Clinical Evaluation of Commercial Conjugates for Direct Immunofluorescence of Herpes Simplex Virus

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Commercially prepared antibody conjugates that are now available allow extensive application of direct immunofluorescence for diagnosing herpetic infections. Five of these conjugates—Microbiological Associates bivalent herpes antiserum (MBA-bivalent), MBA anti-herpes types 1 (MBA-1) and 2 (MBA-2), and Flow Laboratories anti-herpes types 1 (Flow-1) and 2 (Flow-2)—were compared for antibody titer, diagnostic accuracy, and nonspecific background staining on known infected tissue. Four of these were tested simultaneously on 75 clinical specimens. No attempt was made to differentiate between herpes simplex virus type 1 or type 2 infections. All antisera were comparable in titer and exhibited fluorescence with both herpes simplex virus types 1 and 2 antigens. The most significant difference was excessive nonspecific staining consistently seen with the MBA-2 conjugate. Overall, Flow-2 and MBA-bivalent antisera were preferable.

Herpes simplex virus (HSV) is a major cause of genital ulcers, vaginitis, and cervicitis. The last two conditions are rarely diagnosed clinically. The labial mucous membrane ulcers may suggest a herpetic etiology if painful, but they are rarely seen in the classic vesicular stage and are often misdiagnosed as chancroid, syphilis, trauma, or even carcinoma (3). Direct immunofluorescence can rapidly and accurately differentiate herpesvirus infection (7). However, the time and expense of reagent preparation has limited the use of this technique to research laboratories. In the present study, five direct fluorescent-antibody conjugates now commercially available—Microbiological Associates bivalent herpes antiserum (MBA-bivalent), Microbiological Associates anti-herpes types 1 (MBA-1) and 2 (MBA-2), and Flow Laboratories anti-herpes types 1 (Flow-1) and 2 (Flow-2)—were compared, using smears of cells from known infected rabbit kidney (RK) tissue culture and, with the exception of Flow-1, cell scrapings of 75 suspected herpetic genital lesions.

MATERIALS AND METHODS

Clinical specimens. Cell scrapings were obtained by light curettage with a no. 11 scalpel blade from any patient with painful or painless genital ulcers. All specimens were collected by nurse practitioners or physician's assistants at the City of Hartford Venereal Disease Clinic. Specimens were smeared on each of two 1-cm circles on two nonfluorescing glass slides, allowed to air dry, fixed in cold acetone (4°C) for 10 min, and stored at -70°C. After curettage, a sterile

swab from each lesion was taken simultaneously for virus isolation. This swab was immersed in Hanks balanced salt solution with 100 U of penicillin G, 100 µg of streptomycin, and 2 µg of amphotericin B per ml and refrigerated. Within 8 h of collection, the swab was agitated and removed; 0.2 ml was then inoculated into two tubes of RK cell cultures (MBA). After 1 h of absorption at 37°C, the cultures were covered with 1.5 ml of Eagle minimal essential medium supplemented with 100 U of penicillin G, 100 µg of streptomycin, and 2 µg of amphotericin B per ml and 1% fetal calf serum, and 1% L-glutamine. Inoculated tubes were incubated at 37°C and examined daily for cytopathology over a 1-week period.

Control slides. HSV types 1 and 2 (American Type Culture Collection MacIntyre and MS strains) were diluted 10⁻⁴ in phosphate-buffered saline (PBS) and inoculated into two tubes of RK cells. When 2 to 3+ cytopathology was observed (usually 24 to 36 h), the cells were scraped into the medium with a rubber spatula, centrifuged at 1,800 rpm for 4 min, washed twice in PBS, and suspended to 0.5 ml in PBS. Approximately 0.02 ml of the suspension was applied to a nonfluorescing glass slide and fixed in the previously described manner.

Direct fluorescent-antibody stain. One lot of each antiserum was tested. Clinical specimens were numbered randomly to avoid bias from the order of collection, and all antisera were tested simultaneously. Lyophilized antisera were reconstituted with distilled water according to the manufacturer's instructions. Approximately 0.03 ml of antiserum was applied over the entire specimen with a sterile pipette and incubated in a moist chamber at 37°C for 45 min. The slides then were rinsed with PBS (pH 7.6) to remove unbound fluorescein material, washed twice in PBS for 10 min, rinsed with distilled water, air dried, and

overlaid with glycerol-mounting medium (Baltimore Biological Laboratory). Some of the clinical specimens were counterstained with 0.02% Evans Blue for 5 min and rinsed three times with distilled water before mounting. Direct fluorescent-antibody slides were read by two independent observers, using an AO vertical fluorescent microscope with a BG12 exciter filter and an OG1 barrier filter. Control slides of RK cells infected with HSV types 1 and 2 and slides of uninfected RK cells were stained with the conjugates at the same time as the test specimens, along with an additional control of infected cells stained with normal rabbit serum.

