THE TYPING OF COXSACKIE A VIRUSES WITH COMBINATION POOLS OF IMMUNE ASCITIC FLUID

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THE TYPING OF COXSACKIE A VIRUSES WITH COMBINATION POOLS OF IMMUNE ASCITIC FLUID

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INTRODUCTION

The larger number of Coxsackie A viruses isolated in recent years makes the identification of unknown strains difficult, time consuming and expensive. One method of identification is typing each new isolate against specific antisera, currently 24, by the serum neutralization test using suckling mice. Typing antisera are not commercially available and must be prepared by each laboratory interested in identifying Coxsackie A viruses.

The usual sources of sera, such as monkeys and rabbits, are expensive and more likely to produce heterologous antibodies. The use of such sera in pools results in non-specific reactions which are a deterrent to rapid accurate type identification of Coxsackie A viruses.

Mouse serum containing high antibody concentrations can be produced but the small volume available discourages its use. Mouse ascitic fluid has antibody titers comparable to serum and can be obtained in relatively large quantities.

Virus identification is quick and economical when several anti-viral sera are combined in pools. In this way one typing operation will identify an unknown isolate. This is not so when individual samples are tested.

The present studies are concerned with the production of anti-coxsackie A ascitic fluid in adult mice and its use in combination pools for rapid identification of specific strains. So far as presently known, this is the first report of the use of combination pools for typing Coxsackie A viruses.

REVIEW OF LITERATURE

Investigation of several epidemics of poliomeylitis in New York led to the isolation by Dalldorf and Sickles (1948) of an agent from the feces of two children that produced paralysis and degeneration of the skeletal muscles in suckling mice and hamsters. The central nervous system was not affected. The virus was given the name of the village, Coxsackie, where the two patients resided. A similar virus was later isolated from the feces of children with symptoms of poliomeylitis (Dalldorf et al., 1949). This virus also produced degeneration of the skeletal muscles of suckling mice and hamsters. The children had muscle weakness for several months.

A large number of similar viruses were later isolated world wide (Contreras et al., 1952; Findlay and Howard, 1950; Howitt, 1950; Sickles et al., 1955; Sickles et al., 1959).

These viruses could be differentiated into immunologically distinct types by complement-fixation and neutralization tests (Beeman et al., 1952; Contreras et al., 1952; Howitt and Benefield, 1950; Melnick and Ledinko, 1950; Melnick and Kraft, 1950).

Dalldorf (1950) classified the viruses into groups A and B on the basis of lesions produced in experimental

animals. Group A viruses cause paralysis in suckling mice as a result of extensive myositis of skeletal muscles. Group B viruses produce cystic degeneration of the brain and necrosis of the adipose tissue of the skeletal muscles with less extensive myositis.

The suckling mouse is the preferred host for identification of group A viruses, except A 9 and 23 which grow readily in cell cultures (Crowell and Syverton, 1950; Sickles et al., 1959).

Coxsackie A 23 produces symptoms in mice typical of group A viruses but it is neutralized by anti-ECHO virus type 9 serum. Sickles et al. (1959) proposed that this agent should be classified as ECHO 9 virus.

Group B viruses grow readily in cell cultures and isolation and identification is relatively simple (Contreras et al., 1952, Crowell and Syverton, 1959; Hammond, 1960).

The cell culture spectrum, such as monkey kidney (Lennette et al., 1961), human amnion (Lehmann-Grube and Syverton, 1961), and suckling mouse (Moore et al., 1964), and numerous others (Crowell and Syverton, 1955; Dalldorf et al., 1956; Shaw, 1952; Sickles et al., 1955; Weller et al., 1953) supporting multiplication of Coxsackie A viruses is variable in that not all virus strains can be propagated.

A variety of experimental animals have been used for a source of anti-coxsackie A serum (Beeman et al., 1952; Contreras et al., 1952; Dalldorf et al., 1956). Monkey and

rabbit antisera are available in large volumes but are expensive. Mouse sera are in limited supply.

According to Munoz (1957), however, mouse ascitic fluid can be obtained in large volumes. Freund's adjuvant is an irritant when inoculated into the peritoneal cavity of mice. Serous fluid accumulates in response to this irritation in about 50 per cent of the mice inoculated. Antibodies against bacterial antigens found in the blood of these mice, can also be found in the ascitic fluid.

