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Detection of Herpes Simplex Virus DNA from Cerebrospinal Fluid by PCR and a Rapid, Nonradioactive Hybridization Technique

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A molecular assay for the detection of herpes simplex virus (HSV), including a novel, nonradioactive hybridization technique, was evaluated with a total of 123 cerebrospinal fluid specimens. After DNA extraction, specific HSV DNA sequences were amplified with digoxigenin-labeled primers derived from the DNA polymerase gene-coding region from HSV. Amplified products were detected by the Enzymun-Test DNA detection assay (Boehringer, Mannheim, Federal Republic of Germany), which uses biotinylated probes. Amplification with nonlabeled primers and then Southern blotting and nonradioactive detection of hybrids by the digoxigenin technique was the reference system. The sensitivities of the molecular assays were determined with 10-fold dilutions of plasmid pS4 with the *SalI* restriction fragment of the DNA polymerase gene obtained from the HSV type 1 strain Angelotti. The Enzymun assay was able to detect all of the 16 positive samples, giving 100% agreement with the Southern blot hybridization results. Optical density values were widely separated for the positive and negative groups of specimens. Ten copies of plasmid pS4 per microliter could be distinctly detected by the Enzymun assay. The cutoff was determined for the hybridization assay, and an equivocal zone was defined. The whole molecular assay including the Enzymun-Test DNA detection proved to be sensitive and easy to use. It may contribute to the rapid and safe detection of HSV DNA in cerebrospinal fluid.

Herpes simplex virus (HSV) is the most important agent of sporadic encephalitis in industrialized countries (33). HSV type 1 (HSV-1) is commonly associated with oropharyngeal infections, keratoconjunctivitis, and infections of the central nervous system, whereas HSV-2 commonly produces genital infections. However, HSV-2 has been isolated from some patients with HSV encephalitis (1, 19, 20). Since powerful therapeutic management exists today, the prognosis of severe HSV illness has been improved. However, antiviral drugs must be administered early. On the other hand, symptoms mimicking HSV encephalitis could lead to the unnecessary application of drugs. Therefore, a rapid and safe method for the detection of HSV appears to be of paramount importance for decreasing the lethality as well as the sequelae of HCV encephalitis.

PCR is a new in vitro DNA amplification technique that allows for an almost exponential amplification of a well-defined DNA molecule during several cycles (26). DNA amplification by PCR has the advantage that it does not require live organisms and appears to be an optimal tool for enhancement of the sensitivity of detection. Therefore, it has been introduced for the diagnosis of HSV encephalitis (2, 14, 21, 24, 25).

Among nonradioactive hybridization techniques, the biotin-avidin (or biotin-streptavidin) (4, 17) and the digoxigenin (DIG)-antidigoxigenin (10, 11) systems have been widely applied for the direct and indirect detection of nucleic acids, proteins, and glycans on blots, in solution, or in in situ formats. The digoxigenin system detects DNA at less than picogram levels (12).

In the present study, a molecular assay based on a rapid DNA extraction protocol, PCR, and a novel automated nonradioactive hybridization technique were evaluated with cerebrospinal fluid (CSF) specimens. Detection of amplified HSV DNA by the novel hybridization technique was done for the first time. Results were compared with those of conventional Southern blot hybridization, and a cutoff value as well as an equivocal zone were estimated.

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MATERIALS AND METHODS

Clinical specimens. A total of 123 CSF samples were investigated retrospectively. All samples had been collected from 123 patients who were admitted to the University Hospitals in Graz, Austria, or Frankfurt am Main, Federal Republic of Germany. All of them had clinical presentations compatible with HSV encephalitis. Lumbar punctures were done at a mean of 28 h (range, 9 to 74 h) after the onset of illness. All samples were obtained prior to therapy. CSF samples were divided into aliquots and were stored frozen at −70°C. All positive samples ($n = 16$) were subsequently ascertained by the presence of intrathecal synthesis of specific antibodies to HSV and 13 of them were additionally identified to be positive by specific findings on magnetic resonance imaging. No radiologic investigation was done for the three remaining positive samples. Characteristic abnormalities indicative of HSV infection included the predominant affection of the temporal structures and evidence of hemorrhage (3). None of the patients with positive CSF samples had an underlying disease. All negative samples ($n = 107$) were derived from patients with other central nervous system infections such as cytomegalovirus or varicella-zoster virus encephalitis as well as idiopathic conditions mimicking encephalitis. In these patients, diagnoses were made by extended serological and bacteriological investigations supported by measurement of routine parameters such as CSF cell count and determination of CSF proteins.

