Identification of Diagnostic Biomarkers for Infection in Premature Neonates*

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Infection is a leading cause of neonatal morbidity and mortality worldwide. Premature neonates are particularly susceptible to infection because of physiologic immaturity, comorbidity, and extraneous medical interventions. Additionally premature infants are at higher risk of progression to sepsis or severe sepsis, adverse outcomes, and antimicrobial toxicity. Currently initial diagnosis is based upon clinical suspicion accompanied by nonspecific clinical signs and is confirmed upon positive microbiologic culture results several days after institution of empiric therapy. There exists a significant need for rapid, objective, *in vitro* tests for diagnosis of infection in neonates who are experiencing clinical instability. We used immunoassays multiplexed on microarrays to identify differentially expressed serum proteins in clinically infected and non-infected neonates. Immunoassay arrays were effective for measurement of more than 100 cytokines in small volumes of serum available from neonates. Our analyses revealed significant alterations in levels of eight serum proteins in infected neonates that are associated with inflammation, coagulation, and fibrinolysis. Specifically P- and E-selectins, interleukin 2 soluble receptor α, interleukin 18, neutrophil elastase, urokinase plasminogen activator and its cognate receptor, and C-reactive protein were observed at statistically significant increased levels. Multivariate classifiers based on combinations of serum analytes exhibited better diagnostic specificity and sensitivity than single analytes. Multiplexed immunoassays of serum cytokines may have clinical utility as an adjunct for rapid diagnosis of infection and differentiation of etiologic agent in neonates with clinical decompensation. ***Molecular & Cellular Proteomics 7:1863–1875, 2008.***

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Infection, particularly of nosocomial or late onset, is very common in preterm infants (1, 2). The diagnosis of infection in preterm infants can be very difficult. The clinical presentation of neonatal infection is subtle and nonspecific, featuring signs such as jaundice, unstable temperature, difficulty breathing, changes in heart rate, and difficulty in feeding. The diagnostic difficulty is compounded by disease heterogeneity and a lack of reliable, rapid diagnostic tests (3–6). Sources of heterogeneity include etiologic agent, virulence, inoculum, site of primary infection, host genotype, stage of development of host responses, and extraneous clinical interventions. Microbiologic cultures of clinical specimens, the gold standard for diagnosis, have low sensitivity and are not available in time to influence initial therapy. Given the rapid progression and high mortality of sepsis (local infection with evidence of systemic inflammatory response) in preterm infants, broad spectrum antimicrobial chemotherapy is frequently administered at first clinical suspicion of infection (7, 8). Premature infants are at higher risk of drug toxicity because of hepatic and renal organ immaturity, and antimicrobial resistance is an increasing problem in neonatal intensive care settings. Therefore, a reliable and rapid *in vitro* test is urgently needed for early diagnosis and management of infection in neonates. In addition, the availability of a rapid *in vitro* test for etiologic agent in neonatal infection would permit early, targeted treatment.

Recently there has been a considerable interest in the application of host biomarkers for diagnostic tests (9). It appears that biological systems are adaptive and that challenges to host homeostasis cause characteristic topological perturbations of molecular networks. A biomarker is a measurable gene, protein, metabolite, or other indicator of network perturbation that correlates with a specific outcome or clinical state (10). Biomarkers are identified through a four-step process of *discovery* by appropriate multiplex biochemical analysis followed by *replication* ideally in independent cohorts, *validation* of diagnostic sensitivity and specificity, and *translation* into a clinical diagnostic test (9). Numerous candidate biomarkers have been identified in neonatal sepsis: elevated plasma or serum levels of inter-

leukin 6 (IL-6)[1] (11, 13), tumor necrosis factor α (TNFα) (11, 13), neutrophil elastase (NE) (14), C-reactive protein (CRP) (12, 15), soluble CD14 (16, 17), granulocyte colony-stimulating factor (G-CSF) (18), soluble intercellular adhesion molecule-1 (ICAM-1) (12), and soluble L-selectin (19) have shown association with infection in neonates. The value of physiological measurements in this context has also been examined recently (20). However, the positive predictive value or negative predictive value (NPV) of individual analytes has not been adequate for routine use in the diagnosis and management of neonatal infection.

In other clinical conditions where individual analytes lack adequate positive predictive value or NPV, the values of several analytes have been combined, together with an algorithm, to provide a biomarker panel with improved clinical performance (9). Multiplexed measurement of plasma proteins is a logical approach because they constitutively function within networks, pathways, complexes, and families (21–23). Indeed the activity of an individual plasma protein is typically dependent not only upon its abundance but also upon the effects of interacting proteins, modifying proteins, and antagonistic and synergistic proteins. This is especially true of cytokines, which are a group of several hundred small, soluble, plasma proteins relevant to sepsis, that are powerful mediators of target cell activities such as migration, activation, phagocytosis, proliferation, and apoptosis.

Several recent studies have explored the utility of measurement of two or more analytes for diagnosis of neonatal infection (11–13, 17). For example, we have shown that CRP $>$ 6 mg/liter and soluble ICAM-1 $>$ 300 ng/ml in combination in plasma samples collected under endotoxin-free conditions (12, 13) were independent predictors of infection and gave both a high sensitivity for clinical infection (95%) and an NPV of 97% (12). We subsequently demonstrated that routinely collected serum samples reproduced a very high NPV for infection (24). We have further examined the diagnostic utility of the combination of low level CRP and soluble immunological markers.[2] In a recent review, Ng (25) concluded that a combination of several markers can enhance diagnostic accuracy in neonatal sepsis.

In the present study, we used amplified, multiplexed, immunoassay arrays to measure 142 small serum proteins and assess their utility in differential diagnosis of neonatal infection. In addition, we utilized the most informative of these biomarkers to develop a multiplexed biomarker panel with improved diagnostic performance in neonatal infection.

[1] The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; NE, neutrophil elastase; CRP, C-reactive protein; G-CSF, granulocyte colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; NPV, negative predictive value; MFI, mean fluorescence intensity; ANOVA, analysis of variance; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen-activated receptor; IL-2sR, IL-2 soluble receptor.

[2] J. D. M. Edgar, manuscript in preparation.

EXPERIMENTAL PROCEDURES

Patient Population, Samples, and Laboratory Test Results

Serum samples were obtained from acutely ill, preterm (≤36 weeks gestation) infants undergoing intensive care in the neonatal units of the University Hospital, Queen's Medical Centre, Nottingham, UK and the Royal-Jubilee Maternity Service, Royal Hospitals Trust, Belfast, Northern Ireland, UK. These infants were either admitted to the neonatal unit with a suspected diagnosis of infection (early onset and late onset infection) or developed clinical signs suggesting that infection may be complicating their management. As part of their acute management, these infants had blood samples taken for routine laboratory analysis, and an extra aliquot of 0.5 ml of blood was collected for the purposes of this study. At presentation, the culture result, white blood cell count, platelet count, gestational age, primary diagnosis, and clinical signs were also recorded.

