Monoclonal antibodies against Malaysian *Blastocystis hominis* isolates

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Abstract

The immunoblotting technique was used with six monoclonal antibodies (MAbs) to analyze the antigenic profiles of 10 *Blastocystis hominis* isolates [C, H, H6, H7, KP1, Y51, DJ1, RN, 27B05(1), M12] in relation to studies on the pathogenicity of this parasite. MAb RN5-8-5 specifically recognized *B. hominis* isolated from patients with clinical symptoms while MAbs 4F2-1 and 4C4-2 were also potentially useful for this purpose. The other three MAbs (DJ-9, 9D3-3 and Y44F4) recognized *B. hominis* isolates recovered from both symptomatic and asymptomatic patients, as well as cross-reacted with *Escherichia coli* and *Endolimax nana*. All these MAbs did not react with *Entamoeba histolytica* and *Giardia lamblia*.

Key words: *B. hominis*, isolates, monoclonal antibody, immunoblotting

Introduction

Blastocystis hominis (Brumpt, 1912), an intestinal protozoon, appears in various forms and sizes ranging from 7 to 40 µm. Initially, *B. hominis* was thought to be harmless or a common component of the normal human intestinal flora. However, it is now increasingly recognized that this parasite could be pathogenic under specific conditions such as immunosuppression (Garavelli *et al.*, 1990; Cegielski *et al.*, 1993), poor nutrition or with concurrent infections.

Several studies have shown that the clinical presentations range from asymptomatic to chronic (more than a year) gastrointestinal disturbance such as diarrhoea, abdominal discomfort, and joint pains in both immunocompetent and immunocompromised subjects (Garcia *et al.*, 1984; Henry *et al.*, 1986; Telalbasic *et al.*, 1991; Lambert *et al.*, 1992).

Attempts to ascribe pathogenicity to *B. hominis* by epidemiological studies have been criticised because of the difficulty in eliminating all other causes of symptoms, either infectious or non-infectious, as approximately 25% of reported diarrhoea cases have no known aetiology (Edmeades *et al.*, 1978). The lack of an experimental animal model is also another factor in the difficulty to confirm the pathogenic role of this parasite.

Currently the diagnosis of *B. hominis* infection is based on the demonstration of the parasite in stool or culture. The organism was often found together with other common parasitic protozoa such as *Entamoeba histolytica* and *Giardia lamblia* (Zierdt *et al.*, 1995). Identification based on morphology is impossible as both the pathogenic and non-pathogenic strains appear to be similar. In the present study we have produced and used MAbs in an attempt to differentiate parasites from symptomatic and asymptomatic patients.

Materials and Methods

Parasite isolates

Ten isolates of *B. hominis*, an isolate of *G. lamblia* (7404) from a Malaysian patient, *Endolimax nana* (EN1) from a healthy Malaysian, *E. histolytica* (HK9) and *Escherichia coli* (Bac-4) a common bacteria in human intestine, were used in this study. The *B. hominis* isolates included M12 that was isolated from the soft stool of a monkey kept in the Institute for Medical Research animal house. Another four isolates (KP1, Y51, DJ1 and RN) were obtained from patients with diarrhoea, joint pain and stomach discomfort. The subjects were free from other parasites and bacteria that could cause these symptoms. Two axenic isolates (C and H) obtained from the National University of Singapore, and three isolates (H6, H7 and 27B05(1)) from healthy Malaysian aborigines or Orang Asli at Bukit Kemandul village were also used.

Preparation of crude antigens

All parasite isolates were harvested from *in vitro* cultures and the antigens were prepared as reported in our previous study (Init *et al.*, 1998).

Production of monoclonal antibodies

Hybrid cells were produced by fusing NS1/1Ag.4.1 (NS1)(1×10^7) cells with the spleen cells (1×10^8) from immunised mice. The positive hybrids were cultured from 96-well plates to 24-well plates and finally to culture bottles. They were cloned by limiting dilution on a feeder layer of Balb/c peritoneal cells in 96-well plates. The clones producing monoclonal antibody were scaled up to culture bottles. The supernatant with monoclonal antibodies was typed to class and subclass using Mouse

Monoclonal Sub-Isotyping Kit (American Qualex). Larger volumes of MAbs were produced as ascites fluid; Balb/c mice were primed 7 days earlier with an intraperitoneal injection of 0.5 ml pristane, then injected intraperitoneally with 4 - 8 x 10^7 hybrid cells, and ascites fluid collected 10-20 days later. Ascites fluid raised against myeloma cells NS1 was used as negative control.

