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Note

Sensitive resolution of *Giardia lamblia* membrane antigens

EMAD W. MOHAREB*, JAMES B. HUGHES and JOHN I. BRUCE
Center for Tropical Disease, University of Lowell, Lowell, MA 01854 (U.S.A.)

Giardia lamblia is a pathogenic protozoon living in the small intestine of man and other mammals. It is recognized as one of the most common parasitic infections causing water-borne enteric disease in underdeveloped as well as developed countries[1,2]. Giardiasis is particularly common in infants and children[3], travelers[4], homosexual males[5] and hypogamaglobulinemics[6]. Infections range in effect from asymptomatic to acute disease with diarrhea, abdominal cramps, maldigestion, and flatulence.

Despite the considerable morbidity caused by this parasite, relatively little is known about the mechanism of pathogenesis and interactions of the parasite with its host. An understanding of the antigenic composition of *G. lamblia* would help in evaluating the host immune response responsible for controlling giardiasis and might reveal differences between isolates that contribute to the variation of symptoms.

Recently, the surface membrane of the *Giardia* trophozoite was suggested to contain components that could have a profound effect on the hosts' response to infection and may modulate the host–parasite interactions[7]. Previous attempts to identify specific membrane antigens resulted in some conflicting data regarding the number, molecular weights and immunogenicity of these units[7–9].

The aim of the present investigation was to optimize the electrophoretic and blotting conditions for resolving *Giardia* membrane antigens. Particular attention was focused upon the effect of reducing agents and blotting matrices on the resolution and detection of immunologically active membrane proteins.

EXPERIMENTAL

Antigen preparation

Giardia lamblia trophozoites were cultured at 37°C in bile supplemented trypticase-yeast extract-iron-serum (TYI-S-33) medium[10] with Biosate (BBL Micro-biology systems, Cockeysville, MD, U.S.A.) replacing trypticase (BBL) and yeast extract. Trophozoites were harvested in the late log phase (48 to 72 h). Briefly, cultured tubes were chilled on ice for 15 min and repeatedly inverted to dislodge trophozoites, then centrifuged at 1500 *g* for 10 min. Pellets were washed three times in phosphate-buffered saline (PBS) pH 7.4. Pellets were solubilized in a lysing buffer containing 10 m*M* Tris–HCl pH 7.4, 1 m*M* magnesium chloride, 0.25 m*M* dithiothreitol, 1 m*M* N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and 1 m*M* phenyl methyl-sulfonyl fluoride (PMSF) and 0.5% Triton X-100 in the final concentration. The mixture was

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chilled on ice for 30 min with periodic vortexing, then centrifuged at 21 000 *g* and the protein concentration of the supernatant was determined[11].

Electrophoresis and electroblotting

Electrophoresis was carried out basically according to Laemmli's procedure[12] with variable adaptations. To determine the best conditions to dissociate the proteins while maintaining their immunologic activity, samples were subjected to different digestion conditions. A 50-μg sample was either diluted with regular concentration of digestion mixture and incubated at room temperature, or in a 60°C or 100°C water bath for 3 min or with 3× digestion mixture concentration at room temperature. Several gel monomer concentrations were also tested including the homogenous 10% and 12% gels and 5–15% and 10–20% linear gradient gels. Immediately after protein stacking, 5 m*M* thioglycolic acid (TGA) was added to the cathodic chamber. Electrophoresis was performed at 160 V in 4°C. After run termination, gels were equilibrated in Tris–glycine buffer pH 8.3 and electroblotted as described by Towbin *et al.*[13] on nitrocellulose or zeta-probe membranes for 2 h at 40 V. Gelatin (2%) and 5% skim milk in PBS pH 7.4 were used respectively in the overnight blocking of the nitrocellulose and the zeta-probe membranes. Blots were then incubated with 1:500 dilution of rabbit anti-*Giardia* serum, followed by 1:1000 dilution of goat anti-rabbit horseradish peroxidase-conjugated serum (Organon Teknika, Malvern, PA, U.S.A.) and developed with 4-chloro-1-naphthol substrate (Bio-Rad Labs., Richmond, CA, U.S.A.).

RESULTS AND DISCUSSION

The results from the differential treatment of the sample before electrophoresis (Fig. 1) shows that heating in a 60°C bath resolves more immunologically active fractions compared to other conditions. Although incubating the samples in a boiling bath allows more protein to enter the gel by breaking polypeptide aggregates and binding more detergent to the antigen, our results indicate that this could be at the expense of loosing some immunologic activity. Samples kept at room temperature show one major fraction that is partially lost in the 60°C and 100°C samples. Increasing the digestion mixture concentration (sodium dodecyl sulfate–protein) has no further denaturing effect on the proteins and does not enhance the pattern.

