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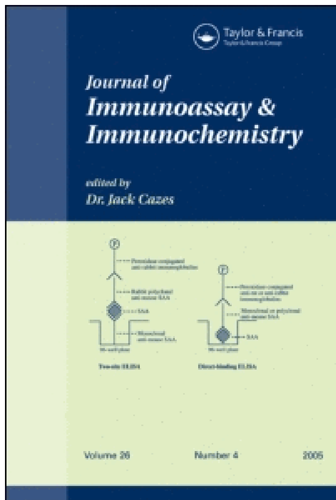

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Diagnosis of Acute Acquired Toxoplasmosis with the Enzyme-Labelled Antigen Reversed Immunoassay for Immunoglobulin M Antibodies

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DIAGNOSIS OF ACUTE ACQUIRED TOXOPLASMOSIS WITH THE ENZYME-LABELLED ANTIGEN REVERSED IMMUNOASSAY FOR IMMUNOGLOBULIN M ANTIBODIES

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ABSTRACT

The reversed enzyme-labelled antigen immunoassay (R-EIA), based on the capture of serum immunoglobulin M antibodies (IgM) and subsequent addition of *Toxoplasma gondii* soluble antigen tagged with peroxidase and substrate, was evaluated comparatively with the IgM-indirect immunofluorescence test (IgM-IIF) for the detection of anti-toxoplasma IgM antibodies in sera from individuals with diagnosed acute acquired toxoplasmosis. Additional serum groups from normal healthy individuals and sera presenting possible nonspecific reactivities were also evaluated. Complete specificity of R-EIA was shown. There was no correlation between the magnitude of R-EIA results and

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IgM-IIF titers, but a positive (although not linear) correlation was found between R-EIA and the IgM-IIF titers obtained after adsorption of sera with Staphylococcus aureus protein A. Direct labelling of the antigen by a simple coupling technique facilitated the assay standardization and improved its signal-to-noise ratio. (Key words: IgM, reversed immunoassay, toxoplasmosis)

INTRODUCTION

Detection of specific IgM antibodies, the first class of immunoglobulins produced during primary immune response, has been widely accepted as an indication of length of time since the onset of infections by parasitic agents. Therefore, serological tests that differentiate between specific IgG and IgM antibodies have become popular diagnostic procedures. The indirect immunofluorescence test (1) has been the test of choice and is widely used in diagnostic procedures. ELISA (2) introduced in 1971 is rapidly gaining acceptance and compares favorably with IIF.

The basis for detection and measurement of these antibodies is the sequential reaction of antigen, serum antibody and labelled anti-antibody in the indirect, labelled reagent technique. Especially in toxoplasmosis (3) and in certain viral diseases (4), this sequence for adding reactants has yielded false results when the anti-antibody is specifically against IgM. The simultaneous presence of IgM anti-IgG rheumatoid factor (RF) in the patient's serum leads to the binding of these macroglobulins to the IgG antibody layer on the antigen and

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subsequent recognition by the anti-IgM labelled antibody, thus yielding a false-positive result in the test (3,5). On the other hand, both IgG and IgM antibodies will compete for binding sites on the antigen, with a selection of the former, perhaps due to the relative numbers of IgG molecules available or a difference in avidity or affinity of the two classes. This leads to false-negative results or a lowering of IgM reactivity in the test system (6) when both immunoglobulin classes are present.

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To avoid this problem as well as interference by RF, Duermeyer et al. (3,7) described an ELISA procedure in which the patient's IgM is captured, reacted with the specific antigen and then labelled anti-antigen and substrate. Now having been used by several others (8,9,10), problems were still encountered when RF, if present, recognized the Fc of the animal serum used for the anti-antigen conjugate and resulted in false-positive reactions. To overcome this, conjugates were prepared using only the Fab_2 fraction which avoids the presentation of the Fc to any RF which might be bound.

