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Interaction of West Nile Virus with Primary Murine Macrophages: Role of Cell Activation and Receptors for Antibody and Complement

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We have measured growth of West Nile virus in mouse primary peritoneal macrophages (resident, thioglycolate elicited, and *Mycobacterium bovis* BCG activated) and in macrophagelike (P338D1) and nonmacrophage (L929, PS clone D) cell lines infected in the absence or presence of specific antibodies (immunoglobulin G ([IgG], IgM), and complement. Monoclonal antibodies directed against Fc receptors (IgG1/2b, 2.4G$_2$) and type 3 complement receptors (Mac-1) were used to define the role of each receptor. Virus yield depended on a balance between enhancement and neutralization and was influenced by the physiologic state of the macrophage, the receptor pathway of viral entry, the mouse strain and age of donor. BCG-activated macrophages displayed a greater ability to restrict West Nile virus than nonactivated cells only in the presence of antiviral IgM, with or without complement; the Fc receptors for various classes of IgG mediated striking enhancement. These studies identify some of the complex innate and acquired factors that determine the interaction between West Nile virus and primary macrophages in vitro.

Flavivirus replication in macrophages (Mϕ) in vitro is potentiated by antiviral antibody (Ab) and complement acting via Mϕ plasma membrane receptors (7, 14, 21). Little is known, however, about the role of Mϕ in enhancement of viral growth in the host and whether Mϕ are also able to restrict flavivirus replication as a result of specific and nonspecific immune activation.

Most studies hitherto have been performed with Mϕ-like cell lines (7, 22), relatively homogeneous permissive cells which express receptors for the Fc portion of immunoglobulin G (IgG), FcR, and for the cleaved complement protein iC3b (CR3) (24). However, these cells lack other functions expressed by primary Mϕ (30), including the ability to generate reactive intermediates of oxygen (Gordon, unpublished data). Moreover, primary Mϕ display considerable heterogeneity as a result of differences in maturity and modulation by various stimuli (9, 16). Peritoneal Mϕ obtained from normal mice after inflammatory stimulation (e.g., injection of thioglycolate broth) or immune activation (e.g., *Mycobacterium bovis* BCG infection) vary markedly in their physiologic properties and in their ability to kill various microbial, parasitic, and cellular targets (1).

In this study we report on the ability of West Nile virus (WNV) to replicate in different peritoneal Mϕ populations after infection in the absence or presence of specific antiviral Ab and complement. We show that the activation state of the Mϕ alters net virus growth profoundly, depending on a balance between enhancement and neutralization mediated by Ab and complement in each target population.

MATERIALS AND METHODS

Mice. Mice were bred at the Sir William Dunn School of Pathology, and both sexes were used indiscriminately. Peritoneal Mϕ were routinely obtained from CBAT6T6 mice between 10 and 12 weeks of age. C57BL6 mice were used in some experiments as noted. DBA/2 mice between 4 and 8 months old were used as a source of complement.

Cells. (i) Cell lines. P388D1 cells, an Mϕ-like cell line derived from DBA/2 mice (17), were grown in Leibovitz (L15) medium supplemented with 100 μg of kanamycin per ml, 50 μg of streptomycin sulfate per ml, 50 U of benzyl penicillin per ml, 10% tryptose phosphate broth, and 5% fetal bovine serum (FBS) which had been heat inactivated (hi) at 56°C for 30 min. P388D1 monolayers were suspended by vigorous shaking of tissue culture flasks. L929 cells, a fibroblastlike line derived from C3H mice (27), were grown in minimal essential medium with 3% hi FBS, L-glutamine, and antibiotics as described above. PS clone D cells, a pig kidney cell line (8), were grown in L15 medium supplemented with antibiotics, tryptose phosphate broth, and 3% hi FBS. Both L929 and PS clone D monolayers were suspended by trypsinization (0.125% trypsin in phosphate-buffered saline [PBS]). Cell lines were free of mycoplasmas.

(ii) Primary mouse peritoneal macrophages. Thioglycolate-elicited peritoneal Mϕ (TPM) were obtained from mice injected intraperitoneally 4 to 5 days previously with 1 ml of Brewer complete thioglycolate broth, whereas BCG-activated peritoneal Mϕ (BCG-PM) were obtained from mice infected intraperitoneally 1 to 3 weeks previously with ca. 10^7 live BCG organisms (9). Resident peritoneal Mϕ (RPM) were harvested from untreated mice. Peritoneal cells were washed in PBS and cultivated at 37°C in the presence of 5% CO_2 in Dulbecco minimal essential medium (DMEM) supplemented with L-glutamine, antibiotics, and 5% hi FBS at 5×10^5 Mϕ per well in 24-well tissue culture trays. Adherent monolayers were washed and infected after 3 to 21 h.

(iii) Virus. WNV was used as a mouse brain stock prepared by a standard method (32) described briefly as follows. Newborn mice were injected intracerebrally with 10^4 to 10^5 PFU of WNV. After 3 days brains were removed aseptically and suspended in 1 ml of PBS each, homogenized in a ground glass homogenizer on ice, and clarified by centrifu-

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gation at 30,000 × *g* for 1 h at 4°C before storage at −70°C in 0.1-ml samples.

