Comparative Complement Fixation and Serum Neutralization Antibody Titers to Herpes Simplex Virus Type 1 and *Herpesvirus simiae* in *Macaca mulatta* and Humans

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The serological relationship of herpes simplex type 1 virus and *Herpesvirus simiae* was studied. Antibody titers to these viruses were determined in 163 *Macaca mulatta* sera and 67 human sera by serum neutralization (SN) and complement fixation (CF) tests. Both groups of sera were also tested by CF with envelope and capsid antigens of herpes simplex type 1. By SN, the majority of the monkeys and all of the humans had a higher titer to herpes simplex type 1 than to *H. simiae*. By CF, with crude antigens the titers in the monkey sera were greater to *H. simiae* than to herpes simplex type 1, although four sera had equal titers to both antigens; the titers in the human sera were conversely higher with the herpes simplex type 1 antigen, except for four sera which had equal titers to both antigens. The capsid CF antigen of herpes simplex type 1 was reactive with the human sera but virtually nonreactive with the monkey sera; the envelope CF antigen of herpes simplex type 1 was reactive with both monkey and human sera but was somewhat less reactive than the crude herpes simplex type 1 CF antigen. In addition, serum samples from a patient recently infected with *H. simiae* were examined by CF and SN for antibody to both herpes simplex type 1 and *H. simiae* viruses. The serological profile indicated a positive correlation with the infecting virus. Although the SN titers did not conclusively reflect an infection with *H. simiae*, the CF titers were higher to *H. simiae* than to herpes simplex in later sera and thus appeared to be compatible with *H. simiae* infection.

The close serological relationship of *Herpesvirus simiae* and herpes simplex virus presents difficulties in determining specific antibody levels to *H. simiae* (6, 12). Serological studies are hindered by the prevalence of antibody to herpes simplex in the general human population (3, 22). It is apparent that antibody against *H. simiae* detected in human sera generally represents a heterotypic response to herpes simplex (3, 29).

Numerous serological surveys of nonhuman primates in wild and captive populations, particularly *Macaca* spp., have been conducted to assay for the prevalence of *H. simiae* and other viruses of possible significance to man (8, 10, 11, 14, 16, 21, 25, 26, 30; S. S. Kalter, A. R. Rodriguez, and J. J. Ratner, Bacteriol. Proc., p. 127, 1964). In addition, Kalter and Heberling (15) have compiled data from several laboratories that tested nonhuman primate sera from various sources for antibody to a number of viruses including *H. simiae* and herpes simplex. In most of the studies, serum neutraliza-

tion (SN) methods were used; complement fixation (CF) data are limited (9). In addition, studies of nonhuman primate sera for antibodies to both herpes simplex and *H. simiae* have been few (3, 28); generally workers looked only for *H. simiae* antibody, with *H. simiae* antigen (8, 10, 21, 30) or herpes simplex antigen (5). However, in at least one study, in monkeys from Uganda (19), a search was made for herpes simplex antibody with herpes simplex antigen.

In view of the fragmentary nature of past serological studies, we attempted to compare antibody titers in sera of a human population and a *Macaca mulatta* population by SN and CF methods with both herpes simplex type 1 and *H. simiae* crude antigens. In addition, both groups of sera were tested by CF with capsid and envelope antigens of herpes simplex type 1 (18). A limited number of serum samples were available from a recent human case of *H. simiae*, and these were also tested against both viruses by SN and CF.

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MATERIALS AND METHODS

Viruses. The VR 3 strain (cell culture passage 30) of herpes simplex virus type 1 (24) and the Lilly E2490 strain (cell culture passage 52) of *H. simiae* virus were obtained from stocks stored at the Virology Division, Center for Disease Control (CDC). The latter strain had originally been obtained from Robert Hull, Eli Lilly and Co., Indianapolis, Ind.

Virus propagation. Both viruses were grown in primary rabbit kidney (PRK) monolayer cultures in 32-ounce glass prescription bottles. Culture growth medium consisted of Eagle minimum essential medium with 10% fetal calf serum. The same medium without calf serum was used on the cell cultures when the viruses were propagated. Monolayer cultures were inoculated with virus at a multiplicity of 5 to 10 and were incubated for about 48 h at 37°C. Infectious viruses were used throughout this study; therefore, because of the biohazard involved, all procedures with *H. simiae* virus were performed in the Maximum Security Laboratory, CDC. This is a special laboratory suite with gas-tight, glove-ported biological safety cabinets similar to that described by Kuehne (17).

