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Anti-dsDNA production coincides with concurrent B and T cell activation during development of active disease in systemic lupus erythematosus (SLE)

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SUMMARY

The objective was to serially analyse T and B cell activation in relation to autoantibody production during the development of relapses in SLE. In a prospective study we serially analysed, by flow cytometry, T cell activation in relation to B cell activation and anti-dsDNA production in quiescent SLE and during the development of a clinical relapse. In addition, we related changes in T and B cell activation to changes in levels of anti-dsDNA and total IgG. During periods with clinically quiescent disease, the expression of activation markers on T cells (IL-2R and HLA-DR) and B cells (CD38) was persistently higher in SLE than in healthy controls ($P < 0{\cdot}001$). Percentages of $CD20^+CD38^+$ B cells were related to levels of total IgG ($P < 0{\cdot}02$), but not to levels of anti-dsDNA. Development of disease activity was paralleled by an increase in the percentages of $CD4^+$ T cells ($P < 0{\cdot}005$) and $CD20^+CD38^+$ B cells ($P < 0{\cdot}001$), which were interrelated. Increases in B cell activation were related to increases in levels of anti-dsDNA ($P < 0{\cdot}005$), but not to changes in total IgG levels. B cells expressing high levels of CD38 spontaneously produced IgG class anti-dsDNA *in vitro*. Persistence of activated B cells during periods with clinically quiescent disease in SLE seems to underly hypergammaglobulinaemia but not anti-dsDNA production. Prior to clinical disease activity, further activation of T and B cells occurs, which is paralleled by rises of anti-dsDNA but not of total IgG. This suggests that the production of anti-dsDNA is a T cell-dependent antigen-driven process, which is independent of the polyclonal activation of the immune system inherent to the disease.

Keywords systemic lupus erythematosus lymphocyte activation anti-dsDNA prospective study

INTRODUCTION

SLE is an autoimmune disease with multiple organ involvement, characterized by relapses and remissions. Irrespective of clinical disease activity, various autoantibodies are present directed against a wide range of antigens, including cellular membrane proteins, and nuclear and cytoplasmic components [1]. Moreover, hypergammaglobulinaemia is frequently observed. These phenomena point to persistent B lymphocyte activity already in clinically quiescent disease. Indeed, B lymphocytes isolated from quiescent SLE patients spontaneously produce anti-dsDNA *in vitro* [2,3]. Signs of persistent T lymphocyte activity are present as well, i.e. increased levels of soluble IL-2 receptor (IL-2R) [4,5], and up-regulation of HLA-DR and IL-2R on circulating T lymphocytes [6,7]. We previously showed that, in clinically quiescent SLE, the proportion of activated B lymphocytes is related to levels of total IgG and IgM, and not to concurrent T lymphocyte activation, nor to levels of autoantibodies directed against double-stranded DNA (anti-dsDNA) [7]. This suggests a 'background' activation of the B cell compartment, probably independent of T cell activity. T cell activity may, however, be involved in the production of pathogenic high-affinity anti-dsDNA antibodies, as shown by the capacity of a HLA-DR-restricted T cell clone to induce lupus B cells to produce anti-dsDNA *in vitro* [8]. Antigen-driven, T cell-dependent anti-dsDNA production is suggested also by our finding that a rise in anti-dsDNA levels before development of a relapse exceeds by far the rise in levels of total IgG [9,10]. In addition, anti-dsDNA are predominantly of the IgG1 and IgG3 subclasses, and V-genes encoding for anti-dsDNA show an increased frequency of T cell-dependent somatic hypermutations [11–13].

We tested the hypothesis that disease activity in SLE is preceded by T cell-dependent antigen-driven production of anti-dsDNA. We analysed whether changes in the percentages of T and B cells expressing activation markers in SLE are inter-related. In addition, we related changes in the percentages of activated B cells from quiescent to active disease to changes in circulating levels of

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anti-dsDNA and total immunoglobulins. To investigate whether activated B cells preferentially produce anti-dsDNA, B cells with high and low CD38 expression were sorted and analysed for their spontaneous production of anti-dsDNA and IgG using an antigen-specific ELISPOT assay.