A simpler and more effective alternative was reported for viral systems (11,12) in which a labelled antigen is used thus eliminating the need for an anti-antigen conjugate and avoiding the RF-animal serum interaction. The sensitivity remained equal to the longer procedure with virtually no loss of the specificity of the assay. Earlier, we described the adaptation

of the labelled antigen procedure to the detection of IgM anti-toxoplasma antibodies (13). In the present report we describe methods which simplify and greatly improve the test system. Evaluation of its sensitivity and specificity is made measuring anti-toxoplasma antibodies in sera from patients involved in an outbreak of toxoplasmosis.

MATERIALS AND METHODS

Sera

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A battery was assembled consisting of 122 sera from 32 patients involved in an acute outbreak of toxoplasmosis among military personnel on maneuvers in Panama (14). Eighty other serum specimens from asymptomatic healthy adults shown to be non-reactive in the indirect immunofluorescence test for toxoplasmosis served as negative controls. An additional group of 23 sera demonstrating one or more serological reactivities such as RF, anti-nuclear antibodies (ANA), paraproteinemia (Waldenstrom's macroglobulinemia) and heterophile antibodies (infectious mononucleosis) was included to better assess specificity of the test system.

IIF Tests

Before being evaluated in the R-EIA, all sera were tested by the IIF test (15) for the presence of IgG and IgM anti-toxoplasma antibodies. Inhibition of IgM reactivity in sera containing high levels of specific IgG was overcome by treating

an aliquant of each serum separately with Staphylococcus aureus containing protein A (Staph A) (Cowan I strain, Pansorbin, Calbiochem-Behring Corp., San Diego, CA) as previously described (13). False-positive IgM reactions were eliminated by adsorption of sera at a 1:10 ratio with a 20% suspension of glutaraldehyde-insolubilized human IgG in phosphate-buffered saline (PBS) (pH 7.6) for 1 h at room temperature.

Antigen-enzyme Conjugate

T. gondii tachyzoites harvested from the peritoneal cavity of mice infected 3 days previously (16) were used for the inoculation of MRC-5 human embryo lung fibroblast monolayers (low-passage number) grown in minimal essential medium (Eagle) supplemented with 10% fetal bovine serum. The maximum number of parasites was observed after 4 days at 37°C. The tachyzoite-rich medium was harvested and fresh medium was replaced in the flasks to allow a second harvest after 24 h. The parasites were washed 3 times by centrifugation in cold PBS 7.2, adjusted to 10^9 organisms/ml and submitted to ultrasonic-pulsed oscillations for 9 min. The supernate after centrifugation at 48 kg for 20 min had a protein concentration of 5.2 mg/ml. Conjugation of this antigen with peroxidase was done as previously described (17) with the following modification: the ratio of enzyme to protein was increased to 4:1 (10 mg of enzyme plus 2.5 mg of protein in 1 ml of the reacting mixture) to minimize the unlabelled antigen proportion in the conjugate,

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since this fraction would decrease the reactivilty of the test and an excess of free enzyme would not affect the noise in the reactions. In addition, the coupling reaction was carried out with continuous gentle agitation on a rotator for 2 h. After the dialysis cycle to remove the glutaraldehyde, the solution was stored without further purification in aliquants in the vapor phase of liquid nitrogen.

R-EIA

The reversed-enzyme immunoassays were performed in disposable polystyrene flat-bottomed plates of 96 wells (Immulon 2, Dynatech Laboratories, Inc., Alexandria, VA). Wells were sensitized with 50 µl of a 10 µg/ml solution in PBS (pH 7.2) of goat IgG against the µ-chain of human IgM (Tago, Inc., Burlingame, CA). This antibody solution had been previously purified by affinity chromatography and shown to have only specific antibody and a slight amount of denatured protein due to the elution process from the column. The sensitization step was carried out for 2 to 3 h at 37°C followed by overnight incubation at 4°C. For testing, the plates were washed 3 times with 0.01M PBS (pH 7.2) with 0.05% Tween 20 (PBS-Tw) (3 min each washing cycle). The plates were then filled with 75 µl per well of PBS-Tw contaning 1% bovine serum albumin (Gibco Laboratories, Grand Island, NY). After adding 25 µl of each serum to the first well of each row, serial 4-fold dilutions were made with 25-µl dilutors. Mixing by rotation of the

