QUANTITATIVE STUDIES OF THE VIRUS-HOST RELATIONSHIP IN CHIMPANZEES AFTER INAPPARENT INFECTION WITH COXSACKIE VIRUSES

II. The Development of Complement-Fixing Antibodies*

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The non-specific, heterologous nature of complement-fixing antibody responses within the group of the Coxsackie (or C) viruses has been demonstrated in man and monkeys. Thus in the course of infection with a single C virus in man, the patient may respond with complement-fixing (c-f) antibodies to several antigenically distinct types (1, 2). In monkeys, immunization with one C virus type may call forth a variable number of heterologous c-f antibodies at the same time as the homologous one (3). The present report records similar non-specificity of the c-f antibody response in chimpanzees which were infected by the oral, intradermal, and intramuscular routes. In the course of a 2 year study period, the animals were serially challenged with a number of homotypic and heterotypic C viruses, and also with the poliomyelitis viruses and the Egyptian strains of the West Nile virus. The accompanying paper in this series describes in detail the production of inapparent Coxsackie infection in these animals, as evidenced by the appearance of virus in the blood, throat, and stools, and by the rapid development of humoral neutralizing antibodies (4).

Materials and Methods

The methods employed and the infectious agents used in feeding and inoculating the animals are described in the accompanying paper (4).

Complement-Fixing (c-f) Antigens.—20 per cent protamine-treated muscle-bone suspensions of infant mice as previously described (1) were prepared as antigen for the following C virus types: Easton-2 (E-2, Dalldorf's Type 1), Easton-14 (E-14), Conn.-5, Ohio-1, Texas-1, Nancy, Easton-10 (E-10), Alaska-5, and Dalldorf's Type 2 (D-2) and Type 3 (D-3). The identification of these prototype strains with others recorded in the literature has been described in a previous communication (5). The first seven antigens were used to test the sera of four of the animals: Beti, Becky, Donna, and Alamo. All ten were used with the sera of chimpanzees 21 to 24. In addition, antigen made in identical fashion from normal infant mice was used. The preparations were standardized for use in the c-f test according to the methods outlined

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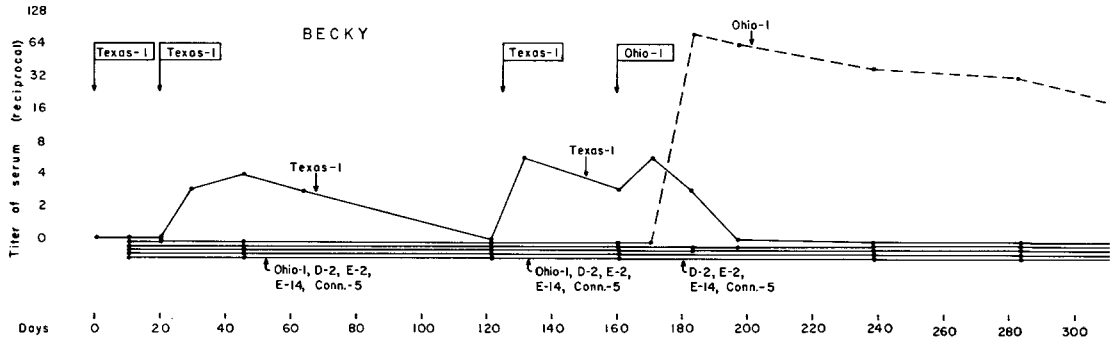


FIG. 1. Complement-fixing antibody titers of chimpanzee Becky. The viruses fed are indicated by the boxes
the E-2 and E-14 viruses were fed to another chimpanzee in the same room at that time. Each serum determination

