Multivariate Analysis of Cytokine Responses Identifies Distinctive Sensitivities to Lipopolysaccharide in Humans

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We describe methods to identify high and low responders in a whole-blood assay of lipopolysaccharide-stimulated cytokine responses. Two multivariate measures of the cytokine responses both captured high and low responses for each of the four individual cytokines that were assayed.

Sepsis is an important health concern (18) that has defied a simple and comprehensive description. Recognition of a familial component in sepsis (24) has prompted the search for predisposing host factors, and because cytokines mediate both protective responses and morbidity in infections (21, 26), they have been examined in this regard. Allelic phenotypes have been observed in vitro for various cytokines, including interleukin-6 (IL-6) (12), IL-8 (13), IL-10 (7, 9, 10), and tumor necrosis factor alpha (TNF- α) (11, 12), but the evidence for a substantial genetic influence on gram-negative sepsis is not conclusive (16). A previous study described only marginal influences of selected host factors and cytokine alleles on lipopolysaccharide (LPS)-stimulated production of IL-6, IL-8, IL-10, and TNF- α (25). However, we observed that these responses were somewhat correlated ($r = 0.438$ to 0.599) and suggestive of interactions or concerted control of the four cytokine responses. Here we describe a simple approach to quantify and characterize the overall cytokine response as reflected by the production of these four cytokines and discuss how this technique may complement efforts to understand and treat sepsis.

The whole-blood culture and cytokine assays have been described previously (25). The time periods for phlebotomy, culture, and assay were strictly followed (phlebotomy, 8 to 9 a.m.; 20 h of culture with or without $0.01 \mu\text{g}$ of *Escherichia coli* LPS/ml). Means (and standard deviations) of the log transforms of LPS-stimulated cytokine levels were as follows: IL-6, 4.289 (0.261); IL-8, 3.900 (0.289); IL-10, 2.910 (0.180); and TNF- α , 2.562 (0.316). Z scores of these transforms were used because of the differing magnitudes and variances of the cytokine responses; without standardization the analyses would be dominated by numerical differences rather than comparative differences in response levels.

Two multivariate measures of the responses were generated, using a recently reviewed approach (15). Log-transformed, standardized cytokine levels were plotted as a vector in four dimensions (IL-6, IL-8, IL-10, and TNF- α), where average responses would be represented by $Z = 0$ on each of the axes. As illustrated in Fig. 1A, we determined the cosine of the angle between a subject's response vector and the vector of each of

the other subjects in turn. The resulting matrix was analyzed, using hierarchical clustering of the Euclidean distances among the cosine values by using complete linkage of the emergent clusters (14). The data were most simply described with high- and low-response clusters ($n = 68$ and 39 subjects, respectively). The subjects were grouped as high or low responders, and as shown in Fig. 2, comparisons for each of the four responses were significant ($P < 0.0001$, Student's t test). The coefficients of determination (20) were 0.18 , 0.26 , 0.50 , and 0.47 for IL-6, IL-8, IL-10, and TNF- α , respectively (as determined by analysis of variance). These proportions of explained variance were notably higher than those for the univariate analyses (25), suggesting that the multivariate analysis provided a more comprehensive model of the responses. Determination of the second multivariate measure, the response index, is illustrated in Fig. 1B. Response indices were determined for all subjects in the study and grouped by high- or low-response category, and these were also found to differ significantly (Fig. 3) ($P < 0.05$, rank sums test). The response index was determined weekly for nine individuals and remained constant for each subject over a 2-month period, indicating that a single determination of the index adequately reflected the response independently of time (data not shown).

It has been observed that the incidence and severity of sepsis is greater in men than women (18, 23, 28). Gender-dependent differences in cytokine responses have also been observed (3, 12, 17, 22). In a subset of the subjects analyzed in this study, we found that men made significantly more IL-6, IL-8, and TNF- α but not IL-10 (25). Comparisons for all 107 subjects in this study gave analogous results (Fig. 4). Of note, the distributions for men and women were similar, except that the IL-6, IL-8, and TNF- α responses for men were right-shifted. Next, the multivariate response measures of men and women were compared. The proportion of men and women within the high- and low-response groups was nearly significant ($P = 0.058$, chi-square test), and the response index was significantly higher in men than women ($P < 0.01$, rank sums test). Interestingly, the index distributions were quite distinctive for men and women (Fig. 5). Men had indices in the intermediate-to-high ranges, and women had a biphasic distribution of high and low indices.

