Detection of Beta-2 Transferrin in Otorrhea and Rhinorrhea in a Routine Clinical Laboratory Setting

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A simple, straightforward, and rapid method for the detection of beta-2 transferrin in otorrhea and rhinorrhea that can be used in a routine clinical laboratory is described. The beta-2 transferrin was detected by agarose gel electrophoresis of the fluid on Beckman Paragon equipment, followed by pressure transfer to a nitrocellulose membrane and then incubation with enzyme-labeled antitransferrin antibody and substrate. The procedure was fast (3.5 h) and sensitive (detected as little as 1 μg/ml) and required only 3 μl of fluid. Beta-2 transferrin was detected in cerebrospinal fluid diluted up to eightfold. No special training or expertise was needed, and all equipment and procedures used are commonly available in a routine clinical laboratory.

Leakage of cerebrospinal fluid (CSF) from the subarachnoid space into the nasal or aural mucosa creates a pathway for life-threatening central nervous system infection, but detection of the leak can be difficult (6). One approach to this problem has been to analyze the leakage fluid for the presence of a transferrin variant, beta-2 transferrin, which is restricted almost completely to the CSF and to the aqueous humor (2, 6, 9, 12). Initially, beta-2 transferrin was detected by electrophoretic separation of nasal or aural fluid on cellulose-acetate membranes followed by immunofixation with antitransferrin antiserum and staining with Coomassie blue (2, 6). Although this procedure worked well, the sensitivity was poor: the fluid needed to be concentrated 20- to 30-fold and small quantities of fluid could not be analyzed. Moreover, heavy contamination with blood caused decreased resolution. Several improvements to the procedure have been introduced to try to overcome these limitations (1, 8, 13). These improvements involved high-resolution electrophoresis and immunoblotting or immunofixation, so that small volumes of uncentrated fluid could be analyzed. However, these highly sensitive procedures can only be done in specialist laboratories and are not suitable for use in routine clinical laboratories.

Here, we report a simple and straightforward electrophoresis and immunoblotting procedure for the detection of beta-2 transferrin in unconcentrated fluids. The test requires only the equipment, reagents, and expertise normally found in a routine clinical laboratory. It is rapid (3.5 h) and sensitive (can detect as little as 1 μg/ml) and requires only a minimal amount of fluid (3 μl).

MATERIALS AND METHODS

Patient samples. Samples for analysis were those sent to the laboratory for routine analysis for beta-2 transferrin content. These were generally fluids but were sometimes cotton swabs or gauze which had been used to soak up fluid. The samples on the swabs and gauze were eluted with small volumes of sterile saline. Positive CSF controls were CSF samples that had been submitted for syphilis testing and were to be discarded after completion of that analysis. Whenever possible, fluid samples were compared with a serum sample obtained from the patient; a human serum sample that showed no abnormal results in laboratory tests was always included as a negative control.

Electrophoresis and blotting. Electrophoresis was performed on a Beckman Paragon electrophoresis kit with immunofixation (IFE) gels (Beckman Instruments, Brea, Calif.). The gels were prepared by soaking for 15 min in excess barbitral buffer (10 mM 5,5-diethyl barbituric acid·50 mM 5,5-diethyl barbituric acid sodium salt containing 2.16 mM calcium lactate). The soaked gel was blotted, the sample template was applied and sealed, and serum, CSF, and fluid samples were applied with the Paragon sample applicator. Serum samples were diluted 1:50 in barbital electrophoresis buffer, whereas CSF and fluid samples were added undiluted (3 μl). After the samples had diffused into the gel for 10 min, the gel was electrophoresed in a Paragon electrophoresis cell at 100 V for 30 min.