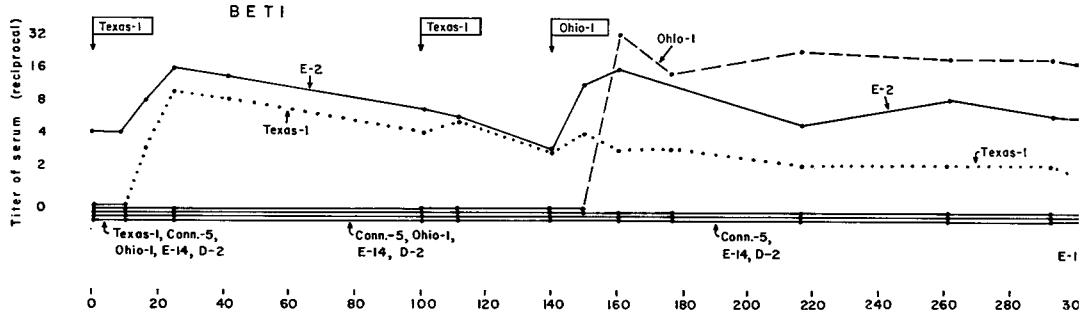
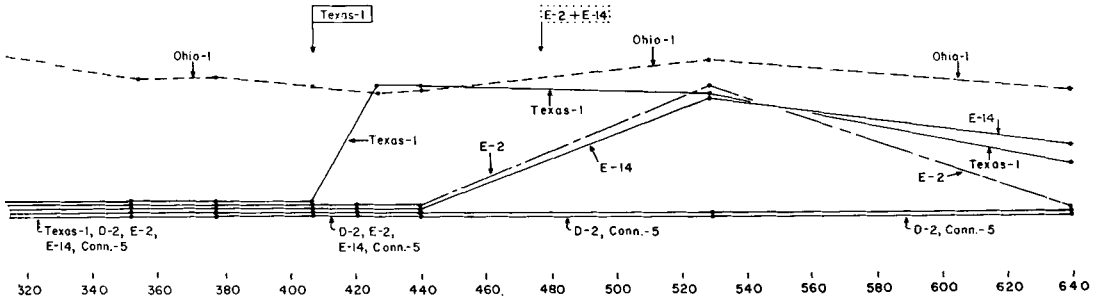
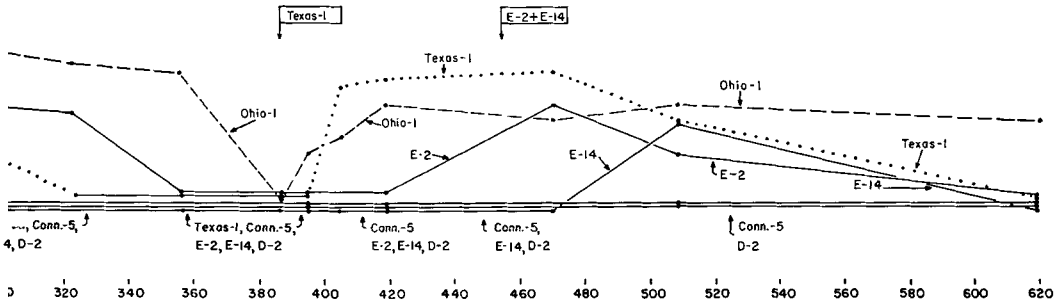


FIG. 2. Complement-fixing antibody titer



oud lines) above the graph and the time of feeding by the arrows. The box composed of dotted lines indicates that is indicated by a solid dot on the graph. See text for explanation of virus types.



of chimpanzee Beti. Legend as for Fig. 1.

previously and the 50 per cent hemolysis method as employed in this laboratory was used to determine the serum titers (6).

Control Sera.—Hyperimmune mouse sera for all the C viruses used were prepared as described previously (7). The antigens were cross-reacted with these sera to ensure specificity of reaction (6).

Serum Inactivation.—All sera were inactivated in a dilution of 1:2 or 1:4 at 56°C. for 30 minutes. Heating the sera at 60°C. for 20 minutes did not alter the titers appreciably.

RESULTS

The titers of the complement fixations obtained are presented diagrammatically in Figs. 1 to 4 for chimpanzees Becky, Beti, Alamo, and Donna; those for chimpanzees 21 to 24 are shown in Figs. 5 and 6 and Table I.

Becky, Beti, Alamo, and Donna.—It is immediately apparent from Figs. 1 to 4 that the picture is different for each animal even though the feeding schedules were practically identical for each group of two animals, *i.e.*, Becky–Beti and Donna–Alamo.

Becky (Fig. 1).—No antibodies to any of the C viruses studied were detectable at the beginning of the experiment. Upon feeding Texas-1 virus, there was a poor c-f response, a maximum titer of 1:4 being observed. The second Texas-1 feeding resulted in a second weak homologous antibody response. When Ohio-1 virus was fed, antibodies against it appeared, rising slowly and reaching an observed peak titer of 1:80. Texas-1 antibodies then became undetectable while the Ohio-1 antibodies dropped slowly. Texas-1 virus was fed a third time on the 405th day of the experiment; thereafter the Texas-1 antibody titer reached the highest level observed in this animal: 1:12. On the 476th day E-2 and E-14 viruses were fed to Beti, Becky's roommate (although not cage mate), and cross-contamination with either or both of these occurred in Becky (4). Her serum titers to each of these rose to about 1:10. On the 640th day Texas-1 and E-14 antibodies were undetectable while Ohio-1 antibodies were still present at the 1:10 dilution. D-2 and Conn.-5 antibodies were absent throughout the study.

Beti (Fig. 2).—This animal arrived in the laboratory with E-2 c-f antibodies (titer 1:4) as well as E-2 neutralizing antibodies (4). All others were undetectable. After Texas-1 virus was fed, Beti manifested both Texas-1 and E-2 responses. A second feeding of Texas-1 virus a short time later had no significant effect upon the titer of either, while the feeding of Ohio-1 virus resulted in an almost immediate fourfold rise of E-2 antibodies, did not affect the Texas-1 antibody, and caused the Ohio-1 antibody to appear and rise to a titer of 1:32. Each gradually became undetectable, until another Texas-1 feeding caused both the homologous antibody and the Ohio-1 to reappear. When E-2 and E-14 viruses were fed, antibodies to both became evident. Beti's final serum sample on the 621st day showed the presence only of Ohio-1 antibodies.