Blood samples were kept at ambient temperature until serum was separated (within 24 h), and serum was stored at −20 °C until analysis. No extra venepuncture beyond clinical requirements was necessary as a result of this study for either patients or control subjects.

Clinical diagnosis was established in a prospective manner, blind to the results of the study measurements. Infants were classified as "clinically infected" or "non-infected" based on the initial clinical presentation and results of routine investigations ((*a*) white cell and platelet counts, (*b*) blood and other cultures, (*c*) radiological imaging, and (*d*) response to antibiotics). The infected group was further subclassified as (*a*) culture-positive infection or (*b*) culture-negative infection.

Written informed parental consent was obtained for study subjects. Ethical approval was obtained from the Ethics Committee, University Hospital, Queen's Medical Centre, Nottingham, UK.

Sample Processing

Aliquots of the samples were thawed, centrifuged to remove particulate matter, and diluted 1:5 with 18.75 units/ml heparin (Sigma) in AD1 diluent (Immunochemistry Technologies, Bloomington, MN), and 0.25 mg/ml Heteroblock (Omega Biologicals, Bozeman, MT), 0.25 mg/ml immunoglobulin inhibiting reagent (Bioreclamation, Hicksville, NY), and 0.1% Tween 20 (Sigma) were added prior to assay.

Antibody Microarray Manufacture

Antibody microarray manufacture has been described in detail previously (26). Briefly glass slides were covered with a Teflon mask to provide 16 sample wells and silanized with 3-cyanopropyltriethoxysilane. Each well was printed with quadruplicate spots of each of 64 capture antibodies using a PerkinElmer Life Sciences SpotArray Enterprise piezoelectric arrayer. Six different antibody arrays were prepared, each containing 25–37 unique capture antibodies (Table I).

Antibody Microarray Assays

Samples were randomized to groups of six, and two groups were randomly assigned to each microarray slide. Each sample was evaluated in duplicate. Quality controls and standards were applied to the four remaining wells on each microarray slide as described previously (26). Assays were performed blinded to the biological identity of the samples. Rolling circle amplification, microarray, sandwich immunoassays were performed on an automated robotic microfluidic station (BioCube, Protedyne Inc., Windsor, CT) as described previously (26, 27). Briefly 15 μl of diluted sample was applied to each well, incubated, and washed. Next a mixture of biotinylated secondary antibodies was applied to each well, incubated, and washed. Then an anti-biotin antibody-DNA conjugate (an anti-biotin antibody with a covalently attached oligonucleotide and an annealed circular nucle-

TABLE I

142 analytes measured by amplified sandwich immunoassays on glass microarrays 1–6

TNFR, TNF receptor; RANTES, regulated on activation normal T cell expressed and secreted.

No.	Abbreviation	Alternate/full name	Analyte class
1	4-1BB	CD137, TNF receptor superfamily, member 9	Cytokine receptor
2	CCL21	6Ckine	Cytokine
3	ACE	Angiotensin I-converting enzyme, CD143	Enzyme
4	ACE-2	Angiotensin I-converting enzyme-2	Enzyme
5	AFP	α-Fetoprotein	Oncofetal antigen
6	AgRP	Agouti-related protein	Cytokine
7	ALCAM	Activated leukocyte cell adhesion molecule, CD166	Adhesion molecule
8	ANG	Angiogenin, ribonuclease 5	Enzyme
9	AR	Amphiregulin	Cytokine
10	BDNF	Brain-derived neurotrophic factor	Cytokine
11	BLC (BCA-1)	B lymphocyte chemoattractant, CXCL13	Cytokine
12	CA125	Cancer antigen 125, mucin 16, MUC16	Antigen
13	CD141	Thrombomodulin, CD141	Clotting factor
14	CD27	TNF receptor superfamily, member 7	Cytokine receptor
15	CD30	TNF receptor superfamily, member 8	Cytokine receptor
16	CD40	TNF receptor superfamily, member 5	Cytokine receptor
17	CNTF	Ciliary neurotrophic factor	Cytokine
18	CNTF Rα	Ciliary neurotrophic factor receptor α	Cytokine receptor
19	CRP	C-reactive protein	Pentraxin
20	CCL27	Cutaneous T cell-attracting chemokine, CTACK	Cytokine
21	D-dimer	D dimer	Clotting factor
22	EGF	Epidermal growth factor	Cytokine
23	ENA-78, CXCL5	Epithelial cell-derived neutrophil-activating peptide	Cytokine
24	Endostatin	Procollagen, type XVIII, α1	Angiogenesis factor
25	Endothelin 3	Endothelin 3	Cytokine
26	Eot	Eotaxin, CCL11	Cytokine
27	Eot-2	Eotaxin-2, CCL24	Cytokine
28	Eot-3	Eotaxin-3, CCL26	Cytokine
29	ErbB1	Epidermal growth factor receptor 1, EGFR	Cytokine receptor
30	ErbB2	Epidermal growth factor receptor 2, HER2, NEU	Cytokine receptor
31	E-selectin	E-selectin, CD62E	Adhesion molecule
32	Fas	CD95, TNF receptor superfamily, member 6	Cytokine receptor
33	FGFR3 (IIIc)	Fibroblast growth factor receptor 3 IIIc isoform	Cytokine receptor
34	FGF2	Fibroblast growth factor-2 (FGF basic)	Cytokine
35	FGF4	Fibroblast growth factor-4	Cytokine
36	FGF6	Fibroblast growth factor-6	Cytokine
37	FGF7	Fibroblast growth factor-7	Cytokine
38	FGF9	Fibroblast growth factor-9	Cytokine
39	FGFR3 (IIIb)	Fibroblast growth factor receptor 3 IIIb isoform	Cytokine receptor
40	FLT3LG	fms-like tyrosine kinase-3 ligand	Cytokine
41	Follistatin	Follistatin	Cytokine
42	GCP-2	Granulocyte chemotactic protein 2, CXCL6	Cytokine
43	G-CSF	Granulocyte colony-stimulating factor, CSF3	Cytokine
44	GDNF	Glial cell line-derived neurotrophic factor	Cytokine
45	GM-CSF	Granulocyte macrophage CSF, CSF2	Cytokine
46	GRO-γ	Growth-related oncogene γ, CXCL3, MIP2B	Cytokine
47	HB-EGF	Heparin-binding EGF-like growth factor	Cytokine
48	HCC4 (NCC4)	Hemofiltrate CC chemokine 4, CCL16	Cytokine
49	HCG	Human chorionic gonadotrophin	Hormone
50	I-309	I-309, CCL1	Cytokine
51	ICAM-1	Intercellular adhesion molecule 1, CD54	Adhesion molecule
52	IFN-α	Interferon α	Cytokine
53	IFN-β	Interferon γ	Cytokine
54	IFN-ω	Interferon ω	Cytokine
55	IGF-1R	Insulin-like growth factor I receptor, CD221	Cytokine receptor
56	IGFBP-1	Insulin-like growth factor-binding protein 1,	Cytokine
57	IGFBP-3	Insulin-like growth factor-binding protein 3	Cytokine
58	IGFBP-4	Insulin-like growth factor-binding protein 4	Cytokine
59	IGFBP-6	Insulin-like growth factor-binding protein 6	Cytokine

