Natural Variability of Circulating Levels of Cytokines and Cytokine Receptors in Patients With Heart Failure: Implications for Clinical Trials

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OBJECTIVES
The purpose of this study was to examine the variability in cytokines and cytokine receptors in patients with heart failure in comparison with a group of healthy control subjects who were free of cardiovascular disease.

BACKGROUND
Despite increasing interest in cytokines as mediators of disease progression in heart failure and the recent interest in suppressing cytokines in clinical studies, the extent of variability in cytokines and cytokine receptors is largely unknown. This information is important for interpreting the results of studies in which changes in cytokine levels are measured in response to a specific form of therapy.

METHODS
Circulating levels of tumor necrosis factor-alpha (TNF-α), and soluble TNF receptors (types 1 and 2), as well as interleukin (IL)-6 and IL-6 receptor were measured on a daily, weekly and monthly basis in heart failure patients (New York Heart Association class IIIa and IIIb; n = 10) and healthy volunteer subjects (n = 10). Measurements of cytokines and cytokine receptors were performed on plasma samples by enzyme-linked immunoassay. The daily, weekly and monthly degree of variability in cytokine and cytokine receptor levels was assessed by determining the coefficient of variation each point in time.

RESULTS
The coefficient of variation for TNF-α and IL-6 levels increased over time in patients with heart failure; moreover, the coefficient of variation in heart failure subjects was significantly greater for IL-6 than for TNF-α. The coefficient of variation in cytokine receptor levels was minimal, and did not differ significantly between heart failure and control subjects.

CONCLUSIONS
In patients with heart failure the degree of natural variability in circulating cytokine levels increases with time, and is greater for IL-6 than for TNF-α. Accordingly, the results of the present study suggest that the sample size needed to show a statistically significant change in the circulating level of a given cytokine will vary depending on the specific cytokine that is being measured, as well as the time period over which that cytokine is being assayed. (J Am Coll Cardiol 1999;33:1935–42) © 1999 by the American College of Cardiology

Despite repeated attempts to develop a unifying hypothesis that explains the clinical syndrome of heart failure, no single conceptual paradigm has withstood the test of time. That is, whereas clinicians have tended to view heart failure as an edematous state, or alternatively a hemodynamic disorder, more recent studies have suggested that the excessive elaboration of biologically active molecules may play an important role in the pathogenesis of heart failure by virtue of the direct toxic effects that these molecules exert on the heart and the circulation (1). In this regard, one of the more recent interesting and intriguing observations in clinical heart failure research is that in addition to the classic neurohormones that are elaborated in heart failure, a second portfolio of biologically active molecules, termed proinflammatory cytokines, are also overexpressed in heart failure (2).

Given that cytokines have been shown to produce pulmonary edema and left ventricular dysfunction in human subjects (3–5), there has been increasing interest in studying the role that these molecules may play as biologic mediators of disease progression in heart failure. Accordingly, it is perhaps not surprising that a number of experimental and clinical studies have begun to examine the levels of cytokines and cytokine receptors in response to various forms of treatment (6–11).
To assess the influence of a given therapeutic intervention from either a clinical or biologic point of view, it is essential to understand the natural degree of variability in the end points that are being targeted for therapeutic intervention. Thus far, however, the extant information with respect to the natural variability in cytokines and cytokine receptors in patients with heart failure has been extremely limited (12,13). Moreover, there is no information with regard to whether or not the degree of cytokine variability in subjects with heart failure is more or less than the degree of variability that is observed in healthy subjects. Therefore, the purpose of this study was to prospectively assess the degree of daily, weekly and monthly variability in cytokines and cytokine receptors in patients with moderate to advanced heart failure in comparison with a group of healthy volunteers who were studied over a similar time period.

