MINIREVIEW

Nitric oxide in dengue and dengue haemorrhagic fever: necessity or nuisance?

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Abstract

Advances in free radical research show that reactive oxygen and nitrogen oxide species, for example superoxide, nitric oxide (NO) and peroxynitrite, play an important role in the pathogenesis of different viral infections, including dengue virus. The pathogenic mechanism of dengue haemorrhagic fever (DHF) is complicated and is not clearly understood. The hallmarks of the dengue disease, the antibody-dependent enhancement, the shift from T-helper type 1 (Th1) to Th2 cytokine response and the cytokine tsunami resulting in vascular leakage can now be explained much better with the knowledge gained about NO and peroxynitrite. This paper makes an effort to present a synthesis of the current opinions to explain the pathogenesis of DHF/shock syndrome with NO on centre stage.

Introduction

Nitric oxide (NO) is an important paracrine and autocrine signal used by different types of cells and produced by a variety of cells in the body, for example macrophages, vascular endothelial cells, Kupffer cells, adrenals and cerebellar tissues (Ignarro, 1991). NO has a wide range of functions in the body, from dilating blood vessels, aggregation of platelets, fighting infections and tumours, mediator of inflammation and macrophage cytotoxic activity to transmission of signals between nerve cells (Moncada *et al.*, 1991; Moncada & Higgs, 1993; Bogdan, 1998). In optimal doses it has protective and regulatory action in cells, but higher concentrations have toxic effects. The enzyme NO synthase (NOS) contributes to signal transmission in different cell systems of body via synthesis of NO from L-arginine in the presence of NADPH and dioxygen (O_2). NOS binds flavin adenine dinucleotide, flavin mononucleotide, haeme, tetrahydrobiopterin and calmodulin. It activates cGMP, which inhibits calcium entry into the cell and decreases levels of intracellular calcium, and K^+ channels, and stimulates a cGMP-dependent protein kinase, etc. NOS has three isoforms (Table 1): the neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3). Cytokines and other proinflammatory stimuli induce iNOS (MacMicking *et al.*, 1997a, b). The activity of nNOS and eNOS is calcium- and calmodulin-dependent, whereas that of iNOS is calcium-independent. NO may regulate NOS expression and activity by negative feedback by the process of *S*-nitrosylation.

Dengue viruses (DV) are transmitted by *Aedes aegypti* mosquitoes. They produce subclinical infection or a mild self-limiting disease, the dengue fever (DF) and a serious life-threatening dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue is the most common arboviral disease of humans and is found in subtropical and tropical areas of the world located between 10°N and 10°S of the Equator. More than 2.5 billion persons are at risk of getting dengue infections. About 50–100 million cases of DF occur every year, with about 250 000–500 000 cases of DHF. The incidence of DHF has increased tremendously in India, Southeast Asia, the South Pacific and the American tropics in the past 25 years, with major epidemics occurring in many countries. The risk factors for developing DHF are cocirculation of all four DV strains in the same area, host factors such as immune status, age and genetic background

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Table 1. Functions of NOS in DV infection

Types of NOS	Location	Cell	Regulation	Dengue disease	
				Lesions	*References
Neuronal NOS (nNOS or NOS1)	Brain	Microglia	Ca^{2+}/CAM	Blood–brain barrier damage	Chaturvedi *et al*. (1991); Winter *et al*. (2008)
Inducible NOS (iNOS or NOS2)	Immune system, cardiovascular system	Macrophage, dendritic cell	Ca^{2+} independent	Virus replication inhibited	Neves-Souza *et al*. (2005); Takhampunya *et al*. (2006)
Endothelial NOS (eNOS or NOS3)	Endothelium	Endothelial cells	Ca^{2+}/CAM	Increased capillary permeability	Khanna *et al*. (1990); Dhawan *et al*. (1994); Basu & Chaturvedi (2008)

*Only selected references have been cited.

and the virus genotype. Patients with DHF may develop capillary leakage, resulting in flow of serum proteins and fluid into the body cavities, for example the pleural and abdominal cavities. Patients have haemorrhages in different parts of the body. Investigations show thrombocytopoenia, neutropoenia and elevated liver enzymes, and sometimes disseminated intravascular coagulation. DHF may progress to a hypotensive state, the DSS, with cold clammy skin and unrecordable pulse and blood pressure. The course of shock is short, but life-threatening. DHF is observed commonly in infants and children who are exposed to a second dengue infection. The pathogenesis of DHF is still not fully understood despite extensive studies and has been a subject of controversy from the time the syndrome was first recognized (reviewed by Chaturvedi *et al*., 2005, 2006a, b; Halstead, 2007; Chaturvedi & Nagar, 2008).

The role of NO in different viral infections has been studied for many years and a number of interesting reviews have been published (Akaike & Maeda, 2000; Akuta *et al*., 2006). Chaturvedi and colleagues first reported the role of NO and peroxynitrite in DV infection in 1996 (Misra *et al*., 1996a, b; Mukerjee *et al*., 1996), but its greater implications in dengue disease have been distinguished only recently. The hallmarks of the dengue disease, the antibody-dependent enhancement (ADE; Halstead, 1970, 2007), the shift from T-helper type 1 (Th1) to Th2 cytokine response (Chaturvedi *et al*., 1999) and the cytokine tsunami (Chaturvedi *et al*., 2000, 2007) resulting in vascular leakage can now be explained much better with the knowledge gained about NO and peroxynitrite (Chareonsirisuthigul *et al*., 2007; Ubol *et al*., 2008a, b). This paper makes an effort to present a synthesis of the current opinions to explain the pathogenesis of DHF/shock syndrome with NO on centre stage. Because of space constraints, all of the papers could not be cited.

NO

NO is a relatively stable free radical. It reacts with other free radicals (O_2^-, superoxide anion; OH, hydroxyl ion) or metals like haeme iron (haemoglobin, myoglobin, cytochromes). NO is short-lived and acts at the site of synthesis (Ignarro *et al*., 1993).

