Rabies Antibodies in Human Serums

Titrated by the Indirect FA Method

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SINCE Coons and his associates (*1*) first reported the immuno-specific staining of tissue with fluorescein-conjugated globulin, this technique has been widely employed. Not only has it been used to demonstrate localized antigen, but it has been used to detect specific antibodies in serum. Goldwasser and Kissling in 1958 (*2*) first reported that the direct and indirect techniques could be used to stain rabies viral antigens and detect rabies antibodies. Since that time the direct technique has been widely used in most laboratories conducting diagnostic tests to demonstrate rabies virus in the brains of suspected animals. The generally accepted procedure, however, for detection of rabies antibodies has been the serum neutralization (SN) test in mice. One disadvantage of this test is that it requires 2 weeks to terminate the experiment. Also, the normal biological variable encountered in using laboratory animals is reflected in varying responses to the antigen. A test which avoided these difficulties would be advantageous.

Use of the indirect fluorescent rabies antibody (IFRA) technique as a screening test for serum antibodies was reported by Thomas and associates (*3*) in 1963. These authors described use of the method for testing only a single dilution of serum (1:5). Results were in accord with those of the serum neutralization test in recognizing positive and negative reactors, and the indirect test was proposed as a screening procedure, to be followed by the SN test on serums

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that were positive in the IFRA test. Because the indirect FA test has been applied to the demonstration and titration of serum antibodies (*4, 5*), usefulness of this test for titration of rabies antibodies was evaluated in our laboratory. Titers obtained with it were compared to the logs of virus neutralized in the serum neutralization test.

Materials and Methods

A total of 84 serums were tested. They were obtained from rabies-vaccinated persons employed in our laboratory and from veterinarians in Texas who, while students at Texas A. & M. University, had participated in a vaccine evaluation program conducted by the Public Health Service's Communicable Disease Center. Also, serums sent to our laboratory for rabies antibody testing were used. Serums from persons who had never received rabies vaccine served as normal controls on specificity of the test.

The conjugate was fluorescein-labeled globulin from horses immunized with human globulin obtained from Baltimore Biological Laboratories, Baltimore, Md., and Difco Laboratory, Detroit, Mich. Because of the globulin's high titer, it did not need to be adsorbed with tissue powder before use.

Diluents for the serums were 20 percent suspensions of normal mouse brain and fixed rabies mouse brain prepared in buffered water containing 10 percent egg yolk (*6*). The conjugate was also diluted in normal mouse brain.

In preparation of the fluorescent slides, two impression smears per slide were made from mouse brains infected with fixed rabies virus (challenge virus standard, strain 24, originally

obtained from the National Institutes of Health, Public Health Service). Smears made from normal mouse brains served as negative controls. All slides were fixed in acetone overnight at −20° C. and stored at that temperature until used.

The indirect fluorescent antibody technique was essentially the same as that described by Thomas and associates (3) with a few modifications. Initially a 1:10 dilution of serum was made, using normal mouse brain and fixed rabies mouse brain, followed by twofold dilutions in the corresponding diluents. The infected brain impressions were covered with 0.05 ml. of these varying dilutions of serum; the serum diluted in normal mouse brain was on one side; the serum diluted in rabies mouse brain, on the other. The slides were incubated in a moist chamber at 37° C. for 30 minutes, then rinsed in phosphate-buffered saline (pH 7.4) for 10 minutes and air-dried. The impressions were covered with antihuman conjugate diluted to the proper predetermined titer. The slides were again incubated, washed and dried as before, then mounted in buffered glycerol (10 percent phosphate-buffered saline, pH 8.5, and 90 percent glycerol). Known positive and negative serums were included in every test as controls.

The preparations were examined under a Reichert monocular microscope with a darkfield condenser, using an Osram HBO 200 light source. The filter system consisted of a BG 12 exciting filter and a GG 9 barrier filter. Upon microscopic examination, reactions were graded 4++, 4+, 3+, 2+, 1+, or negative, depending upon the number of Negri bodies fluorescing and the brilliance of their fluorescence. The following criteria were adopted for grading fluorescence:

4++ = numerous bright fluorescing bodies

4+ = bright bodies fluorescing, reduction in number

3+ = slight reduction in intensity of fluorescence, reduction in number

2+, 1+ = great reduction in intensity of fluorescence, reduction in number

negative = no fluorescence

FA titers were defined as the highest dilution of test serum with which the conjugated antiserum gave any 4+ reaction.

