Diagnosis of *Herpesvirus hominis* Infections in a General Hospital Laboratory

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There is a greatly increased interest in *Herpesvirus hominis* infections especially those of type 2 associated with genital lesions or neonatal disease. Physicians are eager to confirm the clinical impression with a specific virologic diagnosis such as isolation of the agent and its typing, and type-specific antibody responses. Procedures are reviewed here which permit such studies in general microbiology laboratories equipped for simple cell culture and immunofluorescence. This paper recounts experience with several laboratory methods and evaluates their efficiency and practicability in a general laboratory. Virus isolation was optimal if specimens were obtained from visible lesions early in their evolution and it often provided a specific diagnosis, including typing of the isolate by immunofluorescence, within 24 to 48 h. Estimation of serum antibodies to herpes simplex virus type 1 and 2 by indirect immunofluorescence was more sensitive and perhaps also more specific than by microneutralization test. A pilot study of herpes simplex virus antibody titers in mothers and in the cord blood of the offspring suggested the need to evaluate a possible protective role of high titer antibody in the fetus.

During the past decade, there has been a steady increase in the interest in specific laboratory diagnosis of herpesvirus infections in general hospitals. Some major reasons for this development may be found in the following: (i) an apparent substantial increase in *Herpesvirus hominis* (herpes simplex virus [HSV]) type 2 infections, perhaps as a part of the current epidemic of venereal disease; (ii) concern about disseminated neonatal herpes acquired from maternal virus-containing lesions before or at, term; (iii) recognition that disseminated herpes occurs as a severe complication in drug-induced immunosuppression; (iv) the development of treatment modalities for local herpetic lesions (eye, skin, mucous membranes) and the possibility that serious systemic infection with herpes viruses may be susceptible to therapy in the future.

In general, clinicians are interested in the following specific questions. Can virus be isolated from a lesion, confirming a clinical impression? What type of herpesvirus is present, i.e., is it *H. hominis* type 1 or 2, or another herpesvirus? In the absence of lesions, can serologic tests establish the nature and type of infection which occurred in the past? Can immunologic tests predict the likelihood of disseminated herpes infection in an exposed newborn, or in an immunosuppressed adult? If a virus has been isolated, can its susceptibility to available topical or systemic antiviral drugs be assessed?

We have pursued answers to some of these questions, and wish to present our experience in a general laboratory, with emphasis on the success of simple methods, and on the possible interpretation of results in providing answers desired by the clinician. Only methods suitable for *H. hominis* isolation and antibody determinations were employed in these initial studies.

**MATERIALS AND METHODS**

**Patients.** Individuals attended the Univ. of California medical clinics for routine screening, for routine prenatal care, or because of evidence of lesions of skin, eye, or genitalia. Random patients admitted for delivery to one community hospital also participated in the study.

**Isolation of virus.** Specimens were collected by aspiration of fluid from visible vesicles, or by swabbing the site of the lesions, and suspending the material in 1.0 ml of maintenance medium (2% heat-inactivated fetal calf serum and antibiotics in Eagle minimal essential medium). This suspension was inoculated onto monolayers of Vero (African green monkey kidney) cells in stationary tubes, incubated at 37 C, and observed daily during a period of 7 to 10 days for the appearance of typical cytopathic effects (CPE).

**Sera.** Blood was drawn aseptically and permitted to clot, and serum was separated and stored at −20 C
blood was drawn from the mothers until tested. For the studies delivery postpartum antibody levels, cord blood was tested simultaneously without heat inactivation, as were multiple sera from individuals with suspected herpes infections.

**Microneutralization tests.** A modification of the method by Rawls et al. (8) has been described (10) for the neutralizing antibody titrations. Sterile flat-bottom microplates (Cooke) were used throughout. Varying dilutions of serum (1:10 to 1:640) in minimal essential medium were tested in duplicate against a constant dilution of virus (100% tissue culture infective doses) in minimal essential medium on Vero cells. Plates were read after 72 h incubation using an inverted microscope. The CPE was recorded for each virus-serum mixture as either positive (at least one area of typical CPE noted) or as negative (normal cell sheet). Each serum was tested at least twice. Each test included a cell control, complete titrations of each virus inoculum, and known positive and negative sera. The HSV strains used were PH (type 1) and MS (type 2).

