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Neutralizing Antibodies to Cytomegaloviruses in Normal Simian and Human Sera

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Simian and human sera were examined for neutralizing antibodies to simian and human cytomegaloviruses (CMV). Neutralizing antibody to simian CMV was found in sera from 12 of 12 African green monkeys, 8 of 10 rhesus monkeys, and 7 of 7 baboons captured in the wild. The antibody did not cross-react with human CMV strain AD169 but cross-reacted with human strain C87, particularly in the presence of complement. Thirty-six baboons and 10 rhesus monkeys born and hand-reared in captivity remained free of neutralizing antibody both to simian and human CMV for as long as 4 years. Fifteen of 24 human sera (63%) revealed only species-specific neutralizing antibody.

A one-directional cross between the complement-fixing (CF) antigen of simian and human cytomegaloviruses (CMV) has been reported (3, 4). Whereas human sera were found to react in CF tests only with the homologous antigen (3, 4, 9), sera of African green (*Cercopithecus aethiops*) monkeys (3, 4) and of rhesus monkeys (4) captured in the wild possessed CF antibodies reacting with both simian and human CMV. Studies in this laboratory (4) revealed a difference in the CF antigens of human CMV strains AD169 (7) and C87 (2) in that 90% of the monkey sera having CF antibody to simian CMV reacted with the first but none with the second human strain; other human strains revealed intermediate cross-reactivities. This antigenic cross could not be detected in neutralization tests as monkey sera neutralized only simian CMV and human sera neutralized only human CMV (4).

African green monkeys, captured in the wild, have been used for the preparation of hyperimmune sera to different strains of human CMV (7). However, the antibody titers attained were usually low (7). These antisera were reexamined employing the methods described by Yoshino and Taniguchi (12) for the detection of complement-requiring neutralizing (CRN) antibody (B. J. Graham, Ph.D. Dissertation, Baylor College of Medicine, 1971). Although evidence was obtained that these hyperimmune sera contained high titers of CRN antibodies to the immunizing human virus, the preinoculation sera of some of the animals revealed neutralizing antibody to simian CMV that cross-reacted with human strain C87, especially when complement was incorporated into the reaction mixture (Graham, Ph.D. Dissertation). This unexpected finding prompted us to examine in more detail sera of different primates for naturally occurring neutralizing antibody to simian CMV and to human strains AD169 and C87. Evidence will be presented to show that subhuman primates captured in the wild may possess neutralizing antibody to simian CMV that cross-reacts with human strain C87 but not with human strain AD169; animals born and reared in captivity were free of neutralizing antibody to either simian or human CMV. In contrast, human sera contained only species-specific neutralizing antibody.

MATERIALS AND METHODS

Cell cultures and media. Local strains of human embryonic lung (HEL) fibroblasts, propagated as previously described (11), were used. Cells were grown in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and 0.075% $NaHCO_3$ (for cells in stoppered vessels) or 0.225% $NaHCO_3$ (for cells in petri dishes in a 5% CO_2 atmosphere).

Viruses. Two human CMV strains, AD169 (7) and C87 (2), and two simian CMV strains, GR2598 and GR2757, isolated from African green monkeys (4) were used. All four viruses were kept in passage in HEL fibroblasts as described elsewhere (4, 11). Cell-free virus stocks were prepared as described previously (1, 4, 11) and kept at −90 C in the presence of 35% sorbitol (11). Virus infectivity was determined by the modified microplaque technique (11).

Human sera. Human sera were collected from normal adults in the age range of 20 to 45 years. In addition, sera taken from an infant with cytomegalic inclusion disease (S.B.) and from his mother (O.B.) were included in this survey.

Simian sera. Simian sera were collected from normal African green monkeys (*Cercopithecus aethiops*), rhesus monkeys (*Macaca mulatta*), and baboons (*Papio anubis* and *Papio cynocephalus*) captured in the wild. In addition, sera from rhesus monkeys and baboons delivered by caesarean section and hand-reared in captivity were also examined.

