Polyvalent Group B Arbovirus Antiserum Produced in Monkeys.* (30071)

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During the past decade the number of recognized arthropod-borne animal viruses (arboviruses) has increased from approximately 30 prior to 1950 to close to 200. Identification and antigenic classification of new isolates thus currently requires many specific antisera, a resource unavailable to many investigators. However, since some arboviruses are related antigenically and have been classified into groups(1), the possibility of preparing polyvalent grouping antisera for preliminary classification of new virus strains exists. Studies were therefore carried out to learn whether a polyvalent grouping antiserum could be made for one of the largest and most common groups of arboviruses, *viz.*, group B(1), by inoculation of a host with several viruses from the group. The objective was to prepare satisfactory complement-fixing antisera because the complement-fixation test is a more rapid and less expensive procedure than the mouse neutralization test and is applicable to all arboviruses, including those from which hemagglutinins cannot be made by current methods(2). Monkeys were used because they had been shown with enteroviruses to be capable of yielding relatively large quantities of antisera(3), and were known to produce antibodies to at least one group B arbovirus(4). However, since monkeys come from endemic areas for some arboviruses, it was necessary first to screen them for pre-existing antibodies. The results of these tests and of the homologous and heterologous antibody responses of monkeys to 4 selected group B arboviruses are presented herein.

Materials and methods. Viruses. Three strains of Japanese encephalitis (JE) virus were employed, 2 as suspensions of mosquitoes caught in Japan (M7/71 and 76, and M6/Mag 144) and one, M5/596, either as a suspension of infected suckling mouse brains after 6 mouse brain passages or as hamster kidney cell culture fluid after 7 mouse brain, 1 chicken embryonic cell culture and 2 hamster kidney cell culture passages. Other details concerning these strains are presented elsewhere(4). Yellow fever virus, strain 17D, was used as commercial vaccine (National Drug Co.) prepared in embryonated chicken eggs. The infectious virus content of each lot of vaccine was determined by inoculation of suckling mice intracranially with 0.01 ml aliquots of decimal dilutions of virus. Type 1 dengue virus, Hawaii strain, was employed as fluid from infected rhesus monkey kidney cell cultures representing 2 cell culture and 121 or 122 mouse brain passages from its original human source(5). Tick-borne encephalitis (TBE) virus, strain TP 21 (Langat), was inoculated as suspensions of infected suckling mouse brains representing mouse passage 5 from *Ixodes granulatus*(6). Methods of virus titration and storage are presented elsewhere (4).

Monkeys. Those tested for pre-existing arbovirus neutralizing antibodies were *Macaca cynomolgus* (*M. irus*) from Ceylon and *M. mulatta* from India; they were procured in 1959 from the Okati Farms facility of the National Foundation. Monkeys immunized to group B arboviruses were *M. cynomolgus* from the Philippine Islands(4).

Inoculations of monkeys with selected group B arboviruses. Five monkeys were first inoculated intradermally 3 or 4 times with 2 strains of JE virus as mosquito or mouse brain suspensions(4). Dosages of JE virus as $\log_{10}$ suckling mouse intracranial LD_{50} and days before inoculation of a second group B arbovirus were as follows: monkey 57-28, 2.9, 1.8 and 9.0 on days —471, —326 and

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—291; monkey 57-39, 4.0, 6.0 and 5.2 on days —431, —291 and —227 (this monkey also received an intranasal inoculation of 3.5 $\log_{10}$SM ic LD_{50} on day —81 and developed overt encephalitis on day —72); monkey 57-40, 1.9, 1.8, 3.3 and 5.2 on days —470, —325, —290 and —175; monkey 57-36, 1.5, 6.0 and 5.2 on days —663, —290 and —175; and monkey 57-43, 2.9, 3.3 and 5.2 on days —471, —291 and —227(4). Subsequent subcutaneous inocula of group B arboviruses (Fig. 1) were: yellow fever, 5.5 SM ic LD_{50} on day 0 and 5.4 or 5.7 on day 326; dengue, type 1, 3.0 on day 0 and 3.5 on day 340; TP21, 6.3 on day 326; and JE, 1.7 weanling mouse ic LD_{50} on day 340. This JE virus was strain M5/596 harvested from hamster kidney cell cultures.

