BIKEN JOURNAL Vol. 24 109–118, 1981

A SINGLE SERUM DILUTION METHOD FOR THE QUANTITATION OF NEUTRALIZING ANTIBODIES TO VARICELLA-ZOSTER VIRUS

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(Received March 11, 1981

SUMMARY A one-point serum dilution method for determination of neutralizing antibody in human sera to varicella-zoster (V-Z) virus instead of the serial serum dilution method was investigated. Focus counting was performed under a microscope on day 5 to 6 after inoculation of V-Z virus into 6-well plastic trays in which human embryonic lung cells were grown. A table was constructed to estimate the ND_{50} titers by the per cent reduction of the focus count from the control at only one dilution of test sera. The estimated ND_{50} values agreed well with those determined by the serial serum dilution method. Test sera showed a slight nonspecific reactivity at low serum dilutions, but reliable results could usually be obtained at a serum dilution of 1: 8 or more. This method, which saves materials and labor, was applied to the quantification of neutralizing antibody against V-Z virus in human sera with satisfactory accuracy and reproducibility.

INTRODUCTION

Many quantitative methods are available for determining the immune status to varicella-zoster (V-Z) virus, and an accurate and practical method for measuring V-Z neutralizing antibody has been developed since cell-free virus became available (Asano and Takahashi, 1978; Caunt and Shaw, 1969; Grose et al., 1979; Schmidt and Lennette, 1975). However, quantitative measurement of V-Z virus by the standard plaque assay under a solid overlay takes at least 10 days and requires large quantities of materials. Furthermore, cultures may become contaminated with fungi during prolonged culture. Therefore, we tried to devise a modified neutralization test (NT) that would save labor and require smaller samples. This paper reports that virus titration by focus counting is as effective as plaque counting and that relation between focus reduction and the neutralizing antibody titer is linear. A method was developed by which, using a single dilution of serum, the 50% endpoint of neutralization (ND_{50}) could be estimated from a table on the basis of the observed rate of focus reduction.

MATERIALS AND METHODS

1. *Cell cultures*

Human embryonic lung (HEL) cells at the 13th to 26th passage level were used. Cells were grown in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS) and maintained in the same medium with 2% FCS.

2. *Virus strain*

The K9 strain of zoster virus, isolated in 1976 in this laboratory from a patient with herpes zoster, was used in this study.

3. *Preparation of cell-free virus*

Cell-free virus was prepared essentially by the method of Schmidt and Lennette (Schmidt and Lennette, 1976). Tissue culture bottles (150 ml) containing HEL cells were inoculated with V-Z infected cells at ratio of one infected cell to four uninfected cells and were incubated at 36 C for 2 to 3 days. When 70–90% of the infected culture showed cytopathic effects, the monolayers were washed three times with phosphate-buffered saline without Ca^{++} and Mg^{++} (PBS) (pH 7.2) and cells were harvested with a rubber policeman. The cells were suspended at a concentration of 2×10^6 cells per ml in PBS containing 10% sorbitol and 10% FCS, and sonicated at 20 KC/sec for 30 sec. The suspension was then centrifuged at 3,000 rpm for 20 min and the supernatant was stored in 0.5 ml aliquots at −70 C.

4. *Neutralization test*

The focus reduction neutralization test was performed essentially by the method of Caunt and Shaw (Caunt and Shaw, 1969). Cells were prepared in 6-well or 24-well (35 or 16 mm diameter wells) plastic trays (Coster or Linbro 6 and 24 well trays). Each of the wells was seeded with 8×10^5 cells (for 6 well trays) or with 2×10^5 cells (for 24-well trays) and the trays were incubated in a humidified CO_2-incubator at 37 C for 24 h, by which time nearly confluent monolayers were obtained. PBS containing 10% heat-inactivated FCS was used to dilute sera and virus. Serial twofold dilutions of test sera were prepared for the serial dilution method, and only an appropriate one point dilution of serum was performed for the single dilution method. Virus diluted to contain 200–400 focus-forming units (FFU) per 0.1 ml was mixed with an equal volume of diluted serum, and incubated at 37 C for 60 min with occasional shaking. Cell-free V-Z virus diluted 20 to 30 times with PBS containing 10% FCS was stable at 37 C for 2 h. HEL cultures were washed with PBS once, and then 0.1 ml (6-well tray) or 0.05 ml (24-well tray) of serum-virus mixture was inoculated into each well. As a control, diluted virus was mixed with an equal volume of diluent, and inoculated into 6 wells. Trays were incubated at 37 C in a CO_2-incubator for 90 min with shakings every 15 min to permit absorption of unneutralized virus. Then 2 or 0.5 ml of MEM containing 2% FCS was added to each well.