TABLE I—*continued*

No.	Abbreviation	Alternate/full name	Analyte class
60	IGF2	Insulin-like growth factor II, somatomedin A	Cytokine
61	IL-1sR1	Interleukin 1 soluble receptor I, CD121a	Cytokine receptor
62	IL-1sRII	Interleukin 1 soluble receptor II, CD121b	Cytokine receptor
63	IL-10 Rβ	Interleukin 10 receptor β	Cytokine receptor
64	IL-12b (p40)	Interleukin 12β, p40	Cytokine
65	IL-13	Interleukin 13	Cytokine
66	IL-15	Interleukin 15	Cytokine
67	IL-16	Interleukin 16	Cytokine
68	IL-17	Interleukin 17	Cytokine
69	IL-18	Interleukin 18	Cytokine
70	IL-1α	Interleukin 1α	Cytokine
71	IL-1β	Interleukin 1β	Cytokine
72	IL-1ra	Interleukin 1 receptor antagonist, IL1RN	Cytokine
73	IL-2	Interleukin 2	Cytokine
74	IL-2 Rβ	Interleukin 2 receptor β, CD122	Cytokine receptor
75	IL-2sRα	Interleukin 2 soluble receptor α, CD25	Cytokine receptor
76	IL-3	Interleukin 3	Cytokine
77	IL-4	Interleukin 4	Cytokine
78	IL-5	Interleukin 5	Cytokine
79	IL-6	Interleukin 6	Cytokine
80	IL-7	Interleukin 7	Cytokine
81	IL-8	Interleukin 8	Cytokine
82	I-TAC	IFN-γ-inducible T cell α chemoattractant, CXCL11	Cytokine
83	LIF	Leukemia-inhibitory factor	Cytokine
84	LIF Rα (gp190)	Leukemia-inhibitory factor soluble receptor α	Cytokine receptor
85	Lptn	Lymphotactin, XCL1	Cytokine
86	LT βR	Lymphotoxin-β receptor, TNFR superfamily 3	Cytokine receptor
87	MCP-1	Monocyte chemotactic protein 1, CCL2	Cytokine
88	MCP-2	Monocyte chemotactic protein 2, CCL8	Cytokine
89	MCP-3	Monocyte chemotactic protein 3, CCL7	Cytokine
90	M-CSF	Macrophage colony-stimulating factor, CSF1	Cytokine
91	M-CSF R	Macrophage colony-stimulating factor receptor CSF1R	Cytokine receptor
92	MIF	Macrophage migration-inhibitory factor	Cytokine
93	MIG	Monokine induced by interferon γ, CXCL9	Cytokine
94	MIP-1α	Macrophage inflammatory protein 1α, CCL3	Cytokine
95	MIP-1β	Macrophage inflammatory protein 1β, CCL4	Cytokine
96	MIP-1δ	Macrophage inflammatory protein 1δ, CCL15	Cytokine
97	MIP-3α	Macrophage inflammatory protein 3α, CCL20	Cytokine
98	MMP-1	Matrix metalloproteinase 1	Enzyme
99	MMP-10	Matrix metalloproteinase 10	Enzyme
100	MMP-2	Matrix metalloproteinase 2	Enzyme
101	MMP-8 (total)	Matrix Metalloproteinase-8	Enzyme
102	MPIF	Myeloid progenitor-inhibitory factor 1, CCL23	Cytokine
103	NAP-2	Neutrophil-activating peptide 2, CXCL7	Cytokine
104	NE	Neutrophil elastase, elastase 2	Enzyme
105	NGF-β	Nerve growth factor-β	Cytokine
106	NT-3	Neurotrophin 3, NTF3	Cytokine
107	NT-4	Neurotrophin 5, NTF4	Cytokine
108	OSM	Oncostatin M	Cytokine
109	Osteopontin	SPP1	Cytokine
110	PAI-1	Plasminogen activator inhibitor type 1, serpin E1	Enzyme inhibitor
111	PAI-II	Plasminogen activator inhibitor-II, serpin B2	Enzyme inhibitor
112	PARC	Pulmonary and activation-regulated chemokine CCL18	Cytokine
113	PDGF Rα	Platelet-derived growth factor receptor α CD140A	Cytokine receptor
114	PDGF Rβ	Platelet-derived growth factor receptor β CD140B	Cytokine receptor
115	PEDF	Pigment epithelium-derived factor, serpin F1	Enzyme inhibitor
116	PF4	Platelet factor-4, CXCL4	Cytokine
117	PlGF	Placental growth factor, PGF	Cytokine
118	Prolactin	Prolactin	Hormone

TABLE I—*continued*

No.	Abbreviation	Alternate/full name	Analyte class
119	Protein C	Protein C	Clotting factor
120	Protein S	Protein S	Clotting factor
121	P-selectin	P-selectin, CD62P	Adhesion molecule
122	RANTES	CCL5	Cytokine
123	SCF	Stem cell factor, Kit ligand	Cytokine
124	sgp130	Soluble glycoprotein 130, IL6ST, CD130	Cytokine receptor
125	sVAP-1	Soluble vascular adhesion protein-1, AOC3	Adhesion molecule
126	sVCAM-1	Soluble VCAM-1, CD106	Adhesion molecular
127	TARC	Thymus and activation-regulated chemokine, CCl17	Cytokine
128	TGF-α	Transforming growth factor α	Cytokine
129	TGF-β RIII	Transforming growth factor β receptor III	Cytokine receptor
130	Tie-2	TEK, CD202b	Cytokine receptor
131	TIMP-2	Tissue inhibitor of metalloproteinases 2	Enzyme inhibitor
132	TNF-α	Tumor necrosis factor α, TNF	Cytokine
133	TNF-β	Tumor necrosis factor β	Cytokine
134	TNF-RI	TNF receptor superfamily, member 1A	Cytokine receptor
135	TRAILR1	TNF receptor superfamily, member 10A	Cytokine receptor
136	TSH	Thyroid-stimulating hormone	Hormone
137	uPA	Urokinase plasminogen activator, PLAU	Clotting factor
138	uPAR	Urokinase plasminogen activator receptor	Clotting factor
139	VE-cadherin	Vascular endothelial cadherin, cadherin 5	Adhesion molecular
140	VEGF	Vascular endothelial growth factor	Cytokine
141	VEGF-R3	VEGF receptor 3, FLT4	Cytokine receptor
142	VEGF-D	Vascular endothelial growth factor-D	Cytokine

otide) was applied to the arrays, incubated, and washed. Rolling circle amplification was then performed for 45 min using T7 native DNA polymerase as described previously (28) and detected with a Cy5-labeled complementary oligonucleotide probe as described previously (26). A Tecan LS200 scanner was used to assay fluorescent signals on the slides, and microarray images were quantified using the image capture software GenePix 4.0.

