South African Medical Journal : Suid-Afrikaanse Tydskrif vir Geneeskunde

Kaapstad, 14 November 1959 Deel 33 No. 46 Volume 33 Cape Town, 14 November 1959

AN AGENT RELATED TO UGANDA S VIRUS FROM MAN AND MOSQUITOES IN SOUTH AFRICA ‡

K. C. SMITHBURN,* B.Sc., M.D.; H. E. PATERSON†, B.Sc. (HONS.) (RAND); C. S. HEYMANN†, M.B., CH.B. (CAPE TOWN), D.P.H. (RAND), D.T.M. & H. (R.C.P. & S. ENG.); and P. A. D. WINTER†, B.Sc., M.B., B.CH. (RAND), D.P.H., D.T.M. & H.

From the Arthropod-borne Virus Research Unit, P.O. Box 1038, *Johannesburg.*

The purpose of this communication is to report the isolation in the Union of South Africa of an agent closely related to Uganda S virus and to present evidence that this agent is pathogenic for man and is arthropod-borne.

After the Tongaland virus expedition of 1955[1] a permanent field station was established in the Ndumu Game Reserve in northern Natal to serve as a base for long-term virus investigations in the coastal lowlands. One aspect of the programme was to search for patients with virus disease in the hope of isolating the aetiologic agents from them. To accomplish this, clinics were held at the field station and at selected kraals within working distance of it, and all persons who presented themselves were attended medically, although only those with fevers were of special interest to our programme. Temperatures were first taken of all present and those having fevers in excess of 100°F by mouth or 101°F *per rectum* were marked with a piece of adhesive tape showing this temperature so that they could be specially examined. Such persons were carefully queried about their illness and examined physically to detect any obvious cause for the fever. If no obvious cause was found a blood specimen was taken and the donor finger-printed. The blood specimen was placed at once in an iced thermos flask and within a few hours the serum was separated and stored on solid CO_2 until it could be transported to the laboratory and thawed for inoculation. These procedures carried out during March 1956 resulted in the isolation of one of the strains discussed in this communication.

The strain of virus isolated from mosquitoes was encountered during the course of work intitiated in an attempt to discover the aetiologic agent of febrile illness which was occurring in the environs of Johannesburg early in 1958. Here again, attempts were made to isolate virus from patients, but these were unsuccessful; the virus strain encountered was obtained from mosquitoes caught in the study area.

Materials and Methods

The materials and methods used in these studies were essentially those which members of this team have used in investigations already reported.[2]

VIRUS ISOLATIONS

Isolation of strain H336. D.M., Native male aged 9 years, was seen at Maponde's Kraal about 1 mile from the Usutu River on 28 March 1956. He was found to have an oral temperature of 100°F. No cause for the temperature was found on physical examination. The child denied feeling ill, but it is noteworthy that the threshold for feeling ill among these Native people is very high indeed; minor ailments are so prevalent that they are commonly ignored and the individual regards himself as sick only if considerably incapacitated or suffering severely. A blood specimen was taken and the child finger-printed. The serum was separated later in the day, frozen and kept on CO_2 until 31 March 1956, when inoculations into infant and adult mice were made in the laboratories in Johannesburg. The infant mice sickened on day 5 and brain passage was successful, resulting in the establishment of an agent which was readily transmissible in either infant or adult mice. A re-inoculation of the original serum specimen into infant and adult mice again resulted in illness and death of the former. Adult mice inoculated with the original serum specimen always remained well, but it should be reiterated that passage material was readily and highly pathogenic in the adult mice. The agent was found to be easily filterable. The designation H336 applied to the strain is the serial number of the blood specimen from which it was isolated.

On 1 May 1956 D.M. was again bled in order to get a convalescent specimen for immunological tests. When the heat-inactivated acute-phase serum and the convalescent specimen were tested against the newly isolated virus, it was found that the convalescent serum neutralized the agent strongly. Finger prints made when the acute and convalescent specimens were taken were compared and pronounced identical.** Thus, the origin of the virus from the patient was proved by both the immunological response of the donor and by re-isolation from the acute-phase serum.

