IMMUNOLOGICAL REACTIONS OF THE COXSACKIE VIRUSES

I. The Neutralization Test: Technic and Application*

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With the recognition that human infection with Coxsackie (C) viruses may be a relatively common occurrence, it is important to establish methods for determining the immunological reactions of this group of agents. Published work from other laboratories as well as our own has indicated that these agents produce serological responses similar to those of other viruses. Thus tests for neutralizing (1–5) and complement-fixing antibodies (4–8) have already been adapted to these newly discovered agents, but the details of the mechanics of these tests and their reliability have not as yet been described. It is the purpose of this series of three papers to describe in some detail immunological reactions of the C viruses. The first and present paper deals with the neutralization test, the second (9) with the complement fixation test, and the third (10) with cross-protection tests.

Materials and Methods

Virus Suspensions.—Various strains of C virus, isolated from patients, sewage, or flies, were employed usually as muscle-bone suspensions of infected mice. Mice used for production of virus were inoculated within 48 hours of birth either intraperitoneally or subcutaneously, and subsequently sacrificed on the 1st day of illness. Dead mice were discarded and never included in the harvest. In order to accumulate sufficient numbers for harvest, sick mice were etherized and stored frozen at −20°C. These mice were later thawed, washed with ether, and then the head, hands, feet, viscera, and skin removed. The carcasses were again washed with ether and then blended for a few minutes with cold water to make a 10 per cent suspension. The suspension was centrifuged at 2500 R.P.M. for 10 minutes and the supernate distributed in corked or screw-capped tubes and stored at −20°C. Before use a sample tube was thawed, and centrifuged again at 2500 R.P.M. for 5 minutes.

In a few early experiments, brains were used as a source of virus, but this tissue was abandoned in favor of muscle-bone when the latter was found to contain higher concentrations of the virus.

Although no demonstrable protection of the virus could be detected when 1 or 10 per cent normal horse serum was used as a diluent rather than water or saline, 1 per cent horse serum was generally used as a diluent for virus. In making the first dilution to 10^{-2} concentration of infected tissue, a solution containing 20,000 units of penicillin and 100 mg. of streptomycin

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per ml. was added to the diluent, so that the 10^{-2} virus suspension contained 1000 units of penicillin and 5 mg. of streptomycin per ml.

Serum.—Human sera were usually frozen at −20°C. soon after collection; although a few samples were kept at 2°C. without any observed deleterious effect on the antibody. These were obtained from normal people, or as paired samples from patients.

Hyperimmune sera were prepared in 2- to 4-week-old albino mice by intraperitoneal inoculation with 10 per cent suspension of infected murine muscle-bone (or in some instances infected brain). The recommended course was 6 inoculations consisting of 0.1 ml. each twice weekly for 3 weeks. Mice were bled by cardiac puncture 8 to 10 days later. Booster inoculations were given about every 4 or 5 weeks followed by a serum harvest in 8 to 10 days. A similar course was used for preparing serum in hamsters except that infected baby hamster tissue was used as the vaccine.

Monkeys belonging to the following species were used, and all responded with satisfactory antibody development when subjected to the indicated course of vaccination: rhesus (*Macaca mulatta*), cynomolgus (*Macaca irus*, *Macaca cynomolgus*), green (*Cercopithecus aethiops sabaeus*), and sooty mangabey (*Cercocebus fuliginosus*). However, following oral administration of virus, cynomolgus monkeys responded preferentially (11). With these animals a series of 3 to 4 weekly inoculations (each being 2 to 5 ml. of 10^{-1} infected murine tissues) were performed dividing each inoculum intraperitoneally, intramuscularly, and intracutaneously. Animals were given booster inoculations at intervals of 4 to 6 weeks. Serum was collected 8 to 10 days after vaccination. All animal sera were stored frozen.

Method of Carrying Out the Test.—In all instances, serum dilutions (made with saline or 1 per cent horse serum as diluent) were added to the test tubes (10 × 75 mm.) before the addition of virus. The volumes used were 0.2 ml. of each reagent, which were well shaken following their mixture. Incubation was varied as indicated, although the standard test now in use employs a period of 1 hour at room temperature.

Test Mice.—Newborn animals in groups of 8 to 1 mother were used. These animals were usually less than 24 hours and almost never more than 48 hours old. Pregnant albino mice (and on occasion "chocolate" mice) were obtained from four different dealers, and no apparent difference in susceptibility of their young was noticed. The young from several mothers were often mixed and redistributed at random with 8 mice put in the care of each mother. Following inoculation of the serum-virus mixtures, mice were observed daily for 10 to 14 days. Virulent strains of the Texas type have short incubation periods and such tests can be terminated safely by the 10th day. Mice which died within 2 days of the inoculation and mice which disappeared (cannibalism) were not included in the tabulations. Groups of mice in which all died without signs of paralysis being observed in any, were considered unsatisfactory and tests performed with such groups were repeated.

Mice were inoculated with 0.02 ml. either intraperitoneally or subcutaneously (between the shoulders where the skin is loose). It is felt that the latter route is more satisfactory from the standpoint of less leakage of the inoculum.

Calculation of Results.—The Reed-Muench method was used to calculate all 50 per cent end-points, and was used in computing the neutralization index when varying doses of virus were employed. The neutralization index equals the difference in titer between that in the presence of control normal horse serum and the test serum.

The results of serum neutralization tests with varying dilutions of serum against a fixed dose of virus (about 100 ID_{50}) were generally, for a single dilution of serum, either all-or-none neutralization. Serum titers are therefore given as the greatest serum dilution tested which neutralized the virus.

EXPERIMENTAL

Stability of the Virus.—Earlier results in which four strains (Texas-1, High Point, Conn.-5, Ohio-1) in 50 per cent glycerol were found to survive a trip

to Sweden at "room" temperature gave some indication of the stability of the C viruses. To compare the stability under different temperatures the following experiments were carried out.

