CHAPTER 5.

RESULTS & DISCUSSION

(Cloning of scFv Fragment Library in *E.coli* and Screening for Anti-GMCSF Specificity)
Cloning of scFv Fragment Library in *E.coli* and Screening for Anti-GMCSF Specificity.

After obtaining reasonably pure soluble GM-CSF in sufficiently large quantities it was decided to move to the next step which was the immunization of mice with this antigen.

Immunization was the first and the most critical step involved in generating an scFv library suitably enriched for anti-GMCSF specificity. The essence of immunization was to collect antibody producing cells from test animals (mice) at an appropriate time point after immunisation, using the antibody selection and enrichment occurring inside the mice body. Immunization increases the number of cells making an immune response, especially the levels of mRNA. Resting B cells make about 100 copies of Ig mRNA per cell, whereas a hybridoma (and also presumably a plasma cell) makes about 30,000 copies (Schibler, et al. 1978).

The choice of animal model for the study depends upon the type of material to be extracted from the animal. In our case the aim of generating an immune response was to isolate antibody secreting B plasma cells. The best source for these cells is the spleen of the animal. Mice (BALB/c) are small animals, requiring small amounts of the antigen for immunization, easy to handle even in large numbers. An average mouse spleen contains approximately $1 \times 10^8$ cells, enough to give a good mRNA / total RNA yield, as more number of animals can be handled as per the requirement. Hence we chose BALB/c female mice for our study. For most soluble protein antigens, rhGM-CSF in our case, the specific antibody begins to appear in the serum 5-7 days after the animal is injected. The antibody titre continues to rise and peaks around the 12th day. Along with becoming Ab secreting cells B-cells also differentiate and proliferate to form memory B-cells which quickly become activated after a booster dose is administered. Average affinity and degree of specificity of the antibody population for the antigen increases with repeated immunizations (Klinman and Press, 1975). Adjuvants greatly enhance the specific antibody titer as they allow the
antigen to be released slowly and thus ensure the continual presence of antigen to stimulate the immune system. Freund's adjuvants has been reliably and widely used for over 60 years (Fruends, et al, 1937). It is established now that the complete and incomplete Freund's adjuvant's (CFA and IFA respectively) immunization regimen, gives far superior antibody titers to the commonly used commercially available adjuvants (Johnston et al, 1991; Deeb et al, 1992; Smith et al, 1992). Hence we used Freund's adjuvants for immunizations. The schedule of immunization depends upon the final objective of the work. If the objective of immunizations is to obtain antibody secreting plasma cells, as we desired for our work, the intervals between the immunization and final use would be much shorter than if the intention is to generate memory B-cells. Initial injections with antigen emulsified in CFA are only given through the subcutaneous (SC) route while boosters may be given through intra-peritoneal (IP), intra-muscular (IM) or intra-venous (IV) routes. Accordingly we immunized 3 BALB/c female mice with 25μg / 0.1ml / mouse, protein antigen (soluble rhGM-CSF, more than 95% pure) emulsified in CFA, subcutaneously on the abdominal side of the animal. Mice were bled before primary immunization for non-immunized serum preparation. Two weeks later a booster of 20 μg / 0.1ml / mouse was given intra-peritoneally in IFA. After the passage of one week of boosting, mice were again bled to generate immune sera to check antibody titer in mice after immunization. Serum was tested in indirect rhGM-CSF coated ELISA. All of the three immunized mice gave good, comparable (standard monoclonal antibody), antibody titers (Fig. 5.1).

On the second week after the first booster all the mice were given a second booster (intra-venously), at a dose of 15 μg / 0.1ml / mouse, in PBS. On the fourth day after this booster all three mice were sacrificed for their

![Fig. 5.1: Anti-rhGMCSF antibody titer ELISA for BALB/c mice, after first booster immunization.](image-url)
spleen tissue. Lymphocytes were isolated from the spleen tissue and were subjected to total RNA isolation (total of 32.5 μg RNA was generated, using, RNeasy Mini Kit, Qiagen). This total RNA was used as template to prepare cDNA (at a concentration of 2.5 μg per cDNA reaction). Random oligo dT primers were (at a concentration of 1 μM) used to prime cDNA synthesis that generated cDNA:mRNA hybrids (Fig. 5.2).

