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Two-dimensional protein electrophoresis and multiple hypothesis testing to detect potential serum protein biomarkers in children with fetal alcohol syndrome

Fetal alcohol syndrome (FAS) surveillance and intervention efforts are hampered by the lack of a specific biochemical test for diagnosis of the syndrome. Based on the hypothesis that abnormalities in growth and development (key features of FAS) involve altered protein metabolism, we analyzed serum proteins by two-dimensional gel electrophoresis and image analysis to search for potential protein biomarkers of FAS. Serum samples from 12 participants in whom FAS had been diagnosed and 8 sex- and age-matched participants whose mothers did not consume alcohol were analyzed in duplicate to determine whether the integrated intensities of matched proteins are significantly altered in children with FAS. Multiple hypothesis testing on 34 of the gels consisting of more than 1700 spots per gel revealed 21 proteins that we classified as potential protein biomarkers of FAS on the basis of significant t-test differences at $p < 0.02$. We classified 8 of the proteins as candidate biomarkers on the basis of significant concentration differences between case and control subjects at $p < 0.01$. One of the proteins is clearly an isoform of retinol binding protein; two appear in the area of the gel where alcohol dehydrogenase is expected to appear; one appears to be an isoform of alpha-1-antitrypsin; three appear to be isoforms of the beta-chain of haptoglobin; three may be forms of immunoglobulin light chains; and several others have not been associated with known proteins. No single protein differentiated all case subjects from control subjects, but stepwise canonical discriminant analyses revealed four groups of spots that distinguished between FAS case and control subjects with no misclassifications. Because of the small number of samples analyzed, we have not positively identified the proteins. It will be necessary to confirm our observations using additional samples before identifying each protein.

1 Introduction

The leading preventable cause of mental retardation in the world is fetal alcohol syndrome (FAS) [1]. FAS is the most severe of a spectrum of alcohol-related birth defects (ARBD) which have not been categorized, but are probably due to differences in genetic susceptibility of the mother and child, and the level, time, and condition of exposure. The clinical symptoms characteristic of the syndrome, pre- and postnatal growth retardation, craniofacial dysmorphology, and central nervous system abnormalities, have been described in detail by several investigators [2–5]; however, it is often not diagnosed, particularly in the newborn period [6] because recognition requires special training. Postnatal growth retardation, intellectual and neurological abnormalities, behavioral dysfunctions (hyperactivity and poor coordination), and sensory difficulty due to visual, speech, and hearing impairment may not be obvious for some time after birth. There is no clinical test for the syndrome. It is estimated that more than 1200 children are born with FAS

each year in the United States [7]. For many affected children, the syndrome is never diagnosed, so the prevalence of FAS is largely unknown [8].

The lack of a specific biochemical marker for FAS hampers surveillance efforts and clinical intervention. The search for protein biomarkers of FAS in humans is possible and advantageous because (i) several of the proposed mechanisms for FAS implicate altered protein metabolism [9]; (ii) there are a large number and variety of proteins (> 50000) in human tissue and biological fluids [10]; (iii) the technology (two-dimensional gel electrophoresis with silver staining and image analysis) is available to separate, visualize, and analyze the proteins in many types of biological samples and to create and query multiple-gel protein databases [11–14]; and (iv) protein markers are useful for developing high-throughput screening immunoassays. It is necessary to develop tests to detect FAS among children because many mothers still do not receive prenatal care, and attempts to prevent alcohol-related birth defects are often unsuccessful. Knowledge of biomarkers will provide clues to the mechanism by which alcohol-induced fetal damage occurs and may lead to the development of intervention strategies.

The developing fetus is directly exposed to ingested ethanol and its major metabolite, acetaldehyde, and

* Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the US Department of Health and Human Services.

there are numerous indications that alcohol-related birth defects are the result of altered protein synthesis and modification [15–17]. Chronic exposure to ethanol produces an elevation in the enzymes γ-glutamyl transferase, aspartate aminotransferase, alanine aminotransferase, and glutamate dehydrogenase in the serum of humans [18, 19]. Among pregnant women, blood levels of γ-glutamyl transferase and mean cell volume were predictive of FAS in 62% and 41%, respectively, of the cases of FAS reported by Ylikorkala et al. [20], while low levels of maternal serum α-fetoprotein and pregnancy-specific β-1-glycoprotein each predicted FAS correctly in 59% and 56%, respectively, of the cases reported by Hal-mesmaki et al. [21]. However, no studies have evaluated the levels of these enzymes in the human offspring of alcoholic mothers. Apparently, the only proteins that have been examined in the offspring of alcohol-abusing women are renin [22], growth hormone and somatome-dins [23], and plasma albumin [24]. None of the proteins was shown to be altered among children with FAS.

