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Production and analysis of IgG monoclonal anti-DNA antibodies from systemic lupus erythematosus (SLE) patients

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SUMMARY

This study compares recently devised methods for producing IgG anti-DNA MoAbs from patients with SLE and analyses the antibodies generated from one patient at different phases of disease. Lymphocytes from SLE patients were transformed with Epstein–Barr virus (EBV) and/or fused with a heteromyeloma cell line, CB-F7. Direct fusion with CB-F7 resulted in the highest proportion of IgG-secreting lines, whereas EBV transformation resulted in a high percentage of IgM-secreting lines. Using direct fusion, five IgM anti-DNA antibody-secreting hybridomas were generated using lymphocytes from a patient with relatively inactive SLE. Six months later when the disease was active, only IgG anti-DNA antibodies were produced. The antigen-binding patterns of the MoAbs were analysed. Only one of the IgM anti-DNA antibodies reacted with dsDNA by ELISA and none by *Crithidia* immunofluorescence, whereas two of the IgG antibodies reacted with dsDNA by ELISA and *Crithidia* but did not bind to ssDNA. Only the two IgG high affinity anti-dsDNA antibodies bound to histones, and this was enhanced by added DNA, whereas three IgM antibodies bound to cardiolipin. This study supports the notion that MoAbs derived from a patient with SLE represent those found in the serum of SLE patients at different stages of disease activity. The binding to histones by the two IgG anti-dsDNA antibodies supports the recently expressed view that antibodies binding DNA/histone may be important in the pathogenesis of SLE.

Keywords systemic lupus erythematosus anti-DNA MoAbs

INTRODUCTION

SLE is an autoimmune rheumatic disease characterized by the production of anti-DNA antibodies, levels of which often correlate with disease activity and are thought to play a role in the immunopathology of the disease [1]. However, the exact nature of these pathogenic antibodies and how they differ from natural, non-pathogenic autoantibodies have not been fully defined. It seems likely that the degree of cross-reactivity of these antibodies, the isotype and the idiotypes they possess are also of importance. In addition, it is unclear whether these pathogenic antibodies derive from natural autoantibodies.

Investigators have used two different approaches to answer these problems. The first was to examine antibodies present in sera from SLE patients, but it is difficult to determine whether cross-reactivity is due to the range of autoantibodies known to exist in SLE patients or to polyreactivity of individual autoantibodies. The production of MoAbs using hybridoma technology has allowed the analysis of individual antibodies [2,3]. However, monoclonal autoantibodies may not represent the pathogenic anti-DNA antibodies found in disease [4]. In particular, the MoAbs are often selected using an ELISA technique which detects low as well as high affinity antibodies and occasionally gives false positive results. In this study we used fluid-phase assays as well as *Crithidia* immunofluorescence to examine the affinity of the antibodies produced.

Stable human MoAbs have been more difficult to generate than their murine equivalents. Although the precise reasons for this discrepancy have not been elucidated, a number of explanations have been proposed [5]. The source of human lymphocytes is restricted for ethical reasons, in most instances, to peripheral blood, whereas the best source suggested by mouse fusions is the spleen or lymph node. In addition, the human partners are far from ideal, being immunoglobulin secretors with low fusion frequencies. Moreover, most anti-DNA MoAbs analysed have been of murine origin and, for reasons which remain enigmatic, most documented human anti-DNA MoAbs have been of the IgM isotype.

Recently, investigators have used a variety of methods in an attempt to overcome these difficulties and generate IgG anti-DNA antibodies reviewed in [6]. Diamond and coworkers [7], using Epstein–Barr virus (EBV) transformation of peripheral blood lymphocytes (PBL) from SLE patients, generated an

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equal number of IgG and IgM anti-DNA antibodies which expressed only one idiotype. Van Es studied one patient with 'active SLE' and generated, by EBV transformation, one IgG anti-DNA antibody out of 72 IgG-secreting cell lines [8]. IgG anti-DNA antibodies have also been produced using direct fusion of PBL (from patients with active SLE) with the heteromyeloma cell line CB-F7 [9]. EBV transformation of PBL followed by fusing the derived lines with the heteromyeloma partner has been used successfully in the production of human IgG antibodies reacting with cytomegalovirus [10]. We have studied 11 patients with SLE representing a range of clinical activities. In order to establish the conditions most likely to produce IgG anti-DNA antibodies, we first compared the three methods outlined above on three SLE patients.

