Role of Antibodies and Host Cells in Plaque Enhancement of Murray Valley Encephalitis Virus

SRISAKUL C. KLIKS† AND SCOTT B. HALSTEAD‡*

Department of Tropical Medicine and Medical Microbiology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96816

Received 1 November 1982/Accepted 1 February 1983

Chicken antisera to Murray Valley encephalitis (MVE) virus, when incubated with virus and assayed for plaques on chicken embryo (CE) monolayers, neutralized MVE virus at high concentrations of antibody, but caused increases in plaque counts at low concentrations of antibody. Plaque enhancement did not occur when the same virus-antibody mixtures were assayed on a continuous line of rhesus monkey kidney cells (LLC-MK2), nor when the anti-MVE antibody was of mammalian origin and the assay system was CE monolayers. Correspondingly, chicken anti-MVE did not enhance the plaque formation of MVE virus in a stable line of mouse macrophages, P-388D₁, whereas rabbit and mouse anti-MVE did enhance plaque formation. This enhancing activity was associated with noncytophilic immunoglobulin G (IgG). The Fc terminus of the IgG molecule was required, as no plaque enhancement occurred with chicken anti-MVE Fab. These data indicate that there is a requirement for taxonomic complementarity between Fc termini and Fc receptors in the above systems. CE cell monolayers were found to contain approximately 2% of Fc receptor-bearing cells among the fibroblast-like cells. Fc receptor-bearing cells in CE monolayers were isolated and found to be of the mononuclear phagocytic lineage. These mononuclear phagocytes, which originate in lymphoid tissues and blood associated with CE tissue fragments, are integrated into primary CE monolayers and form infectious centers in the presence of virus and low dilutions of antibody.

Antibody-dependent viral plaque enhancement was recognized first during a study on the neutralization of flaviviruses with antisera raised in several vertebrate species (18). On chicken embryo (CE) monolayers, Hawkes observed the partial neutralization of Murray Valley encephalitis (MVE) virus at low dilutions of chicken anti-MVE, whereas at higher dilutions, plaque numbers were greater than in control cultures. Plaque enhancement diminished with further dilutions of the antiserum. The enhancement phenomenon resided in the 7S serum fraction and could be demonstrated only when anti-MVE was raised in avian hosts. Anti-MVE enhanced plaque formation with several flaviviruses, but not with alphaviruses (18, 19).

We have published preliminary evidence that the enhancement of MVE virus plaques on CE cells is a special example of the phenomenon of antibody-dependent enhancement of viral infection in Fc receptor-bearing cells (26). Halstead and co-workers first described this phenomenon

in peripheral blood leukocyte (PBL) cultures when dengue-2 virus was incubated with sub-neutralizing concentrations of dengue antibody (13, 14). Additional examples include the antibody-dependent enhancement of West Nile virus infection in mouse and human macrophage-like cell lines P-388D₁ and U-937, respectively (34), and the 17D strain of yellow fever virus in U-937 cells (42). This paper extends our observations on antibody-dependent plaque enhancement in CE cells. We report studies on the antibody that mediates the enhancement of virus plaque formation, the requirement for homology between the antibody and Fc receptors on cell surfaces, and the identification of a subpopulation of CE cells that are involved in the phenomenon.

MATERIALS AND METHODS

Virus preparations. MVE virus strain 96961/53 was obtained from Ian Marshall of the Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia and prepared as a 10% suckling mouse brain suspension at suckling mouse passage 8.

Tissue cultures and media. CE monolayers were

† Present address: Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, DC 20012.

‡ Present address: U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD 21701.

prepared by the method of Dulbecco (9) with modifications. Washed fragments were suspended in 40 ml of Hanks balanced salt solution (HBSS) in a trypsinization flask for 5 min on a magnetic stirrer. The supernatant fluid, containing light cell debris and some erythrocytes, was discarded. This step was omitted in some preparations to retain as many monocytes as possible. Approximately 60 ml of warm 0.25% trypsin (1:250; Difco Laboratories, Detroit, Mich.) solution in phosphate-buffered saline (pH 7.4) was added, and the tissues were stirred for 15 min. Undispersed tissue was allowed to sediment at the bottom of the flask, and suspended cells were transferred to another flask containing 10 ml of calf serum (GIBCO Laboratories, Grand Island, N.Y.) on ice. Trypsinization was repeated on the tissue sediment. Chicken cell suspensions were pooled, and cells were pelleted by centrifugation at $350 \times g$ for 15 min. The cell pellet was suspended by vigorous aspiration through a syringe and cannula and diluted to a concentration of 0.5×10^6 to 1×10^6 cells per ml in BME Earle BSS (Flow Laboratories, Rockville, Md.)—2% tryptose phosphate broth (Difco)—1% heat-inactivated chicken serum (GIBCO)—1% heat-inactivated calf serum—antibiotics (CE growth medium). Prescription bottles (1 oz. [ca. 30 ml]) were seeded with 1×10^6 to 2×10^6 cells and incubated at 37°C for 48 to 72 h until complete monolayers were formed.

