Neuraminidase augments Fcγ receptor II-mediated antibody-dependent enhancement of dengue virus infection

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Antibody-dependent enhancement (ADE) of dengue virus infection occurs when neutralizing antibodies at sub-neutralizing concentrations or non-neutralizing antibodies form complexes with the virus. These virus–antibody complexes can then attach to a Fcγ receptor-bearing cell, via the Fc portion of the immunoglobulin, resulting in an increased number of infected cells. ADE may be responsible in part for the most severe clinical manifestations of dengue virus infection which include haemorrhage and shock. Three classes of human Fcγ receptors exist, FcγRI, FcγRII and FcγRIII. In this study, we examined the effects of neuraminidase on ADE of dengue virus infection mediated by the low-affinity FcγRII. K562 cells, which express only FcγRII, treated with neuraminidase resulted in augmentation of ADE of dengue virus infection by human anti-dengue antibodies. This augmented ADE of infection could be blocked by anti-FcγRII monoclonal antibody IV.3.

Incubation of neuraminidase-treated K562 cells with IgG-coated human red blood cells resulted in an increase in the percentage of rosette formations compared with the untreated K562 cells. A bispecific antibody directed against FcγRII and dengue virus (IV.3 × 2H2) enhanced virus infection. Neuraminidase also augmented ADE mediated by this antibody, but to a much lesser degree (by 50%) compared with that seen using conventional human anti-dengue antibody (by 200 to 300%). Fluorescence-activated cell sorting analysis of neuraminidase-treated K562 cells showed that the number of FcγRII-specific antibodies that bind to FcγRII increases by 15 to 20% after treatment with neuraminidase. These results indicate that neuraminidase augments ADE of dengue virus infection and that the augmented ADE is mediated through FcγRII.

Introduction

Dengue viruses belong to the family Flaviviridae, genus flavivirus, and exist as four distinct serotypes. Transmission of dengue virus to humans occurs via a mosquito vector. Secondary infection by a dengue virus of a different serotype may result in severe clinical manifestations, including haemorrhage and plasma leakage, the so-called dengue haemorrhagic fever/dengue shock syndrome (Halstead et al., 1970; Sangkawibha et al., 1984; Burke et al., 1988). It is believed that these complications in part may be secondary effects of antibody-dependent enhancement (ADE) of infection which results in an increased number of infected monocytes (Halstead, 1988). The phenomenon of ADE occurs when neutralizing antibodies at sub-neutralizing concentrations or non-neutralizing antibodies bind dengue virus (Henchal et al., 1985; Morens et al., 1987). These virus–antibody complexes bind via the Fc portion of IgG to Fcγ receptor (FcγR)-bearing cells resulting in enhancement of dengue virus infection (Halstead et al., 1977; Peiris et al., 1981).

Human FcγRs are sialoglycoproteins that exist as three distinct classes. Human FcγRI has a high affinity for monomeric IgG and an Mr of 72K (Anderson, 1982). Both FcγRII and FcγRIII are low-affinity receptors of Mr 40K and 50K to 80K, respectively (Rosenfeld et al., 1985; Van de Winkel & Anderson, 1991). FcγRII is the most widely distributed FcγR and can be detected on monocytes, macrophages, neutrophils, platelets, eosinophils and B cells (Unkeless, 1989). We have shown that ADE occurs via FcγRI and FcγRII by using mouse anti-dengue virus antibodies (Kontny et al., 1988; Littaua et al., 1990). However, the affinity of human IgG for FcγRII is low even for antigen–antibody complexes (Tax & Van de Winkel, 1990). Therefore, it is possible that in vivo FcγRII may be modulated in order to be more active biologically. Modulation may occur by increasing the number of FcγRII, by affecting molecules on or around the FcγRII that may interact with IgG.
binding and/or by increasing the affinity of this receptor. It has been reported that various proteases can increase the affinity of FcγRII (Van de Winkel et al., 1989), and that neuraminidase treatment of human monocytes increases the interaction between IgG and FcγRII (Debets et al., 1990).

