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Expression of Monovalent Fragments Derived from a Human IgM Autoantibody in *E. Coli*. The Input of the Somatically Mutated CDR1/CDR2 and of the CDR3 into Antigen Binding Specificity*

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Abstract

A hybridoma producing a polyspecific human monoclonal IgM antibody (named CB03) has been derived from a fusion of mouse myeloma cells with human spleen lymphocytes obtained from an autoimmune patient suffering from chronic idiopathic thrombocytopenia. The antibody was found to be encoded by somatically mutated V_HI and V_{lambda}III genes. To study the input of mutated complementarity regions (CDRs) into antibody specificity, the antigen binding features of the purified complete IgM antibody were compared with (i) a Fab fragment obtained by hot tryptic digestion and (ii) recombinant monovalent fragments expressed in *E. coli*. In detail, vectors were constructed encoding for (i) rFab03 and single chain Fv03 fragments containing the V_H and V_L genes connected by a linker sequence, (ii) scFc1.1. fragments containing the V_H germline equivalent and the CB03 wild-type CDR3 region, and (iii) scFv fragments containing the CDR1 and CDR2 in germline configuration and the CDR3 expressed in the CB253 human fetal B cell hybridoma producing a polyspecific IgM antibody. The expression vectors contained at the 3′ end either a $(His)_6$ motif allowing purification on Ni^{2+}-agarose or a c-myc tag for specifically detecting the expression products by a murine monoclonal antibody. Western blotting and ELISA analyses of the expression

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Abbreviations: CDR = complementarity determining region; *ELISA* = enzyme-linked immunosorbent assay; *Fab* = antibody fragment; *Fd* = V_HC_H1 fragment; *IPTG* = isoprophylthiocyanat-galactose; *PAGE* = polyacrylamid gel electrophoresis; *rFab* = recombinant antibody fragment; *scFv* = single chain antibody fragment, containing the heavy and light chain variable domains connected by a linker peptid

products indicate: (i) recombinant Fab fragments were found in the bacterial periplasm in extremely low amounts (1–10 μg from 1 litre bacterial culture), (ii) scFv fragments were obtained in suitable amounts from bacterial periplasm (800–1000 μg/l), (iii) the monovalent recombinant fragments as well as the Fab obtained by tryptic digestion reflected the polyspecific antigen binding features of the complete IgM antibody, but did bind to the antigens with much lower affinity, and (iv) the CDR3 was found to be of critical importance for the antigen binding pattern of this particular IgM.

We discuss the expression of recombinant scFv fragments in *E. coli* as a suitable method in studying the role of somatic mutation in autoantibody generation.

Introduction

The B cell repertoire of autoimmune patients may be studied using the human hybridoma technique (1, 2). Although this method could be improved by the introduction of a suitable fusion cell line (3), it is still difficult to obtain large amounts of the respective monoclonal antibody due to hybridoma instability, particularly during large scale cultivation (1). The combination of hybridoma and genetic engineering technologies may allow these problems to be circumvented providing the possibility to study the immunochemical and functional properties of autoantibodies as well as the immunoglobulin genes encoding them in more detail.

After identifying hybridomas that produce antibodies of interest, the respective immunoglobulin genes may be obtained by PCR amplification and cloning (4). The nucleotide sequences of the heavy and light chain variable domain genes (V_H and V_L genes respectively) may be studied for V_H/V_L gene family usage and the presence of somatic mutations. To analyze the effects of somatic mutations that result in amino acid replacements, especially in the CDRs, the expression of the respective genes (either germ line genes or somatically mutated variants) in procaryotes should be a useful method due to potentially higher yields of material to be obtained much faster than by conventional hybridoma technique. In this respect, it has been shown that Fab and Fv fragments encoded by murine immunoglobulin genes (5, 6) were able to bind antigen with affinities comparable to Fab fragments produced by proteolytical digestion of the original antibodies. Thus, antibody variable domains which are expressed in *E. coli* and secreted into the periplasmic space appear to be folded into functionally active proteins (7).

By fusing human lymphocytes derived from the spleen of an autoimmune patient with mouse myeloma cells we obtained a human hybridoma cell line producing a polyspecific IgM/lambda antibody (8). Surprisingly, this antibody was found to bind both ssDNA and dsDNA, a feature of pathogenic IgG autoantibodies rather than of polyspecific IgM (9). The V domains were found to be encoded by a V_{lambda}III gene and a V_HI gene which, in comparison with its germline equivalent, the 1.1. germline gene (10), was somatically mutated in CR1 and CDR2 (11). In particular, the Trp50 → Arg50 mutation in the CDR2 could be of interest regarding the binding of the antibody to dsDNA. Ob-

viously, the combination of 2 D gene elements resulted in an unusually long CDR3 found to be expressed in this antibody. The CDR3 was found to contain 2 Arg and 1 Asn residues due to the reading frame, resulting from the VDJ recombination. This and the fact that another Arg had been introduced, most likely by N sequence addition, suggested a role of the CDR3 in binding to dsDNA (9). To study the role of the somatically mutated CDR1/CDR2 as well as the CDR3 for antigen binding specificity, the following experimental approaches were made: (i) vectors have been constructed allowing expression in *E. coli,* (ii) the expression products were analyzed for antigen binding specificity in comparison to the original antibody, (iii) the mutated V_H gene was replaced by the germline equivalent (1.1.), cloned from the autoimmune patient's germline DNA, and (iv) the CDR3 was replaced by another CDR3 found to be expressed in the human hybridoma line CB253 (12) together with the 1.1. germline V_H gene. The vectors constructed for expression purposes contained a pelB leader sequence and at the 3′ end a sequence encoding a peptide (c-myc tag) allowing a detection of the expression products by a murine monoclonal antibody (13), or a $(His)_6$ motif suitable for purifying the expressed material by metal complex chromatography (14).

Materials and Methods

The human monoclonal antibody CB03

Mouse myeloma cells P3X63Ag8.653 were fused with human lymphocytes obtained from the spleen of an autoimmune patient who had undergone a partial splenectomy because of chronic idiopathic thrombocytopenia (8). The hybridoma CB03 produces a human IgM/lambda antibody with polyreactive binding pattern to both endogenous and foreign antigens (Fig. 1). The antibody was purified from an up-scaled culture and Fab fragments were prepared by hot tryptic digestion. The binding properties of the Fab were tested using a solid phase ELISA technique (15).

For sequencing, the variable domain encoding genes of the heavy and the light chain were amplified from cDNA using primers that bind to leader and constant region C_H1 sequences (16) and cloned into pUC18 following routine methods (17).

Bacterial strains and plasmids

E. coli TG1 was used as the expression host. The expression plasmids VαpelB$(His)_6$ and V_HNcoI-poly-tag have been described previously (18, 19). The pSNPT vector (unpublished) was a generous gift from Dr. G. WINTER (MRC, Cambridge, UK). Furthermore, the pSPL vector (B. NIEMANN, Gene, 1994, accepted for publication), the pUC18-V_H1.1. vector, containing a human V_H1 germline sequence (11) identical to the 1.1. gene (10) as well as the pUC18V_H253 plasmid containing an identical germline V_H1 gene expressed in a human fetal B cell hybridoma, gently provided by Drs. HANSEN and SETTMACHER (Charité Berlin, Germany, [ref. 12]) were used for cloning.

