ANTIGENIC ANALYSIS OF CERTAIN GROUP B ARTHROPOD-BORNE VIRUSES BY ANTIBODY ABSORPTION

By D. H. CLARKE, M.D.

(From The Rockefeller Foundation Virus Laboratories, New York)

(Received for publication, September 10, 1959)

As a result of intensified studies during recent years, the number of known arthropod-borne (arbor) viruses has been greatly increased. One of the most valuable pieces of knowledge which has emerged from these studies is that many of these arbor viruses fall into clearly defined antigenic groups (1). Although immunologic relationships between some of them had been previously shown, the introduction of the group concept by Casals (2, 3) clarified and systematized these relationships. While cross-neutralization and complement-fixation tests are also used in establishing group relationships, the technique of hemagglutination-inhibition (HI) has provided the best method for the demonstration of antigenic relationships between members of group B and, indeed, the extent of overlap observed by HI may be so great as to resemble the situation that exists between various strains of type A influenza. As antigenic analysis by antibody absorption has proved fruitful in the study of the latter viruses (4–6), it was decided to apply a similar approach to an analysis of the relationship among certain group B arbor viruses. The results of some of the studies are the subject of the present paper.

Materials and Methods

Viruses.—The viruses used in the study are given in Table I. Their designation in subgroups rests on particularly close antigenic relationships revealed by various serologic procedures (1). The place and date of isolation of certain strains are given when this information is pertinent to the present study.

Immune Sera.—The sera were prepared in either rabbits or mice. Rabbits were immunized with one or more injections of infective virus depending upon the antibody response. Mice received multiple (four or five) immunizing inoculations. With viruses which were pathogenic by the intraperitoneal route, the first injections consisted of inactivated virus followed by one or two inoculations of infective virus. Where multiple inoculations were required, the animals were bled 7 to 10 days following the last dose.

Hemagglutination-Inhibition Tests.—The preparation of test antigens, removal of nonspecific inhibitors from sera, and performance of the tests were done as previously described from these laboratories (7). The test antigens were prepared by either the acetone-ether or sucrose-acetone extraction methods. All tests were carried out in tubes.

Technique of Antibody Absorption.—The absorbing virus was prepared from a 20 per cent homogenate of infected suckling mouse brain made in borate-buffered saline, pH 9.3 (0.05 M borate–0.12 M NaCl). The homogenate was centrifuged for one hour at 11,000 R.P.M. in

the No. 40 rotor of a Spinco model L centrifuge, the supernatant fluid was recovered, and various aliquots were sedimented for 2 hours at 40,000 R.P.M. in the same rotor. The pellets were homogenized in the test serum previously diluted 1/10 in borate-saline, pH 9.0. The latter homogenization was carried out in thick-walled glass tubes equipped with fitted teflon plungers which were motor-driven. All work was carried out in ice baths. The serum-virus mixtures were held overnight in the cold, and the virus then removed by sedimentation for 2 hours at 40,000 R.P.M. This final centrifugation was carried out in small (3 ml.) tubes fitted into the No. 40 rotor by means of appropriate nylon adapters. Little difficulty was

TABLE I

Viruses and Strains Used

Subgroup	Virus	Strain	Symbol
Japanese B– St. Louis– West Nile	Japanese B St. Louis West Nile Ilheus Murray Valley	Nakayama Parton Uganda (1940) Egypt 101 (1950) India IG 2266 (1955)	JBE SLE WN Il MVE
Dengue	Type 1 Type 2 " " " "	Hawaiian New Guinea B Trinidad 1751 India P 8640	
Yellow fever	Yellow fever	African Asibi (1927) French (1928) Ogbomoshe (1946) French neurotropic (vaccine strain) 17 D (vaccine strain) American JSS (Brazil, 1935) Trinidad 4754 (1954) Belem H 70 (Brazil, 1955)	 As Fr Og FN 17 D JSS Tr 4754 Be H 70

encountered in removing all excess virus provided the fluid in the bottom of the tube was sacrificed. It seems probable that the relative ease with which absorbing virus was removed was due to the gummy nature of the virus-containing pellets which tended to trap and immobilize the sedimentable constituents. The absorbed and unabsorbed control sera were then treated with kaolin, and finally, with goose cells to remove non-specific inhibitors and hemagglutinins. Since the sera were in a dilution of 1/10, an equal volume of 12.5 per cent kaolin suspension was used. The initial serum dilution in all HI tests was, therefore, 1/20.

