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Neutralizing Antibody Responses to Varicella-Zoster Virus

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Neutralization of varicella-zoster (V-Z) virus by human sera and immune rhesus monkey sera was enhanced by fresh guinea pig complement. There was no marked difference in the degree to which complement enhanced neutralization by sera from current V-Z virus infections and sera from long-past varicella infections. Immunoglobulin G neutralizing antibody in sera from varicella cases was enhanced by complement to a slightly higher degree than was immunoglobulin M (IgM) antibody, and immunoglobulin G neutralizing antibody in immune monkey sera was enhanced to a much greater degree than was IgM antibody. There was a rapid decline in the complement requirement of IgM neutralizing antibodies over the course of immunization of the rhesus monkeys. V-Z neutralizing antibody titers in the presence of complement were higher than complement-fixing titers of the same sera in all groups of individuals studied. IgM neutralizing antibody for V-Z virus was demonstrable in all cases of varicella but in only 1 of 22 zoster cases, and V-Z IgM neutralizing antibody was not detectable in primary herpes simplex virus infections in which heterotypic antibody titer rises occurred to V-Z virus. Complement-fixing antibody for V-Z virus was absent in 19S serum fractions which contained IgM neutralizing antibody for the virus.

Few studies have been done on neutralizing antibody responses in varicella-zoster (V-Z) virus infections of man. Because of the tendency of cell culture-propagated virus to remain strongly cell associated, difficulty has been encountered in demonstrating neutralization (28). In early investigations on the virus, Taylor-Robinson (25) was able to show neutralization of the cell-free virus present in vesicular fluids, and using a test based upon reduction of focal lesions in V-Z-infected cell cultures, he demonstrated increases in neutralizing capacity of human sera over the course of V-Z virus infections. Caunt (3) found that greater amounts of infectious cell-free virus could be obtained from infected human thyroid cell cultures than from other types of cell cultures, and Caunt and Shaw (4) used cell-free virus from this source to demonstrate neutralizing antibody increases in chickenpox and zoster infections.

A plaque-reduction neutralization test for detection of V-Z viral antibody was developed in this laboratory (21) using cell-free virus prepared from infected human fetal lung fibroblasts as described by Brunell (2). The test has been employed for studies on the antigenic relationship between V-Z virus and herpes simplex virus (HSV) (21), and for investigations on V-Z viral antibody activity in the "slow" and "fast" subclasses of immunoglobulin G (IgG) (15).

This report describes results of further studies on neutralizing antibody responses in V-Z infections. These were concerned with: (i) investigating the possible enhancement of virus neutralization by fresh guinea pig complement; (ii) comparing the levels of neutralizing and complement-fixing (CF) antibody produced in V-Z virus infections; (iii) studying the immunoglobulin M (IgM) neutralizing antibody response in primary (varicella) and secondary (zoster) infections; and (iv) determining the role of IgM antibody in the CF antibody response to V-Z virus infections.

MATERIALS AND METHODS

Neutralization tests. Cell-free virus for use in the V-Z neutralization test was prepared as described by Brunell (2) using the Batson strain (22) of virus propagated in strains of human fetal diploid lung cells developed in this laboratory by J. H. Schieble. When the infected cultures showed a 2- to 3-plus viral cytopathic effect, the cells from each 29-oz (ca. 0.84-liter) culture bottle were scraped into 2 ml of medium consisting of Eagle minimum essential medium supplemented with 10% fetal bovine serum and 10% sorbitol. After sonic treatment at 20 kc/s for 20 s, the virus preparation was clarified by centrifugation at 1,500 rpm for 15 min and stored at −70 C.

Fresh guinea pig serum used as a source of comple-

ment in some of the neutralization tests was placed at −70 C immediately after separation from the clot and held at that temperature until used. The hemolytic activity was determined by the standard CF technique of this laboratory (14). V-Z virus was titrated in the presence and absence of the dilution of guinea pig complement used in the neutralization test in order to assure that the guinea pig serum had no viral inhibitory effect. Each new lot of complement was tested for its ability to enhance V-Z virus neutralization before it was put into routine use.

