Virus Excretion and Neutralizing Antibody Response in Saliva in Human Cytomegalovirus Infection

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The local secretory immune mechanism in infants with cytomegalovirus infection was studied by a measurement of neutralizing antibody in saliva. Neutralizing antibodies were determined by the microculture plaque assay in 65 saliva specimens including 54 samples from cytomegalovirus-infected subjects and 11 from seronegative controls. In addition, cytomegalovirus isolation from saliva or urine or both and antibody determination in serum and saliva were simultaneously performed on seven infants with cytomegalovirus excretion over long periods. Results obtained were as follows. (i) Neutralizing antibodies were detected in 41 (76%) of 54 saliva specimens obtained from infected subjects but in none of the 11 seronegative controls. (ii) Neutralizing antibodies in saliva were of low titer but persistently detectable in all but one of the seven infants. No relationship was recognized between the cessation of virus excretion and the development of neutralizing antibodies in saliva. (iii) Virus-neutralizing activity was specifically found in the immunoglobulin A fraction of pooled saliva by diethylaminoethyl cellulose chromatography.

Recent studies indicate that antibodies of the secretory immunoglobulin A (IgA) class have been shown to be a component of the defense mechanism against viral infections (1, 12). Accumulating evidence suggests that the presence of such antibody in local secretions may be a more important index of immunological resistance to certain viral infections than circulating antibodies (4, 10).

Persistent viral excretions in saliva or urine and latent infections are characteristic of cytomegalovirus (CMV) infection (8, 15). Therefore, it was expected that the local humoral immune response in CMV infection would differ from that of the usual acute viral infections. The status of local secretory immunity to CMV in saliva, however, has not been investigated as yet. The present report describes the development of CMV-specific neutralizing antibody in saliva and its relationship to local CMV excretion.

MATERIALS AND METHODS

Study population. Subjects consisted of 65 infants who visited the outpatient clinic or were hospitalized in the pediatric ward of Sapporo Medical College Hospital. Saliva specimens were collected without stimulation by using sterile Pasteur pipettes. The subjects included 54 infected subjects (CMV seropositive or positive virus isolation or both) and 11 seronegative controls. None of them had immunological disorders. Saliva was centrifuged at 3,000 rpm for 15 min, and the supernatant fluid was stored at -20°C until used. The concentration of the immunoglobulin in each

saliva specimen was not adjusted. Serum specimens were obtained simultaneously and stored frozen.

Tissue culture and virus isolation. Human embryonic lung fibroblasts (HEF) prepared in our laboratory were used for virus isolation and preparation of complement-fixing and neutralizing antigens. Growth medium consisted of Eagle minimum essential medium supplemented with 10% fetal calf serum, and the maintenance medium was minimum essential medium with 2% fetal calf serum. A 0.2-ml sample of fresh urine and saliva specimens, filtered through 400-nm pore-sized membranes, was inoculated into each of two tubes of HEF. Tubes were observed for the appearance of cytopathic effect for at least 4 weeks before being discarded. CMV was identified by the characteristic cytopathic effect and by failure to demonstrate cytopathic effect in nonhuman and epithelial cells.

Complement-fixation test. The AD169 strain of CMV was employed for antigen production. The antigen was prepared by the alkaline-buffer method (6) and stored frozen at -80°C until used. Complement-fixation antibody determinations were performed with the microtechnique (11). Seropositivity was determined by a complement-fixation antibody titer over 1:4.

NT test. The Davis strain was employed for production of antigen for the CMV-neutralization (NT) test. Determination of virus infectivity (8×10^6 plaque-forming units per ml) and NT antibody titrations of saliva and serum were performed by the method of microculture plaque assay (5). The procedure for NT tests was carried out in the absence of complement. Virus was diluted to contain 800 plaque-forming units per 0.1 ml of minimum essential medium. Antibody titers were expressed as the reciprocal of the final dilution exhibiting 60% plaque reduction.

