THE JOURNAL OF INFECTIOUS DISEASES • VOL. 148, NO. 5 • NOVEMBER 1983
 0022-1899/83/4805-0013$00.75

Serodiagnosis of Rocky Mountain Spotted Fever: Comparison of IgM and IgG Enzyme-Linked Immunosorbent Assays and Indirect Fluorescent Antibody Test

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To characterize IgM and IgG antibody responses in Rocky Mountain spotted fever (RMSF), a microtiter enzyme-linked immunosorbent assay (ELISA) using density gradient-purified *Rickettsia rickettsii* as antigen was developed. Sera of vaccinated individuals and patients with RMSF were tested by ELISA and by indirect fluorescent antibody (IFA) tests. Diagnostic agreement between ELISA and the IFA test was 76% and 52% for IgG and IgM antibody, respectively. Diagnostic agreement between the ELISA for IgG antibody and the IFA test for total immunoglobulins was 84%. The ELISAs for IgM and IgG antibody were as specific (100%) and as sensitive (100%) as the IFA test (83%–100%) in detecting antibody increases in paired sera from persons with RMSF and were superior to the IFA test in detecting seroconversions in vaccinees. The ELISA also detected antibodies in a single convalescent-phase serum with sensitivity and reliability. The ELISA for IgG antibody is appropriate for seroepidemiology and serodiagnosis since it permits measurement of antibody at a single dilution of serum up to a year after illness.

Heretofore, tests for serodiagnosis of Rocky Mountain spotted fever (RMSF) had limitations. The highly specific CF and microagglutination tests lack sensitivity [1], and early treatment of RMSF delays rises in antibody [2]. The widely used Weil-Felix test lacks specificity [3]. Indirect HA and latex agglutination are sensitive and specific but not suitable for seroepidemiology, since predominantly short-lived immunoglobulins (IgM) are detected [4]. The indirect fluorescent antibody (IFA) test, although sensitive and accurate [5], is subject to observer bias and is relatively cumbersome to perform.

We employed the IFA test to measure class-specific immunoglobulins to rickettsiae in sera of adult volunteers receiving a new killed vaccine prepared in chick embryo cells. The IFA test was not sufficiently sensitive to detect low levels of IgM antibodies in postvaccination and postinfection sera. The shortcomings of this test prompted us to adapt a microtiter ELISA for detection of antibodies to *R rickettsii* and to compare it with the IFA test.

Received for publication April 29, 1983, and in revised form June 23, 1983.

This work was presented in part at the 20th Interscience Conference on Antimicrobial Agents and Chemotherapy, held in Miami, Florida, in October 1982.

Written, informed consent was obtained from the volunteers, and the study protocols were approved by the Human Volunteers Research Committee of the University of Maryland and by the Clinical Review Subpanel of the National Institute of Allergy and Infectious Diseases.

This work was supported in part by contract NO1AI 12666 from the National Institute of Allergy and Infectious Diseases, and computer support was provided by the Computer Science Center of the University of Maryland.

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Materials and Methods

Sera. During studies of a new formalin-inactivated vaccine for RMSF, sera from 50 volunteers were collected before and after vaccination; also, sera were obtained from 16 vaccinees and six unvaccinated volunteers before and after challenge with *R rickettsii* [6, 7]. Seven pairs of acute- and convalescent-phase sera and 11 single convalescent-phase sera (confirmed serologically by the IFA test) from patients with RMSF were also tested as positive controls. Negative controls were prevaccination and postvaccination sera from 31 healthy adult volunteers who had received attenuated *Salmonella typhi* strain Ty21a oral vaccine

or parenteral influenza virus vaccine prepared in chick embryo cells. In addition, six paired and eight single specimens from patients with confirmed viral exanthems acted as negative controls.

Antigen for the ELISA. Antigen was prepared from the Sheila Smith strain of *R rickettsii* grown in yolk sacs from plaque-purified seed. The yolk sac suspensions were purified by a batch sucrose method and centrifuged to equilibrium in Renografin® (E. R. Squibb, Princeton, NJ) density gradients [8]. Both heavy- and light-banding *R rickettsii* were washed by centrifugation and standardized photometrically by the method of Fiset et al [9] to yield a suspension of *R rickettsii* equivalent to 0.85 mg (dry weight)/ml. This suspension was sonicated for 30 min at maximum speed (Biosonic IV®; Bronwill Corp, Rochester, NY), pooled, and clarified by centrifugation at 20,000 *g* for 30 min. Aliquots of this antigen, preserved with formalin (final concentration, 0.1%) and containing 74 μg of protein/ml as determined by the method of Bradford [10] were stored at −20 C.