PATIENTS AND METHODS

Patients

Consecutive out-patients, fulfilling at least four American College of Rheumatology (ACR) criteria for the diagnosis of SLE [14], were included in this study. Excluded from analysis were patients who were pregnant or patients who showed signs or symptoms suggestive of infection at the moment of blood sampling. Patients were asked to participate in a prospective study during which monthly blood samples were obtained (see below). End point of study for these patients was defined as either an observation period of 6 months, or the occurrence of a relapse. Major and minor disease relapses were defined as shown in Table 1. Eighteen healthy age-matched individuals served as normal controls, of whom six randomly selected individuals delivered monthly blood samples during a 6-month period.

Study design

Patients were seen at the outclinic at least once every 3 months for clinical evaluation. When disease activity was present patients were seen at least once a month. Disease activity was scored using the SLE Disease Activity Index (SLEDAI) [15]. In cases where patients used corticosteroids, therapy was temporarily withdrawn at least 24 h before blood sampling, to exclude direct effects of corticosteroids. Blood samples were drawn every 4 weeks in EDTA (Vacutainer; Becton Dickinson, Mountain View, CA), between 8·30 and 10·00 am to minimize circadian variations of circulating lymphocyte subsets [16]. Samples were subsequently double-stained and analysed the same day by flow cytometry (FACStar). Plasma samples were stored at -20°C until needed.

Table 1. Criteria for major and minor disease exacerbations in SLE

Major exacerbation: fulfilling one or more of the following*:

1. *Severe renal disease:*
   a. recent renal biopsy showing active proliferative lupus nephritis (>50% of glomeruli affected), and/or
   b. decrease of creatinine clearance of >25% within 4 months, accompanied by an active sediment (>5 erythrocytes/HPF, and/or casts) and by proteinuria of >0·5 g/day.
2. *Severe central nervous system disease:*
   seizures, cerebral vascular accident, coma, transverse myelitis, psychosis, choreoathetosis, central nerve palsy.
3. *Haematological disease:*
   haemolytic anaemia (Hb < 60 g/*l*) and/or thrombocytopenia ($<50 \times 10^9/l$).
4. *Severe serositis:*
   pericarditis with (impending) tamponade and/or massive pleural effusion.
5. *Uveitis and/or retinitis.*
6. *Myocarditis with arythmia and/or congestive heart failure.*
7. *Severe myositis with proximal muscle weakness.*
8. *Lung involvement with haemoptysis.*
9. *Major vasculitis:*
   with ulcerations and/or mononeuritis multiplex.
10. *Miscellaneous:*
    fever (>38°C rectally), serositis, haemolytic anaemia (>60 g/*l*), or thrombocytopenia ($>50 \times 10^9/l$), all without improvement after prednisolone at a maximum dosage of 30 mg/day during at least 1 week.

Minor exacerbation: fulfilling all of the following items:

1. Increase of activity index by $\geqslant 2$ points within 6 months, with a minimal activity index of 4 points, accompanied by:
2. The clinically judged necessity to start prednisolone or to start immunosuppressive drugs, and:
3. Not fulfilling the criteria for a major exacerbation.

* Only features present within 2 weeks of the moment of the outclinic visit or the admission under consideration are taken into account.

Antibodies and staining procedure

Staining was done on whole blood samples. In brief, 10 μl of PE- or FITC-conjugated MoAb (Table 2) were added to 100 μl whole blood with subsequent incubation for 15 min at room temperature in the dark. FACS-lysing solution (2 μl; Becton Dickinson) diluted 1 : 10 in Millipore water was added with subsequent incubation for 10 min. Samples were washed with PBS/heparin. After the addition of 150 μl PBS/heparin, 10^4 cells per sample were measured on a FACStar (Becton Dickinson). Cells were gated for lymphocyte characteristics using both forward and right angle scatter, as well as

Table 2. Labelled antibodies used in this study

MoAb specificity	Label	Recognized subsets	Source
CD45/CD14	FITC/PE	All leucocytes/monocytes ('leukogate')	BD
CD3	FITC	T cells	BD
CD4	FITC/PE	Helper/inducer T cells	MCA
CD5	PE	T cells, B cell subset	BD
CD8	FITC	Cytotoxic/suppressor T cells/NK cells	MCA
CD20	FITC/PE	B cells	MCA
CD25	PE	IL-2 receptor; activated T and B cells	BD
CD38	PE	Activated T and B cells	BD
HLA-DR	PE	Activated T and B cells, and others	BD

BD, Becton Dickinson, Mountain View, CA; MCA, MCA Development BV, Groningen, The Netherlands.