Antibody tests. Neutralizing (N), hemagglutination-inhibiting (HI), and complement-fixing (CF) antibody tests with JE virus, strain M5/596 were done with monkey serum or plasma as described elsewhere(7); CF tests employed 4 antigen units, 2 exact units of complement and serum heated undiluted at 56°C for 30 minutes. Identical procedures were used to test for CF antibodies for yellow fever, dengue 1 and TP 21 except that CF antigens were made by the sucrose-acetone extraction method(8) rather than by simple saline extraction and centrifugation as employed for JE virus(9). CF tests at the Rockefeller Foundation laboratory in New York employed antigens prepared by acetone-ether or sucrose-acetone treatment of suckling mouse brains(8), 8-64 antigen units, 1.8-2.2 complement units and serum heated at 60°C for 20 minutes as a 1:4 dilution in saline. At the Rocky Mountain Laboratory, antigens were made by acetone-ether extraction of brain, and 2 antigen units, 2 full complement units and 60°C heating for 20 minutes of 1:4 serum in saline were employed.

Results. Pre-existing arbovirus neutralizing antibodies in Ceylonese cynomolgus and Indian rhesus monkeys. Fifteen different cynomolgus monkeys from Ceylon and 12 rhesus from India were examined for pre-existing arbovirus neutralizing (N) antibodies in serum. Two or three monkeys of each type were tested *vs* selected arboviruses representing the major groups and some ungrouped viruses. In general no N antibodies were detected (Table I), and there was thus no difficulty in finding monkeys suitable for immunization and preparation of antisera. One Ceylonese monkey, positive to St. Louis encephalitis virus, a virus unknown in that part of the world, was either infected while temporarily housed in southeastern United States or infected in Ceylon with an antigenically closely related group B arbovirus such as Japanese encephalitis. Infection of this monkey in Minnesota, another area where SLE exists, was unlikely because monkeys were housed in underground animal quarters, essentially inaccessible to mosquitoes and in the center of Minneapolis. The rhesus monkeys from India positive with Oriboca and Bwamba viruses were of interest because Oriboca and other group C arboviruses are presently known only in Central and northern South America and Bwamba virus only in Africa. Some monkeys with equivocal N test results (Table I) may not represent the presence of N antibody in serum, but the rhesus sera that reacted with Guama, a South American virus, and Sicilian sandfly fever virus, a Middle-eastern virus could indicate the presence of these or antigenically closely related viruses in India.

Antibody responses of monkeys to sequential inoculation of selected group B arboviruses. Five Philippine cynomolgus monkeys without detectable pre-existing N and HI antibodies to JE virus were first inoculated 3 or 4 times with JE virus as described in *Materials and methods* and elsewhere(4), and their homologous antibody responses were measured as part of another study of the anamnestic antibody response of monkeys to JE virus(4). They were then given a second virus, yellow fever or dengue 1, subcutaneously 81-291 days after the last JE virus inoculation, and bled at selected times to measure homologous and heterologous CF antibodies in serum. Each monkey manifested a homologous CF antibody response (Fig. 1, virus II). Heterologous responses to JE, yellow fever or dengue 1 viruses also occurred except in one monkey (57-39) given yellow fever virus in the presence of unusu-

TABLE I. Results of Arbovirus Neutralization Tests of Sera from 15 Different Normal Cynomolgus and 12 Rhesus Monkeys.

Arbovirus group	Virus and strain used in neutralization test		Fraction of monkeys with positive neutralization tests of serum (LNI)[1]	
			Ceylonese cynomolgus	Indian rhesus
A	EE	Riche	0/3	0/1
	WE	RML 1985-60	0/2	?1/3 (*1.1*[4])
B	SLE	Tr 9464	1/2 (*2.0*[2])	0/2
C	Oriboca	BeAn 17	0/3	1/3 (*>1.8*[5])
Bunyamwera	Bunyamwera	original	0/3	0/3
California	California	BFS-283	0/3	?1/3 (*>0.9*[6])
Guama	Guama	BeAn 277	?1/2 (*1.1*[3])	?1/3 (*>1.4*[4])
Bwamba	Bwamba	M459		1/3 (*>1.6*[7])
Naples sandfly fever	Sandfly fever	Naples	0/2	
Anopheles A	Anopheles A	original	0/3	0/3
Ungrouped	" B	"	0/2	
	Sandfly fever	Sicilian	0/1	?1/3 (*1.6*[8])
	Colorado tick fever	RML-N 7180	0/3	0/3

[1] Monkeys with sera positive or equivocal to one virus were negative (LNI <1.0) against the following 2 or more other viruses: [2] Oriboca and Bunyamwera; [3] WE, California, Anopheles A, Naples and Sicilian sandfly fever; [4] Sicilian sandfly fever and Colorado tick fever; [5] SLE and Bunyamwera; [6] Oriboca and Anopheles A; [7] WE and Guama; [8] WE and Colorado tick fever.

ally high pre-existing JE and dengue 1 antibody levels (Fig. 1).

