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Immunoglobulin G and Immunoglobulin M Polar Staining of *Toxoplasma gondii* in the Indirect Immunofluorescence Test

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Polar staining (PS) of *Toxoplasma gondii* in the indirect immunofluorescence test has been considered a nonspecific reaction caused exclusively by certain immunoglobulin M (IgM) antibodies and confined to the anterior end of the parasite. After we observed a patient with positive serology for Chagas' disease who presented an IgG PS reaction, we studied sera from 120 patients with Chagas' disease, 20 sera from patients with *Leishmania donovani* infection, and 30 sera from patients with *Leishmania braziliensis* infection. When only those specimens having no detectable anti-*Toxoplasma* activity were considered, a significantly ($P < 0.01$) higher prevalence of IgG PS was found in the Chagas' disease and *L. donovani* groups than in sera from normal American and Brazilian adults. Those sera also showed higher PS titers (1:64 to 1:1,024) when compared with controls (1:16 to 1:64). IgG PS titers did not decrease after serum treatment with 2-mercaptoethanol. However, the same treatment removed completely IgM PS. IgG PS, but not IgM PS, could be removed by adsorption with *Trypanosoma cruzi* epimastigotes. IgM PS was found in all samples studied, except in 41 of a group of 43 umbilical cord sera. It was found that the antigen source and the microscopy system can influence the detection of PS. It is proposed that after finding an intense IgG PS reaction, the laboratory should screen such serum also for anti-*T. cruzi* antibodies which may be undetected in the sample.

Polar staining (PS) of *Toxoplasma gondii* in the indirect immunofluorescence (IIF) test has been described as a nonspecific reaction confined to the anterior pole of the protozoan and is present in many human sera, regardless of whether the patients were previously infected with this parasite or not (11). Van Renterghem and Van Nimmen (12) found that this phenomenon is caused exclusively by antibodies of the immunoglobulin M (IgM) class and is absent in umbilical cord blood and in sera of children less than 6 months old. Hobbs et al. (6) found a bipolar staining as a related phenomenon which was also caused by certain IgM antibodies. Camargo et al. (3), though referring to the phenomenon as being due predominately to IgM antibodies cross-reacting with certain *Toxoplasma* structures, have found occasional sera that present this reactivity in the IgG antibody class. The present study was prompted by the observation of PS in the anti-IgG IIF test in a serum from a patient with positive serology for Chagas' disease. This index serum was from a patient with long-standing antibody levels of up to 1:512 against *Trypanosoma cruzi*. When tested against *Toxoplasma* antigen with IgG- and IgM-specific conjugates, no *Toxoplasma*-specific antibody was detected, but PS reactions were seen to titers of 1:256 with the anti-IgM system and 1:1,024 with the anti-IgG system. When adsorbed with *T. cruzi* antigen so that all *T. cruzi* antibody was removed, the IgG PS reaction was also completely deleted, but the IgM PS reaction was not reduced. We report here a series of experiments demonstrating an immunological link, through the IgG PS reaction, between *Toxoplasma* and two groups of hemoflagellates, *T. cruzi* and *Leishmania donovani*.

MATERIALS AND METHODS

Serum samples. Six groups of sera were tested for *Toxoplasma* and *T. cruzi* antibodies by the IIF test. The first group consisted of 120 specimens from serologically and clinically proven cases of Chagas' disease obtained from different geographical areas of Brazil. The second group consisted of 20 sera from persons with *L. donovani* infections from India. The third group consisted of 30 samples from patients with *Leishmania braziliensis* infections from Panama and Brazil. The fourth, fifth, and sixth groups were Chagas' disease-negative control samples: respectively, 60 sera

from North American adults, 43 cord blood sera, and 104 specimens from adults from the same regions in Brazil where the samples of the first group were collected.

Toxoplasma antigens. The reference antigen used throughout the study consisted of formaldehyde-killed tachyzoites of the RH strain from infected MRC-5 (derived from ATCC-CL-13) human embryo lung fibroblast monolayers. In one experiment designed to compare sensitivities of antigens from different sources, a small, selected group of sera was tested against four other Formol-fixed antigens of the RH strain: tachyzoites from an infected LLC-MK2 (derived from ATCC-CL-7) rhesus monkey kidney cell culture, a commercial antigen from tissue culture passages (Electro-Nucleonics Laboratories Inc., Bethesda, Md.), and two antigens from mouse peritoneal fluid, one produced locally and the other obtained from a commercial source (Microbiological Research Corp., Bountiful, Utah).

