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Comparison of Rabies Humoral Antibody Titers in Rabbits and Humans by Indirect Radioimmunoassay, Rapid-Fluorescent-Focus-Inhibition Technique, and Indirect Fluorescent-Antibody Assay

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Rabies humoral antibodies were induced in eight New Zealand rabbits by a single intramuscular injection of inactivated suckling mouse brain rabies vaccine. The primary response to immunization was measured in blood samples taken at selected intervals for 6 months. The anamnestic response was measured in blood samples obtained 2 weeks after the rabbits received a booster immunization. The humoral antibody concentrations were measured by the rapid-fluorescent-focus-inhibition technique (RFFIT), indirect fluorescent-antibody assay (IFA), and indirect radioimmunoassay (RIA). The maximal neutralizing antibody titers as measured by RFFIT were attained by the 4th week and persisted into the 24th week. After booster immunization the antibody response was almost 10-fold higher than the highest level attained in the primary response. The antibody levels as measured by IFA and RIA were similar, but the titers as measured by either procedure were almost 10-fold lower than those determined by RFFIT. After booster immunizations the antibody levels, as measured by IFA and RIA, were three- and sixfold higher, respectively, than the maximal levels attained in the primary response. Twenty-two human serum specimens were tested by the same serological procedures, with disparate results. Both RIA and RFFIT effectively differentiated antirabies-positive sera from antirabies-negative sera.

The control and prevention of rabies in man and domestic animals remains a perennial public health problem. Although domestic animal control and immunization programs in the United States have greatly reduced the incidence of canine rabies during the last 20 years from 5,000 to fewer than 200 cases recorded annually, the problem has been perpetuated by a reservoir of wildlife rabies in skunks, foxes, bats, and raccoons (1, 13). Because rabies infection has been characterized as causing an almost invariably fatal encephalomyelitis, persons considered to be at high risk by virtue of their occupations, e.g., veterinarians, dog handlers, field naturalists, etc., should receive preexposure immunization (1). Successful immunization is judged at the present time by the production of measurable neutralizing antibodies (6).

Several convenient procedures for testing neutralizing activity in cell culture by fluorescent antibody have been described (2, 15, 20). Of these, the test of choice appears to be the rapid-fluorescent-focus-inhibition technique (RFFIT) of Smith et al. (20), which is more sensitive than the mouse neutralization test and can be completed in 1 day. The indirect fluorescent-antibody assay (IFA) is widely used, but it may not measure neutralizing activity. Immune globulins induced by whole virus contain a mixture of antibodies with specificities for several component antigens, as described by several investigators (19, 21, 25). However, in many sera the predominant antibody measured by IFA is directed to ribonucleoprotein (RNP) and is not considered to possess neutralizing activity (19, 25).

Indirect solid-phase radioimmunoassay (RIA) has been applied to viral serology (8-10, 17, 18, 27) but has not been used for rabies. A liquid-phase RIA for rabies serology has been described by Wiktor et al. (26) but is not suitable for routine testing. T. J. Wiktor (presented at Food and Drug Administration-Bureau of

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Biologics Rabies Vaccine Workshop, 14 May 1975) also described an indirect RIA procedure for measuring serum antibody levels to rabies. This procedure, like the liquid-phase procedure, required highly purified antigen and is therefore of limited usefulness for routine testing.

A comparative study of rabies humoral antibodies induced in rabbits and in humans was made using RFFIT, IFA, and indirect RIA. The response and duration of these antibodies were monitored during a 6-month period in immunized rabbits after a single injection of rabies vaccine. The anamnestic response was also measured after the administration of a booster dose. Single serum specimens from immunized humans were also tested on a more limited basis, and the results were compared with those from the animal study.

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MATERIALS AND METHODS

Antigen for RIA. The challenge virus standard (CVS-11) strain of rabies was provided by the Viral Zoonoses Branch, Virology Division, Center for Disease Control, Lawrenceville, Ga. Rabies viral antigens were prepared in baby hamster kidney (BHK-13s) cell monolayers. Cell monolayers in 0.5 gallon (ca. 1.89-liter) roller bottles were infected with rabies virus by the procedure suggested by Kaplan et al. (12) and Wiktor (24). The virus-infected cultures and control cultures were incubated for 3 days at 37°C in maintenance medium composed of minimal essential medium supplemented with 2% fetal calf serum (FCS). The spent medium was discarded, and each monolayer was rinsed twice with phosphate-buffered saline (PBS). Fifty milliliters of PBS was added to each culture, and a rubber policeman was used to scrape the cells. The pooled suspensions from the virus-infected cultures and the control cultures were stored at −20°C. Before use, both cell suspensions were thawed and sonically disrupted for 10 s. Volumes of 0.025 ml of the viral antigen or control antigen were added to the wells of siliconized polyvinyl flat-bottom microtiter plates (catalog no. 220-29, Cooke Engineering Co., Alexandria, Va.). The plates were dried overnight at room temperature in a safety hood and then stored in desiccator jars at −20°C in a nitrogen atmosphere. Plates were fixed with 10% buffered formalin for 15 min at 4°C and rinsed twice with PBS.

