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Peripheral B cell abnormalities in patients with systemic lupus erythematosus in quiescent phase: Decreased memory B cells and membrane CD19 expression

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ABSTRACT

B lymphocytes from patients with systemic lupus erythematosus (SLE) are hyperactive and produce autoantibodies. Several B cell phenotype characteristics such as the expansion of activated populations, and of a newly identified memory compartment have already been reported. These results are not easy to interpret because of the clinical heterogeneity of SLE, as well as the difficulties to establish homogeneous and well defined groups taking in consideration the activity of the disease and the various therapies. However, although many mediators and mechanisms can contribute to the clinical presentation and subsequent progression of individuals with SLE, several data suggest that some intrinsic B cells abnormalities may be central to the disease process.

In this view, we have analysed the phenotype of B cells from 18 patients with quiescent diseases (mean SLEDAI score below 2) and from 11 healthy controls. B cell surface marker expression was determined by flow cytometry. We analysed the main B cell sub-populations.

We demonstrate the persistence of plasmocyte-differentiated and -activated B cells even in quiescent patients. However, quiescent patients display a decrease in memory B cells that could reflect the control of their disease. Above all, we describe a lower membrane expression of the CD19 protein on all B cells in every patient compared to controls. This lower CD19 expression is associated with reduced CD45 levels. It is not associated with an evident gene expression alteration and *in vitro* stimulation restores a control phenotype. These findings suggest certain mechanisms of lupus development.

1. Introduction

The presence of multiple autoantibodies in systemic lupus erythematosus (SLE) reflects defective tolerance mechanisms leading to the activation of auto-reactive B cells, and the production of autoantibodies often long before the first expression of the disease [1]. However the nature of the immune abnormalities resulting in these defects remains elusive. Although disease manifestations in SLE are due predominantly to high affinity somatically mutated class-switched IgG autoantibodies, indicating that T-B cell collaboration is essential, increasing evidence suggests that intrinsic B cell abnormalities may play an important role.

B cells from SLE patients are hyper-responsive to a variety of stimuli demonstrating enhanced proliferation to polyclonal activators, increased anti-IgM mediated intracellular Ca^{2+} concentration responses and increased anti-IgM induced protein tyrosine phosphorylation for example [2]. Some studies in human patients with SLE have provided evidence of reduced expression levels of Lyn, a negative regulator of B cell signaling [3,4], and have suggested in some cases alteration of the expression of the CD19 [5,6], and of the expression and the localization of the CD45 molecules [4,7]. Findings in mouse models suggest that defective regulation of intracellular signaling in B lymphocytes could directly lead to lupus-like autoimmunity. For example, mice deficient in genes encoding CD22, or Fcγ receptor type II, which function as negative regulators of B cell receptor (BCR) signaling, produce anti-double-stranded DNA (anti-dsDNA) autoantibodies and develop lupus-like disease [8]. In addition, excessive positive stimulation through membrane proteins, such as BAFF-receptors, promotes B lymphocyte hyperactivity and anti-nuclear autoantibody production [9]. Most importantly, the disease can be transferred in mice by B cells: immunodeficient SCID mice populated with pre-B cells of lupus-prone BW mice develop many of the characteristics of BW mice [10].

Recently, a number of cellular markers that enable classification of peripheral blood B cells into distinct B cell subsets, have been

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identified. Thus, several studies have tried to localize the autoantibody producing cells or "abnormal" B cells in one or the other B cell compartment of SLE patients. Autoimmune B cells could belong to a distinct activated $CD86^+$, and plasma cell differentiated $CD27^{++}$, $CD138^+$, $CD38^+$ subset [11–13]. Furthermore, $CD27^-IgD^-$ memory B cells with an activated phenotype appeared to be increased in patients with active flares [14].

The heterogeneity of the findings is of course partially linked to the complexity of B cell maturation and activation processes. But the difficulties of establishing homogeneous patient cohorts should be underlined when taking into consideration the various potential manifestations of the disease, the various immunosuppressive drugs that can be used, and the fact that frequent B cell lymphopenia in SLE patients makes any study hazardous in its interpretation.

To clarify these points, we have tested the hypothesis that intrinsic abnormalities of B cells leading to auto-reactivity could be easier to determine in quiescent patients. We decided to focus on classical phenotypic markers, in view of a subsequent potential clinical use. We have studied 18 patients, with minor clinical and/or biological manifestations of the disease, for at least 6 months and 11 healthy controls. None of the patients received immunosuppressive drugs or biotherapy during this period and steroid therapy remained under 10 mg prednisone/day.

Altogether, our patients do not display significant B cell lymphopenia. We describe the persistence of differentiated activated B cells even in quiescent patients. We did not find any significant increase of a specific memory B cell compartment as previously seen in patients with active disease but on the contrary we evidenced a decrease of the non-switched memory B cell subset. Above all, we describe a decreased expression of the CD19 surface protein in all patients compared to controls. This CD19 lower expression is associated with lower CD45 levels. It is not associated with an evident lower gene expression and the *in vitro* stimulation restores CD19 levels as in controls. These findings suggest some mechanisms in lupus development.

