

Rheumatoid Factor in Syphilis

ERICH H. CERNY,* CAROL E. FARSHY, ELIZABETH F. HUNTER, AND SANDRA A. LARSEN
Sexually Transmitted Diseases Laboratory Program, Centers for Disease Control, Atlanta, Georgia 30333

Received 7 December 1984/Accepted 16 April 1985

Immunoglobulin M (IgM) antibodies directed against IgG antibodies (rheumatoid factor [RF]) are known to occur often in patients with syphilis and to interfere with serological tests measuring specific antibodies of the IgM class. In this study we examined the occurrence and specificity of the RF and demonstrated a simple method to detect and eliminate the RF for a specific *Treponema pallidum* IgM enzyme-linked immunosorbent assay. We measured the occurrence of the RF with a sensitive enzyme-linked immunosorbent assay and found that it increased with the duration of syphilitic disease: 1 of 13 primary syphilis serum specimens, 3 of 13 secondary syphilis serum specimens, and 10 of 27 latent syphilis serum specimens were reactive in this RF test. Those sera containing IgM RF were immunoprecipitated with anti-human gamma chain antibodies and 2% polyethylene glycol until the RF was removed. One serum specimen from a patient in the secondary stage of syphilis and eight serum specimens from patients with latent disease still presented the RF after immunoprecipitation. Removal of the IgG antibodies also improved the sensitivity of the treponemal IgM test, indicating competition of these antibodies for binding sites of the antigen. The enzyme-linked immunosorbent assays for detection of RF and antitreponemal IgM antibodies are performed on the same plate. Theoretically, only sera positive for both tests have to be immunoprecipitated. But our findings indicated an increase in sensitivity of the IgM enzyme-linked immunosorbent assay after removal of IgG antibodies responsible for competition at the binding sites.

Rheumatoid factor (RF) here is defined as those immunoglobulin M (IgM) class antiimmunoglobulins (IgM anti-IgG or in some cases IgG or IgA anti-IgG) that react with the IgG in the serum of the patient. This complex combined with the treponemal antigen gives, in the case of the IgM RF, a false-positive IgM reaction (4, 18) when the IgM RF-syphilitic IgG reacts with the anti-human IgM conjugate in such sensitive assays as immunofluorescence, enzyme-linked immunosorbent assay (ELISA) (5, 15, 17, 25), or radioimmunoassay (13). Since specific IgM is indicative of active disease, these IgM class RFs must be eliminated. RF has not only been reported in the serum of patients with rheumatoid arthritis but also in individuals with other diseases, such as syphilis (4, 16), as well as in healthy individuals (4).

The interference of the RF with the measurement of IgM antibodies against *Treponema pallidum* was described as early as 1959 by Peltier and Christian (16) and more recently reported by Reimer et al. (18) and Fraser et al. (8).

Different approaches have been used in the past to remove the RF; these include separation of IgG and IgM by ultracentrifugation on a gradient (6), ion-exchange column chromatography (11), absorption of IgG by *Staphylococcus* protein A (7), and adsorption of the RF to cross-linked IgG on latex particles (1, 20). Each of the aforementioned methods has some inherent fault: ultracentrifugation and ion-exchange chromatography are time consuming and dilute the serum sample, whereas aggregated IgG or latex particles only partially remove the RF. We chose to remove the RF by immunoprecipitation of the IgG antibodies (to which the IgM anti-IgG RF attaches), a technique successfully applied to rubella IgM testing (9, 19). An added advantage to this procedure is the elimination of competitive inhibition by specific IgG antibody (9, 18). A 2% solution of polyethylene glycol (PEG) was added to facilitate removal of complexes in

sera from rheumatoid arthritis patients, syphilis patients, and healthy individuals.