RESULTS

Laboratory trial. Five antisera were examined for antibody titer, quality of fluorescence, and nonspecific background fluorescence on control slides of HSV-1- and HSV-2-infected tissue culture cells. Antisera were simultaneously tested in duplicate in dilutions of 1:5, 1:10, and 1:20 in PBS (Table 1). The end point of 3+ fluorescence is arbitrarily defined as dull, yellow-green fluorescence. Various combinations of homologous antisera and infected cells showed no difference in antibody titer, regardless of manufacturer. There was, however, a one-dilution lower titer for heterologous combinations of antisera and cells, again regardless of manufacturer. MBA-bivalent antiserum showed a higher titer with type 2-infected cells than type 1.

There was a major difference in nonspecific background fluorescence when the conjugates stained uninfected RK cells. Both MBA-bivalent and MBA-2 showed background staining that usually appeared as evenly distributed fluorescent background proportional to antiserum concentration. This background staining was so marked with MBA-2 at the 1:5 dilution that it completely obscured the uninfected tissue culture cells. MBA-bivalent antiserum had 1+ background fluorescence at this dilution. The other three antisera at the 1:5 dilution had bright 4+ specific fluorescence.

Clinical trial. In the control slide study, a dilution of 1:10 gave the best balance between desirable fluorescence of infected cells and un-

desirable background fluorescence on uninfected cells. However, with far fewer cells on the clinical smears, more intense immunofluorescence was desired as an end point, so a 1:5 PBS dilution of the conjugates was used. Because of difficulties in obtaining even four adequate scrapings (at least five epithelial cells) from each lesion, one antiserum, Flow-1, was arbitrarily excluded from the clinical trial. Seventy-five specimens, 27 culture positive for HSV, were collected in quadruplicate, stained simultaneously with the four antisera, and read independently by two observers. Table 2 shows the readings of observer 1 (J.G.G.). The second observer read two positives as negative and considered an unreadable slide as positive and a negative slide as unreadable. All other interpretations were the same, resulting in 91% agreement for the two observers. For the majority of specimens, the accuracy of the antisera was determined by the growth or lack of growth of virus in tissue culture. If a specimen showed typical HSV cytopathology in RK cells it was considered positive for HSV. No attempt to type the isolates was made. Two lesions were culture negative but fluorescent-antibody positive with all antisera; these specimens were considered herpetic in origin despite the negative culture.

One major difference between the laboratory and clinical trials was the difficulty in obtaining four specimens with more than five squamous epithelial cells from each genital ulcer. Thirty

TABLE 1. *Antibody titer and background fluorescence of conjugates tested on HSV-infected RK cells*

Conjugate	Maximum dilution for 3+ fluorescence		Back- ground fluores- cence
	HSV-1- infected cells	HSV-2- infected cells	
MBA-bivalent	1:10	1:20	1+
MBA-2	1:10	1:20	4+
Flow-2	1:10	1:20	None
MBA-1	1:20	1:10	None
Flow-1	1:20	1:10	None

TABLE 2. *Evaluation of conjugates on 75 clinical specimens*

Conjugate	No. of specimens					Unreadable ^c
	Inadequate specimen ^a	True pos- itive ^b	True neg- ative	False pos- itive	False neg- ative	
MBA-bivalent	14	19	33	4	3	2
MBA-2	10	15	36	2	6	6
Flow-2	8	18	39	1	7	2
MBA-1	14	15	38	1	7	0

^a Fewer than five epithelial cells.

^b Culture positive.

^c Nonspecific fluorescence obscuring epithelial cells.

percent of the scrapings had few or no epithelial cells, often with excessive obscuring debris and neutrophils, and were tabulated as inadequate specimens. Counterstaining with Evans Blue was initiated in the middle of the study and did reduce this number by aiding the subjective distinction among debris, polymorphonuclear leukocytes, and epithelial cells.

Flow-2 and MBA-bivalent sera detected the highest number of true positives. MBA-bivalent did have a lower false negative rate, and Flow-2 and MBA-1 had lower false positive rates. However, the major consistent difference among antisera was in the degree of nonspecific background staining. In Table 2, "unreadable background" signifies nonspecific fluorescence to the extent that epithelial cells could not be distinguished at all. Some of the specimens could be read but had nonspecific fluorescence interfering with reading the slide (Table 3). As in the laboratory series, MBA-2 showed the most nonspecific background staining. Interestingly, it did not result in a disproportionate number of false positive or negative results; specimens were reported rather as inadequate or unreadable.

