Experimentally Induced Alterations in Antigenic Properties of an Arbovirus Serotype

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Antigenic properties of Getah virus were monitored during 80 serial passages in Vero tissue cultures. The parent virus lost its serotype identity by passage 80. A clone derived from the parent remained antigenically stable during passage. The experimentally induced antigenic alterations in Getah virus were attributable to the tissue culture host selection of constituent subpopulations of virus with properties unlike those of the original parent. The marked differences in neutralization titers of low- and high-passage virus were considered related to or influenced by (i) the variation in subpopulation selection of the virus during passage and (ii) the variation in availability of antigenic stimulants during preparation of antiserum.

Antigenic heterogeneity of the various constituent virus particles comprising a virus strain has been well established for certain enteroviruses (3), myxoviruses (4, 5), and representative arboviruses (9, 10, 27). The extent to which variations in relative concentrations of these antigenically distinct virus components of a strain may influence the antigenic status of the total population is still not well understood. Some investigators have reported results of experiments in which the serological cross-reactive properties of arbovirus strains were experimentally altered by serial passage of parent virus in a susceptible cell culture host (19; T. B. Stim and J. R. Henderson, *Bacteriol. Proc.*, p. 182, 1968). This investigation was initiated to extend these observations with the single objective of studying further the extent to which these alterations in antigenic properties by host passage may influence the final serological identity of a strain. From the data obtained in these experiments, it appears that, by certain passage selection techniques (11-13), a serologically defined arbovirus serotype can be experimentally altered so that its initial specific antigenic properties are unrecognizable by standard serological methods and that the emergent virus does not share identity with any of the serotypes of the common serogroup.

METHODS AND MATERIALS

Viruses. Serogroup A parent strains used for serological experiments, with their mouse passage histories and infectivity titers in mice and tissue culture, are shown in Table 1. Titrations were made by plaquing in tissue culture (14) or by LD₅₀ determinations in in-

fant mice (20). The ICR strain of specific pathogen-free mice was used for preparing virus stocks.

Virus clone. Techniques for cloning viruses by plaque purification have been detailed previously (5). Getah virus (Sagiyama strain) was cloned by three successive plaque-picking and plating procedures. Care was taken to wash cultures at least five times with saline after virus adsorption. At each purification step, plaques were picked from those bottles containing less than 10 plaques.

Tissue culture. A passage line of Vero green monkey kidney cells (24) was used throughout. Stock cells were grown in Eagle's basal medium containing 5% fetal bovine serum and were maintained by using the same medium but with the calf serum concentration reduced to 2%. Tube and bottle cultures were prepared from cell culture stocks in passage generations 135 to 171. All cultures used were free from endogenous mycoplasma contaminants.

Serial passage of virus in tissue culture. The parent Sagiyama strain of Getah and a clone derived from it (Table 1) were each serially passaged in tube cultures. Every 48 hr 0.2 ml of infected tissue culture fluid was transferred to 0.8 ml (1:5 dilution) of medium of an uninoculated culture. The infectious virus inoculum dose varied from passage to passage (see top of Fig. 1). No other virus was handled in the separate laboratory during the period in which the Getah virus passages were being made. Virus stocks were in tissue culture passage 81 (TC-81), and this passage was used in all serological procedures and for immunization of animals.

Antisera. Guinea pigs were hyperimmunized with five doses of infectious virus. Each dose was given by the subcutaneous route at weekly intervals and had a mouse LD₅₀ titer of 10^{6.0} to 10^{9.7} per 0.2 ml. Immunizing antigens consisted of virus from mouse brain (Table 1) or tissue culture (Getah TC-81 virus) sources. Four to five guinea pigs were immunized

TABLE 1. Parent virus strains and infant mouse passage histories with titers in infant mice and tissue culture

Parent virus	Strain	Infant mouse passage ^a	Log ₁₀ titer per 0.2 ml	
			LD ₅₀ of infant mice	PFU in tissue culture
Bebaru	AMM-2354 (26) ^b	9	8.4	8.0
Chikungunya	Ross (21)	177	8.1	7.7
Getah	Sagiyama (23)	14	8.0	7.5
Getah	AMM-2021 (26)	19	9.6	8.4
Getah	Itakura (18)	9	6.0	5.5
Getah	Haruna (17)	9	9.7	7.9
Mayaro	Tr-4675 (1)	15	9.3	7.9
O'nyong nyong	MP-30 (30)	13	7.3	7.3
Ross River	T-48 (8)	12	6.3	6.4
Semliki Forest	Standard (25)	14	9.7	8.3
Una	BeAn-15 (2)	7	8.6	7.7

^a This passage was used for mouse immunization.

^b Parentheses refer to original isolation of the strain.

with each virus antigen. Antisera from animals of a group were pooled and frozen in 0.5-ml samples at about -80 C in a mechanical freezer until used. A fresh ampoule of antiserum was thawed each time tests were performed.

