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A new method for the analysis and production of monoclonal antibody fragments originating from single human B cells

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Abstract

The phage display approach has proven to be a major step forward in studies on the human autoimmune repertoire. However, it remains doubtful whether the heavy and light chains of the antibodies obtained from these libraries resemble original in vivo pairings. Here we describe a novel, simple method for the immortalization of the variable heavy and light chain regions originating from individual, nonboosted, autoantigen-specific human B cells. Our method is based on the clonal expansion of B cells in which cell–cell interactions (CD40-CD40L) as well as soluble factors were shown to be essential. This B cell culture system combined with a selection on antigen (the U1A protein, a frequent autoantigenic target in patients with systemic lupus erythematosus) and single cell sorting resulted in the isolation of U1A-specific human B cells and the subsequent expression of an U1A-specific single chain variable fragment (scFv). Our method circumvents laborious plating and screening and has the advantage that original heavy/light chain pairings can be isolated. Due to the high growth efficiency of single cultured B cells (50–70% outgrowth) even rare B cell activities can be studied using this system. © 1997 Elsevier Science B.V.

Keywords: Autoantibody; B cell stimulation; Single B cell; Phage display; U1 RNA-associated protein

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease in which patients often produce autoantibodies directed against several autoantigens. One of these endogenous antigens recognized by SLE patients is the U1 small nuclear ribonucleoprotein (U1 snRNP or U1 RNP) particle, which is involved in the splicing of pre-mRNA. The U1 RNP consists of a small RNA molecule complexed with two types of protein: namely, the common (or Sm)

Abbreviations: Ab, antibody; Ag, antigen; D, diversity segment; FITC, fluorescein isothiocyanate; H, heavy chain; Ig, immunoglobulin; kDa, kiloDalton; L, light chain; PBMC, peripheral blood mononuclear cells; nt, nucleotides; PCR, polymerase chain reaction; PHA, phytohaemagglutinin; PMA, phorbol myristate acetate; scFv, single chain variable fragment; SLE, systemic lupus erythematosus; snRNP, small nuclear ribonucleoprotein particle; TSN, human T cell/macrophage supernatant; V_H, heavy chain variable domain; V_L, light chain variable domain

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proteins which are also found in other U RNPs and the U1 RNP specific proteins U1-70K, U1C and U1A. The U1A protein is an immunodominant antigen in the autoimmune response against this complex in both humans as well as in animal models (Fatenejad et al., 1994; Mamula, 1995). Mamula (1995) showed very elegantly that tolerance to endogenous U1A could be broken after immunization of mice with a mixture of endogenous and exogenous U1A. This property renders U1A an interesting protein to study with respect to the generation of autoantibodies. The recent development of phage display libraries has proven to be very successful in studying the B cell response against autoantigens (Griffiths et al., 1993, 1994). Previously, we have isolated and characterized U1A-binding antibody (Ab) fragments from several variable (V) gene combinatorial phage libraries (De Wildt et al., 1996). These Ab fragments resemble the Abs present in the sera of patients with SLE with respect to affinity, specificity and epitope recognition. However, due to random combination of the heavy (V_H) and light (V_L) chains during the construction of such libraries, the V_H/V_L pairings occuring in vivo, are lost. Since we were interested in the utilization of the original V_H/V_L pairings of the encoding autoantibodies, we employed a single cell culture system for B cells using mouse thymoma EL-4 B5 cells (Zubler et al., 1985). Our method involves the clonal expansion of B cells by activation via their CD40 molecules (Werner-Favre et al., 1994) and additional factors, e.g. IL-1 (secreted by activated human T cells) which have been shown to be essential for B cell amplification (Zhang et al., 1990). In combination with a preselection on antigen and single cell sorting using a flow cytometer we were able to select individual U1A-specific B cells. V_H and V_L genes originating from single U1A-specific B cells were cloned in a phage display vector and were found to be reactive as a single chain variable fragment (scFv).

