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Factors Affecting the Detection of Enteroviruses in Cerebrospinal Fluid with Coxsackievirus B3 and Poliovirus 1 cDNA Probes

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Enteroviruses are common pathogens of meningitis and encephalitis, and infections are often difficult to distinguish clinically from bacterial and herpetic infections of the central nervous system. An array of enteroviruses added to cerebrospinal fluid in reconstruction experiments were detected by a dot hybridization assay. Optimal handling and processing conditions for infected cerebrospinal fluid were established, and the effect on the hybridization reaction of humoral and cellular components of the inflammatory response was determined. Six hybridization probes, derived from poliovirus 1 and coxsackievirus B3, were then tested, singly and in combinations, to optimize the sensitivity and spectrum of the assay. Implications for enteroviral taxonomy based on these experiments are discussed.

Enteroviruses are the most common etiologic agents causing aseptic (nonbacterial) meningitis (5). Although the prognosis of enteroviral meningitis is generally a favorable one, the initial clinical and laboratory manifestations may be difficult to distinguish from more serious bacterial and herpes simplex viral infections. This results in unnecessary hospitalizations and antibiotic use. Currently, the diagnosis of enterovirus infections depends upon isolation in tissue cultures—a process which may take from a few days to weeks. The sensitivity of tissue culture isolation, moreover, may only be 65 to 70% for enteroviruses (2), with certain serotypes being notoriously difficult to grow (e.g., coxsackievirus A). A rapid, accurate method of diagnosing enteroviruses is necessary to expedite the care of patients with aseptic meningitis.

Recently, it has been shown that nucleic acid hybridization probes derived from the RNA of certain enteroviruses can recognize diverse enteroviral serotypes in tissue culture infections (4, 11). We now report reconstruction experiments in which enteroviruses were detected in cerebrospinal fluid (CSF). These experiments were performed to elucidate the optimal handling and processing conditions for our anticipated testing of clinical CSF specimens and to evaluate the sensitivity and potential pitfalls of viral RNA detection in the presence of an inflammatory response. Additionally, a battery of subgenomic fragments of coxsackievirus B3 (CB3) and poliovirus 1 RNAs was evaluated to select an ideal combination of diagnostic probes for these agents.

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MATERIALS AND METHODS

CSF. Normal CSF was obtained from neurosurgical patients and other patients with noninflammatory illnesses. The CSF contained no erythrocytes or leukocytes and had normal levels of glucose and protein.

Viruses. Polioviruses 1, 2, and 3 (Sabin vaccine strains) were generously provided by Lederle Laboratories, Pearl River, N.Y. Coxsackieviruses A9 (Bozek strain), A16 (G-10 strain), B1 (Conn-5 strain), and B6 (Schmitt strain) and echoviruses 2 (Cornelis strain), 4 (Pesascek strain), 6 (D'Amori strain), 11 (Gregory strain), and 22 (Harris strain) were obtained from the American Type Culture Collection, Rockville, Md. Clinical isolates of respiratory syncytial virus (RSV) and herpes simplex virus (HSV) (strains P.G. and N.M., respectively) were grown in LLC-MK_2 cells (CCL 7.1; American Type Culture Collection) maintained in Dulbecco modified Eagle medium plus 2% fetal bovine serum.

Hybridization probes. pDS 111, a nearly genomic cDNA clone of poliovirus 1 (Mahoney strain), and pDS 14, a 1,809-nucleotide cDNA fragment from the 5′ end of poliovirus 1, are contained in *Escherichia coli* HB101 (pBR325), as recently described (13). pCB111 51, 29, 33, and 35 are subgenomic cDNA clones of CB3, the preparation and characterization of which have been recently reported (14). The CB3-derived clones and the poliovirus-derived clones are shown in Fig. 1.

The six cDNA enterovirus clones were radioactively labeled with ^{32}P by standard nick translation techniques (10) to a specific activity of 10^8 cpm/μg of DNA.

Blood cells. Mononuclear and polymorphonuclear leukocytes were separated on Ficoll-Hypaque gradients by standard techniques (1). Erythrocytes were obtained by centrifuging whole blood in a heparinized tube at 400 × *g* for 20 min, discarding the plasma, and collecting the bottom third of the pellet. All cells were counted in a Coulter counter.

Proteins. Immune serum globulin (ISG) containing 16.5 ± 1.5 mg of protein per dl was obtained from Cutter Biological, Berkeley, Calif. Human serum albumin (25% solution) was obtained from Travenol Laboratories, Glendale, Calif.

