Measurement of Induced Cytokines in AIDS Clinical Trials Using Whole Blood: A Preliminary Report

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Measures of immune function have become increasingly important as endpoints in AIDS clinical trials, with respect to both modulation and reconstitution of immunity by experimental therapies. Measurement of immune function in this setting requires the development of robust analytic approaches suitable for the clinical laboratory. Experiments were performed to evaluate the suitability of using cultured heparinized (“whole”) blood for induction of tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ), two cytokines critical in AIDS pathogenesis. TNF-α expression ranged from 229 to 769 pg/ml in lipopolysaccharide (LPS)-stimulated cultures and was not detected in unstimulated cultures. IFN-γ expression ranged from 0 to 112,000 pg/ml in phytohemagglutinin A (PHA)-stimulated cultures and from 0 to 789 pg/ml in antigen-stimulated cultures. The mean coefficient of variation observed in three weekly determinations was 0.47 for TNF-α and ranged from 0.12 to 1.73 for IFN-γ. These values indicate that sample sizes of 8, 24, and 29 subjects would be sufficient to detect twofold changes in LPS-induced TNF-α and in PHA- and antigen-induced IFN-γ, respectively, if two baseline and two treatment determinations were obtained, and if the interpatient variability of changes in true levels from baseline to follow-up is negligible compared to the variability in the three weekly measurements. Measurement of LPS-induced TNF-α and mitogen- or antigen-induced IFN-γ can be performed simply and reproducibly in human immunodeficiency virus-infected persons by the whole-blood culture method. Further studies are warranted to determine the effect of overnight shipping on assay reproducibility and to determine the extent to which responses can be reliably detected in subjects with low CD4 cell numbers.

An increasing body of evidence suggests that highly active antiretroviral therapy (HAART) can effectively control human immunodeficiency virus (HIV) replication, increase CD4 cell numbers, and prolong survival for AIDS patients. Much less is known regarding the extent to which normal immune function is restored by such therapy. Although one prospective study (AIDS Clinical Trials Group [ACTG] study 315) reported partial immune reconstitution in subjects responding to HAART (15), several cases of disseminated Mycobacterium avium or cytomegalovirus infection have been reported in patients with unusually high CD4 cell counts in the setting of HAART (13, 21), raising the possibility that restoration of CD4 cell numbers may not necessarily lead to improved host defenses against AIDS-associated pathogens.

Measurement of immune function in the setting of a multicenter study requires standardized, robust methods appropriate for a clinical-trials laboratory. In 1996, the Advanced Technology Laboratory (ATL) program of the ACTG was created and charged with the development and validation of such methods. The objectives of the ATL program included development of a simple and reproducible method for measurement of antigen- and mitogen-induced cytokine expression. Cytokine induction is usually performed by using mononuclear cells isolated by density sedimentation. The present study evaluated production of tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) by using an alternative approach which has been termed whole-blood culture. In this method, diluted heparinized blood, rather than isolated mononuclear cells, is placed into culture, and the cytokine content is analyzed after an appropriate period of incubation. These two cytokines were selected based on their role in HIV pathogenesis and immune induction. The objectives of the pilot study were to determine optimal methods for specimen handling and cell culture with respect to sensitivity, reproducibility, and relationship to HIV disease stage.

MATERIALS AND METHODS

Subjects. Subjects included persons with HIV type 1 (HIV-1) infection documented by Western blotting whose CD4 cell counts had been measured within the preceding 6 months, who did not have active opportunistic infections, whose antiretroviral therapy (if any) had not been altered during the preceding 3 months, and who were not receiving immunomodulating agents such as thalidomide, prednisone, or colony-stimulating factors. Healthy volunteers without known HIV-1 infection or risk factors were also recruited as controls. CD4 cell counts were not measured for this group but were assumed to be normal. CD4 cell counts were categorized as normal (>600/μl), moderately reduced (200 to 600/μl), or markedly reduced (<200/μl). Blood was collected into heparinized Vacutainer tubes. Cultures were initiated within 4 h of phlebotomy except as noted. Specimens were kept at room temperature between the times of phlebotomy and culture.