From Fig. 2 it could be concluded that the 5–15% gradient gel promotes sharp resolution of more bands over a broader molecular weight span than the 10% gel. When the 5–15% gradient gel was compared with the 12% and the 10–20% gels (results not shown) it was found that the 5–15% gel provides a relatively better separation between bands than the other two systems.

Gels electrophoresed in the presence of TGA resolved more well-defined bands than those without it (Fig. 3). This could be explained through the reduction effect of TGA on the sulfhydryl groups of the membrane proteins. Most of the integral membrane proteins are known to have reactive sulfhydryl groups[14] and *Giardia* was recently shown to have cysteine-rich surface antigens[15]. The SH groups may be exposed to the environment through the action of detergents on the membrane preparations. Susceptibility to oxidation may induce subsequent protein aggregation. Furthermore, Triton X-100 which is commonly used to strip membrane proteins, is itself known to contain significant amounts of oxidizing agents as impurities. These reactive agents

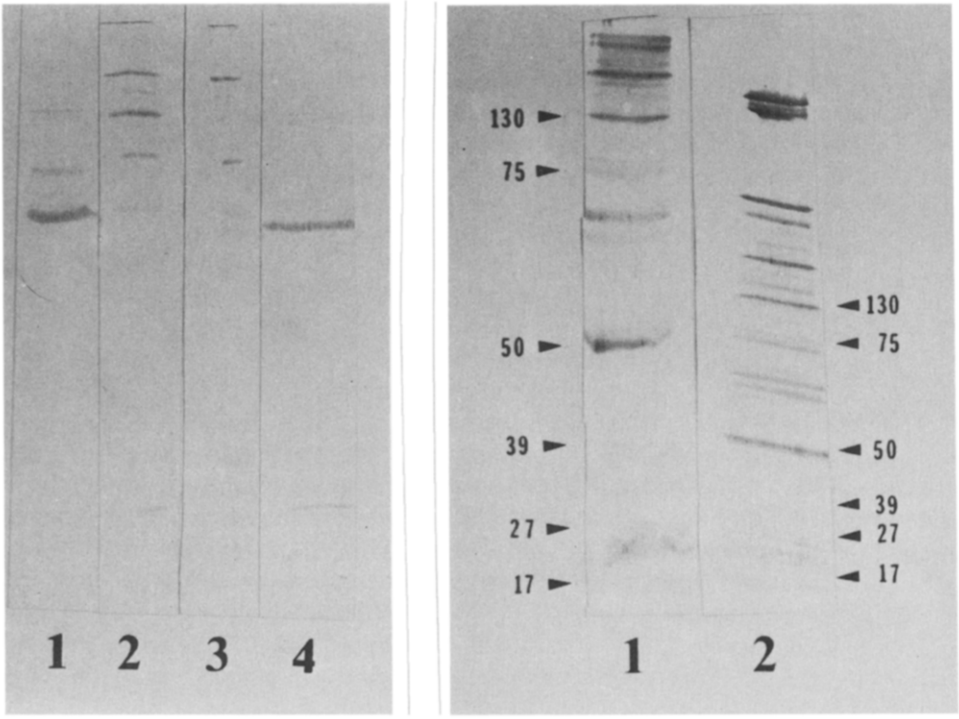

Fig. 1. 10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblot of *Giardia* samples subjected to different digestion conditions. Samples were either diluted with regular concentration of digestion mixture and incubated at room temperature (lane 1), in a 60°C water bath (lane 2), a boiling water bath (lane 3), or with 3 × digestion mixture at room temperature (lane 4).

Fig. 2. Resolution on different gel concentrations and blotting on a zeta-probe membrane (lane 1, 10% homogeneous gel blot; lane 2, 5–15% gradient gel blot). The molecular weights ($\cdot 10^3$) and the positions of standards are indicated by numbered arrows.

increase upon standing in aqueous solutions and therefore may increase the chances of intra-molecular or inter-molecular disulfide bond formation[16]. Thus, TGA presumably helps in maintaining the reduction of the integral protein sulfhydryl groups. The disappearance of some minor cross reactive fractions in the presence of TGA could be attributed to loss of some epitopic sites through the reduction of more disulfide bonds. However, several major antigens are apparently preserved and better resolved in the presence of TGA.