Other reagents. Vanadyl ribonucleoside complex (VRC)

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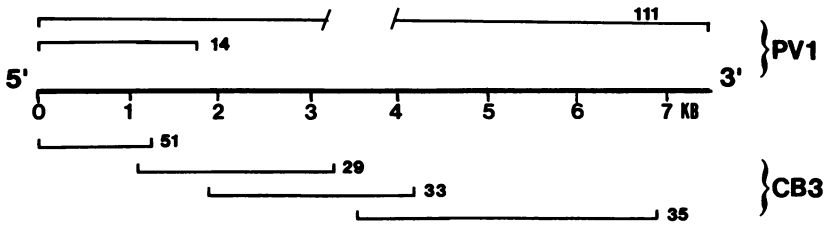

FIG. 1. Schematic representation of the locations of poliovirus 1 (PV1) and CB3 cloned cDNA fragments in relation to the full-length genomes of the parent viruses. PV1-derived clones are pDS 14 and pDS 111; CB3-derived clones are pCB111 51, pCB111 29, pCB111 33, and pCB111 35. KB, Kilobases.

(Bethesda Research Laboratories, Gaithersburg, Md.) was added as an RNase inhibitor to certain specimens at a final concentration of 10 mM. Proteinase K (Sigma Chemical Co., St. Louis, Mo.) was used to hydrolyze added protein in certain specimens at a final concentration of 0.1 mg/ml, followed by incubation at 37°C for 30 min.

CSF reconstruction experiments. Specified titers (see figure legends) of enteroviruses were added to 100-μl volumes of normal CSF. Experimental variables were then introduced to determine the optimal handling and processing conditions for infected CSF as well as the effect of the host inflammatory response on the hybridization assay. Storage times and temperatures were varied in the presence and absence of VRC. Cellular and humoral elements of the host inflammatory response were added individually to virus-containing CSF samples in concentrations spanning the ranges expected in viral and bacterial meningitis.

Fixation conditions. Virus-containing CSF samples were treated with either an equal volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) or an equal volume of a 3:2 mixture of 20× SSC–37% formaldehyde, as has been recommended for preparing cell lysates for hybridization (15), and were incubated at 60°C for 15 min.

Dot hybridization assay. After fixation, the samples were applied to nylon membrane filters (Gene-Screen Plus; New England Nuclear Corp., Boston, Mass.) under suction with a 96-well manifold apparatus (Bethesda Research Laboratories). The filters were dried at room temperature and prehybridized in a sealed plastic bag (Seal-a-meal; Dazey Products Co., Industrial Airport, Kans.) containing 5× SSC and 5× Denhardt solution (3) for 4 h at 50°C with continuous agitation. The prehybridization solution was then removed and replaced with a hybridization solution containing 50% formamide, 5× Denhardt solution, 5× SSC, and 10% dextran sulfate. A 0.1- to 0.15-ml volume of each hybridization probe (5×10^7 to 10×10^7 cpm/μg of cDNA) was then added to 1 mg of calf thymus DNA (in 0.1 ml of 10 mM Tris–1 mM EDTA), denatured at 100°C for 4 min, rapidly cooled, and added to the hybridization mixture in the plastic bag. Hybridization at 50°C for 12 to 15 h was performed with continuous agitation. Washes and autoradiography of the filters were performed as previously described (11).

RESULTS

Storage of reconstructed CSF samples for up to 96 h at room temperature and at 4, −20, or −70°C had no appreciable effect on the hybridization signals obtained. The addition of VRC before storage or immediately before application of the specimen to the membrane filter similarly did not alter the strength of the signal (data not shown). Fixing the specimens in a 3:2 mixture of 20× SSC–37% formaldehyde improved the sensitivity of the hybridization assay by 100-fold over that obtained with fixation in SSC alone (Fig. 2).

The addition of mononuclear or polymorphonuclear leukocytes in concentrations of 3.5 to 3,500 cells per mm^3 and 20 to 20,000 cells per mm^3, respectively, to CSF samples containing identical concentrations of poliovirus 1 had no effect on the hybridization signals (Fig. 3). Furthermore, the highest cell counts tested did not give a false hybridization signal in the absence of virus. Proteinase K treatment in the presence of these cells did not appreciably affect the signal strength. Erythrocytes (10 to 10^4 cells per mm^3) likewise neither interfered with the hybridization signals nor produced a false signal in the absence of virus (Fig. 3). At concentrations of $>10^5$ cells per mm^3, however, counts which rarely occur except with a grossly bloody lumbar puncture, mechanical problems prevented the adequate application of the sample to the filter. Proteinase K did appear to improve the hybridization signal obtained in the presence of these high numbers of erythrocytes (Fig. 3).