Amplification of the $C_H1\mu$ and $C_{lambda}2$ genes

The $C_H1\mu$ and $C_{lambda}2$ gene segments were amplified from hybridoma cDNA (cDNA kit from GIBCO, Germany) using standardized protocols (17). The PCR was carried out using the primer pairs $huC_H1\mu_{back}$/$huC_H1\mu_{for}$ and $huC_{lambda}2_{back}$/$huC_{lambda}2_{for}$ (see

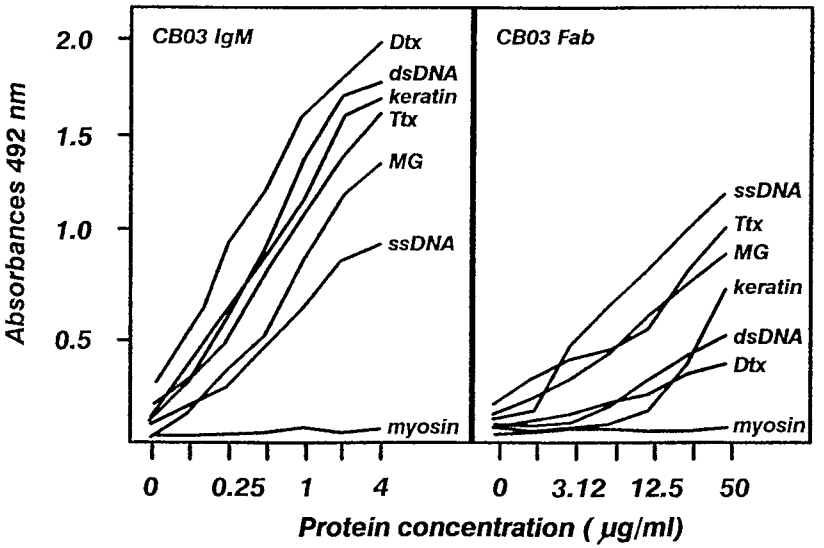

Figure 1. **Polyspecificity of antigen binding by a purified human monoclonal IgM and the related Fab fragment obtained by hot tryptic digestion.** Detection of the antibody or the fragment, bound to the respective antigen by an anti-human-lambda light chain antibody conjugated to HRP.
Dtx = diphtheria toxoid, *Ttx* = tetanus toxin, *MG* = myoglobin. Protein concentrations were measured by UV absorbance determination at 278 nm.

Table 1 for all primer sequences, all primers from TIB MolBiol, Berlin, Germany). For further cloning the respective restriction sites were introduced into primer sequences (see below). PCR conditions were as follows: 3 U Taq polymerase (Promega, Heidelberg, Germany), 5 µl 10× reaction buffer (Promega), 25 pmol of each oligonucleotide primer, 0.2 mM dNTPs (Boehringer, Mannheim, Germany), 2 µl cDNA and water to 50 µl. Cycling conditions were 94 °C (30 sec), 55 °C (30 sec) and 72 °C (1 min). Thirty cycles were performed in a Techne PHC heating block.

Construction of rFab expression plasmids

The V_H03 gene, amplified by the PCR using the primer pair V_H03_{back}/huJ_H6_{for} and the plasmid pUC18V_H03 as the template, was digested with *Nco*I and *Mro*I (all enzymes and reaction buffers from New England Biolabs or Boehringer Mannheim respectively). This fragment was ligated together with a *Mro*I/*Bst*EII digested $C_H1\mu$ PCR fragment into V_HNcoI-poly-tag and VαpelB$_{(His)6}$ to generate pelBFd03$_{c\text{-}myc}$ and pelBFd03$_{(His)6}$. The $C_{lambda}2$ gene was cloned as an *Avr*II/*Hin*dIII-restricted PCR fragment into pUC18V_L03. This ligation mix was used as a template for a further PCR with the primer pair V_L03N_{back}/$huJ_{lambda}2_{for}$. The resulting lambda light chain gene was cut with *Nco*I and *Sph*I and alternatively cloned with *Sph*I/*Eco*RI cut $Fd_{c\text{-}myc(His)6}$ gene fragments into a *Nco*I/*Eco*RI cut VαpelB(His)$_6$ vector (see Fig. 2A).

Construction of single chain Fv expression plasmids

To combine the V_H03 gene with the sequence encoding the $(Gly_4Ser)_3$ linker sequence (20), the *Nco*I/*Mro*I cut V_H03 PCR fragment was inserted into the pSPL vector. Secondly, the V_H03/linker gene was amplified with the primer pair V_H03_{back}/$link_{for}$ and

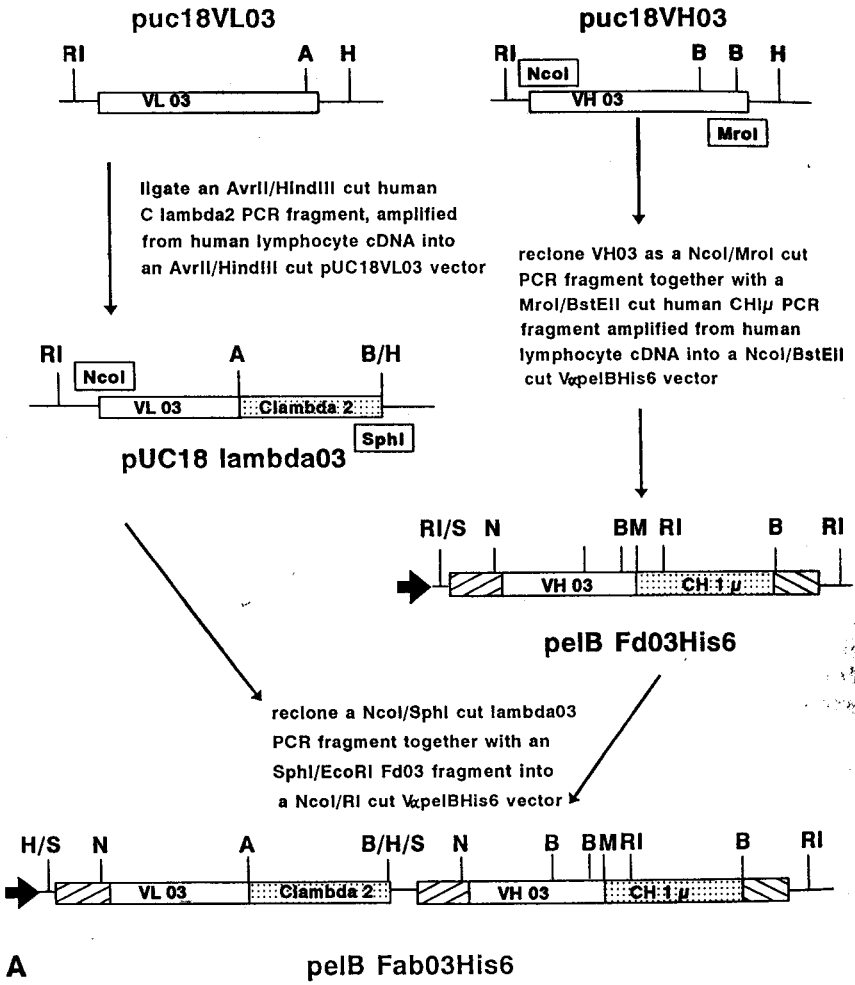

Figure 2 (legend see right page).

the V_H03 gene with the primer pair V_L03X_{back}/CBHis6. After cutting the gene fragments with the enzymes *NcoI*/*XhoI* and *XhoI*/*SalI*, respectively, a three-fragment-ligation with the *NcoI*/*SalI* cut pSNPT vector was carried out to create the plasmid $pscFv_{(His)6}$ (Fig. 2B). To construct the $pscFv03_{c\text{-}myc}$ plasmid the $V_L03_{(His)6}$ gene fragment was removed by cleavage with *XhoI*/*NotI* and a new $V_L03_{c\text{-}myc}$ PCR fragment amplified with the primer pair V_L03X_{back}/$huJ_{lambda}2_{for}$ was inserted (see also Fig. 2B).

