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STUDIES ON NEUTRALIZATION OF VARICELLA-ZOSTER VIRUS AND SEROLOGICAL FOLLOW-UP OF CASES OF VARICELLA AND ZOSTER

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SUMMARY Conditions for the neutralization test of varicella-zoster (V-Z) virus were investigated in detail for the purpose of making this test sufficiently simple and efficient for routine use. It was found that the number of plaques produced under liquid medium 7 to 8 days after infection was comparable with the number produced under solid medium, and that no secondary plaques under liquid medium appeared during a 14-day observation period. Thus plaque counting under liquid medium at 7 to 8 day after infection was a convenient method for assay of viral infectivity and for the neutralization (NT) test. PSGC medium (PBS(−) containing 5% sucrose, 0.1% Na-glutamate and 10% fetal calf serum) was found suitable for keeping infectivity of V-Z virus as stable as possible. With this medium as virus and serum diluent, the most efficient conditions for the NT test are incubation of serum-virus mixture for 30 to 60 min at 37 C with occasional shaking. In a serological follow-up of cases of varicella and zoster, all 4 cases of zoster examined were found to have high NT antibody titers 1 to 2 weeks before the onset of the disease, suggesting that decrease in the NT antibody titer may not be the cause of development of zoster symptoms.

INTRODUCTION

The neutralization (NT) test of V-Z virus has long been hampered by the difficulty of obtaining cell-free virus and the instability of the virus. There have been only a few recent studies on neutralization of this virus (Caunt and Shaw, 1969, Schmidt et al., 1969), and too little is known about the NT test on this virus for it to become a routine test.

In the present study, we investigated the

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conditions necessary for the NT test for the purpose of making this test sufficiently simple and efficient for use as a routine serological test. We then applied the method in serological follow-ups on cases of varicella and zoster.

MATERIALS AND METHODS

1. *Virus*

The Kawaguchi strain of varicella virus isolated in Osaka (Takahashi et al., 1975) was mainly used in this study.

2. *Cells*

Human embryonic lung (HEL) cells at the 3rd to 30th passage level were used. Cells were grown in a mixture of equal volumes of Eagle's MEM and Medium 199 supplemented with 10% fetal calf serum (FCS). Cultures were maintained in similar medium but with only 3% serum.

3. *Preparation of cell-free virus*

Cell-free virus was prepared as follows. When approximately 70% of an infected culture showed cytopathic effects (CPE), the culture fluid was discarded and infected cells were washed with PBS(−). Cells were collected after EDTA treatment, suspended in various media (SPGA, PSGC, and Medium 199 containing 4% FCS), and sonicated at 20 KC/sec for 40 sec. Then the suspensions were clarified by centrifugation at 3,000 rpm for 15 min and the supernatants were used as virus preparations. SPGA medium was composed of 0.218 M sucrose, 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M Na-glutamate, and 1% bovine albumin. PSGC medium was PBS(−) containing 5% sucrose, 0.1% Na-glutamate, and 10% FCS, prepared by mixing sterilized solutions of these components.

4. *Assay of infectivity of cell-free virus*

Plaque assay with agarose-overlay was done as follows. Cell cultures in 60 mm dishes were inoculated with 0.2 ml of virus preparation, usually diluted with PSGC medium, and incubated for 2 hr at 37 C. Then the fluid inoculumn was decanted, fresh Medium 199 containing 0.6% agarose and 10% FCS was added and incubation was continued. The second overlay with 4 ml of solution was done on the 5th day and the third overlay with 3 ml of solution containing 0.01% neutral-red was done on the 10th day. The plaque count was performed on the following day. Plaque assay under liquid medium was carried out in a similar way. After an adsorption period of 2 hr, fresh Medium 199 containing 4% FCS was added, and medium change was done every 3 to 4 days. The number of plaques under liquid medium was usually counted on the 7th to 8th day under a microscope at low magnification, or by naked eye after staining with 0.01% neutral-red.

