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Direct Immunofluorescence Staining for Detection of Herpes Simplex and Varicella-Zoster Virus Antigens in Vesicular Lesions and Certain Tissue Specimens

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Direct immunofluorescence (IF) staining was compared with virus isolation for detection of herpes simplex viruses (HSV) and varicella-zoster virus (VZV) directly in clinical materials. These included 199 vesicular lesion specimens and 280 tissue specimens. Correspondence between IF and isolation results was 88% in testing for HSV in lesion specimens and 98% in testing for HSV in various tissue (mostly brain) specimens. Overall, IF was positive for 82% of the specimens in which HSV was demonstrated, and virus was isolated from 89% of the HSV-positive specimens. IF was markedly more sensitive than isolation for demonstrating VZV in lesion and tissue specimens, detecting all of the specimens positive for VZV, whereas isolation detected only 23%. IF detected VZV antigen in a number of lesion specimens taken late after onset, past the time when they would be expected to yield infectious virus. Specificity of positive IF reactions for HSV or VZV in the absence of virus isolation was supported by the facts that (i) staining was obtained with only a single, presumably homologous, immune conjugate, (ii) clinical symptoms were compatible with infection with the virus for which positive IF findings were obtained, and (iii) positive electron microscopy findings for herpesviruses or positive serological results for VZV were also obtained in some instances. Factors to be considered in achieving specificity of IF staining for these human herpesviruses are discussed.

The potential value of immunofluorescence (IF) staining for rapid identification of herpes simplex virus (HSV) and varicella-zoster virus (VZV) antigens directly in clinical materials has been recognized for many years (2-5, 7-9, 11-13), and this approach has been used routinely in our laboratory for over 15 years. However, general acceptance and use of IF staining as a reliable and useful diagnostic procedure for these herpesviruses has been slow, in part because of concern over the possibility of obtaining false-positive reactions, and also because immune conjugates of adequate sensitivity and specificity have not been widely available. Therefore, despite the fact that a number of publications on the subject have appeared previously, we feel that a further report of successful application of IF staining is justified. This report summarizes some of our experience in applying IF staining to rapid diagnosis of HSV and VZV infections, comparing IF results to those obtained by virus isolation, and it indicates some of the factors which may contribute to sensitivity and reliability of IF staining for virus detection and identification.

MATERIALS AND METHODS

Immune conjugates. Viral antisera free from unwanted antibodies to foreign host proteins were produced by immunization of hamsters known to be free of common laboratory animal infections with suckling hamster brain infected with SK-T7-2980 strain of HSV type 1 (HSV-1) or with the Johnson strain of HSV type 2 (HSV-2) (1), and by immunization of rhesus monkeys with VZV (Batson strain) propagated in monkey kidney cells (11). An immune conjugate to vaccinia virus prepared from antiserum produced by immunization of rabbits with virus propagated in RK13 cells grown and maintained in Eagle minimal essential medium with rabbit serum was used routinely in earlier phases of these studies, but more recently the conjugate was used only in special circumstances. Immunoglobulins were precipitated from the antisera with ammonium sulfate and conjugated with fluorescein isothiocyanate as described elsewhere (3). Optimal working dilutions of each conjugate were determined by titration on cell cultures infected with homologous and heterologous viruses and on uninfected cells from the same lots. A working dilution was selected which gave 3- to 4-plus staining with homologous virus-infected cells and minimal or no staining of heterologous virus-infected cells or of uninfected cells.

Direct immunofluorescence procedures.

