Depression of CD4 T Cell Subsets and Alteration in Cytokine Profile in Boutonneuse Fever


Rickettsia conorii which is endemic in the Mediterranean basin; BF is caused by Rickettsia conorii and transmitted by the dog tick Rhipicephalus sanguineus [1–3]. The disease is characterized by fever, malaise, headache, and frequently, by a local lesion (eschar or tache noire) at the site of the tick bite. About the fourth day of fever, a red maculopapular rash appears. Complications are rare, and death is rare except among aged or debilitated patients [4–6]. Rickettsiae invade and proliferate in the endothelial cells of small vessels, destroying them [1, 2]. The mechanism of host defense is not yet completely understood, although cell-mediated immunity is thought to play a crucial role [7].

In vitro studies in mice indicate that interferon (IFN)-γ and tumor necrosis factor (TNF)-α are important factors for antirickettsial activity [8, 9]. Furthermore, they exert their antirickettsial effect via the induction of nitric oxide synthesis [9]. In vivo studies in mouse models have shown that IFN-γ and TNF-α have a crucial host defense role against R. conorii [10, 11]. T lymphocytes appear to be important effector cells in the successful immune response to rickettsiae in vivo [12, 13], and their presence, together with macrophages, in the perivascular area adjacent to infected endothelial cells suggests that they exert their protective role by paracrine secretion of cytokines such as IFN-γ and TNF-α [14].

Human studies are limited. A reduction in CD4 cells has been described in some patients with Mediterranean spotted fever, but this does not seem to be a general rule [15]. Recently, we observed that IFN-γ is highly and quickly activated in humans naturally infected with R. conorii, and it could be an important factor in the host defense against this infection [16]. Elevated circulating TNF-α levels in humans have also been reported [17]. The aim of this study was to investigate the levels of immune cells and lymphokine production in the sera and in the supernatants of mononuclear cells of Sicilian BF patients during the course of infection and after clinical recovery.

Subjects and Methods

Subjects. Ninety patients with confirmed BF (40 women, 50 men; ages 25–75 years) were studied at the time of the diagnosis and after clinical recovery following successful therapy. They all had characteristic signs and symptoms of active BF (presence of fever, eschar at the site of tick bite, and maculopapular rash). Diagnosis was confirmed by serologic data (high levels of anti-R. conorii antibodies by ELISA and indirect IFA) [18]. Length of illness before diagnosis was <2 weeks. Sera were collected at different stages of the disease: at the time of diagnosis without any specific treatment, every week after treatment began, and after clinical recovery. The day of onset of fever was recognized as the first day of illness. The acute stage was considered to be from days 2–14 after the onset of the symptoms and before the specific treatment; the convalescent stage was from day 21 on. The third week of illness was considered to be borderline between the two stages. Patients were treated with tetracycline (500 mg/4 times a day for 7 days). Healthy laboratory workers and blood donors (n = 21) served as controls.
Materials. RPMI 1640 was from Flow Laboratories (Hertford, UK), and fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT). Ficoll-hypaque (Lagitre, Milan, Italy) was used to isolate peripheral blood mononuclear cells (PBMC).

Antigen preparation. Antigen was obtained from Sicilian patients with BF. The local strain was identified as R. conorii by microimmunofluorescence [19] comparing the local strain with the reference strain of R. conorii (Maless 7; provided by D. H. Walker, University of Texas, Galveston). Rickettsial antigen was obtained by three cycles of freezing (-80°C) and thawing (26°C) followed by sonication of infected VERO cells. The material was centrifuged, and the supernatant was further purified on 25% saccharose gradient by centrifugation at 12,000 g for 15 min at 4°C.

Cell surface phenotype. Phycoerythrin (PE)-conjugated or fluorescein isothiocyanate (FITC)—labeled monoclonal antibodies (MAbs) (Becton Dickinson, San José, CA) recognizing cluster designation antigens were used. Cells (10^6) were incubated for 30 min at 4°C with appropriate MAbs. Cells were then washed twice to remove unbound antibody. Dual-color flow cytometric analyses were done on a FACScan cell analyzer (Becton Dickinson). At least 10,000 cells/sample were analyzed after appropriate gating on forward angle and 90° light scatter to identify the lymphoid component, excluding clusters and debris. Corresponding PE- or FITC isotype-matched negative controls for each strain were used to determine the ranges of positive fluorescence.

