

Separation of β_2 -Transferrin by Denaturing Gel Electrophoresis to Detect Cerebrospinal Fluid in Ear and Nasal Fluids

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Background: Cerebrospinal fluid (CSF) leakage is a critical condition with a substantial risk of meningitis. We investigated the use of transferrin isoform analysis as a diagnostic marker for detection of CSF leakage in fluid samples.

Methods: We analyzed 241 samples from patients with CSF leakage, most commonly presenting as otorrhea or rhinorrhea, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent Western blotting and immunostaining for transferrin. Tears, saliva, nasal fluid, and ear secretions (20 samples each) were analyzed in parallel, and normal human serum served as a control in each experiment. We compared the minimum volume of added CSF that could be detected in secretions by our assay with the minimum volume detected by the prostaglandin-D synthase (β -trace) test. CSF was admixed with blood in different proportions to determine the influence of blood contamination on the transferrin pattern.

Results: In all CSF samples, β_1 - and β_2 -transferrin were present in nearly equal amounts. In tears and ear secretions, β_2 -transferrin migrated in the gel in the same manner as in CSF, but its concentration was noticeably lower than that of β_1 -transferrin, a difference that allowed a clear distinction from the transferrin pattern of CSF. In saliva, both transferrin isoforms were also present but could be distinguished from those of other fluids by electrophoretic migration pattern rather than

relative concentrations. With the β -trace test, a minimum of 5 μ L of CSF was needed for detection, whereas our β_2 -transferrin assay yielded a signal of comparable intensity with a minimum of 2 μ L of CSF.

Conclusion: Analysis of the transferrin microheterogeneity pattern by SDS-PAGE for the identification of CSF leakage is a highly sensitive and specific method that merits consideration as a routine technique.

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Leakage of cerebrospinal fluid (CSF) is a critical condition harboring a substantial risk of meningitis with potentially fatal outcome. Because rhinorrhea and otorrhea may consist of a variety of body fluid secretions, reliable identification of CSF leakage is crucial for adequate clinical management. Various techniques, including x-ray and magnetic resonance imaging analysis, assessment of glucose concentration, and protein-chemical studies, have been used for diagnosis, but none yield entirely satisfactory results. Glucose tests lack sensitivity and specificity and have been largely abandoned. Radiographic and magnetic imaging methods are not always successful and require expensive, time-consuming procedures (1).

High separation efficiency can be achieved with a relatively limited amount of equipment by use of electrophoretic techniques that allow detection, purity verification, and qualitative characterization of oligoclonal bands. Numerous studies have been published on this topic, some using polyacrylamide gels (2–4) or agarose gels (5, 6) combined with immunofixation (7, 8) or with fluorescein (5), Coomassie Brilliant Blue (4), or silver staining (3). In addition to differences in band patterns, 2 proteins, prostaglandin-D synthase (β -trace) and transthyretin (prealbumin), have been identified as possible specific constituents of CSF.

In this study we investigated the utility of transferrin as a diagnostic marker for liquorrhea. Transferrin is an iron-binding monomeric glycoprotein with a molecular

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mass of ~78 kDa and containing 4 negatively charged sialic acid groups (9,10). This protein binds iron very strongly but reversibly, protecting the body against the free radical damage associated with unbound iron (11,12). The location of the transferrin gene together with many of its structural features and the amino acid sequence of its protein product have been established (13,14). In CSF, 2 transferrin isoforms can be detected, and several pathologic and physiologic conditions may induce variations in the microheterogeneity pattern of transferrin (15). Over the years, many experiments were designed to study the relevance of transferrin heterogeneity not only to CSF leakage but also to various disorders of the central nervous system (16–18). These approaches, although useful, did not always succeed in tracing transferrin in cases of small or delayed CSF leaks (19).

