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Preparation of Rabies Fluorescein Isothiocyanate-Labeled Immune Globulin from Mouse Hyperimmune Ascitic Fluids

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Immunization conditions for the production of mouse immune ascitic fluids to be used for the preparation of rabies fluorescent antibody (FA) conjugate are presented. The use of optimal concentrations of ammonium sulfate for precipitation of gamma globulin resulted in a fraction consisting of 75% gamma globulin and 25% alpha-beta globulins with no detectable albumin. Dialysis labeling of the globulin fraction with fluorescein isothiocyanate produced a specific rabies FA conjugate with negligible nonspecific background staining. This procedure represents a simple means of producing rabies FA conjugate.

Mouse immune ascitic fluid (MIAF) obtained from mice immunized with mouse brain-propagated viruses has been shown to be comparable to mouse antiserum in potency and specificity in complement fixation, hemagglutination inhibition, and neutralization tests (12, 13). In this study, MIAF was used to prepare a fluorescent rabies antibody conjugate. MIAF production, gamma-globulin fractionation, and fluorescein isothiocyanate (FITC) labeling are described, and the use of the conjugate in the fluorescent rabies antibody (FRA) test is evaluated. Results indicate that a rabies-specific fluorescent antibody conjugate can be prepared from MIAF containing rabies virus antibody.

MATERIALS AND METHODS

Immunization of mice. Random-bred, 6- to 8-week-old specific pathogen-free white female mice were used for MIAF production. These mice were screened monthly for antibody to the following agents: mouse adenovirus, vaccinia, Theiler's GD VII, reovirus type 3, mouse hepatitis virus, psittacosis, Sendai, minute virus of mice, lymphocytic choriomeningitis, polyoma, newborn mouse pneumonitis (K virus), and pneumonia virus of mice. All tests were negative for this group of agents. The mice were immunized according to the schedule shown in Table 1. On days 1, 7, and 14, two groups of 20 mice per group were inoculated by the intraperitoneal route with 0.5 ml of rabies virus antigen that had been inactivated with 0.3% beta-propiolactone. This 20% suspension of brain tissue from 2- to 3-week-old mice was mixed with an equal volume of Freund complete adjuvant before injection. On day 24, 0.2 ml of a 10% sarcoma 180 TG cell suspension was injected by the intraperitoneal route into the mice. (The sarcoma 180 TG had been checked and found free of the murine viruses previously listed.) On day 29 the mice in group I were challenged by the intraperitoneal route with 0.1 ml of $10^{1.8}$ mouse 50% lethal doses of rabies virus suspended in phosphate-buffered saline, pH 7.3, and those in group II received a similar challenge of $10^{3.8}$ mouse 50% lethal doses of rabies virus. Animals in both groups were then tapped on days 36, 43, and 51. The collected MIAF representing six pools was tested for neutralizing antibody by the serum dilution-constant virus procedure, in which $10^{1.5}$ mouse 50% lethal doses of rabies virus (9) was used. Mouse 50% lethal doses were calculated according to the method of Reed and Muench (11).

Ammonium sulfate fractionation. Gamma globulins from the MIAF were recovered by using ammonium sulfate precipitation as recommended by Hebert (6) for fractionation of mouse serum. A stock solution of saturated ammonium sulfate was prepared and stored at room temperature. A single precipitation for which 70% saturated ammonium sulfate was used with an equal volume of MIAF was followed by two more precipitations with 80% saturated ammonium sulfate to give final reaction mixtures of 35% and 40% saturated ammonium sulfate, respectively. The fractionation procedure is briefly outlined in Fig. 1. The final gamma globulin precipitate was resuspended to one-third of the original volume with buffered distilled water (pH 8.0). The final concentration of globulin was dialyzed until no further sulfate could be detected in the dialysate when tested with saturated barium chloride solution.

Globulin protein content. Protein concentrations of the globulins and conjugates were measured by the biuret method (5) on a Beckman DB spectrophotometer. Protein compositions were determined by cellulose acetate strip electrophoresis (CASE) on Beckman Microzone equipment; the procedure described by Hebert et al. (7) was followed.

Conjugation of globulin with FITC. The immune globulins were conjugated by the dialysis method (2), and varied reaction times were used to obtain the desired fluorescein-to-protein (F/P) ratio (8).

TABLE 1. *Schedule for production of immune ascitic fluids in 3-week-old mice*

Day	Group I	Group II
1	0.5 ml of FCA + CVS rabies virus[a]	0.5 ml of FCA + CVS rabies virus[a]
7	0.5 ml of FCA + CVS rabies virus[a]	0.5 ml of FCA + CVS rabies virus[a]
14	0.5 ml of FCA + CVS rabies virus[a]	0.5 ml of FCA + CVS rabies virus[a]
24	0.2 ml of 10% sarcoma 180/TG	0.2 ml of 10% sarcoma 180/TG
29	0.1 ml of $10^{1.8}$ rabies virus	0.1 ml of $10^{3.8}$ rabies virus
36	1st paracentesis	1st paracentesis
43	2nd paracentesis	2nd paracentesis
51	3rd paracentesis	3rd paracentesis

[a] Inoculum consisted of equal volumes of Freund complete adjuvant and 20% rabies-infected mouse brain inactivated with beta-propiolactone administered by intraperitoneal injection. Abbreviations: FCA, Freund complete adjuvant; CVS, challenge virus standard.