In this report we describe the effects of neuraminidase on ADE of dengue virus infection mediated via FcγRII. We use K562 cells which express FcγRII, but not FcγRI or FcγRIII. Neuraminidase-treated K562 cells infected with dengue virus–antibody complexes, formed using human anti-dengue antibody or a bispecific antibody directed against dengue virus and FcγRII, results in augmented ADE of infection. Incubation of neuraminidase-treated K562 cells with IgG-coated human red blood cells (RBC) results in an increase in the percentage of rosette-forming cells compared with that using untreated K562 cells. Therefore, modulating the function and expression of FcγRII by neuraminidase makes the FcγRII increasingly active in the ADE of dengue virus infection.

Methods

Cell line. The human erythroleukaemia cell line K562 (Lozzio & Lozzio, 1975) was grown in RPMI 1640 medium (Gibco), supplemented with 10% fetal calf serum (FCS) (Gibco), penicillin (100 units/ml) and streptomycin (100 μg/ml).

Viruses and titration of virus. Dengue 2 virus, New Guinea C strain, was supplied by Walter E. Brandt of the Walter Reed Army Institute of Research, Washington, D.C., U.S.A. The virus was propagated in mosquito cells (C6/36) as previously described (Kurane et al., 1984). The titre of virus pools was 3.5 × 10⁷ to 1 × 10⁸ p.f.u. per ml as determined by a plaque titration assay on CV-1 cells. Virus samples were serially 1:10 diluted with MEM/0.5% FCS at 4°C. CV-1 cell monolayers in 24-well plates were washed twice with MEM/0.5% FCS, then 0.1 ml of the serially diluted virus sample was placed on the CV-1 monolayer, and incubated at 37°C for 2 h. The plates were rocked every 15 min. After incubation, the fluids were removed, the cells were washed once with MEM/0.5% FCS, and 1 ml overlay, prepared as stated below, was placed on the cells. The plates were then incubated at 37°C for 7 days prior to plaque quantification.

Preparation of overlay for virus titration. A suspension of 2.3% carboxymethylcellulose in H₂O was prepared and autoclaved. For preparing 100 ml of overlay, 80 ml of 2.3% carboxymethylcellulose, 10 ml of 10×MEM, 4 ml of 7.5% NaHCO₃, 1 ml of 1% DEAE-dextran, 0.6 ml of essential vitamins, 0.6 ml of non-essential amino acids, 1 ml of FCS, 1 ml of the mixture of 10000 units/ml penicillin and 10000 μg/ml streptomycin and 1 ml of 200 mM-L-glutamine were mixed at room temperature.

Antibodies. IV.3 is a murine IgG2b monoclonal antibody (MAb) to FcγRII (Looney et al., 1986). BBM1 is a murine IgG2b MAb to β₂ microglobulin (Brodsky et al., 1979), 2H2 is a dengue complex-specific mouse IgG2a MAb directed against the preM protein (Henchal et al., 1985). IV.3 was kindly provided by Medarex.

The bispecific antibody IV.3 × 2H2 was prepared as previously described (Mady et al., 1991). Fab fragments of IV.3 at 2 to 4 mg/ml were treated with a fourfold molar excess of the bifunctional reagent N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Pharmacia) at 18°C for 2 h, and then dialysed in PBS, pH 7.4. 2H2 Fab in 50 mM-phosphate buffer, 5 mM-EDTA, pH 7-5 (phos/EDTA), was treated with a threefold molar excess of N-succinimidyl S-acetylthioacetate (SATA; Calbiochem-Behring Diagnostics), dissolved in dimethyl formamide at 18°C for 30 min. After dialysis in phos/EDTA, the SATA-2H2 was deacetylated by adding hydroxylamine to 0.05 M and 1 h incubation at 18°C. Hydroxylamine was removed by passage through a 10 ml Sephadex G-25 column (Pharmacia). The SATA-2H2 was mixed at once with an equimolar amount of IV.3 Fab, and incubated at 18°C for 4 h, after which cross-linking was terminated with 1 mM-iodoacetamide. Bispecific antibodies were then dialysed into PBS and purified by HPLC gel filtration chromatography using a Bio-Sil TSK-250 column (Bio-Rad). Coupling one antibody fragment to SPDP and the other to SATA, as described above, allowed the directed preparation of heteroconjugates while minimizing the amount of homocojugates formed. Unreacted monomers were removed by the HPLC purification step. The bispecificity of these preparations was ensured by testing binding to cells using indirect immunofluorescence and by testing binding to dengue virus by ELISA.