According to Herrman and Engle (1958), sarcoma 180 cells also cause an irritation in the peritoneal cavity of mice. When influenza and Newcastle disease viruses are used as antigens, the antibody levels in ascitic fluid and blood plasma were essentially the same.

Coxsackie A antibodies in ascitic fluid fix complement according to Gamble and Kinsley (1963). The ascitic fluid was used by these authors to identify previously unknown Coxsackie viruses.

Three basic antisera pool schemes have been proposed for virus typing: (1) Dalldorf and Sickles (1956) suggest typing an unknown virus against a pool of 5 different antiviral sera. Neutralization of a virus by the pool indicates the presence of a homologous antiserum. Each serum used in the pool is then tested individually. (2) Schmidt et al. (1961) propose an "intersecting" serum scheme employing pools of type specific ECHO sera. A total of 30 sera are

distributed in 10 pools so that each serum appears in 2 pools. Identification is made when a virus is neutralized by 2 pools sharing a common serum. (3) The combination pool suggested by Lim and Benyesh-Melnick (1960) distributes 14 antisera in 4 pools. Each serum appears in 1, 2 or more pools. Virus identification is made when 1 or more of the pools neutralizes the agent.

Munch (1963), using the complement fixation test for the identification of Coxsackie A viruses, employed a scheme similar to that described by Dalldorf and Sickles (1956). Four pools each containing 5 antisera were used.

MATERIALS AND METHODS

Coxsackie A Virus

The 22 prototype strains of Coxsackie A viruses used were received from the Communicable Disease Center, Atlanta, Georgia, as suspensions of suckling mouse tissues (Table 1). Each strain is designated by the name and number assigned by the Virus Type Culture Collection, New York State Department of Health, Albany, New York. Ten of these viruses, A 13, 15 through 22, and A 24 were used to prepare anti-coxsackie A ascitic fluid. Viruses A 1 through A 12 (excluding A 9), and A 14 were used to determine the cross reactions with ascitic fluid from mice inoculated with antigens prepared from the other types. Coxsackie A 9 and A 23 were not used.

Propagation of Coxsackie Virus in Suckling Mice

Live virus to be used as the antigen for the production of antisera in adult mice was prepared in healthy, 6 to 18 hour old mice as follows:

1. Using a 1/4ml syringe with a 27 gauge needle, each mouse was inoculated intraperitoneally with 0.05 ml of the original viral suspension.

TABLE 1.--Prototype strains of Coxsackie A viruses.

Prototype Strains	Number*	Name*	Infectivity Titer**
1	48249	Tompkins	5.50
2	49190	Fleetwood	7.24
3	49191	Olsen	6.24
4	50246	Highpoint	7.50
5	5134	Swartz	8.50
6	5011	G dula	7.50
7	50140	Parker	4.75
8	5010	Donovan	6.50
10	50548	Kowalkik	6.50
11	52148	Belgium	3.75
12	51204	Texas 12	7.24
13	5359	Flores	4.75
14	52113	G 14	6.24
15	52108	G 9	4.50
16	52109	G 10	6.24
17	52111	G 12	6.24
18	52112	G 13	3.75
19	53157	NIH 8663	6.24
20	55166	1 H Pool #35	3.24
21	55161	Kuykendall	3.75
22	5630	Chulman	6.24
24	5720	Joseph	5.50

*Number and name designated by Type Culture Collection, Division of Laboratories and Research, New York State Department of Health, Albany, New York.

**Negative Log MLD_{50} per 0.03 ml.

2. The mice were observed daily. Paralysed mice were chloroformed and stored at 20°C together with those mice that had died.
3. To prepare virus suspensions, the mice were thawed, and the nose, feet, tail and skin removed. The eviscerated carcasses were weighed and ground in a mortar with alundum. A 10% suspension was prepared in Hanks' balanced salt solution (BSS).
4. The suspension was centrifuged at 2500 rpm (International Centrifuge, size 2, head number 831) for 30 minutes, at room temperature.
5. The supernatant fluid was removed and stored at -20°C until used.
6. Mice used as controls were not inoculated.