Mechanisms of the effects of NO

Even at high concentrations NO does not kill cells by itself. For acute toxicity, NO reacts with superoxide anion and forms the peroxynitrite ($ONOO^-$), which has a wider range of chemical targets (Fig. 1). It can oxidize proteins, lipids, RNA and DNA. The toxic effects of NO are due to its ability to modulate mitochondrial respiration, DNA synthesis and energy metabolism. Peroxynitrite inhibits enzymes in the mitochondrial respiratory chain and the functions of manganese SOD (MnSOD), which results in increased formation of superoxide anion. $ONOO^-$ efficiently modifies and breaks DNA strands and inhibits DNA ligase, which increases DNA strand breaks, which activate DNA repair mechanisms, including the nuclear enzyme poly(ADP-ribose) polymerase (PARP). Activation of PARP results in a drop in energy stores that impairs cellular metabolism and causes cell death. Reactions of NO differ between *in vitro* and *in vivo* systems. The main degradation product of NO *in vitro* is NO_2^- (nitrite), whereas *in vivo* the main product is NO_3^- (nitrate). The constitutive enzymes nNOS and eNOS induce low concentrations of NO, which modulates the activity of haeme-containing proteins, for example guanylyl cyclase as well as proteins of the mitochondria such as cytochromes (reviewed by Ignarro, 1991; Ignarro *et al*., 1993; Sharma *et al*., 2007; Tripathi *et al*., 2007). Induction of iNOS generates higher concentrations of NO, which can nitrosylate cysteine residues or produce tyrosine nitration in different proteins and also deamination of DNA. Nitrotyrosine is a marker for the generation of peroxynitrite (Hanafy *et al*., 2001).

Role of NO in the immune system

NO is known to have a strong immuno-regulatory role (Bogdan, 2001). NO either activates or inhibits immune cell activation, proliferation, cytokine synthesis and

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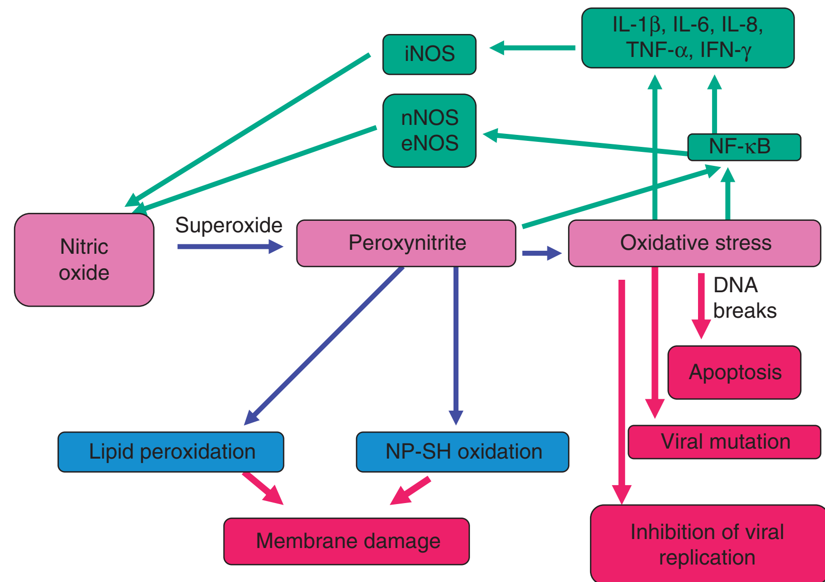

Fig. 1. Mechanisms by which NO inhibits virus replication, induces virus mutation, damages cell membrane and breaks DNA, producing cell apoptosis.

cytokine signaling (Wei *et al.*, 1995; Bogdan, 1998; Huang *et al.*, 1998). NO has been shown to upregulate proliferation and increases glucose uptake by T lymphocytes; on the other hand, it inhibits T-cell activation. NO also modulates apoptosis; it promotes or prevents apoptosis and is involved in alteration of p53 levels (reviewed by Jiménez *et al.*, 2001).

NO has variable effects on cytokine synthesis. Exogenous NO increases synthesis of tumour necrosis factor-α (TNF-α) in human peripheral blood mononuclear cells and lipopolysaccharide-stimulated neutrophil preparations (Van Dervort *et al.*, 1994). Endogenous NO is required for interleukin-12 (IL-12) production, whereas exogenous NO decreases IL-12 production by macrophages (Huang *et al.*, 1998). The production of IL-6 and granulocyte colony-stimulating factor mRNA is decreased in iNOS knockout mice or in normal animals treated with iNOS inhibitors (Hierholzer *et al.*, 1998). NO is involved in cytokine signaling, as in iNOS-deficient mice some cytokine signaling is lost. iNOS is required for IL-12-mediated T-cell proliferation and activation and also for natural killer cell activation by interferon-α/β (IFN-α/β) (Diefenbach *et al.*, 1999). Production of large amounts of NO by macrophages leads to inactivation of lymphocytes and induces a persistent immunosuppression in HIV infection (reviewed by Jiménez *et al.*, 2001). NO plays a key role in the pathogenesis of inflammatory diseases. In the right situations and right concentrations it is anti-inflammatory and has protective effects against various infections. On the other hand, in abnormal situations and higher concentrations NO is a proinflammatory mediator, inducing inflammation and pathological lesions. NO belongs to the labile radical entities, the reactive oxygen species (ROS), and reacts with oxygen and haeme-iron-containing groups, reducing nitrate compounds.

Ding *et al.* (1988) screened the effect of 12 different cytokines on the production of nitrite by macrophages and found that the most effective was IFN-γ. Further, NO production is inhibited by type 2 cytokines [IL-4, IL-10, IL-13, transforming growth factor-β (TGF-β)] (MacMicking *et al.*, 1997a). Therefore iNOS expression may depend on Th1–Th2 shift in the hosts. An enhanced Th1 response is observed in NO-deficient mice during infections and antigenic stimulation, resulting in more production of interferon and less of IL-4 (MacLean *et al.*, 1998; McInnes *et al.*, 1998), indicating that NO selectively inhibits the expansion of Th1 cells by a negative feedback mechanism. Selective inhibition of IL-12 synthesis by activated macrophages may be responsible for this to some extent (Huang *et al.*, 1998). On the other hand, low concentrations of NO produced during the early stages of infection result in a strong Th1-type response, which is effective in host defence against intracellular pathogens (Niedbala *et al.*, 1999). A synergistic effect of IL-18 and IL-12 has been observed in the induction of Th1-type cells (Robinson *et al.*, 1997). The precise mechanism of the action of low concentrations of NO in the Th1 type is not fully known, but recently Niedbala *et al.* (2006) have suggested that the enhancing effect of low concentrations of NO is mediated by cGMP by exerting a direct and selective effect on Th1 cells and not via antigen-presenting cells. Low concentrations of NO in endothelial cells (Umansky *et al.*, 1998) and macrophages (Connelly *et al.*, 2001) upregulates nuclear factor-κB (NF-κB) activity and the high doses downregulates it. cGMP may also act through the P-Raf/MEK/ERK pathway. Thus, NO is also responsible for the induction of T-cell subset response.

