Molecular Cloning and Expression of the Fabs of Human Autoantibodies in Escherichia coli

DETERMINATION OF THE HEAVY OR LIGHT CHAIN CONTRIBUTION TO THE ANTI-DNA/CARDIOLIPIN ACTIVITY OF THE Fab*

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The Fabs of three human autoantibodies (B3/33H11, anti-DNA; UK4, anti-phospholipid) and six related hybrids have been cloned, expressed in Escherichia coli, and purified to homogeneity. SDS-polyacrylamide gel electrophoresis and Western blot analysis of the recombinant Fab demonstrated the purified Fab to be of correct size and in assembled form. Protein expression levels of up to 5–9 mg per liter of culture were achievable. A sensitive and reliable comparative anti-DNA enzyme-linked immunosorbent assay, involving a defined biotinylated 35-mer oligonucleotide in its single- or double-stranded form, is also described. Crithidia assay and anti-DNA or anti-cardiolipin antibody enzyme-linked immunosorbent assay analyses demonstrated convincing binding of the recombinant Fab proteins to DNA/cardiolipin, confirming the expression of functional molecule. The comparative DNA/cardiolipin binding analyses of the nine Fabs revealed that the anti-DNA (light, B3/33H11) or anti-cardiolipin (heavy, UK4) activity lies predominantly on one of the two chains. However, a compatible partner chain is necessary for optimum antigen binding activity of the antibody.

Systemic lupus erythematosus (SLE)† is an autoimmune rheumatic disease affecting principally women during childbearing years, between 40 and 200 per 100,000 women (depending upon ethnic group) in the UK. Virtually all individuals with SLE have joint and/or skin involvement, and between 30 and 70% of the patients have kidney, heart, lung, and central nervous system involvement (1). Anti-dsDNA antibodies are serological markers of SLE often reflecting disease activity (2, 3) and pathogenesis (4). These antibodies have been eluted from both patients with SLE and various mouse models. Anti-phospholipid antibodies, especially anti-cardiolipin, are associated with recurrent thrombosis, miscarriages, and thrombocytopenia as part of the primary anti-phospholipid antibody syndrome (5). Despite considerable improvement in the overall outcome, lupus continues to cause considerable morbidity and mortality.

By using hybridoma technology we and others (6–8) have produced some human anti-DNA and anti-cardiolipin autoantibodies and analyzed their effects in SCID mice (9). Interestingly, although the anti-DNA antibodies studied appeared to be similar in that most bound dsDNA in ELISA and in Crithidia assays, they exhibited distinctive patterns of tissue binding and differing abilities to cause proteinuria (9) and pathogenicity including early histological features of lupus nephritis (10).

The structural basis for antigen specificity and pathogenicity of these antibodies is poorly understood (11). Such an understanding, however, would be of considerable value in the development of therapies that can inhibit or disrupt protein-nucleic acid interactions (12). Computer modeling of some of these antibodies has highlighted possible modes of interaction with DNA (13). However, these models are of limited accuracy. A full understanding of the binding specificities can only be achieved by experimental determination of detailed three-dimensional structure of these antibodies alone and of their complexes with specific DNA antigens. However, a prerequisite of such a study is the ability to produce reasonable amounts (in excess of 5–10 mg) of the antibody protein.

Our initial studies have therefore been focused on the cloning and overexpression of the Fab of a well characterized human anti-DNA antibody B3 (14) in a heterologous cell expression system. We now describe construction of novel vectors pAGP2 (λ) and Blunt™ that allow cloning of virtually any human lambda antibody. We also describe a novel cloning scheme that could be used for the cloning of many lambda antibodies belonging to V,1 and V,2 gene families known to represent a number of pathogenic autoantibodies in a PCR and/or non-PCR manner. The cloning scheme allowed us to clone rapidly two other well characterized antibodies 33H11 (anti-DNA, see Ref. 6) and UK4 (anti-cardiolipin, see Ref. 8) in a non-PCR manner.