Immunoglobulins typically appearing in CSF during meningitis were also added to CSF samples containing poliovirus 1 (Fig. 4). Increasing concentrations of ISG, through the range of values expected for all forms of meningitis, resulted in a progressive attenuation of the hybridization signals. Proteinase K treatment of the mixture restored the hybridization signals even at the highest ISG concentrations (Fig. 4). The effect of ISG appeared to be protein-specific, as albumin in increasing concentrations did not affect the detection of viral RNA, and proteinase K had no appreciable effect on the albumin-treated CSF samples (Fig. 4).

A panel of 14 viruses (12 enteroviruses, RSV, and HSV) was chosen to evaluate our battery of probes. Equal titers of each virus were added to CSF, and each specimen was then processed with VRC, proteinase K, and SSC-formaldehyde as described above. Nine aliquots of each specimen were

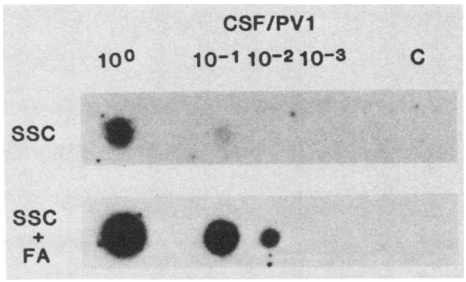

FIG. 2. Dot hybridization of CSF specimens to which serial dilutions of poliovirus 1 (PV1) were added, followed by two different fixation protocols. SSC, a 1:1 mixture of the specimen and a salt solution (see the text); SSC + FA, a 1:1 mixture of the specimen and a solution containing three parts salt and two parts formaldehyde (see the text); C, control CSF to which no PV1 was added. An undiluted (10^0) specimen contained 10^5 $TCID_{50}$ of PV1. The autoradiographs were exposed for 24 h. The detecting probe was pDS111.

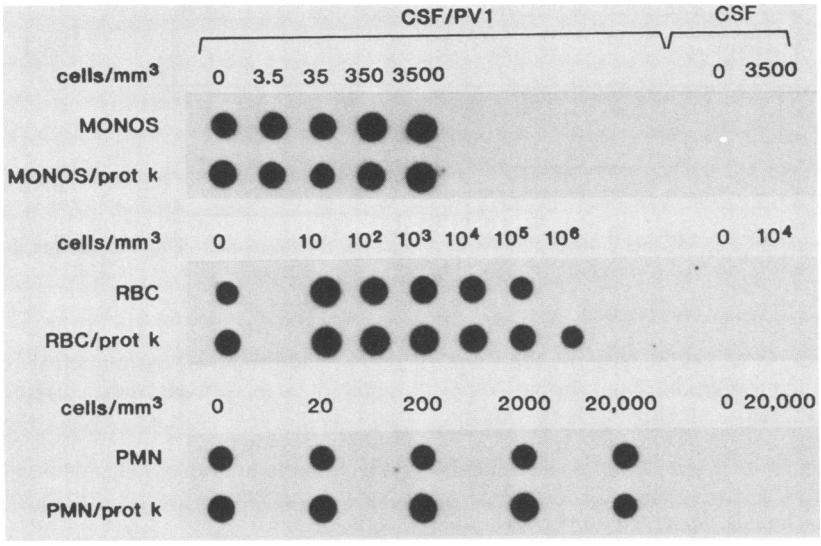

FIG. 3. Dot hybridization of CSF specimens to which the same amount of poliovirus 1 (PV1) ($TCID_{50}$, 10^5) was added, followed by increasing numbers of mononuclear cells (MONOS), erythrocytes (RBC), or polymorphonuclear neutrophils (PMN). Identical samples were treated with proteinase K (prot k). Columns designated CSF indicate control specimens to which no virus was added, with or without cells (in the numbers indicated). The autoradiographs were exposed for 48 h. The detecting probe was pDS111.