The serum from a donor immunized 2 years earlier with the dengue 3 virus strain CH53489 (Innis et al., 1988) was used as a source of anti-dengue virus antibody. The neutralizing titre of this serum was 1:16 for dengue 3 virus and <1:4 for dengue 2 virus. This serum detected dengue 2 virus antigens by indirect immunofluorescence staining.

Treatment of cells with neuraminidase. K562 cells were initially washed in neuraminidase buffer (10 mM-sodium phosphate buffer pH 6.1, 150 mM-NaCl, 5 mM-CaCl₂). Then 1 ml of neuraminidase (Clostridium perfringens, type V, Sigma) at various concentrations diluted in RPMI/10% FCS or medium alone was added to 2 × 10⁶ cells, incubated at 37°C for 30 min and the cells were washed twice with RPMI/10% FCS.

Infection of cells with dengue virus. Virus–antibody complexes were prepared by incubating dengue virus and antibody at 4°C for 1 h. After washing in RPMI/10% FCS, K562 cells were resuspended at 2 × 10⁶ cells/ml. Cell suspension (0.05 ml) was added to 0.2 ml of dengue virus or to the dengue virus–antibody complexes and incubated for 1 h at 37°C. The cells were infected at an m.o.i. of 10 p.f.u./cell or as specified. Following infection, the cells were washed twice with RPMI/10% FCS and cultured in 1 ml of RPMI/10% FCS at 37°C for 24 or 48 h. In the blocking experiments the K562 cells were first incubated with the blocking antibody at room temperature for 1 h and then infected as described above.

Immunofluorescence. Following the 24 or 48 h incubation, the cells were stained for the presence of dengue viral antigens by indirect immunofluorescence as previously described (Littaua et al., 1990). The first antibody was from a murine hyperimmune ascitic fluid raised against dengue 2 virus and the second antibody was a fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (Cappel Laboratories). The neuraminidase-treated and untreated K562 cells were stained with anti-FcγRII MAb IV.3 and then with a second antibody, FITC-conjugated sheep anti-mouse IgG. Fluorescence was determined with a Becton-Dickinson flow cytometer. Quantitative fluorescence microbeads (Flow Cytometry Standards) were used for calibration to convert the fluorescence intensity values obtained from the flow cytometer into the number of molecules of second antibody bound per cell.

Rosetting. K562 cells were washed with neuraminidase buffer and then incubated with RPMI/10% FCS or neuraminidase at 1.25 × 10⁻¹ units/ml as described above. Human RBC were washed three times with RPMI and incubated with various concentrations of human RhD antibody (Cutter Biological) for 30 min at 37°C. The RBC were then washed three times with RPMI and resuspended at a concentration of 1 × 10⁹ cells/ml. K562 cells (0.1 ml, 10⁶/ml) were combined with 0.1 ml of 1 × 10⁹ RBC/ml, centrifuged at 4°C at
1000 r.p.m. for 10 min, and then allowed to stand at 4 °C for 2 h. The cells were then gently pipetted and 250 or more K562 cells were counted. Observation of four or more RBC adherent to a K562 cell was interpreted as positive rosette formation.

Statistical analysis. Differences between the percentage of dengue virus antigen-positive cells were examined by chi-square analysis. Differences yielding P values of < 0.05 were regarded as significant.

