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A NOVEL AND EFFICIENT ROUTE FOR THE ISOLATION OF ANTIBODIES THAT RECOGNISE T CELL RECEPTOR Vαs

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Abstract—Studies of the T cell repertoire have been hindered by the lack of antibodies that recognise V region families, particularly for Vα regions. In this report, single chain Fv (scFv) fragments have been isolated that recognise both recombinant Vαs and native Vαs on the surface of T cells. Mice have been immunised with purified soluble T cell receptors (TCRs) and antibody heavy and light chain variable domain (VH and VL, respectively) genes isolated from splenocytes using the polymerase chain reaction (PCR). The VH and VL genes have been assembled as scFv gene libraries and a bacteriophage display system used to isolate scFvs that recognise a soluble Vα. Five scFvs have been purified and characterised in detail using enzyme-linked immunosorbent assays (ELISAs) and flow cytometry. Three of these five scFvs recognise native Vαs on the surface of T cell hybridomas. This method therefore offers a rapid route to the generation of scFvs that recognise native TCRs and can readily be extended to the production of anti-human TCR antibodies for use in therapy and diagnosis.

Key words: T cell receptor, antibodies, phage display, single chain Fv.

INTRODUCTION

The high affinity and specificity of antibodies makes them attractive reagents for use in diagnosis and therapy (Waldmann, 1991). Recent developments in recombinant DNA technology have resulted in the evolution of powerful methods for the rapid isolation of antibodies in clonal form (for reviews, see Hoogenboom *et al.*, 1992; Ward, 1992a). Diverse libraries of heavy and light chain variable domain (VH and VL, respectively) genes can be isolated from antibody producing cells using the polymerase chain reaction (PCR) (Huse *et al.*, 1989; Larrick *et al.*, 1989; Orlandi *et al.*, 1989; Ward *et al.*, 1989) and cloned for expression as single chain Fvs (scFvs; Bird *et al.*, 1988; Huston *et al.*, 1988) or Fabs using bacteriophage display systems (Breitling *et al.*, 1991; Kang *et al.*, 1991a; McCafferty *et al.*, 1990). Bacteriophage bearing the desired binding specificity can be isolated from libraries by panning on antigen-coated surfaces and the bacteriophage "binders" propagated by reinfection of *Escherichia coli* (Burton *et al.*, 1991; Clackson *et al.*, 1990; Marks *et al.*, 1991). Using these systems, antibody libraries that approach the size and diversity of the immune repertoire (Griffiths *et al.*, 1994; Nissim *et al.*, 1994) can be assembled from either VH and VL genes isolated directly from antibody producing cells, or semi-synthetic libraries can be built using VH and/or VL genes as templates (Barbas *et al.*, 1992; Hoogenboom and Winter, 1992). Thus, large numbers of VH/VL pairs can be sampled for the desired binding specificity, and this is an advantage over hybridoma technology. In addition, the affinities of the selected antibodies can be improved by point mutagenesis (Gram *et al.*, 1992; Hawkins *et al.*, 1992) or chain shuffling (Kang *et al.*, 1991b; Marks *et al.*, 1992a; Ward, 1995). These systems therefore have enormous potential for the generation of antibodies of the desired specificity, and the isolated scFvs or Fabs can be used as building blocks to make complete antibodies for therapy.

The production of antibodies that recognise the V regions of the T cell receptor (TCR) has proved difficult, particularly for Vα domains (designated Vαs) for which there is a paucity of antibodies available. Such antibodies would be of value in analysing TCR V gene expression and for specific immunotherapy in T cell mediated autoimmunity. Until recently, production systems for soluble TCR V regions in reasonable yields using mammalian (Engel *et al.*, 1992; Gascoigne *et al.*, 1987; Gascoigne and Ames, 1991; Gregoire *et al.*, 1991; Lin *et al.*, 1990; Mariuzza and Winter, 1989) and prokaryotic (Novotny

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Abbreviations: scFv, single chain Fv; scTCR, single chain T cell receptor; VH, heavy chain variable domain; VL, light chain variable domain; Vα, T cell receptor alpha chain variable domain; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; SpA, Staphylococcal protein A.

et al., 1991; Soo Hoo *et al.*, 1992; Ward, 1991; Ward, 1992b) hosts have not been available, and this limited the source of antigen for use in immunisations. Transfectomas expressing both α and β polypeptides can be used to generate anti-TCR V region responses, but to date, the antibodies that have been isolated are Vβ-specific (Callan *et al.*, 1993; Choi *et al.*, 1991; Diu *et al.*, 1993; Zumla *et al.*, 1992). Recombinant soluble TCRs (Devaux *et al.*, 1991; Jameson *et al.*, 1989; Traunecker *et al.*, 1986) or TCRs isolated from a T cell leukaemic line (Der-Simonian *et al.*, 1991) have also been used to produce monoclonals, and the use of this approach has resulted in an increased frequency in the production of anti-α chain antibodies. To date, however, the advantages of using bacteriophage display have not been applied to isolating antibodies that recognise native TCRs.