Analysis of monoclonal antibodies by Western blotting
Soluble protein extracts of the above organisms were separated on 12.5% SDS-PAGE using the discontinuous system of Laemmli (1970). Individual polypeptides separated on gels were transferred electrophoretically to nitrocellulose paper (NCP), pore size 0.45 µm (MSI, Westboro, MA) using a modified method of Tsang *et al.* (1986) with a Semidry Blotter II (KEMENTEC) at 0.8 mA/cm gel for one hour. A section of the NCP containing the molecular weight marker was stained with amido black. The remaining NCP was incubated in quenching solution, then treated overnight with 1:100 dilution of MAb. After washing three times in PBS, it was then treated with 1:1000 dilution of horseradish peroxidase goat anti-mouse immunoglobulin (KPL, USA) for three hours and washed as above. Antigenic bands were visualised by addition of the substrate, 4-chloro-1-naphthol (ICN, Biomedicals). The blot was rinsed in distilled water, air-dried and photographed.

Results
SDS-PAGE pattern
The SDS-PAGE polypeptide patterns of *B. hominis* isolates, *E. histolytica*, *G. lamblia*, *E. nana* and *E. coli* have been previously reported (Init *et al.*, 1998)

Monoclonal Antibodies (MAbs)
Six MAbs were used to characterise *B. hominis* isolates in this study. They were 9D3-3, 4F2-1 and 4C4-2 raised against isolate KP1, RN5-8-5 raisedagainst isolate RN, Y44F4 raised against Y51, and DJ-9 raised against isolate DJ1. Three of the MAbs secreted IgM (DJ-9, 9D3-3, RN5-8-5); one secreted IgG1 (Y44F4); and two secreted IgG1+IgG3 (4F2-1, 4C4-2). Ascites fluid raised against NS1 did not show any reaction against all antigens tested in this study (resulrs not shown).

Reactivity patterns of IgM monoclonal antibody (DJ-9, 9D3-3, RN5-8-5)
MAb DJ-9 recognised H6, KP1, Y51, DJ1, RN,