then applied to nine membrane filters. One filter was hybridized with each of the probes separately or in combinations (Fig. 5 and 6). The two poliovirus-derived probes produced similar patterns of hybridization, with the strongest signals resulting from hybridization with polioviruses 1 and 3, echoviruses 2 and 4, and coxsackievirus B6 (Fig. 5A and B). The titer of poliovirus 2 in the virus stock used was calculated to be 2 logs higher than that of poliovirus 3 and 1 log higher than that of poliovirus 1, resulting in a greatly diluted preparation of poliovirus 2 stock being applied to the membrane filter. The inaccuracies of such viral quantitations and the need for RNA titration in judging interrelationships among enteroviruses are discussed below. Coxsackieviruses A9 and A16 were both detected (Fig. 5A and B) by the poliovirus-derived probes, and a longer autoradiograph exposure revealed signals with echovirus 11 and coxsackievirus B1 as well (data not shown). The CB3-derived probes produced an array of hybridization patterns, ranging from weak detection of only coxsackievirus B6 and echovirus 4 by the CB3-specific pCB111 29 probe (Fig. 5C) to the detection of multiple serotypes with the 3′ pCB111 35 and 5′ pCB111 51 probes (Fig. 5E and F). The CB3-derived probes generally produced stronger signals than the poliovirus-derived probes when tested against the echoviruses, coxsackievirus B, and coxsackievirus A9. Coxsackievirus A16 was best seen when hybridized with the pCB111 51 probe but was not detected at all by the other CB3 probes. That same serotype was weakly detected by both poliovirus-derived probes.

Probe combinations which readily detected all serotypes except echovirus 22 could be chosen. When a 5′-end-containing probe and 3′-end-containing probe were used together (Fig. 6), the resulting hybridization signals were stronger and had a broader spectrum than those obtained with any probe used alone. Echovirus 6 was best seen with a longer autoradiograph exposure (e.g., Fig. 6D). However, even a prolonged exposure did not allow the detection of echovirus 22 under the hybridization conditions described.

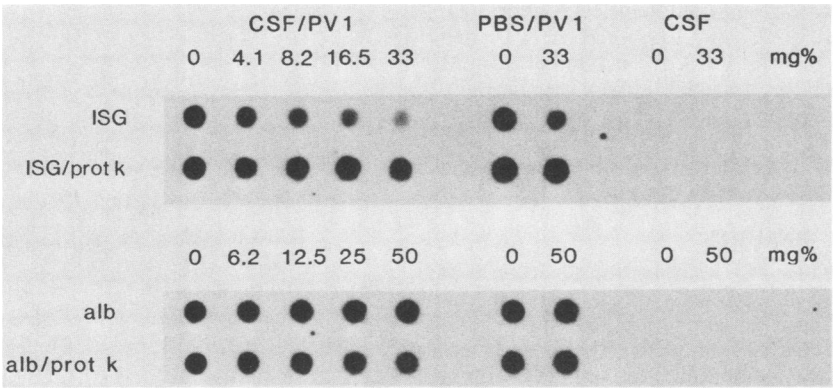

FIG. 4. Dot hybridization of CSF or phosphate-buffered saline (PBS) specimens to which poliovirus 1 (PV1) ($TCID_{50}$, 10^5) was added, followed by increasing concentrations of ISG or albumin (alb). Identical samples were treated with proteinase K (prot k). Columns designated CSF indicate control specimens to which no virus was added, with or without protein (in the concentrations indicated). The autoradiographs were exposed for 24 h. The detecting probe was pDS111.