Smears of cells from vesicular lesions were prepared by collecting cellular scrapings from the base of lesions onto a scalpel blade or cotton swab and making at least three spot smears of about 10 to 15 mm on a clean microscope slide. This permitted staining of each specimen with conjugates to HSV, VZV, and in some cases vaccinia virus. The examination of each specimen against two or more conjugates and demonstration of staining with only a single conjugate served as one specificity control in the test system. Other controls consisted of testing each conjugate against homologous and heterologous virus-infected human fetal diploid kidney cells and against uninfected cells. Smears were air dried for transport to the laboratory, fixed in acetone for 10 min at room temperature, and dried at room temperature before staining. Impression smears of autopsy or biopsy tissue other than brain were prepared by gently pressing the cut surface of a piece of tissue to a clean slide and making a series of impressions. Brain smears were made by placing a small section of tissue 1 to 2 mm in diameter on a slide and crushing and smearing the tissue by drawing a second slide across the first. Again, sufficient tissue smears were made to permit staining with at least two immune conjugates. The tissue smears were dried and fixed as indicated above. Before staining, the smears were ringed with a liquid embroidery pen to retain the conjugate.

Working dilutions of each conjugate were prepared in a 20% suspension of normal mouse or beef brain in 0.01 M phosphate-buffered saline, pH 7.2. The brain suspension reduced overstaining and certain types of nonspecific staining. Each conjugate was added to a smear of the test specimen and to appropriate controls, and slides were incubated in a humidified atmosphere at 35 to 37°C for 20 min and then rinsed twice with phosphate-buffered saline (10 min for each rinsing) followed by a rinse with distilled water. Slides were allowed to dry and then mounted in buffered glycerol solution (one part glycerol and three parts phosphate-buffered saline) for examination with a fluorescence microscope with a UG-1 excitor filter and a 41 barrier filter. Positive readings were based upon the demonstration of 3- to 4-plus fluorescence in the cells, associated with both the cytoplasm and nucleus. Vesicular lesion smears containing too few epithelial cells for definitive examination were reported as unsatisfactory rather than negative. No attempt was made to type HSV by direct IF staining of lesion or tissue specimens.

Virus isolation and identification. For virus isolation attempts, vesicular lesion or tissue specimens, prepared as described elsewhere (10), were inoculated into human fetal diploid kidney (HFDK) cell cultures and primary rhesus or cynomolgus monkey kidney (MK) cell cultures. Inoculated cultures were maintained on Eagle minimal essential medium with 2% fetal bovine serum and were held for a 14-day observation period without a medium change. When cultures showed a 2-plus viral cytopathic effect characteristic of that produced by HSV, VZV, or vaccinia virus, the cells were dispersed with trypsin (10), and cells from one infected tube culture were mixed with trypsin-dispersed cells from two or three uninfected cultures of the same lot to provide good staining contrast of infected cells against uninfected cells. Cells were suspended in 3 ml of phosphate-buffered saline with 2% fetal bovine serum and sedimented by centrifugation at 2,000 rpm for 5 min. Packed cells were suspended in approximately 0.05 ml of phosphate-buffered saline with 2% fetal bovine serum, and smears approximately 5 mm in diameter were made for virus identification by IF staining with the immune conjugates. Typing of HSV isolates was based upon differential staining with type 1 and type 2 conjugates; conjugates were used at dilutions which gave 3- to 4-plus staining with homologous strains and 1- to 2-plus staining with heterologous strains. Over the years we have found results based upon this approach to agree entirely with those obtained by typing isolates by microneutralization tests or by using cross-absorbed, monospecific antisera in IF, radioimmunoassay, or passive hemagglutination inhibition systems.

RESULTS

Table 1 compares results of IF staining and virus isolation attempts on vesicular lesion specimens from 199 patients from whom lesion smears and virus isolation material were submitted. Nineteen, or slightly over 9%, of the smears contained too few cells for IF examination; HSV was isolated from 4 patients in this group. Of the 50 specimens positive for HSV by IF, 9 were negative by isolation, and 13 were positive for HSV by isolation only. The correspondence between IF and isolation results (positive and negative) for detecting HSV was 88%. Of the 50 specimens positive for HSV by IF, 30 were from genital lesions, and 20 were from

TABLE 1. *Correlation between immunofluorescence and isolation results (skin lesion specimens)*

Immunofluorescence	No. of specimens	Isolation (no. of specimens)		
		Positive HSV	Positive VZV	Negative[a]
Positive HSV	50	41 (16 HSV-1, 25 HSV-2)	0	9
Positive VZV	45	0	11	34
Unsatisfactory	19	4 (4 HSV-2)	0	15
Negative	85	13 (5 HSV-1, 8 HSV-2)	0	72

[a] A total of five specimens were positive for vaccinia virus.

