Typing *Herpesvirus hominis* Antibodies and Isolates by Inhibition of the Indirect Hemagglutination Reaction

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Inhibition of the indirect hemagglutination reaction (IHA inhibition) was compared to several other methods for type-specific identification of *Herpesvirus hominis* (HVH) antibodies and isolates. The method appears to have the greatest value for typing antibodies for HVH type 1 and HVH type 2 in human sera; identification of antibody type was relatively simple and results were definitive. The IHA-inhibition test permitted serological diagnosis of HVH type 2 infection in three young adults with meningoencephalitis, thus extending the mounting evidence that nervous system involvement with this virus type is not limited to neonatal infections. II/I indexes of neutralizing or IHA antibody gave an accurate indication of the presence of HVH type 2 antibody in those sera containing type 2 antibody by IHA inhibition, but they indicated the presence of HVH type 2 antibody in one-half or more of the sera shown to contain only HVH type 1 antibody by IHA inhibition. For typing HVH isolates, the IHA-inhibition test gave results identical to those obtained by direct fluorescent-antibody staining using cross-absorbed conjugates, but the IHA-inhibition test was much more cumbersome and time-consuming to perform than was direct fluorescent-antibody staining. A microneutralization technique for virus typing also gave results identical to those obtained with direct fluorescent-antibody staining and IHA inhibition. However, typing HVH isolates by plaque size or the differential effect of incubation temperature was found to be less definitive and accurate.

**MATERIALS AND METHODS**

**Virus strains.** The MacIntyre strain of HVH-1 and the MS strain of HVH-2 were employed for preparation of inhibiting and absorbing antigens, for assay of neutralizing and IHA antibodies, and as reference or control strains of HVH-1 and HVH-2 in all of the test procedures. The field strains of HVH which were typed by the various techniques were isolated from clinical materials in this laboratory by procedures described previously (13). Most of the isolations were made in human fetal diploid lung or kidney cells, but some strains were also recovered in primary rhesus monkey kidney cells.

**Sera examined.** The human sera used for typing HVH antibody by IHA inhibition were from 38 individuals with clinical manifestations of herpetic infections and whose paired serum specimens showed a diagnostically significant increase in HVH antibody by complement fixation, neutralization, or IHA tests. Ten of the patients had HVH isolations as well as positive serological findings.

**IHA and IHA-inhibition tests.** The IHA and IHA-inhibition procedures were conducted essentially as described by Bernstein and Stewart (1a, 2), using sensitizing and inhibiting antigens produced in the L 645 strain of human fetal diploid lung cells.

The antibody type was determined on a dilution of serum containing 8 U of IHA antibody for HVH-1 or
HVH-2. Portions of the 8 U sample were incubated at room temperature for 30 min with inhibiting antigen for HVH-1 and HVH-2 and with uninfected control antigen. HVH-1- and HVH-2-inhibiting antigens were used at dilutions previously shown to completely inhibit 8 U of IHA antibody for HVH-1 or HVH-2, and uninfected antigen was used at the same dilution. The serum samples absorbed with each inhibiting antigen were then tested against sheep erythrocytes sensitized with HVH-1 and HVH-2, and the antibody type in the serum was determined by the patterns of inhibition. The patterns of inhibition given by sera containing HVH-1, HVH-2, or both types of antibody are shown in Table 1. Complete agglutination was read as lack of inhibition, and partial or no agglutination was read as inhibition.

For typing viral isolates by IHA inhibition, inhibiting antigens were prepared by propagating the isolates in human fetal diploid lung cells, and the antigens were examined at dilutions from 1:1 through 1:16 for ability to inhibit the IHA reactions of 8 U of HVH-1 antibody with HVH-1-sensitized erythrocytes or 8 U of HVH-2 antibody with HVH-2-sensitized cells. Type-specific identification was based upon complete inhibition of IHA antibody to one virus type, but not the other. Human sera containing a known, single type of HVH antibody were used for virus typing, since HVH antisera produced in animals gave inconsistent results.