From the blotting experiment using different matrices (Fig. 4), it is clear that the zeta-probe membrane binds and/or retains more fractions, especially the low-molecular-weight antigens (mol.wt. $< 30 \cdot 10^3$ daltons) than did nitrocellulose. The latter matrix binds proteins primarily by hydrophobic interaction. However, Triton X-100 is known to reduce the percentage of polypeptides (especially hydrophobic) bound to nitrocellulose by up to 90%[17]. Since at least some of *Giardia* surface antigens are hydrophobic[7], this could explain why nitrocellulose is not ideal for this purpose. On the other hand zeta-probe binds proteins through the high density quaternary amine

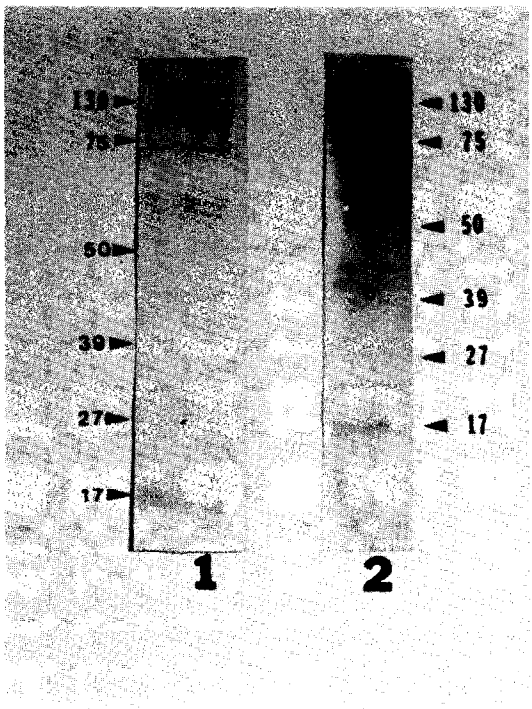

Fig. 3. 10% SDS-PAGE immunoblots for electrophoresis with TGA (lane 1) and without TGA (lane 2). The molecular weights ($\cdot 10^3$) and the positions of standards are indicated by numbered arrows.

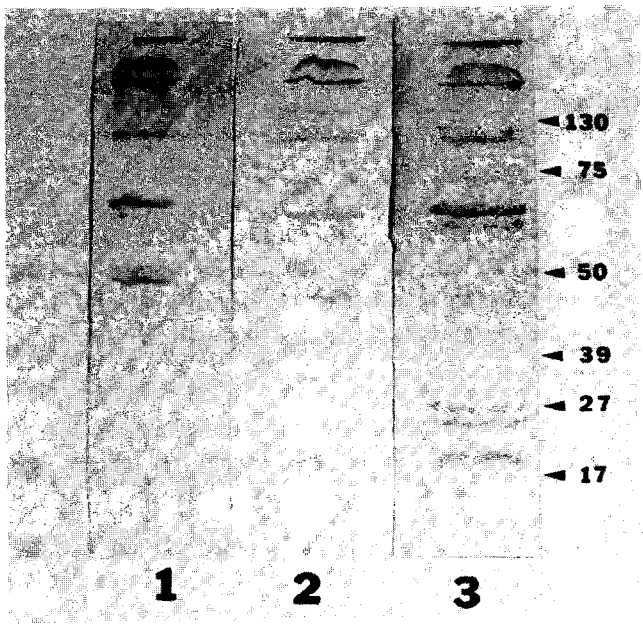

Fig. 4. Affinity of different blotting matrices to *Giardia* antigens after a 10% SDS-PAGE. Lane 1 = 0.45 u nitrocellulose; lane 2 = 0.22 u nitrocellulose; lane 3 = zeta-probe. The molecular weights,($\cdot 10^3$) and the positions of standards are indicated by numbered arrows.

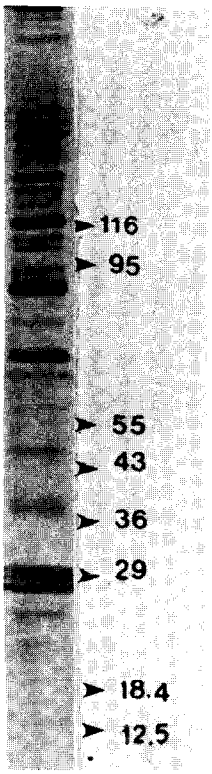

Fig. 5. Zeta-probe immunoblot for the membrane antigens of *G. lamblia* after electrophoresis on a 5–15% gradient gel in the presence of 5 m*M* TGA. The molecular weights ($\cdot 10^3$) and the positions of standards are indicated by numbered arrows.

charge it carries, which makes this matrix particularly useful in detecting trace amount of samples as well as low-molecular-weight antigens.

Finally, it could be concluded that the adaptations employed in this investigation proved to be highly sensitive for the characterization of major and minor *G. lamblia* membrane antigens (Fig. 5).

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