Evaluation of Fluorescent Treponemal Antibody (RFTA and FTA II) and Other Tests (RPCF and TPI) for Syphilis. (27276)

J. F. KENT, S. V. COVERT, H. W. REILLY, W. H. KINCH AND W. B. LAWSON°
Division of Laboratories and Research, New York State Dept. of Health and Albany Medical College, Albany, N. Y.

The Reiter treponeme was recently adapted(1) to a fluorescent test (RFTA) for treponemal antibody. Treating the organisms with dilute hypochlorite was essential to the adaptation, and subsequent trials showed it to be equally effective in conditioning virulent Nichols strain treponemes for the fluorescent treponemal antibody (FTA) test(2). A comparison of the RFTA and modified FTA (FTA II) tests appeared in order. A complement-fixation test using Reiter protein antigen(3,4) was evaluated concurrently because of its increasing use as a screening procedure(5-7) in differentiating persons with latent syphilis from those whose sera react "non-specifically" with cardiolipin antigens. All specimens had been subjected to a critical 40-hour test(8,9) for specific *Treponema pallidum* immobilizing (TPI) antibody.

Materials and methods. Sera were from a collection of the TPI testing unit of the Division of Laboratories and Research; they were from persons verified as syphilitic or non-syphilitic on the basis of clinical or anamnestic evidence, supplemented by a critical 40-hour test(8,9) for TPI antibody; many of the non-syphilitic individuals were "biologic false positive" reactors, their sera reacting with cardiolipin antigens. "Normal", non-reactive sera were obtained from healthy members of the staffs, and from blood donors. All sera were stored at −25°C.

RFTA and FTA II tests. The Reiter strain of *T. pallidum* was grown at 35°C in 23-ml screw-capped tubes containing 18 ml NIH thioglycollate broth* to which 2 ml heated (2 hours at 56°C) rabbit serum was added to make the final concentration 10%. Growth was satisfactory at 2 days, the cultures remaining acceptable for antigen preparation at least to the 5th day. One ml was removed aseptically from the undisturbed upper stratum and delivered into a 15 × 100 mm pyrex centrifuge tube containing 1 ml 0.002% sodium hypochlorite (NaClO)† in 0.85% NaCl solution. The NaClO was essential, the treponemes otherwise clumping, exhibiting atypical morphology, and/or failing to adhere to glass slides. They were mixed well by gentle swirling, then sedimented by spinning 15 minutes at 1,000 *g*. The supernate was decanted, and the organisms were washed once by resuspending in 2 ml 0.85% NaCl solution, centrifuging, and decanting as before. They were suspended finally in enough 0.85% NaCl solution to make the count 10-15/430 × microscopic field, or about 2 × 10^6/ml.

T. pallidum of the Nichols strain was available as suspensions eluted from fresh or frozen(10) syphilomatous rabbit testes in Nelson's medium(11) containing streptomycin and penicillinase(12); they were used on the day of elution or after 1-15 days' storage at 2-4°C. For the FTA test as modified herein (FTA II), 1 ml of suspension was treated like the Reiter organisms with 1 ml 0.002% NaClO in 0.85% NaCl solution. The treponemes were washed once with 2 ml 0.85% NaCl solution, and adjusted finally with 0.85% NaCl solution to approximately the same count (2 × 10^6/ml) as that of the Reiter suspension. It should be noted in this connection that hypochlorite-treated Nichols suspensions could be adjusted to this count with reasonable assurance that the same number/field would remain for final examination in slide films. In the initial experience (1) with Nichols organisms not treated with NaClO, it was necessary to adjust suspen-

° Presented at the Second Latin American Congress of Microbiology, San José, Costa Rica, Dec. 10-17, 1961.

* No. 0257-01, Difco Labs., Inc., Detroit, Mich.

† B·K chlorine-bearing liquid bactericide; active ingredient sodium hypochlorite 5.25%. Pensalt Chemicals Corp., Phila. 2, Pa. A 1:2,625 dilution was made in 0.85% NaCl solution.

sions to 40-50/430 × field to compensate for losses that occurred during treatment of the films with test serum and fluorescent antibody.

