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Human Secretory Immune Response to Fatty Acid-Binding Protein Fraction from *Giardia lamblia*

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The secretory immune response in humans infected with *Giardia lamblia* was studied by using saliva samples and an 8-kDa antigen capable of binding fatty acids. This antigen was not recognized by saliva samples from healthy individuals. The antigen may be useful in diagnostic studies of *G. lamblia* infection.

Giardia lamblia, a flagellate protozoan which infects the human upper intestinal tract, is the most frequent cause of gastrointestinal infection by protozoans (16). *Giardia* can cause giardiosis, a waterborne disease (19) which results in diarrhea, steatorrhea, retarded growth in children, and malnutrition. Host factors are thought to be important in determining the severity of the response to this parasite (17).

Trophozoites do not invade tissues when mucosal surfaces are stimulated by *Giardia* antigens. Under these conditions, immunity to trophozoites is closely associated with the type of immune response generated by mucosal surface-associated lymphoid tissue (9). In a previous paper, we described the specific immunoglobulin A (IgA) secretory response to membrane-associated antigens in a *G. lamblia* infection (28).

Recently, we described purification and characterization of a fatty acid-binding protein (FABP) in *Giardia.* This protein, which has a molecular mass of 8 to 10 kDa, is located principally in the suctory disk and has an affinity constant (K_d) for oleic acid of 3.12×10^{-8} M (14).

In the present study, we examined the secretory immunoresponse to this protein during natural infection by *G. lamblia*, using saliva samples from patients with giardiosis.

Trophozoites of *G. lamblia* strains ATCC 30888 and ATCC 30957 were cultured in a TYI-S 33 culture (20) enriched with 10% fetal bovine serum inactivated by heat treatment at 56°C for 30 min. The parasites were cultured in glass flasks at 37°C for at least 72 h. When they reached the logarithmic growth phase, the parasites were centrifuged at $250 \times g$ for 10 min at 4°C.The pellet was washed in phosphate-buffered saline (0.15 M) (pH 7.4). The viable trophozoites were identified by 0.2% trypan blue staining.

For purification of the antigen, we used a method described elsewhere (S. Hassan, O. Córdova, R. Diaz de la Guardia, M. Maasse, D. Acuña, and A. Osuna, submitted for publication). The pellet containing the protozoan was frozen and thawed three times and then sonicated (5 min, 60 cycles, 0°C) in lysis buffer (20 mM phosphate buffer [pH 7.4], 0.25 mM sucrose, 1 mM EDTA, 0.145 mM KCl, 1 mM dithiothreitol) to break up the cells and treated with protease inhibitors (Complete Mini; Boehringer Mannheim GmbH). The sonicated fraction was centrifuged at $22{,}000 \times g$ for 30 min, and the resulting supernatant was collected. The supernatant was passed through a Lipidex-1000 column (Sigma) by using the method of Lee et al. (22) and was chromatographed (flow rate, 0.1 ml/min) through an affinity column prepared with Sepharose Epoxy 6B (Pharmacia Biotech) as the matrix and stearic acid as the ligand.

The bound fraction was eluted by using 0.1 M Tris HCl buffer containing 1 M NaCl and/or 7 M urea, and the eluted fraction was dialyzed with distilled water. The protein concentration was determined by the Bradford method (3). The sam-