(iv) Media. All experiments were done with DMEM supplemented with L-glutamine, antibiotics, and 5% hi FBS (DMEM-5%) unless stated otherwise.

Infection Protocols. (i) Virus alone. Primary mouse Mϕ monolayers in 0.4 ml of medium per well were inoculated in duplicate with 0.1 ml of WNV at various concentrations. After 2 to 3 h at 37°C, 0.5 ml of fresh medium was added, and the cultures were incubated at 37°C for 2 days before assaying supernatant fluids for virus yield. Control experiments with P388D1 monolayers showed that washing of monolayers did not alter virus yields.

(ii) Virus plus antibody. WNV at 5×10^2 to 10×10^2 PFU/ml in DMEM-5% was incubated for 30 min at 37°C with equal volumes of Ab dilutions and medium; 0.1 ml of each mixture was then added per well to primary mouse Mϕ in 0.4 ml of medium. After 2 to 3 h at 37°C, 0.5 ml of fresh medium was added, and cultures were incubated for 2 days at 37°C before assay.

(iii) Virus plus IgM and complement. DMEM was supplemented with 0.5% bovine serum albumin (DMEM-BSA) instead of hi FBS. WNV at 5×10^2 to 10×10^2 PFU/ml was preincubated for 45 min at 37°C with dilutions of rabbit anti-WNV IgM or DMEM-BSA. Macrophage monolayers in wells containing 0.3 ml of DMEM-BSA were inoculated with 0.1 ml of virus mixture followed immediately with 0.1 ml of a 1:3 dilution (unless specified otherwise) of fresh or hi DBA/2 serum. After 2 to 3 h at 37°C, the monolayers were washed twice with PBS, and 1.0 ml of fresh DMEM-5% was added. Supernatants were assayed after 2 days at 37°C.

Assay for virus yield. Samples (0.1 ml) of 10-fold dilutions of supernatant were added to 0.5 ml of medium containing a suspension of 10^5 PS clone D or L929 cells. After 3 to 4 h at 37°C, an overlay was added to give a final concentration of 0.75% carboxymethyl cellulose. Monolayers were stained with naphthalene black after 2 to 3 days at 37°C, and plaques were counted.

Direct plaque assays with P388D1 and L929 cells. Wells containing 2×10^5 to 2.5×10^5 P388D1 cells or 1×10^5 to 1.5×10^5 L929 cells were inoculated with 0.1 ml of WNV with or without Ab as described previously (22). After 2 to 3 h at 37°C, an overlay of DMEM-5% and carboxymethyl cellulose was added to each well. Cultures were incubated at 37°C for 3 to 4 days, monolayers were stained, and the plaques were counted.

Direct plaque enhancement assays with P388D1 Mϕ and complement. P388D1 cells were plated at 2×10^5 to 2.5×10^5 per well in 24-well trays and allowed to adhere for 2 to 3 hours at 37°C in DMEM-5%. Monolayers were then washed twice with PBS; 0.3 ml of DMEM-BSA was added, followed by 0.1-ml mixtures containing 10 to 50 PFU of WNV which had been preincubated for 45 min at 37°C with an appropriate concentration of rabbit anti-WNV IgM or diluent (DMEM-BSA). This was immediately followed with 0.1 ml of a 1:3 dilution of fresh or hi DBA/2 serum. After 2 to 3 h at 37°C, monolayers were washed gently twice with PBS and overlaid with 1.0 ml of DMEM-5% and carboxymethyl cellulose. After 3 to 4 days at 37°C, monolayers were stained, and plaques were counted.

Inhibition experiments. P388D1 monolayers prepared as described above were washed and overlaid with 0.3 ml of Hanks balanced salt solution with divalent cations and containing the various test reagents and controls. The monolayers were incubated for 60 min at 4°C before infection as described above.

Complement. Fresh serum from DBA/2 mice was obtained by cardiac puncture and used as a source of C5-deficient mouse complement.

Ab. A hyperimmune rabbit anti-WNV antiserum was prepared by an intravenous injection of heat-killed WNV followed by repeated intravenous injections of live WNV, ca. 10^6 to 10^7 PFU. Mouse anti-WNV Ab were isolated from hyperimmune mouse ascitic fluids obtained by inoculating mice immune to WNV, with Erhlich ascites tumor cells. The monoclonal Ab F7/3 and F6/16A (23) were isolated from mouse ascitic fluids. These mouse Ab were purified on a Protein A-Sepharose CL column. Rabbit anti-WNV IgM was purified 6 days after intravenous infection of a rabbit with ca. 10^7 PFU of live WNV. Rabbit IgM was separated by S300 gel filtration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed IgM and no IgG. Monoclonal Ab directed against FcR (IgG1/2b) (33) and CR3 (26) were used as pure proteins or concentrated supernatant, as shown below.

Expression of results. All experiments were done in duplicate or triplicate, and results are expressed as (i) yield of virus per culture (PFU per milliliter), (ii) number of plaques per monolayer in direct plaque assays (plaques per well), or (iii) ratios of numbers of plaques produced in test cultures compared with controls, as defined in the footnotes to the table.

