WESTERN BLOT ANALYSIS OF POLYCLONAL ANTIBODIES FROM EXPERIMENTAL ANIMALS AGAINST BLASTOCYSTIS ANTIGENS

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Abstract: One (1) anti-Blastocestis serum from a monkey naturally infected with isolate M12 and four (4) hyperimmune sera raised in inbred Balb/c mice against crude antigens of two Blastocystis isolates (C and KPI), one each of Entamoeba histolytica (HK9) and Giardia lamblia (7404) were used to react with several homologous and heterologous Blastocystis isolates, E. histolytica, G. lamblia, Endolimax nana and Bac-4 (Escherichia coli) isolated from culture medium of a B. hominis isolate KPI1. All anti-Blastocystis sera did not show cross-reactivity with E. histolytica and G. lamblia by western blotting. Similarly, anti-E. histolytica and anti-G. lamblia sera also did not react against all Blastocystis isolates tested, even though these three protozoa are known to produce diarrhea in humans. Polyclonal sera raised against antigen prepared from xenic culture of Blastocystis produced a smear reaction on the immunoblot, while antibodies raised against antigens prepared from axenic culture (isolate C) gave prominent reaction bands. This may be due to the purity of the immunogen used in inducing the immune response. The cross-reactions of sera from mice immunised with the xenic B. hominis isolates may also due to antibodies against E. coli. Anti-Blastocystis serum from monkey’s with natural infection showed several prominent reaction bands together with a smear at above 40 kD were most probably induced by the excretory-secretory antigens of the parasite. A variety of reaction patterns were obtained with these anti-sera and the antigens from different Blastocystis isolates. These may be reflects from differences in antigenic components from various strains of this parasite. (JUMMEC 2002; 2:142-146)

KEYWORDS: Blastocystis, polyclonal antibodies, immunoblot, experimental animals

Introduction

B. hominis is often found together with other parasitic protozoa (mainly Entamoeba histolytica and Giardia lamblia) and produce similar symptoms (1) but single infections are also seen. In healthy individuals, B. hominis infection is often seen without symptoms or associated with diarrhea, which is usually self-limiting. However, many cases of recurrent diarrhoea associated with B. hominis resolve only on elimination of the parasite after anti-parasitic treatment with metronidazole (2), ketocconazole (3), iodoquinol, quinacrine and paromomycin (4). B. hominis is emerging, as a potential pathogen, and further investigations of this ubiquitous protozoa is warranted (5).

Immunological studies using ELISA and immunoblot analyses against sera from immunized mice were reported by (6) who showed that there is no cross-reactivity between B. hominis and other protozoa. In this study, hyperimmune mouse sera raised against Blastocystis isolates (immunized mice and infected monkey) were used in Western blotting experiments for identification, characterization and study of the antigenic relationship between several homologous and heterologous Blastocystis isolates and other protozoa.

Materials and Methods

Organism isolates

In all, 14 isolates of Blastocystis; one isolate of Escherichia coli (Bac-4), a common bacteria obtained from the culture medium of Blastocystis isolate KPI, and each one isolate of Entamoeba histolytica (HK9), Giardia lamblia (7404) and Endolimax nana (EN1). The Blastocystis in-
cluded 11 isolates (H2, H4, H6, H7, 6105, 10203(1), 27805(1), KP1, YS1, DJ1, RN) were in xenic culture after isolation from Malaysian; 2 axenic isolates (C, H) supplied by the National University of Singapore; and 1 xenic isolate Blastocystis (M12) obtained from soft stool of a monkey kept in the Institute for Medical Research animal house. Isolates KP1, YS1, DJ1, RN were obtained from Malaysian patients with diarrhoea; while isolates H2, H4, H6, H7, 6105, 27805(1), 10203(1) were isolated from healthy Malaysian Aborigines (Orang Asli).

**Preparation of crude antigens from protozoan parasite**

The antigens were prepared as previously reported (7). The antigen concentration was estimated by BioRed assay. The concentrations of antigen used in immunization and Western blotting assay were adjusted to 50-100 mg/ml and 150-250 mg/ml respectively.