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Niedbala *et al.* (2007) have reported a new subset of $CD4^+CD25^+$ regulatory T cells (Tregs) derived from $CD4^+CD25^-$ T cells induced by NO. The induction of Tregs (NO-Tregs) is independent of cGMP but depends on p53, IL-2 and OX40. NO-Tregs produce IL-4 and IL-10, but not IL-2, IFN-γ or TGF-β. The cells are $GITR^+$, $CD27^+$, $T\text{-}bet^{low}$, $GATA3^{high}$ and Foxp3. NO-Tregs suppress the proliferation of $CD4^+CD25^-$ T cells *in vitro* and function in an IL-10-dependent manner. NO-Tregs are also induced *in vivo* in SCID mice adoptively transferred with $CD4^+CD25^-$ T cells in the presence of lipopolysaccharide and IFN-γ, and the induction is completely inhibited by N^G-monomethyl-L-arginine (NMMA), a pan NOS inhibitor (Niedbala *et al.*, 2007).

Role of NO during viral infection

NO has multiple effects during viral infections; for example, it affects the virus or the host. NO can inhibit virus replication or can cause viral mutation and in the host it can cause cell damage and pathology. The induction of iNOS can occur during viral infections (Fig. 2) by two mechanisms, direct and indirect. It can occur directly by viral replication, for example respiratory syncytial virus, or by viral proteins, for example HIV-1 glycoprotein, gp41 (Akaike & Maeda, 2000). The indirect mechanisms include induction by cytokines, the commonest being IFN-γ and by other partly characterized cytokines (Table 2).

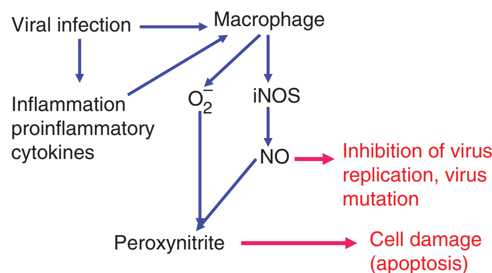

Fig. 2. Mechanisms of the induction of NO during virus infections.

Effects of NO on virus replication

NO has a variable effect on the replication of viruses; it inhibits the replication of most of them, whereas it enhances some viruses, and has no effect on a few of the other viruses (Table 3). NO inhibits viral replication by reversible *S*-nitrosylation of viral proteases (Zaragoza *et al.*, 1997). Another antiviral mechanism of NO is via the formation of peroxynitrite, which blocks viral entry into the host cell (Padalko *et al.*, 2004). NO has strong antiviral effects on hantavirus replication and peroxynitrite has strong antiviral effects on mature free virions, suggesting that different reactive nitrogen intermediates can have different effects on various parts of the replication cycle for the same virus (Klingström *et al.*, 2006). Peroxynitrite enhances Sendai viral mutation *in vitro* and expands the quasispecies spectrum, facilitating the evolution of RNA viruses (Akaike *et al.*, 2000). NO may accelerate viral mutation via formation of 8-nitroguanosine, which may be a substantial contributor to erroneous RNA replication of the virus. NO-generated 8-nitroguanosine may cause viral mutation via two different mechanisms: directly, through incorporation into template RNAs for viral replication, and indirectly, by enhanced oxidative stress because of its potent redox-active property (Yoshitake *et al.*, 2004). NO has a unique biological effect on the genome of both pathogen and host via chemical modification of nucleic acids (Akaike *et al.*, 2000, 2003). NO can act on viral proteins by different mechanisms. NO inhibits Coxsackie virus infection by nitration of the VP1 capsid protein (Padalko *et al.*, 2004) or by *S*-nitrosylation of the cysteine protease 3C (Saura *et al.*, 1999). HIV-1 is inhibited by *S*-nitrosylation of the viral protease by NO (Persichini *et al.*, 1998). Modification of cellular proteins can also inhibit viral infection, for example *S*-nitrosylation of NF-κB downregulates expression of the transactivator

Table 2. Induction of iNOS/NO by cytokines in viral infections

Virus	Cytokine	Test system	References
Dengue	CF	Mouse spleen cells *in vivo* and *in vitro*	Misra *et al.* (1996a)
	Macrophage cytotoxin	Mouse spleen cells cytotoxicity	Misra *et al.* (1996a, b); Mukerjee *et al.* (1996)
	Suppressor factor	Macrophage culture Signal transmission	Khare & Chaturvedi (1997)
Ectromelia, vaccinia, and herpes simplex-1	IFN-γ	Mouse macrophage	Karupiah *et al.* (1993)
Influenza A	IFN-γ	Mouse lung	Akaike & Maeda (2000)
Sendai	IFN-γ	Mouse lung	
Japanese encephalitis (JE)	Macrophage derived neutrophil chemotactic factor	Splenic macrophage of JE virus-infected mice	Saxena *et al.* (2000)

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Table 3. Effect of NO on the replication of viruses grown in different cells*

