APPLICATION OF PEROXIDASE-ANTIPEROXIDASE (PAP) STAINING FOR DETECTION AND LOCALIZATION OF DENGUE-2 VIRAL ANTIGEN. II. OBSERVATIONS FOR THE ANTIBODY ENHANCEMENT ACTIVITY IN HUMAN MONOCYTES

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INTRODUCTION

Antibody dependent enhancement (ADE) phenomenon has been described in some of the flavivirus infection such as Japanese B encephalitis, West Nile, and dengue viruses infection (Halstead *et al.*, 1980). In this phenomenon, an enhancement of virus replication in infected cells may be observed when a small amount of Ig antibody at subneutralizing titer is added to the culture of infected cells such as culture of dengue virus infected monocyte (Halstead and O'Rourke, 1977a). This phenomenon has been suggested as the pathogenetic mechanism in the severe form of Dengue fever i.e. Dengue Hemorrhagic Fever-Dengue Shock syndrome, (DHF-DSS) where cases tend to occur in young subjects with previous infection by one type of dengue virus, followed by the infection of the other type, or in infants less than one year of age where subneutralizing level of maternal antidengue IgG circulates. The usual way to assay the ADE phenomenon is to perform virus plaque assay or Infectious Center Assay (ICA). The plaque assay quantitates the amount of virus yield. The ICA method could titrate the virus in infected monocytes by permitting each infected monocyte to pass the virus into cells susceptible for the particular virus (Halstead *et al.* 1977b). The number of infected foci demonstrated by indirect fluorescent antibody method (IFAT) could be counted to quantitate the number of viral infectious particles which could be compared between different cultures. While ICA method is being used widely, it is time consuming and represents an indirect measurement of the virus produced. We have recently applied successfully the unlabelled enzyme method using Peroxidase-antiperoxidase (PAP) complex for the detection and localization of dengue 2 virus in endogenous peroxidase containing cell system such as peripheral blood leucocytes (Churdboonchart *et al.*, 1984). The fact that it yields a permanent preparation which could be analysed for morphological details in a semiquantitative manner could be very useful for the elucidation of mechanism of ADE at the cellular level. The following presentation compares ADE assay of Dengue 2 infected monocyte between the ICA and PAP method.

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MATERIALS AND METHODS

Virus: Dengue type 2 (strain 16681) was used to infect human mononuclear phagocytes and the Dengue type 2, New Guinea C strain, was used to infect suckling mice.

Immunological reagents: Rabbit anti-dengue-2 sera was prepared from immunoprecipitin bands obtained from countercurrent immunoelectrophoresis and crossed immunoelectrophoresis as described in our previous reports (Churdboonchart *et al.*, 1984, Wong tanyakorn *et al.*, 1985). These bands were excised from agarose gel, washed with normal saline for at least 3 days, grinded, and mixed with Freund's adjuvant. This mixture was

used as immunogen to raise anti-dengue-2 antibody in rabbits.

Swine antiserum to rabbit immunoglobulin (SWAR) was purchased from Dakoimmu noglobulin, Copenhagen, Denmark.

Peroxidase-anti-peroxidase complex was prepared according to Sternberger's method (Sternberger *et al.*, 1970). Briefly, 20 ml of rabbit-anti-peroxidase and 64 mg of lyophilized peroxidase were mixed to form primary complex. These complexes were prepared in an antigen excess condition by the addition of an extra quantity of peroxidase. Final molecular ratio between peroxidase and antiperoxidase was approximately 3 to 2 (3:2).

Antibody-enhanced dengue-2 virus infection in human monocyte: The enhanced antibody was monkey hyperimmune sera with anti-dengue-4 with a hemagglutination inhibition (HI) titer of 1:5, 120. It was titrated and found to have optimum enhancement activity at the dilution of 10^{-4} by conventional ICA method. This antibody was diluted to three dilutions 10^{-3}, 10^{-4}, and 10^{-5} for the study of enhancement activity. Monocytes were collected from the blood of dengue immune blood donors from Ramathibodi Hospital by the usual centri-fugation and sedimentation technique. Twodays old cultures of monocyte with dengue-2 virus (MOI = 0.1). The control were inoculated systems consisted of non-infected, monocyte culture and dengue-2 infected monocyte culture without the addition of any antiserum. RPMI 1640 with 10% fetal calf serum was used as culture media. The cells were harvested at day 1, 2, 3, 4, and 5 and the number of infected cells were measured by ICA and PAP method.

Infectious-Center Assay (ICA)

The number of infected cells was determined by an infectious center assay as previously described (Halstead *et al.*, 1977b). Infected monocytes were washed in 50 volumes of phosphate buffer saline (PBS) and resuspended to 1 ml in 1:10 monkey anti-dengue-2 for 30 min at 37°C. After removal of anti-dengue-2 sera by washing them three times in PBS, the viable cells were adjusted to the final concentration of 1×10^6 cells/ml. 0.1 ml of cell suspensions was applied to 3-4 days old LLC-MK_2 monolayer cells and overlaid with agar immediately. They were incubated at 37°C for 7 days, and then stained with indirect fluorescent antibody method. The number of positive cells were reported in percentages of all cells counted.