To create the plasmids $pscFv1.1._{c\text{-}myc/(His)6}$ the $V_H1.1.$ germline gene was amplified with the primer V_H03_{back}/$pUC_{universal}$ from a pUC18-$V_H1.1.$ plasmid. The V_H03 gene in the $pscFv_{c\text{-}myc/(His)6}$ vectors was replaced by the $V_H1.1.$ gene using the restriction sites *NcoI* (5′) and *BglII*, localized at the 3′ end of the V_H03 gene upstream the CDR3. In a similar way the V_HDJ_H253 genes, amplified with $V_H03$back/$J_H2$for from pUC18-V_H253 and cut with *NcoI*/*MroI*, were exchanged. All expression constructs were sequenced (21) prior to expression analysis using standard sequencing protocols and Sequenase® (USB Corp., Braunschweig, Germany). All cloning procedures were carried out as described (17).

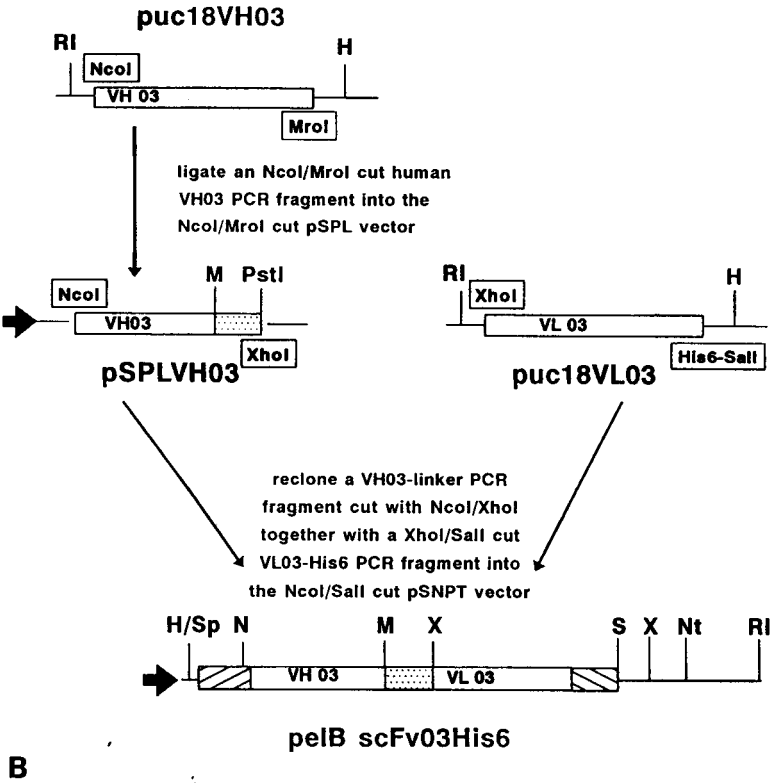

Figure 2. **Vector construction strategies for the expression of recombinant Fab (A) and single chain Fv (B) fragments derived from the human antibody CB03 in *E. coli*.** The black arrows indicate the promotor region. Boxes indicate coding regions: ▨ pelB leader, ▧ c-myc or $(His)_6$ tags and ▭ PCR primers. Restriction sites important for the construction procedure were marked as follows: A: *Avr*II, B: *Bst*EII, H: *Hin*dIII, M: *Mro*I, N: *Nco*I, Nt: *Not*I, P: *Pst*I, RI: *Eco*RI, S: *Sph*I, X: *Xho*I.

Expression of c-myc fusion fragments

E. coli recombinants harbouring $CB03_{c\text{-}myc}$ expression plasmids were grown up at 37 °C to the late logarithmic phase in 50 ml 2×TY supplemented with 100 µg/ml ampicillin and 1 % (wt/vol) glucose. Cells were pelleted by centrifugation, washed once in 2×TY and induced by incubating in 50 ml 2×TY supplemented with 100 µg/ml ampicillin and 0.4 mM IPTG (Roth, Karlsruhe, Germany) for 5 h at room temperature with shaking at 220 rpm. Periplasmic fractions were obtained by osmotically shocking the cell pellet with 2 ml (1/25 volume) ice cold BBS buffer (200 mM Na_2BO_3, 160 mM NaCl, 1 mM EDTA pH 8.0) for 10 min on ice and by centrifugation at 4 °C at 15,000 rpm for 15 min. Pellets were then resuspended in 2 ml TEN buffer (20 mM Tris pH 8.0, 50 mM NaCl and 10 mM EDTA). The TEN and the BBS fractions were analyzed by Western blotting (22) using the murine monoclonal antibody 9E10 directed to the c-myc tag (6, 13) and an anti-mouse-IgG antibody conjugated with horseradish peroxidase (Medac, Hamburg, Ger-

Table 1. Oligonucleotide primers for the construction of plasmids encoding Fab and scFv fragments derived from a human monoclonal IgM/lambda antibody.

Primer	Nucleotide sequence 5′	Restriction site 3′
$huC_H1\mu_{back}$	CTCCTCCGGAAGTGCATCCGCCCC	*Mro*I
$huC_H1\mu_{for}$	CCCGGTGACCCGGGGTGGGACGAAGACGCTCAC	*Bst*EII
$huC_{lambda}2_{back}$	AAGCTGACCGTCCTAGGTCAGCCCAA	*Avr*II
$huC_{lambda}2_{for}$	AAGCATGCAAGCTTAGGTGACCGAACATTCC(T)GTAGGGC	*Sph*I, *Hin*dIII, *Bst*EII
V_H03_{back}	CCCCATGGCCCAGGTGCAGCTGGTGCAGTCT	*Nco*I
huJ_H6_{for}	CCCTCCGGAGGAGACGGTGACCGTGGTCCCTTGGCCCAG	*Mro*II, *Bst*EII
huJ_H2_{for}	CCCTCCGGACGAGACCGTGACCGTGGTCGCTTGGCCCGAG	*Mro*I
V_L03N_{back}	CCCCATGGCCTCTGAGCTGACTCAGGACCC	*Nco*I
V_L03X_{back}	CCCTCGAGTTCTGAGCTGACTCAGGAC	*Xho*I
$link_{for}$	AACTCGAGCCGCCGCCACCCGAGCCGCC	*Xho*I
$huJ_{lambda}2_{for}$	GAGTCATTCTCGACTTGCGGCCGCACCTAGGACGGTCAGCTTGGTCCC	*Not*I
CBHis6	ATCAGTCGACTTAATGGTGATGGTGATGGTGGCCGAGGACGGTCAGCTTGGT	*Sal*I
$pUC_{universal}$	GTAAAACGACGGCCAG	

Restriction sites were underlined. The «for» primers are shown as reverse sequences in 5′–3′ orientation. Parentheses indicate wobble bases.

many) for detection. As a substrate for HRP, diamino-benzidin (Sigma, Deisenhofen, Germany) was used. Lambda light chains in the Fab constructs were detected with an anti-human-lambda antibody (Medac) conjugated to alkaline phosphatase. The use of two different conjugates and substrates enabled the heavy and the light chain polypeptides of the Fab fragment to be detected in the same PAGE gel after transfer to nitrocellulose (23).