The LLC-MK2 line of continuous rhesus monkey kidney cells, originally obtained from Robert N. Hull, Lilly Research Laboratories (22), was cultured in BME Earle BSS containing 10% heat-inactivated calf serum and antibiotics.

Continuous mouse macrophage-like cells (P-388D₁) were grown and maintained in minimal essential medium (MEM)-alpha medium (GIBCO), supplemented with 10% fetal calf serum. Cell monolayers were dispersed with a syringe and cannula, and the cell suspension was diluted to approximately 0.5×10^6 to 1×10^6 cells per ml in MEM-alpha medium. Complete monolayers were formed in 4 days at 37°C in 5% CO₂.

Virus assays. Viruses were quantitated by plaque assays on CE, LLC-MK2, and stable pig kidney (PS) cells. CE monolayers were overlaid with Hsiung medium (21) containing 1.5% carboxymethyl cellulose (medium viscosity; Sigma Chemical Co., St. Louis, Mo.). After incubation at 37°C for 3 days, the medium was removed, and monolayers were rinsed with normal saline and then stained with naphthalene black-acetic acid solution. LLC-MK2 and PS monolayers were overlaid as previously described (13, 30).

Production of antisera. Antisera against MVE virus were raised in young adult White Mountain hybrid chickens of both sexes. Intramuscular inoculations of approximately 1×10^7 and 1×10^8 PFU of a stock MVE virus strain were given to the chickens at an interval of 18 days. Two chickens were bled 2 and 6 weeks post-inoculation and the sera were pooled. An additional three chickens were boosted at 6 weeks and bled 1 week later. Two juvenile white rabbits were given two intramuscular inoculations of approximately 1×10^7 and 1×10^8 PFU of MVE virus at an interval of 3 weeks. Sera were collected at 1 and 3 weeks. Two adult guinea pigs were given two intramuscular injections of 1×10^8 PFU of MVE virus at an interval of 2 weeks. The animals were bled 2 weeks later. Mouse ascitic fluid to MVE virus was prepared in adult Swiss

mice by the immunization method of Brandt et al. (3), using sarcoma 180/TG as ascites inducer (41). Normal chicken, rabbit, and guinea pig sera and normal mouse ascitic fluid were collected before the inoculation of MVE virus. All sera and ascitic fluids were heat inactivated at 56°C for 30 min before use.

Production of antisera to SRBC. Chicken antisera to sheep erythrocytes (SRBC) were raised in two adult White Mountain chickens given eight intravenous injections of a 20% SRBC every other day over a period of 2 weeks. Seven days after the last injection, the chickens were bled, and antisera were pooled. Guinea pig anti-SRBC was produced by the same method. Rabbit anti-SRBC was obtained commercially (glycerinated hemolysin; Difco).

Measurement of antibody activity. The plaque reduction neutralization and the hemagglutination inhibition tests were performed as previously described (5, 16, 17). The enhancement test was similar to the plaque reduction neutralization test except that the incubation temperature of the virus-antibody mixtures was 4°C. Thereafter, the reaction mixtures were assayed for plaques on CE and LLC-MK2 monolayers simultaneously.

Preparation of gamma globulins. Gamma globulins from pooled sera were precipitated with a final concentration of 18% Na₂SO₄ (Matheson, Coleman, and Bell Manufacturing Chemists, Norwood, Ohio), then twice more with 14% Na₂SO₄ (23). Precipitates were dissolved in a borate saline buffer (pH 8.0) and dialyzed against several changes of the same buffer for 48 h. IgM and IgG were separated by elution from a G-200 Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.) column in borate saline, pH 8.0. Each fraction collected was concentrated to its original volume by negative pressure dialysis.

Preparation of aggregated gamma globulins. Aggregated chicken IgG gamma globulin was a gift from A. A. Benedict, Department of Microbiology, University of Hawaii. Human gamma globulin at a concentration of 10 to 15 mg/ml was aggregated by heating at 60°C for 20 min (8). Aggregates were separated by centrifugation on discontinuous sucrose gradients (wt/wt) of 43% (1.0 ml), 25% (1.5 ml), and 10% (1.0 ml) at $100,000 \times g$ for 18 h. The 43% fraction was collected and dialyzed against phosphate-buffered saline (pH 7.4) before use. CE and LLC-MK2 monolayers were incubated with 12.5 mg of aggregated chicken gamma globulin suspension per ml (in 0.25 ml) for 1 h at 37°C and then washed.