2. Materials and methods

2.1. Preparation of lymphocytes

Heparinized blood was washed with an equal amount of PBS/0.3% Na-citrate. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll–Paque (Pharmacia Biotech) and washed with PBS. At this stage PBMC were either used directly or frozen in culture medium containing 10% dimethyl sulfoxide (DMSO) and 50% fetal calf serum (FCS, Gibco). Removal of monocytes by plastic adherence, the enrichment for antigen specific B cells and subsequent culturing were performed essentially as has been described in detail previously (Steenbakkers et al., 1994). Briefly, 6 well culture plates (Greiner) were coated with 5 μg/ml recombinant U1A (Boelens et al., 1991) in 0.1 M $NaHCO_3$, pH 9.6 during an overnight incubation at 4°C. The plates were washed three times with PBS and incubated with $1–5 \times 10^6$ monocyte-depleted PBMC in 4 ml DMEM/HAM's F12 (1/1, Gibco) containing 10% calf serum (CS, Hyclone). Incubation was performed for 1–2 h at 37°C, > 98% humidity and 5% CO_2. Unbound cells were removed by washing six times with PBS and adhering cells were collected from the plates using trypsin treatment (0.05% Trypsin, 1.1 mM EDTA) for 5 min at 37°C. Trypsin treatment was stopped by the addition of DMEM/HAM's F12 (1/1)/10% CS. Cells were harvested and labelled with a mixture of anti-CD19 and anti-CD20 monoclonal Abs conjugated to fluorescein isothiocyanate (FITC, Dako) and viable, single $CD19/CD20^+$ cells were sorted into 96-well plates using a Coulter Epics Elite flow cytometer (Coulter, Hialeah, FL) equipped with an autoclone deposit unit.

2.2. Culture of B cells

Single B cells were cultured in 96-well plates containing 20,000 irradiated EL4-B5 thymoma cells (a kind gift from Dr. R. Zubler, Geneva) per well and 10–15% human T cell/macrophage supernatant (TSN) in DMEM/HAM's F12 (1/1)/10% CS. The TSN was prepared from freshly isolated PBMC (two buffy coats) which were cultured in the presence of 5 μg/ml phytohaemagglutinin (PHA, Murex) and 10 ng/ml phorbol myristate acetate (PMA, Sigma). The TSN was harvested after 48 h. Culture supernatants of the B cell cultures were tested for (Ag-specific) antibody production at day 10 or 11.

2.3. ELISA testing of culture supernatant

Ninety-six well plates (Nunc) were coated with 1 μg/ml recombinant U1A in 0.1 M $NaHCO_3$, pH 9.6, overnight at 4°C. In the case of immunoglobulin (Ig) G, IgM or total Ig detection plates were coated with 1 μg/ml anti-human IgG, IgM or total Ig in 0.1 M $NaHCO_3$, pH 9.2. Unoccupied sites on the wells were blocked with 2% skimmed milk powder/PBS (MPBS) for 2 h at room temperature (rt) and the plates were then washed three times with PBS. Forty μl of culture supernatant were mixed with an equal amount of 2% MPBS and incubated for 1 h at rt in the coated plates. Plates were then washed three times with PBS/0.05% Tween 20 (PBST) and three times with PBS. For the detection of IgG, IgM or total Ig, the corresponding horseradish peroxidase conjugated antihuman IgG, IgM or total Ig (Dako), 1/5000 diluted in 2% MPBS containing 2% CS was used (1/1000 diluted for antigen (Ag)-specific Ab production). Plates were washed as above and ELISAs were developed using H_2O_2 and 3′3′5′5′-tetramethylbenzidine as a substrate. The reactions were stopped with H_2SO_4.

