Brief Definitive Report

COMPLEMENT RECEPTOR MEDIATES ENHANCED FLAVIVIRUS REPLICATION IN MACROPHAGES

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Several laboratories have now demonstrated antibody-dependent enhancement (ADE)[1] of flavivirus replication in cells of the monocyte-macrophage (Mϕ) lineage and in Mϕ-like cell lines (1–4). Antiviral IgG, but not IgM, has been implicated; the phenomenon depends on the Fc portion of the IgG molecule (1, 5), and is blocked by a monoclonal antibody directed against the trypsin resistant mouse Fc receptor (6).

To investigate the possibility that, under physiological conditions, complement receptors might mediate ADE, we have examined replication of the flavivirus West Nile virus (WNV) in macrophages that are known to possess both Fc and complement receptors (7–10). We show here that antiviral IgM can mediate ADE in the macrophagelike cell line P388D1, in the presence of fresh normal serum, and we present evidence that at least one complement receptor, CR3 is involved.

Materials and Methods

Cells. P388D1 cells were grown in Dulbecco's modification of Eagle's minimal essential medium (DME) supplemented with 2 mM glutamine, 10% heat-inactivated (56°C for 30 min) fetal bovine serum (HIFBS) with 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml kanamycin. L929 cells were grown in Eagle's minimal essential medium with 3% HIFBS, and antibiotics as above.

Virus. West Nile virus (WNV; Egypt 101 strain) was used as a 9,000 *g* supernate of infected suckling mouse brain homogenate, frozen in aliquots at −70°C.

Antibodies. Rabbit anti-WNV IgM was prepared from the serum of a New Zealand White rabbit collected 6 d after the intravenous inoculation of 8×10^6 plaque-forming units (pfu) of live WNV. The serum was passed through a Sephacryl S300 superfine column at a flow rate of 13 ml/h. The forward shoulder of the first protein peak was collected and concentrated 10-fold on a PM10 filter in an Amicon ultra-filtration cell. Purity of the IgM was confirmed on an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and by immunoelectrophoresis using goat anti-rabbit whole serum (Miles Laboratories, Slough, U.K.). This preparation of IgM had a neutralization titer ($PRNT_{50}$) of 1:160 as determined in L929 mouse fibroblast cells, and a haemagglutination inhibition titer of 1:80.

F7/3 is a mouse IgG1 monoclonal antibody characterized by Peiris et al. (11) and purified on a protein-A-Sepharose CL 4B column by the method of Ey et al. (12).

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[1] *Abbreviations used in this paper:* ADE, antibody-dependent enhancement; CR3, monocyte-macrophage complement receptors; CVF, cobra venom factor; DME, Dulbecco's modification of Eagle's minimal essential medium; HIFBS, heat-inactivated fetal bovine serum; Mϕ, monocyte macrophage; PBS, phosphate-buffered saline; pfu, plaque-forming units; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WNV, West Nile virus.

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The hybridoma cell lines M1/70 (13) and 2.4G2 (14) were generously provided by Dr. T. A. Springer, Boston and Dr. J. C. Unkeless, New York, respectively.

Complement. Fresh normal serum from DBA/2 C5-deficient mice was used as a source of complement.

Cobra Venom Factor. A purified preparation was generously provided by Professor Otto Götze, Göttingen, FRG.

Enhancement Assay. WNV at 200–300 pfu/ml (as measured by conventional assay on a pig kidney cell line [PS]; reference 15) was mixed with an equal volume of rabbit anti-WNV IgM at 1:40 dilution, or diluent, and incubated at 37°C for 45 min. Aliquots of these mixtures, 0.1 ml, were applied to two to four preformed cell monolayers followed immediately by 0.1 ml of a 1:3 dilution of fresh or heat-inactivated (56°C for 30 min) DBA/2 mouse serum. After 2 h at 37°C monolayers were washed twice with PBS to remove residual virus, a carboxymethylcellulose overlay medium was added, and preparations were incubated at 37°C for 3 d, at which time plaques were stained with naphthalene black and counted.

Results

When IgM at a dilution of 1:40 was incubated with WNV and the residual infectivity of the mixture assayed on L929 fibroblasts, neutralization occurred, which was further potentiated in the presence of fresh normal serum obtained from DBA/2 C5-deficient mice, as would be expected (Fig. 1 *a*) (16). When the Mϕ line P388D1 was infected under the same conditions, however, there was a 5–10-fold increase in plaques produced by mixtures containing IgM, virus, and fresh serum (Fig. 1 *b*). Serum heat inactivated at 56°C for 30 min did not mediate this increase. Control infections in which virus was incubated without IgM and presented to the cells in the presence of fresh normal serum did not show increased viral replication. Fresh serum diluted 1:2 to 1:12 was capable of enhancing viral replication; IgM enhanced viral replication when used at 1:10 to 1:160, neutralization was never observed with P388D1 cells in the presence of fresh normal serum (data not shown).

