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Further Studies on Antigenic Relationships Among the Viruses of the Group B Tick-borne Complex

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This paper is a report on further studies of the antigenic relationships among viruses of the Group B tick-borne complex, carried out by the haemagglutination-inhibition (HI), complement-fixation, absorption-HI and diffusion-in-gel techniques. Earlier work has been extended to include Powassan and Negishi viruses.

Comparisons of a number of strains of tick-borne viruses have revealed inter-strain variation only in the case of Omsk haemorrhagic fever virus, where two subtypes are distinguishable.

This recognition of subtypes of Omsk virus has led the author to review the concept that Central European encephalitis and Russian spring-summer encephalitis viruses are distinct entities. On the basis of their close relationship, as well as new information on vectors and geographical range, she suggests that they be considered variant subtypes of one virus, designated Group B tick-borne encephalitis virus.

The tick-borne viruses under consideration here are those for which ticks are the known or presumed vectors in nature and which belong to Casals' antigenic Group B (Olitsky & Clarke, 1959). Within recent years several new members have been added to the tick-borne complex and much new information gained which has necessitated a change in point of view concerning their interrelationships. In the past it was considered feasible and convenient to treat all of the then known viruses of the complex as strains of one virus on the grounds of their antigenic similarities (Olitsky & Clarke, 1959). More recently, Clarke (1962) reported briefly on studies which showed that various members of the complex were clearly separable on the basis of antigenic structure. These studies were carried out by means of haemagglutination-inhibition (HI) and diffusion-in-gel (DiG) techniques using unabsorbed and virus-absorbed serum. On the basis of these studies 18 strains of viruses from various parts of the world were divided into six viral types, namely, louping-ill, Central European tick-borne encephalitis (CE), Omsk haemorrhagic fever, Kyasanur Forest disease, Far Eastern Russian spring-summer encephalitis (RSSE) and Langat. The present communication describes the methodology used in the past and present studies, extends the previous work to include additional virus strains and data, and proposes a modification of the previously suggested classification.

MATERIALS AND METHODS

Viruses

Table 1 lists all of the virus strains studied, together with their country of origin and the source and year of isolation, where this latter information was available. The first strain of each virus or subtype listed is underlined to indicate that it has been used in this work as the prototype strain. The decision as to choice of prototype was arbitrary, usually depending on early availability or desirable characteristics for antigen and serum production. In the case of the Far Eastern RSSE subtype, although most of our early work was done with the "Parker" strain, the Sophin strain was chosen because it is referred to frequently in the Russian literature and because its history is better documented and it has superior antigen-serum characteristics. The author wishes to express her appreciation of the generous assistance of virologists from various parts of the world in supplying the many strains studied.

Immune sera

In most instances hyperimmune mouse sera were used, although in a few instances a rabbit serum was

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TABLE 1
STRAINS OF TICK-BORNE VIRUSES STUDIED

Virus	Subtype	Strain	Country of origin	Source	Year isolated
Kyasanur Forest disease (KFD)		P 9605	India	Human blood	1957
		W 377	India	Monkey brain	1957
		G 11338	India	*Haemaphysalis spinigera*	1957
Langat		TP 21	Malaya	*Ixodes granulatus*	1956
		TP 61	Malaya	*Ixodes granulatus*	1959
		TP 64	Malaya	*Ixodes granulatus*	1959
Louping-ill (LI)		Moredun	Scotland	Sheep brain	1931?
		VRL 2821/58	Wales	Lamb brain	1952
		VRL 2822/58	Scotland	Lamb brain	1958
Negishi			Japan	Human cerebrospinal fluid	1948
Omsk haemorrhagic fever (Omsk)	I	Kubrin	USSR	Human blood	1947
		Balangul	USSR	*Dermacentor marginatus*	?
	II	Bogoluvovska	USSR	*Dermacentor marginatus*	?
		Guriev	USSR	Human blood	?
Powassan		Prototype	Canada	Human brain	1958
Tick-borne encephalitis (TBE)	Far Eastern (RSSE)	Sophin (Sophy East)	USSR (Far East)	Human brain	1937
		" Parker "	USSR (Far East)	Human brain?	1937?
		Spring N4 East	USSR (Far East)	Human brain	1937
		Khabarovsk-17	USSR (Far East)	Human brain	1957
		1322 *I. persulcatus* II	USSR (?Far East)	*Ixodes persulcatus*	1940?
		Ticks-Ch	USSR (West)	*Ixodes ricinus*	1949
		Riger	USSR (West)	Human blood	1954
		Leningrad No. 6	USSR (West)	*Ixodes ricinus*	1949
		Leningrad No. 8	USSR (West)	*Ixodes ricinus*	1949
	Central European (CE)	Stillerova	Czechoslovakia	Human brain	1950
		Bia M	Poland	Human blood	1954
		Biphasic meningo-encephalitis strain F	USSR	Human blood	1953
		Byelorussian strain 256	USSR	*Ixodes ricinus*	1940
		DKR 22	Poland	Rodent blood	1955
		Graz	Austria	Human CNS	1953
		Hypr	Czechoslovakia	Human blood	1953
		K 185	USSR	Goat's milk	1952
		K 191	USSR	Goat's milk	1952
		Kłodobok	Poland	*Ixodes ricinus*	1953
		Kumlinge A 52	Finland	*Ixodes ricinus*	1959
		Kumlinge A 59	Finland	*Ixodes ricinus*	1959
		Kumlinge A 105	Finland	*Ixodes ricinus*	1959
		Pozelueve	USSR	Human blood	1956
		Slovenia	Yugoslavia	Human blood	1953
		Sweden 20536	Sweden	Human blood	1958
		Sweden T	Sweden	*Ixodes ricinus*	1958
		VI	USSR	Human blood	1953
		Vienna 415BTR	Austria	*Ixodes ricinus*	1961
	?	Strain 119	USSR (Siberia)	Human blood	1952

employed. High antibody levels are required both for the HI absorption technique and for diffusion-in-gel. Since most of the viruses used in this study are highly pathogenic for the mouse when given by peripheral inoculation, the animals first received, at weekly intervals, two or three intraperitoneal injections of a vaccine prepared by inactivation of infective virus with beta-propiolactone.[1] A 10% suspension of infected suckling mouse brain in borate-buffered saline, pH p.3, was treated with beta-propiolactone at a final concentration of 0.05% for 18-24 hours at 4°C. It had previously been established that these conditions could be used to produce non-infective haemagglutinating (HA) and complement-fixing (CF) antigens with little or no loss in *in vitro* activity. (Limited experience with other arboviruses suggests that variation exists in the minimal concentration of beta-propiolactone required to inactivate infectivity. The concentration given here is that found useful for the tick-borne Group B viruses.) One week after the vaccine series the mice were given live virus intraperitoneally as a dilute (10^{-5}) suspension of infected suckling mouse brain, and a week later the same material at a 10^{-2} dilution. After being held for about one month, they were then given a large inoculum consisting of 0.5 ml of 10% infected suckling mouse brain and bled seven days later. To increase the yield of serum the mice were usually held for another month, after which the large booster and seventh day bleeding were repeated and the animals were exsanguinated.

