Quantitation of Antibodies to Varicella-Zoster Virus by Immune Adherence Hemagglutination

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Immune adherence hemagglutination was compared with the complement fixation test as a means of measuring antibodies to varicella-zoster virus. Analysis of acute- and convalescent-phase sera from patients infected with varicella-zoster or with herpes simplex virus showed the immune adherence hemagglutination test to be more sensitive than the complement fixation test, and greater cross-reactivity between the two viruses appeared to be associated with the increased sensitivity. The two assay methods were used to measure antibodies to varicella-zoster virus in 265 sera obtained from patients of different ages as well as sera from 26 patients with leukemia. There were 35 cases where antibodies were detected by immune adherence hemagglutination but not by complement fixation, whereas in five cases the converse was found. Our findings support the contention that immune adherence hemagglutination is the method of choice for detecting antibodies to varicella-zoster virus.

Varicella is usually a benign disease of childhood; however, in conditions associated with compromised host defense mechanisms such as children with leukemia (25), children receiving steroids (6, 7, 9, 13), or subjects with primary immunodeficiency diseases (16, 17), the infection may be fatal or may follow a more malignant and protracted course. The problem of high-risk patients in the pediatric age group is magnified by the fact that immunosuppressive drugs are finding wider usage than ever before for a variety of neoplastic and nonneoplastic conditions. The nature of the primary illness is such that frequent hospitalizations are required as part of routine care. While increasing efforts are being made on the part of physicians and hospital personnel to prevent the admission of known cases or contacts of varicella to hospitals, it is virtually impossible to exclude all such patients. Thus, the outbreak of varicella in a pediatric ward results in a major disruption of activity with accelerated discharge of all possible susceptibles and intensive isolation procedures for cases, contacts, and nondischargeable high-risk patients without regard to definition of susceptibility.

The seriousness of the varicella problem has prompted efforts to employ passive immunization in the prevention of varicella in high-risk patients, and these efforts have produced encouraging results (2, 3, 8, 11, 17, 26). A major limitation has been the short supply of zoster immunoglobulin in the face of large demands

for the product (17), so that strict screening of requests for patients who are truly susceptible has been necessary.

Identification of susceptibility may be based on historical data, which is notoriously inaccurate, or on serological grounds. The complement fixation (CF) test is the method routinely available for detecting antibody to varicella-zoster virus (VZV), but it is not suitable for examining the immune status of patients because of its low sensitivity. The immune adherence hemagglutination (IAH) test is known as one of the more sensitive techniques for detection of antigen and antibody (15). Recently, it has been successfully used to detect antibody to hepatitis viruses A and B (20, 22, 31), cytomegalovirus (5), and VZV (10, 18). The present report describes the application of IAH to quantitate antibodies to VZV in paired sera of VZV-infected patients, in sera of leukemia patients, and in sera of patients of different ages who did not have recent VZV infections.

MATERIALS AND METHODS

Antigens. For IAH, VZV CF and CF tissue control antigens were purchased from Flow Laboratories (Rockville, Md.), and for the CF test, from Microbiological Associates, Inc. Herpes simplex CF antigen was purchased from Flow Laboratories and used in the CF and IAH tests. Before use, it was stored at -70°C . The lyophilized antigen was reconstituted with distilled water as instructed by the manufacturer and then appropriately diluted in Veronal-buffered saline (VBS) supplemented with 0.1% bovine serum albumin

(BSA-VBS). Reconstituted antigen was used immediately or stored at -70°C .

Serum samples. Ten paired sera of VZV- and two paired sera of herpes simplex virus-infected patients taken during both acute and convalescent periods were used. Multiple sera were taken from one case of VZV. A total of 265 sera was collected according to age so that each age group had about 20 sera. These samples were collected as the sera were submitted to the diagnostic Virology Laboratory for confirmation of suspected viral diseases excluding varicella and herpes zoster. All serum samples were heated at 56°C for 30 min before use.