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dilutors in each dilution was done against the walls of the wells to avoid disruption of the optical homogeneity of the plastic, which would be an extra source of variability in the colorimetry. Each plate always included 3 control rows for the blanks, positive and negative controls. After incubation for 30 min at 37°C the plates were washed as described above, shaken dry and incubated with 50 µl of an optimal antigen-enzyme conjugate dilution in the same diluent used for the sera. After another incubation for 30 min at 37°C the plates were washed, shaken dry, and 50 µl of a freshly prepared substrate solution containing 0.1 mg of o-phenylenediamine per ml and 0.003% hydrogen peroxide in 0.08M acetate buffer (pH 4.5) were added to each well. After 30 min of reaction in the dark, the color development was stopped by adding 25 µl of 8 N sulfuric acid. Colorimetric measurements were made in an MR 580 Micro-ELISA auto reader (Dynatech Laboratories) with a 490-nm wavelength filter. Zeroing of the instrument was done against substrate solution blanks included in the test. The definition of a cutoff absorbance value is determined by background absorbance and is highly dependent on the serum dilution in the assay. Since the amount of bound reagent depends upon the protein concentration in each well, we used a cutoff curve determined by nonspecific reactivity at each dilution rather than a line based on the first dilution. The titer was defined as the highest dilution in which the reactivity of the unknown exceeds the

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absorbance value of the same dilution of the control plus 1.96 standard deviations.

RESULTS

Standardization

The optimal concentrations of reactants (except serum) were determined by a checkerboard tridimensional titration in which all three reagents - anti-μ, antigen and substrate - are simultaneously varied. Using 50 μl volumes it was found that the optimal concentration of anti-μ was 10 μg/ml and conjugated antigen was 1:100 to 1:200. When these reagents were tested and each incubation period varied from 30 min to 2 h, 30 min was found to be optimal and used for the remainder of the study. Although increased time increased reactivity, there was a simultaneous increase in nonspecificity and thus no advantage. Titration of the chromogen, OPD, consisted only of testing large variations to determine the effects of minimal, optimal and excess amounts. As expected, no marked differences were observed.

In the evaluation of factors to decrease nonspecific binding, it was found that the additional step of coating the plates with BSA after the sensitization of the solid phase was unnecessary since this inert protein was included in high concentration (1%) in the diluent for sera and conjugate. Likewise, the inclusion of 0.05% Tween-20 in the diluent was an

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important factor in decreasing the nonspecific binding to the plastic but did not totally eliminate this problem. Use of a 10-fold higher Tween-20 concentration in the diluent (0.5%), however, was of no benefit since the specific and nonspecific bound fractions were decreased in equal proportions.

A comparative experiment in which these dilutions were made directly in the plates with microdilutors or in tubes prior to transfer to the plates showed no difference in serum titers between both systems. Although care was taken to avoid scratching the center of the well with the microdilutors, the blanks were also treated in the same manner to avoid any artificial overestimation in the absorbance values by disrupting the light beam pathway through the plastic.

The reproducibility of the assay is shown by the within-assay coefficients of variation of 24 different titrations of 3.2% for positive and 7.8% for negative sera in different plates. The between-assay coefficients of variation were 3.9% for positive and 21.3% for negative sera.

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Interpretation of Test Results

For each serum tested, 2 IgM-IIF titers were obtained, one the preadsorption titer and the second after adsorption with Staphylococcal A protein to maximize the specific anti-toxoplasma IgM expression. No evidence of a clear relationship between R-EIA results and preadsorption IgM-IIF titers was found when