Brains and muscle were harvested from infected mice (Conn.-5, High Point, and Texas-1 strains). Portions from each mouse were cut into small pieces and placed in 50 per cent glycerol. The remainder was ground with water to make a 10 per cent suspension. Part of this was added to an equal volume of undiluted normal monkey serum, and part was frozen without addition of serum at −20°C. Tubes containing the infected tissue in glycerol and in serum were stored at 2°C. and some at 25°C. and aliquots removed for titration at intervals shown in Table I.

TABLE I

Stability of C Viruses at 25°, 2°, and −20°

Negative log of ID_{50}

Strain	Days	Infected mouse brain					Infected mouse muscle				
		50 per cent glycerol		50 per cent serum		Water	50 per cent glycerol		50 per cent serum		Water
		25°	2°	25°	2°	−20°	25°	2°	25°	2°	−20°
Conn.-5 m_6*	0	5.0	5.0	5.0	5.0	5.0	5.8	5.8	5.8	5.8	5.8
	6	>3.0	4.0	2.9	>3.0		4.0	5.3	>2.0	5.2	
	26	1.7		2.1		5.0	3.3		3.1		6.0
	421		3.0					5.0			
Hi.Pt. m_7	0	5.4	5.4	5.4	5.4	5.4	7.5	7.5	7.5	7.5	7.5
	9	>4.0	>5.0	>3.0	>4.0		>5.0	>6.0	>3.0	>5.0	
	56	0		>1.0	>2.0	5.5	2.6		1.7	>3.0	7.0
	427		3.9					6.9			
Texas-1 m_5	0	4.7	4.7	4.7	4.7	4.7	8.0	8.0	8.0	8.0	8.0
	15	>3.0	4.3	>3.0	5.3		>3.0	>7.0	>4.0	>7.0	
	70	0		0		4.5	4.0		>1.0		7.5
	428		2.2					6.5			

* m_6 indicates the 6th passage of the strain in mice.

The virus was found to be stable and retain its titer for several months when frozen at −20°C. When a virus-rich tissue such as muscle was used, virus was still present when stored at 25°C. for 70 days, although the titer decreased by several logs. In glycerol at 2°, the virus, particularly in fragments of infected muscle, decreased only slightly in titer when stored for over 1 year. For work with viruses of known titer, it is now our practice to make up a large batch of virus suspension as 10 per cent suspension of carcass, distribute it in 1 ml. amounts, and freeze these tubes at −20°C. until needed.

Effect of Time, Temperature, and Diluent on Virus Titration End-Points.— To determine the effect of time, temperature, and diluent on the end-point

of a virus titration, the High Point strain (9th mouse passage) was diluted both in saline, and in normal, inactivated 10 per cent horse serum to give the following dilutions: 10^{-6}, 10^{-7}, and 10^{-8}. Aliquots of these dilutions were kept (*a*) at room temperature for 1 hour; (*b*) at 37°C. for 2 hours; (*c*) at room temperature for 1 hour, followed by incubation at 4°C. for 20 hours; and (*d*) at room temperature for 1 hour, followed by incubation at −20°C. for 96 hours. The results shown in Table II indicate that the virus is stable in dilute suspensions (10^{-6} and 10^{-7}) under all the conditions tested. The use of either saline or 10 per cent normal horse serum gave comparable virus titers. Similar results have been obtained with several strains.

TABLE II

Effect of Time, Temperature, and Diluent upon Virus Titrations

Temperature	Time	Concentration of Hi.Pt. virus diluted in saline				Concentration of Hi. Pt. virus diluted in 10 per cent normal horse serum			
		10^{-6}	10^{-7}	10^{-8}	Titer Negative log of ID_{50}	10^{-6}	10^{-7}	10^{-8}	Titer Negative log of ID_{50}
	hrs.								
Room	1	8/8(3)‡	4/6(2)	1/8(0)	7.3	6/8(4)	3/8(0)	0/6	6.6
37°	2	7/8(3)	6/9(5)		7.2				
4°	20*	6/8(4)	2/6(2)		6.5				
−20°	96*	8/8(8)	6/8(2)		7.3	8/8(1)	4/8(2)		7.0

* Prior incubation at room temperature for 1 hour.

‡ Denominator indicates number of mice inoculated, and numerator, mice which succumb to disease with paralysis and/or death. The number in parenthesis indicates mice of the group with observed paralysis.

Effect of Time and Temperature on Serum Neutralization End-Points.—Because the time necessary to inoculate a large series makes it impossible to hold each virus-serum mixture for the same period of time, it was important to determine to what extent this variable influences the neutralization test.

The effect of time and temperature on serum neutralization end-points was investigated by incubating aliquots of various dilutions of 3 immune sera with their homologous viruses (100 ID_{50} doses of each were used) under the following conditions: (*a*) at room temperature for 10 minutes; (*b*) at room temperature for 1 hour; (*c*) at 37°C. for 2 hours; (*d*) at room temperature for 1 hour, followed by storage at −20°C. for 4 days; and (*e*) at room temperature for 1 hour followed by storage at −20°C. for 14 days.

From the results of Table III, it is seen that human serum (NC-77B) heated at 56°C. for 30 minutes, and unheated chimpanzee serum (Becky October 4, 1949) gave the same serum end-points (the maximum dilution to give complete protection) when incubated at room temperature for 10 minutes as when

incubated at room temperature for 1 hour, followed by incubation at −20°C. for 4 days. The Easton-20 acute and convalescent serum end-points were the same when aliquots were incubated at room temperature for 1 hour, and at room temperature for 1 hour followed by incubation at −20°C. for 14 days. Thus, the serum neutralization end-points were the same, when the same serum was compared under varying conditions of incubation. A similar pattern was obtained with other strains, in which the incubation period was allowed to vary between 25 minutes and 3 hours at room temperature. These experiments illustrate the fact that neutralized virus mixtures can be stored and tested at a later date to yield the same results as freshly mixed serum and virus. At the time of carrying out a neutralization test, an aliquot of each virus-serum

TABLE III

Effect of Time and Temperature on Serum Neutralization Titer

Human serum NC-77B* (heated at 56° for 30 min.)						Chimpanzee Becky (10/4/49)* (unheated, post C virus infection)				Easton-20 serum‡						
										Acute serum			Convalescent serum			
Temperature	Time	1:10	1:100	1:1000	Serum titer	1:10	1:100	1:1000	Serum titer	1:10	1:50	Serum titer	1:10	1:50	1:250	Serum titer
Room	10 min.	0/8	2/6(0)	7/7(3)	100	1/8(0)	1/9(0)	8/8(8)	100							
Room	1 hr.	0/5	Inc.	7/7(1)	>10	Inc.	0/7	8/8(1)	100	8/8(7)	8/8(7)	<10	0/8	2/6(0)	0/8	>250
37°	2 hrs.	0/9	0/5	8/8(3)	100	2/8(0)	0/6	8/8(3)	100							
−20°	4 day§	0/8	2/6(0)	7/7(3)	100	0/8	0/7	8/8(3)	100							
−20°	14 days§									7/7(6)	8/8(6)	<10	0/7	0/8	0/8	>250

Inc. = incomplete test. Mice did not survive 2 days after inoculation.