Preparation of Anti-coxsackie A Virus Serum

The immunization procedure was similar to that described by Dalldorf and Sickles (1956). Twenty to twenty-five adult male Webster strain mice, 12 to 14 grams, were inoculated intraperitoneally with the virus suspension as follows: 0.5 ml on day 1; 1.0 ml on day 7; 1.5 ml on day 14; and 2.0 ml on day 21.

Generally, 3 to 4 non-inoculated mice were included for observation as controls.

Production of Ascitic Fluid in Immunized Mice

Sarcoma 180 tumor cells (S-180) to be used for stimulating ascitic fluid, were propagated through 38 serial passages in adult mice during a 30 week period. The tumor cells had been received through the courtesy of Dr. K. Sugiura, Sloan-Kettering Institute for Cancer Research, Rye, New York.

Two to 5 ml of ascitic fluid was removed from 1 mouse by paracentesis (Blood Donor Set number 4475 equipped with a 17 gauge needle. Beckton, Dickinson and Co., Rutherford, New Jersey). Viable cells were counted in a hemacytometer using trypan blue. The cell suspension was diluted in physiological saline to contain 1×10^6 viable cells per 0.1 ml inoculum.

Mice, 18 to 22 grams, were inoculated intraperitoneally in the inguinal region with a 1 ml syringe and a 22 gauge needle. The same procedure was used for each passage of the cells.

Mice used for the production of anti-coxsackie A virus serum were inoculated intraperitoneally with 0.1 ml of ascitic fluid containing 1×10^6 S-180 cells 2 or 3 days after the last dose of virus.

Ascitic fluid accumulated in the peritoneal cavity until the health of the mouse made fluid removal necessary, usually within 8 to 16 days. Further accumulation in 1 to 5 days made it necessary to remove the fluid a second time.

Occasionally, ascitic fluid was removed 3 times from an individual mouse. Each collection was clarified immediately by centrifugation at 2000 rpm (International centrifuge, size 2, head 831) for 10 minutes at room temperature. The supernatent fluid was stored at -20°C in screw cap tubes.

When death appeared imminent, blood was removed by cardiac puncture, using a 5 ml syringe with a 22 gauge needle. Generally, blood was removed after the second or third ascites collection. Occasionally, the poor health of a mouse made it necessary to remove blood after the first ascitic fluid collection. After clotting, serum was removed by centrifugation at 2000 rpm for 10 minutes and stored at -20°C.

Coxsackie A Virus Neutralization Test in Suckling Mice

1. Titration of Virus:
    a. A series of tubes containing 0.9 ml BSS in each was prepared.
    b. To tube 1, 0.1 ml of virus was added with a 1.0 ml serologic pipette. After aspirating and expelling 7 times, 0.1 ml of the mixture was transferred to tube 2. This was continued, serially, through the last tube. Separate pipettes were used for each dilution.

c. Three healthy, 6 to 18 hour old mice, were used for each dilution of virus. The inoculum was 0.03 ml intraperitoneally.

d. Mice were observed for 10 days.

The 50% minimal lethal dose (MLD_{50}) per 0.03 ml was calculated according to the method of Reed and Muench (1938), Table 1. Virus was diluted to contain 200 MLD_{50} doses per 0.03 ml for use in the neutralization test.

2. Dilutions of ascitic fluid or serum:

a. The ascitic fluid or serum was incubated at 56°C for 30 minutes.

b. To a series of 5 tubes, 0.9 ml BSS was placed in the first tube, 1.9 ml in the second tube and 0.5 ml in the other tubes.

c. To the first tube was added 0.1 ml of ascitic fluid or serum. Using a separate 1 ml serological pipette the mixture was aspirated and expelled 7 times and 0.1 ml was transferred to tube 2. After aspirating and expelling the contents of tube 2, 0.5 ml was transferred to tube 3. This procedure was continued through the fifth tube. The final dilutions were 1/10, 1/200, 1/400, 1/800, and 1/1600.