Direct fluorescent-antibody staining. Antisera to HVH-1 and HVH-2 produced in hamsters were conjugated with fluorescein isothiocyanate (5), and the conjugates were absorbed with an equal volume of BHK-21 cells infected with the HVH heterotype. Absorption was conducted at 37°C for 30 min and then overnight at 4°C with constant mixing. The cells were sedimented by centrifugation at 1,000 × g for 10 min, and the supernatant fluid (absorbed conjugate) was passed through a 0.22-mm membrane filter and stored at 4°C until used. The working dilution of conjugate employed for virus typing was the highest dilution giving a 3+ or 4+ specific staining reaction with cells infected with homotypic HVH and little or no staining with cells infected with heterotypic HVH. Direct fluorescent-antibody staining for typing isolates was performed, as described elsewhere (14), by using smears of infected BHK-21 cells. Isolates identified as HVH-1 and HVH-2 conjugate and no staining with the HVH-2 conjugate, whereas isolates identified as HVH-2 gave 2+ to 3+ staining with the HVH-2 conjugate and ± staining with the HVH-1 conjugate.

Neutralization tests. Tests were conducted by a micro method (13) using the BS-C-1 line of grivet monkey kidney cells. Antisera produced in rabbits were employed for virus typing. The antiserum to HVH-1 had a titer of 1:1024 against 100 mean tissue culture infective doses of either HVH-1 or HVH-2, but the antisera to HVH-2 reacted with greater type-specificity, having a titer of only 1:16 against 100 mean tissue culture infective doses of HVH-1 and a titer of ≥1:128 against 100 mean tissue culture infective doses of HVH-2. Isolates were tested at dilutions representing approximately 100 mean tissue culture infective doses of virus, and those which were neutralized to high titer by antiserum to HVH-1, but to a titer of ≤1:16 by type 2 antiserum, were considered to be type 1 strains; strains which were neutralized to high titer by antisera to both HVH-1 and HVH-2 were to be considered to be type 2 isolates.

Plaquing HVH strains. Differences in plaque size (6) and the differential effects of temperature (4, 9, 11) on HVH-1 and HVH-2 were studied by plaquing viral isolates in the NBRL/ME line of normal Swiss Webster mouse embryo cells (obtained from the Cell Culture Laboratory, Naval Biological Research Laboratory, Oakland, Calif.). Monolayers of cells in 30-mm plates were inoculated with virus and incubated at 37°C for 1 h to permit adsorption of virus; the inocula were then removed, and the monolayers were washed with 2 ml of Hanks balanced salt solution. The monolayers were covered with 4 ml of nutrient overlay consisting of 5% fetal bovine serum, 94.5% Eagle minimal essential medium (without phenol red), and 0.5% Isonagar no. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.). Cultures were incubated in an atmosphere of 5% CO2 in air for 3 to 5 days. A 1-ml volume of nutrient overlay containing neutral red sufficient to give a final concentration of 1:50,000 was then added. Plaques were measured or enumerated after an additional day of incubation.

RESULTS

Relationship between clinical manifestation of herpetic infections and type of antibody detected by IHA inhibition. Table 1 shows the type of HVH antibody detected by IHA inhibition in convalescent-phase sera from 38 patients with various clinical manifestations of HVH infection, and illustrates the patterns of inhibition demonstrated by each type of HVH antibody.

Of the eight patients with meningoencephalitis, five were found to have type 1 antibody and three had type 2 antibody. Two of those with HVH-1 antibody also had type 1 virus isolations. Nine of the patients with gingivostomatitis had type 1 antibody, and HVH-1 was isolated from two of these. A single patient with gingivostomatitis had HVH antibody only to type 2 virus. Two of the 19 patients with genital tract infections had antibody to both HVH-1 and HVH-2; one had only HVH-1 antibody in the acute-phase serum and antibody to both types in the convalescent-phase specimen, whereas the other had both types of antibody in acute- and convalescent-phase specimens. The single patient with dermatitis had HVH-2 antibody, and HVH-2 was isolated from a lesion specimen.

Results of HVH antibody typing by IHA inhibition were definitive with human sera, and equivocal reactions were not seen, even with sera containing both types of antibody. How-
ever, inhibition reactions with immune animal sera were not so distinct. Eight units of HVH antibody in rabbit, hamster, or guinea pig sera were not consistently and completely inhibited with the homotypic absorbing antigen. Consequently, controls in each IHA-inhibition run included both human and animal sera of known antibody type.

**Relationship between HVH antibody type detected by IHA inhibition and relative levels of neutralizing and IHA antibody to HVH-1 and HVH-2.** Table 2 relates the antibody type demonstrable by IHA inhibition in convalescent-phase sera from 36 patients with herpetic infections to the relative levels of neutralizing and IHA antibody to HVH-1 and HVH-2 seen in each serum.

