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Time-Resolved Fluoroimmunoassay: a New Test for Rubella Antibodies

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A new solid-phase immunoassay, time-resolved fluoroimmunoassay (TR-FIA), for rubella antibody was developed. The test used polystyrene beads coated with rubella antigen as the solid phase and a chelate of the rare earth metal europium as fluorescent label. A fast light pulse from a xenon flash lamp was used to excite the label, and after a 400-μs delay time the emission fluorescence was measured for 500-μs at 1-ms intervals during a total counting time of 1 s. Background fluorescence of short duration caused by fluorescent serum components and scattering could be eliminated by including the delay time. The TR-FIA was compared with hemagglutination inhibition, single radial hemolysis, and two types of radioimmunoassay (RIA) (a commercial RIA [GammaCoat] and a noncommercial RIA [T-RIA]) by using 60 serum specimens from patients with remote rubella infection. Overall agreement of TR-FIA with hemagglutination inhibition and GammaCoat was 96.7%, with single radial hemolysis 98.3%, and with T-RIA 100%. Linear regression coefficients varied from 0.83 to 0.94, the best being obtained with single radial hemolysis and T-RIA. TR-FIA was also found to be suitable for the diagnosis of acute infections, as significant increases of antibody level were detected in all 30 paired serum specimens tested from patients with an acute rubella infection. Sensitivity and specificity comparable to those of RIA and enzyme immunoassay were obtained with TR-FIA. Furthermore, the advantage that TR-FIA has over RIA is that it incorporates a nonisotopic and stable label; its advantage over EIA is that it is easier to standardize because no additional reaction with substrate is required.

During recent years, various solid-phase immunoassays have found increasing use in the determination of viral antibodies. The most widely applied techniques have been radioimmunoassay (RIA) and enzyme immunoassay (EIA) (3, 11). Although sensitive and specific, both of these assays have certain drawbacks relating to the label used. In RIA the most obvious drawbacks are the inevitable radiation hazards and the short half-life of the iodinated labels. In EIA the toxicity and mutagenicity of some substrates are potential health risks, and the additional reaction with substrate hampers the standardization of the assay.

Conventional immunofluorescence and fluoroimmunoassay methods using organic fluorochromes, e.g., fluorescein isothiocyanate, have not achieved the high sensitivity of RIA and EIA methods. This is mainly because of background problems caused by nonspecific fluorescent components in serum and other organic material and the short Stokes' shift of fluorescein isothiocyanate (20 to 30 nm), which causes considerable interference due to scattering (7, 12).

These problems inherent in conventional fluorescence methods can be mostly avoided by adopting the principle of time-resolved fluorometry, in which a fast light pulse is used to excite the label, and the fluorescence is measured at a certain delay time after the excitation. Thus, direct scattering and nonspecific background fluorescence with a decay time generally less than 10 ns do not interfere with the assay. Time-resolved fluoroimmunoassay (TR-FIA) requires fluorescent labels with a decay time preferably over 1 μs. The labels that fulfill this requirement consist of rare earth metal (Eu, Tb, Sm, Dy) chelates. The chelated ligand strongly absorbs the excitation radiation and transfers it to the chelated central atom. The emission wavelengths are characteristic of the lanthanide used, although the intensity and the decay are ligand dependent. A long Stokes' shift (up to 270 nm) reduces background fluorescence and helps to optimize the measurement of specific lanthanide fluorescence (7, 12).

