Construction of a Recombinant Immunotoxin

by

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Chapter 1

IMMUNOTOXINS IN CANCER THERAPY

Declaration

The experiments presented in this thesis were carried out by myself, except where indicated in the text. None of the material has been presented previously for the purpose of obtaining any other degree.

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ABSTRACT

In recent years a number of therapeutically useful immunotoxins have been produced using recombinant gene technology. In general, this involves fusion of a toxin gene with sequence encoding a variety of clinically relevant proteins or peptides. Using these techniques a recombinant immunotoxin has been engineered by fusing the genes encoding an antibody fragment with the sequence of a small cytolytic peptide, melittin. The antibody fragment consists of the antigen binding site derived from a murine monoclonal antibody K-1-21, which binds to human free *kappa* light chains and recognises a specific epitope (KMA) expressed on the surface of human myeloma and lymphoma cells. The toxic portion of the molecule is melittin, a 26 amino acid, membrane lytic peptide which is a major component of bee venom. Using PCR a single chain Fv (scFv) was constructed by linking V_H and V_L genes with an oligonucleotide encoding a flexible, hydrophilic peptide. The melittin gene was synthesised as an oligonucleotide and extended by PCR. Nucleotide sequence encoding a linker peptide was added to the 5' end and a primer encoding a FLAG peptide was used to extend the 3' end. This gene construct was then ligated into the recombinant expression vector, pPOW scFv, to create the fusion gene encoding the recombinant immunotoxin. The gene construct was expressed in the periplasm of *E.coli* (TOPP2) using the secretion signal pelB. Expression of the foreign protein was monitored by western blot using a monoclonal antibody which recognises the FLAG peptide encoded at the carboxy terminal region of the gene construct. Expression of the recombinant immunotoxin was optimised and the resulting protein was purified using anti-FLAG M2 affinity chromatography. Antigen binding activity was assessed by ELISA and flow cytometry using a human myeloma cell line, HMy2, which expresses the KMA antigen.Binding of the immunotoxin to a control human cell line, K562, which does not express KMA on the cell surface was also assessed. The results indicated that the recombinant immunotoxin retained antigen binding specificity and it was cytotoxic towards the target cell line (HMy2).

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LIST OF ABBREVIATIONS

AEBSF	4-(2-Aminoethyl)-benzenesulphonyl fluoride hydrochloride
dATP	deoxy-adenosine 5'-triphosphate
ABMT	autologous bone marrow transplantation
ADCC	antibody dependent cell mediated cytotoxicity
az	sodium azide
BCIP	bromochloroindolylphosphate
BSA	bovine serum albumin
CD	cluster of differentiation
CDR	complementarity determining region
DMSO	dimethyl sulphoxide
DT	diphtheria toxin
EDTA	ethylenediamine tetra acetic acid
Eth.Br	ethidium bromide
flag	an octapeptide which is recognised by the monoclonal antibody anti-FLAG M2
FITC	fluorescein isothiocyanate
HAMA	human anti-mouse antibody
HMy2	*kappa*-myeloma cell line, LICR LON/HMy2
Ig	immunoglobulin
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
IL-4	interleukin-4
IL-6	interleukin-6
KMA	*kappa* myeloma antigen
mAb	monoclonal antibody
MWt	molecular weight
N	Avogadros number
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	*Pseudomonas* exotoxin
PEG	polyethylene glycol
RTA	ricin toxin A chain
SDS	sodium dodecyl sulphate

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
T-ALL	T-cell acute lymphoblastic leukemia
X-GAL	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

1.1 *INTRODUCTION*

Conventional approaches to the treatment of human cancers include chemotherapy, radiotherapy and surgery. Despite recent improvements in all three areas there are still a number of obstacles to cytoreductive therapy. Firstly, tumour cells can become resistant to chemotherapeutic agents. Secondly, the use of high doses of chemotherapeutic agents results in significant nonspecific side effects in patients. Treatment for these side effects involves autologous bone marrow transplantation (ABMT). These problems suggest that the development of new cytotoxic agents should involve a mechanism for specifically recognising and killing cancer cells whilst sparing normal cells. One group of reagents which potentially fulfil these criteria are the immunotoxins. An immunotoxin consists of a targeting agent which binds to an antigen expressed on the cell surface and is linked to a toxic molecule which is capable of killing the target cell once the ligand has bound. The development of immunotoxins as therapeutic agents will be described in detail in this chapter.

1.2 *THERAPEUTIC ANTIBODIES*

Although the use of antibodies in clinical therapy has been widely accepted in the last two decades the concept of immunotherapy originated many years ago. In 1899, Elie Metchnikoff demonstrated that antiphagocyte serum could block phagocytosis *in vitro* and he proposed that,"the antiphagocytosis serum could be used in order to weaken the reactions of the organism". Several years later Paul Ehrlich extended this idea to include targeted toxins by suggesting that *Zauberkugeln* or "magic bullets" could be used to target a toxic substance or toxophore to tumour cells and selectively kill these cells (Blakey, 1992). Despite these early predictions it wasn't until the pioneering work of Kohler and Milstein (1975) that substantial advances in antibody therapy were made. These investigators achieved the immortalisation of individual B cell clones from immunized mice by fusion with an established mouse plasmacytoma cell line. The resulting cells secreted monoclonal antibodies of defined antigen specificity which could be produced in large quantities *in vitro*. A wide range of monoclonal antibodies have

been produced which bind to tumour associated antigens on the surface of cancer cells and to proteins found on the surface of normal tissue, as well as DNA, haptens and drugs. This technology has resulted in a vast number of monoclonal antibodies which are used in diagnostic and scientific research. For example, in classification of haematopoeitic cells, the definition and characterisation of the "clusters of differentiation" or CD antibody/antigen has led to better understanding of both B and T cell proliferation and differentiation (Barclay *et al.*,1993). Despite the many successes in other areas, the therapeutic applications of monoclonal antibodies have been disappointing. Since Sears *et al.* (1982) first published data related to administration of a monoclonal antibody, 17-1A, to patients with solid tumours at least four major problems associated with this therapy have become apparent. Firstly, solid tumours are not readily penetrated by the relatively large antibody molecules (150 kDa for intact IgG) due to the high interstitial pressure in tumours and their poor vascularisation (Jain, 1990).

Secondly, many monoclonal antibodies generated against cancer antigens crossreact with normal cells to some extent. For instance B72.3, which recognises a glycoprotein mucin-like complex (TAG72) found on 50% of breast carcinomas and 80% of colon carcinomas, reacts with secretory endometrium, transitional colonic epithelium and some human foetal tissues (Adair *et al.*, 1993). Another example of crossreactivity was demonstrated in phase 1 trials of patients with lung and colorectal cancer who received the monoclonal antibody, KS1/4, conjugated to vinblastin. Although the antigen was expressed at high levels on colon carcinoma cells it was also found on a wide range of normal tissue, including the duodenum and crossreactivity of the antibody resulted in serious side effects (Blakey, 1992). Treatment of breast cancer patients with the monoclonal antibody, 260F9, conjugated to ricin was also unsuccessful as the antibody crossreacted with Schwann cells and some patients developed severe neuropathies (Gould *et al.*, 1989).

Thirdly, administration of murine antibodies to humans often results in a human anti-murine antibody (HAMA) response. This response is usually observed 8-12 days after administration of the antibody and reaches a peak at 20-30 days after treatment (Routledge *et al.*, 1993). The production of neutralising human antibodies in the serum of these

patients means that treatment is limited to 10 days and the rapid onset of a secondary response prevents subsequent administration of the murine antibody. There are several examples of this occurrence in the literature. For example, patients treated with three different antibodies produced the HAMA response. These were B72.3 (Adair *et al.*, 1993), anti-Tac which was used to prevent allograft rejection in patients (Queen *et al.*, 1993) and 17-1A which recognises a glycoprotein on the surface of colorectal carinoma cells (LoBuglio and Khazaeli, 1993). An obvious solution to HAMA in patients is to use human monoclonal antibodies. Unfortunately, they have been difficult to produce as the resulting hybridoma cells are usually unstable. Furthermore, it is not possible to immunise humans with cancer cells in order to generate specific human monoclonal antibodies. Another problem with production of human hybridomas is that human tolerance mechanisms would prevent production of antibodies to human proteins.

Fourthly, murine monoclonal antibodies are not effective in activating human complement pathways or triggering antibody-dependent cell-mediated cytotoxicity (ADCC). Although the different murine isotypes functioned properly in animal models they were ineffective in humans resulting in a poor response to treatment. The reason for this was that the therapeutic antibodies could bind to the cell surface antigen but could not ellicit the cytotoxic pathways necessary to kill the cancer cells (Clark, 1993).

Early studies using proteolytic and chemical cleavage of the antibody molecule showed that it could be broken down into a number of fragments containing one or more of the characteristic immunoglobulin domains (Fig 1.1). Some of these fragments such as Fab (L chain + CH1), Fab'_2 (2 x Fab) and Fv (VL+VH) retain antigen binding specificity. Data from X-ray crystals of the Fab and Fv fragments have confirmed the domain structure of the variable region and provided information about the amino acid interactions which stabilise the three dimensional structure of these regions, including the antigen binding site. Knowledge of the primary sequence of a large number of antibodies was derived from amino acid sequencing of these fragments. These data and the subsequent development of the Polymerase Chain

Reaction or PCR (Mullis *et al.*, 1986 ; Saiki *et al.* 1988) have led to the next phase in antibody research, that of antibody engineering (Fig 1.2).

The cloning of immunoglobulin genes was a major turning point in the design of therapeutic antibodies and has led to tremendous development in this field (reviewed in Winter and Milstein, 1991 ; Morrison, 1992). In the last decade advances in antibody engineering and recombinant DNA technology have sought to address the problems associated with immunotherapy. For instance smaller antigen binding fragments such as Fab, Fv and single chain Fv (scFv), have an increased ability to penetrate solid tumours. These fragments can be generated using recombinant DNA technology and can be expressed as active polypeptides in prokaryotic expression systems (Bird *et al.*, 1988 ; Carter *et al.*, 1992 ; Skerra and Pluckthun, 1988 ; Better *et al.*, 1988 ; Huston *et al.*, 1988). Once the antigen binding site has been isolated the specificity can be manipulated by site-directed mutagenesis to improve binding (Denzin and Voss, 1992 ; Routledge *et al.*, 1993). The addition of different genes encoding toxic agents, such as *Pseudomonas exotoxin* (PE) and *Diphtheria toxin* (DT), has also enabled the production of single chain recombinant immunotoxins (reviewed in Blakey, 1992).

Isolation of human immunoglobulin genes and their subsequent expression in mammalian cells produced human antibodies which could potentially be used in therapy. As previously mentioned the advantage of using human antibodies is that the HAMA response can be averted. However, humans cannot be immunised to order and due to tolerance mechanisms there are few human Ig molecules of clinical relevance. For these reasons an *in vitro* selection system which allows random combinations of heavy and light chain variable genes isolated from human peripheral blood cells could supersede hybridoma technology. This artificial immune system has been devised by selecting antigen binding scFv and Fab fragments from large human antibody libraries expressed on the surface of filamentous phage fd (McCafferty *et al.*, 1990 ; Hoogenboom *et al.*, 1991). In theory, this technique should allow selection of unusual VH and VL combinations from the human immunoglobulin repertoire, which bind to novel human antigens. Once selected the antibodies could be further manipulated to improve specificity and affinity. Using this technique human antibodies to self

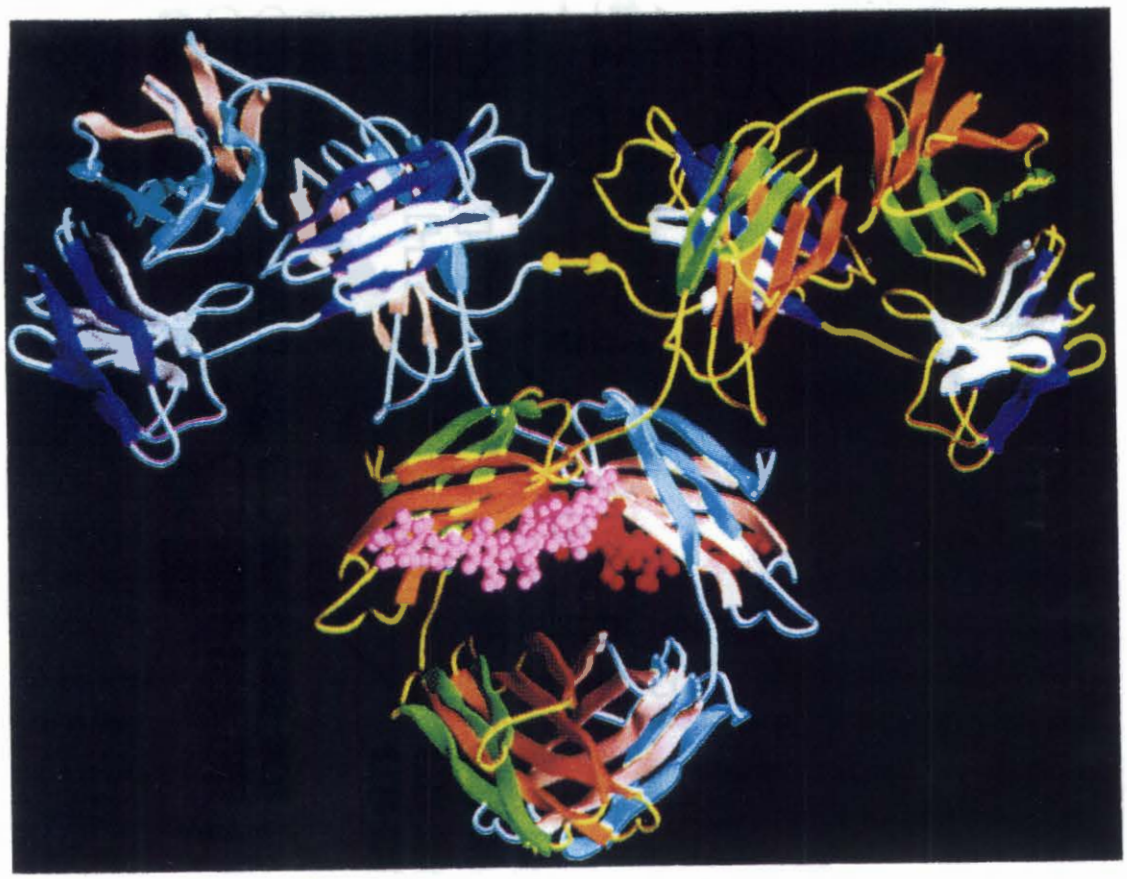


Fig 1.1 THE STRUCTURE OF IgG
The secondary and tertiary structure of the Mcg IgG1 immunoglobulin together with its N-linked oligosaccharides is depicted above as a RIBBONS model. One asymmetric unit consisting of the heavy and light chain is shown by yellow tubular backbone segments and the second heavy and light chain unit is shown white tubular backbone segments. The two V domains are located at the top on the right and left and the five-stranded β-pleated sheets are in green and cyan in the heavy chains and grey in the light chains. The three-stranded β-pleated sheets in the C domains have the same colour code as the five-chain layers in the V domains. Four stranded layers are orange and rose in the heavy chains and dark blue in the light chains. Oligosaccharide models in the Fc region are shown as groups of red or pink spheres. The disulphide bond between the penultimate residues of the two light chains is represented by a yellow ball and stick model. This model and the description of its features is taken from Guddat *et al.* (1993).

