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# Natural Killer Cells in Dengue Hemorrhagic Fever/Dengue Shock Syndrome

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**SUMMARY** **Natural killer (NK) cell activity against K-562 target cells and HNK-1$^+$ cell levels were serially determined in peripheral blood of 62 Thai children with dengue hemorrhagic fever/dengue shock syndrome aged 4-12 years and 59 age-matched normal controls. The studies were performed on febrile stage, 1st and 2nd day of subsidence of fever (shock stage), 3rd and 4th day of subsidence of fever (early convalescent stage) and once again on the late convalescent stage (approximately 14-18 days after subsidence of fever).**

**The study revealed that during the course of disease the NK cell activity was not changed significantly from the normal controls. In contrast, the levels of HNK-1$^+$ cells, which exhibited almost all NK and killer cell functional activities, were significantly decreased in the febrile and the shock stages and were normal in the early and late convalescent stages. The NK cell activity, on the per-cell basis, was significantly increased in the early disease stage when compared to that of the later period of the disease and of the normal controls. The study also revealed that patients with grade III of disease severity exhibited significantly more NK cell functional activities per cell than grade II on febrile stage and the first day of shock. These results suggest that natural killer cells were active in defense against dengue viral infection and might play some role in the pathogenesis of dengue hemorrhagic fever/dengue shock syndrome. Their functions might also determine the severity of the disease.**

Despite extensive scientific research in many aspects of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) in the past decade, DHF/DSS still remains one of the major public health problems in most of the countries in the South-East Asian and Western Pacific Regions.[1] A conceptual understanding at the cellular level of the pathogenesis of DHF/DSS is also far from complete.

The major pathophysiological hallmarks in DHF/DSS are shock and excessive hemorrhage, which at times are fatal complications of dengue viral infection. Previous epidemiological studies convincingly revealed that these complications were commonly observed in individuals with preexisting antibodies either actively or passively acquired.[2] These observations suggest that the immune response of the host may play an important role in the severe complications of dengue infection. Since the immune status of the host may determine the severity of dengue viral infection, it is worthwhile to gain an insight into the mechanism of *in vivo* immunological sensitization and especially of elimination of dengue virus-infected cells so that better treatment and ultimate prevention of this disease can be achieved.

Various cell-mediated immune mechanisms, including specific cytotoxic T cells, K cells mediating antibody dependent cell-mediated cytotoxicity (ADCC), natural killer (NK) cells and macrophages, while perhaps not effective in preventing viral infection, have been found to be effective for the lysis of virus-infected cells *in vitro* and may aid in eliminating viral infection *in vivo*. Evi-

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dence is accumulating for the role of NK cells in resistance against certain infectious agents as well as neoplastic cells. However, little is known of the role, if any, these NK cells play in eliminating dengue viral infection *in vivo*. Recently, Kurane *et al.* [3] were the first to demonstrate significantly increased lysis of dengue-infected Raji cells *in vitro* by peripheral blood mononuclear cells obtained from healthy adult donors in the NK cell cytotoxicity assay. However, many questions still remain to be answered as to the *in vivo* role of NK cells in defense against natural dengue viral infection and, more interestingly, the involvement, if any, of these cells in the pathogenesis of DHF/DSS. In the present study, some of these questions were addressed indirectly by serially determining the changes in both the level and the functional activity of NK cells against K-562 target cells during the course of dengue viral infection in Thai children with DHF/DSS.

## MATERIALS AND METHODS

### Study population

Sixty-two Thai children with DHF/DSS aged 4-12 years who were admitted to the Department of Pediatrics, Siriraj Hospital, Bangkok, during the years 1986-1987 were included in this study. All patients were clinically diagnosed according to the criteria of Nimmannitya *et al.* [4] and serologically confirmed by hemagglutination inhibition test. There were 36 patients with grade II (non-shock) and 26 patients with grade III (shock) of the disease severity as classified by Nimmanitya *et al.* [4]

The studies with the patients were performed on febrile stage (F), first and second day of subsidence of fever or shock stage (S1 and S3, respectively), third and fourth day after subsidence of fever or early convalescent stage (C1 and C2, respectively) and once on day 14-18 after subsidence of fever or late convalescent stage (C3).