Alcohol dehydrogenase, aldehyde dehydrogenase, and monoamine oxidase [25] have been associated with increased risks for alcoholism, but clinical evidence is not sufficient to verify the usefulness of these proteins as biochemical markers for genetic susceptibility to alcoholism or to FAS outcomes among offspring of alcohol-abusing women. Other specific protein modifications have been associated with alcoholism, but few have been investigated as links to FAS, and none has been shown to be sufficiently sensitive or specific to serve as the basis for a biochemical test. Glycoproteins such as transferrin [26] and β-hexoseaminidase, which increases during pregnancy [27], show alterations in the serum of alcoholics. ³H-mannose and galactose have been used to show that the process of glycosylation is altered in isolated hepatocytes exposed to alcohol [17]. Protein-acetaldehyde adduct formation has been shown to occur both in vivo and in cell culture during chronic alcohol exposure [28] and hemoglobin-acetaldehyde adducts have been shown to be elevated in women carrying alcohol-damaged fetuses [29]. Alcohol exposure reduces methylase activity in fetal nuclei [30], causing altered DNA methylation, which may have an important role in gene expression. Even if altered proteins are formed in the fetus, there is no reason to assume that they would be altered in the offspring unless gene expression is altered.

Two-dimensional electrophoresis (2-DE) of proteins with ultrasensitive silver-staining and computerized image analysis allows detection of several thousand proteins simultaneously. Such analyses have been done on serum, urine, whole blood, spinal fluid, and extracts of various types of tissue to detect alterations caused by various diseases or toxic exposures. Several researchers have used 2-DE successfully to search for protein alterations in various diseases or toxicant exposures [31–34]. 2-DE has already revealed abnormal heterogeneity of α-1-acid glycoprotein, α-1-antitrypsin, and an unidentified string of spots (pI 4.4–4.8 and M_r 30000) in the serum of alcohol-abusing patients [34]. However, there have been no reports in the literature in which this technique has been used to search for altered proteins among children in whom FAS has been diagnosed.

Based on the hypothesis that abnormal growth and development involve altered protein metabolism, our aim in this study was to analyze serum samples from children with FAS (case subjects) and from sex- and age-matched children whose mothers did not consume alcohol (control subjects) by two-dimensional polyacrylamide gel electrophoresis, protein silver staining, and computer image analysis. The resulting protein patterns were analyzed, and the database of quantified, matched proteins was analyzed by multiple hypothesis testing to find the proteins which show the highest statistical significance when samples from case and control subjects were compared by t-test. We also wanted to determine whether carbohydrate-deficient transferrin (CDT) is altered in children with FAS.

2 Materials and methods

2.1 Study groups

The case and control groups consisted of 12 children in whom FAS was diagnosed (8 females, mean age 10.1 ± 2.0 years and 4 males, mean age 9.5 ± 1.7 years) and 8 children without symptoms of FAS (5 females, mean age 10.7 ± 1.5 years and 3 males, mean age 10.0 ± 0.6 years). The participants were recruited from a group whose mothers obtained prenatal care at a large inner-city hospital. The criteria for FAS diagnosis included (i) pre- and postnatal growth retardation, (ii) characteristic facial dysmorphism, (iii) central nervous system abnormalities, and (iv) having a mother who drank an average of 340 g of absolute ethanol per week throughout pregnancy. Neurobehavioral characteristics of the participants had been studied over a long period by a developmental psychologist (Coles) who developed a relationship with the participants (both case and control subjects) and their parents [35]. The control group consisted of children from the same geographic and socio-economic population as the case subjects. Mothers of the control subjects did not consume alcohol during pregnancy. We obtained informed consent for sample collection in accordance with established procedures for the study of human subjects.

