Influence of the Human High-Affinity IgG Receptor FcγRI (CD64) on Residual Infectivity of Neutralized Dengue Virus

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We examined dengue virus immune complex–phagocyte interaction with respect to a single Fc receptor class using a transient expression system involving the high-affinity human macrophage receptor, FcγRI. We found that New Guinea C strain dengue 2 virus formed well-defined plaques in normal and transfected COS cells and we analyzed the structural determinants of FcγRI-mediated binding and internalization of dengue 2 virus immune complexes by expressing native or truncated forms of the receptor in COS cells, alone or with its accessory γ chain signaling unit, which bears an immunoreceptor tyrosine-based activation motif (ITAM). The residual infectivity of dengue 2 virus treated with neutralizing human antisera was strikingly higher in FcγRI-bearing COS cells than in controls. Compatible with the IgG subclass specificity of FcγRI, this difference was abrogated quantitatively by treatment of FcγRI-transfected cells with human IgG1 but not IgG2 myeloma protein. The magnitude of receptor-mediated plaque formation after cotransfection with γ chain was also significantly higher than in controls but was less than that observed with FcγRI transfection only, a difference probably explained by reduced levels of FcγRI expression in γ chain cotransfectants. Deletion of the FcγRI cytoplasmic domain had no effect on receptor-mediated immune complex infectivity. We conclude that the FcγRI extracellular domain is sufficient for internalization of infectious dengue virus immune complexes through a mechanism that does not involve classical ITAM-dependent signaling.

INTRODUCTION

Antibody-complexed virus may escape neutralization by preferentially infecting macrophages after Fc receptor (FcR) attachment. This in vitro phenomenon underlies older observations of increased residual infectivity (Kjellen and Schlesinger, 1959) and paradoxically enhanced viral replication (Hawkes, 1964) when partially neutralized vesicular stomatitis virus or several flaviviruses were titrated in primary chick embryo fibroblast monolayers. Enhanced virus plaque formation in these cultures was subsequently explained when they were found to harbor functional macrophages (Kliks and Halstead, 1980). Immune enhancement has since been observed with a number of disparate RNA and DNA viruses grown in cultured mammalian macrophages in the presence of anti-viral antibodies (reviewed in Dimmock, 1993). Despite its apparent ubiquity, immune enhancement remains enigmatic; its molecular basis, like that of neutralization itself, is incompletely understood and its biologic significance is poorly defined. Among viruses whose growth in macrophages can be amplified by antiviral antibody, dengue virus, an enveloped positivesense RNA flavivirus, has received considerable attention because of persuasive seroepidemiologic evidence linking immune status of the dengue virus-infected individual to risk of life-threatening complications; FcR-bearing cells of macrophage/monocyte lineage are believed to be the primary if not exclusive site of replication in the course of human dengue virus infection (reviewed in Halstead, 1988).

Previous in vitro studies of antibody-mediated viral replication in macrophages have exclusively employed cultured cells that simultaneously express multiple FcR classes and isoforms of differing physiology. Such heterogeneity together with evidence of cooperative interactions among FcRs of differing classes (reviewed in Hulet and Hogarth, 1994; Daeron, 1997) presents obvious complexities in the interpretation of experimental results. To examine the nature of dengue virus immune complex–FcR interaction and its consequences with respect to a single receptor class, we have adopted a transient expression system in COS-7 fibroblasts involving human FcγRI (Allen and Seed, 1989; Ernst et al., 1992), one of three classes of macrophage receptors for IgG. FcγRI exists as a 72-kDa integral membrane glycoprotein composed of three domains: extracellular Fc piece-binding, short transmembrane, and cytoplasmic "tail." It is distinguished from other Fc receptors by its high affinity for both monomeric and complexed IgG and by in vivo evidence that it enhances antigen presentation and augments antigen-specific antibody responses in

