Identification of Antigenic Targets for Immunodetection of Balamuthia mandrillaris Infection

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The free-living amoeba Balamuthia mandrillaris causes granulomatous amoebic encephalitis (GAE) in humans. Rapid identification of balamuthiasis is critical for effective therapeutic intervention and case management. In the present study we identified target antigens for the development of a serological assay for B. mandrillaris infection. We demonstrated by silver staining that protein profiles for all eight isolates of B. mandrillaris, independent of human or animal origin or geographic origin, appeared to be similar except for some minor differences, indicating the molecular homogeneity of these strains. The profiles of all isolates, which ranged from 200 to 10 kDa, were similar, with a prominent protein visible around 30 kDa; all appeared considerably different from protein profiles of the control E6 cells and Acanthamoeba castellanii and Naegleria fowleri isolates. Western blot analysis with rabbit hyperimmune serum identified the major immunodominant antigens of 25, 50, 75, and 80 kDa; positive human sera reacted strongly with proteins around 25, 40, 50, and 75 kDa. Proteins around 40 kDa detected by human serum were not recognized by hyperimmune rabbit serum. None of the target proteins were detected by uninfected control sera. Reactivities of five patients’ sera with four different isolates of B. mandrillaris (2 strains of human and 2 strains of animal origins) revealed that patients’ sera reacted slightly differently with different B. mandrillaris isolates, although major proteins of approximately 25, 50, and 75 kDa were present in all extracts.

MATERIALS AND METHODS

Isolates. Eight different B. mandrillaris isolates (five from humans, three from animals) (Table 1) were cultured on monolayers of monkey kidney (E6) cells in Eagle’s minimal essential medium (EMEM) with 10% fetal bovine serum (FBS) and 100 μg/ml gentamicin (M2414; Sigma) in Corning tissue culture flask (25 cm²) at 37°C (18, 20). Human isolates of Acanthamoeba castellanii (CDC/V042) and Naegleria fowleri (CDC/V414) were also grown axenically as described before (17).

Sera. Sera from 5 confirmed B. mandrillaris cases (Table 2) were used for Western blot examination. Serum samples from 3 healthy blood donors (S-BD-1, -2, and -3) were used as negative human controls (negativity confirmed by previous IFAs), and serum from an immunized rabbit served as a positive control (19, 20).

Antigen extraction preparation. Balamuthia cultures were harvested after they cleared the monolayer by ingesting all of the tissue culture cells. The flasks were then chilled on ice for 2 to 5 min, shaken to dislodge the amoebae, and washed 3 times in Hank’s balanced salt solution (HBBS; Gibco catalog no. 14 025, Invitrogen). Amoebae were disrupted using five cycles of freezing on dry ice and thawing in a water bath at 37°C and centrifuged at 24,000 × g for 30 min at 4°C. These amoeba samples were mixed with a solution of 9 N urea and 10% SDS (1:3) and incubated at 65°C for 15 min. The protein concentration of each extract was determined using the BCA protein assay kit (catalog no. 23225; Pierce). Protein concentrations were adjusted to obtain a total concentration of 10 μg per well for Western blotting and 3 μg per well for silver staining. A. castellanii and N. fowleri were harvested from culture vessels and washed with amoeba saline, pH 6.5, and antigen extracts were prepared, as described above.

SDS-PAGE. The antigen extracts were loaded onto a preparative polyacrylamide gel (Criterion precast [Bio-Rad catalog no. 345-0035], 4 to 20% Tris-HCl,
example, (i) a unique band at indicating the molecular homogeneity of these strains. For independent of human or animal origin or geographic origin, 10 kDa for each of the eight strains of plex, with multiple major proteins present, ranging from 200 to seen in any of the other isolates, and (ii) minor differences human isolates, B.m