After incubation at 35 C for 5 to 6 days, foci were counted under a microscope at low magnification. No secondary foci were formed within this period. The titer of neutralizing antibody by the serial dilution method was expressed as the reciprocal of the highest serum dilution producing 50% or more reduction in the mean focus count relative to that of the virus control, while in the single dilution method, the titer was estimated from Table 2, from the percentage reduction in the focus count relative to the control value with appropriately diluted serum.

5. *Test of fluorescent antibody to membrane antigen (FAMA)*

The FAMA test was performed essentially by the procedure of Williams et al. (Williams et al., 1974). HEL cultures infected with V-Z virus K9 strain were harvested with 0.02% EDTA treatment when the CPE reached 70%. Volumes of 0.05 ml (about 2×10^5 cells) of cells were mixed with equal volumes of serial twofold serum dilutions in PBS, and the tubes were incubated for 30 min at 25 C. After several washings with PBS, the cells were treated for 30 min with 0.05 ml of diluted fluorescein-conjugated rabbit antiserum to human immunoglobulin (Miles Laboratory) and washed as before. The cells were mounted in buffered glycerol on a glass slide with a cover slip and examined with a Nikon fluorescence microscope. Isolated cells exhibiting membrane fluorescence were counted as positive.

6. *Human sera tested*

Sera were obtained 8 to 19 days after the onset of clinical symptoms from 6 children of 3 to 13 years old who had contracted varicella and at 8 to 23 days of illness from 4 persons of 3 to 52 years old with herpes-zoster infection. Sera from 11 healthy children of 5 to 6 years old who had had varicella 2 to

3 years before and from 17 adults of 22 to 59 years old who had had varicella many years before were also examined. V-Z virus negative sera were obtained from 7 normal children as described in the text. Sera were stored at −20 C and inactivated by heating at 56 C for 30 min before use.

RESULTS

1. *Quantification of V-Z virus using plastic trays*

For standardization of focus assay for the quantitative determination of V-Z virus under liquid medium without employing solid overlay, experiments were done as follows. Virus was diluted 1:3 to 1:80, and 0.1 ml of each dilution was inoculated into the wells of 6-well trays containing HEL monolayers. After 90 min of adsorption at 37 C in a CO_2 incubator, 2 ml of maintenance medium was added to each well. Then the incubation temperature was lowered to 35 C and the number of foci was counted every day under a microscope at low magnification (×40). Foci appeared from 3 days after inoculation, and the focus count reached a plateau on day 6, and then remaining constant until day 10 when the experiment was terminated (Fig. 1). However, when there were more than 200 foci per well, they gradually decreased in number by fusion as they increased in size. No secondary foci were noticed during the 10-day observation period. Another experiment showed that the number of foci under liquid medium at 8 days after inoculation was approximately equal to the number under solid medium (data not shown).

When the $\log_{10}$ numbers of foci per well counted on day 6 were plotted against the $\log_2$ dilution factor of the inoculum dose, a linear dose-response was obtained, as shown in Fig. 2; a completely proportional correlation was observed in a broad range from 12 to nearly 500 foci per well. Calculation of the standard error of the focus counts of 6 wells inoculated with various virus doses showed that standard errors were within 15% of the mean focus number when the virus dose was more than 47 FFU per well, as seen in Table 1, but that the standard errors increased to about 20% when the virus dose was less than 29 FFU per well. Thus it was concluded that from 50 to nearly 500 foci can be reliably counted on day 6 after

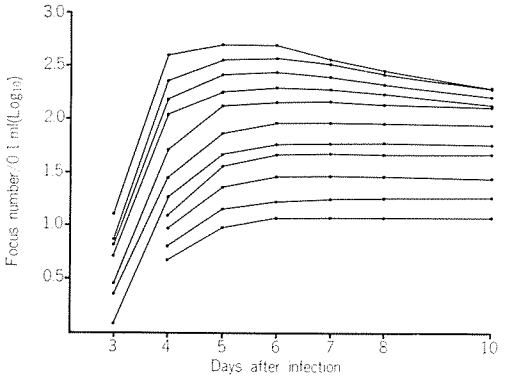

FIGURE 1. Numbers of foci of V-Z virus observed on various days after inoculation.