5. *Neutralization test*

The plaque reduction NT test was performed essentially as described by Caunt and Shaw (1969). Samples of the virus preparation diluted to approximately 100 to 200 plaque forming units (PFU)/0.1 ml were mixed with equal amounts of serial two-, or four-fold dilutions of serum inactivated by heating at 56 C for 30 min, and the mixture were incubated at 37 C for 30 min with occasional shaking. Then 0.2 ml volumes of serum-virus mixture were inoculated onto cell cultures in 36 mm plastic dishes, and these were incubated at 37 C for 2 hr to permit adsorption of unneutralized virus. Then medium was added and incubation was continued for 7 to 8 days until the plaques were large enough to count. Antibody titers were expressed as the reciprocal of the highest dilution of serum producing 50% or more reduction in the plaque count.

6. *Complement fixation (CF) test*

The Kawaguchi strain of varicella virus was generally used as CF antigen. When extensive CPE had appeared in HEL cells in 500 ml bottles, the infected cells were collected, suspended in 5 ml of PBS(−), sonicated for 3 min at 20 KC/sec and centrifuged at 3,000 rpm for 15 min. The supernantant usually had an antigen titer of 1:32 or 1:64, and 4 units were used for the reaction. CF antibody was titrated by the standard microtiter technique, using 4 units of antigen, 2 units of complement, and overnight fixation at 4 C.

7. *Human sera for serological assay*

Human sera were collected from patients with a clinical diagnosis of varicella or zoster and from individuals with a past history of varicella or zoster. The sera were stored at −20 C until assayed.

RESULTS

1. *Investigations on the neutralization of V-Z virus*

1) Comparison of the numbers of plaques under liquid medium and solid medium with regard to the appearance of secondary foci

The virus material was inoculated simultaneously into 2 sets of cells in dishes. After the adsorption period, one set of cells was overlayed with medium containing agarose while the other set was overlayed with maintenance medium. The plaques produced in dishes with agarose overlay were counted on days 7 and 14 and the plaques produced under liquid overlay were counted on days 5, 6, 7, 8, 9 and 14. As shown in Table 1, the number of plaques under liquid medium appearing 7 to 14 days after inoculation was approximately equal to the number under solid medium and no secondary plaques were noticed during a 14-day observation period. Moreover, on day 7, the plaques produced under liquid medium were larger and easier to count than those produced under agarose overlay. Thus counting plaques under liquid medium on day 7 to 8 after inoculation is a convenient method for assay of infectious virus. Because of its simplicity, plaque counting under liquid medium was used exclusively in subsequent tests; the number of plaques counted is referred to as the plaque number.

TABLE 1. *Comparison of the number of plaques under liquid medium and under agarose-overlay*

Days after inoculation	Number of plaques/dish under liquid medium		Number of plaques/dish under agarose-overlay	
5	10,	—	—	—
6	35,	28	—	—
7	40,	37	38,	42
8	40,	39	—	—
9	42,	39	—	—
14	42,	41	41,	42

2) Relationship between virus concentration and plaque number

As shown in Table 2, plaque numbers vary with the dilution of the virus preparation, indicating that each plaque was produced by a single infectious viral particle.

TABLE 2. *Relationship between virus concentration and plaque count*

Dilution	Number of plaques/dish			Mean
10	127,	151,	112	130
50	26,	28,	27	27
100	12,	16,	15	14
500	2,	3,	2	2

3) Adsorption of virus to cultured cells

Cell cultures in 60 mm dishes were inoculated with 0.2 ml of virus diluted 1: 100 with PSGC medium. After various adsorption periods, half of cultures were rinsed gently with 6 ml of PBS(−), fresh medium was added and incubation was continued at 37 C for 7 days. The other half of the cultures were in-

TABLE 3. *Rate of adsorption of virus to HEL cells*

Time of adsorption	Number of plaques/dish								% of virus adsorbed
	Rinsing (−)				Rinsing (+)				
				Mean				Mean	
30 min	166	179	174	173	94	108	112	105	60.7
1 hr	160	181	184	175	121	115	127	121	69.1
2 hr	184	181	158	174	143	—	160	152	87.4
3 hr	180	182	178	180	188	160	—	174	100

cubated with maintenance medium without rinsing the cells. Table 3 indicates that most of the input virus was adsorbed to the culture in 2 hr and that, when the inoculated virus was not rinsed out, an adsorption period of 30 min was enough for assay of infectious virus.