RESULTS

Replication of WNV in primary mouse Mϕ. Previous studies have shown that WNV replicates readily in the Mϕ-like cell line P388D1, although to lower titers than in the non-Mϕ lines L929 and PS clone D (12). To examine virus growth in various primary mouse Mϕ, peritoneal cells were obtained from untreated mice after thioglycollate-broth injection or after infection with BCG. These Mϕ populations have been well characterized in our laboratory with regard to the expression of activation and other phenotypic markers (9, 10). Since it is known that Mϕ from different inbred mouse strains vary in their ability to support replication of flaviviruses and other viruses (3), we compared Mϕ from C57BL6 and CBAT6T6 mice.

All primary Mϕ supported WNV growth, but virus yields varied markedly with the multiplicity of infection, strain of mouse, and Mϕ population (Fig. 1). In general, primary Mϕ were less permissive than P388D1 (ca. 10-fold) and non-Mϕ lines (ca. 10- to 100-fold; data not shown). TPM and BCG-PM from CBAT6T6 mice yielded approximately 100- to 1,000-fold more virus than did cells from C57BL6 mice, unlike RPM, in which similar levels of infectious virus were obtained in both strains. Although BCG-activated mouse Mϕ are able to release reactive intermediates of oxygen when suitably challenged and display an enhanced ability to kill microbial, parasitic, and cellular targets compared with TPM and RPM (1), these Mϕ did not show increased killing of WNV compared with TPM from the same strain.

Role of Ab and FcR. Previous studies with P388D1 cells showed that antiviral IgG can mediate enhanced replication of WNV and that a monoclonal Ab, 2.4G2, directed against the Mϕ FcR for IgG1/2b subclasses (33) prevented such Ab-dependent enhancement (ADE) (21). Since Mϕ also express distinct FcR for the IgG2a subclass (34), and since FcR for different isotypes are modulated independently by immune activation of Mϕ (10), we compared virus growth in primary Mϕ populations after infection in the presence of various antiviral Ab. Table 1 shows the effects of mouse and rabbit anti-WNV polyclonal antiserum and of mouse monoclonal anti-WNV Ab of the IgG1 (F7/3) and IgG2a (F6/16A)

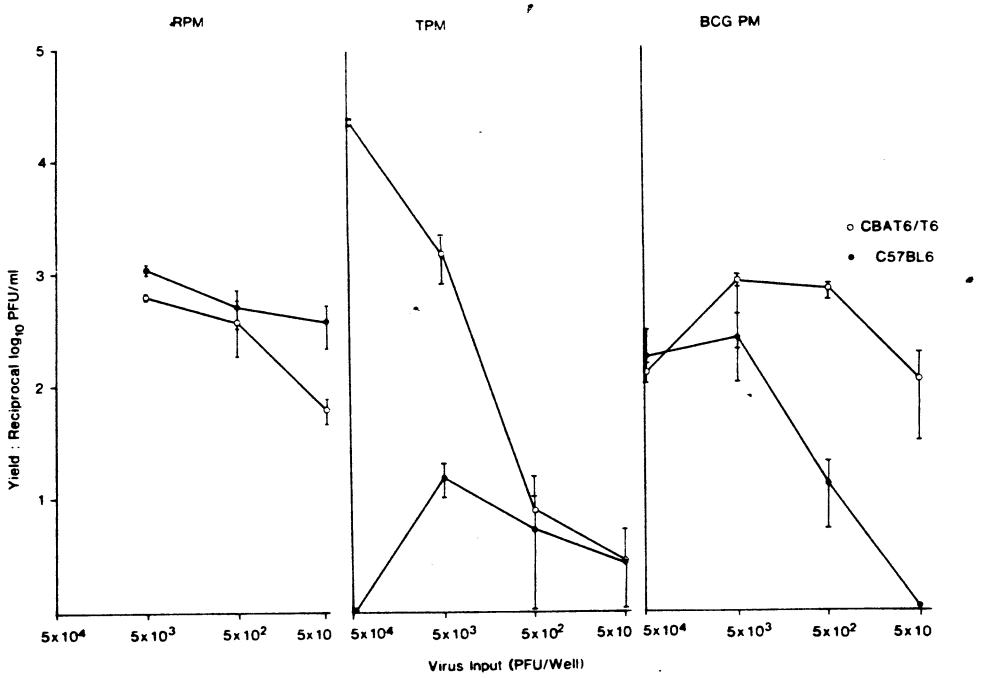

FIG. 1. Growth of WNV in primary peritoneal Mϕ of CBAT6/T6 and C57BL6 mice. RPM, TPM, and BCG-PM (5×10^5) were inoculated with 10-fold dilutions of WNV at a multiplicity of infection ranging from 10^{-1} to 10^{-4} per Mϕ. Virus yields were assayed after 48 h. Similar results were obtained in three different experiments. Virus yields assayed after 72 h were generally higher, but titration curves were similar.

