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Neutralization tests with varicella-zoster virus

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Varicella-zoster (V-Z) virus was first cultivated in tissue culture by Weller & Stoddard (1952). Subsequently the fluorescent antibody technique was used by Weller & Coons (1954) to demonstrate that antibodies to the virus are present in convalescent chickenpox and zoster sera, but few neutralization tests have been done with the agent because of the difficulty in obtaining virus in a cell-free state from most tissue culture systems. Weller & Witton (1958) showed that the ability of intact infected human embryonic fibroblasts to transmit virus to fresh tissue culture monolayers could be partially neutralized by antiserum if the antiserum was incorporated in the medium of the inoculated tissue cultures. The cytopathic effects of the virus were not suppressed completely but the number of foci of infection was greatly reduced and the foci themselves were altered in appearance. Taylor-Robinson (1959) described neutralization tests in which vesicle fluid taken directly from patients was used as a source of cell-free virus. This system gave satisfactory results but its application was limited by the difficulty of obtaining sufficient vesicle fluid. The production of cell-free virus from primary human thyroid cells (Caunt, 1963) provides a more convenient laboratory source of cell-free virus and this paper describes the method we have used for detecting V-Z virus neutralizing antibody in the sera of chickenpox and zoster patients and in pools of human gamma-globulin.

Kapsenberg (1964) and Ross, Subak Sharpe & Ferry (1965) showed some cross-reactions between *Herpes simplex* (HS) and V-Z virus in complement-fixation (CF) tests so we also tested most of our sera with HS virus to see whether any cross-neutralization could be demonstrated. The paired sera were also tested for CF antibody to both V-Z and HS antigens.

MATERIALS AND METHODS

Tissue cultures

V-Z virus was titrated either in primary human thyroid cells prepared as described by Pulvertaft, Davies, Weiss & Wilkinson (1959) or in Vero cells, a continuous line of vervet monkey kidney cells (Liebhaber, Riordan & Horstmann, 1967). In both cases the cells were grown in $6 \times \frac{5}{8}$ in. tubes. HS virus was titrated either in GMK-AH 1 cells, a continuous line of grivet monkey kidney cells (Gunalp, 1965), or in Vero cells.

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Media

Human thyroid cells were grown in 5% calf serum in Parker's 199 medium (Burroughs Wellcome Ltd.) and maintained in the same medium with the calf serum reduced to 2%. Vero cells were also grown in Parker's 199 medium but with 5% and 2% of foetal calf serum (Flow Laboratories) for growth and maintenance respectively. GMK-AH1 cells were grown in Eagle's minimum essential medium (M.E.M., Burroughs Wellcome Ltd.) with 5% and 2% of calf serum for growth and maintenance respectively.

Viruses

V-Z virus was prepared by the ultrasonic disruption of infected primary human thyroid cells as described by Caunt (1963). Several virus strains, all isolated from vesicle fluid of chickenpox or zoster patients, were used. Preliminary experiments showed no antigenic differences between these strains (Shaw, 1968). The virus preparations were stored at −65° C. in small volumes and a fresh portion was thawed for each test.

One strain of HS virus isolated in human amnion cells and subsequently sub-cultured in continuous monkey kidney cell lines was used throughout. Virus preparations were made from infected tissue cultures in which all the cells showed cytopathic effects. The cells from two or three 12 oz. bottles were washed from the glass by pipetting, pooled in 3 ml. of maintenance medium and disrupted by ultrasonic treatment as used in the preparation of V-Z virus. The treated material was then diluted to 35 ml. with the medium harvested from the infected bottles, yielding a preparation with a titre of about 10^8 plaque-forming units (p.f.u.) per ml. which could be stored at 4° C. for 2 weeks with little loss of infectivity. Preparations were made from Vero and GMK-AH1 cells but were always titrated in the same cell system as that in which they were prepared.

Antigens for CF tests

V-Z antigen was prepared by the ultrasonic disruption of infected human thryoid cells (Caunt & Taylor-Robinson, 1964). HS antigen was prepared from infected BHK-21 cells by the method of Grist, Ross, Bell & Stott (1966).