The absorption procedure found most satisfactory was to homogenize 3 ml. aliquots of 1/10 test serum with the pellets obtained from varying volumes of virus suspension. In the early phase of the work, an attempt was made to correlate absorbing efficiency with infective or HA titer, but the limited studies were not encouraging. It seems probable that the total amount of antigenically active, sedimentable material present can be expressed only in terms

of its ability to combine with and remove antibody. For convenience, the amount of absorbing virus is expressed as grams of original infected mouse brain used to prepare the homogenate; this figure is calculated for 1 ml. of undiluted serum.

It might be mentioned that attempts have been made to use virus-coated chick or goose erythrocytes (plain or formol-treated) for antibody absorption, as has been done in influenza virus studies, but with results that were not encouraging. A reasonable explanation appears to be that a major portion of the antigenically active material present in the homogenate does not combine with red cells, at least not to a quantitatively favorable degree.

EXPERIMENTAL

Homologous and Heterologous Absorption Curves.—When an immune serum is absorbed with increasing amounts of the homologous virus and the resultant

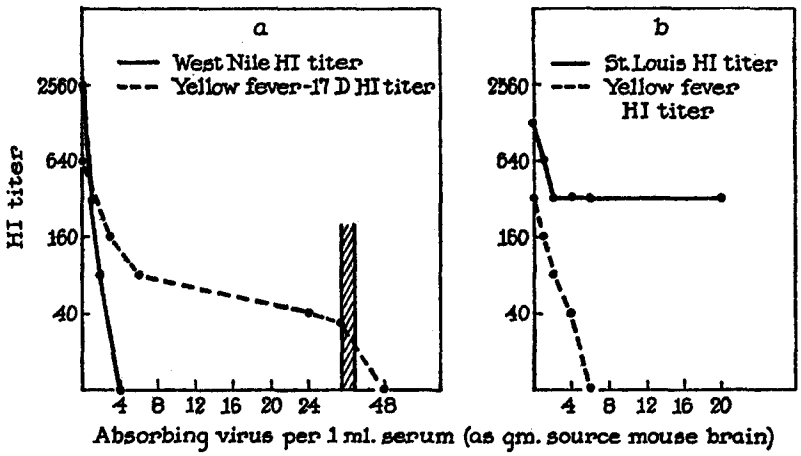

FIG. 1. Homologous and heterologous antibody absorption.
a. Homologous absorption of West Nile (Egypt 101) and yellow fever-17 D sera.
b. Heterologous absorption of St. Louis with yellow fever virus.

homologous HI titers of the serum samples are plotted against the amount of absorbing virus, a curve is obtained, the slope of which varies with the absorbing virus used. Although different viruses differ considerably in their capacity to absorb antibody, there is little or no variation between different preparations of the same virus. Fig. 1 *a* presents two curves which represent the extremes of homologous absorption efficiency. The Egypt 101 strain of West Nile is seen to be a highly efficient absorber, while excessively large amounts of the 17 D vaccine strain of yellow fever were required to effect complete antibody removal. Multiple absorptions with smaller amounts of virus appeared to offer no advantage and were considerably more wasteful of serum, virus, and time. Fortunately, most of the viruses studied resembled more closely the West Nile picture, including strains of yellow fever other than the 17 D.

In Fig. 1 *b* are shown the results of heterologous absorption. In this case, the serum (St. Louis) was absorbed with increasing amounts of yellow fever virus

(JSS strain), and the resultant serum samples were tested against both the absorbing (heterologous) and the immunizing (homologous) virus. The antibodies detected by the yellow fever antigen were removed in a manner resembling a homologous absorption curve, while the St. Louis antibody curve shows a fall followed by a plateau which remains flat regardless of the amount of heterologous absorbing virus used.