Results

Neuraminidase augments FcγRII-mediated ADE of dengue virus infection

K562 cells were used to determine the effect of neuraminidase on ADE of dengue virus infection via FcγRII, because they express only FcγRII and not FcγRI or FcγRIII (Littaua et al., 1990). K562 cells were treated with different concentrations of neuraminidase and then infected with dengue virus at an m.o.i. of 10 or 0.4 p.f.u./cell in the presence or absence of human anti-dengue virus antibody. The percentage of dengue virus antigen-positive cells was examined by indirect immunofluorescence 24 and 48 h after infection. The dengue antibody-positive human serum enhanced virus infection of untreated cells (Table 1). The highest level of enhancement found by using this antibody was observed at a 1:200 dilution (data not presented). When the cells were pretreated with neuraminidase, the antibody enhanced virus infection (as determined by the percentage of dengue virus antigen-positive cells) to significantly higher levels than that seen with untreated cells (Table 1). In addition, the viral titres from the culture supernatant fluids of K562 cells treated with neuraminidase and infected with dengue virus–antibody complexes were higher than in the supernatants of cells not treated with the enzyme but similarly infected (Table 1). Treatment of cells with neuraminidase did not augment infection by dengue virus alone. Human serum from a dengue antibody-negative donor did not augment virus infection even when cells were pre-treated with neuraminidase (data not presented). These results demonstrated that neuraminidase augments FcγRII-mediated ADE of dengue virus infection.

MAb to FcγRII inhibits neuraminidase-induced augmentation of ADE

To confirm that neuraminidase augments ADE via FcγRII, neuraminidase-treated K562 cells were incubated with MAb to FcγRII and then infected with dengue virus–antibody complex. The mouse IgG2b MAb IV.3 directed against FcγRII inhibited the enhancement of infection mediated by anti-dengue antibody (Table 2) and had no significant effect on the infection of K562 cells by virus alone. In addition, the isotype control mouse IgG2b MAb BBMI did not inhibit infection of K562 cells with dengue virus or virus–antibody complex. These results, along with the observation that neuraminidase does not augment infection in the absence of anti-dengue antibodies, indicate that augmentation of ADE by neuraminidase is mediated through FcγRII.

Neuraminidase augments FcγRII-mediated ADE by modulating the function and expression of the receptor

In order to evaluate the mechanisms by which neuraminidase augments ADE of infection, we examined whether enzyme treatment of K562 cells increased rosette formation via FcγRII using human RBC opsonized with human RhD antibody. The percentage of rosette-forming cells increased after neuraminidase treatment (data not presented). We then examined the effect of neuraminidase on ADE mediated by a bispecific antibody (IV.3 × 2H2) which binds to both dengue virus and FcγRII. As previously reported, this antibody can mediate dengue virus ADE in K562 cells (Mady et al., 1991). ADE mediated by the antibody was augmented in neuraminidase-treated K562 cells, but to a much lower degree than that seen using conventional anti-dengue

Table 1. Neuraminidase augments FcγRII-mediated dengue virus infection

<table>
<thead>
<tr>
<th>Neuraminidase (units/ml)</th>
<th>Human anti-dengue antibody</th>
<th>Dengue antigen-positive cells (%)†</th>
<th>Viral titre (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
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<tr>
<td>Experiment 1</td>
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<td>(m.o.i. 10)</td>
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<td>−</td>
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<td>16.9</td>
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<tr>
<td>1:25</td>
<td>−</td>
<td>3.0</td>
<td>30</td>
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<tr>
<td>1:25 x 10⁻²</td>
<td>−</td>
<td>4.3</td>
<td>9.6</td>
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<tr>
<td>× 10⁻³</td>
<td>−</td>
<td>4.6</td>
<td>8.6</td>
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<tr>
<td>× 10⁻⁴</td>
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<tr>
<td>1:25 x 10⁻³</td>
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<td>32.6</td>
<td>50.2</td>
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<tr>
<td>× 10⁻⁴</td>
<td>+</td>
<td>19.1</td>
<td>38.9</td>
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<tr>
<td>× 10⁻⁵</td>
<td>+</td>
<td>13.2</td>
<td>25.4</td>
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<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
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<tr>
<td>(m.o.i. 0.4)</td>
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<tr>
<td>−</td>
<td>−</td>
<td>&lt;0.2</td>
<td>0.8</td>
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<tr>
<td>−</td>
<td>+</td>
<td>2.9</td>
<td>6.2</td>
</tr>
<tr>
<td>1:25 x 10⁻¹</td>
<td>−</td>
<td>&lt;0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>1:25 x 10⁻¹</td>
<td>+</td>
<td>10.3</td>
<td>18.9</td>
</tr>
</tbody>
</table>

