Rapid Typing of Herpes Simplex Virus Isolates by Deoxyribonucleic Acid:Deoxyribonucleic Acid Hybridization

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A method for typing clinical isolates of herpes simplex virus was developed. It utilizes hybridization between unlabeled deoxyribonucleic acid from infected cultures and tritium-labeled virus deoxyribonucleic acid, and it can be completed within a day using a single roller-tube culture of the clinical isolate. The data obtained are inherently quantitative, and the method yields unequivocal identification and typing. Thirty-nine coded clinical isolates were all correctly typed by this method.

A number of different methods have been employed to distinguish between herpes simplex virus (HSV) type 1 (HSV-1) and HSV-2. Some of these have been based upon differences in the biological properties of HSV-1 and HSV-2, such as inhibitor sensitivity (5, 16), pock size on the chick chorioallantoic membrane (31), ability to form plaques in chicken embryo cell cultures (8, 39), temperature sensitivity of replication (18), inactivation by silver nitrate (6), thermal stability of virus-induced thymidine kinase (32), and plaque morphology in HeLa cells (26). In addition, definitive methods based on restriction enzyme analysis of radiolabeled virus deoxyribonucleic acid (DNA) have recently been published (4, 19). Methods based on differences in biological properties require the additional passage of an isolate through one or more cell culture systems and do not distinguish unequivocally between the two virus types.

Other typing methods have utilized such serological techniques as indirect hemagglutination inhibition (2), microneutralization (34, 37), mixed agglutination (13), immunofluorescence (10, 23, 27, 28), solid-phase radioimmunoassay (9), immunoelectrophoresis (14), indirect immunoperoxidase staining (1), enzyme-linked immunosorbent assay (25), and complement-dependent cytotoxicity (33). The widely used immunofluorescence techniques are difficult to quantitate and are subject to observer bias, and all of the serological assays are complicated by the heterologous activity of "type-specific" antisera, a problem which is not unexpected in light of the many antigens shared by HSV-1 and HSV-2 (10, 29, 30, 35). The problem of antigenic cross-reactivity has been approached by using

cross-absorbed antisera (10, 15, 24, 29), but its ultimate solution will come with the wide availability of type-specific antisera, either conventional antisera elicited with purified type-specific antigens or type-specific monoclonal antibodies synthesized by hybridomas.

We report here the development of an alternative HSV typing procedure which differentiates HSV-1 and HSV-2 on the basis of type-specific differences in nucleic acid homology. This approach is practical because: (i) there is only approximately 40% homology between HSV-1 and HSV-2 DNA (3, 17, 22) and perhaps as little as 20% true homology (38); (ii) intratypic variations, though frequent, are limited in their effect on DNA sequence homology (4, 11, 38); and (iii) HSV shows less than 5% homology with varicella-zoster virus (20, 21), Epstein-Barr virus (12), or cytomegalovirus (12). Our procedure is based upon standard methods for DNA:DNA hybridization in solution (7) and utilizes hydroxyapatite chromatography to analyze the reaction products. It has, however, been simplified and adapted to the conditions of the diagnostic laboratory. The test is performed with a single infected roller-tube culture and can yield unequivocal identification and typing within a day.

MATERIALS AND METHODS

Cells. A strain of diploid human fibroblasts (350Q) initiated by M. Myers from newborn foreskin at the virus research unit, Children's Hospital, Boston, Mass., was used between passages 16 and 25. These cells were grown as monolayer cultures in an atmosphere of 5% CO₂ in air in Dulbecco-modified Eagle medium supplemented with 100 U of penicillin per ml and 100 µg of streptomycin per ml (DMEM), plus 10% fetal calf serum. Stationary cultures in glass roller tubes (150 by 14 mm) were seeded with 1×10^5 to 2×10^5 cells in 1.5 ml of DMEM-10% fetal calf serum. Costar flasks (150 cm²) were seeded with 5×10^6 cells

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in 50 ml of DMEM-10% fetal calf serum.

Virus. HSV-1, strain JH (from David T. Kingsbury), was used to prepare the HSV-1 [^3H]DNA probe. The McIntyre VR-3 strain (VR-539, American Type Culture Collection, Rockville, Md.) was used as prototype HSV-1 for the preparation of infected cell cultures. The MS strain (ATCC VR-540) was used for the preparation of the HSV-2 [^3H]DNA probe and as prototype HSV-2 for the preparation of infected cell cultures. Large pools of each virus were prepared in 350Q cells inoculated at a multiplicity of infection of 0.01 plaque-forming unit per cell and stored in aliquots at -90°C .

