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A Serological Study of Eight Nigerian Group Simbu Arbovirus Isolates

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INTRODUCTION

Arthropod-borne viruses (arboviruses) are viruses transmitted by arthropod vectors between vertebrate hosts. Three elements are thought to characterize the interrelationship of virus, host, and vector. The virus multiplies in the tissues of both host and vector, sometimes yielding disease or overt evidence of infection in the former but no detectable effects in the latter. An extrinsic incubation period occurs between ingestion and transmission of the virus by the vector. Presumably the virus also exists at any given time in either arthropod or vertebrate (1).

To be characterized as an arbovirus a virus should ideally be demonstrated to fit the above definition in an orderly, complete fashion. Practically, such demonstrations are generally impossible at this time. Consequently, arbovirus characterization is based on more indirect evidence.

Physical parameters are helpful to a limited extent. Most arboviruses thus far are found to be 20-100 mu in diameter, to be sensitive to diethyl ether or sodium deoxycholate, and to contain RNA (11). Effects of the viruses in vivo are likewise helpful, such as their pathogenicity for laboratory animals, especially the baby mouse, the lesions they produce in tissue cultures, and proof of their asymptomatic multiplication in possible arthropod vectors.

However, even more important as indirect evidence for viruses being arboviruses are the circumstances under which they were isolated originally. Such evidence is present, for example, when viruses are found in materials gathered from large collections of arthropods, both wild and domestic animal populations, sentinel animals, and individual animals and humans that are appropriately ill.

Similarly important to this indirect characterization of a virus as an arbovirus is the serological relationship of a proposed arbovirus to a known one.

Classification of the arboviruses systematically is based primarily on their serological interrelationships, which are stable (9), easily determined, and fairly well understood common properties. On the basis of serological similarities, apparently reflecting sharing of common antigenic structures, most of the 252 arboviruses thus far sufficiently studied have been arranged into twenty-eight groups and one supergroup. Fifty-six, however, are as yet ungrouped (24). Within these groups serological differences among members, thought to reflect differences in antigenic structures, establish the varying degrees of individuality of the viruses.

Serologic means used in characterization and classification of arboviruses include the hemagglutination-inhibition (HI), complement-fixation (CF), and neutralization (NT) techniques. Several degrees of simplicity, rapidity, and inclusiveness exist among these tests, and numerous variations and extensions may be introduced into them as suits the end desired (e.g., more inclusive tests are usually required for grouping, more exclusive for typing) (2). Other techniques are occasionally employed in arbovirus classification, including agar gel immuno-electrophoresis and cross-challenge. These, however, are presently in the stage of development, or of limited applicability, and not in common use.

Importances of the arboviruses are manifold, including both practical aspects, since they are disease agents with medical and economic consequences in man and animals, and academic aspects, as well. Either alone would be sufficient to justify their study.

The Yale Arbovirus Research Unit (YARU), formerly the Rockefeller Foundation Virus Laboratories, is a multi-purpose organization contributing both to the

study of various aspects of arboviruses, in conjunction with other organizations throughout the world, and to the teaching of students of medicine and public health (14, 25). The research dealt with in this paper was undertaken within the teaching program at YARU and as part of its study of arbovirus serology and immunology, here mainly in association with the Arbovirus Research Project of the University of Ibadan in Nigeria.

In its study of the arbovirus ecology of Nigeria, the University of Ibadan Project has collected several arbovirus isolates, including seven of the eight subjects of this paper. The eighth was also isolated in Nigeria by Dr. C.C. Draper and his colleagues of the West African Council for Medical Research, and then studied in part by the East African Virus Research Institute. All of the isolates belong to group Simbu, and their collection, characterization, and preliminary classification were carried out using the various means previously alluded to, in whole or in part. Table 1 contains basic descriptive and reference information relevant to these isolates and their study here. This includes virus numerical designation and provisional name, and virus passage levels employed, as well as date, location, and source of the virus isolation, the isolating organization, and literature reference to the isolation.

The Simbu group of arboviruses belongs in the Bunyamwera supergroup, which also includes groups Bunyamwera, Bwamba, C, California, Capim, Koongol, and Guama, and several unassigned viruses (4, 24). Other members of group Simbu, in addition to the isolates studied here, include Akabane, Buttonwillow, Ingwavuma (SA An 4165), Manzanilla, Oropouche, Sathuperi (IG 10310), Simbu, and Utinga (Be An 84785) (24). Simbu virus originally described by Weinbren, et al., is the group prototype (20, 23). Serological experience with the group has shown that its members often lack easily prepared hemagglutinating activity, and that of the three common serological tests (HI, CF, and NT) CF is the most

inclusive in the relationships it demonstrates, HI and NT being considerably more exclusive. Medical and consequent economic importance of the group is still incompletely studied. Presently it appears that member viruses do not cause dangerous, permanently damaging, or fatal diseases in man. However, Oropouche virus is known to cause a very unpleasant, febrile illness in man, which may be of epidemic proportions, and consequently quite serious economically (21). Furthermore, Ib H 11003, one of the isolates studied here, was isolated from a febrile human child, although nothing more of its medical importance is yet known. The possible role of group Simbu viruses in diseases of livestock, where some of them appear ubiquitously, is also yet to be studied.

Table 1. The 8 Nigerian Simbu group Arbovirus Isolates: Basic Information

Virus			Isolation				
Numerical Designation	Provisional Name	Passage Levels Employed	Source	Location	Date	Organization*	Reference
Yaba 7	- - -	5,6	Mosquito pool (Mansonia africana)	Yaba, Lagos, Nigeria	3/63	1	(18)
Ib An 5077	Sango	4,5,6	market cattle blood	Dugbe slaughterhouse, Ibadan, Nigeria	4/26/65	2	(7)
Ib An 5550	Shamonda	2,3,4	market cattle blood	Ibadan, Nigeria	5/20/65	2	(7)
Ib An 9398	Sabo	2,6,7	Goat blood	slaughterhouse, Ibadan, Nigeria	4/18/66	2	(8)
Ib An 10107	Shuni	1,2,3	Cattle blood	slaughterhouse, Sokoto, Nigeria	5/12/66	2	(8)
Ib An 10598	- - -	3,4,5	Cattle blood	Ibadan, Nigeria	6/27/66	2	(8)
Ib An 10599	- - -	2,3,4	Cattle blood	Ibadan, Nigeria	6/27/66	2	(8)
Ib H 11003	- - -	3,4	febrile human female, 1 and 1/2 years old, heparinized blood	University of Ibadan College Hospital Clinic, Ibadan, Nigeria	7/27/66	2	(8)

* 1: West African Council for Medical Research
  2: University of Ibadan, Arbovirus Research Project

THE PROBLEM

The experiments hereafter described were intended to deal with two problems, essentially, one more academic, the other more practical.