Buffers. VBS, $5\times$ stock, was prepared by dissolving reagents in the following order: sodium 5,5-diethylbarbiturate, 10.19 g; sodium chloride, 83.00 g; 1 N hydrochloric acid, 34.6 ml; stock solution containing 1 M MgCl_2 and 0.3 M CaCl_2 (20.33 g of $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ and 4.4 g of $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ in 100 ml of distilled water), 5.0 ml. Distilled water was then added to a volume of 2,000 ml, and the solution was thoroughly mixed. The pH of the stock buffer was checked before autoclaving, and the stock was stored at 4°C . The working VBS was a 1:5 dilution in distilled water, and the pH of diluted stock was 7.3 to 7.4. The VBS was supplemented with 0.1% bovine serum albumin (BSA) fraction V (Sigma Chemical Co., St. Louis, Mo.) and used as diluent for antigen, antibody, and complement. A 40 mM disodium ethylenediaminetetraacetate (EDTA; Fisher Scientific Co., Pittsburgh, Pa.) solution was prepared by dissolving EDTA in BSA-VBS and used as the diluent for human erythrocytes (RBC) and dithiothreitol (DTT; Baker Chem. Co., Phillipsburg, N.J.). The concentration of DTT solution was 3 mg/ml.

RBC for IAH. Type O, Rh-positive RBC were obtained from healthy volunteers. Since there was marked variation in RBC sensitivity as the indicator cell, RBC from the donor providing the most sensitive cells in a preliminary experiment was used in all subsequent tests. The blood was collected in four times the volume of Alsever solution and kept at 4°C . RBC could be kept for up to 2 weeks after which the sensitivity of RBC in IAH was found to be variable. Before use, RBC were washed twice with EDTA-BSA-VBS and prepared as a 1% (vol/vol) suspension in EDTA-BSA-VBS.

Complement. For the IAH test, guinea pig complement was obtained from pooled guinea pig sera and stored at -70°C . According to the preliminary experimental results, a 1:40 dilution yielded optimum results in the IAH test. For the CF test, complement was purchased from Flow Laboratories. All complement was prepared fresh in BSA-VBS (IAH test) or in VBS (CF test) diluent immediately prior to use.

Hemolysin. Hemolysin was purchased from Flow Laboratories.

Performance of IAH test. Optimum concentration of VZV antigen (or herpes zoster antigen) was determined by checkerboard titration of antigen against reference antiserum. In subsequent tests with the same lot of antigen, the antigen was diluted in BSA-VBS to yield the highest titer of reference antibody in 0.025 ml. VZV antigen was used at 1:8 and HSV antigen at 1:16. Twofold dilutions of serum in 0.025 ml were made in BSA-VBS in U-bottom micro-

titer plates (Cooke Co.). Two sets of dilutions of serum were made for each test: one was reacted with viral antigen, and another was reacted with control antigen. Antigens in a volume of 0.025 ml were added to each well containing the serum dilutions. Each serum was placed in a single cup at a dilution of 1:8 without antigen as a serum control. Plates containing antigens and sera were mixed thoroughly and then incubated in a humid chamber overnight at 4°C . Complement, at the appropriate dilution in BSA-VBS (0.025 ml), was added to all wells. The plates were shaken again and incubated at 37°C for 40 min. Then DTT-EDTA-BSA-VBS (0.025 ml) was added to all wells followed by 0.025 ml of the human RBC suspension. The plates were shaken thoroughly and left at room temperature for about 1.5 h for hemagglutination to develop.

According to the degree of RBC agglutination, the patterns were judged from 0 to +4, and +2 agglutination and above was used as the positive end point. Titers were expressed as the reciprocal of the positive end point dilution. Sometimes a nonspecific reaction was observed with some sera (the degree of hemagglutination in wells containing control antigen was almost equal to the wells containing infected antigen). These sera were omitted from analysis and represented 1.1% of the total sera tested. Each experiment included the antigen and control antigen, as well as a positive serum control that was used to estimate the reproducibility of the assay.

