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TECHNICAL MANUSCRIPT 519

INTERACTION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS WITH NEUTRALIZING ANTIBODY: II. THE PERSISTENT VIRUS FRACTION

Nicholas Hahon

MARCH 1969

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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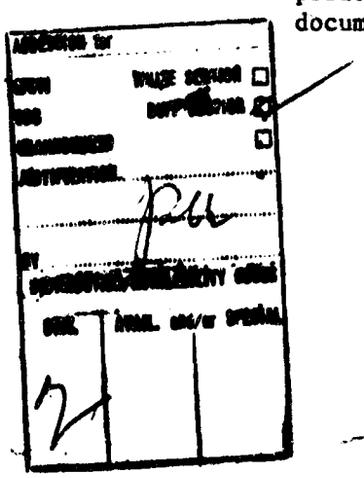

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INTERACTION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS WITH NEUTRALIZING ANTIBODY: II. THE PERSISTENT VIRUS FRACTION

Nicholas Hahon

Experimental Aerobiology Division
AEROBIOLOGY & EVALUATION LABORATORIES

Project 1B562602A059

March 1969

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ACKNOWLEDGMENT

The technical assistance of W. Douglas Zimmerman is gratefully acknowledged.

ABSTRACT

The persistent virus fraction that results from the interaction of Venezuelan equine encephalomyelitis (VEE) virus with antiviral serum is an infectious virus-antibody complex (sensitized virus) that can be neutralized by anti-IgG serum. The quantities of virus sensitized by VEE antiserum and neutralized by anti-IgG serum depend on the concentration of these sera. In contrast to the marked temperature and time dependence of VEE virus neutralization by antiviral serum, neutralization of sensitized virus by anti-IgG serum is more rapid (almost complete within 1 min at 35 C) and less sensitive to temperature. Evidence that virus sensitization preceded neutralization indicates that the persistent virus fraction is formed before virus neutralization has actively begun. Within certain limits, neutralization of sensitized virus by anti-IgG serum is species-specific. Differences in the ability of anti-IgG, anti-IgA, and anti-IgM sera to neutralize sensitized virus indicate that the reaction is also influenced by the specificity of the anti-immunoglobulin.

Sensitized virus was partially neutralized by goat antiserum to monovalent Fab fragments on human IgG and, to a lesser degree, by the Fc fragment. Sensitized virus was neutralized by an in vitro mixture of these fragments to almost the same degree as that neutralized by goat antiserum to intact human IgG. Indirectly, these findings suggest a role for the Fc fragment in virus neutralization.

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I. INTRODUCTION*

Recent studies on the interaction of VEE virus with antibody showed that neutralization reactions were initially first order and then followed curvilinear kinetics.[1] This anomalous behavior, reported with different animal-virus and bacteriophage neutralization systems,[2-9] has been attributed to a small but definite fraction of a viral population that resists neutralization even in the presence of excess antiserum after prolonged treatment. Several hypotheses have been proposed to account for this phenomenon: (i) heterogeneity of viral population of which a distinct portion resists neutralization,[3] (ii) aggregation of virus particles by antibody,[10] (iii) nonavidity of antibody,[6] (iv) dissociation of the virus-antibody complex,[11] (v) preaggregation of virus in suspension,[12] and (vi) formation of infectious virus-antibody complexes that sterically hinder attachment of additional antibody.[13] In view of supportive experimental evidence for each of these diverse hypotheses, the possibility exists that the manifestation of a non-neutralizable virus fraction often observed in kinetic neutralization tests may involve several factors, acting either individually or concertedly: e.g., the state of virus, the quality of antibody, the host cell system, or the circumstances of neutralization. At present, the phenomenon has not been adequately explained or the problem completely resolved.

This report describes an attempt to elucidate the phenomenon of the non-neutralizable virus fraction as it relates to the VEE virus-antibody system.

II. MATERIALS AND METHODS

A. VIRUS

The source and preparation of the Trinidad strain of VEE virus was described previously.[14] The attenuated VEE virus strain[15] was obtained from Dr. William A. Hankins, Fort Detrick, in the form of a suspension derived from infected chick fibroblast cell cultures. Both virus strains were assayed by an immunofluorescent cell-counting procedure.[14]

B. VIRUS ANTISERUM

VEE antiserum was obtained from rhesus monkeys that had been exposed 1 month earlier to an aerosol of approximately 1,000 cell-infecting units (CIU) of virus. Human antiviral serum was obtained from a convalescent patient. Sera were inactivated at 56 C for 30 min.

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C. CELL LINES

The McCoy cell line was used routinely for the assay of virus; its cultivation and maintenance has been described previously.[1] Other established cell lines and their media for growth were hamster kidney and L-929 cells, medium 199 with 10% fetal calf serum (FCS); guinea pig lung cells, basal medium Eagle (BME) with 10% FCS and 0.5% lactalbumin hydrolysate; Chang's human conjunctiva cells, BME with 10% FCS. Hamster kidney and L-929 cells were maintained with medium 199 and 5% FCS; guinea pig lung and human conjunctiva cells with BME and 5% FCS.

D. KINETIC NEUTRALIZATION PROCEDURE

Although the reactants and experimental conditions of neutralization varied; in general, a suspension containing 1 x 10^8 CIU of virus per ml was mixed with an equal volume of appropriately diluted monkey antiviral serum. Both reactants were prewarmed to the temperature employed for incubation (35 C) before mixing. At intervals of incubation, 2 ml of the mixture was withdrawn from the reaction tube and placed immediately into a vial held in ice water to arrest neutralization. Test samples were then diluted in cold phosphate-buffered saline (PBS), pH 7.1, free of calcium and magnesium ions, and assayed for unneutralized virus. A control mixture, consisting of virus and normal monkey serum of the same concentrations and volumes as the test mixtures, was employed with each kinetic neutralization determination. The surviving fraction of virus was determined from the ratio of unneutralized virus in the reaction tube to the virus titer in the control tube at the designated incubation time intervals.

E. IMMUNOLOGICAL REAGENTS

Normal goat serum was obtained from Pentex Laboratories, Kankakee, Illinois. Goat anti-monkey gamma globulin (IgG) serum and goat antiserum to human, chicken, guinea pig, rabbit, bovine, and mouse IgG were obtained from Microbiological Associates, Inc., Bethesda, Maryland. Goat antiserum to human albumin, immunoglobulins, and Fab (1&11) and Fc fragments were obtained from Hyland Laboratories, Los Angeles, California. Fragments were prepared by papain digestion of anti-IgG serum. All sera were heated at 56 C for 30 min and diluted in PBS.

F. VIRUS SENSITIZATION AND ASSAY

The term virus sensitization refers to the interaction of virus with antiviral serum to form an infectious virus-antibody complex.[10] In this report, sensitized virus is used synonymously with persistent virus fraction. VEE virus was sensitized by mixing 1 x 10^6 CIU/ml of virus with an equal volume of a 1:50 dilution of either monkey antiviral serum or of normal monkey serum (control) and incubating at 35 C for 1 hour. Sensitized

virus, in contrast to unsensitized virus, was susceptible to neutralization by goat anti-monkey IgG serum. To test for sensitization, treated virus was generally mixed with an equal volume of a 1:5 dilution of goat anti-monkey IgG serum and incubated at 4 C for 10 min. A control mixture consisted of treated virus and normal goat serum. Appropriate dilutions of test samples were made in cold PBS and assayed for surviving virus. A reduction (0.3 $\log_{10}$ units or greater) of the surviving virus fraction, calculated by subtracting the titer ($\log_{10}$) remaining after incubation with goat anti-monkey IgG serum from the titer ($\log_{10}$) of the control, was indicative of virus sensitization.