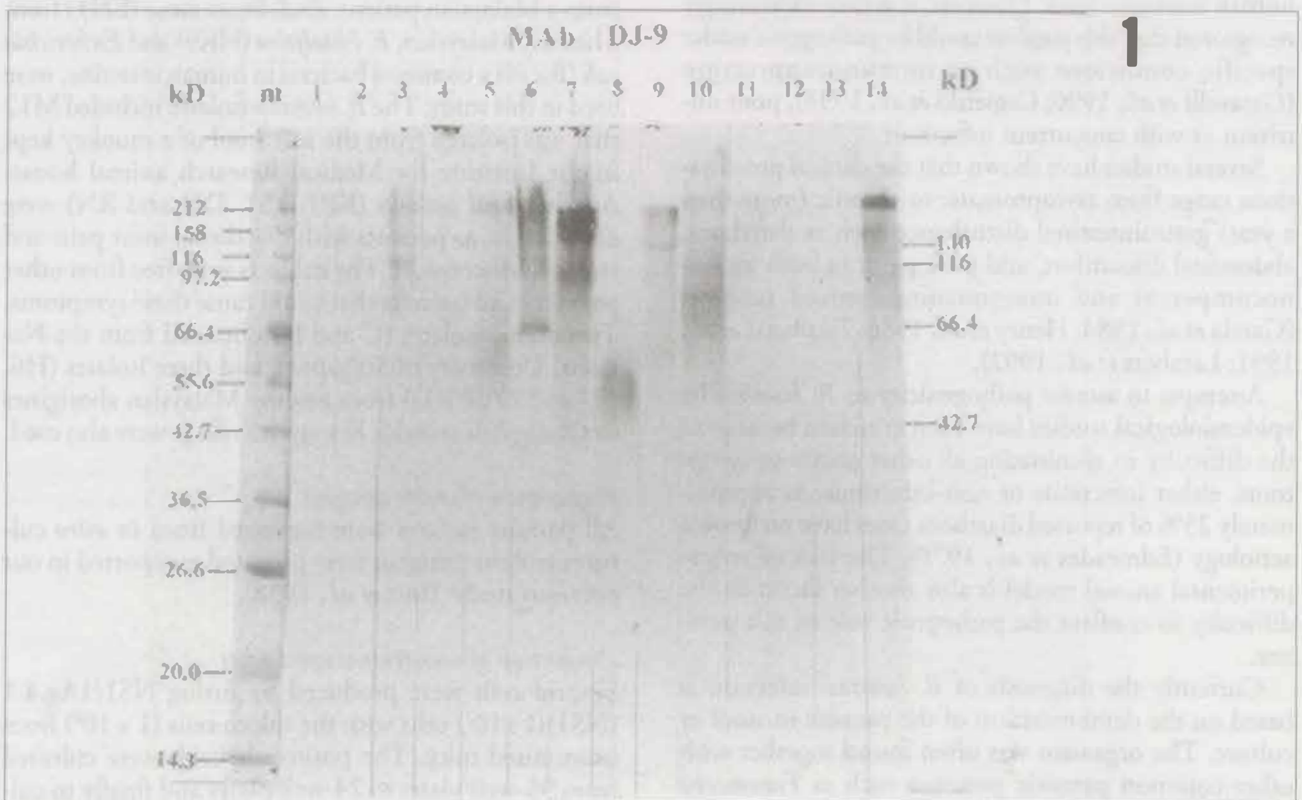

Fig. 1. Immunoblot patterns of SDS-PAGE separated polypeptides of *B. hominis* and other organisms on analysis with MAb DJ-9 (IgM) developed against isolate DJ1.
Standard protein marker (lane m); *B. hominis* isolate C (lane 1); *B. hominis* isolate H1 (lane 2); *B. hominis* isolate H6 (lane 3); *B. hominis* isolate H7 (lane 4); *B. hominis* isolate KP1 (lane 5); *B. hominis* isolate Y51 (lane 6); *B. hominis* isolate DJ1 (lane 7); *B. hominis* isolate RN (lane 8); *B. hominis* isolate 27B05(1) (lane 9); *B. hominis* isolate M12 (lane 10); *Entamoeba histolytica*, HK9 (lane 11); *Giardia lamblia*, 7404 (lane 12); *Endolimax nana*, EN1 (lane 13); *Escherichia coli* isolated from KP1, Bac-4 (lane 14).

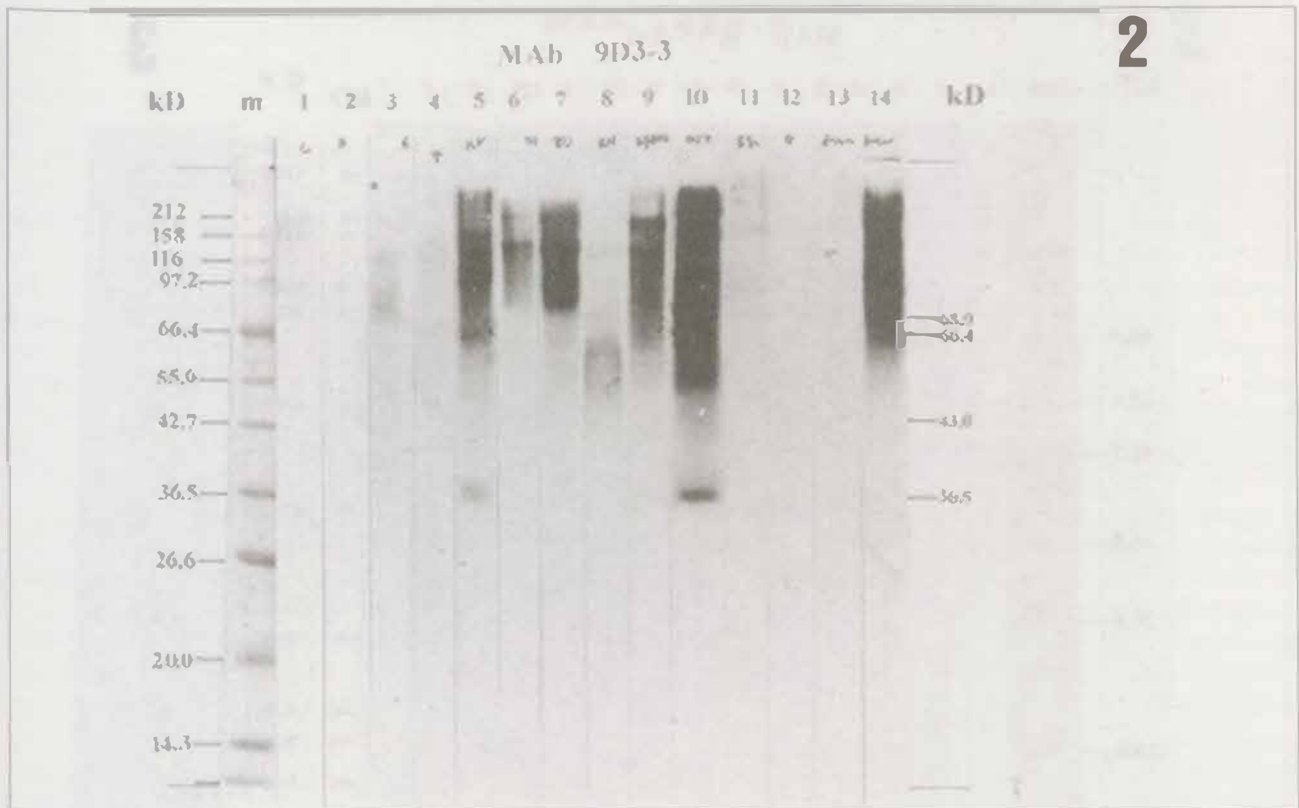

Fig. 2. Immunoblot patterns of SDS-PAGE separated polypeptides of *B. hominis* and other organisms on analysis with monoclonal antibody 9D3.3 (IgM) developed against isolate KP1. Designation of lanes as in Fig. 1.