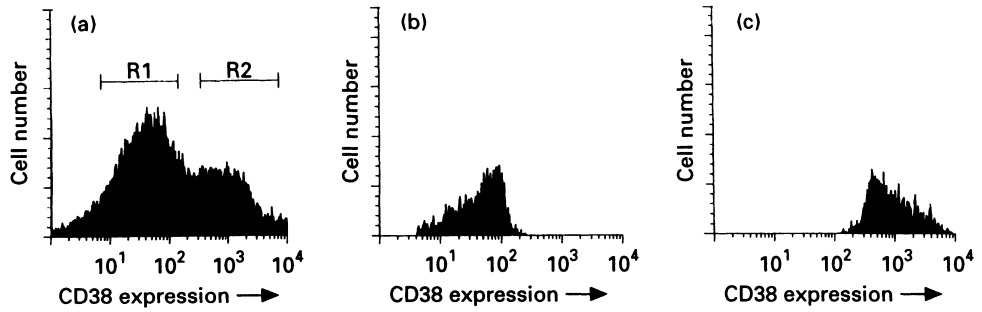

Fig. 1. Expression of CD38 is shown on $CD20^+$ lymphocytes (a). Sorting areas were arbitrarily defined as either low CD38 expression (gate R1) or high CD38 expression (gate R2). Re-analysis after sorting based on gates R1 and R2 is shown (b and c, respectively). Sorting efficiency was higher than 90% in both cases.

a dual staining using CD14 and CD45 (Leukogate; Becton Dickinson) for 'back-gating'. In case of severe lymphopenia, a 'live-gate' was placed to obtain maximal results. Background green and red fluorescence was determined using combinations of CD20 (PE) with CD4 or CD8 (FITC) as a control for calculations in the $CD4^+$ and $CD8^{bright}$ subsets, respectively. As control for calculations within the $CD20^+$ subset, dual staining for CD20 (FITC) and CD4 (PE) was used. Positive cells were determined by setting a region with reference to these controls. The list-mode data were then analysed by the Lysis Stat software package (Becton Dickinson) for calculation of percentages of populations. All analyses were performed consistently by the same persons (G.H. and B.T.F.vdG.), without prior knowledge of the subjects' clinical status.

Cell preparation, sorting procedure, and antigenic specificity of activated B cells

To establish whether IgG class anti-dsDNA-producing B cells are preferentially detected within the subset of circulating $CD38^+$ B cells in SLE, blood was drawn in heparin (Vacutainer; Becton Dickinson). Mononuclear cells were isolated using lymphoprep (density 1·077; Nycomed, Oslo, Norway). Samples were washed twice in RPMI. Labelled CD20 (FITC; MCA, Groningen, The Netherlands) and CD38 (PE; Becton Dickinson) MoAb was added (12·5 and 25 ml/10^7 cells, respectively), with subsequent incubation for 30 min at 0°C in the dark in RPMI/0·3% bovine serum albumin (BSA). Samples were washed in RPMI/0·3% BSA and centrifuged for 2 min at 1000 ***g***. All samples were stored in RPMI/gentamycin/0·3% BSA on ice in the dark until measurement on a FACS. Cells were gated for lymphocyte characteristics using both forward and right angle scatter. Sorting areas were arbitrarily defined in the $CD20^+$ cells expressing high and low levels of CD38 (Fig. 1). During the sorting procedure original samples and sorted samples were kept at 4°C. Sorted samples were analysed for viability and sorting efficiency.