Crude CF antigens. Crude CF antigens of herpes simplex type 1 and *H. simiae* viruses were prepared from infected PRK cultures. After approximately 48 h of incubation, the supernatant medium was decanted from each infected 32-ounce culture, the cell monolayer was washed with 10 ml of 0.85% NaCl buffered at pH 9 with 0.05 M glycine-NaOH, and the cells were scraped into 10 ml of the same buffer. This suspension of cells was treated in a Dounce tissue homogenizer to disintegrate the cells and was centrifuged at $200 \times g$ to remove large cellular debris. The supernatant fluid containing the antigen was dispensed in 1-ml volumes and frozen at -20°C. Control antigens were prepared in the same manner from uninfected cell cultures.

Capsid CF antigens. A virus capsid antigen was prepared from herpes simplex type 1-infected PRK cultures essentially by the method of Martin et al. (18). The supernatant medium was decanted from each culture and replaced with 5 ml of distilled water. After 30 min of incubation at room temperature, 5 ml of 5% Nonidet P-40 (NP-40) was added, and the cultures were incubated for 30 additional min at 36°C. Then the cell suspension was further disintegrated by 15 strokes of a Dounce homogenizer and was cleared of cell debris by centrifuging at $200 \times g$. The supernatant was layered over 30% sucrose cushions and centrifuged in a Beckman SW40 rotor for 60 min at 30,000 rpm. The supernatant from this step, containing NP-40 and sucrose, was decanted, and the walls of the tubes were wiped with cotton applicators to prevent NP-40 from draining into the pellet. The pellet, which consisted of capsids, was resuspended in 0.01 M barbital buffer, pH 7.4, 1 ml per tube, and stored frozen at -20°C.

Envelope CF antigen. A virus envelope antigen was also prepared from PRK cultures infected with

herpes simplex virus type 1 (18). Supernatant fluid was removed from the cultures, and the infected cells of each culture bottle were scraped into 2 ml of distilled water. The cells were disintegrated in a Dounce homogenizer as above, layered over 30% sucrose cushions, and centrifuged in a Beckman SW40 rotor for 60 min at 30,000 rpm. The pellet, which contained the virus particles intact with their envelopes, was resuspended in 1 ml of distilled water per culture and treated with an equal volume of diethyl ether at 0°C for 1 h. Ether was removed by bubbling nitrogen through the treated suspension. The suspension was then dialyzed at 4°C for 24 h against 0.85% NaCl buffered at pH 10.5 with 0.05 M glycine-NaOH, layered onto a 30% sucrose cushion, and centrifuged for 1 h at 30,000 rpm in a Beckman SW40 rotor to separate the envelope material from capsids or capsids with envelope remnants. The supernatant, which contained the envelope antigen, was collected and dialyzed against 0.01 M barbital buffer, pH 7.4, for use in CF tests.

Sera. Human serum samples were obtained in June 1974 from 60 members of the Virology Division, CDC. Seven additional human sera were obtained from members of the Tissue Culture and Media Section, Scientific Services Division, CDC. These latter sera had been frozen at -20°C since collection in June 1968. Serum samples from 163 *M. mulatta* were also tested; these sera were obtained at the time the monkeys were trapped in Jammu State, India, in October 1974 to assure that any herpes simplex virus antibody detected in the animals was acquired in the natural state and not through human exposure after they were trapped. Serial serum samples from a recent human case of *H. simiae* infection (1) were obtained through the generosity of Richard Emmons and Edwin Lennette of the California State Health Department.

CF. CF tests were performed by the microtiter LBCF method (4). All sera were inactivated at 56°C for 30 min before testing.

SN. SN tests were performed by a micromethod essentially as previously described (20, 27). Sterile, 96-well, plastic microtitration plates and the FS 32 strain of diploid human fibroblasts derived at CDC from newborn foreskin were used. The culture medium was Eagle minimum essential medium with 10% fetal calf serum plus 1,400 mg of sodium bicarbonate per liter to maintain the pH in an acceptable range under sealed covers. Trypsinized cells in a concentration of 114,000/ml were dispensed into the wells in 0.1-ml amounts; 20 h later, 0.1 ml (1,000 mean tissue culture infectious doses) of virus dilution or virus-serum mixture was added as appropriate. After 4 days of incubation at 37°C, the supernatant was drained off, and the cells were stained with crystal violet-Formalin for 20 min, rinsed, dried, and read macroscopically as positive or negative for viral cytopathic effect. Serum-neutralizing end points were interpolated by the Reed-Muench method (23).