2.2 Reagents and specialized chemicals

Deionized, 15-MΩ water was used for all steps in the procedure. We used electrophoresis purity reagents as follows: Acrylamide and Servalyt™ (3-10 ISODALT grade) from Serva Fine Biochemicals (Heidelberg, Germany); AcrylAide™ and agarose from FMC (Rockland, ME); urea N,N'-methylenebisacrylamide (Bis), ammonium persulfate (APS), dithioerythritol (DTE), N,N,N',N'-tetramethylethylenediamine (TEMED), and sodium dodecyl sulfate (SDS) from Bio-Rad Laboratories (Richmond, CA). Buffer salts (Trizma base, Trizma hydrochloride, and glycine) and 3-((3-cholamidopropyl) dimethyl-ammonio)-1-propane-sulfonate (CHAPS) were obtained from Sigma (St. Louis, MO). Carbamylated glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was included in each isoelectric focusing gel for internal calibration of the first-dimensional separation [36], and a rat heart preparation was included in each slab gel for

molecular weight calibration [37]. We obtained glutaraldehyde and silver nitrate for staining from Eastman Kodak (Rochester, NY). All other chemicals were American Chemical Society grade from Fisher Chemical Company (Fair Lawn, NJ). Kabi Pharmacia Diagnostics (Piscataway, NJ) provided the kit which we used to measure carbohydrate-deficient transferrin in the case and control sera.

2.3 Collection and preparation of serum samples

Case and control subjects were scheduled for blood collection on one of two consecutive days. We collected blood samples by venipuncture and added aprotinin, a protease inhibitor, to each sample to make the final concentration 67 kallikrein inhibitor U/mL. Each serum sample was separated from the clot and stored frozen at −70°C until processed. After the samples were thawed, a 50 µL volume of each was ultrafiltered and diafiltered three times with 1 mL of water on Centricon-10 microconcentrators (Amicon Corporation, Danvers, MA). We performed total protein anlysis using the UV-difference method of Waddell [38] after diluting 5 µL of each retentate to 1.0 mL with water. Absorbance differences (A_{215}–A_{225}) were measured on a DU-7 spectrophotometer from Beckman Instruments (Fullerton, CA) along with a set of bovine serum albumin calibrators, and total protein values were calculated by nonlinear regression. The ultrafiltered samples were denatured and reduced by diluting them with a solution containing urea (9 mol/L), dithioerythritol (1%, w/w), and 3–10 Servalyt (2%, v/v) to a final protein concentration of 12 µg/µL. The samples were stored at −70°C until analysis.

2.4 2-DE and protein detection

The 2-DE procedure was a modification of the method of O'Farrell [39] that was adapted for the ISO-DALT system (Electro-Nucleonics, Oak Ridge, TN) by Anderson and Anderson [40] and Tollaksen [41]. Myrick *et al.* [32] described the modified procedure for serum. Briefly, it consists of isoelectric focusing on a 1.5 × 135 mm cylindrical gel containing acrylamide/Bis-acrylamide/AcrylAide (0.45%/30%/1.35% v/v, respectively) and a pH 3–10 carrier ampholyte gradient. A sample containing 120 µg of protein was loaded onto each isoelectric focusing gel. We used a 9.8–19.7% acrylamide pore gradient containing 0.2% Bis and 0.75% AcrylAide (v/v) for the second-dimensional separation. After 2-DE separation, the gels were fixed and silver-stained with a modification of the method of Oakley [42] as described in detail by Myrick *et al.* [32].

2.5 Image analysis and database creation

We scanned the back-lighted gels with a Visage 2000™ system (BioImage Products, Ann Arbor, MI) at 1024 × 1024 × 8 bits resolution using a calibrated absorbance step-wedge to convert transmission values to absorbance values. The digitized gel images were analyzed by the GELLAB-II system [11–13, 43–45]. The gel images were converted to the GELLAB-II format before analysis.