Alamo (Fig. 3).—At the start Alamo lacked antibodies to all types of C virus under study. Feeding of Conn.-5 virus caused the homologous antibodies to appear and eventually to reach a titer of 1:128. No other responses were noted until Texas-1 virus was fed. In this animal, too, the response to Texas-1 feeding was poor. Conn.-5 antibodies persisted at a high level before and after that virus was fed on two more occasions. When Ohio-1 virus was given on the 216th day, homologous antibodies rose within 10 days, Conn.-5 antibodies increasing more than eightfold at the same time. Texas-1 and E-14 antibodies as well put in a brief and undramatic appearance. Conn.-5 and Ohio-1 antibodies persisted at relatively high levels for the duration of the study, and Texas-1 antibodies became undetectable. Alamo, a roommate of Donna who was fed E-2 and E-14 viruses on the 531st day, pre-

TABLE I

*Reciprocal c-f Titers of Chimpanzees 21 to 24 before and after Administration of the Viruses Indicated**

Chimpanzee	Viruses	Route	Date 1951	Reciprocals of c-f titers to C virus antigens								
				E-2	E-14	E-10	Nancy	D-2	D-3	Conn.	Ohio	Alaska
21	Leon polio.	Orally	Mar. 8	0	0	0	0	0	0	0	0	0
			14									
			24	0	0	0	0	0	0	0	0	0
			31	0	0	0	0	0	0	0	0	0
			Apr. 7	0	0	0	0	0	0	0	0	0
	7 C viruses‡	s c., i.m.	10									
			17	24	48	48	64	6	12	8	0	32
			27	8	32	64	128	16	16	8	0	48
			May 18	4	0	8	0	12	12	0	0	48
22	Leon polio.	Orally	Mar. 1	0	0	0	0	0	0	0	0	0
			14									
			24	0	64	48	0	0	0	0	0	0
			31	0	64	32	0	0	0	0	0	0
			Apr. 7	0	64	16	0	0	0	0	0	0
	7 C viruses‡	s.c., i.m.	10									
			17	24	96	64	>128	16	24	128	4	48
			27	48	96	96	1024	8	24	512	64	64
			May 18	6	24	32	256	12	12	192	32	6
23	Leon polio., E-2, E-14	Orally	Mar. 8	0	0	0	0	0	0	0	0	0
			14									
			24	0	256	32	0	0	0	0	0	0
			Apr. 7	0	512	48	0	0	0	0	0	0
	7 C viruses‡	s.c., i.m.	10									
			27	192	192	64	128	48	40	16	0	48
			May 18	32	64	64	128	0	0	64	0	
24	Leon polio., E-2, E-14	Orally	Mar. 8	0	0	0	0	0	0	0	0	0
			14									
			24	0	32	16	0	0	0	0	0	0
			31	0	32	6	0					
			Apr. 7	0	16	6	0	0	0	0	0	0
	7 C viruses‡	s.c., i.m.	10									
			17	16	64	32	0	0	12	0	0	32
			27	48	192	64	0	32	24	0	0	48
			May 23	16	8	16	0	12	6	12	0	0

*With the exception of the Texas-1 c-f antibody (see Figs. 5 and 6), all others studied were absent in chimpanzees 21 to 24 from the beginning of the study (August 15, 1950) until March 24, 1951.

‡ E-10, Nancy, D-2, D-3, Conn.-5, Ohio-1, Alaska-5 (see text).

s.c. = subcutaneously.

i.m. = intramuscularly.

0 = titer less than 1:4.