TABLE 4. *Comparison of thermal inactivation of virus in various media*

Temp.	Time	Survival (% of count at 0 hr)		
		PSGC[a]	SPGA[b]	Medium 199-4% FCS[c]
37 C	0	100	100	100
	15 min	—	—	78.6
	30	71.9	71.2	56.7
	1 hr	62.5	61.5	26.7
	2	39.4	38.5	15.0
	6	10.6	29.0	0.5
	12	—	—	0
	24	1.0	2.3	0
24 C	0	100	100	100
	15 min	—	—	99.5
	30	100	100	93.0
	1 hr	100	100	85.0
	2	100	100	67.4
	6	23.8	34.6	21.9
	12	—	—	10.2
	24	4.7	7.9	1.6
4 C	0	100	100	100
	15 min	—	—	99.5
	30	100	100	94.7
	1 hr	100	100	87.7
	2	100	100	69.0
	6	—	100	28.3
	12	—	—	18.2
	24	18.8	38.5	3.7

[a] PSGC: PBS (−) containing 5% sucrose, 0.1% Na-glutamate, 10% FCS

[b] SPGA: 0.218 M sucrose, 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M Na-glutamate, 1% bovine albumin

[c] FCS: fetal calf serum

4) Comparison of thermal inactivation of virus in various media

SPGA medium is reported to be suitable for the preservation of V-Z virus from thermal inactivation (Hondo et al., 1976). We prepared PSGC medium, which is a simplified form of SPGA medium, and compared it with SPGA medium and Medium 199 containing 4% FCS for theraml inactivation of V-Z virus. As shown in Table 4, loss of viral infectivity was not marked in SPGA or PSGC medium. Accordingly PSGC medium was used in subsequent NT tests.

5) Comparison of the NT antibody titers at various incubation periods and temperatures

The NT antibody titers with various incubation periods and temperatures were compared using a convalescent zoster serum. The effect of the incubation period of the serum-virus mixture was investigated under stationary conditions and with shaking. As shown in Table 5, neutralization of the virus was proportional to the incubation period and was more efficient with shaking; but as a drop in the control virus titer was observed after incubation for 2 hr, 30 to 60 min of incubation seems suitable for the NT test.

Next incubation temperatures of 37 C and 24 C in the NT test were compared. As shown in Table 6, neutralization of virus was far more efficient at 37 C than at 24 C. From these results, incubation of the serum-virus mixture at 37 C for 30 to 60 min with occasional shaking seems the most suitable for routine NT tests on V-Z virus.

2. *Serological follow-up of cases of varicella and zoster by the CF and NT tests*

Serological follow-up was made on cases of varicella and zoster and adults with past histories of varicella or zoster. No CF antibody was detectable even 8 months after infection in some children and adults with past histories of varicella (Fig. 1), but NT antibody presisted for longer periods (Fig. 3). Rise of CF antibody was generally faster in cases of zoster than in those of varicella (Fig. 2). In 4 cases

TABLE 5. *Comparison of NT antibody titers after various lengths of incubation of serum-virus mixture with or without shaking at 37 C*

Condition	Incubation time	Serum dilution	No. of plaques/dish				Mean	% of virus neutralized
Shaken	30 min	2	—	1	0	1	1	98.2
		8	12	15	10	12	12	78.2
		32	27	36	39	35	34	38.2
		128	—	48	59	58	55	0
		vc[a]	—	56	59	51	55	
	60 min	2	0	0	1	0	0	100
		8	4	6	4	5	5	90.0
		32	24	25	24	—	24	52.0
		128	—	48	41	—	45	10.0
		vc	—	50	49	51	50	
	120 min	2	0	0	0	—	0	100
		8	0	2	2	2	2	92.9
		32	9	11	7	—	9	67.9
		128	—	31	20	23	25	10.7
		vc	30	28	32	—	28	
Stationary	30 min	2	4	6	4	—	5	91.1
		8	23	22	21	31	24	57.1
		32	41	42	45	49	44	21.4
		128	46	58	60	59	56	0
		vc	51	57	61	56	56	
	60 min	2	1	2	1	1	1	98.0
		8	7	7	10	15	10	79.6
		32	—	33	31	42	35	28.6
		128	—	50	51	49	50	0
		vc	—	48	50	50	49	
	120 min	2	0	0	0	1	0	100
		8	5	4	1	5	4	89.7
		32	—	14	12	17	14	64.1
		128	31	34	35	41	35	10.3
		vc	—	40	38	38	39	

[a]: virus control, the same amount of PSGC medium was added instead of the diluted human serum.

of zoster, high NT antibody titiers were detected 1 to 2 weeks before onset the of the disease (Fig. 4). These patients with past history of varicella had been in hospital with nephrotic syndrome, which enabled us to collect the sera shortly before the onset of zoster symptoms. The CF antibody titier was very low or undetectable at or before onset of zoster.