Plasmid. A plasmid (pS4), kindly provided by K. W. Knopf, German Cancer Research Center, Heidelberg, Federal Re-

public of Germany, containing a single copy of a *Sal*I restriction fragment of the HSV polymerase gene from the HSV-1 strain Angelotti served as a standard for the determination of the sensitivity of the Enzymun-Test DNA detection assay. Tenfold dilutions of the plasmid were subjected to PCR amplification and subsequent hybridization.

DNA extraction. A rapid DNA extraction protocol was used. In a 1.5-ml tube, 50 μl of CSF was added to 150 μl of a solution consisting of 20% (wt/vol) Chelex 100 resin (Bio-Rad Laboratories, Richmond, Calif.) in 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA–0.1% sodium azide. After vortexing for 10 s, the tube was incubated at 56°C for 20 min; this was followed by vortexing for another 10 s. After incubation at 100°C for 10 min, the tube was allowed to cool to room temperature. Following complete settlement of the resin, 20 μl of the supernatant was carefully removed and was used for amplification directly, without further purification.

Design of oligonucleotide primers. Two 22-base oligonucleotides (5′-CATCACCGACCCGGAGAGGGAC [positions in HSV-1, 3140 to 3161; positions in HSV-2; 3309 to 3330] and 5′-GGGCCAGGCGCTTGTTGGTGTA [positions in HSV-1, 3710 to 3731; positions in HSV-2, 3400 to 3379]) deduced from the published sequence of the DNA polymerase gene-coding region from HSV (18, 31) were used. This set of primers, which was chosen within a highly conserved region of the DNA polymerase gene from the herpesvirus group, allows amplification of a 92-bp fragment of the HSV-1 and HSV-2 DNA polymerase genes in clinical samples (5, 6).

DNA amplification. Each DNA extract was amplified twice. One PCR run was performed with nonlabeled primers; the other was performed with 5′-end-labeled DIG primers (MedProbe AS, Oslo, Norway). PCR was done in a 100-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM (each) deoxynucleoside triphosphates, 0.5 μM (each) oligonucleotide primer, 20 μl of extraction supernatant, and 1 U of a thermostable DNA polymerase (Dynazyme; Finnzyme OY, Espoo, Finland). The reactions were performed in specially designed PCR tubes (Sarstedt, Nuembrecht, Federal Republic of Germany) by using a programmable thermal cycler (PHC-2; Techne, Cambridge, United Kingdom). Thirty PCR cycles consisting of 1 min at 95°C, 1 min at 50°C, and 15 s at 72°C were run. After the final cycle, the tubes were incubated for an additional 7 min at 72°C. Each amplification run contained two negative and two positive controls. Negative controls consisted of pooled CSF samples from 20 patients without neurological disease. The supernatants of infected cells served as positive controls: MRC-5 cells derived from vesicle fluid from patients with recurrent herpes labialis were used for HSV-1 (typed with monoclonal antibodies; Syva, San Jose, Calif.), and cells from genital lesions were used for HSV-2. Furthermore, supernatants of commercially available HSV-1- and HSV-2-infected Vero cells (VR-260 and VR-734, respectively; American Type Culture Collection, Rockville, Md.) were used.

Hybridization assays. Nonlabeled amplification products were detected by Southern blotting. DIG-labeled amplification products were analyzed by the novel hybridization technique. Both hybridization systems employed 5′-end-labeled probes (MedProbe AS, Oslo, Norway) of the same sequence (5′-GA CTTTGTCCTCACCGCCGAACTGAGCAG [positions in HSV-1, 3368 to 3697; positions in HSV-2, 3337 to 3365]). For Southern blotting, the probe was labeled with DIG, and for the Enzymun assay the probe was labeled with biotin.