Data Processing

Data processing procedures were as described previously (26). Briefly data points producing outlier events as a result of missing spots, spots with poor morphology, or printed features demonstrating high pixel outliers were removed. Correlation between sample replicates was performed, and sample replicates with correlation coefficient (R^2) values <0.95 were removed. Intensity values were $\log_2$-transformed to minimize dependence of variance on intensity and normalized by analysis of variance to reduce variability observed between replicates (26, 29–32). Sample pass rates following data processing procedures are indicated in Table II.

Statistical Analyses

Single Factor Analysis—Logarithm-transformed mean fluorescence intensities (MFIs) of analyte values in clinically infected and non-infected samples were compared by analysis of variance (ANOVA), Type III, sum of squares (SAS 8.2 general linear model procedure) with a significance level or p value <0.05 as described previously (26). Differences between clinically infected and non-infected samples were stated as effect sizes, which represent a measure of the difference in mean between the two groups, normalized by the within-group standard deviation. This measure is independent of the sample size and is calculated as follows: Estimated effect size = (Mean_Group1 − Mean_Group2)/S.D._Group1_Group2.

The estimated effect size is associated with the predictive ability of a particular analyte. For example, an effect size of 0.3 between two groups is equivalent to a probability of correctly identifying the groups of 0.56. The current study used an effect size ≥ 0.6 as the cutoff for statistical significance based upon previous studies involving analysis of clinical samples on 142-feature antibody arrays (26, 27).

TABLE II
Sample pass rate by array

Sample pass rates following the data processing procedures discussed under "Experimental Procedures" are indicated.

Array	Number of passed samples		
	One replicate	Two replicates	Total
1	20	85	105
2	21	70	91
4	4	77	81
5	16	83	99
6	12	74	86

Pairwise differences in logarithm-transformed MFIs of analyte values in infected neonates with cultures positive for coagulase-negative staphylococci, *Escherichia coli*, *Candida*, and group B streptococci were compared by ANOVA, LSMeans (SAS JMP Genomics 3.2) using a false discovery rate correction and a p value <0.05.

Multivariate Analysis—Because of the rigorous quality controls applied during data processing, the data matrix of samples × analytes contained missing data (Table II). Unfortunately many multivariate analyses are unstable with missing values (33). Model-based methods (34) have long been used to impute values for missing data prior to multivariate analyses, but these methods can introduce bias from incorrectly specified models. To reduce the bias from an incorrectly specified model, we utilized a weighted K-nearest neighbor approach to impute missing values (35). Explicitly we imputed missing values based on corresponding data for the missing analyte from the 20 nearest neighbors irrespective of the clinical diagnosis of the sample. To minimize the impact of imputed data on the discriminant function, we also removed 27 samples and 19 analytes

TABLE III
Demographics of the study population (mean ± S.E.)

	Clinically infected	Clinically non-infected
Number of samples	51	57
Gestational age (weeks)	28.8 ± 0.6	30.2 ± 0.6
White blood cell count	15.1 ± 1.0 (n = 47)	11.8 ± 0.8 (n = 42)
Platelet count	241 ± 17 (n = 46)	251 ± 22 (n = 40)
Positive microbiological culture	32	0
Antibiotic therapy	47	35
Duration of antibiotic therapy	8.5 ± 0.94 (n = 47)	3.0 ± 0.36 (n = 35)
C-reactive protein	24.4 ± 8.0 (n = 41)	3.4 ± 1.2 (n = 51)

in which more than 25% of the data were imputed. A multivariate discriminant function was developed using the STEPDISC procedure of SAS applied to the average values of log-transformed analyte concentrations for 123 analytes from 80 samples (43 infected and 37 uninfected). The criteria for selection of analytes to enter or remove from the model were based on the significance of the F statistic set to $\alpha = 0.1$. The ability of the resulting set of analytes to predict infection status was evaluated using the DISC procedure of SAS. Because of unequal and perhaps non-normal multivariate distributions within the infected and non-infected groups, the discriminant function was based on the nearest neighbor method in which K was set to 15. The resulting discriminant function was then tested for predictive value by 80 iterations of "leave-one-out" cross-validation.

RESULTS

Patient Population—108 serum samples were obtained from 72 infants, comprising 51 samples from 35 clinically infected neonates (early and late onset infection) and 57 samples from 40 non-infected infants. 32 of 51 (63%) clinically infected samples were associated with positive microbiological culture results. Organisms identified in positive cultures were coagulase-negative staphylococci (n = 14), *Staphylococcus aureus* (n = 2), enterococci (n = 3), group B *Streptococcus* (n = 3), α hemolytic streptococci (n = 1), *E. coli* (n = 3), *Enterococcus faecium* (n = 1), and *Candida* spp. (n = 4). Univariate statistics revealed the clinically infected samples to be associated with a marginally higher white blood cell count ($p < 0.05$) and significantly greater C-reactive protein ($p = 1.9 \times e^{-25}$, F-test). Table III shows the demographic characteristics and laboratory values of these groups.

Assay Specifications—Using sandwich immunoassays printed on glass microarrays (26–28), the levels of 142 proteins (Table I) were measured in 108 serum samples from premature neonates. The serum proteins assayed comprised 80 cytokines, 30 soluble cytokine receptors, 12 enzymes or enzyme inhibitors, seven adhesion molecules, six clotting factors, three hormones, and four miscellaneous antigens. The linear dynamic range, sensitivity, specificity, quality metrics, and performance of these antibody microarrays has been described in detail previously (26). Each serum sample was measured in duplicate on 16-well microarray slides following randomization to adjust for location-related bias in values. In addition, the four corner wells in each microarray slide were used to create standard curves for quality control purposes, and slide-to-slide precision was improved with regression-based normalization based upon these standard curves (26). The average slide-to-slide variability (coefficient of variation) was 24, 35, 28, 32, and 20% for Arrays 1, 2, 4, 5, and 6, respectively (Table IV). Analyte levels were expressed as logarithm base 2-transformed MFIs, which show a linear relationship with concentrations (pg/ml) within the linear dynamic range of the assays (26). Because log-transformed analyte concentrations (within the linear range) were of approximately equal scale and variance size, the assumptions underlying classical analysis of variance were approximately satisfied.