The control group consisted of fifty-nine age matched (3-13 years) normal individuals (33 males and 26 females) who were relatives of the studied patients. The studies with this group were performed only once and were done simultaneously with the patients.

### Preparation of peripheral blood mononuclear cells (PBMC)

Blood samples from patients with DHF/DSS and healthy control subjects were collected by venipuncture into heparinized (10 IU/ml of blood) tubes. PBMC were separated by the Ficoll-Hypaque density gradient centrifugation method, [5] washed 3 times with RPMI 1640 medium supplemented with 10% heated-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptromycin (100 $\mu$g/ml), 2 mM L-glutamine and 20 mM HEPES, hereafter referred to as "complete medium".

In order to remove monocytes and some contaminating granulocytes, the method described by Kumagai *et al.* [6] was followed. Briefly, 5-10 $\times$ $10^6$ PBMC in 5 ml of complete medium were introduced into 100 $\times$ 15 mm plastic dishes (Falcon; Becton Dickinson and Co, Lincoln Park, NJ) incubated at 37° C, in a humidified $CO_2$ incubator for 1 hr to allow monocytes and granulocytes to attach to the plastic surface. After incubation, non-adherent cells were harvested, washed twice, counted and adjusted to the desired cell concentration in complete medium and used in immunofluorescent assay and as "effector cells" in the cytotoxicity assay.

### Target cells

The erythroleukemic cell line K-562, originally established by Lozzio and Lozzio [7] from the pleural effusion of a patient with chronic myeloid leukemia in terminal blast crisis, was used as the target cells in this study. A suspension of the K-562 cell line was continuously cultured in complete medium and cells cultured for 48 hours were used as target cells in the cytotoxicity assay.

### NK cell cytotoxicity assay

The NK cell functional activity was determined by a $^{51}$Cr-release assay using $^{51}$Cr-labelled K-562 as target cells at an optimum single effector : target (E:T) ratio of 40:1. In brief, 5 $\times$ $10^5$ K-562 cells were labelled with 50 $\mu$Ci of $^{51}$Cr [sodium chromate ($^{51}$Cr) solution B.P. 250-500 $\mu$Ci/$\mu$g Cr, code CJP.1P, Amersham, Buckinghamshine, United Kingdom] for 1 hr at 37° C, 5% $CO_2$ with occasional mixing. The cells were then washed 3 times in RPMI 1640 supplemented with 5% FBS to remove unincorporated $^{51}$Cr, counted and adjusted to a final concentration of 2 $\times$ $10^5$ viable cells/ml in completed medium. The viability of cells as determined by trypan blue dye exclusion test was always more than 95%. Fifty microliters of $^{51}$Cr-labelled K-562 target cells (1 $\times$ $10^4$ cells) were mixed with 200 $\mu$l of nonadherent effector cells (4 $\times$ $10^5$ cells) in 12 $\times$ 75 mm round-bottomed plastic tubes. Two controls were included in the assays. Control tubes for background (spontaneous) $^{51}$Cr-release received 50 $\mu$l of labelled target cells and 200 $\mu$l of complete medium. Control tubes for total $^{51}$Cr-release received 50 $\mu$l of labelled target cells and 200 $\mu$l of 1.0% Triton X-100. Both the experimental and the control tubes were set up in triplicate. The tubes, after being well mixed, were centrifuged at 400 $\times$ g for 2 min and placed in a 37° C incubator with an automatic supply of 5% $CO_2$ for 4 hr. After incubation, the tubes were placed in a freezing chamber (-20° C) to stop the reaction, and then 100 $\mu$l of phosphate-buffered saline (PBS pH 7.2) was added into each tube. The tubes were again centrifuged for 5 min at 400 $\times$ g and 200 $\mu$l samples of supernatant were carefully removed for counting in a gamma counter.