27B05(1), M12 and Bac-4, but did not recognise isolates C, H and H7. Most of the reactive bands were weak except against Y51 at 66.4 kD, DJ1 at about 180 kD, and Bac-4 at 250 kD (Fig. 1). MAb 9D3-3 showed a prominent smear against the antigens of *B. hominis* isolates KP1, Y51, DJ1, RN, 27B05(1), M12 and Bac-4; a prominent band at 36.5 kD against KP1 and M12; and no reactivity against isolates C, H, H6 and H7. Reactive smears between 60 and 212 kD were common to both *B. hominis* and Bac-4 (Fig. 2). MAb RN5.8.5 only reacted with antigen of *B. hominis*, isolate RN, giving a striking smear between 42.7 and 66.4 kD. It did not show any cross-reaction against other isolates tested (Fig. 3). These three MAbs did not react with *E. histolytica*, *G. lamblia* and *E. nana* antigens.

Reactivity patterns of IgG1 monoclonal antibody (Y44F4)

MAb Y44F4 reacted strongly against antigens of isolates C, Y51, 27B05(1), M12 and *E. nana* and weakly against isolates H6, H7, KP1, DJ1 and Bac-4. It did not show any reaction against isolate H and RN as well as *E. histolytica* and *G. lamblia*. A prominent band seen only with isolate C, was at 26.6 kD, while that seen with *E. nana* was at 29 kD (Fig. 4).

Reactivity patterns of IgG1 + IgG3 monoclonal antibodies (4F2-1, 4C4-2)

MAb 4F2-1 gave similar patterns against all the isolates KP1, Y51, DJ1, 27B05(1) and M12, with prominent bands at molecular weights 26.6 to 116 kD. Against isolate RN it gave prominent bands at molecular weight 26.6 to 48 kD, with a smear at above 48 to 66.4 kD (Fig. 5). MAb 4C4-2 showed similar reactivity patterns against *B. hominis* isolates KP1, Y51, DJ1, RN, 27B05(1) and M12, with prominent bands at molecular weight 26.6 to 97.2 kD (Fig. 6). MAbs 4F2-1 and 4C4-2 did not show any reaction against *B. hominis* isolates C, H, H6, H7 as well as *E. histolytica*, *G. lamblia*, *E. nana*, and Bac-4.

Discussion

In our previous studies, the differences in polypeptide patterns as shown with SDS-PAGE analysis did not provide any information on the pathogenicity of this parasite (Init *et al.*, 1998). However, the results obtained through the use of MAbs raised against several isolates, to analyse antigenic profiles of other isolates by the immunoblotting technique, were of great help in this regard. Currently, six MAbs (DJ-9, 9D3-3, RN5-8-5, Y44F4, 4F2-1 and 4C4-2) were used to identify local *B. hominis* isolates capable of producing symptoms in patients, by the immunoblotting technique. Of these hybrid clones three secreted IgM and one secreted IgG1, while another two clones secreted two subclasses of antibodies (IgG1 and IgG3). Gentry *et al.* (1982)