ELISPOT assay

For ELISPOT, a 96-well plate (Labstar; Greiner, Kremsmünster, Austria) was coated with protamine sulphate (0·5 mg/ml; 45 min) and dsDNA (10 μg/ml; overnight), or goat anti-human IgG (10 μg/ml; overnight) at 4°C as described previously [17], with an additional blocking step (BSA 1% in bicarbonate 0·1 M pH 9·6). The sorted subsets (150×10^3 cells) in 100 ml medium (RPMI/0·3% BSA) were added to each precoated well. If sufficient cell numbers were available, lymphocytes were also incubated on wells coated with protamine sulphate only. After incubation overnight at 37°C, the cells were removed with PBS/0·05% Tween. The plates were then incubated with alkaline phosphatase-labelled anti-human IgG (FIIIAN; American Qualex, La Mirada, CA) for 2 h at 37°C. Individual antibody-producing B cells were visualized by addition of a 5-bromo-3-chloroindolyl phosphate (Sigma, St Louis, MO) solution in a low gelling temperature agarose kept at 44°C. Alkaline phosphatase produces a blue spot without diffusion through the agarose once it solidifies at room temperature. Spots were enumerated using a stereoscopic microscope.

Antibody levels

Anti-dsDNA antibody levels were determined by Farr assay (Diagnostic Products Corp., Los Angeles, CA). Levels of total IgM and IgG were measured by nephelometry (Behring, Marburg, Germany).

Statistical analysis

Analysis was done using the SYSTAT statistical package. Differences in parameters between groups were evaluated with the *t*-test. If paired observations were present in two groups, *t*-test was performed on paired and unpaired data separately. A *P* value was calculated corresponding to the mean of the standardized

Table 3. Cumulative characteristics according to the ACR* criteria of 26 patients with SLE

ACR criterion	*n* (%)
Malar rash	14 (54)
Discoid rash	6 (23)
Photosensitivity	11 (42)
Oral ulcers	4 (15)
Polyarthritis	23 (88)
Serositis	14 (54)
Renal abnormalities	13 (50)
Involvement of CNS	4 (15)
Haemolytic anaemia	4 (15)
Leukopenia	19 (73)
Lymphopenia	23 (88)
Thrombocytopenia	4 (15)
Anti-dsDNA antibodies	22 (85)
Anti-Sm antibodies	3 (12)
Anti-nuclear antibodies	26 (100)

* American College of Rheumatology.

test statistic. Associations between changes in study parameters were evaluated with the sign test. Spearman's test was applied for detecting correlations between different study parameters. If normal distribution could be assumed, Pearson's test was used. $P < 0{\cdot}05$ (two-sided) was considered significant.

RESULTS

Between November 1993 and June 1994, 26 patients (23 female, three male) and 18 healthy controls (16 female, two male) were included in this study. Their mean age was 34 years (range 21–55 years) and 30 years (range 19–45 years), respectively. SLE was diagnosed mean 9 years before study entry. Characteristics of these patients at study entry are shown in Table 3. Patients included were either in remission ($n = 21$) or in relapse ($n = 5$). Eighteen of the clinically quiescent patients were willing to participate in a prospective study during which blood samples were drawn monthly according to the protocol. Eight of these patients remained in a quiescent disease state, whereas 10 developed a relapse during the study period. Samples analysed in this study from the time of relapse were drawn before immunosuppressives were started.

Lymphocyte subsets during quiescent disease

Activation of lymphocyte subsets during quiescent disease was studied and compared with the results of 18 age- and sex-matched healthy individuals. The number of lymphocytes in patients with quiescent SLE was lower than that in healthy controls ($P < 0{\cdot}001$, Fig. 2a). Although six patients were on corticosteroids $\geqslant 5$ mg/day (median 7·5 mg/day, range 5–20 mg/day), no differences in the number of circulating lymphocytes were found between the group of patients with medication (median $0{\cdot}72 \times 10^6$/ml, range 0·10–$1{\cdot}56 \times 10^6$/ml) and that without (median $0{\cdot}90 \times 10^6$/ml, range 0·43–$4{\cdot}18 \times 10^6$/ml). The expression of activation markers on $CD4^+$ T cells, i.e. IL-2R, $P < 0{\cdot}001$, and HLA-DR, $P < 0{\cdot}001$, and on $CD20^+$ B cells, i.e. CD38, $P = 0{\cdot}001$, was increased in patients with quiescent disease in comparison with controls (Fig. 2b,d). Percentages of $CD8^{bright}$ cells expressing HLA-DR (Fig. 2c) and $CD20^+CD5^+$ B cells (data not shown) were comparable in

