

Opsonization of Alphaviruses in Hamsters

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Immune elimination of alphaviruses in immunized hamsters appears to involve formation of virus/antibody aggregates which are subsequently cleared from the circulation by cells of the reticuloendothelial system (RES). Virulent strains of Venezuelan (VEE) and Western equine encephalitis (WEE) viruses which were cleared slowly from the circulation of nonimmune hamsters, were cleared rapidly when inoculated into the blood of immunized hamsters. Likewise, when these viruses were mixed with specific hamster immune serum prior to inoculation, they were efficiently cleared from the circulation of nonimmune hamsters. Virus, mixed with specific immune serum, or inoculated into immunized hamsters, formed virus/antibody aggregates, as demonstrated by density gradient centrifugation, filtration through polycarbonate membranes, precipitation with Staphylococcus protein A, and electron microscopy. Cleared virus was concentrated primarily in liver and spleen, as confirmed by autoradiography. Immune clearance of virulent VEE was demonstrable within 5 to 6 days following immunization of hamsters with live attenuated VEE vaccine, strain TC-83. In these hamsters, a close association was established between formation of virus/antibody aggregates, rapid clearance, and survival of challenged hamsters. Adsorption of virus to hamster macrophages in culture was enhanced by immune serum in the presence of complement. These results are compatible with the hypothesis that immune clearance of virus in the intact hamster involves a complement-dependent interaction of virus/antibody complexes with cells which possess Fc and complement receptors. The clearance of immune complexes by the RES serves to amplify the protective effect of neutralizing antibody alone.

Key words: alphavirus, opsonization, neutralization, macrophage, hamster, immunization

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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INTRODUCTION

Neutralizing antibody to alphaviruses, either induced by immunization or passively administered, is known to protect a wide variety of animal species, including humans, against virulent alphavirus strains [Cole and McKinney, 1971; Houston et al, 1974; McKinney et al, 1963]. While the mechanisms by which certain alphaviruses, such as Venezuelan equine encephalitis virus (VEE), are neutralized by antibody have been studied in some detail *in vitro* [Hahon, 1969], very little is known concerning the mechanisms of virus neutralization in the intact animal. Antiviral antibody could reduce virion infectivity both *in vitro* and *in vivo* by: (i) aggregating infectious virus, and thereby reducing the number of infectious units, (ii) inhibiting attachment, penetration, or uncoating of virus in susceptible cells [Fazekas De St Groth, 1962], or (iii) lysing virus particles in the presence of complement [Berry and Almeida, 1968]. In the intact animal, antibodies might also promote phagocytosis of virus particles, as has been described for mousepox [Mims, 1964], and vaccinia [Silverstein, 1975] viruses, acting analogously to opsonins in bacterial systems. The initial purpose of our studies was to define a model for immune clearance of alphaviruses in animals. We selected the experimental infections of hamsters with virulent strains of VEE and Western equine encephalitis (WEE) viruses to develop this model for several reasons. Attenuated virus strains are available for immunization, infections with the virulent strains are uniformly fatal, and neither virulent strain is cleared from the blood of nonimmune hamsters [Jahrling and Scherer, 1973], thus facilitating comparisons of virus clearance data from immunized versus control groups of hamsters.

MATERIALS AND METHODS

Viruses and Virus Assays

The Trinidad donkey strain of VEE, 1-2AC-8, had been passed 14 times in 11-day-old, embryonated chicken eggs [Hardy, 1959] before being passed one additional time in suckling mouse brain. We refer to this virus strain as VEE-I. The TC-83 vaccine strain of VEE was obtained as a lyophilized preparation from the Merrell-National Drug Co, (Swiftwater, Pennsylvania) lot 4/2. Large plaque (LP) and small plaque (SP) clones of WEE virus were obtained from strain 72V1880 as described previously [Jahrling, 1976]. To prepare concentrated, radiolabeled virus stocks, all viruses were passed once in BHK-21 cell monolayers grown in 800-cm² roller bottles incubated with ³²P as monopotassium phosphate; cell culture supernatants were harvested, concentrated, and purified as described previously [Jahrling and Gorelkin, 1975]. All virus stocks and sera obtained from hamsters were assayed for infectious virus by counting plaque-forming units (pfu) on primary duck embryo cell (DEC) monolayers grown in 10-cm² wells of plastic plates and maintained under medium containing 1% agarose, as described previously [Jahrling et al, 1974]. Radioactive ³²P virus activity was measured by diluting 100- μ l samples in 6 ml of scintillation fluid and counting in a scintillation counter.

Determination of Viral Clearance Rates and Distribution of Inoculum Virus in Hamsters

Male, golden Syrian hamsters weighing 80–90 gm, were obtained from Lakeview Hamster Colony (Newfield, NJ). To determine virus clearance rates, hamsters,

lightly anesthetized with sodium pentobarbital, were inoculated via the intracardiac (ica) route with 1.0-ml vol of the inocula detailed in the text. They were bled from the orbital sinus, usually 1, 5, and 20 min after inoculation, to obtain serum for virus assay. Liver samples were occasionally obtained and assayed for ^{32}P virus content after dissolving them in 5 M NaOH at 80°C for 5 hr, [Jahrling and Gorelkin, 1975]. Specific clearance of virus from the blood was determined by comparing the observed concentrations of virus in the blood with the concentrations expected, if no clearance had occurred [Jahrling and Scherer, 1973; Jahrling, 1976].

Immunization of Hamsters

To immunize groups of hamsters, 4.0 log₁₀ pfu of TC-83 or 6.4 log₁₀ pfu of WEE-SP were inoculated SC. To obtain immune serum for clearance and adsorption studies, groups of 20 hamsters were inoculated and bled under chloroform anesthesia from the heart 28 days after virus inoculation; sera were pooled. Serum pools were heated to 56°C for 30 min before freezing in 1.0-ml aliquots. In a serum dilution plaque reduction neutralization (SDN) test using DEC, a 1:320 dilution of the heated anti-VEE serum reduced 100 pfu of the VEE-I challenge virus titer by 80%; similarly, the anti-WEE serum titered 1:640 against WEE-LP challenge. The heterologous SDN titers for anti-VEE versus WEE and anti-WEE versus VEE were less than 1:10. Where indicated in the text, fresh hamster serum was occasionally added to the heated immune serum to restore complement activity.