Preparation of antisecretory IgA serum. Human colostrum was collected from healthy lactating females within 72 h after delivery. Insoluble material and lipid were removed by centrifugation at 15,000 rpm for 60 min at 4°C, and the whey was collected. Secretory IgA (SIgA) was purified from colostrum by the method reported by Cebra and Robbins (3). An anti-SIgA serum was prepared by immunization of rabbits with 30 mg of concentrated SIgA in Freund complete adjuvant by footpad injection. The purity of the anti-SIgA serum was identified by immunoelectrophoresis after absorption by standard human IgG and SIgA.

Column chromatography. All saliva samples containing CMV-NT antibodies were pooled and dialyzed against 0.01 M phosphate buffer (pH 7.4). The pooled saliva was concentrated by macrosolute concentrators (Amicon A75) and adjusted to 30 mg of protein per ml. Diethylaminoethyl cellulose column chromatography was performed as described by Tomasi et al. (13). Fractions of 3 ml were obtained by stepwise elution of three phosphate buffers: pH 7.4, 0.01 M; pH 6.2, 0.1 M; pH 4.8, 0.3 M. Neutralizing activities to CMV of each fraction were measured. The distribution of immunoglobulins in each fraction was identified by immunoelectrophoresis.

RESULTS

Distributions of salivary NT antibody titer of CMV-infected subjects and controls are shown in Table 1. CMV-NT antibodies were detected in none of the controls. The NT antibody titers were all under 1:4. In CMV-infected subjects, however, salivary NT antibodies were detected in 41 (76%) of 54 subjects. The mean NT antibody titer in saliva of CMV-infected subjects was 1:2^{2.8}. The titers of salivary NT antibody were generally low except for a few subjects.

CMV-NT antibodies were periodically determined in saliva and sera from seven infants with CMV excretion. Virus isolations from urine or saliva or both were performed simultaneously (Table 2). Serum NT antibodies were persistently detected in high titers through the observation period. In contrast, NT antibodies in saliva were of lower titer than that of serum, but again were persistently detected in all cases except for case 5, in which CMV was isolated

TABLE 2. Long-term observations on neutralizing antibodies in saliva and sera of infants with CMV excretion

Case no.	Age	Virus isolation ^a		NT antibody titer ^b		CF ^c titer		
		Urine	Saliva	Saliva	Serum			
1	1 yr	1 mo	+	+	4	256	32	
		5 mo	+	+	4	256	32	
		9 mo	ND	—	4	256	32	
2	3 yr	5 mo	ND	—	16	64	32	
		1 yr	1 mo	+	+	4	512	16
		8 mo	ND	ND	4	512	16	
2	1 yr	11 mo	—	—	<4	256	16	
		2 yr	1 mo	ND	—	8	128	16
		5 mo	+	—	8	256	32	
3	3 yr	5 mo	+	—	4	128	32	
		4 mo	+	+	32	32	8	
		1 yr	3 mo	—	—	<4	512	16
4	2 yr	9 mo	+	—	4	128	32	
		8 mo	+	—	4	64	32	
		2 mo	+	+	8	16	<8	
4	1 yr	7 mo	+	+	4	ND	16	
		1 mo	ND	ND	8	64	32	
		7 mo	+	—	8	256	32	
5	3 mo	ND	+	4	64	32		
		ND	+	<4	128	32		
		9 mo	+	+	<4	512	16	
6	2 yr	3 mo	+	+	<4	512	16	
		1 yr	1 mo	ND	+	<4	ND	16
		11 mo	ND	ND	4	ND	8	
6	2 yr	4 mo	—	—	<4	128	8	
		3 yr	3 mo	+	+	4	ND	32
		9 mo	+	+	4	ND	32	
7	1 yr	10 mo	+	+	8	512	64	
		10 mo	+	+	32	ND	32	

^a +, Virus; —, no virus, ND, not determined.

^b 60% plaque-reduction neutralizing antibody titer.

^c Complement-fixing serum antibody.

persistently from saliva and urine. In cases 2 and 3, the excretions of virus from saliva ceased after salivary NT antibodies were detected. However, detection of salivary NT antibodies and virus isolation were simultaneously observed in cases 4 and 7. Thus, there was no correlation between the cessation of virus excretion and the development of NT antibodies in saliva.