The same serums were tested by the serum neutralization procedure, using the constant-serum, varying-virus method (7). Undiluted serums were inactivated at 56° C. for 30 minutes and mixed with equal volumes of 10-fold dilutions of challenge virus standard 24. The mix-

Table 1. Specimens that were fluorescent-antibody positive and neutralization negative

Patient and date specimen was collected	Vaccination status when specimen was collected	Logs of virus neutralized (SN)	IFRA serum dilution titers
H. H. May 7, 1963	Pretreatment_____	0. 3	Less than 1:10.[1]
June 17, 1963	Post-treatment; collection 4 weeks after last injection_____	[2] 1. 0	1:20.
Aug. 6, 1963	Post-treatment; collection 3 months after last injection_____	[2] .85	1:20.
A. R. R. Apr. 11, 1963	Collection 1 month after last injection of 3 DEV doses Feb. 18 and 25 and Mar. 4, 1963.	[2] 1. 42	1:80.
Sept. 24, 1963	Collection 6½ months after last injection_____	1. 85	1:40.
W. C. May 27, 1964	Prebooster; 14 DEV injections in July 1963_____	[2] 1. 1	1:40.
Aug. 10, 1964	Post-booster; after 14 more DEV injections_____	4. 38	1:640.
D. A. May 19, 1963	Had just completed 8 doses of Semple vaccine, became ill, and switched to DEV; followup specimen not obtained.	[2] 1. 54	1:40.

[1] No titer demonstrable.
[2] Specimen was neutralization negative (less than 1.7 logs of virus neutralized) and IFRA positive.

NOTE: DEV—duck embryo vaccine.

tures were incubated at 4° C. for 2 to 3 hours. Three-week-old albino Swiss mice (random bred, local supplier) were inoculated intracerebrally with 0.03 ml. of the serum-virus mixtures, five mice per mixture. The mice were observed daily for 15 days for death of the animal. The LD_{50} titer of the virus in the presence of each serum was calculated by the

Table 2. Detection of rabies antibodies in paired human serums by the indirect fluorescent antibody test

Patient and date specimen was collected	Vaccination status when specimen was collected	Logs of virus neutralized (SN)	IFRA serum dilution titers
M. B. Dec. 18, 1963	Pretreatment	0	Less than 1:10. [1]
Feb. 14, 1964	Collection 1 month after last of 3 DEV injections	2.33	1:10.
D. C. Mar. 23, 1964	Prebooster; Lilly CEV in past	More than 2.83	1:10.
Mar. 30, 1964		4.15	1:40.
Apr. 9, 1964	Collection after 6 DEV booster injections	4.63	1:320.
K. H. Mar. 27, 1964	Pretreatment	0.75	Less than 1:10.[1]
May 7, 1964	Collected 1 month after last of 14 injections	More than 4.5	More than 1:640.[2]
D. R. May 26, 1964	Pretreatment	0	Less than 1:10.[1]
June 2, 1964	Had just received 7th injection	2.67	1:80.
G. P. June 11, 1964	Prebooster; had 2 complete, 1 partial series	2.66	1:40.
July 3, 1964	Post-booster	More than 4.5	1:1,280.
W. R. B. Apr. 15, 1964	Prebooster	2.28	1:20.
Apr. 23, 1964	Collection 1 week after booster	3.67	1:320.
May 8, 1964	Collection 3 weeks after booster	4.5	1:640.
B. C. Mar. 11, 1963	DEV injections in 1959, 1960, and 1961	3.8	1 : 80.
July 31, 1964	Prebooster	3.18	1 : 160.
Oct. 20, 1964	Post-booster; 3 DEV injections August 1964	3.32	1 : 640.
J. F. Aug. 11, 1961	Prebooster; 6 injections of Semple in fall, 1959	0	Less than 1 : 10.[1]
Sept. 14, 1961	Collection after 14 DEV injections	4.16	1 : 320.
Mc. M. Jan. 15, 1962	Pretreatment	0	Less than 1 : 10.
Feb. 19, 1962	Collection 1 month after vaccine	3.18	1 : 80.
D. Ca. June 11, 1962	Prebooster; 14 injections of DEV in August 1959; 2 boosters since; last 2 months ago.	2.2	1 : 10.
June 27, 1962	Booster June 12, 17, and 22, 1962	2.5	1 : 20.
A. S. Oct. 5, 1960	Pretreatment	0	Less than 1 : 10. [1]
Oct. 18, 1960	Completed 14 DEV injections Oct. 18, 1960	1.9	1 : 80.
Nov. 21, 1960	Collection 1 month after last injection	3.3	1 : 160.
J. R. M. Apr. 11, 1963	Prebooster; November 1960 last of series	3.45	1 : 80.
May 2, 1963	Collection 3 weeks after 1 DEV booster	4.2	1 : 320.

[1] No titer demonstrable.
[2] Endpoint not reached.

NOTE: DEV—duck embryo vaccine; CEV—chicken embryo vaccine.

Reed-Muench (8) method. Serums that neutralized 1.7 logs of virus or more (that is, serums with a neutralization index of 50 or greater) were considered positive (9).