Since this cDNA was to be used as the PCR template for \( V_H \) and \( V_L \) amplifications, double-stranded cDNA was not required. This cDNA:mRNA hybrid was used as a template for the primary PCR amplification of \( V_H \) and \( V_L \) domains. Specific primers designed for amplification of mouse \( V_H \) and \( V_L \) genes, are described in Table 3.1 (see Materials and Methods section). These have been shown to generate a diverse library when used to prepare a repertoire of antibodies from immunized mice (Clackson, et al, 1991). The BACK primer hybridizes to the beginning of the framework 1 of the antibody sequence and the FOR primer (in the case of the mouse kappa light chain the MJKFONX primer) binds to the end of the framework 4 (contributed by the J-segment during antibody rearrangement) (Fig. 5.3a). The mouse \( V_H \) genes were amplified using primers VH1BACK and VH1-FOR2. The primes VH1BACK and VH1FOR2 are consensus primers which covers the majority of mouse heavy chain gene families. The primer VK2BACK was used with a mix of four J region primers (MJK1FONX, MJK2FONX, MJK4FONX, and MJK5FONX) to amplify the light chain kappa families. The primer VK2BACK is a consensus primer that covers most of the
mouse kappa families. Hence to make the mouse repertoire, two primary PCR reactions were run one for \( V_H \) genes and one for \( V_L \) genes. Amplified

\[
\begin{align*}
\text{Primary PCR} \\
\text{Amplification of heavy and light chains from mRNA:cDNA preparation}
\end{align*}
\]

![Fig. 5.3a: Schematic representation of PCR amplification of \( V_H \) and \( V_L \) gene pool.](image)

\( V_H \) and \( V_L \) gene fragments were agarose gel eluted and quantified visually (Fig. 5.3b). Nearly 1 \( \mu \)g each of the gene fragments was amplified. These heavy and light chain domain were linked together to give a complete single chain Fv antibody sequence. For this assembly reaction a linker DNA fragment was used. This linker sequence creates a 15 amino acid sequence \((\text{Gly}_4\text{Ser})_3\) between the heavy and light chain variable domains (Clackson, et al. 1991). The linker molecule was originally prepared by oligonucleotide synthesis to generate the sequence:

\[
5' \text{ GGC ACC ACG GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG GAC ATC GAG CTC ACT CAG TCT CCA 3'}
\]

This oligonucleotide was then used as template for its own PCR amplification with \( \text{LINKBACK} \) and \( \text{LINKFOR} \) primers (Table 3.1, Materials and Methods) converting the single stranded linker oligonucleotide into double stranded linker molecule to be used in \( V_H/V_L \) gene
assembly reaction. The PCR amplified linker gene fragment was agarose gel purified (Fig. 5.4). 400ng of the linker DNA molecule was obtained after gel elution. One end of the linker DNA overlaps with the end of heavy chain (the J region) and the other end with the start of the light chain sequence. The assembly reaction is divided into two sub-PCR reactions. The first PCR reaction has two temperature steps (Denaturation temp. 94°C and assembly temp. 63°C) and 7 cycle’s run. In this reaction no amplifications are achieved and since the linker molecule has overlapping sequences, it holds the VH gene fragment at one end (5’end) and the VL gene fragment on the other end (3’ end) by hybridizing to the complementary sequences and this intermediate molecule serves as a substrate for the Taq polymerase to fill in the gaps and tether VH, linker and VL gene fragments as one complete scFv gene fragment. This assembled reaction mix is then amplified in the second PCR reaction using primers that incorporates sites for restriction enzymes. VH and VL domains were hence combined with the linker fragment in equal molar ratios (50ng VH, 50ng VL and 10ng linker DNA) in the first PCR reaction to get an assembled scFv sequence. Immediately in the second PCR reaction assembled scFvs were further amplified using primers which along with amplification of the assembled scFv also incorporates the sites for the enzymes Nco I and Not I (Fig.5.5a) to allow direct cloning in the pET22b expression vector. PCR amplified scFv gene fragment library was agarose gel purified (Fig. 5.5b) and quantified visually.

Thus this strategy was different from the well established protocol of generating a scFv library by phage display; here instead of cloning in a phagemid vector we directly cloned the assembled scFv gene library in an expression (plasmid) vector to get an scFv expression library in E. coli. This strategy has recently been
used by Stacy et al (2003) who have utilized this method for the isolation of high affinity antibodies directly from human single chain Fv antibody (scFv) expression

**Assembly PCR**

Assembly of heavy and light chains with linker

![Diagram](attachment:image.png)

**Incorporation of restriction sites**

PCR amplification and incorporation of restriction sites

![Diagram](attachment:image.png)