CMV-NT activity in the pooled saliva was specifically found in the fraction which was eluted by 0.1 M phosphate buffer (pH 6.2) in diethylaminoethyl cellulose chromatography (Fig. 1). Immunoglobulin from this fraction was identified as SIgA by immunoelectrophoresis. CMV-NT antibody titers in the pooled original saliva and SIgA fraction were 1:2⁴ and 1:2³, respectively.

DISCUSSION

Cytomegalovirus has a strong affinity for salivary glands, and its persistent infection is common. Although local immunity can protect against upper respiratory tract infections, little is known about a role of local immunity in CMV

TABLE 1. CMV-neutralizing antibodies in saliva from CMV-infected subjects and seronegative controls

Subjects	No. of subjects with neutralizing antibody titer ^a :				
	<4	4	8	16	32
CMV-infected	13 (24)	21 (39)	11 (20)	6 (11)	3 (6)
Seronegative	11 (100)	0	0	0	0

^a Neutralizing antibody titers were determined by 60% plaque-reduction assay. Numbers within parentheses are percentage of total subjects.

FIG. 1. Diethylaminoethyl cellulose chromatography of concentrated pooled saliva from CMV-infected subjects and distribution of CMV-neutralizing antibody activity.

infection. Local humoral immune response is generally effective as the first line of defense against infection in acute viral infection. It was expected that the response in CMV infection differed from that in usual acute viral infections because of the characteristic feature of CMV infection, namely, persistent excretion of the virus in the urine or saliva or both. Since NT antibody cannot inactivate the intracellular viruses, neutralizing immune response to CMV may result in an insufficient effect against cell-to-cell infection of CMV. Neutralizing antibody in serum is unable to stop the local virus excretion (7). Another local immune mechanism may react against persistent CMV excretion.

In our investigation, salivary NT activity to CMV was detected in the majority of subjects with a prior history of CMV infection but in none of the subjects without a history of CMV infection. Accordingly, CMV-specific NT antibodies were supposed to exist in the saliva of CMV-infected subjects. Although CMV-NT antibodies in the saliva were generally of lower titers than those of the sera, the salivary NT antibodies were considered not to be a leakage of the serum antibodies but the products of salivary glands based on their immunological properties. Diethylaminoethyl cellulose chromatography and immunoelectrophoresis revealed that the CMV antibody activity of saliva was carried by SIgA.

The investigation of the CMV excretion and

development of salivary NT antibodies showed inconsistent patterns of immunological response. In cases 2 and 3, virus excretions were stopped earlier in saliva than in urine. Case 5 remained negative in salivary NT antibody except for the low activity at the initial test, and CMV was isolated persistently from saliva and urine. These phenomena may be favorable for the possible role of salivary NT antibody in the cessation of local CMV excretions. On the other hand, case 7 maintained a relatively high titer of salivary NT antibodies, but viral excretion did not cease. This finding may be explained by the presence of an excess of antigen in the saliva.

As CMV isolations were performed on the salivary specimens filtered through the millipore membrane, only cell-free CMV were supposed to be inoculated on to the cultured cells. The incompatible phenomenon that virus recovery and the existence of salivary NT antibody were found simultaneously in the same specimens might be a characteristic feature of CMV infection. According to Notkins (9), the coexistence of NT antibody and infectious virus suggests that antibody might have attached to the virion and that the virus might be circulating in the form of an infectious virus-antibody complex. Although the development of an infectious immune complex was conceivable in the CMV infection, we did not attempt to dissociate the virus from antibody in the saliva.

Secretory CMV-NT antibodies in the saliva of

most subjects persisted for long periods. Our study suggested that locally produced NT antibodies were not able to sufficiently neutralize the infectivity of CMV in the saliva. The study of Waner et al. (14) also failed to show the correlation of the development of SIgA antibody and the CMV excretions in cervical secretions. Thus, the question remains as to whether SIgA antibodies are protective in local CMV infection. Other local immune responses, such as cell-mediated immunity (2), may play a more important role in the defense against local infection with CMV.

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