Filtration of viruses. A 10% suspension of parent Getah virus (Sagiyama strain) of mouse brain source was passed through a 220-nm (APD) membrane filter (Millipore Corp., Bedford, Mass.), and then the filtrate was refiltered through a 100-nm membrane. Undiluted infected tissue culture fluids were filtered in a similar manner. Membranes were "primed" with calf serum before filtration of virus suspensions to avoid excessive adsorption of virus to the filter and consequent loss of titer (16). General methods and equipment used in filtration experiments were similar to those described previously (15).

Neutralization tests. Two types of plaque-reduction neutralization tests were used in experiments, the varying virus method and the varying serum technique. All tests were performed in 2-oz (ca. 60 ml) plastic bottle cultures of Vero cells. The varying virus method was similar to that described previously (10). In one test, each antiserum was mixed with an equal volume of various log dilutions of virus. The virus-serum mixtures were incubated at 37 C for 1 hr, the bottles were inoculated, the inoculum was adsorbed for 1 hr, and all cultures were overlaid (15). In the second test, the varying serum method, procedures were essentially the same as described by Russell and Nisalak (22). Twofold serum dilutions were used, and three repli-

cate cultures were run for each dilution. Data were expressed as the reciprocal serum dilution giving 50% plaque reduction (7).

Hemagglutination-inhibition test. The standard hemagglutination-inhibition test for arboviruses was employed (6). Antisera were treated with kaolin to remove nonspecific hemagglutinin inhibitors. Hemagglutinating antigens were prepared from infected mouse brain (6) or tissue culture fluids (16), and 4 to 8 units of antigen was used in tests.

RESULTS

Antigenic status of parent Getah virus during serial passage in tissue culture. Getah virus (Sagiyama strain) was taken 80 serial passages in Vero tissue cultures. Virus was titrated at each passage, and a plaque-reduction neutralization titer was determined by using antisera prepared against the Sagiyama parent. As shown in Fig. 1, log infectivity titers of the virus at each passage (top of figure) used in the neutralization test were variable and ranged from 4.0 (passage 61) to 7.2 (passage 31). At passage 1, the log neutralization titer was 1.2; at passage 80, the neutralization titer was ≥ 6.4 . From passages 1 to 36, there appeared to be waves of high and low variation in the neutralization titer of virus with peak titers of each wave occurring about every fifth passage. From passages 36 to 80, there was a gradual but steady increase in virus neutralization, but titer fluctuations were less pronounced from passage to passage. Variations in neutralization titers were independent of variations in infectivity titers.

Antigenic status of Getah virus clone during serial passage in tissue culture. A clone derived from the parent Sagiyama strain of Getah virus was taken 80 passages in Vero tissue culture. As with the parent, the virus titer and neutralization test titer were determined at each passage level. There was essentially no change in the neutralization titer during passage, even though, as in the case of the parent, infectivity titers varied considerably (Fig. 2). Log values ranged from 2.4 to 3.1, with passage 1 virus having a titer of 2.8 and passage 80 a titer of 2.9. Again, as with the parent virus series, infectivity titers varied independently of neutralization titers.

Neutralization tests with filtered Getah parent viruses. Neutralization tests were performed by using filtered Getah parent (Sagiyama strain) and a tissue culture passage (TC-81) of this virus. It was thought that perhaps one explanation for the marked discrepancy in log neutralization indexes between mouse brain parent source and the high tissue culture-passaged parent was due to the difference in the non-neutralizable persistent fraction of the mouse brain parent virus, which might have been attributable to virus aggregation

FIG. 1. Plot of the infectivity and neutralization titers of Getah virus (Sagiyama strain) during each of 80 passages in Vero tissue culture.

FIG. 2. Plot of the infectivity and neutralization titers of a clone derived from Getah virus (Sagiyama strain) during each of 80 passages in Vero tissue culture.

(29). Results of these experiments are shown in Table 2. Essentially no differences were found between log neutralization values when comparing filtered and unfiltered virus preparations of mouse brain or tissue culture passage origin. The data indicate that neutralization titers of virus

from mouse brain source (B, Table 2) were not the result of gradual dispersion of the virus population by serial passage in tissue culture as has been suggested by others (19), but instead resulted from a progressive change in neutralizable properties of the virus by passage.

TABLE 2. *Plaque-reduction neutralization tests with filtered and unfiltered Getah parent (P) and tissue culture passage 81 (TC-81) of the Sagiya strain*

Test series	Getah virus	Getah antiserum	Log ₁₀ PFU reduction	
			Unfiltered	Filtered ^a
A	P	P	3.0	3.3
B	P	TC-81	1.5	1.4
C	TC-81	P	≥ 5.9	≥ 5.8
D	TC-81	TC-81	3.2	3.2

^a Virus was passed successively through 220- and 100-nm membrane filters.