Performance of CF test. A microtiter CF test employing optimal dilution of antigen, five times the 50% complement hemolytic end point (5 CH_{50}) of complement, and overnight fixation at 4°C was used to examine the sera (4). Titers were expressed as the reciprocal of the final dilution exhibiting 50% hemolysis.

RESULTS

Detection of antibodies after VZV infections. Paired sera from eight patients with herpes zoster were tested by IAH and CF for antibodies to VZV and herpes simplex virus. All eight patients showed increases in VZV antibody titers of fourfold or greater in both tests (Table 1). An increase in CF antibodies to herpes simplex virus was noted in one of eight patients, whereas five of the eight patients had an increase in antibodies to herpes virus when tested by the IAH test. The antibody responses of three patients with chickenpox are shown in Table 2. A dramatic rise in antibodies to VZV was detected by both methods, whereas minimal rises to herpes virus were found in one of three patients by CF and two of three patients by IAH. Paired sera from two patients with herpes simplex were examined, and cross-reacting antibodies to VZV were observed in the convalescent sera of both of these patients, using the IAH test (Table 2). In all patients examined, the increases in antibody titers were greater to the homologous virus than to the heterologous virus.

Age distribution of VZV antibodies. The occurrence of antibodies to VZV according to

TABLE 1. *Antibody titers to VZV and herpes simplex virus in sera of patients with herpes zoster*

Patient	Age (yr)	Time from onset (days)	Antibodies to VZV ^a		Antibodies to HSV ^a	
			CF	IAH	CF	IAH
AM	34	5	8	64	4	<8
		21	256 (32)	2,048 (32)	4	16 (≥ 4)
CR	69	3	8	32	128	256
		20	128 (16)	1,024 (32)	128	1,024 (4)
KH	67	3	<4	16	32	256
		18	128 (≥ 64)	2,048 (128)	32	256
SE	14	6	16	128	<4	<8
		16	64 (4)	2,048 (16)	<4	16 (≥ 4)
PL	30	5	8	64	<4	16
		24	64 (8)	512 (8)	<4	16
GJ	41	6	32	128	32	32
		19	128 (4)	4,096 (32)	32	256 (8)
CL	72	6	32	256	32	256
		13	512 (16)	8,192 (32)	128 (4)	1,024 (4)
VF	63	5	4	<8	64	128
		21	128 (32)	512 (≥ 128)	64	128

^a Numbers in parentheses represent fold rise in titers between paired sera. HSV, Herpes simplex virus.

TABLE 2. *Antibody titers to VZV and herpes simplex virus in sera of patients with varicella and herpes simplex*

Patient	Age (yr)	Clinical diagnosis	Time from onset (days)	Antibodies to VZV ^a		Antibodies to HSV ^a	
				CF	IAH	CF	IAH
WW	14	Varicella	1	<4	<8	<4	32
			7	64 (≥ 32)	512 (≥ 128)	<4	64 (2)
DM	8	Varicella	1	<4	<8	<4	8
			10	64 (≥ 32)	128 (≥ 32)	<4	8
GT	6	Varicella	1	<4	8	64	512
			3	16	64	64	1,024
			4	64	256	128	2,048
			5	128	512	128	4,096
			7	256 (≥ 128)	1,024 (≥ 256)	128 (2)	4,096 (8)
KO	51	Herpes simplex	1	16	32	16	128
			11	16	128 (4)	128 (8)	1,024 (8)
RO	14	Herpes simplex	7	<4	32	8	128
			8	<4	64 (2)	64 (8)	512 (4)

^a Numbers in parentheses represent fold rise in titers between paired sera. HSV, Herpes simplex virus.

age is shown in Fig. 1. After the loss of maternal antibodies, acquisition of antibodies appeared to be minimal in the first 3 years of life. The percentage of patients with antibodies increased steadily thereafter until ages 16 to 18 years, when essentially 100% of the patients were antibody positive. There was a slight decline in the percentage of positive middle-aged adults, and most patients over age 50 years were positive. Significantly fewer adults aged 31 to 50 were positive for CF antibodies than adults 19 to 30 years of age ($P < 0.01$) and adults 51 years or older ($P < 0.01$). The differences in positivity by IAH were not statistically significant. Except in three age groups (7 to 10 months, 16 to 18 years, and 19 to 21 years), more patients were positive by IAH than by CF.