Peroxidase-antiperoxidase (PAP) staining

The PAP staining was done as described previously (Churdboonchart *et al.*, 1984). Before staining specimens were washed (thrice with EDTA and 5% bovine albumin in PBS), smeared on glass slides, and fixed with 95% ethanol (5 min). They were immersed in 0.6% H_2O_2 in absolute ethanol for at least 24 hr and not more than 48 hr. Normal swine serum (NSS) at 1:20 dilution was used to block non-specific antibody attachment to eliminate non specific background staining. The primary antibody, rabbit anti-dengue-2 (RaD2), was applied for 20 min and the specimens were then washed thoroughly. Secondary or linked antibody (SWAR) was added to link the primary antibody and the PAP complex for 30 minutes. PAP soluble complex was applied for another 20 min. The enzyme substrate was AEC (0.02% 3-amino-9-ethyl carbozole, 0.03% H_2O_2, 5% N, N dimethyl formamide in 0.1 M acetate buffer, pH 5.2). The specific staining appeared as reddish brown color. The slides were counterstained by Mayer's hematoxylin, treated with ammonium hydroxide, and mounted with glycerol under cover slip. These specimens can be kept for months at room temperature.

The specimens were examined under a light microscope. Five non-repeated areas from

the upper right and left. the lower right and left, and middle part of each stained slide were traced for positive cells. Two slides were made for each test. The number of positive cells among total count of 100 leukocytes in each area were noted. Mean value of positive cells from these 10 areas was used as percentage of PAP positive for each specimen.

RESULTS

Result from enhancing experiments are summarized in Table 1. Non-infected cells remained negative from day-1 through day-5. Suspension of infected cells without hyperimmune serum or with the addition of serum with no enhancement antibody were also infected but the count showed lower percen-

Table 1

Enumeration of infected human mononuclear phagocytes by PAP and ICA.

Donors	Days after viral inoculation	Control		D2v*		D2v+aD$_4$**10^{-3}		D2v+aD$_4$10^{-4}		D2v+aD$_4$10^{-5}	
		PAP	ICA	PAP	ICA	PAP	ICA	PAP	ICA	PAP	ICA
No. 1	Day 1	0	ND	0	ND	0	ND	0	ND	0	ND
	2	0	ND	0.1	ND	0	ND	0.2	ND	2.0	ND
	3	0	ND	0.2	ND	3.0	ND	1.0	ND	4.2	ND
	4	0	ND	0.6	ND	8.4	ND	2.0	ND	12.0	ND
	5	0	ND	0.0	ND	13.4	ND	3.8	ND	32.8	ND
No. 2	Day 1	0	ND	0	ND	0	ND	0	ND	0	ND
	2	0	ND	ND	ND	2.0	ND	0.8	ND	0.4	ND
	3	0	ND	ND	ND	4.8	ND	2.4	ND	1.8	ND
	4	0	ND	ND	ND	11.6	ND	10.8	ND	4.2	ND
	5	0	ND	ND	ND	22.2	ND	13.0	ND	5.6	ND
No. 3	Day 1	0	ND	0	ND	0	ND	0	ND	0	ND
	2	0	ND	0	ND	0	ND	0	ND	1.0	ND
	3	0	0	0	2.4	1.3	5.6	2.3	6.9	0.3	6.4
	4	0	ND	0	ND	3.0	ND	7.6	ND	2.7	ND
	5	0	0	1.0	2.9	5.7	3.0	12.6	4.8	3.0	4.0
No. 4	Day 1	0	ND	0	ND	0	ND	0	ND	0	ND
	2	0	0	1.0	2.5	2.0	4.3	5.0	9.1	3.0	5.8
	3	0	0	1.5	2.2	2.5	5.1	3.7	18.1	6.5	6.8
	4	0	0	1.3	0.9	9.7	0.9	7.0	4.5	5.0	3.4
	5	0	0	6.0	1.2	9.7	2.4	6.7	3.9	9.7	2.7
No. 5	Day 1	0	0	0	0.7	0	0.4	0	0.5	0	1.3
	2	0	ND	0	ND	1.0	ND	2.0	ND	1.0	ND
	3	0	0	2.0	0.8	9.0	2.6	27.0	15.8	32.0	32.5
	4	0	0	7.0	1.8	47.0	8.6	30.5	7.0	24.0	5.5
	5	0	0	28.0	2.6	33.0	1.1	37.0	2.2	39.5	0.9

*D2v = Dengue type 2 virus.
** aD$_4$ = monkey hyperimmune sera with anti dengue-4 activity.

tages of infected cells were statistically significant ($p < .001$). An increasing number of infected cells on a day to day basis can also be observed in this non-enhanced group. The positive staining was seen in reddish brown color in various patterns depending on the duration after inoculation. On day 1 small dots or fine granules can be seen. Then they were changed to clumped stains in cytoplasm and perinuclear area. In the late stage (day 4-5), the viral antigen was loaded in the cytoplasm, and the cells may be seen in the distored form.

