SDS-PAGE and immunological analysis of different axenic *Blastocystis hominis* strains

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Summary. Consistent major differences were detected by SDS-PAGE, Western blotting and Ouchterlony immunodiffusion in four axenic and microscopically indistinguishable strains of the anaerobic human parasite *Blastocystis hominis* from different sources. It is concluded that at least two variants with different polypeptide patterns and antigens exist and the biological significance of these findings is discussed.

Introduction

There is no general agreement on the taxonomic position and the pathogenic potential of the anaerobic, eukaryotic parasite *Blastocystis hominis*.^{1–3} Early studies reported its association with diarrhoea,^{4–6} but this is still the subject of debate.^{2,7–9} Since Zierdt *et al.* studied the organism and assigned it to the protozoa,¹⁰ several reports, with increasing frequency, have been published proposing that *B. hominis* is a cause of diarrhoea and abdominal discomfort in man^{11–19} especially in immunocompromised patients.^{20,21} However, the organism is also found in healthy people.^{22,23}

After the application of axenic cultivation methods by Zierdt and Williams,²⁴ *B. hominis* has been described almost completely in morphological terms by several groups of workers.^{3,19–26} Because the development of a direct immunofluorescence assay provided evidence for the existence of serologically different strains (unpublished observations), the present study was performed to analyse polypeptides and antigens of axenic strains of *B. hominis* from different sources with corresponding rabbit antisera.

Materials and methods

B. hominis strains

Strains 1, 2 and 4 were faecal isolates from three patients living in the area of Braunschweig, Germany, and suffering from diarrhoea; strain 5 was from a healthy person with no enteritic symptoms. Despite routine testing for enteropathogens such as *Campylobacter*, *Salmonella* and *Shigella* spp., *Staphylococcus aureus*, *Yersinia enterocolitica*, protozoa, rotavirus and enteropathogenic *Escherichia coli*, as well as for

organisms of questionable pathogenic significance such as *Candida albicans* and *Proteus mirabilis*, no other potentially pathogenic micro-organisms were detected in any of the faecal specimens.

Culture for *B. hominis*

Cultures were incubated on the day that the faecal samples were received; 100 µl of liquid stool from diarrhoeic samples and 100 mg of the normal sample from the healthy person were inoculated into culture medium which had been kept anaerobic in the O₂-free atmosphere of a Gas-Pak jar system (BBL Microbiology Systems) at 36 ± 1 °C for one week before use.²⁴ The cultures were incubated anaerobically for 5 days. *B. hominis* grew as a sediment at the base of the slant of culture medium. For subcultures, half of the cellular sediment after 5 days was transferred to new pre-incubated medium.

Culture medium

Locke's solution, composed of NaCl 8.0 g, CaCl₂ 0.2 g, KCl 0.2 g, MgCl₂ 0.01 g, Na₂HPO₄ · 12 H₂O 2.0 g, NaHCO₃ 0.4 g, KH₂PO₄ 0.3 g in 1 L, was autoclaved at 121 °C for 15 min. After cooling to room temperature it was filtered to remove the precipitate. One part whole beaten egg was added to 3.6 parts (v/v) of Locke's solution; the mixture was filtered through gauze and 2.5-ml portions were pipetted into 12 × 35 mm tubes and inspissated at a 30° slant for 3 h at 80 °C. After cooling, slopes were overlaid with 6 ml of Locke's solution containing sterile rabbit serum 25% v/v and covered with 3.5 ml of mineral oil (Merck 7174).³

Microscopic examination

Before transfer, at the end of a subculture period, a 20-µl sample of culture sediment was examined with a

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Zeiss Axioskope with 100 $\times$ /1.30 Plan-Neofluar oil immersion objectives. The size, shape and number of *B. hominis* cells per examination field were recorded routinely for all strains according to the classification scheme described by Zierdt.²⁷ In all strains, predominantly vacuolated cells of 10–20 μ m diameter, dividing by binary fission, were present in the axenic cultures; granular cells or amoeboid forms were very rare. One third to one half of the total number of cells usually contained intracellular inclusions.