RESULTS

CF and SN tests on monkey sera. Fourteen of the 163 *M. mulatta* sera were so anticomplementary as to preclude their being tested by

CF. The 149 remaining sera were tested by CF against the crude *H. simiae* antigen and the crude, capsid, and envelope herpes simplex type 1 antigens. The distribution of CF titers obtained is presented in Table 1. Only 1 of the 149 sera reacted with the capsid antigen. Although the crude and envelope herpes simplex type 1 antigens were cross-reactive, they were less sensitive than the *H. simiae* crude antigen. For example, 13 (9%) of the 149 test sera reacted with the crude *H. simiae* antigen at a titer of 64, whereas only 3 (2%) reacted with crude simplex antigen at this titer. The envelope antigen was not reactive above serum dilutions of 1:32.

In SN screen tests of the 163 *M. mulatta* sera at a dilution of 1:10, 56 (34.4%) reacted to both *H. simiae* and herpes simplex type 1. Titrations of these 56 SN-positive sera showed that 48 (85.7%) had a higher SN antibody titer to herpes simplex type 1, and 8 (14.3%) had a higher SN titer to *H. simiae*. Only 4 of 163 (2.5%) had a titer >10 to herpes simplex type 1 with a concurrent titer to *H. simiae* of <10. None of the 163 sera had a titer >10 to *H. simiae* with a concurrent titer of <10 to herpes simplex type 1.

Fifty (33.6%) of the 149 sera that were not anticomplementary in the CF test had either SN or CF titers; the relationships of these titers in respect to the *H. simiae* and herpes simplex viruses and antigens are presented in Table 2, along with the titers for human sera. None of these 50 sera had a CF titer greater to herpes simplex type 1 crude antigen than to *H. simiae* crude antigen, although four of the sera had equal titers to the two antigens. By CF, none reacted only to the herpes simplex antigen; however, nine (18%) reacted only to the *H. simiae* antigen. Thirty-four (68%) reacted to both viruses, by SN as well as CF.

The age and sex distribution of the *M. mu-*

latta monkeys is presented in Table 3. Monkeys were considered subadult if they were almost adult size but had not yet sexually matured. The seropositive monkeys were primarily adults; although adult monkeys represented only 49% of the test subjects, they accounted for 92% of the seropositives. Positivity was not related to sex.

CF and SN tests on human sera. CF tests were performed on sera of 67 CDC personnel against the crude *H. simiae* antigen and the crude, capsid, and envelope herpes simplex type 1 antigens as on the *M. mulatta* sera. The results are presented in Table 4. The capsid and envelope antigens of herpes simplex virus type 1 were less sensitive than the crude antigen of the same virus in reactions with human sera; nevertheless, they were reactive to a considerable degree. However, the capsid antigen reactivity was much greater against the human sera than against the monkey sera (Table 1), where it was virtually nil.

Twenty-seven of the 67 human sera (40.3%) had titers of <10 to both viruses by SN and <8 to both crude antigens by CF, 33 (49.3%) had

TABLE 2. Relationship of titers in seropositive *Macaca mulatta* and human sera against indicated antigens

SN		CF		Sera (%)	
<i>H. simiae</i>	Herpes simplex 1	<i>H. simiae</i> crude	Herpes simplex 1 crude	<i>M. mulatta</i>	Human
— ^a	—	+ ^b	+	2/50 (4.0)	0/40 (0.0)
—	+	—	—	1/50 (2.0)	0/40 (0.0)
—	+	+	+	2/50 (4.0)	29/40 (72.5)
—	+	+	—	1/50 (2.0)	0/40 (0.0)
—	+	—	+	0/50 (0.0)	4/40 (10.0)
+	+	—	—	1/50 (2.0)	0/40 (0.0)
+	+	+	—	9/50 (18.0)	0/40 (0.0)
+	+	+	+	34/50 (68.0)	7/40 (17.5)

^a —, Titers <10 in SN tests and <8 in CF tests.

