POLYPEPTIDES ASSOCIATED WITH *IN VITRO* CYST FORMATION OF *BLASTOCYSTIS HOMINIS*

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Abstract. The objective of this study was to characterize the polypeptides associated with cysts of *Blastocystis hominis*. This form is believed to be infective and plays a role in parasite resistance to anti-*B. hominis* drugs currently used for treatment of *Blastocystis* associated diarrhea. Cysts were induced through *in vitro* culture of the parasite in complete medium supplemented with bacterial extract with trypticase, metronidazole or doxycycline. SDS-PAGE analysis showed almost similar polypeptide patterns of parasite extracts obtained from *in vitro* cultured parasites before and after exposure with the three supplements. Polypeptide bands at 76, 58.5, 48, 45, 40, 38, 32, 25 and 22 kDa were constantly seen in all antigenic preparations and no specific cyst-associated polypeptide was present. However, on immunoblot analysis, 3 out of 16 blastocystosis human sera identified a cyst-associated polypeptide at 60 kDa in all parasite extracts prepared from cultures with the three supplements. In addition, there were associated morphological changes detected in these parasites stained with acridine orange and observed under fluorescence microscopy. Metronidazole induced cyst forms (reddish cells) as early as 12 hours post-exposure; more cyst production (with stronger immunoblot bands) occurred after 24 hours exposure. However, cysts rupture with release and destruction of *B. hominis* daughters cells occurred after 48 hours exposure. Doxycycline induced less cyst-like forms at 24 hours (weaker 60 kDa band) and less destruction of the cysts (60 kDa band still present at 72 hours post exposure). Bacterial extract and trypticase also induced cysts at 12 hours with increasing numbers up to 72 hours exposure (corresponding increase in intensity of 60 kDa band from samples harvested at 12 to 72 hours post exposure) without any sign of deleterious effect on the parasite.

INTRODUCTION

The three known forms of *Blastocystis hominis* are the vacuolar, granular and amoeboid forms. The cyst-like forms have also been observed in fresh fecal sample of an HIV-positive patient who was suffering from persistent diarrhea (Mehlhorn, 1988), stored fecal samples and in culture specimens (Stenzel and Boreham, 1991). This parasite can be induced to form cysts *in vitro* by using an encystation medium conditioned with bacterial extract and trypticase (Suresh *et al*, 1993). Vannatta *et al* (1985) and LeBar *et al* (1985) have reported that *B. hominis* reappeared in patients with recurrent diarrhea after treatment with metronidazole. The survival of this parasite after drug treatment may be the source of relapse that is frequently seen in *B. hominis* infection. An effective drug for *B. hominis* is still unavailable. The changes in protein composition during cyst formation are unknown. The SDS-PAGE and Western blotting techniques may be used to detect polypeptide changes as well as their reactive patterns with positive human sera. The information from this study may be useful for selection of effective drugs to eliminate this parasite from the human intestinal tract.

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MATERIALS AND METHODS

Axenic culture of *B. hominis*

Pre-reduced medium was produced by placing 10 ml of complete medium (IMDM + 10%

horse serum) in culture tubes in an anaerobic jar, followed by addition of a packet of dried catalyse (Oxoid). A packet of gas generating kit (Oxoid) was cut open, filled with 10 ml of distilled water and placed immediately in the anaerobic jar. The jar was closed and tightened quickly and then incubated overnight at 37ºC. The pellet (about 10 x 10^6 parasite cells/ml) of axenic *B. hominis* (isolate C) was sub-cultured in pre-reduced medium and incubated in an anaerobic jar (a packet of dried catalyse and gas generating kit was placed as above) at 37ºC.

Encystation of *B. hominis.*

Sub-culture was carried out every alternate day or when 75% of the medium in a test tube had turned yellowish in color. The parasite pellet from several tubes was collected, pooled together, parasite density counted (90-100 x 10^6 parasite cells/tube) and encysted in 10 ml pre-reduced complete medium (IMDM + 10% horse serum) supplemented with (i) 0.1 mg/ml bacterial extract with 0.01 µg/ml trypticase, (ii) 0.01 µg/ml metronidazole and (iii) 0.01 µg/ml doxycycline individually. These tubes were then incubated in an anaerobic jar at 37ºC. The parasites were collected at various intervals after exposure.