What has not been clearly determined is whether the disease activity is an important factor in producing IgG antibodies. Watts *et al.* [5], using a human lymphoblastoid cell line, showed only a marginal difference in the yield of hybridomas from patients with active disease. Winkler *et al.* studied three patients with active disease to obtain IgG anti-DNA antibodies [9]. It is also unclear whether IgM antibodies are obtained from patients with active disease. We have analysed the isotype and binding characteristics of anti-DNA MoAbs derived from the one patient with SLE (out of 11) who produced IgG anti-DNA antibodies and compared the monoclonals produced with the sera simultaneously obtained. This patient was studied on two occasions, once when her disease was relatively inactive and again when it was very active.

PATIENTS AND METHODS

Patients

Eleven patients with SLE under the care of the Bloomsbury Rheumatology Unit were studied. Each of the SLE patients met the American Rheumatism Association's revised criteria for the classification of the disease [11]. Disease activity was assessed by the BILAG activity index [12]. This index, based on the physician's intention to treat, assesses the activity of the disease in eight major organs or systems. The index also yields a global activity score derived from the individual organ scores which has been validated against other activity indices [13]. Patients whose global score is above six are considered to have moderately or severely active disease. The PBL from three patients with similar BILAG global scores were used to compare three methods to generate MoAbs as outlined below, i.e. direct fusion with the heteromyeloma cell line CB-F7, EBV transformation, and EBV transformation followed by fusion with CB-F7. In addition, the PBL from a further eight patients were fused directly with CB-F7. One patient had her PBL harvested twice, once when her disease was relatively inactive and once when her disease was very active. On both occasions her PBL were fused directly with CB-F7.

Peripheral blood was obtained by venesection and the macrophages were removed by carbonyl iron treatment. Mononuclear cells were isolated by centrifugation on Ficoll–Hypaque (Nycomed, Oslo, Norway).

EBV transformation

EBV transformation was achieved by incubating the separated cells with the supernatant of the marmoset cell line, B95.58 (kindly provided by Dr G. Cambridge, Department of Immunology, University College, London, UK) in the presence of 50 ng of cyclosporine. Before transformation the cells were plated out at a density of 10^5 cells/well in 96-well plates (Nunc, Denmark). Transformants grew in each well. Each well was tested for IgM and IgG production as well as IgG and IgM anti-ss and dsDNA activity. In addition 5×10^6 cells of three patients were also incubated in flasks with supernatant of the marmoset B95.58 cell line. These cells were fused 4 weeks later with the heteromyeloma cell line CB-F7 (see below).

Fusion with CB-F7

After separation the PBL were directly fused with the mouse human heteromyeloma cell line CB-F7 (kind gift of Dr S. Jahn, Charité Hospital, Berlin, Germany), a HAT-sensitive, ouabain-resistant non-secreting cell line by PEG 1500 (Boehringer Mannheim, Germany). The fusion protocol was performed according to Grunow *et al.* [14]. Cells were seeded in flat-bottomed microtitre plates (Nunc) with 10^5 cells/well, 100 μl/well. After 24 h, 100 μl well of double concentrated HAT medium containing ouabain were added into the wells. Culture wells were tested as for the EBV-transformed cells. To ensure monoclonality, immunoglobulin anti-DNA-secreting hybridomas were subcloned three times by limiting dilution using mouse peritoneal cells (5×10^3/well) as feeders. Monoclonality was further ensured by chromosome analysis using a method as previously described [3]. Briefly, colchicine was added to growing cells which were then incubated for 2 h and harvested by centrifugation. After fixing in methanol/acetic acid, the cells were air dried on slides, banded with trypsin and stained with Giemsa. Monoclonal hybridomas all appear identical by this method.

ELISA for detecting and quantifying immunoglobulins

Immunoglobulins secreted by the human hybridoma-derived cell lines were detected by a previously described capture ELISA [15], except that $(Fab)_2$ antibodies were used to avoid rheumatoid factor activity.