A double ELISA measuring *T. pallidum* IgM and RF is described, with immunoprecipitation for removal of IgG and IgA for specific *T. pallidum* IgM. The ELISA detecting the RF is performed on the same ELISA plate as the IgM treponemal ELISA. Theoretically, only the sera positive in the treponemal ELISA and containing the RF are then immunoprecipitated with anti-IgG antibodies and retested. However, the IgM titers of all sera from syphilis patients rose after immunoprecipitation removed the source of competitive inhibition.

MATERIALS AND METHODS

ELISA antigen. Treponemal antigen was prepared as previously described (12). Briefly, *T. pallidum* was grown by intratesticular inoculation in adult male rabbits. Tissues were extracted in 0.075 M sodium citrate, washed in phosphate-buffered saline by differential centrifugation, and stored for 1 week before sonication.

Sera. Sera from 53 individuals with syphilis representative of the primary, secondary, and latent categories were obtained from the serum bank of the Sexually Transmitted Diseases Laboratory, Centers for Disease Control. All patients were treated at the time of serum collection or had received previous treatment. All were reactive with the fluorescent treponemal antibody-absorption (FTA-ABS) and rapid plasma reagin card tests (24).

Sera from 39 individuals with clinically diagnosed rheumatoid arthritis were obtained from the same source, as were 10 normal serum specimens from nonsyphilitic individuals. All were obtained with informed consent and had been frozen at -20°C for up to 1 year. All were nonreactive in the RPR card test for syphilis.

Latex test for RF. A latex agglutination test (Rapi/Tex-RF test; Calbiochem-Behring, La Jolla, Calif.) was used as the

* Corresponding author.