The technology further allowed us to construct hybrid Fabs by swapping of the heavy (H) and light (L) chains of the three antibodies with which to investigate the role of the two chains in dictating the autoantigen specificity. The contribution of H and/or L chain to the DNA/cardiolipin binding of an autoantibody has been the subject of considerable debate. Although a number of studies of murine anti-DNA antibodies have been
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FIG. 1. a, construction of pAGP2(λ) vector for the cloning of human lambda light chain. Cλ and B3 Vλ were amplified separately in the first round of PCR, with restriction sites incorporated into the primers (Vλ: NruI at the 5’ end and BstEI at 3’ end; Cλ: BstEI at the 5’ end and EcoR I at 3’ end) using the following primers: 5’-GGGGTCCGATTGCGATGCGACTGGCTGGTTCCCGATAGGGGAAGCGAGGCTGTCGACTACGGCTGCCGCCTCCTGTGC-3’ (5’ primer) and 5’-CCTTTTGGGCTGTTGGGTACGGAGGTGTCGACTACGGCTGCCGCCTCCTGTGC-3’ (3’ primer) for Vλ; and 5’-AGGCGGATTGCGATGCGACTGGCTGGTTCCCGATAGGGGAAGCGAGGCTGTCGACTACGGCTGCCGCCTCCTGTGC-3’ (5’ primer) and 5’-CTTTTGGGCTGTTGGGTACGGAGGTGTCGACTACGGCTGCCGCCTCCTGTGC-3’ (3’ primer) for Cλ. The Vλ 3’ primer also incorporated 36 nucleotides from the 5’ end of the Cλ sequence. These overlapping amplified fragments were then used in a 4-molecule recursive PCR reaction (45) along with the Vλ 5’, 5’, and Cλ 3’ primers to assemble the complete lambda L chain sequence. The final product (in summary: NruI–Vλ–BstEII–Cλ–EcoRI) was purified using PCR purification kit (Qiagen, Slough, UK) for cloning into the above described vectors. However, the original CellTech vector pAGP2 contains Cloning scheme of VH domain. The scheme involves three steps as follows: (i) Cloning of VH domain into pAGP2 (a) as an NruI–BstEII fragment and that of Vλ into pAGP1 as an NruI–ApaI fragment; (ii) subsequence transfer of Vλ–Cλ to pAWtac as an XhoI–EcoRI fragment; and (iii) transfer of Vκ–Cκ1 as an EcoRI fragment to pAWtac containing Vκ–Cκ, kb, kilobase pair.

reported (15–17), similar studies on human IgG anti-DNA antibodies are scarce (18, 19) and provide only limited information. That given that over 90% of murine serum L chains are kappa (20), whereas approximately 40% of human serum L chains are lambda in addition to the human lambda locus being more extensive than that of the mouse (21), the information currently available on the L chain contribution in an autoantigen may not reliably represent similar L chain contribution in a human autoantibody. The autoantibodies studied presently possess lambda light chains encoded by 2A2 gene segment known to be most commonly used among humans (22) and also to exhibit pairing with different heavy chains (23). Furthermore, the 2A2 gene segment has no known mouse homologue (24). The autoantibodies studied presently thus present a relevant set of antibodies to investigate. Such an analysis of lupus autoantibodies might provide clues to the molecular basis for antigenic specificity that might in turn dictate pathogenicity. We report a quantitative assessment of the influence on anti-DNA/cardiolipin binding behavior, conferred to a human IgG antibody by its constituent H/L chain. This is an important step forward toward understanding and in vitro analysis of what could be an in vivo event.

EXPERIMENTAL PROCEDURES

Cloning of B3 Fab

Three plasmid vectors (pAGP1, pAGP2, and pAWtac) and two Escherichia coli strains (LM1035 and W3110) were obtained from CellTech (Slough, UK) for E. coli cloning and expression of the Fab of kappa antibodies. The final expression vector pAWtac is based on pCTR008 (25) involving the secretion of L and H chains under the direction of OmpA signal sequence and involves co-translational coupling of the cistrons (26). The cDNA encoding the variable (VH/Vκ) domains of L and H chains of the B3 antibody (14) were amplified by PCR (95 °C, 2 min; 62 °C, 2 min; 72 °C, 30 s; 30 cycles) using Vent DNA polymerase (New England Biolabs, Hitchin, UK) for cloning into the above described vectors. However, the original CellTech vector pAGP2 contains L chain with kappa constant region. Since B3 is a lambda antibody, the
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Protein Purification—Proteins concentrated using 60% ammonium sulfate were dialyzed against 20 mM sodium phosphate buffer, pH 7, and loaded on a protein G (protein G-Sepharose 4 Fast Flow; Amersham Pharmacia Biotech, Bucks, UK) column. The bound Fab protein was eluted with 100 mM glycine buffer, pH 2.7. Protein G-purified Fab protein was dialyzed against 10 mM sodium phosphate buffer, pH 7.3, and loaded on a heparin (heparin-Sepharose CL-6B; Amersham Pharmacia Biotech) column. The bound Fab protein was eluted with a 100–800 mM NaCl salt gradient. The purity of the Fab purified protein was assessed on SDS-PAGE gels under reducing and non-reducing conditions.