**Immunization and collection of sera from mice**

Four to eight weeks old mice with negative protozoa in stool were used in this study. The pre-immunization sera were obtained from the tail. Antigens of *B. hominis* isolates (C and KP1), *E. histolytica* (isoantigen HK9) and *G. lamblia* (isolate 7404) were used to immunize inbred Balb/C (10 mice were immunized with each isolate). Each mouse was immunized with three doses of antigens subcutaneously at 2-weekly intervals, each dose consisting of 50-100 µg/ml antigens emulsified with Freund’s Complete Adjuvant (FCA). Two weeks after the third immunization dose, the mice were anesthetized with diethyl ether and blood samples were collected by intracardiac puncture. The blood specimens were then transferred to eppendorf tubes and kept at room temperature for 30 minutes to one hour, followed by centrifugation at 4000 rpm for 15 minutes at 4°C. Sera from 10 mice were pooled in one tube, aliquoted in new eppendorf tubes and stored at -20°C until use.

**Collection of serum from a monkey with blastocystosis**

Anti-M12 serum was collected from a monkey naturally infected with *Blastocystis* (isolate M12), while kept in the animal house at the Institute for Medical Research. Blood sample from this monkey was obtained by vane-section and the blood was allowed to clot at room temperature. The serum was separated by centrifugation, aliquoted and kept as above.

**Analysis of Polyclonal Antibodies by Western Blotting**

The western blotting technique used was as previously described (8) using the above collected antigens and animals sera.

**Results**

**Reactivity patterns of sera from immunized inbred Balb/c mice**

Immunized mice sera against *B. hominis*, isolates C (anti-C), showed four reacted patterns against the antigens of all *Blastocystis* isolates tested (Fig. 1). The first pattern is that against isolate C (lane 1); the second pattern against isolate H (lane 2); the third pattern against isolates H2, H4, H6, H7, 6105, INDO (lane 3-8); and the fourth against 27805(1), KP1, RN, YS1, 10203(1), M12 (lane 9-14). Recognition bands against Bac-A were at 26.6, 38.40 kD with a weak smear at high molecular weight (lane 17). Recognition bands appeared in the first pattern were of 18, 36.5, 42.7, 55.6, 66.4 and 68 kD with a striking smear at 36.5 and 66.4 kD. Reaction bands in the second pattern were at 15.5, 23, 26.6, 32, 36, 40, 48, 55.6, 60, 64 kD with a smear from 36 and 68 kD. Those in third pattern were at 23.5, 26.6 and 38 kD: and for the fourth were at 23.5, 26.6, 38, 48, 64.4 and 68 kD. There was an extra major band of 55.6 kD in isolate M12 distinguishing this isolate from the other *Blastocystis* isolates. No reaction against *E. histolytica*, HK9 (lane 15) and G. lamblia, 7404 (lane 16).

![Figure 1. Immunoblot patterns of SDS-PAGE separated polypeptides of Blastocystis and other organisms on analysis with mouse sera immunized against isolate C antigen. Standard protein marker (lane m). Blastocystis isolates C (lane 1), H (lane 2), H2 (lane 3), H4 (lane 4), H6 (lane 5), H7 (lane 6), 6105 (lane 7), INDO (lane 8), 27805(1) (lane 9), KP1 (lane 10), RN (lane 11), YS1 (lane 12), 10203(1) (lane 13), M12 (lane 14). E. histolytica isolate HK9 (lane 15). G. lamblia isolate 7404 (lane 16). E. coli isolate Bac-A (lane 17).](image)