Anti-DNA antibody detection

Culture supernatants were tested for ss and dsDNA antibodies by direct binding solid-phase and fluid-phase inhibition ELISA as previously described [16], with some modifications as follows. The ssDNA was prepared by sonicating calf thymus DNA (Sigma, Poole, UK) and then boiling for 10 min followed by rapidly cooling. Before adding to the plate, the supernatants were pretreated with DNAase 1 (Sigma) in the presence of 1 mM $MgCl_2$ and 0·02 mM $CaCl_2$ for 1 h at 37°C. The reaction was then stopped with 15 mM EDTA. To determine the anti-DNA activity, the background reading (that derived from the wells coated with poly-L-lysine) was subtracted from that derived from the wells coated with DNA.

Antibodies to dsDNA were also detected using an immunofluorescence method. Slides with prefixed *Crithidia luciliae* (Biodiagnostics, UK) were washed in PBS and non-specific binding was blocked with 20% normal goat serum for 30 min. The serum samples and MoAbs (diluted 1:10) were applied to the plates, and after washing the slides were incubated with an anti-human fluoresceinated conjugate. After another washing the slides were viewed using a fluorescent microscope (Nikon). Anti-nuclear antibodies were detected in a similar method to DNA antibodies, except that HEp-2 cells (instead of *Crithidia*)

Table 1. BILAG scores and global score of the patient whose peripheral blood lymphocytes (PBL) were fused on two separate occasions; DNA antibody levels in arbitrary units (normal < 100)

	General	Mucocutaneous	Neurological	Musculoskeletal	CVS/respiratory	Vascular	Renal	Haematology
5/91	D	D	D	C	C	C	E	C
Global score = 4								
dsDNA titre = 296								
11/91	B	C	D	A	B	C	E	A
Global score = 26								
dsDNA titre = 13 140								

A, Most active; E, never active.

were prefixed onto the slides (Biodiagnostics, UK). The staining patterns observed were classified as either cytoplasmic, nucleolar, speckled, or diffuse.

Other antigen ELISAs

Anti-histone activity was detected using an ELISA method as described elsewhere [17]. Briefly, Histone type IIS (Sigma) was coated overnight at 4°C in PBS. The plate was then blocked with 2% casein and any DNA contaminating the histone was removed by treatment with DNAase 1. The supernatants were added to the plate and the antibody bound to the plate was detected as for the DNA ELISA. Anti-cardiolipin activity was detected using an ELISA as previously described [2].

Inhibition studies

In order to determine the relative concentrations of antigen (ss and dsDNA) that would inhibit 50% of the binding of the antibody to ss and dsDNA, varying amounts of ss and dsDNA (S1 nuclease (Sigma) treated) were incubated with the supernatant at a fixed concentration of antibody. Dilutions of the culture supernatant were included on the same plate. Data were expressed as percentage binding to the solid-phase DNA compared with the binding in the absence of inhibitor (%OD). The method of Friguet *et al.* [18], adapted by Winkler *et al.* for anti-DNA antibodies [9], was used to calculate the dissociation constant K_d, to measure the affinity of the antibodies to DNA. The molecular weight of DNA was taken to be 660 kD, which has been shown to give similar affinity measurements whatever the source of DNA [9].

Detection of anti-idiotypes

The IgG antibodies were tested, as described elsewhere with polyclonal rabbit reagents [19], for the presence of three idiotypes first identified on human IgM MoAbs RT-84 Id, RT-72 Id and RT-6 Id [2]. These MoAbs bound ss and dsDNA; ssDNA and an SmD peptide; and Histone H1 respectively.

RESULTS

Six of the patients whose PBL were fused directly with CB-F7 had BILAG global scores of 6 or less, i.e. mild or inactive disease. Three had scores of 7 or 8, whilst two had high scores of 20 and 26, i.e. very active disease. This last patient's PBL were fused on another occasion when her BILAG score was 4, i.e. when her disease was relatively inactive (Table 1).

Three patients' PBL were used to compare the three methods of generating MoAbs as mentioned in Patients and Methods.