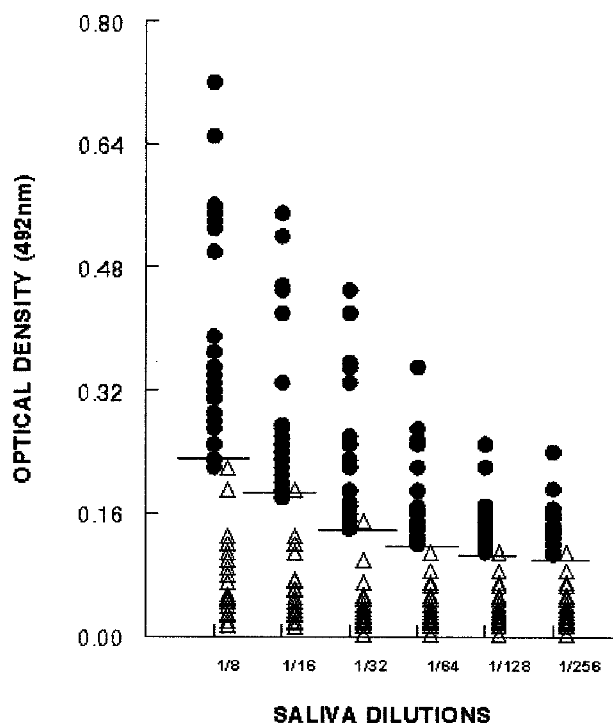

FIG. 1. Presence of sIgA to *Giardia* antigens in saliva samples from patients with giardiosis (●) and in saliva samples from healthy donors (△) with FABP as antigens, as determined by ELISA. The horizontal lines indicate the cutoff values (means of the optical densities for the control healthy saliva plus 3 standard deviations).

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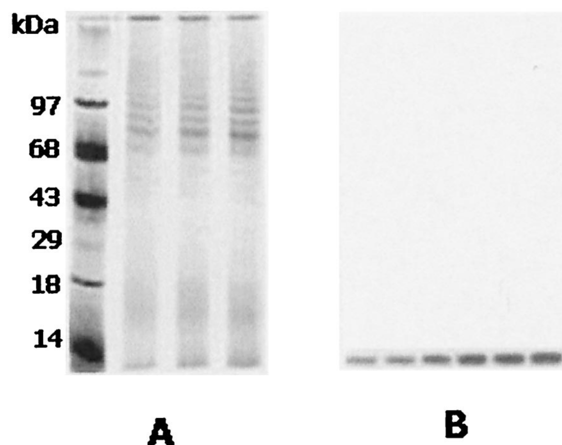

FIG. 2. (A) Silver-stained SDS–12% PAGE gel containing the affinity-purified antigen. The lane on the left contained the standard proteins. (B) Autoradiography of SDS–12% PAGE gel after binding of [U-^{14}C]oleic acid to the purified proteins. The oleic acid bound to the 8- to 10-kDa antigen. The different intensities of the bands in the autoradiograph correspond to the different concentrations of labeled oleic acid used in the incubation mixture (1.5, 0.75, 0.375, 0.18, 0.093, and 0.046 nmol of oleic acid/mg of protein). The labeled oleic acid was incubated for 20 min at 37°C.

ple was divided into aliquots for lyophilization, which were stored until they were needed.

Saliva samples were collected from 20 patients for whom a diagnosis of giardiosis had been confirmed by stool examination. Secretory IgA (sIgA) detection was carried out as described below. All patients had diarrhea with *Giardia* trophozoites or cysts in their feces at the time that the saliva samples were taken. Control saliva samples were obtained from 20 individuals with no *Giardia* cysts in their feces and no history of giardiosis or symptomatic gastrointestinal disease for the preceding 12 months.

The saliva samples were centrifuged at 2,500 × *g* for 15 min, and the supernatants were frozen at −80°C until they were used.

The concentrations of protein in the saliva samples were determined by the Bradford method, and the sIgA concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) in which we used saliva at a dilution of 1/100 in carbonate buffer as an antigen to coat polystyrene microtiter plates (Nunc, Roskilde, Denmark), followed by peroxidase-conjugated affinity-purified goat anti-human IgA (α-chain specific; Sigma), as described below.

The protein concentration obtained for the negative saliva was 32 μg/μl, and the protein concentration obtained for the positive saliva was 82 μg/μl.