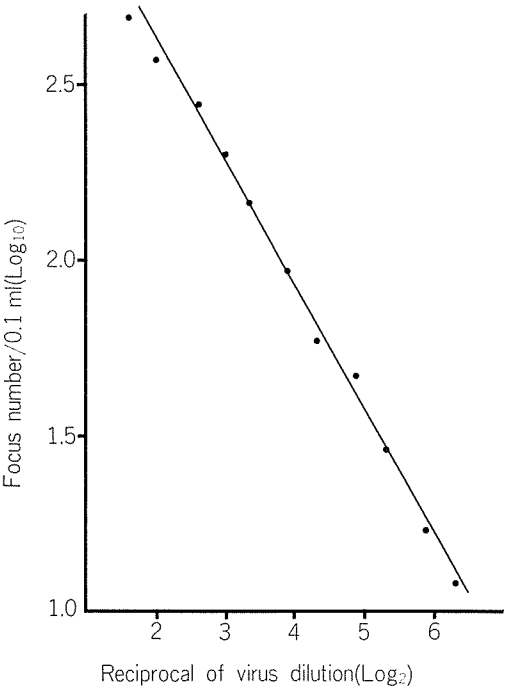

FIGURE 2. Relationship between V-Z virus focus counts and virus dilution.

TABLE 1. *Standard error of focus counting on 35 mm-well trays*

Dilution of seed	Average foci per well ($\bar{X}$)[a]	Standard Deviation (σ)	Standard error ($\sigma/\bar{X}\times 100\%$)
1 : 3	492.8	55.2	11.2
1 : 4	374.3	27.6	7.4
1 : 6	274.2	40.6	14.8
1 : 8	200.0	15.4	7.7
1 : 10	146.0	8.4	5.8
1 : 15	92.5	12.1	13.1
1 : 20	59.2	7.4	12.5
1 : 30	47.2	3.4	7.2
1 : 40	29.2	5.6	19.2
1 : 60	16.8	3.6	21.4
1 : 80	12.0	2.8	23.3

[a] Average of 6 wells.

inoculation; in usual experiments, counts of 50 to 200 foci seem to be convenient.

2. *Construction of a table for estimation of the ND_{50} titer*

Six sera from patients with varicella and four from patients with zoster were submitted to the test. Serial twofold dilutions of the sera were made, and samples containing 200–400 FFU per 0.1 ml were mixed with an equal volume of serum and neutralized. The test was divided into two groups: 0.1 ml volumes of serum-virus mixture were inoculated into duplicate cultures in one group, and in quadruplicate in the other. As virus controls, six cultures in both groups were inoculated. Foci were counted on day 5 or 6. The results are shown in Fig. 3A, B. The percentage reduction in the mean focus number at each serum dilution relative to that of the control are plotted against the logarithm of the reciprocal of the serum dilution, giving sigmoidal dose-response curves. On applying the parallel line method, it was found that neither linearity nor parallelism could be denied with a probability of 95% around the 50% reduction point with the dose-response curves obtained from either

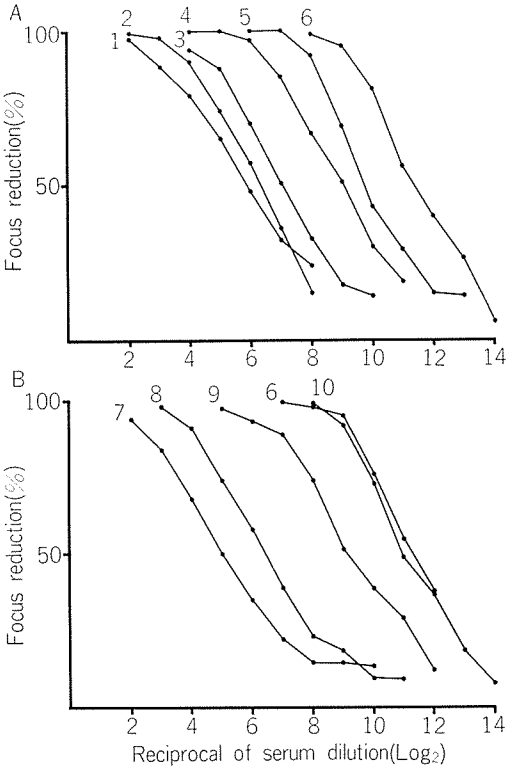

FIGURE 3. Dose-neutralization curves to V-Z virus of various human antisera.