2.4. Cloning of V_H/V_L regions from B cell clones

Total RNA was extracted from the wells with Ag-specific Ab production using RNAzol™ (Biotecx Laboratories) as described by the manufacturer's protocol. Half of the RNA was used for first strand cDNA synthesis using 40 pmol 15-mer oligo *d*(*T*) primer (Boehringer) and SuperScript™ II reverse transcriptase (RT, Gibco) in a total volume of 50 μl. Five μl of this RT-reaction was used in a first PCR to amplify V-regions with family specific primers and constant region primers (Marks et al., 1991). In a seminested second PCR, 1 μl of this first PCR product was used with primers containing appropriate restriction sites for cloning. VHback primers containing a *Sfi*I/*Nco*I site (Marks et al., 1991) and JHforward primers with a *Sal*I site (Figini et al., 1994) were used for the heavy chains. For the light chains Vκ and Vλ primers containing an *Apa*L1 (Jespers et al., 1994) and *J*κ or *J*λ primers containing a *Not*I site (Marks et al., 1991) were used. Heavy and light chains were sequentially cloned in a phagemid vector pHENIX in which C-terminally a peptide epitope of the vesicular stomatitis virus glycoprotein (VSV-G) (Kreis, 1986) was inserted as a tag for easy detection. Individual ampicillin resistant colonies were grown and soluble scFv production was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) as has been described (De Bellis and Schwartz, 1990). After the addition of IPTG the cultures were grown for 3 h at 30°C and periplasmic fractions containing the majority of the scFv were harvested as previously described (De Wildt et al., 1996). Soluble scFv in periplasmic fractions were tested for binding to a panel of recombinant autoantigens U1A, U1C (Nelissen et al., 1994), Ro60 (Slobbe et al., 1992), Ro52 (Slobbe et al., 1992), La protein (Slobbe et al., 1992), CENP-B (Verheijen et al., 1992) and Jo-1 (Rutjes et al., 1997) in ELISA. The proteins were coated as described above. Bound scFv were detected with mouse monoclonal Ab P5D4 which recognizes the C-terminal VSV-G peptide (Kreis, 1986) (kindly provided by Dr. T. Kreis) followed by rabbit antimouse Ig peroxidase conjugate (Dako). ELISAs were developed as described above. Sequencing was performed with fluorescent labelled primers fdSEQ1 (Griffiths et al., 1994) and forlinkSEQ (5′-GCCACCTCCGC-CTGAACC-3′) using the thermo sequenase cycle sequencing kit (Amersham, Life Science). Sequence reactions were analyzed on an automatic sequencer (Pharmacia Biotech). Sequences of V-regions were compared to the sequences present in the V BASE Sequence Directory (Tomlinson et al., 1996) to determine the closest germline counterpart.

3. Results

PBMC from SLE patients as well as from a healthy donor were used for the enrichment of U1A-specific B cells. Ag-specific cells were selected on Ag coated plates and individual $CD19/CD20^+$ cells were plated out using a flow cytometer. The selected B cells were seeded in 96 well plates containing EL-4B5 thymoma cells and 10–15% supernatant of PHA and PMA activated human T cells. Typical percentages of Ig-positive cultures determined by ELISA after 10–11 days culturing ranged between 50 and 70% and the frequency of U1A-specific B cell clones ranged between 1 and 2.5% as

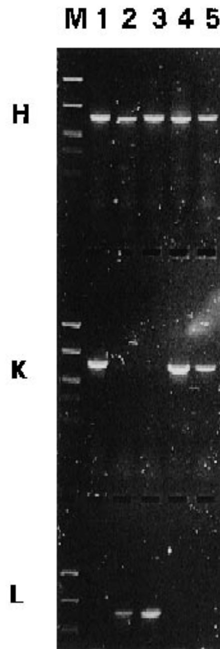

Fig. 1. PCR products obtained from single cell clones (clone 1–5). Three PCR reactions of each clone were performed to amplify heavy chain, lambda and kappa light chains separately. (H) Heavy chain products molecular size = ±750 nucleotides. Either a κ (K) or λ (L) product (molecular size = ±700 nucleotides) from each clone was obtained. (M) DNA marker molecular size 1444, 850, 480 and 342 nucleotides were used as a molecular weight reference for heavy chain (H), κ (K) and λ (L) light chains.

percentage of Ig-positive wells. The absorbance values of wells exhibiting anti-U1A Ab production were between 0.6 and 1.3. As a control, cells from a healthy donor were used and subjected to the same procedure. No U1A-specific Ab production could be detected in these cultures (data not shown), while the percentage of Ig-producing wells was similar to those found with the SLE patient B cells. The relative proportions of IgG, IgM and IgG/IgM double positive isotypes in Ig-producing single B cell cultures were 3:3:1. After culture in the EL-4 B5 system, the B cells showed a plasmablast-like phenotype expressing CD38^{high} and syndecan-1^{moderate}, a plasma cell marker stained with monoclonal Ab B-B4 (data not shown) (Wijdenes et al., 1996).