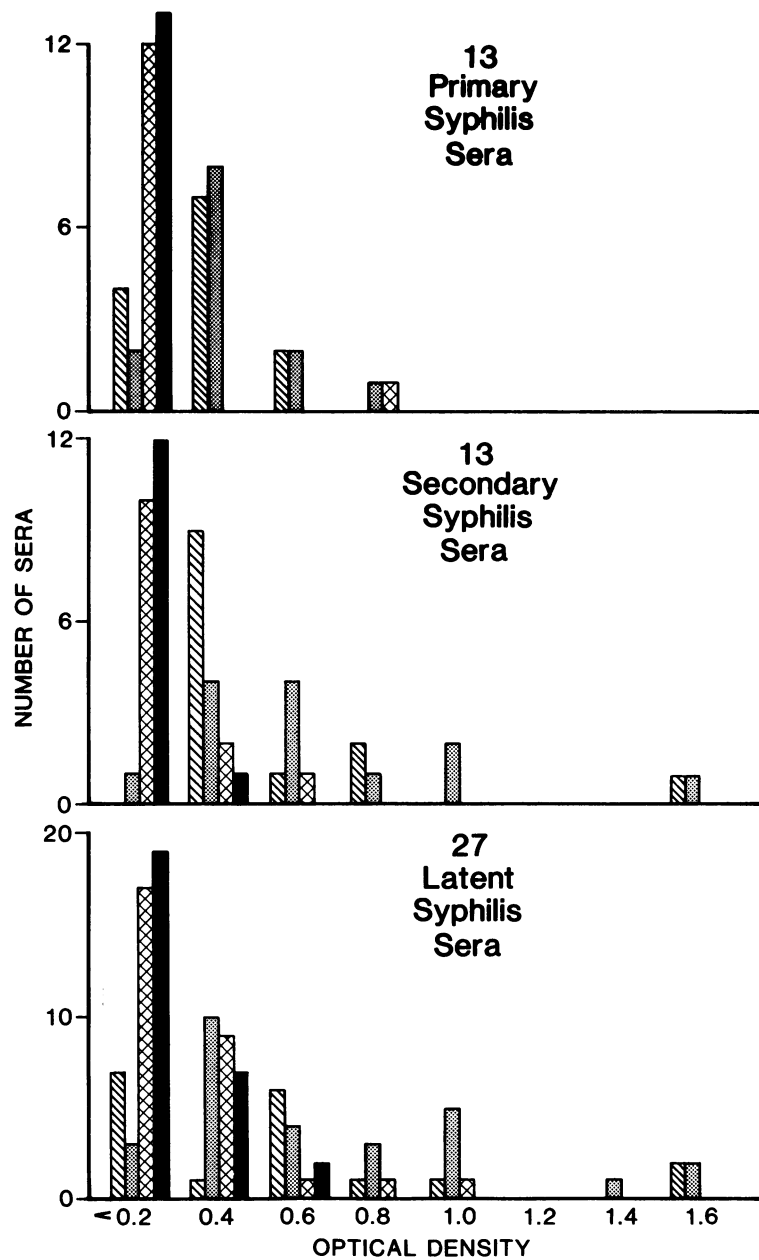


FIG. 1. Results of the treponemal and RF ELISAs for sera from patients with the three stages of syphilis. The ELISA results before and after removal of the RF are shown separately. A total of 1 of 13 primary, 3 of 13 secondary, and 10 of 27 latent syphilis serum specimens showed significant amounts of RF (OD of >0.2). One secondary serum specimen and ten latent serum specimens still showed RF after removal with anti-IgG. This RF could further be reduced by immunoprecipitation with a higher amount of anti-IgG. Symbols: \square , *T. pallidum* ELISA before RF removal; \square , *T. pallidum* ELISA after RF removal; \square , RF ELISA before RF removal; \square , RF ELISA after RF removal.

reference test for the RF. This test was performed on all sera according to the instruction of the manufacturer for the quantitative tube dilution test. A patient was considered seropositive if there was reactivity in the serum at a 1:20 dilution or above.

FTA-ABS IgM test. The FTA-ABS IgM test was performed as reported by Scotti et al. (21) by using fluorescein-labeled anti-human IgM conjugate (Wellcome Research Laboratories, Beckenham, England). This test was performed on all sera evaluated.

Removal of the RF by immunoprecipitation of the IgG in

sera. Glass tubes (12 by 75 mm) were used for the following procedure. Each serum specimen to be precipitated was diluted 1:50 in borate buffer ([pH 8.4] 6.184 g of boric acid, 9.536 g of sodium tetraborate, and 4.384 g of NaCl in 1 liter of water). The diluted serum (50 μ l) was then added to 50 μ l of a 6% solution of PEG 6000 (Fisher Scientific Company, Fair Lawn, N.J.) in phosphate-buffered saline and 50 μ l of a 1:5 dilution (in borate buffer) of rabbit anti-human IgG (gamma chain specific; Dako lot 0625F; Accurate Chemicals, Westbury, N.Y.) for a final dilution of 1:150. After incubation overnight at 4°C or for 3 h at room temperature, the