New precleaned microscope slides (75 × 25 × 1 mm) and cover slips (22 × 22 mm, thinness No. 1) were used, two 1-cm circles being inscribed on the former with a template and diamond stylus. Slides and cover slips were soaked one hour in 95% ethanol, then wiped clean with clean gauze followed by disposable tissue;‡ they were stored in dust-free containers. The technic of testing was that given for the RFTA test (1). A minimum (less than 0.01 ml) of an antigen suspension was delivered into each of 2 circles on a slide and spread to fill the circles with a glass rod. It was important, in both RFTA and FTA II tests, to use as little suspension as possible since excess contributed greatly to background fluorescence. The films were air-dried, after which the slides were immersed 10 minutes in acetone (Certified Reagent), and again air-dried. Sera to be tested were heated 30 minutes at 56°C. A 0.1-ml sample was then diluted 1:200 stepwise by adding 1.9 ml phosphate buffered saline solution (PBS) of pH 7.2,§ mixing well, transferring 0.10 ml to a tube containing 0.90 ml PBS, and again mixing well. The 1:200 dilution served for qualitative tests; for quantitative testing further serial 2-fold dilutions were made in PBS. In the test proper, 0.03-ml aliquants of diluted serum were delivered onto 2 films each of the Reiter and Nichols treponemes, and the slides rotated‖ 30 minutes at 100 rpm in the incubator (37°C); they were protected from evaporation by lids containing moistened blotting paper. Slides were then rinsed in PBS, soaked 5 minutes in each of 2 changes of PBS, and pressed gently on filter paper to remove excess moisture. Fluorescein-labeled rabbit antihuman globulin¶ diluted optimally (1:500) in PBS containing 2% Tween 80** was then added, 0.03 ml being distributed over each film, and the slides again rotated 30 minutes at 100 rpm in the incubator. They were washed twice in PBS and blotted gently as before on filter paper. Finally, a drop of buffered glycerol (9 parts glycerol + 1 part PBS) was placed on each reaction site and surmounted with a cover slip. Slides were examined by darkfield microscope†† using low fluorescence immersion oil‡‡ between condenser and slide. Fields containing treponemes were located first by visible light, and the treponemes then examined for fluorescence by ultraviolet light. Degree of fluorescence was estimated, and the sera were designated reactive (2+ to 4+ fluorescence) or nonreactive (− to 1+). Reactive specimens were diluted serially in PBS and retested. Reactivity restricted to the 1:200 dilution was represented as a titer of 1; reactivity extending to higher dilutions (1:400, 1:800, etc.) as 1/200th the reciprocal of the highest reactive dilution (titer = 2, 4, etc.).

Cardiolipin and Reiter protein complement-fixation tests. The cardiolipin antigen (13,14) contained cardiolipin 0.0175%, lecithin 0.0875%, and cholesterol 0.30%. The Reiter protein antigen(3,4) was a commercial product.¶ The complement-fixation procedure was a natural development of earlier methods(15). Total volume was 0.75 ml, the reagents being used in 0.15-ml aliquants. The reagent diluent(16) was triethanolamine buffered saline solution (TBS) containing Mg^{++} and Ca^{++} in concentrations(17) optimal for immune hemolysis. In practice, 0.05 ml heated (30 minutes at 56°C) serum plus 0.10 ml TBS were incubated 16-18 hours

‡ Kimwipes, Kimberly-Clark Corp., Neenah, Wis.

§ One liter of aqueous solution contained 6.8 g NaCl, 1.48 g Na_2HPO_4, and 0.43 g KH_2PO_4. The formula differed slightly from that prescribed for FTA tests, but had better buffering capacity, and did not affect FTA results(1).

‖ Rotating apparatus, electric, Cat. No. 3623, A. H. Thomas Co., Phila., Pa.

¶ The Sylvana Co., Millburn, N. J.

** Hilltop Labs. Inc., Cincinnati, Ohio.

†† American Optical Co., Model 2; objective, achromatic, 43X; condenser, C234, wide angle, with dark-field stop plate. Illumination, Reichert "Fluorex" with power supply. Subocular filter OG-1 used continuously to exclude visible blue and residual UV light; filter BG-12 interposed for switch from unrestricted to UV illumination.

‡‡ Type A, R. P. Cargille Laboratories, Inc., New York, N. Y.

at 2-4°C with 0.15 ml each of complement (five 50% hemolytic units) and the optimal dilution of antigen (cardiolipin antigen, 1:250; Reiter protein antigen, 1:130); 0.15 ml TBS was substituted for antigen in the serum control. Three-tenths ml of optimally sensitized(18) sheep's erythrocytes (5×10^8 sensitized cells/ml) were then added, and the tests incubated 30 minutes at 37°C for hemolysis. Results were read by visual comparison with standards simulating the possible events, *i.e.*, reaction (40-100% inhibition of hemolysis), weak reaction (10-35% inhibition), or no reaction (0-5% inhibition).