* K562 cells were pre-treated with neuraminidase (where indicated), washed and then infected with dengue virus or virus–antibody complex at an m.o.i. of 10 p.f.u./cell (experiment 1) or 0.4 p.f.u./cell (experiment 2). The percentage of dengue antigen-positive cells was examined at 24 and 48 h after infection.
† The percentage of dengue virus antigen-positive neuraminidase-treated cells infected with virus–antibody complex was compared to the proportion of infected positive cells without neuraminidase treatment by chi-square analysis. *P < 0.001, **P < 0.01, ***P < 0.05; NS, Not significant.
antibody (Table 3). Neuraminidase augmented ADE using human anti-dengue virus antibody by 182% in experiment 1 and 286% in experiment 2, and this augmentation was only 53% in experiment 1 and 42% in experiment 2 when the bispecific antibody was used.

To determine whether more antibody molecules bind to FcγRII after neuraminidase treatment, enzyme-treated K562 cells were stained with IV.3 at various dilutions and then analysed with a fluorescence-activated cell sorter to quantify the number bound to the receptor. Quantitative fluorescein microbeads were used for calibration to convert the fluorescence intensity values obtained from the flow cytometer into the number of molecules of second antibody bound per cell. This number is a relative measure of the number of FcγRII per cell. The number of molecules that bound to FcγRII increased by 15% and 20% at antibody dilutions of 1:10 and 1:10², respectively, after neuraminidase treatment of K562 cells (Table 4). At higher dilutions the number of IV.3 molecules bound per cell approached the IgG2b control number. The discrepancy between the levels of augmentation of ADE by human anti-dengue virus antibody and those by the bispecific antibody, along with the low level of increase in the number of MAb IV.3 molecules bound per cell approached the IgG2b control number. The discrepancy between the levels of augmentation of ADE by human anti-dengue virus antibody and those by the bispecific antibody, along with the low level of increase in the number of MAb IV.3 molecules that bind to FcγRII after neuraminidase treatment suggest that this enzyme may act in a number of ways to augment ADE of dengue virus infection.

**Discussion**

The human IgG Fc receptors mediate a number of cellular functions including antibody-dependent cell-mediated cytotoxicity, phagocytosis and superoxide generation (Van de Winkel & Anderson, 1991). In addition, FcγRs may bind virus–antibody complexes and enhance infection. This phenomenon is termed antibody-dependent enhancement (ADE). ADE of dengue virus infection has been demonstrated to occur via FcγRI (Kontny et al., 1988) and FcγRII by using mouse anti-dengue antibodies (Littaua et al., 1990). Interferon γ up-regulates FcγRI (Guyre et al., 1983; Perussia et al., 1983) and thereby augments ADE of dengue virus infection via this receptor (Kontny et al., 1988). Although it is not known whether modulation of FcγRII leads to augmentation of ADE, it has been reported that the expression and affinity of FcγRII are modulated by
plegetants and enzymes. Granulocyte and granulocyte-
macrophage colony-stimulating factors up-regulate
FcγRII expression (Liesveld et al., 1988). The proteases
trypsin, pronase and elastase have been shown to increase
its affinity without affecting the Mₙ, isoelectric focusing
pattern or expression of this receptor (Van de Winkel et
al., 1989). In addition, neuraminidase also has been
shown to modulate the function of FcγRII (Debets et al.,
1990).