Of the 14 patients with only HVH-1 antibody demonstrable by IHA inhibition, five had identical titers to HVH-1 and HVH-2 in neutralization tests and four had identical IHA titers for both virus types. When higher titers were seen to HVH-1, they were generally only twofold in magnitude. Thus, if only \( \geq 4x \) differences in titer to the two virus types are to be considered significant, relative antibody titers to HVH-1 and HVH-2 were not a reliable indication of the type of antibody present in those sera shown to contain only type 1 antibody by IHA inhibition.

In the group of patients with only HVH-2 antibody demonstrable by IHA inhibition, neutralizing antibody titers to HVH-1 and HVH-2 were not markedly different; only 2 of the 20 patients had \( \geq 4x \) higher titers to HVH-2. However, differences between IHA titers to HVH-1 and HVH-2 were marked; 18 of the 20 patients had \( \geq 4x \) higher IHA titers to HVH-2.

Rawls et al. (12) have suggested the use of a so-called II/I index to express relative neutralizing antibody titers for HVH-2 to those for HVH-1. They found that the index was less than 85 for 93% of the sera from persons with recurrent oral herpetic lesions and no history of genital infections, and 85 or greater in 95% of patients with genital herpetic lesions. Thus, II/I indexes of less than 85 were considered to

**Table 1. HVH antibody type, as determined by IHA inhibition, in human herpetic infections**

| Clinical manifestations | No. of cases | Hemagglutination of HVH-1 sensitized erythrocytes after absorption of serum with: | Hemagglutination of HVH-2 sensitized erythrocytes after absorption of serum with: | HVH antibody type |
|-------------------------|-------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-----------------|
|                         |             | HVH-1 | HVH-2 | Uninfected antigen | HVH-1 | HVH-2 | Uninfected antigen |                         |
| Meningoencephalitis     | 5           | 0*    | +     | +                  | 0     | 0     | +                  | 1                         |
|                         | 3           | 0     | 0     | +                  | 0     | 0     | +                  | 2                         |
| Gingivostomatitis       | 9           | 0     | +     | +                  | 0     | 0     | +                  | 1                         |
|                         | 1           | 0     | 0     | +                  | 0     | 0     | +                  | 2                         |
| Genital tract infection | 1           | 0     | +     | +                  | 0     | 0     | +                  | 1                         |
|                         | 16          | 0     | 0     | +                  | 0     | 0     | +                  | 2                         |
|                         | 2           | 0     | +     | +                  | 0     | 0     | +                  | 1 and 2                   |
| Dermatitis              | 1           | 0     | 0     | +                  | 0     | 0     | +                  | 2                         |

*0, Inhibition; +, agglutination.

**Table 2. Relationship between HVH antibody type detected by IHA inhibition and relative neutralizing and IHA antibody titers to HVH-1 and HVH-2**

| HVH antibody type by IHA inhibition | No. of patients | Neutralization test | IHA test |
|-------------------------------------|-----------------|---------------------|---------|
| HVH-1 and HVH-2 titers same        |                 | HVH-1 titer higher: |         |
|                                     |                 | \( \geq 4x \)       |         |
|                                     |                 | \( \geq 4x \)       |         |
|                                     |                 | \( 2x \)            |         |
|                                     |                 | \( \geq 4x \)       |         |
|                                     |                 | \( 2x \)            |         |
|                                     |                 | \( \geq 4x \)       |         |
| 1                                   | 14              | 5                   | 1       |
| 2                                   | 20              | 7                   | 2       |
| 1 and 2                             | 2               | 2                   | 1       |
indicate the presence of antibody only to HVH-1, and those over 85 were considered to indicate the presence of HVH-2 antibody.

Table 3 relates the type of HVH antibody determined by IHA inhibition to the II/I indexes of the same convalescent-phase sera in neutralization and IHA tests. For sera containing only type 1 antibody by IHA inhibition, the II/I indexes of neutralizing and IHA antibody indicated the presence of HVH-2 antibody in one-half or more of the sera. On the other hand, II/I indexes gave an accurate reflection of the presence of type 2 antibody in sera shown to contain HVH-2 antibody by IHA inhibition.

Comparison of IHA inhibition with other methods for typing HVH isolates. Table 4 compares the results of typing 23 HVH isolates from clinical materials by IHA inhibition, direct fluorescent antibody staining with cross-absorbed conjugates, neutralization tests, plaque size, and differential effects of incubation temperature.

There was complete agreement among IHA inhibition, direct fluorescent antibody staining, and neutralization tests in typing all 23 of the isolates. Sixteen of the isolates were also typed by A. J. Nahmias, and the virus type as determined by the three test procedures agreed with his results in all instances.