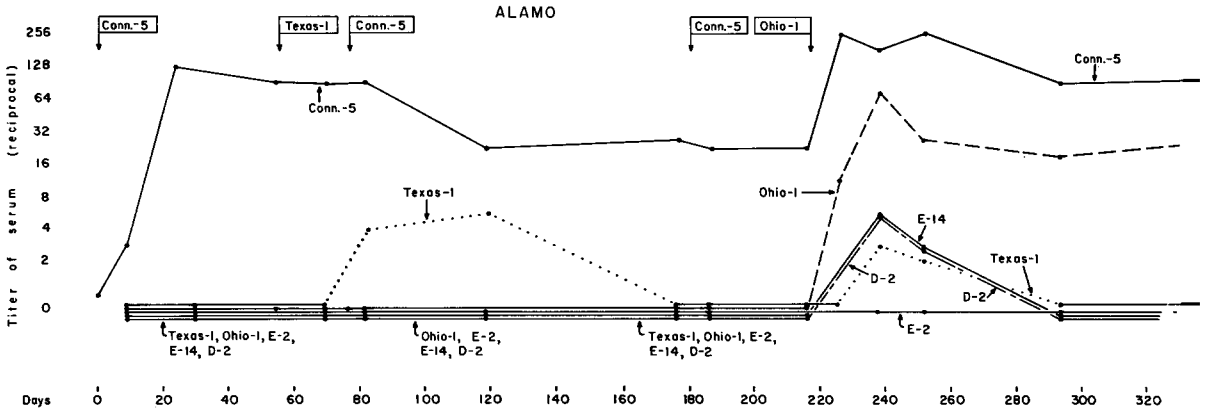


FIG. 3. Complement-fixing antibody titer

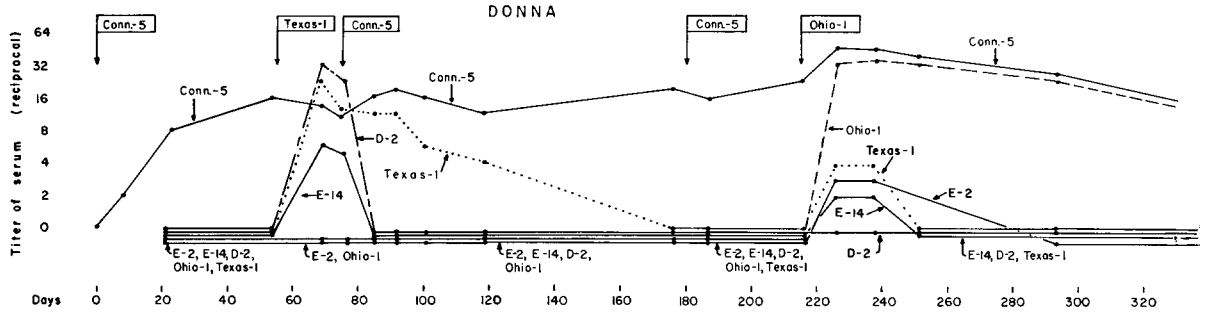
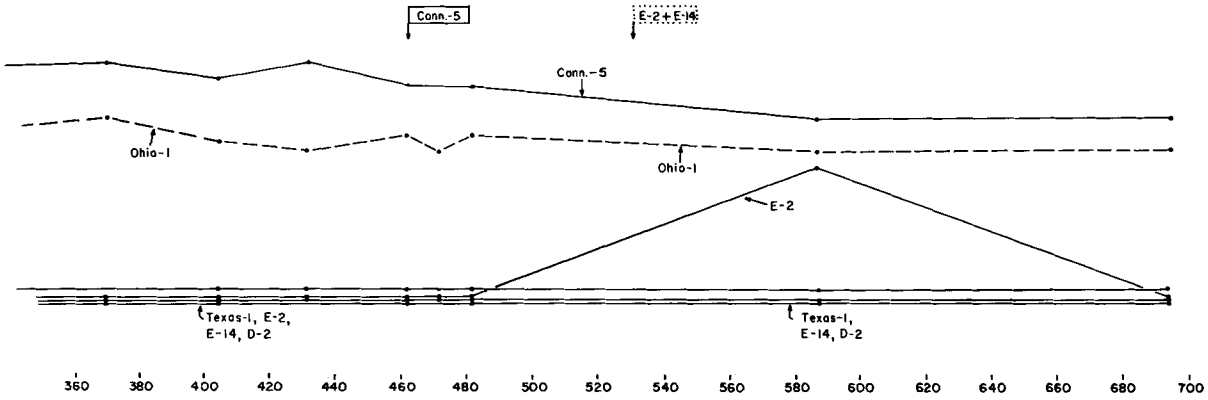
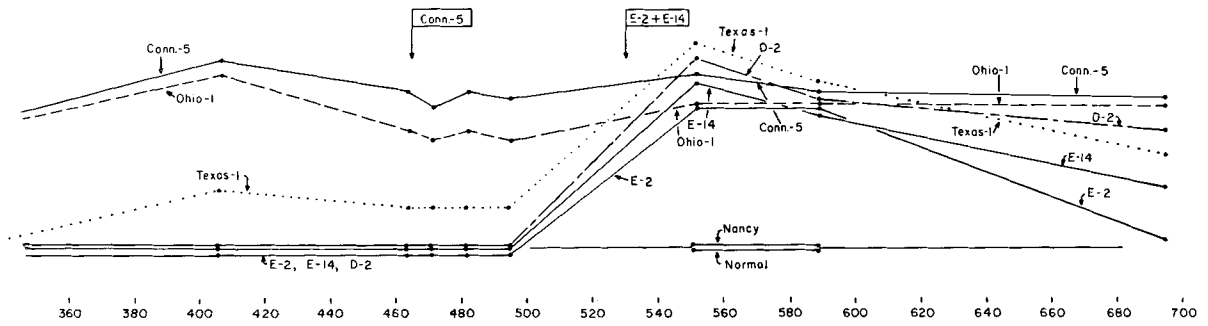


FIG. 4. Complement-fixing antibody titer



chimpanzee Alamo. Legend as for Fig. 1.



of chimpanzee Donna. Legend as for Fig. 1.

sumably developed an infection with the E-2 virus by cross-contamination; the E-2 antibody titer following this infection was 1:12. The final serum sample on the 695th day showed that the E-2 antibody had fallen, but that Conn.-5 and Ohio-1 persisted at rather high levels.