* 100 ID_{50} Hi.Pt. strain added.

‡ 100 ID_{50} Easton-2 strain added.

§ Held at room temperature for 1 hour before being frozen.

mixture may be frozen away to allow for the replacement of those groups of mice which fail to survive for 2 days after inoculation, or which turn out to be unsatisfactory for some other reason.

When the neutralization index is obtained by mixing varying dilutions of virus with an undiluted serum, the virus in the mixture remains neutralized when frozen at −20°C. for several days. In the presence of normal serum, the virus titer is not affected by the freezing. A typical experiment yielding these results, which are shown in Table IV, follows:—

The NHF 1943 strain (2nd mouse passage) was serially diluted and aliquots mixed with an equal volume of NHF immune serum, and as controls, additional samples were mixed with normal inactivated horse serum. Aliquots of the mixtures were incubated at room temperature for 1 hour; and at room temperature for 1 hour, followed by incubation at −20°C. for 5 days. The mixtures with normal horse serum gave the same 50 per cent virus end-point under both of these conditions of incubation ($10^{-8.1}$ and $10^{-8.2}$). Similarly, the 50 per cent

virus end-point was the same, under these conditions, in the presence of the undiluted immune serum ($< 10^{-2}$). The difference between the negative logarithms of the 50 per cent virus end-points obtained in immune and normal serum was > 6.1 when incubated at room temperature for 1 hour, and > 6.2 when incubated at room temperature for 1 hour, followed by incubation at $-20°$C. for 5 days. Similar results have been obtained on the stability of other strains under these conditions.

TABLE IV

Effect of Time and Temperature on the Neutralization Index

Temperature	Time	NHF-43m_2 virus												Log neutralization index
		Plus immune serum						Plus normal inactivated serum						
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titer Negative log of ID_{50}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer Negative log of ID_{50}	
	hrs.													
Room	1	0/8	0/6	0/8	0/8	0/8	<2	8/8(8)	7/7(7)	8/8(8)	4/8(3)	1/8(0)	8.1	>6.1
−20°	120	0/8	0/8	0/6	0/5	0/8	<2	7/7(7)	8/8(7)	6/6(5)	5/8(2)	0/8	8.2	>6.2

TABLE V

Effect of Route of Inoculation on Serum Neutralization Titer

Route of inoculation	Hi.Pt. strain at concentration of 10^{-5} plus Human serum NC-77B (final dilution)			Serum titer
	1:10	1:100	1:1000	
Intraabdominal	2/5(0)	2/5(0)	16/16(7)	100
Subcutaneous	0/8	2/8(2)	6/6(2)	100

	Control horse serum (1:10) plus Hi.Pt. virus at concentration of				Virus titer Negative log of ID_{50}
	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
Intraabdominal	8/8(7)	4/5(0)	3/9(3)	0/8	7.7
Subcutaneous	8/8(8)	8/8(7)	7/9(6)	0/8	8.3

Effect of the Route of Inoculation on Serum Neutralization and Virus End-Points.—It appears from Table V that equally good neutralization can be demonstrated if the serum-virus mixtures are inoculated intraperitoneally or subcutaneously. Human serum NC-77B, when mixed with approximately 100 ID_{50} of the High Point strain gave a serum titer of 100 when injected by either route. The subcutaneous route of injection gives occasional virus titers 1 log higher than by the intraperitoneal route, particularly with the Ohio strain (Table VI). In view of this fact, and also because the inoculum is less apt to leak following the latter route of injection, we use as a routine the subcutaneous route for injections.

Effect of Complement.—To determine whether complement played a role in the *in vitro* phase of the neutralization test, the following experiment was conducted.

Convalescent serum obtained from a patient 1 month after an acute illness characterized by fever, weakness, and abdominal pain and by isolation of a Conn.-5 type of C virus was used. Preliminary results using undiluted sera and serial tenfold dilutions showed that serum taken 1 month before illness contained no detectable antibodies to the Conn.-5 strain, whereas

TABLE VI

Effect of Route of Inoculation on Virus End-Point Titer

Route of inoculation	Negative log of ID_{50} of following virus strains			
	Texas-1	Hi.Pt.	Conn.-5	Ohio-1
Intraabdominal	(*a*) 7.4 (*b*) 7.9	7.7	5.9	(*a*) 5.9 (*b*) 5.4
Subcutaneous	(*a*) 7.8 (*b*) 7.9	8.3	6.7	(*a*)7.2 (*b*)6.5

TABLE VII

Lack of Effect of Complement in C Virus Neutralization Test

100 ID_{50} doses of Conn.-5 strain were present in each serum dilution.

Convalescent human serum	Final dilution of human serum (Virus suspended in unheated guinea pig serum)				Serum titer
	1:16	1:64	1:256	1:1024	
Unheated	0/7	0/8	0/10	8/9(6)	256
Heated at 56° for 30 min.	0/8	0/8	2/8(0)	8/9(5)	256
	(Virus suspended in heated guinea pig serum)				
Unheated	1/9(0)	1/8(0)	1/7(0)	6/8(4)	256
Heated at 56° for 30 min.	0/9	0/9	0/6	6/6(2)	256

serum drawn 6 weeks after illness neutralized 4 logs of virus (neutralization index of 10,000). The following experiment was carried out with the convalescent serum.