2. Material and methods

2.1. Patients

All subjects were recruited for this study under a protocol approved by the institutional CCPRB (Research Ethics Board of Strasbourg Hospital, France) and all gave informed written consent. All SLE patients fulfilled the American College of Rheumatology (ACR) classification criteria for SLE [15]. Disease activity was assessed by the Systematic Lupus Activity Measure (SLAM) [16] and by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [17] at the time of the blood draw. The choice of patients considered having an inactive disease was based on the SLEDAI results (score below 5). Patients were definitely included if their disease was stable for the last 6 months, with no immunosuppressive treatment except for a dose of steroids less than 10 mg/d. Hydrochloroquine therapy was allowed. Healthy controls came from a work-site population. Peripheral blood samples from each subject were collected into tubes containing EDTA.

Assays for anti-nuclear antibodies (ANAs) were performed by indirect immunofluorescence with Hep-2000 cells (Hep-2 substrate slides, Zeus Scientific Inc., NJ, USA). The detection of ANAs at a dilution of 1/160 was considered a relatively positive result. Enzyme-linked immunosorbent assays were used to evaluate serum for anti-cardiolipid autoantibodies (IgG and IgM). Anti-prothrombinase autoantibody was detected with a classical functional test. Anti-double-stranded DNA antibodies (anti-dsDNA antibodies) were screened mostly with Elisa (Kallestad anti-DNA microplate EIA, Bio-Rad Lab. Inc., CA, USA). Serum immunoglobulin levels (total IgG) were routinely tested with Elisa (Normal ranges: 7–14 g/l).

2.2. Flow cytometric analysis

Blood samples were taken as described. For immunofluorescence staining, fresh EDTA whole blood samples were kept at room temperature (RT) immediately after collection and washed in PBS (20 ml PBS for 1 ml of blood). 100 μl were stained at RT using predetermined saturating concentrations of antibodies (Abs) for 20 min. Stains were done as previously described [18] and blood erythrocytes were lysed after staining using FACS Lysing solution (BD, CA, USA) according to the manufacturer recommendations.

Fluorochrome-conjugated antibodies to CD19 (HIB19 clone), IgM, IgD, CD27, CD45, CD38, CD138, CD154 (CD40L), CD180 (RP105) were purchased from Pharmingen (CA, USA). The anti-CD45 antibody (clone HI30) reacts with the 180 (CD45R0), 190, 205, 220 (CD45RA) kD isoforms of the leukocyte common antigen (LCA). Fluorochrome-conjugated antibodies to CD86 and isotypic controls were purchased from Caltag (CA, USA). FITC-conjugated anti-CD20 was purchased from Immunotech (Marseille, France). Two other CD19 monoclonal antibodies (SJ25C1, and 4G7) were used to confirm the decreased expression of the CD19 molecule and were purchased from Becton Dickinson (USA). Antibodies against CD21 and CD81 were used to analyse the CD19 complex and were purchased from BD Pharmingen (USA).

For analysis, the cells were first gated on the lymphocyte population based on forward and side scatter characteristics. Positively stained populations were determined by comparison with isotype controls, gated on the relevant population. B cells were identified based on CD19 expression. All CD19 positive cells were in the viable lymphocyte gate. B cell numbers were calculated based on the white blood cell count, the percentage of lymphocytes and the percentage of CD19 cells identified by flow cytometry. The data acquisition was performed on a FACSCalibur (Becton Dickinson) and the results were analysed with CellQuest software (Becton Dickinson).

2.3. B cell proliferation assays

Blood samples were taken as described. Mononuclear cells were isolated with a Ficoll–Hypaque gradient and stimulated as previously described [18]. Mononuclear cells were cultured in RPMI-1640 medium supplemented with 10% FCS, antibiotics (penicillin and streptomycin, Gibco), HEPES, ph 7.5 (Gibco) and gentamicin (Invitrogen). Briefly, 10^6 mononuclear cells were stimulated with 20 μg/ml of goat anti-human IgM $F(ab')^2$ (Jackson Immunoresearch Laboratories) or LPS, 20 μg/ml (Sigma). After 48 h of culture at 37 °C, B cell phenotype was determined by flow cytometry.

2.4. Statistical analysis

Differences between SLE patients and control populations were determined with a two-tailed unpaired Student's test. The means ± SEM were used for data expression. *P* values less than 0,05 were considered statistically significant.

3. Results

3.1. Patients

We analysed 18 patients with quiescent SLE, fulfilling the ACR criteria revised in 1982 (Tables 1A and 1B) and a control group of 11 healthy donors matched for sex and ages (Table 1C). The mean age of our patients was 40 (23–59). The mean duration of disease was