The data presented throughout the remainder of the paper were obtained by the use of sufficient absorbing virus to remove all detectable antibody or to reach the plateau area.

Strain Homogeneity.—One of the striking observations to emerge from the antigenic analysis of type A influenza viruses has been the great variability of strains, even among those isolated during the course of a single outbreak (8). Since it has been our impression that group B arbor viruses exhibit considerable antigenic stability, it was of interest to subject this impression to closer examination by absorption analysis. For this purpose, three strains of West Nile virus were selected which had been isolated at different times and from different countries. The Uganda strain was the first isolate of this virus and was made by Smithburn and his associates (9). The Egypt strain was isolated 10 years later by Melnick *et al.* (10), while the Indian strain was obtained recently by Work and coworkers (11). The first two strains were obtained from human beings and the third from mosquitoes.

Comparison of the three strains was made by absorbing immune sera prepared against the Egypt and India strains with all three viruses and testing them against the three strain antigens. When a sufficient amount of any of the three strains was used, both sera were completely freed of all antibody (detectable at the 1/20 dilution). Furthermore, the absorbing efficiencies of the three strains, for each serum, were quite similar, although for complete exhaustion the Indian strain serum required larger amounts of the three strains than did the Egyptian.

These studies thus indicate that the three strains of West Nile, despite their diverse origins, are antigenically identical as judged by the absorption technique. The only difference noted among the three was that both sera, before absorption, gave higher HI titers when tested against the Uganda strain antigen than were realized with either the Egyptian or Indian strain antigens. This phenomenon has been observed with different strains of other arbor viruses, an indication that variations in reactivity with antibody, in the absence of antigenic variation, are another factor which must be reckoned with in the interpretation of serologic tests.

Virus Characterization by Heterologous Antibody Absorption.—It became apparent early in our studies that a considerable degree of specificity could be imposed upon an immune serum by absorption with a heterologous group B virus. Such absorbed sera could then be used for the rapid identification of new isolates if these possessed the property of hemagglutination.

The following study was carried out with immune sera prepared against three strains of dengue, the absorption being made, in each case, with the Egypt 101 strain of West Nile virus. The sera were prepared against the Hawaiian strain of type 1 dengue, the New Guinea B strain of type 2 dengue, and a dengue strain designated as Tr 1751 which was isolated from a human case in Trinidad by Anderson and his associates (12). The HA antigens were prepared from the above viruses plus West Nile and a virus, P 8640, isolated from a sick child in India by Work and his associates (13). When this study was initiated, the Trinidad virus was known to be a strain of dengue, apparently closely related to type 2, but knowledge of the Indian virus was limited to the fact that it was a mem-

TABLE II

Comparison of Dengue Strains by Antibody Absorption

Serum		Test HA antigen				
		Dengue 1	Dengue 2	Dengue Tr 1751	India P 8640	West Nile
Dengue Tr 1751	Unabsorbed	80	320	640	640	160
	Absorbed	0*	80	160	160	0*
Dengue 2	Unabsorbed	160	640	640	640	320
	Absorbed	0	80	80	80	0
Dengue 1	Unabsorbed	2560	640	640	640	640
	Absorbed	640	80	80	80	0

Absorption in each case was with the Egypt 101 strain of West Nile virus.

Results expressed as the reciprocal of the serum dilution giving complete inhibition of 4 to 8 hemagglutinating units of each test antigen.

* No inhibition at serum dilution of 1:20.

ber of group B. Table II presents the data from a relatively simple experiment in which unabsorbed and absorbed sera were tested against the five antigens. It can be seen that, following absorption, the group-reactive antibodies detected by the West Nile antigen were eliminated while homologous antibody remained in significant titer, though lower than that of the unabsorbed serum. It can also be seen that the two dengues are related by one or more shared antigens not present in West Nile since West Nile was unable to remove all of the antibody from dengue 1 serum which was cross-reactive with dengue 2. Finally, the two recent isolates from Trinidad and India are clearly shown to be strains of type 2 dengue. It should be noted that cross-complement-fixation studies carried out in these laboratories were in agreement with this interpretation (14).