In this report, we have used soluble TCRs produced in *E. coli* (Ciubotaru and Ward, 1994; Ward, 1992b) to immunise mice. A bacteriophage display library derived from one of these mice has been panned against a soluble Vα. The soluble TCRs are derived from a murine arthritogenic T cell hybridoma (Myers *et al.*, 1993) and have sequences that are representative of the oligoclonal T cell populations associated with collagen-induced arthritis (Osman *et al.*, 1993). Several scFvs that recognise the Vα have been isolated and three out of five bind to native TCRs on the surface of T cell hybridomas. This approach therefore offers an efficient way of producing anti-Vα antibodies for use in therapy and diagnosis, and could readily be extended to the production of scFvs that recognise human Vαs.

MATERIALS AND METHODS

Bacterial strains and vectors

Escherichia coli TG1 [ref. (Gibson, 1984); K12, Δ(*lac-pro*), *sup*E, *thi*, *hsdD5*/F′*traD*36, *pro*A$^+$B$^+$, *lacI*^{q}, *lacZ*ΔM15], HB2151 [K12, *ara*, Δ(*lac-pro*), *thi*/*F′pro*A$^+$B$^+$, *lacI*^{q}ZΔM15], and the cloning vector pHEN1 (Hoogenboom *et al.*, 1991; a generous gift of Drs H. Hoogenboom and G. Winter) were used in this study.

Expression of soluble TCRs

Plasmid constructs for the expression of TCR Vαs and single chain TCRs (scTCRs, in which the Vα is linked to the Vβ by a synthetic peptide linker) have been described (Ciubotaru and Ward, 1994; Ward, 1992b). TCRs derived from the qcII85.33 hybridoma (designated 85.33; Myers *et al.*, 1993) and 1934.4 hybridoma (Wraith *et al.*, 1989) were expressed and purified using the methodology of Ward (1992).

Immunisation of mice

Five BALB/c mice were immunised with purified 85.33 scTCR (Ciubotaru and Ward, 1994) using previously described methodology (Ward *et al.*, 1989). The antibody titres were analysed using sera obtained from tail bleeds and ELISA. The mouse which gave the highest titre was killed for the isolation of immunoglobulin VH and Vκ genes.

Generation of bacteriophage display libraries

Total RNA was isolated from splenocytes and used in cDNA synthesis reactions with the VH1FOR-2, Vκ4FOR-1 and Vκ4FOR-2 primers described previously (Ward *et al.*, 1989; Ward, 1995). Aliquots of the cDNA syntheses were used in the PCR with VH1BACK, VH1FOR-2 (for VH gene library; Orlandi *et al.*, 1989; Ward *et al.*, 1989) and Vκ2BACK, Vκ4FOR-1, Vκ4FOR-2 (Vκ gene library; Clackson *et al.*, 1990; Ward, 1995) and conditions as described (Clackson *et al.*, 1990; Ward *et al.*, 1989). scFv repertoires were assembled in a two-step strategy as follows: a single chain linker duplex with overhangs that are complementary to murine JH genes and 5′ ends of murine Vκ genes was generated using the PCR and primers LINKFOR and LINKBACK (Clackson *et al.*, 1990) using VHD1.3-sc-VκD1.3-myc/pHEN1 (McCafferty *et al.*, 1990; Ward, 1995) as template. The 93 bp fragment was gel purified (GeneClean, BIO 101, La Jolla, CA) and used in a splicing by overlap extension reaction (Horton *et al.*, 1989) with gel purified Vκ library, plus LINKBACK and a mixture of Vκ4FOR-1, Vκ4FOR-2 as primers. The linker tagged Vκ repertoire from this PCR was gel purified and then spliced to the VH gene repertoire using the primer VH1BACKSfi (VH1BACK tagged at the 5′ end with 5′ GTC CTC GCA ACT GCGGCCCAGCCGGCC ATG GCC 3′; *Sfi*I site indicated by underlining) and the following 3′ tagging primers (*Not*I sites indicated by underlining; these primers were generous gifts of Dr R. Mernaugh, Pharmacia):

Jκ1NOT10:
5′ GAG TCA TTC TGCGGCCGC CCG TTT GAT TTC CAG CTT GGT GCC 3′

Jκ2NOT10:
5′ GAG TCA TTC TGCGGCCGC CCG TTT TAT TTC CAG CTT GGT CCC 3′

Jκ4NOT10:
5′ GAG TCA TTC TGCGGCCGC CCG TTT TAT TTC CAA CTT TGT CCC 3′

Jκ5NOT10:
5′GAG TCA TTC TGCGGCCGC CCG TTT CAG CTC CAG CTT GGT CCC 3′

The assembled scFv repertoire was digested with *Sfi*I and *Not*I and ligated into *Sfi*I–*Not*I restricted pHEN1 (Hoogenboom *et al.*, 1991). Aliquots of the ligation mixes were used to transform *E. coli* TG1 cells by electroporation (Dower *et al.*, 1988). The library size was 5×10^4 clones.