Acridine orange staining

A drop of 0.1% acridine orange solution was mixed with a drop of parasite culture before and after culture (at 0, 24, 48 and 72 hours) with 0.01 mg/ml of bacterial extract with trypticase, metronidazole and doxycycline respectively. The mixture was then visualized immediately under fluorescent microscopy and photographed for permanent record.

Harvesting and preparation of *B. hominis* antigens from encystation culture

The culture tubes were maintained at 37ºC for 0, 4, 8, 12, 16, 20, 24, 28, 72 and 96 hours. At the end of each period the respective culture tubes were taken out from the anaerobic jar. The supernatant was discarded and the pellet was transferred to a sterile new test tube followed by addition of 10 ml of sterile 0.85% NaCl, mixed and centrifuged at 2,000 rpm for 5 minutes. The parasite was washed twice with sterile 0.85% NaCl to remove horse serum. The washed pellet was resuspended in a small volume of sterile 0.85% NaCl and transferred to a sterile bijou bottle, then kept at -20ºC overnight. The suspended pellet were thawed slowly at room temperature, sonicated (at 5 rpm for 10 minutes) and kept at 4ºC overnight. The pellets were transferred to Eppendorf tubes and then centrifuged at 12,000 rpm for 15 minutes at 4ºC. The supernatant was transferred into sterile bijou bottles, aliquoted to several smaller bottles and kept at -20ºC until used.

Western blot analysis

The polypeptide patterns separated on the gel were transferred electrophoretically to nitrocellulose paper (Init *et al,* 1998). The transferred polypeptides were probed with sera from 16 patients with blastocystosis (No. 7, 8, 15, 21, 26, 31, 35, 37, 53, 56, 58, 83, 86, 89, 92 and 100) which were known to be reactive with *B. hominis,* isolate C.

RESULTS

Morphology changes after acridine orange staining

Under fluorescence microscopy, almost all the parasites appeared greenish at 0 hour (Fig 1a). There was a mixture of reddish (about 15%) and greenish (about 85%) parasites after 24 hours exposure (Fig 1b). Most of the greenish parasites contained many refractive particles. After 48 hours treatment, more parasites (about 25%) showed reddish (Fig 1c) coloration and more greenish cells with refractive bodies were seen. After 72 hours exposure (Fig 1d), many reddish cells (cysts) ruptured with the release of smaller *Blastocystis*, while most of the greenish cells with refractive bodies seemed to be destroyed. Figs 1a - 1d were from cultures after treatment with doxycycline. Similar findings were obtained after exposure to metronidazole except that there was a slight difference in the percentage of cysts (reddish cells) produced at the respective exposure times. More reddish stained parasites were produced as early as 12 hours after exposure, and parasites started to rupture at 48 hours exposure. Exposure with bacterial extract with trypticase also produced reddish stained parasites but the greenish stained parasites seemed to be without refractive particles. Production of reddish stained parasites (cysts) increased and greenish stained parasites decreased after 12 to 72 hours exposure.

Polypeptide pattern from SDS-PAGE

The SDS-PAGE results showed almost similar polypeptide patterns of parasite extracts pre-

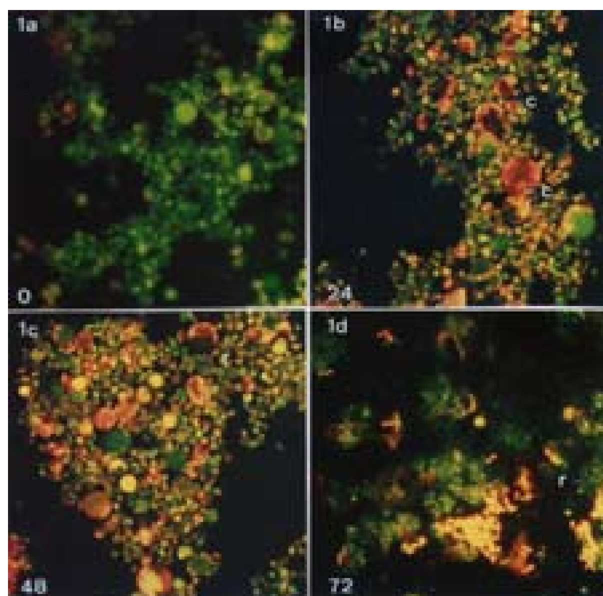

Fig 1–Acradine orange staining on *B. hominis* (isolate C) cells in complete medium supplemented with doxycycline: (a) before exposure or at 0 hour, (b) at 24 hours, (c) at 48 hours, and (d) at 72 hours exposure. Cysts and granular-like forms filled with many refractile particles shown as ‘c’ and ‘r’ respectively.