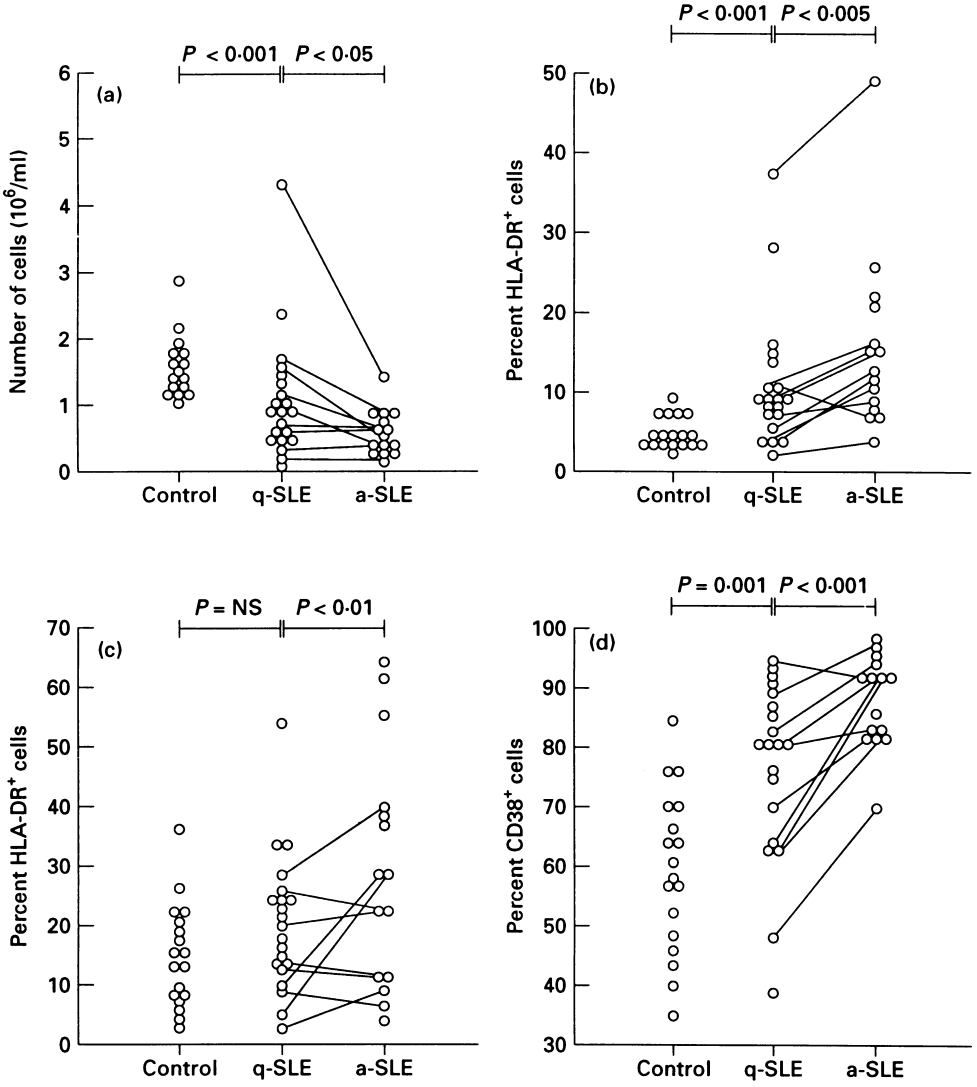

Fig. 2. Numbers of lymphocytes (a) and percentages of $CD4^+HLA\text{-}DR^+$ (b), $CD8^{bright}$ $HLA\text{-}DR^+$ (c), and $CD20^+CD38^+$ (d) subsets in healthy controls ($n = 18$), clinically quiescent SLE (q-SLE; $n = 21$), and at the moment of SLE disease relapses (a-SLE; $n = 15$). Percentages are expressed as percentage of total $CD4^+$, $CD8^{bright}$, and $CD20^+$ subpopulations. Lines denote paired observations.

Table 4. Characteristics of 15 relapses of SLE

	Number of relapses with feature present
Renal involvement	6
Neurological disorder	4
Skin involvement	4
Polyarthritis	12
Serositis	7
Vasculitis	1
Persistent fever >38°C	6

both groups. Despite the apparent activation of $CD4^+$ and $CD20^+$ subsets, the percentages of $CD4^+$, $CD8^{bright}$, and $CD20^+$ cells expressing activation markers were not interrelated. Interestingly, however, the percentages of $CD20^+CD38^+$ cells were related to circulating levels of immunoglobulin IgM ($P < 0{\cdot}02$) and IgG ($P < 0{\cdot}02$), but not to levels of anti-dsDNA.

