RESEARCH REPORT

PREPARATION OF FLUORESCEIN-LABELED ANTIRABIES GAMMA-GLOBULIN AT THE THAI RED CROSS

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Rabies remains a major health problem in most countries of Southeast Asia. In areas where rabies is prevalent, human exposure to an animal must be evaluated as a potential source of rabies infection. Animals suspected of being rabid should be examined by qualified personnel to determine whether post-exposure treatment of the human contacts is indicated. The discovery of the fluorescent antibody (FA) technic in 1958 (Goldwasser and Kissling, 1958) made it possible to quickly detect rabies antigen in brain tissue with nearly 100% sensitivity and specificity. This replaced the Negri body test which gave a sensitivity of only 75% or less (Coons and Kaplan, 1950). The FA test can detect rabies antigen in the brain before Negri bodies have developed. It is also useful in other tissues which do not form Negri bodies. The FA has a high degree of correlation with the mouse inoculation test. Furthermore, it will detect dead virus which is not identified by the mouse inoculation method. FA testing makes possible a rabies diagnosis in a matter of hours rather than days (McQueen *et al*, 1960). In the direct test, a labeled immunoglobulin is employed as a source of antibodies to detect rabies antigen. A positive FA test is indicated by green fluorescent staining rabies antigen. Today, the FA technic is most widely used but virus inoculation in laboratory mice and Sellers technics for detection of Negri bodies are also still used in some laboratories.

The principal procedure for rabies diagnosis in Thailand is the FA test. The main requirements for success in using this technic are well-trained personnel and conjugated antiserum or globulin of good quality. Immunoglobulin of equine origin, labeled with fluorescein isothiocyanate (FITC), is commercially available. The laboratory diagnosis of rabies infection is important since the results influence treatment decisions. They also have public health implications for controlling an epizootic in a community. The difficulty to produce rabies conjugate locally and the high cost of the imported conjugate have resulted in only limited acceptance of the FA test worldwide.

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The Queen Saovabha Memorial Institute (QSMI) is the principal rabies diagnostic and animal bite treatment center in Metropolitan Bangkok. Thirty to forty human animal bite cases are seen daily. Approximately 150 animals are examined clinically and at necropsy every month (Mitmoonpitak *et al*, 1997). In addition, the institution is expanding its range of activities to produce biologicals and diagnostic reagents in an effort to reduce foreign exchange costs. QSMI decided to establish a plasma fractionation plant in the late 1980's to produce equine antirabies immunoglobulin (ERIG), a therapeutic agent used for rabies post-exposure treatment. Purified ERIG has been processed from horse plasma. The plasma is collected from immunized horses by plasmapheresis. The horses had received a series of purified Vero cell rabies vaccine (Institute Merieux, France) and the production of ERIG is carried out as previously described (Luekrajang *et al*, 1996). Locally made ERIG is equivalent to imported products in terms of purity, efficacy and safety (Wilde *et al*, 1991).

Successful attempts to prepare FITC-labeled globulins from plasma or sera have been made previously (Clark and Shepard, 1963; Schneider, 1973; Kawamura and Aoyama, 1982; Perrin, 1996). These experiments were compared. Ammonium sulfate [$(NH_4)_2SO_4$] precipitation is the most applicable method for isolating the globulin fraction. The procedure can be modified for sera or plasma of most mammalian species. In this study, the precipitation procedures used involve variations in

salt concentration and detailed fractionation such as those previously performed by Schneider (1973), Kawamura and Aoyama (1982), Perrin (1996). The precipitations were evaluated in terms of yield (recovery) and electrophoretic 'purity' of the end product. As shown in Table 1 and Fig 1, Kawamura and Aoyama's procedure gave higher yields of globulin and lesser contaminant albumin than did the others. Preparation of globulin by the chromatographic technic according to Levy and Sober (1960) was also carried out. The γ-globulins were thus obtained in relatively pure form (Fig 2). Anion-exchange column chromatography with DEAE-cellulose is a convenient means of isolating γ-glubulin from horse serum or plasma with good yield and a high level of purity. The technic is much more selective than precipitation. Although DEAE-cellulose is expensive, a column that is used and stored properly is stable for years.

Labeling of globulin by FITC was accomplished by adding the powdered FITC directly to a buffered globulin solution as previously described (Goldwasser and Kissling, 1958). Unreacted free FITC was removed from the FITC-globulin mixture by gel filtration on a Sephadex G-50 column. Over-labeled antibodies with FITC could be further separated by ion-exchange chromatography and discarded. In addition to the labeling method, the dialysis technic can offer an alternative method of conjugation by allowing the FITC to diffuse into globulin through a dialysis membrane (Clark and Shepard, 1963). While the conventional method has been considered the most reliable, it is more time consuming than dialysis and needs more care. Staining efficiency of conjugates, prepared from pure γ-globulin or crude $(NH_4)_2SO_4$-precipitated globulin labeled as usual or by dialysis technic, was evaluated on rabies-infected BHK-21 cells.

Table 1

Evaluation of $(NH_4)_2SO_4$ - precipitated globulins prepared by different procedures.

Sample	Protein %	Activity IU/ml	% Ig recovery
Starting plasma	9.5	104.4	-
Ig (Perrin, 1996)	6.6	56.9	13.6
Ig (Schneider, 1973)	4.8	141.4	33.9
Ig (Kawamura and Aoyama, 1982)	5.4	175.6	42.0

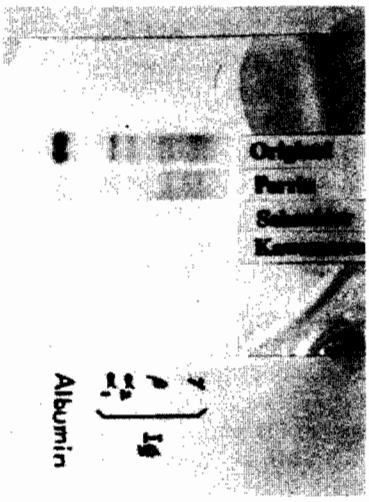

Fig 1–Cellulose-acetate membrane electrophoretic patterns of globulin preparations given in Table 1.

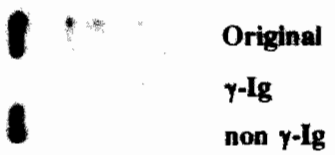

Fig 2–Electrophoretic patterns of γ-globulin and non γ-globulin fractions separated by DEAE-cellulose column.

Rabies conjugate prepared by the dialysis method gave greatly diminished or nonspecific fluorescence. Specific staining titers were two to four times higher than those obtained by the conventional labeling method. Use of globulin solution containing other γ-globulin led to nonspecific fluorescence and lower titers in specific staining. Furthermore, a blinded test comparing our conjugate (pure γ-globulin labeled by dialysis technic) with that obtained from a commercial source (BBL, Becton Dickinson, MD) was conducted on dog brain samples submitted to the diagnostic laboratory of QSMI. Conjugates were applied to clinical necropsy brain impression smears. Impressions prepared from normal dog brains were also included to evaluate specificity of the staining method. The comparisons were made blindly on a total of 300 specimens. The commercially made FITC-labeled $(NH_4)_2SO_4$-precipitated globulin was routinely adsorbed with normal mouse brain suspension before use (Dean *et al*, 1996). The adsorption is an essential requisite for the reduction of nonspecific staining. Our own conjugate was not adsorbed prior to use due to its high purity. There was complete agreement (100% concordance) between the results using both conjugates. No false negative result was found with QSMI conjugate. It had good specific staining quality and no nonspecific fluorescence. Our experience may lead us to nitiate larger scale commercial production.

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