Solid Phase Radioimmunoassay for Identification of Herpesvirus hominis Types 1 and 2 from Clinical Materials

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A solid phase radioimmunoassay (RIA) was developed for typing Herpesvirus hominis (HVH) strains isolated from clinical materials, and it also proved to be applicable to the direct detection and typing of HVH antigen in human and animal brain tissue. The procedure utilized virus-infected human fetal diploid cells or brain tissue smears in the bottom of 1-dram glass vials, antigen was detected through the use of intermediate HVH antisera produced in rabbits or hamsters and cross-absorbed with the HVH heterotype, and 125I-labeled anti-species (rabbit or hamster) globulins produced in goats were used for detection of immune complexes. The cross-absorbed HVH antisera could be used at high dilutions in the RIA test, and they reacted with marked type-specificity in the RIA system. Specificity of the test was also improved by determining and using optimal concentrations of intermediate sera and of 125I-labeled anti-species globulins. Results of typing HVH isolates by the RIA procedure agreed in all instances with those obtained by direct fluorescent antibody staining with cross-absorbed conjugates. The RIA procedure was effective and more sensitive than direct fluorescent antibody for demonstrating and typing HVH antigen directly in smears of infected human brain tissue.

Type-specific identification of Herpesvirus hominis (HVH) isolates from clinical materials is generally accomplished by direct fluorescent antibody (FA) staining (9, 10), and the FA procedure has also been applied to identification of HVH types 1 and 2 directly in smears from lesions (10). However, there are drawbacks to this method for typing HVH. It is usually necessary to cross-absorb the conjugates with cells infected with heterotypic virus to obtain clear-cut specificity of the staining reaction, and this requires large volumes of virus-infected cells. Relatively large amounts of conjugates are used, as they generally cannot be diluted beyond 1:8 or 1:10 for virus typing. Also, smears of lesion materials sometimes do not contain sufficient antigen to give positive results by FA staining (10) or nonspecific staining may be a problem.

Radioimmunoassay (RIA) procedures for detection of viral antigens and antibodies have been found to be more sensitive than conventional serological techniques such as neutralization, complement fixation, and FA staining (2-6, 11-13, 16-18). Hayashi et al. (5, 6) and Rosenthal et al. (12, 13) have shown that 125I-labeled antibodies can be used to detect HVH antigens on the surface of infected cells using either direct (6, 13) or indirect (5, 12) RIA procedures. Therefore, the possibility was considered that RIA might be a sensitive method, relatively economical of reagents, which would be applicable to the typing of HVH isolates recovered in cell cultures and possibly for the direct detection and typing of HVH in clinical specimens. This report describes the development and application of a solid phase RIA technique for type-specific identification of HVH types 1 and 2 (HVH-1 and HVH-2).

MATERIALS AND METHODS

Virus strains. The MacIntyre strain was used as a reference strain of HVH-1, and the MS strain as a reference strain of HVH-2. The field strains of HVH which were typed by RIA were isolated in this laboratory from clinical materials using methods which have been described elsewhere (14). The isolates were also typed by direct FA staining with cross-absorbed conjugates (9, 10, 15). Human brain tissues from which HVH had been isolated, and human brain tissues which had failed to yield a viral isolate, were tested by the RIA procedure; these tissues had been stored for 1 to 12 years at –70 C.

HVH antisera. Antisera to the MacIntyre strain of HVH-1 and the MS strain of HVH-2 produced in hamsters or rabbits were used as intermediate sera in the RIA procedure to detect HVH antigen in infected cells or tissues.
Sera used for typing HVH were first absorbed with human fetal diploid lung (HFDL) cells infected with the HVH heterotype. A 0.1-ml volume of undiluted antiserum was mixed with 0.9 ml of infected cells and physiological saline was added to give a total volume of 5 ml. The mixtures were incubated first at 37 C for 30 min with shaking, and then overnight at 4 C with constant stirring. After centrifugation at 80,000 × g for 1 h the supernatant fluid, representing approximately a 1:50 dilution of absorbed antiserum, was removed. For some of the studies described below 0.1-ml volumes of antisera were absorbed with approximately 1.5 ml of minced HVH-infected mouse brain tissue rather than HFDL cells. Pre-immunization sera were absorbed with uninfected HFDL cells or mouse brain. Absorbed sera were held at −70 C for long-term storage, and working supplies were stored at 4 C for as long as 1 year without any change in activity.