**Indirect immunofluorescence (fluorescent antibody) for antibody titration.** The PH and MS strains of virus were grown in Vero cells in 1-oz prescription bottles in maintenance medium. When 50 to 100% of the monolayer showed typical CPE, cells were removed from the glass (using a solution of pancreatin-trypsin), added to similarly treated cells from three uninoculated bottles, and lightly centrifuged (1,000 rpm/10 min). The mixtures of infected and uninfected cells were resuspended in Hank's balanced salt solution plus 2% calf serum to a volume of approximately 6 ml. Small drops (approximately 0.02 ml) of this cell suspension were placed within the etched circles of microscope slides, with PH strain on one side and MS on the other. Slides were dried at 35°C, fixed in cold acetone for 15 min, rinsed in phosphate-buffered saline, pH 7.2, air-dried, and stored at -20°C. Serum dilutions, prepared as described above or in phosphate-buffered saline, were added to both circles. The slides were incubated in moist chambers at 35°C for 30 min, rinsed in phosphate-buffered saline for 15 min with occasional shaking, and air-dried. A 1:150 dilution in saline of a commercial (BBL) fluorescein-conjugated anti-human globulin was then added to each spot, and the slides were incubated and rinsed as before, and mounted in buffered glycerol saline. The titer of the serum was defined as the highest dilution of serum resulting in 3+ to 4+ fluorescence of infected cells, with less than 1+ fluorescence of the uninfected cells in the same field.

**Immunofluorescence typing of isolates.** Isolates were typed after one or two passages in cells. When 50 to 100% of Vero cells in tube monolayers showed typical CPE, the cells were harvested as described above and pooled with similarly treated cells from three tubes of uninfected cells. Drops of this cell suspension mixture were placed within two etched circles on several slides. After fixation, the slides were stored at -20°C. To identify an isolate as *H. hominis*, a guinea pig serum which reacted with known strains of HSV types 1 and 2, but which did not react with uninfected or vaccinia-infected Vero cells, was used in a dilution of 1:100. To differentiate HSV type 1 and 2, a human serum with a known titer of 1:640 against PH and of 1:160 against MS was used in arbitrarily selected dilutions of 1:50 on one side and 1:500 on the other. All isolates were examined in duplicate by indirect immunofluorescence. Fluorescein-conjugated antiglobulins were obtained from Microbiological Associates (anti-guinea pig) and BBL (anti-human). Slides with a known type 1 and 2 strain were included in each test, together with negative controls. In all tests reported, control HSV type 1-infected cells were stained with both dilutions of the human reagent serum, the control HSV type 2-infected cells were stained only by the 1:50 dilution, and both types reacted with the guinea pig serum. Later in the study a guinea pig antiseraum which in a dilution of 1:100 stained only HSV type 1, and a second serum which in a dilution of 1:100 stained only HSV type 2 were also used for typing. There was complete agreement between these results and the results obtained using the human serum in the two dilutions described above. Neither the conjugated anti-human globulin serum nor the anti-guinea pig globulin serum stained HSV-infected cells in the absence of specific anti-HSV serum.

**RESULTS**

**Isolation of *H. hominis*.** Of 86 specimens received from patients who exhibited lesions suspected of being herpetic, 47 (55%) yielded strains of HSV. This contrasts with results of a survey in which we attempted to isolate HSV from cervical scrapings obtained in a women's clinic devoted to regular examination of patients with cervical dysplasia (9). Of 1,858 cervical scrapings taken in the absence of any visible lesions resembling herpetic vesicles or ulcerations, 48 (2.6%) yielded HSV. Thus the presence of a visible suggestive lesion enhances the likelihood of HSV isolation about 20-fold.

The more clinically typical a lesion, the greater the probability of isolating a virus. Nine of 10 symptomatic patients (90%) with vesicular lesions seen on labia or vaginal wall by one gynecologist yielded HSV, and 13 of 15 (87%) of patients with lesions suggestive of herpes simplex seen on skin or mucous membranes by one dermatologist yielded HSV. By contrast, among surface lesions of doubtful etiology seen on skin or mucous membranes by an experienced infectious disease consultant, only 23 of 43 (53%) yielded HSV. It is probable that the most typical lesions, which had the highest yield of virus, were also the earliest well-developed lesions. It has been shown that the yield of virus tends to be highest within the first 48 h of the
development of a herpetic lesion, diminishing rapidly thereafter (4).