Having performed preliminary experiments to test different electrophoretic separation methods, including isoelectric focusing and disc and agarose gel electrophoresis, we set out to ascertain the reproducibility and reliability of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and to demonstrate the sensitivity and specificity of transferrin heterogeneity and its superiority to β -trace and transthyretin assessment in the diagnosis of liquorrhea.

Materials and Methods

COLLECTION OF SAMPLES

In this retrospective study, CSF samples from 241 patients without acute or chronic infectious diseases were collected from 1998 through 2003 at the Department of Otorhinolaryngology, Head and Neck Surgery at the Christian-Albrechts-University of Kiel. Sample collection was conducted in accordance with the Helsinki Declaration of 1975 as revised in 1996, and written informed consent was given by all patients. All CSF samples were gathered during regular diagnostic procedures. The patients presented with either rhinorrhea or otorrhea suspected to be CSF leakage. CSF leaks were ascertained by fluorescein staining (intrathecal fluorescein application) in all instances. In addition, high-resolution computed tomography and sequential magnetic resonance imaging were performed to locate the defects and to estimate their extent. Of the CSF samples, 173 were from patients with bone fractures of the basal skull. In 98 of these cases, liquorrhea occurred as otoliquorrhea and in 75 cases as rhinoliquorrhea. For the remaining 68 patients, no basal skull fracture could be verified, but 53 of these patients had a history of surgery of either the paranasal sinuses or the middle ear. In these cases, the CSF leak was not recognized during surgical intervention because of the small size of the defect and the absence of an obvious CSF drain. Postsurgical fluorescein assessment nevertheless revealed liquorrhea indicating dural leakage. In 15 cases, the cause of liquorrhea remained unclear.

In all cases, CSF samples were obtained by inserting a dry absorbent piece of cotton into either the nasal cavity

or the auditory canal. Depending on the extent of leakage, the absorbent cotton remained in place for up to 30 min. The cotton was then placed in a collection tube and centrifuged, and the supernatant was analyzed.

For control experiments, tears, saliva, and secretions from the nose and ears were obtained from patients without history of liquorrhea. Twenty samples of serous, mucous, and seromucous secretions were collected during surgery from the middle ears of patients suffering from otitis media. In these cases, the fluid was collected by use of a suction tube with an integrated collection tube. Nasal cavity fluid from 20 healthy individuals was procured as described above from patients with rhinoliquorrhea. In addition, tear samples from 20 healthy volunteers without evidence of ocular-surface disease were collected in glass capillary micropipettes. Plasma samples were obtained from healthy donors. Finally, CSF samples admixed with blood in different proportions were analyzed to determine how contamination of CSF with blood influences the transferrin pattern.

GEL ELECTROPHORESIS

For SDS-PAGE, the samples were centrifuged (20 000g for 5 min) and denatured by heating at 95 °C for 3 min in a modified gel-loading buffer containing 15 g/L glycine, 3.5 g/L Tris-HCl (pH 6.8), 1 g/L SDS, 100 mL/L glycerol, and 1 g/L bromphenol blue but no β -mercaptoethanol. After denaturation, 20- μ L aliquots of the supernatants were loaded on 1-mm thick gels and separated by electrophoresis using the Laemmli (20) discontinuous buffer system at a constant 10 W and 10 °C until the bromphenol blue reached the anodic border of the gel. When different amounts of a sample were tested, phosphate-buffered saline was added to the sample to obtain a loading volume of 20 μ L. For each run, normal human blood serum diluted 1:120 with gel-loading buffer was used as a control.