Isolation of virus strain AR1115. Field investigations in the Johannesburg area were undertaken early in 1958 to attempt the identification of agents responsible for pyrexias

* The Rockefeller Foundation.

† Poliomyelitis Research Foundation, South African Institute for Medical Research, Johannesburg.

‡ The studies and observations on which this paper is based were financed jointly by the South African Institute for Medical Research, the Poliomyelitis Research Foundation, the South African Council for Scientific and Industrial Research, and the Rockefeller Foundation, and were conducted with the collaboration of the Union Health Department.

** The willing collaboration of a member of the South African Police, Detective Head Constable P. F. Retief, Local Finger Print Office, Johannesburg, in making this comparison is gratefully acknowledged.

of unknown origin among people residing especially in the environs of Germiston. Attempts were made to isolate virus from the febrile patients and from mosquitoes caught in regions where some of these patients had presumably suffered exposure to mosquito bites. Three strains representing 2 different viral species were isolated from mosquitoes caught along Germiston Lake. Certain features of the catching area at Germiston Lake were closely paralleled in another region a few miles away at Olifantsvlei, and a mosquito catch was made here in the hope of isolating virus. The 34 *Culex* (*Neoculex*) *rubinotus* Theobald taken in the Olifantsvlei catch were designated AR1115 and were inoculated into infant and adult mice. After a 3-day incubation period newborn mice in both litters were either dead or sick. A suspension of the infected brain material was filterable through a Seitz pad and a serially transmissible agent was readily established. The infective agent in the original mosquito suspension caused illness in the adult mice inoculated, but only after a 5–10 day incubation period.

AR1115 virus strain was found to be readily filterable and easily transmissible and, in due course, it was found to be reciprocally, and approximately quantitatively, cross-reactive with strain H336, as will be seen in a later section of this report.

PATHOGENIC PROPERTIES

In mice the H336 isolate causes diffuse vesicular and pyknotic degeneration of nerve cells throughout the brain, most consistently and conspicuously in Ammon's horn, without any resultant leucocytic reaction. Perivascular infiltration in some instances is moderate, but in other instances does not occur. Inclusion bodies have not been found. Lesions have been found only in the central nervous system.

In mice the agent is pathogenic by whatever route it is introduced and the titres by intraperitoneal and intracerebral inoculation are approximately the same—about $10^{-8.5}$.

Ten guinea-pigs inoculated intracerebrally with 100,000 adult-mouse intracerebral LD_{50} showed no significant febrile or other clinical response during the 10 days immediately following inoculation, but showed a good antibody response.

A non-immune monkey, *Cercopithecus aethiops pygerythrus* F. Cuvier, number M805, was inoculated subcutaneously with 6,600,000 LD_{50} of H336 virus. During the 9 days following inoculation the monkey failed to develop elevated temperature. On 7 of the 9 days, tests for circulating virus were made and none was detected when undiluted serum was inoculated intracerebrally into adult mice. The monkey exhibited a good antibody response as a result of the inoculation.

IMMUNOLOGICAL RELATIONSHIPS

Shortly after the H336 strain was isolated it was tested in neutralization tests against antisera for a number of other viruses having comparable incubation periods. None of the sera tested gave significant neutralization. Subsequently all the monotypic antiviral sera on hand were tested against the H336 virus and neutralization of a low order was obtained with Wesselsbron, Zika, dengue 1 and yellow-fever antisera. In a subsequent test the dengue immune serum failed to cross-react, but confirmatory weak cross-reactions were obtained with the Zika, yellow-fever and Wesselsbron antisera. The sera which failed to show neutralization were the following: Semliki Forest, Bunyamwera, Bwamba, West Nile, Pongola, Rift Valley fever, Uganda S, Spondweni, Ilheus, dengue 2, Mengo (EMC), St. Louis, Simbu, Japanese B, Wyeomyia, California, Anopheles A, Anopheles B, Russian SS, louping ill, WEE, EEE, and VEE. The minor cross-reactions with Zika, yellow-fever and Wesselsbron antisera led us to suspect that H336 is a member of Casals' group B viruses. Support was given to this suspicion when it was found that an antigen prepared from the H336 virus was cross-reactive in complement-fixation and haemagglutination-inhibition tests with group B antisera. Similar observations were made in the laboratories of the Rockefeller Foundation in New York.[3] In those laboratories it was also found* that immune serum against Makonde virus,[4] which was found to be a strain of Uganda S virus,[5] gave strong neutralization of the H336 isolate and that an immune serum from Rhesus monkey M4900 immunized with Uganda S virus likewise gave significant neutralization. Tests done in Johannesburg with Makonde antisera, provided through the courtesy of Dr. A. J. Haddow and Dr. M. P. Weinbren, of Entebbe, confirmed that these antisera had strong neutralizing capacity for H336. However, repeated tests with various ampoules of lyophilized serum from the New York laboratories monkey number M4900 failed in our hands to show significant neutralizing capacity.