T. pallidum immobilization (TPI) test. This was a sensitive 40-hour test(8,9) based essentially on the original method(11), but using an improved survival medium(12). Sera found reactive (more than 50% specific treponemal immobilization) after the usual 18-hour incubation were not examined further. Those found weakly reactive (21-50% immobilization) or non-reactive (0-20%) received supplements of complement, 0.05 ml of active and heat-inactivated guinea-pig serum being added respectively to tests and serum controls, and were reincubated. At 40 hours, the percentage of specific treponemal immobilization was redetermined.

Results. The relative sensitivity of the RFTA and FTA II tests was determined by parallel titration of syphilitic sera; aliquants of the same serum dilutions served for both tests. Comparative titers of 108 individuals appear in Table I. They were identical in the majority (68) of instances. Differences never exceeding 2-fold occurred in the remainder, the RFTA titer exceeding the FTA II in 20 instances, and the reverse being true in another 20 instances. A study was also made of the relative frequency of reactions in qualitative RFTA and FTA II tests of 204 patients representing different stages of syphilis. Table II summarizes the findings; only the RFTA results are represented, those of the FTA II being identical unless otherwise indicated. There were only 4 discrepancies; the RFTA test was reactive and the FTA II negative in one case each of primary, secondary and cardiovascular syphilis, and the FTA II was reactive in one case of unclassified syphilis which was RFTA-negative. As already indicated, a critical 40-hour test (8,9) for specific TPI antibody had been performed to supplement the clinical diagnoses; these received strong support, the only TPI-negative patients being 4 with primary syphilis and one with cardiovascular involvement; in these instances other laboratory and clinical findings provided a definitive diagnosis. Specificity for syphilis was evaluated in tests of 112 persons with non-treponemal infections or non-infectious disorders, sera of 101 healthy individuals serving as controls; 105 of the former reacted in routine tests with cardiolipin antigens, but all were negative in the 40-hour TPI test(8,9). Table III summarizes the findings, the diseases being grouped because of their variety under general nosologic headings. The tests appeared identical in specificity, RFTA and FTA II reactions being encountered in only 2 individuals, one a narcotic addict, the other without apparent disease; the remainder were seronegative.

TABLE I. RFTA and FTA II Titers of 108 Syphilitic Persons Using NaClO-Treated *T. pallidum* of Reiter and Nichols Strains.

RFTA titer	FTA II titer: <1	1	2	4	8	16	32
32							2
16					2	2	
8				2	7		
4			6	10	5		
2		3	20	5			
1	7	27	7				
<1		3					

A complement-fixation test (RPCF) using Reiter protein antigen was evaluated concurrently. In sensitivity for syphilis (Table II) the results agreed generally with those of the tests for fluorescent antibody (FA); 151 of the 204 patients reacted in both RPCF and FA tests, and 21 were negative in both. Some discrepancies were noted, 24 patients reacting only in the RPCF test, 8 only in the RFTA, and 6 only in the FTA II test. In specificity for syphilis (Table III) there was virtual agreement between the tests. Six RPCF reactions were encountered, one in a patient with measles, another in one with melanoma succeeded by basal cell carcinoma,

and the remaining 4 in chronic "biologic false positive (BFP)" reactors, *i.e.*, persons with chronic ailments who reacted persistently with cardiolipin antigens. Only 2 FA reactions were encountered, one in a narcotic addict, the other in a "BFP" reactor. In the group of 101 healthy individuals, there were no reactions with treponemal or cardiolipin antigens.

Discussion. The advantage of pretreating

TABLE II. Relative Sensitivity of RFTA, FTA II, and RPCF Tests of Syphilitic Patients of Known TPI Reaction.

Diagnostic status & TPI reaction		Persons tested	Persons found seroreactive in the given tests: RFTA* & RPCF	RFTA* only	RPCF only	Neither
Primary	—	4	1			3
	+	18	13	1†	4	
Secondary	—					
	+	18	16	1†	1	
Early treated	—					
	+	4	4			
Congenital	—					
	+	10	5		2	3
Neurological	—					
	+	20	14		3	3
Cardiovascular	—	1				1
	+	10	5	1†	3	1
Other late	—					
	+	30	20	3	3	4
Latent	+	77	65	2	7	3
Unclassified	+	12	8		1‡	3
Totals		204	151	8	24	21

* FTA II result was identical except where indicated by † or ‡.
† FTA II negative.
‡ " reactive.

TABLE III. Relative Specificity of RFTA, FTA II, and RPCF Tests of Non-Syphilitic, TPI-Negative Patients.