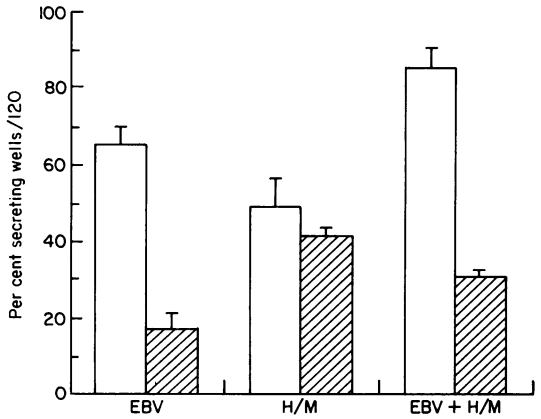

Fig. 1. Percentage number of IgG- and IgM-secreting wells generated from Epstein–Barr virus (EBV) transformation alone, direct fusion with CB-F7 (heteromyeloma (H/M)), and EBV transformation followed by direct fusion. The values represent the mean percentages ± s.e.m. of the three patients studied. □, IgM; ■, IgG.

The percentage of wells with dividing cells that were secreting IgG and/or IgM was calculated (mean of the three patients) (Fig. 1). The highest ratio of wells containing IgG compared with the wells containing IgM was obtained by direct fusion with CB-F7 (H/M). EBV transformation increased the number of wells containing IgM. Fusion of EBV-transformed cells appeared to increase further the number of IgM secretors, which may reflect the increased fusion frequency. Cells that had been EBV-transformed alone proved difficult to subclone and unstable in culture.

The PBL from the 11 patients were fused directly with the CB-F7 line. From only one of these patients were IgG anti-DNA antibodies derived, and these antibodies formed the basis of further study. This patient had the highest BILAG global score of all the patients [26]. This fusion yielded five IgG but no IgM anti-DNA antibodies; however, 6 months earlier when her disease was much less active five IgM, but no IgG antibodies, were generated in exactly the same way. Table 1 compares the BILAG scores on the two occasions.

The binding characteristics of these antibodies were analysed with respect to ss and dsDNA, histones and cardiolipin and compared with the binding of antibodies in the serum taken from the patient at the time of the two fusions. In addition, the ANA activity and binding to *Crithidia* were assessed (Table 2). Analysis of the serum on the first occasion showed no binding to

Table 2. Binding characteristics of the five IgM and five IgG anti-DNA antibodies produced from the patient whose peripheral blood lymphocytes (PBL) were fused on two occasions (see Table 1). Also shown is the binding of the patient's serum taken at the same time.

	Monoclonal antibodies	ssDNA	dsDNA	Cardiolipin	Histones	ANA	*Crithidia*
Fusion 1	IgM 1	++	−	+	−	Cyto	−
Patient	2	+	+	++	−	Cyto + nucleolar	−
inactive	3	++	−	++	−	Cyto	−
	4	++	−	−	−	Neg.	−
	5	++	−	−	−	Neg.	−
Matched serum	IgM sera	0·519	0·402	0	0·386	Cyto	−
results	IgG sera	0·457	0·379	0	0·381	Cyto	−
Fusion 2	IgG B3	−	++	−	++	Speckled	++
Patient	D2	−	++	+	++	Speckled	++
active	E7	+	−	−	−	Cyto	−
	F8	++	−	−	−	Cyto	−
	D5	++	+/−	−	−	Cyto + nucleolar	−
Matched serum	IgM sera	0·243	0·12	0	0·116	Cyto	−
results	IgG sera	0·418	0·391	0	0·527	Speckled	++

The serum results are expressed as optical densities obtained in the ELISA. ANA results are expressed in terms of the pattern observed if positive. Cyto, Cytoplasmic.

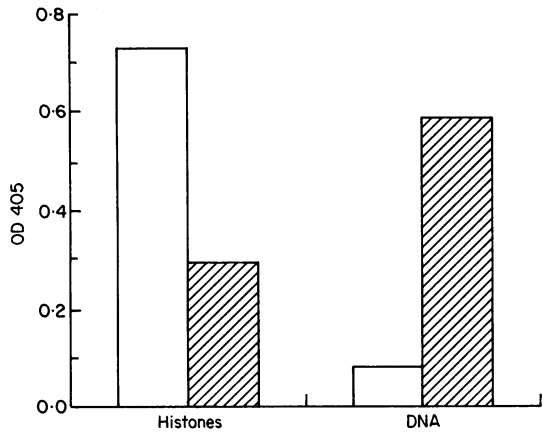

Fig. 2. Effect of DNAase treatment of the MoAb B3 on the binding of B3 to dsDNA and histones in an ELISA. Both dsDNA and histones were coated at a concentration of 10 μg/ml. □, No DNAase; ■, DNAase.