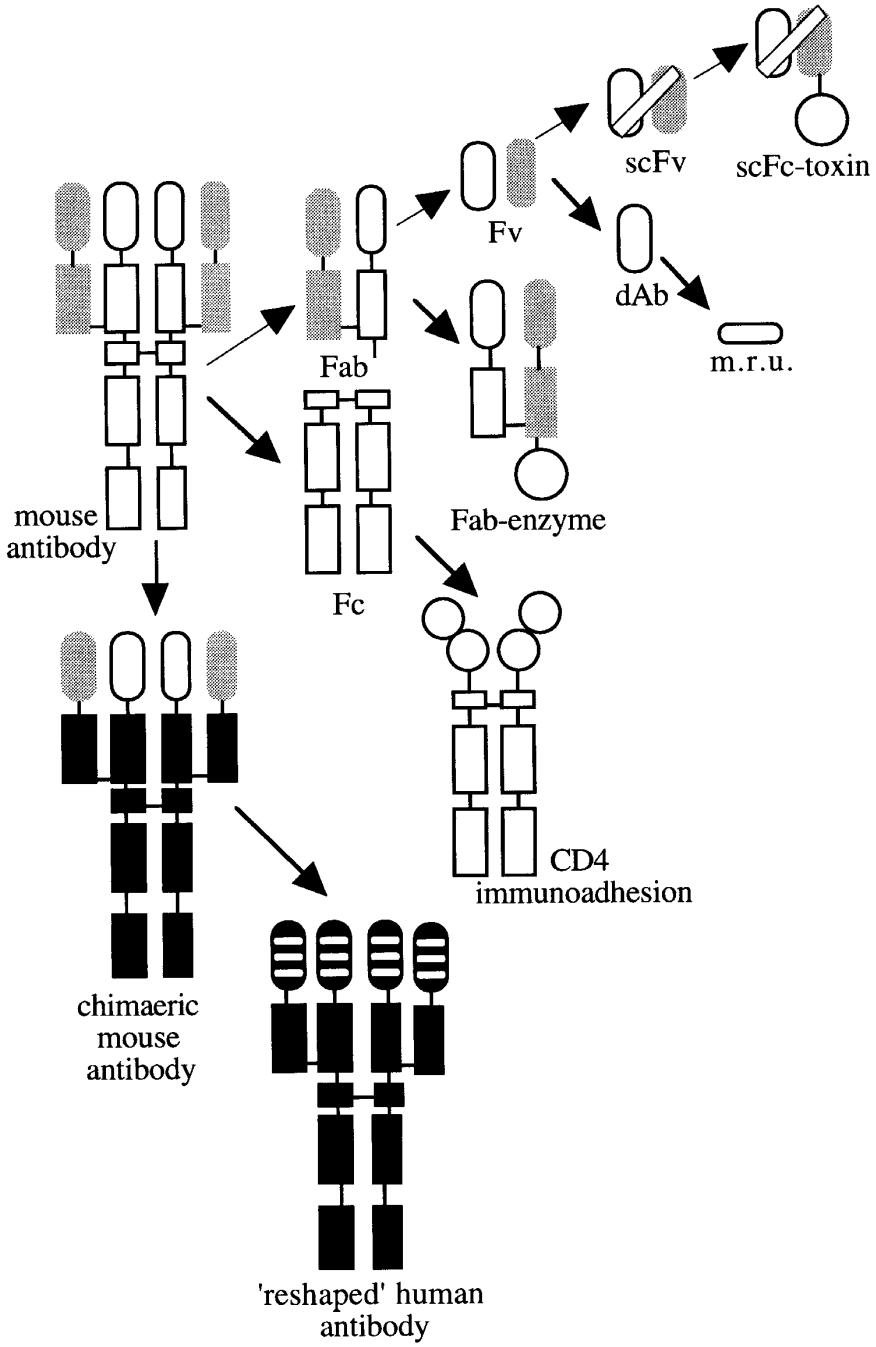


Fig 1.2 ENGINEERED ANTIBODIES AND FRAGMENTS
This is a diagramatic representation of antibody molecules and fragments which have been genetically engineered using recombinant DNA technology (adapted from Winter and Milstein, 1991). The oval shapes indicate the variable domains and the boxes depict the constant regions. A chimaeric mouse antibody consists of the mouse variable region and human constant domains. The reshaped human antibody is a human antibody molecule in which the 6 CDRs have been replaced by grafting the CDRs from a murine monoclonal antibody. The scFv is composed of H and L chain domains which have been linked by a flexible, hydrophilic peptide. Antibody fragments which retain antigen binding activity include Fab, Fv, and scFv. In some cases the VH domain (dAb) has been shown to bind antigen and the minimal recognition units (m.r.u.) can attach to antigen. The Fab has been used to target toxins and enzymes and the scFv fragment has been used to target toxins, drugs and radioisotopes. Fv and Fab fragments have been crystallized and have provided information about the three dimensional structure of the antigen binding site. A CD4 has been used to target an Fc fragment to the viral glycoprotein gp120 expressed on cells infected with HIV and can kill the target cells by elliciting ADCC.

antigen and unusual human related cancer antigens could be generated. This technique has great potential and has produced several antibody fragments from human Ig genes which bind to human specific antigens (reviewed in Griffiths and Hoogenboom, 1993 ; Burton and Barbas III, 1993).

There are several other approaches to reduce the HAMA response in human immunotherapy (Fig 1.2). For example the immunogenicity of murine antibodies can be circumvented by fusing the variable region genes from a murine monoclonal antibody, to the human constant region genes and expressing the chimaeric antibody in a mammalian expression system (Morrison *et al.*, 1984 ; Boulianne *et al.*, 1984 ; Xiang *et al.*, 1990). Alternatively, the complementarity determining regions in a human antibody can be replaced with those from a mouse monoclonal antibody resulting in a humanised antibody with the same specificity (Jones *et al.*, 1986 ; Riechmann *et al.*, 1988 ; Foote and Winter, 1992). Generally, the latter technique results in the loss of binding affinity. However, recent strategies in reshaping framework regions have resulted in improved antigen binding (Routledge *et al.*, 1993). Although further studies are required, *in vivo* administration of therapeutic chimaeric and humanised monoclonal antibodies appear to result in decreased immunogenicity (reviewed in Morrison, 1992). Humanization of the constant regions of murine monoclonal antibodies should also improve activation of effector functions in human therapy (Greenwood and Clark, 1993). Once again these techniques are relatively new and further studies are required to determine their efficacy.

From the literature cited above it can be seen that there are several approaches to improving immunotherapy by manipulating the immunoglobulin genes. However, the studies described in this thesis will concentrate on the development of targeted cancer therapy and then will focus on production of recombinant immunotoxins using antibody fragments, such as scFv, to target a toxin molecule.

1.3 *IMMUNOTOXINS*

As mentioned in section 1.2 the limited efficacy of monoclonal antibodies in clinical therapy has prompted many laboratories to devise more active cytotoxic agents. One way of achieving this is to attach a toxin moiety to the antibody molecule. An immunotoxin is composed of a targeting molecule which can either be an antibody which recognises a specific antigen or a ligand which binds to a specific receptor on the cell surface. This targeting agent is linked to a toxin which is capable of destroying the targeted cell and, ideally, should not bind non-specifically to any other cell. The conjugation of toxin to targeting agent should be stable in the circulation and should not significantly affect cytotoxicity.

1.3.1 TARGETING MOLECULES

There are a number of important considerations when choosing an agent to target an antigen or receptor. As previously mentioned many antigens on the surface of solid tumours are also expressed on the surface of certain normal cells. However, lymphoid malignancies can be targeted through lineage specific antigens which are present on mature and malignant cells but are absent from hematopoietic stem cells (Grossbard and Nadler, 1994). Thus normal cells which express the antigen and are killed by the immunotoxin can regenerate. The target antigen should be present on all clonogenic tumour cells otherwise cells which do not express the antigen can evade the targeted toxin. For instance B cells use hypermutation which generates immunoglobulin idiotypic heterogeneity and these cells can escape targeting by anti-idiotypic antibodies (Waldmann, 1992). The targeted antigen should not be present in the serum. In some interleukin-2 receptor (IL-2R) bearing malignancies soluble IL-2 receptors are shed into the serum (Waldmann *et al.*, 1992). This results in circulating antigen complexing with the immunotoxin and rapid clearance of the complex. In addition, a critical number of antigen molecules should be expressed on the cell surface and the targeted agent should have a reasonably high affinity for the antigen to optimise binding (Grossbard and Nadler, 1994).

Examples of antibodies and receptor ligands used to target lymphoid malignancies are quite varied (Table 1). In some cases the target is

lineage specific such as CD19, a pan B cell marker found on B cell progenitor cells and CD7 a pan T cell marker associated with prethymic T cells. Cytokines have also been used to target cells, for example IL-6, IL-4 and IL-2. A monoclonal antibody (anti-Tac) directed against the IL-2R alpha chain has been used to target cells bearing the receptor. The IL-2R is a heterodimer consisting of a 55 kDa a subunit (p55) and a 75 kDa b subunit (p75). The reason for using the a chain as a target is that normal resting T cells do not express this chain whereas abnormal T cells in some leukemia/lymphomas, certain autoimmune disorders and activated T cells such as those involved in organ allograft rejection do (Queen *et al.*, 1993). Other targets include receptors for growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF) and a-melanocyte stimulating hormone Waldmann (1992). He has also suggested that caution should be taken in therapeutic administration of growth factor-toxin agents as a less than toxic dose might stimulate the cancer cells to proliferate.

The studies presented in this thesis have used a murine monoclonal antibody, K-1-21, to target a specific antigen expressed on the surface of some lymphoma and *kappa* -myeloma cells (Boux *et al.*, 1983). K-1-21 binds to human free *kappa* light chains but does not bind to light chain associated with heavy chain. In addition, K-1-21 binds to the surface of a *kappa* -myeloma cell line, LICR LON/HMy2 (HMy2), and to plasma cells from patients with *kappa* -myeloma. This antibody does not bind to normal B cells nor to malignant cells expressing the lambda isotype. Cell surface expression of the kappa myeloma antigen (KMA), recognised by K-1-21, was *kappa* light chains associated with the cell membrane and in non-covalent association with actin (Goodnow and Raison, 1985). It was also demonstrated that the epitope on free *kappa* light chains, recognised by K-1-21, is dependent on the conformation of the C-*kappa* domain. Dimers of the light chain show decreased binding which is probably a result of conformational changes associated with interactions between the two *kappa* constant regions (Raison and Boux, 1985). Crosslinking of KMA with the monoclonal antibody K-1-21 does not result in internalisation of the antigen (Boux *et al.*, 1985).

From these studies it appears that K-1-21 binds specifically to target cells and recognises a tumour antigen on the surface of *kappa* -myeloma

cells which has a specific conformational epitope. This antigen is not present on either normal cells or non-related malignant B cells and thus should prove to be a specific cell surface target. As the antigen is not internalised after the antibody is bound a toxin which acts at the cell surface would be suitable for targeted cytotoxicity. Although a wide

Table 1 ANTIGEN TARGETS FOR IMMUNOTOXINS

TARGET	IMMUNOTOXIN	DISEASE	
CD22	Fab-RFB4-dgRTA	NHL	Vitteta *et al.*(1991)
	IgG-RFB4-dgRTA	NHL	Vitteta *et al.* (1992)
CD19	anti-B4-bR	NHL	Grossbard *et al.* (1992)
		ALL	
		CLL	
CD5	anti-T101-RTA	CLL	Hertler *et al.* (1989)
	H65-RTA	CTCL	LeMaistre *et al* (1991)
CD25 (IL-2R)	DAB_{486}IL-2	NHL	Strom *et al.* (1993)
	anti-Tac(scFv)-PE40	CLL	Chaudhary *et al.* (1989)
		CTCL	
		Hodgkins disease	
CD7	anti-CD7-R	ALL	Preijjrs *et al.* (1989)
EGF receptor	TGF-*a* -PE40	Lung, breast, head bladder, neck	Pastan and Fitzgerald, (1991)
IL-6 receptor	IL-6-PE40	Myeloma, hepatoma	Siegall *et al.* (1988)
Erb-B2 protein	e23(scFv)-PE40	Breast, ovarian cancer	Batra *et al.* (1992)
B3 antigen	B3(scFv)-PE40	Carcinomas	Brinkmann *et al.* (1992)

Some of the antigens which have been used for targeting immunotoxins are listed above. Abbreviations used are: NHL, non-Hodgkin's lymphoma ; ALL, acute lymphocytic leukemia ; CLL, chronic

lymphocytic leukemia ; CTCL, cutaneous T-cell leukemia. ; dgRTA, deglycosylated Ricin Toxin A chain ; PE40, truncated form of *Pseudomonas* exotoxin ; RFB4, anti-CD22 murine monoclonal antibody range of toxins have previously been used to produce immunotoxins most of them are metabolic inhibitors and exert their effects within the cell.

1.3.2 TOXINS

In general the toxins used in immunotoxin therapy are enzymes of bacterial or plant origin (Table 2). The most commonly used toxins are Pseudomonas exotoxin (PE), Diphtheria toxin (DT) and Ricin toxin A chain (RTA) and these will be discussed in more detail. Several ribosome inactivating proteins (RIPs) have also been used to create chemically linked immunotoxins. An interesting observation from phase I trials with all these immunotoxins has shown that most side effects (vascular leak syndrome, neutropenia and myalgia) are related to the presence of the toxin rather than crossreactivity of the antibody. Administration of these immunotoxins also results in the production of human anti-toxin antibodies and recently it has been suggested that milder human-derived toxins such as RNAases, DNAases and proteases might overcome both these problems (reviewed in Ghetie and Vitetta, 1994).

1. Pseudomonas Exotoxin (PE)

This toxin is the most widely used bacterial toxin and its molecular structure and function are well established. For instance, crystallisation of PE and determination of its three dimensional structure has led to greater understanding of its functional characteristics (Allured *et al.*, 1986). It is a single polypeptide chain of 66 kDa and consists of three structural domains each of which has a defined function. The latter was demonstrated when the PE gene was isolated from *Pseudomonas aeruginosa* and the cloned gene was expressed in a prokaryotic expression system (Hwang *et al.*, 1987). By expressing fragments of the protein which corresponded to the individual domains it was possible to define their functions. They found that domain Ia, which consists of amino acids 1-252, was responsible for cell binding. Domain II (amino acids 253-364) was necessary for translocation from the endosome into the cytosol and domain III (amino acids 400-613) was the ADP

ribosyltransferase which modified elongation factor 2 by ADP ribosylation. A minor domain, Ib, can be deleted without affecting the

Table 2. PLANT AND BACTERIAL TOXINS USED IN IMMUNOTOXINS

PLANT AND BACTERIAL TOXINS

Plant Holotoxins

Ricin
Abrin
Modeccin

Ribosome Inactivating Proteins (RIPs)

Pokeweed antiviral protein
Gelonin
Saporin

Bacterial Holotoxins

Pseudomonas exotoxin A
Diphtheria toxin

In general, the bacterial and plant toxins used in immunotoxins are polypeptide enzymes which catalytically inactivate protein synthesisactivity of the enzyme. Most PE-immunotoxins are derived from a truncated form of the polypeptide (PE40) which does not contain the cell binding domain and therefore does not bind non-specifically to cells. The mutant form PE38 lacks both domain 1a and 1b but retains cytotoxicity. In addition, ligands placed at the carboxy terminus of PE40 bind to antigen but do not kill cells. This is because the carboxy terminus contains the amino acid sequence RDELK which is

required for translocation into the cytoplasm (Fitzgerald *et al.*, 1992). Cloning of the PE gene has facilitated the construction of several fusion genes encoding recombinant immunotoxins and these will be discussed in greater detail in section 1.4.

The mechanism of toxic activity of PE has been elucidated and is initiated by binding of domain Ia to the cell surface followed by endocytosis and delivery to the endosomes (Pastan and Fitzgerald, 1991). In the endosomes the toxin unfolds and is proteolytically cleaved within domain II. This results in disulphide bond reduction and translocation of the 37 kDa carboxy terminal fragment into the cytosol where it inactivates elongation factor 2 and inhibits protein synthesis.

Injection of PE directly into cells showed that only a few molecules were required to kill the cell (Yamaizumi *et al.*, 1978). However, until recently most of the toxin molecules used in immunotoxins were not specifically directed to the cytoplasm but were degraded in the lysosomes (Waldmann, 1992). Thus, a larger number of molecules were required to bind to the cell surface and be internalised due to loss of activity through degradation. Subsequently it was shown that cytotoxicity was increased by changing the carboxy terminus sequence to KDEL which is an endoplasmic reticulum retention sequence and improves delivery of the toxin to the cytoplasm (Seetharam *et al.*, 1991). As this toxin acts in the cytoplasm it must be linked to a targeting agent which is internalised. For instance when conjugated to an anti-CD2 monoclonal antibody and a scFv fragment from an antibody to ovarian cancer cells (OVB3), PE was ineffective because the antigen was not internalised (Waldmann, 1992 ; Chaudary *et al.*, 1990a).

