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$CD4^+$ T-Cell Lymphopenia in Q Fever Endocarditis

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Valvular endocarditis is the most serious complication of chronic Q fever, an infectious disease due to *Coxiella burnetii*. Although its pathogenesis is poorly understood, the role of the immune system has been evoked. The aim of this study was to investigate lymphocyte subsets in the peripheral blood of infected patients by analyzing the distribution of T- and B-lymphocyte subsets. Since various infectious diseases have been found to be associated with modified antigen expression, we also measured the antigen density of the main lymphocyte markers by quantitative flow cytometry. The absolute values of $CD3^+$ T cells and $CD19^+$ B cells were lower in infected subjects than in controls. The decrease in the $CD4^+$ T-cell count was more pronounced than that in the $CD8^+$ T-cell count, leading to a significantly lower CD4/CD8 ratio in patients. The decreases in $CD4^+$ T cells and $CD19^+$ B cells were correlated with levels of *C. burnetii*-specific immunoglobulin G, showing that $CD4^+$ lymphopenia is related to the activity of chronic Q fever. Quantitation of antigen expression on lymphocytes showed that CD3, CD4, CD8, and CD19 were expressed similarly in patients and controls. In contrast, CD2 and CD11a expression levels, which are both related to naive and memory phenotypes, were modified in patients. The study of CD45RO and CD45RA expression by $CD4^+$ T cells provided evidence that lymphopenia preferentially affected unprimed lymphocytes.

Coxiella burnetii, an obligately intracellular microorganism, is the etiologic agent of Q fever. It may exhibit acute or chronic forms (4). Valvular endocarditis is the most serious complication of chronic Q fever (24). Although its pathogenesis is poorly understood, the role of the immune system has been evoked (20). Indeed, endocarditis has been reported in patients with hemolymphatic neoplasia, human immunodeficiency virus (HIV) infection, or immunosuppressive therapy (7, 21, 23). Moreover, a deficiency of cell-mediated immune responses has been found in patients suffering from Q fever endocarditis (10, 11). The evaluation of lymphocyte subsets provides insight into host immune response to infections. Besides HIV infection, for which the depletion of $CD4^+$ T cells is related to the outcome of the disease, the modulation of T-cell count has been reported for some viral and bacterial infections (19). On the other hand, any decrease in $CD4^+$ T cells is known to favor the onset of infection by intracellular pathogens. The aim of the present study was to investigate lymphocyte subsets in peripheral blood of patients with Q fever endocarditis by using flow cytometry and to analyze their distribution according to clinical status. Concomitantly, a recently described method of quantitative flow cytometry, QIFI (3), was used to study the expression of the main cell surface receptors of the lymphocyte during *C. burnetii* infection.

MATERIALS AND METHODS

Patients. Twenty-eight patients (18 men and 10 women) aged 22 to 72 were studied. All were diagnosed as having Q fever endocarditis because their titers of antibodies against *C. burnetii* phase I were higher than 800 for immunoglobulin G (IgG) and 50 for IgA, as revealed by indirect immunofluorescence assay (15). They were all treated with 600 mg of hydroxychloroquine and 200 mg of doxycycline per day. Each patient was investigated every 3 months, since the date of inclusion in the study. Fifteen healthy individuals (eight men and seven women) aged 25 to 60 were included as controls. They had no clinical conditions associated with immune dysfunction, received no corticosteroids or suppressive drugs, and had no abnormal hematological data.

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Blood samples. EDTA anticoagulated venous blood was drawn from healthy volunteers and patients between 9 and 11 a.m. in order to avoid variations from diurnal fluctuations. An automated complete blood count and differential were obtained on an STKS hematology instrument (Coultronics, Margency, France). The absolute lymphocyte count was measured as the product of the leukocyte count and the percentage of lymphocytes in the differential. Samples were labeled for flow cytometry analysis within 2 h of venipuncture.

MAbs. The monoclonal antibodies (MAbs) used in this study are listed in parentheses following the antigens against which they are targeted: CD2 (ST11, IgG1; 10 μg/ml), CD3 (ST3, IgG1; 10 μg/ml), CD4 (ST4, IgG1; 10 μg/ml), CD45 (SLC1, IgG1; 50 μg/ml) (Biosys, Compiègne, France); CD8 (IOT8a, IgG1; 10 μg/ml), CD11a (IOT16, IgG1; 50 μg/ml), CD14 (IOM2, IgG2a; 20 μg/ml), CD45RA-PE (ABL11, IgG1; 10 μl/test), CD45RO-PE (UCHL1, IgG2a; 10 μg/test) (Immunotech, Marseille, France); CD19 (B-4, IgG1; 5 μl/test) (Coulter, Margency, France). An antiendothelial MAb (S-Endo 1), which is nonreactive on hematopoietic cells, was used as an isotype-matched negative control (6).