Complementary DNA made from the RNA of one single B cell clone with positive anti-U1A Ab production was amplified in a PCR with a mixture of V_H family specific primers and a constant region primer (IgG- or IgM-specific) and gave rise to a V_H product of the correct size i.e. 750 nt (Fig. 1). In the case of the light chains a product (700 nt) with either κ or λ primers was obtained, a result suggesting clonality. As a control for the PCR, cDNA isolated from a well in which no B cell was present, was used. Such control reactions never resulted in a PCR product (data not shown). Five U1A-specific B cell clones were isolated. Two U1A-specific B cell clones

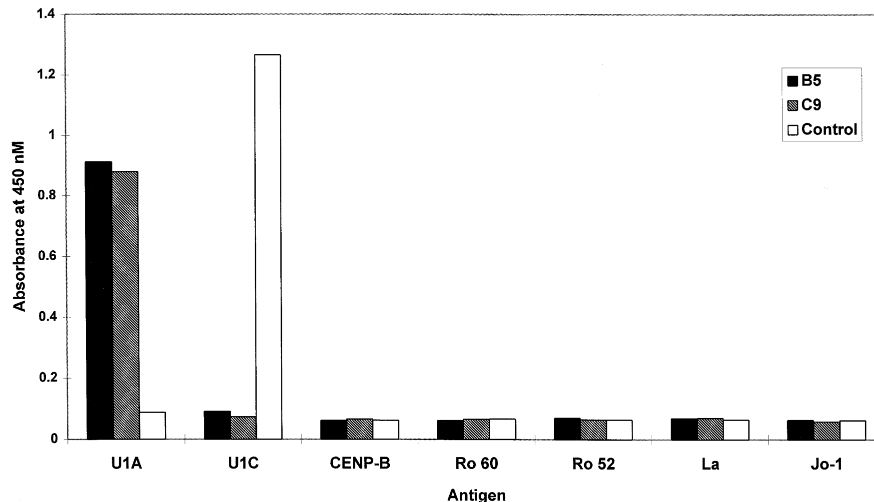

Fig. 2. Specificities of soluble anti-U1A scFv (B5 and C9) on a panel of recombinant autoantigens determined by ELISA. U1C: U1 RNP associated C protein; CENP-B: centromere protein-B; Ro60: SS-A, an autoantigen of 60 kDa; Ro52: an autoantigen of 52 kDa; La: SS-B, an autoantigen of 46.7 kDa; Jo-1: histidyl tRNA synthetase. An anti-U1C scFv was used as a control.

i.e. B5 and C9 were analyzed in more detail and cloned into a phagemid vector for scFv expression. Soluble scFv present in bacterial supernatants or periplasmic fractions were used for specificity tests in ELISA on a number of autoantigens (Fig. 2). The results clearly show that the activity was specifically directed against the U1A protein. The nucleotide sequences of the V_H and V_L genes of these clones were determined and their closest germline counterpart was established using the V BASE Sequence Directory (Tomlinson et al., 1996). Clone B5 uses V_H germline gene DP-51 (V_H3 family) and a light chain, DPK9, from the largest light chain $\kappa1$ family. Clone C9 uses DP-63, a member of the V_H4 family for its heavy chain and also associates with a $\kappa1$ family member (DPK8) for its light chain. Both clones B5 and C9 contain a relatively long CDR3 sequence of 18 (ETLNYDSSGYYYGNAFDI) and 19 (IRTGGSGWYARGGYYGMDV) amino acids, respectively. For both clones D segments could be identified, namely D3-22 (also called DXP3) for B5 and D6-19 for C9 (S.J. Corbett et al., unpublished observations).

4. Discussion

AutoAbs in SLE are targeted predominantly to intracellular nucleoprotein particles. Although much is known about the structure and function of these autoantigens, much less is known about the mechanisms leading to autoAb production. The recent development of displaying Abs on phage (Griffiths et al., 1993, 1994; Winter et al., 1994), has proven to be a valuable tool in the study of B cell responses against self antigens. Previously, we isolated several Ab fragments from patient-derived and semi-synthetic libraries which recognize the U1RNA associated A protein (De Wildt et al., 1996). These Ab fragments showed remarkable similarities with anti-U1A autoAbs present in SLE patients in terms of affinity, specificity and the epitopes which are recognized (De Wildt et al., 1996). It was not known however, whether the V_H and V_L pairings isolated from these phage display libraries are the same as the V_H/V_L pairings present in autoantibodies occurring in vivo.

