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SHORT COMMUNICATION

A MODIFIED PAP (PEROXIDASE-ANTI-PEROXIDASE) STAINING TECHNIQUE USING SERA FROM PATIENTS WITH DENGUE HEMORRHAGIC FEVER (DHF): 4 STEP PAP STAINING TECHNIQUE

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BHK-21 cells infected with dengue virus type 1 were stained by a newly developed 4 step PAP (peroxidase-anti-peroxidase) technique using sera from patients with dengue hemorrhagic fever as anti-virus antibody. The intensity of staining of the sera was proportional to the hemagglutination inhibition and neutralization titers. With this new technique using sera from patients it should be possible to use the PAP technique for serodiagnosis of virus infections.

The immuno-peroxidase (IP) technique has been widely used for detection of viral antigens in infected cells (Dougherty et al., 1972; Sutmoller and Cowan, 1974; Gerna et al., 1976). This technique has many advantages, such as that viral antigens stained by the IP technique can be examined under an ordinary light microscope and the stained specimens can be preserved permanently for further examination.

We have used the PAP staining technique, a type of the IP technique, on cells infected with dengue and Japanese encephalitis viruses for titration of neutralizing antibodies. This technique seems useful in epidemiological surveys where many specimens must be examined at the same time (Okuno et al., 1977; Okuno et al,. 1978).

The PAP technique has higher sensitivity and a clearer background than other IP techniques (direct and indirect methods). Once anti- peroxidase serum has been obtained, the antigen-antibody reaction used to prepare the PAP complex (Sternberger et al., 1970) is easier and more efficient than the chemical conjugation method used in direct and indirect IP techniques (Nakane and Kawaoi, 1974). Moreover, the PAP complex is stable.

As shown in Fig. 1, the ordinary technique requires three antisera (anti-virus rabbit serum as the first serum, anti-rabbit IgG sheep serum

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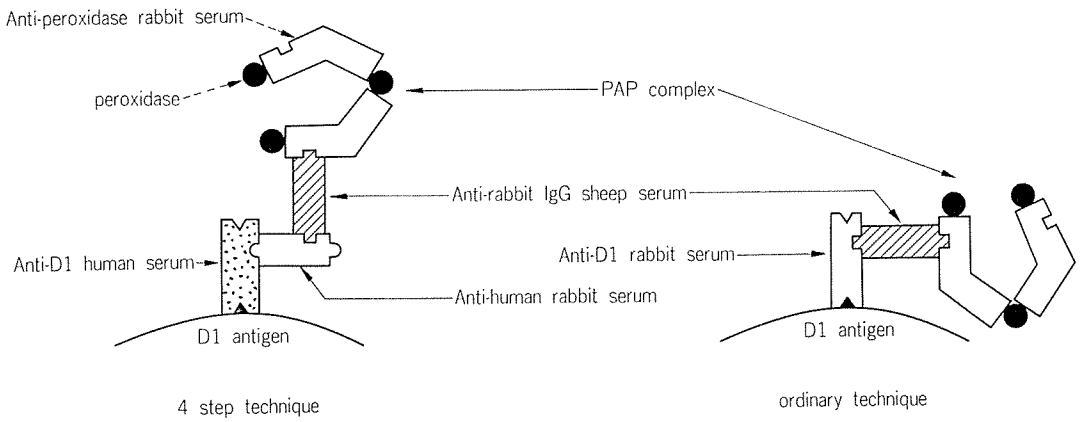

FIGURE 1. Schematic diagrams showing principles of 4 step PAP and ordinary 3 step PAP staining techniques.

as the second serum, and the PAP complex as the third serum). Theoretically it is possible to use sera from patients (anti-virus antibody) as the first serum in the staining technique, and if so, the technique could be used for serodiagnosis of virus infections. There is, however, one problem to using the technique for this purpose. In principle, the first serum (anti-virus antibody) has to be prepared in animals of the same species as those in which anti-peroxidase serum has been made; for instance, the first serum must be obtained from rabbits when the third serum (anti-peroxidase antibody) is prepared in rabbits. Similarly, when serum from patient (anti-virus human antibody) is used as the first serum, anti-peroxidase antibody should also be made in humans. But this is not practical.

To overcome this difficulty, and also to widen the range of application of the technique, we used anti-human IgG rabbit serum to combine anti-virus human antibody and anti-rabbit IgG sheep antibody. Thus, this new technique requires four antisera (antibodies), and is called the "4 step PAP technique" in this paper for convenience. The principles of the 4 step PAP and the ordinary 3 step PAP technique are compared schematically in Fig. 1.