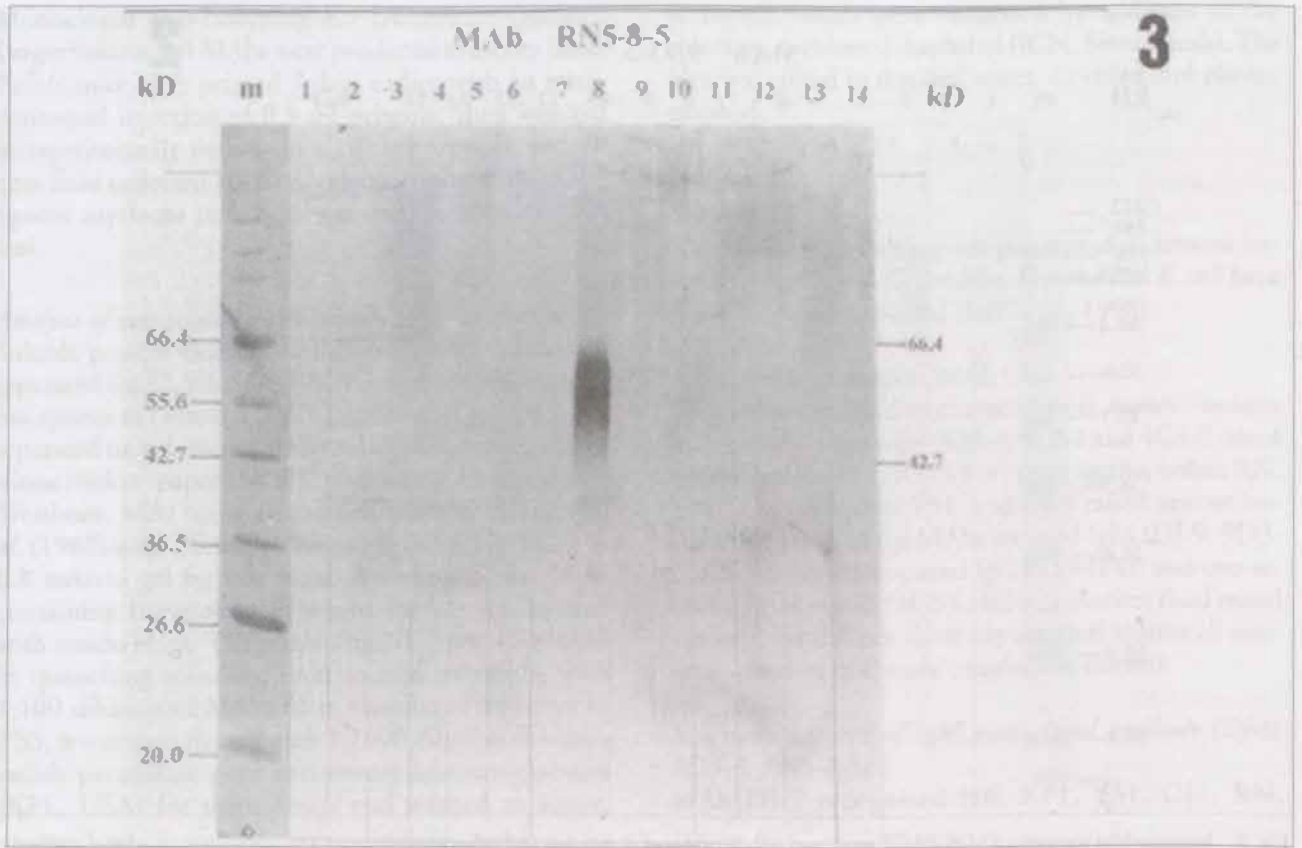

Fig. 3. Immunoblot patterns of SDS-PAGE separated polypeptides of *B. hominis* and other organisms on analysis with monoclonal antibody RN5-8-5 (IgM) developed against isolate RN. Designation of lanes as in Fig. 1.

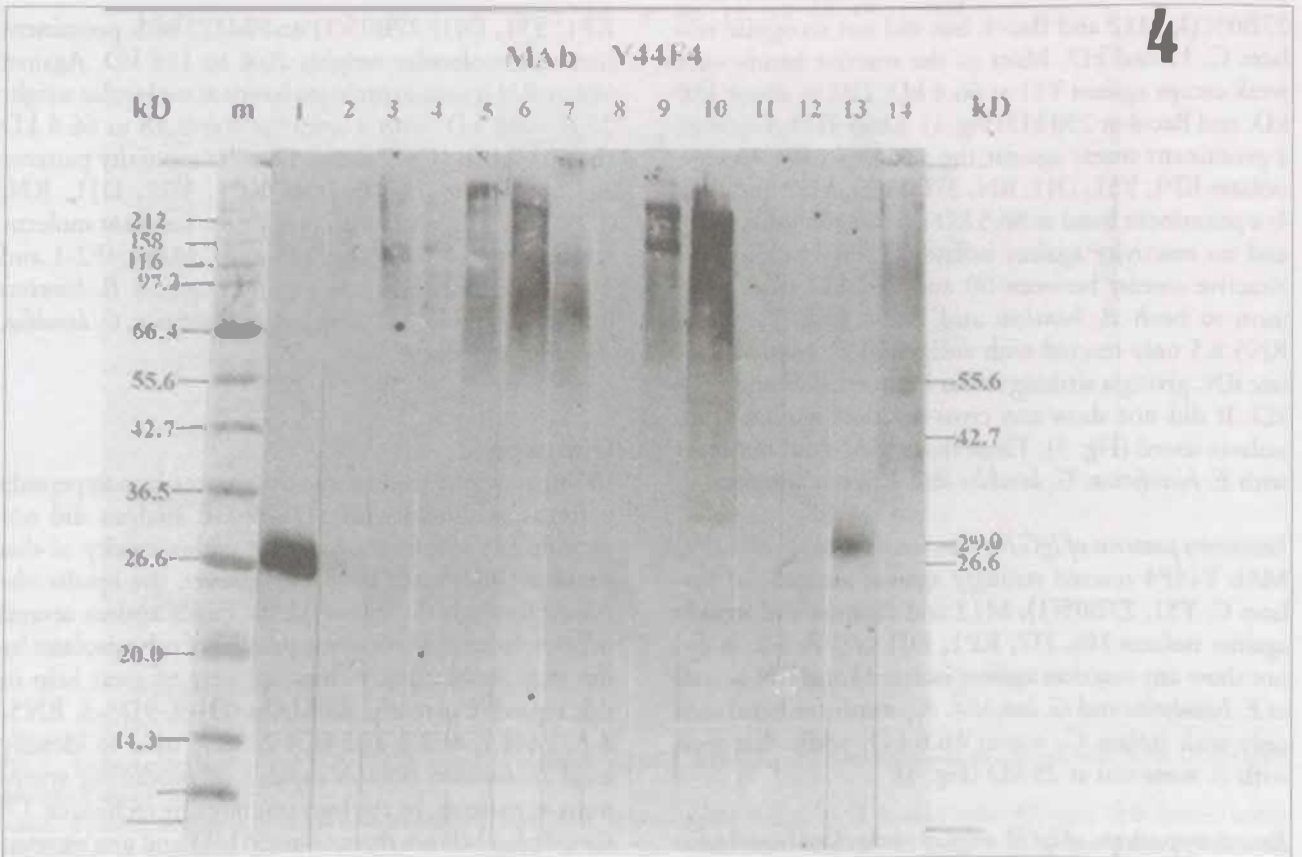

Fig. 4. Immunoblot patterns of SDS-PAGE separated polypeptides of *B. hominis* and other organisms on analysis with monoclonal antibody Y44F4 (IgG1) developed against isolate Y51. Designation of lanes as in Fig. 1.