Boundaries and integrated intensities (sums of absorbance values for each pixel within the boundary) of each silver-stained spot were determined automatically, and the integrated intensity of each spot was corrected by the local, 64 × 64 pixel, background absorbance. Spots were detected and quantified with the following GELLAB-II parameter values:
lowwpass averaging filter = 3 × 3 pixels
Busse-Laplacian filter with central cone = 3 × 3 pixels
saturated spot propagation filter = 99.7%
split saturated spots when the central core > 50 pixels
background size filter = 64 × 64 pixels
central core minimum size = 4 × 4 pixels
draw boundaries and dots in the original gel image
spot area limit = 35–10000000
spot intensity limit = 0.3–10000000
spot OD limit = 0.03–4.5 absorbance
Saturated spots were segmented by a spot splitting algorithm, described recently by Lemkin *et al.* [46]. Fifty-two landmark spots, covering most of the area of the gel, were interactively chosen in a "reference" gel, and matching spots, or x-, y-coordinates (when the spots were missing) were selected in the remaining 33 gel images in the study. All protein spots in each gel were then matched automatically to the reference gel. A composite gel database containing sample information, x-, y-coordinates, and corrected densities for all spots was created. GELLAB-II extrapolated missing spots into each gel, and the extrapolated spots were assigned intensities of zero and x-, y-coordinates by interpolation from the reference gel. A set of computer-selected, robust, nonsaturating spots (absorbance < 1.50), which were found in all gels, was used to normalize spot intensities for each gel image.

2.6 Carbohydrate-deficient transferrin assays

We saturated the serum samples with ferric-citrate and applied them to anion exchange microcolumns to separate the carbohydrate-deficient transferrin (CDT) from other forms of transferrin [26]. We added the eluant containing the CDT to specific antibodies in the presence of ^{125}I-labeled transferrin. The bound transferrin was precipitated by a secondary antibody, and the radioactivity in the precipitate was counted on a gamma-counter (Micromedic Systems, Horsham, PA). The radioactivity in the precipitate is inversely proportional to the quantity of CDT in the sample.

2.7 Statistical analysis

We analyzed images of duplicate gels from the 12 case subjects and the 8 control subjects. We used the GELLAB-II software to perform statistical analyses and to search for spots that were significantly different in case and control subjects on the basis of the Student t-test or the Behrens-Fisher t-test. The Student t-test was also performed on data from the carbohydrate-deficient transferrin assay to determine whether there were significant differences in the results from FAS cases and controls. We performed stepwise and canonical discriminant analyses to determine whether the integrated intensities of specific combinations of significantly altered proteins
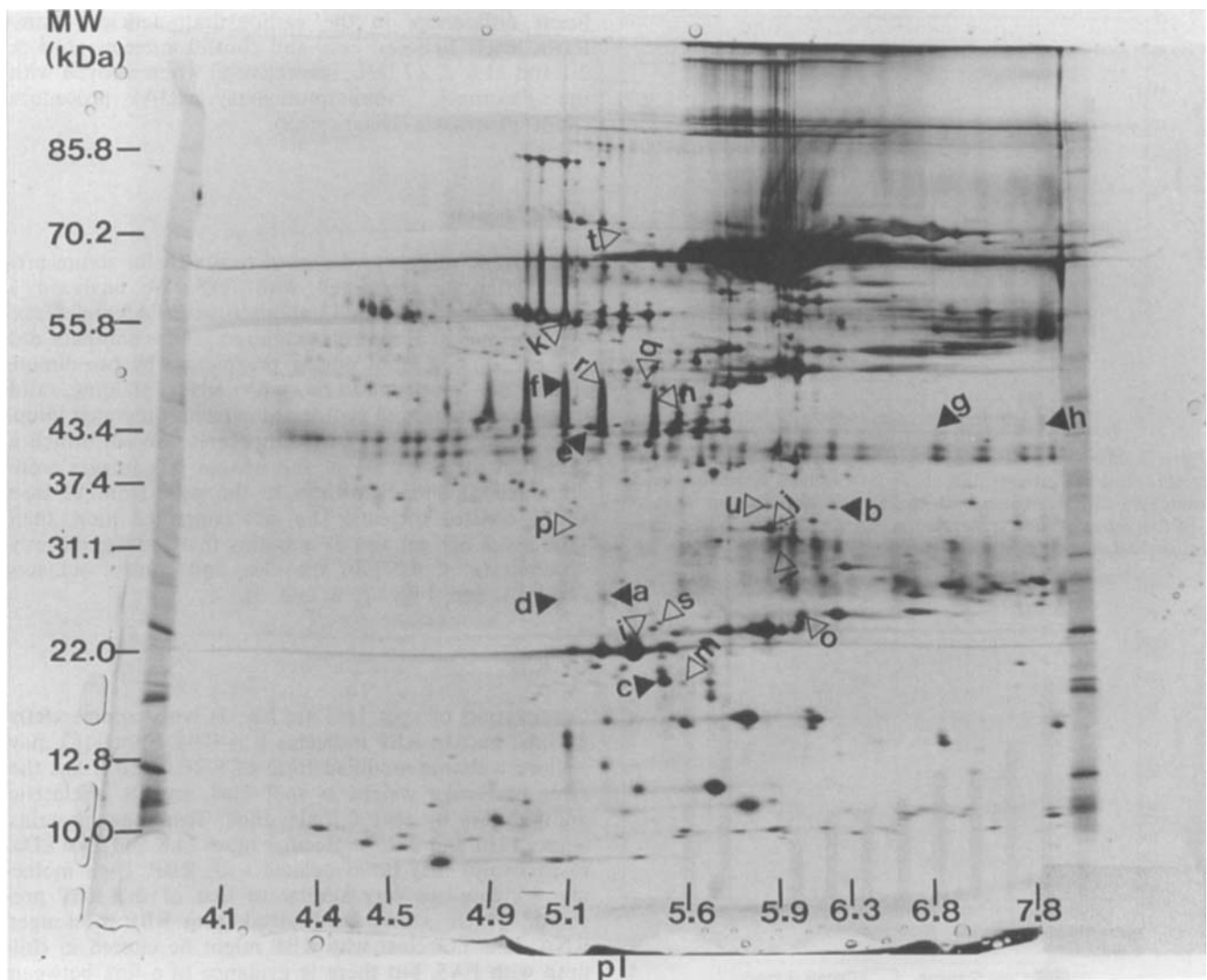
Figure 1. Refernce gel showing serum proteins that are significantly different between FAS case and control subjects. Electrophoresis and staining conditions are described in Section 2.4. ▶ Candidate FAS biomarkers (significantly different at $p < 0.01$ level). ▷ Potential biomarkers (significantly different at p-values between 0.01 and 0.02).