Demonstration of Virus Aggregation

Radiolabeled VEE or WEE containing 10.1 or 10.2 log₁₀ pfu, respectively, were diluted 1:10 into 1:10 dilutions of control or immune serum. The diluent was 1% bovine serum albumin in Hanks' balanced salt solution, pH 7.6 (BSA/H). Virus/serum mixtures were incubated for 1 hr at 37°C in a water bath, prior to density gradient filtration and electron microscope studies. Following incubation, 1.0-ml vol were applied to linear sucrose gradients (10–30% sucrose wt/vol in TNE buffer; 0.1 M NaCl, 0.02 M Tris-HCl, 0.001 M EDTA) poured in 17-ml tubes over 1.0 ml 60% wt/vol sucrose cushions. Gradients were centrifuged in a 27.2 rotor at 25,000 rpm (90,000g) at 0°C for 2 hr. Fractions (1.0 ml) were harvested from the top of the gradients using a mechanical collector. For filtration experiments, 1.0 ml of incubated virus/serum mixture was passed under gravity through Nucleopore polycarbonate membrane filters (13 mm in diameter) rated at 0.10- μm average pore size in Swinnex filter holders with 3-ml disposable syringes attached. Complete filtration required overnight incubation at 5°C. Filters were removed from the holders (they did not require draining) and were dissolved in scintillation fluid for counting. Samples of filtrate (100 μl) were also assayed for ^{32}P activity. Occasionally pre- and postfiltration samples were assayed for pfu. For electron microscopic examination, 25 μl of virus/serum mixtures were applied to carbon-coated grids, stained with 2% phosphotungstic acid (pH 7.2), and examined with a Hitachi electron microscope. To demonstrate the presence of IgG in virus aggregates formed in the virus/serum mixtures, 0.1 ml of staphylococcal protein A, prepared and used as described [Cullen and Schwartz, 1976] was added to 0.45 ml of each mixture, which was then vortexed and centrifuged at 2500 rpm for 10 min at 0°C to precipitate ^{32}P virus/antibody complexes. The proportion of labeled virus precipitated was calculated by comparing the ^{32}P activity in the supernatant with the ^{32}P activity before centrifugation.

Electron Microscopy

Negative stains of VEE virus antibody complexes were made with 2% phosphotungstic acid (pH 7.2) on Formvar carbon-coated grids. Macrophages which were incubated with virus antibody complexes were fixed in 2% glutaraldehyde in sodium cacodylate buffer and sectioned. All preparations were examined in a Hitachi Hu-12 electron microscope.

Preparation and Adsorption of Virus to Hamster Peritoneal Macrophages

Hamsters were inoculated IP with 5 ml of sterile mineral oil, and 3 days later peritoneal macrophages were harvested as described for guinea pig peritoneal macrophages [Kishimoto et al, 1977]. Washed peritoneal cells were suspended at a concentration of 5×10^6 cells/ml in RPMI-1640 growth medium containing 10% heat-inactivated calf serum. Cell suspensions (20 ml) were dispensed into the 10-cm² well of plastic plates, and incubated for 3–4 hr at 37°C in a humidified atmosphere of air containing 5% CO₂. To remove nonadherent cells the plates were gently swirled, and the medium was removed by suction. Cells were washed once with 2 ml of growth medium and incubated overnight before being used for adsorption studies. Adherent cells expressed both FC and C receptors as measured in competitive binding assays. To measure the effects of antibody and complement on the adsorption of virus to these cells, fresh nonimmune serum was added to equal volumes of either heated immune or heated nonimmune hamster serum to restore complement activity to these sera; alternatively, sera were mixed with equal volumes of heated nonimmune serum to obtain 1:2 dilutions of serum without complement. ³²P-Labeled VEE or WEE were then diluted 1:10 into these serum preparations. Virus/serum mixtures were incubated for 1 hr at 37°C; mixtures were sampled for ³²P virus activity and 0.2-ml vol of each mixture were then adsorbed to the drained 10-cm² monolayers of hamster peritoneal cells in triplicate for 1 hr at 37°C in the CO₂ incubator with gentle rocking of the plates at 10-min intervals. Following the adsorption period, residual (nonadsorbed) ³²P virus was determined and the percentage of total ³²P-virus activity adsorbed was calculated.

Autoradiographic Analysis of Liver Sections

Livers from hamsters inoculated with 10.2 log₁₀ pfu of ³H-labeled VEE plus nonimmune or immune sera were removed 20 min following inoculation and fixed in 10% buffered Formalin; 6-μm sections were processed for autoradiography as described [Anderson et al, 1975] using HTB-2 liquid emulsion (Eastman-Kodak, Rochester, NY). Sections were exposed for 8 to 14 weeks and stained through the emulsion with hematoxylin and eosin.

RESULTS

Clearance of Virus in Nonimmune and Immune Hamsters and Virus/Antibody Mixtures in Nonimmune Hamsters

Figure 1 compares clearance of the virulent Trinidad donkey strain of VEE virus (VEE-I) from the circulation of normal (nonimmune) and immune hamsters during the 20-min observation period following intracutaneous inoculation of virus. In nonimmune hamsters, clearance of virus was negligible. However, in hamsters immunized