Analysis of expression of scFvs

To analyse the expression of scFvs tagged with c-myc epitopes, clones were picked, grown up and induced. Culture supernatants were analysed by polyacrylamide gel electrophoresis and immunoblotting, using the 9E10 antibody (Evan *et al.*, 1985) as described (Ward *et al.*, 1989).

Panning of the libraries

Libraries were stored as frozen stocks (Marks *et al.*, 1991), aliquots expanded and superinfected with helper phage VCSM13 (Stratagene, La Jolla, CA) for the production of phage particles bearing scFvs using methodology as described (Marks *et al.*, 1991; Ward, 1995). Phage particles were concentrated by polyethylene glycol precipitation and panned using 30 μl (10 mg/ml) aliquots of tosylactivated Dynabeads M-280 (Dynal, Norway) coated with purified 85.33 Vα at a density of 20 μg Vα per mg of beads. 85.33 Vα was coupled to the beads using the manufacturers' instructions and stored with 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at 4°C. Beads were incubated with 2% w/v milk powder/PBS for 2 hr immediately before panning. Aliquots of concentrated phage were diluted two-fold in 2% w/v milk powder/PBS and incubated with the beads for 2 hr at room temperature, with agitation. Beads were washed 10 times with 0.1% Tween/PBS, once with 50 mM Tris–HCl pH 7.4/0.5 M NaCl and bound phage eluted with 100 mM triethylamine. Eluted phage was immediately neutralised by the addition of a 50% volume of 2 M Tris–Cl pH 7.4 and used to infect exponentially growing *E. coli* TG1 cells as described (Marks *et al.*, 1991). The *E. coli* transfectants that were isolated by reinfection with eluted phage from the second round of panning were transferred onto nitrocellulose as small colonies and grown overnight at 30°C on 2×TY (Miller, 1972) containing ampicillin (100 μg/ml) and 0.1 mM IPTG to induce expression of scFvs. Nitrocellulose filters were probed with biotinylated (Amersham biotinylation kits; Amersham, Arlington Heights, IL) 85.33 Vα and positive colonies replated and reprobed. Bound, biotinylated antigen was detected using streptavidin–horseradish peroxidase followed by 4-chloro-1-naphthol as substrate. Following identification of positive colonies, clones were grown up and induced for expression and culture supernatants analysed using ELISA as described by (Ward *et al.*, 1989). Plates were coated with the following antigens in PBS: 85.33 Vα and 1934.4 Vα (all at 10–20 μg/ml). The sequences of the VH and Vκ genes of positive clones were determined by dideoxynucleotide sequencing and Sequenase (USB Biochemicals Inc, Cleveland, OH).

Purification of scFvs

For purification of secreted scFvs using pHEN1 as expression plasmid, the non-suppressor strain *E. coli* HB2151 was used as host. Cells were grown up and induced for expression as described (Ward, 1992b) and myc tagged proteins purified from osmotic shock fractions using an affinity column made by coupling the 9E10 antibody (Evan *et al.*, 1985) to SpA–Sepharose followed by covalent crosslinking using dimethylpimelidate (Harlow and Lane, 1988). Bound scFvs were eluted using 0.15 M NaCl, 0.1 M glycine pH 3.0 and eluates neutralised by the addition of a 1/10 volume of 2 M Tris–HCl pH 8.0. Alternatively, the SpA–Sepharose and 9E10 antibody was used without crosslinking, and bound scFvs were eluted together with 9E10 antibody as scFv-9E10 complexes.

For purification of scFvs using Ni^{2+}-NTA–agarose, the genes were cloned as *Nco*I–*Not*I fragments into a derivative of Vβpel BHis (Ward, 1992b) with a *Not*I site inserted in translational frame into the *Bst*EII site. ScFvs were isolated from recombinant *E. coli* BMH71-18 cells as described previously (Ward, 1992b).

Biotinylation of scFvs

Purified scFvs were biotinylated using Amersham Biotinylation kits as recommended by the manufacturer.

Enzyme-linked immunosorbent assays

Microtitre plate wells were coated with 85.33 Vα or 1934.4 Vα at a concentration of 10–20 μg/ml in phosphate buffered saline (PBS). ELISAs with culture supernatants and detection of bound c-myc tagged proteins were carried out as described previously (Ward *et al.*, 1989). Purified scFvs were diluted into 3% bovine serum albumin/PBS prior to use in ELISAs. Bound biotinylated scFvs were detected using a 1:1000 dilution of streptavidin–HRP conjugate (ICN Immunochemicals).

Flow cytometry

Three T cell hybridomas were used: 85.33 (Vα11, Vβ8.3) (Myers *et al.*, 1993), 1934.4 (Vα4, Vβ8.2) (Acha-Orbea *et al.*, 1988) and 4G4 (TCR α-β-, a generous gift of Dr J. Forman, UT Southwestern Medical Center, Dallas). Five $\times 10^5$ cells in 100 μl volumes were incubated with 1 μg purified scFv mixed with approximately 0.5 μg 9E10 antibody (to generate bivalent/multivalent scFvs to improve avidity). Alternatively, the 9E10-scFv complexes eluted from the SpA–Sepharose column were used without additional 9E10. After washing, bound 9E10-scFv complexes were detected using anti-mouse IgG conjugated to fluorescein (anti-mouse-FITC conjugate; Southern Biotechnology Associates Inc., U.S.A.) at a dilution of 1:50. Cells were analysed using a Becton–Dickinson FACSCAN and data processed using the program LYSYS II (Becton–Dickinson, Franklin Lakes, NJ). The dead cells were stained with propidium iodide and electronically excluded from the analysis.