Lymphocyte subsets during disease relapses

Fifteen patients were analysed at the time of relapse. Characteristics of the relapses are shown in Table 4. Eight of the 15 patients were on corticosteriods $\geqslant$ 5 mg/day (median 7·5 mg/day, range 5–17·5 mg/day) at the time of analysis.

Numbers of lymphocytes were lower in patients with relapse than in patients with quiescent disease ($P < 0{\cdot}05$, Fig. 2a). Lymphopenia was affecting all subsets: the percentages of $CD4^+$ or $CD8^{bright}$ T cells, and $CD20^+$ B cells did not differ between active and quiescent disease.

Percentages of $CD20^+CD38^+$ B cells were higher at the time of active disease than during quiescent disease ($P < 0{\cdot}001$; Fig. 2b), whereas percentages of $CD20^+CD5^+$ B cells did not differ. With respect to T lymphocytes, percentages of activated cells, i.e. $CD4^+$HLA-DR^+ ($P < 0{\cdot}005$) and $CD8^{bright}$ HLA-DR^+ cells ($P < 0{\cdot}01$), were also higher at the time of active than during quiescent disease (Fig. 2c,d). During active disease, percentages of $CD4^+$ or $CD8^{bright}$ T cells expressing activation markers were not related to percentages of B cells expressing CD38 on their cell surface. In contrast to findings during quiescent disease, percentages of $CD20^+CD38^+$ B cells were related to circulating levels of anti-dsDNA ($r = 0{\cdot}75$, $P = 0{\cdot}005$), but not to levels of total IgG and IgM.

Although the percentages of B and T cells expressing activation markers were not inter-related on cross-sectional examination (see above), the increase in percentage of $CD38^+$ B cells was related to the increase in percentage of activated $CD4^+$ T cells ($P < 0{\cdot}01$), but not to the increase in percentage of activated $CD8^{bright}$ T cells. Also, the rise in percentage of B cells expressing CD38 was related to the increase in levels of anti-dsDNA ($P < 0{\cdot}005$), but not to changes in levels of total IgG or IgM.

Fluctuations of lymphocyte subsets during follow up

From 18 patients blood samples were drawn monthly. Eight of these patients remained in a persistently quiescent disease state, whereas 10 developed a relapse during the study period. In order to assess the kinetics of lymphocyte subset distribution and activation, we analysed individual changes in lymphocyte subsets in patients with persistently quiescent disease in comparison with changes in healthy controls. Percentages of lymphocyte subsets were generally stable over time both in quiescent SLE patients and controls. In particular, fluctuations in activation state of lymphocyte subsets were comparable in quiescent SLE patients and normal controls (data not shown). Typical examples are shown in Fig. 3a,b.

In order to study kinetics of lymphocyte subset activation in the period preceding disease relapses, we calculated percentage changes between monthly time points. In agreement with the data presented in the previous paragraphs, monthly changes in percentages of activated $CD4^+$ T cells were related to changes in percentages of B cells expressing CD38 ($r = 0{\cdot}26$, $t = 2{\cdot}752$, $P < 0{\cdot}01$) and changes in level of anti-dsDNA ($r = 0{\cdot}23$, $t = 2{\cdot}555$, $P = 0{\cdot}01$). A typical example of longitudinal observations in the period preceding a relapse is shown in Fig. 3c.

Activated $CD20^+CD38^{bright}$ cells produce anti-dsDNA antibodies

The expression of CD38 on B cells increased from quiescent to active disease in all but one case (Fig. 2d). This increase was related to rises in levels of anti-dsDNA. To explore whether these $CD38^+$ B cells are the cells that spontaneously produce anti-dsDNA, we analysed sorted $CD20^+$ B cells expressing arbitrarily defined high and low levels of CD38 (Fig. 1) from three patients with quiescent SLE and one patient with active disease (Table 5). Sorting efficiency, measured by analysis of sorted samples, was >90% in all cases. Spontaneously anti-dsDNA-producing B cells were predominantly demonstrated in the sorted $CD20^+$ subset with high expression of CD38. Analysis of this subset showed that the number of anti-dsDNA-producing B cells in the active patient exceeded by far the number of anti-dsDNA-producing cells in quiescent patients. As lymphocyte counts were low, particularly in the patient with active disease, the number of sorted B cells