TABLE 1. IgG-dependent enhancement of WNV infectivity in various cell populations[a]

Cell	Antibody	Enhancement ratio[b] at the following Ab dilution:					No. of expts
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
RPM	Mouse anti-WNV		21.4				2
	Rabbit anti-WNV		0	0	8.5	17.4	2
	F7/3 (IgG1)	23.6	4.0	1.0	1.0		5
	F6/16A (IgG2a)	2.2	10.9	8.9	9.2		4
TPM	Mouse anti-WNV		3.2				2
	Rabbit anti-WNV	0	0.3	0.8	1.3	0.9	3
	F7/3 (IgG1)	10.7	5.0				8
	F6/16A (IgG2a)	2.9	20.0	18.9			7
BCG-PM	Mouse anti-WNV	19.0	19.0	6.3			3
	Rabbit anti-WNV	0	11.3	92.0	112.7	68.1	5
	F7/3 (IgG1)	87.6	12.4	8.5	6.1	0.7	6
	F6/16A (IgG2a)	29.6	69.0	11.2	2.5	1.2	5
P388D1	Mouse anti-WNV	25.8	36.0	13.4	2.4	1.4	2
	Rabbit anti-WNV	0	4.0	59.5	50.5	4.5	3
	F7/3 (IgG1)	14.7	15.3	11.5	2.3		3
	F6/16A (IgG2a)	8.3	23.6	21.9	11.3		3
L929	Mouse anti-WNV	0.4	0.9	1.0			2
	Rabbit anti-WNV	<0.1	<0.1	0.8	0.9		2
	F7/3 (IgG1)	0.8	0.9	0.9			2
	F6/16A (IgG2a)	<0.1	0.4	0.8	0.8		2

[a] RPM, TPM, and BCG-PM from CBAT6T6 mice were inoculated with WNV with or without Ab, and yields were assayed after 48 h. P388D1 Mϕ and L929 cells were infected with the same mixtures, and enhancement was measured by a direct plaque assay.

[b] The results are expressed as enhancement ratios defined as follows: [virus yield (or number of plaques) in cultures + Ab]/[virus yield (or number of plaques) in cultures − Ab]. Results show average enhancement ratios of two to eight independent experiments. Background virus growth in the absence of Ab varied in different experiments in the following ranges: RPM, 0.5 to 21 PFU/ml; TPM, 0.5 to 60 PFU/ml; BCG-PM, <0.25 to 10.5 PFU/ml. Numbers of plaques from direct plaque assays in wells without Ab were as follows: P388D1, 2 to 11; L929, 11 to 22.

subclasses on virus yields in CBAT6T6 primary Mϕ compared with P388D1 and L929 cells. L929 cells lack FcR, and enhancement ratios (virus yields with/without Ab) of <1 indicate neutralization, which was marked in the presence of the rabbit polyclonal antiserum and F6/16A monoclonal Ab. Results with F6/16A and F7/3 confirm results obtained by Peiris et al. with P388D1 cells (23). In the present studies all anti-WNV Ab were able to enhance WNV infectivity in P388D1 cells, although to a different extent (ca. 10- to 60-fold) depending on the Ab, its titer, and its neutralizing activity. Specificity of the FcR in P388D1 Mϕ involved in ADE was confirmed by showing that 2.4G2 blocked enhancement by F7/3 (IgG1) and the polyclonal antisera more efficiently than F6/16A (IgG2a) (results not shown).

All three primary Mϕ populations displayed ADE, although the extent varied with the Ab, its titer, and the functional state of the Mϕ. The profile of ADE in primary Mϕ was broadly similar to that found in P388D1 cells. However, ADE was more readily demonstrable in BCG-PM (10- to 100-fold) than with RPM and TPM (2- to 20-fold). Monoclonal antiviral Ab of both subclasses (IgG1 and IgG2a) were able to mediate ADE in all primary Mϕ populations, approximately to the same extent. In addition, the rabbit polyclonal antiserum and F6/16A monoclonal Ab showed significant Ab concentration-dependent neutralization with all primary Mϕ. Further experiments were done with Mϕ from C57BL6 and CBAT6T6 mice, using the mouse anti-WNV polyclonal antibody at a single concentration, 10^{-2}. Figure 2 shows a similar strain difference in virus yield in the presence of Ab (Fig. 1) and ADE in BCG-PM rather than RPM and TPM of both strains.

These studies indicated that antiviral Ab markedly influenced the yields of WNV, that neutralizing and enhancing effects could occur within the same system, and that primary Mϕ differed markedly in the net enhancement observed.