The Conn.-5 strain was diluted to 2×10^{-4} concentration using as a diluent fresh guinea pig serum for one sample and heat-inactivated (56° for 30 minutes) guinea pig serum for the second. Equal volumes of these virus dilutions were mixed respectively with equal volumes of varying dilutions of the unheated and heated (56° for 30 minutes) convalescent serum. After an incubation period of 1 hour at room temperature, the mixtures were tested for infectivity as shown in Table VII.

It can be seen that complement had no effect on the serum neutralization titer, which was found to be 256 in the absence as well as in the presence of complement.

Effect of Heat on Neutralizing Antibodies.—To determine whether or not the virus-neutralizing substances present in human serum were heat-labile, titrations of various sera, before and after heating, were carried out against different strains of the virus. First a series of tests were performed in which several posi-

TABLE VIII

Resistance of C Virus Neutralizing Antibodies in Human Sera to 56°C. for 30 Minutes

Patient	Area	Date	Strain	Titer against 100 ID_{50}	
				Unheated	56° for 30 min.
Cla.	Maryland	11/10/47	Hi.Pt.	100	100
		1/12/48		10	10
Fre.	Maryland	10/21/47	Hi.Pt.	100	100
		12/ 4/47		<100	100
Mel.	Maryland	10/31/47	Hi.Pt.	10	10
		12/ 4/47		10	10
Bow.	Ohio	9/18/47	Ohio-1	50	250
Cur.	Ohio	9/17/47	Ohio-1	250	250
Bru.	Ohio	8/28/47	Ohio-1	50	50
		9/24/47		>250	1250
Fru.	Ohio	8/24/47	Ohio-1	50	50
Hud.	Ohio	8/25/47	Ohio-1	250	250
		9/22/47		250	250
Mac.	Ohio	8/25/47	Ohio-1	0	0
		9/22/47		0	0
Sha.	Conn.	2/21/49	Conn.-5	250	250
Bot.	Conn.	8/25/48	Conn.-5	0	0
		1/ 8/49		100	100

tive human sera were titrated against 100 ID_{50} doses of the High Point and Ohio strains of virus, before and after they were heated at 56°C. for 30 minutes. Non-specific inhibitors to a variety of viral agents have been shown to be destroyed at this temperature (12, 13). The results shown in Table VIII indicate that the neutralizing antibody to C virus in human sera is stable at 56° C. for 30 minutes.

Further experiments were conducted to determine the heat lability of the neutralizing antibody.

For this purpose, convalescent sera were selected from patients from whom C virus had been recovered during their acute illness, using in the neutralization tests a strain of virus related to that isolated from the patient. In addition, a chimpanzee serum was studied, obtained from an animal following three serial infections with three different immunological

TABLE IX

Heat Inactivation of Neutralizing Antibody to Four Immunological Types of C Virus

Virus*	Serum and dilution‡	Serum held for 30 min. at following temperatures							Titer of unheated serum	Maximum temperature to maintain	
		Unheated	56°	60°	65°	70°	75°	80°		Full titer	Some activity
Easton-2	Patient AG 12/7/49								40	>56°	<70°
	1:8	0/8	0/8			8/8(8)	8/8(8)	8/8(8)			
	1:40	0/8	0/8			5/5(2)	6/6(6)				
	1:200	8/8(8)	8/8(8)			8/8(8)					
	Patient BA 10/5/49								>400	65°	75°
	1:4	0/8	0/8	1/7(0)	0/8	0/8	0/8	8/8(8)			
	1:40	0/8	0/8	1/7(0)	0/8	0/8	5/7(5)				
	1:400	0/6	0/8	0/8	0/6	8/8(2)					
Texas-1	Patient G.J. 8/9/49								>200	>56°	70°
	1:8	0/8	0/8			0/8	8/8(5)	8/8(6)			
	1:40	0/8	0/8			8/8(1)					
	1:200	0/8	0/8			7/7(3)					
	Chimp. Donna 12/20/49								120	70°	70°
	1:12	0/8	0/8	0/8	0/8	1/7(0)	8/8(8)	9/9(3)			
	1:120	0/8	0/8	0/8	0/7	0/8	9/9(9)				
	1:1200	8/8(4)	8/8(2)	6/6(6)	7/7(5)	8/8(1)					
Conn.-5	Chimp. Donna 12/20/49								120	65°	70°
	1:12	0/8	0/8	0/9	0/4	0/7	5/6(1)	6/6(5)			
	1:120	0/8	0/8	1/7(0)	1/5(1)	4/8(3)	9/9(5)				
	1:1200	8/8(4)	7/7(1)	7/7(1)	8/8(3)	8/8(3)					
Ohio-1	Chimp. Donna 12/20/49								120	65°	70°
	1:12	0/8	0/8	Inc.	0/8	2/8(1)	7/7(4)	8/8(7)			
	1:120	0/8	0/8	0/7	1/8(1)	8/8(2)	8/8(6)				
	1:1200	8/8(4)	8/8(1)	8/8(2)	8/8(2)	8/8(2)					

* 100 ID_{50} of each virus, as indicated, was used in each serum mixture.

‡ Patients AG and BA had been infected with Easton-2 virus in September, 1949.

Patient GJ had been infected with Texas-1 virus in July, 1949. Chimp. Donna had been infected with Conn.-5 virus in March, 1949; with Texas-1 virus in May, 1949; and with Ohio-1 virus in October, 1949.

types of C virus (Texas-1, Conn.-5, and Ohio-1). The heat stability of the neutralizing antibody to each strain in this serum containing multiple antibodies, as well as in the convalescent human sera, was studied by heating the sera for 30 minutes at the following temperatures: 56°, 60°, 65°, 70°, 75°, and 80°C. Then the serum titer of the unheated serum was compared to that obtained with the heated serum, using 100 ID_{50} doses of virus.

The results in Table IX illustrate the stability of the neutralizing antibody to four immunological types of virus. With low titered sera (1:40 or less), the

titer was maintained at 56°C.; all detectable antibody was destroyed at temperatures at 70° (or less). With sera having titers above 1:120, the antibody was not altered at 65° and had to be heated to 75°, and in two instances (only one is illustrated in the table) in which the serum titers were above 1:400, the serum had to be heated to 80° before the antibody was no longer detectable.

