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Improved Serological Diagnosis of *Toxoplasma gondii* Infection by Detection of Immunoglobulin A (IgA) and IgM Antibodies against P30 by Using the Immunoblot Technique

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Immunoglobulin M (IgM) and IgA antibodies against the major surface protein of *Toxoplasma gondii* were determined in a total of 195 human sera and five human cerebrospinal fluids by using a P30 membrane extract and the immunoblot technique. By using two different *T. gondii* strains (RH and BK) simultaneously as antigens, we were able to demonstrate diagnostically important strain-specific human antibody responses in 4.5% of the samples tested. A comparison of the immunoblot technique with an IgM immunocapture enzyme-linked immunosorbent assay demonstrated that the IgM and IgA immunoblot seems to be of advantage in the diagnosis of acute toxoplasmosis in certain groups of patients, especially in the diagnosis of cerebral toxoplasmosis in patients with AIDS. The immunoblot technique described is easy to perform and might be useful as an additional serological assay for routine diagnosis of *T. gondii* infections.

Infection with the ubiquitous protozoan parasite *Toxoplasma gondii* generally produces mild or asymptomatic diseases in healthy adults but can cause serious illness in immunocompromised patients and in infants infected in utero (22). Diagnosis is based on detection of the parasite by means of polymerase chain reaction (PCR), mouse inoculation, or cell culture assays (9, 10) and on serological tests which detect specific immunoglobulin M (IgM) and IgG antibodies. Recently, IgA antibodies were shown to be of high diagnostic value in congenital and acute toxoplasmosis (7). One way to determine *Toxoplasma* antigens recognized by different human immunoglobulin classes is the immunoblot technique. It was shown that P30, a major antigen expressed by tachyzoites, provokes an immune response in early stages of infection with *T. gondii* (4, 7, 13, 14, 27).

Naot et al. (19) demonstrated that in congenital toxoplasmosis, specific IgM antibodies might not be detectable in infected newborns by using conventional serological methods like the enzyme-linked immunosorbent assay (ELISA) technique. It was argued that the inability to detect IgM antibodies in these cases might be due to either insufficient sensitivity of the serological method or the possibility that IgG antibodies passively transferred from the mother to the fetus suppress the fetal IgM antibody response to *T. gondii* (1, 22). It was also observed that specific IgM antibodies might not be detectable in human immunodeficiency virus (HIV)-infected patients and patients with isolated ocular toxoplasmosis by using the ELISA or immunofluorescence assay technique (22). Since IgM titers might persist for years, it would be helpful to have additional markers for acute infection (7). It was shown by previous investigations using ELISA and immunoblotting that IgA antibodies seem not to persist as long as IgM antibodies might (7, 20, 28). Therefore, we considered it of interest to determine specific IgA antibodies besides IgM antibodies directed against P30 by using the immunoblot technique. This study analyzed 200 clinical samples from 144 patients to evaluate the diagnostic value of the immunoblot in comparison with conventional serological methods.

MATERIALS AND METHODS

Determination of an optimal preparation of *T. gondii* antigen. Tachyzoites of *T. gondii* RH (23) and BK (36) were maintained and harvested as previously described (8). For direct comparison, several different methods for antigen preparation were applied.

(i) Total cell lysate. To tachyzoites diluted at a concentration of $10^6/\mu l$ in phosphate-buffered saline (150 mM NaCl, 20 mM sodium phosphate, pH 7.2) an equal volume of protein sample buffer consisting of 120 mM Tris (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% mercaptoethanol, and 0.002% bromphenol blue was added. After being boiled for 5 min, the samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

(ii) Disruption by sonication. Following 2 × 15-s pulsed sonication at an output level of 7 in a B15 cell disruptor (Branson, Schwäbisch Gmünd, Germany), the *T. gondii* lysate was centrifuged at 2,000 × *g* for 15 min. The pellet (P1) was stored at −20°C, while the supernatant (S1) was recentrifuged at 50,000 × *g* for 30 min. The final pellet (P2) and supernatant (S2), as well as fractions P1 and S1, were analyzed by SDS-PAGE.