Role of complement and CR3 in P388D1 cells. Earlier

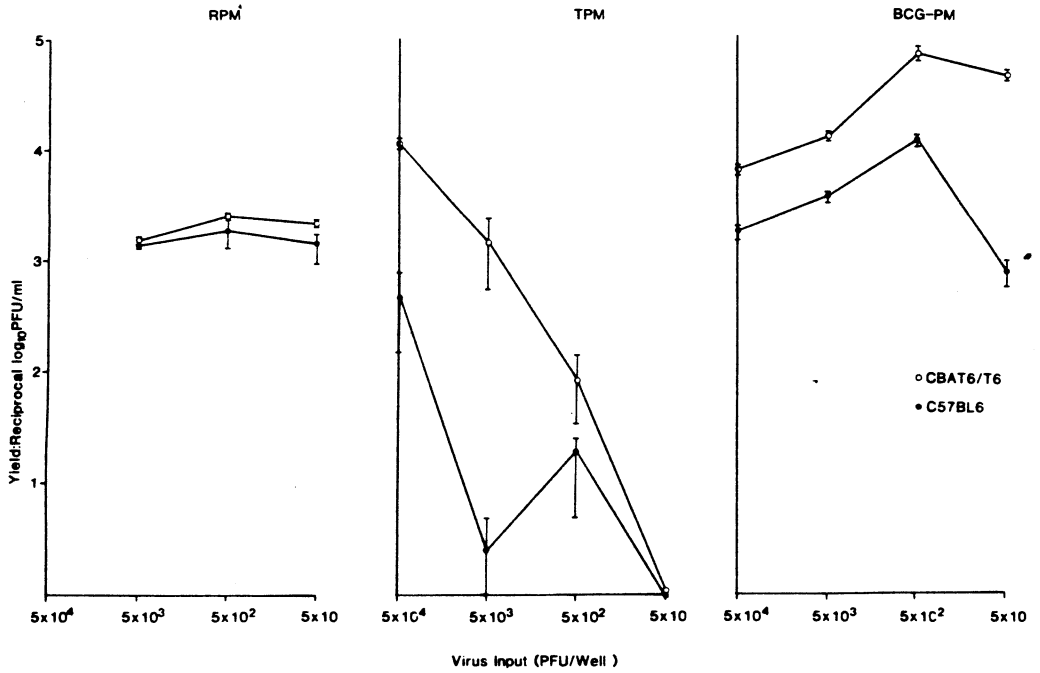

FIG. 2. Virus yields from primary peritoneal Mϕ in the presence of Ab. RPM, TPM, and BCG-PM were inoculated with 10-fold dilutions of WNV pretreated for 30 min at 37°C with a 10^{-2} dilution of mouse anti-WNV IgG. Multiplicity of infection ranged from 10^{-1} to 10^{-4}, and virus yields were assayed after 48 h. Growth in the absence of Ab in these experiments is given in footnote *a* of Table 1. Yields after 72 h were essentially similar.

studies with P388D1 cells showed that a rabbit anti-WNV IgM could enhance WNV infectivity in the presence of fresh mouse serum (7). This effect depended on the concentration of IgM and of fresh serum. Mac-1, a monoclonal Ab directed against the type 3 complement receptor (4) prevented enhanced WNV replication mediated by IgM and fresh serum. We obtained further evidence for the role of complement components and CR3 in this system by using sodium salicylhydroxamate, a nucleophile which inactivates the covalent binding site of C3 (28) and monoclonal anti CR3 Ab. Table 2 shows that 0.7 mM sodium salicylhydroxamate effectively inhibited the enhancement of IgM-virus complexes by fresh serum, without influencing virus growth in control cells. Table 3 shows that purified M1/70 IgG and its $F(ab)_2$ fragment blocked complement-dependent enhancement of WNV replication. Furthermore, a monoclonal Ab M19/23 directed against the Mac-1 α chain, which contains the active site for binding of iC3b-coated erythrocytes (26), also blocked complement-dependent enhancement. In contrast, Ab M18/2.9 directed against the β chain, which does not inhibit CR3 function, showed a reproducible small (30%) additional enhancement. Finally, 2.4G2, which blocks the FcR for IgG1/2b, had no effect on complement mediated enhancement. These findings establish that the Mac-1 molecule participates in complement-dependent enhancement.

TABLE 2. Effect of sodium salicylhydroxamate (SS) on complement dependent enhancement in P388D1 Mϕ[a]

Final SS concn (mM)	No. of plaques per well			
	IgM + fresh serum	IgM + hi serum	Fresh serum alone	hi serum alone
0	50.0	11.5	13.0	15.0
0.04	47.5	12.0	13.0	15.5
0.165	38.0	12.0	14.0	17.5
0.670	17.5	13.0	12.5	16.0

[a] P388D1 cells were inoculated with WNV ± rabbit anti-WNV IgM (1:20) and fresh or hi DBA/2 serum (1:15). After 2 to 3 h at 37°C cultures were washed and overlaid with DMEM-5% with carboxymethyl cellulose. Plaques were counted after 3 to 4 days.

Primary Mϕ. Although all of the Mϕ populations studied here express CR3, the receptors are known to vary with regard to their ability to ingest complement-coated erythrocytes (5, 13, 35). Thus CR3 are actively phagocytic in TPM (5) and BCG-PM (Gordon, unpublished data), but inactive in RPM under the present conditions.

We first established that primary Mϕ also display complement-dependent enhancement of WNV replication. Figure 3 shows a maximum 16-fold enhanced replication in TPM infected with WNV in the presence of IgM and fresh serum, compared with controls. We next examined the role of anti-WNV IgM and complement in WNV replication in different mouse peritoneal Mϕ, compared with P388D1 cells and L929 fibroblasts (Table 4). Results are expressed as different ratios, to analyze the role of each component. L929 cells lack CR3 and showed neutralization (5- to 10-fold) by IgM with fresh or hi serum. This neutralization effect should be taken into account in experiments with Mϕ. Thus complement-dependent enhancement in P388D1 cells was 10-fold in the presence of IgM and fresh serum versus IgM and hi serum, but only 3-fold when neutralization by IgM occurred in fresh serum, relative to fresh serum controls without IgM.