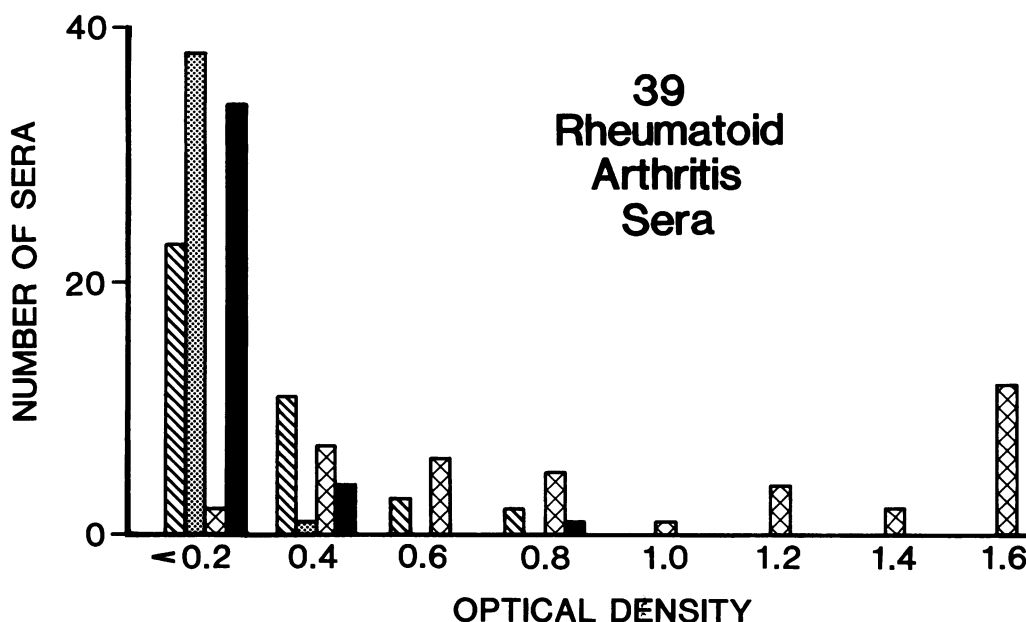


FIG. 2. Serum specimens from 39 patients with rheumatoid arthritis were examined in the treponemal and RF ELISAs before and after removal of the RF. Before removal of the RF, 16 serum specimens showed false-positive treponemal ELISA results (OD of >0.2), with only 1 false-positive result after immunoprecipitation. This was corrected by immunoprecipitation with a double amount of anti-IgG. Symbols: ▨, *T. pallidum* ELISA before RF removal; ▩, *T. pallidum* ELISA after RF removal; ⋯, RF ELISA before RF removal; ■, RF ELISA after RF removal.

mixture was centrifuged at 3,000 rpm for 40 min. The supernatants were then used in the ELISA.

ELISA for RF and antibodies against *T. pallidum*. The principles of the RF ELISA (10) and the treponemal IgM ELISA (5) have been described previously. In this procedure specifically, duplicate vertical rows of polyvinylchloride microtiter plates (U-bottom; Dynatech Laboratories, Inc., Alexandria, Va.) were coated in 50- μ l amounts with a sonicated suspension of *T. pallidum* or rabbit anti-human IgG. Appropriate dilutions were determined before serum assays by block titration (26). Sodium carbonate-bicarbonate at 0.05 M (pH 9.6) was used as a coating buffer. The plates were then tightly covered and stored at 4°C until used or up to 2 weeks. Before use, plates were washed three times with phosphate-buffered saline-0.05% Tween 20. Four wells were designated for each test serum specimen or control: two coated with *T. pallidum* and two coated with anti-human IgG (RF detection). Sera were assayed both before and after immunoprecipitation. Control patient sera (no immunoprecipitation) were diluted 1:150 in borate buffer to be run in parallel with the immunoprecipitated sample. The plates were incubated in a moist chamber at room temperature for 4 h. After a washing step as above, 50 μ l of affinity-purified alkaline phosphatase-labeled goat anti-human IgM (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted in 2% normal goat serum-phosphate-buffered saline-0.05% Tween 20 was added to each well. For this step plates were incubated in humid chambers at 37°C for 1 h. After a washing step as above, the substrate for alkaline phosphatase, *p*-nitrophenyl phosphate (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) in a diethanolamine (26) buffer was added, and the plates were incubated at room temperature for 30 min. The plates were read in a Dynatech MicroELISA MR 580 reader at 405 nm. Based on a previous study with this *T. pallidum* ELISA (15), an optical density (OD) of ≥ 0.2 was considered to be positive.

RESULTS

IgM ELISA for *T. pallidum* and RF before and after treatment with anti-IgG. A total of 10 normal serum specimens, 53 serum specimens from patients with syphilis (Fig. 1), and 39 serum specimens from patients with rheumatoid arthritis (Fig. 2) were tested with ELISA for the presence of antitreponemal antibodies and RF. Both tests were performed before and after removal of the RF by immunoprecipitation.

