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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED: SINGLE-CHAIN ANTIBODY CONSTRUCT OF AUTOANTIBODY

ANTI-ssDNA ANTIBODY BY 04-01

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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OF AUTOIMMUNE ANTI-SSDNA
ANTIBODY BV 04-01

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Most sincere thanks to Dr. Voss for the opportunity to take on an independent research project in his lab. My "hands-on" experience during this project has provided me with a deeper understanding of research far beyond that which is offered in a common classroom setting.

I am also very grateful to Lisa K. Denzin, who offered considerable direction and support throughout the entire project.

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>HEADING</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>4</td>
</tr>
<tr>
<td>RESULTS</td>
<td>10</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>13</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>14</td>
</tr>
</tbody>
</table>
INTRODUCTION

Autoimmunity is a disorder of the immune response which disrupts the ability to distinguish self from non-self. Specifically in the case of Systemic Lupus Erythematosis (SLE), antibodies are directed against the host's DNA. Previously studied monoclonal anti-DNA antibodies derived from inbred murine strains displaying a Lupus-like syndrome has allowed a deeper understanding of antibody-DNA interactions (Theofilopoulos and Dixon, 1985). These monoclonal autoantibodies serve as valuable models for analysis of both binding specificities and structural components of anti-DNA autoantibodies and may, in the future, aid in the clinical treatment of SLE.

The research project I pursued was to construct a single chain antibody (SCA) modeled after the heavy and light chain variable domains of a monoclonal anti-single stranded DNA antibody, BV04-01. Since BV04-01 (IgG2a, kappa) is highly characterized, it is an ideal antibody to use in the construction of a SCA. BV04-01 preferentially binds single-stranded poly(dT) DNA (Ballard et al., 1984; Ballard and Voss, 1985). In addition, the variable region primary structure has been determined as well as the three-dimensional structure through X-ray crystallography (Smith et al., 1989). Antibodies are tetrameric proteins containing two identical heavy and two identical light chains linked by covalent disulfide bonds and non-covalent domain-domain interactions. Single chain antibodies, on the other hand, consist only of the light and heavy chain variable domains (V_L and V_H).
covalently bridged together by a short polypeptide linker (Figure 1). The variable regions maintain the specific activity of the antibody but the lack of constant regions eliminates non-specific interactions as well as complement fixation. The SCA linkers are similar in design to the insertion of short loop sequences between domains in de novo design of proteins (Mutter, 1988; Regan and DeGrado, 1988). SCA proteins have been synthesized with either the light or heavy chain variable regions orientated at the amino terminus (Batra, J.K. et al., 1990; Bird et al., 1988; Huston et al., 1988). In principle, the linker must allow $\gamma_\text{L}$ and $\gamma_\text{H}$ to fold independently in the proper conformation so that interference between the antigen and active site is negligible. However, the role of the linker has not been fully assessed. Studies show that antigen binding properties have not perfectly simulated parental monoclonal antibody binding, but affinities approximating the prototype have been obtained (Huston et al., 1988; Bird et al., 1988; Glockshuber et al., 1990). Single chain antibodies represent an alternative to both $\text{F}_{\text{ab}}$ and $\text{F}_{\text{v}}$ fragments and have already proven valuable in studying antibody-antigen molecular interactions between heavy and light chain variable regions. Thus, the SCA model represents a useful reagent to study variable domain ligand interactions independent of constant regions.
The single-chain derivative of BV04-01 upon eventual expression in E. coli will be a valuable reagent to pursue site-specific mutagenesis studies of an autoimmune antibody. Since the SCA lacks the constant, complement-fixing regions, it may also prove useful as a competitive inhibitor in vivo as a therapeutic reagent in Lupus patients.
MATERIALS AND METHODS

PRIMERS FOR AMPLIFICATION AND SEQUENCE VERIFICATION

Oligonucleotides used for polymerase chain reaction (PCR) amplification, construction and sequence verification of SCA 04-01 were synthesized by the phosphoramidite method (Beaucage and Caruthers, 1981) at the University of Illinois Genetic Engineering Facility and were based on published sequences of BV04-01 variable regions (Smith et al., 1990) and that of polypeptide linker 212 (Fig 2a). For amplification of the $V_L$ region, M13 Universal primer and a primer (0401VL3'-212) complementary to the last 17 nucleotides of the 04-01 $V_L$ was used. The 0401VL3'-212 primer also incorporated a tail which encoded 30 nucleotides of the 212 linker region. For amplification of the $V_H$ region, 0401VH5'-212 and 0401VH3'-SCA primers were used. The 0401VH5'-212 primer was complementary to the first 18 nucleotides of the $V_H$, in addition to incorporating a tail which encoded for the rest of the 212 linker and overlapped with the 0401VL3'-212 primer to facilitate construction of the 212 linker region. The 0401VH3'SCA primer, which was complementary to the last 21 nucleotides of the $V_H$, modified the 3' end of the $V_H$ by addition of two stop codons and a BamHI restriction site.