Preparation of ^3H -labeled HSV-1 and HSV-2 DNA probes. Near-confluent 350Q cell monolayer cultures in 150-cm 2 Costar flasks were washed with DMEM and infected at 36°C with a multiplicity of infection of 20 plaque-forming units per cell. The inoculum was removed after 1 h and replaced with 12 ml of DMEM containing 100 μCi of [^3H]thymidine (New England Nuclear Corp.; NET 027Z; specific activity, 60 to 80 Ci/mmol) per ml. After incubation at 36°C for 36 h, the contents of the flask were brought to 8 M urea by the addition of dry urea, to 0.24 M phosphate by the addition of 4.8 M phosphate buffer (PB; pH 6.8, containing 2.4 M monosodium phosphate and 2.4 M disodium phosphate), and to 1% sodium dodecyl sulfate (SDS) by the addition of 20% SDS. Flasks were held for 5 min at 36°C with this solution covering the monolayer to permit cell lysis to occur, and the contents were then placed in 50-ml plastic screw-capped centrifuge tubes. Flasks were washed with 7 ml of 8 M urea-0.24 M PB, pH 6.8 (buffered 8 M urea) + 1% SDS, which was added to the cell lysate, and the resulting solution was sonicated in a model W185 Sonicator Cell Disruptor (Heat Systems Ultrasonics, Plainview, N.Y.) at 130 W for 5 min at 10°C . The DNA was isolated by hydroxyapatite (HA) column chromatography in buffered 8 M urea (38), and the HSV DNA was separated from the cellular DNA by two cycles of equilibrium density gradient centrifugation in CsCl ($\rho = 1.73$, Beckman type 65 rotor, 45,000 rpm, 48 h, 10°C). The HSV DNA bands from the second cycle, $\rho = 1.728$, were pooled (total volume approximately 2 ml), dialyzed twice against 2 liters of 10^{-3} M ethylenediaminetetraacetic acid in water at 4°C for 4 h, and sonicated for 2 min in a siliconized glass 1-dram (ca. 4-ml) vial immersed in ice water using the microprobe of the model W185 sonicator at 80 W. The [^3H]DNA was then diluted to 40 ml in 10^{-3} M ethylenediaminetetraacetic acid, dispensed in siliconized 1-dram vials, and stored at -90°C . [^3H]DNA probe concentrations were determined by the rate of self-annealing (36). The specific activity of the HSV-1 DNA probe was 1.3×10^7 dpm/ μg , and that of the HSV-2 DNA probe was 1.0×10^7 dpm/ μg . Both probes were found to reanneal to $>95\%$. The probe obtained by the infection and labeling of one 150-cm 2 flask was sufficient for approximately 10^4 reactions. These probes have a functional half-life of approximately 6 months.

Isolation of DNA from roller-tube cultures (Fig. 1A). Roller-tube (150 by 14 mm) cultures of 350Q cells (used for routine virus isolation in the

diagnostic laboratory) contain approximately 5×10^5 cells at confluence, together with 1.5 ml of DMEM plus 2% fetal calf serum. Infected cultures were generally used when $>90\%$ of the cells exhibited cytopathic effects. Six milliliters of 0.24 M PB (pH 6.8), containing 10 M urea plus 1.25% SDS, was added to each uninfected or virus-infected culture to yield a final concentration of 8 M urea and 1% SDS. The tubes were recapped, inverted to insure that all virus present was exposed to 8 M urea (which rapidly destroys its infectivity), and allowed to stand upright for 5 min at room temperature. The contents were further mixed by placing each tube in a Vortex mixer for 30 s and then were sonicated in the cuphorn of the model W185 sonicator at 130 W for 3 min at 10°C . At this point the cell lysate could be stored at -20°C for at least 2 weeks without affecting the subsequent yield of hybridizable DNA (alternatively, the infected culture could be frozen before addition of buffered 10 M urea and SDS).