CPE in the Vero cell system were observed fairly promptly. Of 100 HSV isolates which were identified during the past 3 years, 38% showed CPE in 24 h or less after inoculation of the cell culture, 34% in 48 h, and 22% in 72 h. Thus, 94% of all eventually positive specimens permitted a preliminary report to the physician in 72 h or less. This is not out of line with a variety of other microbiological tests which require the growth of an etiologic agent. The speed of CPE development appeared to be a function of the size of the infectious inoculum and thus related to the point in the development of a lesion when it was sampled.

**Typing of HSV isolates.** Eighteen isolates which had earlier been submitted to typing by other methods to A. Nahmias or W. Rawls (personal communications) since 1971 were typed by immunofluorescence. Eleven of these isolates had been derived from lesions of the eye, or the skin of head, neck, or upper extremities. All were identified as type 1 by both immunofluorescence and the methods employed in other laboratories in the past. Seven isolates previously identified as type 2 by Nahmias or Rawls were typed as type 2 by immunofluorescence.

Of 52 additional strains isolated in this laboratory since 1950 (not submitted to another laboratory for typing) obtained from lesions of or near the genitalia, 50 appeared to be type 2 by immunofluorescence, one appeared to be type 1, and one could not be typed. Of 47 additional isolates from eye, face, or skin lesions, 37 were type 1 by immunofluorescence. Nine (eight isolated from eye) were type 2, and one could not be typed. An apparent HSV type 2 was isolated from both groin and cheek lesions in one individual. HSV type 2 isolates from skin and bronchial washings of a neonate were considered genital in origin. Thus, there seems to be good agreement between typing by immunofluorescence and other methods (1, 3, 6), as well as between type and site of isolation (2, 7).

**Estimation of antibodies to HSV type 1 and 2.** Antibody titrations were performed by microneutralization and by immunofluorescence tests. A comparison of serum titers by neutralization and fluorescent antibody obtained on 20 persons with lesions of skin or mucous membranes on head and neck, and thus presumed by convention to be infected with type 1, are shown in Fig. 1. Titers obtained on 30 persons with lesions on or near genitalia, and thus presumed by convention to be infected with type 2, are shown in Fig. 2.

**Fig. 1.** Antibody titers to HSV-1 and HSV-2 in the serum of persons with herpetic lesions above the waist. Measured by microneutralization and immunofluorescence.

**Fig. 2.** Antibody titers to HSV-1 and HSV-2 in the serum of persons with herpetic lesions below the waist. Measured by microneutralization and immunofluorescence.

Geometric mean titers of sera tested against HSV type 1 and 2 antigens are given in Table 1. In all instances, the mean titer against HSV type 1 antigen was higher than against HSV type 2 antigen. However, the mean titer against HSV type 2 antigen was markedly higher in those individuals who had lesions below the waist. If the formula used by Rawls et al. (9) is applied, the ratio ([Log 10 titer to type 2/Log 10 titer to type 1] × 100) is markedly higher in patients with presumed HSV type 2 infections. It should be noted, however, that the ratio is significantly higher with the immunofluorescence determinations than with neutralization tests. Thus, it seems probable that in a given individual patient the serologic diagnosis of the infecting virus type is more likely to be correct if immunofluorescence titers are employed than if...
neutralization titers are employed. When both isolates for typing and serum for antibody titrations were available from individuals, there was good agreement between the two methods in identifying the type of HSV responsible for the current infection.

As part of an ongoing study on the problem of neonatal herpes (10), a comparison was made between antibody titers in the blood of mothers and the cord blood of their offspring. This was carried out by both microneutralization and immunofluorescence, employing type 1 and 2 antigens. In all cases, there was substantial agreement between the titers in mother and cord blood by either method. Titers to type 1 were higher than to type 2 in both mother and cord blood even when an isolate of HSV type 2 had been obtained from a recurrent lesion of the mother, possibly because of preceding HSV type 1 infection. Examples are given in Table 2.