EA rosette assay. SRBC were sensitized with antisera raised in chickens, rabbits, and guinea pigs by the method described by Ewald et al. (10). SRBC sensitized by the erythrocyte-antibody (EA) rosette assay were washed three times in normal saline. The rosette assay was performed as described by Cerottini et al. (4). A rosette was defined as a leukocyte surrounded by at least three RBC.

Separation of CE EARF cells. EA rosette-forming (EARF) cells were removed from CE suspensions derived either from trypsinized CE tissues or trypsinized CE monolayers by the method described by Moriya and Ichikawa (32).

Identification of cell subpopulations in CE monolayers. Single-cell suspensions were made from 3-day-old CE monolayers by brief trypsinization. EA rosette formation was produced with sensitized SRBC. The

rosettes were separated from nonrosetting cells on Ficoll-Hypaque. Cells bearing SRBC rosettes were suspended in a small volume of hypotonic medium for 3 s and then restored to isotonicity with an equal volume of $2 \times \text{MEM}$. Cells were washed once in phosphate-buffered saline and are referred to as CE EARF cells. As described below, morphological, cytochemical, and biological studies were done on these cells.

Culture and infection of chicken PBL. Heparinized venous blood was collected from chickens. Leukocytes were isolated as described by Lee (29). Cells collected were washed three times in HBSS containing 0.15% disodium EDTA (Sigma) in the first wash, 0.02% EDTA in the second wash, and EDTA-free HBSS in the third wash. Cells were counted and suspended at 1×10^6 to 2×10^6 cells per ml in RPMI 1640 medium with 20 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; Flow Laboratories) and 2 mM of L-glutamine per ml. To infect the chicken PBL, we mixed them with MVE virus at a multiplicity of infection of 0.01. The mixtures were divided into equal portions: one portion was mixed with serial fourfold dilutions of chicken anti-MVE, and the other was mixed with dilutions of normal chicken serum. Cell-virus-antibody mixtures were incubated at 37°C for 1 h and then washed three times to remove residual virus. Cell pellets from the mixtures were suspended in 3 ml of RPMI 1640–10% fetal calf serum. Chicken anti-MVE was readied to appropriate cultures to effect the final concentration added before washing. The mixtures were dispensed at a volume of 1 ml each into three 1-dram vials, incubated at 37°C for 3 to 5 days, frozen at -70°C , and assayed for virus.

Protein-coated silica particles. Protein-coated silica particles were prepared as described by O'Rourke et al. (33). Silica suspensions were always used immediately after preparation since their effectiveness declined rapidly upon storage.

Phagocytosis of latex particles. Latex particles were prepared as described by Halstead et al. (14).

Giemsa stain. Cell suspensions were placed on glass slides, fixed in methanol for 4 min, rinsed, air dried, and stained for 25 min with Giemsa (azure B; Harlec, Gablestown, N.J.) freshly diluted 1:10 in 0.001 M phosphate buffer, pH 6.6. Stained preparations were rinsed and air dried.

Nonspecific esterase staining. A cytocentrifuge preparation of cells was fixed by the method of Knowles et al. (27). Then, the cells were stained for the presence of nonspecific esterase as described by Horwitz et al. (20).

RESULTS

Enhancement of virus plaque formation by chicken antisera. Serial fourfold dilutions of chicken, rabbit, guinea pig, and mouse anti-MVE were incubated with approximately 250 PFU of MVE virus per ml at 4°C for 1.5 to 2 h. Reaction mixtures were assayed on CE and LLC-MK2 monolayers, and the number of plaques per milliliter in virus-serum mixtures (*V*) was compared with that from virus-diluent mix-

tures (*V*₀). The biological effect of antisera was expressed as log₁₀ values of the *V/V*₀ ratio (Fig. 1). *V/V*₀ ratios were inversely proportional to the degree of neutralization (<1.0) and directly proportional to the degree of enhancement (>1.0). On CE monolayers, at high concentrations, chicken antiserum exhibited neutralization, and at lower concentrations, it exhibited plaque enhancement. In contrast, only neutralization was observed when the same virus-serum dilutions were assayed on LLC-MK2 monolayers. Guinea pig antiserum neutralized MVE virus less effectively at a high concentration, but at lower concentrations, it produced a weak enhancement of MVE virus plaques on CE monolayers (*V/V*₀ = 1.12). Marked neutralization, however, was observed on LLC-MK2 monolayers. Rabbit and mouse anti-MVE neutralized without enhancing plaque formation on either CE or LLC-MK2 monolayers. The degree of neutralization demonstrated on LLC-MK2 monolayers was somewhat greater than that observed on CE monolayers (Fig. 2).