WESTERN BLOTTING

After electrophoretic separation, proteins were transferred to nitrocellulose membranes by semidry blotting for 45 min by use of the Biometra electroblotter at a constant 100 mA and 10 °C. The membranes were allowed to react for 30 min with the primary goat anti-human transferrin antibody (Calbiochem) directed against specific amino acid sequences shared by both isoforms (β_1 and β_2) of the transferrin protein. The antigen–antibody complex was visualized by use of biotinylated rabbit anti-goat immunoglobulin and horseradish peroxidase (Dako). The reaction was stopped by rinsing the nitrocellulose with 0.5 mol/L HCl. Transthyretin was detected by use of a primary rabbit polyclonal antiserum (Dako) diluted 1:400 and goat anti-rabbit immunoglobulin as a secondary antibody. For assessment of β -trace, we used a rabbit polyclonal antibody (our own product) and the same secondary antibody as above. The optimum concentration (1:500) for the anti- β -trace antiserum was deter-

mined by use of serial dilutions of highly purified β -trace from human CSF (N protein standard UY). When diluted 1:500, the antiserum detected β -trace at a minimum concentration of 0.1 g/L in Western blots and stained a single band in the Western blot analysis of undiluted CSF and blood serum samples. Band intensity was measured semi-quantitatively by densitometry using the documentation system BioDoc-II (Biometra). Negative controls were obtained by omission of the primary antibody.

STATISTICAL ANALYSIS

The Statistical Package for the Social Sciences (SPSS) software, Ver. 10.0 (SPSS, Inc.), run on a PC computer was used for all calculations. Densitometric profiles were generated for each electropherogram, and mean and median (SD) values were calculated. Sample categories were compared by use of the Mann-Whitney *U*-test, the Kruskal-Wallis test, and χ^2 statistics. Specificity and sensitivity were assessed by use of ROC curve analysis.

Results

The complexity and diversity of the protein composition in CSF, blood serum, tears, saliva, nasal fluid, and ear secretions were assessed by preliminary electrophoretic analysis (Fig. 1). When the individual migration patterns were compared, no similarity was found. In all samples, the most highly stained bands were of relatively high molecular mass (70–90 kDa), and a few of them were present in all samples. However, the majority of proteins showed distinct migration characteristics and thus appeared to be sample specific. Among the samples analyzed, nasal secretions were found to exhibit the greatest protein heterogeneity.

After SDS-PAGE analysis, transferrin was identified in the complex protein mixture of CSF by use of Western

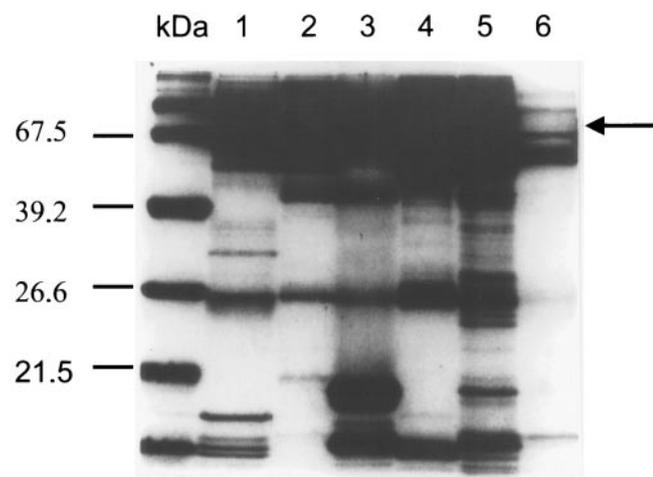


Fig. 1. SDS-polyacrylamide gel stained with Coomassie Brilliant Blue. Distinct protein patterns are seen with CSF (lane 1), serum diluted 1:120 (lane 2), tears (lane 3), ear secretions (lane 4), nasal fluid (lane 5), and saliva (lane 6). The arrow indicates the approximate position of transferrin. The molecular masses of the size markers are shown on the left.

blotting and staining with a transferrin-specific antibody that recognized both the β_1 and β_2 isoforms. The least amount of CSF allowing the detection of transferrin was 0.1 μ L, demonstrating the very high analytical sensitivity of our technique (Fig. 2).