In vitro stimulation of cytokine production. The PBMC preparations from patients at the time of diagnosis and after recovery were cryopreserved in liquid nitrogen. PBMC (10^7) were resuspended in 0.9 mL of 100% FCS in 2-mL screw-cap plastic am­ poules (Cel-Cult, Sterilin, Feltham, UK). Dimethyl sulfoxide (100 µL) was added immediately before the ampoule was stored at −70°C overnight and transferred to liquid nitrogen. On each occasion, 1 or more PBMC preparations from healthy laboratory workers were also cryopreserved in parallel experiments to serve as controls. The cryopreservation technique was used to reduce excessive variations in experimental procedures by stimulating the individual patient samples the same day. The cells were thawed rapidly in a 37°C water bath while 1.0 mL of FCS was added dropwise. The sample was centrifuged at 600 g for 5 min. The cell pellet was resuspended in RPMI 1640 plus 10% FCS, incubated for 10 min at room temperature, and then centrifuged at 600 g for 5 min. The washing was repeated twice. Viable cells (normally >80%) from each frozen batch were carefully counted by trypan blue exclusion [20]. PBMC (10^6/mL) in medium with 5% FCS were studied at diagnosis and during convalescence (data not shown); 8 of these were studied every week (figure 1B). As indicated, the only detectable differences were the weekly decreases in IL-6 and IL-10 levels that were more gradual and constant in individual patients than in all single samples available, so that their reductions were significant starting from the third week of infection.

Antibiotics, given to the patients early in the disease, brought about a rapid decrease in fever but did not modify the levels of cytokines compared with those observed in the patients treated later in the course of infection (e.g., 10 or 12 days; data not shown).

Cytokine levels in supernatants. PBMC from patients and from healthy controls were tested for cytokine production by stimulation with phytohemagglutinin and antigen in vitro. The data indicate that IFN-γ and IL-10 production from PBMC stimulated with mitogen was significantly decreased (P < .05) in the acute phase of infection and gradually returned to normal in convalescence (figure 2). The cytokines in the supernatants from PBMC showed a profile different from that in serum, and IL-6 and TNF-α production were not significantly modified.

Figure 1. Interferon (IFN)-γ, interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α levels in sera of 90 boutonneuse fever patients during infection (A). Paired samples of 8 patients (B). First column (open bar) represents healthy controls. Second, third, and fourth columns (shaded bars) represent first, second, and third weeks of disease, respectively. Fifth column (shaded bars) represents fourth and subsequent weeks of disease. Only significant differences (P values) between groups are shown. Data are expressed as mean ± SE.
during the different phases of infection. We observed the same trend after antigen stimulation of PBMC even though the data were less homogeneous (data not shown).

Mononuclear cell subsets. To investigate the possibility that the differences in the production of cytokines could be caused by numeric alterations in lymphocytes, we analyzed PBMC subsets from both BF patients and healthy controls for cell surface phenotype by FACSscan. The absolute numbers of PBMC were not significantly altered among the groups. In acute BF patients, there was a significant reduction ($P < .05$) in the total T cell population; in particular, there was a marked decrease in total CD4$^+$ cells, whereas CD8$^+$ cells were unmodified. NK (CD16$^+$) and B (CD20$^+$) cells tended to decrease in acute BF, but their numbers did not differ significantly from those of controls (table 1). Furthermore, the analysis of CD4 subsets showed that both CD4$^+$/CD45RO$^+$ and CD4$^+$/CD45RA$^+$ T cells were markedly reduced in acute BF compared with those of healthy controls and returned to normal levels after successful treatment (table 1). However, variability between patient groups masked significant changes in individual patients, as confirmed by the significance of the comparison ($P < .05$) using the within-subject $t$ test. The proportion of monocytes (CD14$^+$) was significantly increased in the patients with acute BF and remained high after recovery. This was essentially due to a contemporaneous reduction both in T cells expressing HLA-DR (table 2) and in B cells (CD20$^+$; table 1).

Discussion

To the best of our knowledge, our report is the largest immunologic study on BF thus far published. Our data demonstrate that during active BF, several PBMC subsets and cytokines differ significantly from control values. These modifications are reversible and gradually return to normal levels after recovery.