Effect of temperature on plaque enhancement. Reaction mixtures were incubated at 4°C at room temperature, and at 37°C for 1.5 h before the assay of residual virus activity on CE and LLC-MK2 monolayers. Plaque enhancement occurred at all temperatures tested. However, there was a slight reduction in the degree of enhancement at higher temperatures (Fig. 3). Therefore, subsequent enhancement tests were performed by incubating virus-antibody mixtures at 4°C.

Class of immunoglobulin mediating plaque enhancement. IgM and IgG fractions were collected and concentrated to levels containing approximately 2.2 and 1.66 mg of protein per ml, respectively. The hemagglutination inhibition titers for the IgM and IgG fractions were 1/10 and 1/160, respectively. Both neutralizing and enhancing activities were found predominantly in the IgG fraction when assayed on CE cells (Table 1). In addition to IgG neutralizing activity, some IgM neutralizing activity was detected, but only on LLC-MK2 monolayers (Table 1). The absence of neutralizing activity associated with the IgM fraction on CE monolayers suggested a possible contamination by enhancing IgG. Therefore, the IgM fraction was treated with rabbit anti-chicken IgG (diluted 1:10) for 1 h at 37°C. The treatment with anti-IgG removed all enhancing activity and allowed the demonstration of neutralization by IgM on CE monolayers (Table 2). The anti-IgG treatment did not change the IgM neutralizing activity on LLC-MK2 monolayers.

Cytophilic antibody. To determine whether noncomplexed antibody which mediates plaque enhancement is adherent to CE cells, we incu-

FIG. 1. Effects of chicken and guinea pig anti-MVE on MVE virus plaque formation in CE and LLC-MK2 monolayers. V , Average plaque counts of MVE virus-antibody dilutions; V_0 , average plaque counts of MVE virus-diluent control mixtures. Each point of $\log_{10} V/V_0$ values is a mean $\pm$ the standard error of three experiments. V/V_0 values from virus-antiserum mixtures assayed on CE and LLC-MK2 monolayers are given.

bated deaggregated chicken anti-MVE on CE monolayers, which were then washed before the addition of virus. Specifically, chicken anti-MVE diluted 1/4 was added to CE monolayers and incubated at 37 or 4°C for 1 h. Monolayers were washed three or six times with HBSS at 37 or 4°C, respectively, and then inoculated with virus for the plaque assay. Results from three experiments (Table 3) indicate that monomeric

antibodies were completely removed from the monolayers after three washes at 37°C. When antibody was incubated at 4°C, some residual enhancing activity was noted after three washes, indicating that some antibody molecules had attached to CE cells. However, this attachment was weak and was reversed by further washing.

Infectious virus-antibody complexes. Differences between the V/V_0 ratios on CE and LLC-

FIG. 2. Effects of rabbit and mouse anti-MVE on MVE virus plaque formation assayed on CE and LLC-MK2 monolayers. Each point represents a mean $\log_{10} V/V_0$ value $\pm$ the standard error of three experiments.

TABLE 1. Enhancement and neutralization activities in whole gamma globulin and IgM and IgG fractions assayed on CE and LLC-MK2 cells

Dilution	No. of plaques ^a in 0.2 ml of inoculum					
	Whole immunoglobulin (4.56 mg/ml)		IgM (2.20 mg/ml)		IgG (1.66 mg/ml)	
	CE	LLC-MK2	CE	LLC-MK2	CE	LLC-MK2
1:50	0	0	29.2	9.7	11.0	0
1:250	12.5	0	35.7	20.0	29.7	1.7
1:1,250	33.7	0.7	38.5	21.7	62.5	9.7
1:6,250	48.5	12.3	31.7	24.7	45.5	20.0
1:31,250	43.7	18.7	29.0	23.7	42.7	18.3
1:156,250	39.7	23.0	22.2	22.7	34.7	23.0
Virus + diluent	21.7	24.4				

^a Mean of triplicates.

MK2 cells suggest that a fraction of virus-antibody complexes recognized by the latter cells as neutralized were infectious to CE cells. To test this hypothesis, we incubated virus-antibody complexes with antiglobulin. Serially diluted rabbit anti-chicken IgG was mixed with an equal volume of a MVE virus-antibody mixture at an enhancing concentration. The mixtures were incubated for 30 min at 37°C and then inoculated onto CE monolayers. The treatment eliminated plaque enhancement.