RESULTS

Generation of the scFv library and isolation of binding specificities

Prior to panning, to analyse the expression of scFvs by individual clones in the library, 14 colonies were picked, grown up and induced and culture supernatants analysed using immunoblotting and the 9E10 antibody. Ten clones expressed detectable levels of myc tagged proteins of scFv size (25–30 kDa) in the culture supernatant (data not shown). In addition, several myc tagged proteins of molecular weight of about 60–70 kDa were also observed in smaller amounts than the major 25–30 kDa species. *E. coli* TG1 was used as expression host in the immuno-

blotting studies, and therefore the higher molecular weight species are probably scFv-gene III fusion proteins due to partial suppression of the amber stop codon in pHEN1 (Hoogenboom *et al.*, 1991). The library was expanded and extruded phage panned using Dynabeads coated with 85.33 Vα. Following two rounds of panning, approximately 5000 *E. coli* clones (obtained by reinfection with eluted phage) were transferred to nitrocellulose and probed with biotinylated 85.33 Vα, and approximately 50% bound this antigen. Two hundred of these colonies that gave the strongest signal were replated, reprobed and 90% were positive. These clones were grown up individually, induced for expression and culture supernatants analysed for binding in ELISA, using the 85.33 Vα as antigen. Of these 200 clones, 20 gave strong signals in ELISA of culture supernatants and ELISA data are shown (Fig. 1) for 12 of these scFvs. To analyse non-specific binding, milk powder coated plates were used. In addition, the 1934.4 Vα which shares 30% amino acid homology with the 85.33 Vα (Acha-Orbea *et al.*, 1988; Ciubotaru and Ward, 1994) was also used. The scFvs that gave the highest and most specific signal (Fig. 1) were chosen for further analysis. To select these scFvs it was necessary to analyse the expression levels of the clones by immunoblotting, as the levels of expression usually differ from one scFv to another. A result of this was that, for example, scFvs 34 and 142 which give relatively weak signals in ELISA, were expressed at significantly lower levels than scFv 118. These scFvs were therefore also chosen for further analysis. Nucleotide sequencing analyses of the scFvs indicated that the VH and Vκ genes of these clones are different (Fig. 2). The VH genes are derived from subsets of the VHII family (A, B or C) and the Vκ genes from VκIII, IV or VI families (Kabat *et al.*, 1991).

To facilitate purification for use in flow cytometry, the scFv genes were recloned as *Nco*I–*Not*I fragments into a derivative of the vector Vβpel BHis (Ward, 1992b) for the expression of the Fvs as His_6-tagged proteins. The scFvs were expressed and purified using Ni^{2+}-NTA–agarose columns as described (Ward, 1992b). The purified proteins were biotinylated, and used in ELISAs with the same antigens as above. However, the level of non-specific binding by the biotinylated proteins was relatively high (Fig. 3), suggesting that the process of biotinylation itself, and/or the incorporation of biotin groups into scFvs resulted in misfolding of the recombinant proteins. Furthermore, storage of the biotinylated scFvs at 4°C for 6 weeks resulted in even higher non-specific binding, suggesting that time-dependent aggregation was occurring. A similar phenomenon has been reported by others (Kipriyanov *et al.*, 1994). Due to the relatively high non-specific binding, these scFvs were not analysed further. As an alternative method of purification and detection, therefore, the scFvs were expressed with carboxy-terminal c-myc tags using the phage display vector, pHEN1, and purified using the anti-c-myc antibody, 9E10, bound to SpA–Sepharose. For all scFvs purified in this way, the binding in ELISA was specific (shown for scFvs 5 and 118 in Fig. 3) and these scFvs were used in flow cytometry.

Flow cytometry

Figure 4 shows the staining of the three hybridomas following incubation with scFvs followed by the anti-myc antibody 9E10 and anti-mouse-FITC conjugate (solid lines). For some experiments, scFvs that were co-eluted with 9E10 from protein A–Sepharose (see Materials and methods) were used in the same way but without the addition of 9E10. For scFv 118, in particular, this method of purification resulted in better staining and this is discussed in more detail below. As controls, the shifts for the same hybridomas incubated with the Vβ8-specific monoclonal antibody F23.1 (Staerz *et al.*, 1985) followed by anti-mouse-FITC conjugate (A, solid lines) and with 9E10 followed by anti-mouse-FITC conjugate (dotted lines) are also shown, and the shifts with 9E10 are the same as for the cells incubated with anti-mouse-FITC conjugate only (data not shown). Three of the scFvs stain the 85.33 hybridoma cells with significant shifts, whereas scFv 5 does not recognize any of the cell lines. The staining pattern for scFv 50 is the same as that of scFv 5 (not shown). ScFv 118 does not show significant binding to either the 1934.4 hybridoma cells bearing a different Vα, or to the TCR negative cell line, 4G4. In addition to binding to 85.33 cells, scFvs 34 and 142 also bind to the 1934.4 hybridoma cells, but to a lower extent than the 85.33 cells. These scFvs do not stain the TCR negative 4G4 cells.