METHODS

Subject demographics. To examine the natural variability of cytokines and cytokine receptors in patients with heart failure, we selected 10 consecutive volunteers from the heart failure clinic at the Houston Veterans Affairs Medical Center. All of the patients had New York Heart Association (NYHA) classification class III heart failure, and were on stable medical therapy for at least one month before enrollment in the study. The patients had to give informed consent and be available for the daily, weekly and monthly follow-up visits during the four-month duration of the study. The cohort of control subjects for this study comprised healthy volunteers selected randomly from the Houston Veterans Affairs Medical Center population. All of these subjects were screened for the absence of organic heart disease by history, physical examination, 12-lead electrocardiography and two-dimensional echocardiography. Heart failure and normal volunteers were excluded from the study if they had a viral or bacterial infection within a two-week period of the study. The research protocol was approved by the Institutional Board for Human Subject Research for Baylor College of Medicine.

After the initial baseline visit (day 1), each of the control and heart failure subjects had a total of seven follow-up visits, including visits on days 2, 3, 8 (week 2), 15 (week 3), 22 (week 4), 50 (week 8) and 78 (week 12). This permitted the assessment of variability in cytokine and cytokine receptor levels on a daily (days 1 to 3), weekly (weeks 1 to 4) and monthly (months 1 to 4) basis.

Circulating levels of cytokines and cytokine receptors. The methods for measuring cytokines and cytokine receptors have been reported previously in detail, and were adhered to throughout this protocol (14,15). All cytokine and cytokine receptor assays were performed using commercially available enzyme-linked immunosorbent assay kits (Quantikine HS, R & D Systems, Minneapolis, Minnesota) as described previously (14,15). All assays for cytokines and cytokine soluble receptors were measured in duplicate. In addition, we performed serial dilutions to be certain that the level of cytokine immunoreactivity declined in a manner parallel to the standard curve (16). All samples for a given patient were analyzed on the same enzyme-linked immunosorbent assay plate to minimize intraassay variability. The intrassay coefficients of variation for tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), soluble type 1 TNF receptor (sTNFR1), soluble type 2 TNF receptor (sTNFR2) and soluble IL-6 receptor (sIL-6R) are 6.1%, 3.7%, 5.0%, 2.8% and 4.5%, respectively. The respective interassay coefficients of variation for TNF-α, IL-6, sTNFR1, sTNFR2 and sIL-6R are 7.8%, 3.6%, 5.2%, 3.6% and 5.1%. The lower limit of sensitivity of these kits for detecting cytokines and cytokine receptors is (pg/ml): TNF-α (0.18), IL-6 (0.09), sTNFR1 (3.5), sTNFR2 (1.0) and sIL-6R (0.5) (14).

For these studies we assessed the variability in circulating levels of TNF-α and IL-6, based on the repeated observation that these cytokines are consistently elevated in heart failure (2,17). We elected not to measure circulating levels of IL-1-beta, based on the observation from this laboratory (unpublished observation) and other laboratories (18–20) that circulating levels of IL-1-beta are not elevated in patients with heart failure. In addition to measuring cytokines, we also elected to measure circulating levels of the soluble cytokine receptors, because these receptors have been shown to regulate the bioactivity of TNF-α and IL-6. For example, sTNFR1 and sTNFR2 have been shown to neutralize the effects of TNF-α (21), whereas sIL-6R has been shown to increase the bioactivity of circulating IL-6 (22).

Variability in circulating levels of cytokines and cytokine receptors. To assess the variability of cytokines, we determined the coefficient of variation for the levels of cytokines and cytokine receptors for heart failure and control subjects (23). The coefficient of variation was defined as the standard deviation of the cytokine or cytokine receptor levels divided by the mean level of the respective cytokine or cytokine receptor level (24). Accordingly, the daily coefficient of variation for individual patients was obtained by determining the mean and standard deviation for the cytokine or cytokine receptor levels on days 1, 2 and 3 of the study. Similarly, the weekly and monthly coefficient of variation for individual patients was obtained, respectively, by determining the mean and standard deviation for the cytokine or
cytokine receptor levels on week 1 (day 1), 2, 3, 4 (weekly variability) and on week 1 (day 1), week 4, week 8 and week 12 (monthly variability) of the study. The average daily, weekly and monthly coefficient of variation for all patients was determined from the mean of the daily, weekly and monthly coefficients of variation for individual patients.

