

Diagnosis of Acute Toxoplasmosis by an Enzyme Immunoassay for Specific Immunoglobulin M Antibodies

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A recently developed enzyme-linked immunosorbent assay for detection of immunoglobulin M (IgM) class antibodies to *Toxoplasma gondii* was evaluated with respect to specificity and sensitivity. By using an antibody capture principle and F(ab')₂ conjugates, interference of rheumatoid factors was absent. No cross-reactions with anti-toxoplasma IgG occurred, and no interference with antinuclear antibodies was found. A large-scale study with about 1,500 clinical specimens revealed a 100% specificity. By testing 79 sera from patients with acute-phase acquired toxoplasmosis, sensitivity was found to be 97%. In routine clinical practice, the IgM-enzyme-linked immunosorbent assay proved to be a more sensitive tool for diagnosis than the immunofluorescent-antibody test. The course of IgM-enzyme-linked immunosorbent assay antibodies in acute patients was studied; IgM reached peak levels within 1 month after onset of illness, and could be demonstrated up to an average of 8 months after onset.

Serological diagnosis of toxoplasmosis is based on a variety of techniques—the Dye test (DT), the immunofluorescence (IF) test, and the indirect haemagglutination test. Each has its limitations, but all are still widely used in clinical medicine. High DT titers and high IF titers have generally been regarded as indicative of acute or active toxoplasmosis, but the interpretation of antibody levels is not always straightforward, nor invariably resolved by serial testing of specimens. IF tests for detecting specific antibodies of the immunoglobulin M (IgM) class greatly assist diagnosis and assessment in suspected toxoplasmosis, but the inherent problems of competitive inhibition by specific IgG and interference by rheumatoid factors (IgM anti-immunoglobulin G) are not easily overcome (2, 5, 15). Recently, enzyme-linked immunosorbent assays (ELISAs) have been described for the detection of specific IgM antibodies which are not affected by rheumatoid factor or IgG interference (3, 13, 14). These methods, in which anti-IgM antibodies are used to coat the solid phase, so-called antibody capture assays, have proved of value in other fields (6, 18, 22). An IgM-ELISA for toxoplasma IgM antibodies developed in the Organon laboratories at Oss, Holland (4), was evaluated on serum specimens from two different populations, one from Scotland through the Toxoplasma Reference Laboratory at Inverness and the other from the Dutch Clinical Laboratory Service in Utrecht.

MATERIALS AND METHODS

Anti-IgM serum. A sheep antiserum to purified human IgM was made μ chain-specific by absorption with human umbilical cord serum. One volume of cord serum was incubated with two volumes of anti-IgM serum for 1 h at 37°C. Precipitates were removed by centrifugation (10,000 \times g for 10 min).

Antigens. Antigens were obtained by sonication of trophozoites of *Toxoplasma gondii*, which had been cultured in peritoneal cavities of mice. Mice were inoculated with 0.3 ml of a parasite suspension (5 \times 10⁶ parasites per ml). After 3 days the mice were killed, and about 1.0 ml of peritoneal fluid was aspirated. Parasites were washed by centrifugation (2,000 \times g for 10 min) and resuspension in an equal volume of saline. The parasites were disrupted by ultrasonic treatment with a Branson B12 Sonifier (Branson Sonic Power Co., Danbury, Conn.) at an output of 80 W for 5 min. The suspension was then stored at -70°C.

Anti-toxoplasma conjugate. A sheep was immunized with toxoplasma antigen by two intramuscular injections of sonicated antigen (1 mg) in Freund complete adjuvant 3 months apart. IgG was isolated by caprylic acid precipitation of non-IgG proteins (16). F(ab')₂ fragments were prepared by treatment of IgG in 0.1 M sodium acetate buffer (pH 4.3) with 1:100 (wt/wt) pepsin (2,700 U/mg; Worthington Diagnostics, Freehold, N.J.) for 20 h. The reaction was stopped by raising the pH to 8.0. F_c and small peptides were removed by gel filtration on Sephacryl S200 (Pharmacia, Uppsala, Sweden). F(ab')₂ fragments were labeled with horseradish peroxidase (RZ 3.0; Boehringer Mannheim GmbH, W. Germany) by means of N-succinimidyl-3-(2-pyridylthion)propionate (Pharmacia) according to the directions of the manufacturer.