TABLE IV
Slide-to-slide variability on a linear scale

Array	CV[a]	Number of measurements	S.D.
	%		
1	23.6	2611	17.7
2	34.5	2098	24.6
4	27.5	3208	21.3
5	31.6	2367	23.0
6	19.9	2681	15.1

[a] Coefficient of variation.

Neonatal Infection Biomarker Discovery—30 of 142 serum analytes showed a significant change (p value <0.05) in expression between clinically infected and non-infected patients based upon ANOVA using logarithm-transformed fluorescence intensities (Table V). 27 of these analytes were in the linear dynamic range of the sandwich immunoassay as evidenced by correlation between concentration (pg/ml) and MFI (data not shown). The measurements of the remaining three analytes (CD40, FGFR3 (IIIb), and IL-12 p40) were below the lower limit of reliable quantitation. For two analytes with statistically significant differences between clinically infected and non-infected samples by microarray immunoassay (CRP and ICAM-1), differences were confirmed by monoplex ELISA retesting (data not shown).

Eight of the 27 analytes (E-selectin, P-selectin, IL-18, IL-2 soluble receptor α chain (IL-2sRα), CRP, urokinase plasminogen activator (uPA), urokinase plasminogen-activated receptor (uPAR), and NE) showed effect sizes greater than 0.6 and mean intensity values in the optimal range of measurement and demonstrated similar trends between fluorescence and

TABLE V

Analytes with significantly different levels in clinically infected and non-infected patients

I-TAC, interferon γ-inducible T cell α chemoattractant; OSM, oncostatin M; PAI-II, plasminogen activator inhibitor-II, NGF, nerve growth factor.

Analyte	Clinically infected			Non-infected			Effect size	*p* value
	Mean	S.D.	*n*	Mean	S.D.	*n*		
E-selectin	10.66	1.57	42	9.26	1.54	51	0.90	0.00004
IL-18	8.38	1.55	47	7.24	0.99	51	0.88	0.00004
IL-2sRα	8.91	0.99	38	8.08	0.99	52	0.84	0.0002
CRP	12.88	0.83	47	12.09	1.15	51	0.77	0.0002
uPAR	9.54	0.88	45	8.95	0.61	39	0.77	0.0007
uPA	7.82	0.40	45	7.53	0.47	39	0.67	0.003
P-selectin	11.65	1.19	41	10.80	1.33	51	0.66	0.002
NE	13.04	0.98	47	12.37	1.25	49	0.60	0.004
CA125	6.82	0.76	45	6.45	0.40	40	0.59	0.008
ICAM-1	12.30	0.87	43	11.83	0.79	37	0.57	0.01
OSM	6.75	0.49	47	6.48	0.47	54	0.57	0.005
PAI-II	6.01	0.89	47	5.55	0.74	50	0.57	0.006
CCL21	8.83	0.71	44	8.43	0.71	40	0.56	0.01
ET-3	5.81	0.57	45	5.52	0.49	41	0.55	0.01
ErbB2	9.07	0.37	43	8.87	0.39	40	0.55	0.01
FGFR3 (IIIb)	6.12	0.25	45	5.97	0.32	41	0.55	0.01
IL-16	7.11	0.75	43	6.73	0.63	37	0.54	0.02
MIP-3α	8.22	1.42	41	7.58	0.99	38	0.51	0.03
ErbB1	9.98	0.32	44	9.79	0.41	40	0.50	0.02
Eot-2	9.48	1.27	36	8.90	1.09	44	0.49	0.03
NGF-β	8.49	0.75	43	8.12	0.83	36	0.48	0.04
IL-1ra	7.31	1.64	45	6.68	1.00	52	0.47	0.02
I-TAC	6.95	1.01	43	6.58	0.53	38	0.46	0.04
MMP-8	12.33	1.88	41	11.41	2.06	50	0.46	0.03
MIP-1β	8.90	2.17	39	8.14	1.12	46	0.45	0.04
CD40	5.12	0.44	48	4.92	0.46	51	0.44	0.03
MPIF-1	6.42	0.77	49	6.13	0.58	55	0.44	0.03
MCP-2	5.97	0.78	48	5.70	0.51	54	0.42	0.04
IL-12p40	5.68	0.59	46	5.47	0.47	51	0.40	0.05
MMP-2	13.55	0.32	44	13.70	0.30	41	−0.48	0.03

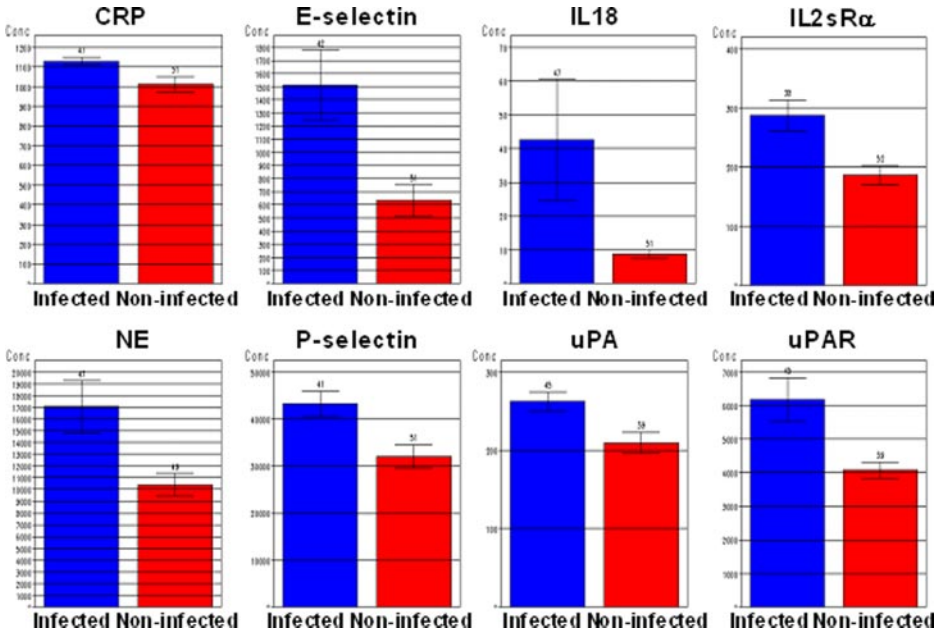

FIG. 1. **Eight serum analytes with greatest differences in levels between clinically infected and non-infected neonates.** Each analyte had significant differences between clinically infected and non-infected neonates ($p < 0.05$ by ANOVA and effect size >0.6 in log-transformed level). Group mean analyte levels are expressed in pg/ml with S.E. (*bars*).

derived concentration data. Additionally an interval censored log-linear model of concentration *versus* group membership resulted in significant difference between infected and non-infected patients for these analytes (data not shown). Fig. 1 shows average concentration (pg/ml) per patient group for each of these eight analytes.