HVH-infected cell cultures. HVH-infected HFDL cells were used for preliminary studies on the use of RIA for detection of HVH antigen in infected cells and for evaluation of cross-absorbed conjugates. A suspension of the L-645 line of HFDL cells containing approximately 50,000 cells/ml was added to 1-dram glass vials in 1-ml volumes. The growth medium consisted of 10% fetal bovine serum and 90% Eagle minimal essential medium prepared in Earle balanced salt solution. After 2 days of incubation at 36 C the cultures contained approximately 100,000 cells and, without a medium change, they were infected with HVH-1 at a ratio of approximately 0.3 infectious particles per cell, or with HVH-2 at a ratio of approximately 0.1 infectious particles per cell. After incubation at 35 C for 20 h for HVH-1 or 15 h for HVH-2, approximately 60 to 65% of the cells were shown to be infected by FA staining. The medium was removed, the cells were rinsed with 1 ml of distilled water, and without preliminary drying they were fixed with acetone for 20 min. The fixed cultures could be stored at −70 C indefinitely.

For typing isolates, HFDL cell cultures in 1-dram vials were inoculated with 0.1 ml of infected cell culture material of unknown titer. The cultures were fixed when microscopic examination showed a 2- to 3-plus cytopathic effect.

HVH-infected brain smears. Smears of mouse or human brain tissue were prepared in the bottom of 1-dram vials by inserting a cotton swab (Clarian cotton tip applicator, Aloe) into the tissue and then making a thin smear by rotating the swab on the bottom of the vial. Five smears were made from each sample, and the similarity of counts per minute on five replicate smears of HVH-infected brain tissue indicated that uniform samples could be prepared in this manner. Smears were dried at room temperature for 2 to 3 h, and then fixed with acetone.

Solid phase RIA technique. To infected cells or brain tissue in 1-dram vials was added 0.2 ml of a 1:4,000 or 1:8,000 dilution of antiserum to HVH. The antiserum dilutions were prepared in 0.01 M phosphate-buffered saline, pH 7.2 (PBS). Incubation was conducted for 2 h at 37 C or overnight at room temperature. The contents of the vials were then aspirated and they were washed two times with 3 ml of PBS. Anti-hamster and anti-rabbit globulins produced in goats were obtained from Antibodies Inc., Davis, Calif. and labeled with 125I by the chloramine T method (7, 8); 125I in carrier free form was added at a concentration of 500 μCi/mg of protein (1). On the basis of the experiments described below, the labeled immune globulins were diluted in PBS containing 1% bovine albumin to contain 40,000 counts/min in a volume of 0.1 ml. 125I-labeled immune globulin for the appropriate species was added to the vials in a volume of 0.1 ml, and incubation was conducted at room temperature for 80 min. The contents of the vials were aspirated and they were washed two times with 3 ml of PBS. Residual radioactivity was assayed with a gamma counter (Abbott Logic series counting system, model 111-A).

All tests included controls consisting of uninfected cell cultures or mouse brain tissue and pre-immunization sera from the same animals used for preparation of HVH antiserum. Binding ratios were expressed in terms of the mean counts per minute in three infected cultures or five brain smears divided by the mean counts per minute in corresponding uninfected materials. In identifying HVH antigen in human brain tissue, binding ratios were expressed in terms of the counts per minute with immune serum divided by the counts per minute with pre-immunization serum. This was done because of the uncertainty of being able to select negative human brain tissue known to be free from HVH antigen.

RESULTS

Developmental studies. Preliminary experiments were conducted in an effort to establish the optimal concentrations of intermediate antisera to use for detection of HVH antigen, the optimal concentration of 125I-labeled antispecies gamma globulin to use for detection of immune complexes, and the optimal incubation time after addition of the 125I-labeled globulin.