(iii) Lysis by using detergents and salt. Tachyzoites were suspended at a dilution of $10^6/\mu l$ in one of the following buffer systems: (i) 150 mM NaCl–1.0% Nonidet P-40–50 mM Tris (pH 8.0), (ii) 500 mM NaCl–1.0% Nonidet P-40–50 mM Tris (pH 8.0), (iii) 1.0% Nonidet P-40–50 mM Tris (pH 8.0), (iv) 150 mM NaCl–1.0% Nonidet P-40–50 mM Tris (pH 8.0)–0.1% SDS–0.5% deoxycholate.

Following incubation at 4°C for 12 h, the suspensions were centrifuged at 50,000 × *g* for 30 min and the pellets and supernatants were analyzed by SDS-PAGE. Following determination of the protein contents of all of the samples as previously described (17), 8 μg of each sample was analyzed by SDS-PAGE.

Serological tests. *T. gondii*-specific antibody titers were

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measured in the dye test (DT) (24), an indirect immunofluorescence test (31) obtained from BAG (Lich, Germany), and the complement fixation test (CFT) (33). Light antigen for the CFT was obtained from the Institute of Medical Parasitology, University of Bonn, Bonn, Germany. Specific IgM antibodies directed against the P30 antigen of *T. gondii* were analyzed by using an IgM capture ELISA (5) obtained from Diagnostic Pasteur (Freiburg, Germany).

Samples from patients. We analyzed 195 serum and 5 cerebrospinal fluid samples for the presence of IgM and IgA antibodies. Of these clinical specimens, 81 were obtained from external hospitals or institutions. The serum collection was classified into six groups depending on clinical diagnoses. Group A comprised 76 samples obtained from 48 pregnant women with serological indications of recent *T. gondii* infection when routinely tested by DT, CFT, and IgM ELISA. Group B consisted of 16 specimens postnatally obtained from 15 infants born of mothers who seroconverted during pregnancy or who had clinical signs of connate infection (i.e., hydrocephalus) at the time of birth. Group C included 45 samples from 29 patients with cervical lymphadenitis. Group D comprised 25 specimens obtained from 22 patients with diseases of the central nervous system, like encephalitis, brain abscess, lymphoma, chorioretinitis, hypacusis, or facialis paresis. Group E consisted of 13 samples from nine HIV-infected patients and four patients with AIDS without cerebral toxoplasmosis and 12 sequential samples from six patients with AIDS with cerebral toxoplasmosis. The diagnosis of cerebral toxoplasmosis was proven by either brain biopsy or autopsy, PCR and mouse inoculation test of cerebrospinal fluid, or clinical improvement following *T. gondii*-specific treatment with pyrimethamine and sulfadiazine, respectively. Group F comprised 13 specimens from 11 patients who had received transplantation surgery or had fever of unknown origin. The 200 clinical specimens included 80 sequential samples obtained from 26 patients of all of the subgroups. The sample collection consisted of 11 sera (5.5%) from noninfected patients, 59 sera (29.5%) from patients with latent infection, 12 sera (6.0%) from patients with AIDS with cerebral toxoplasmosis, and 118 sera (59.0%) from patients with acute infection (for definitions, see below). A positive control consisted of pooled sera of 10 patients with serological evidence of acute toxoplasmosis (DT titer of >1:1,000, CFT titer of >1:10 with rising titers in follow-up sera, and IgM ELISA positive). The negative control consisted of a pool of sera from 10 patients with negative *Toxoplasma* serology (DT titer of <1:2, CFT negative, and IgM ELISA negative).