The more academic efforts were meant to establish the serological distinctions and relationships among the eight group Simbu isolates previously mentioned. Techniques employed were CF, HI, and NT, using hyperimmune mouse ascitic fluid and antigens produced originally for these experiments, where possible. Such creation of an inter-isolate serological classification was intended to enhance and verify basic knowledge regarding these viruses.

The more practical attempts were those made to prepare hemagglutinating antigens for all eight isolates. The intention in this case was to enable performance of the rapid, simple HI test, useful for virus and antibody identification both diagnostically and in serological surveys.

MATERIALS AND METHODS

Virus, Antigen, and Antibody

Lyophilized virus isolates stored in the WHO reference bank at YARU were used to produce isolate-specific complement-fixing and hemagglutinating antigen and hyperimmune mouse ascitic fluids.

Antigens were prepared in essentially the manner described by Clarke and Casals in 1958 (10). Lyophilized virus (of passage level indicated in Table 1) was reconstituted with phosphate buffered bovine albumin (BAP), prepared to 0.75% concentration, pH approximately 7.2, by addition of phosphate buffered saline (PBS) to commercial 7.5% BAP on hand. Final virus concentration was 1% in BAP. Using a 26-gauge needle and glass syringe, 0.015 cc of this reconstituted virus was inoculated intracerebrally into seventy, one- to two-day old, suckling mice. These mice were observed daily until some were dead and the others moribund with signs of encephalitis (paralysis, convulsions; usually within one to three days post-inoculation). The moribund were then collected and frozen in polyethylene bags at -70°C until sucrose-acetone extraction of brain took place.

After thawing the frozen mice and removing their brains, using either a 13-gauge needle on a vacuum line with a bottle trap, or an 18-gauge needle and a 1 cc tuberculin syringe, sucrose-acetone extraction began. The total weight of the brains removed was determined on a triple-beam balance and a 20% suspension (by weight) made of them in 8.5% aqueous sucrose solution. This suspension was homogenized for one minute in a Lourdes blender. The homogenate thus prepared was then rapidly expressed from a 50 cc syringe through an 18-gauge needle into twenty volumes of cold acetone in a reagent bottle,

the mixture shaken vigorously and thoroughly, and the supernate decanted. Twenty more volumes of cold acetone were immediately added to the precipitate in aliquots of three to four volumes, with vigorous shaking between each aliquot. The precipitate was allowed to settle from this supernate for one hour at 4°C. The supernate was then carefully decanted and the precipitate vacuum-dried for one hour. Finally the dried precipitate, still in the reagent bottle, was rehydrated overnight at 4°C with a volume of normal saline equal to twice the total weight of extracted brains.

This sucrose-acetone extracted antigen was stored at 4°C from a few hours to several days before undergoing hemagglutination titration. Thereafter it was either kept frozen at -70°C or lyophilized in ampoules of 0.5 cc amounts, after centrifugation for 10 minutes at 10,000 rpm.

Hemagglutination titration was accomplished using a microtechnique similar to that described by Sever (16). Special equipment involved included reusable lucite plates containing ninety-six 6x6x10 mm wells in an 8x12 well pattern, pipettes calibrated to deliver reagents in 0.025 cc drops, and diluting loops calibrated to deliver 0.025 cc from well to well. Reagents other than antigen included bovine albumin diluent, and a suspension of goose erythrocytes in virus adjusting diluent (VAD). Bovine albumin diluent consisted of a pH 9.0, 0.4% solution of commercial bovine albumin in borate saline. The goose erythrocyte suspension was composed of goose erythrocytes suspended in phosphate buffered saline (VAD) of the pH required by the test. The suspension had an optical density of 0.75 at λ=490.

After centrifugation at 2000 rpm for ten minutes, each antigen was titrated at five different pH levels (5.8, 6.0, 6.2, 6.4, 6.6), in eight, serial, two-fold dilutions from 1:2 to 1:256. One row of eight wells was used for each pH. To each of the second through eighth wells, one drop of bovine albumin diluent was added, and to both the first and second wells, one drop of antigen.

The excess fluid in the second well was then transferred progressively through the succeeding wells, with thorough mixing in each, finally to be discarded after the eighth well. Thus the serial two-fold dilutions were arranged. One drop of bovine albumin diluent and two drops of goose erythrocyte suspension of the proper pH were then added to each of the eight wells, and thorough mixing insured in all wells by gentle tapping of the plate corners.

After a thirty to forty-five minute incubation at room temperature, all wells were scanned for the endpoint indicating the pH level developing the highest titer hemagglutinating activity. This endpoint was defined as the greatest dilution of antigen showing complete or partial hemagglutination.

Hyperimmune mouse ascitic fluids specific for each isolate were prepared. Such fluid as prepared here has been shown elsewhere (15,22) to possess antibody of equally as high titer and specificity as sera from the same animals. It is consequently considered hereafter as the equivalent of such sera.

The fluids used in this research, with one exception, were prepared as follows, sterile technique being observed to the point of inoculation of the adult mice. A virus-containing brain was removed by autopsy from the carcass of a frozen suckling mouse infected as previously described for antigen production. A 10% saline suspension of this infected brain was then made with mortar and pestle. The suspension was emulsified with Freund's complete adjuvant using a syringe, and 0.2 cc of this emulsion was then inoculated intraperitoneally into six week old (adult) male mice. This procedure was repeated seven and fourteen days later using the same adult male mice. At day fifteen they received intraperitoneally 0.2 cc of a 10% saline suspension of Sarcoma 180/TG ascitic fluid, Sarcoma 180/TG being a slow-growing tumor producing large amounts of ascitic fluid. At day twenty-one they once more received the infected brain emulsion.

