A MODEL TO STUDY CYTOKINE PROFILES IN PRIMARY AND HETEROLOGOUSLY SECONDARY DENGUE-2 VIRUS INFECTIONS

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Summary. – Roles of cytokines in primary and secondary Dengue virus (DV) infections are not completely understood. In this study, we challenged mononuclear leukocytes (MNLs) obtained from non-immune and DV-1-infected donors with DV-2 *in vitro* to mimic primary and heterologously secondary DV-2 infections, respectively. We found that MNLs in response to DV-2 could release a large amount of interleukin-1 (IL-1β) and tumor necrosis factor alpha (TNFα). However, IL-1β and TNFα released by MNLs with primary or heterologously secondary exposure to DV-2 were not significantly different. In contrast, MNLs with heterologously secondary DV-2 infections produced significantly higher amount of interferon gamma (IFNγ) than those with primary DV-2 infections. These results suggest that IFNγ, but not TNFα or IL-1β, may in part participate in the pathogenesis of Dengue shock syndrome (DSS) frequently found in heterologously secondary DV-2 infections.

Key words: cytokines; interferon gamma; Dengue shock syndrome; heterologously secondary infections

Introduction

Primary Dengue fever is usually recognized as a benign viral illness. Secondary DV infections with heterotypic Dengue viruses are more frequently associated with serious complications such as DSS, which tends to have a higher mortality (Halstead, 1989; Sangkawibha *et al.*, 1984). Although Dengue fever and its severe form complication, DSS, are prevalent mainly in Southeast Asia and South America, people from Europe or North America who travel to tropical countries may also have an opportunity to get this disease. Data from epidemiologic studies have indicated that the highest risk factor for DSS is an infection with the sequence of DV-1 followed by DV-2 infection (Halstead, 1981; Sangkawibha *et al.*, 1984). The pathogenesis of DSS is not completely understood. Circulating immune complexes had been implicated in the activation of complement (C'), resulting in the disturbance of coagulation and vascular permeability in patients with Dengue fever (Suvatte and Longsaman, 1979; Suvatte, 1987). Immune complexes as assessed by quantitative methods have been shown to be not the major factor in the C' activation of Dengue fever (Malasit, 1987).

MNLs are the principal cells for DV replication (Theofilopoulos *et al.*, 1976; Halstead, 1989) and are known to secrete many proinflammatory cytokines such as TNFα and IL-1β involved in many infectious shock syndromes (Demetri *et al.*, 1989; Fong *et al.*, 1990; Tracey *et al.*, 1988). Furthermore, IFNγ has been reported to augment Fc gamma (Fcγ) receptor-mediated DV infections in monocytic cells (Kontony *et al.*, 1988). It is reasonable to propose that a different cytokine profile may contribute to a different risk incidence of DSS between primary and heterologously secondary DV-2 infections. It has been virologicaly confirmed that an outbreak of DV-1 infections took place in southern Taiwan from 1987 to 1991 (Liu *et al.*, 1989; Shaio *et al.*, 1992; Wu *et al.*, 1993). Thus, it is our privilege to challenge MNLs obtained from normal (non-immune) and previously DV-1-infected donors with DV-2 to mimic primary and heterologously secondary DV-2 infections and to study the cytokine profiles in the *in vitro* DV-2 infections. Employing ELISA, we measured cytokine releasing profiles including

IL-1β, TNFα, IL-2, and IFNγ from MNLs with primary or heterologously secondary exposure to DV-2.

Materials and Methods

Preparation of MNLs was done from peripheral blood drawn from 11 non-immune normal adults living in northern Taiwan and 11 previously DV-1-infected patients who lived in southern Taiwan and were virologically confirmed to have DV-1 infections during 1987-1989 in our previous study (Shaio *et al.*, 1992). Informed consents were completed before blood was drawn. 4.5% dextran T500 (Pharmacia) was mixed with the peripheral blood at a ratio of 1:4 and the syringe was incubated nozzle upward at 37 °C for 30 mins. Leukocyte-enriched plasma was separated into polymorphonuclear leukocytes and MNLs by a gradient centrifugation in Ficoll-hypaque (Pharmacia) at 1,500 x g for 15 mins (Yang *et al.*, 1992, 1994). After centrifugation, interface MNLs (> 98% viability) were collected, washed, and suspended at 2 x 10^6 cells/ml for studies.

Virus. Dengue 2 virus (DV-2), New Guinea C strain obtained from the Institute of Preventive Medicine, Taipei, Taiwan was used in this study. Viruses were propagated in the *Aedes albopictus* C6-36 cells (Igarashi, 1978). Virus titers were determined by a standard plaque assay on BHK-21 cells as described previously (Morens *et al.*, 1985; Shaio *et al.*, 1994). Viruses were pooled and concentrated to 2 x 10^7 PFU/ml in RPMI 1640 medium with 10% foetal calf serum (FCS) pH 7.4, and stored at -70 °C before use.