All of the isolates from brain tissue in cases of encephalitis were typed as HVH-1 by the three tests, as was the isolate from a case of eczema herpeticum. One of three isolates from neonatal herpes was typed as HVH-1, and two were identified as HVH-2. Three of the eleven isolates from genital infections were typed as HVH-1, and eight were typed as HVH-2.

Munk and Ludwig (6) have reported that HVH-1 strains produce only small plaques and that HVH-2 strains produce both large and small plaques, the large ones being two to three times the diameter of those produced by HVH-1.

### Table 3. Relationship between HVH antibody type detected by IHA inhibition and II/I indexes in neutralization and IHA tests

| HVH antibody type detected by IHA inhibition | No. of patients | Neutralization test | IHA test |
|---------------------------------------------|-----------------|---------------------|----------|
|                                             |                 | II/I index < 85     | II/I index ≥ 85 | II/I index < 85 | II/I index ≥ 85 |
| Type 1                                      | 14              | 7                   | 7         | 6         | 8          |
| Type 2                                      | 20              | 1                   | 19        | 0         | 20         |
| Types 1 and 2                               | 2               | 0                   | 2         | 0         | 2          |

### Table 4. Comparison of various methods for typing HVH-1 and HVH-2 isolates

| Clinical manifestation | Virus strain | Virus isolated from | HVH type indicated by: |
|-----------------------|--------------|---------------------|-------------------------|
|                       |              |                     | IHA inhibition | Direct fluorescent antibody staining | Neutralization | Plaque size | Temp differential |
| Encephalitis          | G.Ba.        | Brain               | 1            | 1          | 1         | 1          | 2           |
| Encephalitis          | J.Ho.        | Brain               | 1            | 1          | 1         | 1          | 2           |
| Encephalitis          | I.Se.        | Brain               | 1            | 1          | 1         | 2          | 1           |
| Encephalitis          | M.Ka.        | Brain               | 1            | 1          | 1         | 2          | 1           |
| Encephalitis          | V.Ro.        | Brain               | 1            | 1          | 1         | 2          | 1           |
| Encephalitis          | A.Du.        | Brain               | 1            | 1          | 1         | 2          | 1           |
| Encephalitis          | R.Bo.        | Brain               | 1            | 1          | 1         | 2          | 1           |
| Encephalitis          | S.Ca.        | Brain               | 1            | 1          | 1         | 2          | 1           |
| Eczema herpeticum     | T.Mi.        | Eyelid              | 1            | 1          | 1         | 2          | 1           |
| Neonatal herpes       | E.Va.        | Liver               | 1            | 1          | 1         | 2          | 1           |
| Neonatal herpes       | J.Wi.        | Brain               | 2            | 2          | 2         | 2           | 2          |
| Neonatal herpes       | N.Jo.        | Liver               | 2            | 2          | 2         | 2           | 2           |
| Genital infection     | E.Bi.        | Vulva               | 1            | 1          | 1         | 2          | 1           |
| Genital infection     | D.Lo.        | Vulva               | 1            | 1          | 1         | 2          | 1           |
| Genital infection     | S.Hi.        | Vulva               | 1            | 1          | 1         | 2          | 1           |
| Genital infection     | J.Ga.        | Vulva               | 2            | 2          | 2         | 2           | 2          |
| Genital infection     | J.Mo.        | Vulva               | 2            | 2          | 2         | 2           | 2          |
| Genital infection     | S.Pr.        | Vulva               | 2            | 2          | 2         | 2           | 2          |
| Genital infection     | L.Sh.        | Vulva               | 2            | 2          | 2         | 2           | 2          |
| Genital infection     | P.Ka.        | Vulva               | 2            | 2          | 2         | 2           | 2          |
| Genital infection     | M.Ke.        | Cervix              | 2            | 2          | 2         | 2           | 2          |
| Genital infection     | B.Ro.        | Cervix              | 2            | 2          | 2         | 1           | 2           |
| Genital infection     | D.Be.        | Penis               | 2            | 2          | 2         | 2           | 2          |
strains and the small ones being approximately the same size as HVH-1 plaques. They found that these differences in plaque morphology remained constant in a variety of different host cell lines. By the plaquing procedure described above, with final readings at day 6, reference strains of HVH-1 produced plaques no larger than 2.5 mm in diameter, whereas reference HVH-2 strains produced plaques of 2.5 to 5 or 6 mm in diameter. Typing the 23 isolates on the basis of plaque size provided a correct identification for 10 of the 13 HVH-1 isolates and 8 of the 10 HVH-2 isolates. However, typing by this method was difficult since differences in plaque size sometimes were not definitive. Furthermore, it appeared that the size of plaques produced by certain virus strains might be influenced by their passage history. For example, one preparation of the MacIntyre strain of HVH-1 produced plaques as large as those produced by HVH-2 strains, whereas another with a different passage history produced small plaques characteristic of HVH-1 strains. The possibility of contamination of the former material with HVH-2 was excluded.