Results and Discussion

The following table summarizes results of tests for rabies antibodies on the 84 serums by both the serum neutralization and indirect fluorescent antibody methods:

	Fluorescent antibody	
Serum Neutralization	Negative	Positive
Negative	29	5
Positive	1	49

There was complete agreement between the two tests on 78 (93 percent) of the serums. Five serums were positive in the indirect fluorescent rabies antibody test and negative in the serum neutralization test. Persons who had never received rabies vaccine and served as normal controls gave no false-positive IFRA reactions. In only one instance was a serum negative by the IFRA test but positive by the SN test. This was a single specimen from a person last immunized in 1959.

Of the five serums that were positive by the IFRA test but SN negative, three were taken within 1 month after the last vaccine injection, suggesting a possibly greater sensitivity for the FA method of measuring antibodies. These serums are listed in table 1 with dates of collection, relevant clinical histories, and results. (If known, the type of vaccine used is given in the tables.) The positive IFRA titers on the first specimens from A.R.R. and W.C. were subsequently confirmed by positive SN results when the second specimens were tested. It is possible that D.A.'s blood serum would have given a positive SN titer had a second specimen been obtained.

Not all serums submitted were accompanied by dates of collection and vaccination histories. Table 2, however, lists specimens collected from persons with pertinent histories and from whom more than one specimen had been collected. It will be noted that in all cases an increase in the IFRA titer as well as in the SN titer was clearly demonstrable. From table 2 it is apparent that active antibody response to rabies vaccine can be measured by comparing the IFRA titers

from prevaccine and post-vaccine serums. In no instance did the serum neutralization test detect the initial response before the indirect fluorescent antibody test.

Table 3 lists a group of single specimens from persons whose vaccination status was known. In all seven instances, the SN and IFRA titers were comparable.

There would seem to be a direct relationship between the logs of virus protected and the extent to which a serum could be diluted and still react. Table 4 shows a comparison of the logs of virus neutralized to the IFRA titers obtained. The serums that protected less than 1.7 logs of virus represent those with a neutralization index of less than 50 and which therefore were considered negative. Only serums with an IFRA titer of 1:10 or greater were considered positive. It will be noted that 29 serums were negative by both methods, 5 were positive by the indirect fluorescent antibody test but serum-neutralization negative (with titers ranging from 1:20 to 1:80), and 1 was negative by the IFRA test but positive by the SN test (with 2.2 logs of virus neutralized). The other 49 serums were positive by both methods, although FA titers varied widely within each neutralization group. For example, those serums that neutralized 3.0–3.9 logs of virus had IFRA titers ranging from 1:10 through 1:640. Serums neutralizing 4.0–4,9 logs of virus had IFRA titers ranging from 1:40 through 1:2,560. The serum with a 1:40 titer was 100 percent hemolyzed, and it is possible that the antibody reactive in the fluorescent antibody test was partially destroyed. We have noted that the antibodies in the FA test are more sensitive to adverse conditions, such as freezing and thawing or hemolysis, than the neutralizing antibodies. Although there was variation of FA titers within each group, the mode of the FA titer increased as the logs of virus protected was increased.

Therefore, from the available data, it appears that the indirect fluorescent antibody test potentially could replace the serum neutralization test as a rapid means of examining serums in vaccine evaluation programs. These tests represent two different types of immunological reactions, however, and the real significance of antibodies to actual protection against rabies is still debatable, so that a single titer—whatever method is

used—in fact only assures that the person has responded to the vaccine.

Since persons with any demonstrable titer following the primary series have been shown to respond well to a single booster shot 6 months later, it is important that a test be sufficiently sensitive to show this increase in titer. In all instances where we had several serums from one person, the IFRA method detected increases in titer, and in three cases the increase was first detectable only by this method. The initial response was subsequently confirmed in later specimens by titer rises, both by the indirect fluorescent antibody and the serum neutralization method. It is noteworthy that in no case did the SN test detect the response first.

The disadvantages of the serum neutralization test have been previously mentioned. The main disadvantage of the indirect fluorescent antibody test is that not more than 10 serums can be titrated efficiently by one person in a day. In laboratories testing large numbers of serums, however, this problem could be minimized if all serums were screened at a single low dilution. Specimens from positive reactors could then be titrated the following day. In laboratories test-

Table 3. Detection of rabies antibodies in single specimens by the indirect fluorescent antibody test

Patient and date specimen was collected	Vaccination status when specimen was collected	Logs of virus neutralized (SN)	IFRA serum dilution titers
M. W. February 1963	14 injections, Nov. 19, 1962–Dec. 2, 1962	2.26	1:40.
J. T. February 1963	1 DEV booster, Nov. 27, 1962	3.17	1:640.
L. A. D. March 1, 1963	14 injections, Oct. 23, 1962–Nov. 5, 1962	2.67	1:20.
R. M. R. March 1963	Treatment in March 1962	0.76	Less than 1:10.[1]
R. W. F. April 1963	Injections of nerve tissue in 1926, 1937, 1949, 1952, and 1955; DEV in March 1963.	4. 97	1:2,560.
D. W. E. April 23, 1963	Booster in November 1962	0.45	Less than 1:10.[1]
W. N. June 13, 1964	Had 3 injections, 1 booster; had just finished 14 DEV injections.	More than 4.5	1:320.