In the present report we describe the application of TR-FIA in the determination of rubella

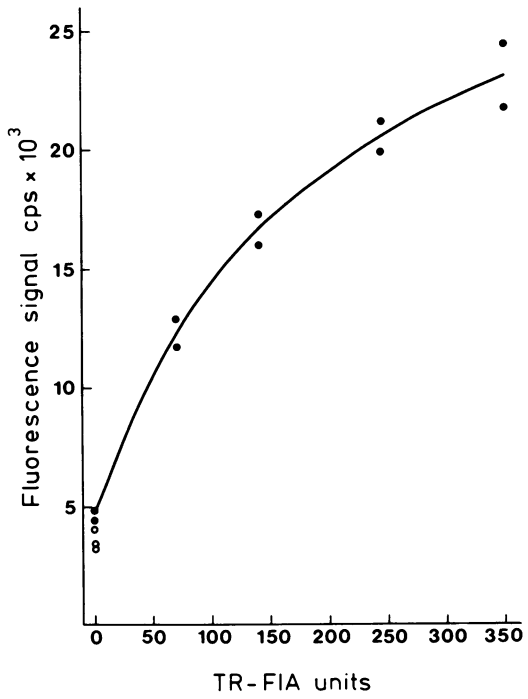

FIG. 1. Standard curve for rubella antibody determination by TR-FIA. Symbols: ●, duplicates of standard specimens; ○, dilution buffer blanks.

antibodies by using swine anti-human immunoglobulin G (IgG) conjugated with an aminophenyl derivative of EDTA which is chelated to Eu.

MATERIALS AND METHODS

Serum specimens. The study material comprised 60 serum specimens from patients with remote rubella infection and paired specimens from 30 patients and serial serum specimens from 3 patients with acute rubella infections.

Antigen. The Therien strain of rubella virus was grown in roller cultures of Vero cells. Extracellular virus from the culture medium was concentrated by ultrafiltration and partly purified by centrifugation through 20% sucrose as described in detail by Meurman and Ziola (6). The optimal dilution of the antigen for TR-FIA was estimated by box titration with a positive and a negative control serum. Polystyrene beads (Precision Plastic Ball Co., Chicago, Ill.) were coated with the antigen by incubating the beads submerged in the antigen dilution overnight at room temperature. The beads were then air dried and stored at 4°C until used.

Eu-labeled anti-human IgG. Swine anti-human IgG (heavy-chain specific) was obtained from Orion Diagnostica (Mankkaa, Finland). Aminophenyl-EDTA-Eu was synthesized by a modification of the method of Sundberg et al. (8) and conjugated to antibody by diazo reaction (4). Unreacted Eu-chelate was then removed by chromatography on a Sephadex G-50 column. The optimum degree of conjugation which produced conjugates retaining sufficient immunological activity for good assay sensitivity was found to be 3 to 5 Eu atoms per IgG molecule.

TR-FIA procedure. Serum specimens were diluted 1:200 with phosphate-buffered saline (pH 7.4) supplemented with 5% normal sheep serum and 2% Tween 20 (PBS-NSS-Tw). Duplicate 200-μl samples of these specimens were incubated with the antigen-coated beads for 1 h at 37°C. The beads were then washed twice with tap water and incubated further for 1 h at 37°C with 250 μl of Eu-conjugated anti-human IgG (100 ng/ml) in PBS-NSS-Tw. After washing as before, 500 μl of 15 μM 2-naphtoyltrifluoroacetone as the light-adsorbing ligand was added, and after 15 min of incubation at room temperature the fluorescence was measured by a single photon-counting fluorometer with a xenon flash lamp (1,000 Hz). In the measurement, a 1-μs excitation pulse at 340 nm was given, and after a delay time of 400 μs the single photon emission was counted for 500 μs at 613 nm. After another delay of 100 μs, a new excitation pulse was given, and this cycle was repeated 1,000 times during the total counting time of 1 s. The delay time and counting time could be adjusted in the fluorometer. The fluorometer used was one of a prototype series made by LKB Wallac (Turku, Finland). This fluorometer should be commercially available in 1983.

The results were expressed as arbitrary units by using a standard curve. An arbitrary value of 350 units was given for a serum pool highly positive for rubella IgG antibody and prepared from sera of 20 persons with long-past rubella infections. This pool was diluted with a rubella antibody-negative serum pool (0 units) to obtain five standards containing 0, 70, 140, 245, and 350 units, respectively. In every test series, duplicates of these standards, diluted 1:200 in PBS-NSS-Tw in the same way as the test specimens, were included, and the results of the test specimens were read from the standard curve of counts per second versus units. As a cutoff level between positive and negative specimens, 5 units was chosen. This cutoff was originally determined by RIA (3) and adjusted to a level which gave a titer of 1:8 in the hemagglutination inhibition (HI) test. Although lower antibody levels could be consistently measured, the protective efficacy of these was uncertain, and therefore, a 5-unit cutoff was chosen.