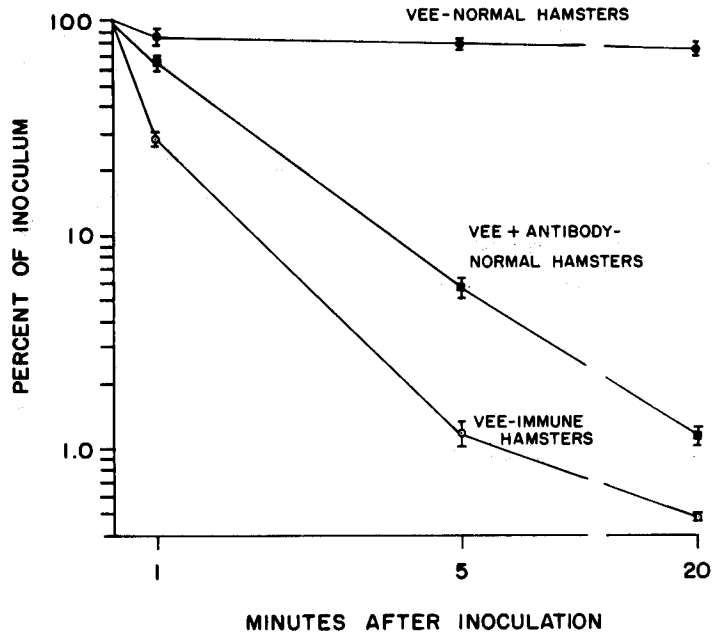


Fig. 1. Clearance of ^{32}P -labeled virulent Venezuelan equine encephalitis (VEE-I) virus from the blood of normal (nonimmune) hamsters and from VEE-immune hamsters (inoculated with TC-83 vaccine 28 days previously) and the clearance of VEE mixed with anti-VEE immune serum from the blood of immune hamsters. Each point is a geometric mean (\pm SE) based on six hamsters.

to VEE 28 days previously, clearance of this virus was greatly enhanced. Similarly, when the inoculum virus was preincubated with hamster immune serum (obtained from hamsters immunized to VEE 28 days previously) prior to inoculation into nonimmune hamsters, the clearance rate was also greatly accelerated (Fig. 1). Since virus concentrations in the blood of inoculated hamsters were measured by ^{32}P label, and not by infectious virus, clearance reflects actual removal of virus from the circulation, and not simply inactivation of infectivity by the combination of infectious virus with neutralizing antibody. The data presented in Table I, however, suggest that the combination of virus with antibody specific for that virus effects the accelerated virus clearance observed in Figure 1.

Data for groups 1, 2, and 5 (Table I) extend the data illustrated in Figure 1 and compare the clearance rates of VEE for immune hamsters (group 1) and nonimmune hamsters inoculated with virus preincubated with VEE-immune serum (group 2) with controls (group 5). In groups 1 and 2, most of the input virus was cleared from the circulation within 20 min, and approximately half of this virus was recoverable from the liver. In contrast, clearance of VEE was not accelerated in hamsters immunized to WEE (group 3) nor did preincubation of VEE with WEE-immune serum accelerate clearance in nonimmune hamsters (group 4). The reciprocal experiment, which compares clearance rates for WEE in WEE- or VEE-immune hamsters or in combination with WEE- and VEE-immune sera (groups 6-10), provides similar information. These data suggest that accelerated clearance with redistribution of virus to the liver depends on an initial interaction of virus with specific antibody.

TABLE I. Clearance and Distribution of ³²P-Labeled Venezuelan (VEE) and Western Equine Encephalitis (WEE) Viruses or Virus/Antibody Mixtures in Normal or Immune Hamsters

Group	Inoculum		Hamster immune to	Inoculum virus recovered ^b (%)			
				Serum			Liver
	Virus ^a + immune serum			1 min	5 min	20 min	20 min
1	VEE	Control	VEE	38.0	4.2	3.3	52.7
2		VEE	Control	56.1	11.9	3.9	46.6
3		Control	WEE	83.2	79.6	75.5	16.2
4		WEE	Control	76.5	78.3	72.9	17.0
5		Control	Control	81.7	82.7	76.8	15.9
6	WEE	Control	WEE	51.2	1.7	<1.0	58.9
7		WEE	Control	29.5	<1.0	<1.0	48.2
8		Control	VEE	92.2	53.7	50.1	19.4
9		VEE	Control	96.6	75.8	51.2	21.0
10		Control	Control	97.7	75.8	68.9	14.7

^aThe 10.1 and 10.2 log₁₀ pfu of ³²P-WEE were diluted 1:10 into 1:10 dilutions of control (nonimmune) hamster, VEE-immune, or WEE-immune serum, and incubated 1 hr at 37°C; 1.0 ml of virus/serum mixtures were inoculated into control, VEE-immune, or WEE-immune hamsters. Six hamsters were inoculated per group.

^bCalculated on the basis of ³²P concentrations in 100 μl of serum or in 100 μl of 10% wt/vol liver homogenate. Standard errors of the mean are omitted to simplify the table, but were negligible.

Demonstration of Virus/Antibody Aggregates

The combination of virus with antibody resulted in the formation of aggregates, as demonstrated by rate zonal centrifugation, electron microscopy, and filtration using polycarbonate membrane filters. Figure 2 represents the sedimentation profile of ³²P-labeled VEE or VEE/antibody aggregates in 10–30% sucrose gradients. Virus was incubated with either nonimmune, 28-day immune, or decomplexed (heated) immune serum for 1 hr at 37°C prior to centrifugation. The sedimentation peak of virus preincubated with nonimmune serum was located in a dense band in fractions 7 and 8 of the gradients. The shoulder seen in fractions 9–12 represents aggregates (ie pairs and triplets) which we see in VEE virus populations purified by the procedures described, and represents approximately 10% of the total virions present. In contrast, virus mixed with VEE-immune serum (either heated or unheated) sedimented faster through these gradients and was recovered primarily from the 60% sucrose cushion at the bottom. The combination of virus with antibody in the presence of complement may have resulted in some minor lysis of VEE virus particles, since approximately 2% of the total label applied was recovered from the top of the gradient containing the virus/immune serum mixture. In contrast, less than 0.3% of the total labels applied to the gradients containing either the virus/normal serum or the virus/heated immune serum mixtures were recovered from the first gradient fraction.