Primers used for sequence verification were: VK2FR3 primer, which was complementary to nucleotides L60 to L65 of the $V_L$; primer L27d, which was complementary to nucleotides L21 to L29 of the $V_L$; and M13 Reverse and Universal sequencing primers. All primer sequences are shown in figure 2b.
Preparation of Template for PCR Amplification

Template (0401VL and 0401VH cDNA clones) for PCR amplification was prepared using a miniprep sequencing template procedure (Krafft et al., 1988) on pUC13 derivatives into which BV0401 was previously cloned (Smith et al., 1990). Concentrations were estimated based upon absorbance at 260nm and then a stock solution of 10 ng/μl plasmid DNA was prepared for use in subsequent polymerase chain reactions.

Construction of SCA Using PCR

All PCR reactions included 30pmoles of each primer (unless indicated otherwise), 10ng of template DNA, 200mM of each dNTP, 1X PCR buffer consisting of 0.1% Triton X-100, 10mM Tris HCl pH 8.8, 2.5mM MgCl₂, 200μg/ml gelatin, 50mM KCl, and 5.0 units of Thermus aquaticus DNA polymerase. Reaction mixtures were brought to a total volume of 0.1 ml and overlaid with 75μl mineral oil. All PCR profiles consisted of one round of denaturation at 92°C for 5 minutes, annealing at 50°C for 3 minutes, and extension at 65°C for 2 minutes. This was followed by 30 cycles of denaturation at 92°C for 1 minute, annealing at 50°C for 1 minute, and extension at 65°C for 2 minutes.

Figure 3a schematically outlines the construction of the SCA using PCR. The M13 Universal and 0401VH3'SCA primers may be viewed as "outside" primers whereas 0401VL3'-212 and 0401VH5'-212 are to be considered "inside" primers whose partial complementarity (18 nucleotides) comprise part of the polypeptide.
linker. Outside primers were added at a concentration of 30pM, while inside primers varied from 1.25 to 5pM in concentration to facilitate the formation of the final product. All four primers were added simultaneously; a method formerly proven successful (Davis et al., 1990).

CLONING OF AMPLIFIED SCA PCR PRODUCT

The resulting PCR product was phosphorylated in the 5' position using T4 polynucleotide kinase in a reaction mixture consisting of 50mM Tris-HCl, pH 7.6, 10mM MgCl₂, 0.1mM spermidine, 0.1mM EDTA, 5mM DTT, and 1mM rATP. Following incubation at 37°C for 30 minutes and heat deactivation at 65°C for 10 minutes, 4.5 units of T4 DNA polymerase were added along with 4mM dNTP’s to generate blunt ends. DNA was incubated at 37°C for 10 minutes, extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. After centrifugation, the DNA was washed with 70% ethanol, dried and resuspended in TE (10mM Tris pH 8.0, 1mM EDTA). The product was purified by electrophoresis in low melting temperature agarose (LMTA·1.2% Seaplaque) in TAE buffer (40mM Tris base, 40 mM acetic acid, 1mM EDTA) and the band of interest excised. The vector, pTZ18u, was digested with Smal and to prevent self- ligation, 1 unit calf intestinal alkaline phosphatase (CIAP) was added and incubated for 1 hour at 50°C. The CIAP was deactivated by the addition of 500mM EGTA at 70°C for 10 minutes and the vector was electrophoresed into LMTA and
excised. Both the vector and the PCR product were melted at 75°C and added in a 1:1 ratio to 2X ligase buffer containing 2 units of T4 ligase. Ligation mixtures were incubated overnight at room temperature and then used to transform 10^6 of competent DH5α E. coli cells according to manufacturer's specifications. The cells were plated onto Luria agar containing 100μg/ml X-gal and incubated at 37°C for 24 hours. Potential clones were verified by isolating plasmid DNA (Maniatis, 1982) from resulting white colonies followed by digestion with BglII and BamHI. A fragment of approximately 800 base pairs indicated a successful clone. This clone was designated pLY1.