Donna (Fig. 4).—Conn.-5 antibodies rose rather rapidly as the result of the first experience with that virus. No other antibodies were seen until Texas-1 virus was fed on the 55th day; this called forth not only Texas-1, but also D-2 and E-14 antibodies. The Texas-1 antibodies had disappeared by the time Conn.-5 virus was fed a second time on the 180th day. This time no increase in any antibodies followed. Ohio-1 virus was fed on the 216th day after which the homologous antibodies reached a level of 1:32. There was no significant effect upon the Conn.-5 antibodies, but E-2, E-14, and Texas-1 antibodies appeared fleetingly in low titers. Both Ohio-1 and Conn.-5 antibodies maintained high levels throughout the remainder of the study, a fourth Conn.-5 feeding having no effect upon them. After E-2 and E-14 viruses were given on the 531st day, 16- to 32-fold rises in Texas-1, D-2, and E-14 antibodies were observed, while antibodies to normal tissue or to the Nancy type failed to appear. The final serum on the 695th day showed that Conn.-5 and Ohio-1 antibodies had not changed in titer, but that the others had fallen.

Chimpanzees 21 to 24 (Figs. 5 and 6, Table I).—Individual variations in response were noted here as well. Of particular interest is the fact that all four animals manifested Texas-1 c-f as well as neutralizing antibodies at the start of the experiment (4). Figs. 5 and 6 depict the variations in titer of this c-f antibody alone as they resulted from the animals' experiences with various agents: poliomyelitis viruses, orally (4), Egyptian strains of West Nile virus, intradermally (8), and C viruses, orally, intramuscularly, and subcutaneously (4). Texas-1 antibodies gradually fell to undetectable levels in all except chimpanzee 23. When different dilutions of Texas-1 virus were fed, chimpanzee 21, which had been given the most dilute suspension ($10^{-8.5}$), was the only one not to show a significant response. It also failed to give any other signs of infection. The others became virus carriers and reacted with c-f rises of four- to fiftyfold. The feeding of Leon poliomyelitis virus alone or in combination with E-2 and E-14 types of C virus was followed by a significant Texas-1 c-f antibody rise only in chimpanzee 23 which had received the two C viruses. After intramuscular and subcutaneous inoculation of seven additional C virus types (excluding Texas-1), all animals showed additional rises in Texas-1 c-f antibody titer. Chimpanzee 21 had the most striking response: a rise of about 200-fold.

As far as the other 9 C virus antibodies are concerned, the feeding of Leon

FIG. 5. Texas-1 complement-fixing antibody titers of chimpanzees 23 and 24 during a course of infections with viruses indicated. The agents and time of administration of each are indicated above the graph by arrows. On February 5, 1951 chimpanzees 24 and 23 were fed Texas-1 virus in the amounts of 5 ml. of a 10^{-1} and $10^{-3.5}$ concentration respectively, of infected murine tissue. Both animals then showed a rise in homologous antibodies. Following infection with the E-2 and E-14 C viruses, chimpanzee 23 alone showed a further boost in titer of Texas-1 antibodies. The inoculation of the group of 7 C viruses in April stimulated a further boost in Texas-1 antibodies in both animals.