Detection of the DIG-labeled amplification product was carried out with the Enzymun-Test DNA detection assay (Boehringer, Mannheim, Federal Republic of Germany) in accordance with the manufacturer's advice. Amplification products were transferred to sample cups provided by the manufacturer and were diluted 1:10 with denaturation reagent containing sodium hydroxide solution. One sample cup containing performance check solution was also prepared. All sample cups were placed into the slots of the rotation block of the ES 300 instrument (Boehringer). After this, adequate amounts of hybridization and conjugate solutions were prepared. Hybridization solution consisted of a mixture of hybridization buffer and stock solution (63 μg/μl) of biotin-labeled capture probe in the relationship 100 + 1. Conjugate solution contained a mixture of conjugate buffer and dissolved anti-DIG antibody labeled with horseradish peroxidase in the relationship 100 + 1. Furthermore, an adequate amount of substrate-chromogen solution, which was previously prepared by adding substrate buffer (phosphate-citrate, H_2O_2 [pH 4.4]) to dissolve the chromogen [di-ammonium 2,2′-azino-di-(3-ethyl-benzothiazoline-6-sulfonate; Boehringer], was used to fill the provided bottle. Finally, an adequate number of streptavidin-coated plastic tubes were placed into the slots of the incubation rotor of the ES 300 instrument. The following procedure was done automatically by the ES 300 instrument. A total of 100 μl of the denatured amplification product and 400 μl of the hybridization solution (including the biotinylated HSV DNA capture probe) were incubated in the streptavidin-coated tube at 37°C for 2 h. After rinsing with washing solution, 500 μl of anti-DIG–horseradish peroxidase conjugate was added; this was followed by incubation for 30 min. After a second wash, the substrate solution was added and the tubes were again incubated for 30 min. The optical density (OD) was read at 420 nm.

The hybridization technique was performed three times on each reaction mixture by using three different test kits from two lots of the Enzymun-Test DNA detection assay. Test kit A was from a commercially available lot. Test kit B was taken from the last lot in the scale-up process of test kit development. Test kits A and B were stored in accordance with the manufacturer's advice (at 4°C) prior to use. A third test kit (kit BS), taken from the same lot as test kit B, was stressed by incubation at 35°C for 10 days and was then stored at 4°C.

For the conventional hybridization protocol, 10 μl of the reaction mixture containing unlabeled amplification products was run on a 1.5% agarose gel; this was followed by transfer of nucleic acids onto a positively charged nylon membrane (Hybond N^+; Amersham, Dreieich, Federal Republic of Germany) as described elsewhere (30). Prehybridization was performed at 42°C in hybridization buffer (SSC [0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide) for 2 h. After washing the membrane twice, immunologic detection of bound probes was done with a DIG-based nucleic acid detection kit (Boehringer). The DNA probe was directly labeled with digoxigenin-11-UTP. After hybridization, the blots were incubated with alkaline phosphatase-conjugated rabbit anti-DIG antibodies, and the DNA probe bound to the filter membrane was visualized with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer) in accordance with the manufacturer's recommendations.

Precautions against contamination. The danger of cross-contamination was avoided by establishing optimized strategies. Precautions taken to prevent contamination included strict separation of the different working areas (reagent preparation area, extraction area, amplification area, hybridization area), division of all substances (extraction reagents, water, 10 × buffer, deoxynucleoside triphosphates, primers) into aliquots before use, application of filtered pipette tips (prevention of aerosol contamination), use of gloves that were fre-

quently changed, and the application of short-wavelength UV radiation to working areas (especially instruments).

Interassay and intraassay variabilities. To evaluate reproducibility, seven of the negative specimens and three of the positive specimens were taken, and the whole molecular assay, including DNA extraction, PCR, and the novel hybridization technique, was repeated five times on different days with a commercially available kit package. Another seven of the negative and three of the positive specimens were additionally amplified, and three aliquots of each of the mixtures containing amplified products were analyzed three times on different days with the novel hybridization system.

RESULTS

From a total of 123 CSF specimens, all 16 HSV-positive CSF samples which displayed the expected 92-bp product by Southern blotting were detected by the novel hybridization assay. A total of 107 samples remained negative by testing by both hybridization techniques. When uninfected fibroblasts and DNAs from human leukocytes and other herpesviruses, including human cytomegalovirus, varicella-zoster virus, and human herpes virus type 6, were subjected to PCR amplification, specific DNA products were not detected by either hybridization assay.