The counts per minutes (cpm)

for the triplicates were averaged and the percent specific cytotoxicity was calculated using the following formula :

$$\% \text{ Specific cytotoxicity} = \frac{(E\text{-}S)}{(T\text{-}S)} \times 100$$

Where E = experimental release (cpm), S = spontaneous release (cpm) and T = total release (cpm). The spontaneous $^{51}Cr$ release was always less than 8% of the total release.

The % cytotoxicity per cell for each sample was then calculated as follows :

$$\% \text{ cytotoxicity per cell} = \frac{C \times 100}{A \times H}$$

Where C = % cytotoxicity, A = actual number of effector cells used ($4 \times 10^5$ cells) and H = % HNK-1$^+$ cells.

### Immunofluorescence assay

Lymphocytes expressing the HNK-1 antigen on their cell surface (HNK-1$^+$ cells) were enumerated by an indirect immunofluorescent method as described by Abo *et al.*[8] A monoclonal IgM antibody, HNK-1 (Leu-7) (a generous gift from Dr. Max D. Cooper of the Tumour Institute, University of Alabama at Birmingham, AL, USA) was used as the first antibody. The second antibody was a fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgM (Southern Biotechnology Associated Inc., Birmingham, AL, USA). Briefly, $1 \times 10^6$ washed nonadherent mononuclear cells were reacted with HNK-1 mAb in a $6 \times 50$ mm glass tube. After incubation at 4° C for 30 min, the cells were washed twice with PBS and reacted with the second antibody for 30 min at 4° C. After washing, a drop of mounting media (30% glycerol in PBS) was added to resuspend the cell pellet and 10 $\mu$l of the completely resuspended cells were placed on a clean glass slide which was then covered with a glass cover slip, sealed with nail enamel and examined under the fluorescence microscope (Ortholux II, Ernst Leitz Wetzlar GMBS, Wetzlar, West Germany). At least 200 total lymphocytes were counted and the percentage and absolute number of HNK-1$^+$ cells were calculated as follows :

$$\% \text{ HNK-1}^+ \text{ cells} = \frac{\text{No of fluorescing cells} \times 100}{\text{Total lymphocytes counted}}$$

$$\text{Absolute number of HNK-1}^+ \text{ cells} = \frac{W \times H \times L}{10{,}000}$$

Where W = white blood count (cells/mm$^3$), H = %HNK-1$^+$ cells and L = % lymphocytes from Wright's stained smear.

### Statistical analysis

Data were assessed by the Kolmogorov-Smirmov One-Sample Test. Nonparametric data were analysed by the Mann-Whitney U test. *P* values of less than 0.05 (two tailed) were considered significant.

## RESULTS

### HNK-1$^+$ cell levels and NK cell activities of normal controls

The HNK-1$^+$ cell levels and NK cell activities of 59 normal controls are shown in Table 1. These values between males and females showed no significant gender difference. Hence, a mean value obtained from these 59 children was used as a normal value for studied patients.

### HNK-1$^+$ cell levels in patients with DHF/DSS

When compared with the normal controls, the patients demonstrated significantly decreased absolute numbers of HNK-1$^+$ cell levels in febrile (F) and shock stage (S1 and S3), but showed no significant difference in the early (C1, C2) and late convalescent stages (C3) (Fig. 1, Table 3).

When the patients were subdivided according to disease severity into grade II and grade III, the patients with grade III of severity showed lower HNK-1$^+$ cell levels in febrile stage when compared with the patients with grade II (Table 2).

**Table 1.** HNK−1$^+$ cell levels and NK cell activity in peripheral blood of healthy male and female children.

| | Males + Females | Males | Females | *P*−value[b] |
|---|---|---|---|---|
| Number | 59 | 33 | 26 | – |
| Age range (mean) | 3–13 (8.6) | 4–12 (8.1) | 3–13 (9.3) | – |
| % HNK−1$^+$ cell | 14.3 ± 4.7[a] | 14.9 ± 5.3 | 13.5 ± 3.9 | NS[c] |
| Absolute HNK−1$^+$ (cells/mm$^3$) | 526.4 ± 231.3 | 549.8 ± 226.8 | 496.6 ± 242.3 | NS |
| % Cytotoxicity | 56.69 ± 11.42 | 57.71 ± 10.82 | 55.39 ± 12,44 | NS |

a : Mean ± standard deviation
b : Mann–Whitney U test was used to test the two independent samples (males and females)
c : NS = Not significant ($P > 0.05$)