15 ml of MIAF + 15 ml of 70% saturated $(NH_4)_2SO_4$; 4 h at 25 C.
↓
Centrifuge 2,500 × *g*, 30 min.
Discard supernatant.
Dissolve packed globulin in distilled water, q.s. to 10 ml.
↓
10 ml of globulin + 10 ml of 80% saturated $(NH_4)_2SO_4$.
Mix gently and centrifuge as above.
Discard supernatant.
Gently dissolve packed globulin; q.s. to 10 ml with distilled water.
↓
10 ml of globulin + 10 ml of 80% saturated $(NH_4)_2SO_4$.
Repeat previous step.
Dissolve packed globulin and q.s. to 5 ml with distilled water.
Dialyze globulin against 0.85% NaCl (pH 8.0).

FIG. 1. *Ammonium sulfate fractionation of MIAF gamma globulin.*

After conjugation, the dialysis tubing was washed under cold running water and then placed into phosphate-buffered saline, pH 9.0, at 5 C. The buffer was changed frequently until all unreacted fluorescein was removed. This was considered to be accomplished if there was no visible fluorescence when the dialysate was observed in the dark under a Wood's lamp.

Evaluation of conjugates. At least two laboratories evaluated each conjugate for staining properties according to the FRA protocol (3, 4). The conjugates were also examined for physicochemical characteristics (8).

RESULTS

The volume of clarified MIAF obtained from each group of mice was approximately 180 ml on day 36, 200 ml on day 43, and 80 ml on day 51. Neutralization titers of these MIAFs are shown in Table 2. Fluids from the 36-day tap had the highest neutralization titer, whereas the 43-day and 51-day fluids had neutralizing titers approximately threefold lower.

The CASE profiles shown in Fig. 2 are characteristic of MIAF and are similar to previous CASE profiles of mouse serum. It can be seen in these profiles that the gamma globulin fraction increased with time; the 36-day MIAF contained 5% gamma globulin, the 43-day MIAF 10%, and the 51-day MIAF 15%.

The CASE profile of rabies FITC conjugate prepared from MIAF seen in Fig. 3 shows a gamma globulin peak of about 75% and a beta-alpha shoulder of about 25%, with no detectable albumin.

Evaluation of the conjugates. The protein levels, F/P ratios, and direct fluorescent antibody (FA) titers of these conjugates are shown in Table 3. For purposes of comparison, the direct titers were mathematically adjusted so that all conjugate titers were based on 10 mg of protein per ml. The ascitic fluid from the mice in group II, which received the higher challenge dose, produced higher-titered conjugates. In a previous series of rabies MIAF conjugates, those with F/P ratios of about 6 gave low staining titers, whereas those conjugates with F/P ratios as high as 20 gave high staining titers but also exhibited a prohibitive amount of background fluorescence. F/P ratios of 10 to 12 proved to be optimal for achieving the highest titers without background fluorescence.

To demonstrate the feasibility of preparing a usable conjugate, we prepared a proportionate pool (pool I) of MIAF from all fluids of group II and MIAF from the 51-day tap of group I. It was fractionated, and the globulin was concentrated to approximately 20 mg of protein per ml. A second FRA conjugate was prepared with a proportionate pool of fluids from all taps of group I mice (pool II). The MIAF was fractionated and concentrated to approximately 20 mg of protein per ml. Both pools were tagged with FITC for an F/P ratio of 8 to 12 (Table 4).

The best working dilutions were achieved

TABLE 2. *Neutralizing antibody levels of MIAF after immunization with rabies virus*

Day of collection	Neutralizing antibody titers	
	Group I	Group II
36	1:21,400[a]	1:36,800
43	1:6,100	1:11,500
51	1:6,100	1:11,500

[a] Highest dilution of MIAF that neutralized $10^{1.5}$ mouse 50% lethal doses of rabies virus.