Statistical analysis. Neither the cytokine nor the cytokine receptor data were normally distributed; therefore, these data were subjected to logarithmic transformation (14,25). However, to permit comparison with results from other studies, both the cytokine and cytokine receptor data are presented as mean ± SEM. A nonpaired t test was used to compare all continuous variables (age, heart rate, blood pressure and ejection fraction) in the heart failure and control subjects, whereas a Fisher exact test or chi-square test was used for the nominal variables (gender, race). The log-normalized coefficient of variation for the daily, weekly and monthly variability in cytokine and cytokine receptor levels between control and heart failure subjects was compared using a nonpaired t test. To confirm that this analysis was robust, we repeated the analysis using the Wilcoxon rank-sum test on non–log-transformed data. Two-way repeated measures analysis of variance (ANOVA) was used to test for mean differences between and within heart failure and control subjects in circulating cytokines and cytokine receptors as a function of time. The Pearson product–moment correlation analysis of the log-normalized data was used to test for significant relationships between cytokines and individual patient age. To confirm that this parametric analysis was robust, we repeated the above analysis using the Spearman rank-order test on non–log-transformed data. Data (Cary, North Carolina) were analyzed using Sigma Stat (Chicago, Illinois), Primer (McGraw Hill, New York, New York) and SAS statistical packages. A significant difference was said to exist at p < 0.05.

RESULTS

Subject demographics. Table 1 summarizes the demographics for the subjects with heart failure (n = 10) and the normal volunteers (n = 10). As shown, the mean age for the heart failure group was significantly higher than that for the control group (p < 0.001). The subjects in the heart failure and control groups were primarily male, reflecting the demographics of the Houston Veterans Administration Hospital; there was, however, no significant difference in the frequency of male and female subjects between the two groups. The racial composition of the two groups was not significantly different (p = 0.48), and consisted of eight white subjects and two African-American subjects in the heart failure cohort, and six white subjects, three African American subjects and one Asian-American subject in the control group. All the heart failure patients had stable NYHA class IIIa or IIIb heart failure and were not edematous at the time they were enrolled in the study. The patients’ functional class remained stable throughout the study. The etiology of heart failure was ischemic in origin in 60% of the patients; the remaining 40% of the patients were considered to have idiopathic dilated cardiomyopathy. The heart failure patients were on standard triple therapy for heart failure, including angiotensin-converting enzyme inhibitors (100%), digoxin (80%) and diuretics (90%). There were no changes in the dosages of these drugs during the three-month period of observation. At the time that these studies were conducted, the use of beta-adrenergic blocking agents for heart failure was not routinely recommended; therefore, only one of the heart failure patients was using a beta-blocker. None of the heart failure patients nor the control subjects was taking medications that were known to alter TNF-α or IL-6 levels. As shown in Table 1, there was no significant difference in the baseline heart rate, systolic blood pressure and diastolic blood pressure between the heart failure and control subjects. However, the mean ejection fraction was significantly lower in the heart failure patients when compared with control subjects (p < 0.001).

Circulating levels of cytokines and cytokine receptors. To determine whether the circulating levels of cytokines and cytokine receptors remained stable in the heart failure and control subjects during the course of the study, we compared the mean levels of cytokines and cytokine receptors at baseline, and then at monthly intervals for a period of four months. Figure 1A to C shows two important findings with respect to the levels of TNF-α or soluble TNF receptors.