The mean titers of antibodies to VZV by IAH

were about two- to fourfold greater than mean titers detected by the CF test (Table 3). The mean titers of the positive sera tended to be higher in children and young adults, lower in the 31- to 40-year-old age group, and higher in older adults.

Comparison of titers obtained by the two assay methods is shown in Fig. 2 for sera from the adult patients surveyed. Titers determined by the two methods tended to correlate, and, with the exception of 6 of the 118 sera, titers obtained by the IAH test were higher than titers obtained by the CF test. Of the 20 of 118 sera without antibodies detectable by the CF test, 14 had antibodies detectable by the IAH test. In the total series of 320 sera examined, 35 were found to contain antibodies by the IAH test but did not have antibodies by the CF test, whereas

the converse was found in five sera.

Antibodies to VZV among leukemia patients. When serum samples from 26 patients with leukemia were examined for antibodies to VZV, 69.2% of the children were without detectable antibodies by both tests (Table 4). Among nonleukemic patients of comparable ages, 55.7% were without antibodies by CF and 39.3% by IAH. Significantly more leukemic patients were found to be susceptible to VZV infection than the nonleukemic patients, if the presence of antibodies at 1:8 or greater in the IAH test reflects immunity.

DISCUSSION

IAH represents an in vitro reaction between normal RBC and antigen complexed with antibody and complement (23). The agglutination of RBC is more sensitive with respect to detect-

ing antibody than is the CF test (11, 15, 18, 20, 24, 31). The method has been used to detect antibodies to VZV and was found to be comparable in sensitivity to antibody detection, using indirect immunofluorescence of membrane antigens of virus-infected cells (10). In our experiments, we also found the IAH assay to be more sensitive than the CF test in detecting antibodies to VZV.

It has been noted that antibodies to VZV in some patients fall below levels detectable by CF within 12 months after infection (1, 12, 32) and the CF test is of limited value in detecting VZV antibodies many years after chickenpox (21). Thus, a negative history of chickenpox and the absence of detectable antibodies to VZV in the CF test does not necessarily mean that a patient has not experienced varicella in the past (21, 34). The use of passive immunization in the prevention of varicella in high-risk patients requires a method of establishing susceptibility so that zoster immunoglobulin can be reserved for those subjects who are truly susceptible. The

FIG. 1. The occurrence of antibodies to VZV by age as determined by IAH and CF test. The differences in positivity by the CF test were statistically significant when adults age 31 to 50 years were compared with adults age 19 to 30 years ($\chi^2 = 3.90$, $P < 0.05$) and with adults age equal to or greater than 51 years ($\chi^2 = 6.96$, $P < 0.01$).

TABLE 3. Mean antibody titers to VZV by age

Age group (yr)	No. tested	CF Test		IAH Test	
		No. +	Mean titer ^a ± SD	No. +	Mean titer ^a ± SD
≤6 mo.	20	15	30.4 ± 33	17	88.5 ± 126
7-11 mo.	10	2	8.0	1	8
1-3	19	1	32.0	3	18.7 ± 12
4-6	18	7	34.29 ± 42	8	89.0 ± 80
7-9	21	7	21.7 ± 20	13	56.6 ± 63
10-12	19	13	20.9 ± 15	16	61.0 ± 63
13-15	20	13	25.8 ± 33	17	65.9 ± 117
16-18	20	20	40.4 ± 74	19	176.4 ± 466
19-21	19	18	29.8 ± 30	18	58.7 ± 57
22-30	20	16	36.3 ± 61	20	287.6 ± 903
31-40	19	12	12.3 ± 9	17	45.2 ± 66
41-50	20	15	20.0 ± 30	18	89.8 ± 68
51-60	20	18	20.4 ± 14	19	81.3 ± 74
≥61	20	19	20.2 ± 18	20	81.4 ± 110

^a Mean and standard deviation (SD) of positive sera.