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FcγRI transgenic mice (Heijnen et al., 1996). The transmembrane portion of FcγRI associates with a short (22 kDa) homodimeric transmembrane subunit, the γ chain, which confers signal transduction capacity by providing an immunoreceptor tyrosine-based activation motif (ITAM) that FcγRI lacks (Allen and Seed, 1989). The γ chain subunit enhances affinity for ligand (Miller et al., 1996) and is necessary for efficient FcγRI-mediated phagocytosis of opsonized large particles (Davis et al., 1995; Lowry et al., 1998), a process that requires protein tyrosine kinase-catalyzed ITAM phosphorylation. The FcγRI extracellular domain alone, expressed in COS cells, has been reported to be sufficient for pseudopod extension (Lowry et al., 1998), for endocytosis of FcγRI-bound radiolabeled IgG, and for FcγRI entry into an internalization-recycling pathway after IgG binding (Harrison et al., 1994; Davis et al., 1995). COS cells that express FcγRI, properly configured with its accessory γ chain, thus exhibit important characteristics of professional phagocytes with respect to FcγR-mediated antigen uptake and metabolic consequences of immune complex-stimulated FcγRI clustering. Additionally, as described herein, they offer a convenient system to examine FcγR-dengue immune complex interaction, since we have found that dengue virus type 2 (New Guinea C strain) forms well-defined plaques in FcγRI-transfected COS cell monolayers, a property of dengue virus not present in cultured macrophages or macrophage-like cell lines (Peiris and Porterfield, 1981; J. J. Schlesinger, unpublished results). In the present study, we analyzed structural determinants of FcγRI-mediated binding and internalization of dengue 2 virus immune complexes by expressing native or truncated forms of the receptor in COS cells, alone or in association with the γ chain signaling unit.

RESULTS AND DISCUSSION

The residual infectivity of partially neutralized dengue 2 virus is increased in FcγRI-transfected COS cells

Figure 1 shows the relative neutralizing effect of two concentrations of dengue virus antiserum in control and FcγRI-transfected COS cells. FcγRI influence on residual infectivity of neutralized dengue virus was examined by displacement titration with human IgG1 or IgG2 myeloma proteins. Preformed virus–antibody complexes and myeloma proteins or albumin were mixed with trypsinized COS cells and the suspensions were delivered to a
24-well cluster plate for plaque assay. The antiserum exhibited potent neutralizing activity that was unaffected by myeloma protein in control COS cells. Residual infectivity of virus–antibody complexes was considerably increased (>100-fold) in FcγRI-transfected cells and, consistent with the relative affinities for FcγRI, receptor blockade with high concentrations of IgG1, but not IgG2, myeloma abrogated this effect. These findings indicate that cell surface FcγRI subserves dengue virus immune complex infectivity in COS cells and that FcγRI-mediated internalization of virus–antibody complexes is independent of protein tyrosine kinase-based signaling.

The γ chain subunit does not augment FcγRI-mediated internalization of infectious dengue virus immune complexes

Although FcγRI was sufficient for internalization of infectious dengue 2 immune complexes, the γ chain’s capacity to markedly increase this receptor’s affinity for ligand (Miller et al., 1996) and uncertainty concerning a possible synergistic ITAM effect on internalization of antibody-complexed virus prompted us to test whether cotransfection with FcγRI and the ITAM-bearing γ chain subunit affected dengue immune complex infectivity. COS cells, transfected with FcγRI and γ chain individually or in combination, were exposed to preformed virus immune complexes. In parallel assays we used antibody-sensitized sheep red blood cells to measure rosetting and phagocytosis as confirmation of FcγRI and FcγRI/γ chain coexpression, respectively. Since cotransfection with γ chain has been shown to reduce total FcγRI concentration circa threefold, as measured by Scatchard analysis (Miller et al., 1996), we varied the cDNA transfectant ratios but found no significant differences in residual infectious virus titers or in rosetting or phagocytosis indices among several FcγRI:γ chain ratios (see Materials and Methods). Therefore, these data were pooled. A total of nine experiments were performed using a fixed amount of virus (1 × 10⁶ PFU) treated with two dilutions of partially neutralizing antiserum. Although plaque counts varied considerably and unpredictably from one experiment to another, a consistent pattern of increased residual infectivity, statistically significant at both antiserum dilutions, was observed with FcγRI and FcγRI/γ chain-expressing cells compared to control cells (Table 1A). As expected, serum neutralizing activity was unaffected in γ chain transfectants. Interestingly, co-transfection was associated with a consistent, approximately twofold reduction in immune complex infectivity at both antiserum dilutions (1/800, P < 0.02; 1/1600, P < 0.001) compared to that found with cells expressing FcγRI only. We postulate that this difference is explained by the previously demonstrated several-fold reduction in FcγRI density that attends γ chain cotransfection (Miller et al., 1996), although we cannot rule out an unrelated "down-regulating" effect of the γ chain on FcγRI-mediated viral entry or subsequent replication events.