Table 1. Demographics and Clinical Characteristics at Baseline

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HF</th>
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<tbody>
<tr>
<td><strong>Baseline Characteristics</strong></td>
<td></td>
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</tr>
<tr>
<td>Age (yr)</td>
<td>33.1 ± 2.4</td>
<td>59.1 ± 2.2*</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>9/1</td>
<td>10/0</td>
</tr>
<tr>
<td>NYHA classification</td>
<td>N/A</td>
<td>III</td>
</tr>
<tr>
<td><strong>Hemodynamics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>70.7 ± 5.8</td>
<td>68.5 ± 4.0</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>119.0 ± 4.7</td>
<td>126.9 ± 7.3</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>68.3 ± 5.4</td>
<td>75.6 ± 3.5</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>57.5 ± 2.2</td>
<td>27.4 ± 2.2*</td>
</tr>
<tr>
<td><strong>Medications (mg/24 h)</strong></td>
<td></td>
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</tr>
<tr>
<td>Lisinopril</td>
<td>N/A</td>
<td>20.0 ± 5.0</td>
</tr>
<tr>
<td>Furosemide</td>
<td>N/A</td>
<td>84.0 ± 27.6</td>
</tr>
<tr>
<td>Digoxin</td>
<td>N/A</td>
<td>0.15 ± 0.03</td>
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</table>

*p < 0.001 compared with values in normal subjects. All data are reported as mean ± SEM. BP = blood pressure; HF = heart failure; N/A = not applicable; NYHA = New York Heart Association.
between the control and heart failure groups (p < 0.001); however, there was no significant difference (p > 0.05) in the mean levels of TNF-α, sTNFR1 or sTNFR2 within the heart failure and control groups as a function of time.

Figure 2A and B depicts the mean values for IL-6 and sIL-6R in the heart failure and control subjects. As shown, the mean values for IL-6 were higher in the heart failure group compared with the control group throughout the course of the study. In contrast, the mean levels of sIL-6R were similar in both the heart failure and control subjects. The mean levels for IL-6 and sIL-6R remained stable in both the heart failure and control subjects. Two-way repeated measures ANOVA showed that there was an overall significant difference in the mean levels of IL-6 between heart failure and control subjects (p = 0.005), whereas there was no significant difference (p = 0.51) in the mean levels of IL-6 within the heart failure and control groups as a function of time. In contrast, there were no significant differences in the sIL-6R levels.

Variability in circulating levels of cytokines and cytokine receptors. To examine the daily, weekly and monthly variability in circulating levels of cytokines and cytokine receptors in the heart failure and control subjects, we examined the coefficient of variation for the cytokines and cytokine receptors. Figure 3 shows two salient findings with respect to the variability in circulating TNF-α levels in heart failure and control subjects. First, the daily (p < 0.05) and weekly (p < 0.02) variability in TNF-α levels was significantly greater in the control subjects than in the subjects with heart failure. Second, there was no significant difference (p = 0.82) in monthly variability between heart failure and control subjects.

Finally, to determine whether the coefficient of variation for circulating levels of IL-6 in heart failure subjects was greater than the coefficient of variation for circulating levels of TNF-α in heart failure subjects, we compared the daily, weekly and monthly coefficient of variation for these two cytokines using a nonpaired t test. This analysis showed that the weekly (p = 0.01) and monthly (p < 0.001) coefficient
of variation was significantly greater for IL-6 when compared with TNF-α, whereas there was no significant difference (p = 0.27) in the daily coefficient of variation for IL-6 and TNF-α levels. Similar findings were obtained when we employed the Wilcoxon rank-sum test on non–log-transformed data.

Table 2 depicts the daily, weekly and monthly coefficient of variation for circulating levels of cytokine receptors for the subjects with heart failure and the healthy control subjects. As shown, the overall variability in circulating levels of sTNFR1, sTNFR2 and sIL-6R was relatively low for both groups of patients, when examined on a daily, weekly or monthly basis. Moreover, there was no statistically significant difference in the coefficient of variation, at any point in time, between either the patients with heart failure or the healthy control subjects.