Filtration of virus/serum mixtures through polycarbonate membranes, rated at an average pore size of 0.10 μm, provided additional evidence that VEE and WEE viruses were aggregated after incubation with homologous, but not heterologous immune serum (Table II). When ³²P-labeled VEE or WEE virus was mixed with its homologous antisera and then filtered, the quantity of ³²P activity trapped on the filter was approximately six times the ³²P activity which passed the filter, indicating

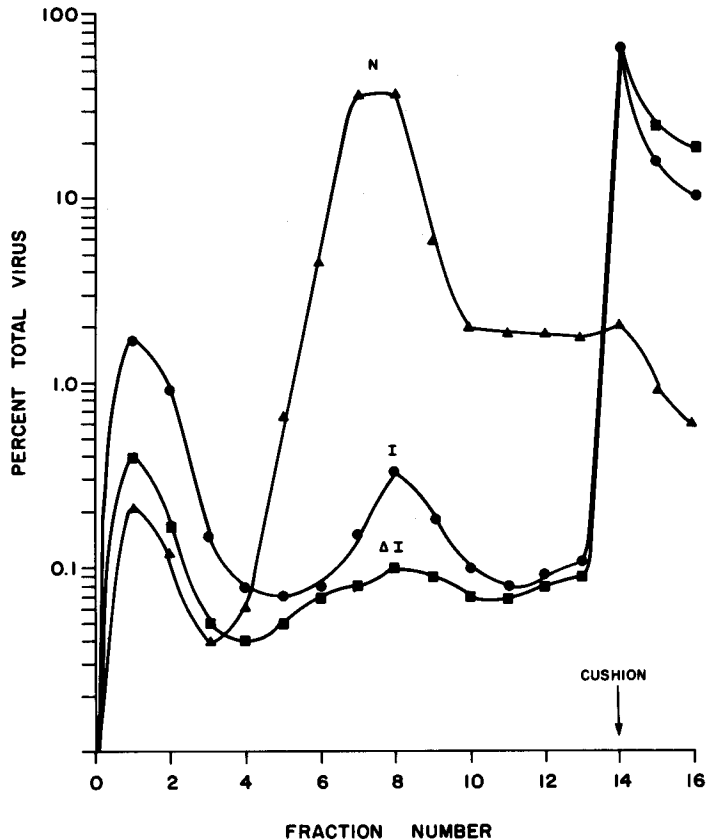


Fig. 2. Distribution of ^{32}P -labeled Venezuelan equine encephalitis virus after rate zonal centrifugation in linear 10–30 sucrose gradients for 2 hr at 90,000g at 0°C . Virus was incubated with 1:10 dilutions of nonimmune serum (N▲), immune serum (I●), or heated (56°C , 30 min) immune serum (Δ I■) prior to centrifugation.

aggregation of ^{32}P viruses. In contrast, when these viruses were mixed either with heterologous immune, or nonimmune (control) serum, less than one-sixth the quantity of ^{32}P virus was deposited on the filters, indicating significantly less aggregation. These conclusions were confirmed by direct observation of virus/serum mixtures by electron microscopy (see Table IV and Figs. 3 and 4). Addition of staphylococcal protein A to the mixtures of virus and immune serum described above resulted in the precipitation of greater than 90% of the total radioactive virus activity, suggesting that the virus complexes also contained IgG antibody. Protein A precipitated less than 9% of virus radioactivity from mixtures of virus and normal serum (data not shown).

One explanation for the accelerated clearance of virulent VEE and WEE viruses from the circulation of immune hamsters is that the viruses become aggregated following introduction into the blood of immune, but not nonimmune, hamsters. We demonstrated that aggregation does occur by passing the six serum samples which comprise the 1-min time points in Figure 1 (for the clearance curves of VEE in nonimmune and immune hamsters), through $0.10\text{-}\mu\text{m}$ Nucleopore filters. Virus recovered from the VEE-immune hamsters was aggregated, since the ratios of counts

TABLE II. Filtration* of Venezuelan (VEE) and Western Equine Encephalitis (WEE) Virus/Serum Mixtures as an Index of Viral Aggregation

Virus ^a	Ratio of cpm filter: cpm filtrate		
	Anti-VEE	Anti-WEE	Normal serum
VEE	5.95	0.81	0.33
WEE	0.87	6.95	1.09

*Filtration of 1.0 ml of virus/serum mixtures under gravity through polycarbonate membrane filters with an average rated pore size of 0.10 μm . Arithmetic means were based on five determinations.

³²P-VEE or ³²P-WEE, containing 10.1 and 10.2 log₁₀ pfu, respectively, diluted 1:10 into 1:10 dilutions of control or immune sera. Percentage of recovery of input (filter + filtrate cpm > 99%).

TABLE III. Clearance and Distribution of ³²P-Labeled Venezuelan Equine Encephalitis Virus* in Hamsters Inoculated Several Days Previously With TC-83 Vaccine

Days after TC-83 immunization	Inoculum virus recovered (%)				
	Plasma			Liver	Spleen
	1 min	5 min	20 min	20 min	20 min
1	95.4	77.6	69.1	9.1	Not tested
2	87.0	79.5	70.2	12.6	Not tested
3	85.1	79.5	66.0	12.2	Not tested
4	69.1	56.2	54.9	15.9	Not tested
5	63.0	49.5	38.1	21.6	Not tested
6	31.6	4.1	3.2	41.5	Not tested
7	22.9	9.1	1.2	49.6	Not tested
28	21.3	3.1	0.9	48.4	45.0

*The 9.1 log₁₀ pfu inoculated in 1.0-ml vol into six hamsters for each group.

per minutes (cpm) [filter/filtrate] ranged from 4.96 to 5.58. In contrast, ratios of cpm [filtrate/filtrate] for virus recovered from nonimmune hamsters ranged from 0.76 to 1.03, indicating negligible aggregation. The estimation of aggregation by the filtration technique correlated closely with aggregation visualized with the electron microscope, as described below.