Multivariate Classification—Although average values of log-transformed analyte concentrations for several individual analytes showed significant differences between infected and non-infected analytes, single analytes were insufficient for prediction of infection status (data not shown). To develop a predictive model we investigated multivariate functions for their ability to discriminantly predict infection status.

Several multivariate classifier panels were obtained using different parameter settings in discriminant analyses. All contained analytes that exhibited significant group differences in univariate analyses, such as uPAR and uPA. Certain classifiers that predicted infection and non-infection correctly in cross-validation studies were rejected because of overfitting concerns due to featuring relatively large numbers of analytes (data not shown). Others were rejected because they featured well annotated analytes that are established biomarkers in other conditions (*e.g.* CA125). A single classifier was chosen that featured the smallest number of analytes and greatest number of analytes with significant group differences in univariate analyses ($n = 5$) and that did not contain analytes lacking biological congruence for neonatal sepsis. This classifier consisted of 13 analytes (analyte, partial R^2 value; CD27, 0.06; CD40, 0.05; ciliary neurotrophic factor receptor α, 0.06; FGF6, 0.10; GRO-γ, 0.07; interferon α, 0.05; MCP-3, 0.07; MMP-2, 0.09; P-selectin, 0.09; plasminogen activator inhibitor-II, 0.06; VAP1, 0.08; VEGF-R3, 0.06; uPA, 0.06). This classifier predicted infection in 38 of 43 infected neonates (P(i|i) 0.88) and predicted absence of infection in 31 of 37 uninfected neonates (P(u|u) 0.84). In cross-validation analyses, the classifier predicted infection in 38 of 43 infected neonates (validateP(i|i) 0.88) and predicted absence of infection in 30 of 37 uninfected neonates (validateP(u|u) 0.81).

Differentiation of Etiologic Agent Class by Examination of Host Serum Protein Levels—Host biomarkers for early differentiation of etiologic agent in neonates with bacteremia were also sought. Log-transformed levels of serum analytes in three neonatal infections with cultures positive for group B streptococci, four with *Candida*, 16 with coagulase-negative staphylococci, and three with *E. coli* were compared. Hierarchical clustering and parallel plots of analytes with largest standardized LSMeans effects are shown in Fig. 2. Host responses differed widely between etiologic agents. Coliform infections evoked little change in the serum analytes examined. In contrast, levels of the proinflammatory cytokines IL-6, IL-8 (CXCL8), MCP1 (CCL2), G-CSF, P-selectin, and prolactin were much higher in group B streptococcal bacteremia than other neonatal infections: average IL-8 levels in group B streptococcal infections were 112,130 pg/ml compared with 40 pg/ml in candidemia and 44 pg/ml in *Staphylococcus epidermidis* bacteremia. Average IL-6 levels were 270,636 pg/ml in group B streptococcal bacteremia, 18 pg/ml in candidemia, and 45 pg/ml in *S. epidermidis*. MCP1 differentiated candidemia (177 pg/ml) from group B streptococcal bacteremia or *S. epidermidis* (216,700 and 109,827 pg/ml, respectively). Fungal infections were associated with elevated levels of the costimulatory receptor 4-1BB, IL-18, IL-2sRα D dimer, uPAR, and chemokines monokine induced by interferon γ (MIG; CXCL9) and MIP3α (CCL23) among others. Cultures positive for coagulase-negative staphylococci were associated with elevated insulin-like growth factor II and TRAILR1.

DISCUSSION

A retrospective clinical study was undertaken to identify a serum biomarker panel to diagnose infection in premature neonates. Antibody microarrays were used to measure the levels of 142 small serum proteins in 107 infected and non-infected patients. Amplified antibody microarrays have several potential advantages over conventional monoplex immunoassays or mass spectrometry-based proteomics assays. Sample volumes available from premature neonates preclude use of conventional immunoassays. The small mass and low abundance (pg/ml) of the analytes together with "masking" by more abundant serum proteins diminish the usefulness of mass spectrometry-based proteomics assays. Sensitivity, specificity, linear dynamic range, and coefficients of variation of miniaturized, multiplexed immunoassays can approach that of monoplex sandwich immunoassays (26). However, very limited sample volumes in the present study required sample dilution and performance of assays in duplicate (rather than triplicate), which impaired sample pass rate (Table II) and coefficients of variation (Table IV). Furthermore the present study was undertaken before some recently reported improvements in the standardization of array manufacture and assays were developed (26), and many of the analytes tested have labile immunogenicity. Nevertheless significant differences between groups were observed for 30 of 142 analytes (Table V). Two of these (CRP and ICAM-1) were also measured by conventional, monoplex immunoassays, yielding congruent results. Eight analytes met additional, more stringent conditions for elevation in serum of infected premature neonates (effect sizes greater than 0.6, mean intensity values in the linear range of measurement, and demonstration of similar trends between fluorescence and derived concentration values). They were P-selectin (CD62P), E-selectin (CD62E), IL-2sRα (CD25), IL-18, NE, uPA, uPAR (CD87), and CRP (Fig. 1).

CRP is a critical, phylogenetically ancient component of the innate immune response. Upon CRP recognition and binding to phosphocholine (a phospholipid expressed on the membranes of many bacteria and damaged host cell walls), the acute phase inflammatory response is initiated (36). Accordingly increased levels of CRP are observed early in response to severe bacterial infection (37). Plasma CRP levels were found to be statistically significantly higher in patients with septic syndrome symptoms, and CRP serum concentration correlated with severity of a patient's clinical state (38). In fatal cases high plasma CRP concentration was recorded during hospitalization, whereas in cases of recovery, rapid CRP decrease was noted (38). In our study, CRP was found to be