Antiglobulins. Guinea pig anti-human and anti-rabbit globulins were precipitated with sodium sulfate and radiolabeled by the Greenwood et al. (5) chloramine-T procedure as described previously (9). The concentration of carrier-free $Na^{125}I$ was 500 or 1,000 μCi per mg of protein.

Antirabies sera. Human sera routinely received as diagnostic specimens were obtained from the Viral Zoonoses Branch, Virology Division, Center for Disease Control, Lawrenceville, Ga. Twenty sera were obtained from persons immunized with multiple injections of duck embryo vaccine for rabies virus. The immunization schedule for these persons is not known. One serum (R) was obtained from a person before rabies immunization and was included as a negative control. Another serum (V) was obtained from a person who was infected by a bite of a rabid bat and was included as a positive control.

Preimmunization sera were obtained from eight male New Zealand white rabbits weighing 2 to 3 kg. Each rabbit was then immunized intramuscularly with 1 ml of inactivated rabies vaccine prepared in suckling mouse brain (Haver-Lockhart Laboratories, Shawnee, Kan.). The rabbits were bled from the ear at weekly intervals during the 1st month and monthly thereafter for the next 5 months. All serum samples were stored at −20°C. Six rabbits received a rabies booster immunization in the 7th month, and hyperimmune anti-rabies sera were obtained by exsanguination 17 days later. Standard rabies-positive and rabies-negative sera were included in each serological test to assure the validity of the results.

Standardization of rabies antigen for indirect RIA. Viral antigen (Ag) and uninfected cell antigen (control) were titrated to determine the optimal concentration for use in RIA. The antigens were diluted in PBS so that 0.025-ml volumes contained protein concentrations as determined by the Lowry procedure (16) ranging from 128 to 1 μg in twofold decrements. Rabbit antirabies serum (AS) or normal rabbit serum was used for the primary antigen-antibody reaction. Both the viral and control antigens were reacted with a 1:10 dilution of rabbit antirabies serum or normal rabbit serum in the primary reaction.

The optimal binding of antirabies globulin in the primary reaction was determined in a secondary reaction by adding ^{125}I-labeled guinea pig anti-rabbit globulin and measuring the bound radioactivity as counts per minute. The results were evaluated by calculating binding ratios which were obtained by the following formula:

$$\text{Ratio} = \text{cpm (AS dilution vs Ag)} - \text{cpm (AS dilution vs control)}/\text{cpm (diluent-Ag)}.$$

A plot of the ratios versus the protein concentrations (Fig. 1) showed that as the protein concentration was increased from 1.0 to 8.0 μg per sample, the ratios increased to a maximal level. Further increases in the protein concentration resulted in a diminution of the ratios. The ratios obtained with normal rabbit serum were <1.0 for all protein concentrations. These results indicated that protein concentrations between 4.0 and 16.0 μg per sample were nearly optimal; therefore, 8.0 μg of antigen or control protein per well was used in all experiments.

RIA procedure. The RIA procedure used was an adaptation of those described by Rosenthal et al. (18) and Hutchinson and Ziegler (8). Uninactivated test sera were diluted twofold in PBS containing 10% FCS. Duplicate samples (0.025 ml) of each serum

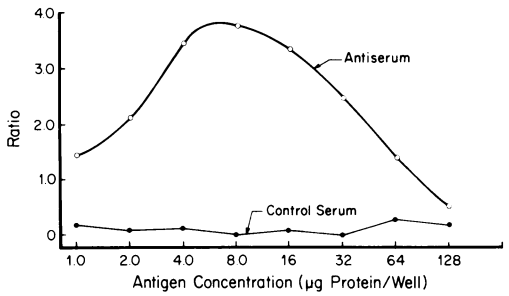

FIG. 1. *Titration of optimal amounts of rabiesvirus antigen and uninfected cell antigen for use in RIA.*

dilution were added to the wells of microtiter plates containing viral antigen or control material. As controls, 0.025-ml volumes of diluent were added to viral antigen-containing wells.

