Demonstration of *Toxoplasma* Antigen Containing Complexes in Active Toxoplasmosis

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With an enzyme-linked immunosorbent assay antigen, specific circulating immune complexes (CIC) were demonstrated in experimental and human toxoplasmosis. In experimentally infected mice, CIC became demonstrable as soon as antibodies appeared after fatal infection. When a nonvirulent strain of *Toxoplasma* was used CIC remained detectable for several weeks. This period was characterized by clinically healthy animals with increasing antibody titers and by cysts growing in the brains of the animals, indicating a subacute stage of the toxoplasma infection. In the human sera, a surprisingly high percentage of CIC was demonstrated. Both immunoglobulin G (IgG) and IgM were found in the CIC; however, IgG was seen in the majority. If the humans were grouped according to other serological results, such as a combination with IgM antibodies, circulating antigens, or both, or a positive complement fixation test, increasingly more CIC were observed. When sera were selected from patients with clinical symptoms generally associated with toxoplasmosis, more CIC were also again demonstrated. On the contrary, in healthy individuals (blood donors), CIC were also regularly observed, suggesting that exacerbations of latent infections or reinfections may regularly occur without leading to clinical signs. In conclusion, we propose that the interpretation of a positive CIC test requires great care but may provide useful information about the activity of a toxoplasma infection.

In the normal immune response, individuals exposed to antigens react by synthesizing specific antibodies which form in vivo immune complexes. Antigens become neutralized, eliminated, or both in this way. This (patho-) physiological phenomenon seldom leads to harmful situations such as the deposition of complexes in tissues. In fact, the immune complex formation and circulation may induce inflammatory and regulatory reactions leading to the elimination of infective agents and systemic immunity (21). Particularly in chronic infections, including parasitic infections such as malaria (9, 10), schistosomiasis (a.o. 1, 8), trypanosomiasis (3), and filariasis (20), circulating immune complexes (CIC) have been described. Although toxoplasmosis is a typically chronic infection, only one recent publication (19) indicates that immune complex-like material is frequently present in sera of patients with toxoplasmosis. However, a few papers deal with glomerular deposititis associated with congenital and acute toxoplasmosis (26, 7, 18). It is very likely that in situations where circulating antigens and circulating antibodies (sometimes at high levels) appear simultaneously, immune complexes are formed as well. In the current study, efforts were made to demonstrate CIC in sera from mice experimentally infected with *T. gondii* strains and in human sera. For this purpose, antiserum was used, which proved to be suitable for the demonstration of free circulating antigen (23, 25). This method is in contrast with generally applied non-antigen-specific methods for detecting CIC but, as a consequence, offers the possibility of a direct association between the complexes detected and a toxoplasma infection.

MATERIALS AND METHODS

Animal model. Female Swiss mice bred at the National Institute of Public Health and Environmental Hygiene (ca. 6 weeks old; free of toxoplasma antibodies) were infected with

either the virulent RH strain of toxoplasma (5×10^7 tachyzoites; injected intraperitoneally) or with a nonvirulent cyst-forming toxoplasma strain (T 626) by injecting 20 brain cysts intraperitoneally. Biological materials for the various examinations were collected per group of five animals at regular intervals (see tables) (from serum, blood smears, peritoneal exudate, and brains). The sera of each group were pooled before testing.

Human sera. Sera of individuals suspected of having toxoplasmosis were submitted for routine examinations to the Laboratory for Parasitology and Mycology. A comparison with other routine serological parameters (enzyme-linked immunosorbent assay [ELISA] for immunoglobulin G [IgG], IgM, and antigen) was made with the help of 984 of such unselected samples. A second group of sera were selected from patients with definite signs of clinical illness which might well be associated with toxoplasmosis. Within that group, those samples which showed a positive titer in the complement fixation test (CF test) were separated from those without. No other conformational information about the actual diagnosis of toxoplasmosis was available. As a control, the sera of 121 healthy blood donors were tested for the presence of CIC.

A serological follow-up study was performed with serum samples from a patient who contracted a toxoplasma infection (laboratory accident). A recurrence of serious clinical symptoms 7 months after the initial infection suggested exacerbation of the infection. All available tests were performed for both sets of data for comparison of the profiles. Finally, some ($n = 10$) sera with a positive immune complex test were treated with 5% polyethylene glycol (PEG 6000; E. Merck AG, Darmstadt, Federal Republic of Germany) and centrifuged for 1 h at 4°C ($30,000 \times g$). The supernatants were retested for immune complexes.