Microtiter ELISA. The method was modified from that described by Young et al for detection of IgG cholera antitoxin [11]. Optimal concentration of antigen, serum, and enzyme conjugates were determined by checkerboard titration [11]. The wells of polystyrene, U-bottom microtiter plates were coated with 100 μl of *R rickettsii* antigen diluted 1:25 in 0.1 *M* sodium carbonate coating buffer (pH 9.6). After incubation for 1 hr at 37 C, the wells were washed three times for 3 min each in 0.2 ml of PBS (pH 7.2) with 0.05% Tween 20 (PBS-Tween) and covered with 100 μl of 0.5% gelatin in PBS. After the plates were reincubated and washed as above, 100 μl of test or control serum diluted 1:400 in PBS-Tween was added. After the plates were reincubated and washed as before, 100 μl of conjugate (either goat antibody to human IgM or IgG diluted optimally in PBS) was added to each well. After 1 hr at 37 C, the triple wash was repeated, and *p*-nitrophenyl phosphate substrate (1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added to each well. After 45 min at 37 C, reactions were stopped with 3 *M* NaOH (25 μl per well), and color intensities were read. All test wells were controlled by parallel blanks. For each specimen, the net OD was calculated by subtracting the mean OD of the specimen tested in duplicate antigen-free wells from the mean OD of the corresponding wells coated with antigen.

A reference standard from a healthy volunteer, negative by the IFA test, was included in each ELISA as a negative control. An early convalescent-phase serum (titer of IgM antibody by the IFA test, 1:80) and a late convalescent-phase serum (titer of IgG antibody by the IFA test, 1:1,280) from challenged volunteers served as positive reference standards for the IgM ELISA and the IgG ELISA, respectively.

Selected sera exhibiting an elevated net OD by the IgM ELISA were screened for rheumatoid factor, and six sera with high titers of rheumatoid factor from patients with active rheumatoid arthritis were tested by IgM ELISA. Rheumatoid factor did not interfere or cause a false reaction in the determination of IgM antibody to rickettsiae.

Selected convalescent-phase sera treated with 0.03 *M* ethanethiol for 3 hr as described by Murray et al [12] had their reactivity in the IgM ELISA eliminated.

IFA test. Sera were tested for IgM antibody, IgG antibody, and total immunoglobulins by a modification of the IFA test of Bozeman and Elisberg [13].[1] Acetone-fixed, *R rickettsii*–infected chick embryo fibroblasts were used as antigen. Specific fluorescence at a serum dilution of ⩾1:80 was considered positive; a fourfold or greater increase in titer was interpreted as significant seroconversion.

Analysis of data. Student's *t*-test (two-tailed), the Wilcoxon rank sum test, the χ^2 test, Fisher's exact test, matched pair analysis, and linear regression analysis were performed on test results where appropriate.

Results

Determination of significant seroconversions for the ELISA. The mean ± SD differences in net OD between 37 pairs of negative control sera tested at 1:400 were 0.02 ± 0.02 by the IgM ELISA and 0.02 ± 0.01 by the IgG ELISA. A significant rise in antibody to rickettsiae was defined as a net OD increase of at least 0.09 for IgM or at least 0.06 for IgG antibody.

[1] C. L. Wisseman, Jr, and A. Waddell, "An Indirect Fluorescent Antibody System for Detection of Rickettsial Antibodies," manuscript in preparation.

Diagnostic agreement between ELISA and the IFA test. All tests were uniformly specific; no seroconversions were detected in pairs of negative control sera by the IgM or IgG ELISA or the IFA test for IgM, IgG, or total immunoglobulins. Significant rises were detected after vaccination in 25 of 50 vaccinees by the IgG ELISA, but in only 16 by the IFA test for total immunoglobulins ($P = 0.10$) and in 12 by the IgM ELISA ($P < 0.01$). The IgG ELISA (or the IFA test for IgG or total immunoglobulins) detected significant postillness seroconversions in all 12 vaccinated volunteers who developed RMSF after challenge; the IgM ELISA detected rises in 11 of these, whereas the IFA test for IgM detected only one. IgM rises were significantly lower in the ill vaccinees (mean rise in net OD, 0.18) than in unvaccinated volunteers with RMSF after challenge (mean rise in net OD, 0.56) ($P < 0.01$). These differences reflect the previous antigenic experience in the vaccinees. Therefore, the IgM results in vaccinees with RMSF were excluded for calculations to determine IgM test sensitivities.