Correlation Between Formation of Virus/Antibody Aggregates, Rapid Clearance, and Protection

Accelerated clearance of VEE in immunized hamsters became detectable within 5 to 6 days following inoculation of live attenuated TC-83, VEE vaccine virus (Table III). The ³²P-VEE virus, cleared from the circulation of these recently immunized hamsters, was recovered primarily from the liver. On day 28, 48.4% of the input virus was recovered from the liver (Table III), while an additional 45% was recovered from the spleen. Table IV provides strong correlative evidence that accelerated clearance is functionally related to aggregation of virus and protection of recently immunized hamsters. Serum specimens obtained from hamsters 5 and 6 days following immunization were tested individually for the presence of vaccine virus, and for their ability to aggregate ³²P-VEE virus as measured by filtration and, in some cases,

TABLE IV. Correlation Between Formation of Venezuelan Equine Encephalitis (VEE-I) Virus/Antibody Aggregates, Rapid Clearance, and Protection of Individual Hamsters Inoculated 5 or 6 Days After TC-83 Vaccine

Days after TC-83 immunization		TC-83 viremia at challenge (\log_{10} pfu ml)	Ratio filter (cpm)/Filtrate	EM observation	Inoculum VEE-I virus remaining in plasma at 20 min (%)	Died after challenge
5	A	2.2	1.90	ND ^a	50	Yes
	B	<0.7	4.10	Aggregates	<1	No
	C	<0.7	2.60	ND	<1	No
	D	4.9	1.17	Singles	100	Yes
	E	3.7	1.19	ND	50	Yes
	F	<0.7	1.12	Singles	100	Yes
	G	4.9	0.82	ND	32	Yes
	H	2.2	1.27	ND	50	Yes
6	J	<0.7	10.97	Aggregates	<1	No
	K	<0.7	6.90	ND	<1	No
	L	<0.7	0.52	Singles	100	Yes
	M	<0.7	5.90	ND	<1	No
	N	<0.7	8.55	Aggregates	<1	No
	O	<0.7	7.47	ND	<1	No
	P	<0.7	7.13	ND	<1	No
Control	None	1.15	Singles	80	Yes	

^aNot done.

by electron microscopy. These parameters were correlated with the clearance of ³²P-VEE from the circulation, and the survival or death of the individual hamsters following the clearance rate determination. The data in Table IV clearly indicate a close association between termination of viremia, formation of virus aggregates which failed to pass the filter and were visualized by electron microscopy, accelerated clearance, and survival.

Figure 3 is an electron micrograph illustrating virus/antibody aggregates, formed by mixing ³²P virus with a 1:10 dilution of serum obtained from hamster J, 6 days after immunization. Only a few single virus particles were visualized. Most of the virus was aggregated; some particles appeared to be undergoing lysis and looked hollow. Most of the virus appeared "fuzzy," which is probably due to the antibody surrounding them. In contrast, the virus in Figure 4, which was mixed with a 1:10 dilution of the nonaggregating serum from hamster 5-D (Table IV), was uniformly dispersed, with sharply defined structures.

Adsorption of Virus/Antibody Aggregates to Macrophages in Culture

Clearance of virus/antibody complexes from the circulation of immune hamsters could be effected by adsorption of the complexes to phagocytic cells of the liver and other reticuloendothelial cells, and could be mediated by Fc and complement receptors. Table V presents data which compare the adsorption efficiencies of VEE and WEE viruses to hamster peritoneal macrophages (which demonstrably possess hamster Fc and complement receptors), and to primary duck cells (which lack these receptors), in the presence or absence of specific homologous antibody and fresh normal serum as a source of complement. Both VEE and WEE viruses, in the presence of antibody plus complement, efficiently adsorbed to hamster macrophages;

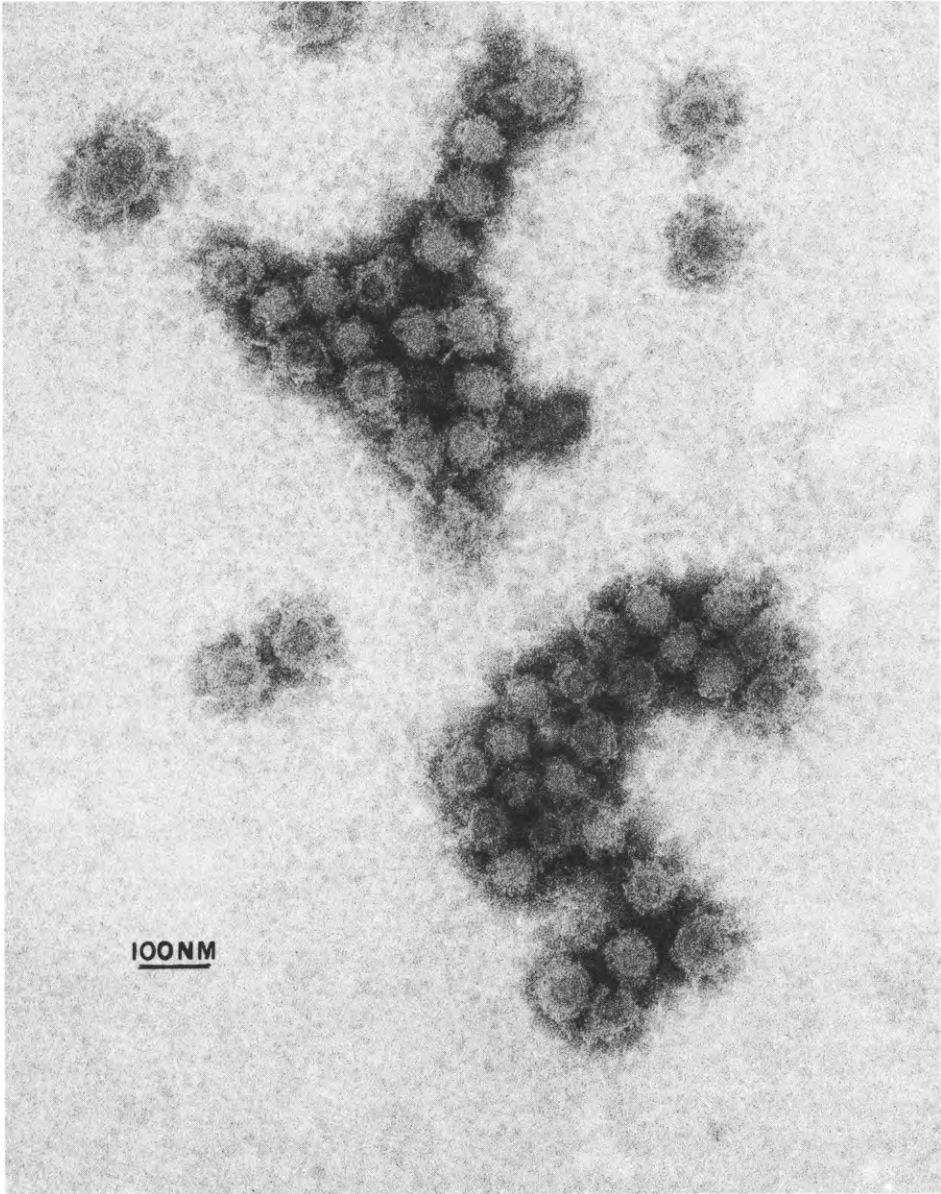


Fig. 3. Electron micrograph of virus aggregates formed by the incubation of Venezuelan equine encephalitis virus with a 1:10 dilution of unheated serum obtained from a hamster 6 days after inoculation of TC-83 vaccine. Most of the virus is aggregated. Some virus particles appear hollow, and may be undergoing degradation ($\times 120,000$).