Quantitative Assessment of the Expression of the Recombinant Fab—Protein concentration of the purified Fab was determined by measuring the absorbance at 280 nm (27) using UV-2401PC (UV-visible recording spectrophotometer, Shimadzu Corp., Japan). The level of expression in individual periplasmic batches was assessed using an enzyme-linked immunosorbent assay (ELISA). Briefly, the polystyrene plate was coated with a monoclonal antibody to human lambda chain (Mo-4, 1:1000; 4 °C, overnight) and blocked (PBS plus 10% Marvel skimmed milk, 1 h, 37 °C). A range of concentrations of the purified Fab (from 2,500 ng/ml, standard) and the periplasmic samples was applied (1 h, 37 °C) in doubling dilutions. Binding of the recombinant Fab was detected using goat α-HA conjugated to alkaline phosphatase and p-nitrophenyl phosphate as substrate. The quantity of the Fab present in the periplasmic samples was determined from the standard curve constructed using the OD values obtained for the standard Fab protein samples.

Functional Assessment of the Recombinant Fab

Periplasmic extracts were tested in ELISA for calf thymus dsDNA binding activities of the recombinant Fabs as described previously (29). The recombinant Fab protein was also evaluated for its binding ability to dsDNA polar body of Crithidia luciae as described elsewhere (30) (Fig. 2b).

Cloning of 33H11 and UK4

The cloning scheme currently involved uses the involvement of unique restriction sites to identify to be naturally present in the framework 1 (5’ end) and framework 2 (3’ end) regions of the L and H chain variable domains. The scheme has the advantage of providing a non-PCR strategy thus avoiding the inclusion of mutations and the time-consuming sequencing steps. Furthermore, the proposed scheme involves the construction of ompA leader (5’ end) and constant (3’ end) regions on the two ends of the variable domain as opposed to replacing one variable domain with the other keeping ompA leader and constant regions intact. This strategy thus minimizes the possibility of the available domain of one antibody not being replaced by that of the other due simply to the inefficient digestion of the DNA by the restriction enzymes employed resulting either in no digestion or in the self-ligation of the singly cut DNA. Furthermore, the proposed cloning strategy involves the use of carefully selected restriction enzymes providing efficient digestion of DNA and releases DNA fragments reliably distinguishable and separable on the gel. The sequences encoding for Vc or VH of 33H11 and UK4 antibodies have been cloned in eukaryotic expression vectors plN10 (Vc) and pG1D1 (VH) for their expression as IgG in our laboratory. Vc or VH were transferred from plN10 and pG1D1 to pAWtac using the cloning scheme described below.