Purification of $(His)_6$ fusion fragments

40 ml periplasmic fractions from 1 litre *E. coli* TG1 cultures harbouring $CB03_{(His)6}$ expression plasmids were prepared as described above. The periplasmic fractions were mixed with 1/10 volume 10 × PBS and passed over a 1 ml Ni^{2+}-NTA-agarose column (Diagen, Hilden, Germany). The column was washed with 5 ml washing buffer 1 (100 mM Tris/HCl, 500 mM NaCl pH 8.0) and 5 ml washing buffer 2 (100 mM Tris, 500 mM NaCl pH 8.3) and the fragments were eluted by 5 times adding 1 ml freshly prepared 0.25 M imidazole. The eluted fractions were pooled and dialyzed against a 50 mM Tris/HCl (pH 6.8) buffer. The proteins were analyzed by UV and fluorescence spectroscopy as well as by PAGE, using 15 % gels and a Midget electrophoretic chamber followed by Coomassie blue staining (24).

ELISA

The binding of both the complete CB03 and the expressed antibody fragments to ssDNA (phage DNA) and dsDNA (pUC 18 plasmid DNA) as well as to a panel of other antigens was tested using established ELISA methods (2). The following experiments were carried out: (i) the BBS fractions of the $CB03_{c\text{-}myc}$ fragments were incubated on antigen-coated plates for 4 h at room temperature. After washing, the 9E10 antibody recognizing the c-myc tag was added and incubated at 4 °C overnight. The reaction was developed by incubation with an anti-mouse IgG antibody labeled with peroxidase (Medac) for 2 h. As a negative control, the BBS fraction of a murine $scFv_{c\text{-}myc}$ fragment (unpublished) was used. (ii) For ELISA experiments with purified fragments, protein concentrations were adjusted to 100 µg/ml. The antigen plates were incubated for 2 h with an $E_{max}/2$ concentration of a CB03-HRP conjugate. Then, different dilutions of the purified fragments were incubated for 2 h at room temperature. The competitive immune reaction was developed using orthophenylene-diamine as a substrate (25).

Results

Comparable antigen binding patterns registered for the Fab fragment and the original CB03 IgM antibody

The hybridoma CB03 was established from the fusion of human splenic B lymphocytes obtained from a patient with chronic idiopathic thrombocytopenia with mouse myeloma cells. The monoclonal IgM/lambda antibody was purified from the supernatant of a hollow fiber fermentation culture (15). This antibody reacts with multiple exogenous and endogenous antigens. A purified Fab fragment was obtained by hot tryptic digestion of this IgM antibody. The antigen binding specificity of the Fab fragments was studied in solid phase ELISAs and the binding pattern to various antigens was detected to be polyspecific, comparable with the original IgM antibody

Table 2. Binding constants of the CB03 antibody to different antigens.

Antigen	K_D (mol/l)
ssDNA (oligo dT_{14})	$1{,}4 \times 10^{-7}$
dsDNA (pUC18 DNA)	$3{,}32 \times 10^{-8}$
Keratin	$5{,}1 \times 10^{-10}$
Myoglobin	$1{,}26 \times 10^{-6}$

Determination of K_D was carried out using a competition ELISA method (26).

(Fig. 1). However, the binding strength of the IgM to at least some of the antigens must be different from what was registered for the Fab fragment. Whereas the complete antibody bound most strongly to diphtheria toxoid and half as much to ssDNA, the opposite was found for the Fab. Furthermore, even 10- to 100-fold concentrations of the Fab did not achieve extinctions comparable to what had been detected for the original IgM antibody. Whereas the affinity of binding the antigens by the complete IgM could be determined to be in the range from 5×10^{-10} to 1.3×10^{-6} mol/l (see Table 2 for dissociation constants), the affinity of binding the respective antigens by the Fab fragments must be much lower since it could not be determined by FRIGUET's technique (26).

Comparing the data obtained from the two different tests we registered one remarkable result: although the binding strength of the complete antibody to the solid phase immobilized dsDNA seems to be higher than to keratin, the dissociation constants for antibody/keratin complexes were lower (Table 2), determined by the method following FRIGUET et al. (26), which is based on an antibody-antigen interaction in the fluid phase. This may be explained by the conformational changes of antigens which can appear by solid phase coating for ELISA experiments (see ref. 15 and 34 respectively) which may yield different consequences for a pentavalent complete IgM antibody (multiple epitope binding), in comparison to a monovalent Fab fragment.

Expression, purification and characterization of the recombinant monovalent fragments

The recombinant $Fab03_{c\text{-}myc}$ and $scFv03_{c\text{-}myc}$ fragments were expressed in *E. coli* TG1 and detected using PAGE and Western blotting technique. For distinction of the two Fab forming protein chains the 9E10 antibody/anti mouse IgG-HRP conjugate (heavy chain) and an anti-human-lambda-AP conjugate (light chain) were used, respectively (Fig. 3A). Both chains were expressed in comparable amounts. However, the distribution of the different chains within the culture medium, the periplasmic fraction and the soluble cell pellet were found to be very distinct. The Western blot photography in Figure 3A shows the relative concentration of the Fd fragment and the lambda chain in the culture medium (lane 1 and 4), in the peri-