Sera

Sera were stored at −20° C. and were heated to 56° C. for 30 min. or 58° C. for 10 min. before use.

Human gamma-globulin

One specimen had been prepared by the Lister Institute and the other specimen, which was vaccinia immune globulin, by E. R. Squibb and Sons, New York.

Diluent for neutralization tests

Viruses and sera were diluted in phosphate buffered saline (Dulbecco & Vogt, 1954) to which 2% inactivated calf serum had been added. The final pH was 7·3.

Neutralization test techniques

V-Z virus

The virus preparation was diluted to contain 1–3 × 10^3 p.f.u./ml. and mixed with equal quantitites of serial dilutions of the serum under test or with 1/10 calf serum as a control. After incubation under various conditions, which are described in the experimental results, 0·2 ml. portions of the serum–virus mixtures were inoculated into each of two or three tubes of tissue culture from which the medium had been removed. The inoculated tubes were stoppered and left stationary at room temperature for 3½ hr. to allow un-neutralized virus to adsorb to the cell sheets before 1 ml. of maintenance medium was added to each tube. The tubes were incubated in stationary racks at 35° C. for 4–5 days until viral lesions were large enough to be counted. No secondary foci occurred within this time. The lesions were counted using a low-power microscope (magnification × 10) and dark-ground illumination.

HS virus

Neutralization tests were carried out by the method described by Ross *et al.* (1965) using the plaque assay method of Russell (1962). The inoculated cells were seeded into 1½ in. diameter glass Petri dishes using three dishes per serum–virus mixture, and these were incubated for 3 days at 35° C. in an atmosphere of 5 % CO_2 in air. The dishes were then drained, fixed in Bouin's fluid, washed under the tap and stained with carbol-fuchsin. The plaques could then be counted by the naked eye.

CF test technique

CF tests were performed by the method described in Public Health Monograph no. 74 (1965).

Table 1. *Comparison of various techniques for neutralization of V-Z virus*

	Number of plaques per tube with serum dilution				Virus control	
Condition of test	1/5	1/25	1/125	1/625	Plaque counts	Mean
Shaken at 35° C.	0, 0, 0	0, 0, 0	14, 7, 13	15, 20, 16	50, 90	70
Shaken at room temperature	0, 0, 0	1, 0, 0	54, 31, 29	59, 40, 63	60, 50	55
Stationary at 35° C.	0, 0, 0	6, 3, 11	51, 53, 67	72, 61, 74	100, 50	75

RESULTS

Comparison of techniques for neutralization tests with V-Z virus

A convalescent zoster serum was used for this test. The serum–virus mixtures were either incubated in stationary racks at 35° C. or were shaken slowly (40 strokes/min.) in a shaking water bath at room temperature or at 35° C. for 1 hr., in an attempt to increase the rate of reaction. The results of these tests are shown in Table 1. The tubes which were shaken at 35° C. gave the highest neutralization titre, with a virus control count not appreciably different from that resulting from

stationary incubation at 35° C. The virus has been shown not to suffer any detectable thermal inactivation at 37° C. in 1 hr. (Shaw, 1968). This technique was therefore used in all subsequent neutralization tests on V-Z virus. The titres recorded are the final dilutions which gave 50 % reduction in plaque count.

Table 2. *Paired sera from chickenpox cases tested for neutralizing and CF antibodies to V-Z and HS viruses*

Case	Duration of rash when serum taken	Serum dilution giving 50 % neutralization with		CF titre with	
		V-Z virus	HS virus	V-Z antigen	HS antigen
1	3 days	< 1/5	1/80	1/40	1/80
	14 days	1/40	1/80	1/1280	1/320
2	2 days	< 1/5	< 1/5	< 1/5	< 1/5
	6 days	1/20	< 1/5	1/1280	< 1/5
	21 days	1/20	< 1/5	1/1280	< 1/5
3	3 days	1/5	1/640	1/320	1/80
	18 days	1/40	1/640	1/2560	1/160
4	3 days	< 1/5	1/1280	1/10	1/20
	28 days	1/40	1/1280	> 1/2560	1/160
5	1 day	< 1/5	< 1/5	a/c	< 1/10
	16 days	1/20	< 1/5	1/1280	< 1/10
6	1 day	< 1/5	< 1/5	1/10	< 1/10
	14 days	1/20	< 1/5	1/320	< 1/10
7	1 day	< 1/5	< 1/5	1/40	< 1/10
	14 days	1/20	< 1/5	1/640	< 1/10

a/c = anticomplementary.