IIF tests. Anti-*Toxoplasma* tests were carried out as previously described (8). *T. cruzi* IIF tests were performed according to Camargo (2) with Formol-killed epimastigotes from cultures. Since the monospecificities of fluorochrome-conjugated antisera were of paramount importance, both anti-IgG (Kallestad Laboratories Inc., Chaska, Minn., lot F204 L084-1) and anti-IgM (Tago Inc., Burlingame, Calif., lot 92-04-02) conjugates, considered to be heavy-chain specific according to the instructions of manufacturers, were further assessed by a fluorometric assay (9) and found acceptable. Slides were read with a Leitz Ortholux fluorescence microscope equipped with a Ploem epi-illuminator and with oil-immersion objectives. A comparative study with some sera was also done with an American Optical epi-illumination microscope with dry objectives. Both instruments were equipped with mercury-vapor lamps.

Treatment of sera. Specific *T. cruzi* antibodies were adsorbed from sera with phosphate-buffered saline (pH 7.6)-washed epimastigotes during 1 h of incubation at 37°C and then overnight at 4°C. The Specificities of PS reactions were assessed by treating sera with 0.1 M 2-mercaptoethanol for 1 h at 37°C and with suspensions of *Staphylococcus aureus* cells from the Cowan I strain, which contains protein A in the cell wall.

Serum fractionation. IgM and IgG fractions were obtained through column chromatography of serum with Sephadex G-200 (Pharmacia, Inc., Piscataway, N.J.). Effluent fractions corresponding to these immunoglobulins were concentrated by membrane dialysis under negative pressure. Each fraction was then used to block the PS reaction of each antibody class in the IIF test by saturating parasite antigenic sites.

IgA and IgM assays. IgA and IgM antibodies in cord sera were assayed by single radial immunodiffusion (7) with commercial low-level plates (Calbiochem-Behring Corp., La Jolla, Calif., and Kallestad Laboratories). Differences in IgM and IgA antibody levels between PS-positive and PS-negative groups of sera were statistically evaluated by Student's *t* test.

RESULTS

Table 1 shows the prevalence of IgG and IgM PS in the groups of sera studied. Reactions of specific anti-*Toxoplasma* antibodies which give peripheral staining may mask polar factor expression. Therefore, when only those sera having no specific anti-*Toxoplasma* activity were considered, the percentages of positive IgG PS reactions were greater in all groups. However, only the Chagas' disease and *L. donovani* groups showed a significantly ($P < 0.01$) higher IgG PS prevalences when each negative group was compared with the negative control group of Brazilian adults by a chi-square test. Moreover, IgG PS titers in the Chagas' disease and *L. donovani* groups ranged from 1:64 to 1:1,024, whereas in all remaining groups titers for this phenomenon ranged between 1:16 and 1:64.

Table 2 shows the importance of the microscopy system in detecting PS reaction. In fact, dry-objective examination gave about 7% less IgM PS detectability, whereas the loss for IgG PS was 42%. Both systems, however, enabled the detection of all IgG PS found in the Chagas' disease and *L. donovani* sera.

Eighteen sera (90%) from the *L. donovani* group presented *T. cruzi* antibodies, whereas only 12 (40%) in the *L. braziliensis* group pre-

TABLE 1. *Frequency of Toxoplasma PS in six groups of sera[a] according to antibody class*

Specific reactivity against *Toxoplasma*	No. of sera showing PS/total no. of sera in:											
	Chagas' disease group		Leishmaniasis group				Control group					
			L. donovani		*L. braziliensis*		American adults		Cord sera		Brazilian adults	
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
Positive	7/92 (7.6)	3/3 (100)	2/3 (66.7)	0/0	1/13 (7.7)	0/0	0/16 (0)	0/0	0/13 (0)	0/0	2/75 (2.7)	0/2 (0)
Negative	28/28 (100)	117/117 (100)	13/17 (76.5)	20/20 (100)	4/17 (23.5)	30/30 (100)	14/44 (31.8)	60/60 (100)	5/30 (16.7)	2/43 (4.7)	3/29 (10.3)	102/102 (100)

[a] Diluted at 1:25 for antigen sensitization in the IIF test. Total number of sera tested in each group: Chagas' disease, 120; *L. donovani*, 20; *L. braziliensis*, 30; American adults, 60; cord sera, 43; Brazilian adults, 104. Numbers in parentheses indicate percentages.