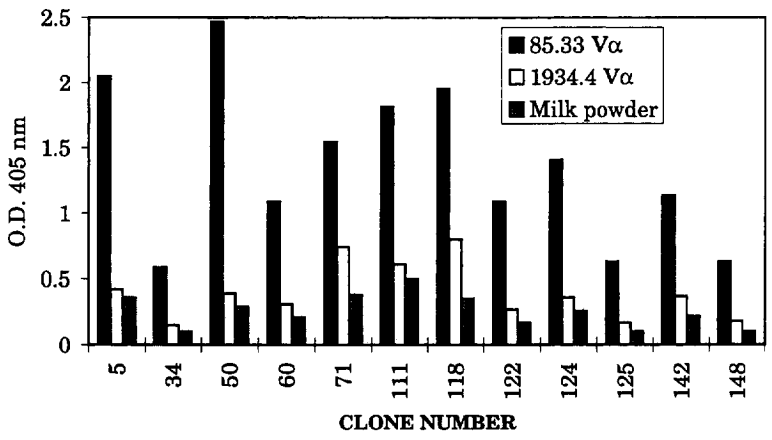

Fig. 1. ELISA data for culture supernatants for *E. coli* clones expressing anti-Vα scFvs.

VH sequences

```
    1         10         20         30         40         50         60

                                      CDR1                       CDR2
5   QVKLQQSGAELVRSGASVKLSCTASGFNIK DYYMH WVKQRPEQGLEWIG WIDPENGDTEY
34  QVQLQESGAGLVKPGASVKLSCTASGFNIK DTYMH WVKQRPEQGLEWIG RIDPANGNTKY
50  QVQLQESGADLVRPGASVTLSCKASGYTFI DYEIH WVKQTPVHGLEWIG TVDPETGGTTY
118 QVKLQESGAELVKPGASVKLSCKASGYTFT SYWMH WVRQRPGQGLEWIG EINLTNGRSNY
142 QVQLQESGPELARPGASVELSCKASGYTFT EYYVN WLKQRNGQGLEWIG EILPGSGSTNY

              70         80         90         100          110

                                                CDR3
    APKFQG KATMTADTSSNTAYLQLSSLTSEDTAVYYCST GTV------AY WGQGTTVTDSS
    DPKFQG KATITADTSSNTAYLQLSSLTSEDTAVYYCAR ANW------D- WGQGTTVTVSS
    NQKFKD KATLTVDKSSNTAYMQLSSLTSEDSAVYYCAR WLNGNYVSPDY WGQGTTVTVSS
    NEKSKN KATLTVDKSSSTAYMELSSLTSEDSAVYYCAR EEG--F---AY WGQGTTVTVSS
    NEKFKG KATFTADTSSNTAYMQLSSLTSEDSVVYYCAR YYRYDF---DY WGQGTTVTVSS
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Vκ sequences

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    1         10         20         30         40         50         60

                                   CDR1                          CDR2
5   DIELTQSPASLAVSLGQRATISC KASQSVDYDGDSYMN WYQQKPGQPLKLLIY AASNLES
34  DIELTQSPAIMSASPGEKVTITC SASSSV-----NYMY WYQQKSDASPKLWIY YTSNLAP
50  DIELTQSPTIIAASPGEKVTITC SASSSV-----SYMN WYQQKPGSSXKIWIY DTSNLAS
118 DIELTQSPAIMSASLGEKVTMSC RASSSV-----NYMY WYQQKSDASPELWIY YTSNLAP
142 DIELTQSPTLMAASPGEKVTITC SVGSSI-----SYMY WYQQKSGTSPKRWMY DTSKLAS

              70         80         90         100        110

                                          CDR3
    GIPPRFSGSGSGTDFTLNIHPVEEEDAATYYC QQNNEDP FTFGSGTKLEIKR
    GVPARFSGSGSGNSYSLTISSMEGEDAATYYC QQFTSSP WTFGGGTKLEIKR
    GVPVRFSNSGSGTSYSLTISRMEAEDAATYYC QEWSSYP WTFGGGTKLELKR
    GVPGRFSGSGSGNSYSLTISCMEAEDAATYYC QQWSSDP LTFGSGTKLEIKQ
    GVPARFSGSGSGTSYSLTISSMEAEDAATYYC HQWSSNP LTFDAGTKLEIKR
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Fig. 2. Sequences of the VH and Vκ genes of scFvs 5, 34, 50, 118 and 142. Sequences encoded by primers are underlined. CDR, complementarity determining region.

DISCUSSION

In this report, the isolation of scFvs that recognise recombinant soluble TCR Vαs is described and, of relevance for their use in T cell studies, a high proportion of these scFvs also bind to native T cells. The scFvs have been derived from mice following immunisation with soluble scTCRs and use of a bacteriophage display system with a recombinant Vα as antigen. Two rounds of panning resulted in the isolation of several hundred positive clones, and five of these were selected for further analysis. These scFvs have been purified and characterised using ELISA and flow cytometry. In the light of the apparent difficulty in making anti-TCR Vα antibodies, it is significant that three of these scFvs recognise the TCR on the surface of native T cells, and one (scFv 118) is specific for the Vα (Vα-11.1-Jα17) borne by 85.33 cells. Thus, using this approach, from a relatively small number of scFvs analysed, one scFv that shows specific recognition of the 85.33 Vα and two others with broader specificity for native Vαs can be isolated.