Diagnostic status	Persons tested	Persons found seroreactive in the given tests: RFTA* & RPCF	RFTA* only	RPCF only	Neither
Non-treponemal infections					
Leprosy	4				4
Viral	4			1	3
Totals	8			1	7
Non-infectious disorders					
Systemic lupus erythematosus	9				9
Hematologic	3				3
Neuropsychiatric	7				7
Cardiovascular	7				7
Pregnancy	10				10
Immunizations	2				2
Narcotism	12		1		11
Miscellaneous	5			1	4
Unclassified	49		1	4	44
Totals	104		2	5	97
Healthy	101				101

* FTA II result was identical.

Nichols strain *T. pallidum* with dilute NaClO, as in preparing Reiter treponemes for the RFTA test(1), is considerable and readily realized. The organisms adhere better to slides, and it is no longer necessary to adjust suspensions initially to counts (cf. 2) several times that anticipated (10-15/430 × microscopic field) following treatment of films with test serum and fluorescent antibody.

The RFTA test as initially evaluated(1) was somewhat more sensitive for syphilitic antibody than the then unmodified FTA test (2). The difference may have been more apparent than real, arising possibly from the fact that the sample of syphilitic sera was smaller than the present one, or that NaClO-treatment of Nichols treponemes somewhat increased the sensitivity of the FTA reaction. Whatever the cause, the RFTA and modified FTA (FTA II) tests appeared on a par with respect to sensitivity for syphilitic antibody (Table I), a conclusion that was borne out in qualitative tests of patients representing different stages of the disease (Table II). It is worth noting, however, that 3 persons with RFTA reactions were FTA II negative, whereas one who was FTA II reactive proved RFTA negative; the differences were confirmed by repeating the tests. As for specificity for syphilis, there were but 2 "non-specific" RFTA and FTA II reactions among 112 persons with non-treponemal infections or disorders (Table III). The findings take on added significance from the fact that 105 of these were chronic "BFP" reactors and so constituted a more critical test of specificity than seronegative individuals.

Attention is drawn here again to the variety of diagnoses, particularly among non-infectious disorders, that were associated with chronic "BFP" reactions in tests with cardiolipin antigens. Leprosy, of course, is a well recognized cause. Among the viral infections were 2 cases of infectious hepatitis, and one each of measles and unidentified upper respiratory infection. In the group of non-infectious disorders, systematic lupus erythematosus, with possibly associated(19, 20) hematological, neuropsychiatric, and cardiovascular symptoms, accounted for 26 cases of "BFP" reactivity. Pregnancy(21) was the apparent cause in 10 instances, and multiple immunizations preceded 2 "BFP" reactions. Narcotic addiction, a comparatively new addition(22) to known causes of such reactions, was implicated in 12 cases.

While the fluorescent antibody tests agreed essentially in sensitivity and specificity, they differed somewhat from the RPCF test. As indicated, the latter was slightly more sensitive for syphilis (Table II), and yielded somewhat more "non-specific" reactions (Table III). The differences in both respects were minor, however, and it appeared that either FA test might serve as well as the RPCF if used as a screening procedure in differentiating patients with latent syphilis from chronic "BFP" reactors; according to recommended practice(5-7), persons found to react with RPCF as well as cardiolipin antigens are considered syphilitic and reported directly, while those found RPCF negative are referred for TPI testing. The present experience justified this practice almost unequivocally. To be sure, the RFTA test (Table II) missed 45 cases of verified syphilis, the FTA II 47 cases, and the RPCF 29, but 3 of these were patients with primary syphilis, already identified by dark-field examination for treponemes, and all but one of the remainder, a patient with cardiovascular syphilis, was identified as suggested(5-7) by reference to the 40-hour TPI test. Obviously fewer would have been recovered if the less sensitive 18-hour test had been used. On the other hand, if reactivity in the RFTA, FTA II, or RPCF test were to be considered conclusively diagnostic(5-7), *i.e.*, not necessitating recourse to the more specific TPI test, a few patients (Table III) would be mislabeled as syphilitic. It is worth noting that the RPCF erred more frequently in this respect than either FA test. In choosing among the 3 tests as screening procedures, a number of ancillary factors deserve consideration. Two favoring selection of the FA tests are elimination of the hemolytic indicator reaction, and rapidity of performance. One advantage of the RFTA over the FTA II or FTA(2) tests is the substitution of a treponeme that can be cultivated simply and inexpensively

in vitro for one requiring time-consuming, uncertain, and expensive maintenance in infected rabbits.