III. RESULTS

A. MULTIPLICITY CURVE

Kinetic curves of VEE virus neutralization with different antiserum concentrations are linear initially and then undergo a decrease in slope that may reach a horizontal line.[14] The degree of this curvature appears to be dependent on the ratio of serum to virus; it is more pronounced when the ratio is increased. This dependency was investigated further by determining the neutralization curve at equilibrium of different concentrations of serum mixed with a constant quantity of virus (1×10^5 CIU/ml). After incubation at 35 C for 2 hours plus 4 C for 1 hour, reaction mixtures were appropriately diluted and assayed for surviving virus.

A multiplicity curve resulted that resembled a kinetic curve when the logarithms of surviving virus were plotted against antiserum dilutions (Fig. 1). From the origin, the multiplicity curve was linear and then became horizontal, even in the presence of high antiserum concentrations. The survival of virus being constant at this portion of the curve indicated that there is a fraction of virus, termed the persistent virus fraction,[3] that resists neutralization.

B. FACTORS INFLUENCING APPEARANCE AND MAGNITUDE OF PERSISTENT VIRUS FRACTION

A report that the persistent fraction of virus populations was caused by aggregated virus[12] prompted an investigation to determine whether the virus preparation used in this study was similarly affected. Kinetic neutralization tests were made with 1:10 dilutions of monkey antiviral serum mixed in equal volumes with unfiltered virus, sonic-treated (20 kc/sec, 2 min) virus, or virus filtered through 0.2-μ or 0.1-μ porosity membranes (Millipore Corp., Bedford, Mass.) in accord with the described procedure.[12] Appropriate tubes containing normal serum and virus served as controls. Reactions were carried out in the manner described earlier. Results in Figure 2 show that neutralization reactions proceeded initially at a linear

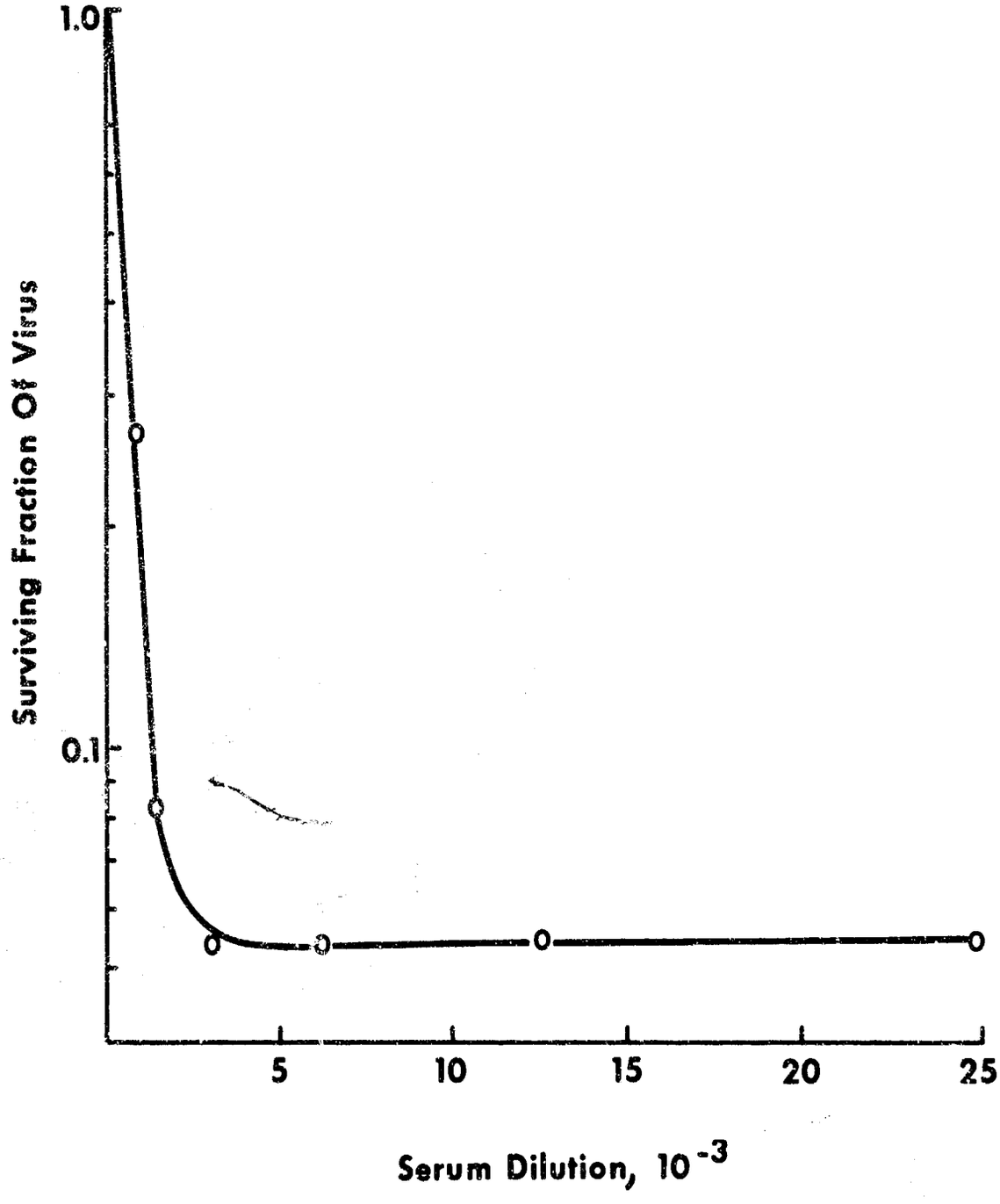

FIGURE 1. Multiplicity Curve of Neutralization of VEE Virus.

and similar rate with all virus preparations employed. Deviations from linearity first occurred with both unfiltered and sonic-treated viruses and resulted in equivalent levels of persistent virus fractions. Although the linearity of the reactions continued slightly longer with filtered virus preparations, the persistent virus fraction still appeared. The level of these fractions was lower, however, than that of the unfiltered and sonic-treated virus preparations. These observations seem to indicate that preaggregation of virus is not the dominant cause of the persistent VEE virus fraction, although aggregation may contribute to the level of the persistent fraction.

In view of numerous reports attesting to the potentiation of virus neutralization by antiserum with the addition of heat-labile serum components,[3,17-20] these additives were investigated to determine whether any change in the quantity of the persistent virus fraction resulted. Kinetic neutralization experiments were made with test mixtures consisting of 5 x 10^8 CIU/ml of virus, 1:10 dilution of monkey antiviral serum, and fresh or inactivated (56 C, 30 min) undiluted guinea pig serum. Control mixtures consisted of virus, fresh or inactivated guinea pig serum, and normal monkey serum. Reactants were prewarmed before mixing; the reactions were carried out at 35 C. Both neutralization curves were linear for the first few minutes and then deviated from first-order kinetics within 10 min after the onset of neutralization (Fig. 3). In the presence of fresh guinea pig serum, the neutralization reaction velocity increased and the persistent virus fraction was reduced but not eliminated.

It is generally recognized that the fraction of virus particles surviving neutralization may depend on the cell type employed for virus assay.[21-33] Different host cell systems were used to assay virus to determine their effect on the quantity of the persistent virus fraction. A kinetic neutralization test was made in the usual manner using a 1:50 dilution of monkey anti-viral serum; reaction samples taken at intervals of incubation were then assayed in parallel on five cell lines: McCoy, L-929, hamster kidney, Chang's human conjunctiva, and guinea pig lung. The human conjunctiva cell line was purposely included in the experiment because it was reported to be less capable of supporting VEE virus growth.[34] Results in Figure 4 show that the kinetic neutralization curves and the level of the persistent virus fractions were similar with all host-cell systems. Among the cell systems tested, surviving virus fractions differed from one another by less than 5% at that portion of the neutralization curve that reaches a constant value. Dulbecco et al.[3] also failed to demonstrate any difference in the shape and slope of multiplicity curves with the limited cell types used for assay of surviving virus. These findings, however, do not preclude the possibility that the choice of host cells used in the assay of virus may influence the level of the persistent virus fraction.