The search for IgA antibodies to *G. lamblia* with an ELISA was performed essentially as previously described (28) by using 1 μg of affinity-purified *G. lamblia* antigen per 50 μl of carbonate buffer (pH 8.3) and 50-μl portions of serial dilutions of saliva samples, followed by addition of peroxidase-conjugated affinity-purified goat anti-human IgA at a 1:1,600 dilution and finally *o*-phenylenediamine dihydrochloride (Sigma) and 10 μl of 30% H_2O_2 per 25 ml. After 15 min the wells were scanned at 495 nm with an ELISA reader (METERTECH Σ 960). Means and standard deviations of the optical density values obtained with the control saliva were used to calculate the cutoff value (the arithmetic mean plus 3× the standard deviation).

The affinity-purified samples were electrophoresed under nonreducing conditions on sodium dodecyl sulfate (SDS)–20 or 12% polyacrylamide gel electrophoresis (PAGE) gels by the Laemmli method (21) and were stained with silver nitrate or Coomassie blue.

Western blotting with nitrocellulose was performed by the method of Towbin et al. (29). Blots were exposed to the same concentration of proteins for the positive and negative saliva samples (32 μg/μl) and then to peroxidase-conjugated affinity-purified goat anti-human IgA (α-chain specific; Sigma) at a 1:1,600 dilution. The blots were scanned and processed by using QuantiScan, version 1.25. The relative molecular masses were calculated by using R_f values.

The purified fraction was incubated with [U-^{14}C]oleic acid (NEN Dupont) at a concentration of 0.1 μCi/μg of protein (4) at 37°C. The incubated protein was separated in SDS–12% PAGE gels, autoradiography was performed with Kodak X-OMAT film, and each preparation was incubated on a Cronex Dupont intensifier screen for 5 days. Fisher's test was used to determine the homogeneity of variance between groups. When the variance was found to be homogeneous, Student's *t* test (10) was used to estimate the significance of the difference between means.

Positive saliva samples from individuals infected with *G. lamblia* contained 7.17 ± 0.55 times more sIgA that the negative saliva samples. The titers were usually greater than 1:8

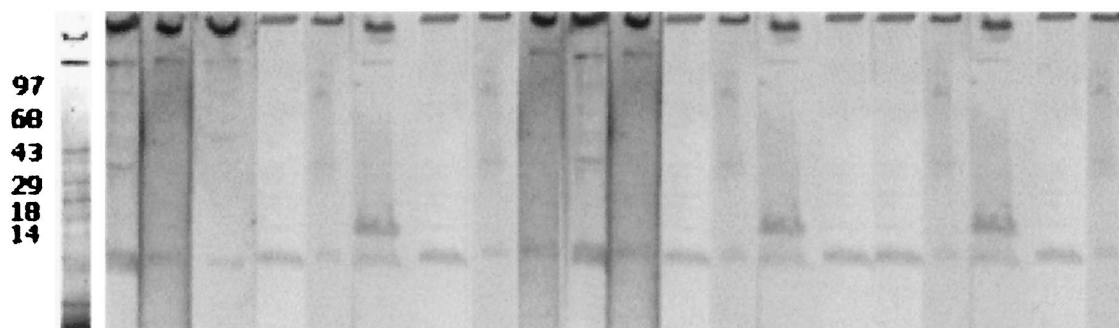

FIG. 3. Immunoblot analysis of the FABP from *Giardia* after SDS–20% PAGE of saliva samples from patients with giardiosis. The gel was stained with Coomassie blue. Molecular masses (in kilodaltons) are indicated on the left.