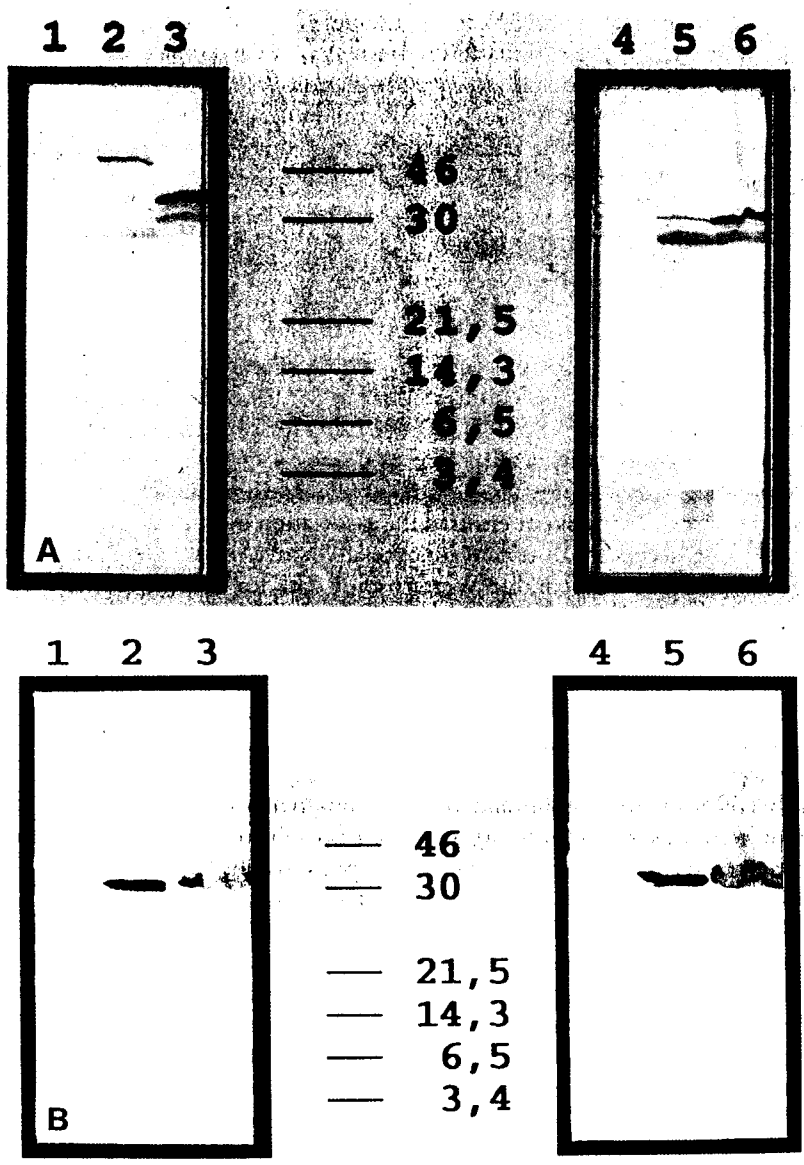

Figure 3. **Immunoblot analysis of the expressed rFab03$_{c\text{-}myc}$ (A) and scFv03$_{c\text{-}myc}$ (B).** Proteins were separated by SDS-PAGE and the fragments were revealed by double staining technique. The Fd$_{c\text{-}myc}$ and the scFv$_{c\text{-}myc}$ fragments were stained using the 9E10 antibody/anti-mouse-IgG-HRP conjugate (black band), whereas the light chain protein was stained using an anti-human lambda antibody conjugated with AP (red band).
A: Lanes 1-3: non-reducing, lane 4-6 reducing SDS-PAGE indicating the distribution of the expressed rFab03$_{c\text{-}myc}$ and lambda dimers between culture medium (lanes 1 and 4), periplasm (lanes 2 and 5) and cell pellet (lanes 3 and 6). – **B:** Lanes 1–3: non-reducing, 4–6: reducing SDS-PAGE indicating the distribution of scFv03$_{c\text{-}myc}$ between the different fractions.

plasmic fraction (lane 2 and 5) and in the insoluble cell pellet (lane 3 and 6) under non-reducing (left part) and reducing (right part) conditions. Most of the light chain was secreted into the periplasmic space and into the culture medium, where the molecules formed lambda dimers (lane 1 and 2). Less than 50 % remained in the bacterial pellet after shocking the bacteria with a hypotonic buffer followed by centrifugation. We could not detect any Fd in the culture supernatant.

Furthermore, the concentration of Fd in the periplasmic fraction was extremely low in comparison with the lambda light chains (lane 5). The main part of the expressed Fd chain remained in the cell pellet without association with a light chain (lane 3). Moreover, we did not find any differences regarding the amounts of soluble rFab03 in the periplasm of *E. coli* when inducing the cultures either at 25 °C or at 37 °C, or by using different concentrations of IPTG (0.1–1 mM) for protein synthesis induction (data not shown in detail).

The covalent linkage of the variable domains by the linker peptid in the case of the scFv03 fragment ensured the association of both domains, which seems to be necessary to obtain functionally active monovalent fragments. Figure 3B shows comparable Western blot data for the $scFv03_{c\text{-}myc}$ fragment, Figure 3A for the $rFab03_{c\text{-}myc}$. Approximately the third part of the expressed scFv03c-myc material was secreted into the bacterial periplasm (Fig. 3B, lane 2 and 5). The soluble scFv03 fragment is monomeric and only a marginal amount of the expressed fragment formed higher-molecular aggregates, which are unsoluble and did not immigrate into the 15 % acrylamide gel.

The recombinant Fab03 and the scFv fragments containing at the carboxyl terminus the $(His)_6$ tag could be purified on Ni^{2+}-agarose from periplasm of *E. coli* achieving a yield of approximately 10 µg/litre of culture and 800 to 1000 µg/litre of culture, respectively. The extremely low yield of purified recombinant $Fab03_{(His)6}$ confirmed the results shown in Figure 3A. The insoluble Fd chain may be purified by solving the harvested cell pellet from a Fab-producing bacterial culture in 6 M GuHCl/8M Urea. In this way a yield of 600–800 µg/litre was obtained. However, this denaturated Fd fragment did not associate with light chains and was, therefore, not suitable for immunochemical experiments (not shown).

The purification procedure of the $scFv_{(His)6}$ fragment becomes evident in Figure 4. The soluble part of the scFv fragment could be received from the periplasmic fraction in a one step procedure using metal complex chromatography. The protein was eluted with 5 column volumes of an imidazol buffer and was analyzed by PAGE (lane 4–8) in parallel with the periplasmic fraction (lane 1) and the fraction containing the proteins not bound by the Ni^{2+}-agarose (lane 2). The pooled fractions were dialyzed overnight against a Tris/HCl buffer. The resulting fragments were more than 98 % pure and the UV absorbance measurements showed a maximum at 277.4 to 278.2 nm, whereas the fluorescence spectral analysis showed an emission maximum at 335 nm indicating a folded protein structure.

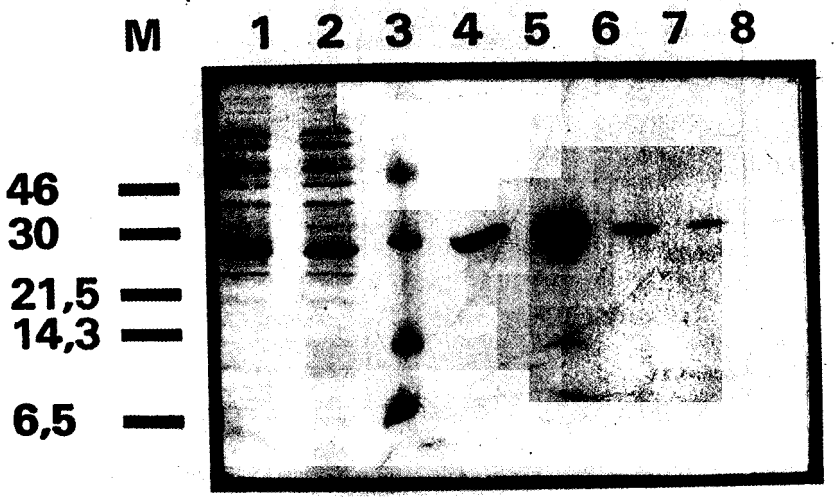

Figure 4. **The purification of the scFv03$_{(His)6}$ fragment from the bacterial periplasm.** The periplasmic fraction obtained from 1 liter bacterial culture has been passed over a 1 ml Ni^{2+} agarose column (see «Materials and Methods» for detail). 1 µl of each fraction was run through a SDS-PAGE (15 %). Lane 1: periplasmic fraction, lane 2: bacterial proteins, after passage through the Ni^{2+} agarose column, lane 3: molecular weight marker (46, 30, 21.5, 14.3 kDa), lanes 4–8: eluted fractions containing the scFv03$_{(His)6}$ fragments.

In a first approach, the immunochemical features of the rFab03 and the scFv03 fragments were determined using the c-myc fusion proteins. For this purpose, different dilutions of crude periplasmic preparations were incubated on ssDNA and dsDNA coated plates and the fragments which bound to the antigens were detected with the 9E10/anti-mouse-HRP conjugate (Fig. 5). Both of the recombinant c-myc fusion proteins reacted with ssDNA and dsDNA comparable to what has been shown for the CB03 antibody and the Fab produced by proteolytical digestion. As expected, the reactivity of the rFab03 containing periplasm was much weaker than in the case of the scFv03 due to the lower concentration of completely associated rFab03 in the bacterial periplasm (see also Fig. 3A).