In CSF, the 2 transferrin isoforms (β_1 and β_2) were regularly present in nearly equal amounts. When the density of the β_1 band was considered to be 100%, the mean (SD) density of the β_2 band was 86.9 (11.2)%. As a rule, 2 μ L of CSF was used for routine analysis, an amount sufficient for confident evaluation of individual samples. All CSF samples were compared each time with sera from healthy donors serving as a control. Control samples showed only the β_1 isoform when diluted 1:120. Thus, the detection of a β_2 -transferrin band with a relative density >60% was sufficient to diagnose CSF leakage with 100% sensitivity and 100% specificity ($P < 0.0001$). The β_2 isoform is also present in blood serum, but its concentration is much lower than that of β_1 ; therefore, it was not detectable at the indicated dilution.

Characteristic transferrin patterns for tears, saliva, and ear and nose secretions are shown in Fig. 3. The band pattern illustrates the presence of 2 transferrin isoforms in tears. Although the β_1 concentration is nearly similar to that in CSF, the β_2 band is barely detectable [relative density, 7.6 (3.3)%; $P < 0.0001$]. In saliva, the migration pattern clearly differed from that of tears and CSF. Both the β_1 and β_2 isoforms were detectable, and the intensities of the corresponding signals were roughly similar [mean density of the β_2 band, 97 (5.7)%; P not significant]. However, a shift was noted in the migration of the β_2 isoform, indicating a physical or chemical modification of this protein. When equal sample volumes were analyzed, the transferrin concentration was \sim 10-fold lower in saliva than in CSF.

In middle ear secretions, the intensity of the β_1 band was 20 times stronger than in CSF when equal volumes were examined, whereas only traces of the β_2 isoform were detectable [mean relative density 3.7 (1.2)%; $P < 0.0001$]. The β_2 isoform usually became evident only when the sample volume was increased, with \sim 3 times

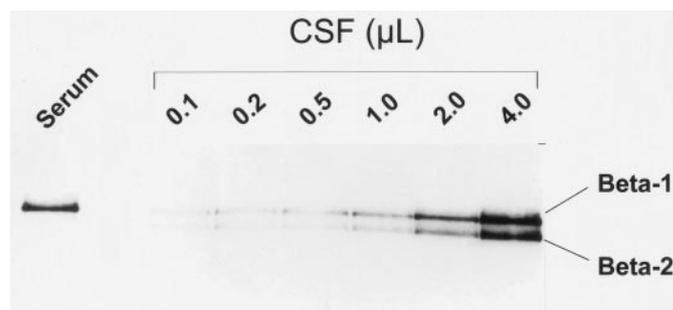


Fig. 2. Immunologic detection of immobilized transferrin isoforms after the proteins were transferred from SDS-polyacrylamide gels to a solid support.

Diluted serum (1:120) used as a control shows only the β_1 isoform, whereas both the β_1 and β_2 isoforms are clearly detectable in different volumes of CSF.

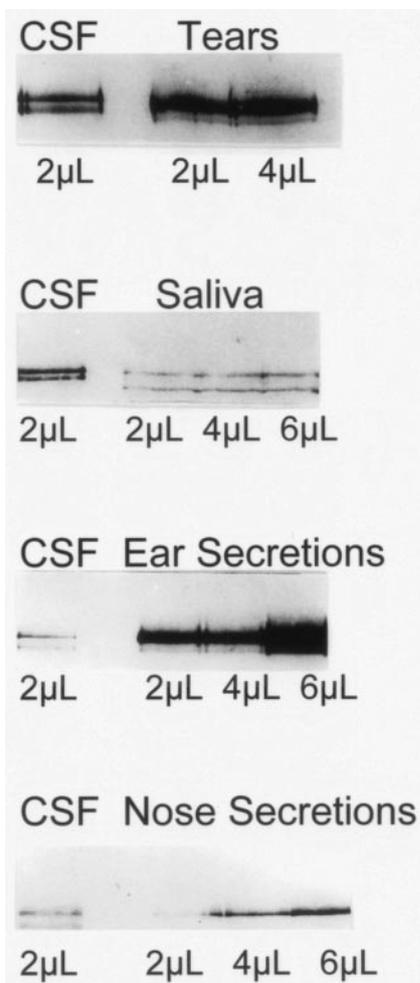


Fig. 3. Typical patterns of transferrin isoforms detected in different fluids and secretions compared with the transferrin microheterogeneity pattern of CSF after SDS-PAGE and Western blotting.

the amount of ear secretions being necessary to produce a signal similar to that seen with 2 μL of CSF.