could be used to distinguish more accurately between case and control subjects.

2.8 Identification of protein spots

We created mosaics (images containing small segments of the same area of several gels displayed simultaneously) for each of the significantly different spots to check the matching visually. Gels containing spots found to be significantly different between cases and controls were compared visually with gels in which specific spots had been identified previously by Myrick *et al.* [32] and with standard serum protein maps [47]. We used constellations of spots around the spot of interest to tentatively identify the spot of interest.

3 Results

Image analysis of the silver-stained protein patterns of 34 gels of 11 children in whom FAS was diagnosed (7

females and 4 males) and 8 control subjects (5 females and 3 males) revealed 1707 ± 182 spots per gel. Six images were discarded from the set due to obvious distortions in the gels. The discarded images included both images from one case subject (female), and one image each from two cases and two controls (all female). Two-tailed Student's or Behrens-Fisher's t-tests of the data set with real and extrapolated spots revealed 32 spots that were significantly different among case and control subjects ($p < 0.02$). We examined the mosaics of each significantly different spot individually and determined that 21 of the 32 spots (numbers: 1163, 1161, 2315, 1150, 1110, 1088, 998, 993, 988, 982, 961, 942, 857, 856, 767, 759, 758, 666, 660, 432, and 133) are potential protein biomarkers of FAS. We classified 8 of the 21 proteins as candidate biomarkers on the basis of significant t-test differences at $p < 0.01$ (spot numbers: 1161, 1088, 982, 942, 857, 832, 817, 758). Table 1 shows the proteins by spot identification numbers and preliminary observations; spot locations are shown in Fig. 1. Mean integrated intensities of the 21 candidate and potential biomarkers in case and control subjects are shown in Figs. 2 and 3.
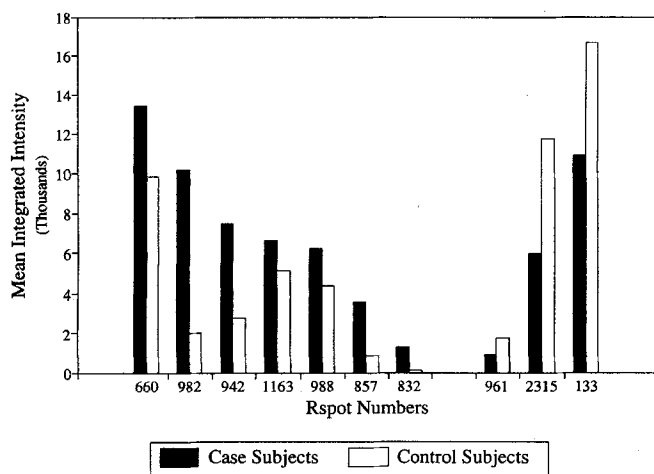
Figure 2. Mean integrated intensity (absorbance) of significantly different, silver-stained protein spots in 2-DE gels of serum specimens from FAS case and control subjects by spot identification numbers. The first group of spots is elevated in case subjects; the second group is elevated in controls subjects.
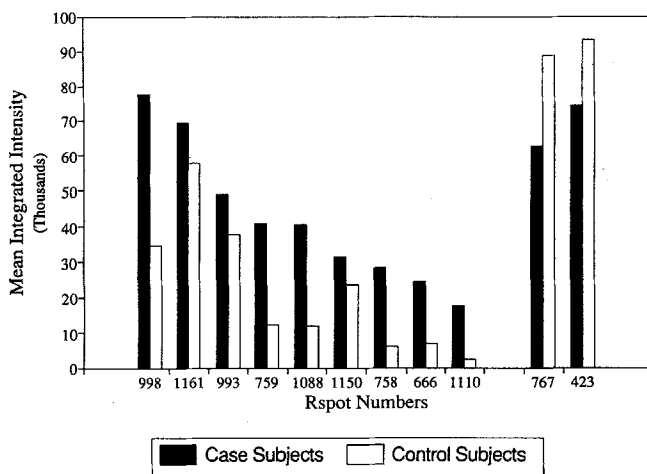