Figure 1 shows the results of studies in which varying dilutions of HVH antisera produced in hamsters were tested against HFDL cells infected with the homologous virus and against uninfected cells. Immune complexes were detected with 125I-labeled anti-hamster gamma globulin diluted to contain 40,000 counts/min in a volume of 0.1 ml. With higher concentrations of intermediate serum there was inhibition of specific binding. The optimal ratios of specific binding to nonspecific binding occurred with the intermediate antisera diluted 1:4,000 to 1:8,000, and these dilutions were selected for experiments on virus typing. Similar results were obtained with a HVH-1 antiserum produced in rabbits, and it was also used as an intermediate antisera at a dilution of 1:4,000.

Figure 2 shows the effect of concentration of 125I-labeled goat anti-hamster globulins on spe-
Fig. 1. Effect of concentration of intermediate serum on binding ratios for HVH-1 (A) and HVH-2 (B).

Fig. 2. Effect of increased $^{125}$I-labeled goat anti-hamster gamma globulin on specific binding.

cific binding. With concentrations up to $4 \times 10^4$ counts/min binding ratios increased sharply, but then reached a plateau. With higher concentrations of $^{125}$I-labeled globulins, both specific and nonspecific binding increased. A concentration of $^{125}$I-labeled globulins containing 40,000 counts/min was selected for use in the test system.

In Fig. 3 it is seen that binding ratios increased with increased incubation time after addition of the $^{125}$I-labeled globulin up to 80 min, and then ratios remained fairly constant. An incubation time of 80 min was adopted.

Another factor which influenced the reliability of the RIA procedure described herein pertained to fixing the HVH-infected HFDL cells. It was found that loss of infected cells from the glass vials could be minimized by washing the monolayers with distilled water and then fixing with acetone without preliminary drying. Washing with PBS or physiological saline resulted in the formation of a precipitate when acetone was added to the cells.

Reactivity of cross-absorbed HVH antisera in the RIA system. Some preliminary experiments were conducted to determine whether absorption of intermediate antisera with uninfected HFDL cells might influence the specificity of RIA results. In Table 1 it is seen that HVH-1 antiserum produced in rabbits and HVH-2 antiserum produced in hamsters, and also pre-immunization sera, showed much less reactivity with uninfected cells after absorption with uninfected HFDL cells, and this resulted in markedly higher specific binding ratios of the HVH antisera for infected cells. On the basis of these findings, pre-immunization sera were ab-
sorbed with uninfected cells or tissue when they were used for establishing binding ratios by comparison with antisera cross-absorbed with infected HFDL cells or brain tissue.

Table 2 compares the reactivity of HVH antisera produced in hamsters after absorption with uninfected HFDL cells and after absorption with HFDL cells infected with the viral heterotype. Antisera absorbed with heterotypic virus-infected cells reacted with marked typespecificity in the RIA system; heterotypic binding ratios of the cross-absorbed antisera were almost as low as those of the pre-immunization sera, and homotypic binding ratios were reduced only slightly.

Table 3 shows that type-specific reactivity could also be obtained by absorption of HVH antisera with mouse brain infected with the HVH heterotype. This finding was considered to be important inasmuch as it is generally easier to produce large amounts of infected brain tissue than large amounts of infected HFDL cells for serum absorption.

Results shown in Table 4 further illustrate the specificity of the reactivity of cross-absorbed HVH antisera in the RIA system. The sera not only reacted type-specifically with HVH-1 and HVH-2 infected cells, but they also failed to react with cells infected with other human herpesviruses, viz. varicella-zoster virus and cytomegalovirus (CMV), and with rubella virus.

**Typing HVH isolates by RIA.** Cross-absorbed HVH antisera were used in experiments to evaluate the RIA system for typing HVH isolates from clinical specimens. The HVH-1 antiserum was produced in rabbits, and the HVH-2 antiserum in hamsters. Isolates were propagated in HFDL cells grown in 1-dram vials as described above under Materials and

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**FIG. 3.** Effect of incubation time after addition of 125I-labeled anti-rabbit gamma globulin on RIA binding ratios for HVH-1.