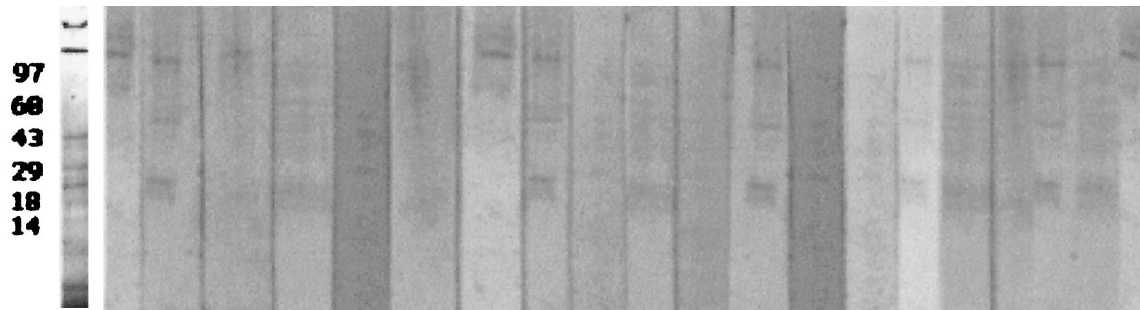

FIG. 4. Immunoblot analysis of the FABP from *Giardia* after SDS–20% PAGE of saliva samples from healthy donors. The gel was stained with Coomassie blue. Molecular masses (in kilodaltons) are indicated on the left.

and differed significantly ($P < 0.001$) from the titers of saliva samples taken from healthy control individuals (Fig. 1).

The results of the SDS-PAGE analysis performed under nonreducing conditions are shown in Fig. 2A. The purified fraction produced a protein pattern which had approximately 11 bands at relative molecular masses ranging from 10 to 90 kDa (90, 78, 69, 65, 60, 55, 49, 41, 29, 25, and 10 kDa) (Fig. 2A). The SDS–20% PAGE gels also contained 11 bands, the lowest at a molecular mass of 8.3 kDa.

The results of autoradiography of the SDS–12% PAGE gels indicated that after incubation with [U-^{14}C]oleic acid, only the bands at a molecular mass of 8.3 kDa were labeled (Fig. 2B). Figure 3 shows the results obtained for the antigenic fraction after incubation with the saliva samples from 20 patients. The saliva samples recognized a total of 10 bands at molecular masses between 10 and 90 kDa. The band at 8 to 10 kDa was recognized by the 100% of the saliva samples from *Giardia*-infected individuals. The negative control saliva samples (Fig. 4) from healthy donors showed no reactivity in the 8- to 10-kDa range. However, the saliva samples from healthy donors reacted with 90-, 78-, 69-, 49-, 29-, and 25-kDa polypeptides at frequencies of 42, 40, 57, 71, 85, and 42%, respectively. Nevertheless, the intensities of the bands were weaker that the intensities of the bands obtained with saliva samples from infected individuals.

The apparent differences in the molecular masses of FABP determined in the immunoblot and autoradiography analyses were due to the concentration of the polyacrylamide used for electrophoresis (20 and 12%, respectively).

The data presented here represent one of the first attempts to characterize the human secretory immune response to natural *Giardia* infection.

Practically all eukaryotic cells studied have a FABP (24). One family of low-molecular-mass (≤14-kDa) proteins is found in many cells of higher animals and also in helminths, in which this family comprises 2 to 3% of the total cytosol protein active in fatty acid metabolism (11). Although apparently located in the cytosol, these proteins are related to microsomal and mitochondrial membranes, providing an interaction that facilitates the ability to synthesize phospholipids (23). The antigenic values for these proteins constitute one of the most important characteristics currently under consideration for use in vaccination against helminth infections (1, 2, 5, 6, 7, 8, 18, 27, 30).

The *Giardia* protein studied by Hassan et al. (14) is located in the suctory disk, in the dorsal membrane, and in the cytosol near the lipid vacuoles, as observed by Gobert (12) for *Schistosoma*. This location implies that there is contact with the intestinal mucosa of the host.

In humans, limited studies have previously detected sIgA antibodies to *Giardia* in breast milk, as well as in saliva. sIgA directed specifically against the parasite was observed in saliva or in urine, and an increase in the intestinal IgA level was directly related to the level of exposure (13, 23, 25, 26). Walterspiel et al. (31) studied the role of sIgA in protecting against the parasite or the symptoms produced by it, and the response of the saliva IgA against *Giardia* is a diagnostic method specific for this parasite (15). Previously (28), some of us determined the molecular masses of antigens recognized by the sIgA in an enriched *Giardia* membrane antigenic fraction.