The DNA was then isolated at room temperature. Dry HA (150 mg) was placed in a glass column (1 by 20 cm) with the outlet closed, and 7.5 ml of cell lysate was added. The HA was stirred into the lysate and allowed to settle by gravity. The sample was then allowed to run through at a flow rate of 1 ml/min, and the column was washed with 7 ml of buffered 8 M urea and drained. Seven milliliters of 0.12 M PB (pH 6.8) was added, the HA was stirred into solution and allowed to settle by gravity, and the buffer was run through at approximately 5 ml/min. The column was washed twice more with 7 ml of 0.12 M PB, leaving the meniscus just at the top of the packed column of HA, and the DNA was then eluted with 0.48 M PB (pH 6.8) at a flow rate of approximately 1 ml/min. The first 350 μl of eluant (the volume in the HA bed plus the column outlet) was discarded, and the next 600 μl , which contained most ($>85\%$) of the DNA that had been bound to the HA, was collected. The resulting phosphate concentration was 0.29 M as measured by refractometry. After collection, 1.2 μl of 0.5 M ethylenediaminetetraacetic acid in water was added to the 600 μl of DNA solution, which was then stored at -35°C . The DNA is stable indefinitely at this temperature. Since slightly less than one-half of the recovered DNA is required to perform the typing reactions, there is sufficient DNA to repeat the typing reaction if desired.

The DNA isolation procedure, which requires approximately 45 min, was performed on two roller-tube cultures at a time, using paired columns. Solutions were run through the column under slight pressure to achieve the indicated flow rates. The pressure was applied at the top of the column with a 10-ml syringe via a needle inserted through a rubber stopper.

DNA:DNA hybridization (Fig. 1B). Reactions were performed in 0.48 M PB in 0.3-ml Pierce Reactivials (Pierce Chemical Co., Rockford, Ill.) maintained at 70°C in a Pierce Reactitherm heating module. Each typing reaction contained 139 μl of DNA solution (isolated from an infected or uninfected roller-tube culture), 4 μl of [^3H]DNA probe (approximately 750 cpm/ μl), and 7 μl of 4.8 M PB (an amount chosen to achieve a final phosphate concentration of 0.48 M in

[A] Extraction of DNA
from infected cultures

Infected roller-tube culture
↓
Add buffered 10 M urea + SDS;
blend and sonicate

Load onto HA column

Wash with buffered 8 M urea
and then with 0.12 M PB

Elute DNA in 600 μ l
with 0.48 M PB; add EDTA

Freeze $\frac{1}{2}$ eluted
DNA for repeat
if desired

[B] Hybridization of DNA
with HSV-1 and HSV-2
[3 H]DNA probes

Set up parallel hybridization
reactions with 3 H-labeled
HSV-1 and HSV-2 DNA probes

Denature at 115°C,
then quickly cool

Incubate at 70°C

Zero-time
sample

5-h sample

24-h sample
(if desired)

Immediately dilute sample 1:50
in 0.14 M PB at 60°C

[C] Analysis of products of
hybridization reactions

Load samples onto HA column at 60°C
and wash with 0.14 M PB at 60°C

Elute ds DNA with
0.3 M PB at 60°C

Collect ss DNA in
void volume and wash

FIG. 1. Rapid typing of HSV isolates by DNA:DNA hybridization. EDTA, Ethylenediaminetetraacetic acid. ds DNA, ss DNA, Double- and single-stranded DNA, respectively.

the reaction mixture). Samples of each DNA were run in parallel with HSV-1 and HSV-2 [3 H]DNA probes, and each test included DNA isolated from uninfected cultures and from cultures infected with prototype HSV-1 and HSV-2. All reaction mixtures were set up, thoroughly mixed, overlaid with mineral oil to prevent evaporation, and tightly capped. The DNA was then thermally denatured by placing the vials in a second Reactitherm heating module maintained at 115°C. After 10 min at 115°C, the vials were quickly cooled in ice water, and a 50- μ l zero-time sample was obtained from each. The vials were then placed in the 70°C heating module, and 50- μ l samples were taken after 5 h and sometimes, in addition, after 24 h. Each 50- μ l sample was pipetted into 2.5 ml of 0.14 M PB (pH 6.8) containing 0.4% SDS in a disposable glass tube, (12 by 75 mm) maintained at 60°C in a water bath. These samples, each of which contained approximately 1,000

cpm, were analyzed by HA chromatography immediately thereafter. However, they could be allowed to remain at 60°C for several hours, since their rate of reannealing under these conditions was only approximately 1/250 that of the original reaction. For example, when such diluted samples were held for 3 h at 60°C, further reannealing was negligible ($r = 0.004$). Alternatively, at this point the diluted samples could be frozen at -90°C for later analysis.