2. Diphtheria Toxin (DT)

Another toxin which has been used in a number of chemically linked and recombinant immunotoxins is the bacterial toxin DT. This toxin is composed of a single polypeptide chain and has a MWt of 62 kDa. The crystal structure of DT has been determined and the gene has been cloned and expressed in *E.coli*.(Choe *et al.*, 1992). It is similar to PE in that the native toxin consists of three domains. Unlike PE, domain I at the amino terminus of the protein contains the enzyme which is responsible for ADP ribosylation of elongation factor 2. Domain II

enables translocation from the endosome to the cytoplasm and domain III contains the cell binding activity (Fitzgerald *et al.*, 1992). Two serine residues at positions 508 and 525 are critical for cell binding. Therefore, to reduce non specific binding the domain at the carboxy terminus is removed from DT and replaced with a relevant binding molecule in order to produce DT immunotoxins. The mechanism of action and intracellular processing of DT is similar to PE and requires internalisation by the target cell where it acts as a potent inhibitor of protein synthesis in the cytoplasm (Pastan and Fitzgerald, 1991). Although several immunotoxins were made using a truncated form of DT the potential health hazard involved in working with this highly toxic protein has hindered the development of DT immunotoxins. Another problem in using DT immunotoxins in human therapy is the presence of neutralising antibodies in the serum of some patients due to active immunisation during childhood (Fitzgerald *et al.*, 1992)

3. Ricin

This toxin has been chemically conjugated to antibodies with a wide variety of specificities and the resulting immunotoxins have been used in several phase I clinical trials (Ghetie and Vitetta, 1994). Ricin is a plant toxin which is similar to PE and DT in that it kills cells by inhibiting protein synthesis. It is also a highly toxic protein. Like the bacterial protein, DT, ricin is composed of two polypeptide chains (A and B subunits) linked together by a disulphide bond and it has a MWt of 62 kDa (Fitzgerald and Pastan, 1989). The A chain is an N-glycosidase that depurinates a single adenine base in the 28S rRNA of the 60S ribosomal subunit. As this position is close to the elongation factor-2 binding site protein synthesis is inhibited. The B chain binds to the surface of cells by the interaction of its galactose residues with cell surface galactose-terminated oligosaccharides (Endo *et al.*, 1987). Internalisation is required for activity and translocation of the A chain across the cell membrane is mediated by the B chain (Olsnes *et al.*, 1989).

Initially it was difficult to produce a recombinant ricin A chain which was expressed in *E.coli* because the cellular proteases could not cleave the A chain from the B chain. However, the introduction of an arginine linker between the chains which is readily cleaved by cellular proteases has facilitated the production of recombinant ricin A chain (O'Hare *et*

al., 1990). At present, most ricin based immunotoxins consist of A chain which has been separated chemically from B chain, or produced by recombinant DNA technology. The ricin toxin A chain (RTA) is then chemically conjugated to targeting agents. Alternatively, non-specific binding to the cell surface is reduced by covalently attaching affinity ligands to the galactose residues of the B chain and blocking non-specific cell binding (Grossbard and Nadler, 1994). This strategy is more effective for an immunotoxin as it contains the B chain which is required for translocation to the cytosol.

It has been suggested that other plant hemitoxins or RIPs such as gelonin, saporin and pokeweed anti-viral protein could be used instead of ricin as they do not have the cell binding domains (Blakey, 1992). These toxins lack the translocation domain and therefore should be less toxic than native ricin but as toxic as the A chain alone.

Although ricin, DT and PE have been used in a wide range of immunotoxins they have several unfavourable characteristics. Firstly, they are all large molecules and even the truncated forms of the toxins are larger than the antibody fragments used for targeting. Secondly, they require internalisation by the cell to exert their toxicity and cannot be used to target cancer antigens which are not endocytosed. Thirdly, they are extremely toxic agents and are therefore difficult to handle in the laboratory. Finally, a significant percentage of the population have circulating neutralising antibodies to PE and DT.

As an alternative, there are several small peptide toxins which could potentially be linked to Ig molecules or antibody fragments to produce immunotoxins. These peptides are called pore-forming proteins (PFP) and are capable of lysing the cell membrane through a 'barrel-stave' mechanism. A number of cytolytic PFPs have been isolated from a wide range of sources including the gram positive bacterium *Staphylococcus aureus* which secretes staphylococcal ∂-toxin, *Xenopus laevis* which produce magainins, insects which produce cecropins and the European honeybee (*Apis mellifera*) which produces the membrane lytic peptide, melittin, (Young and Cohn, 1989 ; Ojcius and Young, 1991). As these PFPs act at the cell surface they do not require internalisation for toxicity. In general, they are less toxic than the metabolic toxins

described previously and are therefore easier to handle. Furthermore, they are small peptides (23-37 amino acids) and are not glycosylated which would facilitate cloning and expression in a bacterial system. For these reasons we have used the toxic peptide, melittin, to produce a single chain recombinant immunotoxin.

4. Melittin (mel)

Previous studies in our laboratory have determined that the membrane lytic peptide, melittin, can lyse human lymphocytes and is therefore a potential candidate for an immunotoxin (Weston *et al.*, 1994). Melittin is the major constituent of bee venom obtained from *Apis mellifera*. It is a basic peptide and consists of 26 amino acids with a MWt of 2840 Da. The amino acid sequence (Fig 1.3) has been determined (Habermann and Jentisch, 1967). The molecule is synthesised in the venom sac as a "prepromelittin" of 70 residues and is activated by cleavage of a 22

residue amino terminus signal peptide (Shipolini, 1984). Melittin is a single chain polypeptide and is shaped like a bent rod with an elbow angle of 120° (Fig 1.4). The first 10 residues form a straight alpha helix which is connected through the bend at residues 11 and 12 to the remaining residues which form a second alpha helix. The first 20 residues are arranged with the polar and charged residues on the lower part of the molecule and the nonpolar residues on the upper part of the molecule. At the carboxy terminal region residues 21-26 are arranged as charged and polar side chains around the entire circumference. The carboxy terminus contains four basic and two polar amino acids (residues 21-26) resulting in a highly charged tail (reviewed in Dempsey, 1990).

In an earlier study the three-dimensional structure of melittin was determined by X-ray crystallographic analysis (Terwilliger and Eisenberg, 1982). These workers showed that the polypeptide chains are packed in a tetrameric unit with two chains arranged anti-parallel to one another (Fig 1.5). The inter-chain interactions are made up entirely of the apolar side chains thus forming a hydrophobic core. The polar residues and the charged C-terminal residues are situated on the surface of the tetramer and form a hydrophilic coat around the structure. At

physiological salt concentrations and pH, melittin is present as a monomer at concentrations less than 4 mmol/L. At concentrations above this oligomers are formed.

Melittin binds to cells with a relatively high affinity (K_d 2 x 10^{-7} mol/L) and the amphiphilic nature of the amino terminal alpha helix results in a high affinity for the lipid/water interfaces. The polypeptide does not penetrate the cell membrane but the amphiphilic alpha helix is believed to insert into the outer laminate parallel to the water/lipid interface, with the hydrophobic portion in the lipid layer and the charged sidechains in the water layer. Lipid bound melittin has a similar conformation to the tetrameric form in aqueous solution. The highly charged C-terminal region is believed to alter the spatial conformation of the aminophosphate dipole making the lipid molecule susceptible to phospholipase A_2 attack. This perturbation of the lipid membrane results in holes in the cell membrane leading to the leakage of intracellular content and ultimately cell death (reviewed in Dempsey, 1990).

It is now apparent that specific residues within the molecule do not account for lytic activity but the overall structure is required for cell lysis. A synthetic peptide, peptide 1, has been produced (Degrado *et al.*, 1982) and is composed of two domains which have a similar structure to melittin but consists of different residues (Fig 1.3). The amino terminal region is hydrophobic and consists of 20 residues which form a amphipathic alpha helix. The carboxy terminal domain is composed of six hydrophilic residues which form a cluster of 3-4 positive charges.

Melittin mellifera:

1 10 20 26

G I G A V L K V L T T G L P A L I S W I K R K R Q Q

Peptide I:

L L Q S L L S L L Q S L L S L L L Q W L K R K R Q Q

Fig 1.3 THE AMINO ACID SEQUENCE OF MELITTIN AND A SYNTHETIC PEPTIDE, PEPTIDE I

The amino acid sequence of melittin was determined by Habermann and Jentisch, (1967). A synthetic peptide, peptide I, which has the same three dimensional structure as melittin but has a different primary sequence was created by Degrado et al., (1981). The amino terminus consists of hydrophobic residues (1-10) which tend to form an alpha helical configuration with amphiphilic character. The carboxy terminus is composed of hydrophilic residues which are predominantly positively charged (20-26). There is a bend in the middle of the molecule at positions 11 and 12 which is responsible for the bent rod shape of the melittin molecule.

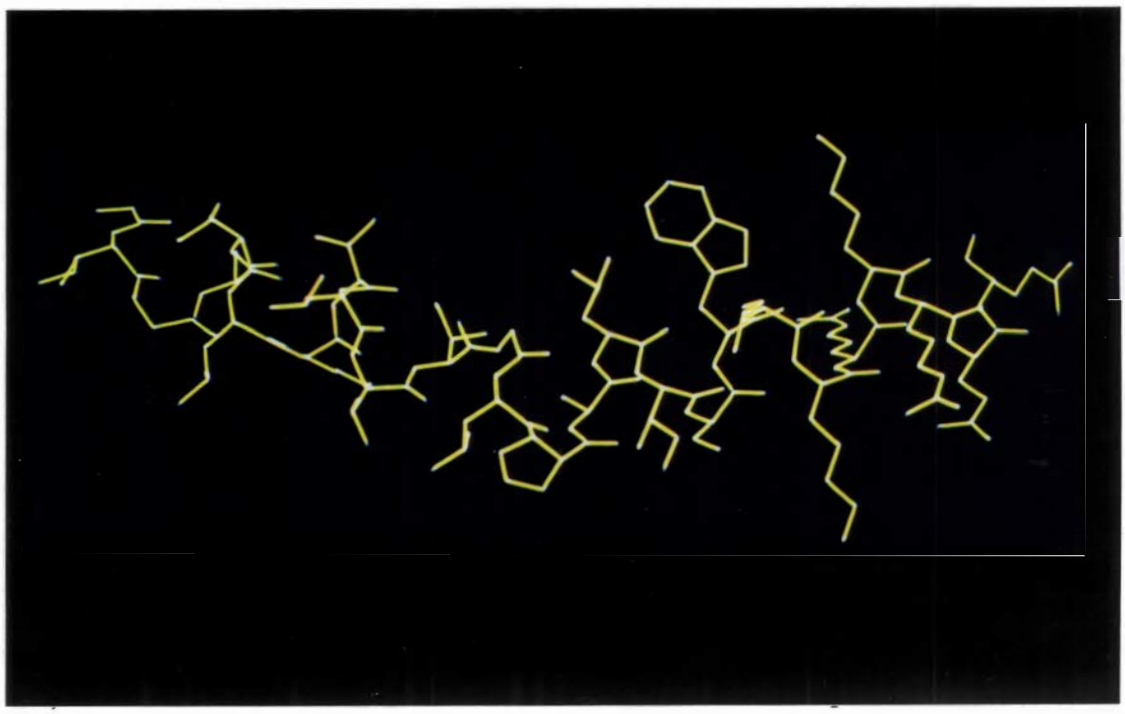


Fig 1.4 A COMPUTER MODEL OF THE CONFORMATION OF MONOMERIC MELITTIN

In this model the melittin monomer is shown as a carbon backbone with the amino acid side chains. Monomeric melittin has an alpha helix at both the amino terminus and at the carboxy terminus which are connected through a bend in the middle of the molecule giving the overall shape of a bent rod. The hydrophobic residues are arranged on the top of the amino terminus in this model and these form the hydrophobic core in the tetrameric structure. Polar and charged residues are situated below the carbon backbone of the amino terminus and these amino acids form the outer hydrophilic coat of the tetramer. After the bend in the molecule, which is quite distinct at the proline residue, the alpha helix is arranged with the residues around the circumference of the carbon backbone. The apolar.amino acids are orientated towards the top of the helix and the polar residues are on the outside of the molecule. Thus the apolar amino acids are aligned on one side of the rod and the polar and charged groups are on the opposite side giving melittin its amphipathic character.

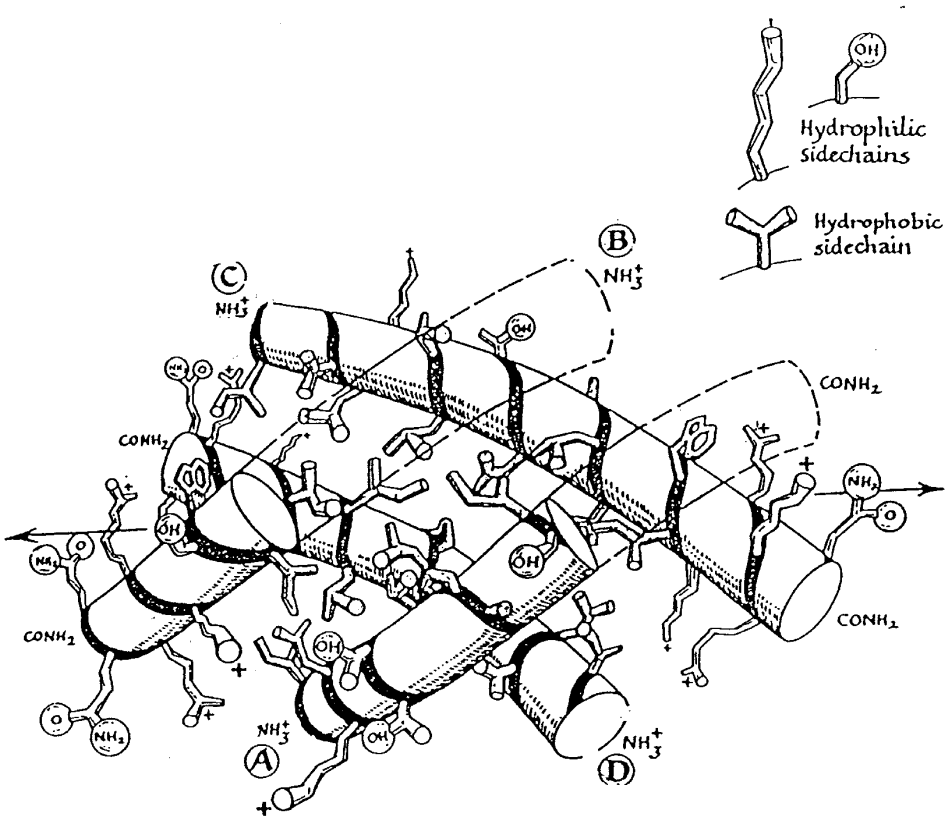


Fig 1.5 PACKING OF MELITTIN CHAINS IN THE TETRAMER
This picture of melittin monomers in the tetrameric conformation is adapted from Terwilliger and Eisenberg (1982). In the diagram it appears that the melittin helices form two antiparallel dimers which are packed together as a tetramer. The hydrophobic side chains (V, L, I and W) are arranged towards the inside of the tetramer and form a hydrophobic core. By comparison the polar and charged amino acids are found on the surface of the structure where they form a hydrophilic coat. Most of the hydrophilic residues are exposed to the solvent whereas the apolar amino acids are buried in the middle of the structure. The highly charged carboxy termini are spatially separated which indicates very little repulsion between the residues.

This synthetic peptide was similar to melittin in both structure and physicochemical parameters but it was 2.5 times more lytic. One explanation for the increased lytic activity is the longer hydrophobic region at the amino terminal.

Studies on 3T3 Swiss mouse fibroblast cells demonstrated that melittin causes 60% hydrolysis of lipids after 30 min and has a half-maximal activation at 3 μg/mL or 10 μmol/L (Shipolini, 1984). Recent work has shown that membrane perturbation due to melittin can be measured by 90° laser scatter on a FACScan flow cytometer (Weston *et al.*, 1994). A human cell line, HMy2, was incubated with a range of melittin concentrations and then analysed for 90° laser scatter. Binding was concentration dependent and using 100 μg/mL melittin the maximum cell death occurred after 2 min. Thus, cell lysis due to disruption of the membrane is fairly rapid.