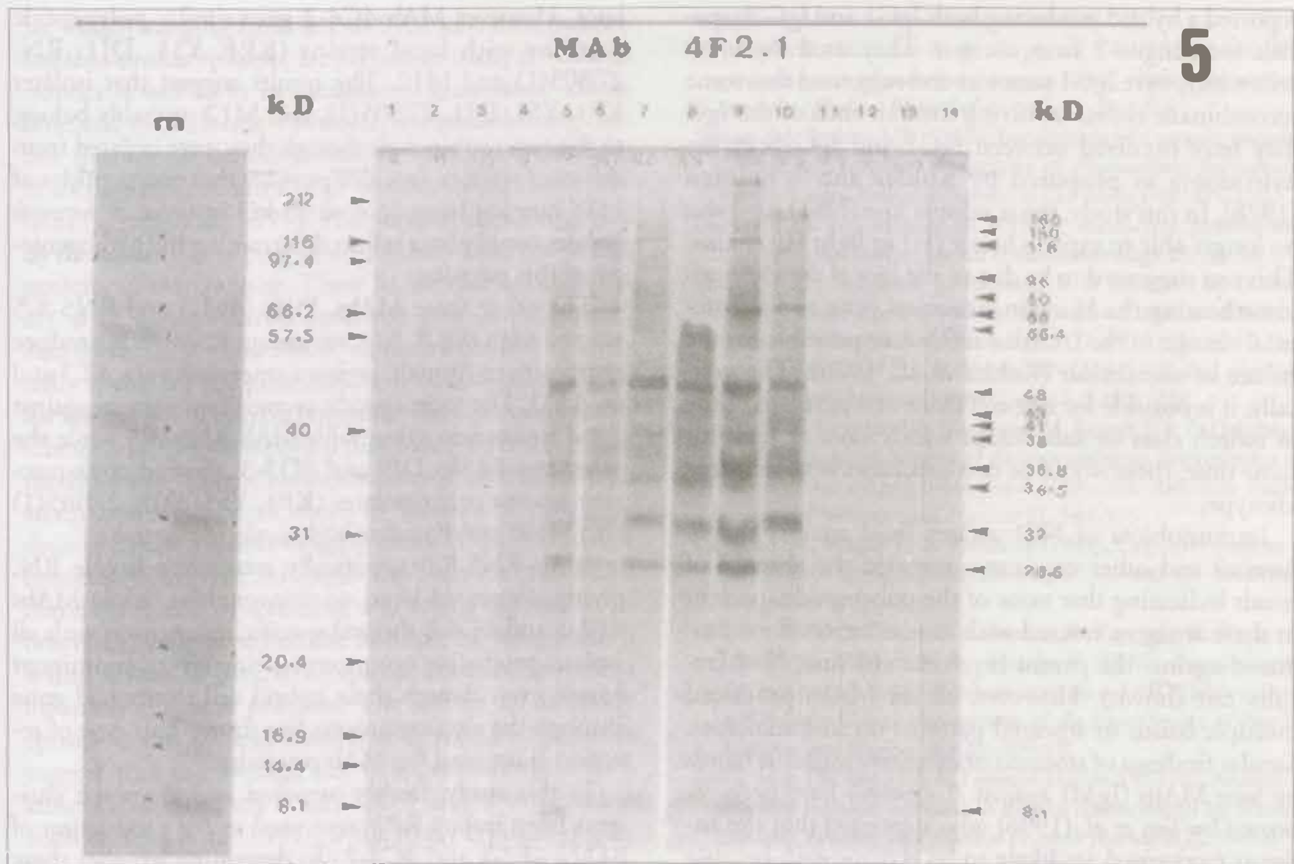

Fig. 5. Immunoblot patterns of SDS-PAGE separated polypeptides of *B. hominis* and other organisms on analysis with monoclonal antibody 4F2.1 (IgG1+IgG3) developed against isolate KP1. Designation of lanes as in Fig. 1.