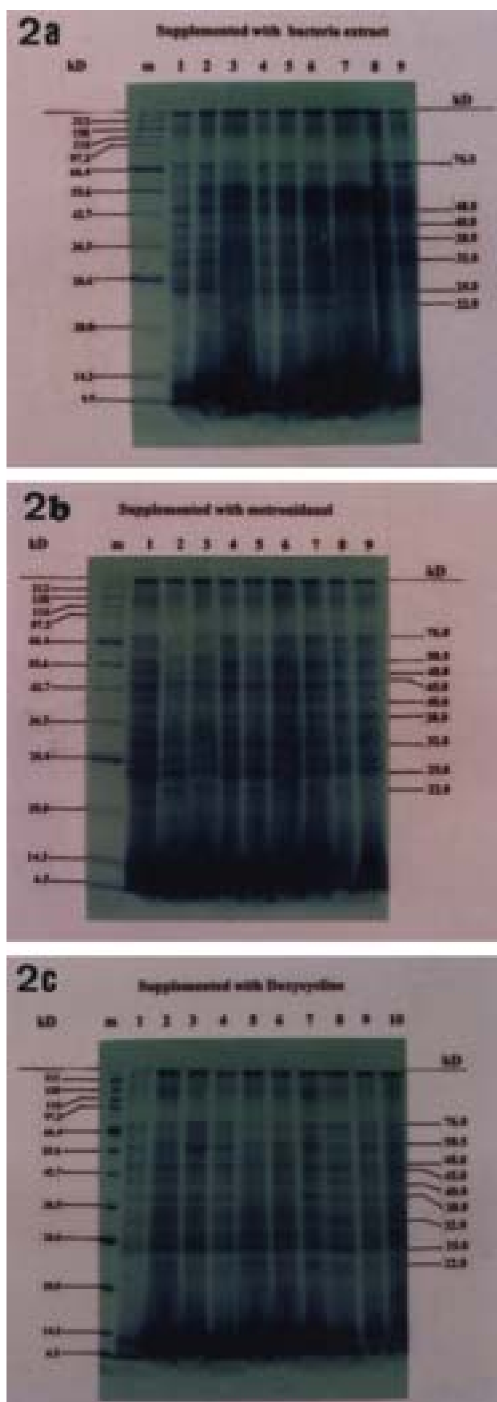

Fig 2–SDS-PAGE separated polypeptides patterns of axenic *B. hominis* (isolate C) antigens prepared before and after exposure with: (a) 0.1 mg/ml bacterial extract and 0.01 µl/ml trypticase, (b) 0.01 µl/ml metronidazol, and (c) 0.01 µl/ml doxycycline. Standard protein markers (lane m) and the antigens prepared at 0 (lane 1), 4 (lane 2), 8 (lane 3), 12 (lane 4), 16 (lane 5), 20 (lane 6), 24 (lane 7), 48 (lane 8), 72 (lane 9), and 96 hours (lane 10).

pared from cultures before and after treatment with bacterial extract (Fig 2a), metronidazole (Fig 2b) and doxycycline (Fig 2c), respectively. This pattern consisted of bands at 76, 58.5, 48, 45, 40, 38, 32, 25 and 22 kDa (those treated with bacterial extract did not show bands at 58.5 and 45 kDa). These bands were consistently seen in all parasite extracts prepared from cultures at different culture intervals (Figs 2a-2d).