In contrast with the results described above for IgG-mediated enhancement, TPM showed marked enhancement by IgM plus complement, 40- to 200-fold, depending on whether neutralization by IgM is taken into account. RPM, on the other hand, showed enhancement with fresh serum alone and only three- to fourfold further enhancement with

TABLE 3. Effect of various anti-CR3 monoclonal Ab on complement-dependent enhancement in P388D1 Mϕ[a]

Antibody	Specificity	Final Ab concn[b]	Plaques per well				Ratio	
			IgM + FS	IgM + HIS	FS only	HIS only	(IgM + FS)/(IgM + HIS)	(IgM + FS)/(FS only)
Control (medium)			55.5	7.0	16.5	36.0	7.9	3.4
2.4G2	FcR IgG2b/1	1:5	53.5	7.5	12.0	34.5	7.1	4.5
M1/70	Mac-1 (CR3)	1:50	2.0	4.5	6.5	32.0	0.4	0.3
M1/70 IgG	Mac-1 (CR3)	1.5 μg/ml	2.5	9.0	8.0	33.0	0.3	0.3
M1/70 $F(ab')_2$	Mac-1 (CR3)	1.4 μg/ml	5.5	10.0	10.0	34.0	0.6	0.6
M19/23	Mac-1 ∝ chain	1:50	2.5	7.0	7.0	24.5	0.4	0.4
M18/2.9	Mac-1 β chain	1:50	78.5	7.5	15.5	25.0	10.5	5.1

[a] P388D1 monolayers were preincubated with various monoclonal Ab and controls before inoculating with WNV with or without IgM and fresh serum (FS) or hi DBA/2 serum (HIS). The table shows the results as number of plaques per well or as two ratios: (IgM + FS)/(IgM + HIS) is the ratio of plaques of WNV plus IgM in the presence of fresh serum to plaques of WNV plus IgM in the presence of heat inactivated serum, which examines the role of complement; (IgM + FS)/(FS only) is the ratio of plaques of WNV plus IgM in the presence of fresh serum to plaques of WNV without added IgM in the presence of fresh serum, which examines the role of IgM in the presence of complement.

[b] Saturating concentrations of Ab.

IgM as well as fresh serum. The direct enhancement seen with fresh serum was found only with low virus inputs (multiplicity of infection, 10^{-4}/Mϕ). Results obtained with BCG-PM also show neutralization (IgM), more marked than with other Mϕ populations, and some evidence of enhancement (IgM plus complement). Other experiments (data not shown) indicated that IgM is 10- to 50-fold more potent at virus neutralization in BCG-PM than in P388D1 or L929 cells.

We concluded from these experiments that complement-dependent (IgM and fresh serum) enhancement of WNV replication could be demonstrated in all three primary Mϕ populations and that the degree of enhancement (TPM >> RPM > BCG-PM) depended on the extent of concomitant neutralization by IgM and on the functional state of the Mϕ.

Effect of animal age on WNV replication. It has been reported (6, 25) that younger mice are more susceptible to flavivirus infection than older animals. In some experiments of our own (Porterfield, unpublished data), intraperitoneal injection of weanling mice (3 weeks) with WNV produced a higher mortality rate than was seen in older mice (5 to 6 weeks). To establish whether Mϕ obtained from mice of different ages showed a corresponding difference in susceptibility to infection, cells were obtained from CBA mice, a permissive strain, after thioglycolate-broth injection to increase yields and infected with 10^{-1} or 10^{-2} PFU/Mϕ. One experiment, representative of three independent experiments, is shown in Fig. 4. Unlike susceptibility of animals to WNV in vivo, TPM from younger mice were consistently more restrictive to WNV (100-fold at 4 versus 16 weeks), and virus yields gradually increased with age to levels observed in adult mice. Although not extensively studied, the addition of antiviral antibody did not overcome restriction by cells from younger animals (data not shown). It should be noted that all other experiments reported in this study employed mice at 10 to 12 weeks of age.