SEQUENCE ANALYSIS

Cloned PCR product pLY1 was sequenced by the dideoxy chain termination procedure (Sanger et al., 1977) using double-stranded plasmid DNA template (Kraft et al., 1988) and Sequenase (U.S. Biochemical Corp., Cleveland, OH). The complete nucleotide sequence was determined utilizing M13 universal and M13 reverse primer sites that flanked the cloned insert in addition to the L27d and Vx2FR3 primers which are complementary to short regions of the V_L.

ADDITION OF OMP A SIGNAL SEQUENCE

Figure 3b illustrates the scheme for the addition of Omp A signal sequence to 04-01 SCA-212. Since SCA 4·4.20·205c, which is an anti-fluorescein single chain antibody, utilizes a V_L region
similar to 04-01 V_L, it was used to add the Cmp A signal sequence to 04-01 SCA. In order to accomplish this, residue L34 of SCA 4-20 had to be changed from arginine to histidine. This was accomplished by using a mutagenesis primer. Using 4-4-20-205C SCA as template along with M13 reverse and L34His mutagenesis primer, amplification of an approximate 200 base pair region, which included the 63 nucleotide signal sequence at the 5' end, was accomplished. The PCR product was cloned into pTZ18u in the same manner as previously described under CLONING OF AMPLIFIED SCA PCR PRODUCT and was designated pLY2. Digestions with ClaI and KpnI resulting in a 200 base pair fragment were sequenced as before. Successful clones were digested with ClaI and KpnI and cloned into similarly digested pLY1 to give the complete BV04-01 SCA-212 product (pLY3). Final verification of the clone was by ClaI/BamHI and ClaI/KpnI digests to give the respective 800 and 200 base pair fragments.

EXPRESSION OF BV04-01 SCA-212

The 04-01 SCA-212 protein was expressed in E. coli from a plasmid (pGX8772) which contained the hybrid lambda phage O_L/P_R promoter sequence (Scandella et al., 1985). The transcribed product includes the SCA gene as well as the E. coli Omp A signal sequence. The 04-01 SCA-212 gene product was digested with ClaI/BamHI and cloned into ClaI/BamHI cleaved pGX8772 which had been prepared in the same manner as previous vectors. Ligations were transformed into E. coli strain GX6712, incubated overnight
at 30°C and resulting colonies verified by digesting with Cla I/Bam HI. To check for the full length over expressed SCA, clones were used to inoculate 5ml cultures of 2XYT broth (Maniatis et al., 1982) and incubated overnight at 30°C. Next, 150 µl of the overnight cultures were used to inoculate 15mls 2XYT which was incubated at 30°C until the optical density at 600nm was approximately 1.0. Expression of the protein was induced by incubation at 42°C for one hour. The cells were pelleted at 6000 rpm for 10 minutes and resuspended in 1.5 ml lysis buffer (50mM TRIS pH 8.0, 1mM EDTA). Each clone was subjected to a French Press twice at 1000psi. Lysed cells were washed twice with lysis buffer including Triton X-100 (0.5%) and finally with lysis buffer alone. Resulting pellets containing over expressed 0.1 SCA were resuspended in 100µl Crack buffer (TRIS, Urea, DTT), boiled for 10 minutes, and analyzed by SDS PAGE (12.5%) (Laemmli, 1970). FAST STAIN (Zolion Research Inc.) was used for a protein detection reagent according to manufacturer's specifications.
The concept of single chain antibodies is new and novel, and in this project, applied to an autoimmune antibody for the first time. In order to accomplish this, it was originally intended to amplify $04\cdot01 V_L$ and $V_H$ segments separately before adding the two products together with only "outside" primers. This method, however, failed to yield acceptable concentrations of the final product. Rather than varying experimental conditions such as time and temperature, it was instead decided to simultaneously introduce all four PCR primers along with the $04\cdot01 V_L$ and $V_H$ templates. Three separate reactions were attempted in which the "outside" primers were introduced at a concentration of 30pM, while the "inside" primers were titrated to 5, 2.5, and 1.25pM. All three reactions gave strongly defined bands of the final product at about 800 base pairs. However, it appeared that the formation of complete product was promoted as the "inside" primers became less concentrated (Fig 5). The gel showed the disappearance of non-specific bands (such as the band slightly larger than the final product) as well as the $V_L$ fragment (the smallest band on the gel). This is probably the result of exhausting the "inside" primers during the first few rounds. After cloning the product into pTZ18u and transforming into E. coli strain DH5α, only 1 of 25 candidates proved to have the correct insert when cut with Cla I/Bam HI (Fig 6). The primary structure of pLY1 was determined and found to have only one
alteration from the predicted sequence. This occurred at L29 in the third nucleotide of the codon and proved to be negligible as it coded for the same amino acid. Also, the mutation will not be present in the final product since during addition of the Omp A signal sequence, this codon was replaced by the correct 04-01 codon.