3. Neutralization Test:

a. To each of 3 tubes containing 0.2 ml of the 1/200, 1/400, 1/800 and 1/1600 dilutions,

respectively, of the ascitic fluid or serum, 0.2 ml of the virus suspension was added. The tubes were shaken vigorously to mix the ingredients and were then incubated at room temperature for 1 hour.

b. For each virus-ascitic fluid mixture and for virus controls, three 6 to 18 hour old mice were used. The virus-ascitic fluid inoculum was 0.06 ml per mouse. For the virus control 0.03 ml was used.

c. The mice were observed for either 10 days or 4 days after virus control mice developed paralysis.

d. As a control on the ascitic fluid an additional mouse received 0.03 ml of a 1/20 dilution of each ascites.

If 2 or more mice receiving end point dilutions died from non-specific causes the entire neutralization test was repeated. Serum and the ascitic fluid from the first, second and third collections were tested separately. One neutralizing unit was expressed as the reciprocal of the highest dilution of ascitic fluid which completely protected the mice against 100 MLD_{50} doses of virus.

4. Cross Reactions:

To determine antigenic relatedness of the virus prototypes, cross reaction tests were performed. A representative ascitic fluid from each group of mice inoculated with the prototype strains was used. Ascitic fluids from first collections that had the highest neutralizing titers were selected. Two pools of the 10 ascitic fluids were prepared with each pool containing 5 separate fluids diluted 1/4. The final dilution of each fluid was 1/20. Each pool was tested with 100 MLD_{50} doses per 0.03 ml of the 22 strains of Coxsackie A virus. A virus neutralized by a pool was then tested against each ascitic fluid appearing in that pool.

Preparation of Ascitic Fluid Pools:

Four anti-coxsackie A ascitic fluid pools, A to D, each containing 4 lots of ascitic fluids were prepared (Figure 1). Ascitic fluid of A 13, 15, 16 and 21 were distributed in pools A, B, C, and D, respectively. Ascitic fluid of A 17 through 20, A 22 and 24 were distributed so that each fluid was contained in 2 pools. Pool A containing A 13, 17, 20 and 22 ascitic fluids was prepared as follows: Each ascitic fluid was diluted with BSS to contain 80 neutralizing units. The 4 fluids were combined in equal proportions and shaken vigorously to mix. Final dilution

COXSACKIE A ANTIBODY TYPE										
ASCITIC FLUID POOL	A13	A15	A16	A21	A17	A18	A19	A20	A22	A24
A 13, 17 20, 22	+				+			+	+	
B 15, 17 18, 24		+			+	+				+
C 16, 18 19, 22			+			+	+		+	
D 21, 19 20, 24				+			+	+		+

Figure 1. Identification of Coxsackie A virus type by neutralization using pooled sera.

of each ascitic fluid represented 20 units. Pools B, C and D were prepared in the same manner.

Preparation of Seed Virus From Specimens Received From Other Laboratories.

Five cultures of Coxsackie A virus isolated from clinical cases were received: Coxsackie A 16 and 19, New York Department of Health, Albany, New York; and A 13, 16 and 20, California State Department of Public Health, Berkeley, California. The A 13 and 16 cultures from California were received as frozen extracellular fluid from infected cell cultures. All other cultures were received as frozen tissue specimens from infected suckling mice.

The tissue specimens were ground in BSS and prepared as inoculum for mice as described under B for preparation of seed virus. The extracellular fluid was inoculated without further processing.

RESULTS

Descriptive details of the collection of ascitic fluid from the several groups of mice at the 3 collection periods are presented in Table 2 and summarized in Table 3. Approximately 1200 ml of ascitic fluid was obtained from 149 mice at 256 collections. The average amount of fluid per mouse was greater at the first collection than at subsequent attempts. For all collections the average was 4.6 ml per mouse. The average yield of serum per bleeding from 77 mice was 0.6 ml per mouse.

Generally, neutralizing antibody titers of the first ascitic fluids collected were higher than for either the subsequent fluids or for serum (Table 4). The highest neutralizing titer of serum and ascitic fluid was 1600.

With A 21 virus, the antibody titer of 200 for the first lot of ascitic fluid and serum was too low for use in typing pools. An additional group of 5 mice was inoculated with a 30% suspension of suckling mouse torso. Ascitic fluid was collected only twice as the mice died before additional samples could be obtained. The antibody titers of the 2 fluid samples were 400 and 100, respectively.

The titer of A 16 ascitic fluid was 800 initially. When incorporated in pools, neutralization of the homologous

TABLE 2.--Yield of ascitic fluid from mice immunized against certain types of Coxsackie viruses and inoculated with S-180 cells.