TABLE X

Effect of Virus Concentration upon Serum Titer

Serum	Virus	ID_{50} of virus negative log	Serum titers at following virus dilutions						Log serum titers with following logs of virus								
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5
Convalescent human	E-2 m_2	7.0	50	250	1250	1250	>1250		1.7		2.4		3.1		3.1		>3.1	
Convalescent human	Conn.-5 m_7	4.0	100	1000											2		3	
Convalescent mouse	NHF-'43 m_1	6.5			100	100	1000							2		2		3
Convalescent mouse	NHF-'43 m_4	8.5			<10	10	100	100		<1		1		2		2		
Convalescent mouse	Texas-1 m_4	8.0				10	10	100					1		1		2	
Hyperimmune mouse	Conn.-5 m_7	6.5	>1000	>1000						>3		>3						
Hyperimmune hamster	Conn-5 m_7	6.5	100	1000						2		3						
Hyperimmune	Conn.-5 m_7	6.5	10	100						1		2						

Quantitative Antibody-Virus Relationship.—To determine this relationship, different series of neutralization tests were performed with human, mouse, monkey, and hamster anti-sera with their homologous C virus strains. Both the quantity of serum and the quantity of virus were varied with respect to each other in order that the quantity of serum required to neutralize each dilution of virus could be determined. The quantity of virus neutralized by the various amounts of serum in a particular series was calculated from the virus titration, which was done at the same time.

On the basis of results in Table X, it appears that the neutralization of C virus strains does not give a straight line relationship between serum titer and

quantity of virus neutralized. However, as the quantity of virus is increased, the quantity of serum required for neutralization likewise increases, but for a uniform increase in the quantity of virus, the serum titer was not found to decrease in similar uniform fashion, as has been reported for other viruses (14, 15).

Dissociation of the Virus-Antibody Complex.—To determine whether neutralized mixtures of the virus could be made infective by dilution, as was first shown by Andrewes for the pox viruses (16), the following experiments were performed.

TABLE XI

Effect of Dilution on Dissociation of Antigen-Antibody Complex

Concentration of infected tissue	Texas-1 strain plus Texas immune serum*	Texas-1 strain plus saline
10^{-1}	0/40	
10^{-2}	0/24	
10^{-3}	16/32(16)	
10^{-4}	29/32(12)	8/8(8)
10^{-5}	18/32(13)	8/8(3)
10^{-6}	1/29(0)	16/16(8)
10^{-7}	1/29(0)	12/16(6)
10^{-8}	0/32	1/16(0)

* A 10 per cent suspension of carcass infected with the Texas-1 strain was incubated with undiluted homologous Texas antiserum for 1 hour at room temperature and the virus-serum then diluted. The original mixture is designated at 10^{-1}.

A 10 per cent suspension of carcasses infected with the Texas-1 strain was mixed with an equal amount of undiluted Texas monkey hyperimmune serum and incubated for 1 hour at room temperature. This was designated as 10^{-1}, and the mixture was then diluted with saline in a series of tenfold dilutions to 10^{-8}. Each dilution was injected into infant mice. At the same time, the virus suspension was incubated with saline under the same conditions, diluted, and 10^{-4} to 10^{-8} concentrations of this control injected. Previous experience showed that higher concentrations of the virus were invariably fatal for mice. The results of two duplicate experiments have been combined and are listed in Table XI.

Complete protection of the 10^{-1} virus was afforded by undiluted serum and the mixture remained non-infective following a tenfold dilution. However, the virus was infective when the mixture was diluted 100, 1000, and 10,000 times designated as 10^{-3}, 10^{-4}, and 10^{-5} concentrations of virus in the table. At the 10^{-6} and 10^{-7} concentrations, containing 20 and 2 ID_{50} doses of virus respectively, and serum concentrations of 10^{-5} and 10^{-6}, the virus was again non-infective. It would appear that at this level of virus, traces of antibody were sufficient to neutralize again this small amount of virus, or that two logs of virus had been destroyed. By titrating the virus in the presence of tenfold

dilutions of serum such as were present in the diluted mixture, (*i.e.*, 10^{-5} infected tissue plus an equal volume of 10^{-4} immune serum, 10^{-6} infected tissue plus 10^{-5} serum, 10^{-7} infected tissue plus 10^{-6} serum, etc.), it was shown that such traces of serum were incapable of influencing the virus titer. Thus although neutralized virus-serum mixtures may be reactivated by simple dilution, some of the virus is rendered irreversibly non-infective.

Antigenic Typing of Strains.—Specific neutralizing antibody is one of the most widely used reagents for identifying different antigenic types of a virus, and for the C group of viruses this statement also holds true. For strains be-

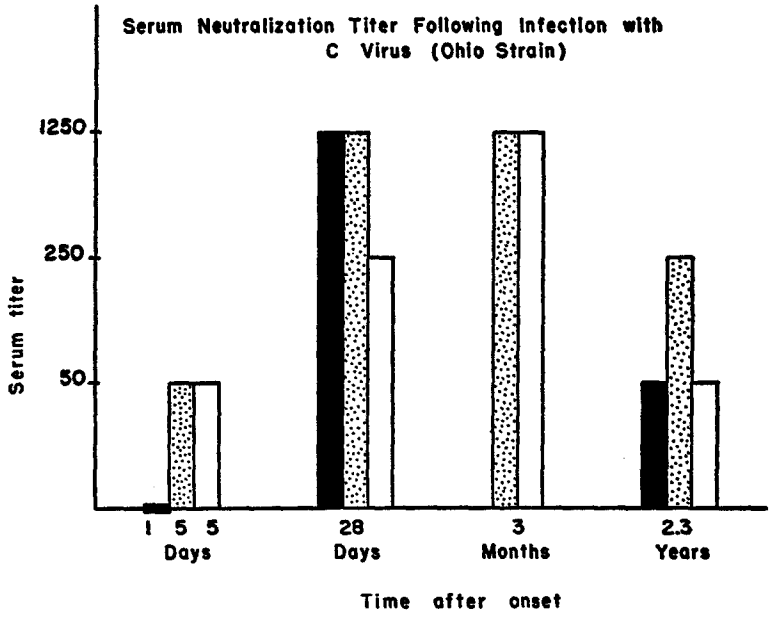

FIG. 1. Duration of antibodies following infection with the Ohio-1 strain. Three patients are represented on the chart by the black, dotted, and white columns respectively. Virus was isolated from each patient at the time the first serum samples were obtained.