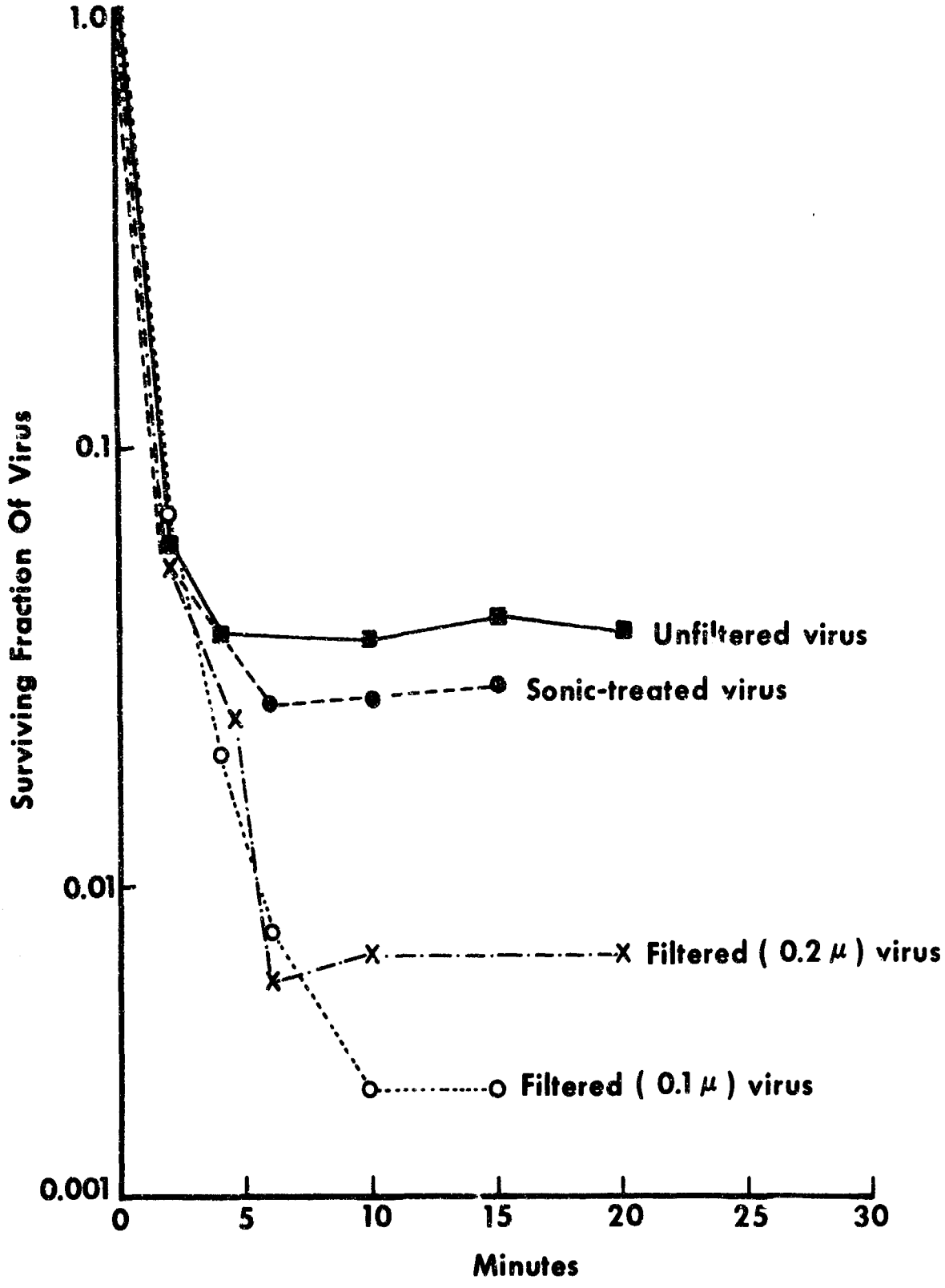

FIGURE 2. Kinetic VEE Virus Neutralization Reactions with Prior Filtration or Sonic Treatment (20 kc/sec for 2 min) of Virus.

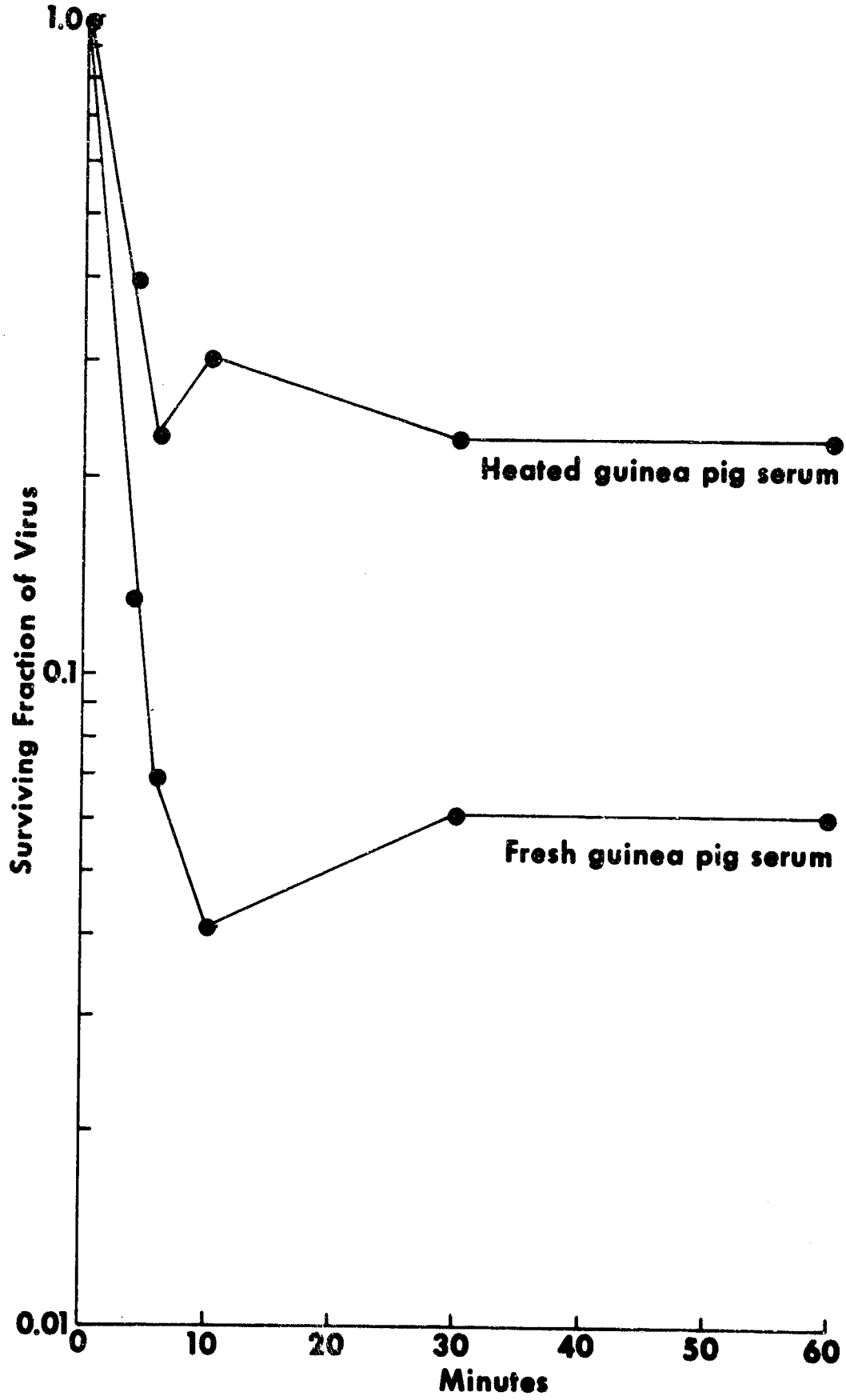

FIGURE 3. Effect of Heated (56 C, 30 min) and Fresh Guinea Pig Serum on the Kinetic VEE Virus Neutralization Reaction.

