Use of Sarcoma 180 to Prepare Hemagglutinating and Complement-Fixing Antigens for Viruses in Adult Mice[1] (35491)

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In the course of a search for a mouse-tissue source of virus antigen it was found that sarcoma 180, as propagated in the live intact mouse, provided an excellent substrate for multiplication of some arboviruses, with associated appearance of infectious, hemagglutinating, and complement-fixing antigens in the ascitic fluid.

Materials and Methods. Sarcoma 180 was obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, where it was certified as free of specific pathogens after *in vitro* passage of the sarcoma 180/TG line described by Sartorelli *et al.* (1). The line had undergone 30–32 intraperitoneal (ip) passages in Charles River CD (R)-1 mice when used in this study. Mice of both sexes, 4–7 weeks old, were inoculated ip with 0.2 ml of fresh sarcoma 180 ascitic fluid. Seven to 9 days later, when most mice had visible abdominal swelling, virus was inoculated ip.

Fifteen different viruses were used: eastern equine encephalitis (EEE) (TenBroeck), Mayaro (BeH 256), Sindbis (EgAr 339), and Una (BeAr 13136) of group A; dengue 4 (Camb. 62-66) and St. Louis encephalitis (SLE) (Parton) of group B; Apeu (BeAn 848) and Marituba (BeAn 15) of group C; Sicilian sandfly fever (Sabin) of the Phlebotomus fever group; Oropouche (Tr 9760) of the Simbu group; Uukuniemi (S-23) of the Uukuniemi group; Hazara (JD 280) of the Congo group; Pongola (SAAr 1) of the Bwamba group; Portillo (Noguera) of the Tacaribe group; and Colorado tick fever (Condon), ungrouped.

These were available as stock virus preparations of 10 or 20% infected mouse brain suspensions, lyophilized and stored at 4° except for the Sicilian sandfly fever virus stock which was kept wet-frozen at —70°. On one occasion (see Table II), a fresh suspension of EEE-virus infected mouse brain was used. Virus was diluted in 0.75% bovine albumin phosphate-buffered saline (BAPS), pH 7.2. Unless otherwise noted, virus was inoculated into groups of five mice as 0.1 ml of a 10% suspension, usually representing a dose of 10,000 suckling mouse intracerebral LD_{50} or greater.

After virus inoculation, 5 ml of ascitic fluid was withdrawn daily from one mouse (rarely more than one) in the group for as long as mice survived. Much larger quantities could have been harvested if desired. The ascitic fluids were routinely stored at —20°.

For experiments in which infectivity titrations were carried out, ascitic fluids of five mice were pooled and defibrinated with glass beads before being stored at —70° in aliquots for infectivity, hemagglutination (HA), and complement-fixation (CF) titrations. In some experiments the cells were separated from the ascitic fluid by low-speed centrifugation, resuspended in 20 vol of 0.75% BAPS (care being taken to fragment particulate masses), and recentrifuged. The supernate was discarded, and the procedure was repeated three times. After the final separation, the sedi-

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ment was resuspended in an equal volume of diluent, stored at —70°, and then frozen and thawed rapidly in an alcohol–Dry Ice mixture, for fragmenting of cells, prior to infectivity titrations. Packed cell volumes were not determined in these experiments, but from other experience, packed cells constitute about 10% of total fluid volume. Infectivity titration end points are expressed in $\log_{10}$ infant mouse LD_{50}, calculated by the method of Reed and Muench (2).

For preparation of antigens, thawed ascitic fluids were withdrawn, with care taken to avoid cellular debris. HA antigens consisted of fluids twice extracted with acetone and then rehydrated with 5 vol of borate saline, pH 9.0, as described for baby mouse serum antigens (3). For CF antigens, the untreated fluids were heated for 20 min at 60° and used either undiluted or diluted 1:2 in Veronal buffer.