Reactivity patterns of *B. hominis* antigens against human blastocystosis sera

After exposure to bacterial extract and trypticase. Three types of patterns were produced from reactivity of the 16 human sera used. Pattern type 1 was from sera No. 7, 31, 35, 86 and 89. Pattern type 2 was from sera No. 83, 53 and 100. Pattern type 3 was from sera No. 58, 8, 15, 21, 26, 37, 56 and 92 (Fig 3a). Pattern type 1 did not show any reactive pattern. Pattern type 2 showed a strong smear between 97.2 and 158 kDa against all the antigens prepared from cultures at 0, 12, 24, 48, 72 hours after exposure. These sera also showed a band at 60.0 kDa which is seen against the antigen prepared from para-

sites at 12 (weak band), 24, 48, and 72 (strong band) hours culture but did not show any reaction at 0 hour exposure. Pattern type 3 showed only one band at 158 kDa, which is weak at 0 hour and stronger at 12, 24, 48 and 72 hours exposure.

After exposure to metronidazole. Four patterns were produced from the 16 human sera used. Pattern type 1 was from sera No. 89, 86, 35, 31 and 7. Pattern type 2 was from sera No. 92, 37, 26 and 8. Pattern type 3 was obtained with sera No. 83, 53 and 100. Pattern type 4 was seen with sera No. 15, 21, 56 and 58 (Fig 3b). Pattern type 1 did not show any reaction. Pattern type 2 produced a smear at about 97.2 and 158 kDa against all the antigens tested except that at 72 hours exposure. Pattern type 3 produced a prominent smear around 158 kDa together with a weak smear from 42.7 to 116 kDa. There was also an extra band at 60 kDa which was prominent with antigen prepared at 12 and 24 hours, but weak against the antigen prepared at 48 hours, and no reaction against the antigens prepared at 0 and 72 hours exposure. Pattern type 4 showed a prominent smear reaction around 158 kDa against all antigens tested.

After exposure to doxycycline. Four (4) types of pattern were produced from 16 human sera used. Pattern type 1 was from sera No. 7, 31, 35, 86 and 89. Pattern type 2 was from sera No. 92, 8, 15, 21, 26, 37 and 58. Pattern type 3 was from sera No. 53, 83 and 100. Pattern type 4 revealed from serum No 56 (Fig 3c). Pattern type 1 did not show any reaction against all antigens tested. Pattern type 2 produced a band at 158 kDa against all antigens prepared at 0, 12, 24, 48 and 72 hours exposure. Pattern type 3 produced a smear between 92.7 to 158 kDa against all the antigens tested, and a band at 60 kDa against the antigens prepared at 24 and 48 hours exposure. The 60 kDa band was not obtained when these three sera were reacted with the antigen prepared at 0 and 12 hours exposure. In addition, when reacted with the antigen prepared at 72 hours, sera No. 83 and 100