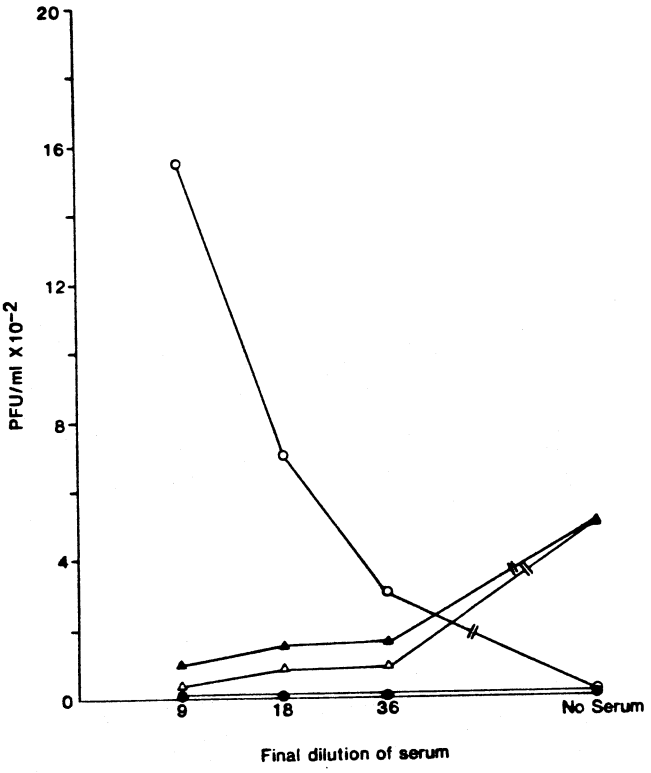

FIG. 3. Serum dependence of WNV-IgM enhancement in TPM. TPM monolayers inoculated with 0.5×10^{-2} to 1×10^{-2} PFU of WNV with or without rabbit anti-WNV IgM with or without dilutions of fresh or hi DBA/2 serum and assayed as described in the text. Symbols: ○, WNV with IgM and fresh serum; ●, WNV with IgM and hi serum; △, WNV with fresh serum only; ▲, WNV with hi serum only.

DISCUSSION

Our main findings can be summarized as follows. (i) Primary Mϕ populations infected with WNV alone supported WNV replication; yields were approximately 10-fold less than in Mϕ cell lines and approximately 100-fold less than in non-Mϕ lines. (ii) Recruited Mϕ populations (TPM, BCG-PM) from two inbred strains differed markedly in their ability to support virus growth, in the absence or presence of specific antiviral Ab (CBAT6T6 > C57BL6). (iii) The age of the donor mouse also influenced viral yield from Mϕ in cell culture with cells from younger animals more restrictive (only TPM tested). (iv) Specific anti-WNV Ab enhanced or neutralized (or both) WNV infectivity depending on the Ab class and isotype, its titer, and the nature of the Mϕ target. ADE was most readily demonstrable in BCG-PM and less so with RPM and TPM. (v) Complement-dependent (IgM and

TABLE 4. Complement-dependent enhancement of WNV infectivity in various Mϕ[a]

Cell	No. of expts	Ratio[b]				Range of yields (PFU/ml) in cultures with HIS only
		(IgM + FS)/(FS only)	(IgM + FS)/(IgM + HIS)	(IgM + HIS)/(HIS only)	(FS only)/(HIS only)	
RPM	2	4.2	167.8	1.0	65.8	<2.5–1
TPM	5	44.3	191.7	0.5	3.3	<2.5–24
BCG-PM[c]	2	0.1	10.4	0.01	0.8	67–196
P388D1	6	2.9	10.2	0.4	1.3	0–20
L929	2	0.1	0.6	0.2	1.2	18–24

[a] RPM, TPM, and BCG-PM were infected with WNV and IgM (1:20), and yields were assayed after 2 days. P388D1 and L929 monolayers were infected, and plaques were assayed directly. The results are pooled for two to six experiments performed in duplicate.

[b] See footnote *a* of Table 3. The results are expressed as four different ratios: (IgM + FS)/(FS only) describes the IgM component of the enhancement, (IgM + FS)/(IgM + HIS) describes the complement dependence of enhancement, (IgM + HIS)/(HIS only) examines the role of IgM without complement, and (FS only)/(HIS only) shows the effect of complement on virus in the absence of IgM.

[c] BCG-PM were infected with a 10-fold higher multiplicity (10^{-3}/MØ) to demonstrate neutralization.

fresh mouse serum) enhancement of WNV replication was found in all primary Mϕ, but its extent depended on the degree of neutralization by IgM alone and on the Mϕ target (marked enhancement in TPM, restriction in BCG-PM). The Mac-1 antigen (CR3) was shown to play a role in complement-dependent enhancement.

We conclude that WNV growth in Mϕ depends on a balance between enhancing and neutralizing effects, innate and immunologically determined, which is regulated independently for distinct receptor-mediated pathways in different Mϕ populations.

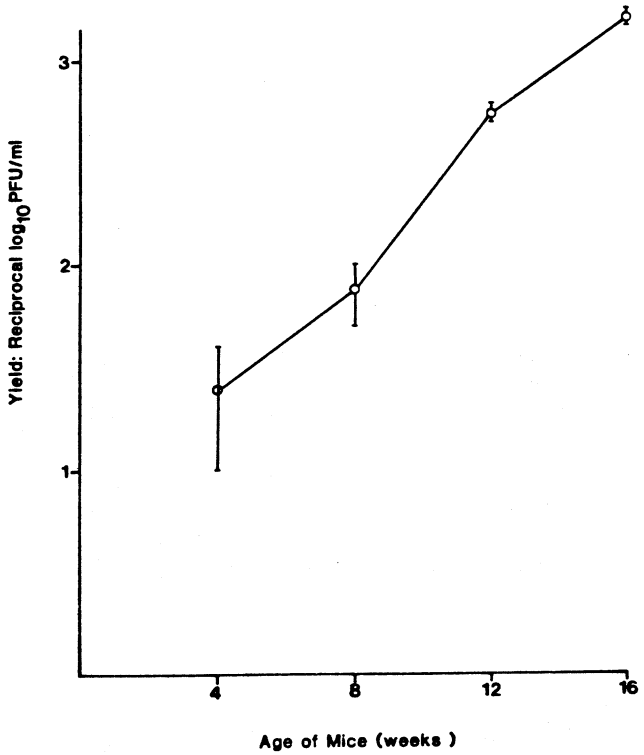

FIG. 4. TPM from younger mice are more restrictive to WNV replication. CBAT6/T6 mice of various ages were injected 4 days previously with 1.0 ml of Brewer complete thioglycolate broth intraperitoneally, and 5×10^5 elicited Mϕ were plated per well. After overnight incubation the monolayers were washed and infected with WNV (5×10^2 PFU per well). Yields were assayed after 2 days. Similar results were obtained in three independent experiments.