1, 2, 3, 7, 8, 9: Sera from children with varicella.

4, 5, 6, 10: Sera from children and an adult with herpes-zoster.

Volumes of 0.1 ml of serum-virus mixture were inoculated into quadruplicate HEL cell cultures (A) or duplicate cultures (B).

duplicate or quadruplicate cultures per dilution. The linearity could be applied from 25% to 85% percentage reduction. The regression coefficient, $\bar{b}$, was 0.533.

From these data, the neutralizing antibody titer, ND_{50} (reciprocal dilution of serum producing 50% reduction in the focus count) of the test serum was calculated from the follow equation:

$$Z = \frac{\log_{10} Y - \log_{10} 50}{0.533} + \log_{10} X$$

where Z, Y and X are the logarithm of ND_{50},

the observed percentage focus reduction and the reciprocal of the serum dilution tested, respectively. Table 2 is constructed from this equation. The ND_{50} of a serum sample can easily be obtained from Table 2 knowing the percentage focus reduction at a single serum dilution.

TABLE 2. *ND_{50} titers obtained from focus reduction rate*

	Reciprocol of serum dilution								
	8	16	32	64	128	256	512	1024	2048
Focus reduction rate (%)									
25	0.338[a]	0.639	0.940	1.241	1.542	1.843	2.144	2.446	2.747
26	0.370	0.671	0.972	1.273	1.574	1.875	2.176	2.477	2.778
27	0.401	0.702	1.003	1.304	1.605	1.906	2.207	2.508	2.809
28	0.431	0.732	1.033	1.334	1.635	1.936	2.237	2.538	2.839
29	0.459	0.760	1.061	1.362	1.663	1.964	2.265	2.566	2.867
30	0.487	0.788	1.089	1.390	1.691	1.992	2.293	2.594	2.895
31	0.514	0.815	1.116	1.417	1.718	2.019	2.320	2.621	2.922
32	0.539	0.841	1.142	1.443	1.744	2.045	2.346	2.647	2.948
33	0.565	0.866	1.167	1.468	1.769	2.070	2.371	2.672	2.973
34	0.589	0.890	1.191	1.492	1.793	2.094	2.395	2.696	2.997
35	0.612	0.913	1.215	1.516	1.817	2.118	2.419	2.720	3.021
36	0.635	0.937	1.237	1.539	1.840	2.141	2.442	2.743	3.044
37	0.658	0.959	1.260	1.561	1.862	2.163	2.464	2.765	3.066
38	0.679	0.980	1.282	1.583	1.884	2.185	2.486	2.787	3.088
39	0.701	1.002	1.303	1.604	1.905	2.206	2.507	2.808	3.109
40	0.721	1.022	1.323	1.624	1.925	2.226	2.527	2.828	3.130
41	0.741	1.042	1.343	1.644	1.946	2.247	2.548	2.849	3.150
42	0.761	1.062	1.363	1.664	1.965	2.266	2.567	2.868	3.169
43	0.780	1.081	1.382	1.683	1.984	2.285	2.586	2.887	3.188
44	0.799	1.100	1.401	1.702	2.003	2.304	2.605	2.906	3.207
45	0.817	1.118	1.419	1.720	2.021	2.322	2.623	2.924	3.225
46	0.835	1.136	1.437	1.738	2.039	2.340	2.641	2.942	3.243
47	0.853	1.154	1.455	1.756	2.057	2.358	2.659	2.960	3.261
48	0.870	1.171	1.472	1.773	2.074	2.375	2.676	2.977	3.278
49	0.887	1.188	1.489	1.790	2.091	2.392	2.693	2.994	3.295
50	0.903	1.204	1.505	1.806	2.107	2.408	2.709	3.010	3.311
51	0.919	1.220	1.521	1.822	2.123	2.424	2.725	3.026	3.327
52	0.935	1.236	1.537	1.838	2.139	2.440	2.741	3.042	3.343
53	0.951	1.252	1.553	1.854	2.155	2.456	2.757	3.058	3.359
54	0.966	1.267	1.568	1.869	2.170	2.471	2.772	3.073	3.374
55	0.981	1.282	1.583	1.884	2.185	2.486	2.787	3.088	3.389
56	0.995	1.296	1.597	1.899	2.200	2.501	2.802	3.103	3.404
57	1.010	1.311	1.612	1.913	2.214	2.515	2.816	3.117	3.418
58	1.024	1.325	1.626	1.927	2.228	2.529	2.830	3.131	3.432