Plaque enhancement by mammalian antisera on mouse macrophage cell monolayers. Class homology between the antibody and Fc receptor-bearing cells is required for successful Fc receptor-ligand binding. This phenomenon may explain why most mammalian antisera fail to enhance plaque formation on avian cells. This was tested as follows: as controls, normal rabbit serum, normal chicken IgG, and normal mouse ascitic fluid were diluted to comparable immunoglobulin concentrations. One milliliter of virus suspension, containing approximately

200 PFU of MVE virus, was mixed with an equal volume of anti-MVE raised in rabbits, chickens, and mice diluted to subneutralizing concentrations. The mixtures were incubated at room temperature for 30 min and then assayed for plaque formation on P-388D₁ mouse macrophage monolayers. P-388D₁ monolayers were only slightly permissive to infection by MVE virus in the absence of antibody. However, in the presence of subneutralizing rabbit and

TABLE 2. Removal of MVE plaque-enhancing activity from the IgM fraction by addition of rabbit anti-chicken IgG serum

Dilution of chicken anti-MVE IgM fraction added to virus	No. of plaques ^a on CE monolayers per 0.2 ml of inoculum		
	IgM + diluent	IgM + rabbit anti-chicken IgG serum	Normal rabbit serum + diluent
Diluent	19.7	21.3	ND ^b
1:25	14.3	3.3	ND
1:50	22.0	10.0	21.7
1:250	39.8	17.7	20.7
1:1,250	28.3	22.3	ND
1:6,250	20.3	20.9	ND

^a Mean of triplicates.^b ND, Not done.

FIG. 3. Effect of temperature of incubation of virus-antibody mixtures on neutralization of MVE virus by chicken anti-MVE immunoglobulin on CE monolayers. Log₁₀ V/V₀ values are expressed as the mean ± the standard error of three experiments.

TABLE 3. Effect on MVE virus plaque formation of preincubation of CE monolayers with chicken anti-MVE, followed by washings

Treatment	No. of plaques ^a per 0.2 ml of inoculum on CE monolayers		Virus control on untreated CE monolayers
	37°C	4°C	
Expt 1			
Antiserum + three washes	15.7	14.0	15.2
Antiserum + six washes	ND ^b	ND	ND
Expt 2			
Antiserum + three washes	13.0	26.3	12.3
Antiserum + six washes	13.7	13.3	
Expt 3			
Antiserum + three washes	17.7	26.3	21.0
Antiserum + six washes	14.3	18.0	

^a Mean of triplicates.^b ND, Not done.

mouse antisera, greatly enhanced plaque formation was observed (Table 4). MVE virus antibody raised in chickens, which readily enhanced MVE virus plaques on avian cells, did not produce the same results in mouse macrophages.

Dependence of plaque enhancement on Fc receptors on cells in CE monolayers. CE and LLC-MK2 monolayers were pretreated with aggregated chicken or human gamma globulin before they were used to assay virus-antibody mixtures in the enhancement test. The preincubation of CE monolayers with aggregated chicken gamma globulin inhibited virus plaque enhancement but

did not affect the neutralization of virus either on CE or LLC-MK2 monolayers (Fig. 4). However, similar treatment with human aggregated gamma globulin did not ablate plaque enhancement, nor did it interfere with the measurement of virus neutralization on LLC-MK2 monolayers. This observation also supports the concept of taxonomic complementarity between Fc termini and Fc receptors.

We have shown that guinea pig antiserum produced a weak enhancement of MVE virus plaque formation on CE monolayers despite the difference between the classes of origin of cells and antibody (Fig. 1). To determine whether Fc

FIG. 4. Effect of preincubation of CE monolayers with normal chicken and human aggregated gamma globulin on MVE virus plaque enhancement by chicken antibody. Each point represents a mean of $\log_{10} V/V_0$ values $\pm$ the standard error of three experiments.

