False-Positive Anti-Toxoplasma Fluorescent-Antibody Tests in Patients with Antinuclear Antibodies

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The indirect fluorescent-antibody (IFA) method for diagnosis of toxoplasmosis is widely used and is considered to be as specific as the Sabin-Feldman dye test. After observing a patient with systemic lupus erythematosus (SLE) who had a positive toxoplasma IFA test but a negative dye test, we studied sera with high titers of antinuclear antibodies from 16 SLE patients and from 2 with rheumatoid arthritis for Toxoplasma antibodies in the immunoglobulin G and M (IgG and IgM) IFA tests and the dye test. Results of these tests were compared with titers of antinuclear antibodies, precipitating antibodies to single-strand deoxyribonucleic acid (DNA), and binding antibodies by use of DNA labeled with ³H-actinomycin D. Of 18 patients, 11 had IgG and 4 had IgM IFA Toxoplasma antibodies; only 2 had antibodies detectable in the dye test. The immunofluorescence patterns in the Toxoplasma IFA test were indistinguishable from those obtained in patients with toxoplasmosis without antinuclear antibodies. Absorption of SLE sera with DNA did not result in a decrease in Toxoplasma IFA titers. When SLE sera were absorbed with live T. gondii, a marked drop in IgG IFA titer was observed as well as a decrease in titers of antinuclear antibodies and ³H-DNA binding. Treatment of Toxoplasma cells with deoxyribonuclease and ribonuclease did not decrease their fluorescence. These results suggest that T. gondii nuclear antigens can absorb antinuclear antibodies but do not have exposed substrates for deoxyribonuclease. Tests in which organisms containing "nuclear" antigens for IFA detection of antibodies to these organisms are used may result in "false-positives" with sera containing antinuclear antibodies.

The IFA technique has recently been adapted for the demonstration of immunoglobulin M (IgM) Toxoplasma antibodies and has proved useful in establishing the diagnosis of acute congenital and acquired infection (9, 10).

The IFA test for toxoplasmosis has been considered absolutely specific, and, because it is very simple to perform, it is being used by many laboratories, rather than the Sabin-Feldman dye test, for routine Toxoplasma serology. The present study was prompted by the serological test results obtained in the patient described below. This patient with systemic lupus erythematosus (SLE) had the unusual finding of a positive IFA test and a negative dye test. This suggested that certain patients with SLE might have "false-positive" reactions in the IFA test on the basis of their antinuclear antibodies rather than on the basis of previous infection with Toxoplasma.

CASE REPORT
M. W. (sera 21 and 22, Table 1), a 19-year-old Caucasian male, was admitted to a local hospital on
11 November 1969 with a 6-week history of progressive weakness, malaise, and a 6.8 kg weight loss. Two weeks prior to admission, he complained of anorexia and sore throat and had documented afternoon and evening fever to 103 °F (39.4 °C). Two days prior to admission, he noted swelling, pain, and a puritic skin rash over both lower legs and ankles. Past history included severe measles at the age of 5 years, with subsequent partial bilateral hearing loss. There was no history of allergy, and he took no medication other than occasional aspirin.

Pertinent physical findings were an oral temperature of 101 °F (38.3 °C), partial hearing loss bilaterally, and enlarged nontender lymph nodes in cervical, axillary, epitrochlear, and inguinal regions. There was mild edema, tenderness, and a discrete macular rash over the anterior tibial area bilaterally.

Laboratory tests revealed a hemocrit of 28%, white blood cell count of 3,500, with 70% neutrophils, 26% lymphocytes, 3% monocytes, and 1% eosinophils, and a sedimentation rate of 45 mm/hr. The Veneral Disease Research Laboratory (VDRL) test was reactive at 1:4, the fluorescent treponemal antibody absorption (FTA-ABS) test was weakly reactive, and a direct Coombs' test was positive. Intermediate tuberculin, histoplasmin, and coccidioidin skin tests were negative. Toxoplasma serology was requested because of the lymphadenopathy. Because of the urgency of the request, an IgM IFA Toxoplasma antibody test was performed and was positive in a titer of 1:40. The Sabin-Feldman dye test, performed several days later, was negative (<1:2). Several early lupus erythematosus preparations were negative; however, an antinuclear antibody titer was 1:20 on 16 November 1969, and subsequent lupus erythematosus preparations were positive. Lymph node biopsy revealed reactive hyperplasia. A muscle biopsy revealed acute and chronic changes of myositis with perivasculitis. The patient's condition improved rapidly after corticosteroids were begun; his fever subsided, the rash, edema, anorexia, malaise, and lymphadenopathy disappeared, and the hematological status slowly improved. Repeat serological tests revealed continued elevation of IgM and IgG IFA Toxoplasma antibody titers and negative dye test titers. Attempts to lower the dose of corticosteroids have been unsuccessful, as the patient has prompt return of presenting symptoms and signs.