Serology. The Sabin Feldman test, direct immunofluorescence, ELISA for the detection of specific IgG, IgM, and circulating antigen, and the CF test were carried out as

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FIG. 1. Application of ELISA for specific antibody-antigen complex detection.

described in detail earlier (14, 15, 17, 22–24; F. van Knapen, Ph.D. thesis, University of Amsterdam, Amsterdam, 1984).

ELISA for CIC. The principle of the ELISA for CIC is schematically presented in Fig. 1. It is based on the same principle as for the detection of circulating antigens. If present, the antigen may be covered partially by immunoglobulins. The sandwich is completed with enzyme-labeled, anti-species-specific immunoglobulin serum (conjugate).

The different steps in the assay were as follows. Rabbit anti-toxoplasma F(ab')₂ was diluted in 0.01 M phosphate-buffered saline (pH 7.2) to a protein concentration of 10 µg/ml. Incubation in microtiter trays was done with 100 µl per well at 37°C for 1 h. A washing was carried out with phosphate-buffered saline containing 0.05% Tween 20 for 5 min and repeated twice. Fresh undiluted patient sera and positive and negative reference sera were incubated at 37°C for 1 h. Washing was carried out as above. The conjugate was diluted optimally in phosphate-buffered saline containing 4% bovine serum albumin and 0.05% Tween 20. An incubation at 37°C for 1 h was used. Depending on whether the objective was to measure IgG or IgM in the immune complexes, the following conjugates were used: sheep anti-human IgG horseradish peroxidase-labeled antibody (heavy chains) commercially obtained from Pasteur Institute, Paris, or rabbit anti-human IgM horseradish peroxidase-labeled antibody prepared in the Institute (Unit of Immunochemistry). After washing (as described above), the substrate was added. Preparation of the substrate was as follows: 80 mg of 5-amino-2-hydroxybenzoic acid was dissolved in 100 ml of distilled water at ca. 70°C. After cooling to room temperature, the pH was adjusted to 6.0 exactly with 1 N NaOH. To 9 parts of the dissolved substrate 1 part of 0.05% H₂O₂ was added. After shaking, 0.1 ml was added to each well. Incubation occurred at room temperature. The visual interpretation was done after 1 h. Optical densities different from those obtained with a negative reference serum (which was suitable as an activator serum in the Sabin Feldman test) were regarded as positive.

RESULTS

The experimental infection of mice with the virulent RH strain of *T. gondii* was given at a dosage resulting in the

TABLE 1. Experimental infection of Swiss mice with a virulent RH strain of *Toxoplasma* (5×10^7 tachyzoites injected intraperitoneally)

Day post-infection	n	Sabin Feldman titer	ELISA with:		Direct immuno-fluorescent blood smear (no. positive/no. tested)	Death (no. dead/no. tested)
			Circulating antigens	CIC		
0	5	<1:4	—	—	—	—
1	5	<1:4	+	—	+	—
2	5	1:16	+	—	+	—
3	5	1:512	+	+	+	—
4	5	NM ^a	NM	NM	+	5/5

^a NM, No material.

death of the animals between days 3 and 4 after infection (see Table 1). Already at day 3, antibodies as well as CIC could be demonstrated (Sabin Feldman titer, 1:512). Circulating antigen as well as free tachyzoites in blood smears was detectable from day 1 onward.

When an infection was given with the nonvirulent T 626 strain, all animals survived (see Table 2). For just a few days after day 7 of infection, the mice looked clinically ill, but all of them recovered. Antibodies were detectable from that time onward, remained for some time at a relatively high level, and seemed to peak around day 35. Circulating antigen was only detected at day 14. From day 13 until at least day 35, CIC were detectable in the peripheral blood. Occasionally some free tachyzoites were observed in blood smears. At a later stage (day 75), only antibodies could be measured as a consequence of the latent (chronic) toxoplasmosis. During the same observation period (until day 35), mouse brains were examined for the presence of toxoplasma cysts. The diameters of the smallest cysts observed were used to express the growth of the cysts once formed. It can be seen (Fig. 2) that, during the observation period (until day 35), the cysts were steadily growing.

In 984 human serum samples of individuals suspected of having toxoplasmosis, immune complexes with IgG, IgM, or both were detected in 123 cases (12.5%). In 96 serum

TABLE 2. Experimental infection of Swiss mice with a nonvirulent *Toxoplasma* strain (T 626; 20 cysts injected intraperitoneally)

Day post-infection	Sabin Feldman titer ^a	ELISA with:		Direct immuno-fluorescent blood smear
		Circulating antigens	CIC	
0	<1:4	—	—	—
3	<1:4	—	—	—
5	<1:4	—	—	—
7 ^b	1:8	—	—	—
9	1:512	—	—	Few
11	1:2,048	—	—	—
13	1:2,048	—	+	—
14	1:1,024	+	NM ^c	Few
21	1:2,048	—	+	—
28	1:1,024	—	NM	—
35	1:8,192	—	+	—
75	1:1,024	—	—	—

^a Mean titer of five pooled sera.