By comparison, melittin is not as toxic as PE, ricin and DT which only require a few molecules to kill cells. Nevertheless, as mentioned earlier there are several advantages to using melittin in an immunotoxin. As it exerts its action at the cell surface it does not need to be internalised and transported to the cytoplasm to be effective. It is a small peptide and is not glycosylated. Therefore, it can be produced using recombinant DNA technology with subsequent expression in a prokaryotic system. The lower toxicity and absence of glycosylation may also reduce side effects such as capillary leak syndrome which is associated with tumour necrosis factor (TNF) release in response to glycosylated ricin immunotoxins. For example, it was shown that deglycosylation of ricin B chain significantly reduced both capillary leak syndrome and non-specific hepatic uptake (Grossbard *et al.*, 1992). The small size of a melittin based immunotoxin might be advantageous as it may be less immunogenic than larger immunoglobulin-toxin conjugates. However, this may not be an accurate assumption as the smaller size of the toxin may not significantly decrease immunogenicity. For instance, an Fab and Ig immunotoxin were equally immunogenic in human subjects (Grossbard and Nadler, 1994).

1.3.3 CHEMICALLY CONJUGATED IMMUNOTOXINS

In the past decade numerous chemically conjugated immunotoxins have been used in clinical trials with various degrees of success (Pastan and Fitzgerald, 1991 ; Ghetie and Vitetta, 1994). Initially toxins were conjugated to the targeting antibody using chemical crosslinkers and, as discussed earlier, all the toxins used in clinical trials have required some chemical modification to remove the cell binding domains (Fitzgerald and Pastan, 1989). These procedures were often inefficient and resulted in low yields as well as heterogeneous product. Recently, it has become feasible to employ recombinant DNA techniques which enable the production of single chain recombinant immunotoxins. However, several chemically conjugated immunotoxins are still being used in phase I and II clinical trials and these will be discussed briefly.

1. Ricin Immunotoxins

Unfortunately recombinant immunotoxins using ricin have been difficult to produce because a disulphide bond between the antibody and toxin is required to enable cleavage of the toxin and subsequent delivery from the endosome to the cytoplasm. As mentioned previously all ricin immunotoxins used in cancer therapy are chemically conjugated to either immunoglobulin (Ig) molecules or the Fab fragment of Ig.

An early ricin immunotoxin, anti-T101-RTA, was constructed from an antibody to the CD5 antigen and the ricin A chain. This conjugate was used to treat patients with chronic lymphocytic leukemia, CLL, (Hertler *et al.*,1989). Another RTA immunotoxin used to target the CD5 antigen was derived from the murine monoclonal H65 and was administered to patients with cutaneous T-cell lymphoma (LeMaistre *et al.*, 1991). In the first case only a transient decrease in white cell count was observed and in the second case only four out of fourteen patients showed partial responses with twelve out of fourteen developing HAMA.

In another study a monoclonal antibody to CD22, RFB4, was conjugated to a deglycosylated A chain to prevent nonspecific hepatic uptake of the immunotoxin. Similarly, an Fab from RFB4 was conjugated to a deglycosylated ricin A chain and both conjugates were used in clinical

trials involving patients with non-Hodgkin's lymphoma (Vitetta *et al.*, 1991 ; Vitetta *et al.*, 1992). Three out of fourteen patients developed human anti-ricin antibody (HARA) and one patient developed HAMA when treated with Fab-RFB4-dgA. Five out of twenty five patients showed both HAMA and HARA when treated with IgG-RFB4-dgA, suggesting that immunogenicity of an antibody fragment is similar to whole Ig. More recently an RTA immunotoxin coupled to a Fab'_2 antibody fragment was shown to be more cytotoxic to target cells compared to the intact Ig immunotoxin (Ghetie and Vitetta, 1994).

An antibody to CD19 linked to ricin, in which the galactose binding sites on the B chain had been blocked (anti-B4-bR), has been used in clinical trials for patients with B cell neoplasms (Grossbard *et al.*, 1992). It was found that continuous infusion of the immunotoxin maintained higher serum levels of cytotoxic agent than a bolus injection. After treatment, the leukocyte count was reduced and complete remissions were observed in two patients and five patients showed partial remissions. However, 50% of patients develped HAMA and/or HARA. Thus, although the response to infusion is encouraging it results in a high level of immunogenicity. Another form of immunotoxin therapy which has been relatively successful involved an anti-CD7 antibody linked to ricin which was used to purge autologous marrow in patients with T-cell adult lymphocytic leukemia (Preijers *et al.*, 1989). A mixture of immunotoxis against CD 5 and CD7 has also been used with limited success as 9 of the 14 patients relapsed at a median of 2.2 months (Uckun *et al.*,1990). An obvious advantage is the lack of a HAMA response in these patients and the direct delivery of the cytotoxic agent to the bone marrow.

The results of clinical trials using RTA based immunotoxins are encouraging and suggest that these agents can safely be administered to patients. Furthermore, they do exert cytotoxicity *in vivo* and unlike conventional therapy, immunotoxins do not kill normal cells. Side effects which have been observed with PE, DT and ricin are vascular leak syndrome, myalgia, neutropenia and in some cases neuropathies (Ghetie and Vitetta, 1994). Perhaps a toxin, such as melittin, which is

slightly less potent than these metabolic inhibitors would reduce these side effects which are apparently toxin related.

2. Melittin immunotoxins

In fact two chemically conjugated melittin immunotoxins have been produced and preliminary data shows that they are capable of specific cell lysis. In the first study a chemical crosslinker, 2,3-dibromopropionyl N-hydroxysuccinimide ester, was used to chemically conjugate K-1-21 and melittin (McKenzie, MSc Thesis 1990). Cellular toxicity of this conjugate was analysed and shown to be specific for HMy2 cells which express the KMA antigen. In a later study the crosslinker, sulphosuccinimidyl 6-[3'-(2-pyridyldithio)-propionamido]-hexanoate, was used to produce a melittin-mouse Ig conjugate that killed malignant B cells, which were CD5+, from patients with CLL via interaction of the immunotoxin with polyreactive sIgM (Weston, PhD Thesis, 1994). These studies confirm that melittin can be targeted to cell suface antigen using either the murine monoclonal antibody, K-1-21, or mouse Ig and the resulting immunotoxin is cytotoxic. Given that melittin is capable of lysing human cells as an immunoconjugate it would probably retain this cytotoxicity if a fusion gene encoding a scFv-melittin was successfully expressed in a prokaryotic expression system. This strategy would result in a single chain recombinant immunotoxin as a homogeneous product. The first step to achieve this would be to isolate the variable region genes of K-1-21 and link them together using antibody engineering techniques.

1.4 *ANTIBODY ENGINEERING*

Over the past decade recombinant DNA technology has been used to isolate the genes encoding a wide range of antibodies. In addition, once these genes have been cloned they can be manipulated by antibody engineering to produce Ig fragments such as Fab, Fv, single chain Fv (scFv) fragments, enzyme fusion fragments, chimaeric antibodies, humanised antibodies and recombinant immunotoxins (Fig 1.2). Furthermore, knowledge of B cell development and the rearrangement of immunoglobulin genes during differentiation has contributed to the isolation and characterisation of these genes.

1.4.1 B CELL DEVELOPMENT AND REARRANGEMENT OF IMMUNOGLOBULIN GENES

The differentiation of B cells is an ordered process in which the rearrangement of the heavy chain genes occurs by fusion of defined germline gene elements, the variable (V_H), diversity (D_H), joining (J_H) and constant (C) region gene segments. Assembly of the light chain gene occurs by rearrangement of the germline variable (V_L), joining (J_L) and constant (C) region genes. The germline DNA of an immature B cell reflects the domain structure of the antibody as the individual V, D J and C gene segments are encoded by separate exons (Fig 1.6). In addition, the genes encoding the heavy and light chains are found on separate chromosomes. In the mouse the heavy chain (H) segments are located on chromosome 12, the *kappa* light chain (V_k) on chromosome 6 and lambda light chain (V_l) on chromosome 16 (Winter and Milstein , 1991).

In the developing B cell, heavy chain gene rearrangement and expression precedes that of the light chain. A site-specific DNA recombination system known as VDJ recombinase, which is composed of products from the RAG1 and RAG2 genes, is responsible for assembly of the V_H and V_L genes (Oettinger *et al.*, 1990). In pre-B-I cells recombination is initiated at the heavy chain locus when the D_H and J_H segments are rearranged on both chromosomes. This is followed by joining of V_H to D_HJ_H segments in the pre-B-II cells and finally joining of $V_HD_HJ_H$ to the constant region gene (Cμ). Once a productively rearranged heavy chain is selected by cell surface expression with the surrogate light chain, encoded by the VpreB and l_5 genes, no further rearrangements occur at that H locus. This selection also prevents rearrangement on the other chromosome and results in allelic exclusion. (Rolink and Melchers, 1993). Rearrangement of the L chain, V_L to J_L segments, occurs once the H chain has productively rearranged in the pre-B-II cells. The *kappa* light chain is rearranged first and if this is not successful then the lambda light chain is assembled. Once a surface Ig molecule, consisting of H and L chain is expressed no further rearrangements can proceed and the cell migrates to the peripheral bloodstream (Chen and Alt, 1993).

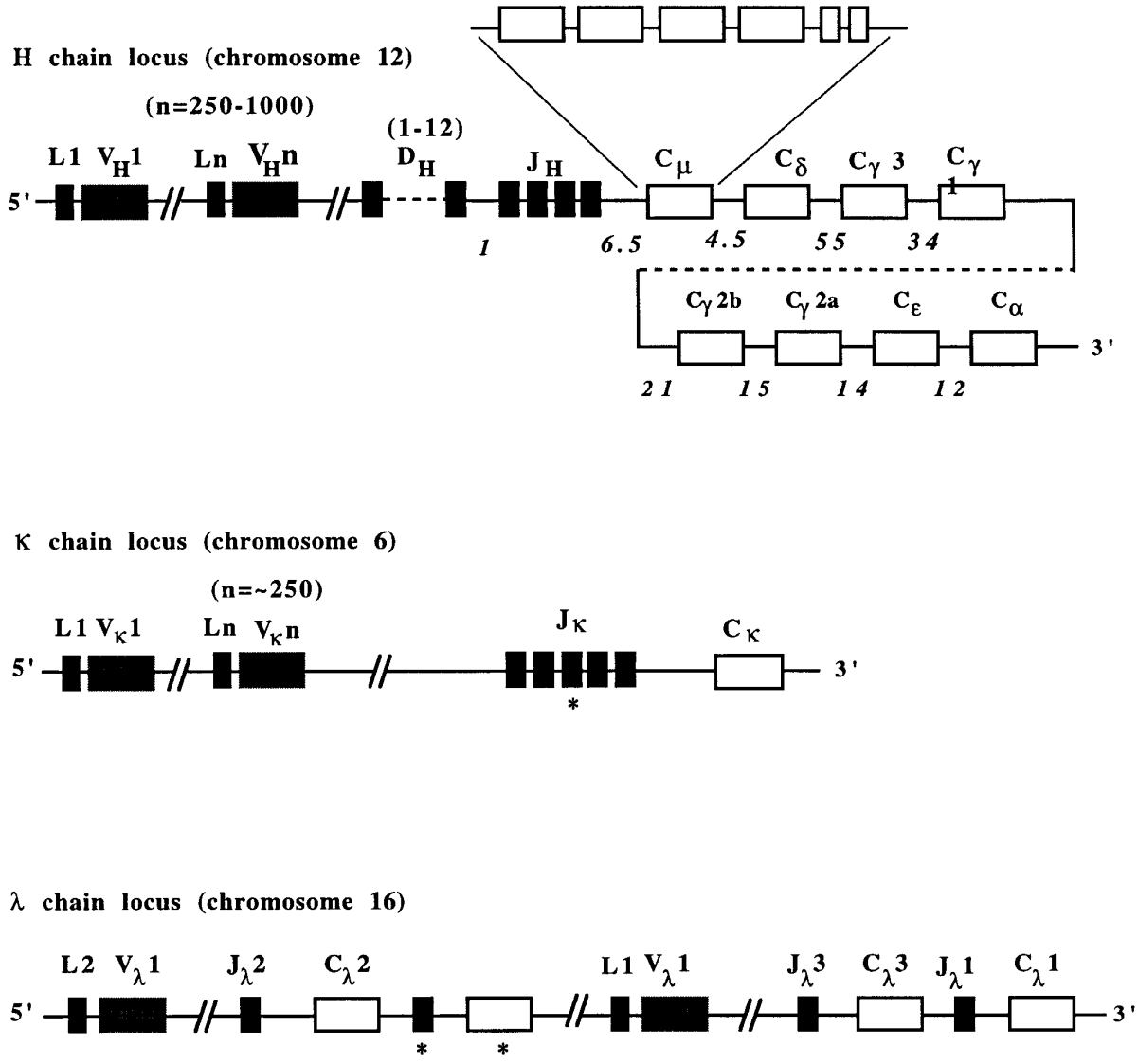


Fig 1.6 THE ORGANISATION OF MOUSE Ig GENES IN THE GERMLINE
In the H and L chains the genes are arranged in the order depicted in the diagrams. However, each C_H gene is depicted as a single box but is composed of several exons, except the μ gene for which the actual exons are shown. The asterisks indicate pseudogenes and numbers in italics refer to approximate lengths of DNA in kilobases. The gene segments are depicted as follows; L, leader ; V, variable ; D, diversity ; J, joining ; C, constant.

1.4.2 ANTIBODY DIVERSITY

Immunoglobulin diversity is achieved in part by these recombination events. For instance, in the mouse there are >200 V_H genes, 15 D_H, 4 J_H and 8 C_H genes from which to choose. For rearrangement of the light chain there are 1000 V_k genes, 5 J_k segments and a single constant region gene (C_k) as well as 2 Vl genes and 4 J-Cl rearrangements, thus the potential diversity due to recombination alone is quite vast (Winter and Milstein, 1991).

Further diversity in the H chain occurs at the joining of the segments V-D and D-J by deletions or insertions as a consesquence of enzymatic breakage and joining. During recombination two types of insertions have been identified. The P nucleotides which have a few extra bases in reversed orientation to the original coding end and N nucleotides which are produced from deoxynucleotide transferase activity at a free 3' coding end (Lewis, 1994).

Additional diversity, designated "somatic mutation", is found in the variable genes of the H and L chains during antigen-driven B cell maturation. In many cases these are point mutations within the V gene in which the mature antibody gene differs from the germline V gene. These mutations generally occur in specific regions of the gene which are known as hypervariable regions or complementarity determining regions (CDR), although some mutations do occur within the framework regions as well. There are three CDR regions in the H chain and three CDR regions in the L chain (Fig 1.6). The sequence between the CDR regions encodes the framework regions which are fairly well conserved between different antibodies. The reason for this conservation is that they have a structural role in the three dimensional arrangement of the antigen binding site. As shown in Fig 1.7, the Fv fragment consists of the V domains of both the heavy and light chains and the framework regions are arranged as ß sheets which form the scaffolding for the six CDR loops. This arrangement of the domains brings the 6 CDRs into close association and forms the antigen binding site.