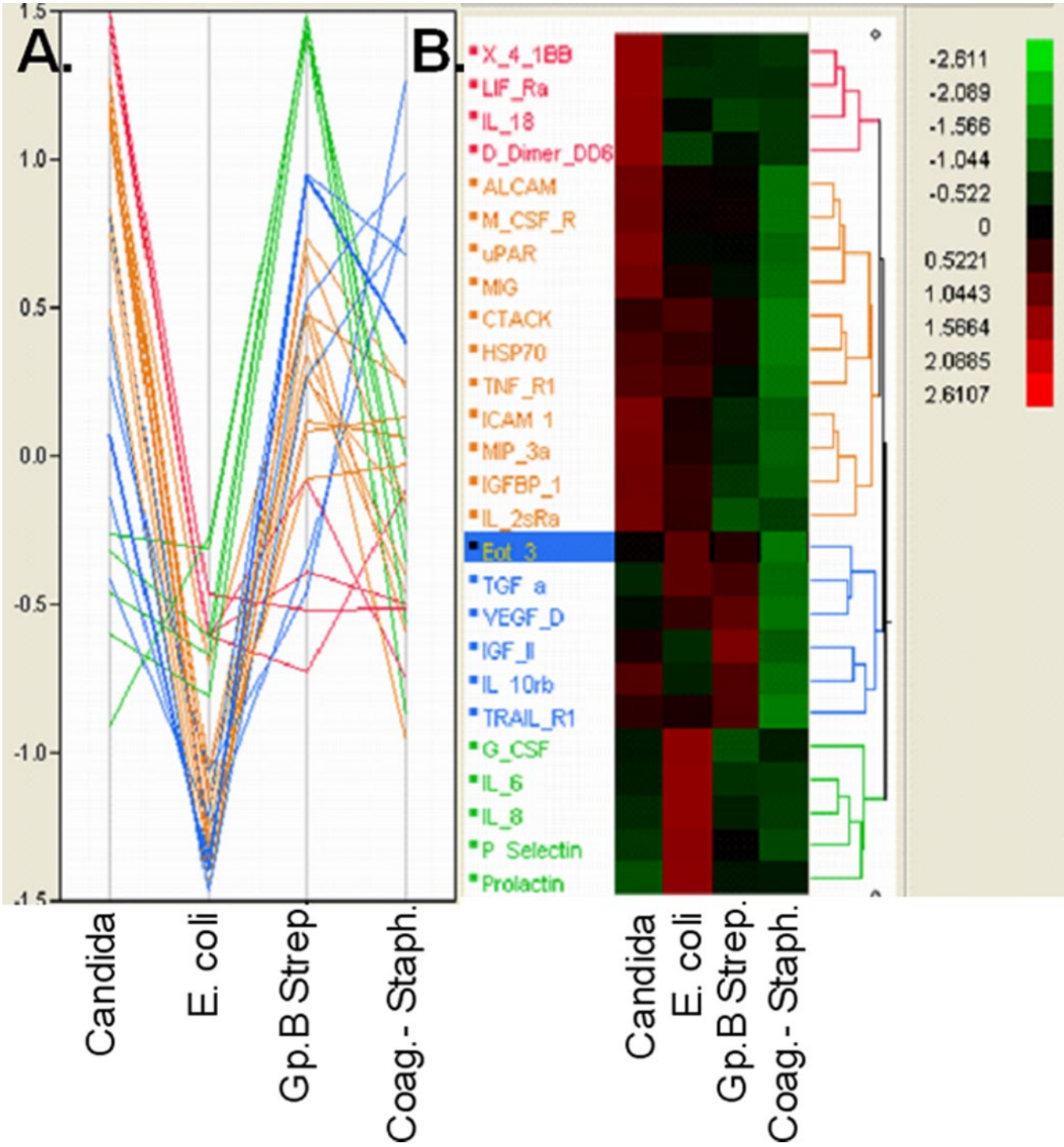

FIG. 2. **Parallel plot (*A*) and dendrogram of hierarchical clustering (*B*) of analytes with largest standardized LSMeans effects in pairwise comparisons of infected neonates with microbiologic cultures positive for coagulase-negative staphylococci (*Coag.–Staph.*), *E. coli*, *Candida*, and group B streptococci (*Gp.B Strep.*).** Eotaxin 3 is *highlighted*; it is elevated in group B streptococcal infection and depressed in *E. coli* infection. See Table I for protein abbreviations and full names.

increased in neonatal patients with infection. Therefore, plasma CRP concentration in patients with bacterial sepsis may be helpful in evaluation of clinical state severity and monitoring of the disease course as well as therapy efficacy. Previously we have shown that plasma CRP $>$ 6 mg/liter has a high sensitivity for clinical infection (95%) and NPV of 97% (12). As a classical acute phase reactant, however, CRP elevation alone has insufficient specificity for diagnosis of neonatal infection.

P- and E-selectins are related cell adhesion molecules expressed and secreted by activated endothelial cells (P- and E-selectins) and platelets (P-selectin). Both P- and E-selectins are components of an adhesion cascade that leads to leukocyte and platelet accumulation at sites of inflammation, infection, and/or injury. Uncontrolled adhesiveness in the microcirculatory system leads to tissue hypoxia (cryptic shock) and eventual organ dysfunction, hallmarks of severe sepsis and septic shock (39). Previous studies of P-selectin have demonstrated elevations in serum and platelets in adult sepsis and septic shock and in platelets in neonatal group B *Streptococcus* sepsis (40–42). However, only elevated levels of E- (but not P-) selectin have been previously documented in neonatal sepsis (43–45).

In part as a result of E-selectin elevation, another early component of the host response to infection is recruitment of neutrophils. Accordingly it was not surprising to find elevated NE in infected neonates. NE is the major serine proteinase secreted by activated neutrophils (or released by damaged neutrophils), and several previous publications have documented NE as an infection parameter for neonatal diagnosis (14, 46–50).

Further evidence of altered endothelial adhesiveness is increased levels of coagulation/fibrinolysis cascade proteins: uPA, an extracellular serine protease, is involved in the catalytic conversion of plasminogen to the active fibrinolytic enzyme plasmin (51). uPA is activated upon binding to its cognate receptor, uPAR, which is a membrane-linked receptor with extracellular protease activity that transduces intracellular signaling pathways. Although in the healthy state, the balance of coagulation and fibrinolysis is tightly regulated, during sepsis coagulation and fibrinolysis are dysregulated as evidenced, for example, by consumption of plasminogen and protein C, elevation of plasminogen activator inhibitor, and occurrence of disseminated intravascular coagulation. Our study, in agreement with others, found increased circulating levels of uPA in the septic state (51). In addition, increased levels of soluble uPAR in response to a variety of infections have been reported and are an indicator of good prognosis in sepsis (52–56). A significant molar excess was observed in the increase in level of uPAR relative to uPA. This raises the possibility that, in neonatal infection, elevated uPAR may act to sequester uPA (despite elevated levels of uPA) and prevent it from binding the membrane-linked uPAR, thus abrogating normal intracellular signaling. Interestingly NE also activates fibrinolysis during sepsis, and its elevation has been suggested as a homeostatic mechanism to overcome impaired fibrinolysis in sepsis (57).