Antibody absorption for HI testing

The method for antibody absorption used was the same as that described previously (Clarke, 1960). In brief, pellets obtained by high-speed centrifugation of different volumes of clarified suspensions of suckling mouse brain were homogenized with a standard volume of immune serum diluted 1 : 10. After overnight incubation in the cold, the mixtures were recentrifuged at high speed and the supernatant fluids, representing the absorbed sera, were tested for residual HI antibody content. In most of the work, special reliance was based on comparison of the curves obtained when the HI titres of aliquots of serum absorbed with increasing amounts of virus were plotted for (*a*) the antigen homologous to the serum and (*b*) the antigen homologous to the absorbing virus. HI titres are expressed as the reciprocal of the highest serum dilution giving complete inhibition of 4-8 HA units. A calculated value is used to express the amount of absorbing virus, and is given as the grams of infected mouse brain of the original source per 1 ml of undiluted serum.

Technique of the HI test

HI testing was carried out using the methods described by Clarke & Casals (1958) with modifications as noted. Sera were treated with kaolin after virus absorption, and since the sera were at a dilution of 1 : 10 an equal volume of 12.5% kaolin was used. One important technical point became apparent in connexion with the use of tick-borne virus antigens. It was found that the titres of antigens extracted with acetone or acetone-ether gradually increased after dilution in the standard bovalbumin-borate/saline diluent, pH 9.0. As a consequence, a preliminary titration made shortly after the dilution of an antigen gave a significantly lower titre than that realized after overnight incubation of the HI test. This resulted in too high a final antigen dosage in the test. Investigation of the phenomenon showed it to be dependent on both pH and temperature. Antigens that were never exposed to a pH above 7.0 showed no HA activity at all while maximal titres were obtained at pH 8.0-9.0. Titre increase occurred much more rapidly at room temperature than at 4°C. The explanation for this effect is not yet known, but it is now our practice to store all antigens at a 1 : 10 or 1 : 20 dilution in the standard pH 9 diluent for at least 24 hours in the cold before use; however, an antigen diluted at pH 9 and held for several hours at room temperature will probably be at maximal titre. Because of their stability in the pH 9 diluent it is convenient and economical to maintain the diluted antigen stocks in the cold for fairly long periods. They are stable for at least one month and working dilutions can be prepared from them at any time.

Finally, mention should be made of the effect of pH on HI titres as opposed to HA titres. This factor has only recently been appreciated and its recognition is due entirely to the observation by Casals (personal communication) that higher HI titres were realized with certain Group A viruses when the final test pH was increased. (The final test pH is that obtained when the goose erythrocytes are added. No change has been made in the pH of 9.0 at which the serum-antigen mixtures are incubated.) This effect has now been observed with Group B

[1] Betaprone, obtained from Testagar & Co., Inc., Detroit, Mich., USA.

viruses also and it must therefore be taken into account to avoid the creation of artificial titre differences. Since Group B viruses characteristically have rather broad pH ranges for haemagglutination, comparison of closely related or identical strains should be made at the same final pH even though this may not be the optimal haemagglutination pH for each antigen used.

Preparation of diffusion-in-gel antigens

Crude antigens. All of the early work, including that presented in the previous report (Clarke, 1962), was done with crude antigens. These were made by a modification of the original sucrose-acetone method used for preparing HA antigens (Clarke & Casals, 1958). The changes in the acetone-extraction steps were suggested by Shope (personal communication), who found that he could produce improved HA antigens with certain arboviruses by these alterations.

Infected suckling mouse brain was homogenized with four volumes of a chilled 8.5% aqueous solution of sucrose. The whole homogenate was added dropwise with brisk mechanical stirring to 20 volumes of chilled acetone in one or more 200-ml centrifuge bottles held in ice-baths. The mixtures were shaken vigorously and left to stand in ice-baths for 30 minutes with occasional shaking. They were then allowed to settle for 10 minutes and the milky supernatant fluid was aspirated off, care being taken to avoid the pink gummy material in the bottom of the centrifuge bottles. A volume of fresh acetone equal to that originally used was added to each bottle and the preparations were allowed to stand in ice-baths for 30 minutes with occasional shaking. The sediments were then readily reduced to a fine suspension by use of a thick glass rod or, preferably, a motor-driven plunger, after which the bottles were centrifuged for 5 minutes at 1800 r.p.m. in a refrigerated centrifuge. The supernatant fluid was aspirated off and the sediments from all bottles were pooled into one with fresh acetone. Centrifugation was carried out as before and, after removal of the supernatant acetone, the sediment was dried rapidly by connexion to an efficient vacuum pump. To the dry powder a volume of borate-buffered saline, pH 9.0, was added which was equal to 0.4 of the total volume of homogenate used, and the stoppered bottle was shaken repeatedly to facilitate even resuspension. After standing overnight at 2°-4°C, the antigen was ready for use *without centrifugation.* It was important to ensure a finely divided, even suspension. For prolonged storage, antigens were lyophilized and kept at 4°C.

Small and large antigens. As a more complete account of the preparation and study of the small and large antigens of the tick-borne viruses will be published separately, the present description will be short. The existence of more than one antigen differing in size was recognized early in our DiG studies from the presence of multiple precipitin lines and from variations in the curvatures (Korngold & Van Leeuwen, 1957) and in the rates of appearance of these lines.

Most of our experience in the preparation of small and large antigens has been with sucrose-acetone-extracted infected suckling mouse brain. Multiple antigens have also been obtained, however, from unextracted brain homogenates made in borate saline, pH 9.0. It has not yet been resolved whether significantly different results are produced by the two starting procedures. In either case the material was held overnight at 4°C and pH 9 at a concentration equivalent to 5% mouse brain. All subsequent steps were carried out in the cold. After clarification by centrifugation at 10 000 r.p.m., the small antigens were obtained as the upper 90% of the supernatant fluid following centrifugation at 40 000 r.p.m. for 90 minutes. This dilute fluid was then concentrated 50-100-fold by means of an LKB 6300 A ultrafilter.[1] The large antigens were obtained by homogenization of the 40 000 r.p.m. pellets in borate saline, pH 9.0. They were subjected to some degree of purification by clarification at 10 000 r.p.m., resedimentation at 40 000 r.p.m. and a final clarification at 10 000 r.p.m. The final product represented a 50-100-fold concentration over the original 5% starting material.

Diffusion-in-gel technique

A micromodification of the Ouchterlony double-diffusion method in two dimensions was used (Ouchterlony, 1958; Mansi, 1957). Ordinary 3×1-inch (76×26-mm) glass microscope slides were prepared by placing a strip of adhesive tape 1 inch (26 mm) from one end to serve as a retaining border, handle and label. To prevent seepage of fluid between the glass and agar surfaces, the 2-inch (51-mm) slide surface beyond the tape was precoated with a thin layer of 0.2% agar in water applied with a small sable paint brush, and allowed to dry in the air.