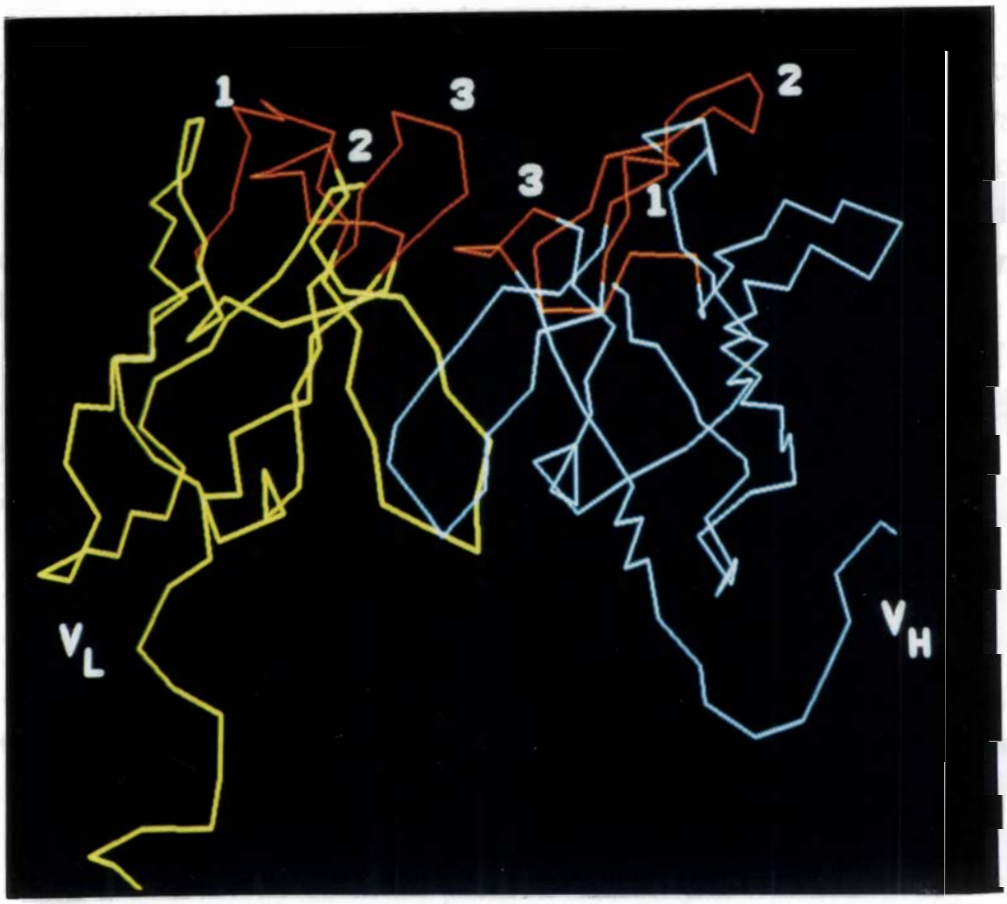


Fig 1.7 THE STRUCTURE OF AN Fv FRAGMENT

This picture depicts the carbon backbone of the POT Fv for which the crystal structure has been solved, however, the general structure can be applied to other Fv fragments (Fan *et al.*, 1993). An Fv fragment is composed of the heavy and light chain variable domains in noncovalent association. The framework section of the VH domain is shown in blue and the framework region of the VL domain is in yellow. Each domain has three complementarity determining regions (CDR) which are numbered and shown in red. The 6 CDRs are brought together by folding of framework regions, which form a series of anti-parallel beta sheets, and provide the scaffolding for the antigen binding site. The antigen binding site is created by the CDRs which form loops at the top of the Fv. In a scFv the carboxy terminus of VH is connected to the amino terminus of VL, seen here just below CDR 2 of VL, by a linker peptide.

The CDR1 and CDR2 regions are encoded by the multiple germline V genes, somatic mutation can account for the additional variability observed in these areas. In contrast, the CDR3 is determined by the joining of VDJ elements in the heavy chain and VJ in the light chain resulting in both insertions and/or deletions as described previously. Additional somatic mutation in this section results in further diversity. In fact the CDR3 of the heavy chain can vary in length from 4 to 25 residues (Griffiths and Hoogenboom, 1993). As this region is the most diverse it accounts for the differences in specificity observed in Ig molecules which are derived from the same germline gene. Once the heavy chain variable gene has rearranged mRNA is transcribed and the constant region gene is joined to the variable region by RNA splicing. The resulting heavy and light chain mRNA produced in the plasma cell encodes a specific antibody molecule.

1.4.3 CLONING OF REARRANGED VARIABLE GENES USING PCR

It has been estimated that the potential antibody repertoire in the mouse is $>10^{10}$ but only a small number of clones, 10^{7}-10^{8}, express antibodies at any given time (Winter and Milstein, 1991). The authors indicate that despite this diversity in antibody production the sequence of the structural frameworks of these proteins is fairly well conserved. These conserved regions could be used to design PCR primers to amplify the variable region genes. In an early study this approach was used to design a large number of PCR primers which were either degenerate or specific for the highly conserved regions within the first framework region of the heavy chain (Sastry *et al.*, 1989). Complementary primers were designed for the prevalent codons in the J_H gene segments. Variable region genes have also been isolated using consensus PCR primers which incorporate the most frequent nucleotides found in the first five codons of the first framework region. Likewise, the reverse primer is complementary to the most common codons found in the J_H gene segments (Orlandi *et al.*, 1989 ; Ward *et al.*, 1989). Similar strategies were used to isolate V_L genes. Some alternative methods include using PCR primers specific for the relatively conserved leader sequence in both murine (Coloma and Larrick, 1991) and human (Campbell *et al.*, 1992) V genes and degenerate primers which are designed from specific V gene families (Marks *et al.*, 1991).

Generally, in PCR cloning, the mRNA is isolated from cells of a monoclonal hybridoma cell line or specific B cell clones. The mRNA is then transcribed into complementary DNA (cDNA) using reverse transcriptase. The mRNA:cDNA hybrid can be used as a template for DNA amplification using PCR and the preferred primers. Amplification of the regions between the PCR primers has permitted the isolation of heavy and light chain rearranged variable genes from a wide range of antibodies. Incorporation of restriction enzyme sites within the PCR primers allows directional cloning into a variety of expression vectors restricted with the same enzymes. Once the variable genes have been isolated they can be manipulated and expressed as a range of different antibody fragments.

1.4.4 ANTIBODY FRAGMENTS WHICH RETAIN ANTIGEN BINDING

The variable genes from the heavy chain of monoclonal antibodies have been isolated using PCR and the VH domains were expressed in *E.coli.* (Ward *et al.*, 1989 ; Power *et al.*, 1992). However, the isolated single domain antibodies showed decreased specificity due to exposed hydrophobic regions which are normally associated with the VL region. The smallest antibody fragment which retains antigen binding is the Fv fragment and it displays the same affinity for antigen as the Fab fragment. Both the H and L chain variable genes of several Fv fragments have been isolated, expressed in *E.coli.* and the expressed product used for x-ray chrystallography (reviewed in Skerra, 1993). Unfortunately, this fragment is labile and tends to dissociate at low concentrations (Glockshuber *et al.*, 1990). This finding has led to a number of different strategies to stabilize the Fv fragment without loss of binding activity.

A flexible, hydrophilic linker peptide was designed to span the distance between the carboxy terminus of the VH domain and the amino terminus of the VL domain without disrupting the association of the Fv structure (Huston *et al.*, 1988). This linker gene encoding fifteen amino acids with three repeats of one serine and four glycine residues, $3(S, G_4)$, was fused to the 3' end of the V_H gene and 5' end of the V_L gene. The construct was cloned and expressed in *E.coli* and the expressed scFv was shown to bind antigen.

A similar approach utilized computer modeling of peptide fragments to design a suitable linker which, in this case, represented a segment of carbonic anhydrase (Bird *et al.*, 1988). The authors also designed another linker by incremental addition of residues which were more flexible and hydrophilic. Both linkers were designed to fit into the groove on the back of the Fv without disrupting the association of the domains. The scFv gene constructs were then cloned and expressed in *E.coli* without loss of antigen binding.

A comparative study, using the monoclonal antibody McPC603, in which the activity and stability of a glutaraldehyde-crosslinked Fv, Fv, a scFv and an Fv in which intermolecular disulphide bonds had been engineered (dsFv) has been described (Glockshuber *et al.*, 1990). The fragments were expressed in *E.coli* and it was found that spontaneous disulphide bond formation of the dsFv occurred in the periplasm. It was also found that all fragments had similar binding affinities for antigen, however, the dsFv was significantly more stable than the Fv and scFv fragments under physiological conditions.

As the monovalency of an Fv, scFv and Fab fragment results in low avidity these fragments can be lost in the washing steps used in an ELISA and in inference immunofluorescent staining (Pack and Pluckthun, 1992). These authors attempted to improve the avidity of a scFv, derived from McPC603 mAb, by engineering "dimerization domains" at the 3' end of the gene. Association of the expressed peptides in the periplasm would result in a bivalent scFv. Two different peptides which formed amphipathic helices were chosen for their ability to self associate. Firstly an 18 amino acid peptide which naturally forms four antiparallel helix bundles and secondly a 33 amino acid leucine zipper which is a coiled-coil helix. The genes encoding the peptides were linked to the 3' end of the scFv gene by the sequence encoding the hydrophilic hinge region of IgG_3 (the latter was chosen to assist soluble expression in *E.coli*). In addition, an oligonucleotide encoding a peptide tail which incorporated a cysteine residue was attached to the carboxy terminus of the gene construct. Expressed products with the cysteine tail region spontaneously formed disulphide-bonded dimers in the periplasm and the fragments without the cysteine tail formed non-covalently associated

dimers. The scFv fragment with the helix bundle showed increased binding to antigen in an ELISA compared to the leucine zipper scFv suggesting that the former peptide formed a more stable bivalent antibody fragment. Both fragments showed a 100 fold increase in antigen binding compared to scFv. Thus these bifunctional or bivalent antibodies bind antigen with the same avidity as the parent antibody but are the size of an Fab fragment.

A similar study using anti-lysozyme (Lys) and anti-phenyloxazolone (pOx) scFv fragments with peptide linkers of 5, 10 or 15 amino acids was carried out by Holliger *et al.* (1993). The shorter linkers favoured the pairing of a VH domain with the VL domain on another chain resulting in formation of two antigen binding sites on a molecule which is the same size as a Fab fragment. An alternative construct involved linkage of the genes from the VH of pOx with the VL from Lys and vice-versa, resulting in production of a bispecific fragment.

Under physiological conditions the most stable Ig fragment is the Fab. Cloning of Fab has been achieved by amplification of the Fd gene and VL gene with subsequent expression in a bicistronic vector (Better *et al.*, 1988). Using a similar cloning strategy an attempt was made to produce a $F(ab')_2$ fragment by secretion of the Fab fragment into the periplasmic space of *E.coli.* (Carter *et al.*, 1992). Unfortunately in this study the Fab did not spontaneously form covalent disulphide bonds between the cysteine residues of the hinge region.

These studies have explored several different methods for creating stable monovalent or divalent antibody fragments. They have also shown that a variety of engineered fragments retain both binding activity and specificity. If these fragments are destined to be used in diagnostics or clinical therapy a combination of avidity, stability and small size would be beneficial. One of the major advantages of using small antigen binding fragments is that they are not glycosylated and therefore can be expressed in a bacterial system.

1.4.5 EXPRESSION OF ANTIBODY FRAGMENTS IN *E.coli*

Early experiments involving expression of eukaryotic proteins in *E.coli* showed that insoluble inclusion bodies were a result of incorrect association of partially folded intermediates. These intermediates were thermolabile and at lower temperatures soluble, correctly folded proteins could be produced (reviewed in Mitraki and King, 1989). There is growing evidence that bacterial growth at high temperatures results in insoluble product and therefore temperature inducible vectors should be avoided (Anthony *et al.*,1992 ; Lilley *et al.*, 1994).

Additional factors were implicated in the production of correctly folded, soluble product and these were prosthetic groups and chaperones, which are important in the correct folding of eukaryotic proteins. Proteins which were capable of folding correctly at high temperatures were shown to be low in proline content or to have regions of acidic amino acids (Schein, 1989). Another factor in optimimal expression of foreign proteins in *E.coli* is the toxicity of the end product. Overexpression of heterologous proteins in bacteria is often lethal whereas moderate levels of induction favour both correct folding and increased yields of expressed product (White and Richardson, 1988 ; Skerra and Pluckthun, 1991 ; Anthony *et al.*, 1992).

A review of promoters used to induce expression in prokaryotic systems suggests that induction of transcription using the lambda promoter system is not easily regulated by heat induction (Darveau *et al.*, 1992). The T7 system is well controlled before induction as the T7 RNA polymerase gene under the control of a *lac* promoter in the BL21 cell line only recognises the T7 promoter and prevents uncontrolled transcription. However, after induction the level of expression is difficult to regulate. Although the *lac* promoter can be regulated by increasing the concentration of ß-D-thiogalactopyraniside (IPTG) the promoter is not completely repressed as induction can occur via cyclic AMP when insufficient repressor is present. Darveau *et al.* (1992) favour the hybrid *trp-lac* UV5 (tac) promoter which was created to improve RNA polymerase binding and included a copy of the *lac* I^q gene on the same plasmid. The latter is a mutant of *lac* I and is known to result in increased expression. Using this system it is possible to achieve

stringent control over repression, regulated induction and a wide choice of host cells for expression.

Despite the obvious advantages and disadvantages of different promoters used in bacterial expression systems there are anomalies in some situations. For example a single amino acid change in the protein, tail spike endo-rhamnosidose of phage P22, can prevent aggregation of the expressed protein (Mitraki and King, 1989). Recently, it has been demonstrated that a single amino acid change in the McPC603 scFv results in decreased aggregation of the protein in the periplasm. A different amino acid change prevents lysis of the host cell during induction with an overall increase in expressed product. However, the amino acid change which increases solubility does not affect lysis of the host cell and vice versa (Knappik *et al.*, 1994). Thus, a single amino acid in the primary sequence of a foreign protein can determine successful expression in *E.coli.*

Many of the antibody fragments produced in earlier studies have been successfully isolated from inclusion bodies and refolded *in vitro*. An alternative strategy using a bacterial secretion peptide to direct transport of the foreign protein to the periplasmic space of bacteria has resulted in the production of soluble antibody fragments (Skerra and Pluckthun, 1988 ; Better *et al.*, 1988). In fact a periplasmic protein which resembles a disulphide oxidoreductase has been implicated in correct folding of proteins by facilitating the formation of disulphide bonds (Bardwell *et al.*, 1993). There are several bacterial signal peptides which facilitate transport of the expressed protein to the periplasm and the most commonly used are ompA (Movva *et al.*, 1980), pelB (Lei *et al.*, 1987) and phoA (Skerra and Pluckthun, 1988). The advantage of secretion into the periplasm is that the correctly folded, soluble protein can be isolated by a single affinity chromatography step. However, there are several reports of aggregated proteins in the periplasm or "periplasmic inclusion bodies" in which the peptide signal has been removed but the protein is associated with the membrane fraction (Malby *et al.*, 1993 ; Pantoliano *et al.*, 1991). Apparently this phenomenon occurs when using the lambda promoter with heat induction and overexpression of the antibody fragment (Skerra, 1993).

The choice of leader sequence is important as not only does the signal peptide interact with the secretory components of the membrane it also influences the rate of folding in the cytoplasm (Randall and Hardy, 1989). It has also been suggested that aggregation of an expressed protein, ß-lactamase, in the periplasm was related to the choice of leader sequence (Bowden and Georgiou, 1990).

From these studies it is apparent that there are a number of criteria which can influence the expression of active proteins. These include bacterial growth at lower temperatures, the choice of promoter and control over induction and also which host cell is compatible. Another option is whether to use the secretion strategy or whether the protein should be isolated from inclusion bodies with subsequent refolding and renaturation *in vitro*. If a secretion system is used the type of signal peptide might also influence the end product. Finally, having optimized all the previous factors the primary amino sequence of the expressed foreign protein may be incompatible with bacterial expression. Despite these difficulties associated with expression of eukaryotic proteins in bacterial cells there have been numerous examples in which VH and VL domains, Fv, scFv and Fab fragments have been expressed in the periplasm or isolated from inclusion bodies resulting in functional, soluble proteins (reviewed in Skerra, 1993 ; Darveau *et al.*, 1992).

In clinical situations the choice of antibody fragment which will be used for targeting is important. As mentioned earlier the Fv fragment is unstable and although it can be expressed at higher levels than the other fragments it is unsuitable for this application. It has been demonstrated that the Fab fragment is more stable than the scFv both *in vitro* and *in vivo* although uptake by tumour cells was similar for both fragments (Colcher *et al.*, 1990). Studies in both humans and animals have shown that Fab and $F(ab')_2$ accumulate in the kidney but the scFv does not (Sandhu, 1992). Therefore, the scFv fragment is probably a better targeting agent for therapeutic applications. At present the *in vitro* studies suggest that the most stable fragment is the disulphide-linked Fv (Glockshuber *et al.*, 1990). Its behaviour *in vivo* has not been ascertained but it is likely to be similar to the Fab fragment and if it does not accumulate in the kidneys it may prove more effective as a targeting molecule.

Over the last decade there have been numerous examples of recombinant immunotoxins which have been expressed in *E.coli*. In general, the antibodies used to target the toxin are derived from well defined molecules which bind to specific antigens on the cell surface. Several different antibody fragments such as scFv, dsFv and Fab have been used to construct these immunotoxins. The most common toxins used are PE and DT which have been genetically manipulated to decrease non-specific binding and increase toxicity.