^b Animals became clinically ill around day 7.

^c NM, No material.

FIG. 2. Size of smallest toxoplasma cysts (in micrometers) in mouse brains after infection with a nonvirulent toxoplasma strain (T 626).

samples, only complexes with IgG were noticed. In 15 serum samples, only IgM complexes were noticed, and in 12 serum samples, both immunoglobulins were demonstrated in CIC. The data of these observations were also related to other serological results obtained with the same sera (Table 3, category 1). CIC were demonstrated in both seropositive and seronegative individuals. Increasingly more CIC were noted when specific IgM antibodies were demonstrable and definitely when circulating antigens were also found. When a selection was carried out for clinical symptoms possibly associated with a toxoplasma infection (category 2), a considerable number of positive CIC determinations was found (37%). Moreover, when a positive CF test result was used as a second selection criterion, as much as 57% of the sera were positive for CIC. In contrast, results of the ELISA for CIC with sera of healthy blood donors (category 3) are also presented. A remarkably high percentage showed positive results.

In Fig. 3, the antibody profiles of specific anti-toxoplasma IgG and IgM of a patient with an active toxoplasma infection and a recurrence of the infection 7 months later are pre-

sented. Also, the profiles of circulating antigen and CIC (both with IgG and IgM) are presented. A striking similarity is observed between the profiles of the CIC and the circulating free antigens. Moreover, the detection of CIC seems to represent an amplification of detectable free circulating antigens. Ten CIC-positive sera treated with polyethylene glycol were retested for CIC. All sera yielded negative results.

DISCUSSION

The study of immune complexes is an important step toward improving serology and understanding the mechanisms of persistent infection and immunopathology of chronic parasitic diseases in which large amounts of parasitic antigen, corresponding antibody, and immune complexes may be present (21). In *Toxoplasma* infections, detection of circulating antigen is possible during the acute stage (2, 23). In the present study the formation of immune complexes was followed in experimentally infected mice.

The principle of the ELISA-CIC method (Fig. 1) allows us to detect immune complexes through an antigen-specific site, in contrast to methods generally applied, i.e., detection through nonspecific precipitation, binding toward immunoglobulins or complement, or both (21). Theoretically, it may be that such an approach is only beneficial if the antigen is present in excess. The latter is certainly true in the acute stage of fulminating infections with *T. gondii* strains. Therefore, the first experiment was carried out by infecting Swiss mice with the virulent RH strain of *T. gondii*. In just a few days, the animals built up antibodies during an overwhelming infection from which they would eventually die (Table 1). This results in detectable immune complexes by day 3 after infection.

Using a nonvirulent strain of *T. gondii* resulting in a chronic (latent) infection, may be more relevant for the situation with human *T. gondii* infections. In this experiment (Table 2), the mice were clinically ill during a few days about 1 week after the inoculation. Antibodies and brain cysts (Fig. 2) became detectable from that time onward. Circulating antigen (indicative of the acute stage) was detectable for a

TABLE 3. Prevalence of toxoplasma antigen containing CIC in sera of various origins

Category	Serology	% Con- taining CIC	No. ex- amined
1. Unselected individuals (suspected of having toxoplasmosis)	IgG positive	11.8	525
	IgG negative	9	242
	IgG + IgM positive	15	181
	Circulating antigen positive	66.6	18
2. Selected patients			
	Clinical signs associated with toxoplasmosis	37	80
	Clinical signs as above in combination with positive CF test	57	28
3. Blood donors		11.5	121

FIG. 3. Specific antibody measurement (IgG, IgM) during a follow-up study of a case of acute infection and exacerbation of chronic human toxoplasmosis. ELISA extinction value was measured at 449 nm after 60 min of incubation at room temperature.

very short period only. However, if more observations would have been included during this period, more positive results for circulating antigen might have been found. Nevertheless, this confirms the hypothesis (23) that free antigens may circulate for limited periods only and supports the idea that immune complexes are formed to eliminate the antigen. In fact, we demonstrated the presence of CIC from day 13 postinoculation until weeks thereafter (Table 2). The latest day on which CIC were detectable in this experiment was day 35 postinoculation. The other characteristics of the toxoplasma infection during this period were increasing antibody titers measured with the Sabin Feldman dye test to relatively high levels (1:8,192) and continually developing brain cysts, at least with regard to diameter (Fig. 2), as an indication of the activity of the parasite. Furthermore, incidentally free circulating tachyzoites were observed in blood smears. Although the animals had all recovered from clinical illness, one could assume that the toxoplasma infection had not yet come to an equilibrium between parasite activity and host immunity. At a later stage (day 75), the only evidence of a past infection was the presence of serum antibodies. In the mouse model, the presence of CIC seems to parallel the acute and subacute stages of a toxoplasma infection.