Immunostaining and flow cytometry. Indirect immunofluorescent staining was performed with 100-μl samples of whole blood and MAbs under saturating conditions as previously described (3). The second layer was a polyclonal sheep anti-mouse Ig $F(ab')_2$ fraction conjugated to fluorescein isothiocyanate (SaM Ig-FITC; Silenus, Eurobio, Paris, France) that was also used at saturating concentration in a 1:100 dilution. Flow cytometry was performed on an Epics XL instrument (Coultronics). This analysis was focused on lymphocytes identified by their forward and right-angle scatter features. The controls showed more than 95% CD45-positive cells and less than 1% CD14-positive cells in the lymphocyte gate; 3,000 events were collected and analyzed. For each marker, the percentage of the positive population was measured. Absolute count was determined by multiplying the flow cytometry percentage by the lymphocyte value obtained from the hematology instrument.

Antigen expression was quantitated by the QIFI technique (3, 18), on the basis of the linear relationship between specific mean fluorescence intensity (MFI) and number of antigenic sites per cell (called antigen density) (16, 17). Specific MFI (MFI of the positive population corrected by nonspecific MFI of the irrelevant antibody) is converted into antigen density by using latex beads covalently coated with known amounts of murine Ig (QIFIKIT; Dako, Trappes, France) as standards. These beads are labeled with secondary antibodies and are used to make a standard regression line in order to calculate antigen expression.

For double labeling, after indirect CD4 staining, phycoerythrin-conjugated CD45RO or CD45RA was added in a second step. The percentage of $CD45RA^+$ cells or $CD45RO^+$ cells was determined within the $CD4^+$ gated population. Absolute values of double-labeled lymphocytes were calculated as previously described.

Statistical analysis. Results are expressed as means ± standard deviations (SD). The cytometric data for Q fever patients and for controls were compared by a two-tailed *t* test. Standard regression and Pearson's correlation coefficient (*r*) were used to determine the relationship between lymphocyte subsets and phase I antibody levels. The likelihood of significant difference was >95%.

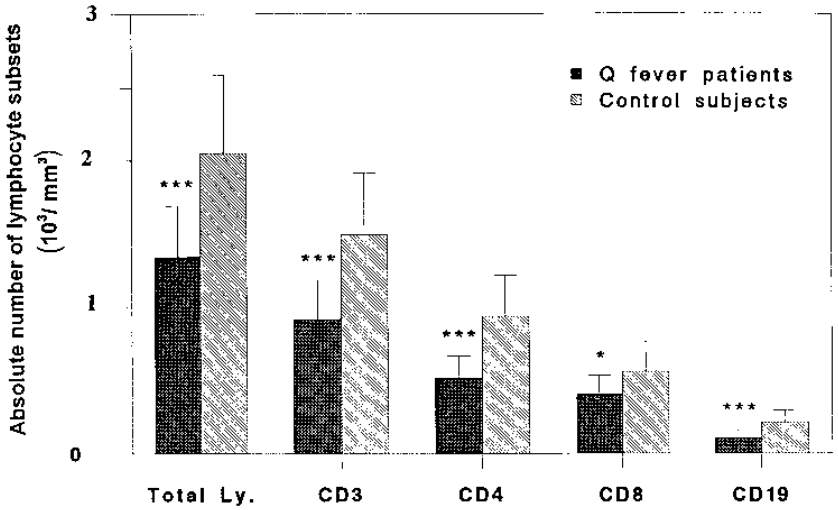

FIG. 1. Lymphocyte subset analysis by flow cytometry. Results are represented as the absolute number of each lymphocyte subset per cubic millimeter (mean ± SD). Levels of significance for comparison between groups are as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Ly., lymphocytes.