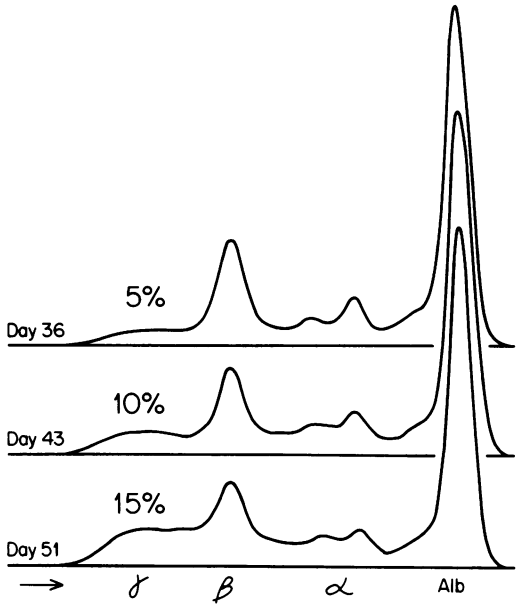

FIG. 2. *CASE profiles of rabies MIAF.*

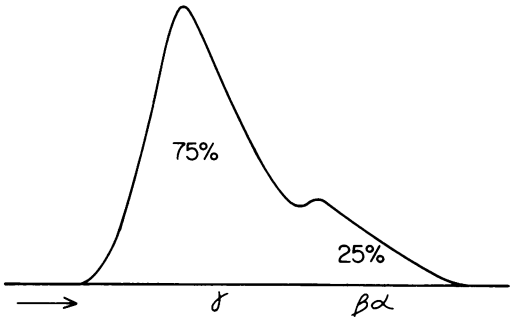

FIG. 3. *CASE profile of rabies FITC-conjugated MIAF.*

with conjugate prepared from the later taps, with the gamma globulin adjusted to 20 mg of protein per ml and labeled with FITC to obtain F/P ratios between 10 to 12. The conjugates were evaluated using the standard FRA procedure (9). The dilutions of conjugate in normal mouse brain gave 4+ staining intensity using both street and challenge virus standard rabies virus mouse brain impression smears. Nonspecific background fluorescence was not observed at the working dilutions (usually 1:40), and no greater than 1+ background staining was observed at the starting dilution of 1:5. Fluorescence of rabies virus was completely inhibited when the conjugate was diluted using a rabies-infected mouse brain suspension. No nonspecific fluorescence of brain myelin tissue was seen.

Normal mouse brain impression smears obtained from different groups of mice stained with the MIAF conjugate were negative.

Impression smears from twenty-two rabies-positive brain specimens, representing 1 human and 10 animal species that had been submitted to the Center for Disease Control for confirmation, were also positive when examined using the MIAF conjugate.

DISCUSSION

These studies indicate that a useful conjugate for the FRA test can be prepared from MIAF. A conservative estimate is that 15 ml of ready-to-fractionate MIAF can be obtained from each mouse. Further, the procedure has been simplified to the extent that it can be performed by laboratories with limited facilities and resources.

The level of neutralizing antibody in MIAF does not appear to indicate its potential as an

TABLE 3. *Protein concentrations, F/P ratios, and comparative titers of rabies FA conjugates*

Day of tap	Protein (mg/ml)	F/P ratio	FA titer[a]	10-mg Protein	
				Factor	Titer
Group I					
36	2.6	12.0	1:10	3.8	1:38
43	5.2	12.0	1:30	1.9	1:57
51	9.0	9.5	1:15	1.1	1:17
Group II					
36	2.9	10.4	1:15	3.4	1:51
43	6.9	11.1	1:80	1.4	1:112
51	8.5	10.3	1:60	1.2	1:72

[a] Titers obtained were based on 4+ intensity of fluorescence.

TABLE 4. *Physicochemical and staining properties of two rabies FA conjugates from pooled MIAF*

MIAF	Protein (mg/ml)	F/P ratio	FA titer
Pool I	10.5	11.3	1:60
Pool II	10.6	8.5	1:60

FA reagent. The highest neutralizing titers were observed in both groups of mice on day 36; however, MIAF collected on day 43 produced conjugates with the higest FA titers for each group. Since higher FA titers were produced from group II MIAF, it appears that the factor responsible for this was the larger booster dose of live rabies virus administered to group II mice on day 29. In both groups, the FA titer declined within 8 days after the 43-day tap.

The evidence suggests that even randomly pooled MIAF from the later taps can result in satisfactory conjugates if the globulin fractions are concentrated to contain 20 mg of protein per ml and labeled at an F/P ratio between 8 to 12.

Comparison of CASE patterns of mouse serum and of MIAF showed that they were essentially the same. The total protein levels of MIAF, however, were one-third that of mouse serum. Therefore, the MIAF antibody globulin protein should be concentrated to correspond to that of mouse serum. This was achieved by reducing the resuspension volume during $(NH_4)_2SO_4$ fractionation until the final volume was one-third or less than that of the starting volume of MIAF. Concentration of the FA antibody two- to fivefold after FITC labeling very often resulted in prohibitive nonspecific background staining.

To avoid immunization with heterologous tissue antigens, some laboratories immunize hamsters with a homologous tissue system (10) and rabbits with purified rabies nucleocapsids (1). In our laboratory, satisfactory results were obtained when a homologous mouse system was used in preparation of rabies MIAF. Rabies-infected mouse brains from 2- to 3-week-old mice were used as immunizing antigen with no evidence of antimyelin antibody. It is suggested, however, that if suckling mice are available, immunizing antigen be prepared in suckling mouse brain to avoid the possibility of obtaining antimyelin antibody in the ascitic fluid.

Although more sophisticated fractionation procedures may yield a purer gamma globulin fraction, ammonium sulfate fractionation with optimum salt concentration is simple and yields a globulin fraction pure enough to label satisfactorily with FITC. Combining this with the dialysis labeling procedure results in a satisfactory rabies FA conjugate.

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