Although scFvs 5 and 50 bind well to recombinant Vα85.33 in ELISA, they do not recognise native Vα85.33 on the surface of T cells. This could be for one or more reasons; first, the epitope that is recognised may be masked by either the Vβ domain or CD3 polypeptides. This is consistent with the difficulties that have been encountered in generating anti-Vα antibodies, particularly when TCR-bearing cells are used as immunogens (Callan *et al.*, 1993; Choi *et al.*, 1991; Diu *et al.*, 1993; Zumla *et al.*, 1992). Second, the Vα region of the 85.33 TCR has a potential glycosylation site and this may mask the epitope. Nevertheless, scFvs 34, 118 and 142 are all able to bind to native Vαs, suggesting that immunisation with recombinant scTCRs is an effective route towards the generation of anti-Vα reagents.

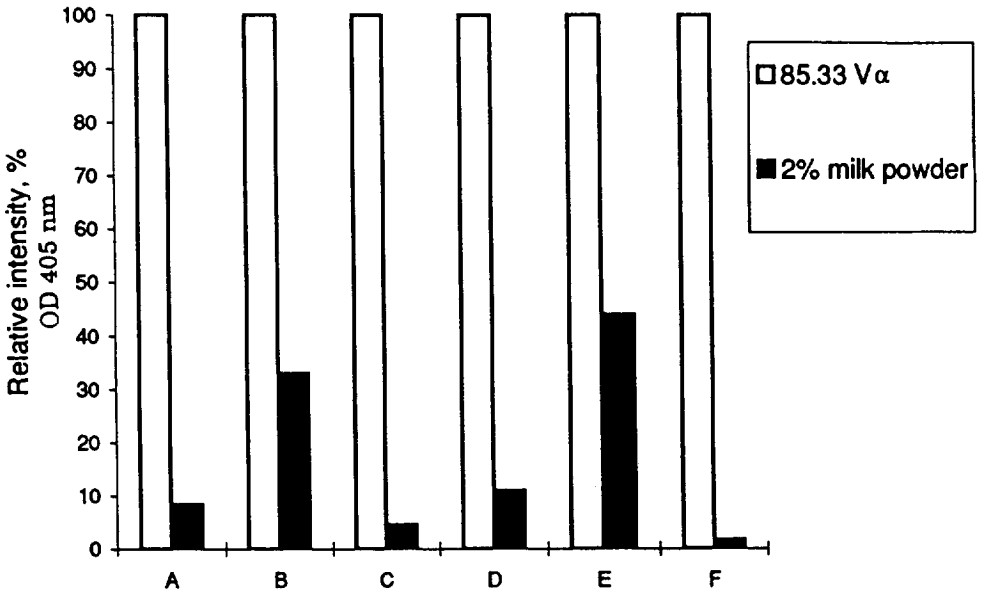

Fig. 3. Binding specificity of biotinylated His_6 tagged scFvs (A, B, D, E) isolated from clones 5 (A–C) and 118 (D–F) compared with c-myc tagged scFvs (C, F). Plates were coated with 85.33 Vα (open boxes) or milk powder (filled boxes). The proteins were stored at 4°C for 1 week (A, C, D, F) and 6 weeks (B, E).

The non-specific binding shown by the biotinylated scFvs may indicate disruption of the native scFv structure as a result of the biotinylation procedure. Although this has not been investigated further, the tendency of scFvs to multimerise and aggregate (Benhar and Pastan, 1994; Essig *et al.*, 1993; George *et al.*, 1994; Griffiths *et al.*, 1993; Holliger *et al.*, 1993; Whitlow *et al.*, 1994) suggests that the biotinylation procedure may result in aggregation. Consistent with this, it has been reported (George *et al.*, 1994) that an aggregated His_6-tagged scFv against the human transferrin receptor bound non-specifically to both the receptor expressing and non-expressing T cells. A further possibility that is not mutually exclusive is that during biotinylation, biotin groups have been incorporated into lysine residues in or in close proximity to the CDRs, and these may affect the interaction with the antigen. Therefore, the small size of scFvs, relative to complete antibodies for example, may limit the utility of biotinylation. This suggests that the use of epitope tags such as the c-myc peptide may be preferable for detection.

ScFvs have been reported to be expressed as monomers, dimers (Griffiths *et al.*, 1993; Holliger *et al.*, 1993) and higher order multimers (Essig *et al.*, 1993; Whitlow *et al.*, 1994), and therefore the data from our ELISAs and flow cytometry probably represent the interaction of bi- or multivalent scFvs with surface-bound antigen (on plastic or the surface of T cells). Furthermore, it is likely that in the presence of IPTG, as in this study, the numbers of gene III-fusion proteins per phage is greater than one and therefore selection on antigen-coated beads results in the isolation of phage that display two scFvs per particle. The need for at least one infective gene III protein per phage excludes the possibility that phages with 3 scFv fusions are selected and propagated (discussed in Marks *et al.