BHK-21 cells infected with dengue virus type 1 (D1) were used throughout this work. The method used for preparation of D1 infected cells is described elsewhere (Okuno et al., 1978). Sera of patients with dengue hemorrhagic fever, used as the first serum in the 4 step PAP technique, were obtained in Chanthaburi Province, Thailand in 1978. Anti-human rabbit serum and anti-rabbit IgG sheep serum were kindly supplied by Kanonji Institute of the Research Foundation for Microbial Diseases, Osaka University. The soluble PAP complex was prepared in this laboratory by the method of Sternberger (1970) with slight modifications (Okuno et al., 1978).

Figure 2 shows the procedure for staining D1 infected BHK-21 cells in the 4 step PAP technique. The infected cells on Lab-Tek 8 chamber tissue culture slides (Miles, I11., U.S.A.) were rinsed with phosphate buffered saline (PBS, pH 7.4) and fixed with acetone for 20 min at 4 C. Then they were treated with 4 antisera (sera of DHF patients diluted 1:400–1:102,400; anti-human rabbit serum diluted 1:2,000; anti-rabbit IgG sheep serum diluted 1:50; and PAP complex diluted 1:20), in the order, for 40 min each. After each step the slides were washed with 3 changes of PBS for 3 min each time. Finally, the specimens were stained by the method of Graham and Karnovsky (1966) using 6 mg of 3,3'-diaminobenzidine tetrahydrochloride in 20 ml of PBS con-

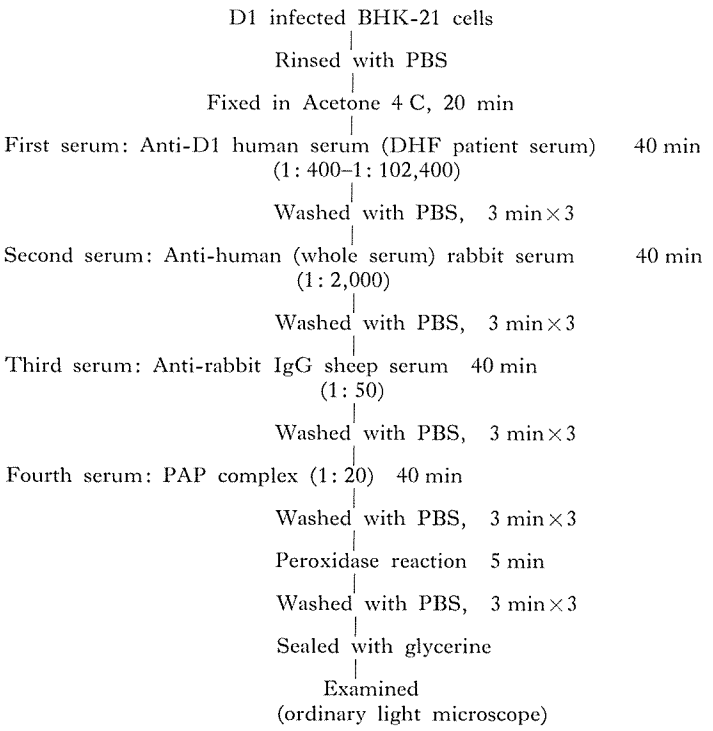

FIGURE 2. Staining procedures used for 4 step PAP technique. Figures in brackets show serum dilutions used in staining.

TABLE 1. *Comparison of antibody titers of sera of patients with aengue hemorrhagic fever (DHF) against dengue virus type 1 (D1) antigens by the N and HI tests and staining activity.*

Serum No.	N titer (FR_{50})	HI titer	Staining activity				
			×400[a]	×1600	×6400	×25600	×102400
1	35	40	−	−	−	−	−
2	130	<20	−	−	−	−	−
3	270	<20	−	−	−	−	−
4	800	1280	++	++	±	−	−
5	4700	5120	+++	+++	++	±	−
6	5300	5120	++	++	+	−	−
7	18000	≧10240	++	+++	++	+	−
8	30000	≧10240	+++	+++	++	±	−
9	58000	≧10240	+++	+++	+	−	−

[a] Reciprocal of serum dilution used for staining.

taining 0.01% H_2O_2. Then they were washed with PBS, sealed with glycerine, and examined under an ordinary microscope.