DISCUSSION

In this work we found that the number of plaques produced in 7 to 8 days after infection

TABLE 6. *Parallel NT tests in which serum-virus mixtures were incubated at 37 C and 24 C with occasional shaking*

Incubation time	Serum dilution	37.0 C				24.0 C			
		No. of plaques/dish		Mean	% neutralization	No. of plaques/dish		Mean	% neutralization
30 min	4	0	0	0	100	0	0	0	100
	8	0	0	0	100	6	3	5	91
	16	2	1	2	96	24	—	24	55
	32	5	3	4	91	21	24	23	57
	64	3	4	4	91	30	33	32	40
	128	—	9	9	80	42	42	42	21
	256	20	24	22	51	60	48	54	0
	vc	42	48	45		60	45	53	
1 hr	4	0	0	0	100	0	0	0	100
	8	0	0	0	100	4	1	3	94
	16	0	0	0	100	9	6	8	84
	32	0	0	0	100	18	15	17	66
	64	4	1	3	92	26	23	25	50
	128	7	6	7	82	40	35	38	24
	256	8	14	11	72	44	53	49	2
	vc	40	38	39		48	51	50	

of HEL cells with V-Z virus under liquid medium is comparable with the number of plaques produced under agarose-overlay, Moreover, no secondary plaques appeared under liquid medium during an observation period of 14 days. Use of liquid overlay is simpler than use of agarose-overlay, and thus seems suitable for use in assay of viral infectivity and the NT test.

We also found that PSGC medium, which is a simplified form of SPGA medium, is as good as the latter for maintaining infectivity of V-Z virus. Using PSGC medium as diluent for virus and serum and also plaque counting under liquid medium, we examined the effect of incubation conditions on neutralization of V-Z virus and found that in the NT test, incubation of mixture at 37 C for 30 to 60 min with occasional shaking was the most suitable for practical purposes.

In a serological follow-up study on 4 cases of zoster, high NT antibody titers were found 1 to 2 weeks before the onset of the disease in all the patients. Caunt and Shaw reported that little or no NT antibody titer was detected at the onset of zoster in 3 patients in tests using PBS(−) containing 2% calf serum as diluent (Caunt and Shaw, 1969). The reason for this discrepancy between their results and ours is unclear, but may be partly due to a difference in sensititivity of the NT tests employed. Although we examined only a few cases, our results indicate that zoster symptoms appear in the presence of NT antibody. Brunell et al. (1975) reported that V-Z IgG was detected by FAMA test (fluorescent antibody to membrane antigen) in 3 cases prior to onset of zoster symptoms. Depressed cellular immunity might be correlated with development of zoster as reported by Russel et al. (1972), and this possibility is now under investigation using a varicella skin test (Kamiya et al., 1977).