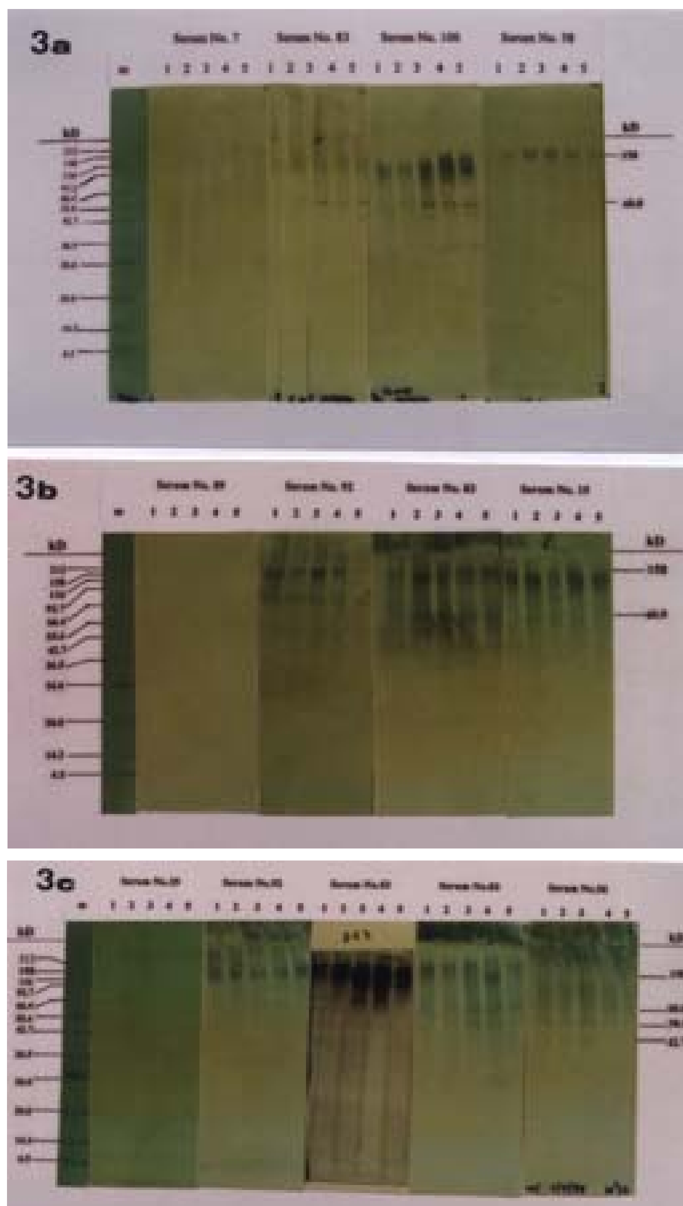

Fig 3–Immunoblotting patterns revealed by the 12 human sera against axenic *B. hominis* (isolate C) antigens prepared before and after exposure with (a) 0.1 mg/ml bacterial extract and 0.01 µl/ml trypticase (the sera shown were No. 7, 83, 100 and 58), (b) 0.01 µl/ml metronidazole (the sera shown were No.89, 92, 83 and 15), and (c) 0.01 µl/ml doxycycline (the sera shown were No.35, 92, 53, 83 and 56). Standard protein markers (lane m) and the antigens prepared at 0 (lane 1), 12 (lane 2), 24 (lane 3), 48 (lane 4), 72 hours (lane 5).

showed a weak band at 60 kDa but serum No. 53 failed to give this band. Pattern type 4 produced a weak smear at 58.5 to 212 kDa, and a weak band at 42.7 kDa with antigen prepared at 24 hours exposure.

DISCUSSION

The cystic stage of *B. hominis* has attracted interest since Stenzel and Boreham (1991) reported its detection in fresh feces of a patient. The cyst stage is probably formed in response to adverse environmental conditions and may be involved in transmission. It will be extremely interesting to see if there are associated changes in antigenic profile as the parasite modifies itself and prepares for transmission. In this study we assessed the changes if any, in polypeptides produced in *B. hominis* during *in vitro* culture to induce cyst formation through the addition of potential inducers of cyst formation, these being bacterial extract with trypticase, metronidazole and doxycycline. The axenic *B. hominis* (isolate C) was used for the study to induce cyst formation. Parasite morphology was observed after 0, 24, 48 and 72 hours while the antigens were prepared after incubation periods of 0, 4, 8, 12, 16, 20, 24, 48, 72 and 96 hours in complete IMDM medium conditioned with the three components listed above.

Both metronidazole and doxycycline showed effects on the *B. hominis* morphological forms. This could be detected through morphological changes induced in the parasite stained with acridine orange and viewed under fluorescence microscopy at 0, 12, 24, 48 and 72 hours of culture using the above conditions. Cysts were first detected at 12 hours after exposure to metronidazole and at 24 hours for doxycycline. Further incubation produced more cysts-like forms after 24 and 48 hours respectively. Finally after 48 and 72 hours of exposure to metronidazole and doxycycline respectively, the cysts ruptured exposing many of *B. hominis* daughters. Greenish appearing cells (granular-like forms) filled with many refractile particles were obviously seen after 24 hours and these almost disappeared after 72 hours exposure. This may be due to the destruction of the surface coat and thin layer cell wall, thus exposing the refractile particles after 24 hours. Following 72 hours exposure the refractile particles disappeared. In contrast, bacteria extract with trypticase could induce the cyst-like formation (at 12 hours exposure) but there was no associated destruction of the surface coat and cell wall of these greenish appearing cells. There was a corresponding increase in the cyst-like forms as well as the greenish appearing cells without refractile bodies after 72 hours exposure. Induction of cyst-like morphology after 24 hours exposure with bacterial extract with trypticase has been reported by Suresh *et al* (1993).