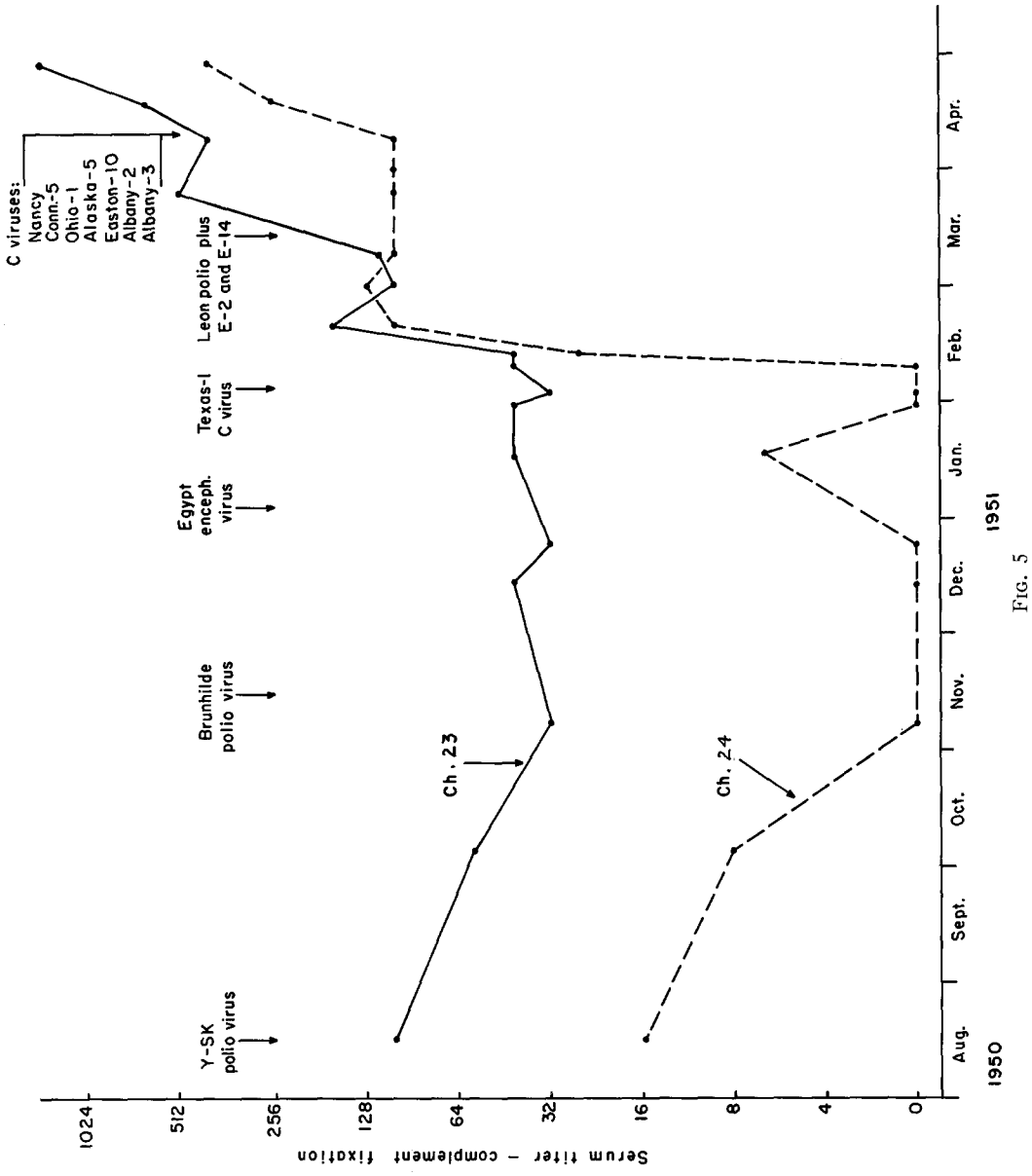

Serum titer – complement fixation
1024
512
256
128
64
32
16
8
4
0
1950
Aug.
Sept.
Oct.
Nov.
Dec.
1951
Jan.
Feb.
Mar.
Apr.
Y-SK polio virus
Brunhilde polio virus
Egypt enceph. virus
Texas-1 C virus
Leon polio plus E-2 and E-14
C viruses:
Nancy
Conn-5
Ohio-1
Alaska-5
Easton-10
Albany-2
Albany-3
Ch. 23
Ch. 24

FIG. 5

virus in combination with E-2 and E-14 viruses in chimpanzees 23 and 24 was followed in both by rises in E-14 and E-10 antibody titers (see Table I).

The feeding of Leon poliomyelitis virus alone in chimpanzees 21 and 22 was followed by rises in E-14 and E-10 antibodies in the latter animal, while chimpanzee 21 did not react. From the data presented in the accompanying paper (4), it also appears that cross-contamination resulted in the infecting of chimpanzee 22.

When the seven additional C viruses were inoculated intramuscularly and subcutaneously (Table I), the responses evoked were somewhat variable. Thus chimpanzee 21 reacted with 6 homologous and 2 heterologous (E-2, E-14) rises; chimpanzee 22 with 7 homologous rises and 1 heterologous (E-2) rise; chimpanzee 23 with 5 homologous rises (E-10 antibodies were already present) and 1 heterologous (E-2) rise; and chimpanzee 24 with 5 homologous and 2 heterologous (E-2 and E-14) rises.

The heterologous c-f responses which occur following infection with a C virus appear to be confined within the family of C viruses. When the chimpanzees were infected with other agents (poliomyelitis viruses or Egyptian strains of West Nile virus), the antibody responses were specific for the latter agents and there was no booster effect in the Coxsackie antibodies. An example of this is shown in Table II for chimpanzees 21 to 24. As shown in Figs. 5 and 6 these animals arrived in the laboratory in August, 1950, with Texas-1 antibodies. Infection with the Egypt virus (8) on January 3, 1951, resulted in a marked c-f response to this virus but there was no significant movement of Texas-1 antibodies. In fact, these antibodies actually declined in titer in some instances while the Egypt antibodies were increasing. Subsequent exposure of the animals to other C viruses as discussed indicated their capacity to respond to heterotypic Coxsackie strains.

DISCUSSION

In chimpanzees, the same picture is seen as concerns complement-fixing (c-f) antibodies to the Coxsackie viruses (C viruses) as was found in man (1, 2) and in monkeys (3), *viz.*, virus types which appear to be immunologically specific as evidenced by their behavior in mice and hamsters (5–7, 9, 10) do not show the same specificity in the c-f reaction when man and the higher primates

FIG. 6. Texas-1 complement-fixing antibody titers of chimpanzees 21 and 22 during a course of infections with viruses indicated. On February 5, 1951, chimpanzees 22 and 21 were fed Texas-1 virus in the amounts of $10^{-6.0}$ and $10^{-8.5}$ concentration respectively, of infected murine tissue. Only chimpanzee 22 responded with a rise in homologous antibodies. Chimpanzee 21 failed to respond because of the low dose of virus (less than one mouse infectious dose). This animal was capable of showing a rise in Texas-1 antibodies, as witness their marked reappearance following the inoculation of 7 heterotypic C viruses in April.