[1] Obtained from LKB-Produkter AB, P.O. Box 1220, Stockholm 12, Sweden, or LKB Instruments Inc., 4840 Rugby Avenue, Washington 14, D.C., USA.

Both Difco Noble agar and Oxoid Ionagar No. 2 [1] have been used, without significant differences in the results, but most of the recent work was done with the latter. The agar was dissolved in water to give a 2% concentration and mixed in the molten state with borate-buffered NaCl to give a final concentration of 1% agar in 0.05 M NaCl–0.05 M borate, pH 9.0; 0.01% final concentration of thiomersal was added as a preservative. The molten agar was pipetted on to the slide in a proportion of 1 ml of agar per square inch of slide surface. The agar was allowed to harden for two or more hours in a humidified chamber and then a central well and a series of peripheral wells, up to a maximum of six, were cut, using a lucite template and a fitted, hollow, stainless steel cutter. The agar was aspirated out through the cutter by insertion of a long blunt 13-gauge (2.40-mm) needle attached to suction. The wells were 4 mm in diameter and approximately 2.5 mm apart.

The wells were filled with the sera and antigens to be studied. We have found it convenient to use a 0.25-ml tuberculin syringe with a ¼-inch (6.4-mm) 27-gauge (0.4-mm) needle for this purpose. The pattern of wells described permits considerable versatility in the experimental approach. Reagents were titrated by placing serial twofold dilutions of sera peripherally and a given dilution of antigen centrally, or *vice versa*. When this was done we used only five peripheral wells in order to avoid reinforcement of the weakest dilution from the adjacent strongest dilution. In the early studies, different viruses or strains were compared by placing crude preparations of homologous and heterologous antigens alternately in the peripheral wells and the serum corresponding to one of the viruses in the centre well. A simple type of absorption procedure was also used and the results of several such studies were shown in the previous report (Clarke, 1962). A series of serum dilutions was prepared and aliquots mixed with either an equal volume of diluent or an equal volume of heterologous antigen. The mixtures were incubated overnight at 4°C and then used to fill the centre wells of a series of slides while alternate peripheral wells were charged with the antigens of the two viruses being compared.

More recently comparisons have been made using the large and small antigen preparations. An optimal dilution was chosen for each antigen on the basis of previous titration with the homologous serum. This antigen dilution was placed in the centre well of a slide and serial dilutions of homologous or heterologous sera were placed in the peripheral wells. For differentiating very closely related viruses, both control sera and sera that had been preincubated with heterologous antigen were used. Each serum dilution was preincubated with an equal volume of undiluted large or small antigen.

The reactions were allowed to proceed at room temperature in a humidified chamber. Precipitin lines with the small antigens were frequently clearly visible within a few hours, whereas the lines attributable to the large antigens required 24-48 hours to become apparent. Maximum development was not always achieved before the second or third day. It has been our practice recently to make final readings and permanent records on the third day. The permanent records were made by placing the slides in an adapter in a photographic enlarger and projecting the image on to maximum-contrast photographic paper. An enlargement of 2.5 times was found convenient.

RESULTS

Two additional viruses of the complex have been studied since the preceding report. These are Powassan and Negishi.

Powassan virus

McLean & Donahue (1959) isolated Powassan virus in 1958 from the brain of a child who died of encephalitis in Ontario, Canada. These investigators showed the virus to belong to Group B of the arboviruses and Casals (1960) subsequently found it to be a new member of the B group more closely related to the RSSE subtype of tick-borne encephalitis than to other viruses not belonging to the tick-borne complex.

In the present study, the relationship of Powassan to all the presently known members of the tick-borne subgroup was shown in two ways. Table 2 gives the HI titres of all sera against both Powassan and the homologous antigens and of Powassan serum against itself and all other antigens. These experiments revealed two points of interest: (1) Powassan was distinct from all other viruses; and (2) heterologous titres were rather uniform.

Table 3 shows the HI titres against various Group B antigens with Powassan serum before and after its absorption with West Nile virus. It can be seen that West Nile was capable of removing all of the crossing antibodies inhibiting its own antigen

[1] Obtained from Consolidated Laboratories Inc., Chicago Heights, Ill., USA.

TABLE 2
CROSS COMPARISON OF POWASSAN WITH THE OTHER VIRUSES OF THE TICK-BORNE COMPLEX

Serum	HI titres of heterologous sera with		HI titres of Powassan serum with heterologous antigens	
	Powassan antigen [a]	Homologous antigen [a]	Antigen [a]	HI titre [b]
KFD	320 [b]	5 120	KFD	320
Langat	160	2 560	Langat	160
Louping-ill	160	2 560	Louping-ill	80
Negishi	320	2 560	Negishi	80
Omsk	320	10 240	Omsk	80
TBE-RSSE	80	1 280	TBE-RSSE	80
TBE-CE	160	2 560	TBE-CE	160
			Powassan	5 120

[a] 4-8 units of antigen.
[b] Reciprocal of highest serum dilution inhibiting 4-8 antigen units.

and those of two other mosquito-borne Group B viruses but was not able to remove completely all crossing antibodies directed against the various tick-borne viruses.

TABLE 3
HI TITRES AGAINST VARIOUS GROUP B ANTIGENS OF POWASSAN SERUM BEFORE AND AFTER ABSORPTION WITH WEST NILE VIRUS [a]

Antigen	Powassan serum	
	Control	Absorbed [b]
Powassan	1 280	640
KFD	160	80
Langat	80	20
Louping-ill	80	20
Omsk	160	40
TBE-RSSE	160	40
TBE-CE	80	20
West Nile	320	0 [c]
Wesselsbron	160	0
Yellow fever	80	0

[a] Antigen units and serum titres as in Table 2.
[b] The amount of absorbing virus was 6 g brain/ml undiluted serum.
[c] No inhibition at dilution 1:20.

From these experiments we conclude that, on the basis of antigenic structure, Powassan is a legitimate member of the tick-borne complex but is quite distinct from all the rest. The rather uniform reciprocal crossing titres observed, together with the results of the West Nile absorption experiment, make it tempting to believe that Powassan is linked to the rest of the subgroup by means of a single antigen which is a relatively minor component of its own structure.

Negishi virus

In the summer of 1948, Dr K. Ando and his associates (1952) isolated Negishi virus from the cerebrospinal fluid of a fatal human case of encephalitis in Tokyo. Some years later, Okuno et al. (1961) showed the virus to be closely related antigenically to the Group B tick-borne complex although distinct from RSSE and Powassan.