Virus	Test system	Mechanism of NO production	References
NO-induced inhibition of virus replication			
Influenza A, B	Kidney cells	Viral RNA synthesis inhibited	Rimmelzwaan *et al*. (1999)
Influenza PR8	Murine macrophage	Induce iNOS RNA	Imanishi *et al*. (2005)
Hantavirus	A549 cells	IFNs induced	Stoltz *et al*. (2007)
	Vero E6 cells	Cytokine induced	Klingström *et al*. (2006)
	iNOS$^{(-/-)}$ mice	NO not produced	
Coronavirus (SARS CoV)	Vero E6 cells	Viral RNA synthesis inhibited	Akerström *et al*. (2005)
Hepatitis B	Liver of transgenic mice	IFN-γ-induced iNOS	Guidotti *et al*. (2000)
LCMV	Liver of transgenic mice	IFN-γ-induced iNOS	Guidotti *et al*. (2000)
Coxsackievirus B3	Macrophage *in vitro*	IFN-γ-induced iNOS	Jarasch *et al*. (2005)
Sendai virus	Mice	NO and peroxynitrite	Akaike *et al*. (2000)
Rabies virus	Neuroblastoma cells	Transcription inhibition	Ubol *et al*. (2001)
Junin virus	Astrocyte culture	iNOS/NO generation	Pozner *et al*. (2008)
Japanese encephalitis virus (JEV)	Primarily JEV-infected N18, human neuronal NT-2, and BHK-21 cells, as well as in persistently JEV-infected C2-2 cells.	Inhibit viral RNA synthesis	Lin *et al*. (1997)
DV	LLC-MK2 monkey kidney cells	RdRp inhibition	Takhampunya *et al*. (2006)
	Neuroblastoma cells	RNA production suppressed	Charnsilpa *et al*. (2005)
NO-induced increase of virus replication			
HIV-1	T-cell lines. Jurkat and MT-2	Activation of LTR-mediated transcription	Jiménez *et al*. (2001)
	Monocytes from PBMC of normal humans	Activation of NF-κB by peroxynitrite	Aquaro *et al*. (2007)
	PBMC of HIV-1 Patients	Decreased expression of iNOS RNA	Cairoli *et al*. (2008)
	Normal PBMC infected with HIV-1	Decreased expression of iNOS RNA	
NO has no effect on virus replication			
Vaccinia virus	Mice	iNOS-deficient mice do not show increased viral replication	van den Broek *et al*. (2000)
	Murine macrophage	Suppress iNOS through soluble viral protein	Bellows *et al*. (2003)
Lymphocytic choriomeningitis virus	Mice	INOS/NO are redundant	Bartholdy *et al*. (1999)
Mouse hepatitis virus	Mice	iNOS-deficient mice do not increase	Wu *et al*. (2000)
Tick-borne encephalitis virus	Mice macrophage	Increased NO production has no effect	Kreil & Eibl (1996)
Hepatitis B virus	Hepatoma cells	Increased iNOS expression has no effect	Proto *et al*. (2008)

*All viruses could not be included.
LCMV, lymphocytic choriomeningitis virus;
LTR, long terminal repeat.

Zta, which is required for reactivation of Epstein–Barr virus (Mannick *et al*., 1994), and tyrosine nitration of microtubules attenuates respiratory syncytial virus infection (Huang *et al*., 2005).

Effects of NO on host during viral infections

NO and peroxynitrite are double-edged swords having both beneficial and harmful effects on the host during infections, resulting in pathological lesions (Table 4). Peroxynitrite is harmful to the host when present in high concentrations, oxidizing lipids and DNA, and oxidizing or nitrating proteins. However, peroxynitrite in low concentrations may be beneficial to the host during pathogen infection (Padalko *et al*., 2004). Nitrative stress-mediated 8-nitroguanosine formation during influenza or Sendai virus infection involves unique biochemical and pharmacological properties such as redox activity and mutagenic potential, which contributes to pathogenic processes during viral infection (Zaki *et al*., 2005). Central nervous system (CNS)

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Table 4. Viral infection in which pathogenesis/protection is through NO production*

Virus	Test system	Site/lesion	Mechanisms of NO production	References
NO-mediated pathology				
Rotavirus	Mice *in vivo*; *ex vivo* ileal loop	Ileum/diarrhoea	Upregulation of ileal iNOS mRNA by virus and by NS4 protein	Borghan *et al*. (2007)
Coxsackie B	Mice	Myocarditis	Increased iNOS/NO	Bevan *et al*. (2001)
Herpes simplex 1	Mice	Liver/apoptosis	Increased production of NO, TNF-α, IL-6, IFN-γ	Irie & Shiga (2005)
HIV	Cortical cell culture	Neurotoxicity	Activation of NOS by HIVgp120 and cytokines	Dawson *et al*. (1993)
	PBMC monocyte	Lymphocyte inactivation	Large amount of NO production	Groeneveld *et al*. (1996)
	Cardiomyocyte culture, animals	Cardiomyopathy	NO induced cardiomyocyte apoptosis by TNF type 1 receptor activation	Monsuez *et al*. (2007)
	Microglia cells	Neurodegeneration	NO-induced oxidative stress	Roy *et al*. (2008)
Influenza A	Mice	Pneumonitis	iNOS presence	Karupiah *et al*. (1998)
Tick-borne encephalitis virus	Mice	Encephalitis	Increased NO	Kreil & Eibl (1996)
Adenovirus	Mice	Lung inflammation	iNOS and peroxynitrite-generated nitrotyrosine	Zsengellér *et al*. (2001)
Murine cytomegalovirus	Mice	Pneumonitis	Increased NO	Tanaka *et al*. (1997)
Hepatitis C	Hepatocyte culture	Hepatitis, oncogenesis	Increased iNOS/NO	Machida *et al*. (2004)
NO-mediated protection of pathology				
Japanese encephalitis	Mice	Encephalitis protected	Production of macrophage-derived neutrophil chemotactic factor increases iNOS	Saxena *et al*. (2001)
Junín virus	Mice, astrocyte culture	Brain damage protected	INOS inhibition increases brain damage	Gómez *et al*. (2003)

*Only a few examples have been cited.

inflammatory response to neurotropic virus infection is likely to be dependent upon the activity of peroxynitrite or its products on the blood–brain barrier (Hooper *et al*., 2001). Infection with virulent Coxsackie virus in mice increases expression of iNOS and produces inflammatory myocardial disease (Bevan *et al*., 2001). Adenovirus infection increases iNOS and peroxynitrite production in the lung, generating nitrotyrosine, and may contribute to inflammatory responses in the lung (Zsengellér *et al*., 2001). Hepatitis C virus (HCV) infection can stimulate the production of NO through activation of the iNOS gene by the viral core and NS3 proteins. NO causes DNA breaks and enhances DNA mutation. This sequence of events provides a mechanism for HCV pathogenesis and oncogenesis (Machida *et al*., 2004). On the other hand, enhanced oxidative stress may be involved in the pathogenesis of viral infections. HIV-infected patients have a higher incidence of oxidative stress, endothelial dysfunction and cardiovascular disease than uninfected individuals (Table 4). Recent reports have demonstrated that viral proteins upregulate ROS, which may contribute to elevated cardiovascular risk in HIV-1 patients (Kline *et al*., 2008). Using an HIV-1 transgenic rat model it has been shown that HIV-1 protein expression decreases NO and causes endothelial dysfunction. Diminished antioxidant capacity increases vascular superoxide levels, which reduce NO bioavailability and promote peroxynitrite generation. Restoring glutathione levels reverses HIV-1 protein-mediated effects on superoxide, NO and vasorelaxation (Kline *et al*., 2008). Experimental murine encephalitis induced by Junin virus increases expression of iNOS by unidentified cells, concomitant with the astrocyte reaction. The specific inhibition of iNOS is associated with greater mortality but lower astrocytosis (Table 4), suggesting that the protective role of NO is related to enhanced astrocyte activation, representing a beneficial cellular response to virus-induced CNS damage (Gómez *et al*., 2003).