HA and hemagglutination-inhibition (HI) techniques were those of Clarke and Casals (3) adapted to a microtechnique (4). Serial 2-fold dilutions of antigens, starting at 1:4, were tested at pH 5.8 through 7.2, at 0.2 intervals. Each HA antigen was tested for specificity in HI test with its homologous and at least two unrelated immune sera or immune ascitic fluids, all extracted with acetone and adsorbed with goose red blood cells.

CF tests were carried out in grid titrations as described by Casals (5). Immune fluids were produced in mice and inactivated for 20 min at 60° before being used. Each immune fluid was shown to be reactive by CF testing with reference sucrose–acetone-extracted mouse brain antigen (mouse liver antigen in the case of Marituba virus).

The specificity of CF antigens made from ascitic fluids was tested using fluids harvested on day 5 after virus inoculation and a homologous and an unrelated immune fluid.

The time of peak CF antigen titer in ascitic fluid antigens was determined in a single test done with two dilutions of immune fluid, 1:8 and 1:16 or 1:16 and 1:32, and serial 2-fold dilutions of antigens from daily harvests. Titers are expressed as the highest antigen dilution reacting with one or both dilutions of immune fluid at 3+ or greater fixation (5).

A comparison by CF test of the reactivity of ascitic fluid antigens with that of sucrose–acetone antigens from newborn mouse brain (Apeu virus) and liver (Marituba virus) was carried out using ascitic fluid antigens harvested on days 3 and 5 after virus inoculation. Serial 2-fold dilutions of immune fluids and 4-fold dilutions of antigens up to 1:256 were employed in a single test.

For comparison of the response in mice, ascitic fluid was induced in four mice by an alternative procedure to inoculation with sarcoma 180, namely, repeated ip injection of Freund's complete adjuvant according to the method described by Munoz (6). After ascitic fluid had appeared, the mice were inoculated ip with virus (Sindbis) and followed in the same fashion as sarcomatous mice inoculated with the virus.

Results. HA. Of the 15 viruses tested, five—EEE, Mayaro, Sindbis, Una, and SLE—produced ascitic fluid hemagglutinins with titers of 1:32 or higher (Table I); HI tests proved these hemagglutinins to be inhibited by the homologous immune fluids and with a sensitivity not less than that of the brain antigens. HA titers of 1:4096 were found with the EEE and Sindbis ascitic fluid antigens (Table I), and the pH range of agglutinating activity was generally wider than with newborn mouse brain tissue antigens. Ascitic fluid HA antigens first appeared on day 2 after virus inoculation, and the highest titers were seen on days 4–6.

With the other 10 viruses tested, ascitic fluids were either negative or, in the case of Hazara, Uukuniemi, and Marituba, gave such low HA titers that further characterization was not carried out.

The relation, if any, between virus dosage and time of appearance and titer of HA antigen was investigated using a freshly prepared suspension of EEE virus (Table II). With an inoculum of 1 ml of 10^{-1} brain suspension, HA was observed 24 hr after inoculation and the titer increased through day 3, when the mice died. HA antigen developed even with an inoculum of 0.1 ml of 10^{-4} brain suspension, the highest dilution used.

Infectivity. Infectivity titers and their relation to the HA titers are shown in

TABLE I. HA Titers of Antigens in Ascitic Fluids from Infected Mice.[a]

Sex of mouse	Virus	Strain	Day after virus inoculation											
			1	2	3	4	5	6	7	8	9	10	11	12
F	EEE	TenBroeck	0	0	0	16[b]	512	4096	64	4	—	—	—	—
M		TenBroeck	0	0	16	2048	2048	4096	1024	—	—	—	—	—
M	Mayaro	BeH 256	0	0	0	128	1024	—	—	—	—	—	—	—
F	Sindbis	EgAr 339	0	32	128	4096	1024	32	8	4	4	—	—	—
F	Una	BeAr 13136	0	0	0	128	32	0	0	0	0	—	—	—
M		BeAr 13136	0	8	32	128	64	64	0	—	—	—	—	—
F	SLE	Parton	0	0	0	64	16	0	8	0	0	0	0	4
M		Parton	0	0	0	256	64	0	0	0	—	—	—	—
M		Parton	0	0	0	4	4	32	16	0	—	—	—	—

[a] Sarcomatous mice were inoculated ip with 0.1 ml of a 10% suspension of infected mouse brain.
[b] Reciprocal of antigen dilution; — = no ascitic fluid available because of death of mice or regression of tumor.

