IN VIVO AND *IN VITRO* STUDIES ON THE MORPHOLOGICAL CHANGE IN THE MONKEY EPIDERMAL LANGERHANS CELLS FOLLOWING EXPOSURE TO DENGUE 2 (16681) VIRUS

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Abstract. A direct comparison of skin Langerhans cell (LC) morphologic change following *in vivo* and *in vitro* exposure to dengue-2 (DEN-2) virus (16681) was performed in the monkey to investigate any differences in functional activity profiles. Time-lapse study of skin biopsy at the intradermal (*id*) virus injection sites, and thin skin sheets removed from the monkey with exposure to virus in culture medium, revealed a highly active migration of epidermal LCs in both sets of experimental specimens. The migration led to a relatively higher number of dendritic cells (DC) which appeared in active migrational profiles, in the superficial dermis. Moreover, obvious cytoplasmic structural changes, corresponding to their immunologic function, were observed in these superficial dermal DCs 2 hours after exposure. Despite their similar changes, early and late endosomes with degraded virus-like particles could be seen in the skin sheets owing to lagging in cellular physiological process *in vitro*, but none in the skin biopsies. Existence of these endosomes, which was extremely difficult to visualize *in vivo*, highlighted the mode of antigen processing by the endocytic pathway. The present study showed that the epidermal LC was a potent antigen-presenting cell for eliciting the success of *id* immunization and carried out the immunological activity *in vivo* or *in vitro* in the like manner, in respect to the physiological conditions.

INTRODUCTION

The Langerhans cell (LC) is a dendritic resident cell of the monocyte-macrophage lineage within the epidermis, which shows itself as the outer guardian of the immunological system. The cell stretches its dendrites between the keratinocytes and makes a spiderweb-like antigen snare, thereby playing the part of a highly specialized macrophage within the epidermal tissue. It appears that the function of the LC has finally been established as serving as a front-line element in immune reactions of the skin (Breathnach, 1980). Furthermore, other studies have shown that LC can take up, process and present antigen to reactive lymphocytes in an extremely effective fashion (Bergstresser *et al*, 1980; Halliday and Muller, 1987). Upon antigenic stimulation, LC is capable of migrating out of the epidermis into the dermis where it is renamed a dendritic cell (DC). The DC further migrates into the local draining lymphatic and transforms into a veiled cell and travels to reach the draining lymph node (Drexhage *et al*, 1979; Hoefsmit *et al*, 1982, Kripke *et al*, 1990; Larsen *et al*, 1990; Silberberg *et al*, 1976). In addition, the LC has been reported to have an important role in the immune surveillance for various diseases associated with certain viral infections in humans (McArdle and Muller, 1986; Morelli *et al*, 1992, 1993) and in defense against *id* inoculated HSV-1 infection in mice (Sprecher and Becker, 1987, 1989). Our experience with mice challenged with DEN-2 virus (Taweechaisupapong *et al*, 1996), a flavivirus which causes dengue hemorrhagic fever and dengue shock syndrome, indicated that *id* injection with DEN-2 virus induced the earliest and highest neutralizing antibody response compared to other routes of administration. Immunofluorescence showed the presence of viral antigen in the epidermis and draining lymph node within 5 minutes, and within 2 hours of challenge, respectively.

Among the experimental animal models of dengue infection, the monkey has shown similar physiologic and immunologic responses to man

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(Halstead *et al*, 1973) indicating the rationale of using the monkey model which would assist towards understanding the processing of dengue antigen in humans when presented by the *id* route. We therefore studied the interaction of monkey epidermal LCs with DEN-2 virus following *in vivo* or *in vitro* challenge in order to explore the local cellular activity responsible for antigen processing and induction of antibody response.