Criteria for acute toxoplasmosis were (i) serology (titers of >1:1,000 by DT or indirect immunofluorescence test and >1:10 by CFT with rising titers in follow-up sera and at least one positive IgM titer), (ii) detection of *T. gondii* DNA by means of PCR (9), (iii) demonstration of parasites in clinical specimens by using the mouse inoculation test (35), or (iv) histological identification of *T. gondii* in biopsies or at autopsy. Demonstration of *T. gondii* by at least two of these three methods was possible in all patients with AIDS with cerebral toxoplasmosis, as well as in five patients with cervical lymphadenitis and one infant with a congenital infection. Among these cases, *T. gondii* was identified in two patients with AIDS with cerebral toxoplasmosis at autopsy. Criteria for latent or chronic infection were as follows: titers of <1:1,000 by DT or by indirect immunofluorescence test or <1:10 by CFT with stable or decreasing titers in follow-up sera, IgM ELISA negative, and no detection of *T. gondii* in clinical specimens. Results obtained for IgM ELISA or immunoblotting were defined as true positive if IgM and/or IgA antibodies against *T. gondii* were present and if the above-mentioned criteria for acute infection were met. IgM ELISA and immunoblot results were defined as true negative if a negative result for IgM and IgA antibodies was obtained and if the above-mentioned criteria for acute toxoplasmosis were not met. Because IgA antibodies start to rise a few days after IgM antibodies rise, determination of results of the IgA immunoblot depended on comparison with IgM, clinical data, detection of *T. gondii* in clinical specimens by PCR and mouse test, or histological confirmation by autopsy. Results obtained for IgM ELISA or immunoblotting were classified as false positive if IgM or IgA antibodies against *T. gondii* were detected although the criteria for acute toxoplasmosis were not met. In contrast, results were classified as false negative if IgM or IgA antibodies were not detected although the above-mentioned criteria for acute infection were met.

SDS-PAGE and immunoblotting. Examination of human sera by SDS-PAGE and immunoblotting was done as described recently (8). Briefly, following separation by SDS–11% PAGE, *T. gondii* antigen was transferred electrophoretically onto 0.45-μm-pore-size nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and incubated for 12 h with human sera diluted 1:200 in phosphate-buffered saline. Cerebrospinal fluid was used at a dilution of 1:4. After 1 h of incubation with alkaline phosphatase-conjugated goat anti-human IgM diluted 1:2,000 or anti-human IgA diluted 1:500 (Sigma, Deisenhofen, Germany), reactive bands were visualized by using 5-bromo-4-chloro-3-indolylphosphate incorporating nitroblue tetrazolium as the substrate (2).

Removal of human IgG antibodies. Human IgG antibodies were removed from sera by immunoprecipitation using RF-absorbens as described by the supplier (Behring, Marburg, Germany).

RESULTS

Preparation of *T. gondii* antigen. As was shown by previous investigations (7, 25, 27), major tachyzoite surface antigen P30 induces an intense and specific antibody response in patients acutely infected with *T. gondii*. Therefore, we tried several preparation methods to identify a time-saving technique for enrichment of this membrane component employed for routine diagnostic immunoblots. A total of 8 μg of protein was used for each preparation method to identify that fraction that exhibits the most intense reactivity of the P30 band with pooled human sera of patients with acute toxoplasmosis. Lysis of *T. gondii* with a low-salt buffer and 1% Nonidet P-40 resulted in enrichment of a protein with a molecular mass of approximately 34 kDa in the supernatant (Fig. 1, method 3SC). The identity of this protein with P30 was proven by using a monoclonal antibody specific for P30 (Diagnostics Pasteur) (data not shown). As was demonstrated by a recent investigation, the molecular mass of P30 ranges from 30 to 35 kDa under reducing gel conditions (15). There were no differences in results obtained with preparations of *T. gondii* BK or RH. Therefore, we used the 3SC preparation for all subsequent routine immunoblots.