An apparent relationship having been demonstrated both in the New York and Johannesburg laboratories between H336 and Makonde strains, guinea-pig immune sera were prepared for a special study of the relationships of these agents. Groups of approximately 10 normal guinea-pigs weighing 300–400 g. were bled and the pre-inoculation serum of those in any one group were pooled. The separate groups were then inoculated with either Uganda S or H336 viruses in relatively small dosage. After an appropriate period the animals were bled and cross-neutralization tests were done with the antisera. Guinea-pigs of groups 17 (Uganda S) and 19 (H336) were exsanguinated at the only post-inoculation bleeding. Animals of groups 54 (H336) and 56 (Uganda S) were, however, each bled on 2 separate occasions and the quantitative differences in antibody content of the sera were determined. Included in the tests were pre- and post-inoculation sera of monkey M805 and rehydrated serum of M4900 immunized in New York with Uganda S virus. Two tests were done with these reagents. In the first, the sera were tested in the usual way without dilution or addition of any accessory substances. In the second test, 0·1 ml. quantities of fresh normal monkey serum were added to equal quantities of test sera, and to these mixtures 0·2 ml. quantities of virus were added. In both tests the mixtures were incubated for 1 hour at 37°C and tested by intracerebral inoculation into young adult mice. Results of the tests are shown in Table I, from which it is clear that there is a close relationship between Uganda S and H336 viruses. There is also evidence that the two are not identical, in that M4900 serum, although it neutralized the homologous virus did not neutralize H336 virus, and in the fact that some of the cross-reactions are not quantitative. As example of the latter it may be seen that, in the tests made without added serum, the two Uganda S guinea-pig antiserum pools neutralized 50 times as much of the homologous virus as of H336.

A vaccination-and-challenge experiment was done to study the relationship of H336 and Uganda S viruses. Uganda S virus is much less pathogenic by peripheral inoculation

* Dr. Max Theiler, personal communication.

TABLE I. RESULTS OF CROSS-NEUTRALIZATION TESTS WITH UGANDA S AND H336 VIRUSES AND THEIR RESPECTIVE ANTISERA

	H336 virus				*Uganda S virus*			
	Nothing added		*Fresh serum added*		*Nothing added*		*Fresh serum added*	
Serum	*Titre*	*Logs neut.*	*Titre*	*Logs neut.*	*Titre*	*Logs neut.*	*Titre*	*Logs neut.*
M 805 pre-inoc.	8·2		7·5					
M 805 post H 336	4·5*	3·7	5·15*	2·35				
M 228 normal					5·6		6·0	
M 4900 post Uganda S † ..			7·4	0·1			3·64	2·36
M 4900 post Uganda S ‡ ..			7·25	0·25			3·48	2·52
GP 17 pre-inoc.	7·5		8·12		6·0		5·36	
GP 17 post Uganda S ..	6·53	0·97	6·36	1·76	3·3	2·7	2·84	2·52
GP 56 pre-inoc.	8·0		7·6		5·33		5·46	
GP 56 post Uganda S 8 days	7·4	0·6	6·62	0·98	3·48	1·85	4·0	1·46
GP 56 post Uganda 30 days	6·4	1·6	5·25	2·35	2·0	3·33	3·33	2·13
GP 19 pre-inoc.	7·36		7·75		5·52		5·6	
GP 19 post H 336	5·14	2·22	4·64	3·11	4·12	1·4	3·4	2·2
GP 54 pre-inoc.	7·25		7·5		6·29		5·87	
GP 54 post H 336 8 days ..	5·5	1·75	6·0	1·5	3·75	2·54	3·4	2·47
GP 54 post H 336 29 days ..	4·5	2·75	4·0	3·5	3·4	2·89	2·5	3·37