Because the patients with heart failure were older than the normal volunteers, we sought to determine whether there was a significant correlation (Pearson product–moment correlation) between the coefficient of variation for TNF-α and IL-6 levels and the corresponding age of the patient. This analysis showed that there was no significant correlation between age and cytokine variability (data not shown) when the data were examined on a daily, weekly or monthly basis (range: r = −0.46 to 0.37; p = 0.18 to 0.99). Similar results were obtained when the nonnormalized data were analyzed with the Spearman rank-order test, suggesting that the parametric analysis was robust. Nonetheless, the effect of age on cytokine variability cannot be completely excluded by this analysis.

DISCUSSION

Despite the increasing interest in the role that cytokines play as potential mediators of disease progression in heart failure, and the increasing interest in modulating cytokine levels in clinical heart failure studies (6–11,26), virtually nothing is known with respect to the natural variability in circulating levels of cytokines and cytokine receptors in patients with heart failure. To address this deficiency we prospectively examined the variability in circulating levels of cytokine and cytokine receptors in subjects with moderate to advanced heart failure (NYHA class IIIa and IIIb). Analysis of the aggregate data permits at least two important conclusions to be drawn. First, the degree of cytokine variability appears to depend not only on the cytokine that is being measured, but also on the time course over which that cytokine is being assessed. For example, we observed that the daily and weekly variability in circulating TNF-α levels was significantly lower in heart failure patients than in healthy subjects (Fig. 3). However, TNF-α variability increased approximately twofold with time in the heart failure subjects, with the result that the monthly variability in TNF-α levels was not significantly different between heart failure and healthy subjects (Fig. 3). In contrast to the findings with TNF-α, there was a great deal of variability in IL-6 levels in both heart failure and normal subjects (Fig. 4), regardless of whether the variability was assessed in both settings. Although there was no difference in the daily and weekly variability in IL-6 levels between subjects with heart failure and normal volunteers, we observed that there was a small but statistically significant increase in monthly variability in IL-6 levels in patients with heart failure when
compared with normal volunteers (Fig. 4). Moreover, there was an approximately fourfold increase in IL-6 variability in the heart failure patients with time. Finally, when we compared the average coefficient of variation for cytokine levels in heart failure patients, we observed that the extent of weekly and monthly variability was significantly greater for IL-6 when compared with TNF-α. The observed differences in variability in circulating cytokine levels in the heart failure and control subjects did not appear to be related to increasing cytokine levels over the three-month period of the study (Fig. 1 and 2), nor to age-related differences between the heart failure and control subjects, insofar as we did not observe a significant relationship between age and cytokine variability for either group of subjects, consistent with previous studies in healthy subjects that have not observed a correlation between age and TNF-α and IL-6 variability (27,28). Furthermore, the natural variability in cytokine levels in the present study was unlikely to be due to intra- or interassay variability alone, since the observed variability in cytokines was well in excess of the analytical variability of the assay methodology used in the present study (14). The second conclusion to be drawn from these studies is that the natural variability in circulating levels of cytokine receptors in heart failure and normal subjects is less than that observed for the respective cognate cytokines (Table 2). Moreover, the variability in cytokine receptors was not significantly different in heart failure patients compared with the normal subjects (Table 2).