Normal sera. Ten normal serum specimens were tested and found to be nonreactive (OD of <0.2) in the treponemal and RF ELISAs. An OD of 0.2 was determined to be the optimal cut-off point between positive and negative values based on previous studies with sera from patients with syphilis and patients without syphilis (12).

Sera from patients with syphilis. Figure 1 represents the results for 53 serum specimens from patients with active syphilis separated by disease categories. The IgM antitreponemal antibodies were detected (OD of ≥ 0.2) in all but 11 of the 53 patients. Only six of these remained nonreactive after removal of the IgG antibodies.

Eight serum specimens from patients with latent syphilis and one serum specimen from a patient with secondary syphilis showed positive results in the RF ELISA after immunoprecipitation with anti-IgG antibodies. A second immunoprecipitation of these sera with increased amount of anti-IgG diminished further the results obtained by the RF ELISA (data not shown).

Sera from patients with rheumatoid arthritis. Figure 2 represents the results for patients with a documented history of rheumatoid arthritis and no history of clinical signs indicating syphilitic disease. Only one serum sample, after attempts to remove RF, still presented a false-positive result in the treponemal ELISA test. After immunoprecipitation, a significant amount of RF was still detectable in this same

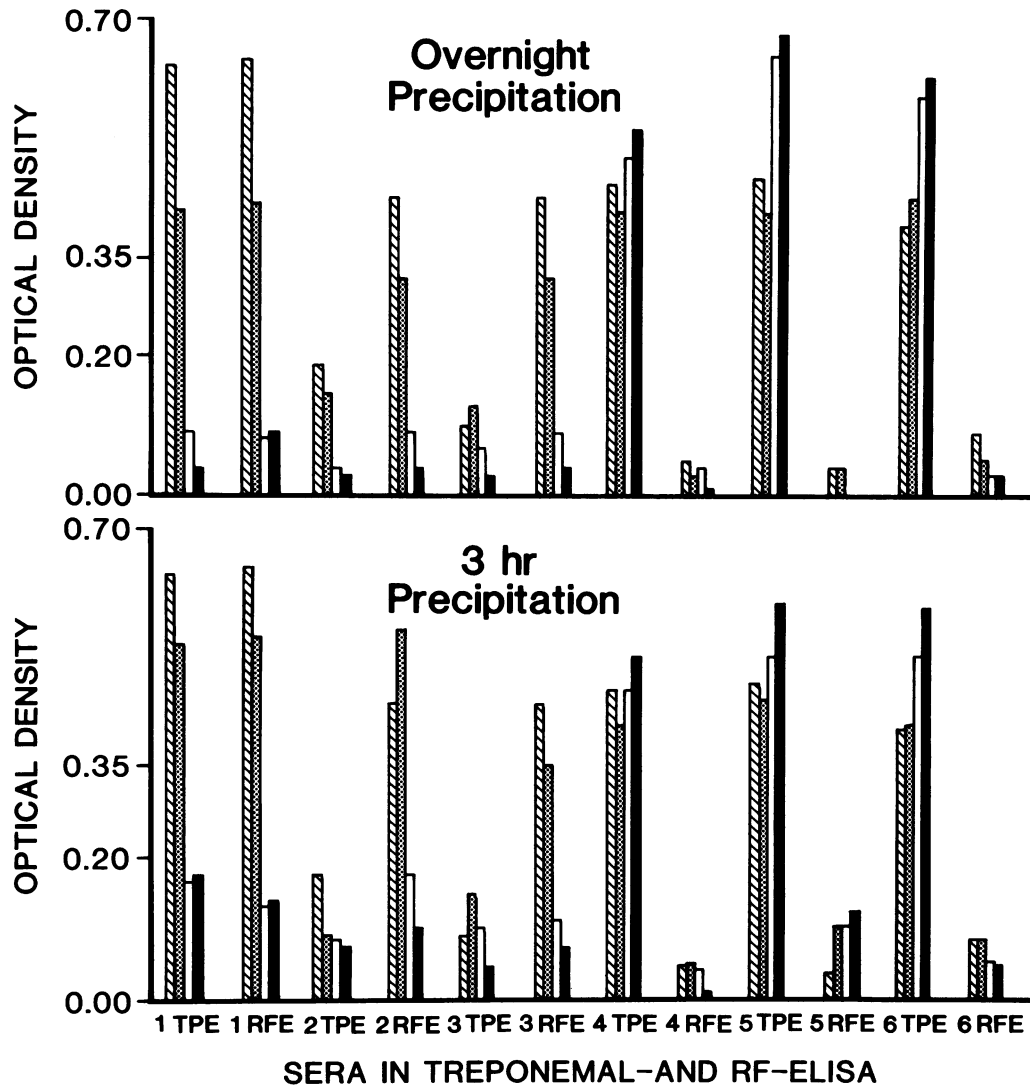


FIG. 3. Three serum specimens from patients with rheumatoid arthritis (specimens 1 through 3) and three serum specimens from patients with syphilis (specimens 4 through 6) were examined in the antitreponemal ELISA (TPE) and the RF ELISA (RFE) under different experimental conditions. Two different incubation times for the precipitation procedure were used: overnight at 4°C (above) and 3 h at room temperature (below). The four different treatments of sera used for removal of the RF are represented. Each serum specimen was not treated (control) (▨), precipitated with only PEG (▩), precipitated with anti-IgG (▧), and precipitated with PEG and anti-IgG (■).

serum specimen (OD of 0.23). Both RF and the false-positive treponemal IgM ELISA results disappeared after treatment with the double amount of anti-IgG. The other four serum specimens still presenting the RF after immunoprecipitation did not cause a false-positive treponemal IgM ELISA when retested. All four of these serum specimens were reactive in the FTA-ABS IgM test.