The plaque reduction neutralization test for V-Z virus was performed essentially as described previously (21) in monolayers of human fetal-diploid lung cells in Linbro FB-6 plates (Linbro Chemical Co., New Haven, Conn.). Hanks balanced salt solution containing 5% inactivated fetal bovine serum was used as a diluent for serum, virus, and guinea pig complement. The test was modified slightly as follows to permit incorporation of guinea pig complement into the reaction mixtures. Twofold dilutions of test serum (inactivated at 56 C for 30 min) were prepared in 0.2-ml volumes; sets of dilutions were prepared in duplicate to permit testing in the presence and absence of complement. To each serum dilution in one set was added 0.1 ml of a 1:8 dilution of heated (56 C, 30 min) guinea pig serum, or 0.1 ml of diluent; to each serum dilution in the duplicate set was added 0.1 ml of fresh, unheated guinea pig serum diluted 1:8 (approximately 10 hemolytic units). Virus diluted to contain approximately 320 plaque-forming units (PFU) per 0.1 ml was added in a volume of 0.1 ml to each mixture, and incubation was conducted at 37 C for 30 min. Each serum-virus mixture was then inoculated into two culture dishes in a volume of 0.1 ml. The inoculated cultures were incubated at 36 C in a CO_2 incubator for 50 to 60 min to permit absorption of unneutralized virus, the inocula were removed, and the cultures were washed and overlaid with nutrient agar as previously described (21). Plaques were counted on days 8 and 9, and the antibody titer was expressed in the highest dilution of serum producing a 50% or greater reduction in plaque count.

Neutralizing antibody for HSV type 1 (MacIntyre strain) and HSV type 2 (MS strain) was assayed by the same plaque reduction technique used for V-Z virus, with the exception that the second overlay containing neutral red was added on day 3 and plaques were counted on day 4.

CF tests. V-Z CF antigen was prepared from the Batson strain as previously described (22). Sera were assayed for V-Z CF antibody by the standard procedure of this laboratory (14).

Separation of IgM and IgG serum fractions. Immunoglobulins were separated by sucrose density gradient centrifugation as described elsewhere (5). The 19*S* (IgM) and 7*S* (IgG) fractions were dialyzed against physiological saline for 18 h at 4 C, and then each was concentrated to a volume representing a 1:2 dilution of whole serum by dialysis against Aquacide I (Calbiochem). The serum fractions were tested for specificity and purity of immunoglobulin content by double immunodiffusion tests (5) against γ-chain-specific anti-human IgG and μ-chain-specific anti-human IgM from Hyland Laboratories. In some instances gradient fractions were also tested by single radial immunodiffusion in Quanti-Plates from Kallestad Laboratories, Inc.

Preparation of V-Z virus immune serum. Rhesus monkeys were immunized with V-Z virus as described previously (23).

Human sera examined. Sera submitted to this laboratory from patients with clinical diagnoses of either varicella (chickenpox) or zoster, and who showed diagnostically significant increases in V-Z CF antibody but not HSV CF antibody, were examined in this study. Acute-phase sera were collected between 2 and 7 days after onset, and convalescent-phase sera were collected 14 to 35 days after onset. Sera were also examined from individuals with a past history (20 years ago or longer) of varicella but no history of zoster. Also included in the study were paired sera from a group of patients with primary HSV infections. In addition to clinical findings in these patients, the diagnosis of primary HSV infection was based upon lack of HSV neutralizing or CF antibody in the acute-phase serum specimen and by production of IgM antibody to HSV over the course of infection. The HSV patients had all experienced past V-Z virus infections, as evidence by the presence of V-Z viral antibody in their acute-phase serum specimens. Typing of HSV antibody in these patients' sera by a solid-phase radioimmunoassay (Forghani et al., manuscript in preparation) indicated that three of the patients were infected with HSV type 1 and eight were infected with HSV type 2.

RESULTS

Enhancement of V-Z virus neutralization by complement. Neutralization of HSV (10, 11, 16, 29) and human cytomegalovirus (8) has been shown by various investigators to be enhanced by fresh guinea pig complement. Accordingly, investigations were conducted to determine a possible enhancing effect of fresh guinea pig complement on neutralization of V-Z virus. Preliminary studies using heated and unheated guinea pig complement in the serum-virus mixtures showed that neutralizing antibody titers were the same in the absence of guinea pig serum and in the presence of heated serum, but that unheated guinea pig complement enhanced neutralization titers to varying degrees.