sera were grouped according to the IIF titer. There was, however, a positive although not strictly linear relationship between R-EIA results and postadsorption titers (Table 1 and Fig. 3A-H) which averaged approximately 4-fold higher than the preadsorption ones. Of interest is the fact that the subgroup of 9 patients' sera with a titer less than 16 in both nonadsorbed and adsorbed IgM-IIF test had a geometric mean titer of 110.6 in the R-EIA. The only indication that these sera were different from the negative control group was that they were specimens from the patients' group which had showed IgM-IIF positive results on at least one date after exposure in the outbreak. The other 113 sera were positive in both adsorbed IgM-IIF test and the R-EIA and were grouped according to the IgM-IIF titers. The differences in reactivity between R-EIA and adsorbed IgM-IIF tests as expressed by the ratio of the mean titers between R-EIA and the IgM-IIF test per group are shown in Table 1. The average weighted ratio of R-EIA mean titer to adsorbed IgM-IIF titer was 11.8. This difference in reactivity would be even higher if the preadsorption titers had been considered.

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Specificity of the Assay

As shown in Fig. 2, the nonspecific reacting sera from the specificity control group (RF, ANA, etc.) reacted in the R-EIA in the same manner as the negative control group. No

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TABLE I

Comparative reactivity between R-EIA and IgM-IIF (Staph A adsorbed) in 122 serum samples from 32 patients involved in an outbreak of toxoplasmosis and 80 control samples from normal individuals

IIF titer group #	Adsorbed IgM-IIF titer	Number of sera	R-EIA mean titer	Titer ratio R-EIA/IIF
NC	<16 (controls)	80	<4	----
1	<16 (patients)	9	110.6	>6.9
2	16	5	371.5	23.2
3	64	12	937.8	14.7
4	256	24	2412.9	9.4
5	1024	55	12816.0	12.5
6	4096	16	31068.6	7.6
7	16384	1	66461.0	4.1

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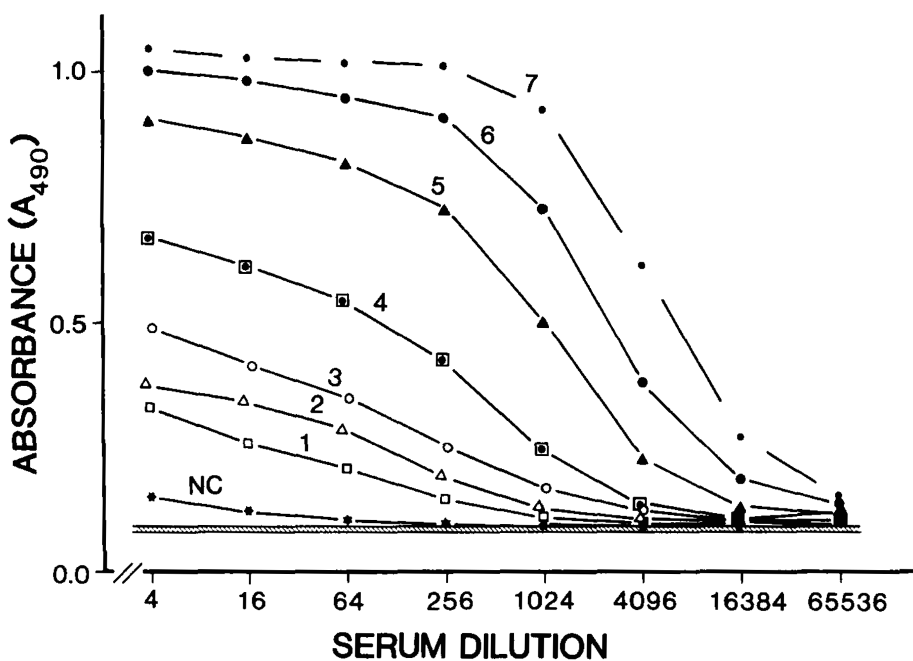

Figure 1. Reversed EIA absorbance values versus serum dilutions for each anti-toxoplasma IgM-IIF group. Shaded area represents blank level (mean $\pm$ 1 standard error). NC = Negative Controls.

overlapping of results was observed even when the control group was compared with the least reactive subgroup (IgM-IIF titers <16) of patients. These samples had been included randomly in the assay because they had yielded false-positive results in the preadsorbed IgM-IIF test. These titers had ranged from 16 to 1024 before adsorption but were rendered negative (<16) after adsorption with insolubilized human IgG. An advantageous effect was the double-binding activity of Staphylococcal A protein