Influence of PEG on the removal of the RF with PEG and anti-IgG alone or combined. Figure 3 shows the influence of 2% PEG on the removal of the RF. Three serum specimens from syphilitic patients known to have antitreponemal antibodies (specimens 4, 5, and 6) and three serum specimens from patients known to have no antitreponemal antibodies but RF (specimens 1, 2, and 3) were incubated for 3 h and overnight with (i) no additive, (ii) 2% PEG, (iii) anti-IgG, and (iv) 2% PEG and anti-IgG. These sera were then retested in the antitreponemal and the RF ELISAs. The control incubation (no PEG or anti-IgG) showed, as expected, the high OD

in the antitreponemal and the RF ELISAs for RF sera. The results after removal of the RF after 3 h of incubation, with exactly the same procedure as before, are shown in the lower part of the Fig. 3.

There is a reasonable correlation between both series. The longer incubation period produces a more complete removal of the RF and should therefore be used whenever feasible.

DISCUSSION

The induction of the RF through infectious disease has been described in relation to a number of infectious agents (2, 20) and also for syphilis (8, 16, 18). We could confirm the induction of the RF by syphilis. Our results showed also an increase in the incidence of the RF with duration of disease. A total of 1 of 13 primary syphilis serum specimens, 3 of 13 secondary syphilis serum specimens, and 10 of 27 latent syphilis serum specimens presented significant (OD of >0.2)

amounts of RF. In all sera the amount of RF was diminished after removal of the IgG with anti-IgG, as expected.

Figure 1 also demonstrates another interesting feature. There were 11 nonreactive treponemal ELISA results before removal of the RF (OD of <0.2) and only 6 nonreactive results after removal. This is best explained by competition between IgG and IgM antibodies for the binding sites on the treponemal antigen (3). This is also reflected by the mean of the OD values, which is higher after removal of the RF than before: for primary syphilis sera the OD was 0.27 (before) and 0.28 (after), for secondary sera the OD was 0.47 (before) and 0.56 (after), and for latent sera the OD was 0.42 (before) and 0.59 (after).