Transferrin pattern analysis in nose secretions revealed only the β_1 isoform ($P < 0.0001$). Although the staining intensity of β_1 increased proportionally to the sample volume, no band corresponding to the β_2 isoform was apparent even when large amounts of sample were loaded.

Electrophoretic examination of CSF is difficult in the event of blood contamination, which may result from a variety of injuries or surgical treatments. Because blood serum contains both the β_1 and β_2 isoforms, with a relative excess of the β_1 isoform, contaminated CSF is not readily distinguishable from blood. The influence of blood contamination on the transferrin pattern in CSF is shown in Fig. 4. An excess of the β_1 isoform can be seen when CSF is admixed with as little as 1% blood (by volume), and the predominance of the β_1 isoform becomes more apparent when increasing proportions of blood are added. This observation is particularly important for the interpretation of β -transferrin patterns. In-

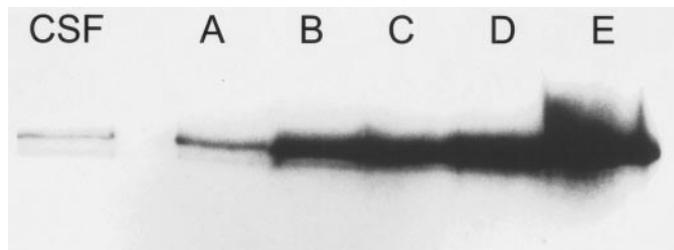


Fig. 4. Transferrin pattern of CSF mixed with serum.

The β_1 signal increasingly overshadows the β_2 signal when the proportion of serum augments: lanes show admixtures of 1% (A), 2% (B), 5% (C), 10% (D), and 50% serum (E) by volume.

deed, a shift in the relative band intensity toward an increment of the β_1 isoform is likely to indicate that blood contamination is blurring the transferrin pattern of CSF so that it is no longer distinctive. Obliteration of the β_2 band by excess β_1 occurs as soon as the proportion of blood contamination attains 2% of the sample volume (Fig. 4).

To demonstrate the utility of β_2 -transferrin detection for the diagnosis of CSF, we compared the sensitivity of the prostaglandin-D synthase (β -trace) and transthyretin protein tests with our β_2 -transferrin assay by simultaneous assaying for the 3 proteins in the same CSF samples by Western blotting and subsequent immunostaining with appropriate antibodies. As shown in Fig. 5, a minimum of 5 μL of CSF is needed for the detection of a β -trace signal, whereas 2 μL of CSF yielded a β -transferrin double band of comparable intensity.

Transthyretin, on the other hand, was detectable in both blood serum and CSF, and the signal intensity obtained with the diluted serum sample corresponded approximately to that detected in 5 μL of CSF (Fig. 6). This finding indicates that transthyretin detection is not CSF specific and that even a quantitative analysis is unlikely to allow differentiation between serum and CSF.