Figure 3. Same as Fig. 2. A second graph with extended *Y*-axis range was used to accommodate proteins with higher integrated intensities. The integrated intensity of spot #423 was multiplied by 0.4. The first group of spots is elevated in case subjects; the second group is elevated in control subjects.

After tentative identification of spot # 1161 as a retinol binding protein (RBP), a commercially purified preparation of human RBP was electrophoresed alone and co-electrophoresed with a small amount of some of the samples. The commercial preparation showed only one spot when electrophoresed alone. The combination of the commercial preparation and the samples, when co-electrophoresed, also showed a single spot at that same location, but the intensity was much greater than that shown in the serum sample without added RBP. No single protein was able to distinguish between all case and control subjects, but the stepwise canonical discriminant analysis revealed four groups of spots ([857 and 1163], [942 and 982], [857, 1161, and 1163], and [423, 988, and 758]) among the set of potential biomarkers that separated case and control subjects with no misclassifications. The score values calculated from linear functions of the logarithms of intensities of spots in each group are shown in Table 2. Serum samples showed no signi-

ficant differences in the carbohydrate-deficient transferrin levels between case and control subjects (13.8 ± 2.1 and 11.8 ± 2.7 U/L, respectively) when assayed with the Pharmacia radioimmunoassay (RIA) procedure (Kabi Pharmacia Diagnostics).

4 Discussion

The current study was designed to search for serum protein variations associated with FAS. We analyzed a serum sample set from 12 children with FAS and 8 sex- and age-matched controls (children whose mothers did not consume alcohol during pregnancy) by two-dimensional gel electrophoresis with silver staining, and created a database of spot locations and integrated intensities from the images of scanned gels. We performed a statistical query on 34 of the images (six images were discarded due to distortions in the gels) from 11 case and 8 control subjects. The gels contained more than 1700 spots per gel and 21 proteins that are significantly different ($p < 0.02$) in the case and control subjects (Table 1 and Figs. 1, 2 and 3).