Several investigators (4, 9, 11) have reported on the differential effect of incubation temperatures on HVH-1 and HVH-2 strains; type 1 strains had equally high infectivity titers when host cell cultures were incubated at 37 and at 39 to 40 C, whereas titers of HVH-2 were markedly lower at 39 to 40 C. In the present study isolates that showed a reduction in plaque count of 90% or greater at 40 C were typed as HVH-2 strains. Six of the 13 strains identified as HVH-1 by IHA inhibition, direct fluorescent antibody staining, and neutralization tests showed marked reductions in plaque counts with incubation at 40 C and, thus, were typed as HVH-2 strains (Table 4). None of the type 2 isolates were incorrectly identified as HVH-1, i.e., they all showed greatly reduced titers at 40 C.

DISCUSSION

The IHA-inhibition test for HVH would appear to have the greatest potential use for type-specific identification of antibodies in human sera. It is a relatively simple in vitro method for typing HVH antibody and yields results sooner than the various types of neutralization tests (10) generally employed for this purpose. Results of antibody typing were definitive, even in human sera containing both HVH-1 and HVH-2 antibodies. The failure of animal antisera to give clear-cut and consistent results in IHA-inhibition tests is puzzling. It is possible that Bernstein and Stewart (2) also observed this, since they used human sera for typing virus by IHA inhibition.

Typing HVH antibody by IHA inhibition should prove to be a valuable tool for epidemiological studies as well as for diagnostic purposes. In the present study, antibody typing by this method provided additional evidence that nervous system involvement with HVH-2 is not limited to neonatal infections (3, 16). The test permitted a serological diagnosis of HVH-2 infection in three cases of meningoencephalitis in young adults for whom virus isolation attempts on cerebrospinal fluids were negative. The test also demonstrated HVH-2 antibody in spinal fluid from one of the patients.

IHA inhibition would appear to be a more accurate method than calculation of II/I indexes of neutralizing or IHA antibody for determining the type of HVH antibody present in human sera. II/I indexes gave an accurate indication of the presence of HVH-2 antibody in those sera containing type 2 antibody by IHA inhibition, but they indicated the presence of HVH-2 antibody in one-half or more of the sera shown to contain only HVH-1 antibody by IHA inhibition. The possibility cannot be completely ruled out that these sera contained HVH-2 antibody that was not detectable by the inhibition technique, but the fact that some of the sera giving discrepant results were from children makes this unlikely. Another limitation of II/I indexes is that they give an indication of the presence of type 1 antibody only or of type 2 antibody, but a combination of antibody to types 1 and 2 cannot be detected.

The type of HVH antibody present in human sera, as indicated by IHA inhibition, was not reflected in the relative neutralizing or IHA titers to HVH-1 and HVH-2, except in the case of IHA titers of sera containing only type 2 antibody. This relative type-specificity of the IHA response in HVH-2 infections has also been noted in a previous report from this laboratory (1) in which the presence of type 2 antibody was assumed based upon the site (genital) of herpetic infections. Typing of antibody by IHA inhibition has confirmed the greater type-specificity of the IHA antibody response in HVH-2 infections than in HVH-1 infections.

The results of these studies confirm and extend those of Bernstein and Stewart (2) on the reliability of IHA inhibition for typing HVH virus isolates. However, typing virus by this method was far more cumbersome and time-consuming than was type-specific identification by direct fluorescent antibody staining using cross-absorbed conjugates. The IHA-inhibition
test does, however, provide the laboratory with an accurate, alternative method for type-specific identification of HVH isolates. Similarly, the microneutralization method used in these studies, based upon differences in heterotypic neutralizing capacity of HVH-1 and HVH-2 antisera, provides the laboratory lacking reagents or expertise in direct fluorescent antibody staining with an alternative procedure for typing HVH isolates. Compared to the above three techniques, HVH typing by plaque size or differential effects of incubation temperature was found to lack precision and accuracy.

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