Neutralization and CF tests on paired sera from cases of chickenpox

The results of neutralization and CF tests on paired sera from seven cases of chickenpox are shown in Table 2. Up to 3 days after the appearance of the rash the titre of neutralizing antibody to V-Z virus did not exceed 1/5. In the only case tested at 6 days the titre had risen to 1/20 and did not subsequently rise further. The maximum titre of neutralizing antibody to V-Z attained by any of the cases was 1/40. There was no rise in titre of neutralizing antibody to HS virus in any of the cases.

The CF antibody titre with V-Z virus rose in all cases and where the patient was a herpetic the CF titre to HS rose also but this latter rise did not occur if there was no HS CF antibody in the acute stage serum.

Persistance of neutralizing antibody to V-Z virus after chickenpox

Sera were tested from nine adults who had had chickenpox many years previously, but who had not had zoster, to see how long neutralizing antibodies persisted. The results are shown in Table 3. Six of these cases had titres of 1/10 or less and three had titres of 1/20, 1/40 and 1/160 respectively. A second sample from this last case taken 10 years after the first still showed a titre of 1/160.

Sera from two adults who had never had chickenpox were also tested and no antibody could be detected at a dilution of 1/5.

No correlation between neutralizing antibody titres to V-Z and HS viruses could be shown.

Table 3. *Sera from adults who had had chickenpox but not zoster tested for neutralizing antibodies to V-Z and HS viruses*

Case	Time since chickenpox (in years)	Serum dilution giving 50 % neutralization with	
		V-Z virus	HS virus
8	40	1/20	< 1/10
9	35	1/10	1/320
10	30	< 1/5	< 1/10
11	30	1/40	1/160
12a	20	1/160	NT
12b	30	1/160	< 1/10
13	28	< 1/5	1/80
14	25	< 1/5	1/100
15	20	1/5	1/160
16	20	1/10	< 1/10
17	not had CP	< 1/5	< 1/10
18	not had CP	< 1/5	> 1/50

NT = not tested.

Table 4. *Paired sera from cases of zoster tested for neutralizing and CF antibodies to V–Z and HS viruses*

Case	Duration of rash when serum taken	Serum dilution giving 50 % neutralization with		CF titre with	
		V-Z virus	HS virus	V-Z antigen	HS antigen
19	3 days	< 1/5	NT	1/10	NT
	9 days	1/320	1/640	> 1/2560	1/320
20	2 days	< 1/5	1/640	1/10	1/160
	23 days	1/40	1/640	1/320	1/160
21	4 days	1/5	< 1/10	1/10	1/80
	15 days	> 1/320	< 1/10	1/2560	1/80

NT = Not tested. Insufficient serum.

Neutralization and CF tests on paired sera from patients with zoster

Paired sera from three cases of zoster were tested for neutralizing and CF antibodies to V-Z and HS viruses and the results are shown in Table 4. It can be seen that although the titre of neutralizing antibody to V-Z virus in the acute stage sera was low or absent, as in the acute stage of chickenpox, two of the convalescent sera show titres of 1/320. No rise in titre of neutralizing antibody to HS occurred in any of the patients.

The titres of CF antibody to V-Z antigen rose in all three cases but no rise in CF titre to HS antigen was seen.

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Persistence of neutralizing antibody to V-Z virus after zoster

Single sera from nine patients who had had zoster at some time were tested for neutralizing antibodies to V-Z and HS viruses and the results are shown in Table 5. A high level of neutralizing antibody to V-Z virus appears to be common after zoster and to persist for many years, although sera from patient 28 showed some fall in titre after 2 years.

Neutralizing antibody titres to HS virus varied and again showed no correlation with those to V-Z virus.