4.1 RBP

Comigration of spot 1161 (c, Fig. 1) with commercially purified human RBP indicates it is RBP. Spot 1163 may well be a charge-modified form of RBP, since it has the same molecular weight as spot 1161, and its isoelectric point differs by only 0.1 pH units. Two other proteins (spots 1110 and 2315 molecular mass 24.8 and 24.6 kDa, respectively) may be associated with RBP. Their molecular weights are very similar to that of the RBP precursor, which is first synthesized from RBP messenger RNA. It is not clear why RBP might be altered in children with FAS, but there is evidence of a link between FAS and retinol. Pullarkat [48] and Duester [49] published separate, independent reports implicating a link between retinoids and FAS; presumably ethanol competitively inhibits retinol dehydrogenase, which catalyzes the rate-limiting step in the oxidation of retinol to retinoic acid. Both retinoic acid and retinol are key regulators of cell growth and differentiation [50–53]. It is also known that a limited supply of retinoic acid, as well as an excess, may lead to effects similar to those seen in children with FAS. Zachman and Grummer [54] indicate that the relationship between the retinoids and FAS may be a complicated one, involving binding proteins and receptors that regulate the transport of retinoids during embryonic development. Among both animals and humans, RBP is one of the first proteins to be synthesized in measurable quantities during early gestation [55–57]. RBP is known to be involved in the transport of retinol, and studies with radiolabeled, free and bound retinol indicate that RBP modifies the rate of uptake of retinol in various types of cells, particularly those of the human placenta [58]. Neurological deficits associated with FAS imply some effect of alcohol on the developing brain. MacDonald *et al.* [59] reported the localization of RBP in the cells of the blood-brain barrier of rats and humans. Our results imply that the relationship between FAS and retinoids may involve RBP.

Table 1. Proteins with significantly different concentrations at $p < 0.02$ in FAS case and control subjects[a]

Symbol	Spot No.	p-Value	pI[b]	Molecular mass[c] kDa	Preliminary observations
a	1088	.001[c]	5.3	26091	IgG light chain area
b	942	.001[d]	6.2	34309	
c	1161	.003[d]	5.5	19877	RBP
d	982	.005[c]	5.1	26241	IgG light chain area
e	767	.005[d]	5.2	42023	
f	758	.006[c]	5.1	47445	Haptoglobin β-chain area
g	832	.006[c]	6.8	43062	ADH area
h	857	.007[c]	7.8	43590	ADH area
i	1110	.010[d]	5.4	24829	Pre-RBP area
j	993	.010[c]	5.9	32317	
k	423	.010[d]	5.1	55760	Alpha-1-antitrypsin area
l	998	.011[d]	5.9	31142	IgG light chain area
m	1163	.011[d]	5.6	19744	RBP area
n	666	.012[c]	5.4	45316	Haptoglobin β-chain area
o	1150	.012[d]	6.1	24976	
p	961	.013[d]	5.2	32317	
q	660	.016[c]	5.4	46876	
r	759	.016[c]	5.2	46313	Haptoglobin β-chain area
s	2315	.017[d]	5.5	24610	Pre-RBP area
t	133	.017[d]	5.3	70168	
u	988	.018[d]	5.9	34221	

a) Serum samples were analyzed by 2-DE with silver staining and image analysis.
b) Estimates
c) Behrens-Fischer *t*-test
d) Student's *t*-test

Table 2. Groups of spots which distinguish between FAS case and control subjects with no misclassifications[a]

Group No.	Spot numbers	Case Scores	Control Scores
1	[857 and 1163]	49.0 to 52.3	52.6 to 55.5
2	[942 and 982]	3.5 to 7.2	1.8 to 3.2
3	[857, 1161, and 1163]	82.9 to 86.2	87.2 to 90.0
4	[423, 988, and 758]	−55.0 to −53.0	−51.3 to −49.0

a) Groups were revealed by canonical discriminant analysis of spot data from 2-DE gels. Score values were calculated from linear functions of the logarithms of intensities of spots in each group.

4.2 Alcohol dehydrogenase

No tentative identifications have been made because alcohol dehydrogenase (ADH) has not been identified on 2-DE maps of human serum, but two spots (832 and 857) may be isoforms of ADH based on literature estimates of the molecular masses (approximately 40 kDa [60]), isoelectric points (approximately 5.6 to 11 [60]), and concentrations (in normal adults, 59 ± 16 ng/mL [61]). The search for differences in ADH among subjects with and without FAS by genetic screening techniques has not led to reports of significant differences. However, our search for differences in protein expression does show quantitative alterations in proteins that are located in the area of the 2-DE gel where ADH is expected to appear. The quantities of the proteins are very low, and the variability in the gels is such that additional studies will be needed both to confirm the elevations among case and control subjects as well as to identify spots. If both RBP and ADH are elevated in children with FAS, it may be that ethanol exposure during embryonic development affects the genes which code for both. The discriminant analysis revealed that spot 1163 with or without spot 1161, when used in combination with one of the proteins located in the area of ADH (857), distinguishes between case and control subjects with no misclassifica-

tions. Is this an indication of some relationship between RBP and ADH? There appears to be sufficient evidence of a need to study the relationships among retinoids, retinoid binding proteins, alcohol dehydrogenases and FAS. Isoenzyme-specific assays for ADH have recently become available [62], and these may be useful for confirming ADH variations and clarifying the relationships, if any, to FAS.