^b +, Titers ≥10 in SN tests and ≥8 in CF tests.

TABLE 1. Distribution of CF titers in 149 *Macaca mulatta* sera against indicated antigens

Titer	<i>H. simiae</i>	Herpes simplex type 1		
	Crude	Crude	Capsid	Envelope
<8	100 ^a	111	148	129
8	4	11	1	11
16	10	15		7
32	20	9		2
64	13	3		
128				
256	1			
512	1			

^a Number of sera with indicated titer against antigen.

TABLE 3. Age and sex distribution of *Macaca mulatta* monkeys used in serological analysis

Results of serological analysis of monkeys (%)		
Category	In study population	In seropositives
Adults	73/149 ^a (49.0)	46/50 ^b (92.0)
Subadults	13/149 (8.7)	2/50 (4.0)
Juveniles	63/149 (42.3)	2/50 (4.0)
Male	35/149 (23.5)	11/50 (22.0)
Female	114/149 (76.5)	39/50 (78.0)

^a Denominator is total number of monkeys in study whose sera were not anticomplementary.

^b Denominator is number of monkeys seropositive by CF and/or SN.

neutralizing titers ≥ 10 to herpes simplex type 1 but a titer < 10 to *H. simiae* virus, and 7 (10.4%) had neutralizing titers ≥ 10 to herpes simplex type 1 and also had titers of ≥ 10 to *H. simiae*. These detectable titers to *H. simiae* occurred only in sera where the titers to herpes simplex type 1 were ≥ 240 (mean = 314), 7.4- to 25-fold higher than the *H. simiae* titers. Of the 40 human sera found positive by one method or the other, 36 (90%) had a CF titer greater to the crude herpes simplex type 1 antigen than to the crude *H. simiae* antigen, and 4 had the same titer to both. Thus, none had a CF titer greater to a crude *H. simiae* than to crude herpes simplex type 1.

The SN and CF titers obtained for the 40 seropositive human sera with the *H. simiae* and simplex viruses and antigens are shown in Table 2, along with comparable data for the seropositive monkey sera. Most of the human sera, 33 out of 40 (82.5%), reacted with herpes simplex by SN and CF; only 7 (17.5%) cross-reacted with *H. simiae* by both the SN and CF tests. In contrast, most of the seropositive monkey sera, 34 out of 50 (68%), cross-reacted with herpes simplex in both tests, and none reacted only to *H. simiae* in both tests.

Eight serial serum samples from a human

TABLE 4. Distribution of CF titers in 67 human sera against indicated antigens

Titer	No. of sera with indicated titer against antigen			
	<i>H. simiae</i>		Herpes simplex type 1	
	Crude		Crude	Capsid Envelope
<8	31		27	44 37
8	11		2	9 13
16	20		14	13 14
32	5		14	1 3
64			10	
128				

case of *H. simiae* virus infection (1) were studied by SN and CF with both *H. simiae* and herpes simplex type 1 viruses and crude antigens as reagents. The results obtained are presented in Fig. 1. By SN, antibody titers to *H. simiae* and herpes simplex type 1 were low to both viruses early in the disease and rose to high levels during the course of the disease; the SN titers to herpes simplex type 1 were consistently higher than those to *H. simiae*. By CF, however, titers were sometimes higher against *H. simiae* than against herpes simplex type 1, depending on the length of time since the infection occurred. In the early stages of the infection, CF titers to herpes simplex type 1 were higher, but in the later stages the *H. simiae* titers were higher.

DISCUSSION

The SN data on human sera presented here concur generally with the data of others (2, 3, 12, 29) which show that the presence of SN antibody to *H. simiae* is dependent on and secondary to the presence of antibody to herpes simplex. Our findings also indicate that the level of SN antibody to *H. simiae* is directly related to the height of the SN antibody titer to herpes simplex, which further bears out the findings of previous authors. Cabasso et al. (3) reported one exception to this relationship, namely, an individual with antibody to *H. simiae* in the absence of antibody to herpes simplex.