All sera were kept at −40 C and inactivated at 56 C for 30 min prior to testing.

Neutralization test. The plaque-reduction neutralization test for CMV (4, 7) was modified to allow for the incorporation of fresh complement in the reaction mixture (12). Unheated guinea pig serum, free of nonspecific viral inhibitors, was employed as a source of complement and kept at −90 C until used. Hemolytic activity of complement was determined as described (6). The detailed procedures for parallel neutralization tests carried out in the presence or absence of complement are described elsewhere (Graham et al., J. Immunol., *in press*). Briefly, the test employed fourfold dilutions of heat-inactivated (56 C, 30 min) test serum (first dilution of 1:8), complement diluted to contain 5 to 10 hemolytic units (usually a dilution of 1:8 or 1:16) and virus diluted to contain 1,200 plaque-forming units per 0.2 ml. MEM, free of $NaHCO_3$ and supplemented with 5% heat-inactivated (56 C, 30 min) FBS was used as diluent for all reagents. Serum, complement, and virus (for tests in the presence of complement) or serum, diluent, and virus (for tests in the absence of complement) were mixed at a ratio of 2:1:1. For virus controls, diluent was used to substitute for either serum (control for the test in the presence of complement) or serum and complement (control for the test in the absence of complement). After incubation for 1 hr at 37 C, residual infectivity was measured as described previously (4, 11). Serum titers were expressed as the final serum dilution producing 60% plaque reduction (4).

CF test. CF tests were performed by the microtechnique described for CMV earlier (4).

RESULTS

Neutralizing antibodies to CMV in normal primate sera. Sera of 12 African green monkeys captured in the wild were tested for the presence of neutralizing antibodies to the two monkey (GR2598 and GR2757) and two human (AD169 and C87) cytomegaloviruses. Neutralization tests in the absence and in the presence of 5 to 10 hemolytic units of complement were carried out in parallel as described above. The results are presented in Table 1. All twelve animals had neutralizing antibodies to both monkey viruses, and no significant difference in antibody titers was noted whether the sera were tested in the absence or the presence of complement. These results confirm our earlier finding on a complete cross between the neutralizing antigens of monkey viruses GR2598 and GR2757 (4). Whereas none of the 12 sera neutralized human strain AD169, 10 of the 12 neutralized human strain C87, especially when

TABLE 1. *Neutralizing antibodies to monkey and human cytomegaloviruses (CMV) in African green monkeys captured in the wild*

Animal[a]	Neutralizing antibody titers[b] to							
	Monkey CMV				Human CMV			
	Strain GR2598		Strain GR2757		Strain AD169		Strain C87	
	−C'[c]	+C'	−C'	+C'	−C'	+C'	−C'	+C'
1	60	250	70	120	0[d]	0	0	0
2	70	170	60	140	0	0	0	0
3	1,000	900	1,000	1,000	0	0	0	60
4	70	380	160	140	0	0	0	40
5	260	300	60	120	0	0	0	20
6	160	450	90	150	0	0	0	100
7	260	120	120	90	0	0	0	70
8	600	600	600	800	0	0	0	50
9	800	4,000	500	700	0	0	20	170
10	1,600	1,000	800	1,000	0	0	20	220
11	2,400	1,000	90	120	0	0	70	80
12	1,000	1,600	600	500	0	0	40	150

[a] Complement fixation (CF) tests revealed CF antibody to monkey CMV, strains GR2598 and GR2727 in 10 of the 12 animals; 9 of the 10 positive sera reacted with human strain AD169; none reacted with human strain C87.

[b] Reciprocal of the highest serum dilution giving 60% plaque reduction.

[c] −C' = test in the absence of complement; +C' = test in the presence of 5 to 10 hemolytic units of complement.