FIG. 3. Rate of neutralization of Getah parent (P) and tissue culture passage 81 (TC-81) viruses by Getah P antiserum. Getah P antiserum (1:50): ●—●, Getah P virus; ●—●, Getah TC-81 virus. Normal serum (1:50): ○—○, Getah P virus; ○—○, Getah TC-81 virus.

Neutralization rate of parent and tissue culture-passaged Getah virus. The rate of inactivation of the parent and tissue culture-passaged virus (both unfiltered) was studied by using antiserum prepared against the mouse brain parent Sagiya strain (Fig. 3). Virus-antiserum mixtures were sampled at the indicated times, and the surviving virus was titrated. Significant differences in the

neutralization rates between the two viruses indicate a difference in the antigen-antibody combining properties of each virus, suggesting an antigenic difference.

Serological relationships between tissue culture-passaged Getah virus and various parent strains. The antigenic properties of the high- and low-passaged viruses were further examined by standard neutralization and hemagglutination-inhibition tests by using Getah parent and Getah TC-81 viruses and antisera (Table 3). There was a consistency in antigenic differences by all methods of analysis, with titers of Getah TC-81 virus against Getah parent antibody considerably higher than homologous titers with the Getah parent virus and antiserum. In contrast, when Getah TC-81 antiserum was cross-reacted with Getah parent virus, titers were significantly lower than the Getah TC-81 homologous system by all tests. These results would indicate an antigenic dissimilarity between Getah TC-81 and Getah parent viruses. Further plaque-reduction neutralization tests were performed by using Getah TC-81 virus and antiserum against other parent strains of Getah. In Table 4 are shown log titers of these cross-reactions. Getah TC-81 virus was related to other strains by one-way cross only (Getah TC-81 virus against Getah parent antisera), and, by existing criteria of identity between arboviruses, Getah TC-81 could be considered a distinct serotype.

Serological relationships between high-passaged Getah virus and other related group A serotypes. Attempts were made to identify further the Getah TC-81 virus, and plaque-reduction neutralization

TABLE 3. *Neutralization and hemagglutination-inhibition tests with Getah parent (P) and tissue culture passage 81 (TC-81) of the parent*

Test	Virus	Antiserum	
		P	TC-81
Plaque-reduction neutralization (serum constant, 1:5 dilution)	P	3.1 ^a	1.4
	TC-81	≥ 6.4	3.2
Plaque-reduction neutralization (virus constant, 100 PFU)	P	20,480 ^b	5,120
	TC-81	40,960	81,920
Hemagglutination-inhibition	P	320 ^c	20
	TC-81	2,560	1,280

^a Log₁₀ PFU reduction.

^b Reciprocal of antiserum dilution giving 50% plaque reduction.

^c Reciprocal of antiserum dilution inhibiting 4 to 8 hemagglutinating units of antigen.

TABLE 4. *Neutralization tests with Getah (Sagiyama parent strain) tissue culture passage 81 (TC-81) and other strains of Getah virus*

Getah virus strain	Antiserum (1:5 dilution)				
	TC-81	Sagiyama	Itakura	Haruna	AMM-2021
TC-81.....	3.2 ^a	≥6.4	4.0	2.2	2.1
Sagiyama.....	1.3	3.2	3.2	2.5	2.5
Itakura.....	0.7	2.5	3.1	2.9	3.0
Haruna.....	0.3	2.8	2.3	3.1	3.2
AMM-2021.....	0.1	3.2	3.9	3.3	3.4

^a Log₁₀ PFU reduction.

ANTISERUM VIRUS	GETAH (SAG-P)	GETAH (SAG-TC-81)	BEBARU	ROSS RIVER	SEMILIKI FOREST	CHIKUNGUNYA	O'NYONG NYONG	MAYARO	UNA
GETAH (SAG-P)	3.2								
GETAH(SAG-TC-81)		3.2							
BEBARU			3.1						
ROSS RIVER				2.2					
SEMILIKI FOREST					2.9				
CHIKUNGUNYA						3.2			
O'NYONG NYONG							2.9		
MAYARO								2.8	
UNA									2.9

■ -ANTIGENICALLY RELATED ▨ -ANTIGENICALLY DISTINCT

FIG. 4. *Cross-neutralization tests with Getah tissue culture passage 81 (TC-81), Getah parent (P), and related serogroup A viruses.*