Cell culture. Culture was performed by diluting either 200 or 100 μl of blood in medium (RPMI 1640 with 25 mM HEPES, endotoxin tested, with added penicillin–streptomycin–L-glutamine solution; Sigma) for a total volume of 1 ml. Cultures were performed in 24-well tissue culture plates (Corning). Cultures...
TABLE 1. Effect of E. coli LPS concentration and dilution of blood on induction of TNF-α by the whole-blood method*

<table>
<thead>
<tr>
<th>LPS concn (ng/ml)</th>
<th>Blood dilution (fold)</th>
<th>Mean TNF-α concn (pg/ml)</th>
<th>Mean CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>10</td>
<td>459</td>
<td>0.47</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>634</td>
<td>0.51</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>348</td>
<td>0.45</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>423</td>
<td>0.46</td>
</tr>
</tbody>
</table>

* Means and CV refer to values obtained from three weekly measurements in specimens from eight donors. All pairwise comparisons of TNF-α concentrations obtained under the conditions described above were significant (P < 0.01 after Bonferroni’s adjustment) except for LPS at 100 ng/ml with a 10-fold blood dilution versus LPS at 10 ng/ml with a 5-fold blood dilution. The CV were not significantly different.

RESULTS

TNF-α. Eight subjects (three healthy, five infected with HIV-1) were studied on 3 consecutive weeks at one site by the whole-blood method. No TNF-α was detected in any of the unstimulated cultures in which blood was diluted 10-fold in medium. One unstimulated culture diluted fivefold had low levels of TNF-α identified (22 pg/ml); the remainder had undetectable levels (<15 pg/ml).

All stimulated cultures contained detectable TNF-α. As shown in Table 1, the mean level of induced TNF-α was 459 pg/ml when blood was diluted 10-fold with medium and stimulated with 100 ng of E. coli LPS/ml. Mean induced TNF-α levels increased to 634 pg/ml when blood was diluted 5- rather than 10-fold and decreased to 348 pg/ml when the LPS concentration was reduced to 10 ng/ml (P < 0.001). However, neither of these modifications in culture conditions significantly affected the mean CV observed over the 3-week interval. Mean TNF-α concentrations obtained with 10 ng of LPS/ml correlated only partially with those obtained with 100 ng/ml (Spearman rank order correlation, 0.64; P = 0.095) when blood was diluted 10-fold; all other combinations correlated moderately (P < 0.04).

Two mycobacterial preparations, one from M. avium and one from M. tuberculosis, were also evaluated for induction of TNF-α. These reagents were selected because of the role of mycobacteria as AIDS-related opportunistic pathogens and because M. tuberculosis filtrate activates monocytes for cytokine production through mechanisms which do not involve the LPS receptor or LPS-binding protein (5, 9). Mean TNF-α concentrations in M. avium filtrate-and M. tuberculosis PPD-stimulated wells were 379 and 437 pg/ml, respectively; these did not differ significantly from those of LPS-stimulated cultures (as determined by pairwise tests of M. avium filtrate versus each concentration of LPS, and of M. tuberculosis PPD versus each concentration of LPS, with Bonferroni’s correction; K = 4). There was a strong correlation between mean LPS-induced TNF-α levels and mean levels of TNF-α induced by M. avium (r = 0.81, P < 0.001), but not with mean levels of TNF-α induced by M. tuberculosis PPD (r = 0.23, P = 0.24). A weak correlation in responses to the two mycobacterial reagents was identified (r = 0.46, P = 0.05).

Mean TNF-α production was strongly correlated with CD4 cell count range in responses to E. coli LPS (r = 0.87, P = 0.02) and M. avium filtrate (r = 0.82, P = 0.03), as shown in Table 2. A trend was identified for M. tuberculosis PPD (r = 0.7, P = 0.07). These relationships must be considered cautiously, however, due to the small sample size.

IFN-γ. The same subjects were studied for induction of IFN-γ. Spontaneous production of IFN-γ occurred in 16 of 46 supernatants collected after 3 days. This occurred only in subjects with normal CD4 cell numbers, was not affected by blood dilution, and resulted in relatively low concentrations of IFN-γ (mean concentration in those with detectable cytokine, 132 pg/ml). In PHA-stimulated cultures, IFN-γ was detected in 36 of 46 cultures, with a mean concentration (including all cultures) of 31,754 pg/ml. The IFN-γ concentration decreased when blood was diluted 5- rather than 10-fold (2,348 pg/ml; P = 0.025). The CV of IFN-γ determinations at 10- and 5-fold dilutions were 278.8 and 229 copies/ml, respectively.