[d] 0 = antibody titer of less than 16.

tested in the presence of complement (Table 1). As indicated in the footnote of Table 1, the CF reactivity of these African green monkey sera was in keeping with our earlier results (4); 10 of the 12 animals had CF antibody (titers of 20 to 320) to both monkey strains. Nine of the 10 positive sera reacted with strain AD169 (titers of 20 to 80), and none of the 12 sera reacted with the CF antigen of human strain C87.

Through the auspices of the Special Virus Cancer Program of the National Cancer Institute, we had access to rhesus monkeys and baboons that had been captured in the wild and served as part of a breeding colony for unrelated studies (5). Sera of such animals and of their offspring, derived by caesarean section and hand-reared in captivity, were available for testing.

Neutralizing antibodies to monkey CMV strain GR2757 were found in 8 of 10 female rhesus monkeys bled at the time of delivery (Table 2). As with the African green monkey sera, the neutralizing antibody was independent of complement. However, the antibody titers were usually low, thus cross-neutralization tests with human CMV were not conducted. (Seven of the 10 animals had CF antibody to monkey CMV that cross-reacted with human strain AD169 but not with strain C87.) Offspring delivered from these rhesus monkeys by caesarean section and hand-reared in captivity failed to show neutralizing antibody to monkey CMV when tested at about 4 years of age (Table 2).

In preliminary tests, sera of seven pregnant baboons that had been earlier captured in the wild revealed neutralizing antibody to monkey strains GR2598 and GR2757 and to human strain C87 but not to human strain AD169. With four of these animals, tests were carried out to determine at what stage after delivery by caesarean section does antibody disappear in offspring that are hand-reared. Maternal sera at time of delivery, cord blood sera, and sera from serial bleedings of the offspring were subjected to neutralization tests with monkey CMV strain GR2757 and with human CMV strain C87 (Table 3). All four mothers had complement-independent antibody to strain GR2757 that cross-reacted with strain C87, but only in the presence of complement. This antibody persisted in the offspring for the first 4 weeks of life; by the 8th week all offspring were free of antibody to either the monkey or the human strain. In further tests, sera of 32 other 1- to 2-year-old baboons, born and reared in captivity, were found to be free of neutralizing antibody

TABLE 2. *Neutralizing antibodies to monkey cytomegalovirus (CMV) in normal rhesus monkeys and their offspring*

Mother[a]			Offspring[a]		
Animal (date serum collected)	Neutralizing antibody titer[b] to monkey CMV (strain GR2757)		Animal (date serum collected)	Neutralizing antibody titer[b] to monkey CMV (strain GR2757)	
	−C′	+C′		−C′	+C′
109 (12/28/63)	50	50	371 (12/28/67)	0[b]	0
333 (1/14/64)	30	30	378 (12/28/67)	0	0
734 (4/6/64)	20	20	433 (12/28/67)	0	0
284 (4/15/64)	30	40	447 (12/28/67)	0	0
787 (4/16/64)	60	60	454 (12/28/67)	0	0
788 (4/16/64)	0	0	455 (12/28/67)	0	0
803 (4/16/64)	40	40	458 (12/28/67)	0	0
310 (4/27/64)	0	0	470 (12/28/67)	0	0
808 (4/27/64)	20	20	471 (12/28/67)	0	0
796 (5/18/64)	20	30	521 (2/1/68)	0	0

[a] Mothers captured in the wild and bled at the time of delivery; offspring derived by caesarean section and hand-reared in captivity.

[b] *See* footnotes to Table 1.

TABLE 3. *Maternal-fetal transmission of naturally occurring antibodies to cytomegaloviruses (CMV) in baboons*

Mother-(baby)[a] animal no.	Sera	Neutralizing antibody titers[b] to			
		Monkey CMV strain GR2757		Human CMV strain C87	
		−C'[b]	+C'	−C'	+C'
1305	M[c]	850	950	0[b]	320
(SA333)	C	900	1000	0	260
	2 W	340	430	0	260
	4 W	150	190	0	30
	8 W	0	0	0	0
A513	M	260	280	0	200
(SA341)	C	130	150	0	150
	2 W	50	60	0	120
	4 W	0	0	0	20
	8 W	0	0	0	0
N242	M	350	640	0	560
(SA331)	C	650	1000	0	360
	2 W	—[d]	—	—	—
	4 W	30	30	0	60
	8 W	0	0	0	0
2235	M	430	550	0	540
(SA299)	C	—	—	—	—
	2 W	130	180	0	150
	4 W	20	30	0	90
	8 W	0	0	0	0
	12 W	0	0	0	0

[a] Mother captured in the wild; baby derived by caesarean section and hand-reared in captivity. Numbers in parentheses are for babies.