Ten copies of the plasmid pS4 per microliter could be distinctly detected by both the Enzymun assay and the reference system. With the Enzymun assay a linear increase in the signal was observed in the range of 1 to 10^3 copies per μl. When more than 10^3 copies of pS4 were added per μl, a saturation effect became evident.

During the whole study, the OD values of the performance check solution included in the Enzymun assay were >2.0. Because three different test kits were used, data from 435 experiments were available. Test kit A gave mean OD values of 5.9 (standard deviation [SD], 0.7) for positive samples and 0.4 (SD, 0.4) for negative samples. The mean OD values of test kit B were 4.7 (SD, 0.7) for positive samples and 0.2 (SD, 0.2) for negative samples. The corresponding OD values of the stressed test kit (BS) were 4.3 (SD, 0.8) and 0.2 (SD, 0.2), respectively (Fig. 1). OD values were widely separated for the positive and negative groups of specimens, making interpretation easy (Fig. 2A to C). All positive specimens tested with test kit A had OD values of >4.25, and OD values obtained by testing with the stressed test kit (kit BS) resulted in OD values of >3.25 with the exception of one sample. A total of 106 of 107 negative specimens tested with test kit A had OD values of <1.25, and none of these specimens reached an OD value of 1.25 when tested with test kit BS. A total of 3 of 435 (0.7%) specimens fell within an OD range of 1.75 and 3.25. One of these was tested with kit A and gave an OD value of 2.43. Retesting of this specimen led to a drop in the OD value to 0.32. The patient from whom this specimen was obtained did not produce intrathecal antibodies and no specific abnormalities were found on magnetic resonance imaging, but a serologic immune response against measles virus was subsequently observed. The other two borderline results occurred with kits B and BS with specimens from a patient who produced intrathecal antibodies against HSV, whose temporal structures were shown to be affected, and who was found to have evidence of hemorrhage at the magnetic resonance imaging investigation. The CSF of this patient was found to be distinctly positive with kit A, but testing with kits B and BS gave OD values of 3.22 and 2.73, respectively. Repeat testing with kits B and BS confirmed the initial test results; i.e., the results were again greater than 2.5 OD units; however, they were still within the equivocal zone.

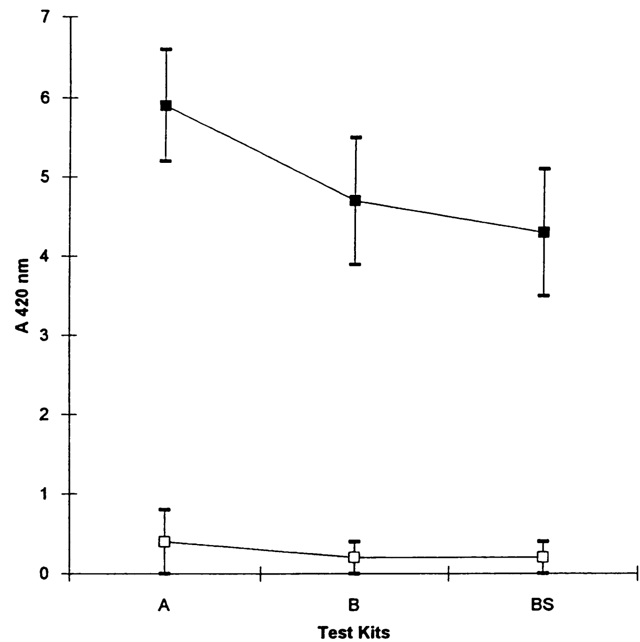

FIG. 1. Mean OD values and SDs for test kits A, B, and BS. ■, positive samples; □, negative samples.

Interassay variability was tested on seven negative and three positive specimens. In negative samples the SDs of the mean OD values ranged from 0.03 to 0.51; in positive samples the SDs of mean OD values ranged from 0.19 to 1.19. Repeat testing of the novel hybridization system on the same amplification mixture gave SDs of mean OD values ranging from 0.01 to 0.06 for negative samples and from 0.04 to 0.08 for positive samples.