MATERIALS AND METHODS

Patients were selected on the basis of their having positive antinuclear antibodies. They represent a cross section of patients seen in the past year by the Rheumatology Unit of the University of California Medical Center at Los Angeles. The additional patient, M. W. (no. 21 and 22, Table 1), is the one with severe SLE described above. Immunofluorescence tests for antinuclear antibodies were performed with ethanol-fixed human peripheral blood smears as a source of leukocyte nuclei, and rabbit antibody specific for human IgG conjugated with fluorescein isothionate obtained from Behring Diagnostics, Inc., Woodbury, N.Y. (1). Double immunodiffusion tests in dilute agarose against single-strand calf thymus deoxyribonucleic acid (DNA; Worthington Biochemical Corp., Freehold, N.J.) were performed as previously described (5). Tests to detect binding antibody by the modification of the Farr technique, in which native calf thymus DNA labeled with 3H-actinomycin D in a concentration of 20 μg/ml is used, were performed as described by Carr et al. (3) with the modification that normal human serum was used as diluent.

More than 20% binding of the labeled DNA is considered abnormal. Values expressed in Table I for DNA 3H binding indicate the fractional increase in binding of the patient's serum tested over normal sera tested on the same day; e.g., a value of 0.5 indicates that the patient's serum bound 30% of the labeled DNA, and the maximal binding of five normal sera on that day was 20%.

The Sabin-Feldman dye test and the IgG and IgM IFA tests for Toxoplasma antibodies were performed as described previously (9). To read the IFA tests, a dark-field condenser with filters BG 12 and no. 50 Zeiss and an HB0200 Osram lamp as the ultraviolet source were used, rather than a bright-field condenser, to gain better definition of the staining of Toxoplasma cell walls. All titers are expressed as final dilutions of serum. The initial serum dilution was undiluted (1:2 final dilution) in the dye test, 1:4 in the IgG IFA test, and 1:10 in the IgM IFA test.

Sera were absorbed with preparations of DNA containing 100 or 500 μg of calf thymus DNA per ml. DNA was dissolved in distilled water and denatured by heating in a water bath at 100 °C for 10 min, followed immediately by cooling in an ice bath. Equal volumes of these DNA preparations and test serum were mixed, allowed to incubate for at least 10 min at room temperature, and then tested in the IgG Toxoplasma IFA test.

Absorption of sera with live T. gondii of the RH strain was achieved by diluting the patient's serum 1:5 in phosphate-buffered saline, pH 7.1 (PBS), and mixing 0.2 ml of this dilution with approximately 6 × 10^6 live Toxoplasma cells obtained from the peritoneal cavity of Swiss-Webster mice which had been infected 3 days earlier. The peritoneal exudate was first centrifuged at 400 × g for 20 min; the supernatant fluid was discarded, and the sediment was resuspended and washed three times with PBS. Sera were inactivated at 56 °C for 30 min before absorption. The absorption was carried out at 4 °C for 24 hr with constant rotation by use of a Fischer Roto-Rack. The organisms were then removed by centrifugation at 400 × g for 20 min at 4 °C. A serum with titers in the dye and IgG IFA tests of 1:1,024 was absorbed as a positive control.

The effects of deoxyribonuclease and ribonuclease treatment on Toxoplasma were investigated by treating IFA slide preparations with these enzymes. Deoxyribonuclease and ribonuclease (Worthington Biochemical Corp.) were used at a concentration of 0.5 mg/ml in buffer (0.0094 M KH₂PO₄, 0.0125 M K₂HPO₄, 0.0015 M NaHCO₃, 0.145 M sucrose, pH 7.1) as recommended by Wilbur and Anderson (14). The enzyme solutions were allowed to react on Toxoplasma IFA test slide preparations for 5 min at room tem-
There titers are distinguishable with sera dye ages with patterns and temperature. Shown in single-strand DNA; IgG (Fig. 272 no. 21). Titers serum ranged to rheumatoid arthritis. The data used in the test, IgG and IgM were males. Dates serum was drawn are shown in parentheses. Zero = <1:8 in dye test, <1:10 in IgM IFA, and <1:4 in IgG IFA test.

RESULTS

Serological data in 16 patients with SLE and 2 with rheumatoid arthritis are shown in Table 1. There were 11 females and 7 males, and their ages ranged from 12 to 57 years.