Cloning of the Light Chain—All the three autoantibodies, B3, 33H11, and UK4 are lambda antibodies and possess similar sequences up to 72 bp at 5’ end of Vc. This region also contains a unique restriction site BsaI at position 42. Furthermore, the Jc region of all the three antibodies possesses a unique restriction site AvrII at position 328 at the 3’ end. Since the region between BsaI and AvrII represents all the differences exhibited by the three antibodies in their Vc domains, it was opportune to exploit the natural presence of these restriction sites for the cloning of their L chain. Although it was achievable by simply replacing the pre-existing BsaI-AvrII fragment of B3 VH earlier cloned in pAGP2 (λ) by that of 33H11 or UK4, most of the available cloning vectors also contained BsaI and/or AvrII site rendering them unsuitable for the proposed purposes. One of the vectors PCR-Blunt used in our laboratory does not have AvrII site, however, possesses a BsaI site in the open reading frame of the codB lethal gene. We therefore con-
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The scheme presently employed for the cloning of the L chains has been summarized in Fig. 3a and can be efficiently used for swapping of \( V_\text{\textit{L}} \), of the antibodies encoded by the same gene segment. However, swapping of \( V_\text{\textit{L}} \) encoded by different gene segments (loci) within the two lambda gene families may result in the change of 1 or 2 (V, I) or up to 4 (V, I) amino acid residues upstream of the restrictions sites \( \text{Bsal} / \text{Bst} \), \( \text{Pvu} / \text{Pvu} \), and \( \text{AvrI} / \text{Pvu} \) II to the 5' end, the use of these enzymes would allow the scheme to be used efficiently for swapping of the \( V_\text{\textit{H}} \) encoded by the same gene segment or different gene segments within a gene family or by different gene families, using the cloning vectors described. The occasional change of a residue upstream of these restriction sites can be eliminated by PCR cloning of a \( V_\text{\textit{H}} \) encoded by the allele of interest via restriction sites \( \text{NruI} / \text{AvrI} \) or \( \text{AvrI} / \text{BstI} \) in our vector V5 (Fig. 3b) using the primers described earlier for the cloning of the \( V_\text{\textit{H}} \) in pAGP1 following necessary modifications incorporating residue changes. The scheme (Fig. 3b) can then be followed for non-PCR cloning of other L chains encoded by the same locus via their transfer to our vector Blunt\textit{z} using appropriate restriction sites (Fig. 3a, III).

Cloning of the Heavy Chain—B3, 33H11, and UK4 each possesses similar sequences up to 66 bp at 5' end of \( V_\text{\textit{H}} \). This region also contains two unique restriction sites \( \text{PvuII} / \text{PvuII} \) at position 10 and \( \text{PpuMI} \) at position 48. Furthermore, \( J_\text{H} \) region of all the three antibodies possesses a unique restriction site \( \text{BstEI} \) at position 346 at the 3' end. Since the region between \( \text{PvuMI} \) and \( \text{BstEI} \) represents all the differences exhibited by the three antibodies in their \( V_\text{\textit{H}} \) domains, it provided an opportunity of convenient cloning of the \( H \) chains in a non-PCR manner involving these naturally occurring restriction sites in the \( V_\text{\textit{H}} \) region. It was achievable by simply replacing the pre-existing \( \text{PvuII/PvuII/BstEI} \) fragment of B3V\textit{H} earlier cloned in pAGP1 by that of 33H11 or UK4. Since \( \text{PvuMI} \) and \( \text{BstEI} \) both generate sticky ends, the use of \( \text{PvuII} \) was preferred over \( \text{PvuII} \) that generates blunt ends. Furthermore, the novel vector Blunt\textit{z} was chosen for the cloning also of the \( H \) chain. The scheme (Fig. 3b) using the primers described earlier for the cloning of the \( H \) chains of 33H11 and UK4, for two reasons. First, it did not possess the restriction sites selected. Second, it allowed the release of distinguishable and conveniently separable fragments for gel purification following the digestions by the selected unique restriction enzymes.

The scheme for the cloning of the \( H \) chain fragment (\( V_\text{\textit{H}} \) and \( C_\text{\textit{H}} \)) domains has been summarized in Fig. 3b. Given the proximity of the restriction sites \( \text{PvuII} \) or \( \text{PvuII} \) which would allow the scheme to be used efficiently for swapping of the \( V_\text{\textit{H}} \) encoded by the same gene segment or different gene segments within a gene family or by different gene families, using the cloning vectors described. The occasional change of a residue upstream of these restriction sites can be eliminated by PCR cloning of a \( V_\text{\textit{H}} \) encoded by the allele of interest via restriction sites \( \text{NruI} / \text{AvrI} \) or \( \text{AvrI} / \text{BstI} \) in our vector V5 (Fig. 3b) using the primers described earlier for the cloning of the \( V_\text{\textit{H}} \) in pAGP1 following necessary modifications incorporating residue changes. The scheme (Fig. 3b) can then be followed for non-PCR cloning of other \( H \) chains encoded by the same locus via their transfer to our vector Blunt\textit{z} using appropriate restriction sites (Fig. 3b).