DISCUSSION

In the present study, a rapid molecular assay for the detection of HSV in CSF was evaluated. An automated hybridization technique for the detection of DIG-labeled amplification products was tested for the first time with 123 CSF specimens, and the results were compared with those of a nonradioactive Southern blotting technique.

A rapid and safe DNA extraction protocol is of paramount importance, because results must be delivered as rapidly as possible, and the probability of false-positive results because of contamination increases in relation to the number of manipulations involved in sample processing (7, 16). In earlier studies, HSV DNA was usually extracted by phenol-chloroform extraction either with (24, 25) or without (14) proteinase K. Recently, Chelex 100 has been successfully used in DNA extraction protocols, guaranteeing the presence of a sufficient amount of extracted DNA (9, 13, 28, 29, 32). The basic Chelex 100 procedure consists of boiling the sample in a Chelex 100 solution and then adding a fraction of the supernatant directly to the PCR mixture. The presence of Chelex 100 during boiling prevents the degradation of DNA by chelating metal ions that may catalyze the breakdown of DNA subjected to high temperatures in low-ionic-strength solutions (29). However, Chelex 100 itself inhibits PCR, mainly by disturbing the optimum magnesium ion concentration. Therefore, the Chelex 100 must settle completely before the extracted DNA is removed to avoid the transfer of resin with the sample.