To further investigate the nature of the heat labile component(s) responsible for this effect, we utilized highly purified cobra venom factor (CVF) which consumes mainly C3 in the C5-deficient mouse serum used here. Fresh serum treated with CVF did not enhance replication of the IgM-virus mixtures (Fig. 1 *c*).

Once activated by limited proteolysis, complement components, C3, C4, and the anti-proteinase α_2 macroglobulin bind covalently to other molecules in a reaction mediated by an activated thiol ester. Nucleophiles that react with this thiol ester prevent covalent binding to other molecules (17). We used the strong nucleophile sodium salicylhydroxamate to compete with virus mixtures for the covalent binding site of the serum components involved. The ADE observed with IgM-virus-fresh serum was decreased with increasing concentrations of sodium salicylhydroxamate and was completely abolished by 0.7 mM of the salt, which did not affect macrophage viability or viral growth in the absence of fresh serum (data not shown). Abrogation of ADE by CVF and by sodium salicylhydroxamate are compatible with a role for C3.

Investigations of the Mϕ membrane receptors that might be implicated indicate that CR3, which recognizes iC3b (a rapidly formed major fragment of activated C3) is involved in the binding step. When P388D1 cells were preincubated with

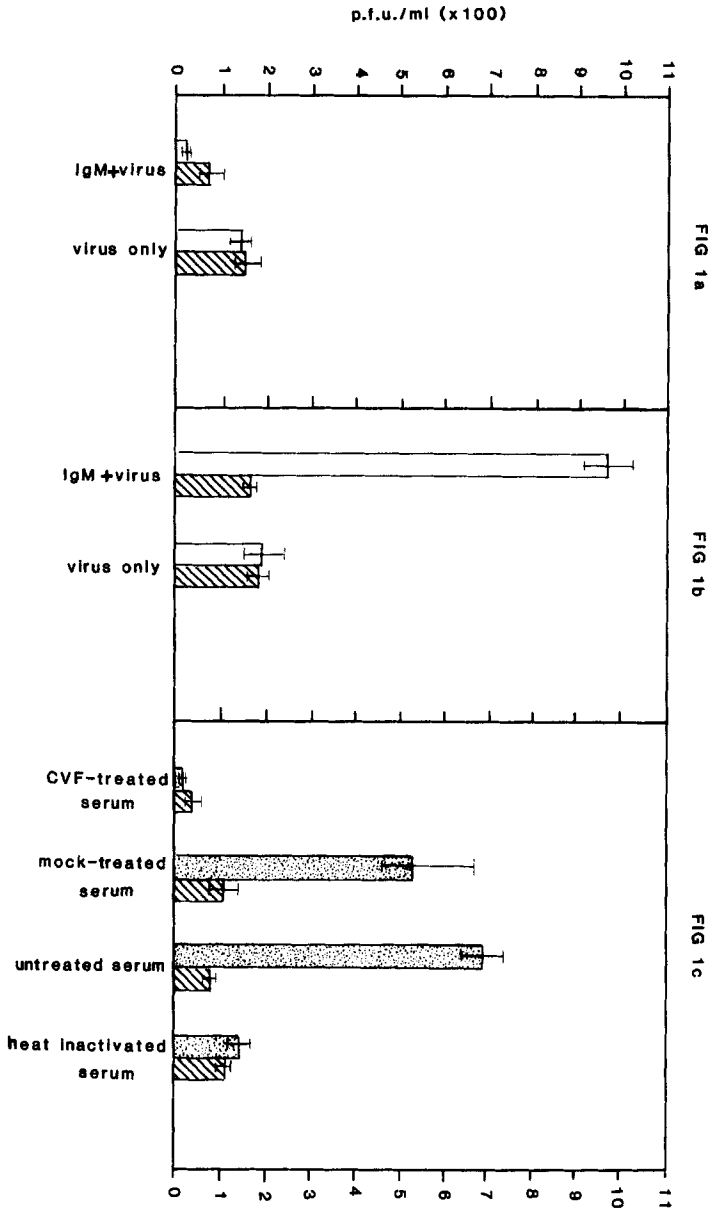

FIGURE 1. Virus yields obtained when cell monolayers were infected with IgM + virus, or virus only, in the presence of fresh (open columns) or heat inactivated (hatched columns) mouse serum; in 1a L929 cells were used, and in 1b P388D1 cells. *c* shows plaque counts obtained when virus + IgM (dotted columns), or virus + diluent (hatched columns) were used in combination with (*a*) fresh DBA/2 serum incubated for 15 min at 37°C with CVF (final concentration 9.1 g/ml); (*b*) fresh DBA/2 serum mock-treated with diluent; (*c*) untreated DBA/2 serum; or (*d*) heat-inactivated DBA/2 serum. In every case the final serum concentration was 1:3. Bars indicate range of three to four assays.

M1/70, which inhibits CR3-mediated binding of opsonized erythrocytes (13) ADE of the IgM-virus-fresh serum mixture was abolished, whereas 2.4G2, directed against the trypsin-resistant Fc receptor (14) had no effect (Fig. 2*a*).