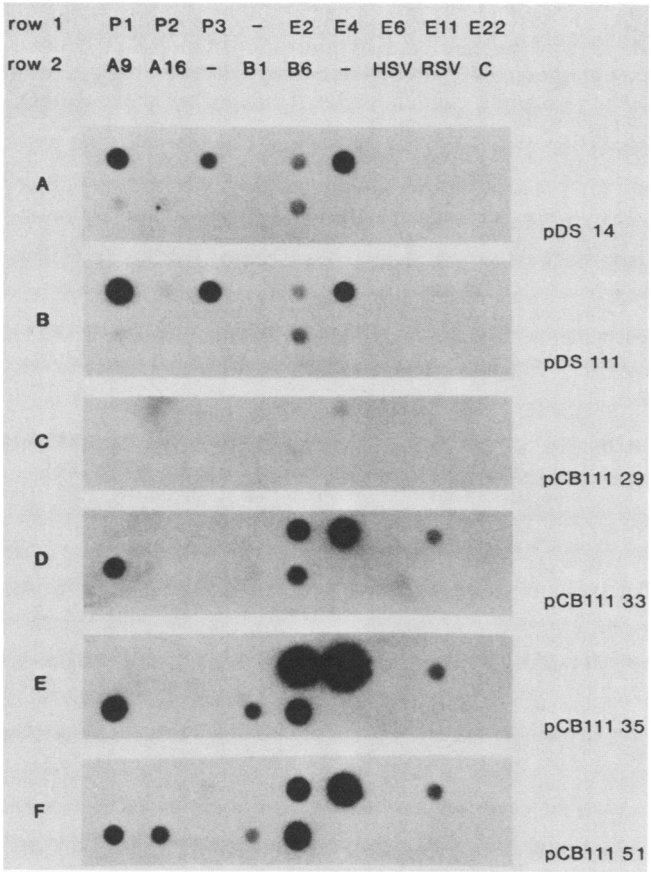

FIG. 5. Dot hybridization patterns obtained with six different poliovirus 1- and CB3-derived probes. Fourteen viral serotypes ($TCID_{50}$, 10^7) were added individually to fourteen 1.5-cm^3 volumes of CSF. Aliquots of each of these infected CSF specimens were then dotted onto nine membrane filters in an identical arrangement and hybridized with individual probes. Each spot thus contained 7×10^5 $TCID_{50}$ of enterovirus. Abbreviations: P1, poliovirus 1; P2, poliovirus 2; P3, poliovirus 3; E2, echovirus 2; E4, echovirus 4; E6, echovirus 6; E11, echovirus 11; E22, echovirus 22; A9, coxsackievirus A9; A16, coxsackievirus A16; B1, coxsackievirus B1; B6, coxsackievirus B6; C, control CSF (no virus added). The autoradiographs were exposed for 24 h.

There was no detectable cross-hybridization with RSV, and an HSV signal was seen only faintly in a single hybridization reaction (with pCB111 33; Fig. 5D). No false-positive signals were obtained with CSF alone. The sensitivity of the assay ranged from 10^2 to 10^5 50% tissue culture infective doses ($TCID_{50}$) for the enteroviruses (exclusive of echovirus 22) tested (data not shown). The actual amount of RNA in the specimens was not determined.

DISCUSSION

Enteroviral meningitis and encephalitis often mimic central nervous system infections caused by bacteria and HSV (5). Although tissue culture isolation of some enteroviruses can be accelerated by the use of multiple cell lines (Dagan and Menegus, 24th ICAAC), most diagnostic virology laboratories require several days to weeks to report positive cultures and as long as 3 weeks before cultures can be reported as negative for enteroviruses. This delay often results in unnecessary hospitalizations and antibiotic use, particularly for young children (2). Furthermore, although potentially effective antiviral drugs are available for enteroviruses (7), clinical trials must await a rapid, reliable diagnostic test.

Nucleic acid probes have been proven useful for the diagnosis of a number of important human viral infections (9). Recent work in our laboratory and elsewhere has demonstrated genetic homology shared among many serotypes of enteroviruses (4, 11, 12, 14), making possible their detection with discrete subgenomic cDNA fragments. The experiments reported herein were undertaken in preparation for testing clinical CSF specimens for enteroviruses. We have determined the optimal handling and processing conditions for experimentally infected CSF and have evaluated numerous probes for future diagnostic applications.

Storage of CSF specimens at room temperature and at 4, −20, or −70°C even for several days did not appreciably affect the hybridization signals. Similarly, VRC did not affect the signals obtained. It is important to note, however, that infected CSF specimens from a patient with meningitis likely contain RNases and other substances of the inflammatory reaction which might interfere with RNA detection, particularly with prolonged exposure. We therefore will transport clinical specimens quickly from the patient to the laboratory for testing or to storage at −70°C. As VRC did not hinder RNA detection, we will add this substance in hopes of inactivating endogenous RNases. The actual need for these measures in testing clinical CSF samples will be evaluated as patient specimens are gathered over the coming months. Fixation with formaldehyde, which significantly increased the intensity of the signal, likely contributes to the inactivation of endogenous nucleases as well as enhances the uncoating of the rather stable enterovirus particles.

