

## Infection Enhancement of Dengue Type 2 Virus in the U-937 Human Monocyte Cell Line by Antibodies to Flavivirus Cross-Reactive Determinants

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Dengue type 2 virus replication was detected in the U-937 human monocyte cell line when the virus inoculum and the culture medium contained flavivirus antibodies diluted beyond their neutralizing titers. This was in marked contrast to yellow fever virus, which replicated very well in the absence of antibodies; however, 10-fold-higher yields of yellow fever virus could be obtained in the presence of flavivirus antibodies. These infection-enhancing antibodies were obtained from either a dengue type 2 human antiserum or reference hyperimmune mouse ascitic fluid. The infection enhancement phenomenon, previously shown to be due to infection of Fc receptor-bearing cells with virus-antibody complexes, was completely blocked by preincubation of the cells with aggregated gamma globulin. The blocking results suggested an Fc receptor-mediated infection of the U-937 cells as well. A panel of monoclonal antibodies, previously characterized as either virus type specific or flavivirus cross-reactive and with mouse immunoglobulin subclasses G1 and G2a in both categories, were tested for their infection enhancement characteristics. A type-specific neutralizing monoclonal antibody preparation that was diluted beyond its neutralization titer did not cause infection enhancement, nor did low-level neutralizing monoclonal antibodies that were dengue serotype specific by the hemagglutination inhibition test. Only flavivirus cross-reactive monoclonal antibodies caused infection enhancement, irrespective of whether the immunoglobulins were G1 or G2a. These cross-reactive flavivirus determinants may reside at the tips of the glycoprotein projections on the virus particles, enabling the Fc ends of the cross-reactive antibodies attached to these determinants to interact with Fc receptors on susceptible cells.

The concept of immune enhancement of dengue virus replication was proposed by Halstead and associates (8) to explain the higher viremias observed in monkeys having heterologous antibodies from a previous infection with another serotype of dengue virus. As an *in vitro* correlate of this concept, greater quantities of dengue virus can be obtained from cultures of peripheral blood leukocytes from dengue-immune donors than from peripheral blood leukocytes from normal donors (9). Greater yields of dengue virus could also be obtained from normal donor peripheral blood leukocytes (10) as well as from the adherent monocyte fraction (3) when subneutralizing quantities of dengue antibodies were mixed with the virus inoculum. In these studies, dilute antibodies were also included in the culture medium to react with progeny virus, thus permitting subsequent cycles of infection by virus-antibody complexes.

The available data suggest that the greater flavivirus yields are due to increased numbers of

monocytes infected by complexes of virus and nonneutralizing immunoglobulin G (IgG) via cell surface Fc receptors. Various studies have shown that: (i) intact IgG molecules enhance dengue infection of monocytes, but F(ab')<sub>2</sub> fragments do not (11); (ii) infection enhancement is restored if the Fc portion is restored to the virus-F(ab')<sub>2</sub> complex by adding an IgG directed against human Fab (5); (iii) pretreatment of human monocytes with aggregated human IgG (5) or of U-937 cells with human IgG1, but not IgG2 (22), partially blocks infection with virus-antibody complexes; (iv) monoclonal antibodies against Fc receptors block infection of mouse macrophage cell lines with virus-antibody complexes (18); (v) the most potent infectious virus-antibody complexes were those which were formed using antiserum with high cross-reactive titers to other flaviviruses (12); and (vi) flavivirus receptors and Fc receptors could be differentiated on human monocytes (5) or on U-937 cells (22) by their sensitivity to trypsin (pretreat-

ment of the monocytes with trypsin before infection removed virus receptors, but not Fc receptors).