Generally by day twenty-eight, two weeks after the sarcoma injection, ascites had developed. Paracentesis was then performed on each mouse, with removal of as much hyperimmune ascitic fluid as possible. Such paracentesis was repeated as fluid reaccumulated, until death of the mice. The ascitic fluid collected was centrifuged at 10,000 rpm for five minutes, the pellet discarded, and the supernatant fluid stored at -20°C until use.

Immune status of the ascitic fluids thus produced was determined by homologous CF testing in all cases and by homologous NT and HI tests as further warranted. Those fluids yielding a homologous titer of at least 1:32 by CF, and 1:10 by HI (as far as determinable), and a $\log_{10}$ neutralization index of greater than 1.6 by NT were used in further heterologous experiments. There was fluid specific for one isolate which did not meet these requirements, that for Ib An 9398. Consequently ascitic fluid hyperimmune to this isolate was withdrawn from the WHO Reference Laboratory stocks for further use here. Although it was prepared in a manner similar to that described above, the schedule followed was different, making it the previously mentioned exception to this description.

Serological Tests

Complement-fixation testing as modified from the method of Casals (3) was used. Prior to each actual test, a titration of complement (C') was routinely performed. Commercial lyophilized guinea pig serum (C') rehydrated with its accompanying diluent was diluted 1:45 using pH 7.4 veronal buffer diluent, the basic diluent of the CF test. A suspension of sensitized sheep cells (SSC) was also prepared by diluting a commercial sheep erythrocyte suspension to 4% in veronal diluent, mixing this suspension with an equal volume of 1:800 hemolysin in veronal diluent solution, and incubating for fifteen minutes in

a 37°C water bath.

The 1:45 C' was serially diluted with veronal diluent to concentrations between 5% and 40% in nine 100x13 mm test tubes. A 0.2 ml aliquot of each dilution was mixed with 0.1 ml aliquots of veronal diluent and SSC in separate test tubes and these incubated, after shaking, for fifteen minutes in a 37°C water bath. They were then reshaken, incubated another fifteen minutes, settled for one hour at 4°C and the results read. The tube containing the least complement (most dilute solution) and still showing complete hemolysis was defined as containing one unit of C'. Since two units of C'/0.1 ml C' solution were requisite for the actual CF test, appropriate adjustment of the 1:45 C' could be made with the veronal diluent, although in actual practice this was never necessary.

A microtiter technique conservative of reagents was used in the actual CF tests, employing disposable plastic plates similar to the lucite plates described in the HA test, and the same reagent droppers as noted there. After thawing or reconstitution of antigens and ascitic fluids, these were appropriately diluted, usually in serial, two-fold fashion, with veronal diluent. The ascitic fluids were incubated twenty minutes in a 60°C water bath while in a 1:4 dilution, their final dilutions being prepared thereafter. To each plate well one drop of the appropriate ascitic fluid was added, then one drop of the C' solution, and one drop of the appropriate antigen. Simultaneous C', uninfected suckling mouse brain antigen, specific ascitic fluid, and specific antigen controls were also included, as well as a repeat, micro-method C' titration. The plates were covered and stored overnight at 4°C.

The following morning the plates were warmed for fifteen minutes in a 40°C oven and one drop of freshly prepared SSC added to each well, one plate at a time. Each plate was gently shaken, and incubated thirty minutes in the 40°C oven, being reshaken every ten minutes. Finally the plates were recovered and the well mixtures

allowed to settle for several hours at 4°C. Reading of the results was then made on a 0 (complete hemolysis) to 4+ (no hemolysis) scale. The endpoint was defined as the last well (most dilute, in terms of ascitic fluid) having a 3+ to 4+ reading. This was recorded as the reciprocal of the ascitic fluid dilution.

Hemagglutination-inhibition testing modified from that described by Clarke and Casals (10) and using a microtechnique similar to that of Sever (16) as previously described for hemagglutination testing, was carried out in a constant antigen, varying antibody system. Only antigen showing at least four units of hemagglutination at a 1:4 dilution (at least one HA unit at 1:16) was included. Ascitic fluid as used in both CF and NT testing was also employed here, after acetone extraction carried out as follows.

Thawed ascitic fluid was diluted 1:10 in a 2% aqueous solution of commercial bovine albumin. This was rapidly expressed from a syringe with small bore needle into twelve volumes of chilled acetone, and the suspension immediately shaken thoroughly. After swift centrifugation to 2,000 rpm, the supernate was discarded and twelve more volumes of cold acetone added to the precipitate, followed again by immediate, thorough shaking. This suspension, after standing five minutes in an ice bath, was centrifuged swiftly to 10,000 rpm, the supernate discarded, and the precipitate dried in a vacuum for one hour. The dried precipitate was then rehydrated overnight at 4°C with volume of borate saline buffer, pH 9.0, equal to that of the original 1:10 dilution of ascitic fluid in bovine albumin. Acetone extracted ascitic fluid not immediately used was stored in 1 ml aliquots at -70°C and thawed only once before use and/or discarding.

A second technique of acetone extracting ascitic fluid was also attempted, but was found to be unsatisfactory and discontinued, never being used in the experiments reported here. It differed from that described above only in

preparation of the 1:10 dilution of ascitic fluid, which was made with saline, not bovine albumin solution.

For HI tests, ascitic fluid was diluted from 1:10 to 1:320 in serial, two-fold steps, with 0.4% bovine albumin in borate saline diluent, pH 9.0 (previously described). Antigen, on the basis of previous HA titrations repeated immediately prior to HI testing, was diluted with the same diluent to contain 4 HA units.

One drop of the appropriate ascitic fluid in appropriate dilution was then added to each well in the test, followed by one drop of the antigen. The plates were incubated overnight at room temperature.