Three patients (16.6%) were positive in the dye test, and the titers were 1:8 in two and 1:64 in one; 11 (61.1%) were positive in the IgG IFA Toxoplasma antibody test, and the titers ranged from 1:4 to 1:256. Four (23.5%) were positive in the IgM IFA toxoplasma antibody test, with titers ranging from 1:10 to 1:40. Each of the sera positive in the IgM IFA test was also positive in the IgG IFA test. The immunofluorescent patterns in the Toxoplasma IFA test were indistinguishable from those obtained in patients with toxoplasmosis without antinuclear antibody (Fig. 1).

In an attempt to determine whether an IFA test titer of 1:4 in our IgG IFA test may be present in the absence of a dye test titer in undiluted sera, we tested 89 sera from outpatients in the general population who had no history of connective tissue disease. In only one instance (1.2%) was the IgG IFA test positive in a dilution of 1:4 in the presence of a negative dye test. This single instance was in a 33-year-old female who was in the fourth month of an apparently normal pregnancy. These results are in marked contrast to the prevalence of 22% of titers of 1:4 in the IgG IFA test in the 18 patients with connective tissue diseases.

Absorption of IFA-positive sera with DNA was performed to determine whether this would remove the antibody reacting in the IFA test. The results of these studies revealed no alteration in the IFA test titers when compared in parallel with dilutions of the unabsorbed sera. Thus, absorption of IgG IFA positive serum
ANTI-TOXOPLASMA FLUORESCENT-ANTIBODY TESTS

FIG. 1. Typical pattern of Toxoplasma fluorescence seen with sera with or without antinuclear antibodies.

TABLE 2. Results of absorption of SLE sera with live Toxoplasma gondii

| Serum no. | ANA-IgG | DNA-H binding (%) | IgG IFA titer |
|-----------|---------|-------------------|--------------|
|           | Before  | After             | Before       | After      |
| 5         | 64      | 20                | ND          | 128        | Negative |
| 7         | 16      | 5                 | ND          | 32         | Negative |
| 20        | 64      | 20                | 33.7        | 17.9       |           |
| Control   |         |                   |             |            |           |

| Serum no. | ANA-IgG | DNA-H binding (%) | IgG IFA titer |
|-----------|---------|-------------------|--------------|
|           | Before  | After             | Before       | After      |
|           |         |                   | Before       | After      |
|           |         |                   |              |            |

* Serum numbers same as in Table 1.
* Expressed as reciprocal of dilution. ANA = antinuclear antibodies.
* Tested at 1:5 serum dilution.
* Not done; insufficient amount.
* Only equivocal immunofluorescence noted at this dilution.

with either 100 or 500 mg of DNA per ml was ineffective in eliminating the positive fluorescent-antibody titers. In contrast, absorption of three patients' sera and one control serum with live Toxoplasma resulted in almost complete disappearance of all fluorescence (Table 2). The IgG IFA titers before absorption ranged from 1:32 to 1:128 for the test sera and was 1:1,024 in the control serum. After absorption with live Toxoplasma, the IgG IFA titer was negative in all but one (no. 20, Table 2), which gave a weak fluorescence at a dilution of 1:10.

To determine whether there is an "inhibiting factor" present in the sera of these patients which might be interfering with the Sabin-Feldman dye test, a known positive serum (dye test titer 1:256) was diluted with serum from patient M. W. (no. 21, Table 1) as a diluent rather than the usual 0.15 M NaCl. No inhibition (reduction in titer) was noted when the serum of M. W. was used as diluent.

Deoxyribonuclease and ribonuclease were employed to determine whether the antigenic receptors on the wall of the Toxoplasma cells are DNA or RNA or both. The enzymes did not cause any detectable decrease in fluorescence; treated and untreated organisms yielded the same results.

Precipitating antibodies to single-strand DNA were noted in only 4 of the 21 sera tested. Titers of IgG antinuclear antibodies were 1:16 in two of them and 1:64 in the other two. The titers of IgG antinuclear antibodies in the 17 sera without precipitating antibodies to single-strand DNA ranged from 1:4 to 1:256. Although none of these 21 sera had precipitating antibodies to native DNA, 18 of the 21 had antibodies detectable by the Farr technique, when native calf
thymus DNA labeled with ³H-actinomycin D was used. Sera 2 and 14 (Table 1), which had no detectable antibodies to DNA by either the precipitin or the binding test, had negative IFA toxoplasma antibody titers as well. Of the 14 sera with titers below 1:32 in the IgG IFA Toxoplasma antibody test, 10 had antibodies to labeled native DNA and 1 of 14 had precipitating antibodies to single-strand DNA. Of the sera with titers of 1:32 or greater (excluding no. 22, Table 1) in the IgG IFA toxoplasma antibody test, six of seven had antibodies detectable with labeled native DNA and three of seven had precipitating antibodies to single-strand DNA.