1.5 *RECOMBINANT IMMUNOTOXINS*

As the targeting agent in an immunotoxin only requires the antigen binding site, antibody fragments are a reasonable alternative to the whole Ig molecule. As mentioned previously these small fragments can be cloned and expressed in *E.coli* and retain significant binding activity. Thus the gene construct for an antigen binding site can be fused to the gene encoding a toxin and the recombinant immunotoxin can be expressed as a single chain polypeptide in *E.coli*. The specificity of the immunotoxin is then determined by the antigen binding site of the parent antibody. As discussed in section 1.1 the genes for both PE and DT have been cloned and both the native toxin and various mutants have been expressed in *E.coli*.

1.5.1 ANTI-TAC-PE RECOMBINANT IMMUNOTOXINS

Using a few simple cloning steps the scFv, derived from a monoclonal antibody to the IL-2R, was fused to the PE40 gene to produce the recombinant immunotoxin anti-Tac(scFv)-PE40 (Chaudhary *et al.*, 1989). As the toxin is a potent inhibitor of protein synthesis the cytotoxic activity of the recombinant immunotoxin was measured by determining the amount of radioactive leucine incorporation into cells and hence the percent inhibition of protein synthesis. Incubation periods varied with different scFv fragments but were generally for 16-20 hr. Comparison of chemically conjugated immunotoxins and this recombinant immunotoxin showed that 0.15 ng/mL anti-Tac(scFv)-PE40 produced 50% inhibition of protein synthesis (ID_{50}) in the human

ATL-derived cell line HUT102. Under the same conditions the chimaeric toxin IL-2-PE40 had an ID_{50} of 5 ng/mL, a chimaera in which 4 basic amino acids are converted to glutamates, IL-2-PE^{4E} , had an ID_{50} of 2 ng/mL and anti-Tac-lys-PE40 which is a chemical conjugate linked through the additional lysine group at the amino terminus had an ID_{50} of 2.5 ng/mL (Kreitman *et al.*, 1990). All these immunotoxins were cytotoxic at pmol/L concentrations and anti-Tac(scFv)-PE40 had the highest toxicity to target cells. These workers also showed that low concentrations of recombinant immunotoxin were able to kill activated mononuclear cells from six patients with ATL.

A mutant form, anti-Tac(scFv)-C3-PE38KDEL, of the immunotoxin has the sequence KDEL at the carboxy terminus instead of the native sequence REDLK. As previously discussed the former is an endoplasmic reticulum (ER) retention sequence which directs the molecule to the ER and facilitates translocation to the cytosol. After expression in *E.coli* the protein was isolated from inclusion bodies and renatured *in vitro*. Cytotoxicity to HUT-102 cells was compared to anti-Tac(scFv)-C3-PE40 which was prepared in the same way. The immunotoxin which did not have the ER binding site had an ID_{50} of 1.7 pmol/L whereas the anti-Tac(scFv)-C3-PE38KDEL had an ID_{50} of 0.8 pmol/L. Several studies have shown that the mutant anti-Tac(scFv)-PE38KDEL has a relatively higher cytotoxicity towards activated T cells, ATL cell lines and fresh ATL cells (Seetharam *et al.*, 1991, ; Kreitman *et al.*, 1993a ; Kreitman *et al.*, 1993b). This improved activity could be accounted for by the additional amino acid sequence which increases the delivery of the immunotoxin from the endosome to the cytosol where inhibition of protein synthesis occurs.

A recombinant immunotoxin with the same specificity but using the Fab fragment was engineered. In this example anti-Tac(Fab)-PE40 fragment was constructed by cloning the Fd gene and the VL gene with the latter fused to the gene for PE40 (Saito *et al.*, 1994). The two genes were co-expressed in *E.coli* and the Fab fusion product was isolated from the periplasm. The anti-Tac(Fab)-PE40 has a larger molecular weight (90 kDa) than anti-Tac(scFv)-PE40 (66 kDa) and it has an eight to ten fold longer half life in mice than the scFv derivative. These authors carried out a comparative study on peripheral blood mononuclear cells (PBMC)

and lymph node cells (LNC) of ATL patients from Japan and ATL patients from the Caribbean. Their results indicated that the anti-Tac-toxin derivatives are equally cytotoxic in different races and that although the Fab has a longer half life in mice there is no short term advantage in its ability to kill target cells. As mentioned in section 1.1, anti-Tac immunotoxins can complex with the IL-2R in the serum of some patients with ATL. In this same study it was reported that despite the high levels of soluble IL-2R in the serum of these patients addition of soluble IL-2R below 1 x 10^4 units/mL to the cytotoxicity assay did not decrease activity. It was suggested that this level was well above the concentration of soluble IL-2R found in the serum of patients after chemotherapy treatment and therefore anti-Tac(scFv)-PE40 and other derivatives should be given post-treatment. These findings suggest that this immunotoxin might not be used as an alternative to chemotherapy but as an adjunct and therefore it is relevant in clinical therapy.

Another major disadvantage of the anti-Tac scFv toxins is their short half life in patients serum *in vivo* compared to native Ig and Fab fragments. Recently, a recombinant disulphide-linked Fv immunotoxin, anti-Tac(dsFv)-PE38KDEL, was created to stabilise the antigen binding site (Reiter *et al.*, 1994a). To construct this two chain polypeptide the glycine residue at position 44 in the V_H gene was converted to a cysteine residue and the serine residue at position 99 in the V_L gene was changed to a cysteine residue. The constructs V_H, V_L-toxin, V_L, V_H-toxin and the scFv-toxin were cloned into separate expression vectors and individually purified from inclusion bodies. After renaturation the V_H -V_L-toxin and V_L-V_H-toxin molecules were mixed in an oxidation step and then correctly folded monomeric material was purified by ion-exchange and size-exclusion chromatography. The anti-Tac(dsFv)-PE38KDEL had the same cytotoxicity as its scFv counterpart and was specific for cells expressing the p55 subunit of IL-2R. A slightly higher yield of correctly folded, soluble dsFv-toxin was achieved compared to the scFv-toxin. From this study it was not apparent whether the disulphide stabilized fragment had a longer half life than anti-Tac(scFv)-PE38KDEL.

In summary a wide range of anti-Tac immunotoxins have been tested *in vitro* and it appears that antibody fragments fused to the mutant form, PE38KDEL, result in increased cytotoxicity whereas the dsFv is the most stable complex. Recently phase I trials for humanised anti-Tac have been reported and the early data shows the absence of HAMA in treated patients (Queen *et al.*, 1993). At present there is no published data of clinical trials using anti-Tac(scFv)-PE recombinant immunotoxins.

1.5.2 ANTI-B3-PE RECOMBINANT IMMUNOTOXINS

Another antigen used in targeted therapy is the B3 antigen associated with many tumour cells including colon, stomach, lung, breast, oesophagus and ovary (Pastan *et al.*, 1991). This monoclonal antibody binds to a carbohydrate epitope on tumour cells and a scFv fragment has been used to construct the recombinant immunotoxins, B3(scFv)-PE40 and B3(scFv)-PE38KDEL. The yields of correctly folded proteins were found to be lower than other scFv-PE immunotoxins obtained from inclusion bodies (Brinkmann *et al.*, 1992). An analysis of several B3(scFv) fragments linked to the mutant toxin PE38KDEL, which differed only in the linker sequence between the antibody and toxin molecules, showed that the peptide linker could influence folding of the various domains. *In vitro* folding of the ribosylation domain at the carboxy terminus of the toxin molecule occurs fairly rapidly whereas the antibody binding domain and toxin translocation domain refold more slowly. Using activity assays it was evident that the peptide linker ASGG allowed independent folding of the scFv and toxin domains resulting in improved yields of correctly folded material. This is an interesting finding as it shows that the amino acid sequence of the peptide linker can influence the recovery of functional material after *in vitro* folding.

A recombinant immunotoxin using the Fab antibody fragment of B3 was shown to improve both the yield of expressed immunotoxin and the stability of the molecule (Choe *et al.*, 1994). A mutant form of the toxin, $PE38^{M}$ which is similar to PE38 but lacks lysine residues, was fused to the carboxy terminus of the Fd gene or the carboxy terminus of V_L gene. Four constructs, Fd-$PE38^{M}$, V_L , Fd and V_L-$PE38^{M}$ were

expressed individually in *E.coli* and purified from inclusion bodies. The isolated recombinant Fd-toxin was renatured in the presence of V_L and vice versa and oxidised to produce the B3(Fab)-PE38^M with the toxin fused to either the heavy or light chain. The yield of correctly folded Fab-toxin was higher when the toxin was fused to the light chain (21-23%) compared to the heavy chain toxin (9-11%). This is probably due to the relatively labile VH domain which tends to denature more rapidly than the VL (Glockshuber *et al.*, 1990). Both constructs had the same cytotoxic activity on the relevant cell lines. However, despite the longer half life the B3(Fab)-PE38^M toxin had the same activity as the scFv construct when tested for antitumour activity in mice. It was found that the B3(Fab)-PE38^M toxin showed a 7-8 fold decrease in binding compared to the B3(scFv)-PE38^M which might account for the decreased anti-tumour activity in mice. This is an interesting result as it suggests that increased binding to the antigen can overcome the problem of a short half life *in vivo*. In a similar study it was observed that the disulphide linked B3(dsFv)-PE38KDEL immunotoxin had the same activity and specificity as its scFv counterpart (Brinkmann *et al.*, 1993b). However, *in vitro* studies indicated that the disulphide stabilized fragment had a longer half life in buffers and serum compared to the scFv. The activity of B3(dsFv)-PE38KDEL was also assessed in mice and it was found that the dsFv-toxin had the same anti-tumour activity as the scFv derivative (Reiter *et al.*, 1994b). These workers also found that the *in vivo* half life of the disulphide-stabilized immunotoxin was similar to the scFv and suggested that clearance and tumour penetration are the same for both molecules. Thus the stability which was demonstrated *in vitro* for B3(dsFv)-PE38KDEL was not reflected *in vivo*. At present it seems the only obvious improvement of this disulphide linked immunotoxin compared to the scFv derivative is the higher yields obtained after purification.

1.5.3 ANTI-erbB2-PE RECOMBINANT IMMUNOTOXINS

A variety of recombinant antibody fragments from the monoclonal antibody e23, which binds to the erbB2 protein, have been linked to mutant forms of PE and their activities have been assessed *in vitro*. The erbB2 protein is expressed in a wide range of cancer cells including lung, breast, ovary and stomach adenocarcinomas. In breast carcinoma

cells gene amplification and overexpression of the erbB2 antigen has been correlated with aggressiveness of the malignancy (Batra *et al.*, 1992). These workers created several scFv constructs which were linked to different mutants of PE (e23(scFv)-PE40, e23(scFv)-PE40KDEL, e23(scFv)-PE38 and e23(scFv)-PE38KDEL). All the gene constructs were expressed in *E.coli* , purified from inclusion bodies and refolded *in vitro*. Cytotoxicity of the immunotoxins was tested on several human cancer cell lines which express the erbB2 antigen including BT474 (breast carcinoma), N87 (gastric carcinoma) and SK-OV-3 (ovarian carcinoma). Cell lines derived from human epidermoid carcinomas, which express low levels of erbB2, were also tested. The recombinant immunotoxins were found to be 2-3 fold more cytotoxic than the chemically conjugated immunotoxin and e23(scFv)-PE38KDEL was shown to be a further 10 fold more cytotoxic. These data are in agreement with the anti-Tac-PE results discussed earlier and confirm that recombinant immunotoxins of different specificities are more potent than their chemically linked counterpart. In addition, the toxicity of PE was increased by delivery to the cytoplasm. Another interesting point is that the toxicity of e23(scFv)-PE38KDEL was directly related to the cellular expression of the antigen. Therefore, the efficacy of the recombinant immunotoxin in clinical therapy is determined by the amount of specific antigen expressed on tumour cells. This implies that tumour cells with low levels of antigen could evade the toxic effects of the immunotoxin. This problem can be overcome in the treatment of lymphoid neoplasms by using a number of antibodies which target different CD antigens on the B or T cells (Grossbard and Nadler, 1994)

Recent studies have compared the immunotoxin, e23(scFv)-PE38KDEL, with a disulphide linked e23(dsFv)-PE38KDEL (Reiter *et al.*, 1994a). To produce the disulphide bond the asparagine residue at position 44 on VH was changed to a cysteine and the glycine residue at position 99 on the VL was converted to cysteine. The refolded immunotoxin was obtained as described for anti-Tac(dsFv). Using cytotoxic assays on a range of cell lines, which expressed the erbB2 antigen in varying amounts, it was found that e23(dsFv)-PE38KDEL was more cytotoxic than the e23(scFv)-PE38KDEL immunotoxin. The former was also more stable than the scFv counterpart. It was suggested that as e23scFv does not bind as well as the e23Fab fragment to target cells the

increased activity of the disulphide linked Fv could be due to increased cell binding and not increased stability of e23(dsFv)-PE38KDEL. This is highly likely, as discussed earlier, both anti-Tac(dsFv)-PE38KDEL and B3(dsFv)-PE38KDEL were more stable than the scFv derivatives but showed the same degree of cytotoxicity. Once again it seems that the binding affinity is more important than the stability of the molecule in determining the cytotoxicity of the immunotoxin.

1.5.4 ANTI-LEWIS-Y PE RECOMBINANT IMMUNOTOXINS

Another PE recombinant immunotoxin was produced using a monoclonal antibody, BR96, which specifically binds to a carbohydrate receptor composed in part by the Lewis-Y antigen (Le^y) expressed on a number of carcinomas (Friedman *et al.*, 1993). Although this antibody binds to certain normal tissues as well it was considered useful as it is rapidly internalised by some solid tumours. The recombinant immunotoxin BR96(scFv)-PE40 was cytotoxic in a range of cell lines which express the Le^y antigen and it was found to be more effective than the chemically conjugated Ig-PE40 derivative. Studies in mice showed a half life of 28.5 min for BR96(scFv)-PE40 and 54 min for Ig-PE40. Despite the longer half life of the latter the recombinant immunotoxin was more effective. This is in agreement with studies of all the other recombinant PE immunotoxins discussed so far. A more recent study has demonstrated that BR96(scFv)-PE40 is capable of tumour regression in human breast carcinoma xenografts in athymic mice (Siegall *et al.*,1994). The tumours remained regressed for longer than 85 days. No toxicity was observed at curative doses (0.25 mg/kg) but at higher doses (4 mg/kg) liver and lung toxicity were observed. It is interesting to note that the authors suggest this recombinant immunotoxin is a potential therapeutic agent despite the possible crossreactivity with normal cells.

Studies of PE recombinant immunotoxins discussed so far have progressed fairly rapidly in the last 5 years and in some cases these agents have been used in animal models with reasonable success. It will be interesting to see if these results can be reproduced in phase I clinical trials. There are also several examples of recombinant immunotoxins using DT and in some cases the antibody specificity is the same as the

PE immunotoxins. However, these examples are not as well characterised as their PE counterparts. An interesting exception is the recombinant immunotoxin $DAB_{486}IL\text{-}2$ which has been used in extensive clinical trials. Some examples of these DT-antibody fragments will be discussed in more detail and their activities will be related to the same PE recombinant immunotoxins.

1.5.5 RECOMBINANT IMMUNOTOXINS USING DIPHTHERIA TOXIN

As mentioned in section 1.2 the first 388 amino acids of DT contain the enzyme activity required to inhibit protein synthesis and the translocation domain for transport to the cytosol. The cell binding domain is located at the carboxy terminus and is removed in recombinant immunotoxins to reduce non-specific binding. Unlike the PE derived immunotoxins, the gene encoding the first two domains of DT is fused to the scFv fragment through the amino terminus of the V_H gene which in turn is linked to the V_L gene.

A single chain DT388-anti-Tac(scFv) recombinant immunotoxin consisting of the first 388 amino acids of DT and a scFv which binds to the p55 subunit of the IL-2R was cloned and expressed in *E.coli* (Chaudhary *et al.*, 1990b). The immunotoxin was specifically cytotoxic to cell lines which express the p55 subunit and had an ID_{50} of 0.37 ng/mL on HUT102 cells which was similar to anti-Tac(scFv)PE40, 0.17 ng/mL, on the same cell line. In addition, DT388-anti-Tac(scFv) was cytotoxic towards proliferating T cells produced in a mixed leukocyte reaction.