The following morning two drops of goose erythrocytes in VAD suspension of the optimum pH (determined in the previous HA titrations) were added to each well, the mixtures shaken by tapping the plate corners gently, and reading made of the results after further incubation for thirty to forty-five minutes at room temperature. The endpoint taken was that well containing the most dilute ascitic fluid which showed no hemagglutination (i.e., complete hemagglutination inhibition), and its dilution of ascitic fluid was subsequently recorded.

Two control systems were included in each HI test. A hemagglutination titration of the antigens involved was prepared, allowed to sit overnight, and read after addition of the goose erythrocyte suspension the next morning. Also, one well for each ascitic fluid used was prepared by adding one drop of the ascitic fluid in its most concentrated form to one drop of the 0.4%, pH 9.0 bovine albumin diluent. After likewise sitting overnight, two drops of pH 6.0 goose erythrocyte suspension were added to each well and the results read. Thus variations in antigen titer and appearance of non-specific hemagglutination were controlled.

Neutralization test technique was a varying virus, constant serum procedure,

carried out in one- to three-day old Swiss mice, Charles River stock (12).

Virus and ascitic fluid stocks and 0.75% phosphate buffered bovine albumin (BAP) prepared for these experiments or withdrawn from the WHO Reference Bank as previously described were used.

Lyophilized virus was first rehydrated with BAP to a 1:50 dilution which was then further diluted serially in ten-fold steps from 1:50 to the level appropriate to the particular test. Aliquots of these dilutions were then added to equal volumes of the various ascitic fluids included in the test, one of which was "normal" mouse ascitic fluid (i.e., ascitic fluid prepared from unimmunized mice). These serum-virus mixtures were then incubated in a 37°C water bath for one hour. In all titrations except that denoted "Test 3", undiluted ascitic fluid was used. In Test 3, all ascitic fluids were diluted 1:4 with BAP before preparing the inoculum.

Each dilution was then inoculated intracerebrally (0.015 cc/mouse) into a separate group of eight of the suckling mice. Prior to the inoculation these mice had been taken from their original litters, randomized, and returned in groups of eight to the mother mice. The groups were checked daily for signs of encephalitic morbidity and for mortality, these observations recorded, and the dead discarded. At the end of fourteen days all mice remaining alive were discarded. All deaths occurring within twenty-four hours after inoculation were considered artefactual and discounted.

Results of neutralization tests are reported here as $\log_{10}$ neutralization index, calculated as described in Methods for Medical Laboratory Technicians (12) and based on values of LD_{50}, the dose of virus needed to kill 50% of the mice, as determined by the method of Reed and Muench (13). The neutralization index measures capacity of an immune ascitic fluid to protect the host system from effects of the virus inoculated into it. This measure compares LD_{50} of the

virus incubated with normal ascitic fluid and LD_{50} of the virus incubated with the immune ascitic fluid. The difference between these two values is the $\log_{10}$ neutralization index of the immune ascitic fluid tested. The anti-log of this value is the neutralization index of the fluid.

Analytic Methods and Criteria of Serological Comparison of Isolates

For all three systems (CF, HI, and NT) the mathematical routine described by Springer (19) was adopted. Due to biological variation in the materials used--virus or antigen, and ascitic fluid--results of both viruses (antigens) and both ascitic fluids were necessary for comparison of any two isolates in any one system. In CF tests, conversion of the endpoints, using the ascitic fluid dilution values, to their rank in order of serial, two-fold dilutions (e.g., 1:4=1, 1:8=2, 1:16=3, etc.), allows these endpoints to be placed on a mathematical grid, as shown.

Antigens	Immune Ascitic Fluids	
	a	b
a	4	4
b	3	3

Since, mathematically, equal values may be added to or subtracted from both values in any one column or row without altering the essential quadrate relationship, variations in antigen sensitivity and antibody titer may be abnegated mathematically by serial performance of such procedures. When all values become equal, the antigens (and hence isolates) may be defined as equal, or indistinguishably similar by the test used. When only three of the four values may be made equal, then the antigens (isolates) may be defined as different from one another by the difference between the common value and the odd value. This difference is stated in "number of two-fold dilutions."

This same system of comparison is applicable to HI results, and to NT

results (substituting $\log_{10}$ neutralization indices for two-fold dilution values). Formal application of this system was actually necessary only with the wealth of data obtained from CF tests. The results of HI tests were so limited that inter-isolate comparisons were made within this system merely by scanning the result table and performing the comparison in the mind. This was also possible with the straightforward NT results, once the differences between homologous and heterologous $\log_{10}$ neutralization indices had been calculated and displayed in Table 7. The values stated there were arrived at by subtraction of $\log_{10}$ neutralization index of heterologous ascitic fluid from $\log_{10}$ neutralization index of homologous ascitic fluid, as determined in the individual test (1, 2, or 3) concerned. Homologous and heterologous values of different tests were not used interchangeably in these calculations.

The criteria for clustering isolate viruses in the inter-isolate serological classification here proposed are not primarily definite, absolute, and requisite of complete similarities, but regard more importantly pattern of evident relationships. Each cluster proposed contains isolates closely related in the system used, and each isolate of a cluster is more closely related to all other isolates in that cluster than to any isolate outside it, in another cluster. However, certain minimal indications of close relationship are considered in each serological system, according to the relative inclusiveness of the system: CF, $\leq$2 two-fold dilutions difference; HI, $\leq$1 two-fold dilution difference; and NT, difference between $\log_{10}$ neutralization indices of homologous and heterologous ascitic fluids $\leq$1.0, reciprocally.

Additional Procedures and Criteria in Preparation of Adequately Active Hemagglutinating Antigens

Several additional procedures were performed on sucrose-acetone extracted

antigens in attempts to elicit adequate hemagglutinating activity. Adequate activity was taken to be complete or partial hemagglutination at an antigen dilution ≥1:16. Such activity made HI testing possible. A successful preparation of antigen was one manifesting such activity in one or more pH ranges at any point in the procedures of preparation. Rare exceptions to these criteria are noted in the reporting of results. HA titrations were carried out on aliquots of antigen collected at various points in the procedure, as noted on Table 8.