[b] *See* footnotes to Table 1.

[c] Abbreviations: M, mother at time of delivery; C, cord blood; W, weeks after birth.

[d] — = Not done.

to both strains of monkey and both strains of human CMV.

Neutralizing antibodies to CMV in normal human sera. The results obtained with sera of primates captured in the wild indicated a cross between the neutralizing antigens of monkey CMV and human CMV strain C87. Our earlier data had indicated that human sera, having neutralizing antibody to human CMV, failed to neutralize monkey CMV (4). It was necessary to ascertain whether this species-specificity of the human antibody would be elicited under the conditions of the test procedures employed in the present study. Human sera were subjected to neutralization tests with both strains of human CMV and both strains of monkey CMV (Table 4). Fifteen of the 24 sera (63%) tested were positive for human CMV. Of these, 12 had neutralizing antibody, in about the same titers, to both human strains; 1 had antibody only to strain AD169; and 2 had antibody only to strain C87. The remaining nine sera were free of antibody. With the exception of the serum of the infant with cytomegalic inclusion disease (S.B.), which revealed a 10-fold increase in antibody titer to strain C87 when tested in the presence of complement (presumably an indication of current infection with CMV), the remaining positive sera appeared to be independent of complement in tests with both AD169 and C87 human viruses. As further shown in Table 4, none of the 24 human sera revealed neutralizing antibodies to the two monkey viruses whether the test was carried out in the absence or in the presence of complement, thus substantiating our earlier finding on the species-specificity of serum neutralization reactions in humans (4).

DISCUSSION

The results of the present study (as summarized in Table 5) revealed that: (i) sera of subhuman primates, captured in the wild, possessed complement-independent neutralizing antibodies to monkey CMV that did not cross-react with human strain AD169 but did cross-react with human

TABLE 4. *Neutralizing antibodies to human and monkey cytomegaloviruses (CMV) in human sera*

Subject (age in years)	Neutralizing antibody titers[a] to							
	Human CMV				Monkey CMV			
	Strain AD169		Strain C87		Strain GR2598		Strain GR2757	
	−C'[a]	+C'	−C'	+C'	−C'	+C'	−C'	+C'
C. B. (21)	30	30	40	160	0[a]	0	0	0
H.M. (27)	20	30	80	260	0	0	0	0
R.P. (37)	40	40	30	130	0	0	0	0
J.Pu. (20)	160	100	40	170	0	0	0	0
A.D. (35)	20	20	260	260	0	0	0	0
G.F. (48)	256	256	256	256	0	0	0	0
S.B. (6 mos.)	320	1,100	520	5,000	0	0	0	0
O.B. (35)	1,100	3,500	170	650	0	0	0	0
F.P. (30)	80	230	170	450	0	0	0	0
G.D. (35)	100	700	200	410	0	0	0	0
G.P. (33)	150	260	150	150	0	0	0	0
M.B. (40)	20	50	60	560	0	0	0	0
E.C. (25)	256	256	0	0	0	0	0	0
L.Q. (20)	0	0	20	64	0	0	0	0
J.Pa. (20)	0	0	20	20	0	0	0	0
R.M. (25)	0	0	0	0	0	0	0	0
L.A. (43)	0	0	0	0	0	0	0	0
E.Bi. (45)	0	0	0	0	0	0	0	0
E.Bo. (21)	0	0	0	0	0	0	0	0
W.V. (36)	0	0	0	0	0	0	0	0
C.H. (21)	0	0	0	0	0	0	0	0
H.L. (20)	0	0	0	0	0	0	0	0
G.W. (26)	0	0	0	0	0	0	0	0
J.H. (25)	0	0	0	0	0	0	0	0