Reactivity of single chain Fv fragments containing mutated CDRs

The advantage in purifying functionally active scFv03 fragments from *E. coli* in comparison with the rFab03 fragment, prompted the construction of the plasmids pscFv1.1$_{c\text{-}myc/(His)6}$ and pscFv253$_{c\text{-}myc/(His)6}$ in which the V_H03 gene is replaced by the respective germline gene (scFv1.1.) or by V_HDJ_H genes expressed by a human hybridoma producing the polyspecific IgM antibody CB253 (scFv253). Figure 6 shows the amino acid sequences deduced for the three scFv fragments without bacterial signal peptides and

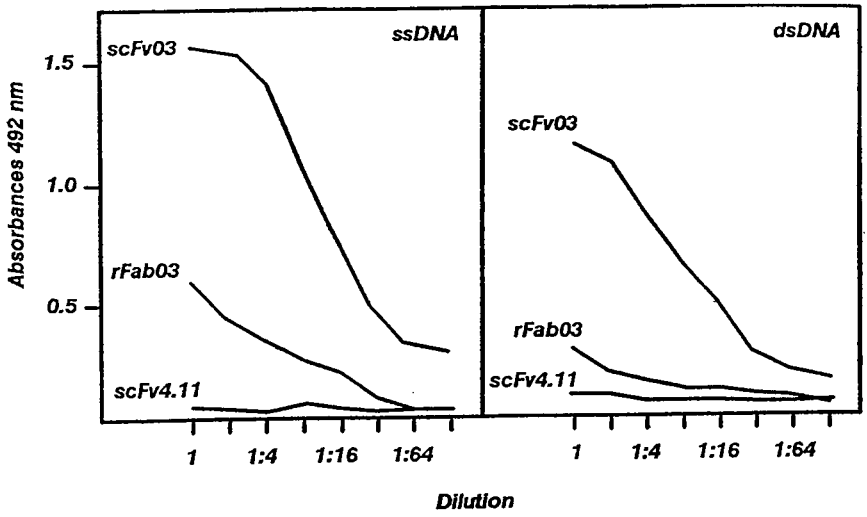

Figure 5. **Binding of recombinant Fab03$_{c\text{-}myc}$ and scFv03$_{c\text{-}myc}$ fragments to ssDNA and dsDNA.** Binding of the recombinant fragments was detected using the 9E10 antibody and an anti-mouse-IgG-HRP conjugate.

fused tags. The fragments scFv03 and scFv1.1. use identical frame work and CDR3 amino acid sequences. The scFv1.1. fragments carry the germline-related CDR1 and CDR2. The scFv03 fragment contains the V_H domain modified by somatic mutations (Tyr→Ser in CDR1 and Trp→Arg in CDR2) expressed in the CB03 hybridoma. The V_H domain of scFv253 fragments consists the same frame work sequences like the other scFv fragments, and furthermore contains the CDR1 and CDR2 in 1.1.-like

Table 3. Antigen binding by scFv fragments in comparison to complete IgM antibodies.

Conjugate antigen/antibody	Anti-huμ-HP			9E10/anti-mouse-HRP			
	CB60	CB253	CB03	scFv03	scFv1.1	scFv253	scFv4–1
ssDNA	–	++	+++	+++	+	+	–
dsDNA	–	–	+++	+++	++	–	–
tetanus toxin	–	+++	+++	+++	+++	+++	–
diph. toxin	–	+	++	++	++	–	–
keratin	–	(+)	+++	++	+	–	–
myoglobin	–	(+)	++	+	–	–	–
myosin	–	–	–	–	–	–	–

The antibody CB60 (8) as well as the murine scFv4.1.1 (unpublished) were used as negative controls. Complete antibodies were adjustet to 1 μg/ml. The expressed scFv$_{c\text{-}myc}$ fragments were adjusted to comparable protein concentrations considering Western blotting data. Symbols indicate: +++ $E_{492} > 1.5$; ++ $E_{492} > 0.8$; + $E_{492} > 0.3$; (+) E_{492} between 0.1 and 0.3; – $E_{492} < 0.1$.

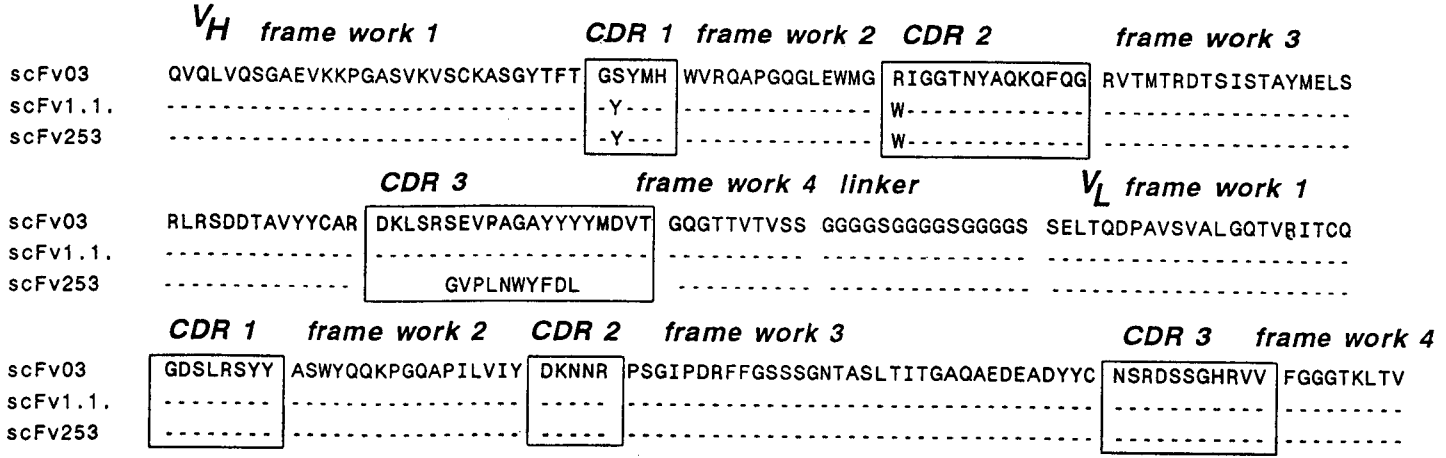

Figure 6. **Amino acid sequences of the expressed single chain Fv fragments.** The sequences are shown using the one-letter-code. Hyphenates indicate identical amino acids. The CDRs were marked by boxes, with regard to KABAT et al. (35).

germline configuration, but another CDR3 (originally found to be expressed in the CB253 hybridoma). The three different V_H domains were combined with the wild type light chain V_L domain. These scFv gene constructs were developed to study the influence on the CB03 specificity of either the somatic mutations found in the V_H03 gene or in the input of the CDR3. In a first approach, all scFv fragments were expressed as c-myc fusion proteins, which had been analyzed for antigen binding by ELISA immediately after preparation. Table 3 shows the results obtained from these experiments in comparison with what had been registered for the complete CB03 and CB253 IgM antibodies. Both CB03 and CB253 antibodies bind to ssDNA as well as to various other antigens. However, only the CB03, but not the CB253 recognize dsDNA. Concluding from ELISA data, the scFv1.1. containing the V_H germline gene without the Arg50 exchange in the CDR2 showed a binding pattern comparable to the scFv03 and to the complete CB03 antibody including the reactivity with dsDNA. The scFv253 (1.1. germline gene plus a shorter CDR3 without Arg residues) fragment, however, reacted only with a limited number of the antigens tested. Only ssDNA and tetanus toxin were bound to proposing an important input of the CDR3 into the polyspecificity of the CB03 IgM antibody.