Virus Type	Ascites Bleeding Number	Number of Mice	Ascites ml	Average per Mouse	Days After S-180
A 13	1	6	55	9.2	16
	2	9	39	4.3	20
	3	2	5	2.5	21
A 15	1	11	78	7.1	13
	2	8	4	0.5	14
A 16	1	17	56	3.3	12
	2	10	18	1.8	15
A 17	1	15	99	6.6	12
	2	5	39	7.8	14
A 18	1	13	50	3.8	13
	2	5	47	9.4	18
	3	1	6	6.0	19
A 19	1	18	36	2.0	8
	2	16	50	3.1	12
	3	10	32	3.2	15
A 20	1	19	93	4.9	10
	2	10	35	3.5	13
A 21	1	16	92	5.8	10
	2	9	61	6.8	13
A 21*	1	5	6	1.2	6
	2	3	11	3.7	11
A 22	1	15	103	6.9	10
	2	10	30	3.0	12
A 24	1	14	85	6.1	12
	2	9	58	6.4	15
TOTAL	25	256	1189		

*Second attempt at producing high titer ascitic fluid using a 30 per cent suckling mouse torso suspension, 5 mice immunized.

TABLE 3.--Yield of ascitic fluid and serum from mice.

Fluid Collection	Number of Mice	Total Fluid ml	Average Yield per Mouse
Serum 1	77	49	0.6
Ascitic Fluid 1	149	753	5.0
2	94	393	4.2
3	13	43	3.3
	256	1189	

Average ascitic fluid yield = 4.6 ml per mouse.

TABLE 4.--Neutralizing antibody titers of ascitic fluid and serum*.

Virus Type	Ascites Collection Number			Serum
	First	Second	Third	
A 13	800	200	400	1600
A 15	800	400	N.D.	400
A 16	<200	<200	N.D.	<200
A 17	800	800	N.D.	400
A 18	800	800	200	400
A 19	1600	1600	800	1600
A 20	800	200	N.D.	400
A 21	<200	<200	N.D.	<200
A 21**	400	100	N.D.	N.D.
A 22	800	400	N.D.	400
A 24	800	200	N.D.	400
AVERAGE	780	590	466	780

* Reciprocal of the test dilution.
** Designates titers obtained with second attempt at antibody preparation using a 30 per cent virus suspension.

N.D. - Not Done.

virus did not occur. The ascitic fluid and sera were retested at a 1/10 dilution with 3 strains of A 16 virus (CDC,New York, California). Only the New York strain was neutralized (Table 5). High antibody titer serum from monkeys inoculated with A 16 virus, obtained from the National Institutes of Health, Bethesda, Maryland, neutralized all 3 virus strains. The New York strain, however, had the greatest homogeneity. This monkey serum was therefore substituted for the ascitic fluid in the combination pools. No other ascitic fluid or serum decreased in antibody titer.

TABLE 5.--Neutralization titers at A 16 ascitic fluid and sera.

Virus Source	Ascitic Fluid	Mouse Serum	Monkey Serum
C.D.C.	<10	<10	200
New York	<10	10	1600
California	<10	<10	200

Antigenic relatedness of the 22 prototype strains of Coxsackie A viruses was determined against the 10 ascitic fluids. No heterologous antibody response was detected with the ascitic fluids when diluted 1/20 (Table 6).

The 4 combination pools were tested with 12 viruses, 8 of which were laboratory strains and 4 were wild strains isolated from the feces of humans from New York and California (Table 7).

TABLE 6.--Immune ascitic fluid neutralizing antibody titers against 22 Coxsackie A antigens.

Virus Type	IMMUNE ASCITIC FLUID A13	A15	A16*	A17	A18	A19	A20	A21	A22	A24
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0
13	800	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0
15	0	800	0	0	0	0	0	0	0	0
16	0	0	800	0	0	0	0	0	0	0
17	0	0	0	800	0	0	0	0	0	0
18	0	0	0	0	800	0	0	0	0	0
19	0	0	0	0	0	1600	0	0	0	0
20	0	0	0	0	0	0	800	0	0	0
21	0	0	0	0	0	0	0	400	0	0
22	0	0	0	0	0	0	0	0	800	0
24	0	0	0	0	0	0	0	0	0	800

0 = No neutralization at a 1/20 dilution of ascitic fluid.
* = Monkey serum received from CDC.