In The Rockefeller Foundation Virus Laboratories, Negishi was first studied by Dr D. E. Carey,[1] who compared it with the other tick-borne viruses by means of the grid CF and standard HI techniques. Table 4 shows the most pertinent results of these studies. It has been the experience in our laboratories that, in the grid CF with Group B viruses, maximal antigen titres are frequently more revealing of antigenic differences than are serum titres. The data indicated that Negishi was distinctly different from most of the other viruses but was not clearly separable from louping-ill (LI) or the Central European (CE) subtype of tick-borne encephalitis virus. To resolve this problem the techniques of antibody-absorption-HI and diffusion-in-gel were used. Results of comparison by the former technique (Fig. 1) showed Negishi to be distinct from both LI and CE, although a significant difference was not revealed when Negishi serum was absorbed with LI. The appearance of plateaux of unabsorbed antibody was seen when LI serum was absorbed with Negishi virus and when Negishi serum was absorbed with CE virus. We consider such plateaux to indicate qualitative differences in that the virus homologous to the serum possesses one or more antigens absent from the absorbing virus. Curve deviation without plateau production is attributed to quantitative differences. The latter picture was seen when CE serum was absorbed with Negishi virus, although the use of larger quantities of absorbing virus might have revealed a plateau. The absence of either curve

[1] Present address: Christian Medical College Hospital, P.O. Box No. 3, Vellore, South India.

TABLE 4

COMPARISON OF NEGISHI WITH OTHER GROUP B TICK-BORNE VIRUSES

A. Cross-grid CF test

Antigen	Maximal antigen titre [a] with	
	Negishi serum	Homologous serum
Negishi	128	—
LI	64	64
TBE-CE	64	128
TBE-RSSE	32	128
KFD	4	128
Langat	4	32
Omsk	16	128
Powassan	64	256

B. Cross-HI test [b]

Serum	Serum titre with	
	Negishi antigen	Homologous antigen
Negishi	160	—
LI	320	640
TBE-CE	1 280	2 560
TBE-RSSE	40	320
KFD	320	1 280
Langat	160	1 280
Powassan	0	40

[a] Reciprocal of antigen dilution giving complete or almost complete fixation of 1.5-2.0 units of complement with the optimal serum dilution.

[b] Antigen units and serum titres as in Table 2.

deviation or a plateau when Negishi serum was absorbed with LI virus suggests that LI possesses all of the antigens of Negishi and in roughly similar amounts.

When crude antigens were used for DiG comparison, it was impossible to differentiate Negishi from either LI or CE virus. When large and small antigen preparations became available, however, distinction was possible using the large antigens. The reason for the failure of the crude antigens became apparent when it was found that the three small antigen preparations were completely cross-reactive. In general, the dominant antigens in crude preparations were the small components.

FIG. 1

ANTIGENIC COMPARISON OF NEGISHI WITH LOUPING-ILL AND TICK-BORNE ENCEPHALITIS, CENTRAL EUROPEAN SUBTYPE, BY THE ABSORPTION-HI TECHNIQUE

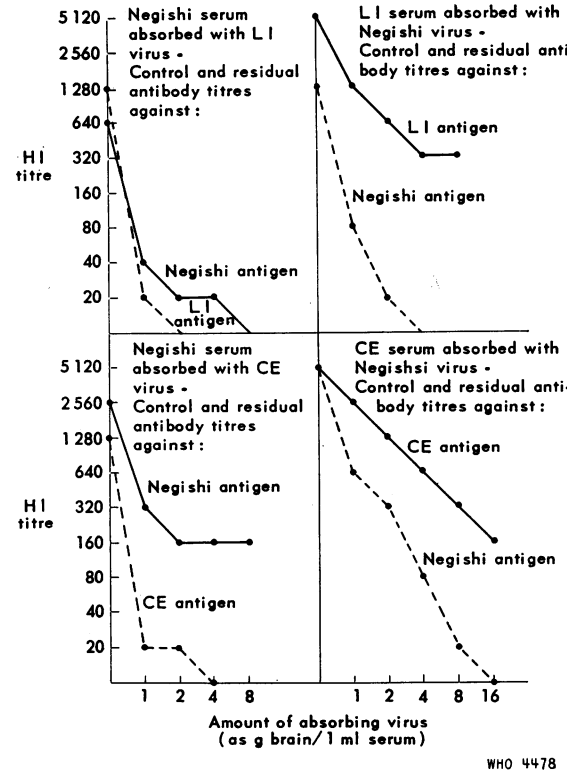


Plate 1 shows the results of comparison of Negishi with LI using large antigen preparations and both control sera and sera absorbed with heterologous large antigen. In Plate 2 the analogous comparison of Negishi with CE is shown. In both instances, differentiation was poor or impossible with the Negishi serum but clear-cut with either LI or CE serum.

Viral strain variation

In order to assess the significance of antigenic variation among closely related viruses it is important to determine the degree of uniformity existing among different strains of what are considered to be the same viruses.

Three strains each of Kyasanur Forest disease (KFD), Langat and louping-ill have been cross-compared by absorption-HI and by DiG with and

FIG. 2
ANTIGENIC COMPARISON OF TWO STRAINS OF LOUPING-ILL BY THE ABSORPTION-HI TECHNIQUE

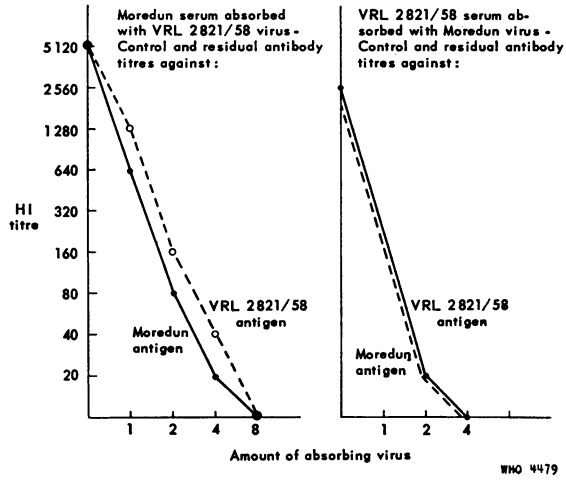
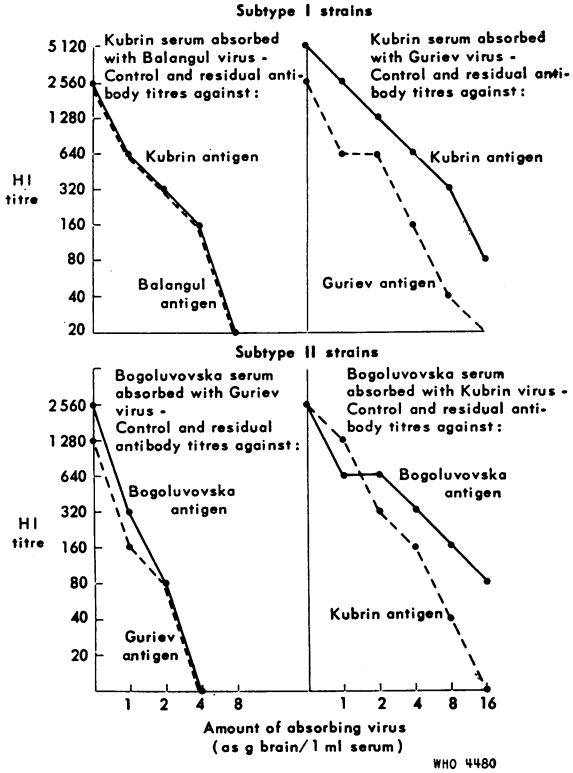