Antigenic Analysis of the Japanese B–St. Louis–West Nile Subgroup.—Several investigators have, in the past, noted that the five viruses comprising this subgroup (see Table I) show immunologic relationships demonstrable by various

procedures (1). Because of their particularly close relationship, these viruses were subjected to antigenic analysis by the absorbed antibody technique. In Table III are presented the results of a series of experiments in which St. Louis and Japanese B immune rabbit sera were absorbed with various viruses and the absorbed and unabsorbed sera tested against the subgroup antigens. The results of absorption of Ilheus immune rabbit serum with one virus are also presented. The titer of each unabsorbed serum against each antigen was found to be quite reproducible, no variation greater than twofold being encountered.

The results of absorption were similar with the St. Louis and Japanese B sera. In both cases, a significant level of homologous antibody remained regardless of the absorbing virus used. The removal of heterologous antibody, however, depended markedly upon the absorbing virus. Absorption of either serum

TABLE III

Absorption Analysis of Japanese B–St. Louis–West Nile Complex Viruses

Absorbing virus	Test antigens																	
	St. Louis serum						Japanese B serum						Ilheus serum					
	SLE	WN	MVE	JBE	Il	YF	JBE	MVE	WN	SLE	Il	YF	Il	MVE	SLE	WN	JBE	YF
West Nile (WN)	80	0	0	0	0		160	0	0	0	0							
Murray Valley (MVE)	160	0	0	0	0		160	0	0	0	0							
Japanese B (JBE)	160	0	0	0	0													
St. Louis (SLE)							160	40	0	0	0							
Ilheus (Il)	160	40	40	0	0		320	160	40	20	0							
Yellow fever (YF)	320	80	80	20	0	0	320	160	40	40	0	0	640	40	20	20	0	0
None	1280	640	1280	320	640	160	1280	1280	640	320	320	80	2560	5120	1280	1280	640	320

Results expressed as in Table II.

with West Nile, Murray Valley, or Japanese B eliminated all cross-reactive antibody, while only antibody against the Murray Valley virus (known to be particularly closely related to Japanese B) remained after absorption of Japanese B serum with St. Louis. From these results, it may be concluded that, although each virus contains a specific antigenic component, there is extensive sharing of other antigenic components between Japanese B, Murray Valley, West Nile and St. Louis viruses. Of these, the first three are apparently most closely related.

In contrast to these results are those obtained from absorption with Ilheus and yellow fever (French neurotropic strain) which appear similarly deficient in some of the antigenic components shared by the other viruses. Since yellow fever virus is not a member of the subgroup, the results obtained with it were not unexpected, but the inadequacy of Ilheus came as something of a surprise. By absorption analysis, Ilheus is thus seen to be less closely related to the other members of the subgroup on the following grounds: (*a*) absorption by yellow

fever virus removed all cross-reactive antibody against Ilheus from both St. Louis and Japanese B sera; (*b*) Ilheus was little if any more effective as an absorber of these sera than was yellow fever; (*c*) absorption of an Ilheus serum with yellow fever virus yielded a preparation having less subgroup reactivity than was true with the other sera although the residual specific (Ilheus) antibody level was high.

Antigenic Analysis of Strains of Yellow Fever Virus.—It has been a generally accepted idea that various strains of yellow fever virus are antigenically identical. That this concept might be incorrect was suggested by the work of a visiting

TABLE IV

Absorption Analysis of Strains of Yellow Fever Virus, Using French Strain Immune Serum

Absorbing strain	Test Antigens								
	African strains					American strains			
	Fr	FN	As	17 D	Og	JSS	Tr 4754	Be H 70	WN
African strains									
YF-FN	0	0	0	0		0			
YF-As	0	0	0	0		0			
YF-17 D	0	0	0	0		0			
YF-Og	0		0		0	0		0	
American strains									
YF-Be H 70	40		40	40		0		0	
YF-Tr 4754	80		160	80		0	0		
YF-JSS	160	160	160	160		0			
West Nile	1280	1280	2560	640		320			0
None	2560	2560	5120	2560	5120	1280	1280	2560	640