B. hominis reappeared in recurrent diarrhea patients after treatment with metronidazole (LeBar *et al*, 1985; Vannatta *et al*, 1985). Our results support the hypothesis that *B. hominis* cysts may be induced by metronidazole and this may be associated with the reappearance of the parasite and recurrent diarrhea after treatment with this drug. We have previously observed that doxycycline and chloroquine can also induce cyst formation (unpublished data). The production of cysts occurred in about 25% of the cells and this may reflect the ability of these surviving parasites to adapt to the adverse environmental conditions through the production of a thick protective surface coat. The rest (75%) of the other parasites were destroyed by the drugs.

The individual polypeptides separated on SDS-PAGE and visualized by Coomassie brilliant blue staining did not show any differences between extracts prepared from cultures at 0 to 96 hours under all the three culture conditions used. The common polypeptides identified in SDS-PAGE were at 76, 58.3, 48, 45, 40, 38, 32, 25 and 22 kDa, with the exception of those at 58.5 and 45 kDa which were absent in parasite prepared from cultures with bacterial extract and trypticase supplementation. This showed that SDS-PAGE analysis could not detect any specific cyst polypeptide although there were cyst-like forms seen under fluorescence microscopy of acridine orange stained parasites harvested 24 hours after exposure to the drugs. This may be due to the low concentration of cyst-associated polypeptides thus failing to produce a band on SDS-PAGE analysis. The polypeptide profile of crude extracts of a mixture of granular and vacuolar forms of *B. hominis* have been reported by other researchers (Kukoschke and Muller, 1991; Mansour *et al*, 1995; Init *et al*, 1998) but to date, there is no report on the *B. hominis* cyst polypeptide profile.

In contrast, the immunoblot assays using sera

from 16 blastocystosis patients showed some interesting results. Three (No. 53, 83 and 100) out of 16 sera produced a common reactive band at 60 kDa with parasite antigens prepared after treatment with bacterial extract with trypticase, metronidazole or doxycycline thus showing the potential of immunoblot analysis for identification of cyst-associated polypeptides. The specific polypeptide band was detected from culture extracts produced at 12 (weak band), 24, 48, and 72 (prominent band) hours after treatment with bacterial extract. This showed that the production of cysts started at 12 hours (weak band), and there was further increase in production of cysts until 72 hours of culture.

This band (60 kDa) also presents in antigens prepared from cultures at 12, 24 (strong band) and 48 (weak band) hours after treatment with metronidazole. This showed that the production of cysts started at 12 hours after treatment, and the process continuing to produce more cysts at 24 hours (strong band). After 24 hours, cysts numbers decreased (because of rupture) resulting in lower concentration of cyst polypeptide and a weak band at 48 hours. As almost all the cysts were ruptured or destroyed by metronidazole at 72 hours of culture, no band was detected in the associated antigens.

In doxycycline treatment, all the three sera showed a strong 60 kDa band at 24 and 48 hours. However at 72 hours, serum No. 53 gave no reaction and sera No 83 and 100 produced a weak 60 kDa band respectively. This may be due to the absence of cysts formation and the associated polypeptide in cultures with doxycycline after 72 hours. Another possibility could be a low level of antibodies directed against *B. hominis* cyst in serum No. 53.

The immunoblot study showed that metronidazole induced cyst formation as early as after 12 hours of exposure but destroyed cysts after 48 hours exposure. In contrast, doxycycline induced cyst production at 24 hours, and could destroyed cysts only after 72 hours exposure.

Most of the sera tested showed smear reaction at high molecular weight (158-212 kDa) against all the antigens prepared before and after exposure (0 to 72 hours) with bacterial extract and trypticase, metronidazole or doxycycline. These smear patterns probably resulted from antigens common to the granular and vacuolar forms of *B. hominis* present in *in vitro* culture. Init *et al* (1998) have also found a smear reaction at high molecular weight when they reacted separated polypeptides of the antigen of isolate C (without treatment) with several human blastocystosis sera. Furthermore, this smear reactivity was suspected to be associated with parasite excretory-secretory antigens believed to have been accumulated to form the capsule of *B. hominis* cells (Zaman *et al* 1994).

Five sera (No. 7, 31, 35, 86 and 89) did not show any reaction against the antigens prepared before or after exposure with bacterial extract and trypticase, metronidazole or doxycycline. The negative results may be due to low antibody titers, even though all the sera used in this study were found to be positive for antibodies against *B. hominis* on screening with ELISA.

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