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Toxoplasma gondii Antigens Recognized by Sequential Samples of Serum Obtained from Congenitally Infected Infants

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The Sabin-Feldman dye test, the immunoglobulin M (IgM) immunosorbent agglutination assay, and the immunoblot technique were used to study the evolution of the antibody response to *Toxoplasma gondii* and to examine antigens of the organism recognized by antibodies in the sera of 12 congenitally infected infants and 7 mothers. In the sera of eight infants, a significant rise was noted in the dye test titers, while the serum of only one infant demonstrated a late increase in the IgM immunosorbent agglutination assay titer. In each infant and mother, antigens with approximate masses of 35,000 and 115,000 daltons were strongly recognized by IgG antibodies. An antigen(s) with an approximate mass of 4,000 daltons was recognized by IgM antibodies in the sera of each of the mothers but was recognized by antibodies in the sera of only two of the infants.

The recognition of certain congenitally acquired diseases is based on the in utero production of antibodies by the fetus (12). Despite the capability of antibody production, the fetal immune response is immature compared with that of adults. It is well established that this gap narrows and that the immune system of the newborn undergoes major developments during the first months of life. Although a great deal of information has accumulated over the years on the clinical course of congenital toxoplasmosis (3, 12), few studies have dealt with the changes in the serological response to this infection during the first months of life (1). Likewise, the available information on the antigens of *Toxoplasma gondii* against which antibodies are formed early in life is scant (11). Such information would be useful not only for the study of the antigenic structure of the organism but also for a better understanding of the humoral immune response during infancy.

We recently used the immunoblot technique to study the antibody response to *T. gondii* in adults acutely infected with the organism (9). We demonstrated that certain bands, indicating antigen-antibody reactions, appeared frequently on the immunoglobulin G (IgG) and IgM immunoblots of all the individuals examined. Because of the evolving nature of the immune response of neonates and infants, we considered it of interest to use the immunoblot technique to examine the changes in recognition of the antigens of *T. gondii* during the first year of life.

MATERIALS AND METHODS

Patients. Our respective laboratories are large referral facilities for *T. gondii* serology. We identified sera from 12 infants previously evaluated by the Research Institute of the Palo Alto Medical Foundation and the Toxoplasmosis Laboratory of the Institut de Puériculture de Paris. Sera from seven of the mothers were available for testing. Sera from the infants were received for testing mainly because their mothers had acquired *T. gondii* infections during gestation. The diagnosis of congenital toxoplasmosis was established by previously described criteria (12). Infants up to 18 months of age were included in the study only if at least two serum samples were available for testing. Clinical and serological follow-up testing was done at various intervals. The infants were usually treated for 1 year with alternating courses of pyrimethamine-sulfadiazine and spiramycin (2). Serological relapse is defined here as a rise in titer not associated with new clinical disease.

Serology. All sera were tested by the Sabin-Feldman dye test (DT) (13) and by the IgM immunosorbent agglutination assay (IgM ISA) (4) (Bio-Merieux, Charbonnieres, Les Bains, France). The results of the IgM ISA are expressed as titers from 0 (negative) to 12. Briefly, sera diluted 1:20 are placed into three wells of microtiter plates previously coated with anti-human IgM. After incubation and washing, different volumes of a fixed concentration of killed tachyzoites are added to the three wells (e.g., 1.5×10^6, 2.0×10^6, and 3.0×10^6 organisms per well). Agglutination in each well is scored from 0 to 4+, and the results are added together (maximum = 12) and expressed as a number from 0 to 12 (titer). The sera from France were shipped diluted 1:2 in glycerol to prevent bacterial overgrowth and stored at 4°C upon arrival at our laboratory. A pool of equal amounts of serum samples obtained from normal individuals who were negative in both the DT and the double-sandwich IgM enzyme-linked immunosorbent assay (7) was used as a negative control for the immunoblots. A serum sample from an acutely infected adult with a DT titer of 154 IU/ml and an IgM enzyme-linked immunosorbent assay titer of 10.8 was used as a positive control.

Polyacrylamide gel electrophoresis. Lyophilized *T. gondii* antigen prepared as previously described (14) was dissolved in sodium dodecyl sulfate buffer to yield a solution containing 1 mg of protein per ml. Gradient slab gels (5 to 15%) with one lane 1 cm wide for application of the molecular weight (MW) standards and another lane 10 cm wide for application of the antigen preparation were made by using an exponential-gradient maker (Hoefer Scientific Instruments, San Francisco, Calif.). *T. gondii* antigen (200 μg) was distributed evenly on the top of the wider lane and electrophoresed as

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previously described (6). Myosin, β-galactosidase, phosphorylase *b*, bovine albumin, egg albumin, and carbonic anhydrase (Sigma Chemical Co., St. Louis, Mo.) were used as MW markers.