The purpose of this paper is twofold. First, it is to show that the U-937 human monocyte cell line does not have dengue virus receptors as measured by a productive infection, a finding which greatly facilitates demonstration of antibody-mediated virus infection. In simultaneous experiments, we have also confirmed that U-937 cells do support yellow fever replication in the absence of antibody (22), indicating that the same receptor does not result in a productive infection by all flaviviruses. Second, it is to show that in the dengue type 2 (dengue-2) U-937 cell system, the infection enhancement phenomenon does not occur with subneutralizing quantities of monoclonal antibodies directed against a type-specific neutralization or a type-specific hemagglutination determinant. Rather, immune enhancement of dengue-2 virus replication in U-937 cells occurs only with antibodies directed against flavivirus-cross-reactive determinants.

#### MATERIALS AND METHODS

**Cell culture.** The U-937 human monocyte cell line was derived from a histiocytic lymphoma (23) and was obtained from Werner Falk (National Cancer Institute). The cells were propagated in suspension culture in medium consisting of RPMI 1640, 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Stock cultures were incubated at 35°C in 100-ml volumes in 100-ml serum bottles attached to a vertical rotary device (Draft Apparatus, Inc., Mineola, N.Y.). The cells were passaged every 3 to 4 days by an approximate 1:4 dilution to  $1.2 \times 10^5$  to  $1.6 \times 10^5$  cells per ml.

**Viruses.** Dengue-2 virus, New Guinea C strain, was used at suckling mouse brain passage 36 or after an additional three passages through BHK suspension cells as described by Malewicz and Jenkin (17). The French neurotropic strain of yellow fever virus had an undetermined number of mouse brain passages and was also passaged through the C6/36 clone of *Aedes albopictus* cells (13). The 17D yellow fever vaccine was lot 0660 CK from Merrell Laboratories, Swiftwater, Pa. (now Salk Laboratories). Virus yields were measured by plaque assay in LLC-MK<sub>2</sub> cells (1), and the identity of the viruses was determined by plaque reduction neutralization tests (PRNT) (1, 21) with reference hyperimmune mouse ascitic fluids.

**Antibodies.** Dengue-2 hyperimmune mouse ascitic fluid and human anti-dengue-2 serum were considered as mixtures of virus type-specific and flavivirus group-reactive antibodies. The production and characterization of dengue-2 monoclonal antibodies by mouse myeloma-spleen lymphocyte hybridomas has been described in detail (6a). All antibody preparations were heated at 56°C for 20 min before use.

**Preparation and use of virus-antibody complexes for infection of U-937 cells.** The human anti-dengue-2 serum (50% plaque reduction neutralization titer to dengue-2 of 1:80 and to yellow fever of 1:10) was diluted to 1:200 (beyond the neutralization titer) and mixed with

an equal volume of dengue-2 or yellow fever virus (final serum dilution of 1:400). The multiplicity of infection (MOI) ranged from 0.01 to 1.0 for dengue-2 and up to 50 for yellow fever. The control inoculum consisted of virus mixed with medium and the same dilution of normal human serum. In some experiments, dengue-2 virus was mixed with equal volumes of 1:4,000 dengue-2 hyperimmune mouse ascitic fluid which had a neutralization titer of 1:1,280. These mixtures were incubated for 15 min at 22°C, after which  $5 \times 10^5$  to  $6 \times 10^5$  U-937 cells in 15-ml plastic centrifuge tubes (Falcon Plastics, Oxnard, Calif.) were suspended in 1-ml samples of the inoculum mixtures. After incubation for 90 min at 35°C, the cells were centrifuged out of the inoculum at  $250 \times g$  for 5 min, washed two times, and suspended in 5 ml of medium containing a 1:400 dilution of human antiserum or a 1:8,000 dilution of the hyperimmune mouse ascitic fluid to achieve the same final concentration of antibody used in the virus inoculum. The centrifuge tubes were incubated in a stationary position at 35°C and agitated twice a day to resuspend the settled cells. After the first agitation each day, the tubes were centrifuged, and 1-ml samples were removed for plaque assays; 1 ml of fresh medium, with or without antibody, was added back to the tubes. Infection enhancement endpoints carried out with human serum or monoclonal antibodies were determined by mixing the virus with equal volumes of fourfold dilutions of the antibody preparations. Samples were harvested only on day 5 of the growth curve, the time of peak virus production.