Serum IFN-$\gamma$ concentrations in Sicilian patients with BF were elevated at the time of diagnosis, decreased significantly within 1 week, and in the normal range after successful therapy. These high serum levels of IFN-$\gamma$ could have relevant protective effects against rickettsiae, as already observed in animal models [10, 11]. Because IFN-$\gamma$, inducing NO production, can kill rickettsiae residing free in the cytosol of infected L-929 mouse fibroblasts [8], a similar process could be taking place in human infected endothelial cells. This increase could be explained by an elevated response of T cells to a continuous high antigen load [21, 22]. The inability of PBMC of acute BF patients to produce adequate amounts of IFN-$\gamma$ after in vitro stimulation with mitogen or antigen contrasts with the observation of high serum levels of IFN-$\gamma$. It is possible that IFN-$\gamma$ is produced at the sites of infection, as observed in leishmaniasis [22, 23], as a result of the accumulation of CD4$^+$ cells in the perivascular area adjacent to infected endothelial cells [12–14].

The latter observation is compatible with the reduction of circulating T cells and, in particular, CD4$^+$ and CD4$^+$/CD45RO$^+$ subsets. This finding is consistent with the observation that in humans, CD45RO$^+$ cells adhere to vascular endothelium, augment endothelial permeability, and enter sites of inflammation more readily than do CD45RA$^+$ cells [24, 25]. On the other hand, in our study, the proportion of the CD45RA$^+$ population was unchanged, whereas only the CD4$^+$/CD45RA$^+$ subset was significantly reduced, as with scrub typhus by 

**Figure 2.** Interferon (IFN)-$\gamma$, interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-$\alpha$ activity in supernatants from phytohemagglutinin-stimulated peripheral blood mononuclear cells of boutonneuse fever patients ($n = 30$) during acute infection (open bars) and in convalescence (solid bars) versus levels in healthy controls (shaded bars). Data are expressed as mean $\pm$ SE.
macrophages might further activate these cells by an autocrine TNF-α loop, thus increasing the antimicrobial activity [31, 32]. Furthermore, TNF-α plays a key role in the inflammatory response, regulating localization of leukocytes to inflammatory sites and modulating the response of resident cells [33]. Just as it directly affects endothelial cells, increasing the production of mediators of inflammation (prostaglandins, leukotrienes, and chemotactic factors), TNF-α itself might also be produced by the same epithelial cells [34, 35]. The increased production of serum TNF-α appeared to be related to CD14+ PBMC levels. In fact, both values were significantly higher in patients with acute disease and recovered patients than in healthy controls and decreased slowly, returning to control levels later than all other parameters. The persistence of high serum levels of TNF-α in recovered patients could be a sign of residual local lesions. Significantly higher levels of TNF-α were also observed in Spanish patients with severe Mediterranean spotted fever [17]. It is of great interest that 2 of our patients with very severe forms of BF, associated with disseminated intravascular coagulation, had very high levels of TNF-α that persisted for a long time and decreased only when the condition of the patients improved. In these 2 patients, IFN-γ was increased over control levels but was below the mean value observed in acute patients with the usual mild form of BF.

IL-10 levels were significantly increased in acute BF but also remained high during the third week of infection, whereas IFN-γ levels returned rapidly to the normal range. IL-10 suppresses the ability of IFN-γ-activated macrophages to produce inflammatory mediators [36–38]. Our finding of high levels of IL-10 in the face of high levels of IFN-γ in BF is consistent with the hypothesis that identifies IFN-γ, together with TNF-α, as the predominant cytokines during the early phase of infection, whereas IL-10 down-regulates the potential tissue-damaging effects of responses induced by IFN-γ and TNF-α [17, 39, 40, 41]. The decrease in CD4+ T cells in PBMC in acute BF patients is consistent with the significant reduction of IL-10 production in vitro, and it can be postulated, as is the case for TNF-α, that IL-10 is produced by infected endothelial cells.