TABLE 4. Effect of mammalian antisera on MVE virus plaque formation in mouse macrophage-like cells (P338D₁)

Addition to MVE virus	No. of plaques in triplicate wells					
	Expt 1			Expt 2		
Diluent	0,	1,	0	0,	0,	0
Normal chicken immunoglobulin (1:5,000)	0,	0,	0	1,	1,	0
Chicken anti-MVE immunoglobulin (1:10,000)	0,	0,	1	0,	1,	0
Normal rabbit serum (1:500)	0,	1,	1	0,	1,	1
Rabbit anti-MVE serum (1:1,000)	50,	47,	43	37,	42,	39
Normal mouse ascitic fluid (1:1,500)	0,	1,	0	1,	1,	1
Mouse anti-MVE ascitic fluid (1:2,500)	35,	41,	43	29,	33,	35

receptors on CE cells were involved in this type of heterologous enhancement, both CE and LLC-MK2 monolayers were treated with 0.25 ml of aggregated chicken gamma globulin (12.5 mg/ml); the monolayers were used to assay virus in reaction mixtures of MVE virus and guinea pig anti-MVE. The treatment did not affect the enhancement mediated by guinea pig anti-MVE.

Enumeration of Fc receptor-bearing cells in CE monolayers. Fc receptor-bearing cells were demonstrated in CE cells by the EA rosette method. Approximately 2% of cells obtained from trypsinized monolayers formed rosettes with SRBC sensitized with chicken antiserum. SRBC sensitized with rabbit antiserum were unable to rosette CE cells even though they did rosette rabbit PBL. SRBC sensitized with guinea pig antiserum did not form rosettes with either chicken PBL or CE cells, but did rosette human PBL. EA rosettes were formed at an incubation temperature of 4°C; when the temperature was raised to 37°C for 30 min, nearly all of the CE cells bearing rosettes phagocytosed sensitized SRBC. Normal SRBC or SRBC sensitized with rabbit antiserum were not phagocytosed.

Association between Fc receptor-bearing cells and antibody-dependent plaque enhancement. EARF cells were removed from trypsinized CE suspensions by rosetting before the preparation of monolayers. In addition to monolayers prepared from untreated CE suspensions, CE monolayers were also derived from a CE suspension that had been treated with unsensitized SRBC (as a control). CE monolayers depleted of EARF cells showed 97.5 and 98.5% reductions in EARF and immune phagocytic cells, respectively. No plaque enhancement was observed on EARF-depleted CE monolayers. Control CE monolayers showed a 27.5 and 21.4% reduction in EARF and immune phagocytic cells, respectively, compared with monolayers prepared from untreated CE suspensions. This was accompanied by a modest reduction in virus plaque enhancement.

Effect of preparation techniques on the propor-

tion of Fc receptor-bearing cells in CE monolayers. Monocytes and B lymphocytes have been detected in chicken embryos as early as 11 days of gestation (11). Chicken monocytes must be present in CE cell suspensions and might be somewhat depleted by the procedures used to prepare monolayers from trypsinized tissues. To test this hypothesis, we prepared CE monolayers from washed and unwashed fragments before trypsinization. These monolayers were compared for plaque enhancement with CE monolayers made from trypsinized tissues washed once with 40 ml of HBSS. Plaque enhancement was significantly higher ($P = 0.01$) when the reaction mixtures were assayed on monolayers prepared from unwashed fragments.

Permissiveness of CE EARF cells to MVE virus. Forty-eight-hour cultures of adherent EARF cells were tested for their interactions with MVE virus in the presence or absence of enhancing antibody. Virus-antibody complexes were formed by mixing and incubating a small volume of a MVE virus suspension (approximately 10^4 PFU/ml) with an equal volume of chicken anti-MVE immunoglobulin at an enhancing concentration (1:10,000). Virus diluent was used as a control. After 30 min at room temperature, 5 μ l containing approximately 20 PFU of MVE virus from each mixture was added to cell cultures in the chambers. Complete MEM-alpha medium was added; the cultures were incubated for 48 h at 37°C, and the virus yield was assayed. In the presence of chicken anti-MVE immunoglobulin, virus production was enhanced 10 times (Table 5).

Antibody-dependent enhancement of virus infection in chicken PBL. Halstead and O'Rourke have demonstrated the antibody-dependent enhanced virus growth of dengue-2 in cultured human and monkey mononuclear phagocytes (13, 14). We sought to determine whether MVE virus replication can be enhanced by chicken anti-MVE in chicken PBL. In addition, a parallel experiment with rabbit anti-MVE was carried out with the same batch of chicken PBL and by

TABLE 5. MVE virus antibody-dependent plaque enhancement on monolayers formed of EARF cells

Treatment	Plaque enhancement (PFU/ml) ^a	
	Expt 1	Expt 2
MVE virus + diluent	90	93
MVE virus + chicken anti-MVE immunoglobulin (1:10,000)	890	1,007

^a Number of plaques per milliliter of inoculum assayed in triplicate on LLC-MK2 monolayers.

the same experimental methods. MVE virus in the presence of normal serum replicated little or not at all in chicken mononuclear leukocytes (Table 6). MVE virus replication in chicken PBL was enhanced at least 10-fold by chicken anti-MVE diluted beyond the neutralizing titer. Rabbit anti-MVE at a dilution that enhanced MVE virus plaques on P-388D₁ mouse macrophages failed to enhance virus production in chicken PBL.