TABLE 2. Relationship of HIV infection status and CD4 count to induction of TNF-α and IFN-γ

<table>
<thead>
<tr>
<th>HIV status</th>
<th>No. of CD4 cells/µl</th>
<th>HIV RNA concn (10³ copies/ml)</th>
<th>LPS-induced TNF-α concn (pg/ml)</th>
<th>Mean CV</th>
<th>PHA-induced IFN-γ concn (pg/ml)</th>
<th>Mean CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos</td>
<td>5</td>
<td>278.8</td>
<td>229</td>
<td>0.10</td>
<td>210</td>
<td>1.7</td>
</tr>
<tr>
<td>Pos</td>
<td>45</td>
<td>38.95</td>
<td>332</td>
<td>0.78</td>
<td>ND</td>
<td>NC*</td>
</tr>
<tr>
<td>Pos</td>
<td>370</td>
<td>25.27</td>
<td>375</td>
<td>0.45</td>
<td>6,010</td>
<td>0.34</td>
</tr>
<tr>
<td>Pos</td>
<td>460</td>
<td>5.74</td>
<td>572</td>
<td>0.08</td>
<td>4,670</td>
<td>0.12</td>
</tr>
<tr>
<td>Pos</td>
<td>1,288</td>
<td>39.08</td>
<td>769</td>
<td>0.79</td>
<td>112,190</td>
<td>0.91</td>
</tr>
<tr>
<td>Neg</td>
<td>NP</td>
<td>NP</td>
<td>406</td>
<td>0.66</td>
<td>52,820</td>
<td>0.21</td>
</tr>
<tr>
<td>Neg</td>
<td>NP</td>
<td>NP</td>
<td>481</td>
<td>0.41</td>
<td>37,500</td>
<td>0.28</td>
</tr>
<tr>
<td>Neg</td>
<td>NP</td>
<td>NP</td>
<td>505</td>
<td>0.50</td>
<td>82,190</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* Means and CV are for results of three consecutive weekly determinations by the whole-blood method. The Chiron bDNA assay was used to determine HIV RNA. Pos, positive; Neg, negative; ND, not detected; NP, not performed; NC, not calculated.

The CV could not be calculated because all values were below the detection threshold of the assay.
TABLE 3. Induction of IFN-γ by C. albicans, M. avium, or M. tuberculosis by the whole-blood culture method

<table>
<thead>
<tr>
<th>HIV status&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of CD4 cells/ml</th>
<th>IFN-γ concr&lt;sup&gt;b&lt;/sup&gt; (CV) induced by:</th>
<th>C. albicans</th>
<th>M. avium</th>
<th>M. tuberculosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos 5</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
</tr>
<tr>
<td>Pos 45</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
</tr>
<tr>
<td>Pos 370</td>
<td>321 (0.15)</td>
<td>32 (1.7)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
</tr>
<tr>
<td>Pos 460</td>
<td>154 (0.88)</td>
<td>174 (0.17)</td>
<td>312 (0.66)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
</tr>
<tr>
<td>Pos 1,266</td>
<td>40 (1.4)</td>
<td>146 (1.4)</td>
<td>1,014 (0.80)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
</tr>
<tr>
<td>Neg NP</td>
<td>248 (0.38)</td>
<td>715 (0.38)</td>
<td>958 (0.96)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
</tr>
<tr>
<td>Neg NP</td>
<td>789 (1.1)</td>
<td>557 (0.55)</td>
<td>1,014 (0.80)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
</tr>
<tr>
<td>Neg NP</td>
<td>31 (1.7)</td>
<td>182 (0.48)</td>
<td>1,063 (0.57)</td>
<td>ND (NC)</td>
<td>ND (NC)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Heparinized blood was diluted 10-fold in medium.

<sup>b</sup>Pos, positive; Neg, negative.

Means (in picograms per milliliter) from three determinations at weekly intervals. NC, not calculated; ND, not detected; NP, not performed.

dilutions did not differ. PHA-induced concentrations of IFN-γ at the two dilutions were very highly correlated (r = 0.95, P < 0.0001).

IFN-γ responses to three antigen preparations—Candida albicans, M. avium, and M. tuberculosis—were also evaluated. Mean concentrations are shown in Table 3. There was no significant relationship among responses to the three antigens. The CV for antigen responses ranged from 0.15 to 1.73 and were not significantly greater than that for PHA. Neither mean IFN-γ concentrations nor CV differed when blood was diluted 5- rather than 10-fold.

Mean IFN-γ production was correlated with CD4 cell count range for cultures stimulated with PHA (r = 0.78, P < 0.05), M. tuberculosis PPD (r = 0.85, P = 0.027), or M. avium filtrate (r = 0.87, P = 0.023), but not for C. albicans-stimulated cultures. Again, these relationships must be considered cautiously because of the small sample size and the added potential variation due to differential exposure.