RESULTS

Absolute numbers of lymphocytes were significantly decreased in patients (1,338 ± 349/mm^3) compared to controls (2,047 ± 540/mm^3) (Fig. 1). In contrast, monocyte and polymorphonuclear leukocyte counts remained similar in patients and controls (data not shown). The counts of CD3$^+$ T cells and CD19$^+$ B cells were lower in infected subjects than in controls (916 ± 264 versus 1,487 ± 422 CD3$^+$ cells/mm^3 and 103 ± 59 versus 212 ± 82 CD19$^+$ cells/mm^3). More specifically, the absolute numbers of CD4$^+$ helper T cells and CD8$^+$ suppressor-cytotoxic cells were decreased in Q fever patients compared with controls (518 ± 148 versus 935 ± 276 CD4$^+$ cells/mm^3 and 403 ± 131 versus 561 ± 204 CD8$^+$ cells/mm^3. It is noteworthy that the reduction in CD4$^+$ T cells was more pronounced than that in CD8$^+$ T cells. When cytometry data were expressed as percentages, patients exhibited a significant decrease in the percentage of CD4$^+$ T cells and a normal percentage of CD8$^+$ T cells (data not shown). As a consequence, the CD4/CD8 ratio was significantly lower in patients than in controls (1.39 ± 0.50 versus 1.77 ± 0.57).

The levels of *C. burnetii*-specific antibodies were related to lymphocyte subsets. The decreases in CD3$^+$ T cells and CD19$^+$ B cells were significantly correlated with phase I IgG levels ($r = 0.293$ and 0.269, respectively). Among T-cell subsets, there was a marked correlation between CD4$^+$ T lymphopenia and antibody titers. In agreement with the moderate effect of Q fever on CD8$^+$ T cells, the correlation between CD8$^+$ T-cell numbers and titers of phase I IgG was less significant than the correlation between CD4$^+$ T cells and levels of specific antibodies ($r = 0.244$ and 0.306, respectively).

To discriminate between modified antigen expression and decreased number of circulating lymphocytes, we further quantitated cell surface density of the main lymphocyte markers. As shown in Fig. 2, the antigens CD3, CD4, CD8, and CD19 were expressed similarly in patients and controls. In contrast, the densities of CD2 and CD11a were significantly higher in patients than in controls (46,470 versus 32,900 sites/cell for CD2 and 39,900 versus 30,400 sites/cell for CD11a) (Fig. 2). On fluorescence histograms, these molecules were heterogeneously distributed; their analysis allowed the discrimination of two cell subsets expressing either high levels (bright) or low levels (dim) of CD2 (Fig. 3) and CD11a. Patients with Q fever and healthy controls exhibited distinct phenotype patterns of CD2 and CD11a subsets (Table 1). Lymphocytes from Q fever patients were characterized by a significant increase in the percentage of CD2 bright cells together with a decrease in the

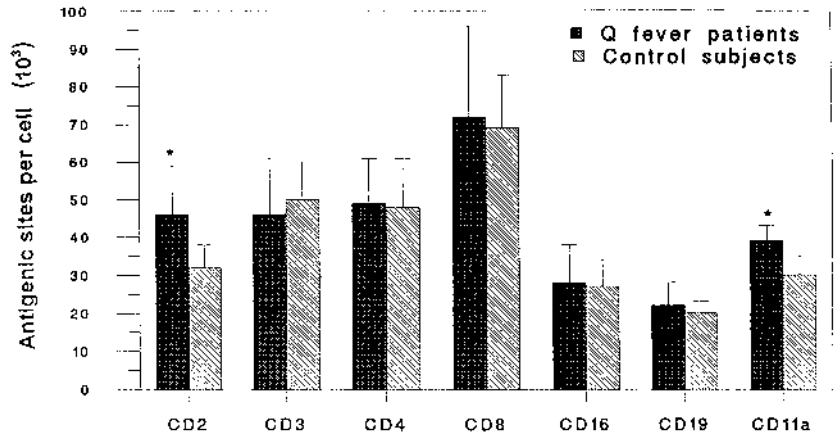

FIG. 2. Quantitative expression of lymphocyte antigens. The surface expression of each marker was determined by the QIFI test and flow cytometry as described in Materials and Methods. Results are expressed as the number of antigenic sites per cell (mean ± SD). *, $P < 0.05$ for comparison between groups.