Silver staining and Western blotting. Separated SDS-treated proteins were either silver stained using Silver Stain Plus (catalog no. 161-0449; Bio-Rad) or subjected to Western blot analysis. Blots were run for 1 h at a constant voltage of 100 V. The nitrocellulose membranes (Schleicher and Schuell, Inc.) were washed four times for 5 min each in phosphate-buffered saline, pH 7.2, containing 0.3% Tween 20 (PBS-T) and incubated overnight at 4°C while being rocked in sera diluted 1:100 in PBS-T with 5% nonfat dry milk. In some cases (when preparative gels were used) membranes were cut into 3-mm strips and the strips were incubated with sera, as described above. The membranes/strips were washed four times, 5 min per wash, in PBS-T and incubated at room temperature for 1 h in either peroxidase-conjugated goat antibody specific for human IgG (Biosource Intl., Camarillo, CA) at a dilution of 1:4,000 or anti-rabbit peroxidase-conjugated goat antibody specific for rabbit IgG (Biosource Intl., Camarillo, CA) diluted 1:1,000 in PBS with 0.05% Tween 20. After three washes in PBS-T and one wash in PBS without Tween, the membranes/strips were developed for 10 min in a solution containing 5 mg of 3,3'-diaminobenzidine tetrahydrochloride and 10 µl of 30% H2O2 in 100 ml of PBS, pH 7.2. The blots were then washed with distilled water and dried at room temperature.

RESULTS

Silver staining. The protein profiles of the B. mandrillaris amoeba extracts are shown in Fig. 1A. The extracts were complex, with multiple major proteins present, ranging from 200 to 10 kDa for each of the eight strains of B. mandrillaris examined. The profiles of all isolates were similar, with a prominent protein visible around 30 kDa; all appeared considerably different from profiles of the control E6 cells and Acanthamoeba and Naegleria isolates. All eight isolates of B. mandrillaris, independent of human or animal origin or geographic origin, appeared to be similar except for some minor differences, indicating the molecular homogeneity of these strains. For example, (i) a unique band at ~60 kDa found in one of the human isolates, B.m-2 (CDC:V416) (Fig. 1A, lane 3), was not seen in any of the other isolates, and (ii) minor differences were also observed in isolates B.m-2 (CDC:V416) (Fig. 1A, lane 3) and B.m-5 (CDC:V188) (Fig. 1A, lane 6), which exhibited a darkly staining protein at ~55 kDa and appeared to show an antigen at this molecular mass with Acanthamoeba. In contrast, all of the other proteins of B. mandrillaris appeared to be distinctly different from those of A. castellanii and N. fowleri.

Western blot analysis. Immunoblot analysis with a hyperimmune rabbit serum was used initially to identify antigenic proteins that may be present in the B. mandrillaris extracts (Fig. 1B and 2A). The immunoblot profiles of Balamuthia antigens showed reactivity with the same proteins that were also present in the silver-stained profile. The hyperimmune rabbit serum reacted with the polypeptides of all 8 strains of B. mandrillaris studied. However, strong reactions were seen in lanes 2, 3, 8, and 9 (all human isolates) and lane 5 (mandrill isolate), whereas lanes 4 and 7 (horse and gibbon isolates) and lane 6 (a human isolate) reacted more weakly (Fig. 1B). Major immunodominant antigens of 25, 50, 70, and 50 kDa were observed. The hyperimmune rabbit serum did not react with any proteins in the control extracts (E6 cells, used for growing Balamuthia or Acanthamoeba or Naegleria isolates).