**Heat-aggregated human gamma globulin.** Stock solutions of heat-aggregated gamma globulin were prepared and used experimentally as previously described (5). U-937 cells were treated for 30 min before infection with 1 to 1,000 µg of the heat aggregated IgG per ml; the latter concentration was shown previously to block infection of adherent human monocytes with virus-antibody complexes (5). U-937 cells were also treated for 1 and 16 h postinfection to determine whether macrophage "triggering," if it occurred (19), would shut off virus production.

#### RESULTS

**Infection enhancement of dengue-2 virus replication in the U-937 human monocyte cell line.** Dengue-2 virus at MOIs from 0.01 to 1 did not result in any progeny virus being produced from U-937 cells. However, the inclusion of dilute human anti-dengue-2 antibody in the virus inoculum and the culture medium resulted in progeny virus being detected in the cell culture medium. As shown in Fig. 1, no virus was produced when the U-937 cells were infected at an MOI of 0.01, whereas the inoculum of the same MOI complexed with dilute antibody resulted in virus production. To determine the potency of two human anti-dengue-2 sera used in this laboratory to demonstrate the infection enhancement effect, fourfold dilutions of the antisera were mixed with the virus inoculum, and the mixtures were used to infect the U-937 cells. The infection enhancement titers of these

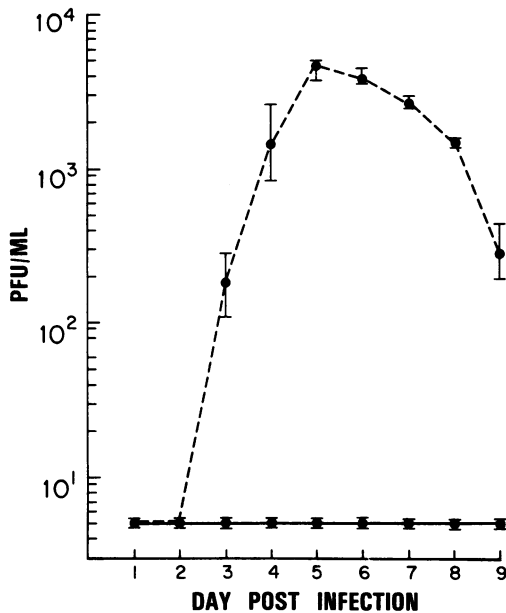


FIG. 1. Dengue-2 virus replication in the U-937 human monocyte cell line. Triplicates of  $5 \times 10^6$  cells were infected at an MOI of 0.01 with BHK cell-passaged dengue-2 virus mixed with normal medium (—) and cultured in normal medium or infected with virus mixed with dilute human anti-dengue-2 (----) and cultured in antibody medium (see text). Graph represents average values, bars indicate range.

antisera were 100- to 1,000-fold higher than the neutralization or hemagglutination inhibition (HAI) titers (4) (Table 1).

**Replication of yellow fever virus in U-937 cells.** Yellow fever virus, another flavivirus, was reported to replicate in U-937 cells cultured in medium without enhancing antibody (22), contrary to the findings with dengue-2 virus in this report. To compare both viruses at the same time in the same cells, samples of U-937 cells were infected with the 17D and French neurotropic strains of yellow fever virus as well as with dengue-2 virus. Infection and culture of the cells were carried out in the presence of control medium or medium containing the diluted human flavivirus antiserum (1:400 final concentration). The yields of virus at 5 days postinfection are shown in Table 2. U-937 cells infected and cultured in the presence of control medium replicated only yellow fever virus, whereas cells infected and cultured in the presence of dilute antibodies replicated both yellow fever and dengue viruses, as expected.