TABLE 2. *Continued*

	Reciprocal of serum dilution								
	8	16	32	64	128	256	512	1024	2048
59	1.038	1.339	1.640	1.941	2.242	2.543	2.844	3.145	3.446
60	1.052	1.353	1.654	1.955	2.256	2.557	2.858	3.159	3.460
61	1.065	1.366	1.667	1.968	2.269	2.570	2.871	3.172	3.473
62	1.078	1.379	1.680	1.981	2.282	2.584	2.885	3.186	3.487
63	1.091	1.392	1.693	1.994	2.296	2.597	2.898	3.199	3.500
64	1.104	1.405	1.706	2.007	2.308	2.609	2.910	3.211	3.512
65	1.117	1.418	1.719	2.020	2.321	2.622	2.923	3.224	3.525
66	1.129	1.430	1.731	2.032	2.333	2.634	2.935	3.236	3.538
67	1.142	1.442	1.743	2.044	2.345	2.647	2.948	3.249	3.550
68	1.154	1.454	1.755	2.057	2.358	2.659	2.960	3.261	3.562
69	1.166	1.466	1.767	2.068	2.369	2.670	2.971	3.273	3.574
70	1.177	1.478	1.779	2.080	2.381	2.682	2.983	3.284	3.585
71	1.189	1.490	1.791	2.092	2.393	2.694	2.995	3.296	3.597
72	1.200	1.501	1.802	2.103	2.404	2.705	3.006	3.307	3.608
73	1.211	1.512	1.813	2.115	2.416	2.716	3.017	3.318	3.619
74	1.223	1.523	1.824	2.125	2.426	2.727	3.028	3.329	3.631
75	1.233	1.534	1.836	2.136	2.437	2.738	3.039	3.340	3.642
76	1.244	1.545	1.846	2.147	2.448	2.749	3.050	3.351	3.652
77	1.255	1.556	1.857	2.158	2.459	2.760	3.061	3.362	3.663
78	1.265	1.566	1.867	2.169	2.470	2.771	3.072	3.373	3.674
79	1.276	1.577	1.878	2.179	2.480	2.781	3.082	3.383	3.684
80	1.286	1.587	1.888	2.189	2.490	2.791	3.092	3.393	3.694
81	1.296	1.597	1.898	2.199	2.500	2.801	3.102	3.403	3.704
82	1.306	1.607	1.908	2.209	2.510	2.811	3.112	3.413	3.714
83	1.316	1.617	1.918	2.219	2.520	2.821	3.122	3.423	3.724
84	1.326	1.627	1.928	2.229	2.530	2.831	3.132	3.433	3.734
85	1.335	1.636	1.937	2.239	2.540	2.841	3.142	3.443	3.744

[a] Nonadaptable.

3. *Reliability of the table*

The ND_{50} values estimated from Table 2 based on the mean per cent focus reduction of 4 cultures were compared with the ND_{50} values determined by the serial dilution method (Table 3). Foci were counted at 5, 6 and 7 days, respectively. The difference between the ND_{50} values estimated by the single dilution method and those obtained by the serial dilution method was not more than 1: 2. In another experiment, the ND_{50} values of the same serum samples were determined from the Table employing different single dilutions of sera. The ND_{50} values thus obtained were in good agreement.