Table 1 summarizes results of tests conducted in parallel in the presence and absence of complement on sera from 4 groups of individuals, namely, 6 varicella (chickenpox) patients, 14 zoster patients, 13 individuals with long-past varicella infections and no history of zoster, and 11 patients with primary HSV infections who had experienced past V-Z infections. In both varicella and zoster infections there was a tendency for complement enhancement of neutralization titers to be greater with convalescent-phase sera than with acute-phase sera. Almost one-half of sera from long-past varicella

TABLE 1. *Enhancement of V-Z viral neutralizing antibody by complement*

Degree of enhancement of neutralizing titers by complement (fold)	Patient group						
	Varicella		Zoster		Past varicella infections	HSV primary infections	
	A[a]	C	A	C		A	C
0	2[b]		2		4	2	
2	2		5	2	2		
4	1	1	6	5	6	3	2
8	1	3	1	4	1	2	3
16		2		3		3	2
32						1	4

[a] A, Acute-phase sera; C, convalescent-phase sera.
[b] Titers <1:8 with and without complement.

infections showed little or no enhancement of neutralizing antibody titers in the presence of complement, but titers of other individuals in this group were enhanced to the same extent as convalescent-phase sera from current V-Z infections. It is of interest that V-Z neutralizing antibody titers of sera from the HSV patients tended to be enhanced to a greater extent by complement than were titers of sera from current V-Z infections.

Figure 1 shows the marked complement enhancement of V-Z neutralizing antibody titers of sera of two rhesus monkeys collected over the course of immunization (23) with V-Z virus. The animals developed maximum levels of V-Z antibody at different rates, but both showed the highest levels of complement-requiring neutralizing antibody at 20 to 40 days after the initial immunization, and with both animals complement enhancement of neutralizing antibody titers decreased over the course of immunization. CF antibody titers more nearly approximated neutralization titers in the presence of complement than those in its absence.

Studies were performed to determine whether V-Z neutralizing antibodies in different classes of immunoglobulins differed in their complement requirements. Sera from varicella patients and early sera from the V-Z-immune monkeys were fractionated on sucrose density gradients, and the 19*S* and 7*S* fractions were examined for enhancement of neutralizing antibody activity by complement (Table 2). The V-Z neutralizing antibody titers of 19*S*, IgM fractions of sera from varicella patients were generally enhanced fourfold by complement, whereas titers of most of the 7*S*, IgG fractions were enhanced eightfold or more. The difference in enhancement of neutralizing antibody titers of IgM and IgG serum fractions was much more marked in the case of immune monkey sera. Titers of IgG fractions were enhanced by complement to a much greater extent than were

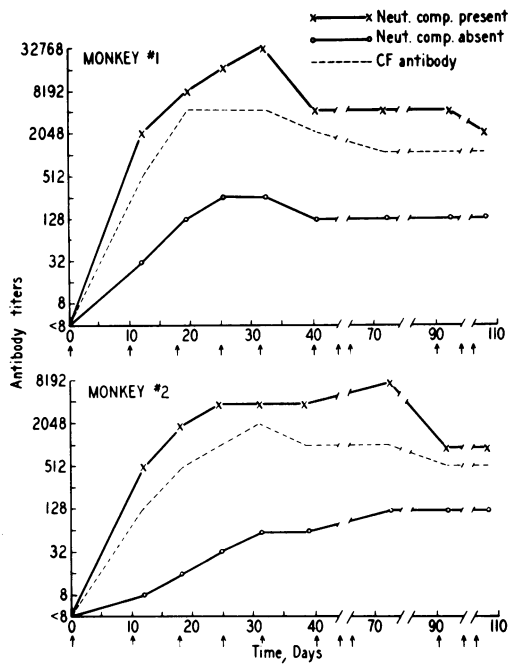

FIG. 1. *Complement enhancement of neutralizing antibody titers of sera from rhesus monkeys immunized with V-Z virus. Arrows indicate times at which immunizing injections were given.*

titers of corresponding IgM fractions. It is seen that enhancement of IgM antibody titers by complement decreased rapidly over the course of immunization, whereas complement enhancement of IgG antibodies decreased more gradually.

Comparison between V-Z neutralizing and CF antibody titers The scattergrams in Fig. 2 compare V-Z neutralizing antibody titers obtained in the absence and presence of complement to CF titers of the same sera. For the varicella and zoster patients, CF titers and neutralization titers in the absence of complement were roughly comparable, but neutraliza-

TABLE 2. *Complement enhancement of V-Z neutralizing antibody in IgM and IgG classes of immunoglobulins*