Purulent or bloody CSF may still be tested without a significant loss of a signal, except at the highest erythrocyte counts. ISG interfered with the hybridization assay at concentrations which can occur in the CSF during meningitis. To further investigate this result, we performed a "double-

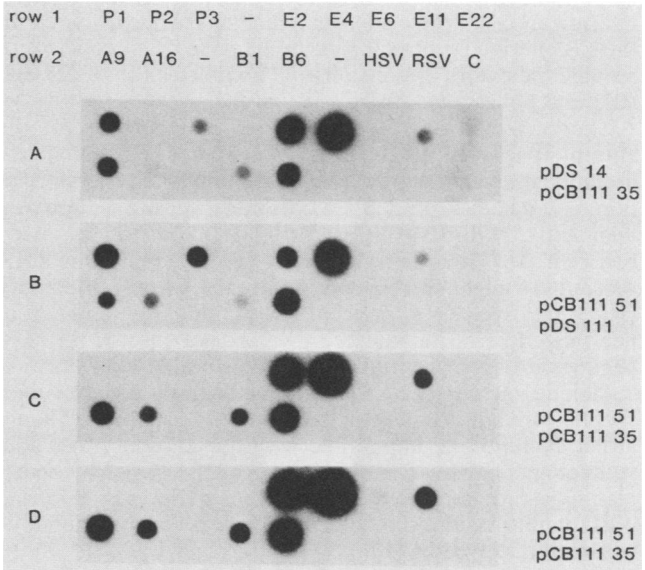

FIG. 6. Dot hybridization patterns obtained with three different combinations of poliovirus 1- and CB3-derived probes. Specimens were prepared as described in the legend to Fig. 5, and the abbreviations are also the same. Autoradiographs A, B, and C were exposed for 24 h; autoradiograph D is of the same membrane filter as that shown in autoradiograph C but exposed for 48 h.

dotting" experiment in which we first applied the entire ISG concentration to the filter in half the CSF volume and then applied, through the same hole in the manifold, all the poliovirus 1 in the remaining half of the CSF volume (12). This reproduced the signal attenuation seen with the original CSF-virus-ISG mixtures, suggesting that the protein was interfering with hybridization at the level of the membrane filter rather than that a specific interaction was occurring in the test tube. We plan to treat all CSF samples with proteinase K, as we have noted no undesirable effects.

A combination of two probes, e.g., pDS 14 plus pCB111 35 or pCB111 51 plus pCB111 35, should detect many enterovirus serotypes. The only exception among those we tested was echovirus 22. This virus is known to be unusual in several respects (6) and may, on further study, be found to be only distantly related to the majority of enteroviruses. This methodology should help further define interrelationships among the enteroviruses and likely will contribute to revisions of current taxonomy. Although the comparative results presented here for multiple enteroviruses studied with multiple specific probes provide preliminary clues to genotypic similarities, the limitations are apparent when the poliovirus 2 signals are considered. Strong hybridization of that serotype with the poliovirus 1-derived clones has previously been shown (11), and nucleotide sequence data show the two serotypes to be quite homologous (8). Using apparently equal amounts of all the enteroviruses studied in the CSF reconstruction experiments described here, however, we found comparatively weak poliovirus 2 signals with the poliovirus 1-derived clones. This is probably the result of inaccuracies in titration of the stock viruses or differences in particle-infectivity ratios among the serotypes or both. Only with RNA purification and titration will accurate homology comparisons be possible. Such work is currently under way in our laboratory. Whatever the actual amount of RNA applied to each dot, however, that same amount was applied to all replicate membranes at the same spot, i.e., echovirus 2 RNA, for example, was at an equal concentration on all membrane strips shown (Fig. 5 and 6). Hence, the stronger hybridizations of the echoviruses and coxsackievirus A with the CB3-derived probes than with the poliovirus probes were reliable indicators of their relative homologies to the cloned CB3 and poliovirus 1 fragments.

The current level of sensitivity of these probes for the enteroviruses—10^2 to 10^5 $TCID_{50}$—may not be adequate for a practical diagnostic test. Although only sketchy information is available on CSF viral titers during enteroviral meningitis, as little as 10^1 to 10^3 $TCID_{50}$ per ml has been reported (16). It is not clear how much actual viral RNA those titers might correspond to; i.e., the particle-infectivity ratio, the free RNA concentrations, and the number of neutralized virions in infected CSF are not known. We have begun a prospective trial of the nucleic acid hybridization technique on clinical CSF specimens from children with aseptic meningitis to assess the utility of such a test. Should direct detection be impossible with our currently available technology, however, amplification of the signal by specimen inoculation for 12 to 24 h in tissue cultures might bring a low viral titer into the detectable range we have found for tissue culture lysates (11).

Whatever the outcome of our direct and amplification trials, we are confident that signifcant technical advances over the next few years will improve both the sensitivity and rapidity of nucleic acid hybridization and that the documented homology among the enteroviruses will make these agents excellent candidates for the application of this technique.

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