**TABLE 1. Reactivity in the RIA system of unabsorbed HVH antisera and of antisera absorbed with uninfected HFDL cells**

| Tissue tested       | Counts/min with intermediate serum* |
|---------------------|------------------------------------|
|                     | Unabsorbed | Absorbed with uninfected HFDL | Unabsorbed | Absorbed with uninfected HFDL |
|                     | Pre-immunized rabbit | HVH-1 immune rabbit | Pre-immunized rabbit | HVH-1 immune rabbit | Pre-immunized hamster | HVH-2 immune hamster | Pre-immunized hamster | HVH-2 immune hamster |
| Uninfected HFDL     | 894        | 1103 | 230 | 246 | 988 | 1265 | 301 | 322 |
| HVH-1-infected HFDL | 1084       | 6178 | 211 | 4832 | 1041 | 4498 | 310 | 3719 |
| HVH-2-infected HFDL | 938        | 4169 | 231 | 3163 | 1008 | 6819 | 226 | 5753 |

Binding ratios:
- **HVH-1:** 1.2 (1.0), 5.6 (3.7), <1.0 (1.0), 19.6 (12.8), 1.0 (1.0), 3.5 (5.3), 1.0 (1.0), 11.5 (17.8)
- **HVH-2:**

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* 125I-labeled goat anti-rabbit gamma globulin used with HVH-1 antiserum; labeled goat anti-hamster gamma globulin used with HVH-2 antiserum.
* Absorbed with approximately 9 volumes of uninfected human fetal diploid cells.
* Counts per minute of infected HFDL cells/cells per minute of uninfected HFDL cells.

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Table 2. Reactivity of cross-absorbed HVH antisera in RIA system

| Tissue tested | Pre-primunized hamster* | HVH-1 immune hamster | HVH-2 immune hamster |
|---------------|-------------------------|-----------------------|-----------------------|
|               | Absorbed uninfected HFDL| Absorbed HVH-2 infected HFDL | Absorbed uninfected HFDL | Absorbed HVH-1 infected HFDL |
| Uninfected HFDL | 261 | 246 | 302 | 301 | 322 | 288 |
| HVH-1-infected HFDL | 280 | 5530 | 4495 | 310 | 3719 | 449 |
| HVH-2-infected HFDL | 226 | 3897 | 353 | 226 | 5753 | 4215 |

Binding ratios:
- HVH-1: 1.0, 22.4, 14.8, 1.0, 11.5, 1.5
- HVH-2: <1.0, 15.8, 1.1, <1.0, 17.8, 14.6

* 125I-labeled goat anti-hamster gamma globulin used to detect immune complexes.
* Counts per minute of infected HFDL cells/counts per minute of uninfected HFDL cells.

Table 3. RIA on H. hominis immune hamster sera cross-absorbed with infected HFDL cells and with infected mouse brain

| Tissue tested | Counts/min with intermediate serum* |
|---------------|-------------------------------------|
|               | HVH-1 immune hamster serum absorbed with HVH-2 infected HFDL | HVH-2 immune hamster serum absorbed with HVH-1 infected HFDL |
| HFDL | Mouse brain | HFDL | Mouse brain |
| Uninfected HFDL | 351 | 150 | 288 | 163 |
| HVH-1-infected HFDL | 4079 | 4444 | 449 | 312 |
| HVH-2-infected HFDL | 301 | 1396 | 4215 | 2662 |

Binding ratios:
- HVH-1: 11.6, 29.6, 1.5, 1.9
- HVH-2: <1.0, 9.3, 14.8, 16.2

* 125I-labeled goat anti-hamster gamma globulin used to detect immune complexes.
* Counts per minute of infected HFDL cells/counts per minute of uninfected HFDL cells.

Methods. Table 5 shows the results of RIA typing on 16 isolates which were also identified by direct FA staining with cross-absorbed conjugates (9, 10). The isolates were examined in the RIA system without knowledge of the FA results. The virus type identified by RIA agreed with that obtained by FA staining in all instances. Further, typing by RIA gave unequivocal results. The type 1 isolates had binding ratios ranging from 5.1 to 11.2 with the type 1 antiserum, and only 1.1 to 1.8 with the type 2 antiserum. Type 2 isolates had binding ratios of 5.5 to 11.1 with the type 2 antiserum, and ratios of only 1.0 to 1.4 with type 1 antiserum.