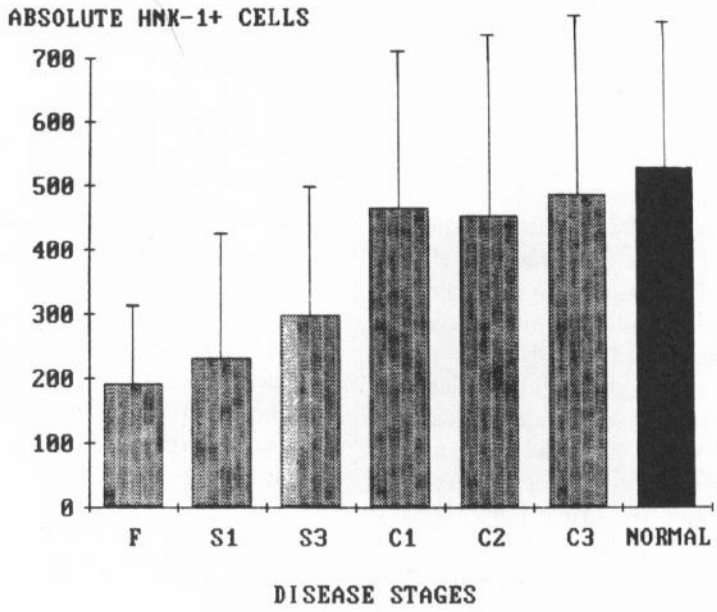


Fig. 1 Absolute numbers of HNK−1$^+$ cells per mm$^3$ at various stages of illness in 62 patients with DHF/DSS compared with the normal controls.

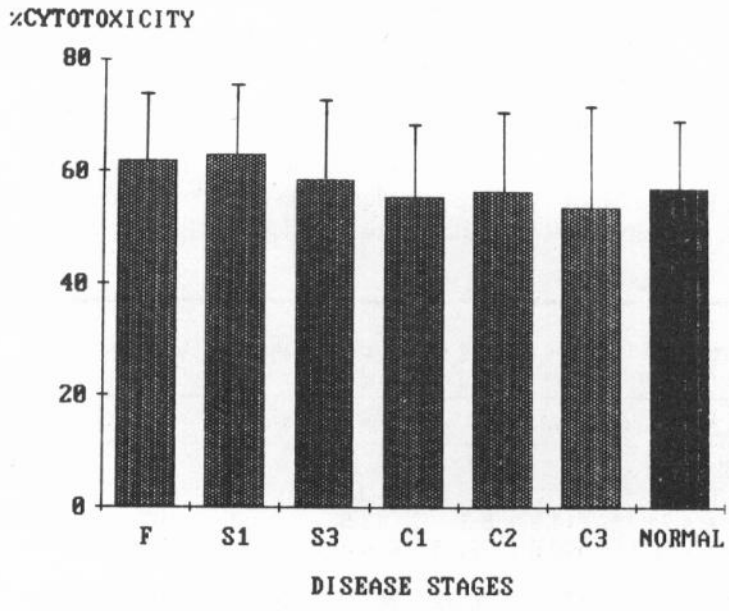


Fig. 2 The percentage of NK cell cytotoxic activity (at E : T = 40 : 1) at various stages of illness in 62 patients with DHF/DSS compared with the normal controls.

### NK cell functional activities in patients with DHF/DSS

The NK cell activity against K-562 target cells was assessed in parallel with HNK-1$^+$ cell enumeration. As shown in Fig. 2, the level of NK cell activity was higher in the early disease stages (F, S1 and S3) than that in the late disease stages (C1, C2 and C3) and the normal controls. However, the differences were not statistically significant. These results, however, were in contrast with those of the HNK-1$^+$ cell levels (Fig. 1). Thus, when the NK cell activity on a per-cell basis was considered, it was found that the activity on a per-cell basis was significantly increased in the early disease stages with a peak in the day of shock (Fig. 3, Table 3).