Immunoblotting. Transfer of proteins to nitrocellulose paper (0.45-μm-pore size; Schleicher & Schuell, Inc., Keene, N.H.) was performed by the method of Towbin et al. (15) with a Trans-Blot-Cell (Bio-Rad Laboratories, Richmond, Calif.). Proteins were transferred at 180 mA over 3 h. A vertical strip of the blots which contained the MW markers and a portion of the separated antigens were cut from the blots and stained with amido black. The remainder of the blots were soaked in a solution of 5% (wt/vol) nonfat dry milk (Carnation Co., Los Angeles, Calif.) in phosphate-buffered saline (PBS) for 1 h at room temperature to block unbound sites and then washed for 10 min in PBS containing 0.05% Tween 20.

Immunochemical detection of antigens. All serum samples from each infant were tested in parallel as previously described (9). Briefly, a 1.4-cm-wide and 14-cm-long nitrocellulose strip cut from a single blot (10 by 14 cm) containing the separated antigens was treated with each serum sample. The strips were incubated overnight with serum diluted 1:50 in PBS containing 1% bovine serum albumin and 0.05% Tween 20 (solution A). In each experiment, the negative and positive controls were tested in parallel with the patient's sera. After incubation, the strips were washed with PBS containing 0.05% Tween 20. Thereafter, the strips were cut in half longitudinally and overlaid for 1 h with horseradish peroxidase-conjugated rabbit anti-human IgG or IgM antibodies diluted 1:8,000 in solution A. The strips were again washed as described above and immersed in the substrate solution (0.1 mg of diaminobenzidine [Bionetics, Charleston, S.C.] per ml and 0.25% H_2O_2 in PBS). The developed bands on the various strips were scored according to their intensity from a (barely visible) to d (intensely stained). Each band was assigned a number corresponding to its MW and a letter from a to d.

The 1:8,000 dilution of the conjugate was used because in preliminary studies (data not shown) we found that higher concentrations markedly intensified the "natural-antibody" bands (10), thereby making the blots more difficult to read. Also, as shown in Results, when using the 1:8,000 dilution of the conjugate, we observed an increase in intensity in these natural-antibody bands concomitant with an increase in the DT titer; this increase was not noted when higher concentrations of the conjugate were used.

RESULTS

Serology. The study group (Table 1) consisted of 12 infants; 5 had congenital toxoplasmosis diagnosed at the time of or soon after birth, and 7 had subclinical *T. gondii* infections. Six of the seven serum samples from the mothers had very high (10 to 12) IgM ISA titers.

A significant (at-least-fourfold) rise in the DT titer at any point in the follow-up period was noted with the sequential serum samples obtained from eight of the infants (no. 2, 4, 7, 8, 9, 10, 11, and 12). In five (infant no. 2, 6, 9, 10, and 11) of seven cases in which at least three samples were available, a drop in the DT titer was followed by an increase. This increase was observed as early as 4 months of age (infant no. 2), while the infant was still being treated. Remarkably high DT titers (reaching 3,200 IU/ml) were found in six of the infants (no. 1, 3, 5, 6, 7, and 9), and in five of these infants, IgM antibodies were demonstrated at the peak of DT reactivity. Six infants (no. 1, 3, 5, 6, 7, and 9) had positive IgM antibody titers, all of which were associated with DT titers of ≥ 1,600 IU/ml. High IgM titers usually declined to undetectable levels after 6 months of age, except for infant no. 7, in whom a high IgM titer was present at 12 months of age even though a previous serum sample collected from the same infant at 9 months of age was negative for IgM antibodies.

Immunoblots. Figures 1 to 3 are examples of the immunoblots obtained with sera from the infants and their mothers. Because many bands on the immunoblots were faint and did not reproduce well in the photographs, the results are also presented in Table 1. The serum samples from each infant and mother were examined by using strips of the same blot to allow for comparison and observation of the evolution with time of the different bands and the overall pattern. Bands present on the blots of the negative control serum stained more intensely on the IgM blots and were mainly in the MW range of 50,000 to 120,000, although occasionally a strongly staining band was seen in the area above the 205,000-MW marker. We have previously observed these bands, which correspond to "naturally occurring antibodies," on immunoblots of most normal seronegative individuals (10).