**Sample size analysis.** The variability in these data was used to calculate the required sample size for two representative studies in which twofold changes from baseline measurements were to be analyzed. In the case of TNF-α, this represents the extent of inhibition following administration of pentoxifylline or thalidomide (4, 18, 26) and is less than the threefold change in lymphoproliferative responses to some antigens identified in ACTG study 315. Estimates were developed for single and double determinations at 1-week intervals, at both baseline and treatment time points. These estimates are based on the assumption that the interpatient variability of change in true level from baseline to follow-up is negligible compared to the combined assay and biological variation in our three weekly measurements. The results of this analysis are shown in Table 4. Fewer subjects were required for evaluation of TNF-α, reflecting the greater stability of this measurement over time. However, even for the most variable measurement—antigen-induced IFN-γ production—meaningful data would likely be obtained from a study of as few as 30 subjects if two determinations were made at each time point.

**Effect of shipping.** To evaluate the potential performance of the method in the setting of a multicenter clinical trial, replicate specimens from two subjects from a second site were processed in three ways. One heparinized Vacutainer from each subject was processed normally. A second Vacutainer was opened in the clinic. One milliliter of blood was removed and was added to a polypropylene tube containing 9 ml of medium. Both tubes were resealed, placed within a biohazard shipping container, and then shipped by Federal Express to the lab, where they arrived the following morning. The results from this experiment are shown in Table 5. The values obtained for both TNF-α and IFN-γ when undiluted blood was shipped were highly correlated with those from blood processed immediately (Spearman r = 0.79; P < 0.001). Those obtained when blood was diluted prior to being shipped correlated less well (r = 0.48, P = 0.08).

**DISCUSSION**

The cytokines IFN-γ and TNF-α are critical in host defenses against a wide range of pathogens, in part through synergistic induction of macrophage production of nitric oxide for the killing of intracellular pathogens (2, 3, 22). The capacity for production of IFN-γ by CD4 cells, particularly in response to soluble recall antigens, is progressively lost in HIV-1 infection (17, 24) and is thought to be a major factor in the pathogenesis of AIDS-related opportunistic infections. The extent to which this capacity is restored by HIV therapy and whether this restoration can be used as a guide to clinical decisions regarding the cessation of preventive therapy are important questions.
for AIDS research. In the case of TNF-α, additional significance may arise from the role of this cytokine in promoting HIV-1 expression by infected cells (14, 25). Thus, TNF-α inhibitors, such as pentoxifylline, thalidomide, and prednisone, may be useful adjuncts to standard antiretroviral therapy, particularly in those clinical circumstances where a strong stimulus for TNF-α expression may be present, as in some opportunistic infections.

The standard method for cytokine induction involves isolation of mononuclear cells by density sedimentation. This requires collection of cells at the interface of two intermiscible liquids, after which the cells must be washed, counted, and resuspended in medium with added serum prior to culture. The method may not be well suited to a busy laboratory. However, removal of erythrocytes, neutrophils, and platelets appears not to be necessary in order to study mononuclear-cell function. This is the basis for the whole-blood culture, which was originally described for the study of lymphocyte proliferation and has since been used to study the expression of several cytokines in clinical and laboratory studies (1, 8, 12, 16, 20, 23, 26). The method is simple and robust and requires only a small volume of blood. However, it is blood volume, rather than the number of input cells, which is held constant in these cultures. This may add an additional source of potential variation, both among individuals and within individuals over time. Some researchers have suggested that data from whole-blood cultures be reported on a per-cell basis, adjusted according to the number of input responding cells in the specimen (7). Such a calculation for PHA-induced IFN-γ production in this study yields values of 0 to 860 pg/10^3 CD4 cells in HIV-positive subjects and estimated values of 470 to 1,030 pg/10^3 CD4 cells in control subjects (based on an estimated CD4 cell count of 800/μL). However, total cytokine production (rather than that on a per-cell basis) may be more relevant in terms of assessment of clinical immunity in a patient. Per-cell calculations may also not be appropriate for situations in which the number of responding cells cannot be determined (e.g., antigens) or in cases in which a cytokine is produced by cells of multiple phenotypes.

The number of mononuclear cells in whole-blood cultures is only 10 to 20% of that in cultured isolated mononuclear cells. For this reason, whole-blood cultures may be less sensitive in detecting IFN-γ responses to antigens with low precursor cell frequencies, particularly in individuals with low total numbers of lymphocytes. This suggests that there may be a threshold below which no responses will be detected. The small sample size of the current report limits any conclusions as to the possible lower limit of CD4 cell numbers for detection of antigen-stimulated responses, but this may be 100 to 250 cells/μL.