To determine whether the presence of Toxoplasma antibodies will result in positive serology for SLE, the serological tests for SLE were performed on dye test-positive sera obtained from 25 persons in the general population. The dye test titers ranged from 1:8 to 1:64,000. All were negative for antinuclear antibodies, and their binding of ³H-DNA was within normal limits (less than 20%). Tests for rheumatoid factor with human gamma globulin sensitized latex particles and rabbit amboceptor sensitized sheep red blood cells revealed titers seen in the normal population. All of the sera were negative for heterophil antibody.

DISCUSSION

The results described above suggest that some sera from patients with SLE may give what appear to be false-positive reactions in the IgG and IgM IFA tests for toxoplasmosis. The sera studied in the present report all had antinuclear antibodies by the immunofluorescence technique, and most had antibodies detected against tritium-labeled native DNA. Precipitating antibodies against single-strand calf thymus DNA were less prevalent, and none of the sera had demonstrable precipitating antibodies against native calf thymus DNA antigen. Positive IgG or IgM IFA Toxoplasma antibody tests or both were noted in 11 sera in which the dye test was <1:2. Eight of the 18 patients had IgG IFA test titers of 1:16 or greater, and in some instances the IgG IFA titer was as high as 1:128 and 1:256. In our laboratory, the titers obtained in the IgG IFA Toxoplasma test usually do not vary more than one twofold dilution from those obtained in the dye test.

In view of the negative Sabin-Feldman dye test titers, it appears unlikely that any of the other serological manifestations in these cases would be attributable to Toxoplasma infection. Neither the presence nor the titer of IFA Toxoplasma antibodies was found to correlate with the presence of antibody to native or single-strand DNA. These results are consistent with the hypothesis that Toxoplasma organisms contain antigens indistinguishable by immunological techniques from some of those found in human leukocyte nuclei, as well as in native and denatured calf thymus DNA. These hypothetical antigens may be quite heterogenous, and the cross-reactions with Toxoplasma antigens may depend on different specificities in the various patients' sera. For example, a single patient who has a mixture of antinuclear antibodies including a large amount of antinuclear protein antibody might have a positive IFA Toxoplasma antibody test on the basis of identical nucleoprotein moieties in Toxoplasma and human leukocyte nuclei, whereas another lupus patient might have cross-reactive antibodies based on antinucleotide specificities indistinguishable in Toxoplasma and denatured calf thymus DNA. The inability to decrease the IFA Toxoplasma antibody titers after treatment of the Toxoplasma cells with nucleases suggests that T. gondii cells contain nuclear antigens capable of absorption of antinuclear antibodies but do not have exposed substrates for the nucleases.

Although antinuclear antibodies have been found in the sera of a variety of patients with collagen-vascular disorders, their greatest incidence in high titers has been reported in SLE (1). By use of immunofluorescence techniques with human leukocyte nuclei as antigens, antinuclear antibodies have been demonstrated in all four immunoglobulin classes: IgG, IgA, IgM, and IgD (6). They may be directed against a variety of antigens including DNA, nucleoproteins, ribonucleic acid, phosphate-extractable protein of the nucleus, and other even less well-defined nuclear antigens (1). Sera containing antinuclear antibodies from patients with diseases of unknown etiology frequently contain antibodies directed against cytoplasmic constituents, clotting factors, and membrane antigens (12). It has been well documented that such patients also may have antibodies directed against the cardio-lipid antigen, and consequently such patients are said to have "false-positive" serological tests for syphilis (2). Although such patients may have antibodies detectable in the VDRL test, they rarely have neutralizing antibodies against Treponema as defined by positive T. pallidum immobilization tests (2).

While these studies were in progress, Kraus, Haserick, and Lantz (7) published their interesting observations on an atypical "beading" and probable false-positive reaction for syphilis in SLE. The VDRL slide test, the FTA-ABS test, and the T. pallidum immobilization (TPI) test were performed in sera from 150 patients with...
SLE. The VDRL test was positive in 24 of them, and the TPI test was positive in 1. Some degree of fluorescence in the FTA-ABS test was noted in 23 patients; 4 had definitely positive reactions, 8 were borderline, and the other 11 had an atypical "beading" pattern in contrast to the homogeneous pattern of fluorescence usually seen. None of the 23 had clinical or historical evidence of syphilis. It is important to recognize that tests which utilize organisms containing "nuclear" antigens for IFA detection of antibodies against these same organisms may result in "false-positive" reactions with sera containing antinuclear antibodies.

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