The development of type-specific antibody in relation to a primary lesion is illustrated in six patients shown in Table 3. Specific isolates had been obtained from each patient before the antibody determinations were performed. All patients evidently had primary infections. An antibody titer rise appeared as early as 9 days after virus was isolated from a primary lesion which had been present for only 2 to 3 days. In all cases, the antibody titer rise was detected more readily by immunofluorescence than by neutralization. In this series, antibody to type 1 was at least eightfold higher than to type 2 in proven type 1 infections. In type 2 infections, the titer to the two types was similar or identical.

**DISCUSSION**

The developing methods of simplified diagnostic virology make it possible for most hospital laboratories to undertake tests in support of a diagnosis of viral infection with little more investment in time, equipment, and materials than are required in tests for microbial infections. In particular, simplified methods of cell culture, commercially available cell lines and media, and the use of immunofluorescence in serologic determinations have drastically reduced the complexity of laboratory studies in viral infections.

The availability of simplified laboratory techniques coincides with a greatly increased need for rapid and accurate diagnosis of common virus infections, particularly those due to herpesviruses. This need has developed as a result of the greatly increased frequency of herpes infections observed in hospitals and of the development of actual or potential antiviral drugs. The toxicity of such drugs makes it
mandatory that they be considered for use only when the diagnosis can be substantiated by laboratory results.

This paper summarizes the methods employed in our laboratory for the diagnosis of herpes virus infections and illustrates several practical points regarding the limitations and the possible advantages of these methods. H. hominis is recovered most readily from early, typical lesions. Thus, physicians must be encouraged to secure specimens promptly and to keep them frozen if necessary, rather than to delay obtaining the specimen until the need for diagnostic assistance is more pressing (e.g., in disseminating herpetic lesions). With an adequate specimen from an early herpetic lesion, virus growth in cell culture is usually evident in 48 h or less, a period comparable to the growth and tentative identification of many bacterial pathogens.

Our laboratory's experience with establishing an isolate as H. hominis of either type 1 or 2 by immunofluorescence suggests that this, too, is a simple and reliable procedure. However, the reading of immunofluorescent preparations always requires experience and the inclusion of good controls before a high degree of reliability can be expected. The determination of antibodies is also significantly simpler, more rapid, and more sensitive by immunofluorescence than by microneutralization tests.

The determination of type-specific antibodies to H. hominis has not been well standardized. It is widely accepted that persons infected with HSV type 1 regularly have a four- to eightfold higher antibody titer to the homologous type than to HSV type 2. Conversely, persons infected with HSV type 2 tend to have a relatively higher level of anti-type 2 antibody. The ratio of anti-type 1 to anti-type 2 is not rigidly set. The geometric mean titers and their ratios (shown in Table 1) provide a framework for the statement that type-specific antibody levels found in a given patient probably indicate type 1 or type 2 infection. In early infections (Table 3), serologic tests may not permit unequivocal conclusions regarding the type of infecting herpesvirus.

One of the major implications of the current epidemic of venereal diseases is the great increase in the number of HSV type 2 genital infections diagnosed in women. This carries the potential risk of disseminated herpes infection in the newborn. Many women with herpetic lesions during the last few weeks of pregnancy are undergoing cesarean section in the hope of avoiding contact between newborn and a virus-containing lesion in the birth canal. The risk of such a disastrous infection of the newborn appears to be high if the mother sustains a primary genital infection just prior to the expected date of confinement. However, chronic, recurrent H. hominis type 2 infection in the mother may not carry such a serious prognosis.

It has been shown that specific neutralizing antibodies to herpesviruses have similar titers in mother and offspring, if the infection (clini-
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cally apparent or latent) has been present for some time in the mother (5, 10). It is possible that a substantial titer of antibodies in the fetus, present at the time of potential exposure to HSV type 2, has some protective effect against the initial virus reinfection. Two babies (Table 2) had such antibodies, and normal delivery (without cesarean section) was not followed by evidence of herpes infection, although virus-containing lesions had been observed in the mothers' genital tracts less than 8 to 30 days before delivery. This question merits further investigation to avoid unnecessary cesarean sections.

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Literature Cited