Another comparison of recombinant immunotoxins produced against the transferrin receptor using both DT and PE has been described (Batra *et al.*, 1991). Both the V_H gene and the V_L gene were isolated from the monoclonal antibody, HB21, which binds to the human transferrin receptor. The scFv was linked to DT to produce DT388-anti-TFR(scFv) and to PE40 to give anti-TFR(scFv)-PE40. In cytotoxicity assays using a wide range of cancer cell lines it was found that anti-TFR(scFv)-PE40 was a 100 fold more cytotoxic than the DT derived toxin. These cell lines included A431 (epidermoid carcinoma), KB (epidermoid

carcinoma) and MCF7 (breast carcinoma). In two cell lines HUT102 (ATL) and HT29 (colon carcinoma) the immunotoxin DT388-anti-TFR(scFv) was 3 fold more effective than anti-TFR(scFv)-PE40. Overall the anti-TFR(scFv)-PE40 was more effective but the reason for the difference in activity was not apparent.

A recombinant immunotoxin which has been used successfully in phase I and II clinical trials is targeted to the IL-2R using the cytokine IL-2. It is well known that the interaction of IL-2 with the IL-2R mediates T cell proliferation. Immunosuppression aimed at proliferating T cells can be achieved by inactivating these cells with immunotoxins derived from monoclonal antibodies to the IL-2R (eg anti-Tac(scFv)-PE) or from IL-2-toxin conjugates. It has been suggested that IL-2 is a better targeting agent than anti-Tac or humanised anti-Tac as it has a 100 fold higher binding affinity for the receptor (Strom *et al.*, 1993). These workers have carried out extensive phase I/II clinical trials using the recombinant immunotoxin DAB_{486}IL-2. This fusion toxin was produced by joining the gene encoding the first 486 amino acids of DT with the gene encoding the ligand, IL-2. A total of 109 patients with refractory IL-2R expressing leukemias and lymphomas were treated. Responses were noted for patients with Hodgkin's lymphoma, cutaneous T cell lymphoma, leukemia and low grade non-Hodgkin's lymphoma. No responses were observed in patients with high grade non-Hodgkin's lymphoma, possibly due to the large tumour mass. In all patients DAB_{486}IL-2 was well tolerated with only transient side effects occurring. However, it was found that 26% of patients had antibodies to diphtheria toxoid and DAB_{486}IL-2 before treatment and 67% had antibodies after treatment. In general, a large percentage of the population would be expected to have antibodies to DT as a result of active immunisation (Blakey, 1992). The presence of these antibodies is likely to reduce the effectiveness of DT derived immunotoxins. As mentioned earlier, a further problem in treating patients with cancers which express high levels of IL-2R is that large amounts of the receptor are shed into the serum and this would lead to rapid clearance of the immunotoxin (Grossbard and Nadler, 1994). Nevertheless, the results of this clinical trial are the first published data in which a fully recombinant immunotoxin was used to treat lymphoid neoplasms. It is interesting to note that despite the high levels of neutralising antibodies

in some patients before and after the trial only 4 patients developed hypersensitivity to DAB_{486}IL-2. These findings suggest that treatment with DAB_{486}IL-2 was effective in a range of cancers and despite the presence of neutralizing antibodies there were encouraging responses. This study has also demonstrated that one of the problems associated with using PE and DT as therapeutic agents is the presence of neutralising antibodies in the serum of some patients.

1.5.6 IMMUNOGENICITY IN IMMUNOTOXIN THERAPY

As discussed in section 1.1 the production of a HAMA response to murine antibody therapy in humans is a major problem. Originally it was thought that the smaller antibody fragments would be less immunogenic. However, it has been shown that two different variable regions have vastly different immunogenic properties (Adair *et al.*, 1993). In a phase I clinical trial it was demonstrated that 16 out of 24 patients treated with the chimaeric antibody B72.3 (cB72.3), which recognises the carcinoma antigen TAG72, developed a strong anti-variable region response. Similarly it was shown that in studies with the murine antibody at least 80-90% of HAMA positive patients produced antibodies to the B72.3 variable region and in the overall study 62% of patients treated with the murine antibody produced antibodies to the variable region. The authors suggested that the variable domain of B72.3 has a particularly immunogenic epitope compared to the chimaeric antibody, c17-1A, as only 1 out of 25 patients showed human anti-chimaeric antibodies (HACA) to the latter. In a related clinical trial 5 patients received ^{131}I-c17-1A and a week later were given ^{131}I-cB72.3. It was found that 4 out of 5 patients produced HACA to B72.3 and none produced antibodies to 17-1A (LoBuglio and Khazaeli, 1993). This study confirms that some murine variable domains are more immunogenic than others. A possible solution would be site directed mutagenesis or CDR grafting onto less immunogenic variable domains, if the immunogenic epitopes are not located in the antigen binding site.

An additional problem in immunotoxin therapy is the production of antibodies to the toxin as well as the antibody. As discussed previously a large percentage of the population has pre-existing neutralising antibodies to DT. A smaller percentage (5%) have antibodies to PE

following exposure during bacterial infections (Fitzgerald and Pastan, 1989). This means that patients with circulating antibodies to these toxins will have a decreased response to therapy. Earlier studies have shown that patients treated with ricin based immunotoxins also develop antibodies to the toxin (Vittetta *et al.*, 1991 ; 1992 ; Ghetie and Vitetta, 1994). It was thought that a smaller targeting agent such as a Fab or scFv might reduce the HAMA response *in vivo*. Unfortunately this has not been the case as a Fab-ricin conjugate, Fab-RFB4-dgA, produced a similar HAMA response as the IgG-RFB4-dgA toxin (Grossbard and Nadler, 1994).

If we intend to use the recombinant immunotoxin K-1-21(scFv)-mel in therapy we should consider the immunogenicity of melittin in humans. An early study on the immunogenicity of melittin in human subjects who were honeybee venom (HBV) sensitive and nonallergic beekeepers has been analysed (Paull *et al.*, 1977). Elevated IgE antibody levels to melittin were found in 29% of allergic subjects and 25% of nonallergic subjects who were exposed to HBV. This study suggests that in some cases melittin acts as an allergen and circulating antibodies to the molecule would be present in certain individuals who have previously been exposed to HBV. However, the overall number of individuals with antibodies to melittin is likely to be relatively low compared to DT. A further study in which rabbits were immunized with melittin showed that antigenic sites were present at the carboxy terminus, in the middle of the molecule and at the amino terminal region (Von Grunigen and Schneider, 1989). Thus the entire melittin molecule is antigenic in rabbits. Whether this antigenicity can be related to the human immune system is unpredictable. However, melittin is relatively small compared to other toxins used in immunotherapy, such as PE and DT, and this might make it less immunogenic. Furthermore, its small size means that its sequence could be manipulated to produce a less immunogenic molecule which retains cytotoxicity.

The mAb, K-1-21, has previously been used to treat a patient with prolymphocytic leukemia. The antibody was administered by infusion and no HAMA response was detected by EIA, which also included a HAMA positive serum control (Mulligan SL, 1988 PhD thesis). Despite this favourable result, further studies are required on the

immunogenicity of K-1-21 in humans before discussing its efficacy in clinical therapy.

1.6 CONSTRUCTION OF THE SINGLE CHAIN RECOMBINANT IMMUNOTOXIN, K-1-21(scFv)-MELITTIN

A summary of antibody fragments for use in clinical therapy shows that these molecules can be used for targeted therapy and in most cases they produce more potent immunotoxins than the immunoconjugates. An overview of the different antibody fragments used to create recombinant toxins suggests that the scFv, dsFv and Fab can all be cloned and expressed in *E.coli* resulting in functional protein. Both the dsFv and Fab are more stable than the scFv under physiological conditions. However, recombinant immunotoxins using the three stabilised Fv fragments are equally cytotoxic and therefore this stability was not directly related to function. It appears from *in vivo* studies that the scFv does not accumulate in the kidneys and would probably be a better targeting agent for clinical purposes.

In the present study a single chain recombinant immunotoxin using a scFv fragment derived from the mAb K-1-21 and a novel membrane lytic peptide, melittin was created. To do this we isolated the heavy and light chain variable region genes from the murine monoclonal antibody, K-1-21, using PCR. The variable region genes were joined by an oligonucleotide which encodes the hydrophilic, flexible peptide linker 3(S, G_4). The resulting scFv gene construct and the single domain K-1-21 VH were cloned and sequenced in the expression vector p(POW).

To produce the recombinant immunotoxin, the gene encoding the lytic peptide melittin was synthesized and then extended by PCR to include the nucleotide sequence for a flexible peptide linker at the 3' region of the gene. This linker was the same sequence as the peptide used to create the scFv gene. It was designed to span the approximate distance of an Fv fragment so that once the scFv fragment bound to antigen, melittin would be able to associate with the cell membrane. It should also be

flexible enough to allow self association of the melittin molecules resulting in membrane disruption. A peptide flag which is recognized by a monoclonal antibody, anti-FLAG M2, was encoded at the carboxy terminus of the melittin gene and enabled subsequent analysis of protein expression and isolation of the expressed product.

The melittin gene was fused to the gene encoding K-1-21 scFv resulting in the fusion scFv-toxin gene, K-1-21(scFv)-mel, in the expression vector pPOW. Expressed protein was directed to the periplasm in bacterial cells using the secretion signal *pelB* and purified from the soluble whole cell fraction by anti-FLAG M2 affinity chromatography.

A theoretical model of the recombinant immunotoxin, K-1-21(scFv)-mel, is shown in Fig 1.9.

Antigen binding activity of the isolated recombinant immunotoxin, scFv-mel, was assessed by ELISA using the soluble antigen, human free *kappa* light chains (BJP). In addition, antigen binding to target cells was determined by flow cytometry using a human myeloma cell line (HMy2) which expresses the specific cancer antigen KMA. Specificity of the cytotoxic activity was determined by incubating the immunotoxin with HMy2 cells and a human cell line (K562) which does not express the surface antigen. Percentage cell death was determined using flow cytometry by measuring ethidium bromide uptake by dead cells.

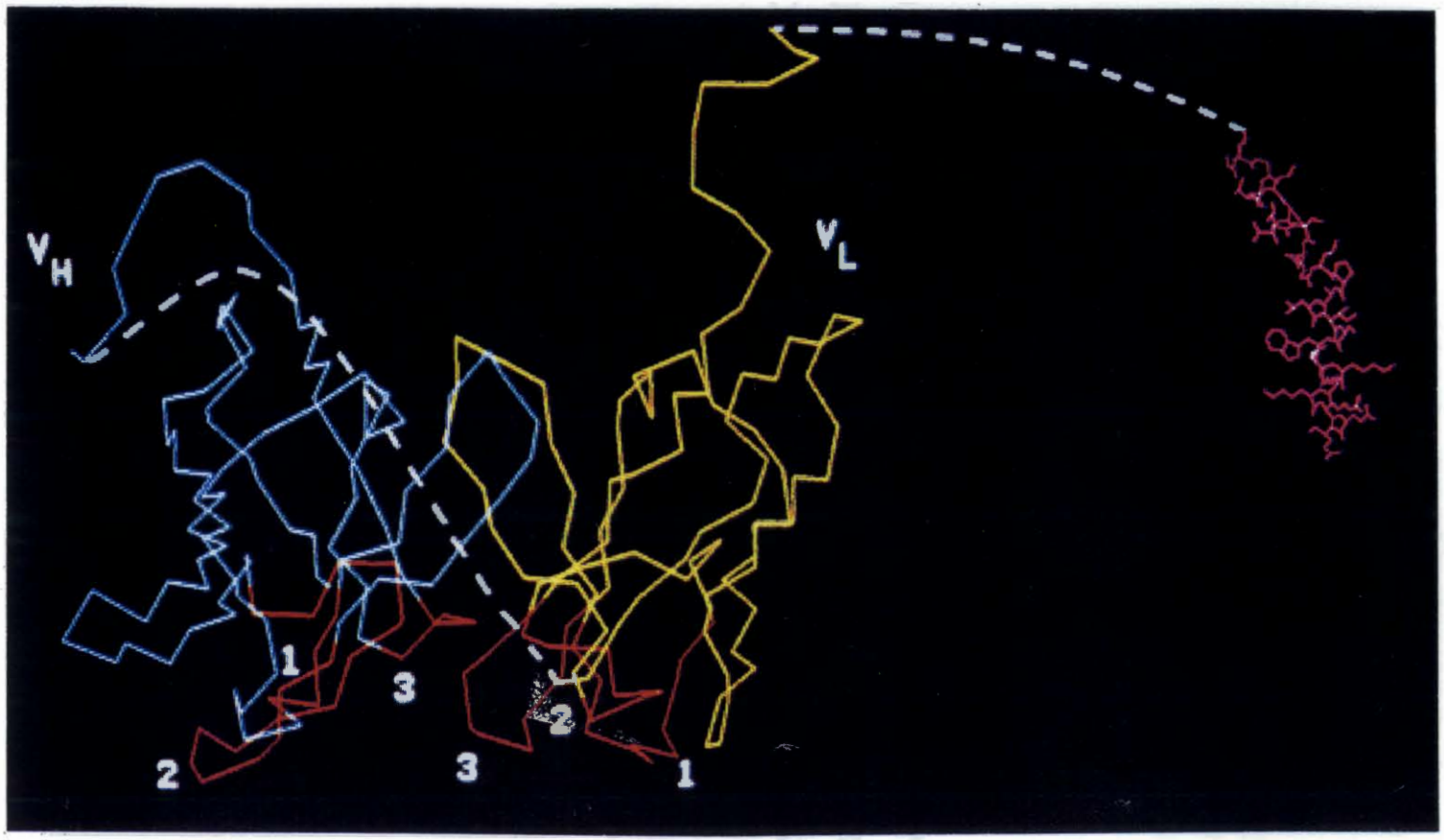


Fig 1.8 A THEORETICAL MODEL OF THE RECOMBINANT IMMUNOTOXIN, K-1-21(scFv)-mel.
This theoretical model has been adapted from the x-ray crystalographic structure of the POT Fv fragment (Fan *et al.*, 1992) and the melittin monomer. The carbon backbone of the VH domain is shown in blue and the VL domain is in yellow. Melittin is depicted as the carbon backbone with the side chains and is shown in purple. A broken white line represents the linker peptide between VH and VL, and also between VL and melittin. The relative scale of the scFv, melittin and the peptide linkers is approximate.

Chapter 2

CONSTRUCTION AND CLONING OF A RECOMBINANT IMMUNOTOXIN FUSION GENE DERIVED FROM THE MONOCLONAL ANTIBODY K-1-21 AND MELITTIN

2.1 INTRODUCTION

As previously discussed in Chapter 1 a number of chemically linked immunotoxins have been produced from monoclonal antibodies with a variety of different specificities. Several types of metabolic toxins or their mutant forms have also been used in these immunoconjugates. Administration of these reagents to patients in phase 1 trials has produced promising results and suggests that immunotoxins are a potential alternative to chemotherapy. The major disadvantage of chemically conjugating the antibody and toxin molecule is that this procedure is often inefficient and results in heterogeneous products. A solution to these problems would be the construction of a fusion gene encoding a single chain recombinant immunotoxin. The subsequent expression of this recombinant immunotoxin in *E.coli* would result in the production of a single, homogeneous protein molecule.

Previous studies have shown that antibody fragments such as VH, Fv, scFv, dsFv and Fab can be cloned using recombinant DNA techniques. The resulting fragments can be expressed in bacterial cells and retain antigen binding activity. Although these smaller fragments can penetrate tissues more readily than whole Ig molecules there are a number of inherent problems associated with certain fragments. The Fv fragment, for example is formed by noncovalent association of the heavy and light chains and therefore dissociates at low concentrations. In general, the scFv, Fab and dsFv have the same binding affinity although the scFv is less stable (Glockshuber *et al.*, 1990). Recombinant immunotoxins using the scFv and dsFv fragment have similar cytotoxicity to target cells and therefore it seems that the longer half life of the latter, both *in vitro* and *in vivo*, offers little advantage. A scFv fragment is probably the preferred molecule for creating a small targeted toxin reagent for use in clinical therapy (Sandhu, 1992).