At the completion of sucrose-acetone extraction (sa extr.), antigens were subjected successively to sonication (son.), trypsinization (tryp.), and resonication (reson.), in that order. Sonication is a method of disrupting particulate matter by subjecting it to high intensity, high frequency sonic energy. Ten to twenty cc of antigen were placed in a 16x160 mm glass test tube suspended in a cooling bath of either brine, or dry ice, water, and ethyl alcohol. The sonic energy horn attached to a Sonifier Cell Disruptor power source (Heat Systems Co., Melville L.I., N.Y.) was inserted into the antigen and sonication at approximately twenty watts carried out for a two minute period, this being followed by a one minute silence, allowing the antigen to cool. This pattern of sonication and silence was repeated for a total of three cycles in those antigens sonicated but not trypsinized or resonicated. In a few instances, hydrogen gas was bubbled through the antigen prior to sonication and left layered over it in bubbles during the cycles.

Trypsinization is a method of particle disruption depending on the lytic action of the enzyme trypsin on chemical bonds. After four cycles of sonication two minutes, silence two minutes, without hydrogen, the antigen was centrifuged for five to ten minutes at 2,000 rpm, and the pellet discarded. Commercial trypsin (Difco Laboratories) was prepared in a 512 γ/ml solution with borate saline, pH 9.0. After remaining at room temperature for five minutes, this solution was added to an equal volume of the sonicated, centrifuged antigen, yielding

a solution with the final trypsin concentration of 256 ɣ/ml. This remained at room temperature for fifty minutes. Then commercial soybean trypsin inhibitor (Worthington Biochemical Corporation) was added, 128 ɣ/ml of solution. This last solution was left for at least one hour at room temperature before HA titration. If not titrated immediately it was stored in 1.0 cc aliquots at -70°C and allowed to be thawed only once before titration and use or discarding.

If acceptable hemagglutinating activity did not emerge after trypsinization, the antigen was finally sonicated once more (resonicated), without hydrogen, for a one minute period, and re-evaluated.

Between performance of all the various procedures or tests, the antigens were stored either at 4°C or -70°C, in bulk or in small ampoules. The only controls included with these empirical attempts at development of HA activity were those inherent in simultaneous HA titration of variously treated aliquots of the same antigen.

RESULTS

Inter-isolate Serological Classification

CF Tests

Results of the homologous CF titration of hyperimmune mouse ascitic fluids appear in Table 2. All ascitic fluids titered $\geq$1:128 except Ib An 10107 and Ib An 9398, which titered 1:32.

Results of heterologous CF testing of the eight isolate antigens against their ascitic fluids appear in Table 3. One simultaneous test yielded all the results shown. A homologous titration was coincidentally repeated, being included as a control. Through dilution 1:1024 all antigens except Yaba 7 manifested 3+ to 4+ CF reactions with their most concentrated homologous ascitic fluid. Yaba 7 antigen manifested such a reaction only through dilution 1:256. It was assumed that maximum antibody titers were developed by antigen dilutions between 1:4 and 1:1024. Those ascitic fluids showing no evidence of diminution of antibody titer peak by 1:1024 antigen dilution were assumed to be at peak, and this value recorded.

A comparison of the eight isolates based on the information contained in Table 3 and employing the analytical methods described on page 14 is presented in Table 4. The apparent CF serological grouping of isolate viruses, according to the stated criteria, is also delineated in Table 4.

This classification by CF serology includes three groups of viruses in which all members are similar to within 1 two-fold ascitic fluid dilution of one another. There is one exception: the separation of Ib An 5077 and Ib An 10599 by 2 two-fold dilutions. However both of these viruses are still far more closely related to one another than to any isolates outside the group.

Table 2. Homologous CF Titration of Hyperimmune Mouse Ascitic Fluid Produced and/or Used for Serological Comparison of 8 Nigerian Simbu group Viruses

	Ascitic Fluid Hyperimmune to Virus							
Antigens[2]	Ib An 5077	Ib An 10598	Ib An 10599	Ib H 11003	Yaba 7	Ib An 5550	Ib An 10107	Ib An 9398[1]
Ib An 5077	≥128							
Ib An 10598		≥128						
Ib An 10599			≥128					
Ib H 11003				≥128				
Yaba 7					≥128			
Ib An 5550						≥128		
Ib An 10107							32	
Ib An 9398								32

[1]Mouse ascitic fluid from WHO Reference Laboratory stocks: Ib An 9398 (MG 48872, Source 48743)

[2]Antigens diluted 1:40

Table 3. Homologous and Heterologous CF Titration of 8 Nigerian Simbu group Viruses

Antigen	Antibody (Ascitic Fluid) Ib An 5077	Ib An 10598	Ib An 10599	Ib An 10107	Ib H 11003	Yaba 7	Ib An 9398	Ib An 5550
Ib An 5077	512	128	32	32	128	64	8	64
Ib An 10598	512	64	64	32	256	32	8	32
Ib An 10599	128	64	32	32	128	32	8	32
Ib An 10107	256	64	16	16	128	32	4	16
Ib H 11003	256	64	32	16	128	32	4	32
Yaba 7	32	<32 (64)*	<8 (<8)	<4 (8)	16	128	16	32
Ib An 9398	64	32	4	4	32	128	32	32
Ib An 5550	64	<32 (64)	4	8	16	32	8	128

*Values in parentheses represent titers obtained in repeat tests, and are not used in creating Table 4.

Table 4. Serological Comparison of 8 Nigerian Simbu group Viruses, CF Technique (Source of Data, Table 3)

Virus Isolate	Ib An 5077	Ib An 10598	Ib An 10599	Ib An 10107	Ib H 11003	Yaba 7	Ib An 9398	Ib An 5550
Ib An 5077	0	1*	2	0	1	5	5	4
Ib An 10598		0	1	1	1	>3	3	>3
Ib An 10599			0	0	0	>4	5	5
Ib An 10107				0	0	>4	5	4
Ib H 11003					0	5	5	5
Yaba 7						0	1	4
Ib An 9398							0	4
Ib An 5550								0

*Values shown are the number of two-fold dilutions difference between ascitic fluids.

HI Tests

Results of both homologous and heterologous HI titrations of those isolates tested are found in Table 5. Tests 1 and 2 were performed on separate days.