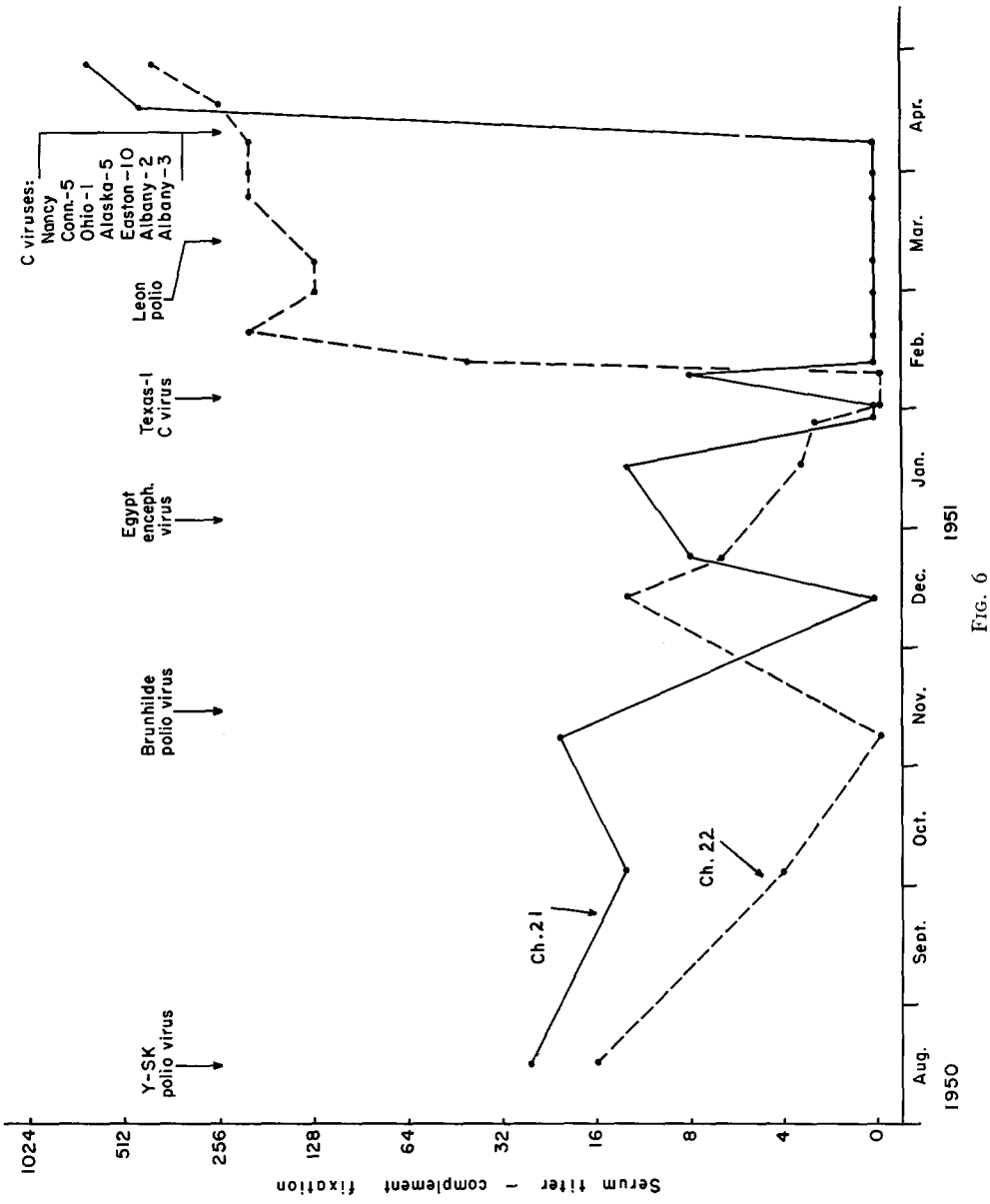

Serum titer – complement fixation
1024
512
256
128
64
32
16
8
4
0
Aug.
Sept.
Oct.
Nov.
Dec.
Jan.
Feb.
Mar.
Apr.
1950
1951
Ch. 21
Ch. 22
Y-SK polio virus
Brunhilde polio virus
Egypt enceph. virus
Texas-1 C virus
Leon polio
C viruses:
Nancy
Conn.-5
Ohio-1
Alaska-5
Easton-10
Albany-2
Albany-3

FIG. 6

are infected or immunized with them. On the other hand, neutralizing antibodies seem to respond type-specifically, and they do not appear or are not present except in the face of active infection or as the result of specific immunization (1–4). In contrast complement-fixing antibodies appear to follow no set rule, appearing and disappearing almost at random. However, the

TABLE II

Specificity in Chimpanzees of c-f Antibody Responses to Egyptian Strains of West Nile Virus and the Texas-1 Coxsackie Virus