Mechanisms of flavivirus penetration, replication, and restriction in Mϕ are poorly understood. In the mouse models used here, we compared Mϕ populations which differ in plasma membrane, endocytic, and secretory properties and in their ability to kill various intra- and extracellular organisms and cellular targets, mainly by oxygen-dependent mechanisms (1). However, the role of the respiratory burst in Mϕ antiviral activity is unknown. Furthermore, the ability of different Mϕ populations to produce interferon and respond to γ-interferon produced by immunologically activated T lymphoctyes has not been well defined (17a, 19). With these considerations in mind, we consider the results obtained in relation to Mϕ heterogeneity and known receptor function. Characteristic reproducible differences in Mϕ-virus interaction were noted among different Mϕ populations, but the present analysis does not take into account possible heterogeneity within each population. Single cell analysis (unpublished) shows that few cells in a Mϕ population are productively infected, although more cells yield virus in ADE. It is not known whether the same Mϕ within a population restrict or promote virus replication. Although the role of FcR and CR3 has been clearly documented here and in previous studies (7, 21), and it is known that virus adsorption to Mϕ is enhanced via these receptors (12), the differences observed among various Mϕ populations cannot be simply correlated with receptor expression alone. Striking evidence was obtained for distinct intracellular pathways mediated via different receptors within the same cell population. For example, TPM express high levels of FcR (IgG1/2b) (10) and active CR3, yet these cells show little ADE by IgG and marked enhancement by IgM and complement. BCG-PM, in contrast, express lower levels of FcR (IgG1/2b) (10), but efficient enhancement occurs via this receptor. The small degree of enhancement via CR3 observed in BCG-PM is opposed by potent neutralization mediated by IgM alone. It is known that TPM store undegraded material (agar?) in secondary lysosomes (20) and that mycobacteria can interfere with phagolysosomal fusion (2). It is likely that WNV penetrates Mϕ via an endosomal compartment (15), but the complex alterations in plasma membrane flow and receptor turnover in these Mϕ populations which influence the route of viral entry require further study (31).

The interactions between immunologically activated BCG-PM and WNV are also of considerable interest. BCG-PM are known to kill a variety of intracellular microorganisms and parasites, and lymphokine (γ-interferon) can activate other Mϕ populations in vitro to display enhanced killing activity toward such targets and to release high levels

of H_2O_2 (19). In our studies BCG-PM were not more restrictive to WNV than nonactivated Mϕ even when exposed to WNV-IgG complexes, which mediated striking ADE of virus growth. This was a surprising result since the FcR can be an effective trigger of a respiratory burst in BCG-PM (18). WNV-IgM complexes did result in marked neutralization even in the absence of complement. There are no known receptors for IgM complexes on BCG or other Mϕ (34), and the effect of IgM, which was free of detectable IgG, is likely to be in promoting viral aggregation. Particle size could play an important role in triggering a respiratory burst in BCG-PM. Consistent with this line of argument are our unpublished observations that an additional signal is required to elicit killing of a viral target by BCG-PM. This can be provided by fresh serum or phorbol myristate acetate added to IgG-WNV complexes, which reduce the level of enhancement mediated by IgG substantially and which, in combination, eliminate all virus growth. BCG-PM display evidence of γ-interferon (19) effects, as demonstrated by enhanced respiratory burst activity and Ia antigen (9) and persistent low levels of mannosyl-fucosyl receptors (9, 11). However, the present studies highlight our ignorance of the regulation and function of respiratory burst products, interferon(s), and other possible antiviral activities in activated Mϕ. A common feature of BCG-PM and TPM which should also be taken into account is the substantial recruitment of less mature Mϕ into the peritoneal cavity. Resident peritoneal Mϕ displayed both Ab- and complement-mediated enhancement, but the striking difference observed between the two mouse strains studied here was found only in TPM and BCG-PM. It is known that the ability to mobilize Mϕ to sites of inflammation is under genetic control (29), but the present studies indicate that newly recruited Mϕ display genetically determined differences in their ability to replicate or restrict WNV. Several other examples are known of genetic host susceptibility to virus infection which is expressed at the level of the Mϕ (3), although to our knowledge the role of Mϕ maturity has not been previously defined.

In contrast with studies in which isolated Mϕ reflect the susceptibility or resistance of the host, Mϕ from immature animals were more restrictive to flavivirus infection, unlike the intact animal. This indicates that factors other than Mϕ function alone influence virus replication and spread within the host. Further studies are needed with Mϕ obtained from animals at different stage of flavivirus infection to define the role of Mϕ in the immune response to these viruses.

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