Discussion

Although several recent studies showed that CSF can be identified by use of different electrophoretic methods designed to separate transferrin isoforms (21–25), the differential diagnosis of CSF otorrhea and rhinorrhea remains notoriously difficult. Transferrin occurs in a wide

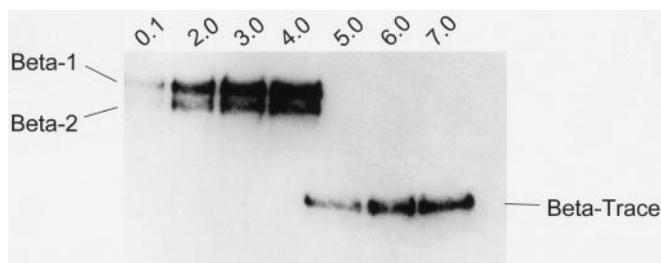


Fig. 5. Western blot illustrating the immunologic detection of transferrin isoforms in comparison with the β -trace protein.

β -Trace is undetectable in $< 5 \mu\text{L}$ of CSF, whereas a strong double band corresponding to transferrin is already apparent in 2 μL of CSF.

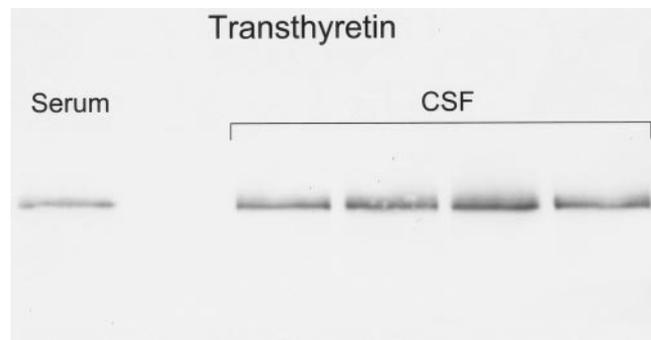


Fig. 6. Transthyretin is detectable in both CSF and serum diluted 1:120.

The protein concentration in CSF may be higher, but this feature does not appear to allow differentiation.

variety of isoforms (26), and its microheterogeneity patterns may vary under several pathologic and physiologic conditions (15). Nevertheless, an initial diagnosis of CSF leakage can be made by examination of the microheterogeneity pattern, which also depends on a genetically determined variation in the amino acid sequence (15) or on different degrees of sialylation and galactosylation (27). Thus, analysis of the relative band intensities of the β_1 and β_2 isoforms in CSF can make possible early diagnosis of CSF leakage and consequent prevention of meningitis.

This study demonstrated that highly resolving SDS-PAGE for the analysis of β -transferrin in CSF, a method that allows the simultaneous analysis of up to 20 probes in 4–5 h, is highly sensitive and reproducible. The particular advantage of this technique is that no proteins are lost, whereas with other staining methods proteins frequently diffuse out of the gel because they are not irreversibly fixed in focusing or native gels (28).

Our method requires fresh CSF samples that are free of solid particles or precipitates that interfere with the separation by blocking the pores of the matrix. Another critical factor is the sample buffer. Sample buffers for SDS electrophoresis usually contain mercaptoethanol, which reduces the disulfide bridges of the proteins and would cause dissociation of both the β_1 - and β_2 -transferrin isoforms into 2 subunits each. Therefore, a sample buffer without mercaptoethanol must be used to detect the 2 nondissociated transferrin isoforms in CSF.

According to reports from numerous studies dealing with CSF otorrhea or rhinorrhea, β_2 is the only characteristic transferrin isoform for CSF and is not encountered in tears, nasal fluid, saliva, or serum (26, 29, 30). In contrast, the results presented here document the invariable presence of both the β_1 and β_2 isoforms in blood, tears, ear secretions, and saliva. Only nose secretions were found to lack the β_2 isoform.

In tears and ear secretions, β_2 -transferrin migrates in the gel in the same manner as in CSF, but its concentration is noticeably lower than that of β_1 , a difference that allows a clear distinction from the transferrin pattern of CSF.