The patients with grade II and grade III of disease severity were found to exhibit comparable NK cell activity in all but the febrile stage which is higher in grade III. When the NK cell activity per-cell was considered, the patients with grade III showed more NK cell activity significantly on febrile stage and the first day of shock (Table 2).

## DISCUSSION

Most studies have shown that NK cells which lyse virus-infected cells have the same phenotypic and morphologic characteristics of those active against K-562 cells.[9,10] However, heterogeneity of NK cells has also been reported in human systems.[11] Fitzgerald *et al.* reported that the human NK cells lysing K-562 and herpes simplex virus type 1 (HSV-1)-infected target cells had somewhat different characteristics, although both cell types appeared within the large granular lymphocyte population.[12] Kurane *et al.* also showed that some of the effector cells responsible for lysis of dengue-infected cells were phenotypically different from those responsible for lysis of K-562 cells.[3,11] The

**Table 2.** The HNK−1$^+$ cell levels, % NK cell cytotoxicity and % cytotoxicity per 1 HNK−1$^+$ cell at various stages of illness in DHF/DSS patients with grade II and grade III of disease severity.

| Disease stages | % NK−1$^+$ cells | | | Absolute HNK−1$^+$ cells (cells/mm$^3$) | | | % Cytotoxicity | | | % Cytotoxicity per 1 HNK−1$^+$ cell (x $10^{-4}$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Grade II | Grade III | *P*–value[c] | Grade II | Grade III | *P*–value | Grade II | Grade II | *P*–value | Grade II | Grade III | *P*–value |
| F | 9.7 ± 3.8[a] (n = 13)[b] | 10.4 ± 5.1 (n = 2) | NS[d] | 207.4 ± 123.4 | 87.6 ± 13.3 | $P < 0.05$ | 59.21 ± 11.69 | 76.76 ± 0.4 | $P < 0.05$ | 17.8 ± 7.2 | 23.9 ± 11.5 | $P < 0.05$ |
| S1 | 9.5 ± 5.6 (n = 18) | 7.2 ± 3.6 (n = 18) | NS | 252.2 ± 226.8 | 209.9 ± 183.9 | NS | 65.70 ± 10.75 | 59.51 ± 12.64 | NS | 20.9 ± 7.9 | 24.0 ± 9.1 | $P < 0.05$ |
| S3 | 8.9 ± 2.7 (n = 20) | 8.6 ± 4.0 (n = 12) | NS | 330.6 ± 169.7 | 244.0 ± 219.0 | NS | 58.72 ± 13.73 | 57.59 ± 12.73 | NS | 17.7 ± 5.1 | 19.8 ± 7.9 | NS |
| C1 | 9.5 ± 2.4 (n = 17) | 10.4 ± 4.3 (n = 17) | NS | 467.5 ± 210.4 | 459.2 ± 274.1 | NS | 53.82 ± 11.79 | 56.60 ± 11.39 | NS | 14.7 ± 3.6 | 14.6 ± 3.5 | NS |
| C2 | 10.7 ± 3.1 (n = 23) | 11.3 ± 4.2 (n = 13) | NS | 481.1 ± 233.6 | 397.1 ± 310.7 | NS | 57.36 ± 12.97 | 54.18 ± 12.88 | NS | 14.2 ± 4.5 | 13.1 ± 4.3 | NS |
| C3 | 14.3 ± 4.3 (n = 36) | 13.7 ± 3.3 (n = 26) | NS | 509.2 ± 270.5 | 454.4 ± 226.0 | NS | 53.09 ± 14.41 | 53.91 ± 15.33 | NS | 9.8 ± 3.3 | 10.1 ± 3.2 | NS |

a : Mean ± standard deviation
b : Number of cases
c : Mann–Whitney U test was used to test the two independent samples (grade II and grade III values)
d : NS = Not significant ($P > 0.05$)

**Table 3.** The HNK–1$^+$ cell levels, % NK cell cytotoxicity and % cytotoxicity per 1 HNK–1$^+$ cell at various stages of illness in 62 DHF/DSS patients and 59 normal controls.