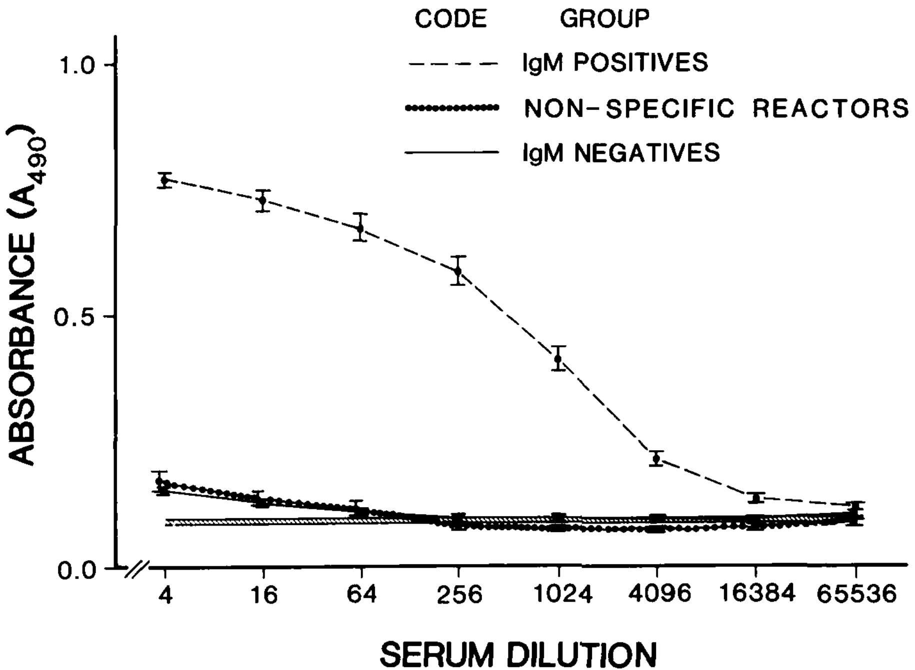

Figure 2. Reversed EIA absorbance values versus serum dilutions for 3 groups of sera. Bars represent the mean ± 1 standard error. Shaded area represents blank level as in Fig. 1.

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which removed RF activity in many samples, particularly if incubation time was extended to 1 h.

Correlation with the IIF Test

Although Fig. 1 shows a definite relationship between the absorbances of R-EIA and the Staphylococcal A protein adsorbed IgM-IIF titers by the absence of overlapping among the IIF curves, clearer evidence of a direct although nonlinear relationship is shown in Fig. 3, where the absorbance values in