MATERIALS AND METHODS

Animals

Four cynomolgus (*Macaca fascicularis*), 1 rhesus (*Macaca mulatta*) and 1 pigtailed (*Macaca nemestrina*) monkeys, which were seronegative for dengue virus types 1-4 and Japanese encephalitis virus by plaque reduction neutralization test (PRNT), were used in this study. All animals were obtained from local traders in Thailand. They were of both sexes, 4-10 years old, weighing 1.5-3.0 kg, and housed in a mosquito-proof room, in individual cages measuring $70 \times 62 \times 70$ cm^3, and fed monkey pellets (Pokphand animal feed, Bangkok, Thailand), fresh fruit, and water *ad libitum*.

Virus strain

DEN-2 virus (16681) recovered from the serum of a DHF patient in Bangkok, Thailand in 1964 (Halstead *et al*, 1970 a,b) was obtained from the Center for Vaccine Development, Mahidol University. This strain is the parental strain of the DEN-2 PDK 53 candidate vaccine virus. The titer of the virus was 2.7×10^5 pfu/ml.

Injection of virus to monkey for *in vivo* study

Monkeys were numbered 1-6 as shown in Table 1 and anesthetized with Ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, New York 13201) 10 mg/kg body weight, intramuscularly. They were injected subcutaneously (*sc*) or intradermally (*id*) with 0.1 ml of DEN-2 virus (2.7×10^4 pfu) into their right arms respectively (Table 1). Production of antibodies to DEN-2 virus were checked weekly for 3 weeks. Approximately 2 ml of blood was drawn from the femoral venous plexus of the monkeys on day 0, 7, 14, and 21 after DEN-2 inoculation and allowed to clot for serum collection. Neutralizing antibodies were estimated by using the 50% plaque reduction end-point (Russell *et al*, 1967).

Monkeys no. 3 and 5 were re-injected *id* with the same dose of DEN-2 virus (2.7×10^4 pfu) into their right arms and with 0.1 ml of control fluid (uninfected culture fluid) into their left arms, on day 22 after the first injection. Skin biopsies of the injected sites were made for transmission electron microscopy (TEM) at 0, 15 minutes and 2 hours post-injection (*pi*).

Table 1

Plaque-reduction neutralizing antibody (PRNT) titers of monkeys after subcutaneous and intradermal injection with DEN-2 virus.

Monkey No.	Monkey spp	Routes	Reciprocal PRNT titers			
			Day 0	Day 7	Day 14	Day 21
1	Cynomolgus	*sc**	< 10	< 10	1,150	1,700
2	Cynomolgus	*sc*	< 10	< 10	2,500	3,300
3	Cynomolgus	*id***	< 10	> 20	> 2,560	1,280
4	Cynomolgus	*id*	< 10	25	6,000	2,500
5	Rhesus	*id*	< 10	2,100	> 2,500	3,500
6	Pigtailed	*id*	< 10	54	1,500	700

All monkeys were injected with 0.1 ml DEN-2 virus (2.7×10^4 pfu).
* subcutaneous injection
** intradermal injection

Removal of thin skin sheet and *in vitro* exposure to DEN-2 virus

One day prior to the experiment, the lower back regions of the cynomolgus monkeys, no. 1 and 2 (Table 1), were shaved and cleansed with antiseptic surgical soap. The animals were anesthetized with Ketamine hydrochloride. An area of about 6 × 6 cm^2 of the buttock skin was aseptically and carefully sliced off with a razor. The thin sheets of skin obtained were immediately suspended in a small volume of Iscove's modified Dulbecco's medium (IMDM; Gibco, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 50 mg/ml gentamicin. They were then vertically minced into pieces about 4 × 4 mm^2 with scissors and washed in 3 changes of wash solution (IMDM supplemented with 10% FCS, and penicillin-streptomycin mixture). The small pieces of skin were divided into 8 portions and transferred into 8 wells of a 24 well-plate, each containing 2 ml of culture medium, incubated at 37°C in 5% CO_2 for 1 hour. An inoculum of 0.5 ml of DEN-2 virus (1.35×10^5 pfu) was added to each of 6 of the wells containing the skin sheets, and the other 2 wells were omitted for control. The skin sheets in the virus added wells were harvested for TEM and immunofluorescence at 15, 30 minutes, and 2 hours after addition of the virus. The controls were harvested at 30 minutes.