TABLE 2. *Influence of microscopy system on detection of PS in IgG PS-positive sera according to antibody class*[a]

Serum group (no. of sera tested)	No. of sera presenting PS by:			
	Oil-immersion objective (Leitz)		Dry objective (American Optical)	
	IgG	IgM	IgG	IgM
Chagas' disease (3)	3	3	3	3
L. donovani (4)	4	4	4	3
L. braziliensis (3)	3	3	2	3
Cord sera (4)	4	0	0	0
Controls (adults) (5)	5	5	2	5

[a] All sera were diluted at 1:25 to sensitize antigens. A 40× objective and a 10× ocular were used in each system.

sented cross-reactions. No sera in the remaining groups reacted with *T. cruzi* antigen. Adsorption of 10 sera of the Chagas' disease group with *T. cruzi* epimastigotes markedly reduced IgG PS titers without any change in the IgM PS titers. However, complete negation of the IgG PS phenomenon was achieved only when *T. cruzi* antibodies were also completely adsorbed.

Treatment with 2-mercaptoethanol did not change IgG PS titers. However, the same treatment canceled the reactivity of any IgM PS or IgM-specific antibodies, if present. Nevertheless, adsorption with *S. aureus* containing protein A only partially decreased IgG PS titers. The same treatment in a serum presenting a high titer of specific IgG *Toxoplasma* antibodies yielded a 64-fold decrease in the original titer.

Different *Toxoplasma* antigens sensitized with IgG PS sera all showed the phenomenon, though in various degrees of sensitivity (Table 3). Even the least sensitive antigen tested, antigen D, however, exhibited reactivity with three sera of the Chagas' disease group and failed to detect the phenomenon in the remaining six sera. This antigen also showed a decreased sensitivity when tested against positive specific IgG anti-*T. gondii* sera. Despite the differences in detecting IgG PS, all antigens behaved similarly in reacting with IgM PS antibodies.

Serum fractionation by gel chromatography isolated PS reactivity in different fractions corresponding to IgG and IgM antibodies. Assessment of effluent fractions with both monospecific conjugates revealed no overlapping of results. Antigens previously sensitized with concentrated serum fractions of each antibody class, when compared with phosphate-buffered saline-treated antigens, reacted similarly with concentrated immunoglobulin fractions of the other class when the conjugate was specific for the second antibody coat.

IgM PS was observed in all groups of sera. When only specimens presenting no specific *Toxoplasma* antibodies were considered, its prevalence reached 100%, except in the cord sera. This group presented a nonsignificant difference in IgG PS prevalence, but showed a low percentage of IgM PS sera. IgA and IgM levels were determined to detect possible contamination with maternal blood. However, no significant differences were found between positive and negative sera, either for IgG PS or for IgM PS at the 5% level of significance.

DISCUSSION

Since the PS reaction has been said to be only in IgM, it is possible that IgG PS could be misinterpreted as IgM in routine examination of specimens for *Toxoplasma* antibodies in the IIF test. In fact, our earlier findings led us to investigate the purity of our IgG-specific conjugate when we observed PS reactions in tests of a negative control serum in which it was used.

Although previously described as an uncommon phenomenon (3), IgG PS in the *Toxoplasma* fluorescent antibody test has not been implicated with any infectious diseases. Its presence in serum seems to be independent of the

TABLE 3. *Sensitivities of four different T. gondii antigens for use in the IIF test in showing PS of each antibody class as compared with the reference antigen*[a]

No. of sera showing PS with reference antigen (antibody class)	No. of sera showing PS with antigen from:							
	Cell line				Mouse peritoneal exudate			
	A		B		C		D	
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
7 (IgG and IgM)	6	7	7	7	6	7	3	7
2 (IgG only)	1	0	2	0	1	0	0	0
3 (IgM only)	0	3	0	3	0	3	0	3