The extracellular FcγRI domain is sufficient to augment internalization of infectious dengue virus immune complexes

Finally, we analyzed whether deletion of the FcγRI cytoplasmic domain (tail) affects infectious immune complex internalization by comparing results with COS cells expressing native and truncated forms of the receptor. The function of the FcγRI tail has not been well defined. Its removal is associated with a slight increase in binding affinity of the truncated form (Miller et al., 1996) and

### Table 1

<table>
<thead>
<tr>
<th>COS transfectant</th>
<th>1/800</th>
<th>1/1600</th>
<th>Percentage of rosetted cells</th>
<th>Phagocytic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control</td>
<td>2 ± 1</td>
<td>9 ± 3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>γ chain</td>
<td>2 ± 1</td>
<td>8 ± 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FcγRI</td>
<td>21 ± 5 P = 0.001*</td>
<td>110 ± 16 P &lt; 0.001</td>
<td>30 ± 4</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>FcγRI + γ chain</td>
<td>9 ± 2 P = 0.017</td>
<td>51 ± 8 P = 0.002</td>
<td>28 ± 2</td>
<td>115 ± 8</td>
</tr>
<tr>
<td>B. Control</td>
<td>2 ± 1</td>
<td>8 ± 2</td>
<td>0</td>
<td>nd*</td>
</tr>
<tr>
<td>FcγRI</td>
<td>26 ± 5 P = 0.003</td>
<td>143 ± 17 P &lt; 0.001</td>
<td>29 ± 3</td>
<td>nd</td>
</tr>
<tr>
<td>FcγRI tailless</td>
<td>17 ± 4 P = 0.011</td>
<td>121 ± 24 P = 0.003</td>
<td>23 ± 4</td>
<td>nd</td>
</tr>
</tbody>
</table>

* Rosetting was expressed as the percentage of cells binding three or more red blood cells sensitized with a subagglutinating amount of rabbit anti-sheep erythrocyte antiserum.

* The phagocytic index was defined as the number of SRBC internalized by 100 FcγRI-expressing COS cells.

* Mean ± SEM.

* Compared to control condition (Student’s t test).

* Not determined.

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Miller et al., 1996
limited data suggest that a tailless FcγRI construct sub-
serves endocytosis, at least as measured by FcγRI in-
ternalization after receptor cross-linking by antibody
(Davis et al., 1995). However, it is uncertain whether
mechanisms of endocytosis involving FcR aggregated by
IgG cross-linkage are the same as those that govern
FcR-mediated internalization of infectious antibody-com-
plicated virus. Table 1B shows results of four experiments
performed in duplicate that demonstrate essentially
equivalent mediation of immune complex infectivity by
native FcγRI and mutant FcγRI tailless-transfected COS
cells after exposure to preformed dengue immune com-
plexes.

Molecular dissection of FcγRI biology indicates that
phagocytosis (internalization of large opsonized par-
ticles) and endocytosis (internalization of soluble immune
complexes) are biologically distinguishable. In both pro-
cesses ligand-clustered FcγRI is internalized, but only for
efficient phagocytosis are protein tyrosine kinase activa-
tion and ITAM phosphorylation essential. Taken together,
our results with an opsonized virus are compatible with
and extend earlier work of Davis et al. (1995), who
showed ITAM-independent FcγRI internalization (endo-
cytosis) after IgG cross-linking, and of Mady et al. (1991),
whose findings with bispecific antibodies against cell
membrane-associated proteins other than FcR on human
macrophage-like U937 cells suggested that FcR attach-
ment of dengue immune complexes facilitated infectivity
by *"focusing"* virus onto the cell surface. It remains un-
known whether a specific virus receptor acts in concert
with FcγRI to effect internalization of infectious antibody-
complexed dengue virus; whether FcγRI accompanies,
and if so, directs the complexes on their probable jour-
ney to lysosomes; or if, in *vivo*, dengue virus immune
complex-induced FcγRI clustering (that likely includes
coaggregation of other FcR classes) might coincidentally
activate ITAM-dependent pathways of immunopathologic
importance.