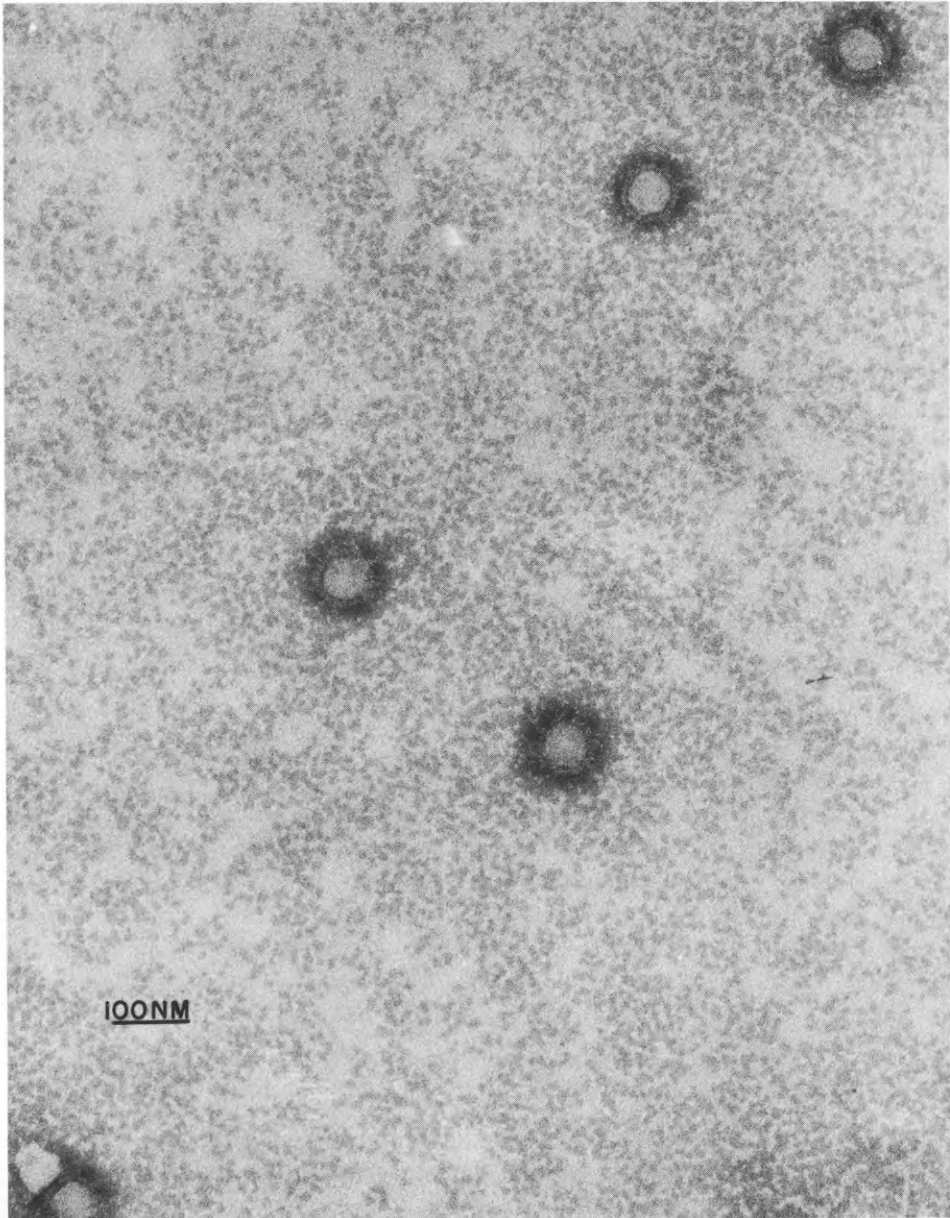


Fig. 4. Electron micrograph of single, well-dispersed Venezuelan equine encephalitis virus particles following incubation of the same dilution of the same virus used in Figure 3 with serum obtained from a hamster 5 days after inoculation of TC-83 vaccine, and suspected to lack virus-aggregating activity based on filtration and clearance data. ($\times 120,000$).

TABLE V. Adsorption of ³²P-Labeled Venezuelan and Western Equine Encephalitis Viruses to Hamster Peritoneal Macrophages in the Presence or Absence of Immune Serum and Complement

Virus	Heated serum immune to	Complement ^a added	Virus adsorbed (\pm SE) ^b (%)		
			Macrophages	BHK-21	Duck cells
VEE	VEE	+	45.4 \pm 6.0	16.5 \pm 2.3	17.3 \pm 1.6
	VEE	0	22.2 \pm 2.1	14.9 \pm 0.6	15.9 \pm 1.8
	Control	+	16.6 \pm 4.2	5.4 \pm 0.4	16.4 \pm 0.8
WEE	WEE	+	47.2 \pm 5.0	12.3 \pm 0.8	13.6 \pm 1.2
	WEE	0	23.3 \pm 2.0	11.9 \pm 1.1	18.2 \pm 1.1
	Control	+	20.8 \pm 1.9	10.9 \pm 0.3	18.2 \pm 0.7

^aFresh nonimmune serum was added as a source of complement.