Chimpanzee	Virus	Date	Egyptian antibodies	Texas-1 antibodies
21		Dec. 26, 1950	0	8
	West Nile (Egypt), 10^{-1}	Jan. 3, 1951		
		16,	8	12
		30,	128	0
	Texas-1, $10^{-8.5}$	Feb. 5,		
		Mar. 2,	64	0
22		Dec. 26, 1950	0	6
	West Nile (Egypt), 10^{-1}	Jan. 3, 1951		
		16,	16	4
		30,	64	3
	Texas-1, $10^{-6.0}$	Feb. 5,		
		Mar. 2,	64	128
23		Dec. 26, 1950	0	32
	West Nile (Egypt), 10^{-1}	Jan. 3, 1951		
		16,	16	40
		30,	64	40
	Texas-1, $10^{-3.5}$	Feb. 5,		
		Mar. 2,	64	160
24		Dec. 26, 1950	0	0
	West Nile (Egypt), 10^{-1}	Jan. 3, 1951		
		16,	32	6
		30,	64	0
	Texas-1, 10^{-1}	Feb. 5,		
		Mar. 2,	64	128

homologous c-f antibody can usually be found after an experience with a particular immunological type.[1]

[1] The cases of the present study in which this did not occur after inoculation of C viruses intramuscularly and subcutaneously (chimpanzees 21 to 24), and the absence of an immediate homologous response in chimpanzees 23 and 24 after the feeding of E-2 virus, cannot be accounted for either on the basis of virus dosage or of bleeding time. The apparent failure of the viruses to infect when they were administered concurrently also is difficult to explain,

The events occurring in chimpanzees 21 to 24, in which natural immunity to one type of C virus, the Texas-1, was found, are noteworthy. Chimpanzee 21 was the only one of these failing to respond with a rise in titer of the homologous antibody after the Texas-1 virus was fed experimentally. It is recalled that this animal had received the most dilute suspension of virus ($10^{-8.5}$). This finding coincides with the results of virus carrier state and neutralizing antibody studies performed in these animals, for chimpanzee 21 was the only one that failed to show the tenfold neutralizing antibody rise and failed to become an intestinal virus carrier after the feeding (4). But a most striking increase in the Texas-1 c-f antibody titer occurred in chimpanzee 21 after the intramuscular and subcutaneous inoculations of 7 additional virus types: a 200-fold increase. The Texas-1 type was *not* among those inoculated. The same heterologous rise was also seen, but was less marked, in the other animals of this group at the same time, probably because of the high levels of Texas-1 antibodies already present at the time of the injection. Thus c-f antibody for a Coxsackie virus may increase as the result of a heterologous or homologous stimulus, and such an increase does not necessarily take place only on one occasion, never to appear again. The E-2 antibody in Beti, first present as the consequence presumably of a natural experience, appeared again after a second infection with the same agent; and the rise of the D-2 antibody after Donna's first laboratory infection with the Texas-1 virus was followed by another appearance after infection with the E-2 and E-14 viruses.

The widespread heterotypic response within the Coxsackie family observed in the present study and in a previous one on human sera (1) has involved strains of both the so called A and B groups. An illustrative example from the present study may be cited:

> Chimpanzee Beti following challenge with both Texas-1 (Type 4, Group A of Dalldorf) and Ohio-1 (Type 2, Group B of Dalldorf), responded with a fourfold rise of Easton-2 (Type 1, Group A of Dalldorf) antibodies. On the 380th day of the experiment, these antibodies had all fallen below a detectable level. On this day Beti was challenged orally with Texas-1 virus and both Texas-1 and Ohio-1 c-f antibodies reappeared.

Similar examples may be seen on examination of Figs. 1 to 4. Even though the heterotypic response is broad within the Coxsackie family, there appears to be a significant difference in the longer duration of Conn.-5 and Ohio-1 antibodies over that seen for the other types studied. Infection with agents

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for the injected animals did show rises in neutralizing antibodies (4) even when the c-f results were negative. The homologous c-f antibody appeared each time a new virus was fed alone in these experiments, and perhaps the results with two or more of them together reflect an interference limited to the c-f response. But this is mere speculation.

outside of the Coxsackie family (poliomyelitis viruses and Egyptian strains of West Nile virus) had no effect on the Coxsackie antibodies.

These findings have a bearing on the response of an immune animal to re-exposure to the homologous virus. On several occasions, immune animals were fed again a virus to which they originally responded with a subclinical infection. As expected, these animals failed to become virus carriers again and the level of neutralizing antibodies remained constant. However, in many cases there was a boost in level of the c-f antibody and this was notably dramatic in those chimpanzees in which this antibody had fallen below a detectable level.

SUMMARY

Eight chimpanzees were fed and/or inoculated with a single Coxsackie (C) virus or combinations of different ones. The responses in terms of complement-fixing (c-f) antibodies to 6 to 10 such viruses were measured throughout periods of 1 to 2 years. Although the animals usually responded with rises in homologous antibodies after the feeding or the inoculation of viruses of special immunological types, a variable number of heterologous c-f antibodies were observed to increase significantly at the same time.

When immune animals were challenged with homologous virus, they failed to become virus carriers again and no rises in neutralizing antibodies were detected. However, the challenge usually resulted in a boost in the titer of the homologous c-f antibody and often of heterotypic c-f antibodies. This was particularly striking in those chimpanzees in which the c-f antibody had fallen below a detectable level.

In contrast to this, the infection of chimpanzees with agents outside the Coxsackie family (poliomyelitis viruses and Egyptian strains of West Nile virus) failed to influence the level of Coxsackie antibodies even during the periods when c-f antibodies to these non-Coxsackie viruses were rapidly rising.

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