However, it may also be incorrect to assume that cytokine responses in whole-blood culture are affected by blood dilution in a simple linear relationship, particularly with respect to cultures in which precursor frequencies do not approach limiting dilution. Doubling of the cell density in this study resulted in increases in production of only 38% for LPS-induced TNF-α and 22% for PHA-induced IFN-γ. Many immunoregulatory factors are present in blood, including antibodies, other plasma factors, and products of other cell types. Platelets, for example, are a potential source of transforming growth factor β, a potent immunoregulating factor (10, 11). Platelets are partially but inconsistently removed from mononuclear cells during cell separation but are not removed in whole-blood culture. Other factors may be removed during whole-blood culture by adsorption onto erythrocyte membranes or interaction with antibody. In all these cases, however, one may argue that the responses measured in cultured whole blood may more accurately reflect those expressed in vivo. Thus, conventional cell culture may be useful for defining the function of isolated cells but may not reflect their interaction with other host factors in a patient in vivo.

The variation observed in this study may have arisen from one or more of three potential sources: biological variation, variation in cell culture technique, and variation in analysis of cytokine content. The high correlation of replicate wells in the cytokine ELISAs (which generally differed by <5% [data not shown]) suggests that variation in analysis of cytokine content is not a major factor. In other data which were not presented, <5% variation was also observed in TNF-α responses in three specimens drawn in immediate succession in three healthy individuals. These suggest that biological variation may account for most of the variation that we observed. This may be particularly true for CD4 cell responses, for which we found greater variability than for TNF-α production (which mainly reflected monocyte activation in the short-term cultures). The reported variation in CD4 cell numbers may be as high as 0.19 within a 3-day interval (6, 19). Most of this variation has been attributed to variability in blood leukocyte counts, differential fractions of lymphocytes, and total lymphocyte counts rather than in flow cytometry per se. The variation in CD4 cell numbers over a 3-week interval has not been studied, but these data suggest that much of the variation in IFN-γ production observed here may represent biological variation in CD4 cell numbers.

The factors which may affect cytokine responses following shipping are complex and include temperature, oxygen tension, pH, and nutrient depletion. These factors are thought to be responsible for the reduced lymphoproliferative responses in shipped specimens, particularly when the specimens are held at 4°C (7). In addition, some adherent cells (monocytes) may be lost to the surface of the tube during this time. These factors were expected to result in reduced expression of IFN-γ and TNF-α in shipped blood and were the rationale for the evaluation of shipped blood which had been diluted in a polypropylene tube. Surprisingly, cytokine responses were generally lower in specimens that had been shipped diluted rather than neat. Further studies are required to determine the source of the variation arising during shipping. Although the data presented suggest that overnight shipping of undiluted blood does not interfere with the rank order of results when stimulated and unstimulated specimens are compared, the extent to which shipping will hinder quantitative comparisons in clinical trials also requires further investigation.

Two approaches to measuring induced cytokines in the setting of a multicenter trial, in which either blood or supernatants may be shipped to a central location, may be considered. These two methods would each introduce different types of variation. The effects of shipping may in essence reflect the effects of time and temperature, which in turn may depend on season, location, altitude of air transport, and unpredictable factors such as traffic delays. To minimize these effects, shipping containers should include additional mass (such as an “ice pack” at room temperature) inside an insulated vessel to provide maximum temperature stability. The alternative approach, induction of cytokines on site, would necessitate a training and certification process at each laboratory and might restrict participation in a study. In the limited experience of the Induced Cytokine Focus Group to date, laboratories which have not had previous experience in long-term cell culture may experience unexpected contamination and spurious results during initial attempts at cell culture. For this reason, the certification process should include documentation of low values of TNF-α in unstimulated cultures (since this is the most sensitive indicator of contamination), as well as documentation...
of adequate expression of IFN-γ in PHA-stimulated cultures. It may be appropriate for initial clinical trials to both ship and perform cultures locally so that the variability introduced by these methods may be directly compared.

In summary, measurement of LPS-induced TNF-α and mitogen- or antigen-induced IFN-γ can be performed simply by the whole-blood culture method. The method is sufficiently reproducible and stable over short intervals that relatively small sample sizes may be sufficient to find meaningful changes in clinical trials, although the sensitivity may be a limiting factor in the analysis of IFN-γ production in subjects with advanced disease. Additional studies are necessary to determine whether cultures should be performed locally or centrally (on shipped blood) in the context of multicenter studies and to determine the relationship of HIV disease stage to cytokine production.

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REFERENCES