**Blocking of the infection enhancement effect with heat-aggregated IgG.** Since the enhancing effect of dilute antibody on virus infection is attributed to a nonneutralizing antibody-virus

TABLE 1. Dengue-2 infection enhancement titers of human antiserum compared with conventional serological tests with the homologous virus

Donor	50% plaque reduction neutralization	HAI	Infection enhancement <sup>a</sup>
1	80	80	12,800
2 <sup>b</sup>	300	80	250,000

<sup>a</sup> Reciprocal of highest dilution producing  $1.7 \log_{10}$  virus by day 5 postinfection.

<sup>b</sup> Two-year post-immunization serum from volunteer no. 4 in a dengue-2 vaccine study (2) and also used in an in vivo infection enhancement attempt (15).

complex interacting with an Fc receptor on monocytes, we evaluated the ability of aggregated IgG, which binds to Fc receptors, to block infection by antibody virus complexes. U-937 cells pretreated with 1,000 and 100  $\mu\text{g}$  of aggregated IgG per ml did not produce dengue virus after infection with virus-antibody complexes (Table 3). Increasing yields of dengue-2 virus were obtained from monocytes pretreated with 10 and 1  $\mu\text{g}/\text{ml}$ , respectively, demonstrating a dose-response effect of the aggregated IgG. It was reported that aggregated IgG triggers monocytes to release lysosomal enzymes (19); to counter the argument that this activity would destroy ingested virus rather than block infection via Fc receptors, we added heat-aggregated IgG after infection with virus-antibody complexes. Short-term (1-h) or long-term (16-h) postinfection treatment of infected U-937 cells with 100 or 1,000  $\mu\text{g}$  of aggregated IgG per ml had no effect on virus production.

**Evaluation of dengue-2 monoclonal antibodies directed against dengue-2 serotype-specific determinants to effect infection enhancement.** Monoclonal antibodies type specific by PRNT (with very little hemagglutination inhibition [HAI] activity) and type specific by HAI (with very little neutralizing activity) are listed in Table 4. Infection enhancement could not be demonstrated above or below the neutralization titer of these monoclonal antibodies.

**Evaluation of dengue-2 monoclonal antibodies directed against flavivirus-cross-reactive determinants to effect infection enhancement.** Infection enhancement titers of the cross-reactive monoclonal antibodies ranged from 1/400,000 to 1/400 and appeared to decrease with decreasing cross-reactive PRNT titers rather than with the cross-reactive HAI titers (Table 5). For example, the dengue-2 monoclonal 1B10 and 4D6 had similar HAI antibody titers to dengue and Japanese encephalitis virus, yet 1B10 (PRNT, 1/35 to 1/120) has an enhancement titer of 1/400,000 whereas that of 4D6 (PRNT, <1/10 to 1/20) was only 1/1,600.

TABLE 2. Yields of dengue-2 and yellow fever viruses from U-937 cells cultured in control medium or antibody medium<sup>a</sup>

Medium	Dengue-2 New Guinea C BHK cell passage (MOI 0.6)	Yellow fever French neurotropic C6/36 cell passage (MOI 0.5)	Yellow fever 17D vaccine egg passage (MOI 0.01)	Yellow fever French neurotropic mouse brain passage (MOI 50)
Control	<5	$3.4 \times 10^3$	$4.8 \times 10^2$	$1.3 \times 10^4$
Antibody	$1.5 \times 10^3$	$6.0 \times 10^4$	$5.5 \times 10^3$	$1.2 \times 10^5$

<sup>a</sup> Expressed as PFU per milliliter 5 days postinfection at the indicated MOI.

TABLE 3. Dengue-2 virus yields from U-937 cells pretreated with heat-aggregated IgG and then infected with virus-antibody complexes<sup>a</sup>

Treatment time	IgG ( $\mu$ g/ml)	Virus yield <sup>b</sup>
Preinfection	1,000	<5
	100	<5
	10	$1.2 \times 10^3$
	1	$1.1 \times 10^4$
1 h postinfection	100	$5.0 \times 10^4$
Virus plus medium (control)		<5
Virus plus antibody (control)		$3.5 \times 10^4$

<sup>a</sup> Mouse brain passage virus plus dilute dengue-2 hyperimmune mouse ascitic fluid.