Conjugates were stored at -70°C or were lyophilized.

Human sera. The evaluation of the IgM-ELISA was performed on two collections of sera. Collection 1 consisted of sera from patients in Scotland in whom toxoplasmosis had been confirmed by standard serological tests, together with selected sera from patients suffering from other conditions including glandular fever, lymphoma, rheumatoid arthritis, and related disorders (320 sera). Acute toxoplasmosis had been confirmed by the following criteria: in 33 patients by characteristic clinical glandular symptoms accompanied by a positive IgM-IF test (titer, $>1:16$) and indirect hemagglutination and DT positive results in IgM sucrose gradient fractions; in 7 patients by IgM-IF and IgM sucrose gradient positive results; and in 7 remaining patients by clinical symptoms accompanied by either a greater than fourfold rise in DT titer, a positive parasite isolation, or a positive IgM-IF test and a positive IgM sucrose gradient result.

Collection 2 from the Netherlands consisted of sera sent in for toxoplasmosis diagnosis to the Clinical Laboratory Service during a 5-month period and comprised about 1,500 serum specimens.

Other assay systems. Sera from collection 1 had been tested by DT and IgM-IF, and about two-thirds of them had been subjected to sucrose gradient fractionation followed by indirect hemagglutination and DT assays of the IgM- and IgG-containing fractions.

Sera from collection 2 had been tested in an IgG-IF assay (Wellcome, Beckenham, England).

All sera were tested by a toxoplasma IgG-ELISA (TOXONOSTIKA, Organon Teknika, Belgium).

Procedure for IgM ELISA. Wells of polystyrene microtiter plates (Greiner Labortechnik, W. Germany) coated with μ chain-specific sheep anti-human IgM were filled with 1:100 diluted test serum and incubated for 1 h at 37°C . After aspiration and washing, antigen and anti-toxoplasma conjugate were incubated simultaneously for 1 h at 37°C . After aspiration and washing, substrate was incubated for 30 min at room temperature; the reaction was stopped by addition of 4 NH_2SO_4 . Reactions giving extinctions twice the mean value of negative control sera were considered to be positive.

RESULTS

Specificity. The specificity of the IgM-ELISA was investigated by testing a panel of sera, which consisted of sera known to be capable of interfering in IgM assays such as rheumatoid factor-containing sera, high-titered IgG sera, and sera containing antinuclear antibodies. Also, a large number of sera from heterophile antibody-positive infectious mononucleosis patients were tested. This latter group was included since the symptoms of infectious mononucleosis may mimic those of toxoplasmosis.

All of 15 rheumatoid factor-positive sera, all of 15 high-titered IgG anti-toxoplasma sera, and all of 4 antinuclear antibody-containing sera reacted negatively.

One out of 200 heterophile antibody-positive sera reacted positively. This serum was submitted to sucrose gradient fractionation and was

proved to be positive for anti-toxoplasma IgM according to this method and is therefore likely to represent a double infection.

Sensitivity. Collection 1, which consisted of 320 sera, included 79 sera from 47 cases of confirmed toxoplasmosis (0 to 4 months after presumed onset). The confirmation was done according to criteria which combined clinical, serological, and other laboratory evidence, as well as the results of biopsies in some instances.

Seventeen of the 79 acute-phase sera were single specimens, and all were found to be positive by IgM-ELISA. The remaining 62 specimens were from 30 patients from whom serial samples were available. Sixty of these specimens were positive by IgM-ELISA. The two negative sera were old specimens and were the earliest of the series still available, although the diagnosis of acute infection had originally been confirmed by the results of a specimen collected 4 to 6 weeks previously. In these two sera, the IgM-IF test was negative as well. Results of all four tests in this group of sera are given in Fig. 1a.

The sucrose gradient method and the IgM-IF test had sensitivities comparable to that of the IgM ELISA. For the DT, half the specimens had titers below the threshold regarded to be indicative of acute infection (1:512).