Using human anti-dengue virus antibody and
neuraminidase-treated K562 cells there was a significant
augmentation of ADE of infection as determined by the
percentage of dengue antigen-positive cells and viral
titres. Similar results were also seen using murine anti-
dengue virus antibody (data not presented). This ADE
could be significantly blocked when the cells had been
incubated with anti-FcγRII MAb IV.3 before infection
by virus-antibody complexes.

There are a number of possible mechanisms by which
neuraminidase may augment this ADE. Firstly,
neuraminidase may act by affecting the as yet
unidentified dengue virus receptor. At high enzyme
concentrations (5 units/ml) there was a decrease in the
number of infected cells when virus alone was used;
however, dengue virus-antibody complexes were able to
overcome this effect. At the concentration of
neuraminidase (1.25 x 10⁻⁶ units/ml) used here, there
was no effect on the infection of K562 cells with dengue
virus alone. We conclude that the augmentation of ADE
is secondary to the effects of neuraminidase on FcγRII
itself.

This conclusion is supported by the fact that in ro-
setting experiments using human RBC, human RhD
antibody and K562 cells showed an increase in rosettes in
neuraminidase-treated K562 cells compared to untreated
cells, confirming the results of Debets et al. (1990). In
addition, the augmented ADE of infection could be
blocked with anti-FcγRII MAb IV.3.

Another possible mechanism of action of
neuraminidase is that it increases the number of FcγRII
molecules. Quantitative fluorescence analysis
demonstrated an increase in the amount of MAb IV.3
binding to the receptor after neuraminidase treatment
but the increase approached only 20% between
neuraminidase-treated and untreated K562 cells. The
augmentation of ADE using human anti-dengue anti-
body was 182 to 286% more in the neuraminidase-
treated than in the untreated cells, and the augmentation
of ADE using bispecific antibody was only slightly
greater than 50%. Therefore, it is unlikely that the
augmentation of ADE can be accounted for purely by an
increased amount of FcγRII.

Neuraminidase may also increase the affinity of
FcγRII. The bispecific antibody consists of a Fab portion
of IV.3 covalently linked to a Fab portion of 2H2 (anti-
dengue virus MAb). If the augmentation of ADE with human anti-dengue virus antibody is exclusively
mediated by an increase in the affinity of FcγRII for the
Fc portion of IgG, then one would not expect ADE by
the bispecific antibody to be augmented after
neuraminidase treatment. However, such ADE of infec-
tion was clearly augmented in neuraminidase-treated
K562 cells, although to a lesser degree than that seen
with whole human anti-dengue virus antibody.

Lastly, neuraminidase may increase the accessibility of
FcγRII. We speculate that neuraminidase cleaves sialic
acid residues on FcγRII, allowing more efficient binding
of both the Fc portion of IgG and the Fab of MAb IV.3
to its epitope on FcγRII. In areas of inflammation
various enzymes including proteases and neuraminidase
have been reported to be present (LaMarco et al., 1986;
Tax & Van de Winkel, 1990). It is possible that in vivo,
during dengue virus infection, these enzymes modulate
the function and expression of FcγRII allowing it to be
active in ADE of infection, and to play an important role
in the pathogenesis of dengue virus infection.

We thank Jurand Janus for technical assistance and Marcia
McFadden for fluorescence activated cell sorter analysis. This work
was supported by grants from the United States Army Medical Research and Department Command (DAMD 17-86-C-6208), and
from the NIH (NIH-RO1-AR0624, NIH-T32-AR07722). The opinions
contained herein are those of the authors and should not be construed
as representing the official policies of the Department of Army or the
Department of Defense.

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(Received 7 September 1992: Accepted 5 January 1993)