All tests were highly sensitive; seroconversion was detected in 100% of unvaccinated volunteers or patients with RMSF by the IgM or IgG ELISA or by the IFA test for total immunoglobulins and in 83% by the IFA test for IgG or IgM. Overall, the results of the IgM ELISA and the IFA test for IgM agreed in only 13 (52%) of 25 paired sera, whereas the IgG ELISA and the IFA test for total immunoglobulins gave concordant results in 85 (84%) of 101 paired sera, and the concordance of the IgG ELISA and the IFA test for IgG was 76% (19 of 25 paired sera). The linear correlation coefficients between the results of the IgG ELISA and the IFA test for total immunoglobulins ($r = 0.72$) and between the results of the IgM ELISA and the IFA test for IgM ($r = 0.75$) were both highly significant ($P < 0.006$). Results of the IgG ELISA and the IFA test for IgG also correlated significantly ($r = 0.55$, $P < 0.0001$), as did the IFA tests for IgG and total immunoglobulins ($r = 0.68$, $P < 0.0002$), but the results of the IFA test for IgM and total immunoglobulins did not ($r = 0.12$).

Serodiagnosis by ELISA using a single serum specimen. For use in seroepidemiologic surveys or for serodiagnosis when only a single serum specimen was available, a net OD value was selected as a cutoff between negative and positive antibody levels. The mean ± SD net OD of serum specimens from 101 negative controls was 0.11 ± 0.062 in the IgM ELISA and 0.06 ± 0.037 in the IgG ELISA. Any net OD of ⩾0.30 for the IgM ELISA or ⩾0.18 for the IgG ELISA was regarded as positive.

Using these criteria, there was little overlap between the ELISA results of those sera from individuals with RMSF and those from negative controls (figure 1). Serum IgG antibody was generally lower in patients with naturally acquired RMSF than in experimentally infected volunteers, in part because of earlier specimen collection in the former group (six of 11 were collected within two to three weeks after the onset of RMSF) than in the latter group (four of six were collected three months after the onset of illness). Again, peak net OD levels in the IgM ELISA were lower in those who had previous antigenic experience. The sensi-

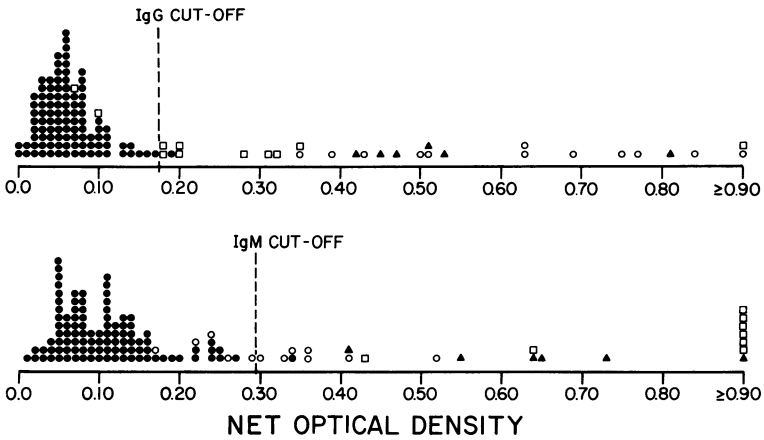

Figure 1. Distribution of IgG ELISA (*top*) and IgM ELISA (*bottom*) net OD values (at 410 nm) in 101 single serum specimens from individuals with no history of RMSF (●) and in a single convalescent-phase specimen from 12 vaccinated (○) and six unvaccinated (▲) volunteers with experimentally induced RMSF and from 11 persons with naturally acquired RMSF (□). The cutoff values between positive and negative samples are indicated.

tivity for serodiagnosis of RMSF was very good (100% in the IgM ELISA, 93% in the IgG ELISA).