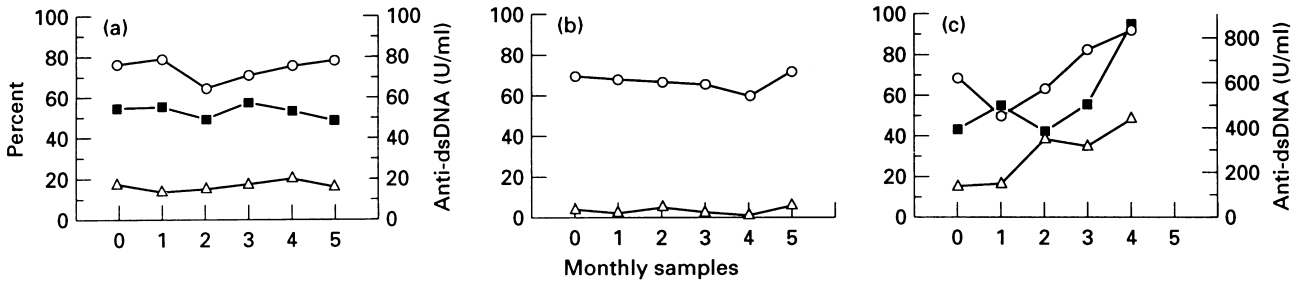

Fig. 3. Longitudinal observations of percentages of $CD4^+$HLA-DR^+, $CD20^+CD38^+$ cells, and anti-dsDNA levels in a patient with persistently quiescent SLE (a), a matched healthy control (b), and a SLE patient in the period preceding a disease relapse (c). Percentages are expressed as percentage of total $CD4^+$ and $CD20^+$ subpopulations. △, % $CD4^+$ HLA-DR^+; ○, % $CD20^+$ $CD38^+$; ■, anti-dsDNA (U/ml).

Table 5. Number of spots indicating the spontaneous production of total IgG and IgG class anti-dsDNA antibodies by sorted $CD20^+$ B cells expressing arbitrarily defined high ($CD38^{bright}$) and low ($CD20^+CD38^{dull}$) levels of CD38 as detected by ELISPOT, analysing 150 000 cells/well in four patients with SLE (three quiescent, one active) and one healthy control

	Number of spots within		Circulating anti-dsDNA (U/ml)	Circulating total IgG (g/*l*)
	$CD20^+CD38^{bright}$ subset	$CD20^+CD38^{dull}$ subset		
	dsDNA-coated wells			
Control	0	0	<2·5	9·7
Patient A, quiescent SLE	0	1	53	22·6
Patient B, quiescent SLE	8	2	243	17·0
Patient C, quiescent SLE	8	0	63	20·1
Patient D, active SLE	387	15	1499	29·0

Results are means of duplicate experiments. In addition, circulating levels of anti-dsDNA and total IgG are shown.

producing IgG could not be analysed in conjunction with the number of B cells producing anti-dsDNA.

DISCUSSION

Patients with SLE are characterized by the presence of hypergammaglobulinaemia, even during periods without clinical disease activity. This suggests activation of the B cell system. In this study, consecutive patients from a large cohort of SLE patients followed in a university hospital outclinic were prospectively analysed for cellular B cell activation and compared with matched healthy controls.

The proportion of B cells expressing CD38, a marker that is constitutively expressed on all B cells [18] and increases during B cell activation [19], was higher in quiescent SLE than in controls. The percentage of $CD20^+CD38^+$ B cells was related to levels of immunoglobulins, not to levels of anti-dsDNA, supporting the hypothesis of polyclonal B cell activation already in quiescent SLE [20,21]. Persistent activation of B cells may in part be sustained by activated T cells, in concordance with the finding that *in vitro* both $CD4^+$ helper and $CD8^{bright}$ cells are required for polyclonal B cell activation in SLE [22]. Indeed, the proportions of activated $CD4^+$ helper cells were higher in quiescent SLE than in healthy controls. However, neither the proportion of activated $CD4^+$ helper cells, nor that of $CD8^{bright}$ cytotoxic/suppressor cells were related to the proportion of activated B cells.