Axenisation

Subcultures were made by transferring half of the cellular sediment in 1.5 ml of supernate to a new pre-incubated tube. Subcultures were incubated for 5 days before the new transfer. Extracellular bacteria were eliminated in the presence of ampicillin 4 g/L, streptomycin 1 g/L and colistin 1.5–4.0 mg/L after 8–10 transfers.²⁴ Cultures that appeared to contain no extracellular bacteria after microscopic examination were checked for microbial growth by subculture on sheep-blood agar plates incubated at 36°C for 3 days in aerobic and anaerobic conditions. Those cultures without microbial growth in these conditions were designated as axenic. To minimise the production of antibodies directed against components of the culture medium, rabbit serum 25% v/v was used for cultivation. The axenic cultures were grown for 3–6 months before immunisation.

Preparation of antisera

Antigenic materials were handled under sterile conditions. The sediments of four parallel axenic cultures of strains 1 and 2 were pooled and pelleted by centrifugation (Hettich-Mikroliter centrifuge, type 2025) for 5 min. The supernates were discarded, and the pellets were washed twice with 0.5 ml of FTA-buffer (pH 7.2) consisting of 10 mM Na₂HPO₄·2H₂O, 1.5 mM KH₂PO₄ and 120 mM NaCl. Then the cells from each strain were suspended in 2.0 ml of FTA-buffer. The suspensions were checked again for microbial growth by culture of a sample on sheep-blood agar. No microbial contamination was detected in any case. The cells were killed by heat treatment (65°C, 30 min) and the stock suspensions were divided into 0.1-ml volumes and kept frozen at –20°C. For immunisation, 0.1 ml of a stock suspension was diluted with 0.9 ml of FTA to give a turbidity reading of McFarland standard No. 2. These suspensions were each mixed with 1.5 ml of incomplete Freund's adjuvant (Sigma F5506) as a water-in-oil-emulsion and 1.5 ml of this emulsion was injected into skeletal muscles of 4-month-old male rabbits at weekly intervals. The animals were bled after 9 weeks; the sera were collected and kept frozen at –20°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Axenic culture sediments were collected with a pasteur pipette, pelleted by centrifugation and washed twice in FTA. Proteins of *B. hominis* were extracted by boiling one washed pellet in 40 μ l of the sample buffer (glycerol 10% v/v, SDS 1% w/v, 0.001 M edetic acid, β -mercaptoethanol 2% v/v, Bromophenol blue 0.05% w/v in double-distilled water) for 10 min. After centrifugation and cooling, another 2% v/v of β -mercaptoethanol were added to prevent rearrangement of disulphide bridges. A 40- μ l sample containing c. 20 μ g of protein was electrophoresed in polyacrylamide 8% or 10% gels (stacking gels 3.5%) according to the method of Laemmli.²⁸ Gels were 1.5 mm thick and the apparatus GE-2/4 (Pharmacia, Uppsala, Sweden) was equipped with gel cassettes 18 $\times$ 14 cm and a power supply ECPS 3000/150. Gels were run, with cooling, for 1.5 h at 80V and 15 mA (stacking gels) and for 4 h at 300 V and 10 mA (separation gels). They were calibrated with Sigma MW-SDS-70-L and MW-SDS-200 mol.-wt marker kits. Gels were stained by gentle agitation at room temperature overnight in a mixture of methanol 150 ml, 100% w/v trichloroacetic acid 100 ml, Coomassie Brilliant Blue R 250 0.6 g in 560 ml of double-distilled water. Gels were destained in a 50/50/7 v/v/v mixture of water, methanol and acetic acid for 6 h, changing the solution five times.