These results make plain the identity, or near identity of Ib An 10598 and Ib An 5077, at most only 1 two-fold ascitic fluid dilution distant from one another. Ib An 10107, however, is seen to be entirely separate from both, by HI serology, being 3 two-fold dilutions distant from Ib An 10598, and 2 from Ib An 5077. Since no homologous HI reaction took place between Ib An 10599 antigen and ascitic fluid, no positive control was present in comparison of this ascitic fluid heterologously with Ib An 10598 and Ib An 5077 antigen. Therefore, no absolutely definite statement is possible about the relationship of these two with the former. However, the other results present in Test 2 indicate that the three are probably quite similar.

Thus it appears that the virus isolates mentioned here, on the basis of HI serology and stated criteria, should be arranged in two mutually exclusive groups, one containing Ib An 10598 and Ib An 5077, and possibly Ib An 10599, the other containing Ib An 10107.

Table 5. HI Comparison of Simbu group Viruses from Nigeria

Antigen	Units[1]	pH	Antibody (Ascitic Fluid) Ib An 5077	Ib An 10598	Ib An 10107	Ib An 10599	Ib H 11003	Yaba 7	Ib An 9398	Ib An 5550
Test 1										
Ib An 5077	4	6.2	1:10	1:10	0[2]	0	0	0	0	0
Ib An 10598	4	6.0	1:20	1:20	0	0	0	0	0	0
Ib An 10107	4	6.0	0	0	1:10	0	0	0	0	0
Test 2										
Ib An 5077	4	6.2	1:20	1:10		0				
Ib An 10598	4	6.0	1:20	1:20		0				
Ib An 10599	8	6.0	1:20	1:20		0				

[1]Units of hemagglutination, as demonstrated by simultaneous HA control

[2]0=<1:10 dilution

NT Tests

Results of both homologous and heterologous NT titrations are displayed as $\log_{10}$ neutralization indices in Table 6 and the isolates compared by their use in Table 7. Revelation by Test 1 that isolates Ib An 5077 and Ib An 10599 were indistinguishably similar, within the limits of experimental variation, led to substitution of Ib An 5077 for both in all subsequent tests (Tests 2 and 3). In computing data for Table 7 from that of Table 6, $\log_{10}$ neutralization indices stated as having values greater than x were assumed to have the value x, on the basis of qualitative trends evident in the titrations. Lines of division shown on Table 7 delineate the apparent NT serological grouping of the isolates tested, in light of the stated criteria.

This classification by NT serology includes five groups. The first contains Ib An 5077 and Ib An 10599, indistinguishably similar viruses within limits of the test, having ascitic fluids with reciprocal neutralization capacities no more distant from each other than 0.2 $\log_{10}$ neutralization index. The second group contains two indistinguishable virus isolates, Ib An 10107 and Ib H 11003, whose ascitic fluids appear the same in reciprocal neutralizing capacities. The three remaining groups are made up of one individual virus isolate each, none apparently closely related to any of the other isolates tested here. These three groups are comprised of Yaba 7, Ib An 9398, and Ib An 5550, respectively.

Table 6. Homologous and Heterologous Neutralization Titration of 7 Nigerian Simbu group Viruses

Virus	Test Number	Ascitic Fluid Hyperimmune to Virus: Ib An 5077	Ib An 10599	Ib An 10107	Ib H 11003	Yaba 7	Ib An 9398	Ib An 5550
Ib An 5077	1	>3.8	3.8	0.6	0.3			0.1
	2	>4.3				0	0	
Ib An 10599	1	1.6	1.8					
Ib An 10107	1	0		3.2				
	3			>3.5	>3.5	0	0.2	0.4
Ib H 11003	1	0.1			>3.5			
	2				>4.0	0.4	0.4	0.2
	3			>3.5	>3.5			
Yaba 7	1					>3.5	0	0.1
	2	0		0	0	>3.6		
Ib An 9398	1					0.4	2.6	
	2	0		0	0		3.3	0.7
Ib An 5550	1	1.5				0		3.1
	2				0		0	3.8
	3			0.4				>3.4

Table 7. Serological Comparison of 7 Nigerian Simbu group Viruses by Neutralizing Capacities of Homologous and Heterologous Ascitic Fluids (Source of Data, Table 6)

Virus	Ascitic Fluid Hyperimmune to Virus						
	Ib An 5077	Ib An 10599	Ib An 10107	Ib H 11003	Yaba 7	Ib An 9398	Ib An 5550
Ib An 5077	0	0	3.2	3.5	4.3	4.3	3.7
Ib An 10599	0.2*	0					
Ib An 10107	3.2		0	0	3.5	3.3	3.1
Ib H 11003	3.4		0	0	3.6	3.6	3.8
Yaba 7	3.6		3.6	3.6	0	3.5	3.4
Ib An 9398	3.3		3.3	3.3	2.2	0	2.6
Ib An 5550	1.6		3.0	3.8	3.1	3.8	0

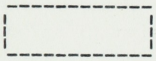
*Values stated represent the difference between $\log_{10}$ neutralization index of the indicated ascitic fluid and the homologous ascitic fluid of the virus concerned.

Serological Classification Summary

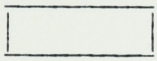
A summary of the proposed inter-isolate serological classification, based on the results of CF, HI, and NT tests presented is displayed in Graph 1, below.

Graph 1.

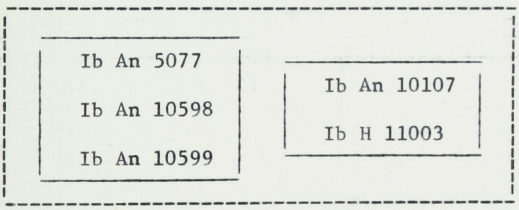
Serological Inter-isolate Classification

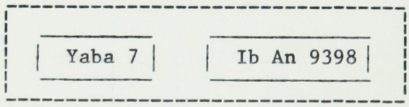


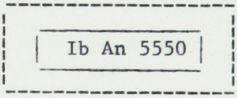
closely related or equal by CF



closely related or equal by NT and/or HI







Use of Sonication, Trypsinization, and Resonication for Preparation of Hemagglutinating Antigens

Results of the additional procedures used in attempts to produce adequate HA activity in sucrose-acetone extracted antigens of the eight isolates appear in Table 8. Using these data, with regard to the stated criteria, successful and unsuccessful preparations of HA antigen are listed in Table 9.

