ACTIVE SYSTEMIC LUPUS ERYTHEMATOSUS IS ASSOCIATED WITH THE RECRUITMENT OF NAIVE/RESTING T CELLS

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SUMMARY

The aim of this study was to determine whether active systemic lupus erythematosus (SLE) is associated with recruitment of resting CD45RA+ T cells or reactivation of CD45RO+ memory T cells. Three-colour immunofluorescence was used to determine CD45 isoform expression by CD4+ T cells from 28 patients with SLE. Newly recruited and highly differentiated primed T cells were distinguished by their CD45RB expression. The pattern of CD45 isoform expression varied directly with time since the onset of symptoms in patients with active SLE. Shortly after symptoms appeared, most cells were CD45RA+ resting cells or CD45RO+RB bright early primed cells. However, over the course of active disease, patients accumulated CD45RO+RB dull cells which represent an advanced state of differentiation. The switch from an early to late primed phenotype correlated significantly with time since the onset of symptoms. The recruitment of resting T cells in active SLE, rather than the simple reactivation of existing memory clones, has implications for understanding the pathology of this disease and for treating it.

KEY WORDS: SLE, T-cell differentiation, Memory, Resting, CD45, Active, Phenotype.

DIFFERENT stages of T-cell differentiation can be distinguished by the patterns of CD45 isoform expression on the cell surface. The different isoforms are derived from variable mRNA splicing of a single gene product of CD45 and can be identified using antibodies [1-3]. Naive or resting CD4+ T cells express high levels of CD45RA and CD45RB. Following activation, T cells rapidly lose CD45RA and acquire CD45RO expression. CD45RB expression is lost more slowly and decreases sequentially as CD45RO expression increases over a large number of cycles of activation. Thus, recently activated CD4+ T cells are CD45RB bright RO dull and highly differentiated memory cells are CD45RB dull RO bright [3]. These changes in CD45 epitope expression are associated with functional changes: CD45RA naive/resting cells produce high levels of mostly IL-2, but little else, CD45RO+RB bright early primed cells produce a lot of interferon (IFN)-γ and IL-2, but low levels of IL-4, and highly differentiated CD45RO+RB dull cells produce very little IL-2, some IFN-γ and a large amount of IL-4 [3]. There is also a clear increase in susceptibility to apoptosis with T-cell differentiation [3].

Analysis of T-cell phenotypes in patients with active systemic lupus erythematosus (SLE) is complicated by the presence of lymphocytotoxic antibodies that are frequently directed against CD4+ T cells and which result in lymphopenia [4]. Previous studies have shown a reduced proportion of CD45RA+ T cells, particularly in active disease with renal and neurological involvement [5-7]. Subsequently, antibodies directed against CD45RA were identified in the serum of patients with SLE [8, 9]. These antibodies may have been partially responsible for skewing the results in favour of primed rather than naïve resting CD4+ T cells in active lupus. In contrast, another study reported a reduction in primed cells in active disease using antibodies against the adhesion molecule CD29 to identify primed cells [and a correlation between reduced CD29+ cells and anti-double-stranded (ds) DNA antibody production] [10]. More recently, cells simultaneously expressing both CD45RA and CD45RO have been identified in SLE patients, and were thought to represent recently activated cells in transition from CD45RA to CD45RO [4].

Following the observation that recently activated CD4+ cells are CD45RO+RB bright, while those that have traversed many cycles are CD45RO+RB dull [3], we have used CD45RB expression to assess the relationship between disease activity and CD4+ T-cell differentiation state to determine whether active disease is associated with recruitment of naïve/resting T cells or reactivation of existing memory T-cell clones. In particular, we have investigated CD45 exon expression by CD4+ T cells in relation to time since onset of symptoms in patients with active lupus.

PATIENTS AND METHODS

Patients and controls

Peripheral blood samples were obtained at routine SLE clinic visits from 28 patients with SLE, all of whom met the ACR (formerly ARA) classification criteria for SLE [11]. Six patients with active disease

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were newly diagnosed cases of SLE. The remaining patients were attending the SLE clinic regularly. Careful note was made of the duration of symptoms of SLE in all cases. Patients varied in how quickly they reported new symptoms and did not increase their steroid dose without review or telephone discussion. Changes in therapy were not delayed by the study.