Results expressed as in Table II.

investigator to these laboratories, Dr. M. Bruno-Lobo of Brazil, who observed that the HI results with human sera obtained with a JSS strain antigen (see Table I) were consistently inferior to those obtained with other strains such as the Asibi, the French or the 17 D vaccine strains (15). An examination of various strains by antibody absorption was, therefore, undertaken, and it became apparent that the JSS strain was, indeed, antigenically defective as compared with certain others. Although absorption by the JSS strain of an immune serum prepared against the French strain resulted in the removal of more homologous, French, antibody than was obtained by absorption with another group B virus (West Nile), a significant level of antibody reacting with the French and other strains still remained (Table IV).

It was initially assumed that the JSS was simply an odd variant, but since it

was the only American strain among those under study, it was decided to compare other strains on the basis of their place of origin, America or Africa. Table IV presents the data obtained when the same pool of immune mouse serum (French) was absorbed with various yellow fever strains with the rather surprising result that all the American strains were found to be incapable of completely removing from an African strain immune serum all antibody detectable by any African strain antigen. It should be noted that although five African yellow fever viruses appear to be represented, including the French strain producing the serum, these correspond to only three original isolates, the French neurotropic being a mouse-adapted variant of the parent, French, and the 17 D a tissue culture variant of the parent, Asibi. However, within the limitations of this study, there appears to be a genuine difference between randomly chosen strains, depending upon whether they are of African or American origin.

TABLE V

Antigenic Analysis of the 17 D Vaccine Strain of Yellow Fever by Absorption of a 17 D Immune Serum

Absorbing strain	Test antigens				
	17 D	FN	Fr	As	JSS
Asibi (As)	40	0		0	0
French neurotropic (FN)	40	0	0		0
JSS	80	40	80		0
None	640	640	1280	640	160

Results expressed as in preceding tables.

A comparable converse study has not been carried out in which an American yellow fever immune serum prepared in the same way has been studied for the possible presence of antibody which cannot be absorbed by African strains. However, absorption of a JSS strain immune monkey serum with the French neurotropic African virus did remove all homologous, JSS, antibody. Since it is quite possible that the pattern of antibody response of the monkey to a single small dose of virus may differ significantly from that of the mouse following multiple large doses, this is considered to be suggestive but by no means conclusive evidence that American strains are truly antigenically defective and do not, in turn, have a characteristic "American" antigenic marker.

The final demonstration of antigenic variation among yellow fever strains was with the 17 D vaccine virus, which was shown to possess an antigenic component absent from other strains, including the parent, Asibi (Table V). Absorption of a 17 D immune mouse serum with the American JSS virus showed the characteristic failure to remove the "African" antibody, but neither the

French neurotropic nor the Asibi virus was able to remove residual 17 D antibody. This gain in an antigen on the part of the vaccine strain does not appear to have been attended by any demonstrable concomitant loss, since the 17 D virus proved capable of removing all detectable antibody from an Asibi immune mouse serum in a manner entirely analogous to its effect upon its own, 17 D, immune serum.

It is significant that the alteration in pathogenicity for man of a strain of yellow fever is not necessarily attended by any antigenic modification demonstrable by the absorption technique. The French neurotropic vaccine strain is one which was freed of its visceral pathogenicity for man by prolonged mouse brain adaptation. When this attenuated variant was compared, by cross-absorption experiments using both sera, with its parent, French strain, the two showed complete antigenic identity.