Experimental design and measurement of cytokines production. MNLs (2 x 10^6 cells/ml) obtained from non-immune and DV-1-infected donors were incubated in 24-well culture plates. Experiments were run in a paired control-experiment design in duplicates. MNLs obtained from non-immune donors were challenged with DV-2 at the multiplicity of 1 PFU/cell to mimic primary DV-2 infections; in contrast, MNLs obtained from the donors with a previous DV-1 infection during 1987 and 1989 (Shaio *et al.*, 1992) were challenged with DV-2 at the same multiplicity to mimic heterologously secondary DV-2 infections. Both groups of MNLs were also incubated with medium alone as negative controls. Culture supernatants were harvested at 16 and 72 hrs post infection (p.i.). The samples were aliquoted and stored at -70 °C until assayed. Supernatants harvested from 16 hrs incubation were tested for TNFα and IL-1β using ELISA kits purchased from Cistron Biotechnology (Pine Brook, NJ). Supernatants harvested from 72 hrs incubation were measured for IL-2 and IFNγ by ELISA kits obtained from Genzyme Biotechnology (Cambridge, MA).

Results

MNLs obtained from non-immune donors (non-immune MNLs) or from previously DV-1-infected donors (DV-1-infected MNLs) cultured alone in medium did not release significant amount of TNFα (24 to 79 pg/ml) or IL-1β (0.0 to 5.3 pg/ml). Non-immune MNLs as well as DV-1-infected MNLs in response to DV-2 for 16 hrs released significant amount of TNFα and IL-1β, as shown in Table 1. However, DV-1-infected MNLs in response to DV-2 did not produce higher amount of TNFα or IL-1β than non-immune MNLs (t test, $p > 0.05$; Table 1).

Table 1. Production of TNFα and IL-1β by MNLs with primary and heterologously secondary exposure to DV-2

Reactions	TNFα (pg/ml)[c]	IL-1β (pg/ml)[c]
Non-immune MNLs		
+RPMI[a]	48 ± 25	2.0 ± 3.5
+DV-2[b]	1059 ± 218	756 ± 327
DV-1-infected MNLs		
+RPMI[a]	54 ± 38	2.8 ± 4.0
+DV-2[b]	829 ± 251	766 ± 112

RPMI – RPMI 1640 medium with 10% FCS, pH 7.4.
[a]Spontaneous production of IL-1β and TNFα by DV-1 infected MNLs was not significantly different from that by non-immune MNLs (t test; $p > 0.05$; n=11). [b]Production of IL-1β and TNFα by MNLs was not significant different between primary and heterologously secondary infections with DV-2 (t test; $p > 0.05$; n=11). [c]Mean ± SD.

Table 2. IFNγ production by MNLs with primary or heterologously secondary exposure to DV-2

Reactions	Non-immune MNLs + RPMI	Non-immune MNLs + DV-2	DV-1-infected MNLs + RPMI	DV-1-infected MNLs + DV-2
IFNγ (pg/ml)	0.0	31.9	0.0	124.1*
(SD)	(0.0)	(26.9)	(0.0)	(37.7)

RPMI – RPMI 1640 medium with 10% FCS, pH 7.4. *Production of IFNγ by MNLs obtained from previously DV-1-infected donors was significantly higher than that by those obtained from non-immune donors (t test; $p < 0.05$; n=11).

Experiments were next performed to determine the production of IL-2 and IFNγ by non-immune and DV-1-infected MNLs exposed to DV-2 for 3 days. As shown in Table 2, non-immune MNLs in response to DV-2 released low levels of IFNγ (31.9 ± 26.9 pg/ml). In contrast, DV-1-infected MNLs in response to DV-2 released significantly higher amount of IFNγ (124.1 ± 37.7 pg/ml). Both groups of MNLs, however, released undetectable IL-2, no matter whether infected with DV-2 or not.

Discussion

Proinflammatory cytokines such as TNFα and IL-1β have been implicated in the pathogenesis of shock-related syn-

dromes including septic shock (Fong and Lowry, 1990; Tracey *et al.*, 1988). It remains uncertain whether cytokines are also involved in DSS, which is more frequently associated with an infection with DV-1 followed by another sequential DV-2 infection (Sangkawibha *et al.*, 1984). In this study, we exposed MNLs obtained from normal and previously DV-1-infected donors to DV-2 *in vitro* to mimic primary and heterologously secondary DV-2 infections, respectively. The fact that production of TNFα and IL-1β by MNLs from previously DV-1-infected donors in response to DV-2 was not significantly higher than that from non-immune donors suggests that proinflammatory cytokines may be not directly involved in the pathogenesis of DSS. In contrast, MNLs with heterologously secondary exposure to DV-2 significantly augmented IFNγ production in comparison to those with primary exposure to DV-2. IFNγ, but not IFNα or IFNβ, is known as a cytokine that is not directly induced by virus. The fact that MNLs in heterologously secondary infections with DV-2 for 3 days released higher levels of IFNγ than those in primary infections with DV-2 suggests that a memory immune response may be involved in the augmentation of IFNγ production. Another possibility is that the heterologously secondary DV-2 infections may trigger an immune response along Th1 reaction, which tends to release predominantly IFNγ, rather than Th2 reaction, which tends to release less IFNγ.

IFNγ is known to inhibit viral replication and to augment antibody-dependent cell cytotoxicity against virus-infected cells. IFNγ, however, could also prime monocytes/macrophages for Fcγ-receptor-mediated enhancement of DV infection (Kontony *et al.*, 1988). Enhancement of Fcγ-receptor-mediated phagocytosis may augment release of procoagulant factors, amplify production of cytokines, and induce nitric oxide production, resulting in DSS associated with extravascular leakage, intravascular coagulopathy, hemorrhage, and hypotension. To differentiate these two possibilities, additional studies to measure IFNγ levels in the sera obtained from patients with and without DSS are needed.

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