DV

DV belong to the family *Flaviviridae*, genus *Flavivirus*, and have four genetically and antigenically related serotypes known as DV-1 to DV-4. Each serotype of DV contains abundant genetic variation and can be subdivided into subtypes or genotypes that may be responsible for the varying severity of clinical symptoms. DV replicates mainly in cells of monocyte/macrophage lineage (Chaturvedi *et al*., 2006a).

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DV proteins and immune response

DV genome is a single-stranded RNA of positive polarity that encodes a single polyprotein, which is translated into three structural proteins – C, prM and E – and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, which are important for virus replication. Anti-E antibodies are the main response against DV. The antibodies inhibit viral binding to cells, neutralize viral infectivity *in vitro*, protect mice from DV challenge on passive transfer and show a variable degree of cross-reactivity among the DV serotypes. NS1 is a key glycoprotein involved in the production of infectious virus and the pathogenesis of dengue diseases. During the replication of DV, NS1 associates with the membrane on the cell surface and in the RNA replication complex (Noisakran *et al.*, 2008). NS1 is expressed on the surface of the virus-infected cells and is secreted into the circulation as a soluble multimer; it is an important target of antibodies against DV. Antibodies against NS1 can trigger complement-mediated lysis of DV-infected cells *in vitro* and protect mice from DV challenge. But the cross-reaction of these antibodies with endothelial cells leads to expression of cytokine, chemokine and adhesion molecules, resulting in cell damage. NS3 is a multifunctional protein; it is the main antigen that stimulates DV-reactive $CD4^+$ and $CD8^+$ T cell (reviewed by Chaturvedi *et al.*, 2006b; Chaturvedi & Nagar, 2008). NS3 has located at the active site of a trypsin-like serine protease and, together with the NS2B cofactor, forms an active protease that is required for polyprotein processing. NS3 also has an RNA-stimulated NTPase and RNA helicase that is important for viral RNA replication, as well as 5′-RNA triphosphatase activity. NS5 is required for viral RNA replication and is an RNA-dependent RNA polymerase (RdRp) (O'Reilly & Kao, 1998). In flavivirus-infected cells, NS3 and NS5 exist in a complex and are thought to be components of the viral RNA replicase complex (Kapoor *et al.*, 1995; Westaway *et al.*, 2003). A role of NS3 and NS5 in virus replication and 5′-capping has been suggested due to their enzymatic activities but the functions of the other nonstructural proteins are not clear (Benarroch *et al.*, 2004).

Pathogenesis of DHF

The precise mechanism of DHF is not yet fully known. The important hypothesis put forward regarding the role of host factors are ADE of DV replication, shift of Th1- to Th2-type cytokine response and other T-cell responses resulting into cytokine tsunami. The viral factors include genotypic mutation, for example the Southeast Asian type of DV produces DHF in children, whereas the American genotype does not. Some of these mechanisms are discussed briefly. Non-neutralizing anti-DV antibodies from an earlier infection by a heterologous serotype of DV mediate ADE. They form complexes with the infecting DV leading to greater uptake by macrophages and consequent greater numbers of DV-infected cells. The first suggestion of an association of increased risk for DHF with a secondary DV infection was made in 1970s (Halstead, 1970). A subpopulation of $CD4^+$ T cells produce a unique cytokine, cytotoxic factor (CF) during DV infection of mice and man (hCF). CF induces H2-A-positive macrophages to produce another cytokine, macrophage cytotoxin (CF2), which amplifies its cytotoxic effects on target cells (Fig. 3). The hCF purified from the sera

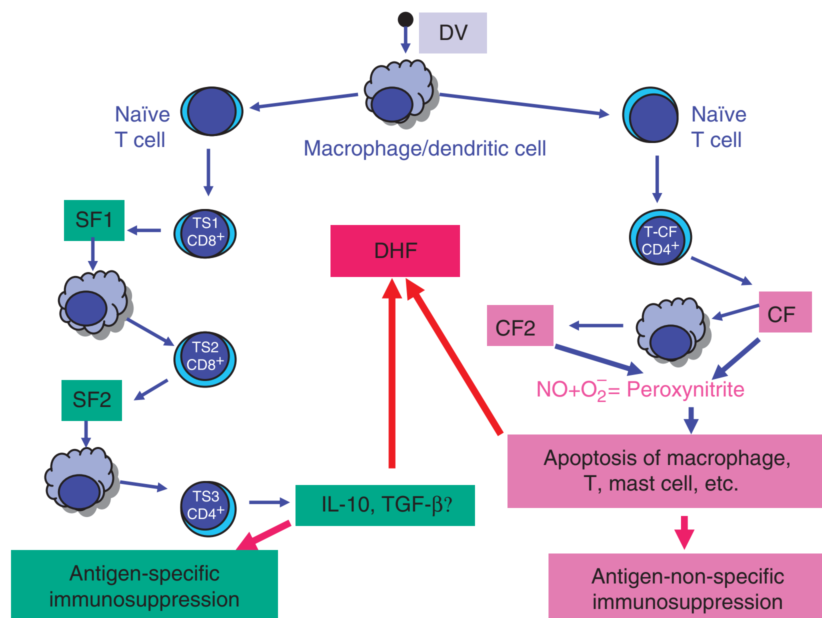

Fig. 3. Role of NO in the proposed mechanisms of immunosuppression during DV infection.

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of DHF patients appear to be pathogenesis-related proteins, capable of reproducing DHF-like pathological lesions on inoculation in mice, such as increased capillary permeability, cerebral oedema and blood leukocyte changes (Chaturvedi *et al*., 1991, 1997; Mukerjee *et al*., 1997; Agarwal *et al*., 1998). The highest levels of hCF-autoantibodies are seen in sera of patients with mild illness (DF) and the lowest in patients with DHF grade IV (Chaturvedi *et al*., 2001; reviewed by Chaturvedi *et al*., 2006a, b).