The detection of circulating antigen in sera from patients suffering from acute toxoplasmosis (2, 23), in which specific antibodies were frequently detectable, also prompted us to consider the possible formation of CIC. In a relatively high proportion (12.5%) of 984 serum samples from individuals suspected of having toxoplasmosis, complexes were determined. The selection of sera was not based on firm clinical indications and included many sera from individuals in whom toxoplasmosis had to be excluded. Therefore, the relatively high prevalence was even more remarkable. When compared with other serological findings in the same sera, it was obvious that in cases in which evidence for active toxoplasmosis was observed, more positive CIC were observed (Table 3, category 1).

In sera obtained from individuals showing clinical evidence of active although unconfirmed toxoplasmosis, a much higher percentage (37.5%) of CIC was demonstrated (Table 3, category 2). When this criterion was combined with a positive result in the CF test, even more sera became positive. This is an indication that the finding of CIC is associated not only with clinical illness but also with the onset of a toxoplasma infection as characterized by a positive CF test. This is in accordance with the experience of Siegel and Remington (19), who showed immune complex-like material particularly in patients with a more severe clinical course in acute toxoplasmosis. It is remarkable that in healthy blood donors a considerable number of positive CIC was found. In fact, this is more or less in agreement with the unselected sera from category 1 (Table 3), inasmuch as no other serological indications were found for an active toxoplasma infection.

It may be concluded that in a relatively high proportion of clinically healthy individuals (whether suspected or not of having a toxoplasma infection), CIC may be found. This was also suggested by Siegel and Remington (19) and merits more discussion. Chronic toxoplasmosis is characterized by a situation called infection immunity of premunition (4). Effective immunity is probably maintained and boosted by relapsing infections, e.g., by ruptured tissue cysts (12). The activation of latent toxoplasmosis by other diseases or by immune suppression are well known (o.a. 5); however, relapsing toxoplasmosis in immunocompetent individuals is often suggested but difficult to prove (4, 13). Reports of exacerbations of chronic eye toxoplasmosis (o.a. 16) and recurrent parasitemia in asymptomatic patients (11) support the hypothesis that, in a human population with an average seropositivity, up to 50% asymptomatic recurrences can occur regularly. The detection of CIC containing IgG immunoglobulins in by far the majority of CIC-positive cases in this study also supports this idea. Whether CIC with IgM are reflecting primary infections remains to be investigated. Seronegative individuals showing a positive CIC test have to

FIG. 4. Interrelation between patterns of circulating antigen and circulating complexes with IgG or IgM during a follow-up study of a case of acute infection and exacerbation of chronic human toxoplasmosis.

undergo a follow-up study before conclusions can be made about a possible toxoplasma infection.

A follow-up study was done with the serum of a patient with an active toxoplasma infection (laboratory accident) and a recurrence of the infection, as was indicated by severe clinical symptoms and rising antibody levels (both IgG and IgM antibodies). A remarkable similarity was observed between the patterns of CIC and unbound antigens. In fact, the actual level of circulating antigen was too low to be considered as positive in the ELISA. This certainly was caused by the presence of a high level of both IgG and IgM antibodies to toxoplasma, leading to the formation of CIC. At the initial infection and later on for an even more prolonged period, CIC were detectable. From Fig. 4 it may be suggested that the detection of CIC represents an amplification of detectable free circulating antigens. In other words, CIC represent the presence of active parasites or acute toxoplasmosis.

To exclude the possibility that, in a certain percentage of healthy individuals, nonspecific reactions or anti-idiotypic antibodies might be responsible for the positive CIC findings, 10 of such serum samples were treated with polyethylene glycol (5%; PEG 6000; Merck). In this way, the immune complexes were precipitated, which resulted in a complete negative CIC test of the supernatant.

In conclusion, it can be stated that CIC are detected frequently and may reflect a short, often local recurrence of a toxoplasma infection both in asymptomatic individuals and in patients with obvious clinical symptoms of toxoplasmosis, albeit much more often in the latter. Although the detection of toxoplasma CIC as such may be indicative of an active process, it is of little clinical use since asymptomatic processes are often involved. In addition, the combination of clinical symptoms and the presence of CIC may be brought about by other infections which are immunosuppressive themselves and hence induce CIC with toxoplasma antigen. Therefore, the interpretation of a positive CIC test requires great care and should be combined with the results of other serological tests.

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