Clinical disease activity was assessed using the BILAG index [12] at each visit and data were stored on the BILAG computer program. Active disease was defined as BILAG grade A in any of the eight systems or at least three grade B scores [12]. A disease flare was defined as the occurrence of new symptoms and signs sufficient to score BILAG grade A or B in at least two systems. The onset of a disease flare was defined as the time at which new symptoms and signs sufficient to score BILAG grade A or B in at least two systems were first noted. Patients who did not meet these criteria were labelled 'inactive' as they did not require a change in major disease-modifying therapy. The grade scores for each of the eight BILAG systems were converted to a numerical score using A = 9, B = 3, C = 1, D = 0, and E = 0, and were summed to give a total BILAG score for each visit.

There were 12 patients with SLE in the active group (median score 18.5, interquartile (IQ) range 14.5–31.5) and 16 patients in the inactive group (median score 4.0, IQ range 2.0–5.0). The most commonly affected systems in the active patients were the musculoskeletal, mucocutaneous and renal systems. Five out of the 12 active patients had four or more systems scoring BILAG grade A or B. There was no significant difference in age between the active and the inactive group (median 32 and 35 yr, respectively). The active group had a shorter total disease duration (median 3.5, IQ range 0.75–6.5 yr) compared with the inactive group (median 7.0, IQ range 3.5–14.0 yr), but this difference did not reach statistical significance (Mann–Whitney test). Seven of 12 (58%) patients in the active group and 15 of 16 (94%) patients in the inactive group were taking prednisolone (P = 0.057, Fisher’s exact test). Two patients with active disease had a multisystem flare despite being on more than 10 mg of prednisolone. One of 12 (8%) patients in the active group was taking azathioprine compared with 8 of 16 (50%) patients in the inactive group (P = 0.04, Fisher’s exact test). There was no significant difference in the number of patients in each group taking anti-malarial agents (one active and two inactive patients). Patients on cyclophosphamide and cyclosporin A were excluded from the study.

Sixteen healthy volunteers with an age range of 20–66 yr (median 44.5 yr) served as controls. CD45 isoform expression did not vary with age or sex.

**Cell preparation**

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood by density-gradient centrifugation.

**Monoclonal antibodies**

Leu3a (Becton Dickinson, Oxford) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or biotin was used to label CD4. PE-conjugated 2H4 (Coulter, Hialeah, FL) was used to identify CD45RA, PD7/26-FITC (a gift from Dr D. Y. Mason) was used to identify CD45RB, and biotinylated UCHL1 (a gift from Prof. P. C. L. Beverley) was used to identify CD45RO. Biotin conjugates were detected using streptavidin-conjugated allophycocyanin (Becton Dickinson).

**Flow cytometry**

Analysis of CD45 isoform expression within the CD4+ T-cell subset was performed using three-colour immunofluorescence with a Coulter EPICS Elite flow cytometer (as described previously) [13]. All samples were labelled with anti-CD4, together with combinations of antibodies specific for CD45RA, CD45RB and CD45RO. Conjugated irrelevant mouse antibodies of all isotypes (Dako) were used to establish the specificity of staining. Fluorescence compensation was adjusted using samples of cells that were labelled individually with anti-CD4 conjugated to each fluorochrome. All samples were gated on forward and side scatter to exclude dead cells. CD45 isoform expression was analysed within the population gated as CD4+, as described previously [3, 13].

**Statistical analysis**

Data were analysed using Fisher’s exact test and non-parametric methods: Mann–Whitney U-test, Dunn’s rank sum test for multiple comparisons and Spearman rank correlation coefficient (corrected for ties).