Morphologic confirmation of inoculum virus

A LLC-MK_2 cell monolayer in a 32 oz bottle was inoculated with 3.9×10^6 pfu of DEN-2 (16681). The culture media was discarded and the cell monolayer was collected for TEM when a direct immunofluorescence check using FITC-conjugated anti-dengue indicated 90% infected cells after incubation for 9 days.

Electron microscopy

Skin biopsies were fixed with 1% glutaraldehyde + 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and further processed for TEM as described elsewhere (Sriurairatana *et al*, 1973). DEN-2 infected LLC-MK_2 cells were fixed, pelleted, and processed through the same procedure as the skin biopsies. Skin sheets in culture wells were fixed for 3 hours by adding 2.5 ml of a fixative containing 2% glutaraldehyde + 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4), and processed for TEM as the skin biopsies. One micron semi-thin sections of all specimens were stained with toluidine blue (Trump *et al*, 1961), and examined by light microscopy. Ultrathin sections were examined in a Hitachi H7100 TEM at 100 kV.

Immunofluorescence

Skin sheets for immunofluorescence examination were embedded in Tissue-tex (Miles Inc Diagnostics Division, Elkhart, IN, USA), cut in a cryostat at 5 μm thickness and stained with FITC-conjugated anti-dengue by the direct immunofluorescence staining method as described (Kuberski and Rosen, 1977) and mounted in buffered glycerol (pH 8.0). The sections were observed under an immunofluorescent microscope (Nikon, OPTIPHOT, Episcopic-Fluorescence attachment EF-D, Japan).

RESULTS

All of the 4 monkeys that received *id* injection with DEN-2 virus developed PRNT titers to DEN-2 in a range of > 20-2,100 detected on day 7 (Table 1). Three of these monkeys had peak PRNT titers on day 14 and then the PRNT titers declined on day 21. The 2 monkeys that received sc injection developed increasing PRNT titer on day 14.

LCs in virus injected skin

Toluidine blue stained semi-thin sections of skin biopsies from control fluid, or virus injected sites, showed obvious differences from the normal skin biopsied before any treatment. The normal skin showed some epidermal LCs with distinctly clear cytoplasm regularly located between the keratinocytes, and a few dendritic cells (DC) were singly present in the collagenous substance of superficial dermis (Fig 1A). The skin injected with control fluid showed edematous change due to inoculum deposition. The microvasculatures in the upper dermis appeared to be pushed downwards away from the superficial dermis (Fig 1B). The virus-injected skin showed marked alteration which varied with time. In addition to the edematous change, there was a marked increase in the numbers of both the epidermal LCs and the superficial dermal DCs,

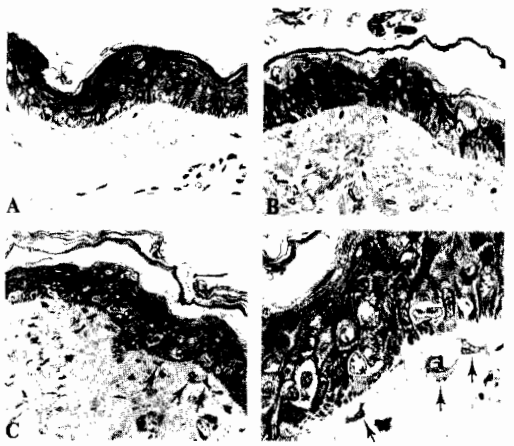

Fig 1–Photomicrographs of toluidine blue stained semithin Epon sections of skin biopsies from cynomolgus monkeys. (A) Normal skin biopsy before viral injection. ×400 (B) Control fluid *id* injected skin biopsy, 15 minutes *pi*, sectioned through the blister formed on the injection site showing separation of stratum corneum from stratum granulosum which is less stained than normal. The collageneous superficial dermis is slightly edematous due to inoculum deposition. ×400 (C) DEN-2 virus *id* injected skin biopsy, 15 minutes *pi*, showing increased numbers of both epidermal Langerhans cells and superficial dermal dendritic cells (arrow). ×400 (D) High magnification of the right part in C showing the morphology of epidermal Langerhans cells (★) and dermal dendritic cells (arrow) ×1,000.