**MATERIALS AND METHODS**

**Cells and viruses**

COS-7 cells (obtained from Dr. Clark Anderson, Ohio
State University, Columbus, OH), maintained in D-MEM
supplemented with 10% fetal bovine serum, were plated
at a density of ca. 1–2 × 10^6 cells in 60- or 100-mm
plastic petri dishes and transfected the next day when
monolayers were ca. 80% confluent. C6/36 Aedes albo-
pictus mosquito cells, grown at 28°C in MEM supple-
mented with 5% fetal bovine serum, were used to prop-
agate New Guinea C strain dengue 2 virus, obtained in
suckling mouse brain from Dr. Walter E. Brandt (Walter
Reed Army Institute of Research, Washington, DC). Virus
was titered by plaque assay in COS and Vero cells:
plating efficiency was equivalent in both cell types.

**Plasmid constructs and transfections**

The human FcγRI (isform A) cDNA and its tailless
mutant (missing 61 amino acids from the protein’s c-
terminus), each cloned into the pCDM vector, have been
previously described (Ernst et al., 1992; Miller et al., 1996)
and were generously provided by Dr. Clark L. Anderson.
The human γ chain cDNA in vector pSVL and monospee-
cific rabbit serum 934 against it have been described
(Kuster et al., 1990; Letourneur et al., 1991) and were gifts
from Dr. Jean-Pierre Kinet (Harvard University, Cam-
bridge, MA). Mouse monoclonal anti-FcγRI antibody 32.2
(Anderson et al., 1986) was obtained from Dr. John
Looney (University of Rochester, Rochester, NY). Plasm-
ids were propagated in transformed JM109 strain *Esch-
erichia coli* and purified using Qiagen affinity columns.
The cDNAs were individually or cotransfected into COS-7
cells by the diethylaminoethyl—dextran method (Ernst et
al., 1992) as described by Lowry et al. (1998). In some
cotransfection experiments several FcγRI-γ chain ratios
(3:6, 3:3, 3:1 µg plasmid DNA/100-mm dish) were used.
Cells were used 48–72 h after transfection when expres-
sion was generally on the order of 20–50% as estimated
by indirect immunofluorescence using anti-FcγRI or an-
ti-γ chain antibodies. Phase-contrast and fluorescence
microscopy were used as described by Lowry et al.
(1998) to measure FcγRI expression or coexpression
with the γ chain subunit by rosetting or phagocytosis of
antibody-sensitized FITC-labeled sheep red blood cells,
respectively. Rosetting was expressed as the percentage
of cells binding three or more SRBC sensitized with a
subagglutinating amount of rabbit anti-sheep erythrocyte
antiserum. The phagocytic index was defined as the
number of SRBC internalized by 100 FcγRI-expressing
COS cells.

**Dengue 2 virus immune complexes and COS cell
plaque assay**

Convalescent anti-dengue virus sera from Thai and
Puerto Rican dengue fever patients were generously
provided by Drs. Eric Henchal (Walter Reed Army Insti-
tute of Research, Washington, DC) and Gladys Sather
(Centers for Disease Control, Puerto Rico), respectively:
a single antiserum pool that neutralized dengue 2 virus
was used with C6/36 mosquito cell-passaged New
Guinea C strain dengue 2 virus (pool titer: ca. 1 × 10^6
PFU/ml) to prepare antibody–virus complexes. These
were formed by incubating 1–5 × 10^5 PFU virus with
antiserum for 90 min at 37°C; antiserum diluted ca.
1/1000 to 1/5000 gave maximally enhanced virus plaque
formation in FcγRI-transfected COS cells. Immune com-
plexes were added to trypsinized COS cells in suspension
[FcγRI is trypsin-resistant (Anderson and Abraham,
1980)] and the mixture was immediately transferred to
60-mm dishes or tissue culture cluster plates, 1 × 10^5
cells/60-mm dish, proportionately fewer for smaller for-
mats. These were incubated overnight to allow monolayer formation before agarose overlay. Neutral red-containing agarose was added 4 days after infection and plaques were photographed with Kodak LPD4 Precision Line high-contrast, high-resolution transparency film for counting by projection onto a screen. Data were analyzed for statistical significance using Student’s t test.

**FcyRI blockade by myeloma proteins**

Highly purified human IgG1 and IgG2 myeloma proteins were gifts from Dr. George Abraham (University of Rochester, Rochester, NY). Myeloma proteins or control human serum albumin were mixed with immune complex solutions before incubation with trypsinized control or transfected COS cells.

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**REFERENCES**