Determination of optimal conditions for immunoblotting. *T. gondii* RH and BK lysates were separated by preparative SDS-PAGE. Following immunoblotting and blocking with 1% bovine serum albumin, pooled human sera from patients with acute toxoplasmosis were used at different dilutions and incubated for different periods of time to determine optimal conditions for immunoblotting. It was shown that dilution of

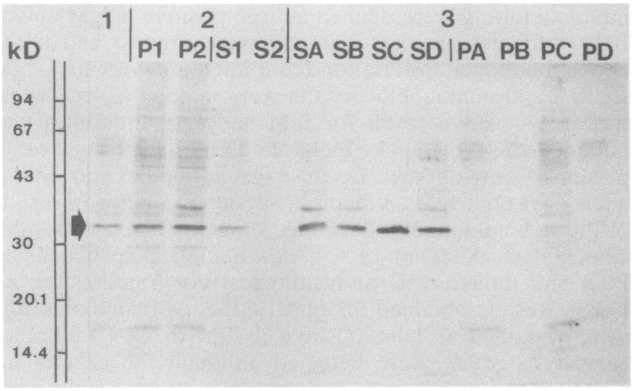

FIG. 1. Immunoblot demonstrating the reactivities of different *T. gondii* antigen preparations with a pool of sera from patients with acute toxoplasmosis. The arrow indicates reactive protein P30. Methods: 1, total cell lysate; 2, disruption by sonication; 3, lysis by using detergents and salt. S, supernatant; P, pellet. For details, see Materials and Methods.

human sera at 1:200 and incubation for 2 h were sufficient for detection of anti-P30 antibodies. However, the intensity of the antiserum-reactive P30 band was enhanced when 1:200-diluted human serum was incubated for 16 h (Fig. 2). Therefore, and for convenient routine diagnostic scheduling, we recommend incubation of 1:200-diluted human sera for about 16 h or overnight. Dilution of alkaline phosphatase-conjugated goat anti-human IgM or IgA had to be determined for each new charge of antibody conjugate.

Evaluation of IgM and IgA immunoblots for routine diagnosis. It was demonstrated that a P30-specific immunocapture IgM ELISA was of high specificity and sensitivity in the diagnosis of acute toxoplasmosis in patients without AIDS (5, 11, 18, 25). Therefore, we used Platelia-Toxo IgM (Diagnostics Pasteur) for direct immunoblot comparison of 200 clinical samples from 144 individuals. In sequential samples of seven patients of groups A, C, and E, IgA antibodies were detected a few days following the appearance of IgM antibodies. By analyzing 73 sequential samples of 18 patients of groups A, C, and D, we did not demonstrate the presence of IgA antibodies beyond 5 months. Of these patients, 11 were observed for at least 7 months. Therefore, IgA antibodies might be missed in the early-acute and late-acute phases. Considering these observations, we evaluated the IgA immunoblot on the basis of follow-up sera, serology, clinical data, detection of *T. gondii* antigen by mouse inoculation (35) or PCR (9), and autopsy. Differences between true-positive results obtained for IgM ELISA and IgM or IgA immunoblotting were statistically significant (chi-square test, $P < 0.01$) (Table 1). These results were similar in all groups of patients, with an interesting exception. Twelve samples from patients with AIDS with proven cerebral toxoplasmosis were analyzed. IgM antibodies in these samples were detected in only one sample by using the ELISA technique, whereas the immunoblot was able to detect IgM antibodies in 10 samples and the IgA immunoblot detected 11 positive samples, 4 of which were cerebrospinal fluids (Table 1). Therefore, differences between true-positive results obtained for the ELISA technique and immunoblotting in patients with AIDS with cerebral toxoplasmosis were highly significant (chi-square test, $P < 0.001$). However, in one HIV-infected patient with autopsy-proven multicentric lymphoma a false-positive result was obtained for IgM antibodies by the ELISA technique, as well as for IgA