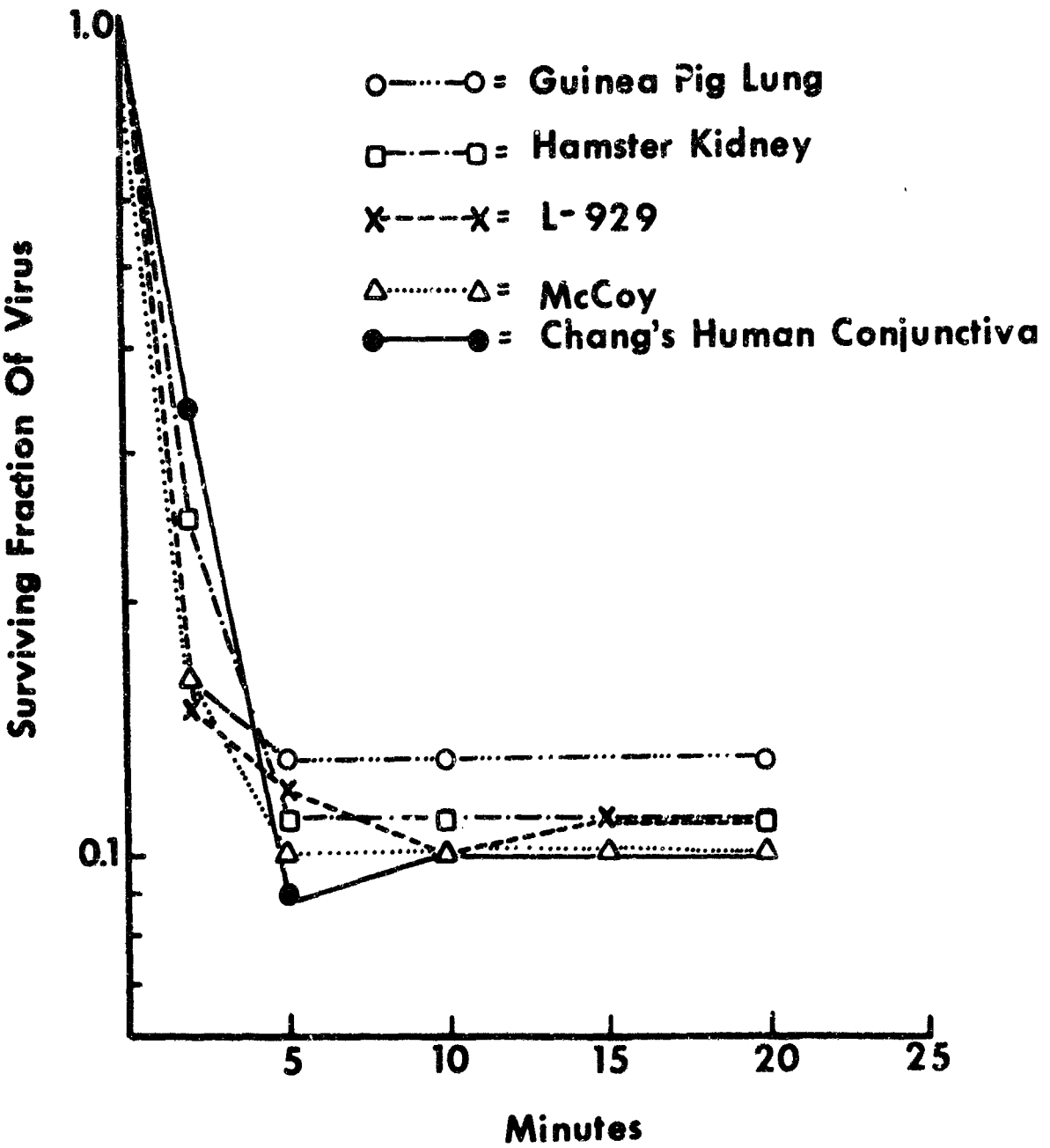

FIGURE 4. Levels of Persistent Virus Fractions in Kinetic VEE Virus Neutralization Reactions Assayed on Different Cell Lines.

To determine whether the persistent virus fraction constituted a genetically resistant population, surviving virus was passed twice intracerebrally in mice. Parallel kinetic neutralization tests were performed using a 1:50 dilution of monkey antiviral serum mixed with parent virus (original virus suspension) or progeny virus (persistent virus fraction passed twice in mouse brains). A similar test was also carried out with an attenuated VEE virus strain. Both the parent and progeny viruses showed almost identical neutralization curves (Fig. 5); compared with both these viruses, the rate of neutralization of attenuated virus was greater. A persistent virus fraction was also manifested with the attenuated virus; however, it was lower in quantity than that noted with the other two viruses. Kinetic neutralization tests performed under similar conditions with parent or attenuated viruses mixed with attenuated monkey antiviral serum resulted in neutralization curves comparable to those shown for the corresponding viruses in Figure 5. These experiments indicate that resistance of virus particles to neutralization, manifested by a constant quantity of surviving virus, is not the result of a stable genetic mechanism. Experimental results obtained with different systems of virus-antibody and host cells support this conclusion.[4,5,25]

The persistent virus fraction incubated at 35 C for 30 min with additional antiserum of high concentration was refractory to further neutralization. Similarly, it was unaffected after incubation with ribonuclease (2 μg/ml). The latter, in agreement with findings with Newcastle disease virus,[4] indicates that the persistent virus fraction is not caused by the presence of infectious viral RNA.

C. NEUTRALIZATION OF PERSISTENT VIRUS FRACTION BY ANTI-IgG SERUM

Persistent virus fractions have been observed when lactic dehydrogenase or herpes simplex viruses were incubated with their respective antisera.[13,16] These fractions are in the form of infectious virus-antibody complexes that may be neutralized by goat anti-mouse IgG serum. In a kinetic neutralization experiment to determine whether these findings could be extended to the VEE virus-antibody system, dilutions (1:50) of monkey antiviral or normal serum were mixed with equal volumes of 1×10^8 CIU/ml of virus suspension and then incubated at 35 C. Reaction mixtures were sampled at different time intervals and assayed for unneutralized virus. Samples were also mixed with equal volumes of 1:5 dilutions of either goat anti-monkey IgG serum or normal goat serum to test for sensitized virus as described earlier. Results in Figure 6 show that the initial rate of neutralization in the mixture of virus and antiviral serum was linear and then deviated as a result of a persistent virus fraction. The quantity of virus that was neutralized by VEE antiserum was approximately 1.0 $\log_{10}$ unit. Although the addition of normal goat serum to virus-antiserum mixtures did not alter the kinetic curve, goat anti-monkey IgG serum significantly reduced the persistent virus fraction by