Both transferrin isoforms are also present in saliva, in which the significant difference is the mobility of the β_2 isoform, which is attributable to an altered molecular mass. Serum also contains both transferrin isoforms, but β_1 is strongly predominant. Therefore, a reliable examination of CSF rhinorrhea or otorrhea is not possible when CSF is contaminated by blood. As indicated in Fig. 4, even traces of blood produce an overload of the β_1 isoform, making diagnostic examination difficult. This phenomenon is known from a previous study, which examined the detectability of β_2 -transferrin in different mixtures of CSF and blood serum (31).

Another caveat is that certain pathologic conditions may disturb the typical microheterogeneity pattern of transferrin in serum. Severe chronic alcoholism was shown to produce an increase in relative β_2 -transferrin concentrations (32), apparently as a result of diminished activity of liver glucosyltransferase (33). Liver function tests may therefore be advisable when the differential diagnosis of liquorrhea is assessed.

Earlier studies investigated prostaglandin-D synthase (β -trace protein) for the diagnosis of liquorrhea and various neurologic diseases (21–23). We therefore compared this test with our β_2 -transferrin assay by simultaneous detection of both proteins within the same CSF aliquot. The results showed that although the β -trace test is specific, the detection limit is much higher than that for β_2 -transferrin. This observation is in line with the report of a proportion of false-negative results in a β -trace assay using Laurell rocket immunoelectrophoresis (34).

Because the absence of CSF fistulas in our control cases was not verified with clinical or radiologic methods, false-negative results cannot be excluded in our transferrin assay or the assays for the other proteins examined in this study. However, we consider the probability of CSF leakage in healthy individuals to be low enough to justify our study design.

Recently, an automated procedure for quantification of β -trace protein in body fluids was developed (34, 35). This nephelometric assay has shown high specificity, rapid performance, and sensitivity in the range of 92%–100% (35–37). In one study, qualitative assessment of β_2 -transferrin in nose secretions showed a sensitivity of 93% and specificity of 97% (36). Although this observation clearly indicates a lower sensitivity of the immunofixation method compared with SDS-PAGE and Western blot analysis, the finding of false-positive results (~3%) is difficult to interpret in light of our observations documenting the absence of β_2 -transferrin in nasal fluid. The detection of small amounts of the β_2 isoform in nose secretions of patients without CSF leaks could nevertheless be attributable to contamination with serum or tears.

Transthyretin (prealbumin) has also been proposed as a diagnostic marker for CSF (32). In this study, we regularly detected transthyretin in diluted blood serum samples at concentrations that were similar to those in CSF, indicating that this protein not likely to provide

diagnostic information when assayed with our method. Our results are attributable to protein denaturation during SDS-PAGE, which hampers differentiation between the CSF-specific monomeric form and the tetrameric form present in blood (38). This distinction would require more sophisticated techniques (39) that are unlikely to become routine procedures but may be helpful in difficult situations, e.g., in the case of blood contamination.

In summary, we found that CSF leakage in otorrhea or rhinorrhea can always be detected by identifying the β_2 isoform of transferrin. Although saliva, tears, and ear secretions also exhibit transferrin microheterogeneity, the concentration pattern of the protein isoforms differs from that found in CSF. Therefore, CSF can also be detected in a mixture containing these fluids and secretions. The method presented here is easily performable and therefore well suited for routine applications. Although it is more time-consuming than the nephelometric β -trace test, our assay may be particularly useful for the detection of small amounts of CSF in nose and ear secretions, which may fall into a diagnostic gray zone with the nephelometric approach (35, 36). Nevertheless, our method shares with all others based on immunologic reactions the problem of identifying the β_2 isoform in blood-contaminated CSF samples. At present, all analyses to detect CSF otorrhea or rhinorrhea are carried out with polyclonal antisera that recognize all existing genetic variants of transferrin. Consequently, it is not possible to predict the efficiency with which a given polyclonal antiserum will detect different antigenic epitopes of an immobilized, denatured target protein. With a view toward increasing the specificity of our technique, we are currently developing an immunologic assay based on the reactivity of a monoclonal antibody directed solely against the β_2 isoform.

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