The RF may produce false-positive results by two different mechanisms. It may bind to IgG antibodies which attach to the antigen and thus mimic antigen-specific IgM antibodies. Or it may bind by means of unsaturated binding sites directly to the enzyme-labeled conjugate and produce false-positive results regardless of the specificity of the labeled antibody. The binding sites for the RF on immunoglobulins are located on the Fc fragment, and the use of labeled Fab fragments as conjugates has been advocated (14, 23). Another approach to circumvent this problem was used in our assay. A 2% solution of serum belonging to the same animal species as the conjugate (goat) was used in all incubation steps to saturate all free binding sites of the RF. Despite this procedure, the high number of false-positive results with sera from patients with rheumatoid arthritis indicates clearly that this is not totally adequate: the RF must be removed from the test specimen to omit RF interference of the first type mentioned above. We used an IgG precipitation with anti-IgG to remove IgM bound to IgG (IgM RF). A small number of sera still show the RF as measured by the RF in the RF ELISA after immunoprecipitation with anti-IgG. This means RF removal is not always throughout. These samples need further immunoprecipitation until all the RF disappears. We would therefore not advocate use of the immunoprecipitation procedure without monitoring the sera for complete removal of the RF by RF ELISA.

There are samples needing excessive amounts of anti-IgG to diminish the RF-like activity. We are currently investigating whether factors other than the RF may be responsible for this phenomenon.

The use of PEG to facilitate precipitation of immunocomplexes has been used in many tests. We selected the concentration of 2% because other investigators have shown that concentrations over 2% may precipitate noncomplexed immunoglobulins (22). Addition of PEG alone did not significantly change the results since there was essentially no precipitation of the RF. Removal of IgG by anti-IgG diminished significantly the amount of RF, and these results were further improved when anti-IgG and PEG are added together. We observed also that addition of PEG facilitated the separation of the supernatant from the precipitate.