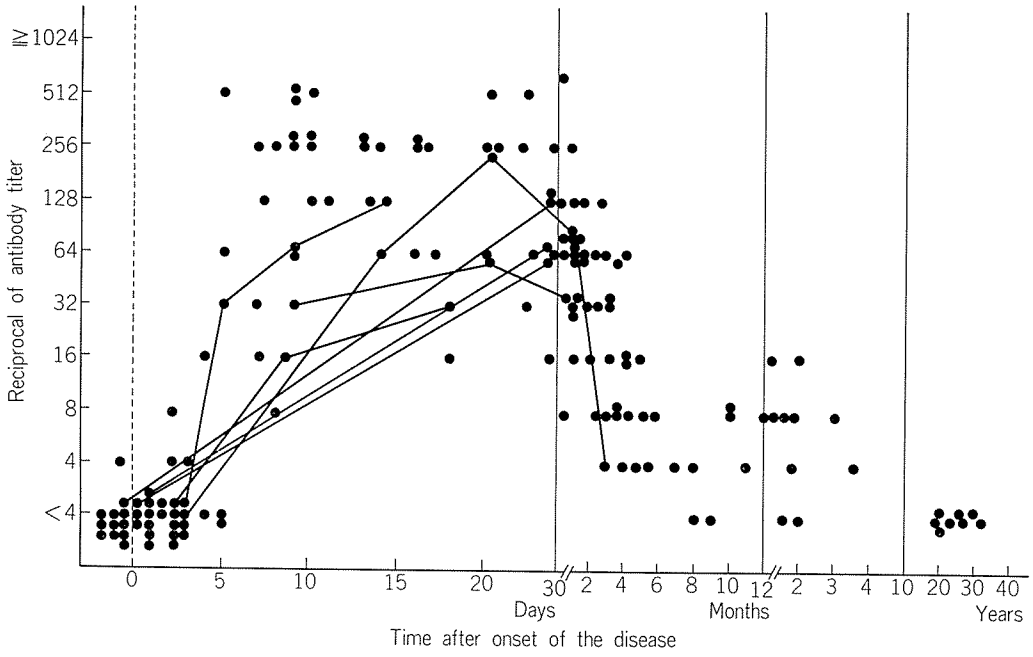

FIGURE 1. CF antibody titers of sera from patients with varicella at various times after appearance of the rash

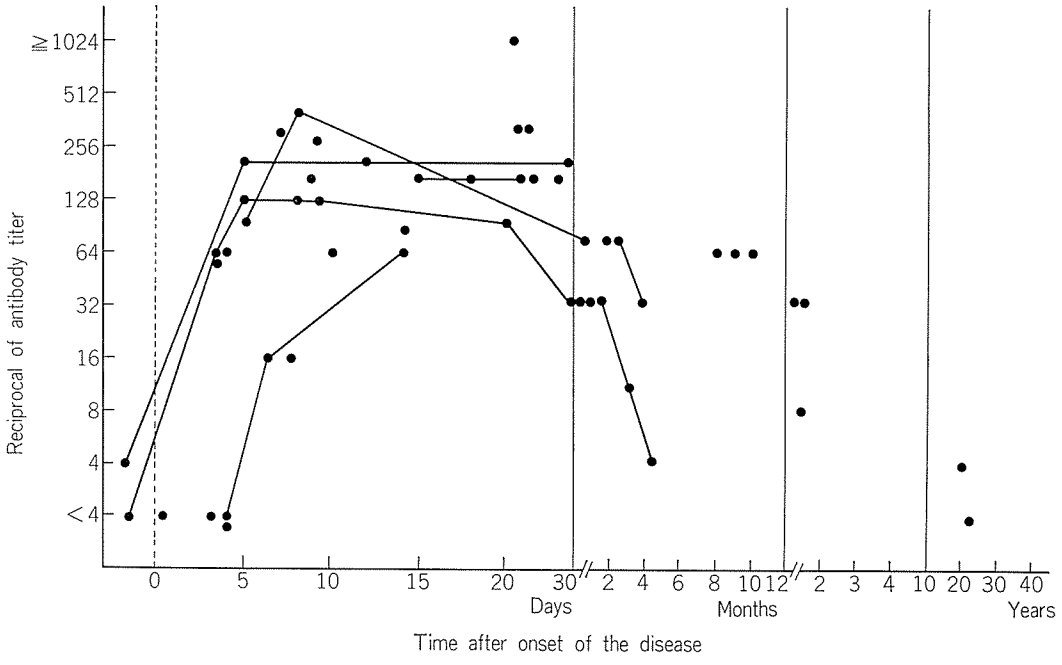

FIGURE 2. CF antibody titers of sera from patients with zoster at various times before and after appearance of the rash