The fraction of [3 H]DNA remaining single stranded was determined by HA chromatography, using a water-jacketed analytical column (1 by 15 cm) maintained at 60°C (Fig. 1C). A pair of samples could be assayed in 15 min using two such columns. All buffers were at pH 6.8 and maintained at 60°C. A 100-mg sample of DNA-grade HA (Bio-Rad Laboratories; catalog 130-0520) was placed in the column, and 4 ml of 0.14 M PB was added. The HA was stirred into solu-

tion, allowed to settle by gravity, and compacted as the buffer ran out under slight pressure at a flow rate of 8 ml/min. When the meniscus reached the top of the HA column, 2.5 ml of buffer containing the DNA sample was added, and the eluate was collected at a flow rate of 4 ml/min in a 20-ml scintillation vial. The column was then washed with 4.5 ml of 0.14 M PB, and the eluate was collected at 4 ml/min in the same vial (single-stranded DNA does not bind to HA at this temperature and ionic strength, and is thus eluted into this vial). The column was washed with an additional 3.5 ml of the same buffer, which was discarded. The double-stranded DNA was then eluted with 3.0 ml of 0.30 M PB at a flow rate of 4 ml/min. The column was run dry, and the eluate (3.0 ml of added 0.30 M PB plus 1 ml of 0.14 M PB wash retained in the HA and column deadspace) was collected in a second scintillation vial. The volume in each scintillation vial was brought to 8 ml by the addition of water, and then 10 ml of Scintiverse (Fisher Scientific Co.) was added and mixed into the aqueous phase by vigorous shaking. The vials were cooled to room temperature and counted for sufficient time to accumulate 10^4 counts per vial.

Expression of data in terms of reactivity (r) values. The hybridization of single-stranded DNA depends upon a bimolecular collision between two complementary strands, and thus can be expressed kinetically by the second-order equation: $-dc/dt = kc^2$, where t = time, c = concentration of single-stranded DNA, and k is a rate constant. This equation has the solution $c = c_0/(1 + kc_0t)$, where c_0 is the initial concentration of single-stranded DNA at time zero. Since the fraction of DNA remaining single stranded at time t is equal to c/c_0 , the fraction of DNA which has become double stranded at this time is $1 - c/c_0$, which equals $(c_0 - c)/c_0$. Consequently, the ratio (r) of double- to single-stranded DNA at time $t = (c_0 - c)/c$. If $c_0/(1 + kc_0t)$ is substituted for c , the equation becomes $r = kc_0t$; r is thus a linear function of the initial DNA concentration and of time. This parameter, r , called "reactivity," is chosen for the expression of data because of the ease with which it is determined experimentally (it is simply the ratio of the net counts per minute eluted at high and at low PB concentrations) and because when samples of an unknown DNA are hybridized for the same period of time with different DNA probes, the values of r obtained are measures of the degree of homology between that DNA and the probes.

The parameters r_1 and r_2 are defined at a specified time as the values of r obtained in reactions performed, respectively, with the HSV-1 and HSV-2 probes, minus the appropriate negative control value (which is obtained by performing an identical reaction with that probe and the DNA from an uninfected culture). Thus r_1 (or r_2) is called the "reactivity with the HSV type 1 [or 2] probe." In all experiments the negative control value with each probe was less than 0.02 at 5 h. Consequently, the correction was negligible. However, this correction would become more important with probes of lower specific activity.

Because the rate of these reactions is rapid initially, but falls off according to second-order kinetics, it be-

comes extremely slow as the reaction nears completion. Thus, little was gained by permitting reactions to run for times leading to r values greater than 1.00, the point at which the hybridization reaction was 50% complete. Allowing the reaction to proceed for twice as long ($r = 2.00$) only led to a percent completion of 67%, and even when the reaction was allowed to proceed for four times as long it was only 80% complete. On the other hand, at times less than that required to achieve an r value of 1.00, the reaction was more complete than one might expect. Thus if the reaction was only run for one-half the time required to reach $r = 1.00$, such that $r = 0.5$, the reaction was still 33% complete. Since the negative control value of r at 5 h with the present probes is ≤ 0.02 , a percent completion at this time of only 17% ($r = 0.20$) represents a signal-to-noise (background) ratio of 10:1. Thus even at such a lower percentage of completion, the reaction may yield data permitting the identification and typing of a clinical isolate.

Definition of the reactivity ratio (R). The reactivity ratio, R , of a DNA sample is defined as the ratio of the reactivity (r) of a specimen with the HSV-1 probe (r_1) to that with the HSV-2 probe (r_2), i.e., $R = r_1/r_2$. Accordingly, R should be greater than 1 for DNA isolated from cultures infected with HSV-1 and less than 1 for DNA from cultures infected with HSV-2.