longing to one antigenic type, such a relationship may be established either by using a typed virus with a serum to an unknown type or *vice versa*. Two experiments with strains isolated in two epidemic areas (Ohio, 1947, and Easton, Pennsylvania, 1949) serve to illustrate the reciprocal crossing between strains belonging to a single type. The results, listed in Table XII, illustrate that strains isolated from different patients in an area may belong to a single type, but that the two types shown differ from each other.

Further experiments in strain classification in which varying dilutions of virus have been set up against undiluted immune sera are summarized in Table XIII. We now recognize at least seven different immunological types.[1]

[1] Dr. Gilbert Dalldorf kindly supplied us with his 3 antigenic types referred to in Tables XIII and XV.

Antibody Development in Relation to Human Disease.—The available evidence suggests that neutralizing antibodies to different strains of C virus develop early in the course of the disease (1, 4). Further information on the specificity of the neutralizing antibody response has been obtained from an extensive

TABLE XII

Cross-Reactions of Homotypic Strains

Virus	Control ID_{50}	Log of neutralization index of immune serum					
		Ohio-1	Ohio-2	Ohio-3	Easton-2	Easton-6	Easton-18
Ohio-1	$10^{-5.2}$	>4.2	>4.2	>4.2	0		
Ohio-2	$10^{-4.4}$	>3.4	>3.4	>3.4			
Ohio-3	$10^{-4.7}$		>3.7	>3.7			
Easton-2	$10^{-6.5}$	0			>4.5	>4.5	>4.5
Easton-6	$10^{-6.0}$				>4.0	>4.0	
Easton-18	$10^{-6.3}$				>4.3		>4.3

TABLE XIII

Cross-Neutralization of Strains of C Virus and Demonstration of Seven Antigenic Types

Virus	Control ID_{50}	Immune serum: Log of neutralization index								
		Conn.	Ohio	Texas	Hi.Pt.	Easton	D-1	D-2	D-3	Nancy
Conn.-5	$10^{-5.7}$	>4.7	0	0	0	0	0	0	0	0
Ohio-1	$10^{-5.3}$	0	>4.2	0	0	0	0	0	0	
Texas-1	$10^{-7.0}$	0	0	6.0	5.0	0	0	0	0	
Hi.Pt.	$10^{-6.5}$	0	0	4.6	5.0	0	0	0	0	
NHF-'43	$10^{-7.5}$	0	0	6.0	6.0	0	0	0	0	
Easton-2	$10^{-6.0}$	0	0	0	0	5.0	5.0	0	0	
NY-5	$10^{-5.5}$	0	0	0	0	>4.5	>4.5	0	0	
Dalldorf 1	$10^{-6.0}$	0	0	0	0	5.0	5.0	0	0	
Dalldorf 2	10^{-5}	0	0	0	0	0	0	>4	0	
Dalldorf 3	10^{-5}	0	0	0	0	0	0	0	>4	
Nancy	10^{-6}	0	0	0	0	0	0	0	0	5.0

study of the occurrence and development of antibodies in 6 patients who contracted infections with one or another of the C viruses while working with them in the laboratory (17).

From each patient a virus was isolated during the acute phase of illness which was typed by following the reactions with specific hyperimmune animal sera (see bottom three rows of Table XIV). The data in Table XIV also show that no patient before his illness had detectable antibodies to the strain subsequently isolated from him or to the prototype strain. But soon after illness, there developed simultaneously antibodies to the strain isolated from the patient and to the prototype strain to which it was related.

The reactions of the sera of the laboratory patients with strains not related to the one iso-

TABLE XIV

Antibody Response in 6 Patients Infected While Working with C Viruses

Patient and date of onset	Serum	Serum titer obtained against 100 ID_{50} of virus								
		Virus strain used in neutralization test								
		ES m_1*	JLM m_1	NL m_1	LK m_1	FL m_1	GJ m_1	Conn.-5 m_5	Texas-1 m_4	Ohio m_2
EWS	1/11/49	0						0	0	0
1/9/49	2/21/49	50						100	100	0
	3/25/49	250						100		0
JLM	11/29/48		0					0	0	100
5/16/49	2/21/49		0					0	100	10
	5/18/49		0					0	100	100
	5/26/49		250					100	100	1000
	6/12/49		1250					100	100	100
	10/20/49							100	100	100
NL	2/10/49			0				0	0	100
5/21/49	5/26/49			1250				100	100	100
	11/ 2/49							1000	100	100
LMK	3/25/49				0			10	10	0
10/20/49	5/24/49				0			10	1000	0
	10/28/49				100			1000	100	100
	12/27/49							100		1000
FL	6/15/49					0		0	0	0
7/16/49	7/25/49					100		0	1000	0
GJ	6/15/49						0	0	0	0
7/6/49	7/25/49						250	0	100	0
Animal immune	Conn, mouse 10/20/49	>100	>100	>100	0	0	0	1000	0	0
	Texas, mouse 10/22/49	0	0	0	0	>100	>100	0	1000	0
	Ohio, 4332 8/11/49	0	0	0	>100	0	0	0	0	1000

* Subscripts after letter m indicate the number of passages the virus had undergone in mice before it was used in the neutralization tests.

lated from each patient are instructive. Only 2 of the 6 patients had an illness due to Texas type infection, yet the other 4 must have had a subclinical infection with this type. The results with the serial samples of blood taken from patient JLM starting several months before

his illness emphasize this point: On November 29, 1948, antibodies were absent to both the Conn. and Texas types. By February 21, 1949, antibodies to the Texas type had appeared in titer of 1:100 and this serum level was maintained in the next 4 bleedings carried out in May, June, and October of that year. The patient became ill (diagnosis compatible with epidemic pleurodynia or myalgia) on May 16 at a time when he had no antibodies to his own strain or to the Conn. prototype. By May 26, such antibodies were present.