Development of IgM and IgG antibodies after R rickettsii infection. Serial specimens from six unvaccinated challenged volunteers who developed RMSF and received treatment with tetracycline within 6 hr after the onset of fever were tested by ELISA and the IFA tests. Figure 2 illustrates the pattern of antibody responses in these volunteers. By each test, IgM and IgG antibody levels rose together beginning one week after the onset of RMSF. IgM antibody levels peaked by three weeks; IgM antibody levels by the IFA test fell sooner than did IgM antibody levels by ELISA. IgG antibody levels by ELISA peaked sooner than did levels of total immunoglobulins by the IFA test; both declined gradually, remaining elevated for ~12 months after illness.

By use of the serodiagnostic criteria, significant antibody rises by ELISA were detected by six days in four volunteers (two by the IgM ELISA and two by the IgG ELISA), but seroconversions detected by the IFA test were not demonstrated until the second week of illness. All were able to be serodiagnosed by three weeks, and rises could still be demonstrated at three months by the IgM ELISA and at 14 months by the IgG ELISA.

Discussion

The results of our studies indicate that the ELISA is highly sensitive and accurate for the determination of antibody responses to RMSF. Moreover, the ELISA is more sensitive than the IFA test for detection of low levels of antibody that are present after vaccination and during late convalescence. However, our findings, based on a small number of volunteers experimentally infected with *R rickettsii* and treated early with tetracycline, suggest that, as with other serologic tests for RMSF [1, 3–5], the value of the ELISA in rapid diagnosis is limited since neither IgM nor IgG seroconversion could be demonstrated before six days after the onset of illness.

It was gratifying to discover the applicability of

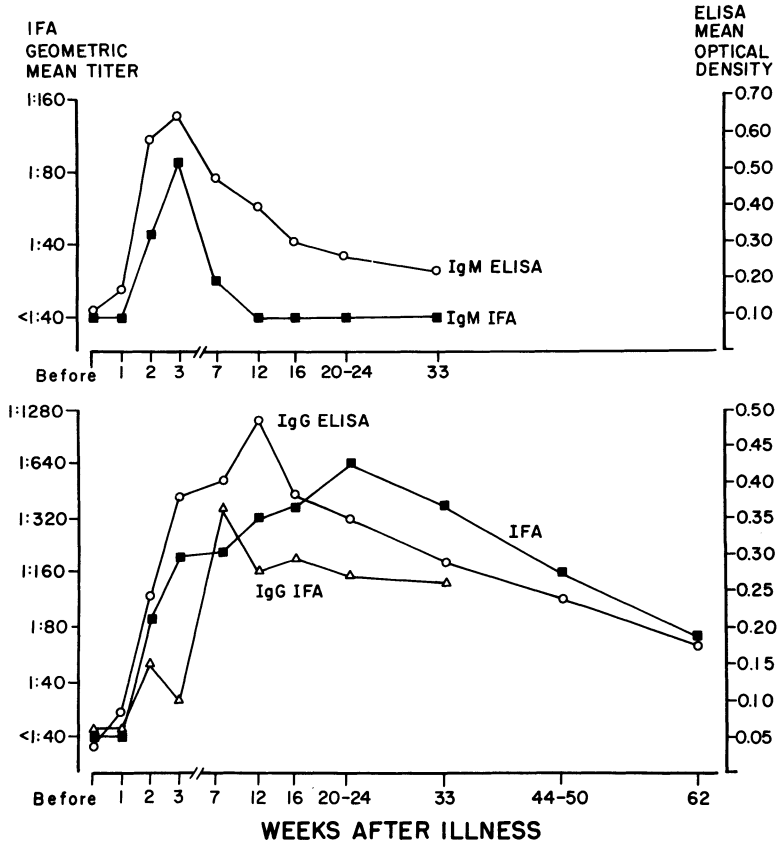

Figure 2. Serum IgM (*top*) and IgG (*bottom*) antibody responses in six unvaccinated volunteers who developed clinical RMSF after intradermal challenge with *R rickettsii*. Antibody responses were measured by an ELISA and by the IFA test.

the ELISA when only a single serum specimen was available for diagnosis. The ease with which the test can be performed and its high sensitivity (100%) and specificity (99%) should encourage its use in seroepidemiology, vaccine evaluation, and perhaps the diagnosis of natural infection. The IgG ELISA may be especially suitable for seroepidemiology, since it detected antibody to rickettsiae at a single dilution of serum and in a single serum specimen (in experimentally infected volunteers) collected at least a year after illness. Further study is required to determine how long ELISA remains positive in patients convalescent from naturally acquired RMSF.

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