Construction of the Final Expression Vector for 33H11 and UK4 and the Hybrids—The L (ompA-, \( V_\text{\textit{L}} \), and \( C_\text{\textit{L}} \)) chain variable and constant domains along with the ompA signal sequence were inserted into the expression vector pAWtac via our new vectors V4 (pAGP2 \( \lambda \) equivalent) and V8 (pAGP1 equivalent) employing the scheme described in Fig. 1h. In brief, the \( L \) chain of the three antibodies (B3, 33H11, or UK4) were singly inserted into pAWtac as \( \text{Xhol} / \text{EcoRI} \) fragments thus constructing the three intermediate vectors, one for each antibody L chain: pAWtac-L (B3), pAWtac-L (33H11), and pAWtac-L (UK4), respectively. The three H chains were singly inserted in each of the three constructs as EcoRI fragments, thus allowing the construction of the two-antibody (in addition to the earlier cloned B3) and three-hybrid final expression vectors. The key to the legends used in the figures is as follows: letters B, R, and U denote B3, 33H11, and UK4 respectively; the first of the two letters name of the Fab construct denotes the \( L \) chain and the second the letters the \( H \) chain.
Expression, Detection, and Functional Assessment of the Fabs

The final expression vectors for the eight constructs were transferred to the expression strain W3110. The induction experiments, preparation of periplasm, dot blot, SDS-PAGE gel, Western blot analysis, and the functional assessment by anti-DNA antibody ELISA of the expressed Fabs were conducted as earlier described for B3 Fab. Anti-cardiolipin activity of the nine Fabs was determined using an anti-cardiolipin antibody ELISA as described previously (8). Periplasmic extracts prepared from E. coli with or without IPTG induction for individual constructs were tested in a sandwich ELISA for the binding of the recombinant Fabs to anti-human lambda antibody as earlier described for the quantitative ELISA assay of B3 Fab. The binding of the recombinant Fabs to DNA and cardiolipin was compared using a novel ELISA immunoassay as described below.

Anti-DNA Activity—Biotinylated or nonbiotinylated 35-mer oligonucleotides (5’-biotin-CCGAATCCGTTCACTTCCAGCCAGCGTATTAGCC-3’ and its nonbiotinylated complementary strand) were synthesized. These oligonucleotides are known to bind to B3 and have been used previously for the evaluation of the affinity of DNA-binding proteins to DNA (31, 32). Biotinylation of polystyrene plates were coated (4 °C, overnight) with streptavidin (50 μg/ml). These oligonucleotides are known to bind to B3 and have been used previously for the evaluation of the affinity of DNA-binding proteins to DNA (31, 32). Biotinylation of polystyrene plates were coated (4 °C, overnight) with streptavidin (50 μg/ml). The nonbiotinylated oligonucleotide was applied to the streptavidin-coated wells (50 μg/ml, 1 h, 37 °C). An immobilized double-stranded oligonucleotide was generated by annealing the nonbiotinylated oligonucleotide (5’-GGCTAATACCTGGCCTGAACGTTCCCGG-3’, 20 μg/ml) to the immobilized oligonucleotide in situ, in half of the wells coated with the first strand of the oligonucleotide. Hybridization was performed in 1 M sodium chloride, 20 mM monosodium phosphate, 0.1 M EDTA, pH 7 (1 h, 37 °C), as recommended by Amersham Pharmacia Biotech Biosensor AB (Application Note 306, 1995). The plate was then washed with 10% fetal calf serum in PBS (1 h, 37 °C). Anti-DNA binding was assessed by the OD405 of the DNA in the wells of the plates (data not shown), suggesting the molecule was a monomeric molecule (data not shown), suggesting the molecule was a monomeric protein G column could be efficiently eluted with 200 mM NaCl and resolved as a clean band at around 45 kDa on SDS-PAGE gels under non-reducing conditions (Fig. 2a). Bands at approximately 45 kDa (unreduced, Fab) and 22 kDa (reduced, free H and L chains) on Western blots (Fig. 4) suggest the purified Fab to be of correct size and in assembled form. Expression levels of up to 5 mg of Fab protein per liter of culture were recorded using the quantitative ELISA. C. elegans assay on the purified Fab confirmed the binding of the recombinant B3 Fab protein to DNA (Fig. 2b) comparable to that of the whole B3 IgG molecule (data not shown), suggesting the molecule was functional.