| Disease stages | % HNK–1$^+$ cells | *P*–value[c] | Absolute HNK–1$^+$ cells (cells/mm$^3$) | *P*–value | % Cytotoxicity | *P*–value | % Cytotoxicity per 1 HNK–1$^+$ cell (x $10^{-4}$)[b] | *P*–value |
|---|---|---|---|---|---|---|---|---|
| F | 9.8 ± 4.0[a] | $P < 0.05$ | 191.5 ± 122.0 | $P < 0.05$ | 61.6 ± 12.4 | NS | 18.6 ± 8.2 | $P < 0.05$ |
| S1 | 8.4 ± 4.8 | $P < 0.05$ | 231.1 ± 207.5 | $P < 0.05$ | 62.6 ± 12.1 | NS | 22.5 ± 8.6 | $P < 0.05$ |
| S3 | 8.8 ± 3.3 | $P < 0.05$ | 298.1 ± 194.3 | $P < 0.05$ | 58.3 ± 13.4 | NS | 18.5 ± 6.4 | $P < 0.05$ |
| C1 | 10.0 ± 3.5 | $P < 0.05$ | 463.3 ± 244.4 | NS | 55.2 ± 11.7 | NS | 14.6 ± 3.6 | NS |
| C2 | 10.9 ± 3.5 | $P < 0.05$ | 477.0 ± 268.8 | NS | 56.2 ± 13.0 | NS | 13.8 ± 4.5 | NS |
| C3 | 14.0 ± 4.0 | NS[d] | 486.2 ± 254.2 | NS | 53.5 ± 14.9 | NS | 10.0 ± 3.5 | NS |
| Normal controls | 14.3 ± 4.7 | – | 526.4 ± 231.3 | – | 56.7 ± 11.4 | – | 10.2 ± 2.3 | – |

a : Mean ± standard deviation
b : Average of calculated values for individual samples
c : Mann–Whitney U test was used to test the values at each disease stage with the normal controls
d : NS = Not significant ($P > 0.05$)

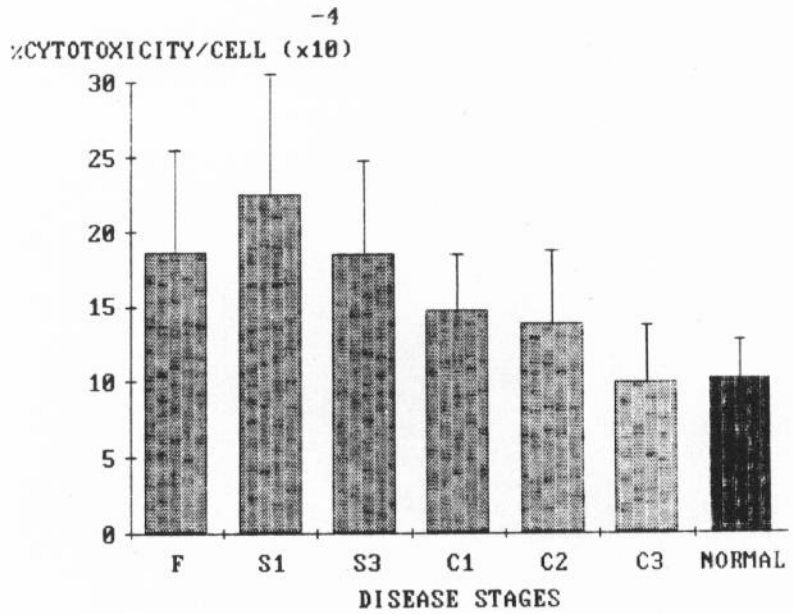


**Fig. 3** The percentage of NK cell cytotoxic activity on a per–cell basis at various stages of illness in 62 patients with DHF/DSS compared with the normal controls.

present study has demonstrated in febrile and shock stages of the patients with DHF/DSS significant increment in the per cell activity of HNK-1$^+$ cells, previously shown to exhibit almost all NK and killer (K) cell functional activities.[8] Hence, further studies are needed to demonstrate the function of other NK cell phenotypes in this disease for confirmation of such previous findings. This study also revealed that NK cell activity per 1 HNK-1$^+$ cell was higher on febrile stage and the first day of shock in grade III patients when compared with those in grade II, which suggests that NK cell function might also determine the severity of disease.