Crithidia, implying the absence of high affinity anti-dsDNA antibodies, and a cytoplasmic staining pattern on HEp-2 cells (ANA). On the second occasion IgG antibodies were identified in the serum that bound to *Crithidia*. Moreover, the ELISA reading of IgM anti-dsDNA antibodies reduced by almost four-fold, whilst the IgG anti-dsDNA reading increased slightly, when the patient had active disease compared with the ELISA readings when the patient had inactive disease.

Two out of the five IgG MoAbs bound only to dsDNA and were *Crithidia*-positive, whilst none of the five IgM antibodies bound to *Crithidia*. These two anti-dsDNA antibodies did not bind to dsDNA unless the DNA in the supernatant was removed by prior treatment with DNAase. The antibodies that only bound to ssDNA were not affected by DNAase treatment. In addition, the IgM antibody that bound to dsDNA in the ELISA was unaffected (data not shown). In contrast, the binding to histones by the IgG anti-dsDNA antibodies was reduced by removal of the DNA in the supernatant (Fig. 2).

The affinities of the IgG and one of the IgM antibodies were compared. The two IgG anti-dsDNA antibodies bound strongly to dsDNA, but did not bind to ssDNA. Figure 3a shows the fluid-phase inhibition to dsDNA on the plate of one of these antibodies, B3, that survived three rounds of cloning (unfortunately the other antibody did not). The apparent affinity to dsDNA was calculated to be $K_d = 6 \times 10^{-10}$, but ss DNA did not inhibit its binding to dsDNA. Figure 3b shows that the binding of B3 to histones is increased with the addition of dsDNA, but when more than 10 μg of dsDNA is added the binding diminishes. The three IgG antibodies (D5, F8 and E7) that bound almost exclusively to ssDNA all had similar affinities (Fig. 4) of approximately $K_d = 5 \times 10^{-7}$. The IgM anti-ss and dsDNA antibody had similar binding curves for ss and dsDNA in the fluid phase; the K_d for both was calculated as $1{\cdot}8 \times 10^{-5}$ (Fig. 4). None of the IgG anti-DNA antibodies bound the three anti-Ids described in Patients and Methods.

DISCUSSION

The advent of hybridoma technology has allowed investigators to analyse the fine specificity, idiotypes and origins of antibodies present in the sera of SLE patients. In particular, anti-DNA antibodies have been the focus of attention because their presence is characteristic of SLE. Anti-DNA antibodies have also been found in normal individuals [20]. These tend to be natural autoantibodies, polyreactive, of the IgM isotype and bind predominantly to ssDNA. In contrast, some natural autoantibodies are of the IgG isotype, and it is important to identify differences between these natural IgG anti-DNA antibodies and pathogenic antibodies. Most of the human MoAbs produced to date have been of the polyreactive IgM isotype,

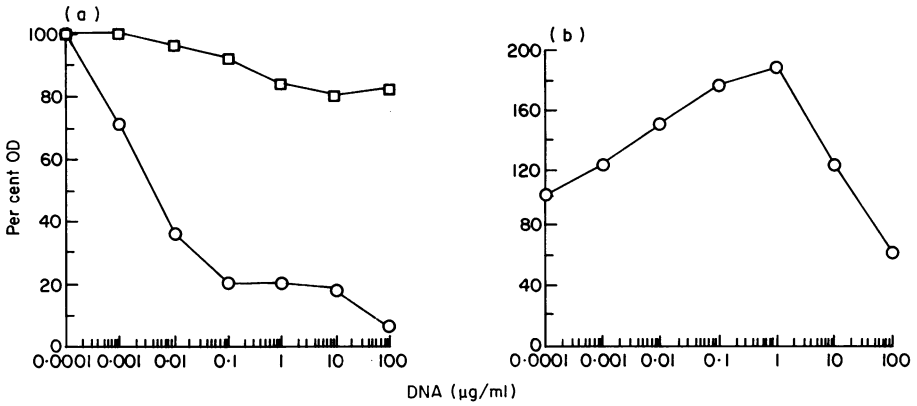

Fig. 3. (a) Inhibition of the binding of B3 to solid-phase dsDNA by dsDNA and ssDNA. ○, dsDNA; □, ssDNA. (b) Effect of the binding of B3 to histones by the addition of dsDNA in fluid phase. ○, Histones. Per cent OD, per cent binding of B3 to (a) dsDNA, (b) histones, compared with that without inhibiting antigen.