FIG. 2. Comparison of antibody titers to VZV detected by IAH and CF in patients (A) 19 to 30 years of age, (B) 31 to 50 years of age, and (C) greater than 50 years of age. Titers are expressed as the reciprocal of the end dilution showing +2 agglutination and above (IAH) and 50% hemolysis (CF).

TABLE 4. Comparison of antibody titer to VZV among leukemic and nonleukemic patients

Age (yr)	No. tested	Leukemia										Nonleukemia									
		Antibody titer by:					No. tested					Antibody titer by:					CF				
		IAH										IAH									
		<4-4	8	16	≥32	<8	<4-4	8	16	≥32	<8	<4-4	8	16	≥32	<8	<4-4	8	16	≥32	<8
3-4	7	7				7															
5-6	5	3	1		1	4															
7-8	8	6		1	1	5															
9-10	5	2	2		1	2															
11-12	1			1																	
Total (%)	26	18 ^a (69.2)	3	2	3 (30.8)	18 (69.2)															

^a Differences in occurrence of antibodies between leukemic and nonleukemic patients are not statistically significant by CF test but are significant by IAH ($P < 0.025$).

^b Differences in occurrence of antibodies as determined by IAH and CF test are statistically significant for nonleukemic patients ($P < 0.01$).

increased sensitivity of the IAH test suggests that this test system may be suitable for this purpose. However, analysis of sera from 26 children with leukemia by the CF and IAH tests failed to yield a difference in positivity. This was in contrast to the analysis of sera from 61 similarly aged patients among whom 10 had antibodies detected by IAH but not by CF. This finding and the greater percentage of leukemic patients without antibodies as compared with nonleukemic patients is not understood.

It has been observed that patients with VZV infections may have a rise in antibody titers to herpes simplex virus in the CF test (27), and a heterologous CF antibody rise to VZV may occur in certain patients who have experienced a prior infection with VZV and (19, 27, 29) who are subsequently infected with herpes virus. In contrast to the report of others (10), we found that the increased sensitivity of the IAH test was associated with an increased detection of heterologous reactivity between VZV and herpes simplex virus. Although the rise in titers to the homologous virus was always greater than the rise to the heterologous virus, the cross-reactivity was sufficiently great in some cases to suggest that the inclusion of both antigens in assays performed on paired sera obtained from atypical cases may be appropriate.

Recently, Schmidt et al. (28) used various subunit antigens of VZV to examine complement-fixing activity with sera from homotypic infections and from herpes simplex virus infections. They failed to find a subunit antigen that was more suitable than crude VZV antigen for serological differentiation of VZV and herpes virus infections. Whether or not these subunit antigens might be more specific than crude antigens in the IAH test requires further examination.

The distribution of antibodies to VZV according to age suggests that most primary infections occurred between the ages of 4 and 12 years in the population from which our samples were taken. By late teenage, essentially all of the patients had detectable antibodies to VZV. It is of interest that the occurrence of antibodies as the mean titers were lower in the middle-age groups with the lowest values noted in the 31- to 40-year-old age group. The prevalence of antibodies detected by both the CF test and the IAH test approached 100% among patients over 60 years of age. Antibodies in early life probably result from primary infections by VZV, and these antibodies may decline over the years, whereas antibodies may be synthesized in response to reactivation of the latent virus later in life. Since varicella usually occurs in the first 20 years of life and the incidence of herpes zoster

is greatest after age 50 years, Hope-Simpson (14) postulated that reactivation of VZV can only occur when immunity to the virus falls below a certain critical level. The observations of Tomlinson and MacCallum (30) and our data support this hypothesis and are at variance with the observations of Wentworth and Alexander (33). Since cross-reactivity with herpes simplex virus could have been measured in the VZV assays, the conclusions regarding the distribution of VZV antibodies in the population must be tempered. Though it is unlikely that this cross-reactivity contributed significantly to the data presented, careful analysis of sera adsorbed with herpes simplex virus antigen would be required to clarify this point.

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