FIG. 5. *Diagram illustrating relationships between a serogroup A "complex" of viruses as determined by two-way cross-neutralization with hyperimmune serum. Taken from data of Fig. 4.*

tests were performed by using other serotypes of the group A "complex" of which Getah is a member. Viruses and antisera of Getah parent, Getah TC-81, Bebaru, Ross River, Semliki Forest, Chikungunya, O'nyong nyong, Mayaro, and Una were tested by plaque-reduction neutralization (Fig. 4). Criteria for antigenic relatedness or distinctness were the same as established previ-

ously (9). Antisera prepared against Getah TC-81 failed to neutralize significantly any of the serogroup "complex" viruses, but Getah TC-81 virus was significantly neutralized by Getah parent, Ross River, Chikungunya, and Mayaro viruses. Thus, Getah TC-81 virus shares less of an antigenic relationship (a probable loss in subpopulation antigen types) with other serotypes of the "complex" than the serotypes share with one another. A simplified diagram of such antigenic relationships, taken from the data of Fig. 4, is shown in Fig. 5. These relationships were established on the basis of two-way cross-neutralization by using hyperimmune sera. Getah TC-81 has no place in this relationship scheme among viruses of this serogroup complex, indicating further the distinctness of its antigenic property.

DISCUSSION

In a recent report, Ozaki and Kumagai (19) described alterations in the antigenic properties of Japanese encephalitis virus after 29 passages in cultures of a stable line of porcine kidney cells. They found, as was the case in these experiments, that the amount of virus neutralized by certain antisera was much less with low-passage than with high-passage virus (Fig. 1 and Table 3). These workers also found this neutralization phenomenon obtained by passage of the virus to occur when plaque-purified virus was passaged. This finding is in contrast to our results; no changes in neutralizing properties were observed by tissue culture passage of our clone (Fig. 2). It is possible that Ozaki and Kumagai were not passing true clones, which may explain this discrepancy in results.

The data of this report tend to support further and confirm previous descriptions of host-induced antigenic alteration of strains due to mechanisms of subpopulation selection and ultimate predominance of antigenically distinct particles which comprise the parent. In the case of Getah virus, host selection ultimately yielded a total virus population with unusually different antigenic properties compared to the original parent. The gradual increase in the log neutralization titer of Getah parent virus started at about tissue culture passage 40 with a titer of 2.8, and by passage 80 the titer was ≥6.4. One possible explanation for this phenomenon might be the variation in subpopulation selection during passage, which would influence the antigenic properties of the virus, and the selection occurring during immunization of the animals. This might best be characterized by the hypothetical illustration presented in Fig. 6.

At least two host selection factors appear to influence results obtained in the neutralization

FIG. 6. Hypothetical scheme illustrating virus subpopulation variation in antigenic properties during tissue culture passage and antibody specificity variation during animal immunization.

test: the selection and predominance of certain antigenic subpopulations of virus during tissue culture passage and the types of specific determinants associated with the antibody produced as the result of hyperimmunization of animals with a heterogeneous antigen (multiple subpopulation antigen types). The former phenomenon, as shown by the tissue culture passage at the left of Fig. 6, has been detailed before (12) and will not be discussed here. The latter, however (right side of figure), represents a concept which may have some significance in the magnitude of cross-neutralization of virus with antiserum.

As animals are immunized with the antigenically heterogeneous Getah virus parent, the homotypic antibody concentration resulting from each type of antigenic stimulus may change. Such concentration changes may be due to the availability of antigenic stimulants (unneutralized virus) after inoculations 2 through 5. Or, if the infectious virus antigen multiplies in the guinea pig host, there could be a host selection of subpopulation types (13) independent of antibody

type or concentration. Lastly, perhaps both immune status and degree of virus multiplication after antigen injection greatly influence antibody type represented in the final hyperimmune serum. Thus, the final result of the neutralization of parent and tissue culture passage virus would indicate that the antibody population resulting from hyperimmunization with parent virus may be more compatible with the tissue culture-passaged virus population than the original parent virus population.

The antigenic relationships between high-passage Getah parent virus and the mouse brain parent (Tables 3 and 4) and related serotypes (Fig. 4) would indicate that the high-passage virus is sufficiently distinct by existing criteria to justify designating it a new serotype. Thus, on the basis of this experimental model, it may be possible to change significantly the antigenic properties of other arbovirus serotypes by using the proper host and a suitable number of passages. Results of host passage could culminate in an arbovirus with distinct serotype properties as shown here or with antigenic properties more related to known serotypes other than the parent from which it was derived (T. B. Stim and J. R. Henderson, *Bacteriol. Proc.*, p. 182, 1968).

One can only speculate regarding the significance or importance of these phenomena in the natural evolution of new arbovirus serotypes. Because of the close antigenic relationships of certain viruses, it is very possible that some strains may be comprised of the same subpopulations in different concentrations and are thus expressed in different ways, as proposed before (12). However, it is now possible to experimentally induce heretofore unknown arbovirus serotypes from known ones by host selection techniques, and it may be possible to revert the new virus back to the original parent type under suitable experimental conditions and if there was no loss in the original antigenic subpopulation components during passage.

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