*, 1992b). Consistent with the concept that in solution the scFvs multimerise or dimerise, size exclusion chromatography indicated that they formed higher order multimers (data not shown). Our attempts to express the scFvs as monovalent Fvs (unlinked VH and VL domains) by recloning the VH and Vκ genes into an Fv expression plasmid (Ward, 1995) were unsuccessful. For these Fvs, the VH:VL association appears to be weak and no heterodimers could be isolated from osmotic shock fractions, although both VH and Vκ polypeptides were expressed (data not shown). The higher avidity of dimeric or multivalent scFvs induced by the presence of the single chain linker (Essig *et al.*, 1993; Griffiths *et al.*, 1993; Holliger *et al.*, 1993; Whitlow *et al.*, 1994) has obvious advantages when surface-bound antigens are being considered, particularly if the surface-bound antigen is at sufficient density to allow crosslinking. Furthermore for scFv 118, in particular, we observed that co-elution of the recombinant protein with the 9E10 antibody from SpA–Sepharose was an efficient way of generating a scFv that stained 85.33 cells specifically and well. The reasons why this was improved over the other method of purification as a myc tagged protein, i.e. elution without complexed 9E10, is not clear, but may be due to an increased efficiency in multimerisation of the scFvs due to the bivalent nature of the 9E10 antibody. The heterogeneity of the staining by scFv 118 is also of note, and interestingly this suggests that the epitope recognised by this scFv is either differentially exposed or in different conformations on the surface of individual T cells.

The yields of the five scFvs that have been characterised in this study differ from each other over a wide range (0.2–2 mg/l). These yields are lower than those that we have observed for other scFvs (for example, D1.3 scFv, which is isolated in yields of five or more milligrams per litre of culture; E.S.W., unpublished data). The "rules" that govern the expression levels of (sc)Fvs are as yet not clear, but are probably related to stability of the folded

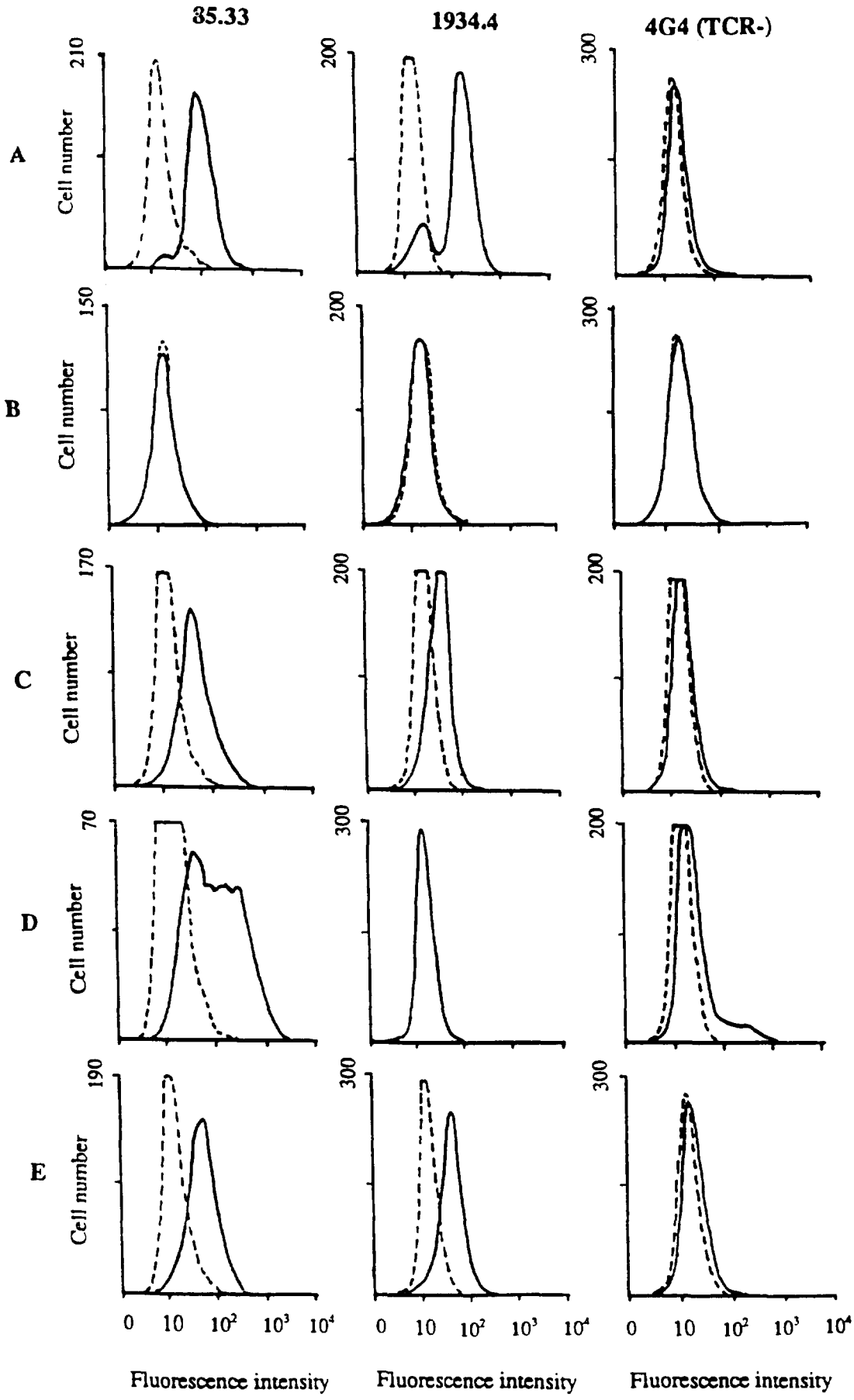

Fig. 4. Flow cytometry data for scFvs with three different hybridomas. Solid lines represent the fluorescence shifts for cells treated with scFv and 9E10 followed by anti-mouse-FITC conjugate. Dotted lines represent fluorescence shifts for cells incubated with 9E10 followed by anti-mouse-FITC conjugate. A, Vβ8-specific monoclonal antibody F23.1 (Staerz *et al.*, 1985); B, scFv 5; C, scFv 34; D, scFv 118 and E, scFv 142.

protein, its solubility and whether the linker peptide interferes with the folding pathway. In studies related to an anti-carbohydrate scFv, it was observed that mutation of Ile77 to Thr77 or Asn77 resulted in a 10-fold increase in expression yield (to 120–150 mg/l; Deng *et al.*, 1994). However, in scFvs 5, 34, 50 and 142, position 77 of the VH is Asn and for scFv 118, is Ser (Fig. 2). This suggests that threonine (or closely related serine) or asparagine at position 77 in VH sequences does not always result in high expression levels, but there are two possible reasons for this apparent discrepancy. First, the VH sequences of scFvs 5, 34, 50, 118 and 142 are quite different to that of the anti-carbohydrate scFv, and it may be necessary to have threonine/serine/asparagine in the "correct" context to have an effect. Second, different expression plasmids and conditions have been used in this study and the study of Deng *et al.*, 1994 and this makes direct comparison of expression yields difficult.