Twenty-three post-acute-phase sera (5 to 9 months) were selected. Approximately 50% were positive in the IgM-ELISA, and this proportion was similar to that found by the other methods (Fig. 1b). A total of 172 sera from patients with long-standing infections (>10 months) were selected; these sera included a disproportionate number from three types of patients: a small group with chronic general malaise, usually accompanied by a persistent high DT titer; antenatal patients; and patients with choroiditis. These three groups were of special interest because many appeared to contain specific IgM, although the results of the IgM-IF and sucrose gradient assays were often in conflict and therefore difficult to interpret. In the group as a whole, 11% were found positive by IgM-ELISA, and virtually all were concentrated in the special subgroups (Fig. 1c). The results are very similar to those obtained by using the sucrose gradient method, but less than half the proportion was found positive by IgM-IF.

Application of the IgM-ELISA in routine clinical practice. Collection 2 consisted of 1,486 sera which had been sent in for toxoplasmosis diagnosis. All sera were tested in the IgM-ELISA and in an IgG-IF test.

Forty-two sera were found positive in the IgM-ELISA; 35 of these sera had IF titers equal to or higher than 1:300, which is considered

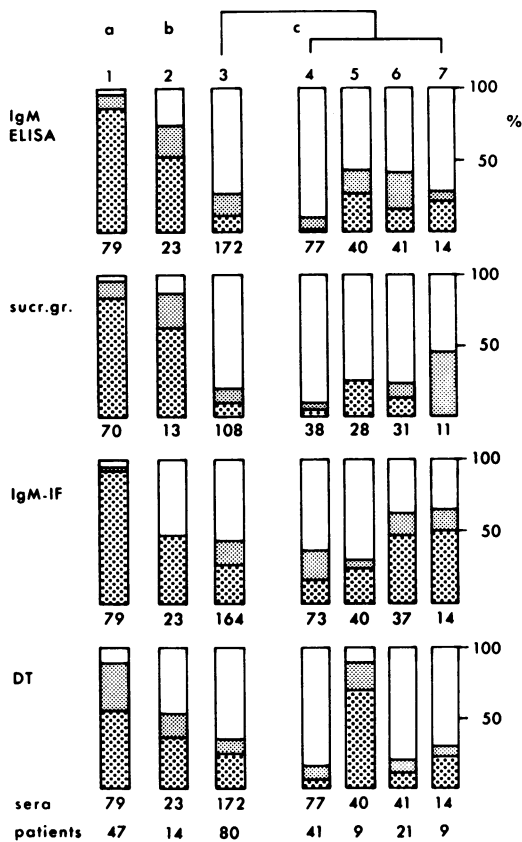


FIG. 1. Comparison of methods for detecting specific IgM to *Toxoplasma gondii* in collection 1. Top row, IgM-ELISA; second row, sucrose gradient method; third row, IgM-IF; bottom row, total antibody (DT). The collection is divided into acute sera (0 to 4 months postinfection, column 1), postacute sera (5 to 9 months, column 2), and sera collected >9 months postinfection (column 3). The number of sera involved is indicated underneath each column. The remaining columns (4 through 7) are divided into a general group (column 4) and three groups of special interest: sera from patients with persistent symptoms and high DT (column 5); sera from antenatal patients (column 6); and sera from choroiditis cases (column 7). Symbols: heavy dots, positive; light dots, borderline; open area, negative. For IgM-ELISA: positive, optical density at 450 nm 2.5 times the mean negative control; borderline, optical density at 450 nm 2.0 to 2.5 times the mean negative control. For sucrose gradient DT, indirect hemagglutination, or both in IgM fractions: positive, >1:4; borderline, 1:4. For IgM-IF: positive >1:16; borderline, 1:16. For the DT: positive, >1:512; borderline, 1:512.

indicative of acute infection. Seven sera had low or negative IF titers, and five of these could be investigated further and were confirmed to be from acute toxoplasmosis cases by the sucrose gradient method or by testing follow-up sam-

ples. From the two remaining sera no additional data were available.

Ten sera from collection 2 gave borderline results in the IgM-ELISA. Seven of these had IF titers equal to or greater than 1:300. Two of the other three low-titered sera could be confirmed as acute toxoplasmosis, but no additional data were available concerning the remaining serum.