Before development of disease relapses, levels of anti-dsDNA rise in many cases [9]. This rise exceeds the rise in levels of total IgG and anti-tetanus toxoid (TT) antibodies [9,21], suggesting T cell-mediated oligoclonal B cell expansion when anti-dsDNA is produced. These data offer a rationale for studying changes in the activation of lymphocyte subsets at a cellular level. The distribution of these subsets was stable in patients with persistently quiescent disease and in healthy controls. In contrast, patients with changes in clinical disease activity showed changes in their lymphocyte subset distribution as well. The proportion of activated B cells, when evaluated at a cellular level, reflected the development of clinical disease activity. Moreover, circulating B cells expressing high levels of activation markers proved to be capable of spontaneously producing anti-dsDNA *in vitro*. These findings are in agreement with previous studies analysing spontaneous immunoglobulin [2,3] or anti-dsDNA [8,17] production by lupus B cells *in vitro*. Using a plaque-forming cell assay, Fauci & Moutsopoulos showed that the number of immunoglobulin-secreting cells in active SLE was higher than in quiescent SLE, which was again higher than in healthy controls [2]. Schwab *et al.* reported limiting dilution analysis of activated B cells in lupus patients and TT-boosted healthy controls. In their study, IgG class anti-dsDNA were only produced by SLE B cells. The number of anti-dsDNA-producing clones was comparable to the number of IgG-anti-TT antibody-producing clones in the boosted controls [23]. In the present study, the increase in B cell activation from quiescent to active disease was related to the increase in levels of anti-dsDNA and the increase of activated $CD4^+$ helper T cells. In contrast, no relation was found between changes in the proportion of activated B cells and changes in the percentages of activated $CD8^{bright}$ cytotoxic/suppressor cells. These data point to a T cell-mediated production of anti-dsDNA, and are in agreement with the previously proposed 'two-step' mechanism in the induction of high-affinity anti-dsDNA [24,25], i.e. a background of polyclonal B cell activation with a superimposed antigen-stimulated oligoclonal response.

B cell activation may, however, also occur independently of T cells. In particular $CD5^+$ B cells, the human counterpart of Ly-1 B cells in the mouse, have been mentioned in this respect [26–28]. This small B cell subset seems to be confined to the production of predominantly IgM class low-avidity antibodies, amongst which are the so-called natural autoantibodies [26]. Accordingly, high-affinity IgG class anti-dsDNA are produced *in vitro* by $CD5^-$ B cells only [17,29]. In the present study, B cells expressing CD5 could be detected during quiescent disease, but percentages were comparable to healthy controls. Moreover, the proportion of B cells expressing CD5 remained stable before development of a relapse. These findings agree with previous data [30], although some authors report higher numbers of $CD5^+$ B cells in lupus [31,32].

The persistent presence of a high proportion of activated B and T cells during periods with persistently quiescent SLE is of interest with respect to our understanding of the possible pathogenesis of this autoimmune condition. Although viral agents have been proposed [33–36], the factors that may sustain immunoactivation during quiescent lupus are as yet unknown. As a consequence of

the persistently high level of activation of all lymphocyte subsets they are prone to an increased rate of apoptosis [37,38]. Indeed, lymphocytes from lupus patients show an increased rate of apoptosis *in vitro* [39]. Moreover, circulating DNA in SLE patients has a length of 200 bp and its multiples [25], a phenomenon which can only be explained by cellular apoptosis, not by necrosis [40]. These multimeric DNA structures are bound to nucleosomes, which were demonstrated at higher concentrations in lupus patients than in healthy controls [41]. The possible importance of these phenomena in the pathogenesis of SLE is underlined by the finding that nucleosomes are released during apoptosis, due to inefficient binding of apoptotic cells [42,43]. These nucleosomes may subsequently act as antigenic targets inducing, or sustaining, the production of autoantibodies reacting with nucleosomes or DNA [44].

In conclusion, this study shows that B and T cell subsets are persistently activated during clinically quiescent SLE. The development of disease activity is associated with an increase in the proportion of $CD4^+$ T cells and B cells expressing activation markers, and an increase in circulating levels of anti-dsDNA. This suggests T cell-mediated production of anti-dsDNA antibodies.

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