Fig. 5.5a: Preparation of a library of inserts encoding single chain Fv fragments
libraries. Since the same restriction sites are present in the MCS of the pET22b vector (as were introduced in the scFv insert) both vector and insert were digested with these restriction enzymes and agarose gel eluted. Ligation was set with a 3:1 insert to vector ratio (Fig.5.6). The ligation mix was used to directly transform BL21 (DE3) competent cells. However a low number of transformants was obtained primarily because the transformation efficiency of these cells is quite low. Since the success of finding high affinity antibodies lies in screening a larger library, we switched to cloning in DH5α cells which are known to have high transformation efficiencies. Competent DH5α cells were made and transformed with the ligase mix., a fairly large number of clones were obtained as colonies on LB ampicillin plates after over night incubation. To check whether these colonies contained the desired scFv gene inserts, plasmids were isolated from a few colonies and checked for size and insert fall out upon restriction digestion with Nco I and Not I. All the colonies checked contained the plasmid of the desired size and showed insert fall out. Cloning of the scFv gene fragment was also confirmed by PCR based amplification of the inserted scFv gene using the plasmid as template (Fig. 5.7). V gene repertoires when derived from the mRNA of lymphocytes (after immunization) are known to contain specific, antigen binding fragments which are created at a frequency of ~1/500 (Mullinax, et al. 1990). This is the probability of finding a desired specificity for binding to one single epitope whereas a single protein antigen displays a large number of epitopes on its surface depending upon its size and structural features. Hence, given that this scFv library was obtained with enriched VH and VL pools it was felt
that the scFv with the desired specificities would constitute at least 1% of the total scFv population. Therefore it was decided to screen 300 colonies in the first round so as to be able to isolate the desired scFv clone.

**Fig. 5.6:** Schematic representation for the construction of recombinant scFv library in pET22b expression vector.
Fig. 5.7: Cloning of scFv in pET22b vector

Lane1: Mol. Wt. marker.
Lane2: Restriction enz. digested and agarose gel eluted scFv gene library (insert).
Lane3: Restriction enz. digested and agarose gel eluted pET22b vector molecule.
Lane4: Clone 5 plasmid DNA.
Lane5: scFv PCR amplification with clone-5 plasmid DNA as template.
Lane6: Clone 9 plasmid DNA.
Lane7: scFv PCR amplification with clone-9 plasmid DNA as template.
Lane8: Clone 15 plasmid DNA.
Lane9: scFv PCR amplification with clone-15 plasmid DNA as template.
Lane10: Restriction digestion with clone-5 plasmid DNA (showing 730-735bp fallout fragment).
Rather than isolate a plasmid pool from all the transformants taken together. It was decided as safer strategy to isolate pure plasmid from single colonies and use them individually to transform BL21 (DE3) cells. Plasmid was therefore isolated from 300 such colonies of *E. coli* DH5α and used to transform BL 21 (DE3) cells separately in order to retain the genetic diversity of the library.

A few transformed BL21 (DE3) cells were checked for expression of the cloned scFvs (Fig.5.8a and 5.8b). It was observed that among the three, PCR confirmed clones; one did not express the scFv protein. This could be the situation with other clones as well, where expression level would be poor or non existent. However we have factored in this probability when we chose a fairly large number (300) of clones for expression studies. Therefore it was decided to proceed directly with ELISA screening. Thus the transformed BL 21 (DE3) cells were (after revival in LB) used directly for primary culture inoculations (thereby by-passing the plating step). We also increased the antibiotic selection pressure three times (ampicillin 300μg/ml) to repress the growth of untransformed cell. These primary cultures were grown in LB medium O/N and then used to inoculate secondary cultures in 5 ml LB medium supplemented with ampicillin (100μg/ml). Cultures were grown at 37°C with continuous shaking at 220rpm, and induced in mid log phase (which was obtained 2-4 hours post inoculation) with 1 mM IPTG. Cells were harvested at the 4th hour post induction since we had observed that the expression levels usually peak at this time point (Fig.5.8 a, b). 1 ml of the culture of each of the 300 clones expressing scFv was retained for ELISA screening. It was observed (Fig.5.8a, b) that the expressed recombinant protein (from most samples) was seen as a single protein band on 12% SDS-PAGE, corresponding to a molecular weight of ~ 30kDa, suggesting that the pelB signal sequence was not processed. Usually the expression of a recombinant antibody in the reducing environment of the cytoplasm of *E. coli* leads to the formation of inclusion bodies (Sanchez, et al, 1999; Tsumoto, et al, 1998). It has been shown that mild detergents that do not bind too strongly to the protein (Tanford, 1968) can be used to solubilize inactive proteins (Lacks and
Springhorn, 1980; Kurucz et al, 1995). Hence primary ELISA samples were made by solubilizing the cell pellet itself in 2% SDS (sodium dodecyle sulphate detergent). We had already observed that the level of expression of the scFvs...
Cloning of scFv