For transblotting, the gel was placed onto three pieces of thick filter paper (3M; Whatman, Hillsboro, Ore.) soaked with 0.1 M Tris·0.77 M glycine·20% methanol·0.025% sodium dodecyl sulfate (Sigma, St. Louis, Mo.) and then covered with a slightly larger piece of soaked nitrocellulose. A glass pipette was used to roll out any air bubbles, three pieces of buffer-soaked 3M filter paper were placed on top of the nitrocellulose, and the sandwich was rerolled to eliminate air bubbles. A 2-in.-thick (ca. 5-cm-thick) wafer of dry brown paper towels was placed on top of the wet sandwich, and pressure was applied by placing a 2-in.-thick laboratory catalog on top. The pile was left undisturbed for 1 h at room temperature for transfer to occur. After transfer, the top surface of the nitrocellulose was marked to identify it (this was the back), and the membrane was placed face up in a shallow dish and washed twice with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST: 0.01 M potassium phosphate [pH 7.4] in 0.15 M sodium chloride) (Sigma).

Immunostaining. The nitrocellulose membrane was blocked by immersion in PBS containing 1% bovine serum albumin (PBS-BSA) (Sigma) for 15 min, and then 20 ml of a solution containing 2 ml of normal goat serum (GIBCO BRL, Gaithersburg, Md.), 0.1 ml of horseradish peroxidase-labeled goat anti-human transferrin (E-Y Laboratories, Inc., San Mateo, Calif.), and 17.9 ml of PBS-BSA was added and incubated with rocking for 60 min in the dark. The membrane was rinsed briefly twice with PBST, twice with substrate buffer (0.01 M Tris buffer, pH 7.4) with rocking, and then with the substrate

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diaminobenzidine (Sigma; 20 mg per 50 ml of buffer) plus 50 μl of 30% hydrogen peroxide (Sigma). When bands became visible, the reaction was stopped by pouring off the substrate solution and rinsing the membrane twice with deionized water. The membrane was then blotted dry.

**Rate nephelometry.** Rate nephelometry was performed with the Beckman Array rate nephelometer and Beckman antibodies and calibrators according to the manufacturer’s instructions.

**Sensitivity.** The sensitivity of the method was determined by analysis of known concentrations of serum transferrin by rate nephelometry. Human serum with a low normal level of beta-1 transferrin (200 mg/dl; normal reference range, 252 to 429 mg/dl) was diluted to appropriate levels with electrophoresis buffer and analyzed. To check that these results applied to CSF, two samples of CSF with a normal total transferrin level were diluted with electrophoresis buffer and analyzed.

**Interpretation.** Since the usual answer required was simply whether beta-2 transferrin was present in the fluid, visual examination of the membranes was sufficient.

**RESULTS**

Representative results of fluid analyses for beta-2 transferrin are shown in Fig. 1. Clear separation between beta-1 and beta-2 transferrin was achieved; samples a and b contained beta-2 transferrin, whereas samples c and d did not.

The limit of detection of the assay was 1 μg of transferrin per ml (Fig. 2). This assay was repeated several times with different serum and CSF samples.

The normal level of total transferrin, both beta-1 and beta-2, in CSF was determined by rate nephelometry to be 2.6 ± 1.2 mg/dl (N = 9). On average, about 25% of the transferrin in CSF was the beta-2 variant, as determined by visual estimation, suggesting a level of about 0.8 mg of beta-2 transferrin per dl in normal CSF, or 8 μg/ml. Since the limit of detection was 1 μg/ml, beta-2 transferrin should still be detectable in CSF diluted up to eightfold by other fluids. To test this point, two CSF samples with average levels of total transferrin were diluted with barbital electrophoresis buffer and analyzed for beta-2 transferrin. As shown in Fig. 3, each was clearly positive for beta-2 transferrin at a 1:8 dilution.

The assay has been in use for 12 months, and during this time, 63 samples from 41 patients have been examined. Of these 63 samples, 31 (49%) were positive for beta-2 transferrin. Most of these samples were submitted because of suspected CSF leakage secondary to a primary diagnosis which involved surgery. Of seven patients with known CSF leakage, all were positive for beta-2 transferrin in the leakage fluid before surgery to correct the fault and negative after surgery. Many requests for the assay now come from the emergency room. The average time for completion of the test is 3.5 h, but this time can be decreased by shorter incubation times if necessary. Conversely, the incubation steps can be left longer if needed to fit with work schedules. Each of the on-call technologists in the clinical immunology laboratory is proficient with the assay; training has required only one observed run and one actual run for proficiency.