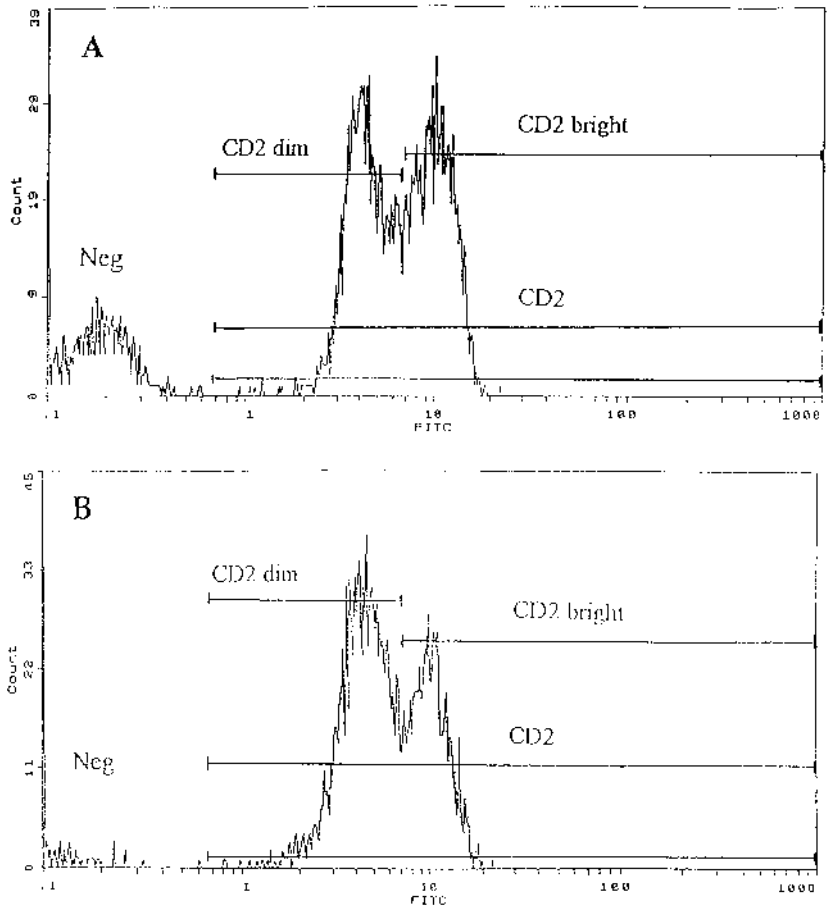

FIG. 3. Heterogeneity of CD2 expression on lymphocytes. Samples from Q fever patients (A) and controls (B) were tested for CD2 expression. Antigen densities of CD2 dim and CD2 bright subsets are, respectively, 27,000 and 64,000 sites/cell.

percentage of CD2 dim cells. Similar patterns were found within cells expressing CD11a molecules. The imbalance was also reflected by the decreased absolute values of CD2 dim and CD11a dim subsets in patients (505 ± 270 and 541 ± 247 cells/mm^3, respectively) compared with controls (1,026 ± 360 and 1,152 ± 359 cells/mm^3, respectively). Since low and high densities of CD2 and CD11a have been suggested to serve, respectively, as naïve and memory markers of lymphocyte subsets (1, 13), we confirmed these results by analyzing CD45RO and CD45RA expression by CD4$^+$ T cells. Both percentage and absolute values of naïve CD45RA$^+$ cells were significantly lower in infected patients (27% ± 13% and 174 ± 114 cells/mm^3, respectively) than in the age-matched control group (43% ± 14% and 425 ± 206 cells/mm^3, respectively). Reciprocal changes in the memory subset, i.e., an increased percentage of CD45RO$^+$ cells (68% ± 13% versus 53% ± 15%) and an unchanged absolute count of double-labeled CD45RO$^+$ CD4$^+$ lymphocytes (402 ± 227 versus 492 ± 115 cells/mm^3) were noted.

TABLE 1. Heterogeneity of CD2 and CD11a expression on lymphocytes[a]

Lymphocyte subset[b]	Q fever patients		Controls	
	%	Absolute no.	%	Absolute no.
CD2	79 ± 6	1,040 ± 243***	80 ± 5	1,649 ± 456
CD2 dim	37 ± 13**	505 ± 270***	49 ± 9	1,026 ± 360
CD2 bright	42 ± 12**	540 ± 152	31 ± 8	622 ± 197
CD11a	90 ± 9	1,222 ± 324***	91 ± 7	1,852 ± 497
CD11a dim	39 ± 13***	541 ± 247***	57 ± 9	1,152 ± 359
CD11a bright	51 ± 13***	680 ± 218	34 ± 9	700 ± 248

[a] Results are expressed as means ± SD. ***, $P < 0.001$. **, $P < 0.01$. *, $P < 0.05$.