The role of NK cells in resistance against and recovery from viral infection *in vivo* has been demonstrated in experimental rats. Infection with viruses induces interferon (IFN) synthesis and it has been shown that IFN enhances NK cell activity.[13] It has also been reported that NK cell activity is raised during infections with several viruses in experimental animals[14] and man.[15] In the present study, we did not find an overall significant increase in NK cell activity, however, a slightly increase in NK cell activity in the early disease stage was noted. Possible explanations for this finding are: 1) all studied patients were admitted to the hospital rather late after the onset of fever, *i.e.*, day 4 or later. Hence, the NK cell activity on the febrile stage represented the activity at day 4 or later. Had we been able to examine the NK cell activity earlier, *i.e.*, day 1-3 after the onset of fever, we might have found a significant increase in NK cell activity. In this regard,

available data indicated that NK cells peak early in the first few days following viral infection.[16] Nevertheless, it is necessary to determine the levels of NK cells in individuals before they get viral infection and make correlations between these pre-existing NK cell activities and severity of infection if the important role of NK cells in viral infections *in vivo* is to be established. 2) There is a probability that significant NK cell activity may occur locally at the site of dengue infection and local activity may be reflected poorly or not at all by the examination of circulating cells in the peripheral blood. High levels of NK cell activity have been reported to occur in organs that are the sites of virus replication.[17,18] Attraction of NK cells to sites of infection is likely to be at least partially due to chemotaxis, which has recently been demonstrated for both human and rat NK cells.[19,20] From the pathogenic studies in the Rhesus monkey,[21] it was found that, following subcutaneous inoculation of dengue virus in the forearm, the virus replicated in histiocytes at the skin inoculation site, then in macrophages in regional lymph nodes, next in Küpffer cells in the liver and cells with a macrophage morphology in spleen, lung and Peyer's patches. At the end of the viremic period, however, the dengue-infected monocytes passed regularly through the blood. Thus, NK cell activity may be increased primarily in these infected organs while in the peripheral blood the activity was still unchanged. Not until the dengue-infected monocytes are found in the peripheral blood can the circulating NK cells be activated to efficiently kill the infected cells. In this regard, dengue virus has been successfully recovered from the peripheral blood monocytes collected at the time of shock or defervescence from large number of DHF/DSS patients.[22] Interestingly, it was found in the present study that the NK cell activity on a per-cell basis was significantly increased and peaked on the first day of shock. At present, it is not definitely known about the role of NK cells in the lysis of dengue-infected human monocytes. Monocytes, one of the sources of replicating dengue virus in humans, are hypothesized to release various mediators leading to dengue shock syndrome.[23] Thus, the results of this study suggest the possibility that NK cells play a role in the pathogenesis of DHF/DSS by killing the infected monocytes which cause the release of such vasoactive mediators.

Based on the mouse work,[14] it can be predicted that human dengue viral infections should lead to an increase in NK cell numbers either in the peripheral blood or at the site of viral infection. However, in the present study, we found a significant decrease in HNK-1$^+$ cell levels in the peripheral blood at the early disease state. A likely explanation for this finding is that during an acute infection these cells were attracted by some chemotaxis to the dengue-infected organs, thus, causing a relative reduction of the level of these cells in the peripheral blood. However it is possible that, at this period NK cells were activated by interferons or lymphokines to increased cytotoxic activity, as evidenced by an increase in NK cell cytotoxicity on a per-cell basis. It is also suggested that NK cells at the convalescent period, though present in higher levels, are functionally less active than those at the acute period.

This is the first report to demonstrate the NK cell functional activity and levels at various stages of dengue viral infection. Though a definite conclusion as to the role of NK cells in DHF/DSS can not be made, it is hoped that future research will use the findings in this study as a guideline for further improving our understanding of the role of NK cells in defense against dengue viral infection in general and of the involvement, if any, of NK cells in the pathogenesis of DHF/DSS.

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