In order to determine the role of the L and/or the H chain in conferring the anti-DNA and/or anti-cardiolipin activity to the molecule, two more antibodies 33H11 (anti-DNA) and UK4 (anti-cardiolipin) were cloned as described (Fig. 3). This allowed the construction of the six hybrid Fabs by swapping of the chains of the three antibodies B3, 33H11, and UK4. The expression of the Fabs was achieved by IPTG induction of the W3110 cells containing the final expression vector. All the recombinant Fabs resolved in around 45-kDa region in their unreduced forms (Fig. 4, lane a) on the Western blots, whereas upon reduction, the dissociated H and L chains resolved in around 22 kDa region (Fig. 4, lane b) as expected. This observation suggests that all the expressed Fabs were in assembled form.

The recombinant Fabs exhibited variable binding to calf thymus dsDNA (Fig. 5a) or cardiolipin (Fig. 5b) suggesting that the Fabs were functional and that different H and L chains and their combinations had affected the binding patterns of the Fabs. Relative anti-DNA activity of the nine Fabs is summarized in Fig. 5c. The native H and L chain combinations of the Fabs bound to ssDNA in the following decreasing order: BB > RR > BU (Fig. 5c). Binding of BB to dsDNA was comparable to its binding to ssDNA (Fig. 5c), whereas RR and BU exhibited weak binding to dsDNA. These observations are in line with the fact that B3 is predominantly an anti-dsDNA (13), whereas UK4 is mainly an anti-cardiolipin antibody (8). However, 33H11 would appear to be a predominant binder of ssDNA in the assay system used. The Fabs exhibited anti-DNA activities in the following decreasing order of their percent binding to ssDNA: BR > BB ≈ BU > BR > BU ≈ UB > RU or to dsDNA: RU > BB ≈ BU > BR > BB ≈ UB > RU ≈ UB > RR (Fig. 5c). B3 or 33H11 L chain in association with any of the three H chains studied, except for 33H11 L chain in combination with 33H11 H chain, conferred significant anti-ssDNA antibody to the Fab.

Relative anti-cardiolipin activities of the nine Fabs have been summarized in Fig. 5d. The Fabs exhibited anti-cardiolipin activities in the following decreasing order of their percent binding: BB > BR > RR > RU ≈ BU > BB > BR > UB ≈ UB ≈ UR. In contrast to BB or RR L chain, UU L chain conferred significant anti-cardiolipin activity to the Fab only in the presence of UU H chain. BB and RR though predominantly anti-DNA antibodies, also exhibited significant anti-cardiolipin cross-re-

FIG. 4. Western blot analysis of the recombinant Fab proteins demonstrating the binding of the assembled Fabs (approximately 45 kDa, unreduced, lane a) and of free light chains (reduced Fab, lane b) to anti-human λ antibody. Key: 1, BB; 2, BR; 3, BU; 4, RB; 5, UB; 6, UR; 7, RU; 8, RR; 9, UU. Letters B, R, and U denote B3, 33H11, and UK4, respectively; the first of the two letters name of the Fab construct denotes the light chain and the second of the letters denotes the heavy chain.

RESULTS

Dot blot analysis demonstrated the presence of the recombinant Fab in the periplasm, supernatant, and cell extracts from IPTG-induced cells but not in those from uninduced cells. The expression of the Fab was confirmed by Western blot analysis. Fab protein precipitated at 60% saturation of ammonium sulfate and, following elution from the protein G column, resolved as about 45-kDa band on SDS-PAGE gels along with some contaminating bands. Protein G-purified Fab protein loaded on
activity. The percent binding of the Fabs of the two antibodies to cardiolipin was, surprisingly, relatively higher than the binding of UU previously shown to be a predominant anti-cardiolipin antibody in its IgG form.