Typing of HVH antigen in human brain tissue by RIA. The feasibility was explored of using the RIA system for detecting and typing HVH antigen directly in human brain tissue. Cross-absorbed HVH antisera produced in hamsters were used for experiments. Tests were conducted on human brain specimens from which HVH had been isolated and, for control purposes, on brain specimens which had failed

Table 4. Specificity of RIA for H. hominis

| Tissue tested | Intermediate serum* |
|---------------|---------------------|
|               | HVH-1 immune rabbit serum absorbed with HVH-2 infected HFDL cells | HVH-2 immune hamster serum absorbed with HVH-1 infected HFDL cells |
| HFDL | Counts/ min | Binding ratios* | Counts/ min | Binding ratios* |
| Uninfected HFDL | 303 | 12.5 | 288 |
| HVH-1-infected HFDL | 3816 | 449 | 1.5 |
| HVH-2-infected HFDL | 411 | 1.3 | 4215 | 14.6 |
| Varicella-zoster-infected HFDL | 368 | 1.2 | 305 | 1.0 |
| CMV-infected HFDL | 366 | 1.2 | 395 | 1.3 |
| Rubella-infected HFDL | 396 | 1.3 | 295 | 1.0 |

* 125I-labeled goat anti-rabbit gamma globulin used with HVH-1 antiserum; labeled goat anti-hamster gamma globulin used with HVH-2 antiserum.
* Counts per minute of infected HFDL cells/counts per minute of uninfected HFDL cells.
to yield a viral isolate. These specimens had been stored for 1 to 12 years at −70°C. At the time RIA tests were performed, the tissues were also examined for HVH antigen by direct FA staining.

RIA results are shown in Table 6. None of the nine brain specimens which had failed to yield a viral isolate gave a positive RIA reaction with the HVH antisera; counts per minute were similar with the immune and pre-immunization sera. Of 16 specimens from which HVH-1 had been isolated initially, 14 gave clear-cut positive RIA reactions with the HVH-1 antiserum, but not with the HVH-2 antiserum. The two specimens which failed to give a positive RIA reaction had been stored for 9 and 10 years. A specimen (cerebral tissue) from which HVH-2 had been isolated and which had been stored for only 1 year failed to give a positive RIA reaction. However, spinal cord, temporal lobe, and medulla from the same patient failed to yield HVH on initial testing, and it is possible that virus was present only in low concentration and was inactivated by storage, or that sampling failed to select tissue containing antigen.

Only eight of the fourteen specimens positive by RIA gave a positive FA staining reaction for NVH, and none of the three specimens from which HVH had been isolated initially but which gave a negative RIA reaction were positive for HVH antigen by FA staining.

**DISCUSSION**

These studies have confirmed the sensitivity of RIA for specific detection of HVH antigen in infected host cells, and they have illustrated the practical application of RIA procedures for HVH in diagnostic virology.

Although homotypic binding ratios of unabsorbed HVH antisera, or antiserum absorbed with HVH-2 antigen, were slightly higher than those obtained with HVH-1 antiserum, it is likely that this finding may be a result of the low concentration of HVH antigen in the specimens tested. It is also possible that HVH antigen may be associated with other tissue components, such as human globulin or antibodies, which could influence the results of the RIA. Further studies are needed to clarify these findings.

### Table 5. Typing *H. hominis* isolates by RIA

| HVH type by direct FA staining with cross-absorbed conjugates | Virus isolated from: | No. | Counts/min | Binding ratios | Counts/min | Binding ratios |
|-------------------------------------------------------------|----------------------|-----|------------|---------------|------------|---------------|
| 1                                                           | Brain                | 6   | 1501–3351†| 5.1–11.4†    | 495–790    | 1.1–1.8       | 1            |
|                                                            | Eyelid               | 1   | 2893      | 9.8          | 655       | 1.5          | 1            |
|                                                            | Genital lesion       | 1   | 2027      | 6.9          | 532       | 1.2          | 1            |
|                                                            | Liver (neonate)      | 1   | 413       | 1.4          | 3554      | 8.3          | 2            |
|                                                            | Genital lesions      | 7   | 307–438   | 1.0–1.4      | 2293—4753 | 5.5—11.1     | 2            |
| MacIntyre (Reference HVH-1)                                |                      |     | 4031      | 13.3         | 606       | 1.4          | 1            |
| MS (Reference HVH-2)                                       |                      |     | 390       | 1.3          | 4889      | 11.4         | 2            |