Other tests. The HI test was carried out by the standard method with kaolin absorption to remove the nonspecific inhibitors of hemagglutination (2).

Single radial hemolysis (SRH) tests were carried out on undiluted serum specimens with commercial SRH plates (Orivir Rubella, Orion Diagnostica) (9).

TABLE 1. Intraassay variation in TR-FIA[a]

Serum specimen	cps × 10^3 (mean ± SD)	Units (mean ± SD)
Negative	5.28 ± 0.15	0 ± 0
Low positive	8.90 ± 0.48	14 ± 3
Medium positive	22.50 ± 1.49	123 ± 14
High positive	33.41 ± 1.63	297 ± 18

[a] Four serum specimens with variable amounts of antibody were tested in 10 parallel determinations.

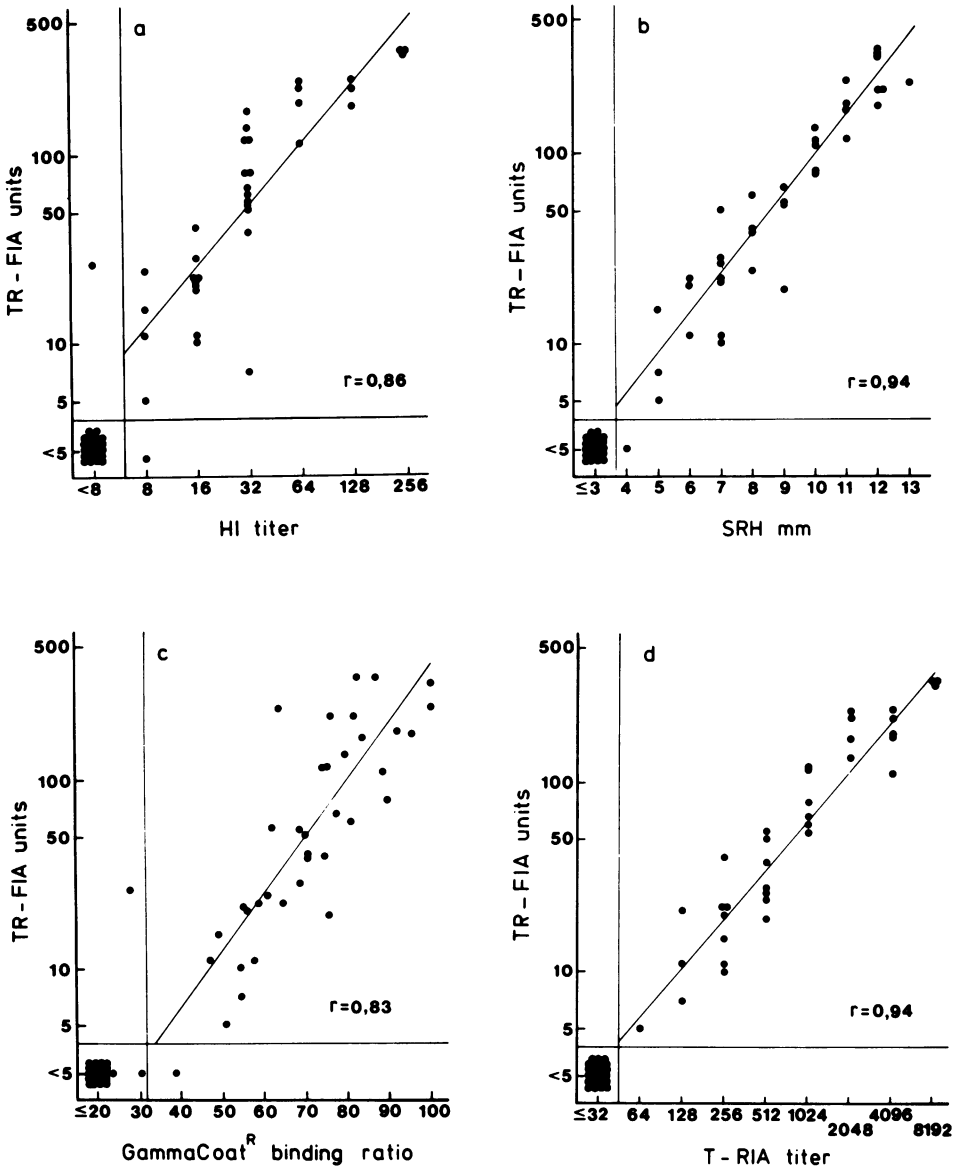

FIG. 2. Comparison of antibody determination results by TR-FIA with those obtained by the HI (a), SRH (b), GammaCoat (c), and T-RIA (d) tests. The straight lines and linear regression coefficient (r) values were obtained by linear regression analysis from the values of positive specimens.

A commercial RIA test for rubella antibody (GammaCoat, Clinical Assays, Cambridge, Mass.) was carried out according to the instructions of the manufacturer. The specimens were tested at a single dilution of 1:20.

An RIA test for rubella antibody that was developed in this laboratory (T-RIA) was carried out as described previously (5, 6). A counts-per-minute value 2.1 times that of the negative control was used as a cutoff level.

RESULTS

A typical standard curve obtained is shown in Fig. 1. To decrease the nonspecific binding of proteins to the solid phase during the immunological reactions, the serum specimens and Eu-conjugates had to be diluted in buffer containing inactive carrier protein and detergent. In preliminary experiments, different concentrations of bovine serum albumin, bovine globulin, fetal calf serum, pig serum, and sheep serum were tested in phosphate-buffered saline and Tris-hydrochloride buffers. The best results were obtained with PBS-NSS–Tw.