To stimulate antibodies to the tick-borne encephalitis (TBE) complex of Group B, arboviruses, TP 21 (Langat) virus(6), a relatively avirulent strain of TBE virus, was given 326 days later to 3 monkeys (57-28, 57-39 and 57-40) and as a control, yellow fever was given to 2 monkeys (57-36 and 57-43). Again homologous and heterologous CF antibody responses occurred (Fig. 1, virus III). Antibody titers to TP 21 virus rose to highest levels after its inoculation, but interestingly, TP 21 antibody levels also rose 16-fold in one control monkey (57-36) that received yellow fever and no TP 21 virus. Subsequent inoculation 14 days after TP 21 or yellow fever viruses, of relatively small doses of JE or dengue 1 viruses did not detectably increase antibody levels (Fig. 1, virus IV), suggesting that proper spacing of inoculations and dosage of virus are essential to effect rises in antibody titers. These results thus showed that certain group B arboviruses could engender formation of antibodies in monkeys which reacted by CF test with some other uninoculated viruses of the group.

To learn more fully the spectrum of heterologous group B arbovirus CF reactions of these post-immunization sera, tests were kindly performed by Dr. J. Casals (Rocke-

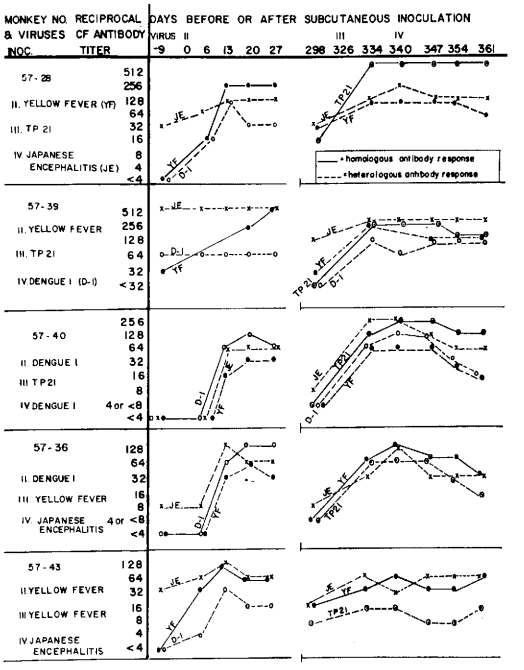

FIG. 1. Antibody responses to selected group B arboviruses given subcutaneously to monkeys previously hyperimmunized with Japanese encephalitis virus.

TABLE II. Heterologous and Homologous Group B Arbovirus CF Antibody Titers in Sera of Cynomolgus Monkeys Inoculated Subcutaneously with Japanese Encephalitis, Yellow Fever, Dengue 1 and/or Tick-Borne Encephalitis (TP 21) Viruses.

Virus as CF antigen	Reciprocals of CF antibody titers by immunizing viruses*, monkey numbers and laboratories testing sera (A, B and C)														
	JE, YF, TP21						JE, D-1, TP21			JE, D-1, YF			JE, YF		
	57-28†			57-39			57-40			57-36			57-43		
	A‡	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Controls—saline —normal	<8	<4	4	<8	<4	8	<8	<4	<4	<8	<4	<4	<8	<4	<4
mouse brain	8	<4	8	<8	<4	16	<8	<4	<4	<8	<4	<4	<8	<4	<4
Japanese encephalitis‖	*128*§	*64*	*64*	*256*	*128*	*256*	*128*	*32*	*32*	*32*	*32*	*32*	*64*	*32*	*64*
Yellow fever‖	*128*		*16*	*128*		*32*	**64**		<4	*64*		*4*	*64*		*8*
Dengue, type 1		**16**	8	**128**	**64**	32	*128*	*32*	*16*		*16*	<4		4	<4
TP21	*512*		*128*	*128*		*128*	*256*		*32*	**32**		**16**	16		<4
Bussuquara		**32**			**64**			**32**			**32**			4	
Dengue 2		**32**	8		**64**	16		**64**	**16**		**32**	4		8	<4
" 3		8			**32**			**32**			8			4	
" 4			8						<4			<4			<4
Ilheus			**64**			**128**			**32**			**32**			8
Modoc		8			**16**			4			<4			<4	
Murray valley encephalitis			*32*			**128**			4			4			8
Ntaya			16			**128**			<4			4			8
Powassan			**32**			32			**16**			<4			<4
Rio Bravo			8			8			<4			<4			<4
SAH 36					**64**									8	
St. Louis encephalitis			16			**64**			4			<4			4
Spondweni		8			**16**			<4			<4			<4	
Uganda		**32**	8					**16**	<4		**16**	<4		4	<4
Wesselsbron		**32**			**64**			**16**			**16**			4	
West Nile			16			32			<4			<4			4
Zika		4	16		8	32		<4	4		<4			<4	4