Choice of day 5 to 7 for counting did not affect the ND_{50} values; the same ND_{50} values were obtained from the per cent reductions determined on day 5–7 as shown in Table 3. The ND_{50} value could not be determined in one of nine sera, because results on the reduction rate were beyond the range of Table 2. In such cases, the test must be repeated at an appropriate dilution. However, if 2 dilutions

TABLE 3. *Comparison of VZV ND_{50} titers estimated by the single dilution method and the serial dilution method*

Serum No.	Age (yr)	Sex	Clinical diagnosis	Days after onset	ND_{50} titers determined by serial dilution	ND_{50} titers determined by single dilution after		
						5 days	6 days	7 days
1	6	M	Varicella	12	32	32	32	32
2	13	M	Varicella	7	64	64	64	64
3	3	M	Varicella	19	64	64	64	64
4	3	F	Zoster	18	256	256	ND[a]	256
5	14	F	Zoster	23	512	512	512	512
6	3	F	Zoster	8	2048	2048	2048	2048
7	5	M	Varicella	13	32	64	64	32
9	3	F	Varicella	14	512	256	ND	ND
10	52	F	Zoster	22	2048	2048	2048	1024

[a] Not determined.

of 8 times difference in dilution are used, Table 2 is applicable for a wide range of ND_{50} values and the antibody titers of most serum specimens can be determined in a single test.

4. *Focus reduction at low serum dilution*

Negative sera were collected from 6 babies of 7 to 10 months old in a nursery who had no history of varicella or other herpes virus diseases and from one healthy 2-year-old child who contracted varicella several months later. All seven sera were seronegative, showing titers of less than 1:2 by the FAMA test. At a dilution of 1:2 in the NT assay, focus reduction of more than 50% was observed in 7 of the 8 sera; at a dilution of 1:4, only one serum gave focus reduction of more than 50%, and at a dilution of 1:16 focus reduction of all sera was less than 20%. Therefore, in the neutralization test, a false positive value will not be obtained at a serum dilution of more than 1:8. Nonspecific focus reduction can be neglected when more than 35% focus reduction is observed at a serum dilution of 1:8 (Fig. 4).

Most of the sera obtained from healthy persons who had contracted varicella 2 to more than 10 years previously showed ND_{50} titers of 1:8 or more at serum dilutions of 1:8 or 1:16 (Table 4), indicating the applicable range

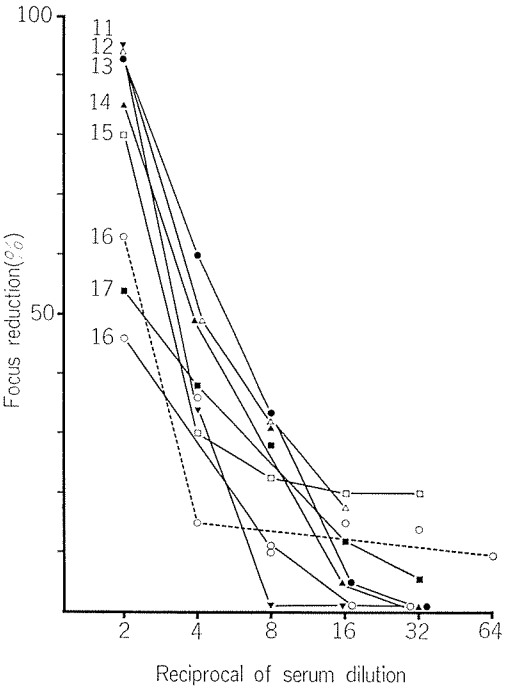

FIGURE 4. Nonspecific reduction of V-Z virus foci with normal sera.

V-Z virus was mixed with negative control sera (11, 12, 13, 14, 15, 16, 17). Volumes of 0.1 ml and 0.05 ml of serum-virus mixtures were inoculated into 6 well trays (- - - -) and 24 well trays (——), respectively.

of focus reduction per cent. Therefore, the neutralization test with a single dilution is applicable to a variety of human sera, not only from patients but also from persons with histories of past infection.

TABLE 4. *Determination of VZV ND_{50} titers in subjects with negative or past history of varicella infection by the single dilution method*

Expt. No.	History of VZV infection	Serum No.	Age (yr)	Dilution of serum	Focus reduction %	ND_{50} titer
1	Positive-adults	18	25	8	50.1	8
		19	51	8	67.9	8
		20	37	8	88.9	≧16
		21	29	8	75.9	16
		22	42	8	59.9	8
		23	39	8	65.7	8
		24	22	8	93.7	≧16
		25	46	8	81.3	16
	Negative-infants	15	2	8	33.6	<8
		16	8 mo.	8	32.7	<8
2	Positive-adults	26	42	8	74.4	8
		27	33	8	59.2	8
		28	43	8	78.4	16
		29	50	8	67.9	8
		30	29	8	50.1	8
		31	27	8	49.2	<8
		32	59	8	85.3	16
		33	22	8	76.2	16
		34	44	8	68.9	8
	Positive-children[a]	35	6	8	72.1	8
		36	6	8	56.0	8
		37	6	8	88.6	≧16
				16	61.0	16
		38	6	8	78.9	16
		39	6	8	56.9	8
		40	6	8	69.7	8
		41	5	8	64.7	8
		42	5	8	85.8	≧16
				16	72.9	16
		43	6	8	90.8	≧16
				16	67.0	16
		44	5	8	59.7	8
		45	5	8	55.1	8
	Negative-infant	13	8 mo.	8	15.8	<8