Hypermune sera raised against *B. hominis* isolate-KP1 (Fig. 2), showed reactivity against all *Blastocystis* isolates (lane 1-10) tested, E. *nana* (lane 13) and Bac-A (lane 14) but did not react with *E. histolytica*, HK9 (lane 11) and *G. lamblia*, 7404 (lane 12). The reactivity was seen as a striking/weak smear or with several prominent bands. The antigens from isolates H2, H4, 6105, 7106, 10203(1) were excluded in this study. The reactivity consisted of
Figure 2. Immunoblot patterns of SDS-PAGE separated polypeptides of Blastocystis and other organisms on reaction with polyclonal mouse antiserum against isolate KPI antigens. Standard protein marker (lane m), Blastocystis isolates C (lane 1), H (lane 2), H6 (lane 3), H7 (lane 4), KPI1 (lane 5), Y51 (lane 6), DJ1 (lane 7), RN (lane 8), 27B051 (lane 9), M12 (lane 10), E. histolytica isolate HK9 (lane 11), G. lamblia isolate 7404 (lane 12), E. nana isolate EN1 (lane 13), E. coli isolate Bac-4 (lane 14).

Figure 3. Immunoblot patterns of SDS-PAGE separated polypeptides of Blastocystis and other organisms on reaction with polyclonal mouse antiserum against E. histolytica isolate HK9 antigens. Standard protein marker (lane m), no reaction pattern against Blastocystis isolates C (lane 1) (similar result when reacted with H, H2, H4, H6, H7, 6105, INDO, 27B051, KPI, RN, Y51, 102031 and M12), E. histolytica isolate HK9 (lane 2), G. lamblia isolate 7404 (lane 3), E. nana isolate EN1 (lane 4), E. coli isolate Bac-4 (lane 5).

Figure 4. Immunoblot patterns of SDS-PAGE separated polypeptides of Blastocystis and other organisms on reaction with polyclonal mouse antiserum against G. lamblia isolate 7404 antigens. Standard protein marker (lane m), no reaction pattern against Blastocystis isolates C (lane 1) (similar result when reacted with H, H2, H4, H6, H7, 6105, INDO, 27B051, KPI, RN, Y51, 102031 and M12), E. histolytica isolate HK9 (lane 2), G. lamblia isolate 7404 (lane 3), E. nana isolate EN1 (lane 4), E. coli isolate Bac-4 (lane 5).

Figure 5. Immunoblot patterns of SDS-PAGE separated polypeptides of Blastocystis and other organisms on reaction with serum from a monkey with Blastocystis (isolate M12) infection. Standard protein marker (lane m), Blastocystis isolates C (lane 1), H (lane 2), H6 (lane 3), H7 (lane 4), KPI1 (lane 5), Y51 (lane 6), DJ1 (lane 7), RN (lane 8), 27B051 (lane 9), M12 (lane 10), E. histolytica isolate HK9 (lane 11), G. lamblia isolate 7404 (lane 12), E. nana isolate EN1 (lane 13), E. coli isolate Bac-4 (lane 14).
a striking smear at 26.6 to 32 kD against Blastocystis isolates C, H, H6, KPI, YS1, DJ1, RN, 27B05(1), M12 as well as E. nana and Bac-4. Another striking smear was present between 48 to 66.4 kD against isolates C and H (lane 1 and 2) as well as against E. nana and Bac-4. Several prominent bands at 36.5 and 48 kD were present against H6 (lane 3); 31 and 36.5 kD against H7 (lane 4); 38 kD against KPI, DJ1, RN, 27B05(1), M12 (lane 5, 7, 8, 9, 10), E. nana and Bac-4. Isolates RN, 27B05(1) and M12 (lane 8, 9 and 10) also showed a weak smear between 40 and 212 kD.

Mouse anti-E. histolytica sera showed major bands at molecular weight 38, 50, 52 kD and minor bands at 34 and 39 kD against E. histolytica (HK9) antigens (lane 2). The sera did not show reactivity against all Blastocystis isolates (lane 1), G. lambia (lane 3), E. nana (lane 4) and Bac-4 (lane 5) antigens tested (Fig. 3).

Mouse anti-G. lambia sera showed reactivity against G. lambia antigen at molecular weight 32, 33, 53, 57 and 66.4 kD (lane 3). No reaction was seen against all Blastocystis isolates (lane 1), E. histolytica (lane 2), E. nana (lane 4), and Bac-4 (lane 5) antigens tested (Fig. 4).