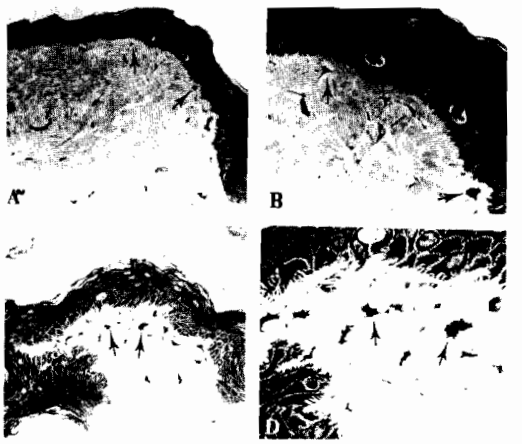

Fig 2–Photomicrographs of toluidine blue stained semithin Epon sections of monkey skin sheet aseptically removed from buttock of a cynomolgus monkey, minced and cultured in culture medium. (A) A control skin sheet kept in culture medium for 30 minutes, showing few epidermal Langerhans cells and a few dermal dendritic cells (arrow). ×400 (B) High magnification of the upper right in A showing morphological change in the epidermal Langerhans cells (✶) and dermal dendritic cells (arrow). ×1,000 (C) Minced skin sheet from the same monkey as A after cultured with DEN-2 for 30 minutes, showing very few Langerhans cells in the epidermis in contrast to higher number of dendritic cells (arrow) in the dermis. ×400 (D) High magnification of the right part of C showing the dendri-tic cells with their dendritic process (arrow) ×1,000.

at 15 minutes *pi* (Fig 1C). Furthermore, the superficial dermal DCs appeared in active migrational profiles, evident by their stellate cell bodies and a space within the collagenous ground substances left by the contraction of cell body during movement towards draining lymph vessels (Fig 1D). By 2 hours, the edematous change subsided, and there were very few LCs in the epidermis while the number of superficial dermal DCs increased to an even higher number than at 15 minutes *pi*.

LCs in skin sheets exposed to virus *in vitro*

Semithin sections of skin sheets generally showed the epidermis and the thin upper dermis. The cutaneous cells were not as well stained as those of the biopsies. Also, the *in vitro* manipulation of skin sheets brought about a 20% loss of epidermal LCs in the unexposed control (Fig 2A) compared with those of the normal or control fluid-injected skin biopsies. A cytopathic change, which appeared as a clear space that almost surrounded the cytoplasmic contour of the LC (Fig 2B), was noted in the epidermal LCs among other normal epidermal cells. Many of the DCs were stellate in shape. For the skin sheets exposed to the virus, marked changes were first noted at 30 minutes *pi*. The number of epidermal LCs diminished, whereas the number of DCs apparently increased (Fig 2C) when compared with the control. Under high magnification, the DCs were highly stellate and had long processes (Fig 2D). The 2 hours exposure

specimen showed very few LCs in the epidermis, but a relatively higher number of DCs in the superficial dermis.

Immunofluorescence of the skin sheets exposed to DEN-2 in culture medium showed clumps of antigen reacting with FITC-conjugated anti-dengue within both epidermis and dermis in the early period after viral exposure. In the later periods, the antigen was in small specks. It was not clear whether these specks were intra- or extra-cellular. However, there was no such fluorescence in the skin sheets unexposed to the virus.

Ultrastructural changes

By TEM, the epidermal clear cells seen in the normal skin were confirmed to be LCs by their characteristic ultrastructural features, *eg* Birbeck granules, prominent vimentin microfilaments and moderate numbers of lysosomes. The DCs in superficial dermis exhibited abundance of vimentin microfilaments but no Birbeck granules.