Infection of humans with *H. simiae* virus more often than not results in death. Therefore, comparative studies of *H. simiae* and herpes simplex antibodies in humans who have had overt *H. simiae* infection are rarely possible. Although SN titers for the single human case of *H. simiae* infection presented in this study were consistently higher to herpes simplex type 1 than to *H. simiae*, the CF results showed a reversing relationship in the convalescent

FIG. 1. Serological profile of human case of *H. simiae*. Symbols: herpes simplex type 1, CF (■), SN (●); *H. simiae*, CF (▨), SN (●).

phase and thus provide supporting evidence of an infection with *H. simiae*. Although we did not investigate dual infections of *H. simiae* and herpes simplex in monkeys, the results of our CF tests with monkey sera relate well to those with the human case in that the CF titers were highest with crude antigen of the suspect field-infecting virus, *H. simiae*. To our knowledge this serological study reports, for the first time, profiles of antibody to both viruses by both CF and SN which can serve as a basis for study and evaluation of future cases.

There are few reports of SN tests for both herpes simplex and *H. simiae* antibodies conducted concurrently on the same monkey sera. In a study of eight monkeys with naturally acquired SN antibody, Burnet et al. (2) found the levels to be either the same to herpes simplex and *H. simiae* or higher to herpes simplex, with one exception where the titer was higher to *H. simiae*. Ueda et al. (28), in a study with 19 monkeys, found that all the monkeys with antibody to both viruses had higher titers to herpes simplex. Cabasso et al. (3) tested five lots of simian gamma globulin and found the titers to be higher to herpes simplex virus in four lots and higher to *H. simiae* in one lot. Our results are similar; in 149 *M. mulatta*, 48 of which had SN antibody to one or both viruses, we found that 40 had a higher level of antibody to herpes simplex and 8 had a higher level to *H. simiae*. Apparently, SN antibodies found in *Macaca* spp. will generally neutralize herpes simplex virus as well or better than *H. simiae* virus, but exceptions do occur. Our data are also in agreement with those of Shah and Southwick (26) that antibody incidence is higher in adult monkeys; they detected SN antibodies to *H. simiae* in 7 of 8 adult monkeys but in none of 39 juveniles.

Ueda et al. (28) could not be sure that the monkeys they studied had not been exposed to herpes simplex virus during their period of contact with humans after capture; such exposure could account for the antibody that was detected. We avoided this problem by having the sera used in our study collected at the time the monkeys were captured. We cannot, however, preclude the possibility that the monkeys had been infected in the wild with an unknown herpes-like virus which cross-reacts with both herpes simplex and *H. simiae*.

Some workers have stated that the CF test is not capable of detecting antigenic differences between *H. simiae* and herpes simplex (6, 7, 13) viruses. However, to our knowledge there is only one report of CF tests performed with *H. simiae* antigen (9). Falke (9) found cross-reactivity with CF antigens of three herpes simplex strains and one *H. simiae* strain, but, in testing the sera of 42 rhesus and cynomolgus monkeys, he found the sera to be more reactive with the *H. simiae* antigen. The highest titers were obtained with this antigen. He also reported that in one human serum tested, equivalent titers were obtained to two herpes simplex antigens and one *H. simiae* antigen. Our study with a larger number of monkeys supports the data of Falke. We found antibody levels by CF to be higher against *H. simiae* antigen in 44 of 48 monkeys. In the other 4 monkeys, titers were equal with *H. simiae* and herpes simplex type 1 crude antigens.

In our study of 67 human sera, 40 of which had CF antibody to one or both crude antigens, 36 had titers higher to herpes simplex and 4 had equal titers with both antigens. It is apparent that, although cross-reactivity does occur between the two viruses, the CF antigens react best with homotypic antibody, that is, with antibody to the virus which infected the monkey or human individual. This was also shown to be the case with the single human *H. simiae* infection which was studied.

Although the cross-reactivity of the envelope antigen of herpes simplex type 1 was essentially the same as with the crude antigen of herpes simplex type 1, the capsid antigen was of strikingly low reactivity with the monkey sera. The implications are that a capsid antigen is more specific than either a crude or an envelope antigen and that a capsid antigen prepared from *H. simiae*-infected cells might be similarly specific for *H. simiae* antibody. If this is actually so, definition of *H. simiae* infection would be greatly simplified. Obviously, the next step is to prepare an *H. simiae* capsid antigen. Its preparation was not possible during the course of this study because of the biohazards involved; however, suitable containment for such work with *H. simiae* is now being developed at CDC.

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