Not unexpectedly, the intensity of the staining of the bands on the IgG blots correlated well with the DT titers. When a significant decrease in DT titers occurred, most bands on the corresponding IgG blots decreased in intensity when compared with the immunoblots developed with a previous serum sample (e.g., Fig. 1, IgG strip 8, and Fig. 3, IgG strip 34). The converse was true when an increase in DT titers was noted (e.g., Fig. 1, IgG strips 8 and 9). Insofar as the bands on the IgM blots of the infants were substantially fainter than the bands on their IgG blots, it was difficult to establish a correlation between the IgM ISA titers and the change in the band pattern on these blots.

It is interesting that bands were frequently observed on immunoblots developed with sera that were negative in the IgM ISA and that in one case (infant no. 3), no bands were observed when the serum sample had a titer of 12. Of the relatively few bands found on the IgM blots of the infants who had positive IgM ISA titers, the most prevalent band (five of six cases) had an approximate MW of 60,000; other bands were found at MWs of 240,000, 200,000, 180,000, 115,000, and 35,000.

The most consistent finding on the immunoblots was the appearance of bands of the IgG blots which corresponded to the approximate MWs of 35,000 and 115,000. Bands corresponding to these MWs were seen in the IgG blots of all 12 infants and 7 mothers. A faint band corresponding to an MW of 115,000 was also noted on most strips developed with the negative control serum. However, the intensity of the staining of this band on the latter strips was always lower than the intensity of the corresponding band on the patient's strip. In contrast, a band corresponding to an MW of 35,000 was infrequently found on strips developed with the negative control serum, and when present it was very faintly stained. The 35,000- and 115,000-MW bands were notably darker than the other bands (Fig. 1 to 3). In all of the immunoblots of infant sera, these bands scored at least c.

Interestingly, a band(s) with an approximate MW of 4,000 was found in all IgM blots of adult patients with acute *T. gondii* infections (9) but was only occasionally seen in the IgM blots of the infants. Whereas this band(s) was detected in each of the IgM blots of the seven mothers, it could be detected in the IGM blots of only two of the infants (no. 6, third sample, and 8, second sample); in five other infants (no.

TABLE 1. Results of serological tests and antigen recognition by antibodies in the sera of infants congentially infected with *T. gondii*

Origin of serum[a]	Mo from birth in which serum was obtained[b]	IgM ISA titer	DT titer (IU/ml)	Antigen(s) recognized by[c]: IgM	Antigen(s) recognized by[c]: IgG	Comments
M1		12	400	4c, 35b, 62b, 75b, and 105b (7)	35c, 62b, and 95b (4)	
B1	0	12	1,600	None	35d and 115d (7)	Chorioretinitis
	0.5	11	3,200	250a	35d and 115d (7)	
	2	0	800	None	3c and 115b (3)	
	12	0	400	4a (4)	35b and 115b (4)	
M2		11	400	4c, 28b, 90b, and 205b (4)	25d and 115b (5)	
B2	0.5	0	200	None	35d and 115b (5)	IgG synthesis delayed
	2	0	40	None	25c and 115a (5)	
	4	0	800	4a	25c and 115d (6)	
M3		3	3,200	4c, 35b, 85b, 90c, and 210d (5)	29b, 35d, 48b, 90b, and 115c (6)	
B3	0.5	12	3,200	35d (3)	35d and 115c (11)	Subclinical infection
	3	12	1,600	None	None	
B4	6	0	200	35a and 115a (5)	35d and 115c (2)	Subclinical infection
	9	0	800	115a (4)	35d and 115c (6)	
M5		12	1,600	4c, 35c, 50c, 62c, 85c, 110b, and 115b (6)	4c, 28b, 35d, 62b, 72b, 95b, 140b, 195b, and 200b (4)	
B5	0	9	3,200	(4)	4c, 35d, and 115d (15)	Optic and cerebral lesions
	0.5	4	3,200	35a (3)	4c, 35d, and 115d (15)	
	7	7	1,600	None	35d, and 115b (1)	
B6	1	12	3,200	145b (6)	35d and 115c (11)	Serological relapse after 6 mo
	6	0	800	35a (1)	35d and 115c (5)	
	10	0	1,600	4b (7)	35d and 115d (14)	
B7	9	0	400	(2)	(11)	Subclinical infection and serological relapse after 9 mo
	12	11	3,200	4a (2)	4c, 35d, and 115d (12)	
B8	14	0	100	35a (3)	35d and 115a (3)	Subclinical infection and serological relapse after 14 mo
	18	0	1,600	4a (3)	4a, 35d, and 115d (15)	
M9		12	1,600	4c, 32b, 35b, 97b, 115b, 200b, and 240b (3)	29b, 35d, 58b, 65b, 95c, and 115d (5)	
B9	1	3	1,600	(3)	35d and 115d (14)	Subclincal infection and serological relapse after 5 mo
	5	5	200	None	35d and 115b (8)	
	10	10	3,200	None	4a, 35d, and 115d (18)	
M10		10	1,600	4c, 35b, 62b, 97b, and 115b (1)	29c, 35d, 44b, 48b, 67b, 95b, 115d, and 170b (7)	
B10	0	0	800	115a	35d and 115d (15)	Serological relapse after 3 mo
	3	0	80	None	35d and 115b (5)	
	5	0	400	4a	4b, 35c, and 115d (14)	