Percentage of infected cells by PAP staining and ICA showed good correlation up to day 3. At day 4 and 5 percentages of antigen bearing cells by PAP method kept on increasing while the ICA showed declining percentage of infected cells.

Fig. 1 shows Dengue antigen bearing cells by PAP method. The pattern of staining can be seen different among the positive cells. Some of them had fine granule stains in the whole cytoplasm with certain area of darker staining and some only had fine granules staining in cytoplasm. Certain cells demonstrated none of the basophilic stain of the nucleus.

DISCUSSION

ADE assay by PAP gave a rapid result and the slide can be made permanent offering chances for comparison among specimens. Several specimens could be stained and studied at one time. The use of light microscopy on PAP stained slides allow a better and detailed morphological study even when the number of the infected cells is relatively low (0.1 %). It is also possible to localize the antigen and to observe the pattern of antigen in the infected cells. Several dilutions of immune sera could be made to measure the enhancing titer at one time. In comparison with ICA, PAP is above par for its simplicity and rapidity points of view with an equal level of sensitivity.

Our results were in general greement with those previously reported that enhancement phenomenon could be observed after 2-3 days of the inoculation of the virus. In our experiment, it was also noted that the percentages of PAP positive cells continue to increase upto day 5 post inoculation while the number of infected cells by ICA began to decline on day 4. This indicates that PAP may detect non-infectious structural and non-structural part of the virion which existed in the positive cells leading to the higher number of positive staining cells. The other merit is the fact that the infectious units could be shown by both ICA and virus plaque assay, but the total antigenic mass could be assessed only by this technique. The non-infectious structural and non-structural viral antigen(s) may be very important in term of the ability to activate the complement system which may lead to complement mediated cell injury and in the formation of "phlogistic" immune complexes in dengue infection as suggested (Halstead, 1981).

Another important finding is that by using the same batch of hyperimmune sera at different dilutions, the peak of enhancement activity was shifted from 10^{-3}, 10^{-4} to 10^{-5}. These results suggested that for an optimum study of ADE phenomenon, better results may be obtained when several dilutions of antibody were used eventhough the antiserum may have been already titrated for maximum ADE activity. Besides the *in vitro* application of PAP, this technique may be used to stain mononuclear cells obtained from dengue patients which may lead to the assertion for the presence of dengue viral antigen directly from patients' peripheral blood, whether it is a complete virion or not.