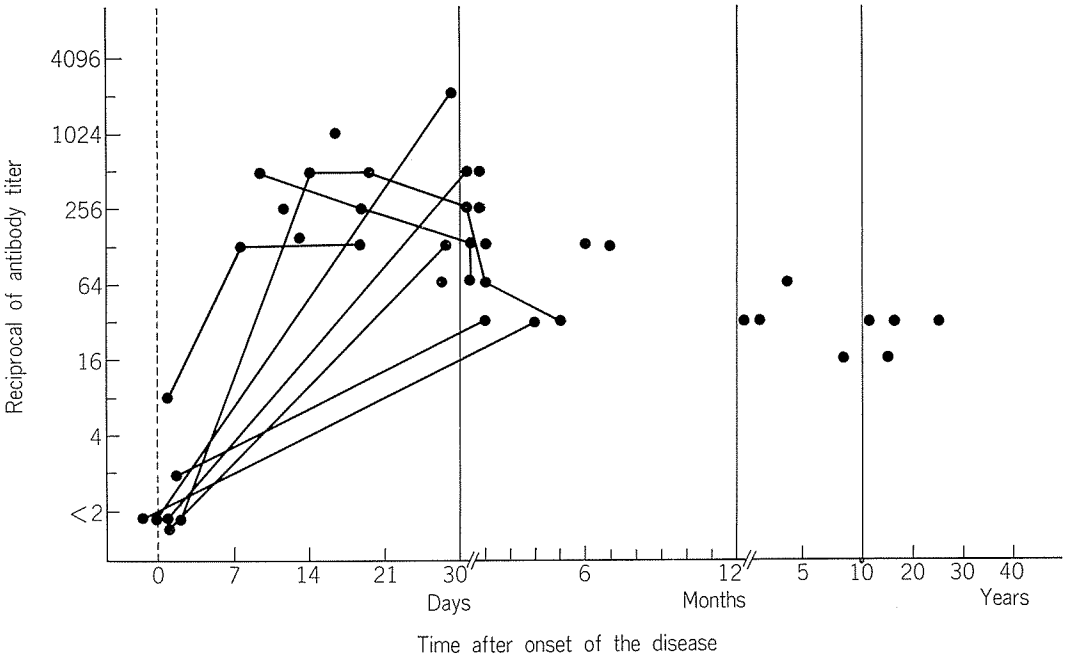

FIGURE 3. NT antibody titers of sera from patients with varicella at various times after appearance of the rash

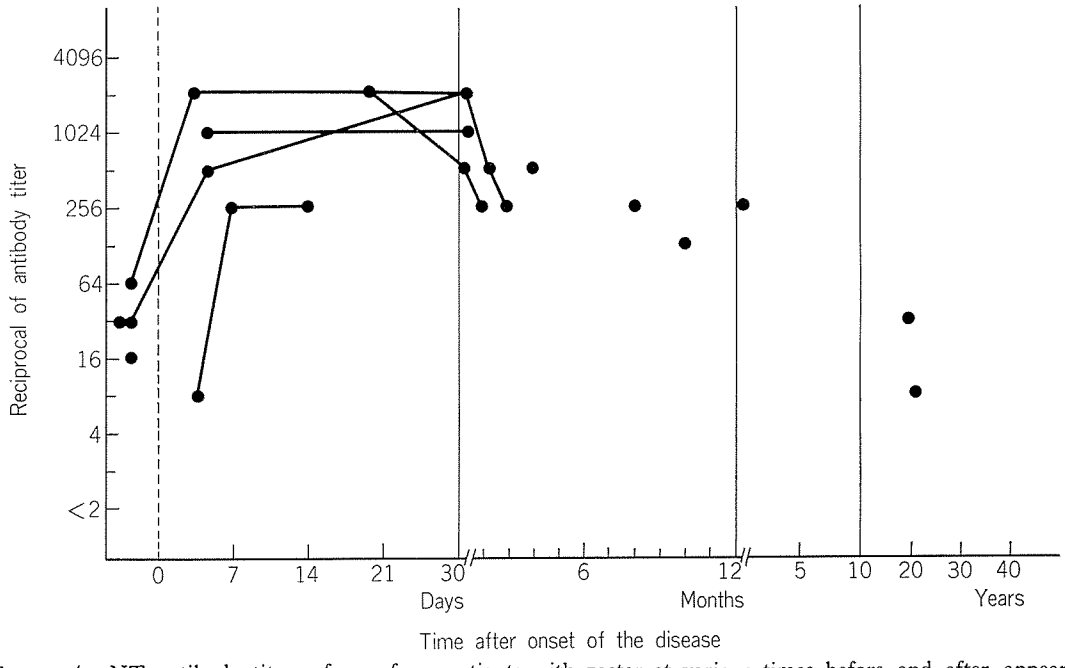

FIGURE 4. NT antibody titers of sera from patients with zoster at various times before and after appearance of the rash

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