The profile of serum IL-6 production in patients with active BF and after recovery was similar to that of IL-10. In acute BF, high IL-6 levels could be due to synthesis at the sites of infection by infiltrating T cells and monocytes [12–14] or by activated and damaged endothelium [42–44], and IL-6 could act as an inflammatory cytokine rather than as a growth factor for B cell differentiation and immunoglobulin production [44]. The fact that B cells (CD20+) are reduced in acute BF in the face of high levels of IL-6 is consistent with this hypothesis. Later, when IL-10 starts to inhibit T cells that produce IFN-γ (Th1-like) [22, 37, 39, 43], favoring the proliferation of those CD4+ T cells secreting lymphokines such as IL-6 (Th2-like) [44], the terminal differentiation of B cells to immunoglobulin-producing cells could be favored. In accordance with this, there is the normalization of B cells in recovered patients.

On the other hand, in previous studies, we have demonstrated that the antibody titer only increased 2 weeks after infection and peaked in 4 weeks [18]. Afterwards, IgM decreased and IgG remained high for several months [18]. Tetracycline treatment appears to reduce fever, but it does not seem to modify cytokine production. It is likely that tetracyclines act on the fever through their bacteriostatic effect, but the persistence of the rickettsiae could be responsible for the continuous activation of the cells producing cytokines.

In conclusion, in the normal evolution of BF, IFN-γ appears to have an important role in antirickettsial activity, acting during the early phase of infection. Later, the effect of IL-10 becomes significant, acting by down-regulating

### Table 1. T cell subset analysis of PBMC from patients with boutonneuse fever during acute infection (A) and in convalescence (B) versus healthy controls (C).

<table>
<thead>
<tr>
<th>Subject</th>
<th>CD3+</th>
<th>CD4+</th>
<th>CD8+</th>
<th>CD16+</th>
<th>CD20+</th>
<th>CD45RO+</th>
<th>CD45RA+</th>
<th>CD3+/CD45RO+</th>
<th>CD4+/CD45RA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>47 ± 16*</td>
<td>28 ± 13*</td>
<td>33 ± 13</td>
<td>9 ± 5</td>
<td>5 ± 3</td>
<td>25 ± 18*</td>
<td>47 ± 10*</td>
<td>18 ± 9*</td>
<td>16 ± 9*</td>
</tr>
<tr>
<td>B</td>
<td>85 ± 5</td>
<td>40 ± 12</td>
<td>39 ± 8</td>
<td>9 ± 4</td>
<td>8 ± 3</td>
<td>49 ± 10</td>
<td>66 ± 8</td>
<td>28 ± 16</td>
<td>23 ± 10</td>
</tr>
<tr>
<td>C</td>
<td>75 ± 7</td>
<td>40 ± 8</td>
<td>32 ± 8</td>
<td>15 ± 7</td>
<td>10 ± 5</td>
<td>40 ± 6</td>
<td>50 ± 8</td>
<td>24 ± 7</td>
<td>30 ± 9</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD of 47 patients. PBMC, peripheral blood mononuclear cells. Cell surface phenotype was determined by FACScan (Becton Dickinson, San José, CA). Total PBMC/mm³: A = 2310 ± 580; B = 2418 ± 494; C = 2690 ± 390.

* Significantly different from healthy controls (P < .05).

† Significantly different from convalescent patients (P < .05).

### Table 2. Percent HLA-DR+ cells in PBMC of patients with boutonneuse fever during acute infection (A) and in convalescence (B) versus healthy controls (C).

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total HLA-DR+</th>
<th>CD3+/HLA-DR+</th>
<th>CD14+/HLA-DR+</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22 ± 10</td>
<td>10 ± 7</td>
<td>18 ± 9*</td>
</tr>
<tr>
<td>B</td>
<td>16 ± 8</td>
<td>12 ± 7</td>
<td>22 ± 11*</td>
</tr>
<tr>
<td>C</td>
<td>35 ± 10</td>
<td>16 ± 3</td>
<td>7 ± 3</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD of 47 patients. PBMC, peripheral blood mononuclear cells. Cell surface phenotype was determined by FACScan (Becton Dickinson, San José, CA). Total PBMC/mm³: A = 2310 ± 580; B = 2418 ± 494; C = 2690 ± 390.

* Significantly different from healthy controls (P < .05).
inflammatory mediators and, together with IL-6, activating the immunoglobulin response against the rickettsiae. TNF seems to be useful as an accurate marker of complete resolution of the infection. Finally, the data suggest that T cells may have an active role in regulating cytokine production in BF.

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