FIG. 3
ANTIGENIC COMPARISON OF DIFFERENT STRAINS OF OMSK HAEMORRHAGIC FEVER VIRUS BY THE ABSORPTION-HI TECHNIQUE



without absorption. The strains of LI were isolated over a period of approximately 27 years and the mouse-passage levels varied from an unknown, presumably very high number for the Moredun strain to third-passage levels for the two recent isolates. No significant strain variations in antigenic structure were found. The same statement can be made for nine strains of the RSSE subtype and 19 strains of the CE subtype of tick-borne encephalitis; the one exception, strain 119, will be considered later. Fig. 2 compares the curve of absorption-HI for the Moredun strain of LI with that of the 1952 isolate (VRL 2821/58). Plate 3 shows the results by DiG when the " Parker " strain of RSSE was compared with the 1957 isolate, Khabarovsk-17. The DiG test was carried out with unabsorbed and cross-absorbed sera and with crude antigens. The results are presented as examples of the many reactions of identity obtained by the two methods. Other examples were presented in the previous report (Clarke, 1962). In the case of absorption-HI, the reaction of identity consists of closely parallel or superimposed falling curves with increasing quantities of absorbing virus. Reactions of identity by DiG are symmetrical equal reactions with both antigens against unabsorbed sera and the simultaneous disappearance of both lines with cross-absorbed sera.

Quite a different situation was found with the virus of Omsk haemorrhagic fever. Four strains of the virus were studied. Strain differences were first suspected from DiG comparison using crude antigens, but they were so subtle as to be open to question. Cross-absorption-HI, however, clearly revealed the variants, as can be seen in Fig. 3. The four strains fell into two distinct subtypes, the two members of each subtype being indistinguishable. The subtype designated I (Table 1) consists of the Kubrin and Balangul strains. Strains Bogoluvovska and Guriev make up subtype II. It is of interest that in each subtype one strain was isolated from man and the other from ticks. With only four strains examined it is, of course, impossible to assess the extent of variation which may be characteristic of this virus.

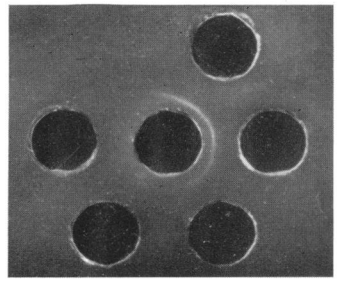
It has been a controversial question for some time whether to consider that tick-borne encephalitis is caused by one virus or by two different viruses, in the latter case an eastern (RSSE) and a western (CE) virus. In the previous paper, a position was taken in support of the concept of two distinct viruses on the basis of observed differences in anti-

PLATE 1

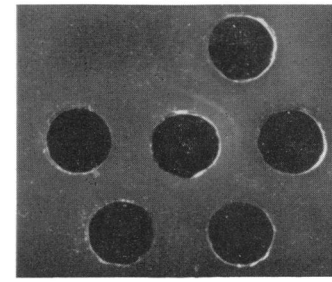
COMPARISON OF NEGISHI WITH LOUPING-ILL BY THE DIFFUSION-IN-GEL TECHNIQUE[a]

NEGISHI CONTROL SERUM

Negishi Large Antigen

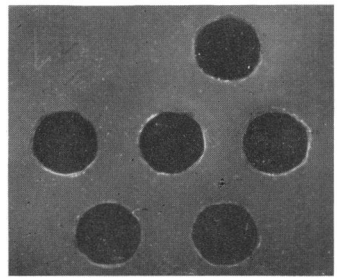


LI Large Antigen

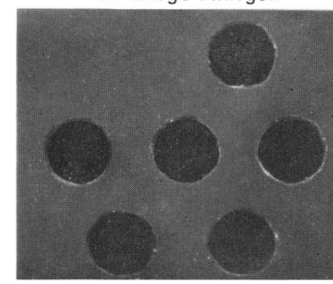


NEGISHI SERUM ABSORBED WITH LI

Negishi Large Antigen

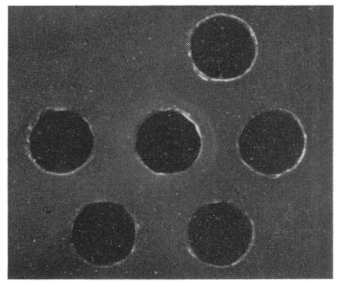


LI Large Antigen

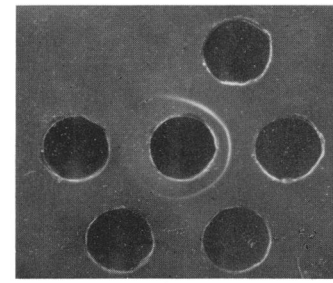


LI CONTROL SERUM

Negishi Large Antigen

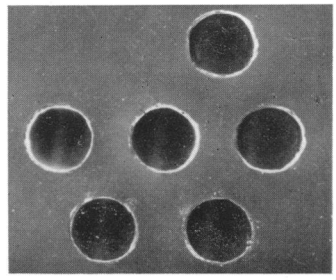


LI Large Antigen

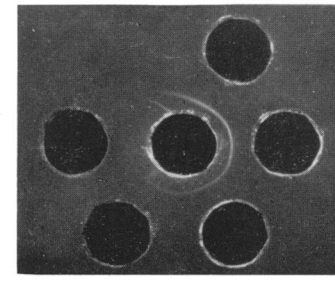


LI SERUM ABSORBED WITH NEGISHI

Negishi Large Antigen



LI Large Antigen



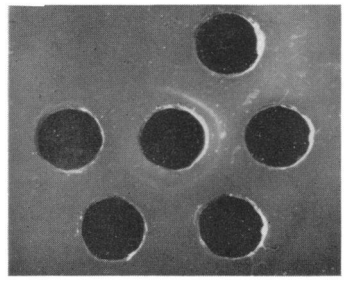
[a] Antigens in centre wells; serial dilutions of serum peripherally.