**DISCUSSION**

The cloning strategy and the vectors used in the present study have for the first time allowed overexpression of human anti-DNA antibody Fab fragments in a heterologous cell system. Expression levels of approximately 5 (B3) to 9 (UR) mg of Fab protein per liter of culture were achievable. Stollar (33) has recently reported an *E. coli* expression of scFv antibody fragments ranging from 30 μg to 15 mg per liter of culture. However, Fab fragments remain the favorite for their crystallization which is the long term objective of the present studies. B3 recombinant Fab protein was purified to homogeneity. The initial step to purify Fab protein was an ammonium sulfate precipitation at 60% saturation. The presence of the CH1 domain was an intermediate purification step. Like many DNA-binding proteins, the main in the Fab facilitated the use of protein G as an intermediate purification step. The cloning scheme described in Fig. 3 allows swift cloning of the antibodies primarily encoded by the gene segment following the cloning of one antibody belonging to that locus. The novel cloning scheme allowed us the flexibility of rapid cloning of 33H11 and UK4 antibodies in a non-PCR manner and of constructing their hybrids. This cloning scheme allows rapid transfer of antibody chains cloned, for example, in Cambridge Antibody Technology eukaryotic expression vectors pLN10 (light) or pG1D1 (heavy) to CellTech prokaryotic expression vector pAWbac via the newly constructed shuttle vectors pAGP2 (λ), Bluntxe, V2, V3 (light chain), V5, and V6 (heavy chain) thus also providing a bridge between the two extensively used vectors for antibody expression. The restriction sites used occur naturally in the framework regions of the studied autoantibodies and are relatively conserved in the antibodies belong to the following gene families: VH1 (heavy chain) family (35). Although we have created a *Bst* site at the 5’ end of the lambda constant region present in pAGP2 (λ), the cloning scheme described overcomes the problem of the presence of this additional *Bst* site in the context of the cloning of *V*λ as the insertion of the lambda constant region at the 3’ end of the variable domain does not involve the use of *Bst*EII. The described cloning scheme allowed us to rapidly swap and make novel combinations of H and L chains with which to investigate the contribution of the two chains to the antigen binding.

An extensive comparative study of conventional anti-DNA antibody assays led to the conclusion that more than one assay was required to confirm the anti-DNA activity of the antibodies reliably (36). These assays involve coating of complex DNA (e.g. calf thymus, salmon sperm, human placental, or others) either

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**Fig. 5.** Binding of the nine recombinant Fab proteins in ELISA to calf-thymus dsDNA (a) or cardiolipin (b) demonstrating that they exhibited functional activity. The ELISA system used could not detect convincing binding of RR to dsDNA, due to its low level of expression. All the eight constructs exhibited convincing anti-DNA activities, and those containing B3 light chain possessed relatively higher levels of DNA binding. Different OD scales are therefore used in b for the two sets of the constructs allowing depiction of convincing binding also of the poor DNA binders. Letters B, R, and U denote B3, 33H11, and UK4, respectively; the first of the two-letters name of the Fab construct denotes the light chain and the second of the letters denotes the heavy chain. Quantitative comparative assessment as percent binding in relation to total binding to anti-human lambda monoclonal antibody, of the nine Fab proteins in ELISA, to a 35-mer biotinylated oligonucleotide in its single or double stranded (c) form or to cardiolipin (d).
directly or via poly-L-lysine on UV-treated polystyrene microplates (37). However, these methods involve the risk of inconsistency in the amount of DNA coated on the plate, and the use of poly-L-lysine may result in high back ground levels. Immobilization of dsDNA on the solid phase may also expose determinants associated with ssDNA (18). Furthermore, the preparation of ssDNA from such complex DNA molecules may not guarantee complete elimination of determinants associated with dsDNA. Given the limited reliability of the conventional ELISAs in the quantitative assessment of the reactivity of anti-DNA antibodies, we utilized a defined biotinylated oligonucleotide as the antigen in an ELISA for the comparisons required presently. It has the advantage that the DNA is coated reliably in its single- or double-stranded form in reproducibly consistent quantities on streptavidin-coated polystyrene plates and significantly reduces background binding. Interestingly, most of the autoantibodies tested do not seem to exhibit cross-reactivity against streptavidin used to immobilize the oligonucleotide in the novel ELISA immunoassay which is probably the explanation for the observed low background levels. A comparison of the sensitivity of the two immunoassays revealed that the novel ELISA is able to detect a sticky antibody exhibiting high background level (over 70%) in conventional assays even when present in concentrations as low as 78 ng/ml. The novel ELISA thus allowed us to assess the anti-DNA activity of RR antibody (Fig. 5c) which was earlier undetectable in conventional immunoassay (Fig. 5a). The sensitivity of the conventional assay for a reliable assessment of anti-DNA activity of an antibody was determined to be 200–300 ng/ml of immunoglobulin protein. The novel ELISA thus provides significant improvement and overcomes most of the drawbacks inherent in the conventional ELISA and has allowed us to compare the binding of all the Fab proteins studied to ss- or dsDNA. Such a quantitative analyses of autoantibodies would be difficult to carry out on a BIAcore system.