In the skin of *id* control fluid injected site, a moderate widening of the intercellular spaces between epidermal cells was only noted at 15 minutes *pi*. In the skin of the virus injected site, a cytopathic change was noted in addition to the edematous change in some of the epidermal LCs and keratinocytes at 15 minutes *pi*. The LCs often exhibited cytopathy on the cell periphery, as the plasma membrane opposing the keratinocytes was hardly visible (Fig 3). Instead, a clear halo that almost surrounded the inner cytoplasmic organelles was seen. In the keratinocytes, a cytoplasmic clear zone located next to the nucleus and extending along the nuclear envelope for about half of the nuclear surface was occasionally seen. A veiled cell, characterized by its long cytoplasmic veils and abundant vimentin-microfilaments, was sometimes seen in the superficial dermal lymph vessel. By 2 hours *pi*, DCs in the superficial dermis consistently showed moderately to greatly distended rough endoplasmic reticulum (RER) cisternae filled with fibrillar proteinaceous elements (Fig 4, Inset A). Moreover, a few clusters of peculiar spherical particles of varying diameters were often observed in the perinuclear area. These clusters of peculiar spherical particles were confined within thin membrane bounded vesicles (Fig 4, Inset B).

TEM examination of the skin sheets that were not exposed or exposed to DEN-2 for 15 and 30 minutes revealed a cytopathic change in the epidermal LCs comparable to those of the *id* injected skin at the same periods *pi*. In the DCs, endosome-like vesicles with scanty, ill-defined contents could be occasionally seen. By 2 hours of viral exposure, substantial changes were noted in the cytoplasm of the superficial dermal DCs (Fig 5A). A few clusters of peculiar spherical particles (Fig 5B) similar to

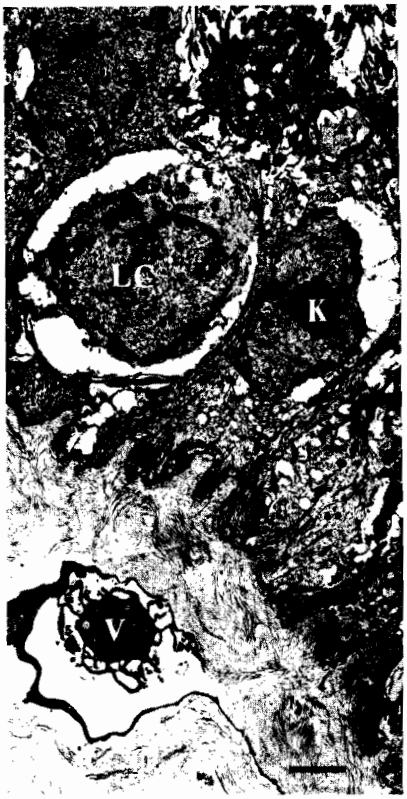

Fig 3–Electron micrograph of a dermo-epidermal zone in the cynomolgus monkey skin biopsy of DEN-2 virus *id* injected site, 15 minutes *pi*. The keratinocytes (K) show widening intercellular bridges and sometimes with a large and long cytoplasmic clear space next to the nucleus. The Langerhans cells (LC) exhibit cytopathic changes on the cell periphery with a clear space that almost surrounds the inner cytoplasm. The plasma membrane is hardly visible. Part of a veiled cell (V) with numerous thin and long surface membrane projections is seen within a lymphatic channel. Scale bar = 2 μm.