RESULTS

Roller-tube cultures as a source of DNA for HSV typing. DNA was isolated from confluent roller-tube cultures of 350Q cells infected with prototype HSV-1 or HSV-2 and from sham-infected cultures. The average concentration of DNA ($\pm$ standard deviation) in the 600- μ l eluates from 10 sham-infected cultures was 5.7 ± 0.7 μ g/ml, and the average cell count was $5.6 \pm 0.4 \times 10^5$ cells per tube. The average concentration of DNA in the 600- μ l eluates from 14 HSV-infected tube cultures was 8.1 ± 0.85 μ g/ml. There was no significant difference between the concentration of DNA isolated from HSV-1- and HSV-2-infected cultures, but the infected cultures yielded significantly more DNA than did the sham-infected cultures.

Binding and recovery of DNA upon column isolation. To determine the efficiency with which HSV DNA is isolated from roller-tube cultures by HA column chromatography, a small amount of 3 H-labeled HSV-1 probe was added to HSV-1-infected roller-tube cultures showing 4+ cytopathic effect, and the DNA in these cultures was isolated. It was found that 90% of the labeled HSV DNA (and, presumably, of the unlabeled HSV DNA as well) bound to the HA, while 10% failed to bind and passed through during the loading step. The fraction failing to bind upon loading increased rapidly when the lysate from more than one culture was passed through a single column. Loading the lysate from two or three cultures led to recovery

of only 1.5 times and only 1.8 times the DNA recovered from a single culture, respectively. This is apparently due to interference with the binding of DNA by the cell debris, ribonucleic acid, and protein present in the lysate, because the HA was in excess and should have been capable of binding the total DNA present in at least five infected cultures.

Although the proportion of applied DNA retained in the column varied with the number of cultures loaded, the proportion of the retained DNA that was recovered in the first 600 μ l of eluate remained constant at >85%.

Sensitivity of the DNA:DNA hybridization reaction assessed with purified HSV DNA. Unlabeled HSV-1 and HSV-2 DNA, purified and sheared into small fragments by microprobe sonication as described for the preparation of ^3H -labeled HSV DNA probes, was hybridized with HSV-1 and HSV-2 [^3H]DNA probes in the standard DNA:DNA hybridization reaction, and the reaction products were analyzed by HA chromatography. The concentrations of unlabeled HSV DNA employed, 0.5, 0.1, and 0.02 $\mu\text{g}/\text{ml}$, were chosen to approximate conservatively the concentrations of HSV DNA that might be obtained in the 600- μ l eluate if the DNA isolation procedure used for infected roller-tube cultures was applied, respectively, to 1, 1/5, and 1/25 of an infected culture (assuming that one infected culture yields approximately 5 μg of DNA per ml 10% of which is HSV DNA). As shown in Table 1, with 0.5 μg of unlabeled HSV DNA per ml, more than 30% of the homologous [^3H]DNA probe was in the form of double-stranded DNA after 5 h of hybridization.

With 0.1 μg of unlabeled HSV DNA per ml, 8% of the homologous probe was in double-stranded DNA, whereas with 0.02 $\mu\text{g}/\text{ml}$ the proportion of probe hybridized at 5 h was not significantly above background (Table 1). These results suggested that the hybridization technique employed could detect as little as 1/5 of the HSV DNA present in a single infected roller-tube culture with a 5-h period of hybridization. Furthermore, the relative rates of hybridization of the ^3H -labeled HSV-1 and HSV-2 DNA probes clearly differentiated HSV-1 from HSV-2 DNA. The reactivity ratio ($R = r_1/r_2$) was >3 with unlabeled HSV-1 DNA and ≤ 0.34 with unlabeled HSV-2 DNA.

Sensitivity of the DNA:DNA hybridization reaction assessed with DNA isolated from HSV-infected roller-tube cultures. Hybridization reactions were next performed with the DNA eluates obtained from single, prototype HSV-infected cultures, as well as with 1:5 and 1:25 dilutions of those eluates in eluates of DNA from sham-infected cultures. The results of these experiments indicated that HSV DNA detection and typing could be accomplished with as little as 1/5 of the DNA from a single infected roller-tube culture in hybridization reactions carried out for 5 h, and with as little as 1/25 of the DNA from a single infected culture if the hybridization reaction was carried out for 24 h (Table 1). The relative rates of hybridization of the HSV-1 and HSV-2 probes clearly distinguished between DNA from HSV-1- and HSV-2-infected cultures: the HSV-1:HSV-2 reactivity ratio (R) was >2.5 for DNA from HSV-1-infected cultures and <0.5 for DNA from HSV-2-