PLATE 2

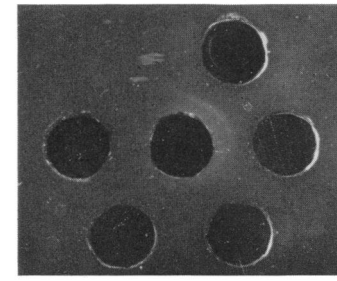
COMPARISON OF NEGISHI WITH TICK-BORNE ENCEPHALITIS, CENTRAL EUROPEAN SUBTYPE, BY THE DIFFUSION-IN-GEL TECHNIQUE [a]

NEGISHI CONTROL SERUM

Negishi Large Antigen

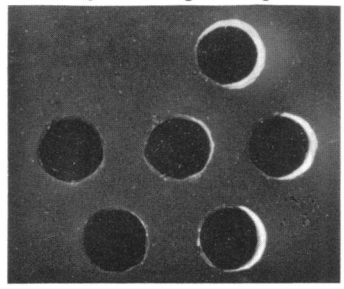


CE Large Antigen

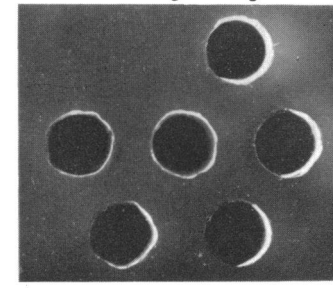


NEGISHI SERUM ABSORBED WITH CE

Negishi Large Antigen

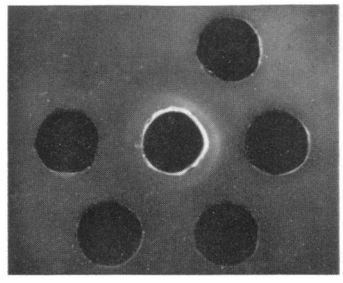


CE Large Antigen

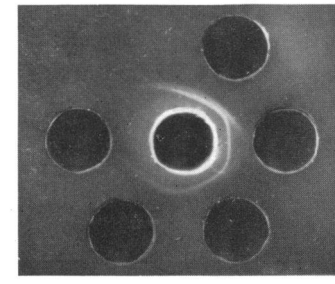


CE CONTROL SERUM

Negishi Large Antigen

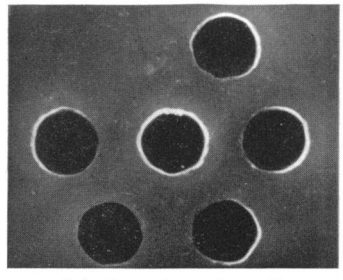


CE Large Antigen

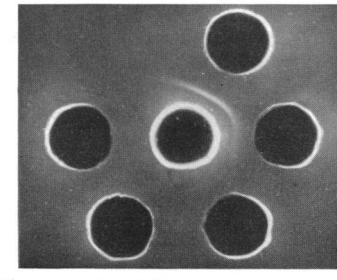


CE SERUM ABSORBED WITH NEGISHI

Negishi Large Antigen



CE Large Antigen

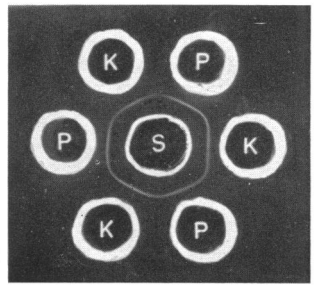


[a] Antigens in centre wells; serial dilutions of serum peripherally.

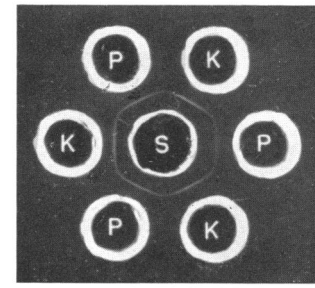
PLATE 3

ANTIGENIC COMPARISON OF TWO STRAINS OF TICK-BORNE ENCEPHALITIS, RSSE SUBTYPE, BY THE DIFFUSION-IN-GEL TECHNIQUE

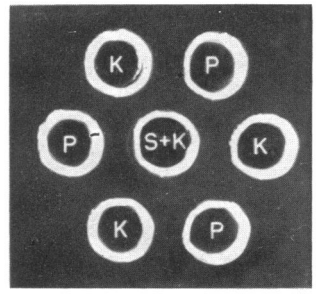
S = "Parker" serum alone
1:8



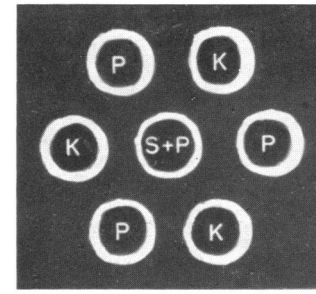
S = Khabarovsk - 17 serum alone
1:8



S + K = "Parker" serum +
Khabarovsk - 17 antigen



S + K = Khabarovsk - 17 serum +
"Parker" antigen



P = "Parker" antigen
K = Khabarovsk - 17 antigen

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PLATE 4A

LARGE AND SMALL ANTIGENS OF OMSK HAEMORRHAGIC FEVER VIRUS WITH MOUSE SERUM OR HUMAN CONVALESCENT SERUM IN THE DIFFUSION-IN-GEL TECHNIQUE [a]

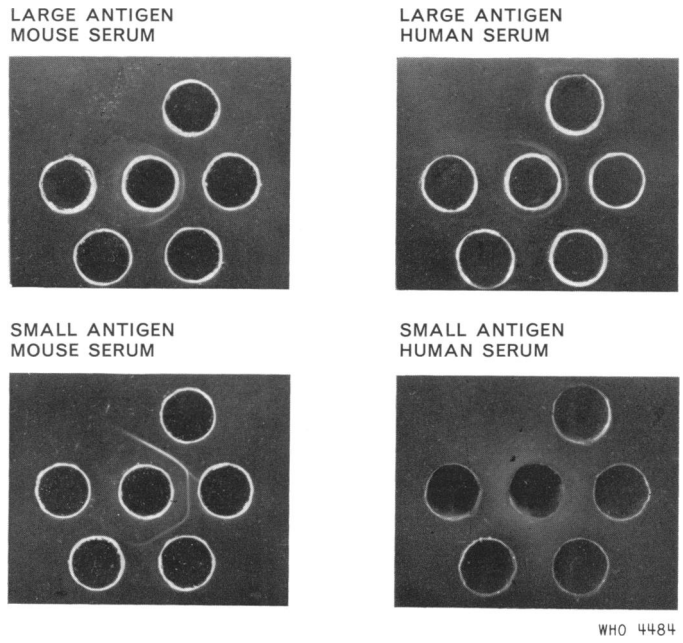
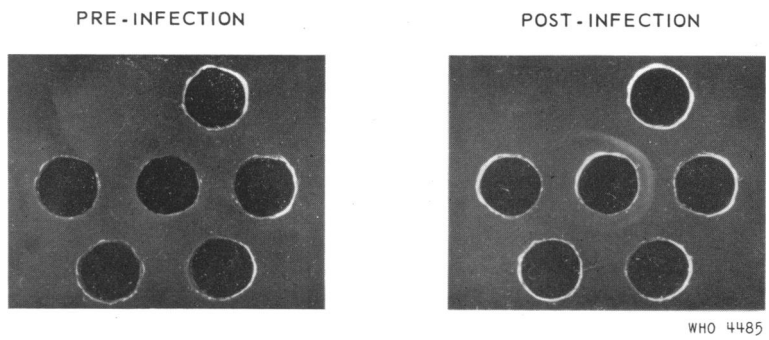