Summary. Fluorescent treponemal antibody tests (RFTA and FTA II) using hypochlorite-treated Reiter and Nichols strain treponemes, respectively, were compared with each other and with a Reiter protein complement-fixation (RPCF) test in a study of sera previously subjected to the *Treponema pallidum* immobilization (TPI) test. The fluorescent antibody (FA) tests were closely comparable in sensitivity and specificity for syphilis. The RPCF test was slightly more sensitive than either FA test, but yielded somewhat more "non-specific" reactions. It appeared that either FA test might serve as well as the RPCF test as a screening procedure in differentiating persons with latent syphilis from those whose sera react "non-specifically" with cardiolipin antigens. Considerations favoring selection of an FA test are simplicity of performance and the need for fewer reagents. Moreover, the RFTA test substitutes an organism that can be cultivated simply *in vitro* for one requiring maintenance in infected rabbits.

We are grateful to Jacob B. DeWeerdt and Janet G. Jennings, who performed the essential TPI tests, and to Willard B. Olmstead who assisted with the fluorescent antibody tests.

1. Covert, S. V., Kent, J. F., Stevens, R. W., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 729.

2. Deacon, W. E., Freeman, E. M., Harris, A., *ibid.*, 1960, v103, 827.

3. D'Alessandro, G., Dardanoni, L., *Am. J. Syph., Gonor., and Ven. Dis.*, 1953, v37, 137.

4. Cannefax, G. R., Garson, W., *Pub. Health Rep.*, 1957, v72, 335.

5. Carpenter, C. M., Miller, J. N., Boak, R. A., *N. Eng. J. Med.*, 1960, v263, 1016.

6. Fiumara, N. J., *Pub. Health Rep.*, 1960, v75, 1011.

7. Garson, W., Portnoy, J., Harris, A., Kent, J. F., Widelock, D., in *Diagnostic procedures and reagents*, Am. Pub. Health Assn., N. Y., 4th ed., in press.

8. Rosenau, B. J., Kent, J. F., *J. Lab. and Clin. Med.*, 1958, v51, 664.

9. Kent, J. F., DeWeerdt, J. B., *Am. J. Clin. Path.*, 1961, v35, 526.

10. Anderson, R. I., Kent, J. F., Sanders, R. W., *Am. J. Syph., Gonor, Ven. Dis.*, 1954, v38, 527.

11. Nelson, R. A., Jr., Diesendruck, J. A., *J. Immunol.*, 1951, v66, 667.

12. Borel, L. J., Bentejac, R., Durel, P., *Brit. J. Ven. Dis.*, 1958, v34, 241.

13. Maltaner, E., Maltaner, F., *J. Immunol.*, 1945, v51, 195.

14. Kent, J. F., Burke, J. C., Carroll, D. P., Simonton, L. A., Otero, A. G., *J. Chronic Dis.*, 1958, v7, 36.

15. Kent, J. F., Bukantz, S. C., Rein, C. R., *J. Immunol.*, 1946, v53, 37.

16. Kent, J. F., Otero, A. G., Harrigan, R. E., *Am. J. Clin. Path.*, 1957, v27, 539.

17. Mayer, M. M., Osler, A. G., Bier, O. G., Heidelberger, M., *J. Exp. Med.*, 1946, v84, 535.

18. Kent, J. F., *Science*, 1947, v105, 316.

19. Moore, J. E., Shulman, L. E., Scott, J. T., *J. Chronic Dis.*, 1957, v5, 282.

20. Miller, J. L., Brodey, M., Hill, J. H., *J.A.M.A.*, 1957, v164, 1461.

21. British Co-operative Clinical Group, *Brit. J. Ven. Dis.*, 1959, v35, 162.

22. Boak, R. A., Carpenter, C. M., Miller, J. N., *J.A.M.A.*, 1961, v175, 326.

Received September 16, 1961. P.S.E.B.M., 1962, v109.

Effects of Mannoheptulose on Glucose Metabolism of Isolated Tissues. (27277)

SIDNEY S. CHERNICK, ROBERT O. SCOW, ERNST SIMON* AND FRANCES A. STRICKER

Laboratories of Nutrition and Endocrinology and of Biochemistry and Metabolism, National Institute of Arthritis and Metabolic Diseases, National Inst. Health, Bethesda, Md.

Parenteral administration of mannoheptulose produces hyperglycemia and hyperketonemia in rats(1,2). It has been suggested

* Visiting Scientist on leave of absence from Dept. of Exp. Biol., Weizmann Inst. of Science, Rehovoth, Israel.