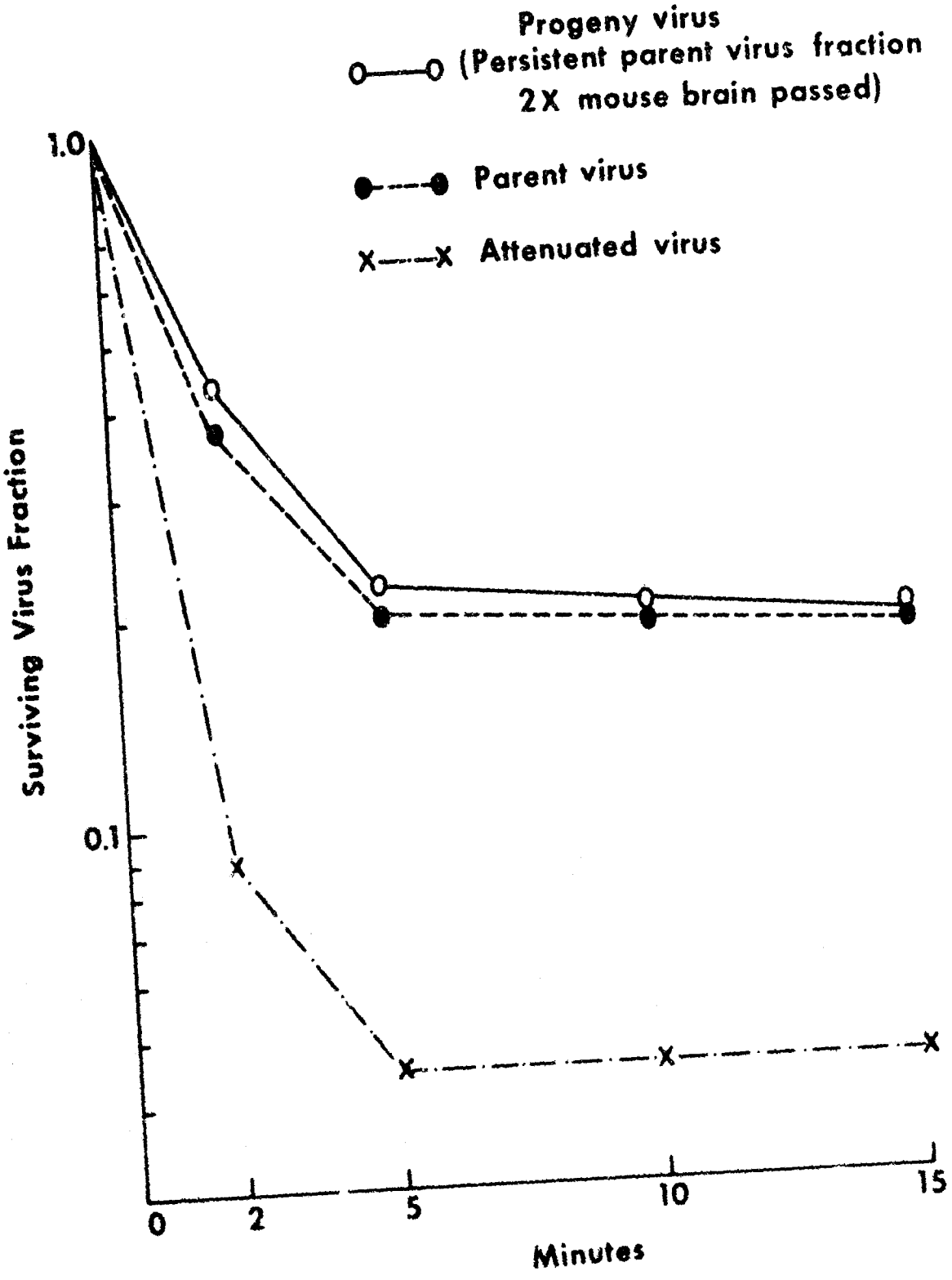

FIGURE 5. Kinetic Neutralization of VEE Parent, Progeny, and Attenuated Viruses by Monkey Antiserum to Parent Virus.

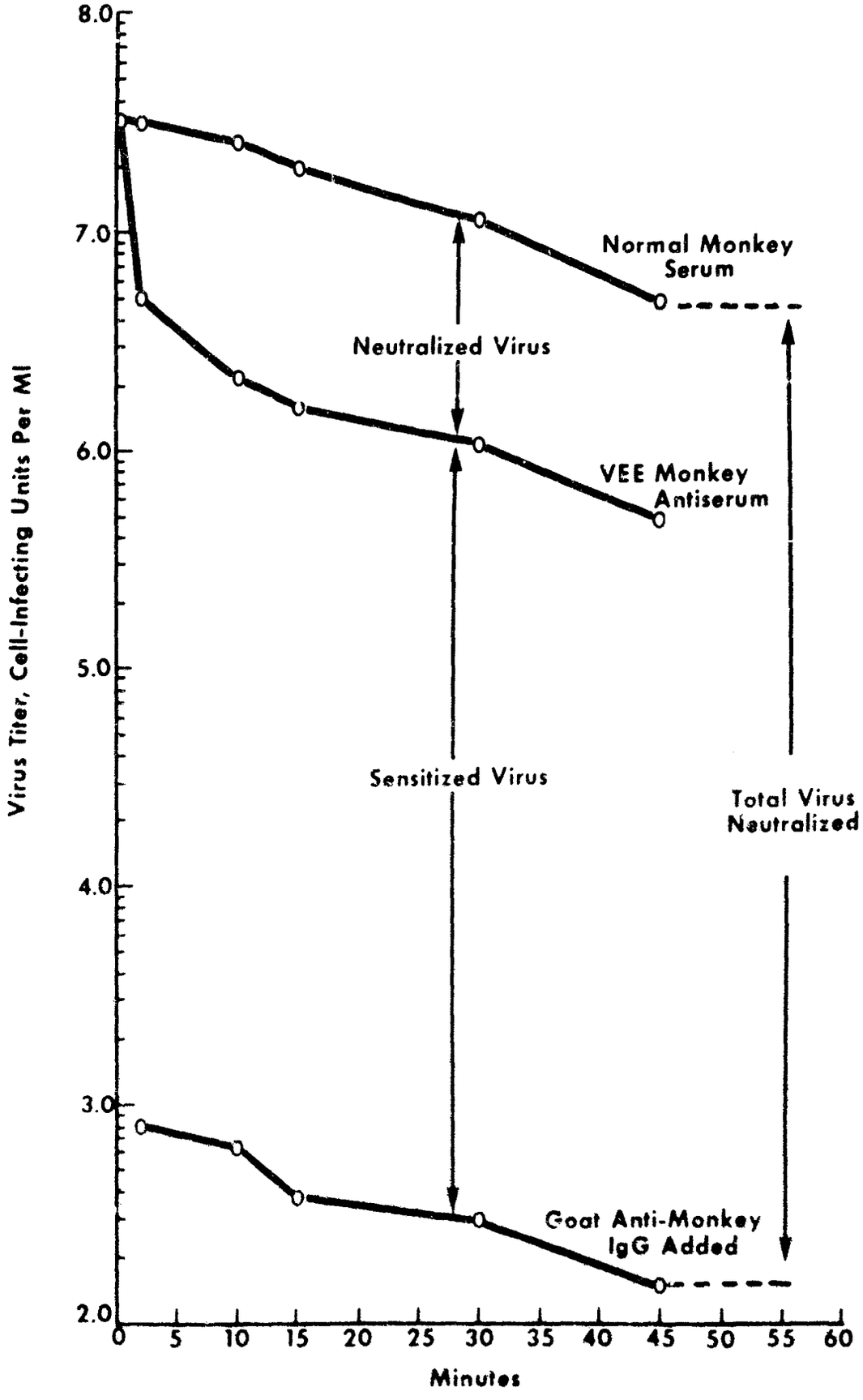

FIGURE 6. Kinetic Neutralization of VEE Virus by Monkey Antiserum and the Neutralization of the Persistent Virus Fraction by Goat Anti-Monkey IgG Serum.

more than 3.0 $\log_{10}$ units. The total quantity of virus neutralized exceeded 4.0 $\log_{10}$ units. These findings imply that the persistent VEE virus fraction existed in the form of an infectious virus-antibody complex (sensitized virus) and that it could be neutralized by antibody not directed against the virus.