TABLE 2. *Patients with positive IF and negative isolation results for HSV (skin lesion specimens)*

Clinical diagnosis	No. of patients	Time of specimen collection
Genital lesions	4 (3 male, 1 female)	4 days, 7 days, ?, ?
Stomatitis	2	4 days, 5 days
Congenital disease	1	1 month
Vesicular rash	1	4 days
Herpes zoster	1	3 days

TABLE 3. *Relationship between time of specimen collection and ability to isolate VZV from skin lesions*

Results	No. of specimens collected on day after onset									
	1	2	3	4	5	6	7	8	9	≥10
VZV IF positive, isolation positive		1	2	2	2					
		1	1	1	1					
VZV IF positive, isolation negative		1	2	2	2	2	3	1	1	3
							3			3
							1			1

nongenital sites. Of the 58 specimens positive by isolation, 38 (32 HSV-2 and 6 HSV-1) were from genital lesions, and 20 (15 HSV-1 and 5 HSV-2) were from genital sites.

Table 2 gives additional information on the 9 patients with positive IF and negative isolation results for HSV. Clinical syndromes were compatible with HSV infection, and the specimens reacted only with the HSV conjugate and not with the VZV conjugate. For the most part, failure to isolate virus could not be associated with the late specimen collection.

Of the 45 specimens positive for VZV by IF, only 11 were positive by isolation (Table 1). Several pieces of evidence would support the specificity of the positive VZV staining results obtained in the absence of virus isolation. The specimens showed staining only with the VZV conjugate and not with HSV or vaccinia conjugates, and none of the specimens yielded HSV in cell culture. Four of the specimens were positive for herpesvirus particles by electron microscopy, and 4 additional patients had positive serological findings for VZV. Of the 34 patients, 28 had clinical diagnoses of herpes zoster or varicella, 2 had a diagnosis of possible smallpox, 1 had a diagnosis of vaccinia, and 3 had diagnoses simply indicating a vesicular rash.

Table 3 relates the time of specimen collection to ability to isolate VZV from vesicular lesion specimens. All of the positive isolations were obtained with specimens collected no later than 5 days after onset. Some early specimens which were positive for VZV by IF were negative by isolation, but it is more striking that a number of positive IF results were obtained with specimens taken long after they would be expected to yield infectious virus.

TABLE 4. *Correlation between immunofluorescence and isolation results (tissue specimens)*

Immunofluorescence	No. of specimens	Isolation (no. of specimens)		
		Positive HSV	Positive VZV	Negative
Positive HSV	26[a]	25 (24 HSV-1, 1 HSV-2)	0	1
Positive VZV	11[b]	0	2	9
Negative	243	4[c] (HSV-1)	0	239

[a] Specimens: 19 brain, 3 lung, 2 kidney, 1 liver, 1 trachea.
[b] Specimens: 5 lung, 4 liver, 1 spleen, 1 kidney.
[c] Brain specimens.

TABLE 5. *Comparative sensitivity of IF and virus isolation for detection of HSV and VZV in positive specimens*

Virus	Specimen	No. of positive specimens	No. of specimens (%) positive by:	
			IF	Isolation
HSV	Lesion	63	50 (79)	54 (86)
	Tissue	30	26 (87)	29 (97)
VZV	Lesion	45	45 (100)	11 (24)
	Tissue	11	11 (100)	2 (18)

In Table 4 IF is compared with virus isolation for detection of HSV and VZV in 280 tissue specimens. These included 230 brain, 30 lung, 9 liver, 6 kidney, and 4 heart specimens and 1 trachea specimen. Of the 26 specimens positive for HSV by IF, 25 were also positive by isolation, and 4 specimens were positive by isolation only. Correspondence between IF and isolation results in testing for HSV in tissue specimens was 98%. Again, IF was much more sensitive than isolation for detection of VZV.