PLATE 4B

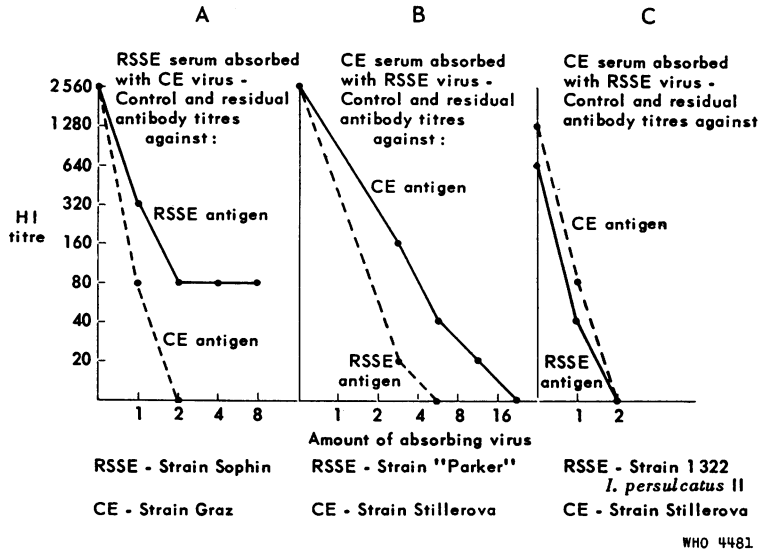
LARGE KFD ANTIGENS WITH PRE-INFECTION AND POST-INFECTION HUMAN SERA IN THE DIFFUSION-IN-GEL TECHNIQUE [a]



[a] Antigens in centre wells; serial dilutions of serum peripherally.

FIG. 4

ANTIGENIC COMPARISON OF RSSE AND CENTRAL EUROPEAN SUBTYPES OF TICK-BORNE ENCEPHALITIS BY THE ABSORPTION-HI TECHNIQUE

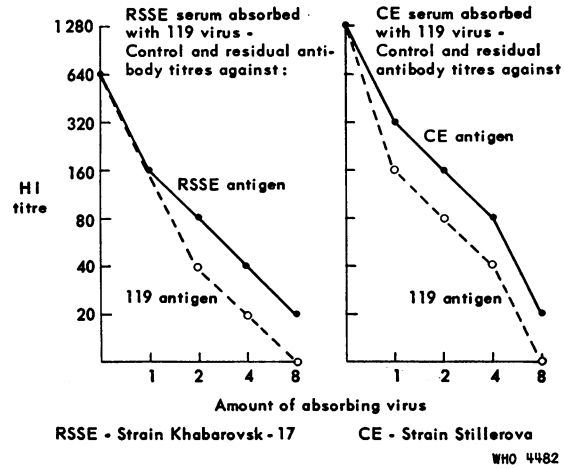


genic structure in conjunction with the then apparent difference in geographical distribution and in species of tick vector. At that time the RSSE virus was believed to be confined to the Far Eastern regions of the USSR and *Ixodes persulcatus* was considered to be the principal vector.

With the recognition of antigenically different strains of Omsk virus a re-evaluation of the previously stated position seemed indicated, especially since further work had disclosed that differentiation between RSSE and CE could present certain problems. Fig. 4 exemplifies various types of curves that have been obtained in comparing RSSE and CE strains. Fig. 4A is quite characteristic of the results obtained when RSSE sera were absorbed with CE viruses; when CE sera were absorbed with RSSE viruses, however, the results were as shown in either 4B or 4C. In all but one instance it was possible to make the identification by using RSSE sera, but the converse was not necessarily true, as is seen in Fig. 4C. Finally one virus, strain 119, was encountered which, both by DiG and by absorption-HI (Fig. 5), could not be differentiated from either type. From the data presented and much additional supporting information, it appears that RSSE strains possess one or more antigens absent from CE and are thus qualitatively distinct. CE strains, on the other hand, appear to contain no antigens lacking in RSSE but to be quantitatively stronger in one or more antigens than their RSSE counterparts. The distinct RSSE antigen (or antigens) is indicated by the plateau produced in Fig. 4A. The quantitatively different picture of the CE type, where curve diver-

FIG. 5

ANTIGENIC COMPARISON OF TICK-BORNE ENCEPHALITIS, STRAIN 119, WITH RSSE AND CENTRAL EUROPEAN SUBTYPES BY THE ABSORPTION-HI TECHNIQUE



gence was obtained but no plateau, is shown in Fig. 4B. With lower-titred sera (Fig. 4C) this divergence could not be seen. A more detailed antigenic description of strain 119 would require further studies.

It is now my desire to alter my previous position in favour of the designation of one viral type, Group B tick-borne encephalitis, with two subtypes, RSSE (eastern) and CE (western). The choice of RSSE and CE as identifying names is based in part on long usage in the case of RSSE and in part on a desire to avoid confusion with the Eastern (EEE) and Western (WEE) encephalitis Group A arboviruses. The change in point of view is due not to any errors in the previously recorded data but to a number of considerations: (1) the close antigenic relationship between the two subtypes, especially in one direction; (2) the demonstration of antigenic variations in Omsk strains; (3) the identification of isolates from *I. ricinus* ticks as RSSE subtypes (Table 1); (4) the identification of isolates from western USSR as RSSE subtypes (ticks-Ch, Riger, Leningrad No. 6, Leningrad No. 8); (5) the fact that although descriptions from the Far Eastern USSR indicate the disease there to be generally of a more severe nature, the clinical picture produced by the two types does not appear to be distinguishable in the individual case. It would thus appear to be more of a disservice than a service to all concerned to continue to treat such similar entities as different viruses despite the fact that they can be unequivocally differentiated. In this connexion the realistic and helpful concept of Casals (1961) should be kept in mind: " that a virus type or species is a cluster of different individualities grouped around and resembling a prototype or model, rather than a number of strains all identical with a prototype ".

Laboratory infections

Since the initiation of our studies on the tick-borne viruses, it has been our practice to immunize all of our personnel at risk with formolized RSSE vaccine (generously supplied by the Walter Reed Army Institute of Research). Vaccinees were tested for the development of neutralizing antibodies and only those showing a good response were allowed to work closely with the tick-borne viruses. Retesting was done annually and booster inoculations were given to any individuals who had lost their pre-existing neutralizing capacity. Despite these precautions, three rather severe infections occurred. From the blood of two cases the virus of KFD was isolated and from the third the virus of Omsk haemorrhagic fever. In addition to these three overt infections, two inapparent infections were diagnosed retrospectively by serological study. Owing to antigenic overlap it was not possible to determine which tick-borne virus caused the latter two infections but it was most probably KFD.