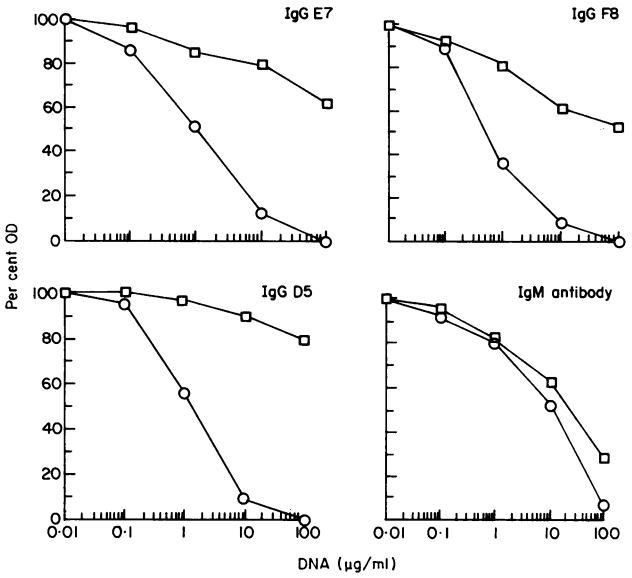

Fig. 4. Inhibition of the binding of three IgG anti-ssDNA antibodies (D5, E7 and F8) and one IgM anti-ss/dsDNA antibody to solid-phase ssDNA by ss and dsDNA. ○, ssDNA; □, dsDNA.

whereas IgG anti-DNA antibodies are those associated with active SLE [1].

To establish and study a set of IgG anti-DNA antibodies derived from SLE patients and compare them with IgM antibodies, we analysed recently devised methods to produce IgG anti-DNA antibodies to determine which was the most successful. EBV transformation and direct fusion with a heteromyeloma partner, with or without prior EBV transformation, were used. EBV transformation generated more IgM antibodies of all specificities than direct fusion, particularly when EBV transformation was combined with fusion. Casali & Notkins [20] maintain that this apparent bias towards the production of IgM antibodies reflects the higher proportion of IgM- to IgG-producing cells in peripheral blood. However, two groups using an ELISA spot to enumerate the number of IgG- and IgM-secreting cells in the peripheral blood found a roughly equal proprtion of IgG- to IgM-secreting cells in normal individuals and SLE patients [21,22]. Klinman *et al.* [22] found that 20% of the IgG and 8% of the IgM-secreting pool were committed to anti-DNA antibody production in patients with very active disease, but roughly equal numbers were detected in the patients with less active disease. Direct fusion with CB-F7 resulted on average in a higher proportion of IgG-secreting hybridomas than when using EBV transformation, and fewer IgM-secreting hybridomas. One explanation for the differences seen may be that EBV transformation and fusion select a population of B cells. Casali & Notkins [20] found that a proportion of activated B cells, those in the S1 phase (i.e. dividing cells), were resistant to EBV transformation compared with cells in the G1 phase (activated, premitotic cells) and resting cells. Thus, antibodies produced in this way may not be wholly representative of the primed B cells in SLE patients. Fusion of B cells selects for activated cells and thus may generate hybridomas from a pool of proliferating B cells which become plasma cells producing pathogenic antibodies [23].

There is debate at present about the nature of pathogenic anti-DNA antibodies. Nephritogenic antibodies are predominantly of the IgG isotype [24,25]. Some argue that monospecific high avidity anti-dsDNA antibodies represent pathogenic antibodies [4], whereas others have found polyreactive antibodies eluted from the kidneys of SLE patients [25]. In addition, IgM antibodies have been found at sites of tissue damage which bear anti-DNA antibody idiotypes [26]. Brinkmann *et al.* have challenged the notion of cross-reactivity being due to direct binding, and have proposed that DNA–histone complexes mediate the polyreactivity [4].