DISCUSSION

A number of studies have established that direct (5, 7) or indirect (9, 10) immunofluorescence performed directly on lesion scrapings is a useful technique for diagnosis of HSV infection. We evaluated five commercially available conjugated HSV antisera. One antiserum was designated as a bivalent antiserum (MBA-bivalent). The other antisera were designated as anti-HSV-1 (MBA-1, Flow-1) or anti-HSV-2 (MBA-2, Flow-2).

Titers of the antisera on RK cells were identical regardless of manufacturer. The cross-reactions in the heterologous titration are due to the common antigen shared by HSV-1 and -2 (8). Although there was a consistent one-dilution difference between homologous and heterologous combinations of antigens and antisera, the difference was not sufficient, in our opinion, to show differentiation of viral types on the clinical

specimens containing few cells and relatively large amounts of debris.

Nahmias et al. (5) were able to distinguish HSV-1 from -2 in clinical specimens by diluting the conjugated antiserum to a point where type-1 antiserum reacted only with HSV-1. The type-2 antiserum reacted with both HSV-1 and -2. However, possibly due to differences in antisera, viral strains, or tissue culture systems, this technique for typing HSV has not proved generally useful for others (4).

Intensity of fluorescence may distinguish HSV-1 from HSV-2 when slides are made from specimens first grown in tissue culture, but, in our hands, not on direct immunofluorescence on clinical specimens. Reliable differentiation of HSV-1 and -2 might be possible if the antisera were absorbed with heterologous infected cells (8, 10).

There was a lower false positive rate for Flow-2 and MBA-1, and a lower false negative rate for MBA-bivalent. This may be an artifactual result of the inclusion of specimens adequate for only two or three antisera examinations in our study. For example, certain lesions gave barely adequate specimens with much debris and few, but greater than five, epithelial cells for two antisera, and inadequate specimens for the other two. Thus, these last two antisera would be spared testing on a difficult specimen with a high risk of a false result. The apparently high false negative rate in general, in comparison to other reports of herpes immunofluorescence, is in part due to the use of a single scraping for each antiserum tested in our study. This rate does not provide an estimate of the chance of a false negative result in a clinical setting, since then at least two cellular specimens read jointly would usually be used.

The most important consistent difference among commercial conjugates was the nonspecific background fluorescent staining seen with MBA-2. All specimens were collected in quadruplicate and stained simultaneously, excluding inadequate rinsing or tissue autofluorescence as explanations. The concentration or purity of fluorescein isothiocyanate used in labeling globulins affects the degree of nonspecific background staining and may explain the difficulties with MBA-2. Another possible explanation might be the presence of conjugated, nonantibody, serum proteins in this particular conjugate (2). This type of background staining can be minimized by centrifugation of the conjugate prior to staining, but at the expense of a lowered antibody titer and a greater cost per specimen.

Counterstaining with a dilute Evans Blue solution subjectively aided the distinction between positive cells and fluorescent debris. We recom-

TABLE 3. *Specimens with excess background fluorescence*

Conjugate	Excess background ^a no. ^b
MBA-Bivalent	8 (11) ^c
MBA-2	22 (29)
Flow-2	8 (11)
MBA-1	4 (5)

^a Nonspecific fluorescence obscuring some epithelial cells but still permitting evaluation.

^b Total of 75 specimens examined.

^c Numbers in parentheses represent percentages.

mend its use, at least until a large number of clinical specimens have been examined. It does diminish intensity of specific fluorescence, and so should be used only with hypocellular or debris-laden specimens stained with antiserum in relatively low dilutions.

With all commercial conjugates approximately equal in expense and potency, the ideal antiserum for routine use in community hospital laboratories and clinics would be that detecting the most true positives by intense specific fluorescence, while showing minimal false positive reactivity or unreadable results from nonspecific background staining. Overall, Flow-2 and MBA-bivalent antisera most closely approached this ideal. Our laboratory is presently using the MBA-bivalent antiserum, although the Flow-2 reagent is certainly equal.

The lower cost of the immunofluorescence test compared with viral culture has increased the interest in attempting a laboratory diagnosis of genital lesions. Since this procedure has been available, both the clinical diagnostic acumen of those collecting specimens as well as the quality of the slides submitted have improved.

Although we did not evaluate them, positive and negative control tissues are commercially available (MBA). The availability of these reagents takes this procedure out of the realm of the research virus laboratory to that of the routine clinical hospital laboratory. It is hoped that more widespread use of the direct fluorescent-antibody technique for HSV on clinical specimens will provide important diagnostic and epidemiological formation.

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