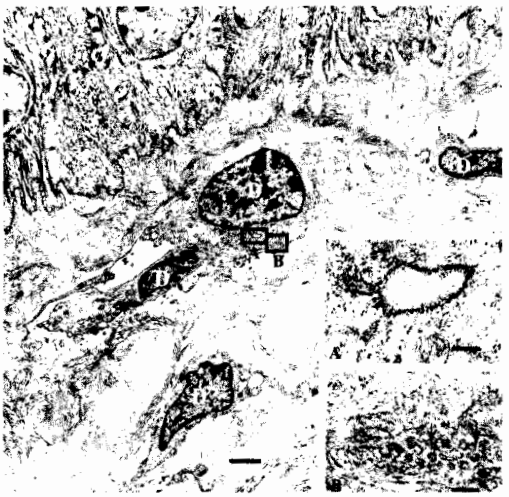

Fig 4–Electron micrograph of cynomolgus monkey skin biopsy of DEN-2 virus *id* injected site, 2 hours *pi*. Parts of four dendritic cells (D) are shown in the superficial dermis. They all exhibit similar features of migrating cells evident by the clear spaces generated in the migrating tract. Two types of cytoplasmic structures (A, B) consistently observed in these dendritic cells are shown at high magnification in inset A and B. Scale bar = 2 μm. Inset A depicts a greatly distended rough endoplasmic reticulum cisternae filled with fibrillar proteinaceous elements. Scale bar = 200 nm. Inset B depicts a cluster of DEN-2 virus-like particles (arrow) in thin single membrane vesicle. Scale bar = 200 nm.

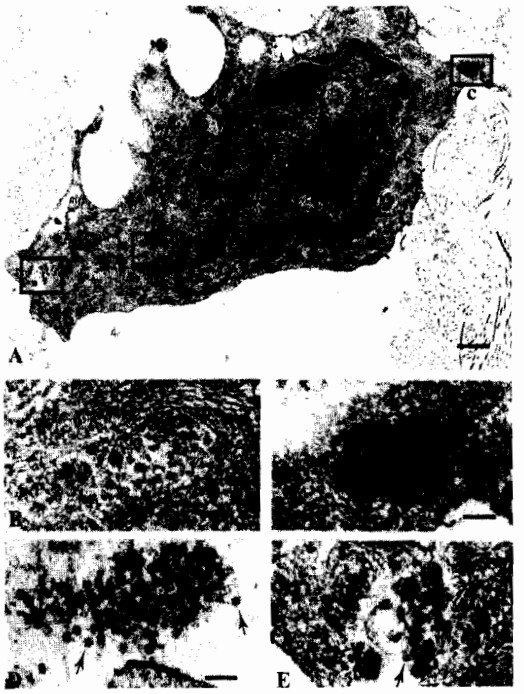

Fig 5–(A) Electron micrograph of a superficial dermal dendritic cell in skin sheet removed from cynomolgus monkey after *in vitro* exposure to DEN-2 virus for 2 hours showing three interesting cytoplasmic structures (b, c, e). They are shown in high magnification in B, C, and E. Scale bar = 500 nm. (B) A cluster of virus-like, peculiar particles (arrow) that resemble incomplete DEN-2 virus particles at early stages of virus assembly. Scale bar = 100 nm. (C) The aggregate of numerous electron dense virus-like particles (arrow) confined in the thin membrane bounded vesicle. Scale bar = 100 nm. (D) A large cluster of DEN-2 virions mixed with unidentified tiny particles that probably derived from the culture medium is lying in the extracellular milieu close to the cell membrane of a LLC-MK_2 cell in DEN-2-LLC-MK_2 culture. The virus is well-enveloped (arrow). Scale bar = 100 nm. (E) some proteinaceous particles of moderate electron density with no specific morphology (arrow) appear to be at a stage of partial degradation. Scale bar = 100 nm.

those observed in the *id* injected skin were observed in the perinuclear zone. At the same time, there were two types of endosomes present in these DCs. The first type contained aggregates of electron-dense, virus-like particles (Fig 5A in box c, and 5C) closely resembling the dengue virions in the DEN-2 infected LLC-MK_2 cell culture (Fig 5D). The second type of endosome was prevalent. It contained various proteinaceous structures of moderate electron density with no specific morphology which appeared to be at different stages of degradation (Fig 5E).

DISCUSSION

Serological evidence from the present study showed that *id* injection of the monkey with DEN-2 virus induced a more rapid and higher antibody response than did the *sc* route. This paralleled our previous observation in mice (Taweechaisupapong *et al*, 1996) showing that *id* immunization induced the best serological response compared to other

routes of administration. In the present study, the histologic and ultrastructural study on cellular events occurred in the *id* virus injected site shortly following the injection, and in the thin skin sheets following an *in vitro* viral challenge indicating that the epidermal LC in both specimens played its fundamental role in antigen processing in like manner. Its functional activity corresponded to physiological conditions and could be observed morphologically by the particular structural change.