TABLE 1. Sensitivity of typing reactions with purified prototype HSV DNA and with DNA isolated from roller-tube cultures infected with prototype HSV

Virus	Type of DNA	Concn or dilution ^a of DNA used in reaction	Duration of incubation ^b					
			5 h			24 h		
			r_1	r_2	R	r_1	r_2	R
HSV-1	Purified viral	0.50 $\mu\text{g}/\text{ml}$	0.46	0.11	4.2	1.56	0.45	3.5
		0.10 $\mu\text{g}/\text{ml}$	0.09	0	—	0.43	0.13	3.3
		0.02 $\mu\text{g}/\text{ml}$	0.02	-0.02	—	0.13	0.02	6.5
HSV-2	Purified viral	0.50 $\mu\text{g}/\text{ml}$	0.14	0.43	0.33	0.42	1.67	0.25
		0.10 $\mu\text{g}/\text{ml}$	0.02	0.08	0.25	0.13	0.38	0.34
		0.02 $\mu\text{g}/\text{ml}$	0.01	-0.02	—	0.05	0.06	—
HSV-1	Infected-cell	Undiluted	1.51	0.38	4.1	4.20	0.75	5.7
		1:5	0.30	0.12	2.5	1.01	0.32	3.2
		1:25	0.10	0.02	4.4	0.40	0.10	3.6
HSV-2	Infected-cell	Undiluted	0.42	1.26	0.33	0.83	3.62	0.24
		1:5	0.10	0.38	0.26	0.34	1.21	0.30
		1:25	0.02	0.07	0.28	0.12	0.26	0.46

^a Diluted with DNA eluate from sham-infected cells.

^b Each value represents the average of two determinations.

infected cultures. Even at 5 h the HSV type could be correctly identified with 1/25 of the DNA from a single infected culture, although the homologous hybridization reactions had proceeded only to 6.5 to 9% of completion. In addition, these reactions proceeded at a rate approximately three times that of the reactions containing purified HSV DNA only. This suggests that the concentration of HSV DNA in the eluates from infected tube cultures is approximately 1.5 $\mu\text{g}/\text{ml}$.

In an additional experiment, 1:25 dilutions of the DNA eluates from triplicate cultures infected with prototype HSV-1 or HSV-2 were hybridized for longer periods. HSV DNA at a concentration 1/25 of that in the eluate from a single infected roller-tube culture could be identified and accurately typed, in general, by hybridization for 24 h and certainly by hybridization for 48 h (Table 2).

The quantity of HSV DNA recovered from a single roller-tube culture infected with either prototype HSV-1 or prototype HSV-2 is thus well in excess of that required for reliable identification and typing by this method when a 5-h period of hybridization is used. Although primary isolates may not replicate as efficiently as the prototype strains, the use of the undiluted eluate from a single infected roller-tube culture should still provide a substantial margin of safety.

Specificity of the DNA:DNA hybridization reaction performed with DNA isolated from HSV-infected roller-tube cultures. The capacity of the DNA:DNA hybridization reaction to discriminate between HSV-1 and HSV-2 was determined with DNA isolated from single roller-tube cultures infected with prototype HSV-1 or HSV-2. All reactions were carried out for 5 h and, in some cases, were also carried out for 24 h. In separate experiments involving a total of 32 individual HSV-1-infected cultures and 19 individual HSV-2-infected cultures, the

homologous reactions proceeded to approximately 47% completion by 5 h for HSV-1 and 68% completion for HSV-2, and the reactivity ratio with the two ^3H -labeled HSV-DNA probes ($R = r_1/r_2$) at 5 h clearly discriminated between cultures infected with HSV-1 and with HSV-2 (Table 3). Although discrimination was slightly better after 24 h of hybridization, the reactions were not linear over this period of time, and the longer hybridization was unnecessary for reliable typing.