The addition of the 4-4-20-205C SCA's Omp A signal sequence to 04-01 SCA-212 required construction of a mutagenic primer which substituted a histidine at L34 in place of arginine in native 4-4-20. The PCR conditions for amplification were immediately favorable and ample amounts of DNA were produced (Fig 7). Dideoxy sequencing indicated no aberrations from the expected sequence. Successful integration of pLY2 clones into pLY1 were indicated by cutting plasmid DNA with ClaI/BamHI (900 bp fragment) and ClaI/KpnI (200 bp fragment). Only two of nine attempted clones (pLY3) appeared to contain the correct insert (Fig 8). Subcloning into expression vector pGX8772 via ClaI/BamHI restriction sites and subsequent transformation into E. coli strain GX6712 proved successful in that 11 of 12 clones subjected to ClaI/BamHI contained the correct insert size (Fig 9). Finally, to insure over-expression of the full length SCA in E. coli, three clones were examined for production of the SCA gene product as described under MATERIALS AND METHODS. All three gave a correct protein length on the 12.5% SDS PAGE gel. Both a negative control--E. coli strain GX6712 containing no plasmid, and a positive control--4-4-20-205C, were used. As Figure 10
illustrates, the 4·4·20·205C product is larger due to the 25 amino acid linker.
Based on the results presented it is plausible that the 04·01 SCA-212 should now be grown on a large scale basis, refolded and purified out of E. coli inclusion bodies in the same manner as former SCA isolation procedures (Bird et al., 1988). The expressed protein should then be subjected to binding studies as well as compared to the parent molecule in terms of affinity. Should 04·01 SCA-212 prove to have similar activity to the prototype, site specific mutagenesis studies would then be feasible and appealing. Also, crystal structures of both liganded and unliganded SCA can be used in comparison to the parent molecule and perhaps illustrate the role of the linker. Another possible application for the 04·01 SCA-212 construct would be to test germ line sequences obtained with the CDR2 heavy chain of 04·01 to determine whether all detected variants bind DNA.

The 04·01 SCA construct, as well as other SCAs, will provide a superior reagent to the F_v counterpart. F_v fragments are generally considered the minimal substructural Ig component possessing intrinsic binding properties of the parent antibody molecule (Ward et al., 1989). However, the absence of an interchain covalent linkage in the F_v fragment when used therapeutically in vivo, will probably lead to dissociation with little chance for reassociation. In contrast, the SCA linker prevents such separation, thus rendering it a superior molecule.
BIBLIOGRAPHY


FIGURE 1

LIGHT CHAIN

HEAVY CHAIN

NORMAL ANTIBODY

ANTIGEN BINDING SITE

LINKER

SINGLE CHAIN ANTIBODY

LIGHT CHAIN

HEAVY CHAIN
FIGURE 2a

**BV04-01 SCA-212 LINKER**
(From SCA 4-4-20)

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<tr>
<td>14</td>
<td>GGT</td>
<td>GLY</td>
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**FIG 2b**

PRIMERS USED FOR SCA AMPLIFICATION AND SEQUENCE VERIFICATION

0401VH3'SCA:

5'--GGG-ACT-CTG-GTC-ACT-GTC-TCT-TAA-TAG-GGA-TCC-TA--3'

stop stop Bam HI

0401VH5'-212:

5'--GGT-TCT-GGT-AAA-TCT-TCT-GAA-GGT-AAA-GGT-GAG-GTG-

CAG-CCT-GTT-GAG--3'

0401VL3'-212:

5'--CC-AAG-CTG-GAG-CTG-AAA-GGT-TCT-ACC-TCT-GGT-TCT-GGT-

AAA-TCT-TCT--3'

M13 UNIVERSAL:

5'--GTA-AAA-CGA-CGG-CCA-GT--3'

L34 HIS:

5'--G-CAG-GTA-CCA-ATG-TAA-ATA-GG--3'

M13 REVERSE:

5'--AAC-AGC-TAT-GAC-CAT-G--3'

L27d:

5'--TGC-AGA-TCT-AGT-CAG-AGC-CTT-GTA-AAG-AGT-AAT-GG--3'

Vk2FR3:

5'--ACT-GCC-ACT-GAA-CCT-GTC--3'
PCR Cloning Procedure

A) PCR 0401 V in pUC19

B) PCR 0401 V in plCI3

C) PCR reaction including all 4 primer

D) Clone SCA construct into pTZ18u (p.Y1)

Sequence

E) Digest with ClaI/KpnI
Addition of Omp A Signal Sequence

A) PCR 4420 205C SCA

B) Clone product into pIZ18u (pl.Y2) Sequence

C) Digest with ClaI/KpnI

D) Clone ClaI/KpnI fragment from pl.Y2 into digested pl.Y1

E) Clone construct into Expression Vector (pl.Y4)
FIGURE 4
BV04-01 SCA-212 SEQUENCE

NH₂--ATG-AAA-AAG-ACA-GCT-ATC-GCG-ATT-GCA-GTG-GCA-CTG-GCT-
fM K K T A I A T A V A L A
|-----------------------------| OmpA signal sequence-----

GGT-TTC-GCT-ACC-GTA-GCG-CAG-GCC-GAC-GTC-GTT-ATG-AGT-CAG-ACA-
F A T V A Q A D V V M T Q T
-----------------------------

P L S L P V S L G D Q A S I S
L10

TGC-AGA-TCT-GTG-GGT-GTA-GAT-CTA-GAG-AAA-GGT-GAT-TAT-TAT-
C R S S Q S L V H S N G N T Y
Bgl II  L27  A  B  C  D  E  F  G  H  I  J  K  L  M  N  O  P  Q  R  S  T  U  V  W  X  Y  Z
L30

TTA-CAT-TGG-TAC-CAG-AAG-CCA-GGC-CAG-TCT-CCA-AAG-CTC-CTG-
L H W Y L Q K P G Q S P K L L
L40

ATC-TAC-AAA-GTT-TCC-AAC-CGA-TTT-TCG-GGG-GTC-CCA-GAC-AGG-TTC-
I Y K V S N R F S G V P D R F
L50  L60

AGT-GGG-AGT-GGA-TCA-GGG-ACA-GAT-TTC-ACA-CTC-AAG-ATC-AGA-
S G S G S G T D F T L K I S R
L65  L70

GTG-GAG-GCT-GAG-GAT-CTG-GGA-GTT-TAT-TTC-TGA-TCT-CCA-AAG-ATC-
V E A E D L G C Y F C S Q S T
L80  L90

CAT-GTG-CCG-CTC-ACG-TTC-GGT-GCT-GGG-ACC-AAG-CTG-GAG-GAG-CTG-
H V P L T F G A G T K L E L K
Figure 5. Ethidium Bromide Stained Gel of 04-01 SCA-212 PCR Products.

Lane 1, "inside" primers at 5pM.
Lane 2, "inside" primers at 2.5pM.
Lane 3, "inside" primers at 1.25pM.
Figure 6. Ethidium Bromide Stained Gel of Possible pLY1 Clones Cut with Cla I/Bam HI.

Lane 4, pGEM (Promega) DNA markers
Lane 6, only clone containing correct insert size of approximately 800 b.p.
Figure 7. Ethidium Bromide Stained Gel of 4-4-20
SCA-205C's Omp A Signal Sequence PCR Product.

Lane 1, pGEM (Promega) DNA Markers
Lane 2, amplified product of approximately 200
b.p.
Figure 8. Ethidium Bromide Stained Gel of Possible pLY3
clones cut ClaI/BamHI (top lanes) and ClaI/KpnI
(bottom lanes).

Lane 1, pGEM (Promega) DNA Markers.
Lanes 8 & 10, Only clones containing 800 b.p.
ClaI/BamHI fragment as well as 200 b.p. ClaI/KpnI
fragment.
Figure 9. Ethidium Bromide Stained Gel of Possible pLY4 Clones Cut with Clai/BamHI.

Lane 1, pGEM (Promega) DNA markers. Lanes 2-10, 12, & 13, clones containing correct insert size of approximately 900 b.p.
Figure 10. SDS PAGE Gel of Over-expressed Full Length 04-01 SCA-212 Stained with FAST STAIN (Zoion).

Lanes 1-3, clones containing 04-01 SCA-212.
Lane 4, molecular weight markers of following sizes (bands from bottom to top): 3, 6.2, 14.3, 18.4, 29, 43 KD.
Lane 5, positive control (4-4-20 SCA-205C)
Lane 6, negative control (E. coli strain GX6712 containing no plasmid).