In collection 2, no sera which had high IF titers ($\geq 1:300$) were found to be negative by IgM-ELISA.

Course of IgM-ELISA response during acute toxoplasmosis. From the study performed with routine clinical samples (collection 2), serial samples were available from various patients. The course of IgM-ELISA responses is depicted in Fig. 2, starting with the first specimens obtained. In most cases, IgM levels were already maximal in the first specimens obtained and disappeared between 4 and 7 months later.

From collection 1, 17 patients could be selected from whom several serial samples were available. These specimens had all been tested by DT, IgM-IF, and IgM-ELISA.

From these data a mean course for each parameter could be derived, and these are represented in Fig. 3, starting from the presumed day of onset of illness. The mean IgM-ELISA response is almost maximal already in the first specimens available, which is about 1 to 2 months before the mean DT titer reaches its peak value. In the first specimens, the mean DT titer is only slightly above the threshold indicating acute infection. The IgM-ELISA response declines to nothing in about 6 to 10 months. The mean IgM-IF titer follows roughly a similar course.

Maximum IgM-ELISA response. From both collection 1 and collection 2 acute toxoplasmosis cases could be selected from which serial samples were available in a way as to allow us to determine the maximum levels of the IgM-ELISA responses. Large individual differences in the maximum amounts of IgM appear to exist, ranging from just above the cutoff to more than four times the cutoff.

IgG-ELISA response. For the sera of collection 2 for which the IgM responses are shown in Fig. 2, IgG-ELISA courses were determined as well. The various patterns are shown in Fig. 4. In most cases, IgG-ELISA antibodies were already present at a moderate level in the first specimens obtained and rose in the next 2 or 3 months to their maximum levels. In some cases, IgG-ELISA values were still negative in the first specimens.

DISCUSSION

The advantage of using an antibody capture assay with an F(ab')₂ conjugate is clearly evi-

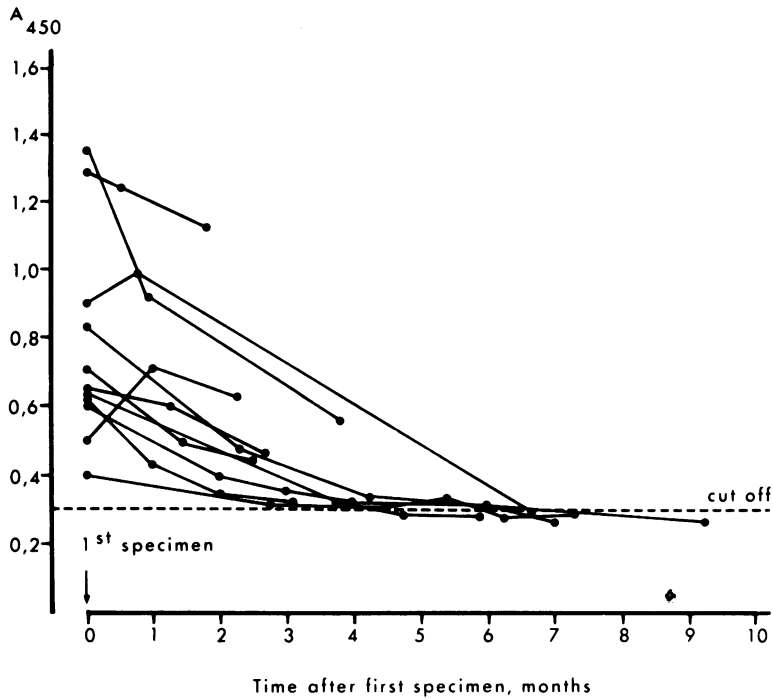


FIG. 2. Courses of IgM-ELISA responses in 11 patients (collection 2).

dent from the results on the specificity of the IgM-ELISA. The test was shown to be completely free from rheumatoid factor interference. This is in contrast to the classical IgM assays, which use anti-IgM conjugates and which there-

fore suffer from false-positive reactions caused by rheumatoid factors. Many reports exist about this phenomenon and about the necessity of pretreatment of sera to remove either IgG or rheumatoid factor (7-9). Findings similar to our