was fairly high, between 10-20% of the total cellular protein. Hence the 1 ml pellet was calculated to have at least ~ 50 μg scFv protein (a visual estimation of the induced protein band on SDS PAGE of the total cell lysate also gave estimates). The pellet was therefore diluted prior to testing by ELISA. It was felt that the dilution with buffer (PBS) would also help partial refolding and thus allow better binding to GM-CSF. 96 well ELISA plates were coated with 100 μl of antigen (rhGMCSF) solution at a concentration of 5μg./ ml. The SDS solubilized cell pellets were diluted 1000 fold and added to the coated wells. The wells were washed PBS containing 0.05% Tween 20. cloning in the pET22b expression vector produces scFv proteins with a C-terminal His tag which can be used for detection. Antigen bound scFvs were therefore picked up through this His tag by using anti- His tag antibodies as the secondary detection reagent. Thus primary screening by ELISA was done with all the 300 transformed BL21 (DE3) cells expressing scFvs. This primary screening gave 15 positive clones. Absorbance at 450nm of all positive clones ranged from 1 to 0.1 against the standard antibody absorbance of 1.6. We picked clones with varying absorbance values because false candidates would be screened out in the subsequent stringently conditioned ELISAs. These 15 clones were again expressed in BL21 (DE3) cells. A few induced cell cultures were sonicated to break the cells and SDS-PAGE analysis of the supernatant and pellet fractions (generated after centrifugation of the sonicated samples) showed that the scFv protein was obtained in the pellet fraction confirming that the scFv proteins were indeed expressed as inclusion bodies inside the cell (data not shown). This observation provided us with the means to purify the expressed scFvs. All 15 scFv clones were therefore expressed in BL21 (DE3) cells and inclusion bodies were isolated. IBs were washed with 1% DCA to get fairly pure IBs. scFvs were quantified on 12% SDS-PAGE visually. 5 μg of all IBs were solubilized in 100 μl of denaturation buffer (50mM Tris Cl, 8M urea, 1.5mM DTT), incubated 30 minutes at room temperature and dilution refolded with PBS to a final concentration of 0.5 μg/ ml. Disulfide bonds were allowed to be formed by simple air oxidation (Anfinsen et al. 1961). In some cases disulphide bond formation has been shown to be promoted
by the presence of metal ions (Saxena and Wetlaufer, 1970), hence we did not include EDTA in our renaturation buffer (PBS). We could expect correct refolding as the final protein concentrations were kept very low. These refolded scFvs were directly used in the second ELISA screening where they were added at a final concentration of 0.5 μg/ml and hence 0.05 μg/well. The renaturation buffer contained no denaturants hence the 100 fold dilution reduced the initial 8 M urea to a final concentration of 0.08 M and the initial 1.5 mM DTT to a final concentration of 0.015 mM making them ineffective to interfere with the ELISA. This second round of screening with ELISA was made stringent by lowering the level of antigen concentration. Compared to the first screening, rhGM-CSF was coated at 10 fold lower concentration (0.5 μg/ml i.e. 0.05 μg/well). Also the scFv proteins were better refolded this time and almost no contaminating E. coli proteins were present allowing better binding and hence selection of the specific antibody by ELISA. This second ELISA screening resulted in the selection of four anti-GMCSF scFvs from among the 15 primary clones (Fig. 5.9).
The clone selected after the second round of screening were subjected to a final confirmatory ELISA with the same reaction conditions as expressed earlier. These four clones were thus expressed again in BL21 (DE3) cells and subjected to inclusion body isolation. Inclusion bodies were given 1% DCA wash and were then subjected to dilution refolding. All four scFv clones tested reproduced similar binding specificities (Fig. 5.10).

All four clones were then sequenced and as expected each had a unique DNS sequence demonstrating that completely different $V_H$ and $V_L$ combinations can give rise to the same specificity w.r.t. a particular antigen (for complete DNA sequence, see appendix). These clones were then used for further experiments in an attempt to improve their binding specificity to GM-CSF.

As is clear from the Fig. 5.10 the binding specificities shown by these clones were lower than that of the anti-GMCSF monoclonal antibody used as a positive control in the ELISA screenings.