Optimal concentrations of either ^{125}I-labeled guinea pig anti-human or anti-rabbit globulin were determined by a procedure described by Hutchinson and Ziegler (9). The optimally diluted ^{125}I-labeled indicator was added to each microtiter well in a volume of 0.025 ml. The reciprocal of the highest dilution of an antiserum that caused twofold or greater binding of ^{125}I-labeled indicator than the background controls was designated as the antibody titer (9). A titer less than 2 was considered negative.

RFFIT procedure. Neutralizing antibody titers were determined by RFFIT as described by Smith et al. (20). In this procedure, appropriately diluted serum specimens were mixed with a standard amount of CVS-11 rabies virus in Lab-Tek tissue culture chamber slides (Miles Lab. Inc., Naperville,. Ill.). The serum-virus mixtures were incubated for 1.5 h at 37°C in an atmosphere containing an air-CO_2 (95%:5%) mixture, and upon termination of the neutralization period, BHK-13s cells were added to the chambers. After 24 h of incubation the culture fluid was removed, and the monolayers were rinsed with PBS and fixed with acetone. After staining with fluorescein-labeled equine antirabies globulin, neutralization of the virus was assessed microscopically by examining 20 fields/chamber for fluorescence of appropriate intensity and character indicative of specific staining. The reciprocal of the dilution factor of an antiserum which caused a reduction of at least 50% of the number of fields containing fluorescing cells indicated neutralizing antibody activity and was designated as the titer (20). A titer of less than 5 was considered negative.

IFA procedure. Antigen for the IFA assay was prepared by adding CVS-11 rabies virus (10 mean tissue culture infective doses/0.1 ml) to Lab-Tek chambers containing 1×10^5 BHK-13s cells, which were pretreated with 25 µg of diethylaminoethyl-dextran per ml. After 24 h of incubation at 35°C in a 5% CO_2-95% air atmosphere, the infected cells were fixed with acetone. Test sera were diluted in growth medium (80% minimal essential medium, 10% FCS, and 10% tryptose phosphate broth), and 0.1-ml volumes were added to each chamber. The slides were incubated for 30 min at 37°C and then rinsed for 10 min in PBS (pH 7.4) and air-dried. Caprine anti-rabbit conjugate (BBL, no. 2031648, Bioquest, Div. of Becton, Dickinson and Co., Cockeysville, Md.) was diluted 1:40 in 20% normal mouse brain diluent and added to each specimen. The slides were then reincubated for 30 min at 37°C, washed 10 min in PBS, rinsed once with distilled water, and then air-dried. The slides were examined microscopically with a Leitz ultraviolet microscope equipped with a UG-1 exciting filter and an ultraviolet-excluding barrier filter. The titer was defined as the reciprocal of the highest dilution factor of test serum in which 50% or more of the fields examined contained specifically fluorescing cells. Titers less than 10 were considered negative. A reference rabbit antiserum was used with each test as a positive control.

RESULTS

Humoral antibody induction in immunized rabbits. Serum samples from eight immunized rabbits were collected over a 6-month period, and antirabies titers were determined by RFFIT, IFA, and RIA (Table 1). In each of the serological tests, all of the serum specimens taken from an individual rabbit during this 6-month period were tested simultaneously. A standard reference antiserum was included with each test to insure that the combined test results were valid for the group of animals. For each serological procedure, titers were determined by testing replicate samples of each antiserum dilution. Coefficients of variation for standard reference sera were calculated from results of a large number of tests conducted over a period of several months and were 35.6, 45.3, and 32.1% for RFFIT, IFA, and RIA, respectively. Test results were considered valid when titers were within the quality control limits established from these data.

The sequential changes in the geometric mean titers determined by RIA, RFFIT, and IFA are shown in Fig. 2. Antibody activity was demonstrable by all three tests 1 week after immunization, and the maximal titers as determined by each of the tests were attained 4 weeks after immunization. After reaching a maximal titer at 4 weeks postimmunization, the titers steadily declined through the 24th week as measured by each test. A significant fourfold decline in RFFIT titer was detected by the 24th week. In contrast, a significant decline in IFA and RIA titers was detected by the 16th week, almost 2 months earlier than by the RFFIT titrations.