IL-2sRα is a circulating form of one of three components of the high affinity membrane receptor for interleukin 2 present on activated T and B cell thymocyte subsets, pre-B cells and regulatory T cells. Although the biological function of IL-2sR is not completely understood, it is known to indicate T lymphocyte activation during disease. Several previous studies have demonstrated elevated serum levels of IL-2sRα in neonatal sepsis (39–44).

IL-18 is a proinflammatory member of the IL-1 cytokine superfamily. Although IL-18 elevation has not been described previously in neonatal sepsis, recent studies have associated it with other disease morbidity. Neonatal necrotizing enterocolitis (58) and respiratory syncytial virus (59) infection have been associated with genetic polymorphisms in IL-18. In a mouse model, elevated IL-18 levels have been reported in neonatal group B streptococcal infections (60). Furthermore elevated levels of IL-18 have been documented in adult sepsis patients and correlate with adverse outcome and Acute Physiology and Chronic Health Evaluation (APACHE) II score (45, 46).

Interestingly it appears that the eight analytes identified as elevated in the infected neonates function in a network that links several processes, which are well known to be important in the response to systemic infection, *e.g.* coagulation, cell adhesion, and inflammation (Fig. 3). For example, it has been shown that IL-18 induces E-selectin expression on endothelial cells (61); IL-18 release is stimulated by CRP (62). CRP also induces significant E-selectin expression in human endothelial cells (63). Similarly the endothelial expression of E-selectin was increased by human neutrophil elastase (64). In contrast, uPA (urokinase) administration reduces the serum levels of soluble E-selectin in patients with acute myocardial infarction (65, 66). The presence of uPA increases platelet surface P-selectin expression in a concentration-dependent manner (67). It has also been suggested that intravascular fibrinolysis induced by uPA may induce P-selectin (68). uPA itself, as well as uPAR, are cleaved by neutrophil elastase (69, 70). Adhesion of human monocytes to P-selectin via its surface ligand, P-selectin glycoprotein 1, induced uPAR expression (71). Other studies have reported additional links between the above molecules, *e.g.* colocalization of neutrophil elastase, uPA, and uPAR (72) or colocalization of uPA and IL-2sRα (73). Finally pathogens are known to interact in a specific manner with components of fibrinolytic pathways as well as with CRP (74).

Multivariate analyses were undertaken to derive a multiplexed biomarker panel with improved diagnostic sensitivity and specificity relative to single analyte determinations. Derivation of a multivariate classifier was impeded by the small number of patients studied and by missing values. Several methods for imputation of missing values were utilized but were unsatisfactory. A 13-analyte classifier was developed without missing value imputation whose membership had biological congruence with neonatal infection. In cross-validation analyses, however, the classifier predicted infection in only 38 of 43 infected neonates (validateP(i|i) 0.88) and absence of infection in only 30 of 37 uninfected neonates (validateP(u|u) 0.81). These values provide a proof of concept for the utility of host biomarker

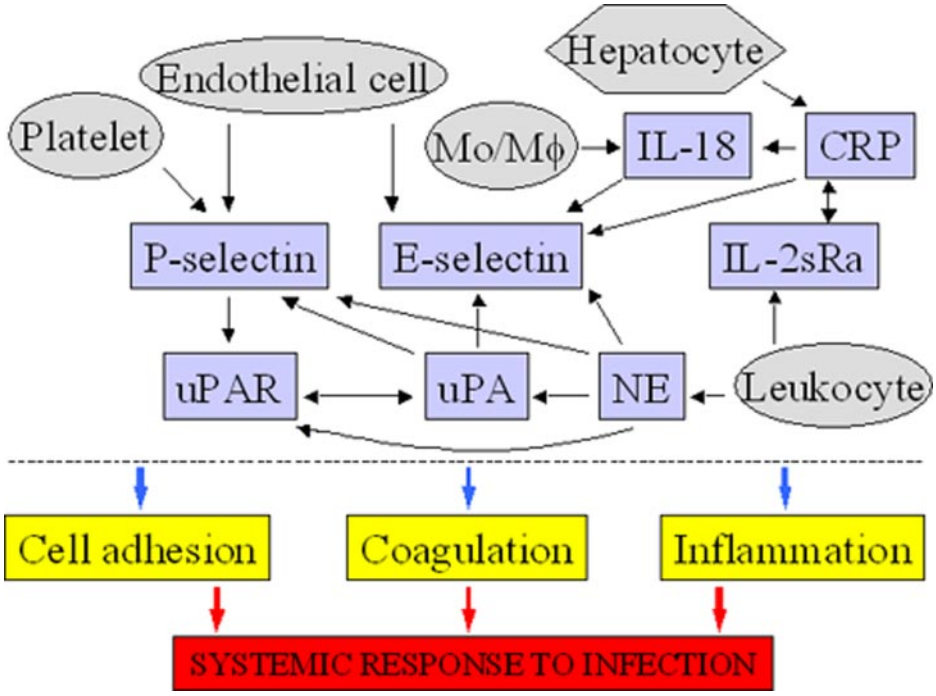

FIG. 3. **Eight serum proteins (P-selectin, E-selectin, IL-2sRα, IL-18, NE, uPA, uPAR, and CRP) that are elevated in infected neonates function in a molecular network that links coagulation/fibrinolysis, cell adhesion, and inflammation, which are critical determinants of the response to systemic infection.** *Mo/Mϕ*, monocytes macrophages.

measurement in diagnosis of neonatal infection but are insufficient for routine diagnostic use and attest to the extreme heterogeneity of neonatal infection. Additional prospective studies are needed. Future studies should feature larger numbers of patients with matching of acute illness scores and other clinical covariates between groups and/or with synchronization of infection progression through use of a longitudinal study design. In addition, a variety of miniaturized, multiplexed immunoassay formats are now available with improved precision, and a large set of candidate biomarkers are available for testing. Such a study is currently in progress.

Finally host biomarkers for early differentiation of etiologic agent in neonatal infection were sought. Host responses were compared for the four most common species in positive microbiologic cultures: group B streptococci, *Candida*, *E. coli*, and coagulase-negative staphylococci. The rationale for differentiation of etiologic agent based on host serum protein levels is that etiologic agents differ predictably in lifestyle and membrane lipid and protein content, evoking characteristic host responses. We found unique signatures of host response to each etiologic agent (Fig. 2). For example, levels of the proinflammatory cytokines IL-6 and IL-8 were more than 1000-fold higher in group B streptococcal bacteremia than in other neonatal infections. These novel findings will require validation in larger case series but offer the exciting prospect of rapid differentiation of at least some etiologic agents in neonatal sepsis, which would enable early institution of targeted antimicrobial therapy. Differences of the magnitude observed with IL-6 and IL-8, if substantiated, would permit etiologic agent differentiation with rapid, point-of-care, semiqualitative immunoassays.

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