Patient	Days after onset	V-Z neutralizing antibody								
		Whole serum			IgM			IgG		
		−C[a]	+C	Enhancement (fold)	−C	+C	Enhancement (fold)	−C	+C	Enhancement (fold)
HeMc	3	16[b]	32	2	8	32	4	4	8	2
AnCa	7	64	512	8	32	128	4	8	128	16
	21	128	1,024	8	4	16	4	32	256	8
AnKu	14	512	2,048	4	16	64	4	256	1,024	4
LeWi	4	4	16	4	<4	8	4	4	32	8
	14	64	1,024	16	4	32	8	64	512	8
V-Z-immune monkey S-2372-1	13[c]	32	2,048	64	8	128	16	16	1,024	64
	25	256	16,384	64	16	64	4	256	8,192	32
	32	256	32,768	128	32	64	2	256	8,192	32
V-Z-immune monkey S-2372-2	13	8	512	64	8	128	16	<4	256	128
	25	16	2,048	128	2	8	4	32	4,096	64
	32	64	4,096	64	2	4	2	64	4,096	32

[a] −C, Complement absent; +C, complement present.
[b] Reciprocal of neutralizing antibody titer.
[c] Days after beginning of immunization.

tion titers in the presence of complement tended to be markedly higher than CF titers. With sera from long-past varicella infections, neutralization tests, even in the absence of complement, demonstrated antibody in a number of individuals with CF titers of ≤1:8. In contrast to patients with current V-Z virus infections, patients with HSV infections had V-Z CF titers which were much higher than neutralizing antibody titers in the absence of complement; however, neutralization titers in the presence of complement were generally higher than CF titers.

IgM neutralizing antibody responses in primary and secondary V-Z virus infections. In a study on a small number of patients, Leonard et al. (15) demonstrated V-Z neutralizing antibody in IgM serum fractions from varicella (chickenpox) patients but not in patients with zoster infections. However, using the indirect fluorescent antibody staining technique for detection of IgM antibody, Ross and McDaid (18) found V-Z IgM antibody in the sera of 20 of 40 zoster patients examined, and it was suggested that detection of IgM antibody might not be a reliable method for distinguishing between primary and secondary infections with V-Z virus.

Studies were performed in this laboratory to determine whether V-Z neutralization tests might also detect IgM antibodies in zoster patients. Sera from varicella patients, zoster patients, and patients with primary HSV infections were fractionated by sucrose density gradient centrifugation, and the 19*S* and 7*S* fractions were examined for V-Z neutralizing antibody in the presence and absence of complement (Table 3). Neutralizing antibody for V-Z virus was demonstrable in the IgM serum fractions of all varicella patients examined. Only 1 of the 22 zoster patients had IgM neutralizing antibody for V-Z virus. The IgM serum fraction of this patient was shown to be free of IgG by both gel double diffusion and radial immunodiffusion tests, and when fractionation was repeated on the serum, V-Z neutralizing antibody was again demonstrable in the IgM fraction. The possibility was considered that the patient's infection might actually have been atypical chickenpox, but the patient, a 53-year-old female, gave a history of having had chickenpox at age 8 or 9. IgM serum fractions from two additional zoster patients produced some reduction in plaque count at the 1:4 dilution, but the reduction was less than 50%. In all zoster patients except the one with demonstrable IgM antibody, the V-Z neutralizing antibody titers of 7*S* serum fractions were comparable to those of corresponding whole sera. None of the HSV patients had IgM antibody for V-Z virus; however, they all produced IgM antibody for HSV, and a large proportion of the patients had heterotypic neutralizing and/or CF antibody titer rises to V-Z virus.

Attempts to demonstrate V-Z IgM antibody with complement-fixing activity. Most studies on immunoglobulin classes of viral CF antibodies have shown them to be exclusively IgG in nature (1, 9, 12, 20). However, some workers have made brief mention of the presence of CF activity for human herpesviruses in IgM serum fractions (7, 26; Farris et al., Abstr.