Continued on following page

TABLE 1—*Continued*

Origin of serum[a]	Mo from birth in which serum was obtained[b]	IgM ISA titer	DT titer (IU/ml)	Antigen(s) recognized by[c]: IgM	Antigen(s) recognized by[c]: IgG	Comments
B11	0	0	1,600	35a (6)	4a, 35d, and 115b (13)	Subclinical infection and serological relapse after 6 mo
	6	0	80	35b and 115a (6)	35d and 115d (13)	
	12	0	1,600	4a, 35a, and 115 (6)	35d and 115d (10)	
M12		12	3,200	4b and 115b (5)	35c, 43b, 50b, 60b, and 115c (13)	
B12	4	0	80	None	35 and 115b (9)	Subclinical infection and chorioretinitis at 10 mo
	10	0	800	35a and 115b (1)	35c and 115d (10)	

[a] M1 = mother no. 1; B1 = baby no. 1.
[b] 0, Serum was collected at birth.
[c] Numbers correspond to appropriate MWs (in thousands) of well-defined, major antigen-antibody bands; letters correspond to the intensity of staining of the bands as defined in the text; numbers in parentheses correspond to the numbers of additional, faintly stained, minor bands with various MWs.

1, fourth sample, 2, third sample, 7, second sample, 10, third sample, and 11, third sample) the presence of this band was questionably present.

The band patterns on the IgM blots of the infants whose mothers' sera were available for comparison were rarely different from those of the mothers. In three cases, bands that were present on the IgM blots of the infants were not present on the IgM blots of the mothers. These bands corresponded to approximate MWs of 250,000, 200,000, 105,000, and 97,000. The following bands were found exclusively on the IgG blots of the infants: a 250,000-MW band on strip no. 16 (Fig. 2), 45,000- and 47,000-MW bands on strip no. 33 (Fig. 3), and a 200,000-MW band on strip no. 12 (second serum sample) (data not shown). It is also of interest that the IgG strip developed with the first serum sample from infant no. 1 (data not shown) had 35,000- and 115,000-MW