Table III for SLE and EEE viruses. As shown, multiplication of virus took place and titers obtained in the ascitic fluid could be as high as those in the infected mouse brain tissue used as inoculum. Although infectivity titers of the ascitic fluids showed a peak, virus was present at all times in ascitic fluid or ascitic cells, or both.

CF. Ten of the viruses tested produced satisfactory ascitic fluid CF antigen, and the other five did not yield antigen (Table IV).

When ascitic fluid antigens were compared for reactivity with sucrose–acetone antigens from brain (Apeu virus) and liver (Marituba virus), the differences observed were not marked.

TABLE II. HA Titers of EEE Virus Antigens in Acetone-Extracted Mouse Ascitic Fluids.[a]

Inoculum		Day after virus inoculation					
Dilution	Vol (ml)	1	2	3	4	5	6
−4[b]	0.1	0	0	64[c]	8192	256	256
−3	0.1	0	0	32	1024	4096	0
−2	0.1	0	0	0	0	512	4096
−1	0.1	0	0	0	4	128	0
−1	0.5	0	128	128	1024	—	—
−1	1.0	64	256	8192	—	—	—

[a] Sarcomatous mice were inoculated ip with a fresh infected mouse brain suspension containing $10^{9.7}$ LD_{50}/0.02 ml.
[b] Expressed in $\log_{10}$.
[c] Reciprocal of antigen dilution.

Preliminary checking of HI antibodies in the ascitic fluid of mice inoculated with SLE virus (Table IV) showed that the fluid harvested on day 12, in which CF antigen was still present, had an HI antibody titer of 1:1280.

In the comparison of mice in which ascitic fluid had been induced by repeated injection of Freund's complete adjuvant and mice given sarcoma 180, both groups subsequently inoculated with Sindbis virus, none of the mice on the Freund's adjuvant regimen developed HA antigen in their ascitic fluid, whereas all mice on the sarcoma 180 regimen did.

Regression of the sarcomatous process was observed irregularly in mice inoculated with some of the arboviruses studied.

Discussion. Brain tissue of infected newborn mice has become a standard source of HA and CF antigens for most arboviruses (3), with serum of such mice or of infected hamsters representing a source of HA antigen in some instances (3, 7). For certain viruses, however, the yield of antigen is low and the preparation at times costly.

The method described here provides a simple, inexpensive way to obtain antigens of reasonably good titer for both tests, with a number of viruses. In addition, as shown by the titrations of virulence done with the crude fluids, ascitic fluids so prepared may constitute a source of virus stocks, free or relatively free from contaminating tissue par-

TABLE III. Infectivity and HA Titers of Ascitic Fluids from Mice Inoculated with SLE or EEE Virus.

Virus	Dose inoculated[a]	Test	Day after virus inoculation 1	2	3	4	5	6	7	8
SLE	7.0	HA	0	0	0	4[b]	4	32	16	0
		Infectivity: cells	3.3[c]	4.5	5.3	5.7	7.4	7.2	5.9	4.4
		fluid	2.7	3.9	3.7	5.6	7.3	7.8	6.8	4.9
EEE	7.1	HA	0	0	0	0	8	—	—	—
		Infectivity: cells	3.9	4.9	5.5	6.8	5.0	—	—	—
		fluid	5.5	5.5	5.8	7.4	7.0	—	—	—
	9.4	HA	0	0	0	4	128	0	—	—
		Infectivity: fluid	6.0	6.5	8.4	7.8	9.4	7.6	—	—
	10.4	HA	64	256	8192	—	—	—	—	—
		Infectivity: fluid	9.3	9.7	9.6	—	—	—	—	—

[a] Dose expressed in $\log_{10}$ LD_{50}, as determined by intracerebral inoculation of infant mice.
[b] HA titer expressed as reciprocal of antigen dilution.
[c] Infectivity titer expressed in $\log_{10}$ LD_{50}/0.02 ml, determined as in footnote *a*.

ticles, for use in neutralization tests or as antigen in immunization schedules.