To be clinically useful, a typing method for HSV must not identify other human herpesviruses as HSV. We therefore hybridized DNA isolated from roller-tube cultures of 350Q cells infected with cytomegalovirus (strain AD-169) or varicella-zoster virus (strain CP 5262) with the ^3H -labeled HSV-1 and HSV-2 DNA probes in the standard HSV typing reaction. The DNA was isolated from these cultures when approximately 90% of the cells exhibited cytopathic effect, and the reactions were carried out with DNA from each of four replicate cultures infected with each virus. The mean reactivity with cytomegalovirus was $r_1 = 0.02 \pm 0.02$ and $r_2 = 0 \pm 0.01$. For varicella-zoster virus these values were $r_1 = 0.02 \pm 0.02$ and $r_2 = 0.02 \pm 0.02$. Thus after a 5-h incubation, no reaction between DNA from cytomegalovirus- or varicella-zoster virus-infected cultures and the HSV probes had proceeded beyond 2% of completion. In contrast, the reaction between DNA from HSV-infected cultures and the homologous probe had proceeded to 47 to 68% completion (Table 3).

The role of sonication in the DNA isolation procedure. Since some diagnostic laboratories may not have access to a sonicator with a cuphorn attachment, DNA was isolated from 30 HSV-infected roller-tube cultures as described in Materials and Methods except that for approximately half of the cultures the cuphorn sonication step was omitted. The DNA from each culture was then hybridized for 5 h with

TABLE 2. Sensitivity of typing reactions performed for prolonged periods with 1:25 dilutions of the DNA eluates from prototype HSV-infected roller-tube cultures^a

Virus	Length of incubation ^b											
	24 h			48 h			72 h			96 h		
	r_1	r_2	R	r_1	r_2	R	r_1	r_2	R	r_1	r_2	R
HSV-1	0.34	0.12	2.7	0.64	0.17	3.8	0.76	0.19	4.1	0.95	0.20	4.8
HSV-2	0.16	0.48	0.33	0.27	0.75	0.36	0.28	0.90	0.32	0.26	1.06	0.25

^a Diluted with DNA eluates from sham-infected cultures.

^b Each value represents the average of results from three replicate cultures.

TABLE 3. *Specificity of typing reactions with prototype HSV strains: reactivity (r) and reactivity ratio (R)^a*

Virus	5-h incubation				24-h Incubation			
	<i>n</i>	<i>r</i> ₁	<i>r</i> ₂	<i>R</i>	<i>n</i>	<i>r</i> ₁	<i>r</i> ₂	<i>R</i>
HSV-1	32	0.88 ± 0.61 (69%)	0.24 ± 0.13 (54%)	3.50 ± 0.62 (18%)	13	2.26 ± 1.12 (50%)	0.48 ± 0.16 (33%)	4.46 ± 0.77 (17%)
HSV-2	19	0.52 ± 0.20 (38%)	2.08 ± 1.11 (53%)	0.28 ± 0.05 (17%)	7	0.79 ± 0.15 (19%)	4.01 ± 1.33 (33%)	0.21 ± 0.04 (20%)

^a Values represent mean ± standard deviation. *n*, Number of roller-tube cultures. Parentheses indicate coefficient of variation.

the ³H-labeled HSV DNA probes, and the reaction products were assayed by HA chromatography. Omission of the sonication step reduced the rate of hybridization with the HSV [³H]-DNA probes by more than 25% (Table 4). In all cases, however, the lower rate was still adequate to permit virus typing after 5 h of hybridization, and there was little or no loss of specificity.

Application of this detection and typing procedure to clinical isolates of HSV. Thirty-nine clinical isolates of HSV which had previously been typed by microneutralization (37) were coded and used to infect roller-tube cultures of 350Q cells, and the DNA was isolated and hybridized by the standard procedure for 5 h. For 12 of the 39 cultures, the cuphorn sonication step was omitted from the isolation procedure. Typing by hybridization was correct in all 39 cases (Table 5). The reactivity ratio (*R*) of the 25 HSV-1 isolates was 3.3 ± 0.4, and that of the 14 HSV-2 isolates was 0.31 ± 0.05. Although omission of the sonication step reduced the reactivity with both probes by 40 to 50%, the reactivity ratios obtained still permitted reliable typing with a 5-h period of hybridization.

DISCUSSION

The method of typing HSV by DNA:DNA hybridization presented here takes advantage of the large difference between the nucleotide sequences of HSV-1 and HSV-2 DNA (3, 17, 22, 38). It yields results which are inherently quan-

titative and reliably differentiates HSV-1 from HSV-2. Because there is little homology between either HSV-1 or HSV-2 DNA and the DNA of the other human herpesviruses (12, 20, 21), this HSV typing method will also unequivocally identify an isolate as HSV. When 39 coded clinical isolates of HSV were typed by the hybridization method described, the results agreed in every case with those obtained by microneutralization.