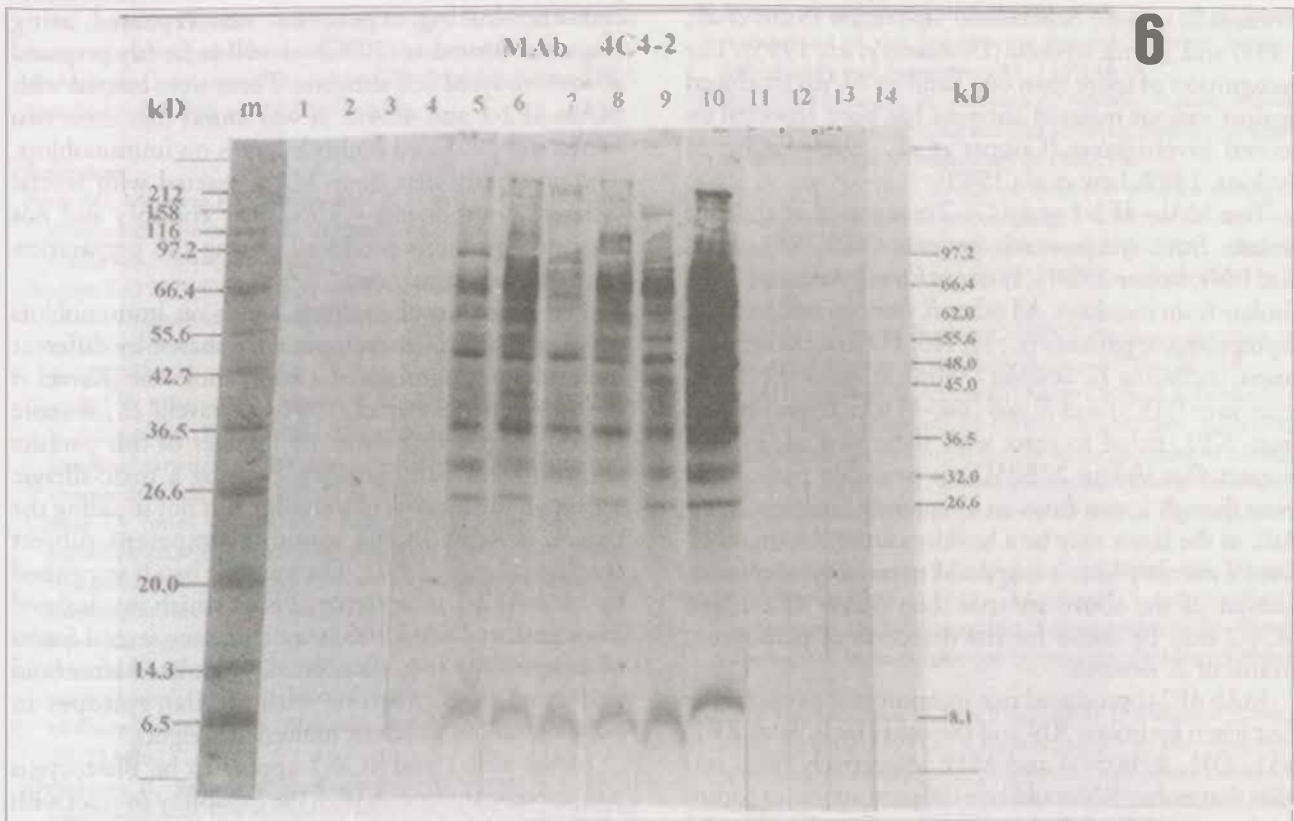

Fig. 6. Immunoblot patterns of SDS-PAGE separated polypeptides of *B. hominis* and other organisms on analysis with monoclonal antibody 4C4-2 (IgG1+ IgG3) developed against isolate KP1. Designation of lanes as in Fig. 1.

reported a hybrid producing both IgG1 and IgG2b specific for dengue-2 virus antigen. They used myeloma cells which were IgG1 secretors and suggested that some recombinant events involving the Fab ends of the IgG may have occurred between IgG1 and IgG2b in the hybridoma as proposed by Kohler and Schulman (1978). In this study, the myeloma line (NS1) used was no longer able to express heavy (H) or light (L) chains. This was suggested to be due to the loss of the chromosome bearing the H chain structural gene and a structural change in the L chains mRNA responsible for the failure of translation (Kohler *et al.*, 1976). Theoretically, it is possible for one cell clone at a particular time to switch class or subclass in which case, at least for some time, there would be two subclasses with the same idiotype.

Immunoblots of NS1 ascites fluid against the *B. hominis* and other organisms revealed the absence of bands indicating that none of the polypeptides present in these antigens reacted with mouse ascites fluid produced against the parent myeloma cell line, NS1 (results not shown). However, all six MAbs produced multiple bands or smeared patterns on immunoblots. Similar findings of smeared or extensive multiple bands by four MAbs (IgM) against *B. hominis* have been reported by Tan *et al.* (1996) who suggested that the antigens recognised are likely to be glycoprotein because of their sensitivity to pronase and periodate treatments. Extensive banding patterns were also observed in characterisation studies of MAbs to *Trypanosoma cruzi*, *Trichinella spiralis*, *Schistosoma japonicum* (Saito *et al.*, 1994) and *Taenia saginata* (Draelants *et al.*, 1995). The recognition of more than one band by MAbs produced against various malarial antigens has been reported by several investigators (Cooper *et al.*, 1988; Etzion & Perkins, 1989; Lew *et al.*, 1989).