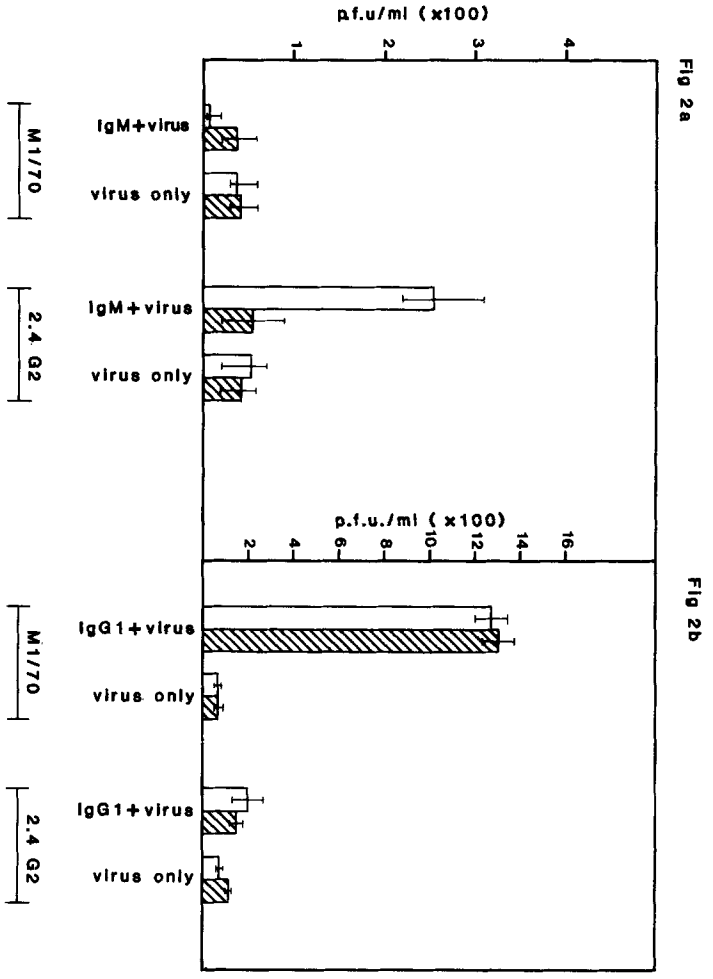

FIGURE 2. Receptor blocking studies. Preformed P388D1 monolayers held on an ice tray were treated for 60 min with DME containing 20% supernatant fluids from hybridomas producing antibodies (*a*) M1/70 (anti Mac1) or (*b*) 2.4G2 (anti-trypsin-resistant Fc receptor), then washed with ice-cold DME and infected with West Nile virus with or without anti-WNV antibodies, in the presence of fresh □ or heat-inactivated ▨ DBA/2 serum. In *a*, the antibody was anti-WNV IgM. In *b*, the antibody was a monoclonal anti-WNV IgG_1. Bars indicate range of duplicate assays.

Under our experimental conditions, neither M1/70 nor 2.4G2 affected controls in the absence of IgM. In contrast, ADE mediated by IgG1 was not affected by either fresh serum or M1/70 but was abrogated by 2.4G2 antibody. Since the cells were preincubated with the monoclonal antibodies M1/70 and 2.4G2 and washed free of unbound antibody before introduction of virus, these studies implicate macrophage CR3 in IgM-serum enhanced replication of WNV.

Discussion

In these studies the magnitude of the enhancement system is much less than in IgG-mediated ADE. However, 20–50-fold enhancement has been observed when thioglycollate-elicited, but not resident, mouse peritoneal macrophages are

infected with virus-IgM-fresh serum (unpublished observations). These differences may be related to observations that certain Mϕ ingest opsonized particles readily via Fc receptors (7, 9) but fail to ingest particles bound via complement receptors (7, 9, 10) unless the cells are stimulated by various treatments (9, 18–20) or the particles coated with both IgG and complement (10).

In the experiments presented here, we have deliberately separated Fc receptor-dependent ADE, mediated by IgG, from enhancement via complement receptors and IgM. The two enhancement pathways are not mutually exclusive, but may act synergistically when certain classes of IgG fix complement. Complement receptors are found on many cell types (21) (erythrocytes, lymphocytes, neutrophils, monocytes, macrophages, kidney epithelial cells) and there may be several different complement receptors on each cell type. Although it remains to be shown that other complement receptors can also mediate enhanced viral replication, we suspect that enhancement via complement receptors may be a general phenomenon. Since some enveloped viruses are able to activate complement in the absence of antibody (22, 23), such a route of virus penetration should be considered seriously in other systems.

Summary

Evidence is presented that Mϕ complement receptors (CR3) mediate IgM-dependent enhancement of flavivirus replication in the presence of complement. Enhancement is blocked by pretreatment of macrophages with monoclonal antibody M1/70, which inhibits CR3 binding, but not by pretreatment with monoclonal antibody 2.4G2, which inhibits FcR binding.

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