The patient we studied generated only IgM anti-DNA antibodies when her disease was inactive, suggesting that IgG anti-DNA antibodies were not being produced *in vivo*, or that the cells producing them were not activated, or that the IgG antibodies were being produced in a different site. IgG antibodies were identified by ELISA in the patient's sera when her disease was inactive and active, but only on the second occasion were there anti-DNA antibodies of sufficient affinity to bind to *Crithidia*. Moreover, the amount of IgM anti-dsDNA antibodies as measured by ELISA was reduced almost four-fold when the patient's disease was active, consistent with the idea of an increased switch towards IgG anti-DNA antibodies. This increased switch to IgG was also observed when examining the

anti-histone response (Table 2). The increased switch to IgG anti-DNA antibodies during a disease flare supports the notion that it is this isotype which is produced during active disease. The production of anti-DNA MoAbs is not a non-specific phenomenon. We have fused PBL from patients with diagnosis other than SLE, e.g. vasculitis [27], and not produced anti-DNA MoAbs despite the generation of MoAbs of other specificities.

Only by analysing the monoclonals produced (as opposed to the sera) are we able to demonstrate that, concomitant with this apparent increased switch of isotype, the binding specificities of the antibodies also changed. The IgM antibodies bound predominantly to ssDNA, and none bound to *Crithidia*, indicating that the anti-dsDNA activity of the monoclonals produced was of low avidity. Two out of five of the IgG anti-DNA antibodies bound to *Crithidia* and all bound to HEp-2 cells, indicating a positive ANA. Not all of the IgG antibodies generated in this study were of high affinity to DNA, supporting the view that switching is not always associated with high affinity antibodies. The high affinity anti-dsDNA antibodies did not bind significantly to ssDNA, which is unusual. Stollar & Papalian [28] have shown that ssDNA, prepared in a similar though not identical manner to the ssDNA we used, does maintain some secondary, native DNA-like structure. However, the immunological reactivity is reduced at 37°C (the temperature at which the experiments were performed), contrasting with native DNA. The binding to ssDNA may be lost at high affinities, suggesting that dsDNA is the target antigen in affinity maturation. Although IgG anti-DNA antibodies described by others [8] are somatically mutated and therefore presumably antigen-driven, the relevant antigen remains unknown.

These anti-dsDNA antibodies would not have been identified if they had not been pretreated by DNAase. Change in the reactivity of anti-DNA antibodies by DNAase has been reported by other workers, but not to the extent that without prior DNAase treatment the antibodies were undetectable [4,17]. Thus it is important when screening for monoclonals for DNA reactivity by ELISA to include DNAase treatment as routine, otherwise very high affinity antibodies may be missed. Unlike other workers we did not find that lower affinity antibodies of both isotypes, particularly those that bound predominantly to ssDNA, were affected by DNAase treatment (even to a minor extent, data not shown). This may be due to the type of DNA present in the supernatant, i.e. it is in a dsDNA form. Bell and colleagues have demonstrated that cells in culture release DNA containing nucleosomes *in vitro* and that these nucleosomes stimulate proliferation and immunoglobulin synthesis of normal mouse lymphocytes [29]. Of interest, a recent report indicates the presence of these nucleosomes in much higher concentrations in the plasma of SLE patients compared with normal controls [30].

Three IgM anti-DNA antibodies, but only one IgG antibody, bound to cardiolipin. This did not represent a reduction in cross-reactivity to all autoantigens, since two of the IgG antibodies bound to histones. However, over 50% of the binding to histones could be explained by anti-DNA/DNA complexes present in the supernatant and the binding could be enhanced by the further addition of DNA. This reactivity to histones by high affinity anti-dsDNA is in keeping with the reports suggesting that it is antibodies to the DNA/histone complex that cause tissue pathology [4,31]. The antibodies were able to bind to histone directly and, by complexing to DNA, could provide a dual mechanism for the formation of DNA/histone/antibody complexes. We are at present sequencing these antibodies to determine whether important differences exist.

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