^bBased on six replicate determinations, three in each of two experiments.

however, adsorption of virus/antibody mixtures to these macrophages in the absence of either complement or specific antibody was less efficient. Figure 5 illustrates the observed association between VEE virus/antibody aggregates and hamster macrophages following the 1-hr incubation protocol described for Table V. In the presence of complement, numerous virus/antibody aggregates were observed within vacuoles of the majority of cultured peritoneal macrophages. Cells incubated with virus plus normal serum or with virus plus immune serum in the absence of complement did not contain these virus/antibody aggregates. These data are compatible with the hypothesis that the clearance of virus/antibody aggregates from the circulation of immune hamsters depends on a complement-mediated interaction of virus/antibody aggregates with cells that possess Fc and complement receptors.

Autoradiographic Distribution of ³H-Labeled Virus

To define more precisely the fate of radiolabeled virus in VEE-immune hamsters, autoradiographs of liver and spleen sections, obtained 20 min after inoculation, were developed. As predicted from Table III, virus in immune hamsters was concentrated in livers and also in spleens. Figure 6 illustrates the high concentrations of labeled virus associated with unidentified cells (presumably Kupfer cells), in the livers of immune hamsters. In contrast is the sparse distribution of labeled virus in livers of nonimmune hamsters. In spleens of immune hamsters, virus was concentrated in the marginal zones of white pulp, while in nonimmune spleens very little labeled virus was detected.

DISCUSSION

The data presented suggest that neutralization of virulent VEE and WEE viruses in the intact immune hamster depends on two initial events. The combination of virus with specific immune serum results in some apparent virus inactivation, due primarily to the aggregation of virions, which in effect reduces the number of infectious units [Benacerraf and Miescher, 1960]. Subsequently, the virus/antibody aggregates are efficiently eliminated from the circulation by interaction with cells of the RES. The recovery of virus/antibody aggregates (precipitable by Staphylococcus protein A) from the circulation of immune hamsters 1 min after the inoculation of ³²P-VEE, confirms that formation of virus/antibody complexes precedes the elimination of virus. The termination of viremia following a primary infection with a nonlethal VEE

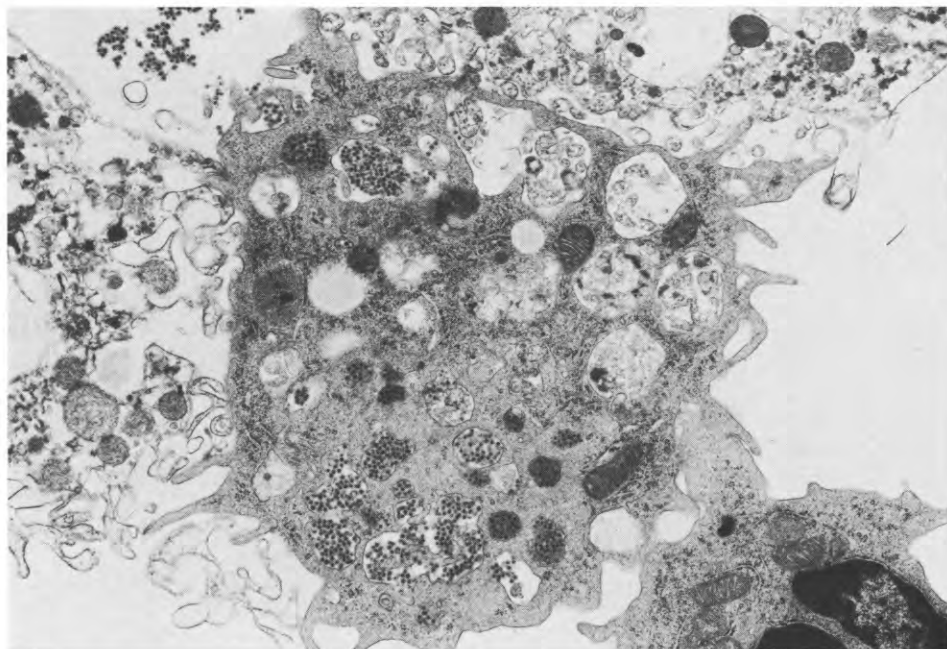


Fig. 5. Electron micrograph of VEE virus/antibody aggregates within vacuoles of hamster peritoneal macrophages following incubation of VEE virus plus immune serum and complement ($\times 13,050$).

virus strain in hamsters similarly appears to depend on the formation of virus/antibody aggregates which are subsequently eliminated.

The efficient clearance of virus/antibody aggregates dramatically increases the effectiveness of neutralizing antibody alone. A 1:10 dilution of the VEE-immune hamster serum used in Figure 1 reduced the infectious VEE inoculum virus titer from $9.1 \log_{10}$ pfu/ml to $7.8 \log_{10}$ pfu/ml. Yet when this virus antibody mixture, with an effective virus titer of $7.8 \log_{10}$ pfu, was inoculated *ica* into hamsters, none of the hamsters died, and the inoculum virus was completely cleared from the blood. A dose of $7.8 \log_{10}$ pfu of virulent VEE virus, if not complexed with antibody, would not be cleared at all [Jahrling and Scherer, 1973; Jahrling and Gorelkin, 1975], and would rapidly kill hamsters in 3 days or less. Similarly, immune clearance amplifies the effect of low levels of antibody, such as the levels obtained soon after immunization. The sera from the hamsters immunized 6 days previously (Table IV) had SDN titers of 1:10 or less, yet greater than 99% of the high titered input VEE virus was eliminated from the circulation within 20 min. Immune elimination of antigen from the blood has long been recognized as a sensitive indicator of an early immune response [Benacerraf and Miescher, 1960; Talmage et al, 1951].