Reactivity patterns of serum from monkey with Blastocystis natural infection

This serum reacted with all Blastocystis isolates tested (lane 1-10), E. nana (lane 13) and Bac-4 (lane 14), but did not react against E. histolytica, HK9 (lane 11) and G. lambia, 7404 (lane 12) antigens. Reactivity against isolates C and H (lane 1 and 2) showed different patterns from the other antigens tested. Anti-M12 produced three major bands at 42.7, 55.6 and 97.2 kD, with a smear between 36.5 and 116 kD and a minor band at around 14.3 kD against isolate C (lane 1); and a smear between 42.7 and 66.4 kD against isolate H (lane 2). Reactivity against local Blastocystis isolates (H6, H7, KPI, YS1, DJ1, RN, 27B05(1) and M12) (lane 3-10) was similar. They showed a smear pattern from 33.5 kD and above. The major recognition bands were at 55.6 kD against YS1 (lane 6) and M12 (lane 10); 158 kD against M12; 20 and 35.5 kD against DJ1 (lane 7); 212 kD against DJ1 and 27B05(1) (lane 9); 6.5, 42.7 and 55.6 kD against E. nana (lane 13); and 25, 26.6, 30 and 55.6 kD against Bac-4 (lane 14). They also showed many other minor bands (Fig. 5).

Discussion

In our previous SDS-PAGE analysis, several Blastocystis isolates, E.histolytica (HK9) and G. lambia (7404) showed at least 15 polypeptide bands while E. nana (EN1) showed only 4 polypeptide bands ranging from 6.5 to 212 kD. Among these protozoa, several polypeptide bands showed similar molecular weight on SDS-PAGE but gave different polypeptide patterns on immunoblot analysis (7). In this study, immunoblot analyses with polyclonal antibodies (sera) showed that there is no cross-reactivity between the isolates of Blastocystis, E. histolytica and G. lambia even though these three protozoa are known to produce diarrhoea in humans. The result concurs with the report by (6) and (7).

Cross-reactivity against all Blastocystis isolates tested by polyclonal anti-C was showed as clear-cut bands, while polyclonal anti-KPI gave a smear reaction. This may due to the purity of antigens used in mice immunization; antigen C was prepared from axenic culture while antigen KPI was from xenic cultures. The differences in polypeptide patterns between the axenic and xenic isolates are probably due to E. coli polypeptides (7). Cross-reactivity between the isolates of Blastocystis and E. nana could be seen in both anti-C and anti-KPI sera but the reactivity patterns were obviously different. Results also showed that cross-reactivity occurred among isolates of Blastocystis; some showed similar cross-reaction patterns, but there were obvious differences in reaction patterns. These patterns are clearly seen with polyclonal anti-C (Fig. 1), which gave four types of patterns; first given with isolate C, second with isolate H, third with isolates H2, H4, H6, H7, 610S; and fourth with isolates 27B05(1), KPI, RN, YS1, 10203(1), M12. These could be due to biochemical differences among different strains of B. hominis as previously reported (9, 10).

In this immunoblot study, the reaction pattern type-4 was shown with antigen from Blastocystis isolates KPI, RN, YS1 (from patients with diarrhoea), M12 (from a monkey’s mushy stool) and isolates 27B05(1), 10203(1) (from aborigines, Orang Asli). In other report, all these Blastocystis isolates produced a PCR product at 280 bp, which was suspected to be one of the markers of pathogenic Blastocystis (8). Therefore, if this suspected is true then the pathogenic protein produce by the pathogenic Blastocystis could be in the range from 48.0 kD and above.

Anti-serum from infected monkey (M12) showed reaction with all the Blastocystis isolates tested. This showed that there is immune response against Blastocystis in the infected monkey. As with sera from human-Blastocystis natural infection (7), this monkey’s serum (anti-M12) showed prominent smears reaction together with several clear-cut bands at above 40 kD. We suspect that these reactions at above 40 kD were most probably induced by the excretory-secretory antigens of the parasite. The excretory-secretory antigens are believed to be associated with capsule formation through their continuous accumulation on the surface of the parasite (11).
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References