The migrational profiles of the superficial dermal DCs, as were shown in Figs 1 and 2, and the presence of veiled cell in lymph vessel (Fig 3), supported the concept of migration of LC/DC from the antigenic challenge site towards the draining lymphatics, from where they further travelled to the draining lymph node, presenting the processed antigen fragments to the reactive lymphocytes, and triggering the effector phases of immune response.

According to the current knowledge of antigen processing, the antigen presenting cell degrades the antigen by the endocytic pathway following endocytosis of exogenous antigen (Neefjes and Ploegh, 1992). The peptide fragments of the antigen associate with the newly synthesized major histocompatibility complex (MHC) class II molecules (Davidson *et al*, 1991). The peptide: MHC class II complex takes 1-3 hours to traverse the endocytic route and appears at the cell surface for recognition by T-helper cells. Our consistent observation of greatly distended RER cisternae in the superficial dermal DCs of virus injected skin 2 hours *pi* (Fig 4, inset A) is in accordance with an increase in assembly of MHC class II molecules in the RER cisternae after endocytosis of antigen. The RER cisternal contents indicated the DC possessed the newly synthesized pool of MHC class II molecules. However, there were few such DCs seen in the skin sheets. Instead, by 2 hours of viral exposure, DCs in the skin sheets often showed two types of endosomes distinguished by their proteinaceous contents (Fig 5). The first type of endosome as was shown in Fig 5C was likely to represent an early endosome. The aggregated virus-like particles in the endosome presented here was similar to those large aggregates of West Nile virus particles demonstrated in the endocytic compartments of the macrophage-like cell line by Gollins and Porterfield (1985). Some degree of proteolysis may have just occurred in the early endosome, as the endocytosed electron-dense particles still bore resemblances to the dengue virions that were present in the DEN-2 infected LLC-MK_2 cell culture (Fig 5D). The second type of endosome was shown in Fig 5E, and it logically represented a late endosome where a more advanced proteolysis occurred. On the other hand, the progressive antigen degradation readily occurred *in vivo* resulting in the absence of these two types of endosomes in the virus-injected skin.

The fate of the degraded DEN-2 virus particles – the proteolysis-resistant, nucleic acid-rich components of the virus – in the LC/DC was not known. It would be interesting to know if they were retained in any of the "late" or "very late" endosomes, or if they were pooled in any particular cytoplasmic structure in the professional antigen-presenting cell by 2 hours of viral challenge. However, there has been no reported study on the fate and localization of the viral nucleic acid-rich components in the LC/DC. The present study appears to be the first to observe the perinuclear clusters of peculiar spherical particles in the DCs following viral challenges *in vivo* and *in vitro* (Figs 4 inset b, and 5B). Considering the particle size and morphology, the arrangement of regularly spaced particles that were clustered in the thin membrane-bounded vesicle and their chronological appearance, it is worthy to note their resemblance to the incomplete dengue virus particles seen at early stages of viral assembly within the RER cisternae which was described and illustrated by Sriurairatana *et al* (1978). The particles in clusters could be the viral nucleic acid-rich, proteolysis-resistant components of dengue virions – the partially degraded nucleocapsids, observed at a stage when the viral peptide: MHC class II complexes had left the endocytic compartments. This speculation can be further verified by in situ hybridization at ultrastructural level.

In the present study, ultrastructural findings, together with the rapid production of neutralizing antibody following *id* injection of DEN-2 virus, strongly indicate the effective role of LC as a potent antigen-presenting cell. Our evidence supports the current knowledge of the endocytic pathway and cellular antigen processing by the LC. The functional activity of the epidermal LC suggests potential development of a topical vaccine on the basis of effectiveness of LC activity in presenting the antigen required for provoking a good antibody response.

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