All the Fabs containing the BB L chain (BB, BR, and BU) exhibited significant binding to ss- or dsDNA (Fig. 5c). The replacement of the UU L chain with that of BB resulted in a nearly 4-fold increase (BU), whereas substitution of the BB L chain with that of UU resulted in a >50% drop (UB), in the percent DNA binding of the hybrid Fabs suggesting that the B3 L chain plays an important role in conferring Fab binding to DNA. RB exhibited nearly 3-fold (dsDNA) or nearly 25% (ssDNA) increase in the binding to DNA suggesting better compatibility of BB H and RR L chains. The replacement of UU L chain with that of RR resulted in nearly 3- (ssDNA) or 5- (dsDNA) fold increase in the DNA binding of RU. However, the substitution of the RR L chain with that of UU resulted either in a drop (ssDNA) or no effect (dsDNA) in the DNA binding of UR. The replacement of the RR L chain with that of BU resulted in a significant drop in the binding of the hybrid Fab UB or UR suggesting that in the context of UU L chain, the anti-cardiolipin activity of UU predominately lies on the H chain. A significant drop in the anti-cardiolipin activity of BR as compared with that of BB and a significant increase in the anti-cardiolipin activity of RB as compared with that of RR, in the presence of poor anti-cardiolipin activity of UR, suggests the anti-cardiolipin activity lies predominantly on RR light chain. A comparison of the binding activities of the hybrid Fabs BB, RB (good binders), and UB (poor binder), all containing BB H chain, would suggest that the BB L chain like the RR L chain, probably also has the potential to confer anti-cardiolipin activity when present in association with an appropriate H chain. BB and RR L chains earlier observed to have the potential of conferring anti-DNA activity would also appear to be involved in conferring anti-cardiolipin cross-reactivity to the Fab in the presence of an appropriate H chain.

The present results that anti-DNA activity of B3 lies on the L chain are supported by our computer modeling studies predicting the B3 L chain arginines (CDR-L1–27A, CDR-L2–54) to interact directly with the docked DNA (13). Mutation of B3 arginine at L1–27A to serine results in a significant loss in the anti-DNA activity of the mutant IgG compared with that of the wild type IgG. A number of studies using mouse models suggest that the L chain confers or modulates DNA binding (16, 17, 38, 39) or expands the specificity of (15, 16), or confers second cross-reactivity to (40), the autoantibody. Some studies have also implicated components of VH alone or those in combination with VL in conferring DNA binding activity (17, 41–44). However, such studies are only scarcely available on human IgG autoantibodies. The requirement of restrictive pairing of a heavy chain with lambda light chains (18) and the involvement of basic residues of CDR3 and L chain (19) have been suggested to confer DNA binding activity to human autoantibodies. No information is available in the literature on the L or H chain determination of anti-cardiolipin activity of an antibody. The present results would suggest that although the anti-DNA (L chain) or anti-cardiolipin (H chain) activity predominantly lies on one of the two chains, the partner chain probably also plays an important role in determining the final outcome of the pairing. Correct conformation of the assembled molecule required for antigen binding may not be achieved in the presence of an incompatible partner chain. Kieber-Emmons et al. (45) demonstrated that multiple L and H chains contain residues that can facilitate DNA binding, reaffirming the notion that there are multiple ways that different amino acids combine to form an antigen-binding pocket with affinity for dsDNA and ssDNA or cardiolipin. It was later shown that the DNA binding affinity of the autoantibody was in part dependent on the particular H/L chain pairing (46).

Our study describes the use of newly constructed vectors and provides a technology allowing cloning in a PCR and/or non-PCR manner and overexpression and purification of virtually any human lambda antibody. The expression system described would, in particular, be useful for the production of human anti-DNA antibodies known to be difficult for their overexpression in a heterologous cell system, to a quantitative level required for their functional or structural studies. The novel comparative ELISA immunoassays described presently allow a reliable evaluation of relative anti-DNA or anti-cardiolipin activities of the autoantibodies in a controlled manner. The technology described encourages further studies to determine whether the observed light (anti-DNA/cardiolipin) or heavy (cardiolipin) chain involvement in dictating antigen specificity is a general phenomenon among human autoantibodies.

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