A microorganism-induced suppressor T-cell (TS) cascade was delineated in DV-infected mice in the late 1980s (Chaturvedi, 1984) and it has now been confirmed in a large number of viruses (Mills, 2004). The antigen-specific TS cascade in DV-infected mice consists of three generations of TS and their secretary soluble suppressor cytokines (SF), with in between macrophages transmitting the signals (Fig. 3). DV-induced suppressor pathway suppresses antigen-specific antibody production, including that of enhancing antibody. Thus increased replication of the virus mediated by the enhancing antibody and the immunopathology mediated by the immune complex is prevented. On the other hand, suppression of neutralizing antibody would delay elimination of DV from the body, causing pathological lesions. TS can also increase the severity by producing IL-10/TGF-β (Chaturvedi, 1984; Chaturvedi *et al*., 2007). Both IL-10 and TGF-β suppress NO production (Cunha *et al*., 1992; Vodovotz *et al*., 1993) and may increase the virus load and the severity of dengue disease. The outcome may depend upon the fine balance between them.

Different types of T cells that become activated during DV infection and contribute to the development of DHF are summarized in Fig. 4. Serum levels of IFN-γ and IL-2 are the highest in DF and the lowest in severe DHF cases. On the other hand, serum levels of IL-4, IL-10 and IL-6 are maximum in DHF patients and negligible in patients with DF. The levels of TNF-α in the sera increase with the increase of severity of DHF (Chaturvedi *et al*., 1999). The most significant finding was a shift of the predominant Th1-type response observed in 66% of the DF patients to Th2-type response seen in 71% of DHF grade IV, indicating a role of Th2 cells in the pathogenesis of DHF (Chaturvedi *et al*., 1999). During acute infection in children, few dengue-responsive $CD8^+$ T cells are recovered that show an activated phenotype and undergo apoptosis. These DV-specific T cells have a low affinity for the infecting virus and a higher affinity for previously encountered strains. Cross-reactive DV-specific T cells show high cytokine production, which may contribute to the vascular leakage. DV-specific memory lymphocyte response has been detected even 20 years after a primary infection by DV. Induction of cross-reactive memory T cells (mainly NS3-specific) during a secondary infection leads to increased incidence of DHF and DSS. When viral peptides complexed with HLA molecules are presented to memory T cells, proliferation and the production of proinflammatory cytokines follow, which can directly affect vascular endothelial cells, resulting in plasma leakage (Mongkolsapaya *et al*., 2003, 2006). Recently, Ubol *et al*. (2008b) have shown that during DF, genes in the interferon system and complement inhibitor play a role in lowering virus production and reducing tissue damage. In patients with DHF, the dysfunction of immune cells, complement, and cytokines increases viral load and tissue damage. Profound T-cell activation and death may contribute to the systemic disturbances leading to DHF, and original antigenic sin in

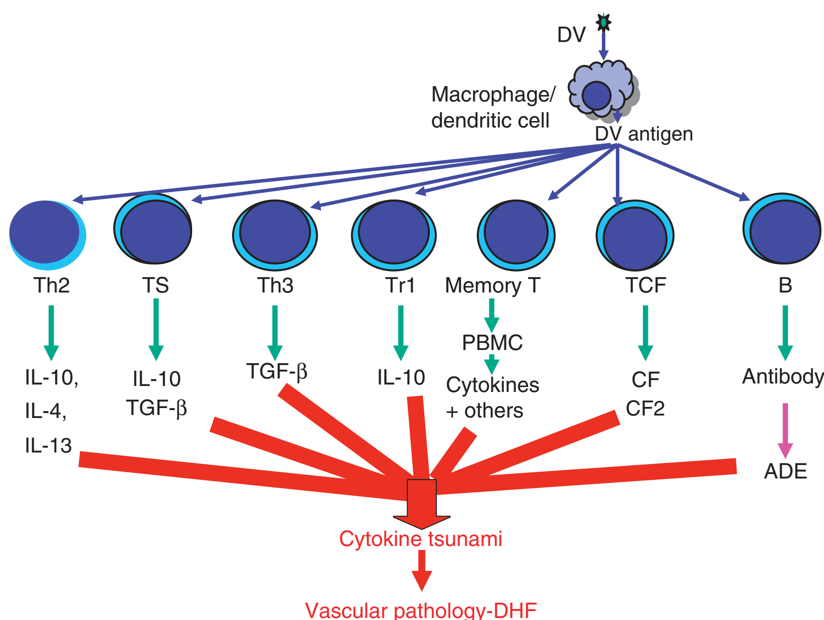

Fig. 4. Role of various subsets of T cells in the pathogenesis of DHF through generation of cytokine tsunami. ADE (B cell) is included to emphasize its importance.

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the T-cell responses may suppress or delay viral elimination, leading to higher viral loads and increased immunopathology (reviewed by Green & Rothman, 2006; Chaturvedi *et al*., 2007; Halstead, 2007; Chaturvedi & Nagar, 2008; Hatch *et al*., 2008). Further, the increased serum levels of IL-8 (Raghupathy *et al*., 1998) and TGF-β1 (Agarwal *et al*., 1999) are associated with increasing severity of DHF and death. Thus, the cascades of T-cell activation resulting in a 'tsunami' of cytokines and other chemical mediators ('cytokine tsunami') released mainly from T cells, monocytes/macrophages and endothelial cells, ultimately cause an increase in vascular permeability and lead to DHF/DSS.

Role of NO in DV infection

During the 1990s a number of papers were published that showed some association between NO and viral infections, both *in vivo* and *in vitro* (reviewed by Reiss & Komatsu, 1998). But the role of NO and peroxynitrite in DV infection and the role in the pathogenesis of DHF was first suggested by Chaturvedi and colleagues (Misra *et al*., 1996a, b; Mukerjee *et al*., 1996). This generated a lot of interest in dengue virologists, resulting into interesting studies that are presented here briefly.

Studies in mice

Production of nitrite by the spleen cells of mice followed inoculation of DV or the DV-induced cytokines CF and CF2. In DV-infected mouse spleen, maximum NO_2 production occurred at 8–11 days postinfection, which correlated with the peak appearance of CF in the spleen. Maximum NO_2^- production occurred in the spleen cells 45 min after intravenous inoculation of mice with CF. The NO_2^- was produced by macrophages (Misra *et al*., 1996a). In a similar study, CF2 was also shown to induce production of NO_2^- in the macrophages of spleens of mice (Mukerjee *et al*., 1996). Further, production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by the spleen cells was shown following intracerebral inoculation of DV or intravenous inoculation of CF/CF2 in mice. It was concluded that the cell apoptosis was mediated by NO^+O^- (peroxynitrite) and not by H_2O_2 (Misra *et al*., 1996b, 1998). NO also transmits the DV-specific intracellular suppressor signal in macrophage (Khare & Chaturvedi, 1997).