Cytokine variability in heart failure. Although the precise reason(s) for the differences in the variability in circulating TNF-α and IL-6 levels in heart failure subjects is not known, there are several potential explanations that warrant discussion. One possible explanation for the differing degrees of variability between these two cytokines may relate to the differing degree of stabilization of these proteins in the peripheral circulation. That is, although TNF-α and IL-6 are known to bind to, and to be stabilized by their circulating cognate receptors (29–31), the degree of stabilization may be quantitatively different for each cytokine. For example, we observed that the ratio ([mol/liter]/[mol/liter]) of (sTNFR1 + sTNFR2)/TNF-α ranged ~2,100 to 2,700 for the healthy volunteers and heart failure subjects in this study, whereas the ratio of sIL-6R/IL-6 ([mol/liter]/[mol/liter]) ranged ~3 to 6. Thus, the increased variability that we and others have observed for IL-6 may relate, at least in part, to the possibility that IL-6 is degraded more rapidly in the periphery than TNF-α is. Further support for the point of view that increased stability may account for the differing degrees of variability in TNF-α and IL-6 levels is suggested by the observation that the absolute levels of circulating TNF receptors increase in direct response to increased TNF-α levels (“receptor shedding”) (32), whereas the response of IL-6 receptors to increasing levels of IL-6 is quite variable, and may increase, decrease or remain the same (33–35). Indeed, in the present study we noted that there was an approximately twofold increase in sTNFR1 and sTNFR2 in heart failure patients, where the levels of sIL-6R were not significantly different in the healthy subjects and the patients with heart failure (Fig. 2) (36,37). The previous statements notwithstanding, it should be recognized that a number of proteins other than sIL-6R may serve as chaperones for IL-6, and that the transport and stabilization of this molecule is likely to be very complex (31). A second, intriguing explanation for the decreased variability in TNF-α levels in the heart failure subjects may relate to the general loss of biologic variability that has been observed repeatedly in heart failure patients. That is, previous studies have reported a loss in heart rate variability (38), and loss in neurohormonal variability in patients with heart failure (39,40). Indeed, it has been suggested that loss of “variability reserve” (41) is a sign of a biologic system that is persistently activated, and may therefore respond less well to superimposed physiologic/pathologic stimuli (42). However, although this concept may explain the loss of variability in TNF-α, it does not explain the higher degree of variability in IL-6 levels that we observed in the heart failure patients. A third explanation for the difference in variability between TNF-α and IL-6 in our study might simply relate to the inherent differences in their circadian or seasonal variation in IL-6 levels.

Although a full discussion of the natural variability in cytokines and cytokine receptors in health and disease is beyond the intended scope of this discussion, it bears emphasis that the results of the present study are qualita-

Table 2. Natural Variability in Cytokine Receptors

<table>
<thead>
<tr>
<th>Coefficient of Variation (%)</th>
<th>sTNFR1</th>
<th></th>
<th>sTNFR2</th>
<th></th>
<th>sIL-6R</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HF</td>
<td>Control</td>
<td>HF</td>
<td>Control</td>
<td>HF</td>
</tr>
<tr>
<td>Daily</td>
<td>5.7 ± 1.0</td>
<td>4.1 ± 1.3</td>
<td></td>
<td>4.9 ± 0.8</td>
<td>4.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Weekly</td>
<td>8.3 ± 1.3</td>
<td>5.5 ± 1.1</td>
<td></td>
<td>8.2 ± 1.1</td>
<td>6.3 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Monthly</td>
<td>7.8 ± 1.2</td>
<td>7.4 ± 2.0</td>
<td></td>
<td>10.8 ± 2.7</td>
<td>9.6 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>(2.9–12.3)</td>
<td>(0.6–14.7)</td>
<td></td>
<td>(1.4–8.6)</td>
<td>(0.5–15.1)</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>(1.4–16.6)</td>
<td>(1.6–12.7)</td>
<td></td>
<td>(4.2–14.3)</td>
<td>(2.3–10.5)</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>(2.3–16.0)</td>
<td>(2.3–24.5)</td>
<td></td>
<td>(5.8–34.7)</td>
<td>(3.9–13.3)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM and as the range for the coefficient of variation (shown below in parentheses).
HF = heart failure; sIL-6R = soluble interleukin-6 receptor; sTNFR1, sTNFR2 = soluble TNF receptor types 1 and 2.
tively similar to those reported by Masson et al. (13), who studied the natural variability in IL-6 and sTNFR1 levels in 18 heart failure subjects (NYHA class II/III) over a three-week period. The calculated coefficient of variation for IL-6 and sTNFR1 in their study was ≈195% and ≈7%, respectively, which was somewhat higher than we observed in the present study. Although the reasons for the greater degree of variability in IL-6 levels in the study by Masson et al. (13) are not known, they may relate to the fact that these investigators determined cytokine variability based on two measurements three weeks apart, whereas we assessed IL-6 variability based on cytokine determinations at four separate time points, each one week apart. The results of the present study differ somewhat from those reported by Dutka et al. (12), who measured TNF-α levels serially every three months for one year in patients with NYHA class IV heart failure. These investigators did not directly determine a coefficient of variation for TNF-α levels in their study, but did report that TNF-α levels were below the limit of detection of their assay on at least one occasion in all 16 of their patients, thus leading them to conclude that there was “considerable between and within patient variation” in TNF-α levels in their study. The most likely explanation for the difference between their report and the present study is that the TNF-α assay they used was 150-fold less sensitive than the one used in the present study. Thus, it is possible that the inability to detect a circulating TNF-α level in their study was the result of the assay that was employed, as opposed to a high degree of variability in TNF-α levels in their patients with heart failure. However, we cannot exclude the possibility that the NYHA class IV patients in their study may have had a greater degree of variability in TNF-α levels than the NYHA class III patients we examined in the present study.