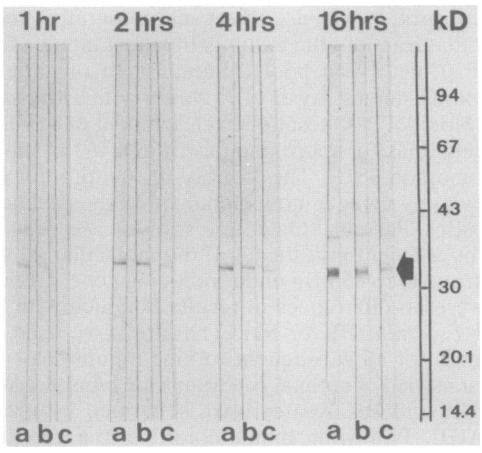

FIG. 2. Determination of optimal conditions for P30-specific immunoblotting. Incubation times of human sera diluted 1:100 (a), 1:200 (b), and 1:500 (c) are given at the top. The arrow indicates reactive protein P30.

TABLE 1. Comparison of ELISA and immunoblotting for detection of IgM and IgA antibodies against *T. gondii*[a]

Group (no. of patients) and test	No. (%) of results			
	True positive	False positive	False negative	True negative
A (76)				
IgM ELISA	61 (80.26)	3 (3.80)	4 (5.06)	8 (10.13)
IgM blot	65 (85.53)	2 (2.53)		9 (11.39)
IgA blot	42 (55.26)	1 (1.27)		33 (43.42)
B (16)				
IgM ELISA			1 (6.25)	15 (93.75)
IgM blot			1 (6.25)	15 (93.75)
IgA blot	1 (6.25)			15 (93.75)
C (45)				
IgM ELISA	34 (75.56)		2 (4.44)	9 (20.00)
IgM blot	34 (75.56)		2 (4.44)	9 (20.00)
IgA blot	23 (51.11)			22 (48.89)
D (25)				
IgM ELISA	8 (32.00)		1 (4.00)	16 (64.00)
IgM blot	9 (36.00)			16 (64.00)
IgA blot	5 (20.00)	2 (8.00)		18 (72.00)
E (25)				
IgM ELISA	1 (4.00)	1 (4.55)	11 (44.00)	12 (48.00)
IgM blot	10 (40.00)		2 (9.09)	13 (52.00)
IgA blot	11 (44.00)	1 (4.55)	1 (4.55)	12 (48.00)
F (13)				
IgM ELISA	7 (53.85)			6 (46.15)
IgM blot	7 (53.85)	1 (7.69)		5 (38.46)
IgA blot	6 (46.15)			7 (53.85)
Total (200)				
IgM ELISA	111 (55.50)	4 (2.00)	19 (9.50)	66 (33.00)
IgM blot	125 (62.50)	3 (1.50)	5 (2.50)	67 (33.50)
IgA blot	88 (44.00)	4 (2.00)	1 (0.50)	107 (53.50)

[a] For classification of patient groups and definitions of true positive, false positive, false negative, and true negative, see Materials and Methods.

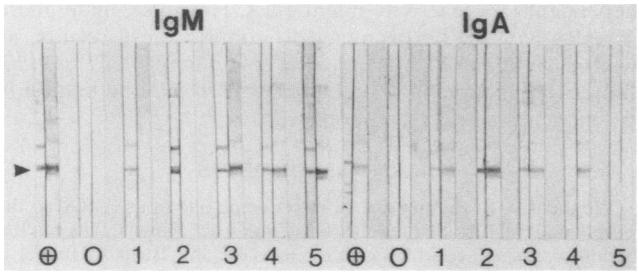

FIG. 3. Representative P30-specific immunoblot using antigen preparations of *T. gondii* BK (left) and RH (right) simultaneously. Cases 1, 2, and 4 correspond to patients 1, 2, and 3 of Table 2. ⊕, positive control; O, negative control. The arrowhead indicates reactive protein P30.

antibodies by immunoblotting. This patient had a DT titer of 1:64. No IgM or IgA antibodies were detectable in any HIV-positive patient with a negative DT titer.