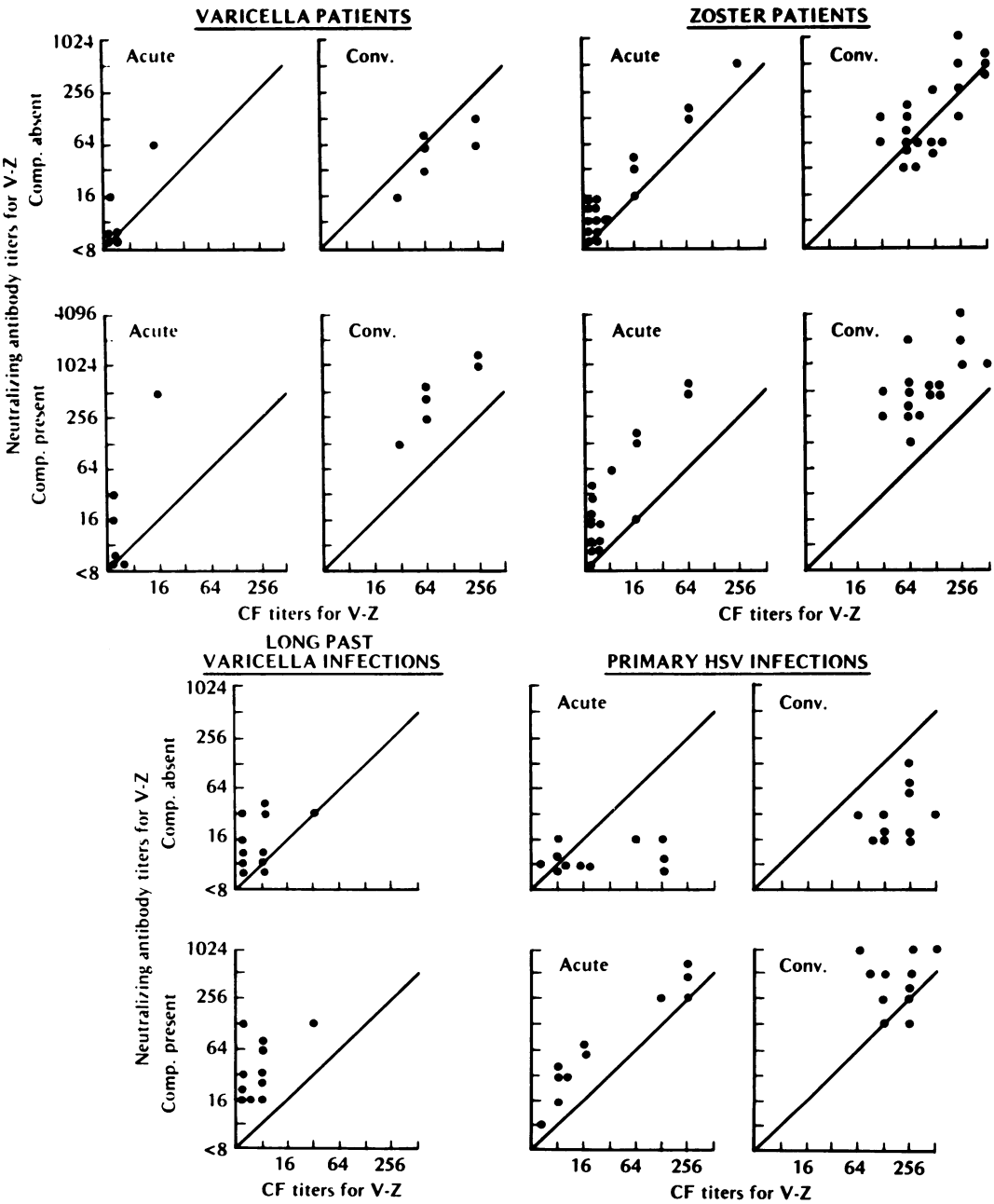

FIG. 2. *Comparison of V-Z CF antibody titers and neutralization titers obtained in the absence and presence of complement.*

Annu. Meet. Am. Soc. Microbiol. 1974, V337, p. 256). Efforts were made in the present study to demonstrate specific viral CF activity in 19S serum fractions shown to contain neutralizing antibody to V-Z virus. CF activity was demonstrable only in a 19S gradient fraction which was found by immunodiffusion to be contaminated with IgG (Table 4). Thus, it appears that as in other viral infections, CF antibodies produced by infection or immunization with V-Z virus are IgG in nature, and if CF activity is demonstrable in IgM preparations the possibility of contamination with IgG should be considered.

DISCUSSION

The present study adds V-Z virus to the list of herpesviruses for which neutralization may be strongly enhanced by fresh guinea pig complement. Martos et al. (17) noted a twofold enhancement by complement of neutralizing antibody titers of V-Z immune sera produced in rabbits, but in our studies a greater degree of enhancement was seen with most of the human sera tested and with the V-Z immune sera produced in rhesus monkeys. Whether this reflects differences in complement requirements of V-Z neutralizing antibody produced by different species remains to be determined.

TABLE 3. *IgM neutralizing antibody response to V-Z virus in varicella, zoster, and HSV infections*

Patient group	No. tested	No. with V-Z neutralizing antibody titer ≥ 1:4 in 19S gradient fraction
Varicella	5	5
Zoster	22	1
HSV[a]	11	0

[a] All patients produced IgM neutralizing antibody to HSV; eight had fourfold or greater CF rises and six had fourfold or greater neutralizing antibody titer rises to V-Z virus.