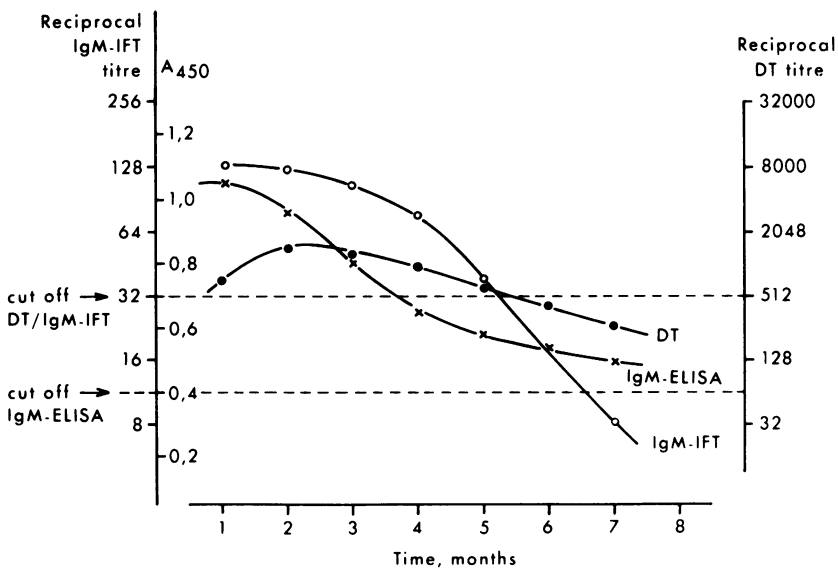


FIG. 3. Mean courses of DT, IgM-IF, and IgM-ELISA in acute toxoplasmosis.

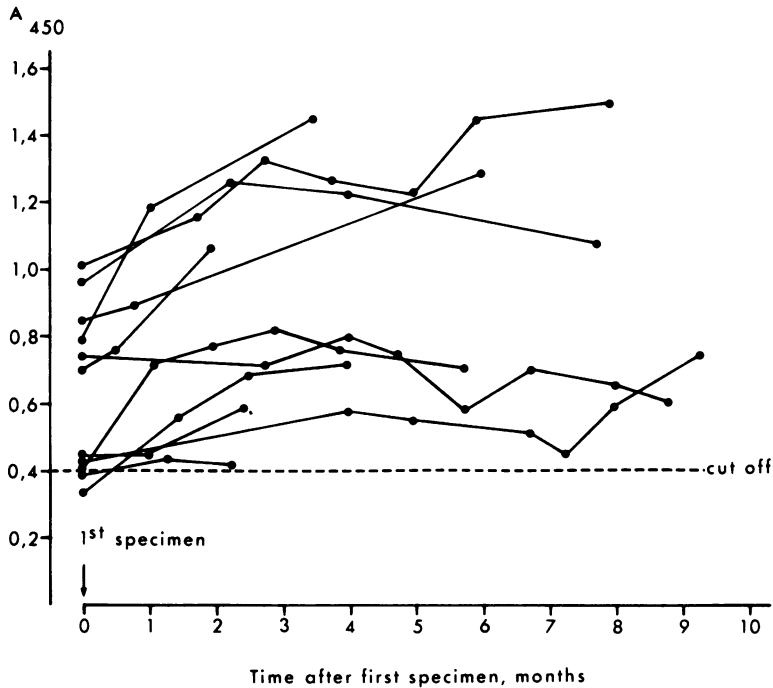


FIG. 4. Courses of IgG-ELISA responses in 11 patients (collection 2).

results when using $F(ab')_2$ conjugates have been reported by others (10).

Specificity with respect to IgG was shown to be high, which indicates that any cross-reactivity with IgG had been effectively removed from the anti-IgM serum by absorption with cord serum. In a group of heterophile antibody-positive sera, all but one serum reacted negatively. Investigation of the specificity of the test with these kind of sera is meaningful since many heterophile antibody-positive sera occur among sera sent in for diagnosis of toxoplasmosis due to the similarity of the symptoms of both disorders. The serum in this group that was positive for anti-toxoplasma IgM antibodies probably reflects a case of true acute toxoplasmosis since it appeared to react positively in the sucrose gradient method. In view of the incidence of acquired toxoplasmosis in the general population, 1 IgM-positive among 200 sera can very well be expected.