[a] This group had contracted varicella 2 to 3 years previously.

DISCUSSION

The complement fixation (CF) test is commonly used for assay of antibody to V-Z virus. However, it is well recognized that the antibody titer to V-Z virus by CF is low and decreases to an undetectable level within a year after infection, and that this test is unsuitable for examining previous V-Z infection. The immune adherence hemagglutination (IAHA) test seems to be more sensitive than CF and this test is rather simple and rapid. However, it is reported to be relatively insensitive for detecting V-Z antibody from past infections (Kalter et al., 1977). Moreover, false negative reactions at lower dilutions of high-titer sera and nonspecific reactions against control antigens were occasionally seen (Forghani et al., 1978; Gershon et al., 1976). The FAMA technique is sensitive enough to measure low levels of antibody to V-Z virus (Zaia and Oxman, 1977) and is easy to perform and does not require cell-free virus. Radioimmunoassay (RIA) is thought to be the most sensitive antibody assay technique (Friedman et al., 1979), but both FAMA and RIA need special equipment. The NT has been used as the most reliable method for detecting specific antibody to V-Z virus, but the test requires storage of cell-free virus and a large numbers of fresh tissue cultures of human diploid cells. Moreover, it is laborious and time-consuming. Gerna et al. reported that NT antibody could be determined in a short time by applying the immunoperoxidase antibody (IPA) technique (Gerna et al., 1977; Gerna and Chambers, 1976).

The single dilution method using 6-well trays reported here is an accurate, reproducible and economical test that saves material and time. As foci could be counted on day 5 after inoculation, results could be obtained at least 3 days earlier than by the classical solid overlay method. Moreover, this technique is not complicated by fungal contamination occasionally observed in long-term cell cultures. The neutralizing antibody titers determined by this method were not affected by variation in the challenge dose of virus from 50 to several hundred FFU per well. Comparison of the titers obtained with 4 and 2 cultures per dilution of test serum showed that the variance ratios (F) from both linearity and parallelism were somewhat smaller using 4 cultures, but even with 2 cultures, F values were not large enough to be significant. Therefore, 2 cultures can be used for a serum sample in routine testing. One disadvantage of this method is that foci must be counted under a microscope, and so it is difficult to handle large numbers of specimens. However, it is possible to count the foci at any time between day 5 and 7, and it should also be possible to count them by naked eye on day 8–10 after neutral red staining. Cell-free virus for the NT test, 10^4 to 10^5 FFU per ml, is easily obtained by the procedure described in Materials and Methods.

With the recently developed enzyme immunoassay (EIA) and IAHA test, some nonspecific reactions were observed at serum dilutions as low as 1:8 (Forghani et al., 1978). Grose et al. noted nonspecific focus reductions at dilutions of 1:2 and 1:4 in the complement requiring NT assay (Grose et al., 1979). In our study, similar nonspecific focus reductions were seen. It is uncertain whether this nonspecific reaction was due to our procedures, the activity of the serum itself or the effects of other agents. On the contrary, the FAMA technique has been found to be relatively free of nonspecific reactions at low serum dilutions, and long-lasting antibody titers were observed after infection. In an experiment on administration of live varicella vaccine, we observed that the titers of FAMA antibody against V-Z virus in sera from children who had received live varicella vaccine 6 to 7 weeks before were about twice the NT titers and two or four times the CF titers. The FAMA test seems most suitable for identifying the immune status of high risk persons to V-Z virus infection in immunocompromised conditions.

ACKNOWLEDGMENTS

The authors thank Dr. M. Minamitani and Dr. N. Takayama, Tokyo Metropolitan Hospital of Komagome, and Dr. T. Ogata of this Institute for providing serum specimens.

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