Twenty-eight weeks after the primary immunization, six surviving rabbits received a booster immunization. Seventeen days later, or about 30 weeks after initial immunization, the rabbits were exsanguinated, and the sera were

TABLE 1. *Humoral antibody levels in immunized rabbits as measured by indirect RIA, RFFIT, and IFA*

Time postvaccination (weeks)	Titer (GMT range)[a]		
	RIA	RFFIT	IFA
0	<10 (<10-10)	<20 (<10-<20)	<10 (<5-<10)
1	18 (<10-75)	160 (40-320)	45 (20-160)
2	42 (28-114)	403 (160-640)	50 (20-80)
3	54 (16-207)	452 (160-640)	95 (40-160)
4	47 (18-212)	640 (320-1,280)	113 (40-320)
8	31 (21-78)	320 (160-640)	37 (20-80)
12	30 (11-127)	238 (160-320)	34 (10-80)
16	28 (<10-111)	246 (80-640)	22 (10-80)
20	20 (<10-76)	207 (80-640)	18 (10-80)
24	19 (<10-56)	134 (40-320)	16 (10-40)
30[b]	330 (180-500)	5,747 (2,560-10,240)	359 (160-640)

[a] Titers are reciprocals of end point dilution factors. Geometric mean titers (GMT) were calculated for sera from eight individual rabbits.

[b] Six rabbits received booster inoculations of rabies vaccines at 28 weeks and 17 days later were exsanguinated.

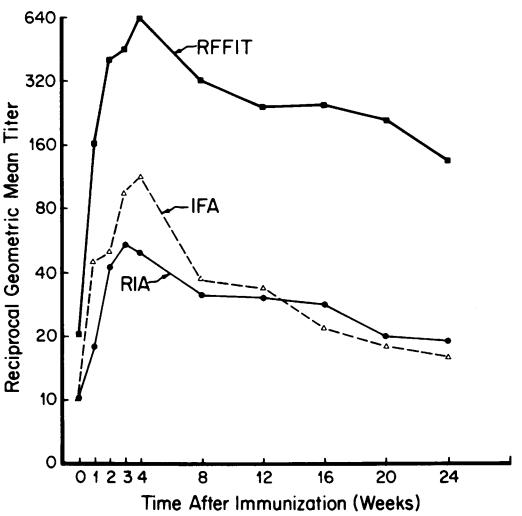

FIG. 2. *Sequential changes in the antirabies titers as measured by RFFIT, RIA, and IFA. Each point is the geometric mean titer of eight rabbits.*

tested for antibodies to rabies by each of the serological methods (Table 1). The titers of the hyperimmune sera determined by RIA, RFFIT, and IFA were 7.0-, 9.0-, and 3.2-fold higher, respectively, than the peak titers attained at 4 weeks postimmunization. With both the 4-week sera (peak titer of primary response) and the hyperimmune sera, the RFFIT titers were 13- to 17-fold greater than the RIA titers. On the other hand, the IFA titers were not appreciably different than the RIA titers.

Humoral antibody in immunized humans. Twenty-two human serum specimens were assayed by RFFIT, IFA, and RIA (Table 2). Four of the 22 sera tested were negative by RFFIT (<5), whereas six were negative by RIA (<2) and 11 of 21 were negative by IFA (<10). Two of the sera with negative RIA titers had very low

TABLE 2. *Humoral antibody titers in humans as measured by RFFIT, mouse neutralization, IFA, and RIA*

Serum identification[a]	Titer[b]			
	RFFIT	Mouse	IFA	RIA
A	145	NT	80	135
B	13	<5	<10	<2
C	<5	NT	<10	<2
D	16	NT	20	94
E	145	NT	40	78
F	50	NT	<10	33
G	>50	NT	<10	10
H	8	NT	NT	<2
I	125	>100	40	49
J	16	NT	<10	52
K	20	NT	10	31
L	16	10	<10	40
M	<5	NT	<10	<2
N	<5	NT	<10	<2
O	480	830	10	69
P	16	NT	<10	22
Q	210	NT	40	145
R	<5	<5	<10	<2
S	280	>333	<10	3
T	210	NT	40	138
U	625	NT	20	180
V	3,125	3,333	5,120	1,220
GMT	47		21	24

[a] Serum R was obtained as a preimmunization serum and was used as a negative control. Serum V was obtained from a person infected by a rabid bat bite. Sera A to Q and S to U were obtained from persons immunized with multiple injections (intramuscularly) of duck embryo vaccine.

[b] Reciprocal of end point dilution factors. NT, Not tested.

RFFIT titers (B = 13, H = 8). In contrast, seven sera were negative as measured by IFA but were positive by RFFIT. Four of the sera with negative IFA titers had very low RFFIT titers (B = 13, J = 16, L = 16 and P = 16), but three had moderately high RFFIT titers (F = 50, G = $\geq$ 50, S = 280). The agreement between RIA and RFFIT in distinguishing positive and negative specimens was excellent (20 out of 22 [91%]), whereas the agreement between RIA and IFA was only fair (15 out of 21 [71%]).