Primers and probe sequences have been selected from the

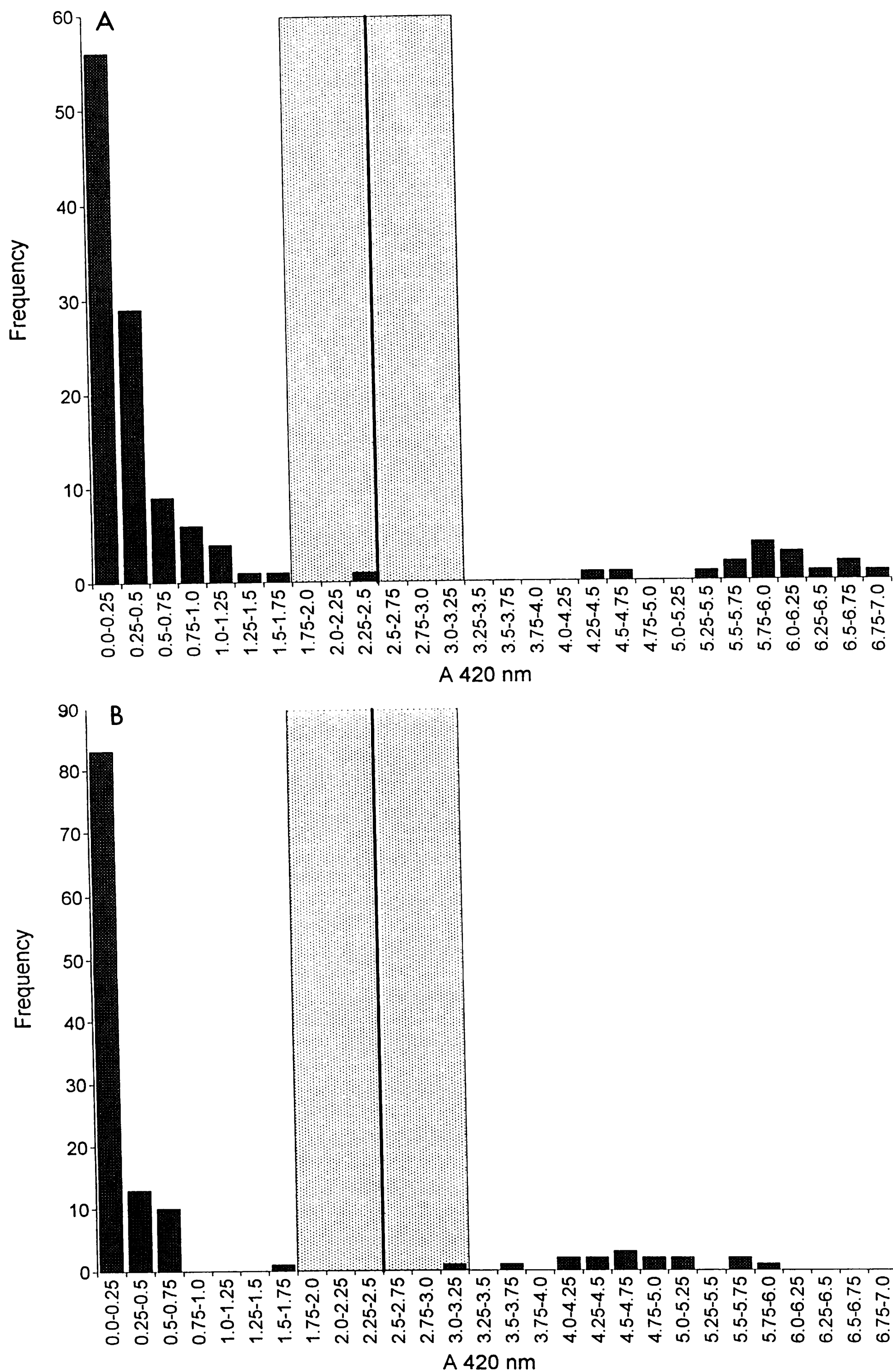

FIG. 2. OD values, cutoff, and equivocal zone used in the study. The results demonstrate the wide separation of negative and positive samples. Results for test kits A (A), B (B), and BS (C) are presented.

DNA polymerase gene-coding region identifying the genomes of HSV-1 and HSV-2. Thus, the primers that we used do not discriminate between these virus types (31). Furthermore, no cross-reaction with other herpesviruses could be found when using these primers (6). Because therapeutic management for HSV-1 infections does not differ from that for HSV-2 infections, the primers that we selected are suitable for use in routine clinical diagnosis. However, a prognostic significance associated with the type of HSV infection in neonates and infants has recently been reported (8, 34). Therefore, differ-

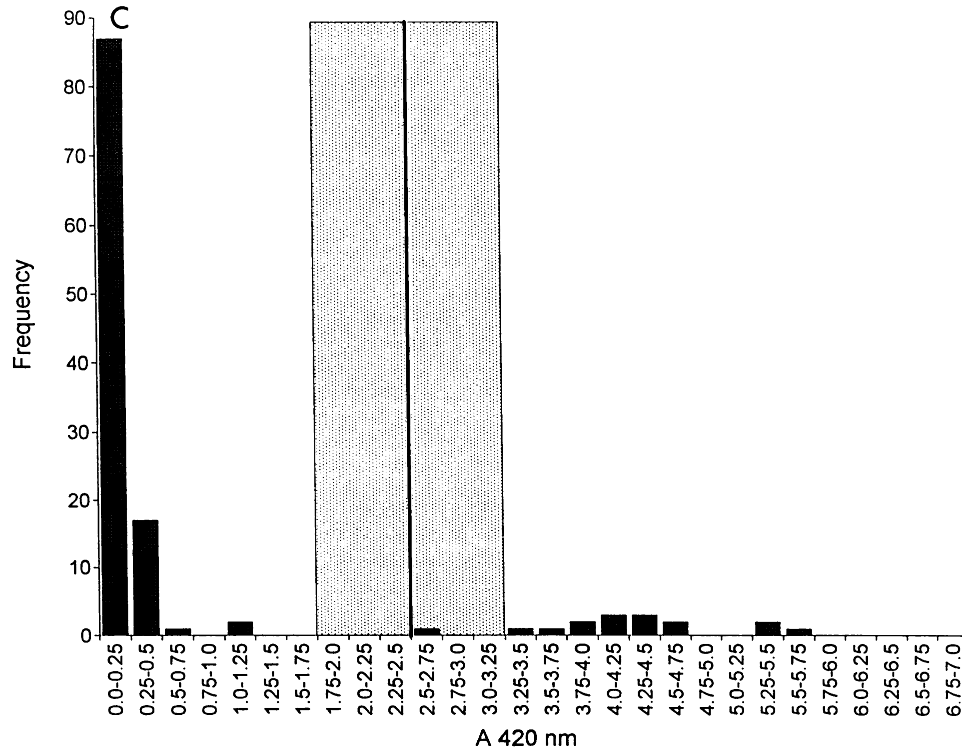

FIG. 2—*Continued.*

entiation of HSV-1 from HSV-2 by using restriction enzymes or by amplification with primers specifically dedicated to HSV-1 or HSV-2 would be of interest in these groups of patients. Because of the potential of PCR to amplify even one molecule of DNA to detectable levels, desirable PCR work practices must be maintained to prevent cross-contamination (15). Cross-contamination creates false-positive PCR results, which may lead to unnecessary treatment.