TABLE 7.--Viruses from clinical cases of Coxsackie A infection.

Source	Virus	Host	Generation	Titer
New York	A 16	SM*	First	5.24
	A 19	SM	Second	0.75
California	A 13	HFD**	N.I.++	No virus
	A 16	MK+	First	3.50
	A 20	SM	Eighth	3.24

*SM = Suckling Mice
**HFD = Human Fetal Diploid
+MK = Monkey Kidney
++N.I.= No Information

Extracellular fluid, presumed to contain A 13 virus (California) when inoculated into suckling mice, did not induce paralysis. The fluid was then inoculated into cultures of WI 38 human lung diploid cells and cultures of monkey kidney cells. No cytopathogenic change was observed in 10 days and the cells were discarded. All other viruses produced paralysis in suckling mice and seed virus for each was prepared as described under B. Infectivity titers of the seed viruses were high enough to prepare dilutions containing 100 MLD_{50} doses per 0.03 ml, except A 19 virus which had to be used undiluted.

All viruses tested against the 4 pools were identified by the combinations of pools neutralizing each virus. Only A 12 virus was not neutralized as no antibodies against this virus were in the pools (Table 8).

TABLE 8.--Neutralization pattern of Coxsackie A viruses tested against the 4 pools.

Virus Type	Source	Pools Neutralizing Virus A	B	C	D	Neutralization Pattern
A 12	CDC					None
A 13	CDC	X				A
A 15	CDC		X			B
A 16	NY			X		C
A 16	Calif.			X		C
A 17	CDC	X			X	AD
A 18	CDC	X	X			AB
A 19	NY		X	X		BC
A 20	Calif.			X	X	CD
A 21	CDC				X	D
A 22	CDC	X		X		AC
A 24	CDC		X		X	BD

DISCUSSION

The results indicate conclusively that ascitic fluid, stimulated by sarcoma 180 tumor cells, in mice immunized against Coxsackie A viruses, is an excellent substitute for mouse serum as a source of antibodies. The fluid is obtainable in large quantities. The neutralizing antibody titers are comparable to those of mouse serum and can be used reliably for identification of Coxsackie A viruses when combined in pools.

The availability of large volumes of ascitic fluid is of practical importance in preparing typing fluids. About 1 ml of serum is the maximum that can be obtained from a mouse. The 4.6 ml ascitic fluid harvested per mouse is about 7 times the average yield of serum.

The ascitic fluid accumulates in the peritoneal cavity of a mouse in response to an irritation of the peritoneum by sarcoma 180 cells, which increase about 1000 fold in 8 days. According to Drinker (1942) the blood supply in an injured or irritated region increases and arterioles and capillaries become overdilated allowing effusion of serous fluid. Removal of this fluid by the lymphatics is sluggish and further slowed by the formation of fibrin in the inflamed area, causing stagnation of the fluid.

As observed by Anacker and Munoz (1961) the ascitic fluid and serum of mice have similar electrophoretic patterns and almost identical antibody titers. Coxsackie A antibodies in the blood of a mouse before inoculation of the sarcoma cells, will be extruded into the peritoneal cavity as part of the serous fluid that responds to the irritated peritoneum.

The sarcoma cells contribute to the mortality of the mice. About 60% of the mice inoculated with sarcoma cells died during the course of the experiment. According to Goldie (1956), a high death rate may be the result of the pathological condition induced by malignant cells and metastasis.

The lower antibody titer in the second and third ascitic fluid collections, and in serum, is probably a reflection of a sampling error. Ascitic fluid was removed arbitrarily from groups of mice that had moderate to extensive abdominal distension. A large volume of low titer fluid from 1 mouse may have been added to a small volume of high titer fluid from another mouse, giving misleading titers when compared to sera which were removed in equal volumes.

One difficulty of preparing anti-viral serum is in the preparation of suitable antigens free of tissue elements and extraneous protein material. For the production of specific antibody for diagnostic and research work, especially where typing serum is required, antigen purification is

extremely important. Fortunately, the difficulty encountered with extraneous tissue elements has been overcome, in the present study, by using antigen prepared in suckling mice to be inoculated into adult mice.