† 125I-labeled goat anti-rabbit gamma globulin used with HVH-1 antiserum; labeled goat anti-hamster gamma globulin used with HVH-2 antiserum.

### Table 6. RIA for typing *H. hominis* antigen in human and animal brain tissue

| Specimens examined | Virus isolated | No. | Counts/min with intermediate HVH immune hamster sera | Binding ratios |
|--------------------|----------------|-----|-----------------------------------------------------|---------------|
|                    |                |     | Preimmunized | HVH-1 | HVH-2 | HVH-1 | HVH-2 |
| Human brain        | None           | 9   | 231–348†    | 226–417 | 260–316 | <1.0–1.2 | <1.0–1.2 |
| Human brain        | HVH-1          | 14  | 202–375     | 884–2084 | 233–529 | 2.9–6.4  | <1.0–1.7 |
| Human brain        | HVH-2          | 2   | 299–325     | 296–386 | 238–382 | <1.0–1.1 | <1.0–1.1 |
|                   |                | 1   |            |          |            |          |          |
| Uninfected mouse brain | None   | 370  | 365        | 350     | <1.0      | <1.0      |
| HVH-1 infected mouse brain | HVH-1 | 339  | 3957       | 497    | 11.6       | 1.4       |
| HVH-2 infected mouse brain | HVH-2 | 358  | 457        | 3179   | 1.2        | 8.2       |

† Absorbed with uninfected HFDL cells or HFDL cells infected with HVH heterotype.

* Counts per minute with immune serum/counts per minute with pre-immunization serum.

* Range.
uninfected HFDL cells, were somewhat higher than heterotypic binding ratios, cross-absorption of the sera with cells infected with the HVH heterotype imparted marked type-specificity to the RIA system, and permitted unequivocal typing of HVH-1 and HVH-2. The use of cross-absorbed antisera and pre-immunization serum absorbed with uninfected cells to increase type-specific binding ratios is considered to be a major factor in the success of typing isolates or clinical materials containing low levels of antigen.

Since the intermediate HVH antisera can be used at high dilutions in the RIA system, much less virus-infected cells or tissue is required for cross-absorption than for cross-absorption of conjugates used in direct FA staining for typing HVH. Problems associated with nonspecific fluorescence and autofluorescence, which may limit the reliability of FA staining, are avoided by the use of the RIA system. Further, RIA results based upon counting isotope emissions are more quantitative and less subjective than those based upon microscopic reading of degrees of immunofluorescence.

The solid phase RIA procedure described herein would also be adaptable to use in microtiter plates or with cells or smears on coverslips. However, 1-dram vials were used for these preliminary and developmental studies with a view toward greater safety in handling potentially hazardous viruses and radioisotopes.

The RIA method would appear to offer considerable promise as a rapid in vitro procedure for detection and typing of HVH antigen directly in clinical materials. It was markedly more sensitive than FA staining for detection of antigen in HVH-infected human brain tissues which had been stored for prolonged periods of time. It is possible that RIA tests on fresh human brain tissue would approach the sensitivity of virus isolation as a diagnostic procedure. None of the brain tissues which failed to yield a viral isolate gave false-positive reactions in the RIA system. Further studies are anticipated on the ability of RIA to detect and type HVH antigen directly in vesicular lesion materials or in blood leukocytes.

The use of RIA for virus identification need not be restricted to larger laboratories, since equipment such as that used in the present study is, in fact, less expensive than that required for fluorescence microscopy. Investment in RIA equipment would also appear to be warranted on the basis of the fact that it can be expected to become applicable to an increasing number of microbiological procedures in the near future.

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