A number of strategies have been used to modulate antibody:antigen interactions. These mostly include a) site specific mutagenesis based on the crystal structure or homology models of the antibody: antigen complex and (b) chain shuffling. Chain shuffling and site-directed mutagenesis involve alterations to the intrinsic affinity of the monovalent antibody fragment. In chain shuffling, the scFv molecule is subjected to cycles of manipulations in which the gene for one chain (e.g., $V_H$) is cloned into a repertoire for the second chain ($V_L$) (Marks et al., 117).
Cloning of scFv

1992). The resulting library, that contains scFv-phage with $V_H$ chains specific for the antigen and random $V_L$ chains, is panned against the target antigen to identify clones with improved binding characteristics. This method provides additional diversity, particularly in the framework regions of the antibody fragments and hence, the potential for significant increases in affinity. Using this methodology, one group have increased the affinity of the anti-HER2/neu C6.5 scFv, 6-fold, from $1.6 \times 10^{-8}$ to $2.6 \times 10^{-9}$ M (Schier et al., 1996).

The chain shuffling experiments are generally performed by combining e.g. VH of one specific antibody with the repertoire of light chain and vice-versa. After having identified four clones that react to our antigen of interest i.e. rhGM-CSF we wanted to create one clone, which would show the highest affinity. We devised a simple protocol, applying the principles of chain shuffling but at a smaller scale. The aim of this particular experiment was to find out a better combination of $V_H$ and $V_L$ among these four clones itself. For this chain shuffling experiment, $V_H$ and $V_L$ of all four clones were again PCR amplified (same primers were used as for the first $V_H$, $V_L$ amplification) (Fig. 5.11).

scFv joining with the linker peptide was performed using the same protocols as were used for generation of primary library of scFvs. The new combinations are depicted in table (5.1). All of the 12, light chain shuffled, scFvs were cloned in pET22b vector. Inclusion bodies were isolated and purified (Fig. 5.12a and b). Among shuffled clones, clone I and clone III did not express any recombinant scFv protein. ELISA was performed with the rest of the shuffled clones and
Table 5.1: shuffled light chain combinations

<table>
<thead>
<tr>
<th>Combination</th>
<th>Heavy chain</th>
<th>Light chain</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>5L</td>
<td>clone I</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>56L</td>
<td>clone II</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>196L</td>
<td>clone III</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>4L</td>
<td>clone IV</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>56L</td>
<td>clone V</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>196L</td>
<td>clone VI</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>4L</td>
<td>clone VII</td>
</tr>
<tr>
<td>8</td>
<td>56</td>
<td>5L</td>
<td>clone VIII</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>196L</td>
<td>clone IX</td>
</tr>
<tr>
<td>10</td>
<td>196</td>
<td>4L</td>
<td>clone X</td>
</tr>
<tr>
<td>11</td>
<td>196</td>
<td>5L</td>
<td>clone XI</td>
</tr>
<tr>
<td>12</td>
<td>196</td>
<td>56L</td>
<td>clone XII</td>
</tr>
</tbody>
</table>

Fig. 5.12a: SDS PAGE of purified inclusion bodies:
Lane M: Mo. wt. marker; Lane 1: scFv IBs of clone-4; Lane 2: scFv IBs of clone-5; Lane 3: scFv IBs of clone-56; Lane 4: scFv IBs of clone-196; Lane 5: scFv IBs of clone-II; Lane 6: scFv IBs of clone-IV; Lane 7: scFv IBs of clone-V; Lane 8: scFv IBs of clone-VI; Lane 9: scFv IBs of clone-VII;
the four original clones (Fig. 5.13). Finally the three best clones were selected: clone 4, clone 196 which were the clones obtained in the original screening and light chain shuffled clone VL (5 heavy chain + 196 light chain). The one shuffled clone selected among 10 different shuffled clones showed lesser absorbance signal than the original clone 4 and clone 196. Thus it is clear that the shuffling of \( V_H \) and \( V_L \) gene pools in the initial step had given rise to scFvs with moderate binding specificities which implies that the chain repertoire to be shuffled should be increased to find the high affinity binding scFvs. These three clones were further subjected to expression studies at shake flask level to select a clone with high expression in \( E. coli \) as one important aspect and aim of this work was to achieve high level expression of the specific scFv.
Cloning of scFv

Fig. 5.13: ELISA analysis of GM-CSF binding scFvs (four original clones selected in the primary and secondary ELISAs and 10 light chain shuffled (generated from light chain shuffling between the original four clones) scFv clones.