Ability of Enzyme-Linked Immunosorbent Assays to Detect Early Immunoglobulin G Antibodies to Toxoplasma gondii

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Two ELISA procedures, one using sonicated antigen coated with carbonate buffer and the other formalin fixed trophozoites with dry coating, differ in their ability to detect early antibodies in toxoplasmosis. In order to identify factors responsible for this difference, seven ELISA systems differing from each other in antigen used and/or coating procedure were compared. Both fixation of the trophozoites with formalin and air-drying of the antigen in the microtiterplate were important factors determining the ability of the assay to detect IgG antibodies in the early stage of infection. Differences in the results of the two ELISA procedures can be used to distinguish between the acute and chronic stages of infection.

Diagnosis of acquired Toxoplasma gondii infection is usually based on serological demonstration of IgG or IgM antibodies. Since the first description by Voller et al. (1) of a microtiterplate enzyme-linked immunosorbent assay (ELISA), the ELISA has become an increasingly popular procedure for detection of Toxoplasma gondii-specific IgG antibodies. Many reports have been published comparing ELISA with conventional serological methods, especially the widely used indirect immunofluorescence assay (IF). Some authors found good agreement between results of the two techniques (2–5), others found discrepant results (6, 7). The reasons for these discrepancies are still unclear, and they are mostly attributed to antigenic differences: antigen used in the immunofluorescence assay consists mainly of membrane antigens, whereas the ELISA contains both membrane and cytoplasmic antigen components. This explanation is supported by the results of Picher et al. (8) and Van Knapen (9) who found good agreement between ELISA and IF when the same antigen was employed for both techniques.

The aim of the present study was to identify the variables responsible for the test discrepancies, and to establish whether the differences in sensitivity could be used to determine the stage of infection.

Materials and Methods

Toxoplasma gondii Antigen. Toxoplasma gondii trophozoites, RH strain, were cultivated in cell cultures according to the method of Braveny (10) with slight modifications. Briefly, 5 × 10⁶ human larynx carcinoma cells (Hep-2) were suspended in 100 ml Minimal Essential Medium (MEM) with 5% fetal calf serum and seeded into a Roux flask. After 4–6 h of incubation at 37°C, the suspension was inoculated with 2.5 × 10⁶ trophozoites and incubated further at 37°C. On the fifth day the culture medium containing Toxoplasma gondii was collected in centrifuge tubes. By renewing the culture medium on the fifth day, the harvest could be repeated on the eighth day. The medium containing the parasites was centrifuged for 10 min at 3000 g. After resuspension of the pellet in phosphate buffered saline (PBS), the suspension was washed three times in PBS. The pellet was finally resuspended in saline to a concentration of 2 × 10⁶ trophozoites per ml. From this suspension the following six antigen preparations were prepared, each containing the same initial concentration of trophozoites: (a) whole untreated trophozoites in saline; (b) preparation (a) with addition of formalin to give a final concentration of 1%; (c) preparation (a) subjected to freezing five times (liquid nitrogen) and thawing (37°C); (d) preparation (a) subjected to sonication (5 × 1 min, 50 Watt); (e) supernatants of (d) subjected to centrifugation (3000 g, 10 min) to remove membrane fragments; (f) preparation (c) with addition of formalin to give a final concentration of 1%.

Sera. The 35 serum samples used consisted of 21 chronic-stage sera from patients who had a constant IgG titre for at least one month and no detectable IgM antibody levels (IgM-IF < 1/20), and 19 acute-stage sera from patients with serological evidence of recent toxoplasmosis, i.e. significant amounts of IgM antibodies (IgM-IF > 1/64), and a rise in IgG levels in follow-up sera.

Immunofluorescence Assay. One drop (5 × 10⁶ organisms) of formalin-fixed trophozoites was placed in each well on a multispot microscope slide and air dried. One drop of two-fold dilutions of patient sera was added to the antigen coated wells. After incubation in a humidified chamber for 30 min at 37°C the slides were washed in PBS and dried. Twenty-five µl of anti-human IgG-FITC (Wellcome, UK) diluted in PBS with 0.03% Evans blue as counterstain was added to the wells. After further incubation at 37°C for 30 min, the slides were washed, dried and mounted in glycerol-PBS under coverslips. The endpoint titer was the highest serum dilution giving clear fluorescence. Sera with IF titers > 1/20 were considered positive.
Sonicate/Carbonate-Buffer-Coating ELISA. The sonicate ELISA was performed by the procedure described by Voller et al. (1). The 96 well microtiterplates (Immunoplate I; Nunc, Denmark) were coated with 100 µl of the sonicated antigen diluted 1/100 in 0.01 M carbonate buffer (pH 9.6) and left overnight at 4 °C. Before use plates were washed three times with PBS + 0.5 % Tween-20 (PBS-T) to minimize unspecific adsorption in subsequent steps. Fifty µl of the serum sample diluted 1/200 in PBS-T + 0.5 % gelatin was added to each well, and plates were incubated for 2 h at room temperature. After three washings 50 µl of horseradish peroxidase conjugated anti-human IgG (Sigma Chemical, USA) diluted 1/2000 in PBS-T was added to the wells. Plates were incubated for 1 h at room temperature and washed. Substrate solution (100 µl of o-phenylenediamine 0.8 mg/ml + 0.4 µl/ml of 30 % hydrogen peroxide solution in citric acid buffer at pH 5.0) was added and the plates were incubated for approximately 20 min after which the reaction was stopped by adding 100 µl 4N sulphuric acid. Absorbance was read at 492 nm on a Behring ELISA Processor II.

Whole Trophozoites/Dry-Coating ELISA. Microtiterplates were sensitized with 100 µl of formalin-fixed trophozoites diluted 1/100 in saline. The plates were air dried. Before use plates were washed three times with PBS-T and 50 µl of the serum dilution (1/200) was added to each well. Further performance of the ELISA was as described above.

Other ELISA Procedures. Five other ELISA procedures were used, differing only in antigen preparation and/or coating procedure from the ELISAs described above. The seven procedures thus used contained respectively: (1) sonicated antigen (e) with carbonate buffer coating; (2) complete sonicate (d) with carbonate buffer coating; (3) freeze-thaw antigen (c) with carbonate buffer coating; (4) sonicated antigen (e) with dry coating; (5) sonicated formalin-fixed antigen (f) with dry coating; (6) whole untreated trophozoites (a) with dry coating; and (7) whole formalin-fixed trophozoites (b) with dry coating. For each preparation and each coating procedure a 1/100 antigen dilution was used to coat the plates. This concentration was determined to give an antigen excess for all preparations in both coating procedures.

Standardization of Results. Absorbance readings obtained in the ELISA were converted into international units per milliliter using a standard curve. This standard curve was the dilution curve of a positive serum pool in a pool of negative sera, calibrated against the WHO reference sample. The same reference samples were used in all assays.

Statistical Methods. Differences between the results of two ELISA procedures were evaluated using Student's t-test. To obtain normally distributed results the logarithms of the units were used for all calculations. The Spearman Rank test was used for non-parametric regression analysis.

Results

For 21 chronic-stage sera the results obtained in an ELISA using sonicated antigen showed good correlation with the IF results. In contrast, for 12 acute stage sera the correlation was extremely poor (Figure 1). When however an ELISA was performed using the same antigen as in the IF assay, i.e. formalin-fixed whole trophozoites dried on the microtiterplate, the results for acute-stage sera were in much better agreement with the IF results (Figure 2).