In the present study we have employed a novel strategy for generating monoclonal antibodies from individual autoantigenic peripheral B cells retaining the original V_H/V_L pairings. This approach has the advantage over classical hybridoma technology that laborious cell culturing and unstable hybridomas are circumvented. On the other hand it also prevents the random pairings of V_H/V_L chains which are introduced during the preparation of combinatorial phage libraries. Lymphocytes were selected for binding to autoantigens and single $CD19/CD20^+$ cells were sorted using a flow cytometer. These selected B cells were cultured with human T cell supernatant and murine thymoma helper cells. The clonally expanded B cells were subjected to an RT-PCR and subsequently expressed as an scFv in a phagemid vector. The cloned V regions were found to recognize specifically the U1A protein in an ELISA procedure. Clone B5 uses the V_H germline segment DP-51 from the V_H3 family and clone C9 uses the DP-63 gene (V_H4 member) for its heavy chain. Interestingly, we have isolated previously several V_H4 family members, from different phage display libraries which recognize the U1A protein (De Wildt et al., 1996, and unpublished results). This finding is consistent with the observation of several groups that the V_H4 family encoding for pathogenic autoAbs in SLE patients appears to be overrepresented (Pascual and Capra, 1991; Demaison et al., 1996). This preferential V_H4 usage does not correspond to its frequency in the expressed healthy repertoire. The involvement of a B cell superantigen specific for the V_H4 family has been suggested as one of possible factors accounting for pathogenesis in SLE (Demaison et al., 1996; Domiatisaad et al., 1996). It is has to be noted that clones B5 and C9 both use a $\kappa1$ family member for its light chain. This frequent usage of $\kappa1$ segments in anti-U1A Abs has been observed by us previously (De Wildt et al., 1996 and unpublished results). Although the light chain is thought to play a minor role in antigen recognition, $\kappa1$ members probably support binding to U1A in these Abs.

The Ag selection was performed on Ag-coated plates but immobilization of Ag on superparamagnetic minibeads has also been found to be effective. A major advantage of these magnetically sorted cells (MACS) is that they can be used directly for flow cytometry analyses. The expansion step results in an increase of mRNA levels derived from one clone,

which avoids the risk of contamination in downstream procedures. The increased amount of RNA also makes it more convenient to analyze single peripheral B cells which, in most cases, are resting cells with low mRNA levels. One major consideration in the study of peripheral B cells is often the lack of other available patient material. The high efficiency of the culture system (50–70% Ig^{+} cultures) renders this method attractive for the isolation of rare, nonboosted, (auto) antigen-specific B cells. The frequency of U1A-specific B cell clones after antigen selection ranged between 1 and 2.5% as a percentage of Ig-positive wells. Assuming that the frequency of Ag-specific B cells in the periphery ranges between 10^{-4} and 10^{-5} this would indicate an enrichment factor of 100–1000. Recently, other groups have also succeeded in the isolation of Ag-specific B cells from peripheral blood using an expansion B cell culture system. However, these results were obtained with cells from virally infected donors (Steenbakkers et al., 1993) or donors vaccinated with bacterial Ags (Lagerkvist et al., 1995). Others have analyzed Igs at the level of single cells with either known or unknown antigenic specificity. V-regions of known antigenic specificity were amplified from single hybridomas (Embleton et al., 1992) or from B cells present in immunized rabbits or mice (Babcook et al., 1996) and V regions from single B cells of unknown specificity were analyzed in lymphoid organs of immunized animals (McHeyzer Williams et al., 1991, 1993; Kuppers et al., 1993). The frequency of these cells in the periphery or in lymphoid organs is likely to be much higher compared to the autoAg-specific B cells used by us.

In summary, we have described a convenient method for the study of V_H/V_L pairings originating from individual (autoAg-specific) B cells. The combined use of Ag-selection, single cell sorting and an efficient culture system makes it possible to analyze large numbers of B cells, circumventing laborious plating and screening procedures.

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