<sup>b</sup> PFU per milliliter; average yield from two cultures 5 days postinfection.

## DISCUSSION

The U-937 human monocyte cell line supported the replication of dengue-2 virus only when the infecting virions were complexed with antibodies in very dilute homologous antiserum or complexed with monoclonal antibodies that were cross-reactive by the HAI and PRNT tests to other dengue virus serotypes. Antibodies were also included in the culture medium so that complexes with progeny virus could initiate subsequent cycles of infection during the multicycle growth curve required to detect dengue viruses that replicate slowly and produce typically low yields of infectious virus particles. Fc receptors were implicated in antibody-mediated dengue infection of human monocytes in peripheral blood leukocyte suspensions, as well as in adherent monocytes, by methods referred to above. As an indirect demonstration of Fc receptor-mediated infection, we used aggregated IgG to block infection of U-937 cells by virus-antibody complexes; aggregated IgG binds to Fc receptors in the same manner as antigen-antibody complexes as previously reviewed (5). Infection of U-937 cells was not observed when these cells were pretreated with high concentrations of aggregated IgG (1,000 and 100  $\mu$ g/ml), whereas only partial blockage of virus Fc-mediated

entry occurs at these same aggregated IgG concentrations in experiments with fresh human adherent monocytes (5). Adherent monocytes differ from the U-937 monocyte cell line in that the adherent cells produce up to 500-fold more virus (3, 5), and they have altered biochemical activities associated with the process of adherence; the increased biochemical activity (14, 16, 20) may result in increased membrane receptor generation or turnover that influences efficiency of Fc receptor blockage by aggregated IgG. It has been reported that adherent monocytes have almost eightfold more Fc receptors than do U-937 cells (7).

The lack of dengue virus replication in the absence of antibodies indicated that the U-937 cells were much more useful than fresh adherent monocytes for demonstrating the phenomenon of infection enhancement. The latter replicated variable quantities of dengue virus without the addition of antibody to the infected culture system (3, 5). Dengue virus receptors can be removed from these adherent monocytes with trypsin; these trypsin-treated cells still support dengue virus replication, however, if they are infected with virus-antibody complexes (5). Thus, the U-937 cells constitute an excellent system for studies of immune enhancement since (i) they do not have a significant number of dengue virus receptors that must be removed with trypsin, (ii) infection enhancement can be completely blocked with IgG, and (iii) fresh blood donations are not required.

It is curious that the U-937 cells do have receptors for the flavivirus type species yellow fever virus, as recently reported (22) and as shown here in a simultaneous comparison with dengue virus. Whereas in our studies dengue did not replicate in U-937 cells unless the inoculum was complexed with antibody, significant quantities of yellow fever virus were produced in the absence of antibody, and the addition of dilute antibody to the culture system in our laboratory increased the yield about 10-fold. Schlesinger and Brandriss (22) performed their infection enhancement studies at 4°C and showed that infection enhancement could be demonstrated predictably at 37°C only when the U-937 cells

TABLE 4. Antibody activities of dengue-2 type-specific monoclonal antibodies

Hybridoma	HAI titer <sup>a</sup>	PRNT titer <sup>b</sup>	Infection enhancement <sup>c</sup>
3H5	20	32,000	<10,000
1C7	80	40	<10
1C12	80	40	<10
2H3	160	40	<10
3H1	160	40	<10

<sup>a</sup> Taken from Gentry et al. (6a). All numbers represent the reciprocal of the dilution.

<sup>b</sup> Virus, antibody, and U-937 cells were incubated at 35°C for 1.5 h.