Identification of cells supporting MVE virus replication in PBL. Silica particles coated with protein are not toxic to nonphagocytic PBL (2) but selectively kill mononuclear phagocytes (33). To determine whether mononuclear phagocytes were involved in the enhancement of MVE virus production in chicken PBL, we added protein-coated silica particles to final concentrations of 200, 100, and 25 µg/ml to reaction mixtures of virus, antibody, and cells. The PBL were incubated at 37°C for 3, 4, and 5 days and then assayed for virus. Silica at 100 µg/ml and above significantly inhibited virus replication

enhancement by chicken antiserum (Table 7). Virus growth in PBL treated with normal serum was also significantly reduced by silica.

CE morphology. A cytocentrifuge preparation of EARF cells was made, fixed in methanol for 4 min, stained with Giemsa, and examined microscopically. Cells varied in diameter from 15 to 40 µm and were round to oval. The majority of cells had single nuclei with occasional binucleated cells. The cytoplasm contained prominent vacuoles. Cell membranes were irregular with many villous projections. Approximately 35% of these cells ingested latex particles.

Cultivation. CE EARF cells were isolated and suspended in MEM-alpha medium at a concentration of 5×10^6 cells per ml. Approximately 0.3 ml of this cell suspension was placed in each chamber (1 by 1 cm) of a glass tissue culture slide. After 24 h of incubation at 37°C, cells firmly attached to the glass surface assumed a stellate form with elongated processes. Approximately 45% of these cells ingested latex particles.

Staining. Approximately 65% of EARF cells stained for nonspecific esterase. A majority of positive cells demonstrated the diffuse reddish-brown cytoplasmic stain characteristic of monocytes (20, 27, 45). A small portion of the cells displayed discrete granule-like cytoplasmic staining.

DISCUSSION

In the present report, primary CE monolayers were shown to be a mixture of at least two cell types: a majority of CE fibroblasts and approximately 2% EARF cells having Fc receptors. We

TABLE 6. Effect of chicken and rabbit anti-MVE on production of MVE virus in chicken PBL cultures

Serum added to PBL culture	No. of plaques per 20 µl in triplicates on:								
	Day 3			Day 4			Day 5		
Normal chicken									
1:500	0,	0,	1	1,	0,	0	27,	32,	25
1:5,000	0,	0,	0	7,	5,	9	0,	0,	2
Chicken anti-MVE									
1:500	64,	60,	61	69,	71,	75	81,	96,	76
1:2,500	>100			>100			>100		
1:12,500	>100			>100			>100		
1:62,500	>100			>100			>100		
1:312,500	76,	80,	73	>100			>100		
Normal rabbit									
1:500	0,	0,	0	0,	0,	0	26,	31,	25
1:5,000	0,	0,	0	11,	8,	9	1,	0,	0
Rabbit anti-MVE									
1:100	0,	0,	0	0,	1,	0	5,	3,	7
1:500	0,	0,	0	0,	0,	0	1,	2,	3
1:2,500	0,	0,	0	0,	0,	0	0,	0,	0
1:12,500	0,	0,	0	0,	0,	0	0,	0,	0
1:62,500	2,	1,	6	0,	0,	0	0,	0,	0

TABLE 7. Effect of silica on antibody-mediated enhanced production of MVE virus in chicken PBL cultures

Concn. of silica ($\mu\text{g/ml}$)	No. of plaques per 20 μl in triplicates					
	PBL + chicken anti-MVE (1:5,000) on:			PBL + normal chicken serum (1:5,000) on:		
	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
200	0, 0, 0	0, 0, 0	0, 1, 0	0, 2, 0	0, 1, 0	0, 0, 1
100	0, 0, 0	0, 1, 1	0, 0, 0	0, 0, 1	0, 0, 0	0, 0, 0
25	7, 9, 9	12, 9, 8	6, 6, 6	0, 0, 0	0, 0, 0	0, 0, 0
—	>100	>100	>100	0, 3, 6	13, 14, 15	8, 8, 10

propose that the EARF cells (about 4×10^4 in a monolayer of approximately 2×10^6 total cells) have the potential to become infectious centers after infection by virus-antibody complexes. The progeny virus from these cells then infect the neighboring cells to form the extra plaques observed (plaque enhancement). LLC-MK2 cells, which lack Fc receptors, do not become infected by that portion of the inoculum containing infectious immune complexes. Since particle-to-PFU ratios of 100:1 or more have been described for flaviviruses (1), complexing by antibody may serve to bring some of these unmeasured viruses to the surfaces of cells bearing Fc receptors.