Sensitivity of the test was demonstrated by testing 79 sera from 47 confirmed acute cases of acute toxoplasmosis. A negative result was found in only two sera, both of which had been obtained at least 3 months after the presumed onset of illness, which may account for their unreactivity. They also produced negative IgM-IF results.

The overall performance of the IgM-ELISA on acute and postacute sera compared favorably

with the sucrose gradient method, and on sera collected at a longer time interval after infection it appeared to be more specific than the IgM-IF method.

All three methods detected IgM class antibody in a small proportion of sera from patients with chronic infections. These results may be genuine, the result of reactivation rather than poor test specificity, because they were only found for patients who maintained high DT titers with persistent symptoms (20), for those with choroiditis from clinically reactivated congenital disease, or for asymptomatic antenatal patients in whom some suppression of cell-mediated immunity occurs, possibly leading to the synthesis of detectable amounts of specific IgM. The evidence in favor of this hypothesis, however, would have been more convincing if there had been better agreement among the three assays. Only 20% of the IgM-ELISA-positive sera in this group were also positive by both of the alternative methods. It would therefore appear that further studies on fresh sera from these particular types of patient are required to ascertain whether trace amounts of specific IgM are really present.

The course of IgM-ELISA antibodies during acute toxoplasmosis (Fig. 3) advocates the use of this assay instead of the DT. The detection of specific IgM by IF might be equally valuable as suggested by the course of these antibodies, but

the disadvantages of the IgM-IF test (described above) still remain.

Maximum IgM responses during the acute phase of toxoplasmosis are shown to vary widely among different individuals. Quantitative determination of IgM for diagnosis is therefore of doubtful value, and the mere presence of specific IgM, irrespective of level, may be regarded as indicative of a recent infection. However, an accurate assessment of the stage of infection can only be made if the IgM-ELISA results are read in conjunction with measurements of specific IgG antibody levels, which have been shown to rise relatively slowly, only to reach maximal levels after 2 to 5 months after onset of infection (Fig. 4).

The application of the IgM-ELISA in routine clinical practice was demonstrated by testing samples in collection 2 from individuals suspected to have toxoplasmosis. According to the IgM-ELISA, 42 out of about 1,500 samples were from recent infections, whereas the IgG-IF test indicated only 35 cases. Six out of seven discrepant samples could be confirmed to represent recent toxoplasmosis infections. No high-titered sera as measured by IF were found which did not have detectable amounts of specific IgM. Therefore, IgM-ELISA was shown to be a more sensitive tool for diagnosis of acute toxoplasmosis than the IF test.

The results obtained with the IgM-ELISA correlate very well with those presented by Naot et al. (12) and Naot and Remington (13) who used a similar test. They too have found a very high sensitivity and specificity and observed a similar pattern of decline of IgM-ELISA antibodies. They also observed wide variation in the maximal IgM response in different individuals (personal communication).

The data presented here and those recently published by others (7, 12, 13, 19) allow some evaluation of the diagnostic methods now available for the diagnosis of toxoplasmosis.

Methods based on the detection of antigen, either from biopsies or as circulating antigen in blood, are neither widely practicable nor sufficiently sensitive (1, 17). Methods based on detection of a high total antibody titer, such as a high DT or Ig-IF titer, are shown to be of limited diagnostic value in acute infection, which confirms findings of Welch et al. (19). Also, DT and IF have several drawbacks as to their practicability.

Methods to detect specific IgM appear to be the best available for the purpose as is now increasingly recognized to be the case for many other infectious diseases (21). As to the three methods investigated in our study, the antibody capture IgM-ELISA has the advantage of being apparently more specific than IgM-IF, which is

supported by many reports from the literature (11, 13). Also, it is easier to perform, particularly with respect to sucrose gradient fractionations.

Although it will be necessary to assess the performance of the IgM-ELISA on larger populations, the indications are that these IgM assays can eventually replace most other forms of testing for *Toxoplasma* antibodies, which will mean an important improvement in the ease of the diagnosis of acute toxoplasmosis.

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