**RESULTS**

The expression of CD45 isoforms by CD4+ T cells in peripheral blood of patients with SLE

As noted in previous studies [4], the SLE patients were lymphopenic (data not shown) and the proportion of CD4+ cells in the PBMC of patients with active and inactive SLE was significantly reduced compared with the control group (Table I). Among the CD4+ population, the active patients tended to have less CD45RA+ and more CD45RO+ cells than the controls, although the proportion of CD45RA+ and CD45RO+ cells was not significantly different between the active and inactive SLE patients and the healthy controls (Table I). The proportion of CD45RO+RB\textsuperscript{bright} cells was reduced in the active SLE group compared with the healthy controls (Table I). There was no significant difference between CD45RO+RB\textsuperscript{bright} expression in active and inactive SLE patients nor between the inactive SLE and healthy control groups. The daily dose of prednisolone taken by the patients in the active and inactive SLE groups was not significantly different (Table I).
TABLE I
Proportion of CD4\(^+\) cells in peripheral blood and proportion of the CD4\(^+\) cells expressing CD45RA, CD45RO and CD45ROB\(^{\text{br}}\) in active and inactive SLE patients and healthy controls

<table>
<thead>
<tr>
<th>Median and interquartile range</th>
<th>Active (n = 12)</th>
<th>Inactive (n = 16)</th>
<th>Controls (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CD4(^+) cells</td>
<td>32.2* (24.3–35.7)</td>
<td>37.9* (29.4–41.7)</td>
<td>42.5 (40.5–53.5)</td>
</tr>
<tr>
<td>%CD45RA(^+) cells</td>
<td>35.0 (20.0–63.8)</td>
<td>56.0 (45.3–65.0)</td>
<td>42.0 (29.5–52.5)</td>
</tr>
<tr>
<td>%CD45RO(^+) cells</td>
<td>65.0 (39.5–78.8)</td>
<td>39.0 (33.0–55.3)</td>
<td>50.5 (44.5–60.0)</td>
</tr>
<tr>
<td>%CD45ROB(^{\text{br}}) cells</td>
<td>22.2* (18.2–25.9)</td>
<td>26.0 (20.3–35.0)</td>
<td>32.5 (26.5–37.0)</td>
</tr>
<tr>
<td>Time since flare (weeks)</td>
<td>6.0 (3.5–8.0)</td>
<td>35.0 (16.0–119.0)</td>
<td>–</td>
</tr>
<tr>
<td>Prednisolone dose (mg)</td>
<td>5.0 (0.0–10.0)</td>
<td>8.8 (5.0–12.3)</td>
<td>–</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with control, Dunn's test.

Relation of CD45 isoform expression to time since onset of lupus symptoms

The patients with active SLE were found to have a clear pattern of CD45 isoform expression within the CD4\(^+\) population which correlated with time since the onset of symptoms of active lupus disease. Patients with a short duration of symptoms had more CD45RA\(^+\) and CD45ROB\(^{\text{br}}\) cells, and less total CD45RO\(^+\) cells, than patients whose symptoms had been present for a longer time (Fig. 1). Patients with more prolonged active disease had low levels of CD45RA\(^+\) cells and predominantly CD45R0\(^+\) cells, most of which were CD45ROB\(^{\text{br}}\) cells. There was no significant correlation between the expression of any of the CD45 isoforms and prednisolone dose (data not shown). The patients in the inactive group did not show any correlations between CD45 isoform expression and time since last lupus flare (Fig. 2), prednisolone dose or BILAG score (data not shown).

DISCUSSION

Over the last few years, it has become clear that CD4\(^+\) T cells express several isoforms of the leucocyte common antigen, CD45, depending on the state of differentiation of the T cell. Unprimed CD4\(^+\) cells can be stained with antibodies to CD45RA, CD45RB and CD45RC, but lack the CD45RO isoform [2, 3]. Following activation, CD45RA and CD45RC are rapidly lost from the cell surface, but CD45RB expression is lost more slowly and in a reciprocal relationship to the increase in CD45RO expression associated with multiple cycles of activation and rest in primed T cells. This inverse relationship of CD45RB and CD45RO