Table 5 summarizes the comparative sensitivity of virus isolation and IF for detection of HSV and VZV in positive specimens. Overall, isolation was positive for 89% of the specimens in which HSV was demonstrable, and IF was positive for 82% of the specimens positive for HSV. In the case of VZV, IF detected all positive specimens, whereas isolation was positive for only 23%.

DISCUSSION

Although we recognize the serious implications of making a false-positive diagnosis of genital HSV infection based upon IF staining, our long experience with IF staining, in which a high proportion of specimens examined for HSV have been from genital lesions, together with the close

correspondence which we have shown between IF and isolation for detection of HSV, have led us to the conclusion that direct IF staining can be a reliable and rapid method for diagnosis of HSV infection when performed by experienced personnel using good reagents and equipment. There would appear to be little reason to doubt the specificity of the few IF positive results for HSV which were obtained on specimens failing to yield infectious virus, since the specimens gave positive staining only with the HSV conjugate, and clinical symptoms in the patients were compatible with HSV infection. It should be noted that all specimens examined in this study were sent from elsewhere in the state; thus, there was greater opportunity for virus to lose viability than would occur when virus examinations are done directly in a clinical setting.

The use of at least two immune conjugates on each specimen is considered to be an important specificity control in IF staining of clinical materials. In our total experience with IF staining on clinical specimens or cell culture isolates, we have encountered only a single specimen which gave positive staining for both HSV and VZV. This occurred in a lymphoma patient who clearly had disseminated zoster together with an active HSV infection. VZV was identified by positive IF staining of tissue from skin lesions, lung, liver, and kidney, whereas HSV was isolated from tissue from other skin lesions, brain, lung, bone marrow, and kidney. A specimen of stomach tissue from this patient gave positive staining for both HSV and VZV, but negative isolation results.

Some of the problems encountered by other workers with nonspecific IF staining on various types of clinical specimens might be attributable to the use of the indirect, rather than the direct, IF procedure and particularly to the use of immune serum of human origin for indirect IF examination. Comparative studies by Olding-Stenkvist and Grandien (8) have clearly shown the greater specificity of direct IF staining compared with that of the indirect method for detecting HSV and VZV in skin lesions. In an indirect IF system employing human serum as the primary antiserum, there is the possibility that the anti-human IG conjugate might react directly with any human immunoglobulins present in the test specimen and thus give a false-positive result for the virus being sought or mask specific fluorescence.

Preparation of HSV and VZV antisera by immunization with virus propagated in a homologous host system avoids the production of antibodies to foreign host proteins which might give misleading staining results, and this is considered to be an important factor in ensuring specificity of IF results. Although immune monkey sera to VZV are not widely available, immunization of rabbits with density gradient-purified VZV (6) is an alternative approach for preparation of an immune reagent for direct IF staining. We have found some lots of commercially available fluorescein-conjugated VZV antisera to be suitable for virus detection and identification.

IF staining for VZV showed remarkably greater sensitivity than did isolation for detection of this virus in lesion and tissue specimens. It seems likely that difficulty in isolating VZV from either type of specimen might be due to the fact that this virus is much more strongly cell-associated than is HSV. In the present study, a high proportion of lesion specimens which were positive for VZV by IF but negative by isolation were collected long after the time when they would be expected to yield infectious virus. This ability of IF to detect VZV in late specimens, as well as in some early specimens which fail to yield infectious virus, greatly enhances the capability for laboratory diagnosis of VZV infections. Again, clinical findings, together with positive electron microscopy and serological findings in some cases, would support the specificity of positive VZV IF results obtained in the face of negative isolation results.

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