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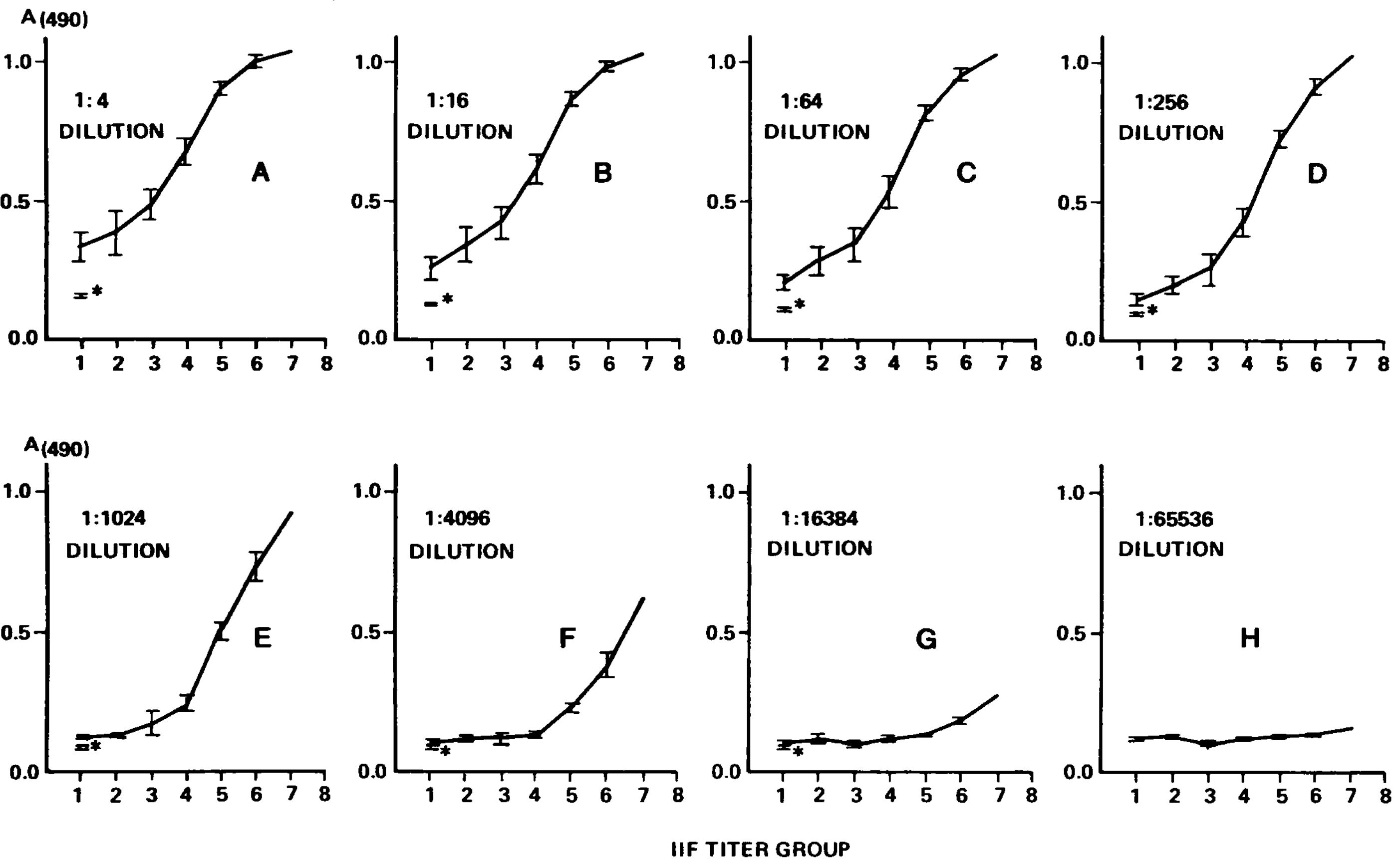

Figure 3. Reversed EIA absorbance values for each antitoxoplasma IgM-IIF titer group (See Fig. 1) at 8 different serum dilutions. Bars represent the mean ± 1 standard error. NC (Negative Control) group.

the R-EIA at each of the 8 dilutions tested are plotted against the titer levels of the six IIF serum titer groups. A sigmoidal dose-response curve is observed in the 4 initial dilutions (Fig. 3A-D) which shows the possibility of interpolation of IIF equivalent titers from R-EIA absorbance values in a single well or tube reaction. For highly reactive sera there will be a loss in resolution in the upper part of the curves because there is a tendency toward plateau formation, which can be avoided by testing the serum in a higher dilution (e.g., 1:1024) that will tend to linearize the second half of the dose-response curve. A logit-log transformation as used in many radioimmunoassay systems could be used satisfactorily for the interpolation of IIF-equivalent titers in the range of the first 4 dilutions (1:4 to 1:256).