DISCUSSION

The rapid increase in the number of arbor viruses which have been and are being isolated has posed the serious problem of how to deal with their classification in any systematic and practical fashion. It is our belief that the single most reliable and convenient method for classification of these agents is the one introduced and developed by Casals (2, 3), which is based on antigenic relationships, preferably demonstrated by *in vitro* techniques. That antigenic structure can, indeed, be a stable property is indicated in the present study. The three strains of West Nile virus compared had been obtained from three different countries and two different hosts, and their isolations covered a span of 15 years. In addition, their passage level in mouse brain ranged from the 2nd to the 35th. Yet, despite their varied histories, no evidence of antigenic dissimilarity was obtained. Furthermore, although African and American yellow fever strains differ, each geographical type appears to maintain a constant antigenic make-up as demonstrated by the fact that, in each case, isolations separated by a period of approximately 20 years yielded viruses which behaved in an indistinguishable manner. The 17 D vaccine strain presumably acquired its characteristic antigen during its subsequent history in tissue culture or the chick embryo. It is well recognized that prolonged adaptation of a virus to a tissue or host may result in marked alteration of certain biologic properties such as tissue or host pathogenicity. Certainly this is true of the French neurotropic yellow fever vaccine strain with its 200 to 300 mouse brain passages as compared with the pantropic Asibi and French strains which were maintained in monkeys and kept at low mouse passage levels. Yet no demonstrable antigenic alteration resulted from this profound biologic modification.

A second aspect of the problem of virus classification, and one which promises to be more difficult to resolve, is the question of what criteria are needed to justify the designation of a new isolate as a discrete entity or as a variant of a pre-

viously isolated virus. Two extreme stands can be taken on this question: (*a*) all viruses which can be shown to be antigenically related should be considered as variants of one virus; (*b*) any reasonably marked antigenic variation justifies the designation of such a virus as a separate entity. (Proponents of these points of view have been described, respectively, as "lumpers" and "splitters.") The application of absorption analysis to such viruses as those making up the Japanese B–West Nile–St. Louis complex yields results which appear to explain why each is recognizably different, because of the possession of a specific antigen, as well as why serologic overlaps have been encountered, because of the prevalence of shared antigens. However, it is of interest that there is an identical degree of antigenic difference, as judged by residual antibody titers after absorption, between Japanese B and Murray Valley or West Nile, on the one hand, and an African (French) strain of yellow fever and an American (JSS) strain, on the other (Tables III and IV). Yet, Japanese B, Murray Valley and West Nile are generally accepted as distinct viruses while the difference between African and American yellow fever previously went unrecognized. It is apparent from the same tables that the extent of removal of heterologous antibodies from St. Louis, Japanese B, and Ilheus sera by a distantly related virus (yellow fever) is generally somewhat greater than is the converse removal of "American" antibodies from an African strain immune serum by the distantly related West Nile virus. Apparently, when the level of shared antigens is sufficiently high, conventional serologic procedures may fail to distinguish between two viruses even though the more sensitive technique of absorption analysis reveals consistent differences. Furthermore, conventional serologic techniques vary in their specificity. For example, Smith found the HI and complement-fixation tests unreliable for distinguishing between dengue and Japanese B following dengue infections in Malaya and considered only the neutralization test sufficiently specific (16); Goldblum found, however, that the neutralization test gave similar results with West Nile and Japanese B viruses following West Nile infections in Israel and considered the complement-fixation test the procedure of choice (17). Although at the present time it seems impossible to assess the full significance of these similarities and differences, it would appear necessary ultimately to resolve the problem of classification in a manner which will be meaningful and useful to all of those concerned with arbor viruses and the diseases caused by them. Absorption analysis appears to provide more detailed knowledge of the antigenic structure of these viruses than any other technique so far utilized. Such information would seem to be essential to the development of any scheme of classification, although it may be that more than one approach will be necessary and that antigenic structure will have to be related to other viral properties in order to realize an entirely satisfactory picture.

The demonstration that a rather stable type of antigenic variation may occur between different strains of a virus should prove of value in the selection of

strains for serologic studies and in clarifying our knowledge of the natural history of viruses as they occur in different parts of the world. Also, with regard to the selection of virus strains for serologic studies, the problem of strain variation in avidity for antibodies should be kept in mind. This was noted in the comparison of West Nile strains and is believed to play a role in the poor performance of the JSS strain of yellow fever as an antigen. Comparative studies with human survey sera in our laboratories (14) as well as the data presented by Groot and his associates (18) have provided evidence for low avidity as an attribute of the JSS antigen. This avidity is presumably unrelated to antigenic structure.