Western blots

Proteins were transferred from the gels to a PVDF membrane (Immobilon 0.22 μ m; Millipore IPVH 000 10) by established methods²⁹ with a Pharmacia GD-4 II device. The transfer buffer ($\times$ 5) consisted of 0.125 M tris(hydroxymethyl)-aminomethane (Merck 8382), 0.96 M glycine (Merck 4201) and 0.5 M NaCl, pH 8.3; 200 ml of transfer buffer concentrate, 100 ml of methanol and 700 ml of double-distilled water were mixed prior to use. After protein transfer, the remaining gel was stained with Coomassie Blue as described above. The blots were washed for 5 min in transfer buffer and then incubated for 1 h in blocking solution (PBS with 10 mM potassium phosphate, 130 mM NaCl, pH 7.4, + Tween 20 0.05% v/v and defatted milk powder 5% w/v). Then whole rabbit antiserum at final dilutions of 1 in 100 and 1 in 66 was added, and the mixture was incubated overnight at room temperature with gentle agitation. The next morning, the blots were washed four times, for 5 min each, in PBS-buffer and then incubated for 1 h with peroxidase-conjugated second antibody (anti-rabbit IgG from goat, Sigma 0545) at a final dilution of 1 in 1000 in blocking solution. The immunoblots were then washed four times, for 5 min each, with PBS-buffer and developed in a solution of 4-chloro-1-naphthol (Sigma C 8890) 0.5 mg/ml in a mixture of PBS 80 ml and mercaptoethanol (MeOH) 16 ml after addition of

0.1 ml of H_2O_2 . After development, the blots were washed extensively with PBS-buffer and photographed. Photographs were taken with an Olympus OM.2 fitted with a macro-objective OM 50 mm and Agfa PAN 25 film.

Immunodiffusion experiments

These were done by the method of Ouchterlony³⁰ with antisera directed against strains 1 and 2. The axenic cellular material (100 μ l) from the *Blastocystis* strains was suspended in 300 μ l of FTA and sonicated for 40 s ("high 278") with a Labsonic 2000 sonicator (B. Braun, Melsungen, Germany). Measurements of OD₂₈₀ indicated, for all sonicated strains, protein concentrations in the range 1.0–1.2 mg/ml. The diffusion holes were filled with 50- μ l portions of the sonicated material. Diffusion was for 12 days with 100 μ l of the undiluted rabbit antisera 1 and 2 at 4°C.

Results

SDS-PAGE of axenic culture sediments from strains 1 and 2 indicated that both polypeptide patterns were related, but not completely identical (fig. 1). Based on these apparently different patterns, and on evidence of serologically different strains provided by the direct immunofluorescence assay (unpublished observation), a more detailed immunological investigation was performed with rabbit antisera raised against axenic cell suspensions from strains 1 and 2.

Western blot analysis revealed that the antiserum raised against axenic cells from strain 1 reacted with protein components from strain 1 at working dilutions of 1 in 100, but only a very weak cross-reaction, yielding no discrete bands, was observed with protein

extracts derived from strain 2 (fig. 2A), indicating that strains 1 and 2 were immunologically different. In conformity experiments, a protein extract from strain 2 was found to react with antiserum obtained by immunisation with a cell suspension from strain 2 at a working dilution of 1 in 66; again, only a very weak cross-reaction was observed with a protein extract from strain 1 (fig. 2B).

Further immunological evidence for the existence of two different strains was provided by the results of Ouchterlony immunodiffusion. A precipitin band was observed with sonicated cells from strain 1 and antiserum raised against strain 1; no precipitin band was detected with sonicated material from strain 2. Immunodiffusion experiments with antiserum directed against strain 2 yielded a precipitin band with cells from strain 2 but not with material from strain 1 (fig. 3).