The optimal amount of Eu-labeled anti-human IgG was titrated by testing the rubella antibody

TABLE 2. Increases of antibody levels detected by TR-FIA in paired serum specimens from 30 patients with an acute rubella infection

Patient sex	Age (yr)	Days after onset of rash	TR-FIA units	Patient sex	Age (yr)	Days after onset of rash	TR-FIA units
M	1	1	<5	F	16	4	<5
		15	118			14	66
M	4	2	7	M	19	3	9
		18	96			17	154
M	6	1	13	F	21	0	<5
		12	130			13	60
F	6	2	<5	M	21	1	<5
		12	151			15	70
F	7	1	<5	F	21	3	<5
		14	91			17	128
M	8	0	16	M	22	0	<5
		15	117			8	129
F	9	1	12	F	22	1	<5
		16	60			11	64
F	10	0	<5	F	22	3	<5
		11	53			14	106
F	10	0	<5	F	23	0	<5
		14	121			8	60
M	11	2	36	F	23	1	<5
		12	228			8	33
F	11	1	31	F	23	1	<5
		14	96			13	79
M	12	3	7	M	24	2	<5
		19	68			13	14
F	12	5	46	F	28	1	<5
		19	157			12	40
M	13	1	14	F	29	2	<5
		14	196			12	42
F	14	2	15	F	43	4	7
		12	93			18	47

standards with different concentrations of label. The 100 ng/ml concentration (25 ng per bead) chosen corresponded to a total activity of approximately 300×10^3 cps per bead. Of this, about 60×10^3 cps (20%) was bound when incubated with a polystyrene bead coated with 2 μg of purified human IgG. As a comparison, the optimal amount of ^{125}I-labeled anti-human IgG used in T-RIA was 160×10^3 cpm per bead, of which 5,000 cpm (3%) was bound to a bead coated with human IgG. The background of about 5,000 cps obtained with negative specimens was largely dependent on the photometer and varied between the individual instruments of the prototype series. It will apparently be decreased by further technical improvements on the instrumentation.