To quantify their binding behaviour we purified the $scFv1.1._{(His)6}$ and the $scFv253_{(His)6}$ fragments following the procedure, described in detail for $scFv03_{(His)6}$. The following approach was used to analyze their immunochemical features: The antibody CB03 was coupled to HRP and incubated on microtiter plates coated with ssDNA and dsDNA for 2 h at room temperature. Then, different dilutions of purified fragments (100 µg/ml to 1.5 µg/ml) were incubated at 4 °C overnight and the substrate reaction was carried out. We found that only high concentrations (100 and 50 µg/ml) of the scFv fragments as well as of the Fab fragment produced by proteolytical digestion could compete with the binding of the CB03-HRP conjugate to both antigens, suggesting a rather unspecific effect (data not shown in detail). We did not find any measurable differences between the tested fragments. Most likely, the binding constants of monovalent fragments obtained from these particular IgM antibodies seem to be too low to be registered by the competition ELISA technique.

Discussion

Pathogenic autoantibodies were shown to be encoded by V_H/V_L genes carrying somatic mutations in the complementarity determining regions, suggesting a role for autoantigens in selecting B cell clones for the production of high-affinity autoantibodies (9), causing cell and tissue damage in autoimmune patients. Such autoreactive B cells could be immortalized (2) to study the immunochemical features of the autoantibodies, as well as the related immunoglobulin genes. In previous experiments, using the human

hybridoma technology we obtained monoclonal IgM antibodies from a fusion of spleen lymphocytes from a patient with chronic idiopathic thrombocytopenia with the myeloma cell line P3X63Ag8.653 (8). From the same spleen we established two independent hybridomas (CB03 and CB15) producing an IgM antibody binding to multiple auto- and foreign antigens as well. Analyzing the fine specificity of these autoantibodies we determined a binding to both ssDNA and dsDNA, a feature of pathogenic rather than of naturally occurring autoantibodies (27). Sequence analysis of V_H/V_L genes and comparison with the respective germline genes cloned from the patient's genomic DNA (11) showed: (i) the two independently obtained hybridomas express identical V_HDJ_H and V_LJ_L genes suggesting a clonal expansion of the respective clone in that particular immune compartment, (ii) the CDR1 and CDR2 were modified by replacement mutations, one of which, most likely, resulting from autoantigen-driven B cell selection; we hypothesized that the Trp50→Arg replacement plays a role in affinity maturation of the autoantibody, since Arg has been shown to be of importance for the interaction of murine monoclonal autoantibodies with dsDNA (28). Furthermore, a mouse V_H gene belonging to the J558 family (the murine equivalent for human V_HI genes), expressed in a murine hybridoma producing a high-affinity autoantibody against DNA, does carry comparable replacement mutations (9). (iii) The unusually long CDR3 contains two additional Arg residues, one of which had been introduced, most likely, by N-nucleotide insertion.

The question has been raised about the input of either the somatic mutations in CDR1/2 or of the CDR3 into the autoantibody specificity. This may be of interest since somatic mutations that result in amino acid replacement in the antigen-binding regions of the antibody molecule have been shown to play a crucial role in affinity maturation during an immune response (29). To prove such suggestions regarding a human monoclonal autoantibody, the expression of antibody fragments in procaryotes might be a useful method. The advantages of this technique are: (i) based on the high growth rate of *E. coli*, a large quantity of the expressed material may be obtained in a much shorter time period, at least in comparison with the eukaryotic expression system, and (ii) the method allows an easy handling of site-directed-mutagenesis techniques (7). Two different approaches were used for the expression of antibody fragments: (i) cloning into a vector containing the c-myc tag allowing the detection of the expression products in the different compartments of *E. coli* using a murine monoclonal antibody (13), and (ii) cloning into a vector containing a 3′ sequence encoding a $(His)_6$ peptide which may be used for purification of the product on Ni^{2+}-NTA-agarose. These expression strategies have been used for murine antibodies (5) as well as for other proteins of immunological interest like TCRs (18, 19). For the construction of the Fab it was necessary to isolate the C_H1 domain and the $C_{lambda2}$ gene (30) from the hybridoma DNA. The difficulties encountered in expression and purification of the rFab fragment probably resulted from the poor solubility of the Fd

fragment in the periplasmic space of *E. coli*. This fact may be a feature of this particular V_HC_H1 fragment obtained from the CB03 antibody, since the successful production of other antibody Fab fragments in *E. coli* had been described (31). In contrast, it has been also shown (32) that the C_H1 domain in the recombinant Fab fragments was responsible for much lower yields of functionally active rFab fragments in comparison with Fv fragments expressed in the same vector system.

The ELISA analysis of scFv fragments contained in crude lysates showed a polyspecific binding to the antigens tested. Therefore, the ability to bind to multiple antigens by polyspecific antibodies may not only be the result of the pentavalency of the IgM since the monovalent fragments showed a polyspecific antigen binding pattern as well. The results obtained from these experiments prompted us to study the immunochemical features of purified scFv products containing the $(His)_6$ motif (33). To test the antigen binding we tried to use a competition assay in which the scFv fragments compete with the purified Fab (proteolytically obtained) and the complete IgM antibody for binding to the solid phase-coupled antigen, following the method described by FRIGUET et al. (26). However, the scFv fragment failed to inhibit the binding of both Fab or IgM, even when added in a much higher concentration. Nevertheless, we suggest the expressed scFv fragments to be immunochemically intact for the following reasons: (i) an anti-idiotypic antibody recognizing a non-conformational epitope on the light chain of the antibody had been raised, showing binding to CB03 IgM, to Fab and to scFv fragments as well (M. SEIFERT and ROGGENBUCK, unpublished), (ii) the fluorescence spectral analysis of the purified fragments indicated folded proteins, and (iii) kinetic measurements on a Biocore™ equipment (Pharmacia) basing on plasmon surface resonance phenomenon implicated a significant, but 10-fold lower binding strength of the scFv in comparison to Fab fragments (34). Taking these data into account, the competition ELISA technique may not yield measurable results. Therefore, we used the approach to express c-myc tag fusion proteins to analyze, at least qualitatively, the antigen binding by the different scFv fragments. From these data we conclude: (i) The ability of the CB03 antibody to bind both ssDNA and dsDNA may not be due to the somatic mutations detected in the CDR1 and CDR2 since the scFv fragments containing the germline V_H equivalent did bind to dsDNA as well, and (ii) more responsible for this binding may be the CDR3 region, containing in the case of the scFv03 and the scFv1.1. two Arg residues, one introduced by N-sequence addition, which are known to play a role in protein-DNA interaction (28). The replacement of the CDR3 encoding region by a D gene, found to be expressed in a fetal B cell hybridoma CB253, recombined with the germline-identical 1.1. V_H gene, resulted in a failure to recognize dsDNA. Nevertheless, other antigens, bound by the complete maternal antibody (ssDNA, tetanus toxin) were recognized as expected. We suggest, based on these results, that CDR3 plays an important role in generating reactivity of this antibody in recognizing both ssDNA and dsDNA. We cannot con-

clude at this point the influence of mutations in CDR1 and CDR2 on the affinity of binding. However, the selection of this B cell clone may be the result of the antigen binding specificity created by V_HDJ_H recombination during B cell ontogeny, before any somatic mutations took place (28). Experiments using the plasmon surface resonance phenomenon (34) are in progress to verify whether the mutations in CDR1/2 could play a role in affinity maturation of dsDNA binding by the CB03 antibody.

Methodologically it may be a useful approach to express human antibody fragments in *E. coli* to analyze the significance of somatic mutations for binding specificity. In the future, we will use this technique to screen at least qualitatively somatic mutations introduced into the CDRs by site-directed mutagenesis for their influence on antigen-binding specificity. Quantitative data, furthermore, may be obtained by cloning the respective V_H/V_L genes into eukaryotic expression vectors to use complete IgG antibody molecules for more detailed immunochemical analysis.