TABLE XV

Family Infection with Two Types of Coxsackie Virus

Serum		Serum titer obtained agianst 100 ID_{50} of virus					
		JLM m_1	NM m_1	SM m_1	Conn.-5 m_6	Texas-1 m_4	Ohio-1 m_3
JLM*	2/21/49	0	0	0	0	100	10
	5/18/49	0	0	0	0	100	100
	5/26/49	250	1250	250	100	100	1000
	6/12/49	1250	1250	250	100	100	100
NM	3/23/49	0	0	0	0	250	0
	6/ 3/49	0	100	50	0	250	0
SM	6/ 3/49	0	0	0	0	250	0
	12/29/49	0	0	0	0	250	0
Conn.-5		>100	0	0	1000	0	0
Texas-1		0	0	0	0	1000	0
Ohio-1		0	0	0	0	0	1000
Dalldorf-1		0	0	0	0	0	0
Dalldorf-2			0	0	0	0	0
Dalldorf-3			0	0	0	0	0

* JLM had fever from May 18 to May 23. NM had fever from May 14 to May 16 and recurrence on May 27 and 28.

Concerning antibodies to the Ohio type, 2 patients had such antibodies before they became ill (to a heterologous type). Three of the remaining 4 persons did not develop Ohio antibodies during the period studied, whereas a fourth, LMK, did—at the time of illness. The LMK strain proved to belong to the Ohio type.

At the time of JLM's infection with the Conn. type, his family was infected with yet another type. His daughter, Nancy aged 8, was ill with 2 bouts of fever (maximum 103.5°), the first from May 14 to 16 and the second from May 27 to 28. Virus was isolated from fecal samples collected May 21 and June 5. Her strain belonged to a hitherto unknown type, shown by the data in Table XV. Nancy's mother was a healthy carrier of this strain as evidenced by isolation of the virus from her stools collected on June 10. However, unlike Nancy, she did not develop neutralizing antibodies to her own virus, nor to the Conn. type which was the

causative agent of her husband's illness. Nancy developed antibodies to her own virus, to her mother's virus, but not to her father's virus. Between May 18 and 26, her father developed antibodies not only to his virus and to the Conn. type to which it was related, but also to the type which infected the other two members of the family. The only reason for interpreting his illness as due to the Conn. type and not to Nancy's type was the isolation of a Conn. type virus during his illness. Actually, strains isolated early in his disease (stools of May 19) and late (throat swab of May 31) were typed and both belonged to the Conn.-5 type.

Duration of Neutralizing Antibodies in Man and Their Occurrence in Normal Populations.—In order to attempt an answer to this question 3 patients, proven to be infected with C virus by means of virus isolation plus antibody responses (4), were followed for a period of over 2 years.[2] The data of Fig. 1 indicate that neutralizing bodies appear early (by the 5th day of onset), increase rapidly to titers of 1:1250 which are maintained during the period of 1 to 3 months following infection, and may still be present 2 years later, although at lower levels (titer of 1:50 to 1:250). Studies with strains belonging to other types have not been as extensive, but fit into the same pattern. The frequency of the finding of neutralizing antibodies in normal adult human sera and gamma globulin also supports the view that these antibodies persist for long periods.

The largest series in this study is concerned with over 200 children in Winston-Salem, North Carolina, who were bled in May, 1948, and again 6 months later. During this period, an epidemic of poliomyelitis occurred in the area, having its peak incidence in July. Antibody tests were carried out with the High Point strain, isolated from a sample of urban sewage collected in July of that year from High Point, a small city about 20 miles from Winston-Salem. All sera were inactivated at 56°C. for 30 minutes before use. Serum dilutions were set up against 100 ID_{50} doses of virus. It was soon discovered that when antibodies were present the serum titer was over 10 and usually about 100. When a serum dilution of 10 was negative, the undiluted serum was usually also negative. Consequently, in Fig. 2 we have plotted the percentage of sera with titers of 10 or over. From the determinations on 20 placental sera, it was found that a high percentage of children start life with antibodies to this C strain. The level falls rapidly to a minimum of 14 per cent positive at the age of 1, and then it quickly rises to reach the adult level of 80 per cent at the age of 7. It is of interest that a sizable number of healthy children who were devoid of antibodies in May developed them by November. The relationship of these antibody levels, and changes in them during the summer, to other antibodies forms the subject of another report (18).

Neutralizing antibodies to specific types may occur in certain normal populations as shown by the finding in one area that 60 normal sera all contained antibodies to the Texas-1 type but not to the Conn.-5 type. That the latter strain is not localized to the state in which it was isolated in 1948, is suggested by its crossing with a strain isolated in Winston-Salem, North Carolina, and by the finding of antibodies to the Conn. strain in adults in Sweden. Furthermore, gamma globulin collected in different parts of this country and in Denmark has been found to contain antibodies to at least four antigenic types of C virus (Table XVI).

[2] We are indebted to Dr. Albert B. Sabin for making these sera available to us.