The reproducibility of TR-FIA was studied with four serum specimens with variable amounts of rubella antibody. The intraassay variation of 10 parallel determinations is shown in Table 1. The assay showed good reproducibility, with coefficient of variation values of the fluorescence varying from 2.8 to 6.6%, and those of the unit values varying from 6.1 to 21.4%. The same four sera were used for the

TABLE 3. Antibody responses in three patients with an acute rubella infection as measured by HI, SRH, GammaCoat, T-RIA, and TR-FIA tests

Patient	Days after onset of rash	HI titer	SRH (mm)	GammaCoat-binding ratio[a]	T-RIA titer	TR-FIA units
1	0	16	<3	22.5	<32	<5
	7	128	9	42.7	8,192	47
	15	128	10	66.0	8,192	104
	30	256	11	69.8	8,192	104
	56	256	11	96.1	8,192	227
	176	256	11	99.8	4,096	241
2	1	8	<3	19.8	<32	<5
	8	256	9	46.4	8,192	60
	15	256	10	57.7	16,000	76
	28	256	10	59.3	16,000	78
	58	256	11	75.4	16,000	240
	170	256	12	81.7	8,192	214
3	1	16	<3	18.6	32	5
	7	256	11	66.1	8,192	195
	14	256	11	80.9	8,192	260
	28	256	12	82.3	16,000	340
	63	512	12	100.5	16,000	318
	189	256	12	86.5	8,192	346

[a] Positivity limit, 33.0.

determination of interassay variation. The mean ± standard deviation values of 10 subsequent assays during a 4-month period were 0 ± 0, 13.5 ± 2.5, 125 ± 19, and 297 ± 39 units, respectively.

TR-FIA was compared to HI, SRH, and two types of RIA tests (GammaCoat and T-RIA) by parallel testing of 60 serum specimens from patients with remote rubella infection. A very good overall correlation was found (Fig. 2). One serum which was negative by HI and GammaCoat tests was low positive by SRH, T-RIA, and TR-FIA. One serum was low positive by HI, SRH, and GammaCoat but negative by T-RIA and TR-FIA. Total agreement of TR-FIA with HI and GammaCoat was 96.7%, with SRH 98.3%, and with T-RIA 100%. The linear regression coefficient values calculated from the positive specimens varied from 0.83 to 0.94, the best being obtained with T-RIA and SRH (Fig. 2).

The detection of titer increases by TR-FIA was studied with paired serum specimens from 30 patients with an acute rubella infection, 15 of whom were children aged 1 to 14 years and 15 adults aged 16 to 43 years (Table 2). In all cases, over twofold increases in antibody levels were found, the difference between acute- and convalescent-phase specimens taken about 10 to 15 days apart being generally 40 to 150 units, and the convalescent serum value being at least 3.1 times that of the acute phase value.

The appearance of TR-FIA antibodies was further studied and compared with other tests by assaying serial serum specimens from three patients with an acute rubella infection (Table 3). Maximum TR-FIA titers were detected 30 to 60 days after the onset of rash. The general patterns of antibody development noted with the different tests showed good agreement, although TR-FIA and GammaCoat reached maximum titers later than the tests based on endpoint titration.

DISCUSSION

Recently, several new tests have been introduced for rubella serology to replace the old HI test (1, 5, 9, 10). Especially promising have been the highly sensitive and specific RIA and EIA tests for which several commercial kits are now available. These solid-phase immunoassays also permit the separate determination of class-specific antibodies if labeled antisera to the heavy chains of human immunoglobulins are available.