Table 5. *Sera from patients who had had zoster tested for neutralizing antibodies to V-Z and HS viruses*

Case	Time from onset of rash when serum taken	Serum dilution giving 50 % neutralization with V-Z virus	Serum dilution giving 50 % neutralization with HS virus
22	5 days	1/160	1/320
23	14 days	> 1/160	NT
24	18 days	1/40	1/40
25	18 days	1/80	< 1/10
26	1 month	1/80	1/40
27	3 months	> 1/640	1/40
28a	2 months	> 1/160	> 1/320
28b	2 years	1/80	NT
29	15 years	1/160	1/40
30	40 years	1/160	1/80

NT = not tested.

Table 6. *Neutralization tests with V-Z virus on paired sera from contacts of chickenpox or zoster*

Case	Time from contact when serum taken	Serum dilution giving 50 % neutralization with V-Z virus
31	0 days	1/80
	32 days	1/80
32	1 day	< 1/10
	41 days	< 1/10
33	5 days	1/40
	49 days	1/40
34	5 days	1/20
	4½ months	1/20
35	2 days	< 1/10
	29 days	1/80

Neutralization tests with V-Z virus on sera from close contacts of chickenpox and zoster

Hope-Simpson (1965) suggested that adults who had had chickenpox in childhood might become reinfected on contact with chickenpox cases and show an antibody response to the virus even though they did not develop clinical illness.

In order to test this hypothesis paired sera from five close household contacts of chickenpox or zoster were supplied to us by Dr Hope-Simpson. The first serum in each pair was taken on the day of contact or very shortly afterwards. The results of these tests are shown in Table 6. The only rise in neutralizing antibody to V-Z virus occurred in case 35, a mother whose three children developed chickenpox. Her first serum was taken 2 days after the first child developed the disease. Cases 31 and 32 were also the parents of children who developed chickenpox. Case 33 was a boy whose mother had chickenpox and case 34 was a boy whose mother had zoster

The sera from case 35 were tested for CF antibodies to V-Z. The first serum showed no fixation and the second one was anticomplementary.

Neutralization tests against V-Z virus on pooled human gamma-globulin

The gamma-globulin solution was first diluted 1/10 and then serial twofold dilutions were made. Up to 1/40 both batches were toxic to the tissue culture cells but at higher dilutions neutralization occurred and the 50 % end-point was 1/160 for the Lister Institute specimen and 1/320 for the other.

DISCUSSION

The results presented in Table 2 show that the neutralizing antibody response immediately after primary varicella infection is low and the maximum titre demonstrated was 1/40. Among people who had had chickenpox many years previously six of nine tested had neutralizing antibody titres of 1/10 or less which would be consistent with a gradual decline from an original low level. Two of the others had titres of 1/20 and 1/40 which could be explained by a variation in the original antibody response, but case 12, with a neutralizing titre of 1/160, is more difficult to explain in this way and will be discussed more fully below. The results in Table 4 show that zoster arises in people who have low or undetectable amounts of V-Z neutralizing antibodies but a high level of such antibody develops rapidly and, from the evidence in Table 5, it persists for many years. This typical secondary antibody response in zoster supports the theory that zoster represents a recurrence or second experience of V-Z virus. A similar secondary response in CF antibody in zoster cases was reported by Weller & Witton (1958) and Taylor-Robinson & Downie (1959). If it does represent a recurrence of a latent infection then clearly the mechanism of latency is different from that in recurrent *Herpes simplex* infection where a high level of neutralizing antibody persists after the primary attack and local clinical disease recurs despite the presence of circulating antibody. Hope-Simpson (1965) postulated that, after chickenpox, V-Z virus becomes latent in the sensory root ganglia of the spinal nerves in a 'pro-virus' state or in some way sequestered from the reticulo-endothelial system so that it does not provide a continuous antigenic stimulus and as a result the host's immunity falls to a negligible level. In such a carrier of latent virus reversions to virulence of the virus might occur from time to time. If the patient still possessed circulating, neutralizing antibody then no disease would occur but a rise in antibody titre would result. If the reversion took place when the antibody titre was below a critical level, however,

clinical zoster would result. The low titres of antibody in the acute stage of the paired zoster sera we have tested support this view and the high titre of neutralizing antibody in case 12 (Table 3), who had not had zoster, might have arisen because of a reversion to virulence of endogenous virus.