The multivariate response measures described in this report have two useful features. First, they captured significantly different responses for each of the four cytokines (Fig. 2) while summarizing them in a single measure. Second, they provided

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FIG. 1. The two multivariate analyses used in this study. In this illustration, two of the four cytokine responses are plotted on the x and y axes for three subjects, a, b, and c. (A) Categorical analysis. The angles between the response vectors of subjects a and b and a and c are illustrated. The cosine of the angles between all paired combinations of the subjects' response vectors was determined, and the resulting matrix was analyzed by clustering. (B) Quantitative analysis. A low-response reference vector was plotted as $Z = -5$ in all four dimensions; two dimensions are shown. The angle between the response vector of subject a and the reference vector is indicated. The response index is the cosine of this angle. For subject a, this angle is approximately 180° and the response index is approximately -1 . The approximate angle and response index for b would be 90° and 0 , respectively, and for c they would be 0° and 1 , respectively.

a more comprehensive model of the cytokine responses, since they accounted for a greater proportion of the response variation than the univariate analyses. A comparison of Fig. 4 and 5 provides a striking visual contrast as well: the univariate analyses indicated that men tended to have higher cytokine responses than women, but the multivariate analysis showed that the difference could be attributed very specifically to differing frequencies of high and low responders among men and women.

Consequently, we anticipate several applications for this technique. Conceivably, the correlations among the cytokines point to a dimension in the responses greater than that of the individual cytokines. By identifying subjects differing at this level, it becomes possible to characterize the basis of the rela-

tionships among the individual responses. For example, inter-individual differences in the TLR4 signaling pathway are likely to influence many aspects of the response to LPS (1, 19), and in fact, greater NF κ B mobilization has been seen in nonsurviving septic patients than in survivors (2, 4). Alternatively, interactions among the cytokines may intensify their correlation, as, for example, TNF- α -enhanced production of IL-8 and IL-10 (8, 27). In any case, stratification of subjects by their overall response would facilitate the evaluation of such explanations. There may be clinical applications for the multivariate measures as well. There have been many attempts to correlate prognosis in sepsis with circulating levels of various pro- and antiinflammatory cytokines, with various degrees of success. Although the complexity of cytokine responses in sepsis is

FIG. 2. Comparisons of cytokine responses of high- and low-responding groups. The angles among the four-parameter response vectors were clustered on the basis of their similarity. Subjects were grouped into one of the final two clusters, and their cytokine responses were compared. Each comparison was significant ($P < 0.0001$, as determined by Student's t test).

FIG. 3. The response index. The cosine of the angle between each subject's response vector and a low-response reference vector was determined. Subjects were grouped by high- or low-response cluster, and the frequency distribution of the response indices of these two groups was plotted.

FIG. 5. Comparison of the overall cytokine response of men (solid line) versus that of women (broken line).

appreciated, to our knowledge there have been few attempts to capture multiple cytokine responses in a summarizing metric (5, 6). Since it has been suggested that production of pro- and antiinflammatory cytokines together can lead to complications in sepsis (21), representation of the concerted cytokine response may help clarify the role cytokines play in the disease. In particular, stratification by the multivariate response measures could facilitate experimental evaluations of targeted therapies where it is possible to determine the subjects' response level prior to endotoxin exposure. However, it remains to be seen if our observations apply to the ex vivo responses, or cytokine levels in plasma, of sepsis patients. We have very preliminary evidence that responder status may have some relevance to cytokine levels in plasma: a comparison of IL-8 levels in the unstimulated control cultures of high and low responders was significant ($P < 0.05$, Student's t test; no other cytokines were consistently detected in the control cultures).

Lastly, note that discriminant functions (14) were generated by using the clustered data as a training sample. With these functions, subjects can be classified as high or low responders solely on the basis of results from the whole-blood assays, without the need for vector analysis and clustering. There was 95.3% agreement in classification by clustering and the discriminant functions, even without adjustments for unequal frequencies of high and low responders or for cost of misclassification, both of which can increase the agreement between the two methods (14). Thus, it would be possible to generate a reference set of data in a primary lab, cluster the responses, and generate the functions. The discriminant functions could then be used for classification by any labs whose methods are consistent with those of the reference lab. However, this approach requires careful consideration of the reference population: it should include subjects whose responses span the clinical spectrum with regard to endotoxemia to ensure that

FIG. 4. Comparison of the individual cytokine responses of men (solid line) versus those of women (broken line). Significant comparisons are indicated (determined by Student's t test).

the four-parameter-response space encompasses the responses of interest, e.g., responses from subjects that may be susceptible or resistant to sepsis because of extreme levels of cytokine production.

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