[1] No titer demonstrable.

NOTE: DEV—duck embryo vaccine.

Table 4. Antibody content in human serums determined by the neutralization and indirect fluorescent antibody methods

Logs of virus neutralized	Less than 1:10	Number of serums with indirect FA titers of—									Mode of indirect FA titer
		10	20	40	80	160	320	640	1,280	2,560	
0–1.69	29	0	2	2	1	0	0	0	0	0	Less than 1:10.
1.7–2.9	1	5	6	5	3	0	0	0	0	0	1:20.
3.0–3.9	0	1	3	4	5	2	2	2	0	0	1:80.
4.0–4.9	0	0	0	[1] 1	0	0	4	3	2	1	1:320.

[1] Serum 100 percent hemolyzed.

ing only a few specimens per week, this limitation is no problem, and indeed the IFRA test becomes more economical in time and cost than the SN test. Thus, not only does the indirect fluorescent antibody test avoid the disadvantages imposed by the serum neutralization test; it also is a definite aid to the physician in determining the immune status of a patient following rabies exposure, when time is so valuable; and it helps the physicians compare titers of prevaccine and post-vaccine serums.

Summary

The indirect fluorescent rabies antibody (IFRA) test was evaluated for its usefulness in titrating rabies serum antibodies. The titers obtained were compared to the logs of virus protected in the serum neutralization (SN) test. There was complete agreement between the two tests for 78 (93 percent) of the serums. Five serums were IFRA positive, SN negative; in only one instance was a serum SN positive, IFRA negative.

In all instances where there were several serums from one person (prevaccination and postvaccination), the indirect fluorescent antibody method detected the increases in titer, and in three instances the FA response was noted first. This initial response was subsequently confirmed in later specimens by rises in titer by both the IFRA and SN methods. In no instance did the serum neutralization test detect the initial response before the IFRA test. These results suggest that the indirect fluorescent antibody method may be the more sensitive. If so, it could ultimately supplant the neutralization test as a sensitive, rapid, high-resolution diagnostic method in comparing titers of prevaccine and post-vaccine serums.

REFERENCES

(1) Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E.: The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J Immun 45: 159–170 (1942).

(2) Goldwasser, R. A., and Kissling, R. E.: Fluorescent antibody staining of street and fixed rabies virus antigens. Proc Soc Exp Biol Med 98: 219–223, June 1958.

(3) Thomas, J. B., Sikes, R. K., and Ricker, A. S.: Evaluation of indirect fluorescent antibody technique for detection of rabies antibodies in human sera. J Immun 91: 721–723 (1963).

(4) Goldman, M., Gordon, M. A., and Carver, R. K.: Comparison of titers of dye and fluorescence-inhibition tests in the serologic diagnosis of toxoplasmosis. Amer J Clin Path 37: 541–550 (1962).

(5) Riggs, J. L., and Brown, G. C.: Application of direct and indirect immuno-fluorescence for identification of enteroviruses and titrating their antibodies. Proc Soc Exp Biol Med 110: 833–837 (1962).

(6) U.S. Public Health Service: Fluorescent antibody techniques in the diagnosis of communicable diseases. PHS Publication No. 729. U.S. Government Printing Office, Washington, D.C., 1960, pp. 66–67.

(7) American Public Health Association: Diagnostic procedures for virus and rickettsial diseases. Ed. 2. New York City, 1956, p. 46.

(8) Reed, L. J., and Muench, A.: A simple method of estimating fifty percent endpoints. Amer J Hyg 27: 493–497 (1938).

(9) Smadel, J. E.: Serological reactions in viral and rickettsial infections. In Viral and rickettsial infections of man, edited by T. M. Rivers. J. B. Lippincott Company, Philadelphia, 1948, ch. 3, pp. 67–96.

Nutritional Quality of Convenience Foods

Researchers at Cornell University's Department of Food Science and Technology have been awarded a $26,766 grant by the Public Health Service to determine the exact effect various food processing procedures have on plant protein.

The researchers, L. Ross Hackler and Robert L. LaBelle, hope to learn how heat, dehydration, and fractionation processes in the preparation of precooked and easy-to-prepare "convenience" foods affect amino acids and their utilization and availability in the body.