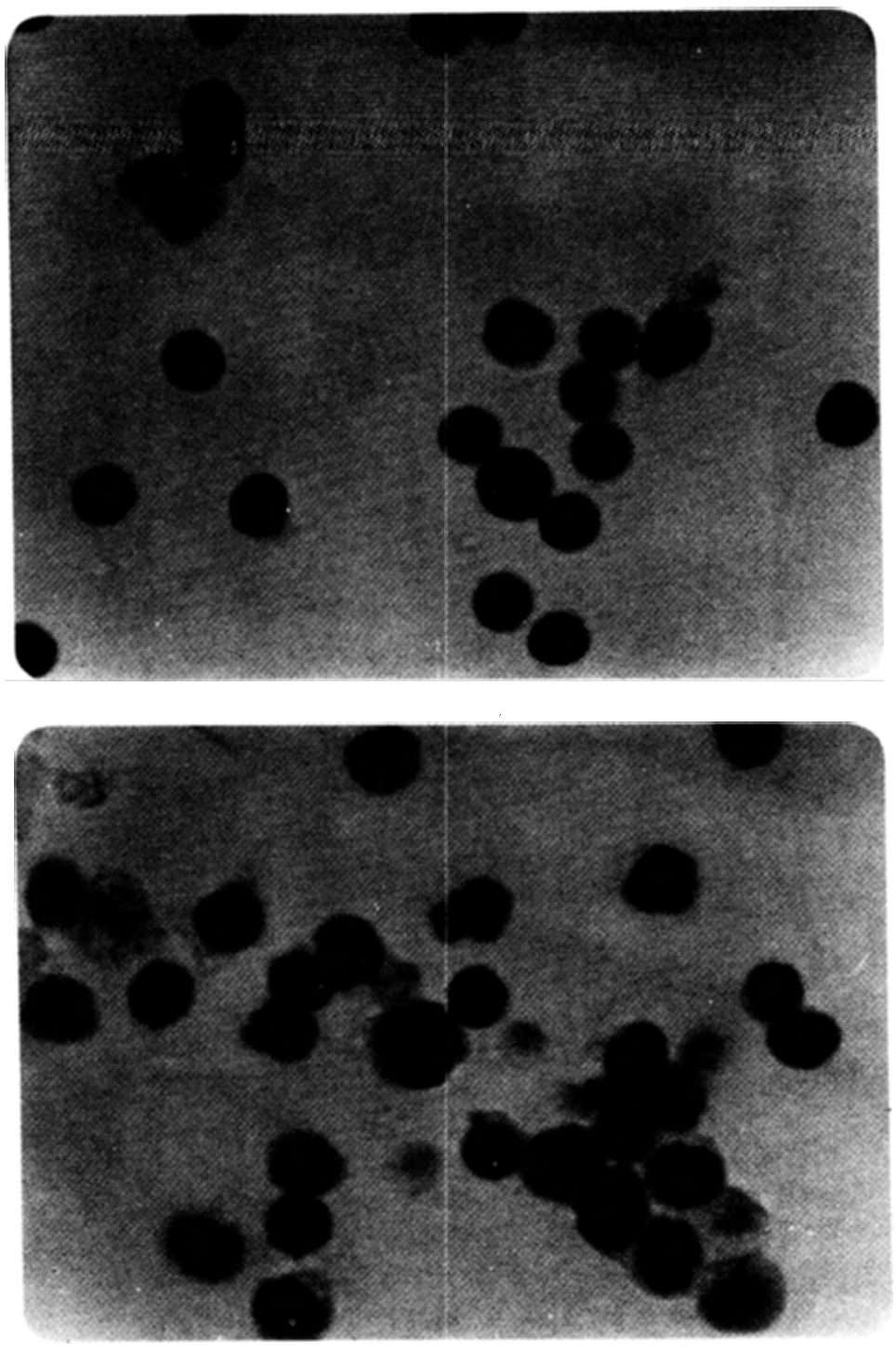

Fig. 1-PAP staining of human mononuclear phagocytes. (a) noninfected cells, day 5, negative control, x 1000.(b) Dengue type 2 virus strain 16681 infected cells, enhanced with 10^{-4} dilution of hyperimmune sera, day 3, x 1000.

SUMMARY

Peroxidase-antiperoxidase (PAP) staining was applied to measure the antibody enhancement activity in human monocytes. Increasing in number of infected cells can be seen with increasing of staining intensity of the cells by ordinary light microscope. Shifting of the optimum enhancement activity was found in previously tritated antiserum indicated that for titration of antibody enhancement activity several dilutions of antiserum should be included in each experiment. Validity of the PAP method was made by the comparison of the results with Infectious Center Assay (ICA). With this technique, titration for antibody enhancement for dengue virus infection can be done with non-expensive equipment and can be kept for comparison for months.

REFERENCES

CHURDBOONCHART, V., KAMSATTAYA, K., YOKSAN, S., SINARACHATANANT, P. and BHAMARAPRAVATI N., (1984). Application of peroxidase-antiperoxidase (PAP) staining for detection and localization of dengue-2 antigen. I. In an endogenous peroxidase containing cell systems. *South east Asian J. Trop. Med. Pub. Hlth., 15*: 547.

HALSTEAD, S.B. and O'ROURKE, E.J., (1977a). Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J. Exp. Med., 146* : 201.

HALSTEAD, S.B., O'ROURKE, E.J. and ALLISON, A.C., (1977b). Dengue viruses and mononuclear phagocytes. II. Identity of blood and tissue leukocytes supporting *in vitro* infection. *J. Expt. Med., 146* : 218.

HALSTEAD, S.B., PORTERFIELD, S. and O' ROURKE, E.J., (1980). Enhancement of dengue virus infection in monocytes by Flavivirus antisera. *Amer. J. Trop. Med. Hyg., 29* : 638.

HALSTEAD, S.B., (1981). The pathogenesis of dengue molecular epidemiology in infectious disease. *Amer. J. Epidemiol., 14*, 632.

STERNBERGER, L.A., HARDY, P.H., CUCULIS, J.J. and MEYER, H.G., (1970). The unlabelled antibody enzyme method of immunohistochemistry: Preparation complex (horseradish peroxidase anti-horseradish peroxidase) and its use in the identification of spirochaetes. *J. Histochem. Cytochem., 18* : 315.

WONGTANYAKORN, N., CHURDBOONCHART, V., BHAMARAPRAVATI, N. and FUTRAKUL, P., (1985). Precipitating antibodies to non-treated dengue type 2 viral antigens in sera of Thai Hemorrhagic Fever patients by crossed immunoelectrophoresis. *Southeast Asian J. Trop. Med. Pub. Hlth., 16* : 1.