Our studies on EARF cells isolated from CE monolayers indicate that they manifest immune phagocytosis and approximately 35% ingest latex particles and 65% contain nonspecific esterase. These properties and their morphological appearance indicate that CE EARF cells are of mononuclear phagocyte lineage. The contamination of rosetting cells with nonmacrophage cells having the same density as the rosettes or a functional heterogeneity among mononuclear phagocytes (37–39) could explain the observed heterogeneity in the macrophage-like properties of the rosetted population. In this study, we have demonstrated that chicken peripheral blood mononuclear phagocytes are permissive to the replication of MVE virus. Virus entry into mononuclear phagocytes is facilitated by the attachment of virus-antibody complexes to the cells. Mononuclear phagocytes are differentiated and distributed in lymphoid organs and CE blood as early as 11 days of gestation (11). This is the probable origin of the 2% EARF cells in CE monolayers. Extra washing of trypsinized CE fragments resulted in some reduction in EARF cells and, correspondingly, in MVE virus plaque enhancement. Further evidence that EARF cells in CE monolayers are mononuclear phagocytes is their possession of trypsin-resistant IgG Fc receptors that survived trypsinization during these experiments.

This study confirms the observations of Hawkes (18) and Hawkes and Lafferty (19) that enhancing antibody belongs to the IgG class and does not bind directly to the surface of target

cells in its monomeric form. Rather, the interaction of enhancing antibody with Fc receptor-bearing cells is of the opsonic type, requiring preformed virus-antibody complexes. MVE virus–chicken anti-MVE complexes assayed on CE monolayers produce two opposite effects, neutralization and enhancement; at low dilutions, the neutralizing effect overrides enhancement, whereas at higher dilutions, enhancement is manifested. In the CE system, plaque enhancement is regulated by class homology between the species of antibody and the cells used to assay the virus.

The enhancement phenomenon is an important consideration in virology because of its bearing on two widely studied phenomena: underneutralization and the persistent fraction. Host cell dependence of the degree of neutralization of virus has been reported for many systems (25, 28, 31). The phenomenon was observed in this study in that MVE virus–anti-MVE mixtures consistently yielded higher V/V_0 ratios in CE monolayers than they did when assayed on LLC-MK2 cells (Fig. 1). We propose that a frequent explanation of this phenomenon is the ability of Fc receptor-bearing cells to recognize immune complexes as infectious, which parenchymal cells do not do. The effect of these infections is to reduce the number of virus particles neutralized and so reduce the neutralization titers of antiserum. Several features are common to the enhancement phenomenon and the persistent fraction: (i) both occur in virus-antibody mixtures during the neutralization process; (ii) both are associated with virus-antibody complexes; and (iii) both are associated with IgG, not IgM. Persistent fractions have been described as being formed with antisera from many vertebrate hosts assayed on a wide array of cell cultures. The enhanced infection of immune complexes depends on the presence of Fc-bearing cells in the assay system. Since few studies on underneutralization or the persistent fraction have focused on the properties of the assay system, at present, it is not possible to quantify the proportion of these phenomena attributable to immune enhancement. Certainly, those studies employing CE cells are suspect. In the future, studies on virus neutralization will

require a careful definition of the assay system.

Alternative non-Fc receptor-dependent explanations have been proposed for the existence of a persistent fraction (6, 7, 18). In studies of a few other viruses, enhancement has been observed in tissue culture systems which would not be expected to contain Fc receptor-bearing cells (6, 7, 24, 25); however, Kimura et al. (24) demonstrated the Fc receptor-mediated enhancement of Getah virus replication in BHK-21 cells. We have observed enhanced MVE virus infection on CE monolayers by immune complexes formed by guinea pig antiserum. Since neither SRBC sensitized by guinea pig antiserum attached to CE monolayers nor aggregated IgG of chicken origin was able to block this enhancement phenomenon, it must be concluded that guinea pig anti-MVE-virus complexes do not attach to CE cells via a chicken Fc receptor. The existence of Fc-like receptors capable of reacting with guinea pig Fc termini has not been ruled out. Our observations, together with those of others (13, 14, 18, 34, 42), suggest that antibody-mediated virus replication enhancement in Fc receptor-bearing cells is a generalized biological phenomenon among flaviviruses, as well as viruses of other taxons (35, 36, 44), which may play a regulatory role in the severity of a number of viral diseases.

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