Analysis of two further axenic *B. hominis* strains (4 and 5) indicated that the protein pattern of strain 1 corresponded to that of strain 5, and the pattern of strain 2 corresponded to that of strain 4. Strain 4 gave the same immunological reactions as the cellular material from strain 2 (by both Western blot and immunodiffusion techniques), but not the same as that from strain 1. Contrary to that finding, strain 5 reacted with antiserum raised against strain 1 in the Western blot and immunodiffusion assay, but did not react with material from strain 2 (data not shown).

Reactivity against homologous material in both Western blot and immunodiffusion systems was lost after absorption of the antisera with the appropriate antigenic strains. The results of the immunological analysis are summarised in the table.

Discussion

Four axenised strains of *B. hominis* from independent sources were separated into two groups by three methods—SDS-PAGE, Western blotting and immunodiffusion. Strains 1 and 5, in one case, and strains 2 and 4 in the other, showed indistinguishable polypeptide patterns and immunological reactions. The detection of numerous bands, as was found in the case of corresponding sera and antigens by Western blots, and almost no cross-reactions except for weak, undefined smears (probably from lipid- or glycosyl-components) with heterologous sera treated by the same extraction procedure, confirms that there is no close immunological relationship between the two pairs of strains. The latter findings match completely the results of the immunodiffusion experiments. All results together lead to the conclusion that the specific name *Blastocystis hominis* describes at least two different variants.

Based on ultrastructural investigations, Dunn *et al.*¹ recently described a variation in *B. hominis* stocks from different sources in culture. Taking this into account, our conclusion that *B. hominis* includes at

Fig. 1. SDS-PAGE (8% gels) of protein extracts from axenic *B. hominis* strains 1 and 2 under reducing conditions; Coomassie Brilliant Blue staining. Mol.-wt Kda markers: trypsinogen 24, pepsin 35, ovalbumin 45, bovine serum albumin 66, phosphorylase b 97, β -galactosidase 116, myosin H-chain 205. The arrows indicate significant differences between strains.

Fig. 2. Western blots after SDS-PAGE under reducing conditions of protein extracts from axenic *B. hominis* strains 1 and 2 with antiserum against strain 1 (**A**, 8% gel) and against strain 2 (**B**, 10% gel). Secondary antibodies were anti-rabbit IgG from goat, HRPO-conjugated. For markers see legend to fig. 1, except for carbonic anhydrase 29 Kda. With homologous sera numerous bands are present, whereas with heterologous sera only barely visible cross-reaction occurred.

Fig. 3. Immunodiffusion of sonicated cellular material from strains 1 and 2 with rabbit antisera obtained by immunisation with strain 1 and strain 2. Photographs were taken after incubation for 12 days at 4°C.

least two different variants seems to be reasonable, although the relationships between the ultrastructural variations and the differences in antigen and protein patterns reported in the present study remain to be established.

In a previous study³¹ we reported that the parasites were found more frequently in stool specimens from healthy persons than in those from patients with diarrhoea and this observation was in agreement with the results of other investigators.²² Such findings were rationalised by the hypothesis that different types of *B. hominis* exhibiting the same morphology but with a different pathogenic potential might exist.³¹ Our finding that at least two biochemically and immunologically different strains exist supports this theory. However, the number of axenic strains examined is too small for a general classification following such criteria and needs to be enlarged. Further work is needed to elucidate the complex relationships in the hitherto homogeneous species *B. hominis*. Our findings

Table. Immunological reactions of four different axenic strains of *B. hominis* with two rabbit antisera in Western blots and immunodiffusion assays

Method	Rabbit antiserum	Reaction between antiserum and antigens from strains			
		1	2	4	5
Western blot	Against strain 1	Yes	Very weak	Very weak	Yes
Immunodiffusion		Yes	No	No	Yes
Western blot	Against strain 2	Very weak	Yes	Yes	Very weak
Immunodiffusion		No	Yes	Yes	No

contribute to an improved understanding of *B. hominis* infection and do not support the view that no strains of *B. hominis* play an aetiological role in enteritis.

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