4.3 Other observations

Of the 21 spots that were significantly different in case and control subjects, three (758, 759, and 666) appear to be associated with variants of the acute-phase protein, haptoglobin; all 3 appear within the β-chains and were elevated in cases. Alterations in the isoforms of haptoglobin are not unique to FAS because isoforms of this protein were recently found to be elevated among patients with both Alzheimer's disease and schizophrenia [33] and among mothers who gave birth to children with Down Syndrome [32]. Alzheimers disease, schizophrenia, and Down syndrome affect the central nervous system, and some cases of schizophrenia are currently thought to be caused by fetal alcohol exposure [63]. These proteins are not expected to be useful as biomarkers of FAS, but they may be useful as biomarkers of neurological damage. Three proteins are present in areas of the gel where immunoglobulin light chains appear. Although ample evidence exists that alcohol interferes with B-cell proliferation and immunoglobulin production [64], there is no firm theoretical basis for differences in immunoglobins of offspring due to prenatal ethanol exposure. IgG does cross the placenta and enter fetal circulation, but it disappears by the age of nine months [65]. Most protein, including IgM and IgA from the mother, do not cross the placenta into fetal circulation. Long-term effects of prenatal ethanol exposure on immunoglobulin

synthesis have not been reported in the literature. Spot 423 was tentatively identified (on the basis of its location in the gel) as an isoform of alpha-1-antitrypsin (AAT), which was also identified in 2-DE gels of alcoholics [34]. The significance of these findings is not known, and it is not known why AAT might be altered in people with FAS.

4.4 CDT

Although there was no theoretical basis for assuming that CDT would be different in humans with FAS and those without the syndrome, studies in rats [66] suggest that prenatal ethanol treatment during one of the vulnerable periods of brain development leads to altered incorporation of sialic acid into synaptosomal membrane-bound sialoglyco-compounds. Since CDT has been shown to be a useful marker of alcohol consumption in populations of men and women and is being investigated as a marker for alcohol consumption in pregnant women, we wanted to determine whether CDT is significantly different in FAS case subjects than it is in control subjects. The CDT assays confirmed that the analysis of carbohydrate deficient transferrin is not useful for distinguishing between those children with FAS and those without the syndrome.

4.5 Discriminant analysis

It is significant that the stepwise canonical discriminant analysis found four sets of proteins that distinguish between case and control subjects with no misclassifications (Table 2). It is not unusual for multiple proteins or other biomolecules to be altered in a given disease or syndrome, and many diseases are diagnosed using multiple tests. The discriminant analysis provides a technique for searching for unknown substances that may be used together to provide more powerful discrimination between test results that indicate the presence or absence of disease. The stepwise canonical discriminant analysis selects the most significantly different spot and examines each spot in the set to find one or more which provide better discrimination between cases and controls using a linear function of the logarithm of the integrated intensities of the spots in each group. This procedure did not examine other spots which were not significantly different according to our criteria. It is possible that there are other combinations of proteins that would provide excellent discrimination when used in combination with the proteins we identified or with other proteins. Finding four groups of proteins that distinguish between subjects with FAS and control subjects indicates that this technique may be a useful and powerful one for designing diagnostic procedures for many diseases and syndromes.

The fact that none of these proteins, alone, could differentiate all of the case subjects from control subjects, is probably an indication of the complexity of this disease. It is likely that protein alterations reflect susceptibility rather than causation, but such proteins would still be useful as clinical tools. Of the 21 proteins found to be significantly different between FAS case and control subjects, 8 are candidate protein biomarkers of FAS. We will

continue to monitor the other spots and re-evaluate their significance with additional samples. Because of the small sample size we used in this study, it will be necessary to confirm these alterations in a larger study and to identify, specifically, each of the proteins that we confirm as candidate biomarkers of FAS.

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