In summary, our results show that IgA from saliva samples of human patients with giardiosis recognized 8-kDa antigens in enriched antigenic FABP fractions of *G. lamblia*. On the other hand, these antigens were not recognized by IgA from saliva samples from members of a healthy control population. The 8-kDa antigens appear to be the same as the 14-kDa antigen described by Rosales-Borjas et al. (28) and to have a frequency of 70% in positive samples and 100% specificity. The differences in molecular mass may be a consequence of the differences described in the percentage of polyacrylamide in the SDS-PAGE gels used and the frequencies at the highest concentration of antigens used in our study.

Our results were obtained with a limited number of saliva samples. If they are substantiated by further analysis, they offer hope for a new vaccination strategy or for diagnosis with *G. lamblia* antigens.

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REFERENCES

1. **Abane, J. L., A. Oleaga, V. Ramajo, P. Casanueva, J. L. Arellano, G. V. Hillyer, and A. Muro.** 2000. Vaccination of mice against *Schistosoma bovis* with a recombinant fatty acid binding protein from *Fasciola hepatica*. Vet. Parasitol. **91:**33–42.
2. **Becker, M. M., B. H. Kalinna, G. J. Waine, and D. P. McManus.** 1994. Gene cloning, overproduction and purification of a functionally active cytoplasmic fatty acid-binding protein (Sj-FABPC) from the human blood fluke *Schistosoma japonicum*. Gene **48:**321–325.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of

microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Campbell, F. M., M. J. Gordon, and A. K. Dutta-Roy.** 1994. Plasma membrane fatty acid-binding protein (FABP) of the sheep placenta. Biochim. Biophys. Acta **1214:**187–192.
5. **Chabalgoity, J. A., J. A. Harrison, A. Esteves, R. Demarco de Hormaeche, R. Ehrlich, C. M. Khan, and C. E. Hormaeche.** 1997. Expression and immunogenicity of an *Echinococcus granulosus* fatty acid-binding protein in live attenuated *Salmonella* vaccine strains. Infect. Immun. **65:**2402–2412.
6. **Espino, A. M., and G. V. Hillyer.** 2001. Identification of fatty acid molecules in a *Fasciola hepatica* immunoprophylactic fatty acid-binding protein. J. Parasitol. **87:**426–428.
7. **Esteves, A., L. Joseph, M. Paulino, and R. Ehrlich.** 1997. Remarks on the phylogeny and structure of fatty acid binding proteins from parasitic platyhelminths. Int. J. Parasitol. **27:**1013–1023.
8. **Estuningsih, S. E., P. M. Smooker, E. Wiedosari, S. Widjajanti, S. Vaiano, S. Partoutomo, and T. W. Spithill.** 1997. Evaluation of antigens of *Fasciola gigantica* as vaccines against tropical fasciolosis in cattle. Int. J. Parasitol. **27:**1419–1428.
9. **Faubert, G. M.** 1996. The immune response to *Giardia*. Parasitol. Today **12:**140–145.
10. **Fisher, R. A.** 1934. Statistical methods for research workers, 5th ed. Oliver and Boyd, Edinburgh, United Kingdom.
11. **Frolov, A., T. H. Cho, E. J. Murphy, and F. Schroeder.** 1997. Isoforms of rat liver fatty acid binding protein differ in structure and affinity for fatty acids and fatty acyl-CoAs. Biochemistry **36:**6545–6555.
12. **Gobert, G. N.** 1998. Immunolocalization of schistosome proteins. Microsc. Res. Tech. **42:**176–185.
13. **Granot, E., D. T. Spira, D. Fraser, and R. J. Deckelbaum.** 1998. Immunologic response to infection with *Giardia lamblia* in children: effect of different clinical settings. J. Trop. Pediatr. **44:**241–246.
14. **Hasan, S., R. Diaz de la Guardia, M. Maasse, O. Córdova, C. Mascaró, and A. Osuna.** 2002. Isolation and purification of a fatty acid binding protein in *Giardia*. Rev. Iber. Parasitol. **61:**59–67.
15. **Hashkes, P. J., D. T. Spira, R. J. Deckelbaum, and E. Granot.** 1994. Salivary IgA antibodies to *Giardia lamblia* in day care center children. Pediatr. Infect. Dis. J. **13:**953–958.
16. **Heyworth, M. F.** 1996. *Giardia* infections, p. 227–238. *In* L. J. Paradise et al. (ed.), Enteric infections and immunity. Plenum Press, New York, N.Y.
17. **Hopkins, R. S., and D. D Juranek.** 1991. Acute giardiasis: an improved clinical case definition for epidemiologic studies. Am. J. Epidemiol. **133:**402–407.
18. **Janssen, D., and J. Barrett.** 1995. A novel lipid-binding protein from the cestode *Moniezia expansa*. Biochem. J. **311:**49–57.
19. **Jarroll, E. L., A. K. Bingham, and E. A. Meyer.** 1981. Effect of chlorine on *Giardia lamblia* cyst viability. Appl. Environ. Microbiol. **41:**483–487.
20. **Keister, D. B.** 1983. Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. Trans. R. Soc. Trop. Med. Hyg. **77:** 487–488.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
22. **Lee, J., X. Jiao, J. F. Hejtmancik, M. Kaiser-Kupfer, and G. J. Chader.** 1998. Identification, isolation and characterization of a 32-kDa fatty acid-binding protein missing from lymphocytes in humans with Bietti crystalline dystrophy (BCD). Mol. Genet. Metab. **65:**143–154.
23. **Miotti, P. G., R. H. Gilman, L. K. Pickering, G. Ruiz-Palacios, H. S. Park, and R. H. Yolken.** 1985. Prevalence of serum and milk antibodies to *Giardia lamblia* in different populations of lactating women. J. Infect. Dis. **152:**1025–1031.
24. **Murphy, E. J., R. D. Edmondson, D. H. Russell, S. Colles, and F. Schroeder.** 1998. Isolation and characterization of two distinct forms of liver fatty acid binding protein from rat. Biochim. Biophy. Acta **1436:**413–425.
25. **Nash, T. E., D. A. Herrington, G. A. Losonsky, and M. M. Levine.** 1987. Experimental human infections with *Giardia lamblia*. J. Infect. Dis. **156:**974–984.
26. **Nayak, N., N. K. Ganguly, V. Walia, S. S. Kanwar, and R. C. Mahajan.** 1987. Specific secretory IgA in the milk of *Giardia lamblia*-infected and uninfected women. J. Infect. Dis. **155:**724–727.
27. **Paulino, M., A. Esteves, M. Vega, G. Tabares, R. Ehrlich, and O. Tapia.** 1998. Modelling a 3D structure for EgDf1 from *Echinococcus granulosus*: putative epitopes, phosphorylation motifs and ligand. J. Comput. Aided Mol. Des. **4:**351–360.
28. **Rosales-Borjas, D. M., J. Diaz-Rivadeneyra, A. Dona-Leyva, S. A. Zambrano-Villa, C. Mascaro, A. Osuna, and L. Ortiz-Ortiz.** 1998. Secretory immune response to membrane antigens during *Giardia lamblia* infection in humans. Infect. Immun. **66:**756–759.
29. **Towbin, T., H. T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
30. **Waine, G. J., J. C. Scott, D. Mazzer, and D. P. McManus.** 1998. Mapping of linear B-cell epitopes on the 14-kDa fatty-acid binding protein of Chinese *Schistosoma japonicum*. Int. J. Parasitol. **28:**303–308.
31. **Walterspiel, J. N., A. L. Morrow, M. L. Guerrero, G. M. Ruiz-Palacios, and L. K. Pickering.** 1994. Secretory anti-*Giardia lamblia* antibodies in human milk: protective effect against diarrhea. Pediatrics **93:**28–31.

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