Studies in patients

Rodriguez-Ortega (1998) has discussed the clinical features of dengue that link NO with the pathology of the severe dengue disease. Valero *et al*. (2002) reported increased levels of NO in patients with DF, whereas in the patients with DHF, levels similar to those of healthy controls were found. In contrast, Trairatvorakul *et al*. (2005) reported that the levels of serum NO in DF patients were significantly lower than those of normal controls. Patients with DSS had higher NO levels than those with DHF I/II. Neves-Souza *et al*. (2005) studied the expression of DV antigens and iNOS in human blood monocytes analysed by flow cytometry using cells either from patients with acute DF or after DV infection *in vitro*. Activation of DV-infected monocytes based on induction of iNOS occurred both *in vivo* and *in vitro*, and the susceptibility of DV to NO production was noted. NO inhibits aggregation, recruitment and adhesion of platelets to the vascular endothelium. Mendes-Ribeiro *et al*. (2008) have shown that an elevated rate of L-arginine transport in DF patients is associated with enhanced NOS activity and elevated plasma fibrinogen levels, resulting in reduced platelet aggregation. Oxidative stress mediated changes in plasma proteins; for example, protein carbonylation and the ratio of protein carbonylation and protein-bound sulphydryl group levels can be an early biomarker for prediction of severe dengue infection (Soundravally *et al*., 2008).

Studies *in vitro*

Production of nitrite by mouse spleen cell cultures was studied following inoculation of DV or CF/CF2. DV-stimulated spleen cell culture supernatants showed a peak production of both CF and NO_2^- at 72 h. Pretreatment of spleen cells with NMMA inhibited NO_2^- production. NO_2^- production was abrogated in a dose-dependent manner by treatment of spleen cells with the Ca^{2+} channel blocking drug nifedipine. Thus, DV-induced CF induces production of NO_2^- in spleen cells, probably in a Ca^{2+}-dependent manner, and may be a mechanism of target cell killing (Misra *et al*., 1996a). Further, DV, CF or CF2 induce production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by the mouse spleen cell cultures. It was suggested that O_2^- and nitrite are necessary for cell killing by CF/CF2 in a Ca^{2+}-dependent manner and the killing may possibly be by generation of peroxynitrite (Misra *et al*., 1996b, 1998). In another study it was shown that NO transmits the DV-specific suppressor signal intracellularly in macrophages (Khare & Chaturvedi, 1997). DV infection induces a biphasic activation of Kupffer cells in cultures: there is one peak of activation shortly after infection which involves the production of NO and IFN-α, and a second peak after a few hours involving IL-6 and TNF-α synthesis. DV-replicating Kupffer cells undergo apoptosis and are cleared by phagocytosis. The timing of the synthesis of these soluble mediators suggests the involvement of a very early step of infection in their activation (Marianneau *et al*., 1999). Valero *et al*. (2002) reported that *in vitro* incubation of human platelets with DV did not increase levels of NO. Chen & Wang (2002) reported that after DV infection, the *in vitro*-differentiated monocyte/macrophages secrete multiple innate cytokines and

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chemokines, including TNF-α, IFN-α, IL-1β, IL-8, IL-12, MIP-1α and regulated upon activation, normal T cell expressed and secreted (RANTES) but not IL-6, IL-15 or NO. Human monocyte cultures infected with DV show increased numbers of apoptotic cells and increased production of TNF-α. No increase in production of NO was observed. These results may be related to early primary viral infection in which virus could induce apoptosis in monocytes, but monocytes may contribute to host defence mechanisms against virus by viral phagocytosis, phagocytosis of infected apoptotic cells, and the release of proinflammatory cytokines (Espina *et al*., 2003).

Chareonsirisuthigul *et al.* (2007) reported that DV infection of THP-1 cells via an ADE pathway suppresses NO radicals, by disrupting the transcription of the iNOS gene transcription factor, IRF-1 and blocking the activation of STAT-1. Further, it suppresses the transcription and translation of IL-12, IFN-γ and TNF-α, whereas the expression and synthesis of the anti-inflammatory cytokines IL-6 and IL-10 are enhanced. Thus, besides facilitating the entry process, ADE infection also modifies innate and adaptive intracellular antiviral mechanisms, resulting in unrestricted DV replication in THP-1 cells (Chareonsirisuthigul *et al*., 2007). Brown *et al.* (2009) have shown that antibody-enhanced DV infection of the FcR-bearing mast cell/basophil KU812 cell line results in a massive induction of apoptosis.

Effect of NO on DV replication

Charnsilpa *et al.* (2005) investigated the effects of NO on DV production and RNA replication in mouse neuroblastoma cells in the presence of an exogenous NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP). NO inhibited viral replication via suppressed RNA synthesis, resulting in reduction of NS1 synthesis. Thus, NO may serve as a defence that diminishes viral load in patients. Further, the activity of recombinant DV NS5 in negative-strand RNA synthesis is affected in the presence of 5 mM SNAP in *in vitro* RdRp assays, whereas the RNA helicase activity of DV NS3 is not inhibited (Takhampunya *et al*., 2006). Thus, the inhibitory effect of NO on DV infection is partly via inhibition of the RdRp activity, which then downregulates viral RNA synthesis. The clinical isolates of DV have been divided into two groups on the basis of their sensitivity to the inhibitory effect of NO radical on viral replication. Ubol *et al.* (2008a) have studied genotypic differences at the level of the amino acid sequence of the NS5 gene between NO-sensitive viruses isolated from DF patients and NO-resistant isolates from DHF patients. They found that these two groups of viruses contain different amino acid sequences at position 621–646 in the active site of NS5, suggesting that the response to the inhibitory effect of NO radical may, at least in part, be regulated by NS5. They have also studied the differences in global gene expression of NO-producing host cells in response to infection by the two groups of isolates using cDNA array analysis. NO-resistant viruses have a stronger influence on gene expression of the infected host cells in both the number and the type of genes.