Strain-specific human antibody response. Immunoblotting was carried out by using equivalent concentrations of antigens of *T. gondii* BK and RH simultaneously. It was demonstrated that 9 (4.5%) of the 200 samples contained antibodies to P30 that were reactive with only one of the two *T. gondii* strains (Fig. 3 and Table 2). These samples were tested at least twice to confirm the striking results. In addition, to exclude the possibility that IgG antibodies compete for reactive epitopes, IgG antibodies were removed by immunoprecipitation. Strain-specific human antibody responses for IgM were observed in five samples (2.5%), whereas this phenomenon was present for IgA antibodies in four samples (2.0%). These strain-specific human antibody responses seemed to be independent of patient diagnosis, age, or sex and were obtained with sera as well as with cerebrospinal fluid samples.

DISCUSSION

Antibodies directed against protein P30 are highly specific for acute infection with *T. gondii* because this major surface protein of tachyzoites is highly immunogenic and induces an intense antibody response in patients with acute toxoplasmosis (7, 13, 14, 26). It was shown by previous investigations using ELISA or immunoblotting that IgA antibodies seem not to persist as long as IgM antibodies might and that they do not interfere with IgM rheumatic factor (7, 20, 28). In addition, since IgA antibodies are not transmitted in utero, they might be helpful in determining the stage of infection in congenital toxoplasmosis (7).

We developed an immunoblot for routine determination of IgM and IgA antibodies to major surface protein P30 of *T. gondii* in human sera and cerebrospinal fluids. By analyzing sequential samples from certain patients, we confirmed previous findings that IgA antibodies to P30 seem not to persist as long as IgM antibodies might (7, 20, 28), because in our study IgA antibodies were not detectable by immunoblots beyond 5 months. However, it must be mentioned that IgA antibodies were detected in four samples of chronically infected patients belonging to groups A, D, and E. Since these patients did not meet the criteria for acute toxoplasmosis, we classified the detection of IgA antibodies as false positive. Huskinson et al. (14) also demonstrated the presence of IgA antibodies to P30 in the sera of four chronically infected individuals by using immunoblots with nonreduced antigen. Huskinson et al. analyzed only five patients with chronic toxoplasmosis, whereas our study comprised 59 samples drawn from 36 chronically infected individuals. Therefore, it is not known whether the data provided by Huskinson et al. were representative for chronic infection. However, we cannot exclude the possibility that IgA is present in some cases of chronic infection. In addition, it also might be possible that these surprising differences between the investigation of Huskinson et al. and our study are due to treatment of the membrane extract with SDS and mercaptoethanol in our immunoblots.

Two hundred clinical samples were analyzed. The P30 immunoblot seemed to be superior to a commercial P30 IgM ELISA, because significantly more true-positive results for detection of IgM antibodies were obtained by the immunoblot technique. This result is in contrast to those of a previous investigation by Verhostede et al. (30) demonstrating that immunoblotting is not useful as a reference method. Since Verhofstede et al. used a total cell lysate of *T. gondii* as the antigen, further processing of the antigenic extract might be critical for immunoblotting, as was shown by our results.

It was demonstrated by Potasman et al. (21) that naturally occurring human antibodies against P30 also exist. However, the accuracy of the results obtained by immunoblotting in our study was proven by either other serological tests, detection of *T. gondii* in clinical specimens, or improvement of clinical symptoms following specific treatment. We suggest that immunoblotting cannot replace other serological test systems but can serve as an additional method to improve diagnosis. Remarkable is the finding that P30 immunoblotting, in contrast to IgM ELISA, revealed the presence

TABLE 2. Serological data for patients with *T. gondii* strain-specific antibody responses[a]

Patient no., sample	Diagnosis	CFT titer	DT titer	IgM ELISA result	IgM blot result		IgA blot result	
					BK	RH	BK	RH
1, serum	Transplant	1:10	1:256	0	+	0	+	+
2, serum	Pregnancy	1:80	1:4,000	+	+	0	+	+
3, serum	Pregnancy	1:40	1:16,000	+	+	+	0	+
4, serum	Pregnancy	1:10	1:256	+	0	+	+	+
5, serum	Pregnancy	1:20	1:1,000	+	+	+	0	+
6, serum	Lymphad.	1:80	1:4,000	+	0	+	0	0
7, serum	AIDS	0	1:256	0	0	0	+	0
8, CSF	AIDS	0	1:4	0	0	0	+	0
9, CSF	Cong. inf.	0	1:64	0	+	0	0	0