Table 1 shows the staining activity of sera of DHF patients by the 4 step PAP technique against D1 virus antigens. Nine sera of pa-

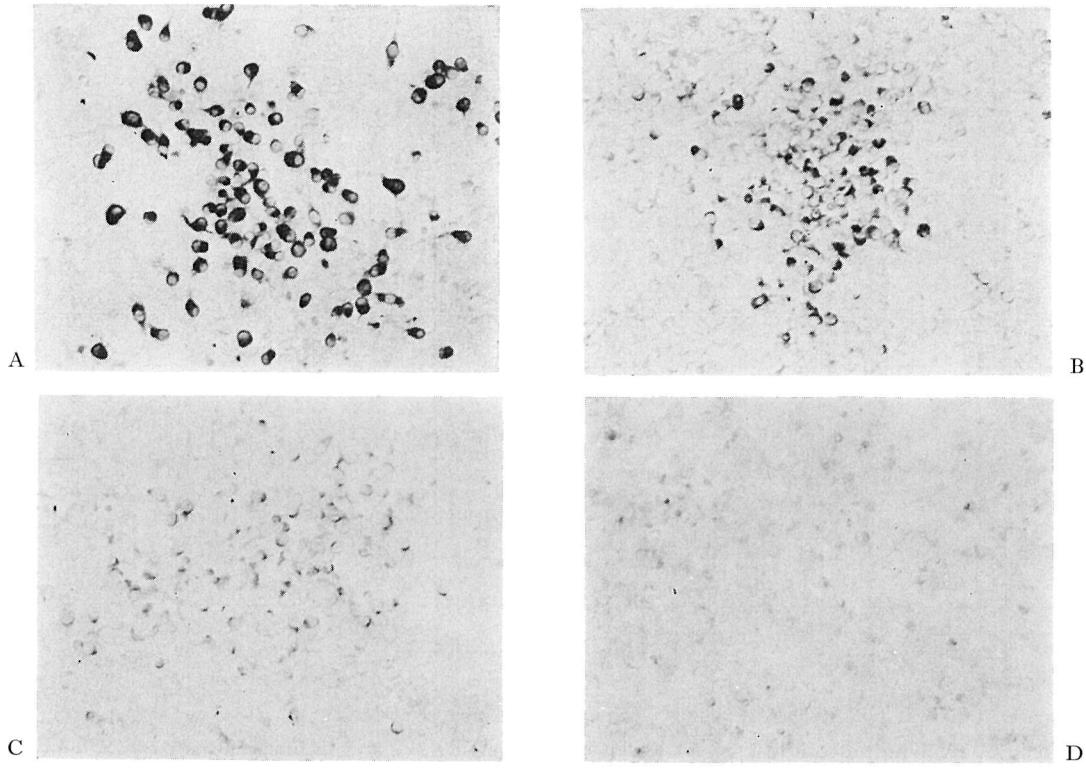

FIGURE 3. Comparison of staining grades by the 4 step PAP technique. A: Strongly positive (卌), B: Definitely positive (+), C: Slight staining (±), D: Negative (−). Dengue virus type 1 (D1) infected BHK-21 cells were stained using human serum No. 5 at 1: 1,600 (A), No. 9 at 1: 6,400 (B), No. 5 at 1: 25,600 (C) and No. 5 at 1: 102,400 (D), respectively, as the first serum.

tients in the convalescent phase were diluted serially 4-fold from 1: 400 to 1: 102,400 with PBS. The intensity of staining was graded from strongly positive (卌) to negative (−). Examples of the staining grades are shown in Fig. 3. Some sera stained the infected cells as strong as the anti-D1 rabbit serum prepared in this laboratory for the ordinary PAP staining technique.

The hemagglutination inhibition (HI) and neutralization (N) titers of the sera are also shown in the table. HI tests were performed by the method of Clarke and Casals (1958) with microtiter modification (Sever, 1962). N tests were performed by the focus reduction method using PAP staining (Okuno et al., 1978) and expressed as the 50% focus reduction titer (FR_{50} titer).

In general the titers of the staining activity of the sera seemed to be proportional to the N and HI titers. Therefore, when the reciprocals of the maximal dilution of the first serum that caused distinct staining of given infected cells is defined as the staining titer of the serum, this titer by the new technique could be used, like the N and HI titers, as an indicator in serodiagnosis.

The 4 step PAP technique, introducing anti-human rabbit serum, eliminates the limitation of the ordinary 3 step PAP technique that human serum cannot be used as the first serum. Moreover, the new technique enables us to use sera of any animal species as the first serum, provided the second serum, which combines

the first antibody (anti-virus antibody) and the third antibody, is chosen appropriately.

Furthermore, the 4 step PAP technique with some modifications could be used for rapid diagnosis of virus infections, in the same way as the so-called " enzyme-linked-immunosorbent assay (ELISA) ".

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