Another possible reason for this high neutralizing titre in the absence of zoster is that reinfection might occur from an external source and result in a rise in the level of antibody. This possibility was also proposed by Hope-Simpson (1965) and it was tested using paired sera from close contacts of chickenpox or zoster. In one case the titre rose from < 1/10 to 1/80, thus showing that this mechanism can operate. However, case 12 was unaware of any contact with chickenpox or zoster about the time when the serum samples were taken and CF tests using V-Z antigen proved negative, thus making it unlikely that he had had recent experience of the virus. If the high level of antibody in case 12 is the result of reinfection by contact with chickenpox or zoster or of recrudesence of latent virus then either of these events must have occurred sufficiently long ago for the CF antibody to have waned although the neutralizing antibody persists.

Ross *et al.* (1965) showed a rise in CF antibody to HS in 48 % of their chickenpox patients and in 26 % of their zoster patients. They also showed slight rises (less than fourfold) in neutralizing antibody to HS in some chickenpox cases. They argued that concurrent HS infection could be discounted because in recurrent HS there is no rise in CF antibody and the presence of neutralizing antibodies in the acute stage sera eliminates the possibility of primary HS.

We have confirmed the rise in CF antibody to HS occurring during the course of chickenpox in patients who are already herpetics but we could not show it during zoster in the two patients tested although they did have HS antibodies. No rise in titre of neutralizing antibody to HS was shown in any of our chickenpox patients, even in those who showed a rise in CF antibody to HS during their illness.

There does not appear to be any relationship between neutralizing antibodies to HS and V-Z in the sera we have tested and we could not attribute low levels of neutralizing antibody to V-Z virus to the possession of high titres to HS virus. We have not been able to examine the effect of primary HS infection on levels of neutralizing antibody to V-Z virus.

There have been many conflicting reports about the value of human gamma-globulin in the prevention of chickenpox. Abrahamson (1944) used plasma from a convalescent zoster patient and Trimble (1957) used pooled human gamma-globulin to protect susceptible contacts of chickenpox from the disease. Ross (1962) claimed that the use of gamma-globulin produced significant modification of chickenpox in family contacts. However, Schaffer (1965) stated that gamma-globulin was ineffective in chickenpox when used in the same dosage as that which is effective against measles, hepatitis and poliomyelitis and Shaw & Grossman (1966) also found gamma-globulin unreliable in protecting susceptible contacts of chickenpox in a hospital ward.

One of the possible explanations for these discrepancies is that many adults have very low levels of neutralizing antibody to V-Z virus in their serum and if a gamma-globulin pool contained only such sera then it would be ineffective in the

prophylaxis of chickenpox but other batches of gamma-globulin might have a much higher titre and be correspondingly more effective. We tested two batches of human gamma-globulin and found neutralizing antibody titres of 1/160 and 1/320 respectively. The first batch had been used in the treatment of severe chickenpox in a child whose immunological responses had been impaired by radiotherapy and drugs and it was thought to have made an important contribution to her recovery (D. Mainwaring, personal communication). It is now possible to test batches of gamma-globulin for neutralizing antibodies to V-Z virus and select, if necessary, those with high enough titres to be useful in the prevention or treatment of chickenpox.

SUMMARY

A technique for neutralization tests using varicella-zoster virus propagated in primary human thyroid cells is described. The level of neutralizing antibody following chickenpox does not usually exceed a titre of 1/40 and in adults many years after infection it may be very low. After zoster a much higher and more persistent antibody response occurs. Contact with chickenpox also produced a rise in neutralizing antibody in one out of the five patients tested. One case who had had chickenpox but not zoster had a high level of neutralizing antibody and the possible reasons for this are discussed. No cross-neutralization with *Herpes simplex* virus was demonstrated but the rise in titre of complement-fixing antibody to HS occurring in herpetic subjects with chickenpox (Ross *et al.* 1965) was confirmed. Two samples of human gamma-globulin were shown to have high levels of neutralizing antibody to V-Z virus and one was known to have been found effective clinically.

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