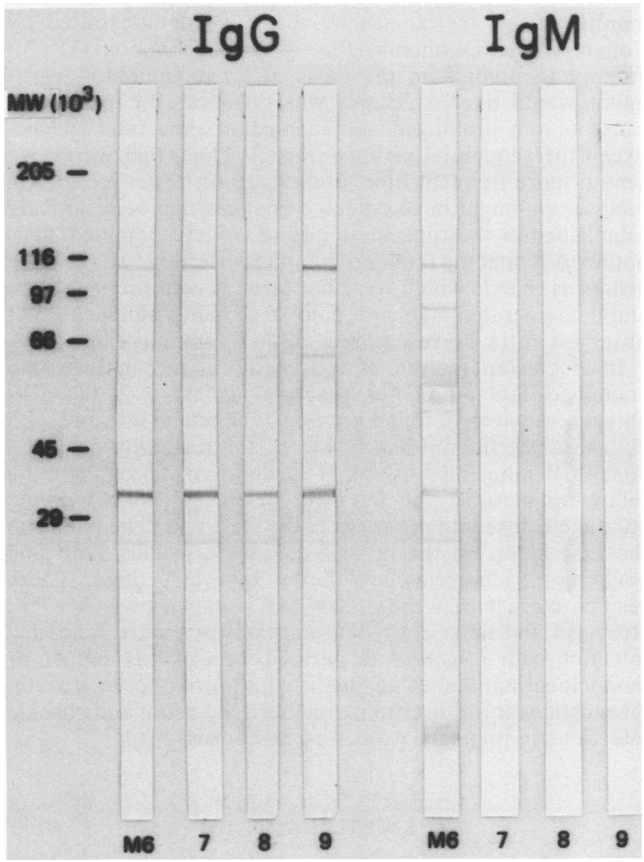

FIG. 1. Protein blot of sequential serum samples (strips 7 to 9) obtained from case 2 (corresponding to infant no. 2, first, second, and third samples, respectively, in Table 1); M6 was obtained from the mother's serum. MW markers are depicted on the left.

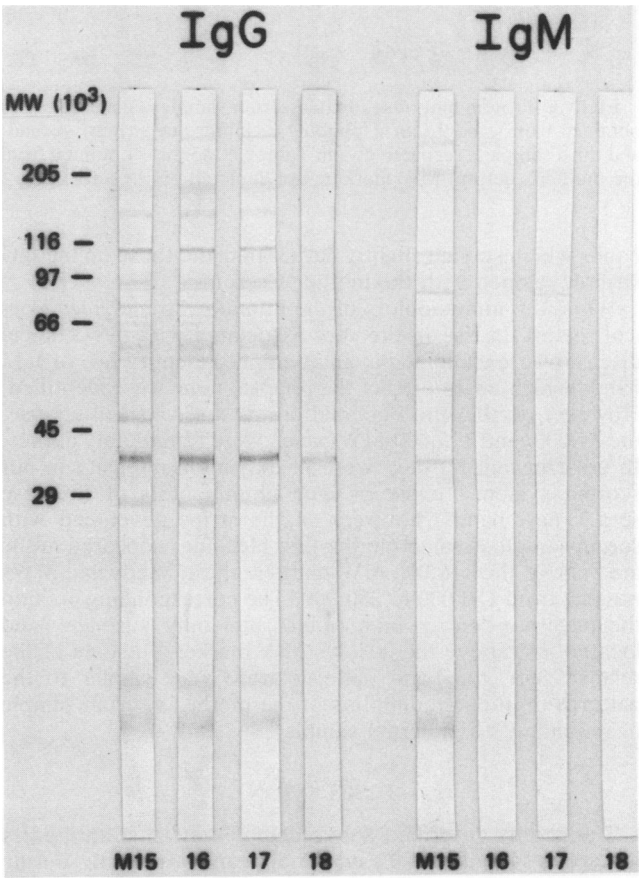

FIG. 2. Protein blot of sequential serum samples (strips 16 to 18) obtained from case 5 (corresponding to infant no. 5, first, second, and third samples, respectively, in Table 1); M 15 was obtained from the mother's serum. MW markers are depicted on the left.

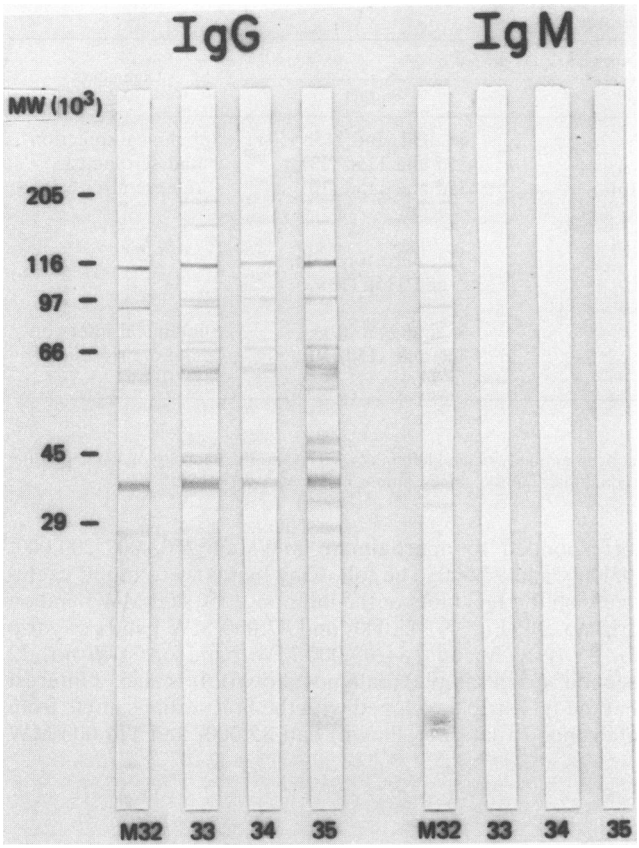

FIG. 3. Protein blot of sequential serum samples (strips 33 to 35) obtained from case 9 (corresponding to infant no. 9, first, second, and third samples, respectively, in Table 1); M32 was obtained from the mother's serum. MW markers are depicted on the left.

bands which stained slightly darker than did those on the IgG strip developed with the mother's serum.