In the case of the Omsk infection, an acute-phase serum was shown to be fully protective against 160 LD_{50} of RSSE virus in an intracerebral neutralization test using weanling mice. The same serum was devoid of neutralizing activity for the Omsk virus, as might be anticipated. A similar study with acute-phase sera was not done for the KFD infections but the individuals involved had been shown to possess RSSE neutralizing antibodies within a few months prior to their illnesses. Following these infections, either overt or inapparent, antibodies capable of neutralizing Omsk, KFD and RSSE viruses have been shown to persist for several years at least. From these experiences it is obvious that the degree of antigenic overlap among some members of the complex is not sufficiently extensive for a killed vaccine, effective against one, necessarily to prevent infection and illness with another. The persistent cross-neutralization demonstrable for years after even inapparent infections suggests, however, that a live attenuated vaccine might protect against all the Group B tick-borne viruses with the probable exception of Powassan.

The potential usefulness of the DiG technique as a diagnostic procedure was explored using post-infection sera from laboratory personnel. Plate 4A shows results of the study in which the large and small antigens prepared from suckling mouse brains infected with Omsk virus were tested against serial twofold dilutions of mouse hyperimmune serum and human convalescent serum. Although a strong precipitin reaction was obtained with both sera against the large antigen, only the mouse serum reacted with the small antigen. The explanation for this difference will be considered in a separate communication, but obviously for diagnosis it is necessary to ensure the presence of an adequate quantity of the large antigen component. In one attempt at the preparation of antigen from HeLa cell tissue-culture fluid infected with Omsk, only the small antigen component could be visualized. This material was therefore of no use for diagnostic purposes.

Plate 4B shows the pre- and post-infection sera from one of the inapparent KFD infections. This

test was carried out only with KFD large antigen. The presence of precipitating antibodies is clearly shown in the post-infection specimen. It has been found that antibodies detectable by DiG are demonstrable for only a few weeks. This technique thus provides a useful means of demonstrating recent infections but would be of no value as a survey tool. No attempt has been made to assess the specificity of the gel-precipitin reaction with the sera available since all individuals had been previously immunized with yellow fever and RSSE vaccines and could be assumed to respond to their laboratory infection with broadly cross-reactive antibodies.

DISCUSSION

As new methods are developed and old methods modified to increase their sensitivity, we are becoming more and more aware that we can increase or decrease the closeness of observed relationships between different viral isolates by selecting the method of comparison. As a consequence, the problem of arboviral classification seems to become more rather than less bewildering with the passage of time. For just this reason it would seem to be a responsibility of the virologist to try to assess the significance of antigenic relationships and variations as they are observed to occur.

The demonstration of the relationship of Powassan virus to the tick-borne complex was of ecological importance in indicating the probable type of vector to be expected for this virus. This was subsequently confirmed by the identification of Powassan isolates from *Dermacentor andersoni* ticks in Colorado (Thomas et al., 1960) and from *Ixodes marxi* ticks in Ontario (McLean & Larke, 1963). The magnitude of difference, however, between the antigenic make-up of Powassan and that of all other members of the complex makes it easy to decide that Powassan should be considered a separate and distinct viral entity.

Similarly, Langat virus and the viruses of Omsk haemorrhagic fever and Kyasanur Forest disease all have distinctive antigenic structures which not only make differentiation fairly easy but also are of sufficient degree to permit infection of individuals possessing neutralizing antibodies to other members of the complex. In addition, each of these viruses exists in a distinct geographical area and the typical clinical pictures described for human infections with Omsk and KFD viruses differ from those produced by the other tick-borne human pathogens (no natural human infection with Langat virus has been reported). It thus seems reasonable to consider these three as discrete viral entities.

Within the Group B tick-borne complex, Negishi, LI and the CE and RSSE subtypes of tick-borne encephalitis virus form an especially compact group from the standpoint of antigenic structure. In the past this has caused difficulties in making identifications and differentiations, although Casals (1944), nearly twenty years ago, showed that RSSE and LI could be distinguished readily by intracerebral cross-resistance tests in mice. With the techniques of absorption-HI and DiG, all of these viruses or types can be separated, as has been shown by the data presented in this and the preceding report. In some instances, however, differentiation with either technique was clear only undirectionally. In other cases, definite antibody plateaux were not realized even though curve divergence was obvious. The general impression gained is that especially extensive sharing of antigens occurs among these viruses which serves to set them somewhat apart from the other members of the complex. One of the practical results gained from the comparative study of these four, therefore, is the knowledge that conventional serology frequently fails to distinguish one from another and that, as a consequence, more discriminating techniques must be used.

The question might quite legitimately be raised as to why LI and Negishi should not also be regarded as subtypes of tick-borne encephalitis virus since they are so closely related antigenically. By way of answering the question, consideration might be given to the fact that these two viruses occur in widely separated geographical areas. In addition, LI is primarily a disease of sheep. Information concerning Negishi infection is very limited since it has never been seen subsequent to the initial isolation. In the opinion of the author, however, it might indeed be logical to classify these four viruses together as variant subtypes, although the decision to do so would obviously require formal agreement among interested virologists.

In general, the thesis held in the preceding report still obtains—namely, that variation in antigenic structure occurs when there is discontinuity in geographical distribution. Antigenic variants can, however, exist within a region, as has been seen in the case of the two types of Omsk virus and in the occurrence of both CE and RSSE types of tick-borne encephalitis virus in western USSR. In connexion

with the latter observation, it is of some interest that no strain of tick-borne encephalitis isolated outside the USSR has proved to be of the RSSE type. Thus the argument that these viruses do not tend to move readily over long distances from one area to another still appears to be valid.

RÉSUMÉ

Les virus transmis par les tiques ont été répartis, d'après les études de Clarke (1962), en 6 groupes d'après leurs caractères antigéniques: louping ill, l'encéphalite de l'Europe centrale, la fièvre hémorragique d'Omsk, la maladie de la forêt de Kyasanur, l'encéphalite verno-estivale russe et celle de Langat. Les tests employés pour différencier et identifier ces virus sont: l'inhibition de l'hémagglutination, la fixation du complément, l'inhibition avec sérums absorbés, et la diffusion sur gélose.

L'auteur décrit les méthodes utilisées et les modifications qui leur ont été récemment apportées et expose de nouvelles acquisitions, en particulier en ce qui concerne deux virus d'encéphalites découverts depuis le dernier recensement: Powassan et Negishi.

A mesure que les méthodes deviennent plus sensibles et que l'on en met au point d'autres, les ressemblances et différences entre les virus peuvent être atténuées ou accentuées, selon la méthode employée. Il s'ensuit que le problème de la classification des arbovirus se complique plus qu'il ne se simplifie.

Dans le cas des virus Powassan et Negishi, les différences avec les autres virus sont telles qu'elles justifient l'attribution à ces virus d'une entité propre.

Par ailleurs, l'auteur discute les relations antigéniques des divers virus constituant le complexe du groupe B des virus transmis par les tiques, et leur signification. Elle souligne le rôle de la distribution géographique dans l'individualisation des virus.

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