Two MAbs 4F2-1 and 4C4-2 recognised all the four isolates from symptomatic patients (KP1, Y51, DJ1, and RN), isolate 27B05(1) (from Orang Asli) and M12 (isolate from monkey). All other *B. hominis* isolates from asymptomatic patients (C, H, H6, H7) and other protozoa, including *G. lamblia* (7404), *E. nana* (EN1), *E. histolytica* (HK9) and *E. coli* (Bac-4) which was isolated from KP1, failed to react with these two MAbs. We suspect that isolate 27B05(1) is probably pathogenic even though it was from an apparently healthy Orang Asli, as the latter may be a healthy carrier. Isolate M12 from a monkey host is suspected to cause symptoms in human. If the above are true then MAbs 4F2-1 and 4C4-2 may be useful for the detection of pathogenic strains of *B. hominis*.

MAb 4F2-1 produced two immunoblot patterns: the first given by isolate RN and the other by isolates KP1, Y51, DJ1, 27B05(1) and M12 respectively. This implies that isolate RN could be a different strain (in pathogenic potential) of *B. hominis* even though it has the same morphology and protein profile as the other isolates. However MAb 4C4-2 gave similar polypeptide patterns with local strains (KP1, Y51, DJ1, RN, 27B05(1) and M12. The results suggest that isolates KP1, Y51, DJ1, 27B05(1), and M12 probably belong to the same strain even though they were isolated from different sources. It is also possible that polypeptides of MW ranging from 26.6 to 65 kD in these *B. hominis* isolates could play a role in determining the pathogenicity of this parasite.

The other three MAbs, DJ9, 9D3-3 and RN5-8-5 reacted with the *B. hominis* isolate RN which produce symptoms in human, giving a smear between 42.7 and 66.4 kD. The most specific recognition pattern against these strains was given with MAb RN5-8-5 while the other two MAbs DJ9 and 9D3-3, showed cross-reaction against other isolates (KP1, Y51, DJ1, 27B05(1) and M12) and Bac-4 at high molecular weight.

MAb RN5-8-5 specifically recognised isolate RN, giving a smeared band on immunoblot, while MAbs 4F2-1 and 4C4-2 showed specific recognition with all isolates producing symptoms, giving several prominent bands, even though these hybrid cell clones had gone through the cloning process five times. This type of reaction is unusual for MAb reactivity.

In this study, freshly prepared and aliquoted antigens from isolate KP1 were used in the production of MAbs 4F2-1 and 4C4-2. To determine whether these various components recognised on immunoblots by these MAbs were degradative products of crude antigen from lysed *B. hominis* cells, a similar immunoblotting experiment was repeated using aliqouted (stored at -20°C), as well as freshly prepared *B. hominis* lysed cell antigens. These were reacted with MAbs 4F2-1 and 4C4-2. It was found that these two MAbs still produced multiple bands on immunoblots. The possibility that these MAbs reacted with several different components with similar epitopes and not degraded products produced during the preparation must also be investigated.

The detection of multiple bands on immunoblots suggests that similar epitopes were shared by different molecules or fragments of a larger molecule (Kamel *et al.*, 1989; Mencke *et al.*, 1991). Garavelli & Libanore (1993) suggest that there are families of this parasite with different pathogenicity, exerting a toxic-allergic action on the mucosa of the colon but not invading the bowel, at least in the immunocompetent subject (Lakhanpal *et al.*, 1991). The multiple bands recognised by MAb 4F2-1 in antigens of KP1 which was isolated from patient DIAG 106, may represent several forms of morphology (e.g. vacuolated, granulated amoeboid and cyst) of *B. hominis* with similar epitopes in polypeptides of different molecular weights.

MAbs 4F2-1 and 4C4-2 appear to be Blastocystis pan-specific antibodies with the capability to react with all *B. hominis* isolates recovered from patients with symptoms. They recognised various polypeptides of molecu-

lar weight ranging from 8.1 to 97.2 kD, thus indicating that similar epitopes are present in many *B. hominis* isolates as well as in its vacuolated, granulated, amoeboid and cystic forms. However, immunoblotting cannot distinguish between multiple processed products of a single protein and a large number of unrelated proteins (Cheng *et al.*, 1991). The polypeptides recognised by these MAbs may play an important role in the pathogenicity of this parasite. These MAbs did not cross-react with other related protozoa tested, such as *E. histolytica*, *G. lamblia* and *E. nana* and could be potentially useful for the purification of *B. hominis* antigens for further studies on pathogenicity. In a previous study, Cheng *et al.* (1991) reported that MAb M26-32, a Plasmodium pan-specific antibody, reacted with all species, strains and erythrocytic stages by Western blotting producing reaction bands ranging from 20 to 230 kD. A common epitope of this MAb was then identified and based on results of screening a genomic expression library, they suggested that the multiple banding pattern is due, at least in part, to an epitope commonly present on many different proteins. The size of an antibody epitope is still unclear. Studies with synthetic peptides suggest that the average size is approximately 4 to 5 amino acids (Geysen *et al.*, 1988) and 5 to 6 amino acids (Cheng *et al.*, 1991), while X-ray crystallography of protein antigen-antibody complexes suggests a much bigger size (Amit *et al.*, 1986).

Acknowledgements

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