When the immunoblots of the infants who had a serological relapse (a rise in titer not associated with new clinical disease) were examined in an attempt to identify IgG or IgM bands which might predict the relapse, none were identified. However, in the sera obtained during a serological relapse, the 35,000- and 115,000-MW bands were remarkably darker. In most instances, they were of maximum intensity in our scoring system. In six of nine immunoblots of "relapse sera," new bands, not seen in the strips developed with serum sample obtained in the first bleeding, appeared in the area above the 116,000-MW marker; these bands had MWs ranging from 150,000 to 250,000. The corresponding area on the negative control immunoblot had only a single band located just above the 205,000-MW marker. In some of the relapse sera, the band patterns were very similar to the patterns on the immunoblots of the mother's serum sample or the infant's first serum sample.

DISCUSSION

The results described above demonstrate that antibodies to certain antigens of *T. gondii* appear consistently during the first 18 months of life in infants infected in utero. Each of the 12 infants and 7 mothers had IgG antibodies against 35,000- and 115,000-MW antigens, and the bands corresponding to these antibodies were consistently the most darkly stained. The results also revealed a direct correlation between the intensity of this band pattern and the DT titer.

We recently discovered a group of adults with acute *T. gondii* infections in whom the most common and intensely staining band represented an antigen having an MW of 35,000 (9). A similar 35,000-MW band has also been reported by others in mice and humans (5, 8). This band was rarely detected on immunoblots of noninfected controls. Each of the immunoblots of the sera of the mothers we studied had an IgM band corresponding to an antigen(s) with an MW of 4,000. This band was previously reported in acute acquired *T. gondii* infections (9). In contrast, this 4,000-MW band was infrequently found or barely seen in the immunoblots of the sera of the infants we studied. Because the volume of most serum samples was too small, the immunoblot could not be repeated at a higher serum concentration. Of particular interest was the lack of this band(s) in the immunoblots of some of the infants who had remarkably increased IgM ISA titers (e.g., infant no. 1, first sample, and 3, second sample). A similar discordance in the recognition of low-MW antigens was observed in another study from our laboratory (11). In some cases, the absence of the 4,000-MW band(s) may have been due to a low concentration of the specific antibody, to an inability of the infant's immune system to recognize these low-MW antigens, or to the possibility that these low-MW bands do not represent antigens necessary for a positive IgM ISA.

Passively transferred maternal antibodies gradually decline during the first month of life. In five of the infants we studied, a substantial increase in DT titers occurred at 6 to 12 months of age (serological relapse). As demonstrated by Couvreur and Desmonts, this is not uncommon (1). An attempt to predict on the basis of an immunoblot which infant would have a relapse was unsuccessful, partly because of the prolonged and inconstant time intervals between the sequential serum samples. Thus, had we drawn sera at more frequent time intervals prior to the serological relapse, we might have detected changes that were already established at the time the study sera were obtained. It is noteworthy that the immunoblot of relapse sera had, in some instances, bands which were not present with previous sera and demonstrated a general pattern of bands similar to that observed in the corresponding mother's immunoblot.

In conclusion, the sera of each of the children in this group contained IgG antibodies reactive against a 35,000-MW antigen, as we have found previously in adults infected with *T. gondii* (9). In contrast, however, the reactivity against a 4,000-MW antigen was weak in the children. Throughout the follow-up periods, the intensity of the 35,000-MW band paralleled closely the changes in the DT titers. The bands on the IgM blots, on the other hand, were usually faint and could not be correlated with the IgM ISA titers. These results suggest that the 35,000-MW antigen provokes the strongest and most consistent antibody response following infection with *T. gondii*. A purified form of this antigen or monoclonal antibodies against it could prove to be a useful diagnostic test for discriminating between acute and chronic infections in pregnant women or newborns.

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