The procedure can be performed with a 5-h period of hybridization using as little as 1/10 of the DNA from a single infected roller-tube culture. By using a longer incubation period (24 to 48 h), the typing can be accomplished with as little as 1/50 of the DNA from a single infected culture. Since such roller-tube cultures are routinely used for primary virus isolation in most diagnostic laboratories, and since radiolabeling of the isolate is not involved, this method should be applicable to primary isolates with no need for additional tissue culture passage.

We have employed HA chromatography rather than S1 nuclease digestion to analyze the products of the hybridization reaction. Substitution of the latter technique may further simplify this HSV typing method. Furthermore, it is likely that type-specific HSV DNA probes of high specific activity will soon become generally available as a result of the enormous progress now being made in cloning specific fragments of the HSV-1 and HSV-2 genomes. The availability

TABLE 4. *Effect of omission of cuphorn sonication on reactivity (r) at 5 h and reactivity ratio (R) of DNA from prototype HSV-infected cultures^a*

Virus	Standard procedure				Cuphorn sonication omitted			
	<i>n</i>	<i>r</i> ₁	<i>r</i> ₂	<i>R</i>	<i>n</i>	<i>r</i> ₁	<i>r</i> ₂	<i>R</i>
HSV-1	11	0.69 ± 0.12 (17%)	0.20 ± 0.03 (14%)	3.37 ± 0.41 (12%)	10	0.50 ± 0.08 (15%)	0.17 ± 0.02 (11%)	3.03 ± 0.40 (13%)
HSV-2	4	0.61 ± 0.03 (5%)	2.11 ± 0.16 (7%)	0.29 ± 0.03 (9%)	5	0.41 ± 0.11 (27%)	1.59 ± 0.37 (23%)	0.29 ± 0.04 (14%)

^a Values represent mean ± standard deviation. *n*, Number of roller-tube cultures. Parentheses indicate coefficient of variation.

TABLE 5. Results of typing 39 clinical isolates

DNA isolation procedure	HSV-1 ^a			HSV-2 ^a		
	<i>r</i> ₁	<i>r</i> ₂	<i>R</i>	<i>r</i> ₁	<i>r</i> ₂	<i>R</i>
Standard procedure	0.49	0.20	2.4	0.26	1.01	0.26
	0.58	0.20	2.9	0.43	1.43	0.30
	0.68	0.21	3.2	0.44	1.46	0.30
	0.76	0.23	3.3	0.44	1.61	0.27
	0.79	0.25	3.2	0.48	1.91	0.25
	0.83	0.25	3.3	0.50	1.76	0.28
	0.87	0.26	3.3	0.50	1.90	0.26
	0.89	0.24	3.7	0.53	1.32	0.40
	0.96	0.32	3.0	0.59	1.90	0.31
	1.12	0.31	3.6	0.59	2.16	0.27
	1.13	0.29	3.9			
	1.14	0.32	3.6			
	1.33	0.34	3.9			
	1.34	0.38	3.5			
	1.41	0.35	4.0			
	2.02	0.57	3.5			
	2.25	0.60	3.7			
	1.09 ± 0.47 ^b	0.31 ± 0.12	3.4 ± 0.4	0.48 ± 0.10	1.65 ± 0.34	0.29 ± 0.04
Cuphorn sonication omitted	0.25	0.78	0.32	0.37	0.14	2.6
	0.29	0.79	0.37	0.44	0.15	2.9
	0.30	0.78	0.38	0.46	0.15	3.1
	0.34	1.05	0.32	0.46	0.17	2.7
				0.57	0.17	3.4
				0.57	0.19	3.0
				0.59	0.20	3.0
				0.64	0.19	3.4
	0.30 ± 0.04	0.85 ± 0.13	0.35 ± 0.03	0.51 ± 0.09	0.17 ± 0.02	3.0 ± 0.3
Inclusive statistics	0.91 ± 0.48 (53%) ^c	0.27 ± 0.12 (44%)	3.3 ± 0.4 (13%)	0.42 ± 0.12 (28%)	1.41 ± 0.48 (33%)	0.31 ± 0.05 (16%)

^a Isolates typed as HSV-1 or HSV-2 by hybridization and microneutralization, listed in order of increasing reactivity with HSV-1 DNA probe. For HSV-1, *n* = 25; for HSV-2, *n* = 14.

^b Mean ± standard deviation.

^c Parentheses indicate coefficient of variation.

of such type-specific DNA probes will greatly facilitate the application of nucleic acid hybridization techniques to clinical virology.

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