<sup>c</sup> The starting dilution of monoclonal antibody was a dilution which neutralized the virus inoculum (a dilution below the neutralization titer). The dilutions were carried out to 320,000 for 3H5 and to 2,560 for the others.

were first treated with trypsin, similar to the situation with dengue virus receptors on fresh adherent human monocytes (5). Flavivirus receptors have been studied in cell cultures as a general phenomenon (6), but the receptors on U-937 cells that bind yellow fever virus apparently do not bind dengue virus as measured by a productive infection.

The concept of infection enhancement *in vitro* had been demonstrated previously by mixing subneutralizing quantities of IgG in homologous sera with the virus inoculum. Thus, it was assumed that a few type-specific IgG molecules attached to an infectious virion enhanced infection via an Fc receptor, whereas more type-specific IgG would prevent infection by neutralizing the virus. The first evidence that virus type-specific IgG was not involved was in the report by Halstead et al. (12), where it was shown that the infection-enhancement potency of an antiserum was related to its cross-reactivity with other flaviviruses. The results in our study strongly suggest that this is the case: five monoclonal antibody preparations specific for dengue-2 virus, and devoid of any cross-reactivity with the other dengue serotypes or with Japanese encephalitis virus (another flavivirus), did not enhance virus infection of U-937 cells; however, monoclonal antibody preparations that exhibited cross-reactions with the other dengue serotypes, as well as Japanese encephalitis virus, were the only ones to enhance dengue-2 virus infection. Infection enhancement did not appear to be related to a particular IgG subclass, since IgG1 and IgG2a were represented among the dengue-2 monoclonal antibodies (6a) that did and did not produce infection enhancement.

The differential reactivity of the monoclonal antibodies in the various tests suggests that an antigenic map can be constructed. The infection-

TABLE 5. Dengue-2 infection enhancement titers in U-937 cells of monoclonal antibodies cross-reactive with the dengue viruses and Japanese encephalitis virus

Hybridoma	Range of HAI titers <sup>a</sup>	Range of PRNT titers <sup>a,b</sup>	PRNT titer <sup>c</sup>	Infection enhancement titers to dengue-2
1B10	200–400	35–120	100	409,600
1C10	800–1,600	25–120	200	409,600
4G2	800–1,600	30–230	250	204,800
2C4	100–400	25–125	50	25,600
4F1	200–800	10–60	25	1,600
4D6	160–320	<10–20	80	1,600
4G1	20–160	<10	10	400

<sup>a</sup> Range of titers to Dengue-1, -2, and -3 and Japanese encephalitis virus, adapted from Gentry et al. (6a).

<sup>b</sup> The PRNT for serotyping dengue viruses was incubated at 22°C for 30 min.

<sup>c</sup> See footnote *b* of Table 4.

enhancing determinants may be near the top of the projections on the virus particles where the Fab portions of the IgG can interact in such a way that the Fc portion of the IgG projects outwards allowing interaction with an Fc receptor on the cell surface. These determinants were associated with cross-neutralization and cross-HAI, and such determinants would be expected to be near the tips of the projections to interact readily with virus receptors on susceptible cells. Since monoclonal antibodies against the type-specific HAI determinants do not produce infection enhancement, the type-specific HAI determinants may be located just off the shoulders of the projections, so that the Fc portion of the attached IgG is lying askew, not able to reach an Fc receptor, but able to block the hemagglutinin determinant from interacting with erythrocytes in the HAI test. The type-specific neutralization determinant may be closer to the base of the projections, near the viral membrane, since the type-specific monoclonal antibody 3H5 did not produce infection enhancement and was not as efficient at inhibiting hemagglutination as was neutralization. Future studies to test this hypothesis will include preparation of radioactive virions to determine which monoclonal antibodies produce complement-dependent virolysis (which determinant is closest to the viral membrane), if there is internalization or binding of nonenhancing virus-antibody complexes, and the use of labeled and unlabeled monoclonal antibodies to determine whether some will block the attachment of others.

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