References

1. Jahn, S., R. Grunow, S. T. Kiessig, U. Settmacher, and R. von Baehr. 1990. Strategies in the development of human monoclonal antibodies. Developm. Biol. Standard **71**: 14.
2. Winkler, T., S. Jahn, and J. R. Kalden. 1991. IgG human monoclonal anti-DNA autoantibodies from patients with systemic lupus erythematosus. Clin. exp. Immunol. **85**: 379.
3. Grunow, R., S. Jahn, T. Porstmann, S. T. Kiessig, H. Steinkellner, F. Steindl, D. Mattanovich, L. Gürtler, F. Deinhardt, H. Katinger, and R. von Baehr. 1988. The highly efficient human B-cell immortalizing heteromyeloma CB-F7. J. Immunol. Meth. **106**: 257.
4. Marks, J. D., M. Tristem, A. Karpas, and G. Winter. 1991. Oligonucleotide primers for polymerase chain reaction amplification of human Ig variable genes and design of family-specific oligonucleotide probes. Eur. J. Immunol. **21**: 985.
5. Skerra, A., and A. Plückthun. 1988. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. Science **240**: 1038.
6. Ward, E. S., D. Güssow, A. D. Griffiths, P. T. Jones, and G. Winter. 1989. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. Nature **341**: 544.
7. Plückthun, A., and A. Skerra. 1989. Expression of functional antibody Fv and Fab fragments in *Escherichia coli*. Meth. Enzymol. **178**: 479.
8. Jahn, S., S. T. Kiessig, R. Grunow, A. Hlinak, O. V. Rochlin, and R. von Baehr. 1987. Cell biology of human IgM-producing hybridomas derived from a fusion with mouse myeloma cells. Hybridoma **6**: 679.
9. Shlomchik, M., M. Mascelli, H. Shan, M. Z. Radic, D. Pisetsky, A. Marshak-Rothstein, and M. Weigert. 1990. Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. J. exp. Med. **171**: 265.
10. Berman, J. E., S. J. Mellies, R. Pollock, C. C. Smith, H. S. Suh, B. Heinke, C. Kowall, U. Surti, L. Chess, C. R. Cantor, and F. Alt. 1988. Content and organization of the human IgV_H locus: Definition of three new V_H families and linkage to the IgH locus. EMBO J. **7**: 4141.
11. Jahn, S., B. Niemann, T. Winkler, J. R. Kalden, and R. von Baehr. 1994. Expansion of a B lymphocyte clone producing IgM auto-antibodies encoded by a

somatically mutated V_HI gene in the spleen of an autoimmune patient. Rheumatol. Internat. **13:** 187.

12. HANSEN, A., S. JAHN, A. LUKOWSKY, G. GRÜTZ, J. BOHN, R. VON BAEHR, and U. SETTMACHER. 1994. VH/VL gene expression in polyreactive-antibody-producing human hybridomas from the fetal repertoire. Exp. Clin. Immungenet. **11:** 1.
13. EVANS, G. I., G. K. LEWIS, G. RAMSAY, and B. M. BISHOP. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol. **5:** 3610.
14. HOCHULI, E., H. DÖBELI, and A. SCHACHER. 1987. New metal chelate adsorbance selective for proteins and peptides containing neighbouring histidine residues. J. Chromatogr. **411:** 177.
15. ROGGENBUCK, D., U. MARX, S. T. KIESSIG, S. JAHN, and T. PORSTMANN. 1994. Fermentation, purification and immunochemical characterization of a natural human polyreactive monoclonal antibody. J. Immunol. Meth. **167:** 207.
16. LARRICK, J. W., and C. BORREBAECK. 1989. Rapid cloning of rearranged immunoglobulin genes from human hybridoma cells by using mixed primers and polymerase chain reaction. BBRC **160:** 1250.
17. SAMBROOK, J., E. F. FRITSCH, and T. MANIATIS. 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor Lab. Press.
18. WARD, E. S. 1991. Expression and secretion of T-cell receptor V alpha and V beta domains using *E. coli* as a host. Scand. J. Immunol. **34:** 215.
19. WARD, E. S. 1992. Secretion of T-cell receptor fragments from recombinant *Escherichia coli* cells. J. Mol. Biol. **224:** 885.
20. CHAUDHARY, V. K., C. QUEEN, R. P. JUNGHANS, T. A. WALDMAN, D. J. FITZGERALD, and I. PASTAN. 1989. Recombinant immunotoxin consisting of two antibody variable domains fused to pseudomonas endotoxin. Nature **339:** 394.
21. SANGER, F., S. NICKLEN, and A. R. COULSON. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463.
22. TOWBIN, H., T. STAEHLIN, and J. GORDON. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:** 4350.
23. CORDELL, J. L., B. FALINI, W. N. ERBER, A. K. GOSH, Z. ABDULAZIZ, S. MACDONALD, K. A. PULFORD, H. STEIN, and D. Y. MASON. 1984. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J. Histochem. Cytochem. **32:** 219.
24. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680.
25. PORSTMANN, B., T. PORSTMANN, E. NUGEL, and U. EVERS. 1985. Which of the commonly used marker enzymes gives the best results in colorimetric and fluorimetric enzyme immunoassays: horseradish peroxidase, alkaline phosphatase or beta-galactosidase? J. Immunol. Meth. **79:** 27.
26. FRIGUET, B., A. F. CHAFOTTE, L. DJAVADI-OHANIANCE, and M. E. GOLDBERG. 1985. Measurement of the true affinity constant in solution of antigen-antibody complexes by enzyme linked immunosorbent assay. J. Immunol. Meth. **77:** 305.
27. TRON, F., and J. F. BACH. 1989. Molecular and genetic characterization of pathogenic autoantibodies. Autoimmunity **5:** 151.
28. RADIC, M. Z., J. MACKLE, J. ERIKSON, C. MOL, W. F. ANDERSON, and M. WEIGERT. 1993. Residues that mediate DNA binding of autoimmune antibodies. J. Immunol. **150:** 4966.
29. BEREK, C., and C. MILSTEIN. 1987. Mutation drift and repertoire shift in the maturation of the immune response. Immunol. Rev. **96:** 23.
30. HIETER, P. A., G. F. HOLLIS, S. J. KORSMEYER, T. A. WALDMAN, and P. LEDER. 1981.

Clustered arrangement of immunoglobulin lambda constant region genes in man. Nature **294:** 536.

31. BETTER, M., C. P. CHANG, R. R. ROBINSON, and A. H. HORWITZ. 1988. *Escherichia coli* secretion of an active chimeric antibody fragment. Science **240:** 1041.
32. SKERRA, A., and A. PLÜCKTHUN. 1991. Secretion and in vivo folding of the Fab fragment of the antibody MOPC603 in *Escherichia coli:* influence of disulfides and cis-prolines. Protein engineering **4: 971.**
33. SKERRA, A., I. PFITZINGER, and A. PLÜCKTHUN. 1991. The functional expression of antibody Fv fragments in E. coli: improved vectors and a generally applicable purification technique. Bio/Technol. **9:** 273.
34. ROGGENBUCK, D., H. KÖNIG, B. NIEMANN, G. SCHOENHERR, S. JAHN, and T. PORSTMANN. 1994. Real-time biospecific interaction analysis of a natural human polyreactive monoclonal IgM antibody and its Fab and scFv fragments with several antigens. Scand. J. Immunol. **40:** 64.
35. KABAT, E. A., T. T. WU, M. REID-MILLER, H. M. PERRY, and K. S. GOTTESMAN. 1987. Sequences of proteins of immunological interest. 4th ed. U.S. Department of Health and human services, Natl. Inst. Health, Bethesda, USA.

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