Although we have no direct evidence that immune clearance leads to destruction of virus, the survival of hamsters inoculated with infectious virus/antibody complexes suggests that such complexes are degraded. If so, neutralizing antibody would function analogously to opsonins in bacterial systems. Accelerated adsorption of virus antibody aggregates has been described previously for Newcastle disease virus [Silverstein and Marcus, 1964; Brunner et al, 1960], mousepox [Mims, 1964], polio

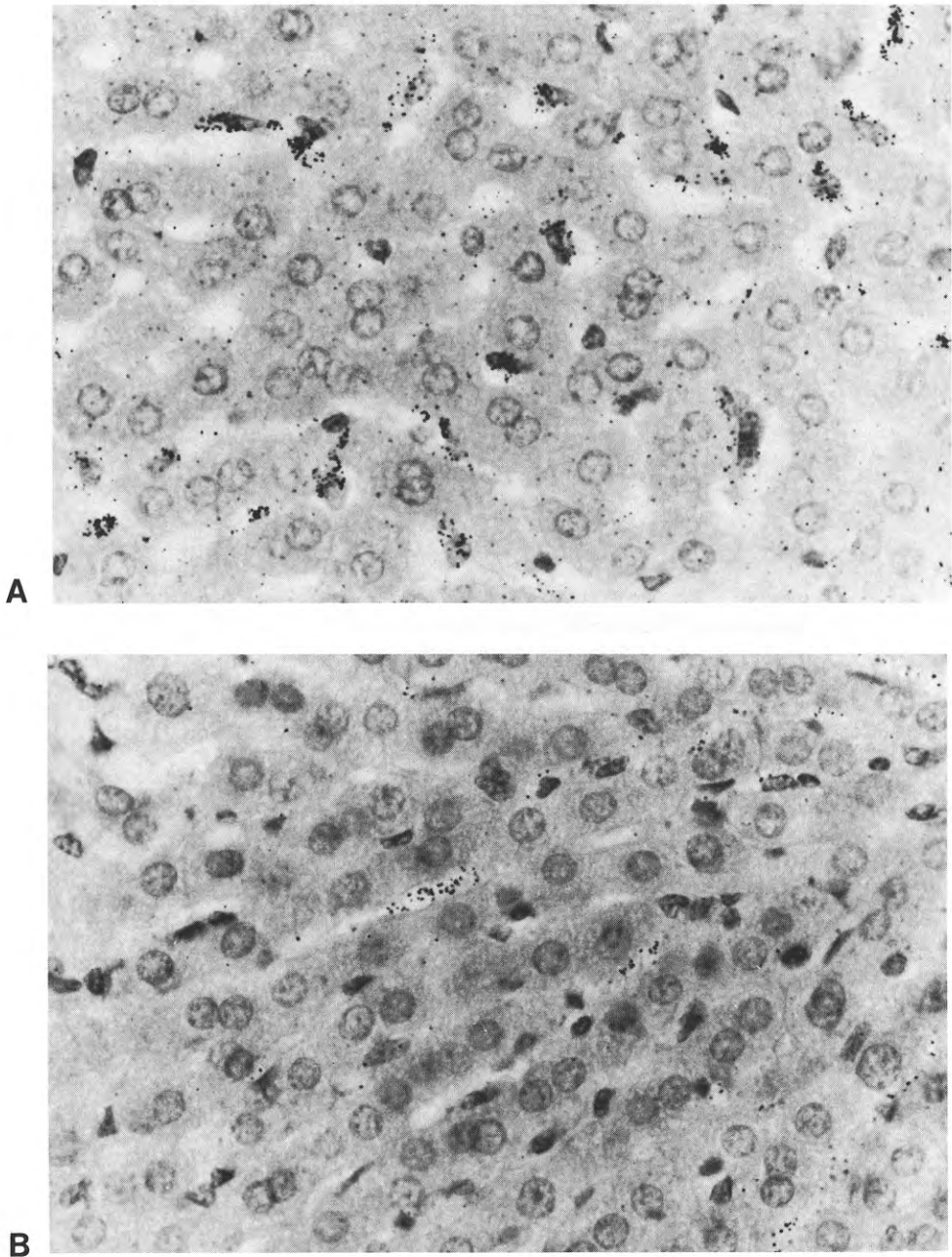


Fig. 6. Autoradiographs of liver sections obtained from hamsters 20 min after ica inoculation of ³H-labeled VEE virus in (A) immune hamster and (B) nonimmune hamster (autoradiograph plus hematoxylin and eosin, $\times 440$).

[Mandel, 1967], and vaccinia [Silverstein, 1975] viruses. It is reasonable to expect that alphaviruses, complexed with antibody and similarly presented to RES cells, would be degraded analogously through interactions with lysosomal enzymes. Presumably, virus/antibody complexes, were they not removed from the circulation by interaction with RES cells, would be available to infect susceptible target cells in the intact hamster.

Many factors have been described which influence the adsorption and interactions of antigens with RES cells, and these factors should now be evaluated for the interactions of alphaviruses with hamster macrophages. Sheep red blood cell (SRBC) antigens complexed with IgM antibody in the presence of C3 tend to remain attached to mononuclear phagocytic cell membranes, while SRBC-IgG complexes in the presence of C3 are effectively interiorized and lysed by intracellular enzymes [Huber and Holm, 1975]. Certain components of complement, especially C3, appear to be necessary for efficient phagocytosis of certain antigens, and a marked synergism between C3 and IgG in inducing phagocytosis of opsonized particles has been reported [Ehlenberger and Nussenzeig, 1977]. It is reasonable to expect, but remains to be proved, that C3 and IgG play similar roles in the phagocytosis of opsonized alphavirus particles. In the present study, accelerated clearance was observed not only in hamsters immunized 28 days previously, but also in hamsters immunized only 6 days previously. This suggests that IgM might also be an effective inducer of immune clearance. Both IgG and IgM have opsonic activity for bacteria [Smith et al, 1967]. Alternatively, low levels of IgG occurring as early as day 6 could be responsible for the early immune clearance observed. Further studies should investigate the relative efficiencies of IgM and IgG in effecting adsorption of virus to hamster macrophages in culture; also, the events following adsorption (eg interiorization, degradation, and replication) should be examined. Likewise, the roles of the components of complement (especially C3) in the interactions of alphaviruses with both unstimulated and activated macrophages should be defined. In the present study, addition of fresh serum (as a complement source) to heated immune serum dramatically increased the proportion of virus adsorbed to activated hamster macrophages in culture.

It is anticipated that data from more refined experiments with alphaviruses and macrophages in culture will lead to a more precise understanding of alphavirus neutralization in the intact immune animal. Once the conditions for immune clearance or true opsonization are more precisely defined, effective treatment of primary alphavirus infections might be achieved by manipulation of critical host immune responses or by timely intervention with appropriate quantities of immune plasma.

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