is poorly expressed by amoebic trophozoites in the gut lumen. A recent study by Tachibana *et al.*²⁰ indicated that amoebae isolated from patients with invasive amoebiasis strongly expressed a 30-Kda antigen, whereas amoebae isolated from asymptomatic amoebic cyst passers expressed little or none of this antigen. It is possible that hyperexpression of the (29–30)-Kda molecule may correlate with the invasive potential of the parasite, and thus may be important in virulence.

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