In these experiments, panning of the phage using antigen-coated Dynabeads was more effective for the isolation of scFvs with binding activities than the use of Immuno-tubes (Nunc, Naperville, IL). The reasons for this are not clear, but we have observed that for some plastic surfaces the level of non-specific binding (despite blocking with milk powder) appears to be quite high. With such surfaces panning results in the isolation of a high proportion of irrelevant phages that frequently do not express detectable levels of scFvs, due either to a lack of a complete open reading frame or very low expression (S.P. and J.G.H., unpublished observations). Moreover, *E. coli* clones that produce no scFvs/low levels outgrow the clones that produce higher levels of scFvs (S.P., unpublished observations). These irrelevant clones are therefore further enriched, possibly at the expense of the "binders", during the grow-up phase prior to the next round of panning.

The bacteriophage display system offers advantages over hybridoma technology (Köhler and Milstein, 1975) as many more clones can be sampled for the desired binding activity. This may account for the isolation of scFvs that bind to Vαs on native T cells, because when using other methods such antibodies have been difficult to produce and as a result, relatively few anti-Vα antibodies are available. It is also possible that the smaller size of the scFvs allows better access to the cell surface-bound TCR. However, this is unlikely as binding of 9E10-scFv complexes rather than, for example, scFvs labelled directly with fluorescein, was detected in the flow cytometry experiments.

In summary, this approach offers a rapid and convenient route for the production of anti-TCR V region antibodies. The isolation of scFvs that recognise native Vαs indicates that the (Vα) determinants on the recombinant scTCRs are representative of those on native T cells and is consistent with other data suggesting that the soluble TCRs are folded into a native state (Fields *et al.*, 1994; Ward, 1992b). The approach could readily be extended to the isolation of scFvs that recognise human TCR V regions, and the similarities between human and murine TCR sequences (Kabat *et al.*, 1991) suggest that human V domains will be expressed efficiently in the *E. coli* system (Ward, 1991, 1992b). Future experiments will be directed towards using complementarity determining region-derived peptides as antigens in panning in an attempt to isolate scFvs of higher specificity. Such antibodies could have value in analysing surface TCR expression by oligoclonal T cell populations at disease sites (Acha-Orbea *et al.*, 1988; Gold *et al.*, 1991; Howell *et al.*, 1991; Oksenberg *et al.*, 1990, 1993; Osman *et al.*, 1993; Paliard *et al.*, 1991; Wucherpfennig *et al.*, 1990) and ultimately may have uses as highly specific therapeutic tools to treat T cell mediated autoimmunity.

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