In two instances the criteria described were felt inadequate. Ib An 5550 antigen, sucrose-acetone extracted 8/14/67, only showed trace hemagglutination at dilution 1:256, pH 5.8, after extraction. This only improved to partial hemagglutination at the same dilution and pH and to trace hemagglutination at the same dilution in two other pH ranges after sonication. This zone phenomenon was felt to represent unusable HA activity because of its unstable pattern, and the antigen was consequently counted as an unsuccessful preparation. Yaba 7 antigen, sucrose-acetone extracted 8/17/67, was found to have an unstable pattern, also, at pH 6.4, with complete hemagglutination at a 1:4 dilution, but no hemagglutination at dilutions greater and less than 1:4. The pattern was even more unstable at pH 6.6, showing partial hemagglutination at dilutions 1:2, 1:128, and 1:256, with only trace hemagglutination in between. This pattern deteriorated after sonication. As a consequence, all of these results were counted as unsuccessful in an otherwise also unsuccessful preparation.

In reviewing the results on Tables 8 and 9 one may observe that adequate HA antigen was successfully prepared always for Ib An 5077, never for Ib An 9398, Ib H 11003, and Yaba 7, and occasionally for Ib An 10598, Ib An 10107, Ib An 10599, and Ib An 5550.

Table 8. Hemagglutinating Antigen Preparations of 8 Nigerian Simbu group Viruses

Virus	Date of SA Extr.	Date of Son. ($-H_2$)	($+H_2$)	Date of Tryp.	Date of Reson.	Date Tested	Reactive pH	Greatest Dilution Showing Hemagglutination After: SA Extr.	Son.	Tryp.	Reson.
Ib An 5077	8/10/67	8/11/67				8/11/67	6.4	1:16	1:16		
							6.6	1:8	1:8		
Ib An 5550	8/14/67	8/15/67				8/15/67	5.8		...1:256[1]		
Ib An 10598	8/16/67	8/17/67				8/17/67	6.2	1:16	1:16		
							6.4	1:4			
Yaba 7	8/17/67	8/18/67				8/18/67	6.2	1:4			
							6.4	1:4[1]			
							6.6	1:2... 1:128, 1:256[1]	1:4... 1:128[1]		
Ib An 9398	8/25/67	12/26/67	9/1/67			12/28/67	5.8		1:2		
Ib An 10107	8/28/67	12/26/67				12/28/67	5.8		1:8		
							6.0		1:16		
Ib An 10599	8/29/67		9/1/67			12/28/67	5.8		1:4		
							6.0		1:4		
							6.2	1:4			
Ib H 11003	8/30/67		9/1/67			12/28/67	5.8		1:4		
Yaba 7	12/24/67	12/26/67				12/28/67	5.8		1:4		
Ib An 5550	12/26/67	12/26/67				12/28/67	5.8		1:2		
							6.2	1:2			
							6.4	1:2			
							6.6	1:4			
Ib An 5077	6/11/68	6/14/68		6/14/68		6/14/68	5.8			1:4	
							6.0		1:32	1:4	
							6.2	1:16	1:64	1:4	
							6.4	1:8	1:32	1:2	
Ib An 10598	6/11/68	6/14/68		6/14/68	6/19/68	6/14,19/68[2]					
Ib An 10107	6/11/68	6/14/68		6/14/68	6/19/68	6/14,19/68	5.8		1:4		
Ib An 5550	6/13/68	6/14/68		6/14/68	6/19/68	6/14,19/68	5.8	1:2		1:4	1:16
Ib An 10599	6/13/68	6/14/68		6/14/68		6/14/68	5.8	1:4			
							6.0	1:4	1:32	1:8	
							6.2	1:4	1:16		
Ib An 9398	6/13/68	6/14/68		6/14/68	6/19/68	6/14,19/68	5.8	1:8			
Yaba 7	6/13/68	6/14/68		6/14/68	6/19/68	6/14,19/68	6.2	1:4			
Ib H 11003	6/13/68	6/14/68		6/14/68	6/19/68	6/14,19/68					

[1]See text.
[2]Second of double dates indicates date of HA testing after resonication.

Table 9. Successful and Unsuccessful Preparations of Hemagglutinating Antigen of 8 Nigerian Simbu group Viruses
(Listed by antigen isolate number and date of sucrose-acetone extraction; source of data, Table 8)

Successful		Unsuccessful	
Ib An 5077	8/10/67		
Ib An 5077	6/11/68		
Ib An 10598	8/16/67	Ib An 10598	6/11/68
Ib An 10107	8/28/67	Ib An 10107	6/11/68
Ib An 10599	6/13/68	Ib An 10599	8/29/67
Ib An 5550	6/13/68	Ib An 5550	8/14/67
		Ib An 5550	12/26/67
		Ib An 9398	8/25/67
		Ib An 9398	6/13/68
		Ib H 11003	8/30/67
		Ib H 11003	6/13/68
		Yaba 7	8/17/67
		Yaba 7	12/24/67
		Yaba 7	6/13/68

DISCUSSION

Occasional technical problems were encountered in the course of serological testing to determine the relationships of the various isolate viruses. These appeared to be due to expected biological variations and will be mentioned briefly.

Hyperimmune mouse ascitic fluids manifested different antibody titers in homologous CF testing due to variations in virus virulence, degree of antigenic stimulus present, and immunologic competance of the mice involved in their production. Minimal variations in biological materials and technique involved account for slight variations in results obtained occasionally in heterologous CF titrations (as recorded parenthetically in Table 3).

Lack of several hemagglutinating antigens prevented positive control of the corresponding ascitic fluids used in HI serology. Casals has noted that such uncontrolled ascitic fluids are useful in HI titrations (1), but this is obviously so only if positive reactions are obtained. Exclusively negative reactions could be consistent with a lack of even homologous inhibitory antibody in the fluids, as seen in the case of fluid for Ib An 10599, Test 2, Table 5. Consequently the uncontrolled sera of HI Test 1 were considered not interpretable (Table 5).