D. PARAMETERS OF NEUTRALIZATION OF SENSITIZED VIRUS BY ANTI-IgG SERUM

To define the conditions of neutralization by anti-IgG serum, the effect of antibody concentration on virus sensitization was first investigated by mixing different dilutions of monkey antiviral serum with 5 x 10^8 CIU/ml of virus in equal volumes and incubating the mixtures at 35 C for 1 hour. Portions of the mixtures were assayed for unneutralized virus; other portions were mixed with an equal volume of a 1:10 dilution of goat anti-monkey IgG serum, incubated at 35 C for 1 hour, and then assayed for surviving virus. Normal monkey or goat sera served as controls. The data in Table 1 show that 1:10 dilution of VEE antiserum neutralized the greatest amount of virus (2.0 $\log_{10}$); the quantity decreased in the presence of higher antiserum dilutions. The addition of goat anti-monkey IgG serum neutralized 3.7 $\log_{10}$ units more of virus with both 1:50 and 1:100 dilutions of VEE antiserum. In the presence of higher VEE antiserum dilutions (1:500 to 1:5,000), where little virus neutralization occurred, more than 1.5 $\log_{10}$ units of virus were neutralized by anti-IgG serum. Virus sensitization, therefore, had also resulted with the high VEE antiserum dilutions. With the 1:10 dilution of antiviral serum, anti-IgG serum was slightly inhibited in its neutralization of sensitized virus. That excess unbound IgG in the reaction mixture can prevent anti-IgG from reacting with sensitized virus has been suggested as the cause of the observed inhibition.[13] In general, however, greater concentrations of antiviral serum sensitized more virus.

The effect of dirferent concentrations of goat anti-monkey IgG serum on the neutralization of sensitized virus was determined by first mixing 1 x 10^8 CIU/ml of virus with an equal volume of 1:50 dilution of antiviral monkey serum. Mixtures were incubated at 35 C for 1 hour to effect virus sensitization. In equal portions, sensitized virus suspension was mixed with different dilutions of goat anti-monkey IgG serum or normal goat serum (control), incubated at 35 C for 1 hour, and then assayed for surviving virus. Results show that neutralization of sensitized virus is dependent on the concentration of anti-IgG serum (Table 2). Greater amounts of sensitized virus were neutralized with increased concentrations of anti-IgG serum.

The effects of time and temperature on the neutralization of sensitized VEE virus by goat anti-monkey IgG serum were investigated. Virus was sensitized as described earlier and then mixed with anti-IgG serum. Mixtures were incubated at 35 C or 0 C, sampled at prescribed time intervals, and then assayed for surviving virus. Results in Table 3 show that sensitized virus was neutralized by anti-IgG serum almost completely within 1 min at 35 C but less rapidly at 0 C.

TABLE 1. EFFECT OF VEE ANTISERUM CONCENTRATIONS ON VIRUS SENSITIZATION[a/]

Dilutions of VEE Monkey Antiserum	Incubation with VEE Monkey Antiserum		Incubation with Goat Anti-Monkey IgG Serum		Total Surviving Virus Fraction
	Virus Titer	Surviving Virus Fraction	Virus Titer	Surviving Virus Fraction	
1:10	6.2	-2.0	4.8	-2.8	-4.8
1:50	6.8	-1.4	3.9	-3.7	-5.1
1:100	7.2	-1.0	3.9	-3.7	-4.7
1:500	7.3	-0.9	5.9	-1.7	-2.6
1:1,000	7.4	-0.8	5.8	-1.8	-2.6
1:5,000	8.0	-0.2	6.0	-1.6	-1.8
1:10 (control)	8.2	0.0	7.6	0.0	0.0

a. Incubation with each phase was 35 C for 1 hour; all data indicate $\log_{10}$ CIU/ml. Virus survival was calculated by subtracting the titer remaining after incubation with VEE monkey antiserum or goat anti-monkey IgG serum from the titer of controls (normal monkey or goat sera).

TABLE 2. NEUTRALIZATION OF SENSITIZED VEE VIRUS BY DIFFERENT CONCENTRATIONS OF GOAT ANTI-MONKEY IgG SERUM[a/]

Dilution of Goat Anti-Monkey IgG Serum	Virus Titer, $\log_{10}$ CIU/ml	Surviving Virus Fraction, $\log_{10}$ CIU/ml
1:2.5	2.4	-4.3
1:5	3.1	-3.6
1:12.5	4.1	-2.6
1:25	4.6	-2.1
1:50	4.7	-2.0
1:250	5.3	-1.4
1:2.5 (normal goat serum)	6.7	0.0

a. Approximately 1 x 10^8 CIU/ml of virus was mixed with an equal volume of 1:50 dilution of VEE monkey antiserum and incubated at 35 C for 1 hour to effect virus sensitization. Anti-IgG was mixed with sensitized virus and incubated at 35 C for 1 hour.

TABLE 3. EFFECT OF TIME AND TEMPERATURE ON THE NEUTRALIZATION OF SENSITIZED VEE VIRUS BY GOAT ANTI-MONKEY IgG SERUM[a]

Time, min	35 C		0 C	
	Virus Titer	Surviving Virus Fraction	Virus Titer	Surviving Virus Fraction
1	4.4	-2.3	4.0	-2.5
3	4.5	-2.2	4.2	-2.3
5	4.4	-2.3	4.3	-2.2
15	4.4	-2.3	4.3	-2.2
Control	6.7	0.0	6.5	0.0

a. Approximately 5 x 10^8 CIU/ml of virus was mixed with an equal volume of 1:50 dilution of VEE monkey antiserum and incubated at 35 C for 1 hour to effect virus sensitization. Portions of sensitized virus were mixed with equal volumes of 1:10 dilutions of goat anti-monkey IgG serum and incubated (35 C or 0 C) for prescribed time periods. Both reactants were at the desired incubation temperature before mixing. Sensitized virus mixed with 1:10 dilutions of normal goat serum and incubated (35 C or 0 C) for 15 min served as controls. Samples were diluted in ice-cold PBS and assayed for surviving virus. All data indicate $\log_{10}$ CIU/ml.

To determine the time relationship between neutralization and sensitization of VEE virus by antibody, a kinetic neutralization test was carried out in the usual manner; the concentration of reactants was similar to that of the preceding experiment. Reaction mixtures were sampled at different time intervals and assayed for unneutralized virus. A portion of each sample was treated with anti-IgG serum and then assayed for sensitized virus. Results reveal that virus sensitization preceded virus neutralization (Table 4). During the 0.5- to 4-min incubation time interval virus neutralization continued but the quantity of sensitized virus remained constant. Cursory evidence of these events was observed in the early sample period of a preceding experiment (Fig. 6). These data suggest that a major portion of the persistent virus fraction was an entity at the onset of virus neutralization by antiviral serum.

TABLE 4. SENSITIZATION PRECEDING NEUTRALIZATION OF VEE VIRUS BY ANTIBODY[a]

Time, min	Surviving Virus Fraction	
	Neutralization	Sensitization
0.5	-0.3	-4.3
1	-0.4	-4.3
2	-0.6	-4.3
4	-0.7	-4.3
Control	0.0	0.0

a. Approximately 1 x 10^8 CIU/ml of virus was mixed with an equal volume of 1:50 dilution of VEE monkey antiserum or normal monkey serum, incubated at 35 C and sampled at designated time intervals. Samples were held at 0 C, diluted in ice-cold PBS, and assayed for surviving virus. Portions of undiluted virus-antiserum mixtures were mixed with equal volumes of 1:5 dilutions of goat anti-monkey IgG serum or normal goat serum, held at 0 C for 10 min, and assayed for surviving virus. All data indicate $\log_{10}$ CIU/ml.

b. Normal monkey or goat serum.