* Tests done with different bleedings taken 38 days apart. † Added fresh serum was heat-inactivated.
‡ Added fresh serum was unheated.

than H336. The immunization of mice was attempted by the intraperitoneal injection of 0·5 ml. quantities of virus reconstituted to 0·5% from lyophilized Uganda S infected mouse brain stock. There was a good deal of mortality following the inoculation of the living virus. However, extra mice had been vaccinated and enough remained for the challenge inoculations. 21 days after the injection of living virus as vaccine, vaccinated mice and unvaccinated mice of the same age, which had been reserved for this purpose, were challenged by intracerebral inoculation of serial decimal dilutions of Uganda S or H336 virus. Results of the tests were as follows:

Titre Uganda S in normal mice	7·0
Titre Uganda S in vaccinated mice	4·58
Titre H336 in normal mice	8·0
Titre H336 in vaccinated mice	6·5

The immunity induced by the Uganda S vaccine was thus approximately ten-fold as effective against the homologous virus as against H336.

Another approach to the study of the relationship of Uganda S and H336 viruses was made by testing the same human survey sera against both agents. 280 sera were tested against the two viruses by identical methods. The results of these tests are shown in Table II, from which it may be seen that, although there is agreement in the results in 76% of cases, there was, nevertheless, disagreement in 10%. Critical analysis of the tests showing the discordant results indicates that this was not due to differences in virus dosage, for the 30 sera which were protective against H336 virus and negative against Uganda S virus, were actually tested against virus dosages averaging 421 and 212 LD_{50} respectively.

TABLE II. RESULTS OF PROTECTION TESTS WITH H336 AND UGANDA S VIRUSES AGAINST THE SAME HUMAN SERA

Virus		*H336*			*Total*
		Protective	*Inconclusive*	*Negative*	
Uganda S	Protective	24	2	0	26
	Inconclusive	26	2	2	30
	Negative	30*	4	190	224
	Total	80	8	192	280

* Mean virus dosage: H336, 421 LD_{50}. Uganda S, 212 LD_{50}.

For comparison with the afore-mentioned results a study was also made to determine the correlation between results of routine protection tests with H336 and the mosquito strain AR1115. 120 human sera were tested against both these agents by identical methods, and in this instance there was complete agreement in 91·67% and disagreement in only one of the 120 sera tested, as shown in Table III. This result, together with HAI and cross-neutralization tests which showed quantitative neutralization of AR1115 virus by H336 antisera, led to the conclusion that the H336 and AR1115 strains are identical with each other, and related to but not identical with Uganda S virus. The latter relationship is, however, closer than the relationship of most group B viruses to each other and there is doubt whether the differences observed justify the application of a separate specific name for the South African isolates.

TABLE III. RESULTS OF PROTECTION TESTS WITH H336 AND AR1115 STRAINS AGAINST THE SAME HUMAN SERA

Virus strain		*H336*			*Total*
		Protective	*Inconclusive*	*Negative*	
AR 1115	Protective	29	1	0	30
	Inconclusive	6	1	1	8
	Negative	1	0	81	82
	Total	36	2	82	120

TRANSMISSION OF H336 STRAIN BY *Culex* (*Culex*) *univittatus* THEO.