[a] Antigens were sensitized with sera diluted at 1:25. Sources of antigens: reference, infected MRC-5 monolayers; A, infected LLC-MK2 monolayers; B, unknown cell line (commercial); C, exudate from 72-h-infected mice; D, peritoneal exudate (commercial).

ubiquitous IgM PS, since the frequencies and titers of IgM PS were comparable in the groups here studied. IgG and IgM PS antibodies react with the anterior end of the protozoan in similar patterns. There seem to be different antigen-binding sites in the cell pole, however, since attempts to block PS of each immunoglobulin class with concentrated serum fractions of the other failed. The microscopy system and the antigen source and resulting antigen sensitivity to reaction with IgG antibodies seem to play an important role in the detection of the phenomenon. This difference may be due in part to the greater optical efficiency of the oil-immersion objectives. A similar effect was found by Hobbs et al. in comparing halogen with more efficient mercury-vapor illumination (6). Neither IgG nor IgM PS is likely to be caused by the presence in the antigen of mouse host substances reacting with certain antibodies in human sera, since antigens from cell line and mouse ascites sources gave comparable results. Studies on the specificity of PS reactions have been done by others with the RH strain (6, 12) and the M strain (E. L. F. Franco, E. F. Duarte, J. A. C. Magrin, I. T. Higuti, and A. V. R. Souza, Proc. Annu. Meet. Braz. Soc. Parasitol. 4th P. 32, 1979) of *T. gondii*. R. W. Higby (Dr.P.H. thesis, University of North Carolina, Chapel Hill, 1980), using the tachyzoites of the RH strain harvested from infected cell lines, also failed to detect PS with antibodies of the IgA and IgG classes, though these previous studies did not include sera from persons infected with the hemoflagellates. However, weak IgG PS reactions were also observed in some normal sera with the reference antigen used in this study.

Sera from patients with Chagas' disease and leishmaniasis are known to cross-react (1). It was found that the *L. donovani* sera studied had a higher percentage of cross-reactions with *T. cruzi* than did the *L. braziliensis* sera, which is in agreement with a concomitant greater prevalence of IgG PS in *L. donovani* sera. A possible explanation for the high prevalence of IgG PS in these patients could be the presence in the *Toxoplasma* anterior end of antigens also shared by *T. cruzi* and *Leishmania*. Therefore, infections by these hemoflagellates elicit the production of antibodies as IgG polar reactors with *T. gondii* organisms. This constitutes an immunological link between these three species. We have found no indication of *T. gondii* infections stimulating antibodies that cross-react with hemoflagellates. Perhaps a different humoral mechanism is involved in that case than is required for the production of IgG PS reactions.

Unlike IgG PS, IgM PS does not seem to be caused by cross-reactions with antibodies against other parasites, since it is highly prevalent in many different populations. Nevertheless, we have found IgM PS in a few cord sera which were unlikely to be contaminated with maternal blood. This may possibly indicate that the antigenic stimulus for the production of IgM PS can be available to the fetal immune system before birth. Its rare presence in cord sera seems to be caused by the impossibility of these macroglobulins crossing the placenta.

Even with the new data provided above on polar reactivity caused by IgG antibodies, the nature and specificity of the PS reaction remain obscure. Previous studies (Franco et al., Proc. Annu. Meet. Braz. Soc. Parasitol. 4th, p. 32, 1979; Higby, Dr.P.H. thesis, University of North Carolina, Chapel Hill, 1980) failed to explain the nature of antigenic stimulus for the PS response. The similarity of the phenomenon to the capping mechanism of antigen migration (4, 5) is also unlikely to be the right explanation, because cultured parasites which have multiplied in an environment free of host-specific antibodies present PS. Lectin-binding sites have been described in *Toxoplasma* cysts, but not in the tachyzoites (10). This speaks against the possible saccharide nature of polar antigens. Also, studies in this laboratory (unpublished data) have shown that IgG and IgM PS reactions are not present after reaction with trypsin-treated parasites.

As pointed out by Van Renterghem and Van Nimmen (12), for the correct interpretation of *Toxoplasma* IIF tests, it is necessary to exclude PS reactions to avoid false-positive reactions often of serious consequences. We would add that in endemic areas, where a particularly intense IgG PS reaction could be found, the laboratory should also screen such sera for antibodies to *T. cruzi* which might be present, though undetected, in the sample.

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