In the present study we showed that by using a labeled antibody conjugated with chelated Eu, a time-resolved fluoroimmunoassay for rubella antibodies can be developed with sensitivity and specificity comparable to those of RIA and other tests. The test was technically simple and could be performed in about 3.5 h with a single dilution of the test sera. By expression of the results in relation to a standard curve, a quantitative determination could be made. The validity of these determinations was further proven by the very good correlation of the TR-FIA results with those of the other tests, the linear regression coefficients being about 0.9. A very good overall agreement with the TR-FIA and the other tests

was observed, discrepant results being detected in 2 of 60 specimens tested. One marginally positive serum was not detected by TR-FIA, whereas a low antibody titer was detected by TR-FIA and SRH in one serum negative in the HI test. These discrepancies may be at least partly due to the different types of antigens used in the tests. The quantitative determination of antibodies also made TR-FIA applicable for the diagnosis of acute infections, and clear increases in antibody levels were observed in all paired serum specimens tested.

Our results suggest that TR-FIA offers a good alternative to RIA and EIA in solid-phase immunoassays in virology and microbiology. The Eu labels are stable and nontoxic. The determination of fluorescence is simple, and the assay is probably easier to standardize than EIA, where the temperature sensitivity of the additional enzyme-substrate reaction and instability of the colored products cause additional difficulties. Since Eu chelates are much smaller than enzymes and the detection limit of lanthanides by fluorescence is as low as 10^{-13} M (13), TR-FIA may also be suitable for the assay of small-molecular-weight substances, with a sensitivity probably comparable to that of RIA and higher than that of EIA.

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LITERATURE CITED

1. **Brody, J. P., J. H. Binkley, and S. A. Harding.** 1979. Evaluation and comparison of two assays for detection of immunity to rubella infection. J. Clin. Microbiol. **10**:708–711.
2. **Center for Disease Control.** 1970. A procedural guide to the performance of the standardized rubella hemagglutination inhibition test. Center for Disease Control, Atlanta, Ga.
3. **Halonen, P., and O. Meurman.** 1982. Radioimmunoassay in diagnostic virology, p. 83–124. *In* C. Howard (ed.), New developments in practical virology, vol. 5. Alan R. Liss Inc., New York.
4. **Leung, C. S.-H., and C. F. Meares.** 1977. Attachment of fluorescent metal chelates to macromolecules using "bifunctional" chelating agents. Biochem. Biophys. Res. Commun. **75**:149–155.
5. **Meurman, O. H.** 1978. Persistence of immunoglobulin G and immunoglobulin M antibodies after postnatal rubella infection determined by solid-phase radioimmunoassay. J. Clin. Microbiol. **7**:34–38.
6. **Meurman, O. H., and B. R. Ziola.** 1978. IgM-class rheumatoid factor interference in the solid-phase radioimmunoassay of rubella specific IgM antibodies. J. Clin. Pathol. **31**:483–487.
7. **Soini, E., and I. Hemmilä.** 1979. Fluoroimmunoassay: present status and key problems. Clin. Chem. **25**:353–361.
8. **Sundberg, M. W., C. F. Meares, D. A. Goodwin, and C. I. Diamanti.** 1974. Selective binding of metal ions to macromolecules using bifunctional analogs of EDTA. J. Med. Chem. **17**:1304–1307.
9. **Väänänen, P., and A. Vaheri.** 1979. Hemolysis-in-gel test in immunity surveys and diagnosis of rubella. J. Med. Virol. **3**:245–252.
10. **Vejtorp, M.** 1978. Enzyme-linked immunosorbent assay for determination of rubella IgG antibodies. Acta Pathol. Microbiol. Scand. Sect. B **86**:387–392.
11. **Voller, A., A. Bartlett, and D. E. Bidwell.** 1978. Enzyme immunoassays with special reference to ELISA techniques. J. Clin. Pathol. **31**:507–520.
12. **Wieder, I.** 1978. Background rejection in fluorescence immunoassay, p. 67–80. *In* W. Knapp, K. Holubar, and G. Wick (ed.), Immunofluorescence and related staining techniques. Elsevier/North-Holland Publishing Co., Amsterdam.
13. **Yamada, S., F. Miyoshi, K. Kano, and T. Ogawa.** 1981. Highly sensitive laser fluorimetry of europium (III) with 1,1,1,-trifluoro-4-(2-thienyl)-2,4-butanedione. Anal. Chim. Acta. **127**:195–198.