There are several limitations of the present study that bear emphasis. First, the overall sample size of the present study was relatively small, which was due in large part to the difficulty in finding stable heart failure patients who were willing to return to the hospital for the frequent visits. Nonetheless, this relatively small sample size did not preclude obtaining statistically significant results with respect to the variability in TNF-α and IL-6 levels. Second, all patients had moderate heart failure (NYHA class IIIa and IIIb); accordingly it is unclear if our results will be relevant for patients with milder or more advanced degrees of heart failure. Nonetheless, the levels of cytokines in our study overlap with those reported for patients with NYHA IV heart failure (15). Thus, it is possible that our findings may also apply to patients with more advanced heart failure.

Conclusions. In assessing the impact of a given therapeutic intervention from either a biologic or a statistical point of view, it is necessary to first understand the natural degree of variability in the end points that one has chosen to target. Given the recent interest in cytokines as therapeutic targets in the setting of heart failure, the results of the present study would appear to be important for at least two reasons. First, the results of the present study suggest that the sample size needed to show a statistically significant change in the circulating level of a given cytokine after a therapeutic intervention will vary, depending on the specific cytokine that is being measured, as well as the time period over which that cytokine is being assayed (Fig. 3 and 4, Table 3). As one theoretical example, the data in the present study suggest that for a given therapeutic intervention to detect a 15% change in either circulating TNF-α or IL-6 levels over a four-month period (alpha = 0.05; power = 0.80), it would require a sample size of 21 and 377 patients, respectively (Table 3) to show a statistically significant difference. Thus, the sample size needed to show a statistically significant difference may vary at least 18-fold, depending on the particular cytokine that one intends to target. A second important, and potentially disconcerting implication of this study is that it may not be possible to relate specific changes in circulating levels of certain cytokines with specific changes in outcome measures in individual heart failure patients with any degree of confidence, because of the tremendous amount of intrasubject variability in cytokine levels. The above statements are in no way intended to offer a disparaging or discouraging commentary on the importance of assessing changes in circulating cytokines in the setting of heart failure; however, they do suggest that studies that are designed to assess changes in circulating cytokine levels in patients with heart failure should not be undertaken casually, and that all such studies should be appropriately designed to avoid drawing conclusions that may lack biologic or clinical significance.

Acknowledgments
The authors gratefully acknowledge the secretarial assistance of Jana Grana, as well as the spirited technical
assistance of Donna Espada, Adrienne Chee, Wendy Skinner and Dorellyn Lee-Jackson. We also would like to thank Dr. Andrew Schafer for his past and present support and guidance.

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REFERENCES