Obviously, additional investigations are needed along several lines: for example, extension of these observations to other viruses, not limited to arboviruses; determination for each virus of the optimal time for harvesting the ascitic fluid for HA and CF antigens; possible improvement of titers and expansion of the list of viruses by adaptation of a virus to the sarcomatous mouse system by serial passage in sarcomatous mice; determination of the relation between amount of inoculated virus and time and amount of antigen production; and determination of the mechanism involved in the production of the antigens.

A probable association of virus with the sarcoma or other peritoneal cells is suggested

TABLE IV. CF Titers of Antigens in Ascitic Fluids from Infected Mice.[a]

Virus	Day after virus inoculation 1	2	3	4	5	6	7	8	9	10	11	12
EEE	0	0	0	0	16[b]	64	4	4	—	—	—	—
Mayaro	0	0	0	0	0	0	0	0	0	—	—	—
Sindbis	0	0	8	4	8	16	8	8	8	—	—	—
Una	0	0	0	8	4	0	0	0	0	—	—	—
Dengue 4	0	0	0	0	0	0	0	0	0	—	—	—
SLE	0	0	2	≧128	≧128	16	32	32	16	32	16	8
Apeu	0	0	32	64	128	128	—	—	—	—	—	—
Marituba	0	4	32	128	128	128	0	—	—	—	—	—
Sicilian sandfly fever	8	8	16	8	4	0	0	—	—	—	—	—
Oropouche	4	≧64	32	≧64	16	—	—	—	—	—	—	—
Uukuniemi	0	0	0	0	64	≧128	8	—	—	—	—	—
Hazara	0	0	0	32	0	0	—	—	—	—	—	—
Pongola	—	0	0	0	0	0	—	—	—	—	—	—
Portillo	0	0	0	0	0	0	0	0	0	—	—	—
Colorado tick fever	0	0	0	0	—	—	—	—	—	—	—	—

[a] Mice were females inoculated with 0.1 ml of dilution 10^{-1} of virus suspension.
[b] Reciprocal of antigen dilution; 0 = no fixation at dilution 1:2, lowest used.

by the parallel titrations of virus in cells and fluid carried out with SLE and EEE viruses. Possibly the virus present in the cell fraction is virus attached to cell membranes by simple adsorption. Further experiments to localize cell-associated virus should clarify this point.

Thus far, preparation of ascitic fluid for HA has been attempted only by acetone extraction. For CF, an ascitic fluid antigen prepared by acetone extraction was not better than one prepared by heating of the fluid for 20 min at 60°. As compared with heated ascitic fluid used as CF antigen, the unheated fluid usually has a titer one or two 2-fold dilutions higher. Heating of the antigen, however, results in (a) the disappearance of the slight anticomplementarity present in undiluted, unheated ascitic fluid, (b) the elimination of any complement activity present, and (c) the inactivation of any live virus present.

The ascitic fluid obtained with the present method may provide a system for studying parallel development of antigens and antibodies in the same fluid system.

Summary. When sarcomatous adult mice were inoculated intraperitoneally with certain viruses, virus replication took place and the ascitic fluids subsequently harvested provided a good source of virus stocks and antigens for hemagglutination and complement-fixation tests. Satisfactory antigens were obtained for arboviruses of groups A, B, C, Phlebotomus fever, Simbu, Uukuniemi, and Congo.

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