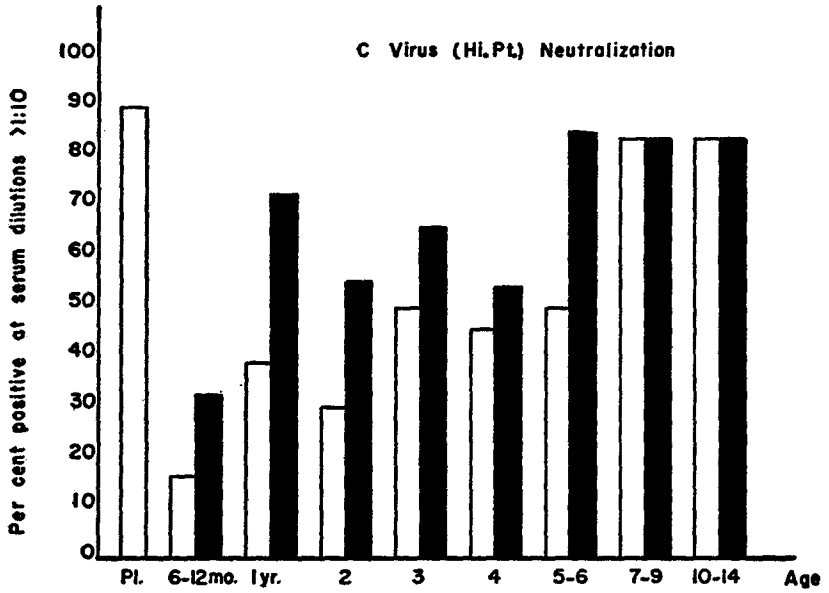

FIG. 2. Neutralizing antibodies in the normal population of Winston-Salem, North Carolina, in 1948. The white columns represent samples collected in the spring and the black columns, samples from the same persons collected in the fall.

TABLE XVI

Titer of Gamma Globulin Samples against Four Different Antigenic Types of C Virus

Year	Area	Titers obtained with 100 ID_{50} virus			
		Conn.-5 m_7	Hi.Pt. m_8	Easton-2 m_8	Ohio m_4
1944	Northeastern United States	64	100	100	100
1945	Eastern United States	>64	1000	100	100
1945	Central United States	>64	100	100	100
1945	Western United States	16	100	100	1000
1949	Eastern United States	>64	1000	100	1000
1949	Denmark	50	>1000	50	100

DISCUSSION

In the course of work with the new group of Coxsackie viruses, it became necessary to develop reliable tests for neutralizing antibodies in order to proceed with the typing of new strains and to obtain some insight into the immune response in man. Certain of the variables inherent in such tests have been investigated, such as effects of time, temperature, complement, and route of inoculation of test mice.

Several strains of virus have been typed. At present they fall into seven

distinct immunological types, three of which have been isolated and previously typed by Sickles and Dalldorf (19). Certain types may be widespread in a particular period. For example the Conn.-5 type in 1947 and 1948 was present in eastern United States, having been recovered from patients and sewage in Connecticut, from patients and flies in North Carolina (5), and from patients in Boston (20) and New York City (21). It is noteworthy that this virus type was isolated from cases of epidemic myalgia (epidemic pleurodynia, Bornholm's disease) (20–22). Recently in Britain, Findlay and Howard (23) have confirmed these findings, having produced infectious myalgia by intranasal instillation of the Type 2 virus in a human volunteer, and having isolated a C virus (untyped) from a naturally occurring case.

Another type which has appeared in widely scattered areas is the Texas-1. Originally isolated from flies trapped in the Rio Grande Valley, this type of virus has been recovered from "poliomyelitis" patients in Texas, from sewage in North Carolina (High Point strain), and from flies trapped in Connecticut in 1943. Serological evidence points to the widespread occurrence in North America and in Europe of this type as well as other types. Furthermore, gamma globulin collected between 1944 and 1949 from several areas in the United States as well as in Denmark has been found to contain antibodies to the 4 types arbitrarily selected for testing.

Although a single type may predominate in a given area, it has been our experience in three poliomyelitis epidemic areas to find more than one immunological type of virus in the population. This has been true of the epidemics in 1948 in Texas and in North Carolina and in 1949 in Easton, Pennsylvania. It is of interest that two antigenic types of virus were isolated from one family. However the exposure of this family was unusual in that one member was actively engaged in laboratory work in the C viruses.

The six infections which occurred in laboratory workers at Yale—as well as the infections reported among British workers—serve as good illustrations of the specificity of the response, each patient responding in a matter of days with antibodies to the strain isolated from his intestines or throat. These experiences serve as an accidental but nonetheless fine example that strains belonging to the Conn.-5, Ohio-1, and Texas-1 types can cause disease in man. To these should be added the Type 2 virus in light of the British work (23) with human volunteers.

SUMMARY

The neutralization test is a reliable and useful procedure for following immunological reactions of the Coxsackie viruses (C virus).

The standard procedure has been an incubation period of 1 hour at room temperature followed by subcutaneous inoculation into newborn mice. However, this time and temperature are not critical, for the virus in neutralized within 10 minutes of mixing with immune serum and remains neutralized for

long periods. During the variable incubation periods used, the control virus remained active, even in dilute suspensions.

The neutralization test is not affected by the presence or absence of complement.

Neutralizing antibody is stable at 65°C. for 30 minutes, and immune serum has to be heated to 80°C. for 30 minutes before the antibody is no longer detectable.

As the quantity of virus is increased, the quantity of serum required for neutralization likewise increases, but not in a regular or predictable fashion.

Neutralized mixtures of the virus can be made infective again by simple dilution before inoculation.

The neutralization test is a satisfactory means for typing Coxsackie viruses. At least seven antigenic types have been identified. Similar antigenic types have been found to be scattered over wide areas. Thus the Conn.-5 type was present in 1948 in Massachusetts, Connecticut, New York, and North Carolina. The Texas-1 type was present in 1943 in Connecticut and in 1948 in North Carolina and Texas.

Further information on the specificity of the neutralizing antibody response has been obtained from a study of the occurrence and development of antibodies in 6 patients who contracted infections with one or another of the C viruses while working with them in the laboratory. From each patient a virus was isolated during the illness. No patient had detectable antibodies to his strain before his illness, but each soon thereafter developed antibodies to his own strain and to the prototype strain to which it was related.

By means of the neutralization test, it has been shown that a family epidemic may include two different immunological types of virus.

Neutralizing antibodies appear at the time of or soon after onset of illness, increase rapidly to titers of about 1:1000 which are maintained during the period of 1 to 3 months following infection, and are still present 2 years later, although at lower levels.

Neutralizing antibodies are present in the normal population. In North Carolina, over 80 per cent of the children have antibodies at birth. The level falls rapidly to a minimum of 14 per cent at the age of 1, and then it quickly rises to reach the adult level at the age of 7. Gamma globulin collected in various parts of the United States between 1944 and 1949 and in Denmark in 1949 neutralizes at least four antigenically different Coxsackie viruses.

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