![Fig. 1.—Proportion of CD4\(^+\) cells which were either (A) CD45RA\(^+\), (B) CD45RB\(^{\text{br}}\) (CD45RB br), (C) CD45RO\(^+\) or (D) CD45ROB\(^{\text{br}}\) (CD45ROB br) in the peripheral blood of 12 patients with active SLE expressed as a function of time since the onset of flare. The proportion of CD45RA\(^+\), CD45RB\(^{\text{br}}\) and CD45ROB\(^{\text{br}}\) cells correlated inversely and CD45RO\(^+\) cells correlated directly with time since onset of active disease (r = Spearman rank correlation coefficient).]
expression appears to account for the pattern of CD45 epitope expression observed in peripheral blood CD4+ cells from healthy individuals [3].

In this study, we provide evidence that CD45 epitope expression on CD4+ T cells changes during active disease in SLE patients. Soon after the onset of a flare, there are relatively few primed (CD45RO+) cells and many of these CD45RO+ cells appear to be recently recruited from the CD45RA+ population as they are both CD45RO+ and CD45RB+ alike. With increasing time since the onset of symptoms, the proportion of CD45RO+RB+dull cells increases and there is a fall in the number of CD45RA+ and CD45RO+RB+bright cells. These observations would be difficult to explain by the development of anti-CD45RA antibodies alone. Instead, they suggest that recruitment and subsequent differentiation of unprimed (CD45RA) T cells occur during a lupus flare, analogous to the changes observed in vitro over several weeks with stimulation of T cells from healthy individuals [3]. The considerable variation in CD45 isoform expression in patients with inactive disease is hardly surprising, as with increasing time since last lupus flare, there is likely to be more influence on T-cell phenotypes in peripheral blood from coincidental illnesses such as infection. The increased predisposition of terminally differentiated CD4+ cells (CD45RO+RB+dull cells) to apoptosis [3] may contribute to the persistent T lymphopenia in SLE patients and is currently being investigated.

The observation that CD45 isoform expression varies with time since onset of symptoms may help to explain previous discrepant results. Morimoto et al. [5] and Sato et al. [6] originally reported a reduced proportion of CD45RA cells in SLE patients, especially those with active renal and neurological disease. In contrast, Gorla et al. [10] reported reduced CD29+ cells (an alternative though less specific marker to CD45RO for primed cells) in active lupus. The duration of active disease was not discussed in these studies. It is possible that the patients reported by Gorla et al. had been active for less time than those studied in the earlier reports. Renal disease, in particular, may be present for some time before patients are assessed, as it is not associated with specific symptoms that will cause the patients to seek medical attention early. In addition, there has been conflicting evidence for IL-2, IFN-γ and IL-4 production in vivo and in vitro by mitogen-stimulated PBMC from SLE patients compared with healthy controls [4, 14, 15]. The production of these cytokines is likely to be influenced by the state of differentiation of the T cells present. As the composition of the CD4+ T-cell population varies during the course of a flare, the cytokines produced by the cells are likely to vary.

This study shows that the proportion of CD4+ cells expressing different CD45 isoforms varies with the time since onset of active disease. The relatively high proportion of CD4+CD45RO+ cells which are
CD45R\textsuperscript{bright} in the peripheral blood of SLE patients with recent flares suggests recent T-cell recruitment and is analogous to the observation that these cells are present at significant levels in the synovial fluid of patients with reactive arthritis [13]. In contrast, CD4\textsuperscript{+} cells from the synovial fluid of patients with rheumatoid arthritis, a more chronic condition, are almost exclusively CD45RO\textsuperscript{+}RB\textsuperscript{dim} [13]. Although the recruitment of naive/resting CD4\textsuperscript{+} cells in active SLE may reflect viral infection triggering the flare, the patients did not have obvious clinical infection or raised CRP at the time of assessment or in the preceding weeks. However, these results suggest that T-cell activation involving resting CD45RA\textsuperscript{+} cells, not just reactivation of primed or memory cells, may play a role in the development of active lupus. This has implications for future specific immunotherapy, as treatment directed at primed cells alone is unlikely to be sufficient to control the disease.

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