SUMMARY

A method for carrying out antibody absorption studies for antigenic analysis of group B arthropod-borne (arbor) viruses is described and examples of homologous and heterologous absorption curves are presented. Evidence that antigenic structure can be a stable property was obtained with three strains of West Nile virus isolated from different hosts in different countries over a period of years. Comparative studies with viruses of the Japanese B–St. Louis–West Nile subgroup indicate that each virus contains a completely specific antigen as well as one or more cross-reactive components. Strains of yellow fever virus isolated in America were shown to lack an antigen present in strains of African origin although no differences were found between isolates from the same geographical area. The attenuated 17 D vaccine strain of yellow fever was found to have acquired an additional antigen not present in the unadapted parent or in other strains tested. However, alteration in pathogenicity for man was not found to be necessarily attended by any antigenic modification, as shown by the antigenic identity of the French neurotropic vaccine strain with its pantropic parent.

BIBLIOGRAPHY

1. Rivers, T. M., and Horsfall, F. L., Jr., Viral and Rickettsial Infections of Man, Philadelphia, J. B. Lippincott, 1959, chapters 12 and 14.
2. Casals, J., and Brown, L. V., Hemagglutination with arthropod-borne viruses, *J. Exp. Med.*, 1954, **99,** 429.
3. Casals, J., The arthropod-borne group of animal viruses, *Tr. New York Acad. Sc.*, series 2, 1957, **19,** 219.
4. Friedewald, W. F., Qualitative differences in the antigenic composition of influenza A virus strains, *J. Exp. Med.*, 1944, **79,** 633.
5. Hirst, G. K., Strain specific elements in influenza antigens, *J. Exp. Med.*, 1952, **96,** 589.
6. Jensen, K. E., and Francis, T., Jr., The antigenic composition of influenza virus measured by antibody absorption, *J. Exp. Med.*, 1953, **98,** 619.
7. Clarke, D. H., and Casals, J., Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses, *Am. J. Trop. Med. and Hyg.*, 1958, **7,** 561.

8. Jensen, K. E., The nature of serological relationships among influenza viruses, *Advances Virus Research*, 1957, **4,** 279.
9. Smithburn, K. C., Hughes, T. P., Burke, A. W., and Paul, J. H., A neurotropic virus isolated from the blood of a native of Uganda, *Am. J. Trop. Med.*, 1940, **20,** 471.
10. Melnick, J. L., Paul, J. R., Riordan, J. T., Barnett, V. H., Goldblum, N., and Zabin, E., Isolation from human sera in Egypt of a virus apparently identical to West Nile virus, *Proc. Soc. Exp. Biol. and Med.*, 1951, **77,** 661.
11. Work, T. H., Rajagopalan, P. K., and Shah, K. V., Isolation of the Tamilnad strain of West Nile virus from *Culex vishnui* mosquitoes in North Arcot District of Madras State, India, in preparation.
12. Anderson, C. R., Downs, W. G., and Hill, A. E., Isolation of dengue virus from a human being in Trinidad, *Science*, 1956, **124,** 224.
13. Work, T. H., Pereira, S., Dandawate, C. N., Webb, J. K. G., and Clarke, D. H., Isolation of type 2 dengue virus from the blood of a febrile child in Madras State, India, in preparation.
14. Casals, J., personal communication.
15. Bruno-Lobo, M., personal communication.
16. Smith, C. E. G., A localized outbreak of dengue fever in Kuala Lumpur. Serological aspects, *J. Hyg.*, 1957, **55,** 207.
17. Goldblum, N., Sterk, V. V., and Gasinska-Klingberg, W., The natural history of West Nile fever. II. Virological findings and the development of homologous and heterologous antibodies in West Nile infection in man, *Am. J. Hyg.*, 1957, **66,** 363.
18. Groot, H., Kerr, J. A., Sanmartin, C., and Vidales, H., Antibodies to yellow fever and other arthropod-borne viruses in human residents of San Vicente de Chucuri, Santander, Colombia, *Am. J. Trop. Med. and Hyg.*, 1959, **8,** 175.