* Immunizing viruses = those producing a detectable homologous CF antibody response after inoculation.

† Sera tested here were from days 354, 361, 341, 354 and 361 for monkeys 57-28, 57-39, 57-40, 57-36 and 57-43 respectively (Fig. 1).

‡ A = Univ. of Minnesota; B = Rockefeller Foundation, New York; C = Rocky Mountain Laboratory.

§ *Italics* = homologous CF antibody titers to inoculated viruses.

Bold face = heterologous CF antibody titers ≧ 4 times the titer with normal brain, control antigen.

‖ Strains of JE and yellow fever viruses used as CF antigens in laboratories B and C were different from strains used and inoculated in laboratory A.

feller Foundation Laboratories, New York) and by Dr. L. A. Thomas (U. S. P. H. S., Rocky Mountain Laboratories, Montana) for antibodies to 17 additional group B arboviruses. Sera from 2 monkeys (57-28 and 57-39) which received 3 or 4 inoculations of JE virus, then yellow fever and TP 21 virus displayed numerous heterologous group B CF reactions (Table II). CF antibody titers against many group B arboviruses were in a usable range, ≧ 4 times the normal brain control titer; the observed variations in titers among laboratories may have been related to differences in technics of performing CF tests (see *Materials and methods*). Monkeys which received JE, dengue 1 and TP 21 (57-40), or JE, dengue 1 and yellow fever (57-36) produced sera that reacted with fewer group B viruses and often to lower titers than were observed with sera from monkeys given JE, yellow fever and TP 21. Interestingly, serum from monkey 57-36 which did not receive TP 21 virus, reacted with TP 21 but not with Powassan virus, another member of the group B tick-borne virus complex. Only the 3 monkeys inoculated with TP 21 yielded serum that reacted with Powassan. In contrast, monkey 57-43 which received only JE and yellow fever viruses failed to produce satisfactory heterologous antibody titers. The group specificity of these reactions was documented

by Dr. Casals who showed that no serum reacted with western encephalitis and/or chikungunya CF antigens, representing arbovirus group A.

Discussion. The results of these studies showed that sequential inoculation of the viruses of Japanese encephalitis (strains M5/596, M7/71 and 76 and M6/Mag144), yellow fever (strain 17D) and tick-borne encephalitis (strain TP 21), produced broad group B arbovirus CF antiserum in monkeys. Inoculation of both yellow fever and TBE viruses were essential to broaden reactivity of group B CF antibodies of monkeys immunized initially with JE virus. Combinations of dengue 1 and TP 21 viruses, and dengue 1 and yellow fever viruses to supplement initial JE virus immunization produced sera with some heterologous reactivity but not sufficient for group identification of new isolates. It is quite likely that a more convenient immunization schedule could be devised using shorter intervals between inoculations and more inoculations of yellow fever and TP viruses than employed here. However, as in these studies, it would seem advisable to avoid as much as possible the use of immunizing viruses grown in mouse brains so that antisera will not react nonspecifically in CF tests with mouse brain antigens commonly employed in identification of new isolates. Viruses grown in cultures of nonmurine cells or in embryonated chicken eggs readily serve this purpose. Also in the future it would seem wise in preparing polyvalent group B arbovirus antisera, to test serum from each monkey against all group B arboviruses after the initial series of inoculations of JE, yellow fever and TP 21 viruses, and then to inoculate the monkey with the viruses against which the serum fails to react. In such a manner, a single monkey could be made reactive to all group B arboviruses and bled repeatedly over many months (perhaps using plasmaphoresis) to yield large quantities of polyvalent serum. If similar grouping antisera can be prepared for other major groups of arboviruses, the potential number of antisera against which newly isolated arboviruses have to be tested will be significantly reduced from its current level of over 100 sera.