[a] Abbreviations: CSF, cerebrospinal fluid; Lymphad, lymphadenitis; Cong. inf., congenital infection. Test results: +, positive; 0, negative.

of IgM, as well as IgA, antibodies in all six patients with AIDS with proven cerebral toxoplasmosis. Therefore, detection of IgM antibodies in this group of patients might depend on the diagnostic technique, as was also shown by a recent investigation comparing DS-ELISA and ISAGA (12). In contrast to a recent report (28), Darcy et al. (6) demonstrated the presence of specific IgA antibodies in sera of HIV-infected patients with or without cerebral toxoplasmosis. By analyzing samples from patients in group E, we were able to demonstrate that in 91.66% of the samples drawn from patients with AIDS with cerebral toxoplasmosis, P30-specific IgA was detected by the immunoblot technique. The diagnosis of cerebral toxoplasmosis in these patients was proven by detection of *T. gondii* in brain biopsies or cerebrospinal fluid samples by PCR or the mouse inoculation test, by clinical improvement following specific therapy, or by demonstration of *T. gondii* at autopsy. However, it must be mentioned that in group E we obtained one false-positive result for IgA antibodies by immunoblotting, as well as for IgM antibodies by ELISA, i.e., with samples from a patient with autopsy-proven multicentric lymphoma. The IgA response has widely been associated with the route of infection, i.e., involvement of the gut-associated immune system (3). Our results, as well as those of a recent investigation (20), demonstrated that a specific IgA antibody response can also be observed after parenteral immunization or infection and thus seems not to depend on the route of infection. An unproven mechanism for the occurrence of specific IgA antibodies in cerebral toxoplasmosis also might be isotype switching of B cells (29).

It has been suggested that the serology of HIV-infected patients with cerebral toxoplasmosis is not helpful diagnostically (32). As could be demonstrated in our study, detection of P30 IgA antibodies might be a useful additional serological assay for determining the stage of infection in these patients. However, more samples have to be examined to confirm these preliminary results. As was shown by a study with 20 patients with toxoplasmosis and AIDS, sampling of the cerebrospinal fluid was found to be of little clinical value (12). Nevertheless, in certain cases it was possible to demonstrate local intrathecal production of antibodies to *T. gondii* in HIV-infected patients with cerebral toxoplasmosis (16, 37). As was shown by us, demonstration of IgA or IgM antibodies in cerebrospinal fluids from patients with proven cerebral toxoplasmosis might be possible by the P30 immunoblotting technique.

In contrast to previous studies, we used two different strains of *T. gondii* simultaneously in consideration of possible strain-specific human immune responses. Indeed, it was shown that about 4.5% of all clinical samples contained IgA or IgM antibodies to P30 that reacted exclusively with only one *T. gondii* strain in the immunoblot. These strain-specific human antibody responses might explain different test results when tests are performed in laboratories using different *T. gondii* strains as antigenic sources. This is the first report describing this diagnostically important phenomenon. This finding is in agreement with a recent observation demonstrating that *T. gondii* strain variations may account for antibody diversity among patients with AIDS and cerebral toxoplasmosis (34). However, the observed differences in antigenic recognition of human sera in that study were independent of diagnostically important major surface protein P30. Although P30 was thought to be present in tachyzoites of all of the *T. gondii* strains tested so far (4), our results demonstrated that strain-specific differences in the epitope repertoire of P30 which are responsible for strain-dependent P30 reactivity might exist. However, many more samples have to be examined to confirm these results, as well as to get more insight into the epidemiology of *T. gondii*. Moreover, monoclonal antibodies to P30 may be helpful in defining strain-specific epitopes.

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