This mosquito—already known from the work of Taylor *et al.*[6] to be a vector of West Nile virus—is one of the dominant mosquitoes in the coastal lowlands of Natal. It was decided to test its potential as a vector of certain agents isolated there. Female adults reared from eggs collected from wild-caught gravid females were used in the test. Since little was then known concerning the viraemia produced by H336 virus in any laboratory animal and because difficulty was often experienced in getting *Culex univittatus* to bite mice, the mosquitoes were exposed to infection by allowing them to take a meal consisting of infected mouse brain, defibrinated rabbit blood and sterile glucose solution. The infective feed was administered in a piece of glass tubing. The mosquito was first caught up in the tube, its exit being barred by a cotton pledget at the centre of the tube and another at one end. Virus was introduced onto the absorbent pledget in the centre of the tube from the open end opposite to that in which the mosquito was confined and soon soaked through to become available to the mosquito. The mosquito could be manipulated to a certain extent by moving the cotton pledgets. When each mosquito had engorged, it was released into a cage and then transferred for maintenance to a 3×1 inch glass vial containing at the bottom a wad of damp paper. The vial was closed by gauze held in place by adhesive tape and was stored in a humid atmosphere. The tubes in which the mosquitoes were exposed to the infective material were sterilized by boiling. An aliquot of the virus used to infect the mosquitoes was titrated and an endpoint of $10^{-8.5}$ was obtained. Fourteen *Culex univittatus* took the infective feed.

Attempts to transmit by bite were made by manually restraining baby mice over the gauze covering the tubes in which the individual mosquitoes were kept. Much difficulty was experienced in inducing the mosquitoes to feed, but visible blood was taken by separate arthropods on days 6, 7, 8, 9, 13 and 14. All these bitings apparently resulted in transmission, for the bitten mice sickened and those not sacrificed died. Specificity tests were done on the first 3 and these were all positive, showing conclusively that the cause of illness in the mice was H336 virus.

DISCUSSION

Regardless of the fact that the youth from whom the H336 strain was isolated disclaimed feeling ill, it is obvious that the agent obtained from his blood is pathogenic for man. Unfortunately the patient was seen only once during his illness and did not then exhibit any specific symptoms. It is probable that the illness he suffered was of very mild nature and it may be that this is a characteristic of infection with this agent. The fact that considerable numbers of human sera from residents of the Tonga lowlands[7] and various localities in Mozambique[8] exhibit neutralizing antibodies for H336 virus indicates that it not uncommonly attacks man.

The isolation of an apparently identical strain of virus from mosquitoes is tentative evidence of the fact that the agent is arthropod-borne. Conclusive proof of this fact is the successful transmission of the agent by *Culex univittatus* in laboratory experiments. Whether *Culex rubinotus*, from which the virus was isolated, is a vector is not proved by these results, but in view of the fact that *Culex univittatus*, a known vector of a related virus, can be shown capable of transmitting H336, it seems likely that a natural vector relationship exists.

SUMMARY

1. A virus was isolated from the blood of a Native child during an access of fever. Comparison of acute and convalescent sera from the virus donor showed the development of specific neutralizing antibodies during convalescence and indicated the aetiologic relationship of the virus to the febrile episode.

2. A strain of virus apparently identical with the aforementioned strain was isolated from *Culex rubinotus* mosquitoes caught at Olifantsvlei, near Johannesburg.

3. These two strains, apparently identical with each other, are closely related to but apparently not identical with Uganda S virus. The relationship is closer than that between most viruses of Casals' group B (to which the new agents belong) and for this reason a separate specific name is not to be applied at this stage.

4. The new agent has been successfully transmitted in the laboratory by the bite of *Culex univittatus* mosquitoes, and is therefore capable of being arthropod-borne.

REFERENCES

1. Smithburn, K. C. and de Meillon, B. (1957): S. Afr. J. Med. Sci., **22**, 41.
2. Kokernot, R. H., Smithburn, K. C., de Meillon, B. and Paterson, H. E. (1958): Amer. J. Trop. Med. Hyg., **7**, 579.
3. Casals, J. (1957): Trans. N.Y. Acad. Sci., **19** (II), 219.
4. Ross, R. W. (1956): J. Hyg., **54**, 177.
5. Dick, G. W. A. and Haddow, A. J. (1952): Trans. Roy. Soc. Trop. Med. Hyg., **46**, 600.
6. Taylor, R. M., Work, T. H., Hurlbut, H. W. and Rizk, F. (1956): Amer. J. Trop. Med. Hyg., **5**, 579.
7. Smithburn, K. C., Kokernot, R. H., Heymann, C. S., Weinbren, M. P. and Zentkowsky, D. (1959): S. Afr. Med. J., **33**, 555.
8. Kokernot, R. H., Smithburn, K. C, Gandara, A., McIntosh, B. M. and Heymann, C. S.: In preparation.