Although it could be presumed that monkeys coming from tropical endemic areas for arboviruses, might have high prevalences of pre-existing arboviral antibodies from previous infections, most monkeys tested here had no detectable arboviral N antibodies in serum. These results fit with the absence in 8 rhesus monkeys of pre-existing HI antibodies to 4 group B arboviruses reported by Groot(10). The fact that a few Southeast Asian monkeys were positive for antibodies to viruses currently unrecognized in Ceylon and India suggested that further serologic survey of monkeys from various parts of the world might provide useful new information concerning geographic distributions of arboviruses.

Summary. Polyvalent group B arbovirus antiserum reactive by complement-fixation test was prepared in monkeys by sequential inoculation of Japanese encephalitis (JE), yellow fever, and TP 21 (Langat) viruses. Such serum should facilitate identification of newly isolated arboviruses as members of group B. Combinations of JE, dengue 1 and TP 21 or yellow fever viruses produced sera with lesser degrees of polyvalency, and use of only JE and yellow fever viruses yielded serum with only low CF titers to a few heterologous group B arboviruses. Examination of 27 monkeys for pre-existing arboviral neutralizing antibodies revealed most to be negative; however, one rhesus monkey from India was positive to Oriboca virus and another to Bwamba virus, suggesting that these or antigenically closely related arboviruses exist in India even though they are currently recognized to occur only in South and Central America or in Africa respectively.

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Isolation of Mucopolysaccharide from the Precartilaginous Embryonic Chick Limb Bud.* (30072)

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During an investigation of cartilage differentiation in the embryonic chick limb bud, embryos from stage 17 to stage 26(1) were injected with radioactive sulfate and the pattern of sulfate uptake in their limb buds examined by autoradiography(2). An uptake of radioactive sulfate was observed at every stage. An uptake of radioactive sulfate has been reported in the chick embryo as early as stage 4 (primitive streak)(3). The first histological evidence of cartilage in the limb bud of the embryonic chick is found at stage 25(4). An uptake of radioactive sulfate well before the first histological evidence of cartilage has been observed in other vertebrate embryos(5,6,7). It has been proposed that an uptake of sulfate before the first histological evidence of cartilage might be due to compounds other than chondroitin sulfate, perhaps to a chondroitin sulfate precursor(6).

The pattern of radioactive sulfate uptake observed in the autoradiographs and the changes of this pattern with embryonic development suggested that the uptake might be concerned with the differentiation of limb bud cartilage. Before the uptake of radioactive sulfate could be used to study the differentiation of limb bud cartilage, some knowledge had to be gained about the chemical nature of the material to which the incorporated radioactive sulfate was bound.

While this work was in progress, Franco-Browder, De Rydt, and Dorfman reported the presence of chondroitin sulfate A and/or C in the chick embryo at stage 11 to 12 and of the same material in the embryonic chick limb bud at stage 22 to 23(8). This paper verifies and extends that report.

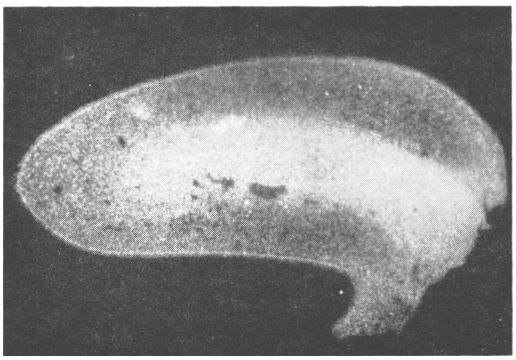

FIG. 1. Stage 23 limb bud which has been allowed to accumulate radioactive sulfate for 5 hr. Photographed in darkfield at 54×. Length of limb is 1.15 mm.

Materials and methods. Fertilized White Leghorn or White Cornish Cross† eggs were incubated for 3½ days at 37.5°. S-35 sulfate (carrier-free, Oak Ridge National Laboratory) was injected through a window in the shell(9) directly onto the *area vasculosa* as was done for the autoradiographs(2). Most of the eggs were injected with radioactive sulfate during stage 22, just before the differential rates of sulfate uptake in the limb bud mesoderm became established. The eggs were then incubated at 37.5° for 5 hours. During this further incubation the embryos were able to reach a stage at which the differential rates were clearly established (Fig. 1). The limbs were removed from the embryos in Tyrode's solution.

In most of the experiments, the limbs were

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† Brown Cornish male × Arbor Acres White Rock female.