In a comparative study Smith et al. (20) concluded that both RFFIT and mouse neutralization measured antibodies that prevented lethal infections. We, likewise, compared the titers of seven sera by RFFIT and by a mouse neutralization test (Table 2) for assurance of the RFFIT results with an established virus neutralization test. With one exception (B) the titers obtained by the two procedures were in agreement. This discrepancy probably was due to the minimally detectable antibody concentration (B = 13, RFFIT antibody titer).

DISCUSSION

Rabies antibodies appearing in either immunized or naturally infected animals include mixtures of immunoglobulins induced by both ribonucleoprotein (RNP) internal antigens, and glycoprotein surface antigens (19, 25). Experience has indicated that these two antibody populations are measured disparately by different serological procedures, and the significance of antibody titers obtained by each of the methods has been the subject of several studies (4, 14, 22).

Neutralization tests, such as mouse neutralization, RFFIT, and plaque reduction neutralization tests, measure only antibodies that inactivate rabies virus. Recent investigations (19, 25) suggest that neutralizing antibodies are induced only by the glycoprotein antigen. On the other hand, complement fixation, IFA, and precipitation tests probably measure antibodies induced by RNP antigen. Furthermore, the amounts of each of the two antibody populations measured depend upon the relative proportion of RNP and glycoprotein antigens used in a test procedure.

The indirect solid-phase RIA test performed with cell-associated viral antigen did not appear to be measuring virus neutralizing antibodies in immunized rabbits but rather antibodies to the group-specific RNP antigen. In this respect, it was similar to other serological tests such as IFA, complement fixation, and enzyme-linked immunosorbent assay (23).

There was a similarity between RIA and IFA tests in the rabbit study. Two possible interpretations of the RIA results can be made. (i) Like IFA tests, the RIA test measured antibodies induced by the RNP antigen; (ii) the RIA test results are similar to the IFA results because the viral antigens used were cell-associated and may have consisted principally of nonenveloped RNP. The latter interpretation is most likely because enveloped viruses bud from the cell surface during viral multiplication and subsequently are released (11, 24). In our RIA, these extracellular viruses were probably lost in preparative procedures; therefore, the resultant antigen was predominantly intracellular or RNP antigen. Most antisera would be expected to contain antibodies to both enveloped and nonenveloped viruses. However, in the primary reaction, when cell-associated antigen is used, only antibody to the nonenveloped RNP would be absorbed and subsequently measured. Hence, for determining neutralizing antibody activity, the antigen preparation should consist of viral particles harvested from the extracellular material. This was confirmed by Wiktor et al. (26). With an ^{125}I-labeled purified rabies virus preparation, he demonstrated excellent agreement between antibody titers measured by RIA and neutralization tests.

We observed that with rabbit antisera IFA and RIA titers were similar to each other, but the titers of each were severalfold less than the titers determined by RFFIT. With human antisera the IFA and RIA geometric mean titer values were quantitatively similar. In spite of the similarity, there appeared to be a qualitative difference between the two tests. A comparison of RIA and IFA titers with RFFIT titers shows that the IFA test failed to detect antibodies more frequently than did the RIA test. RIA failed to detect antibodies in only two sera for which antibodies were detected by RFFIT. IFA, on the other hand, failed to detect antibodies in seven sera for which antibodies were detected by RFFIT. The differences between the titration results for human and rabbit antisera may reflect a difference in immunization procedures. A single dose of inactivated mouse brain rabies vaccine induced an excellent antibody response in rabbits. However, the duck embryo vaccine used for human immunization required several doses and induced only a moderate antibody response (3). The responses to the two immunization schedules may have resulted in differences in the specificity of the antibodies of the two species. Among the human specimens was a single serum specimen (V) taken from a recorded case of human rabies (7). The excellent immune response was evidenced by high

concentrations of antibodies measurable by RFFIT, IFA, and RIA.

These results indicate that the RIA procedure as described is not the procedure of choice for measuring neutralizing antibodies. However, the results suggest that sera which possess antibodies measurable by RIA will also have neutralizing antibodies. Thus RIA can be a useful procedure for screening large numbers of sera.

Investigations reported by Schneider et al. (19) further suggest that antibodies specific for the glycoprotein envelope are responsible for virus neutralization. Thus, an RIA procedure performed with undenatured rabies virus or purified rabies glycoprotein antigen should yield titers comparable to neutralizing titers.

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