[b] Dim, expressing low antigenic density; bright, expressing high antigenic density.

DISCUSSION

We show that chronic Q fever is specifically associated with a lymphopenia involving mainly CD4$^+$ T cells. In addition, the CD4$^+$ T-cell count is recognized as a marker of disease activity. The analysis of T-cell subsets showed that absolute numbers of CD4$^+$ and CD8$^+$ lymphocytes were significantly lower in patients than in controls. Nevertheless, when results were expressed as relative percentages, they clearly indicated that the imbalance between regulatory T-cell populations was in favor of suppressor-cytotoxic cells, with a profound alteration of helper cells. Thus, this imbalance is reflected by a decrease in the CD4/CD8 ratio. Transient abnormalities of T-cell subsets have been reported for other infectious diseases, such as tuberculosis, hepatitis, Epstein-Barr virus infection, toxoplasmosis, and pneumocystosis, but the CD4/CD8 ratio was always higher than 1.00 (2, 9, 12). CD4 lymphopenia with a low CD4/CD8 ratio has been found with cytomegalovirus infection; it resulted from an increase in CD8$^+$ T cells (9). Thus, the mech-

anisms involved in $CD4^+$ lymphopenia appear distinct from those implicated in the modification of T-cell subsets in several infectious diseases. The mechanisms of this $CD4^+$ lymphocytopenia are hypothetical. First of all, it was not the consequence of chloroquine treatment, since similar treatment did not induce such cytopenia in patients with rheumatoid arthritis (data not shown). It is more probable that the infectious process affects the generation of T cells and/or their peripheral survival. The alteration of bone marrow cytokines involved in hematopoiesis (8) is very unlikely because it would result in more profound cytopenia affecting several leukocyte subsets, such as is encountered in HIV infection (25). It is more tempting to speculate that lymphocyte changes are the consequence of peripheral mechanisms. One hypothesis, sustained by the frequency of autoimmune manifestations with *C. burnetii* infection, is lymphocyte autoimmune destruction (22). The most likely explanation is the selective margination of lymphocytes from blood, followed by their transient accumulation in tissues. In patients with Q fever endocarditis, the production of tumor necrosis factor alpha and interleukin-1β has been reported to be enhanced (5), which might favor peripheral causes of lymphopenia. This $CD4^+$ T-cell lymphopenia is related to the outcome of Q fever endocarditis. Since IgG levels are recognized as a marker of disease activity (5, 27), the strong correlation between IgG levels and $CD4^+$ T-cell count clearly indicated that CD4 lymphopenia is related to the activity of chronic Q fever.

On the other hand, it is becoming evident that cell surface antigens are expressed on T cells at different levels, which can be modified in various pathological conditions (14, 26). The recent development of antigen quantitation by flow cytometry made it possible to study the expression of lymphocyte cell surface molecules. Expression profile analysis of CD2 and CD11a showed that their distributions were heterogeneous and could be mapped into different subsets displaying low (dim) or high (bright) expression, with a lower proportion of dim lymphocytes in infected patients. Thus, the higher expression of CD2 and CD11a observed in the whole population reflected the decrease in subsets with low CD2 or CD11a expression rather than an absolute increase in global antigenic density. It seems logical to associate the modification of CD2 and CD11a densities with enhanced margination as a possible mechanism of $CD4^+$ and $CD8^+$ T-cell depletion. CD2 and CD11a are two of several T-cell surface molecules capable of delivering the necessary costimulatory signal to produce activation when a T cell recognizes an antigen via T-cell antigen receptors. Because increased expression of T-cell adhesion molecules CD2 and CD11a was found to be functionally important in the enhancement of their reactivity in an immune response, such expression is used to characterize cells with memory phenotype. Using CD45RA and CD45RO expression by $CD4^+$ T cells as markers of unprimed and primed lymphocyte subsets, respectively, we demonstrated that the absolute count of cells with a naïve phenotype was decreased in patients whereas the number of cells with a memory phenotype was not significantly different in patients and controls. These results led us to conclude that the CD4 lymphopenia observed in patients preferentially affected unprimed cells, while primed cells appeared to be protected.

In conclusion, the analysis of T-cell subsets in Q fever endocarditis revealed that decreased counts of $CD4^+$ T cells are a marker of the activity of the infectious process. In addition, the discrimination between unprimed and primed lymphocytes provides new insights into the physiopathology of Q fever.

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