The direct cost of the test was determined to be $87.10, allowing $0.96 per minute for technologist time.

**DISCUSSION**

CSF leakage may present as otorrhea or rhinorrhea or may be suspected from repeated episodes of meningitis. Chemical detection of CSF in these fluids depends on the finding of a higher glucose content than expected for mucosal secretions, but this finding is unreliable if serum is present (4, 5). The
direct observation of leakage after intrathecal injection of a dye or radioisotope may be difficult and possibly hazardous to the patient and is not easily repeated (7). Thus, detection of a leak by means of a CSF-specific component is an attractive alternative procedure.

Beta-2 transferrin is not absolutely specific for CSF. It is also found in the aqueous humor (12) and in the serum of patients with liver disease due to chronic excessive alcohol ingestion (11). However, these findings should not influence the detection of beta-2 transferrin in cases of otorrhea or rhinorrhea. Allelic variants of transferrin can have altered mobility and may lead to false-positive detection of beta-2 transferrin in leakage fluids (10). The frequency of such variants, especially cathodic variants, is very low, however (3), and the different mobility should be obvious compared with that of the normal serum transferrin control. We have not encountered any of these variants in the samples studied so far. Nevertheless, the possibility of error due to these variants remains, and it would clearly be best to analyze a serum sample from the patient as well as the normal serum transferrin control at the same time.

The methods initially developed to detect beta-2 transferrin as a marker of CSF leakage were not very sensitive (2, 6), but improvements to the technique have made it difficult for the routine laboratory to use (1, 8, 13). For example, the procedure of Reisinger and Hochstrasser (8) requires electrophoresis on polyacrylamide gels, electroblocting of the bands onto a nitrocellulose membrane, and development of the protein bands with antitransferrin antibody and enhanced protein A-gold solution or with horseradish peroxidase-labeled antitransferrin antibody. Fransen et al. (1) used agarose gel electrophoresis followed by immunoblotting onto a nitrocellulose membrane coated with the immunoglobulin G fraction of goat anti-human transferrin antibody. Transferrin which bound to the coated membrane was then detected with biotinylated antitransferrin (the same antibody used to coat the membrane) followed by streptavidin-biotin-horseradish peroxidase complex and substrate. These authors claimed a 10-fold increase in sensitivity over the Reisinger and Hochstrasser (8) procedure, with a turnaround time of 8 h. Zaret et al. (13) used electrophoresis on Panigel high-resolution agarose membranes, immunofixed the membranes with antitransferrin, and stained them with Coomasie blue. The percentage of transferrin in the beta-1 and beta-2 regions was estimated by densitometry, and the amount of beta-2 transferrin was calculated from the total transferrin content, obtained for a separate aliquot by nephelometry. Zaret et al. obtained the clearest results with fluid concentrated at least twofold or when sequential aliquots were applied to the gel. Unfortunately, the Panigel electrophoresis equipment used by these authors is not in general use in routine clinical laboratories, and Zaret et al. obtained unsatisfactory results with the more common Beckman IFE agarose system. Thus, none of these three procedures is suitable for use in a routine clinical laboratory.

Our objective was to develop a flexible procedure for the detection of beta-2 transferrin in fluids with routine equipment and procedures. Thus, we did not want to use specialized equipment or procedures, we did not want to use polyacrylamide gels, and we did not want to have to pretreat nitrocellulose membranes with specific antibody or perform silver staining, since these procedures would not be appropriate for a routine laboratory. We were willing to sacrifice some sensitivity for speed and ease of procedure, but we believe that a sensitivity of 1 μg/ml, which correlates with the ability to detect beta-2 transferrin in CSF which has been diluted up to eightfold with other fluids, is adequate.

The value of this procedure is that is uses equipment and expertise found in routine clinical laboratories, provided that these laboratories perform serum protein electrophoresis. The reagents are widely available and easy to use. No special training or expertise is required, and we have found that technologists learn the procedure very quickly.

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REFERENCES