Nucleic acid hybridization leads to increased sensitivity (23) and excludes unspecific amplification products (22, 27). Nonradioactive hybridization systems are increasingly used instead of techniques that use radioactive agents. The DIG system is based on a specific interaction between the cardenolide steroid DIG and a high-affinity, DIG-specific antibody. DIG occurs exclusively in different species of *Digitalis*. Therefore, nonspecific interactions with endogenous cellular substances in other biological materials are strongly reduced. Nonradioactive hybridization with the DIG system allows specific detection of subpicogram levels of DNA (10). Recently, the Enzymun-Test DNA detection assay has been introduced. This assay allows an automated hybridization and detection procedure on the ES 300 instrument and is convenient for use in testing large numbers of samples. This novel hybridization technique proved to be as sensitive as the reference system and was easy to use. Depending on the number of samples, it was possible to carry out the procedure described here in less than 4 h. Sample preparation consisted of only one step. A minimum of 30 μl of PCR mixture containing amplified DNA is required for a single determination. In the present study, three different test kits of the Enzymun-Test DNA detection assay were tested, which increased the amount of mixture containing amplification products only to 50 μl. Both hybridization and conjugate solutions were freshly prepared. This was done within a few minutes. The formation of foam had to be avoided by gentle pipetting. The conjugate solution had to be protected from direct sunlight. The hybridization solution as well as the conjugate and substrate-chromogen solutions were brought to 20 to 25°C before use. After starting, the system ran automatically. Interassay as well as intraassay variabilities gave excellent results, which is evidence of the stability of the whole molecular assay.

Results of all tests agreed 100% with the results of Southern blot hybridization. During the whole study, the absorbance of the performance check solution was >2.0. If the absorbance of the performance check solution is <1.5 after testing under the specified conditions, the results should be regarded as unreliable. The OD values of the tested specimens were widely separated for the positive and negative groups of specimens. Within the three tested kits a continuous decrease of absolute values was seen, but the separation of positive and negative groups of specimens was still distinct, demonstrating the stability of the conjugate even under inferior conditions. This wide separation between positive and negative readings is an advantage of the assay tested, making determination of the cutoff value easy. Considering the distributions and the mean absorbances of negative samples, a cutoff value of 2.5 OD units is proposed. The presence of HSV DNA in the sample is determined by relating the absorbance of the unknown specimen to that of the cutoff value. For the present study, an equivocal zone of ±30% was used. Clinical specimens with A_{420} readings of less than 1.75 OD units were interpreted as presumptive negative results, and specimens with A_{420} readings of greater than 3.25 OD units were interpreted as positive for the presence of HSV DNA. During the whole study, only 3 of 435 specimens fell into the equivocal zone. These specimens were repeat tested, and in all cases the initial result was confirmed by repeat testing, giving evidence of clear separation between positive and negative values in the Enzymun assay.

For routine use of the Enzymun assay, we recommend that at least one positive control and three negative controls be run each time that the test is performed. The OD value of the positive control should be >3.25, and the OD value of the negative control should be <1.75. Pooled CSF specimens from patients with negative serologies are proposed to be used as negative controls. If the OD value of one of the controls falls into the equivocal zone, samples for the entire run should be discarded, and the entire run, including DNA extraction and amplification, should be repeated. Specimens giving absorbances showing up to ±30% deviations from the cutoff value must be repeat tested. The final test result for these specimens should be determined by using 2.5 OD units as the cutoff.

In summary, application of the Enzymun molecular assay described in this report proved to be suitable for the rapid and safe detection of HSV in CSF specimens. The novel hybridization technique included in the procedure was found to be very quick, easy to use, and sensitive and gave distinctly separate readings. The system will help clinicians make safe and early diagnoses and should be readily accepted for routine use.

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