Serological classification of arboviruses is a rather nebulous subject presently. Terminology is evolving and is not always used in a standard way, especially below serological group level. Certainly set criteria are not available to define serological relationships, as well.

Casals, however, has reviewed these difficulties nicely, introducing a great deal more order to the situation (5). He notes the possibility of a totally unmanageable classification system, with individual viruses considered separate entities unless exactly identical. He also indicates a range of

alternative systems, and finally describes a quite practical system comprised of individual viruses clustered according to their close resemblance to a model, or prototype virus, each cluster defining a type of virus or virus "species".

The inter-isolate serological classification proposed for the eight viruses studied here is largely modeled after this last system. Criteria for the relationships demonstrated by each serological system were established to be mutually inclusive of all "species" members and exclusive of all members of other "species". Each member of a "species" cluster was more closely related to all the other members of its "species" than it was to any member of any other "species".

Within the CF system three "species" were determined in this manner. The HI system was found more exclusively definitive of "species", demonstrating at least two distinct types entirely within one of the clusters previously determined by CF serology. The NT system also seemed to define "species" more exclusively than the CF system, demonstrating at least five within the three the latter had shown. And again, as with the two HI clusters, none of these five clusters crossed CF-determined "species" boundaries.

Such variation in classification results reflect different serological system specificities, CF being the most inclusive, and HI and NT the most exclusive systems for group Simbu viruses. Such variation also presents the question of which system to use in defining serological "species" clusters. This ultimately seems to depend upon the situation requiring "species" classification, and consequently, upon the pragmatic decision of the investigator involved.

Ramifications of such pragmatism are manifold since the classification of arboviruses is important in several ways. Results of serological surveys and diagnoses are analyzed with regard to serological classification, and consequently it serves as basis for decisions made in identification, control,

and therapy of arboviral diseases, as well as epidemiological interpretation regarding ecology, geographical distribution, and evolution of these viruses.

Some epidemiological speculation seems germane at this point, since it has recently been reported by Causey, Kemp, and Causey that a herd of dairy cattle studied in Nigeria has progressively developed NT antibody to several of the group Simbu viruses studied here (6). That report noted that all animals over 52 months of age had developed neutralizing antibodies for Ib An 9398, Ib An 10107, Ib An 5550, and Ib An 5077. It is immediately evident that these represent all three CF "species", and four of the five NT/HI "species" determined in this investigation. However, Shope, et al., have hypothesized that Bunyamwera supergroup viruses have evolved from a single ancient predecessor and should vary antigenically in different geographical areas or in different cycles in the same ecosystem (17). With this hypothesis in mind it is curious that there is now evidence of several supergroup Bunyamwera, group Simbu viruses existing in individuals of the same cycle of one ecosystem in one geographical area. One might hypothesize from such a curiosity that the original home of an ancestral virus (of group Simbu, or groupings of higher or lower level) had been found by chance, in the vicinity of which one might expect more coexistent antigenic varieties. Or one might otherwise think that in the passage of time viruses evolved in different milieus from one ancestor might have returned, perhaps in cattle driven to market or in migratory birds to once more cohabit in the same milieu.

Such speculations as these would perforce bear in mind possible lack of significant correlation between in vitro serological reactions and in vivo immunological reactions. Repeated indication of past infection by two viruses of the same CF "species" (Ib An 10107 and Ib An 5077) in an individual animal seems serologically inappropriate, since it is reasonable to expect the infection by the first to provide immunologic intolerance to infection by the second.

However, indication of past infection by four different NT/HI "species" viruses in one animal appears quite appropriate serologically, since it is expected that these would produce no immunologic intolerance to one another. Thus there does appear to be a lack of correlation between *in vitro* CF testing and *in vivo* immunologic reactions. This must call into question the proper place of CF technique in arbovirus evaluation, such question rendering the technique valueless in considering the hypothesis of note here.

However, there appears no such lack of correlation or question regarding NT/HI determined virus "species". Consequently one might also postulate that perhaps the serological tests or criteria used here define "species" too exclusively for the particular purpose of establishing the evolutionary hypothesis presented. A more inclusive definition of species may be indicated, based on host range and other ecological considerations instead of serology, perhaps treating the serological clusters determined here as "sub-species" and "varieties". This seems especially possible since the serological information reported from dairy cattle may be evidence of past infection in incidental hosts, rather than in hosts of the ecosystem usually maintaining the viruses in the geographical area.

In the investigation immediately at hand, where merely a serological inter-isolate classification was intended, it was felt sufficient in the end to cluster the virus isolates studied into five NT/HI "species", contained within three CF "super-species", as illustrated in Graph 2.

Scrutiny of results of the procedures employed in producing adequately active HA antigens reveals that successful preparation correlated uniformly only with the individuality of the antigens themselves. Uniform correlation of successful preparations with other factors, such as variations in techniques or individual procedures was impossible.

Such observations are probably explicable on the basis of variations in titer and/or structure of the individual antigens, and the response of their structural components to the procedures. Some of the antigens, such as Ib An 5077, may have a great number of antigenically active hemagglutinating sites per unit of antigen, all relatively superficially accessible for serological interaction. Others, such as Ib An 9398, Ib H 11003, and Yaba 7, may be considerably less optimally endowed, having fewer sites or less accessible arrangements of them, or both. Physical disruption of such units by the high intensity sonic energy of sonication, or chemical disruption of the peptide bonds within such units by proteolytic enzyme trypsin could conceivably alter either the number or accessibility of such active antigenic sites. The original structure (accessibility, complexity) and number of antigenically active sites would moderate response to such procedures, however. Creation and augmentation or destruction and diminution of these sites would respectively increase or decrease the overall activity of the antigen. The results obtained with these procedures contain numerous examples in which such alterations may have occured.

Should this simplified explanation be essentially correct, it is easily conceivable that with further empirical experimentation, technical proficiency will be developed and other special procedures discovered which will improve and facilitate consistently successful preparation of hemagglutinating activity from all the antigens studied here.

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