The neutralization of viral infectivity by specific antibody is used as a practical means of identifying viruses. Like other serologic tests it is based on the antigen-antibody combination measurable by a definite reaction or response. Technics for performing the neutralization test have been modified for diagnostic purposes to permit large-scale examination of virus-antibody combinations.

The specificity of the neutralization test makes it particularly valuable in dealing with viruses in the entero-virus group, such as ECHO and Coxsackie where plurality of antigenic types are encountered. Some viruses in these groups possess antigenic components of immunologic specificity contained in the viral particle and specific antisera can be produced. When several sera are pooled the combined non-specific antibody becomes more evident. One solution to this problem is to dilute the sera to reduce the effect of the non-specific antibodies without significantly reducing the effect of the specific antibody. This principle was utilized in the present study, although little non-specific activity was present. For practical purposes the combined pools were rendered completely specific for Coxsackie A viruses.

Gamble et al. (1963) described a method of identifying Coxsackie A viruses by the complement fixation test using pooled ascitic fluid, but they resorted to individual fluid samples for identification of each sample. This technic has certain drawbacks. Potent complement fixing antigens and antibody are difficult to prepare. In addition, when an unknown virus is tested against a battery of antisera the absence of a positive response may be the result of low potency antigen rather than the absence of a homologous antibody. In the neutralization test, however, virus controls can be employed to indicate virus activity. The presence of a homologous antibody is quickly indicated by failure of the host to develop symptoms when inoculated with a virus-antibody mixture.

Identification of the 24 types of Coxsackie A viruses is a cumbersome task when individual antisera are tested. The pool scheme described in this study minimized the work involved in identifying 10 of these viruses by utilizing 4 pools of anti-coxsackie ascitic fluid. Neutralization of a virus when only one of the pools is tested has no significance. When 4 of the pools are tested, however, neutralization by 1 or 2 of the pools permits identification of a suspect Coxsackie A virus. This is accomplished by a process of elimination. For example, neutralization of a virus by pools A and D identifies the virus as A 17, since antibody against A 17 virus is in pool

A and D but not B and C (Figure 1). Likewise, neutralization by pool D only identifies a virus as A 21, since this is the only pool containing antibody against A 21 virus.

This type of pool scheme, for identification of 10 Coxsackie A viruses can be expanded to identify all types. The maximum number of virus types (N_v) that can be identified by N_p pools is determined by the equation:

$$N_v = \sum_{i=1}^{p-1} \frac{N_p!}{(N_p-N_i)!N_i!} = \sum_{i=1}^{p-1} C_{N_p}^{N_i}$$

where N_i = the number of types in each pool. All 24 serotypes can be determined by increasing the proposed scheme to 7 pools.

Few virus laboratories are equipped to identify Coxsackie A viruses. If immune sera were available commercially, the large number of suckling mice necessary to carry out a typing program would still present a major problem. By using combination pools, such as described here, diagnostic laboratories can offer a wider range of services for identification of Coxsackie A viruses.

SUMMARY

Relatively large volumes of ascitic fluid containing anti-coxsackie A antibodies were prepared. Adult mice, vaccinated with 4 weekly intraperitoneal injections of infected suckling mouse torso, were inoculated with sarcoma 180 tumor cells to stimulate the production of ascitic fluid.

An average of 4.6 ml of ascitic fluid was harvested per mouse in 256 collection periods. The average ascitic fluid yield per mouse was about 7 times the average serum yield.

Generally, antibody titers of first ascitic fluid collections were higher than subsequent collections. The highest titer of ascitic fluid and serum was 1600.

The anti-coxsackie A ascitic fluids were characterized by a strong homologous antibody content and a lack of heterologous antibody for other Coxsackie A viruses when used at a dilution of 1/20.

Four pools of anti-coxsackie A ascitic fluids were prepared. The 4 pools were used to identify 12 viruses from laboratory strains and from clinical infections. All viruses were neutralized by the pool containing the homologous

antibody. Identity was determined by the combinations of pools that neutralized each virus.

A pool scheme devised with type-specific fluids has proved to be a useful tool in typing Coxsackie A viruses and in reducing the time and materials required by other methods.

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