Role of NO in the pathogenesis of DHF

Alteration in NO production is associated with a variety of pathological effects, such as vasodilation, inflammation, thrombosis, immune response and neurotransmission. NO production may initiate or suppress apoptosis but the mechanism is not fully understood. Macrophage lineage cells replicate DV and are professional NO producers. NO produced by them downregulates replication of DV. DV-infected mice spleen cells produce NO_2^-. CF/CF2 induce production of NO_2^- in the spleen cells in a Ca^{2+}-dependent manner, which may be a mechanism of target cell killing (Mukerjee *et al*., 1996). CF2 also induces production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by the spleen cells of mice *in vitro* and *in vivo* and the killing (Fig. 3) appears to be by generation of peroxynitrite (Misra *et al*., 1996a, b, 1998). Neves-Souza *et al.* (2005) reported that activation of DV-infected monocytes is based on induction of iNOS both *in vivo* and *in vitro*. A mechanism through which NO acts is via endothelin (ET-1), which is produced by endothelial cells. Thrombin, cytokines and ROS, etc., stimulate the vascular endothelium to produce and release ET-1. Release of ET-1 is inhibited by prostacyclin and NO. Binding of ET-1 to endothelial ET_b receptors stimulates the formation of NO. In the absence of smooth muscle endothelin receptor stimulation, this NO causes vasodilation (reviewed by Iglarz & Clozel, 2007; Basu & Chaturvedi, 2008). DV infection of human umbilical cord endothelial cells inhibits the production of ET-1 and prostacyclin 2, thus affecting their normal functions of secretion of vasoactive substances, resulting in increased vascular permeability and impairment of homoeostasis and blood coagulation (Jiang *et al*., 1999).

Anti-NS1 antibodies act as autoantibodies that cross-react with noninfected endothelial cells and trigger the intracellular signaling leading to the production of NO and to apoptosis. Endothelial cell damage may cause vascular leakage that contributes to the pathogenesis of dengue disease (Lin *et al*., 2002). Further, the mitochondria-dependent pathway that is regulated by NO production causes endothelial cell apoptosis in this system (Lin *et al*., 2004). Yen *et al.* (2008) have reported macrophage infiltration into the vicinity of the endothelium, increased TNF-α production and endothelial cell apoptosis in the tissues with increased expression of iNOS and nitrotyrosine in endothelium in the haemorrhagic tissues in DV-infected mice.

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In vitro studies showed that primary mouse and human endothelial cells are productively infected by DV and produce reactive nitrogen and oxygen species (RNS and ROS) and apoptotic cell death, which is greatly enhanced by TNF-α. N^G-nitro-L-arginine methyl ester and *N*-acetyl cysteine reverse the effects of DV and TNF-α on endothelial cells. Development and the severity of haemorrhage are markedly reduced in mice lacking iNOS or p47(phox) or in mice treated with oxidase inhibitor, indicating a possible use of antioxidant as a therapeutic agent for DHF (Yen *et al.*, 2008). This has been confirmed in patients with DHF/DSS where antibodies against NS1 present in patient sera cross-react with endothelial cells and induce apoptosis via a caspase-dependent pathway and cause cell lysis in the presence of complement. DHF/DSS patient sera show higher percentages of cytotoxicity than DF patient sera (Lin *et al.*, 2003). Antibodies directed against DV NS1 have been shown to cross-react with human platelets and endothelial cells, leading to endothelial cell damage and inflammatory activation (Lin *et al.*, 2006).

Conclusions and future perspectives

Multiple roles of NO in the pathogenesis of DV infections have been discussed as regards mutation of DV, nonspecific inflammatory responses and immunological host reactions modulated by NO that may cause DHF. A synthesis of the current understanding of the role of NO in the pathogenesis of DHF is presented in (Fig. 5). Replication of NO-sensitive DV is inhibited by the generation of NO, resulting in lower virus load and consequently a milder dengue disease, DF (Fig. 5). The NO-resistant DV are virulent, have a higher replication rate and have a stronger influence on host genetic response as compared with the NO-susceptible DV. NO-resistant DV induce greater expression of immune response-related genes, for example genes involving cytokines/chemokines, activation of T cells, B cells, platelets and inflammatory cells. This may result in the interaction between platelets, vascular endothelial cells and inflammatory cells that induces vascular leakage. Further, NO-resistant DV significantly upregulate IL-6, IL-7, IL-8, RANTES and MCP-3, which are correlated with increased DHF. Our findings in the sera of the patients with dengue and DHF show an inverse relationship between IL-4 and IL-10 to IFN-γ (Chaturvedi *et al.*, 1999). Higher levels of IL-6 and IL-8 that may be involved in vascular leakage and haemorrhage are found in DHF/DSS patients but not in DF patients (Raghupathy *et al.*, 1998; Chaturvedi *et al.*, 1999). Thus, NO has a central role in DV infection; inhibition of replication of DV by NO results in DF and failure to inhibit results in severe disease, DHF (Fig. 5).

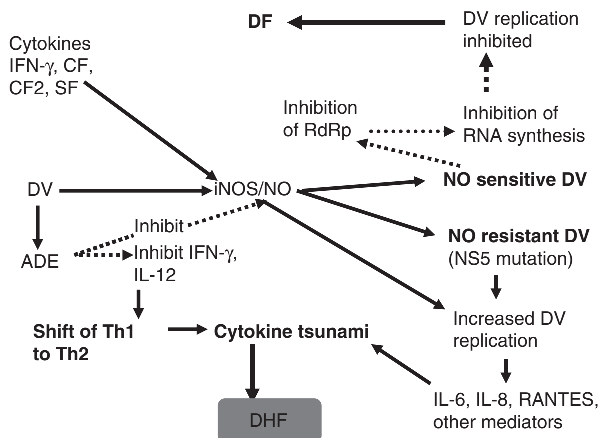

Fig. 5. Roles of NO during DV infection that may determine whether it will lead to DF or DHF.

The ultimate role of NO on DV pathogenesis is still far from completely understood and a lot more work remains to be done. To determine whether the severity of DV infection is determined by mutation of NS5, in-depth studies on NS5 genetic manipulation are needed. Are the phenotypes of DV determined by the differences in genotype of NS5? Effects of NO on DV NS5 have been investigated but what about other structural and nonstructural proteins of DV? In DV endemic areas all four serotypes are circulating in the population, resulting in concurrent infection with more than one DV or with other viruses. What is the role of NO in such infections? Infection of macrophages by DV–ADE complex suppresses innate immunity in infected cells, resulting in the higher production of virus per cell. Suppression of innate immunity is regulated by IL-10, but is the master molecule NO? More studies are required to understand these aspects.

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Professor Umesh C. Chaturvedi has retired from the position of the Head of the Department of Microbiology, K.G. Medical College (now CSM Medical University), Lucknow. The financial assistance to U.C.C. as INSA Honorary Scientist for the preparation of this manuscript by the Indian National Science Academy, New Delhi is gratefully acknowledged.

Statement

U.C.C. has retired and is now an INSA Honorary Scientist.

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