LITERATURE CITED

1. Avrameas, S. 1969. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoabsorbents. *Immunochemistry* 6:53-66.
2. Bartfeld, H. 1969. Distribution of rheumatoid factor activity in nonrheumatoid states. *Ann. N.Y. Acad. Sci.* 168:30-40.
3. Cohen, J. R., L. C. Norins, and A. J. Julian. 1967. Competition between and effectiveness of IgG and IgM antibodies in indirect fluorescent antibody and other tests. *J. Immunol.* 98:143-149.
4. Cremer, N. E., and J. L. Riggs. 1979. Immunoglobulin classes and viral diagnosis, p. 191-195. In E. H. Lennette and N. J. Schmidt (ed.), *Diagnostic procedures for viral, rickettsial, and chlamydial infections*. American Public Health Association, Inc., Washington, D.C.
5. Farshy, C. E., E. F. Hunter, S. A. Larsen, and E. H. Cerny. 1984. Double conjugate enzyme-linked immunosorbent assay for immunoglobulins G and M against *Treponema pallidum*. *J. Clin. Microbiol.* 20:1109-1113.
6. Forghani, B., N. J. Schmidt, and E. H. Lennette. 1973. Demonstration of rubella IgM antibody by indirect fluorescent antibody staining, sucrose density gradient centrifugation and mercaptoethanol reduction. *Intervirology* 1:48-59.
7. Forsgren, A., and J. Sjoquist. 1966. Protein A from *S. aureus*. I. Pseudoimmune reaction with human gamma globulin. *J. Immunol.* 97:822-827.
8. Fraser, K. B., P. V. Shirodaria, and C. F. Stanford. 1971. Fluorescent staining and human IgM. *Br. Med. J.* 3:707.
9. Gispen, R., J. Nagel, B. Brand-Saathof, and S. De Graaf. 1975. Immunofluorescence test for IgM rubella antibodies in whole serum after absorption with anti-gamma Fc. *Clin. Exp. Immunol.* 22:431-437.
10. Gripenberg, M., F. Wafin, H. Isomaki, and E. Linder. 1979. A simple enzyme immunoassay for the demonstration of rheumatoid factor. *J. Immunol. Methods* 31:109-118.
11. Hornsleth, A., J. Leerhoy, P. Grauballe, and H. Spanggaard. 1974. Rubella-virus-specific IgM and IgA-antibodies. The indirect immunofluorescence (IF)-technique applied to sera with reduced IgG concentration. *Acta Pathol. Microbiol. Scand. Sect. B* 82:742-744.
12. Hunter, E. F., C. E. Farshy, S. L. Liska, D. D. Cruce, J. A. Crawford, and J. C. Feeley. 1982. Sodium desoxycholate-extracted treponemal antigen in an enzyme-linked immunosorbent assay for syphilis. *J. Clin. Microbiol.* 16:483-496.
13. Miller, J. M., E. M. Donowho, D. W. Trent, and E. E. M. Moody. 1979. A solid-phase radioimmunoassay for the detection of antibodies of *Treponema pallidum*. *Sex. Transm. Dis.* 6:43-49.
14. Naot, Y., E. V. Barnett, and J. S. Remington. 1981. Method for avoiding false-positive results occurring in immunoglobulin M enzyme-linked immunosorbent assays due to presence of both rheumatoid factor and antinuclear antibodies. *J. Clin. Microbiol.* 14:73-78.
15. Pedersen, N. S., C. S. Pedersen, and N. H. Axelsen. 1982. Enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody against the Reiter treponeme flagellum in syphilis. *J. Clin. Microbiol.* 16:608-714.
16. Peltier, A., and C. L. Christian. 1959. The presence of the "rheumatoid factor" in sera from patients with syphilis. *Arthritis Rheum.* 2:1-7.
17. Pope, V., E. F. Hunter, and J. C. Feeley. 1982. Evaluation of the microenzyme-linked immunosorbent assay with *Treponema pallidum* antigen. *J. Clin. Microbiol.* 15:630-634.
18. Reimer, C. B., C. M. Black, D. J. Phillips, L. C. Logan, E. F. Hunter, B. J. Pender, and B. E. McGrew. 1975. The specificity of fetal IgM: antibody or anti-antibody? *Ann. N.Y. Acad. Sci.* 254:77-93.
19. Schmitz, H., H. Shimizu, D. Kampa, and H. W. Doerr. 1975. Rapid method to detect rubella immunoglobulin M and immunoglobulin A antibodies. *J. Clin. Microbiol.* 1:132-135.
20. Shirodaria, P. V., K. B. Fraser, and F. Stanford. 1973. Secondary fluorescent staining of viral antigens by rheumatoid factor and fluorescein-conjugated anti-IgM. *Ann. Rheum. Dis.* 32:53-57.
21. Scotti, A. T., and L. Logan. 1968. A specific IgM antibody test in neonatal syphilis. *J. Pediatr.* 73:242-243.
22. Soltis, R. D., and D. E. Hasz. 1982. The effect of serum immunoglobulin concentration on immune complex detection by polyethylene glycol. *J. Immunol. Methods* 57:275-282.
23. Tarkowski, A., L. M. Bjursten, L.-A. Nilsson, and H. Nygren. 1983. False positive results in class-specific rheumatoid factor assays due to interaction between RF and Fc fragments of anti-immunoglobulin indicator reagents. *J. Immunol. Methods*

- 58:171-182.
24. U.S. Department of Health, Education, and Welfare. 1969. Manual of tests for syphilis. Publication no. 411. U.S. Government Printing Office, Washington, D.C.
 25. Veldkamp, J., and A. M. Visser. 1975. Application of the enzyme-linked immunosorbent assay (ELISA) in the serodiagnosis of syphilis. *Br. J. Vener. Dis.* **51**:227-291.
 26. Voller, A., D. Bidwell, and A. Bartlett. 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506-512. *In* N. R. Rose and H. Friedman (ed.), *Manual of clinical immunology*. American Society for Microbiology, Washington, D.C.