The capacity of goat anti-IgG serum of different animal species to neutralize virus that had been sensitized by monkey antiviral serum was tested. Sensitized virus was mixed with anti-IgG serum of different species, incubated at 35 C for 10 min, and then assayed for surviving virus. Results in Table 5 show that maximal neutralization of sensitized virus occurred with goat anti-monkey serum and, to a lesser degree, with goat anti-human IgG serum. That goat anti-human IgG serum reacting with virus sensitized by monkey antiserum was capable of cross-neutralization is suggested by the data. Anti-IgG serum of other animal species did not significantly neutralize sensitized virus.

To explore further the cross-neutralization phenomenon, a reciprocal neutralization test was made by reacting virus that had been sensitized with either monkey or human antiviral serum with goat anti-monkey or anti-human IgG serum. Almost complete reciprocity of neutralization of sensitized virus by homologous and heterologous anti-IgG sera resulted (Table 6). The exception was virus that had been sensitized with monkey antiviral serum and reacted with anti-human IgG serum. The quantity of sensitized virus neutralized by this combination was less than that with the other test combinations.

TABLE 5. NEUTRALIZATION OF SENSITIZED VEE VIRUS BY GOAT ANTI-IgG SERUM OF DIFFERENT SPECIES[a]

Goat Serum	Virus Titer	Surviving Virus Fraction
Anti-monkey IgG	3.1	-3.6
Anti-human IgG	5.7	-1.0
Anti-chicken IgG	6.7	0.0
Anti-guinea pig IgG	6.6	-0.1
Anti-rabbit IgG	6.5	-0.2
Anti-bovine IgG	6.5	-0.2
Anti-mouse IgG	6.5	-0.2
Normal	6.7	0.0

a. Approximately 1 x 10^8 CIU/ml of virus was mixed with an equal volume of 1:50 dilution of VEE monkey antiserum and incubated at 35 C for 1 hour to effect virus sensitization. Portions of sensitized virus were then mixed with equal volumes of 1:2.5 dilutions of goat antiserum to IgG serum of each different species and incubated at 35 C for 10 min. Samples of each mixture were appropriately diluted in PBS and assayed for surviving virus. All data indicate $\log_{10}$ CIU/ml.

TABLE 6. SENSITIZATION OF VEE VIRUS BY HUMAN OR MONKEY ANTISERUM WITH CROSS-NEUTRALIZATION BY HOMOLOGOUS AND HETEROLOGOUS GOAT ANTI-IgG SERUM[a]

Antiserum for Virus Sensitization	Neutralization Serum	
	Goat Anti-Human IgG	Goat Anti-Monkey IgG
Human	-3.6	-3.5
Monkey	-1.0	-3.6

a. Approximately 1 x 10^8 CIU/ml of virus was mixed with an equal volume of 1:50 dilution of VEE monkey or human antiserum and incubated at 35 C for 1 hour to effect virus sensitization. Portions of sensitized virus were then mixed with equal volumes of 1:2.5 dilutions of goat anti-human or anti-monkey IgG serum or of normal goat serum (control) and incubated at 35 C for 10 min. Samples were diluted in PBS and assayed for surviving virus.

b. All data indicate $\log_{10}$ CIU/ml.

The neutralization of sensitized virus by different classes of immunoglobulins was determined by first sensitizing virus with human antiviral serum and then reacting the virus with different goat anti-human immunoglobulins. Maximal neutralization of sensitized virus was observed with anti-IgG serum and, to a lesser extent, with anti-IgA serum (Table 7). Virus was not neutralized by either anti-IgM or anti-albumin sera. These results are similar to those reported by Notkins et al;[26] who additionally noted neutralization of lactic dehydrogenase virus by anti-mouse IgF serum.

TABLE 7. NEUTRALIZATION OF SENSITIZED (VEE HUMAN ANTISERUM) VIRUS BY DIFFERENT CLASSES OF GOAT ANTI-HUMAN IMMUNOGLOBULINS[a]

Goat Antiserum to Human Immunoglobulin	Virus Titer	Surviving Virus Fraction
IgG	3.3	-3.6
IgA	4.5	-2.4
IgM	6.9	0.0
Albumin	7.0	0.0
Normal goat serum	6.9	0.0

a. Approximately 1 x 10^8 CIU/ml of virus was mixed with an equal volume of 1:25 dilution of VEE human antiserum and incubated at 35 C for 30 min to effect virus sensitization. Portions of sensitized virus were then mixed with equal volumes of undiluted goat antiserum to different human immunoglobulins, albumin, or normal goat serum and incubated at 35 C for 10 min. Samples were diluted in PBS and assayed for surviving virus. All data indicate $\log_{10}$ CIU/ml.

To determine whether antiserum to monovalent fragments of IgG has the capacity to neutralize sensitized virus, the virus was sensitized with human antiviral serum and then incubated with goat antiserum to either Fab or Fc fragments of human IgG. A mixture of antisera to Fab and Fc fragments was also reacted with sensitized virus. Results in Table 8 reveal that antibody to monovalent Fab fragments was capable of neutralizing sensitized virus. Antibody to the Fc fragment also neutralized virus that had been sensitized, but the quantity was 1.0 $\log_{10}$ unit less than that neutralized by antibody to Fab fragments. The mixture of antisera to both Fab and Fc fragments neutralized virus to almost the same degree as intact anti-IgG serum. Indirectly, the evidence suggests that the Fc fragment, devoid of combining sites but containing structural features necessary for other biological functions,[27] plays a role in virus neutralization. These findings, in relation to available evidence on the neutralization of viruses by IgG fragments, are discussed subsequently.

TABLE 8. NEUTRALIZATION OF SENSITIZED (VEE HUMAN ANTISERUM) VIRUS BY GOAT ANTISERUM TO PAPAIN-DIGESTED FRAGMENTS OF IgG[a/]

Goat Antiserum to Human IgG Fragments	Virus Titer	Surviving Virus Fraction[b/]
Fc	6.1	-0.6
Fab	5.1	-1.6
Fc + Fab	3.9	-2.8
IgG	3.7	-3.0
Normal goat serum	6.7	0.0

a. Approximately 1 x 10^8 CIU/ml of virus was mixed with an equal volume of 1:25 dilution of VEE human antiserum and incubated at 35 C for 30 min to effect virus sensitization. One part undiluted goat antiserum to IgG fragments of normal goat serum were mixed with two parts sensitized virus and one part PBS diluent. Mixtures were incubated at 35 C for 10 min, appropriately diluted in PBS, and assayed for surviving virus. All data indicate $\log_{10}$ CIU/ml.

IV. DISCUSSION

The studies reported here indicate that the persistent virus fraction manifested when VEE virus interacted with neutralizing antibody was not the direct result of aggregation of virus particles, heterogeneity of virus population, infectious viral RNA, or the host cell system employed for virus assay. Nor was dissociation of the virus-antibody complex a factor, because previous studies with VEE virus demonstrated that neutralized virus was not appreciably reactivated under physiologic conditions.[1] The fact that prolonged incubation of virus-serum mixtures in the presence of excess virus antiserum did not result in further virus neutralization is additional evidence against a dissociation phenomenon. The level of the persistent virus fraction was only slightly lowered when heat-labile serum factors from fresh guinea pig serum were added to virus-antibody mixtures. The persistent fraction of VEE virus was in the form of an infectious virus-antibody complex (sensitized virus) that could be neutralized by anti-IgG serum, an antibody not directed and inactive against the virus itself.