[a] *See* footnotes to Table 1.

strain C87, especially in the presence of complement. (ii) Primates born and hand-reared in captivity remained free of neutralizing antibodies to either monkey or human CMV, and (iii) the neutralizing antibody to CMV in normal human sera was also complement-independent and was species-specific. These findings indicate a one-way cross between the neutralizing antigens of CMV (strains GR2598 and GR2757) isolated from African green monkeys and human strain C87 but not human strain AD169.

Our earlier studies (4) had indicated that the neutralization reaction in both monkey and human systems was species-specific (4). It should be noted, however, that in the present study the neutralization tests were carried out at 37 C instead of at room temperature and that complement was incorporated into the reaction mixture; and indeed complement had to be added to detect antibodies to C87 in simian sera.

Thus, through the use of this more sensitive test, the detection of the above described cross-reactivity was made possible. The finding that native simian (Tables 1 to 3) or human (Table 4) antibody to the homologous virus was not enhanced by the incorporation of complement in the test and that human sera remained negative for the monkey strains even in the presence of complement indicate that the cross-reacting antibody detected in simian sera was not due to a non-specific effect of complement. Furthermore, as indicated above, only lots of complement free of viral inhibitors were used throughout the study.

Our earlier studies (4) had indicated that various human strains of CMV contained a spectrum of overlapping CF antigens as monitored by their reactivity with normal monkey sera, with strain AD169 being the most reactive and strain C87 not reacting at all. In regard to the neutralizing antigens of these two human strains, the reverse appears to be true. Antisera prepared to different strains of human CMV as well as to the two strains of monkey CMV in primates reared in captivity and free of preexisting antibody (Graham et al., J. Immunol., *in press*) are now being analyzed to elucidate further the make-up of neutralizing antigens of these viruses.

TABLE 5. *Neutralizing antibodies to simian and human sera to monkey and human cytomegaloviruses (CMV)*

Sera	No. tested	No. of sera positive[a] for							
		Monkey CMV				Human CMV			
		Strain GR2598		Strain GR2757		Strain AD169		Strain C87	
		−C'[b]	+C'	−C'	+C'	−C'	+C'	−C'	+C'
Simian									
Animals captured in the wild									
Baboon	7	7	7	7	7	0	0	0	0
African green monkey	12	12	12	12	12	0	0	4	10
Rhesus monkey	10	—[c]	—	8	8	—	—	—	—
Animals reared in captivity									
Baboon	36	0	0	0	0	0	0	0	0
Rhesus monkey	10	—	—	0	0	—	—	—	—
Human	24	0	0	0	0	13	13	14	14

[a] Antibody titers of 16 or greater.
[b] *See* footnote in Table 1.
[c] — = Not done.

CMV isolated from African green monkeys appears to be less species-specific (3, 4, 9) than any of the known cytomegaloviruses (10). The virus replicates in cells of human origin (3, 4, 9), and CF antibodies to the virus are found in sera of rhesus monkeys that had not been in contact with African green monkeys (4, 9). The present study revealed that sera of normal rhesus monkeys have also neutralizing antibodies to the African green monkey CMV strains. To our knowledge, CMV has not been isolated from baboons. However, the finding of high neutralizing antibody titers to monkey CMV in the sera of the animals examined in the present study indicates a latent infection with the same or a closely related virus.

In the primates studied, the antibody appears to transverse the placenta and within about 8 weeks after birth the newborn loses the antibody and remains free of it for as long as 4 years when reared free from contact with naturally-infected animals. Such antibody-free animals have been used with success for the preparation of specific antisera to human and monkey strains of CMV (Graham et al., J. Immunol., *in press*).

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