Some workers (29) have demonstrated high levels of complement-requiring neutralizing (CRN) antibody for HSV in early sera from primary infections and have suggested that these high titers of CRN antibody may have diagnostic significance. However, other studies (11, 16) have shown levels of CRN antibody to be variable in primary HSV infections and have not lent strong support to the diagnostic significance of such antibodies. From our studies it appears that CRN antibodies for V-Z virus have no diagnostic significance in differentiating antibody elicited by current V-Z infections from that produced by past infections, or even from the V-Z neutralizing antibody which may be boosted by heterotypic infections with HSV. However, increasing the sensitivity of the V-Z virus neutralization test through the use of guinea pig complement may have other practical applications. The more sensitive neutralization test may prove to be useful in determining immunity status to V-Z virus in individuals at high risk of infection; the present studies showed that the neutralization test, particularly with complement, was more sensitive than the CF test for detecting V-Z antibody from past infections. Also, a sensitive neutralizing antibody assay for V-Z virus will undoubtedly prove useful in studies aimed at determining the relative roles of humoral and cell-mediated

TABLE 4. *Absence of CF antibody for V-Z virus in 19S sucrose density gradient fractions containing IgM neutralizing antibody*

Serum tested	Fraction	Antibody titer to V-Z virus by:		
		Neutralization		CF
		−C[a]	+C	
Human varicella 3-5236	Whole	64[b]	512	64
	19S	32	128	<4
	7S	8	128	64
Human varicella 3-7597	Whole	512	2,048	256
	19S	16	64	<4
	7S	256	1,024	256
V-Z-immune monkey S-2372-1	Whole	32	2,048	512
13 days post-immunization	19S	8	128	<4
	7S	16	1,024	512
18 days post-immunization	Whole	128	8,192	≥4,096
	19S	16	64	4[c]
	7S	128	4,096	≥4,096
V-Z-immune monkey S-2372-2	Whole	8	512	128
13 days post-immunization	19S	8	128	<2
	7S	<4	256	64
18 days post-immunization	Whole	16	2,048	512
	19S	8	128	<4
	7S	16	2,048	256

[a] −C, Complement absent; +C, complement present.
[b] Reciprocal of antibody titer.
[c] Fraction contaminated with IgG.

immunity in resistance to infection and in latency and activation of V-Z virus.

Although there is much evidence to indicate that V-Z virus and HSV share common antigens (13, 19, 21, 24, 27), heterotypic antibody increases have generally been demonstrable only in humans who have experienced a previous infection with the viral heterotype, and not in laboratory animals immunized with one virus or the other. By using complement to enhance the sensitivity of the test, attempts were made as part of this study to demonstrate V-Z neutralizing antibody in the sera of two HSV (type 1) immune rabbits with homotypic neutralizing antibody titers of $\geq$1:2,048, but neutralizing antibody titers to V-Z virus were $<$1:4 with and without complement.

Our findings on complement enhancement of V-Z neutralizing antibodies in different classes of immunoglobulins were somewhat at variance with those reported for HSV immune rabbit sera by Hampar et al. (10). In both studies a decrease of CRN antibody was noted over the course of immunization, but the CRN antibody for HSV in immune rabbit sera was predominately IgM in nature, whereas CRN antibody against V-Z virus in our immune monkey sera was predominately IgG. However, in both studies the complement requirement of IgM neutralizing antibodies was seen to decrease over the course of immunization.

Results of the present studies would suggest that production of IgM neutralizing antibody to V-Z virus is a rare occurrence in secondary (zoster) infections. These findings differ rather markedly from those obtained by Ross and McDaid (18), who used the indirect fluorescent antibody technique for detection of V-Z IgM antibody and found positive IgM staining reactions with sera from one-half of the zoster patients studied. No indication was given as to whether sera showing positive IgM staining were tested for presence of rheumatoid factor, which can give false positive reactions in fluorescent antibody tests for viral IgM antibody (6). The possibility exists that the fluorescent antibody test may be capable of detecting IgM antibodies specific for V-Z virus which lack neutralizing activity and which are produced in secondary (zoster) infections. Direct comparison of the relative sensitivity and specificity of neutralization tests on IgM gradient fractions and indirect fluorescent antibody tests on whole sera for detection of V-Z IgM antibodies will be required to resolve this point.

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