Investigation of determinants that may affect the formation and neutralization of the infectious complex showed that the quantities of virus sensitized by antiviral serum and neutralized by anti-IgG serum increased with higher concentrations of these respective sera. With the highest antiviral serum concentration employed for virus sensitization, however, neutralization of sensitized virus by anti-IgG serum was slightly inhibited. This phenomenon has been attributed to unbound IgG that prevents anti-IgG antibodies from reacting with sensitized virus.[13] In this study, the reverse situation was also noted, with high concentrations of anti-IgG serum inhibiting neutralization of virus by VEE antiserum. When virus, monkey antiviral serum, and goat antiserum to monkey IgG or normal goat serum were mixed simultaneously and incubated, virus neutralization by antiviral serum was markedly inhibited in reaction tubes containing anti-IgG serum but not in tubes containing normal goat serum.

Virus sensitized with VEE monkey antiserum was not neutralized by anti-IgG sera of different animal species. An exception to these findings was the partial neutralization of sensitized virus by goat anti-human IgG serum. Reciprocity of neutralization of sensitized virus (formed with monkey or human antiviral serum) was almost complete by homologous and heterologous anti-IgG sera (Table 6). The cross-reactivity is probably the consequence of a common globulin antigenic determinant shared by species of the Primate order. In general, however, neutralization of sensitized virus by anti-IgG serum appears to be species-specific.

The specificity of the anti-immunoglobulin also affected the neutralization of sensitized VEE virus. Maximal neutralization was noted with anti-IgG serum and to a lesser extent with anti-IgA serum; anti-IgM serum had no neutralizing effect. Although this suggests that VEE virus was not sensitized with IgM antibody, additional tests are needed to determine whether virus can be sensitized with IgM antiviral antibody and then neutralized by anti-IgM serum before this assumption is warranted.[26]

In contrast to the marked temperature and time dependence of VEE virus neutralization by antiserum,[1] neutralization of sensitized virus by anti-IgG serum was rapid and less sensitive to temperature. Sensitized VEE virus was neutralized almost completely within 1 min at 35 C and within 3 to 4 min at 0 C. Previous studies showed that sensitized lactic dehydrogenase[26] and herpes simplex viruses[13] were neutralized by anti-IgG serum at 4 C within 1 min and 15 sec respectively. Differences in temperature did not significantly affect the reaction. Evidence that sensitization of VEE virus precedes neutralization (Table 4) is highly pertinent to the problem of the persistent virus fraction. Apparently, a major portion of the persistent VEE virus fraction is formed before virus neutralization by antiviral serum has actively begun. The formation of virus-antibody complexes, which are not necessarily neutral, prior to viral inactivation has been reported in other virus neutralization systems.[26,28] These findings suggest that virus sensitization in some way prevents additional antiviral antibody, which is

necessary for neutralization, from attaching to virus particles. That aggregation of sensitized VEE virus particles may be a factor in the inhibition of virus neutralization is not supported by this study or by findings with other viruses.[13,26,29] Antibodies to both monovalent fragments of IgG and intact IgG were capable of reaching and neutralizing sensitized VEE virus rapidly and effectively. It is also relevant that monovalent Fab fragments of papain-digested antisera to influenza or herpes simplex viruses (not prone to produce aggregates) sensitized and reduced the neutralization of these viruses by undigested antiviral sera.[26,30] The neutralization of low concentrations of sensitized VEE virus by anti-IgG serum precludes lattice formation as a possible cause of inhibition of virus neutralization.

Although the quality of antibody participating in VEE virus sensitization and neutralization was not investigated, it has been postulated that the persistent virus fraction arises, at least in part, from the presence of nonavid antibody in antiserum.[6,31,32] This type of antibody can combine reversibly with the virus surface but does not form a stable union. Results of a recent study on the properties of both early and late 7S and 19S neutralizing antibodies to herpes simplex virus indicate that both common and distinctive properties exist in the ability of these antibodies to sensitize virus for subsequent neutralization by anti-IgG serum or complement.[33] It appears that the quality of antibody participating in virus neutralization reactions could be a factor contributing to the phenomenon of the persistent virus fraction.

The assessment of individual antibody subunits in the interaction between virus and antibody has been facilitated within recent years with the demonstration that antibody molecules may be degraded enzymatically.[34] Monovalent Fab fragments of goat anti-mouse serum or goat anti-rabbit IgG serum have been shown to neutralize, in part, lactic dehydrogenase virus[26] and poliovirus,[35] respectively. The former virus was not neutralized by the Fc fragment. Although the Fc fragment does not contain antigen-binding sites and has not been specifically related to antigen-binding activities,[36] limited virus neutralization activity of this fragment has been reported.[36] It has been assumed that contamination with undigested IgG or other active fragments is responsible for the activity exhibited by the Fc fragment.[35,36] In this study on VEE virus neutralization, sensitized virus was partially neutralized by goat antiserum to monovalent fragments of human IgG and, to a lesser degree, by the Fc fragment. It was also noted that VEE virus sensitized with antiviral human serum was neutralized by a mixture of goat antisera to Fab and Fc fragments of human IgG (Table 8). Sensitized VEE virus was neutralized to almost the same degree as that neutralized by goat antiserum to intact human IgG. Indirectly, these results suggest a role for the Fc fragment in virus neutralization. Observations by others on the inability of Fab fragments to establish and maintain neutralization of virus[36-39] favor the concept that the Fc fragment may complete the

structural configuration necessary for antibody to exert its most efficient neutralizing power. Effective virus neutralization, therefore, is not only a function of the antigen-binding sites of the antibody but may depend on the entire structure of the IgG molecule.[35]

Although the experiments reported here do not clearly define the mechanism by which neutralization of sensitized virus by anti-IgG serum occurs, the evidence adds support to hypotheses proposed by others.[16,25,26] A secondary reaction is involved in the interaction of anti-IgG with sensitized virus. Neutralization of the infectious virus-antiserum complex by anti-IgG is effected by an increase in size of the complex or the formation of bridges that cover or block critical infective sites on the virus particle. The inefficiency of monovalent Fab antibody fragments to block completely the reactive sites on the infectious virus-antibody complex may be related to their reduced size or the need for the Fc antibody fragment to provide a complete and effective configuration on the complex. Electron microscopy studies have revealed changes in the size of virus-antibody complexes that may be related to the valency of Fab fragments.[40] Fractionation techniques of antibody molecules, the use of anti-IgG serum to assay sensitized virus, and electron microscopy examinations of virus-antibody complexes offer the means for elucidating further the stages and mechanisms involved in virus neutralization.

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13. ABSTRACT

The persistent virus fraction that results from the interaction of Venezuelan equine encephalomyelitis (VEE) virus with antiviral serum is an infectious virus-antibody complex (sensitized virus) that can be neutralized by anti-IgG serum. The quantities of virus sensitized by VEE antiserum and neutralized by anti-IgG serum depend on the concentration of these sera. In contrast to the marked temperature and time dependence of VEE virus neutralization by antiviral serum, neutralization of sensitized virus by anti-IgG serum is more rapid (almost complete within 1 min at 35 C) and less sensitive to temperature. Evidence that virus sensitization preceded neutralization indicates that the persistent virus fraction is formed before virus neutralization has actively begun. Within certain limits, neutralization of sensitized virus by anti-IgG serum is species-specific. Differences in the ability of anti-IgG, anti-IgA, and anti-IgM sera to neutralize sensitized virus indicate that the reaction is also influenced by the specificity of the anti-immunoglobulin.

Sensitized virus was partially neutralized by goat antiserum to monovalent Fab fragments on human IgG and, to a lesser degree, by the Fc fragment. Sensitized virus was neutralized by an in vitro mixture of these fragments to almost the same degree as that neutralized by goat antiserum to intact human IgG. Indirectly, these findings suggest a role for the Fc fragment in virus neutralization.

14. Key Words

*Venezuelan equine encephalomyelitis virus		Neutralizers	Sensitivity
*Antibodies	*Neutralizing	Antiserums	Host virus relations
		Viruses	Cell lines

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