Human sera from cases of balamuthiasis were also used to further identify antigenic proteins in the B. mandrillaris extracts (Fig. 2B and 3). The positive human serum S-1 (Fig. 2B and 3) reacted strongly with proteins around 25, 40, 50, and 75 kDa. Interestingly, proteins around 40 kDa detected by human serum were not recognized by hyperimmune rabbit serum (Fig. 1B and 2A). The immunoblot profiles of B. mandrillaris antigens showed reactivity with the same proteins that were also present in the silver-stained profile. The hyperimmune rabbit serum reacted with the polypeptides of all 8 strains of B. mandrillaris studied. However, strong reactions were seen in lanes 2, 3, 8, and 9 (all human isolates) and lane 5 (mandrill isolate), whereas lanes 4 and 7 (horse and gibbon isolates) and lane 6 (a human isolate) reacted more weakly (Fig. 1B). Major immunodominant antigens of 25, 50, 70, and 50 kDa were observed. The hyperimmune rabbit serum did not react with any proteins in the control extracts (E6 cells, used for growing Balamuthia or Acanthamoeba or Naegleria isolates).

To further investigate the diagnostic utility of the proteins, we also evaluated a panel of sera collected from B. mandrillaris-infected persons (Fig. 3). When four different isolates of B. mandrillaris, two strains of human origin (B.m-1, B.m-8) and two of animal origin (B.m-6, B.m-3), were examined using five different patient serum samples (S-1 to S-5) and two uninfected blood donors (S-BD-1 and -2), we observed no reaction with uninfected blood donors’ sera (S-BD-1 and -2), while patients’ sera reacted with various proteins ranging from 10 to 200 kDa, including strong reactions with proteins around 25 and 50 kDa (Fig. 3). Patients’ sera reacted differently with different B. mandrillaris isolates, although major proteins of approximately 25, 50, and 75 kDa were present in all extracts (Fig. 3, arrowhead). Two sera were collected from persons who survived B. mandrillaris infection (S-2 and S-4). These sera
detected very similar sets of proteins in all extracts. However, the S-4 serum reacted with a protein in the 18-kDa range (Fig. 3, arrowhead) that was present in the extracts from gibbon and horse isolates but not from the two human isolates. Some other differences were observed: serum S-5 reacted with the extracts of the two human isolates (B.m-1 and B.m-8) and produced dark-staining proteins at about 25, 50, 75, and 100 kDa. However, reactions with the two animal (gibbon and horse) isolate extracts revealed no proteins at the 25-kDa region, but reactivity was seen at the 50-, 75-, and 100-kDa regions as well as a strong band at the ~150-kDa region (Fig. 3, arrowhead). Serum S-4 reacted strongly with the antigen extracts of all isolates. The strongest reactivity was seen using the S-3 serum, which reacted with all four isolates at almost the same magnitude as the rabbit serum. All of the sera tested, except S-4 and S-5, reacted with an additional protein around 15 kDa with B.m-3 (the horse isolate). Serum S-4 reacted with the same protein around 15 kDa with B.m-1 and B.m-8 (human) isolates; S-5 reacted only with B.m-1. Based on these results it appears that human isolates were similar to each other, whereas the animal isolates were more similar to each other than to the human isolates. However, the major reactive antigens identified by the human immune sera resided overall at ~25, 50, 75, and 100 kDa.

**DISCUSSION**

SDS-PAGE is a powerful technique for the separation of proteins and thus is very helpful in comparing different isolates/strains of eukaryotic organisms. Using this technique we have shown that when probed with the hyperimmune rabbit serum, the electrophoretically separated proteins of all eight isolates of B. mandrillaris studied exhibited similar antigen patterns, confirming that all isolates, independent of geographic or host origin, are antigenically similar. Interestingly, Booton et al. (1) found no variation in the nuclear ribosomal DNA (rDNA) gene among B. mandrillaris isolates by using many of the same isolates (B.m-1 to B.m-5, B.m-7, and B.m-8) that we used in this study. Limited sequence variation in the mitochondrial 16S rDNA genes of all isolates was observed, but the range of dissimilarity was low across the entire gene, suggesting that infections caused by B. mandrillaris are due to a single species with a cosmopolitan distribution.