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DISCUSSION

For some time the IgM-IIF test has been accepted as the test of choice for the detection of IgM antibodies as a measure of recent or early infection. The major pitfalls of reactivity with antinuclear antibodies and rheumatoid factor have stimulated research into other techniques. As shown by the data presented here, the R-EIA is a more sensitive and specific test than the IIF test for detection of anti-toxoplasma IgM antibodies. The direct labelling of the antigen with peroxidase, as proposed in an earlier report (13) by using the

periodate method (18), is simplified in the present modification in which glutaraldehyde was used as a coupling agent. Although such a method is based on a random coupling process, the increase in the ratio of enzyme to antigen decreased the proportion of unlabelled antigen which in turn contributed to the improvement of the sensitivity of the test. Use of chromatography-purified conjugates in preliminary experiments (data not shown) did not improve the characteristics of the assay since free enzyme did not affect the noise in the test system. Another important modification in the test procedure since its initial description was the use of a lower pH in the substrate buffer. In fact, the signal-to-noise ratio improved considerably at the pH of 4.5 compared to pH of 6.0 proposed in the original report (13).

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The use of a purified solution of anti-IgM antibodies in the sensitization of the solid phase was a crucial step to achieve maximum sensitivity. Since it has been demonstrated that there is a limit for the saturation of the polystyrene with IgG (19), the binding capacity of the first layer will be limited to the proportion of specific anti-IgM antibody molecules used for sensitization. In fact, in preliminary experiments, we used the IgG fraction of a goat serum against human IgM. Despite its high titer, such preparation obviously had a small proportion of truly specific antibodies and therefore, the second layer of IgM molecules bound in the solid phase was less dense, decreasing

the activity of the assay. Using an antibody preparation purified by affinity chromatography yielded much better results by maximizing the binding of IgM molecules from the serum.

In their reversed 5-step EIA for anti-toxoplasma IgM, Naot and Remington found consistent correlation between IgM-IIF and R-EIA titers (9). We corroborated these data for nonadsorbed (Staphylococcal A protein) IIF titers; as expected, a correlation was evident when R-EIA results were compared to adsorbed IIF titers, since we had removed with the Staph A adsorption the interference of excess IgG on the true IgM expression. This adsorption makes the IIF test comparable to the R-EIA, which selects in the first phase of the assay the antibody class to be examined in the patient's serum.

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Nevertheless, despite the relationship found between the two tests, 9 sera from the patients' group that had titers less than 16 in the IgM-IIF test had significantly higher absorbance values in the R-EIA. This disagreement was attributed to the greater sensitivity of the R-EIA, which could detect the specific anti-Toxoplasma IgM levels in those sera even though they were undetected in both the unadsorbed and the adsorbed IgM-IIF test. The other remaining 113 sera with adsorbed IgM-IIF titers ranging between 16 and 16384 were all positive with the R-EIA. Among those sera there were 26 specimens which were completely negative in the unadsorbed IIF test for IgM antibodies with Staphylococcal A protein, however, those samples

were all positive, yielding titers between 16 and 1024. Although not completely blocked in their specific IgM expression, several other sera presented lowered IgM titers as compared to results obtained after Staphylococcal A protein adsorption. This competitive blocking by IgG of the IgM expression, the major cause for the poor correlation between the IgM-IIF and the R-EIA, was overcome with the adsorption step, thus normalizing the IIF titers.

An interesting finding was the side effect of Staphylococcal A protein which also removed the RF activity of most sera in the group with nonspecific interfering activity. There are two possible explanations for this phenomenon; first, the bacterial cells would adsorb the IgG molecules which would form a second immunoadsorbent for the IgM RF molecules; therefore, extending the incubation time with the cells up to 1 h would maximize the efficiency of the process, which was indeed observed. Second, by removing most of the IgG (and therefore the specific IgG) from the serum, the antibody layer formed on the protozoan surface during the incubation with serum was less dense, thus adsorbing fewer RF molecules onto its surface.

Although an apparent absolute specificity of the assay was observed even when testing sera from the group of nonspecific reactors, we agree with previous authors (9) that ANA antibodies of the IgM class, if present in the serum, might lead to

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false-positive results in the R-EIA if nuclear antigens are present in the antigenic preparation used for enzyme conjugation.

A common phenomenon in the IIF test, the polar staining of the parasites by nonspecific IgM antibodies (20), did not interfere in the R-EIA, although we took no special precautions to remove membrane antigens from the preparation used for tagging with the enzyme.

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