Similarities in the surface proteins of different B. mandrill-
laris isolates were detected by Schuster et al. (15). Using an enzyme immunoassay, they found no significant variations in the titers of a positive control serum when tested against different isolates of *Balamuthia*. Further, they found no differences whether the isolates used came from different geographic areas or whether they were isolated from different animals (humans or other mammals). Additionally, all sera tested by them (whether they came from Georgia, southern or northern California, or Australia) reacted equally well against all isolates. Hence they concluded that all *Balamuthia* isolates express a similar set of protein antigens. Using a different technique, Western blot analysis, we have confirmed the observations by Schuster et al. (15), who also observed a lack of antigenic cross-reactivity between *Balamuthia mandrillaris* and *Naegleria* and *Acanthamoeba* species. In our study, sera from both *Balamuthia*-immunized rabbit and naturally infected human sera recognized only specific proteins from *Balamuthia* and not proteins from *N. fowleri* or *Acanthamoeba* species. Kiderlen et al. (8) also found no cross-reactivity between *Acanthamoeba* and *Balamuthia* in their immunohistological studies when they used different rabbit anti-*Acanthamoeba* sera. They stated that no cross-reactivity was observed between the different rabbit antisera and heterologous amoebae used in any combination.

According to a number of articles published over the years, antibodies to the small free-living amoebae such as *Naegleria fowleri*, *Acanthamoeba* spp., and *Balamuthia* have been detected in a wide range of humans and animals in the absence of demonstrable infection (17). The presence of such antibodies in humans and animals is probably due to exposure to amoebae in the environment, as they are presumed to be ubiquitous (21). For example, Huang et al. (6), using flow cytometry, detected anti-*Balamuthia* antibodies in healthy adults, children, and even cord blood from South Australia. Some of the sera they tested had titers ranging from 1:64 to 1:256. Using an IFA, Schuster et al. (14, 16) detected antibodies to *Balamuthia* in patients hospitalized with encephalitis and found high titers of 1:128 and 1:256 only in sera that were collected from patients that were known to be infected with *B. mandrillaris*. Using an ELISA, Schuster et al. (15) confirmed their previous findings, detecting the presence of anti-*Balamuthia* antibodies in sera from patients known to be infected with *B. mandrillaris*.

Using a flow cytometry-based assay, Kiderlen et al. (9) tested 237 sera from various groups of people: German blood donors, people working in West African rainforests, and patients with atypical encephalitis, pneumonitis, visceral amoebiasis, and toxoplasmosis. Out of 59 German blood donors, 19% (11/59) had elevated titers of anti-*Balamuthia* antibodies according to a flow cytometry assay. In comparison, 92% (23/25) of persons that had been involved in a primate project in West Africa had elevated *B. mandrillaris*-specific antibody titers. Of these, 15 (50%) had very high titers, comparable to sera from proven cases of balamuthiasis. Five of the 30 West African donors were Europeans, and their sera showed only low reactivity to *Balamuthia* amoebae, within the range of German blood donors. The other 25 individuals were West Africans who belonged to traditional farming and hunting communities. In their next study Kiderlen et al. (7) studied the prevalence of *B. mandrillaris* in West Africa in a larger collection of sera (n = 192). All of the sera from the West Africans contained anti-
bodies that were well above the average level of the German reference sera. In this study *B. mandrillaris*-specific antibody levels tended to increase with age. Of the nine individuals with the highest titers, most were elderly men professing intensive outdoor activity. According to Kiderlen et al. (7), these West Africans had no indication of being infected with *Balamuthia* but may have had constant contact with antigenically related soil amoebae and therefore developed high antibody titers to *B. mandrillaris*. These authors concluded that such high titers might stem from actual infections with *Balamuthia* that were successfully overcome, indicating that not all infections with *B. mandrillaris* are lethal or equally pathogenic.

In conclusion, methods are needed for seroprevalence studies to better understand the transmission and epidemiology of *B. mandrillaris* infections. Recombinant protein antigens are needed for standardization and wider adoption of methods. In this study we identified several protein antigens that were present in detectable quantities in amoeba extracts and that can be targeted for further analysis and evaluation as serodiagnostic reagents.

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