From the reports discussed in Chapter 1 most recombinant immunotoxins have used either PE or DT as the toxic moieties. Although the cloning and manipulation of these toxin genes has allowed the production of highly toxic mutant forms these are relatively large molecules. In addition, these toxins are metabolic inhibitors which exert their effects within the cell and therefore need to be endocytosed (Pastan and Fitzgerald, 1991). These toxins would therefore not be effective agents

against a cancer antigen, such as KMA, which is not internalised after antibody binding. However, a small membrane lytic peptide such as melittin could be targeted by an antibody fragment and destroy the cancer cell by disrupting the cell membrane. Melittin is a small peptide consisting of 26 amino acids, therefore, an oligonucleotide encoding the peptide sequence could be readily synthesised. Addition of a linker sequence between the scFv and melittin genes could be generated by PCR.

As mentioned in the previous chapter, the variable region genes of both the heavy and light chains can be isolated using a PCR strategy. Primers designed to bind to conserved regions of the rearranged gene, such as the first framework region at the amino terminus and the J_H gene at the carboxy terminus of the antibody, have been used successfully in the PCR.

In the present study, recombinant DNA techniques were used to isolate the heavy and light chain variable region genes from the hybridoma cell line K-1-21. The V_H gene was amplified using a consensus PCR primer complementary to the first five codons of the framework region at the amino terminus of the antibody. A reverse V_H primer which is complementary to the conserved nucleotides in the J_H gene region at the carboxy terminus was used for amplification by PCR. A second reverse primer incorporated the sequence encoding a small octapeptide, flag, which is recognised by the monoclonal antibody anti-FLAG M2 and facilitated identification of the expressed protein. Once the heavy chain variable gene had been amplified by PCR it was isolated and cloned into the expression vector pPOW at the appropriate restriction sites to keep the nucleotide sequence in the correct reading frame.

To amplify the V_L gene a degenerate PCR primer was designed from known amino acid sequence of the amino terminus region of the light chain. The carboxy terminus primer was complementary to the first ten codons of the murine Cκ gene. The V_L gene was sub-cloned into a sequencing vector and the nucleotide sequence of both V_H and V_L was determined before construction of the scFv fusion gene.

To create the scFv the 5' end of the V_L gene was extended using three consecutive PCR primers which sequentially add the oligonucleotide

encoding the linker sequence. The extended light chain gene was then cloned into the pPOW VH construct at the appropriate sites to create the scFv fusion gene. The resulting pPOW scFv construct was subsequently used to produce the scFv-mel fusion gene construct.

The recombinant immunotoxin fusion gene was created in several stages. Firstly, the oligonucleotide encoding the melittin gene was extended using a PCR primer complementary to the first four codons at the 5' end of the melittin oligonucleotide. This primer also contains the nucleotide sequence encoding a 15 amino acid linker peptide. The reverse primer encoded the sequence for the flag peptide and was complementary to the 3' end of the melittin oligonucleotide. After amplification the melittin fusion gene was isolated and cloned into the pPOW scFv construct at compatible restriction enzyme sites. This strategy created the fusion gene construct pPOW scFv-mel 1.

A second strategy was required because it was later discovered that although 5 clones containing the pPOW VH construct showed identical nucleotide sequence, only one of these clones expressed the VH domain. Unfortunately, the V_H gene chosen to construct the scFv-mel 1 fusion gene was one of the domains which was not expressed in *E.coli*. The linker-VL-linker-melittin-flag fusion gene was therefore removed from the pPOW scFv-mel 1 construct by digestion with the appropriate restriction enzymes. The gene fragment was then isolated and ligated into the pPOW VH construct which expressed the single domain antibody.

The final construct, pPOW scFv-mel 2, was sequenced to confirm that the gene construct was in the correct reading frame for subsequent expression in *E.coli*. Recombinant DNA techniques used in the construction of the fusion gene encoding the single chain recombinant immunotoxin, K-1-21(scFv)-mel, and the single domain antibody fragment, K-1-21 VH, are described in detail in this Chapter.

2.2 *METHODS.*

2.2.1 CELL CULTURE

The hybridoma cell line K-1-21 was produced by fusion of spleen cells from BALB/c mice, hyperimmunised with purified human *kappa* Bence Jones proteins (BJP), and mouse myeloma cells, P3-NS1-1-Ag4-1 (Boux *et al.*, 1983). Hybridoma cells were grown in suspension in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), HEPES buffer (4.76 g/l), sodium bicarbonate (8.5 mg/l), penicillin (100 iU/ml) and streptomycin (0.1 mg/ml). Cells were harvested during log phase growth and a total of 10^8 cells were collected by centrifugation, washed in serum-free medium and resuspended in sterile phosphate buffered saline, pH 7.4 (PBS).

2.2.2 RNA EXTRACTION

The precautions recommended for the isolation of non-degraded RNA were strictly adherred to and RNA grade reagents were used throughout the extraction (Sambrook *et al.*, 1989). Total RNA was extracted according to the method of Chomczynski and Sacchi (1987). With some amendments made to the recommended volumes to account for a 10 fold higher cell number. Briefly, 3.5 ml of guanidinium thiocyanate solution (solution D, containing 4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol) was added to 10^8 cells. The solution was drawn up in a sterile syringe and dispelled through a 21 gauge needle 10 times. Afterwards 0.35 ml of 2 M sodium acetate pH 4.0, 3.5 ml of water saturated phenol and 0.7 ml of chloroform : isoamyl alcohol (49:1) were added sequentially. Following centrifugation at 10,000*g* for 20 min at 4°C, the aqueous phase containing RNA was isolated and to this 5 ml of isopropanol was added. The solution was then placed at -20°C for 1hr. Precipitated total RNA was isolated by centrifugation at 10,000*g* for 20 min at 4°C. The RNA pellet was dissolved in 0.3 ml of solution D and transferred to a microfuge tube. Isopropanol (0.3 mls) was added to the solution and RNA was precipitated at -20°C for 1 hr. After centrifugation in an Eppendorf 5415C microcentrifuge at 12,000*g* for 15 min at 4°C the pellet was washed with 75% ethanol, vacuum dried and resuspended in diethyl pyrocarbonate (DEPC) treated water (Sambrook *et al.*, 1989). The concentration of total RNA was determined by measuring the absorbance at 260 nm (1 A_{260} unit of RNA=40 ug/ml). Increasing

amounts of RNA were visualized by electrophoresis on a 1% denaturing agarose gel containing 2.2 M formaldehyde and MOPS buffer (Appendix). RNA molecular weight markers representing a range of 0.24-9.5 kb (Promega Corporation, Madison, USA) were co-electrophoresed to identify the major ribosomal RNA fractions of 28S and 18S.

The PolyATtract mRNA Isolation kit (Promega Corporation) was used to purify mRNA from the total RNA preparation. As the yield of total RNA was approximately 500 µg, the protocol for small scale mRNA isolation was followed. Isolated mRNA was eluted in a total volume of 250 µl, concentrated by alcohol precipitation, and resuspended in 20 µl of RNAase-free water. As the amount of total RNA used was less than 500 µg, the concentration of isolated mRNA was too low to be determined by absorbance at 260 nm.

2.2.3 FIRST STRAND cDNA SYNTHESIS FOR PCR.

First strand cDNA was synthesised from 10 µl of purified mRNA using the cDNA CYCLE kit (Invitrogen, San Diego, USA). Random primers provided in the kit were used for cDNA synthesis according to the recommended protocol and the final product was resuspended in 20 µl sterile water and aliquots stored at -70°C. The approximate yield of cDNA was determined by incorporation of 10 µCi [α-^{32}P] dATP (Amersham, UK). The amount of [α-^{32}P] dATP retained on Whatman DE-81 filters before and after cDNA synthesis was determined on a Beckman scintillation counter and calculated as described in Sambrook *et al.* (1989). According to this method the concentration of cDNA was 1.4 ng/µl. The cDNA:mRNA hybrid was subsequently used to amplify the V_H and V_L genes by Polymerase Chain Reaction (PCR) using a series of oligonucleotide primers. Table 3 lists the PCR primers used to isolate the genes and extend the melittin oligonucleotide.

2.2.4 POLYMERASE CHAIN REACTION

1. Amplification using *AmpliTaq*

Amplification by PCR was carried out using the cDNA template and specific primers for the V_H or V_L genes. The oligonucleotide encoding melittin was extended using suitable PCR primers which are listed in Table 3. A GeneAmp DNA Amplification Reagent kit containing

Table 3 PCR PRIMERS

VH1 for	5'-AGG TSM ARC TGC AGS AGT CWGG -3'
	S = C or G M = A or C R = A or G W = A or T
VH2 for	5'- T ATC GCG CAG GTG CAG CTG CAG -3'
VH1 back	5'- TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CCAG -3'
VH2 flag	5'- ATG ATG GAA TTC TTA TTA TTT ATC ATC ATC ATC TTT ATA ATC TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CCA -3'
VL1 for	5'- CCC GCC AGA CGT/C GAT/C ATT/C GTG/C ATG -3'
VL1 back	5'- CC GAA TTC GAT GGA TAC AGT TGG TGC AGC ATC AGC CCG -3'
VLLK1 for	5'- TCA GGA GGA GGA GGT TCG GGT GGT GGT GGT TCG GAC ATC GTC ATG - 3'
VLLK2 for	5'- CCC ACG GTC ACC GTC GCC TCC GGT GGT GGT GGT TCA GGA GGA GGA GGT - 3'
MEL	5'- GGC ATT GGA GCT GTG CTA AAA GTC CTC ACC ACA GGT CTT CCA GCA TTG ATA TCC TGG ATC AAG CGT AAA CGG CAG CAG - 3'
MELLK for	5'- ATG ATG GAA TTC TCC GGA GGC GGT GGC TCG GGC GGT GGC GGC TCG GGT GGC GGC GGC TCT GGC ATT GGA GCT GTG - 3'
MELflag	5'- ATG ATG GTC GAC TTA TTA TTT ATC ATC ATC ATC TTT ATA ATC TGA GGA GAC CTG CTG CCG -3'

The primers listed above were used for the construction of the gene encoding the K-1-21(scFv)-mel recombinant immunotoxin. Individual primers are discussed in detail in the Results section. Restriction enzyme sites incorporated in the primers have been underlined.

10x Reaction buffer, 10 mM dNTP's and *AmpliTaq* DNA polymerase was obtained from Perkin Elmer Cetus, Norwalk, USA.
The PCR mixture contained:

cDNA:RNA hybrid	10-14ng cDNA (from section 2.2.3)
for primer	25 pmol
back primer	25 pmol
dNTP's	200 µM each
10x PCR Buffer	
sterile water to	100 µl

The mixture was overlayed with paraffin oil and placed in a HYBAID heat cycler for 5 min at 95°C and then held at 92°C whilst 2.5 units of *Taq* DNA Polymerase was added to the tube. After addition of the enzyme the reaction was cycled for 1 min at 55°C, 2 min at 72°C and 1 min at 93°C for 35 cycles. Amplified products were resolved by polyacrylamide gel electrophoresis (PAGE) on a 10% polyacrylamide gel (Appendix). Gels were stained with ethidium bromide (Eth.Br; 0.2 µg/ml) in TBE pH 8.0 (0.09 M Tris, 0.09 M boric acid and 1 mM EDTA) and then viewed under ultra violet (UV) light (Sambrook *et al.*, 1989).

2. PCR using *Pfu* DNA polymerase

In a PCR using the heat stable DNA polymerase, *Pfu*, the recommended reaction mixture was followed and contained 50 pmol of each primer (Stratagene, La Jolla, CA). The DNA was initially denatured at 95°C for 5 min, annealed at 50°C for 5 min and the enzyme, *Pfu* DNA polymerase was then added. The reaction was cycled 35 times at 72°C for 1 min, 94°C for 30 s and 55°C for 30 s. Afterwards the products were separated by PAGE on a 10% acrylamide gel and viewed under UV light.

2.2.5 DNA PURIFICATION

1. Extraction with phenol:chloroform (Phe/Chl)

The solution containing DNA was either from a PCR reaction or enzyme digestion. An equal volume of a 1:1 solution of Tris buffered phenol pH 8.0 and chloroform:isoamyl alcohol (24:1) was added to the DNA solution in a microfuge tube. After mixing, the solution was centrifuged at 12,000*g* in a microcentrifuge for 5 min at room temperature. The aqueous layer was removed and extracted with chloroform:isoamyl

alcohol (24:1), centrifuged at 12,000*g* for 5 min and once again the aqueous layer was isolated. An aliquot of 3 M sodium acetate was added to the aqueous layer to give a final concentration of 0.3 M sodium acetate. This was followed by adding 2 volumes of absolute ethanol to the mixture which was then placed at -80°C for 30 min. Afterwards, the mixture was centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was carefully removed with a drawn out pasteur pipette and the DNA pellet was rinsed with 500 µl of 75% ethanol. After centrifugation at 12,000*g* for 5 min at 4°C the pellet was vacuum dried and then resuspended in sterile water (Sambrook *et al.*, 1989). Where stated, DNA was precipitated from solution directly by the addition of 2 volumes of absolute ethanol, washed with 75% ethanol, dried and resuspended in sterile water.

2. Proteinase K treatment of the PCR mixture

To remove the enzyme, *Taq* DNA polymerase, from the reaction mixture the aqueous layer was treated with Proteinase K (Promega Corporation). The aqueous layer of the PCR mixture was removed and 1 µl EDTA (0.5 M), 5 µl 10% SDS and 5 µg/100 µl Proteinase K were added to the PCR product and incubated at 50°C for 30 min. Proteinase K was then heat denatured at 68°C for 10min. The DNA was extracted with phenol:chloroform (1:1) and ethanol precipitated as outlined above.

2.2.6 DIGESTION WITH RESTRICTION ENDONUCLEASES

Digestion of the PCR products and cloning vectors was carried out using restriction endonucleases (RE) from Toyobo, USA unless otherwise stated. In all experiments digestion buffers were also obtained from Toyobo and the conditions recommended by the manufacturers were followed.

2.2.7 ISOLATION OF DNA FROM AGAROSE GELS

1. Purification using NA-45 membranes

Separation of the DNA was carried out on agarose gels (Appendix) by electrophoresis at 120 volts. NA-45 membranes were obtained from Schleicher & Schuell, Dassel, Germany. Strips of membrane (1 x 5 cm) were cut and then activated by soaking them in 10 mM EDTA pH 8.0 for 10 min followed by 0.5 M potassium hydroxide. The membrane strips were then washed in sterile water until pH 7.0 was reached and stored at 4°C in sterile water.

The DNA to be isolated was resolved by electrophoresis on an agarose gel, stained with Eth.Br and then viewed with a UV monitor to determine the position of the DNA. A slit was cut in the agarose gel in front of the DNA band. A piece of activated membrane was cut to the required size and then inserted into the slit. The DNA was collected on the membrane by electrophoresis at 100 volts for 10-15 min. The membrane was then removed and washed with TBE pH 8.0. The DNA associated with the membrane was eluted with TEN buffer pH 7.5 (1 M NaCl, 10 mM Tris, 1 mM EDTA and 50 mM arginine) at 50°C and precipitated by addition of 2 volumes of absolute ethanol and stored at -20°C overnight. Precipitated DNA was centrifuged at 12,000*g* for 15 min at 4°C, washed with 75% ethanol, dried and resuspended in sterile water.

2. Purification of DNA using an ELUTIP-d column

ELUTIP-d columns were obtained from Schleicher & Schuell, Dassel, Germany. Preparation of the column was carried out as directed by the manufacturers using a high salt buffer (1.0 M NaCl, 20 mM Tris-HCl, pH 7.4 and 1 mM EDTA) followed by a low salt buffer (0.2 M NaCl, 20 mM Tris-HCl, pH 7.4 and 1 mM EDTA).

Isolation of the DNA was carried out by electrophoresis on an agarose gel followed by electro-elution (Sambrook *et al.*, 1989). Briefly, a small portion of the gel was excised immediately in front of of the DNA band and rinsed with TAE buffer, pH 8.0 (0.04 M Tris, 57.1 ml/L glacial acetic acid and 1 mM EDTA). The gel was then electrophoresed for 5 min at 100 volts and then the solution was collected from the cavity. Fresh TAE buffer (approximately 200 µl) was added to the cavity and the gel was electrophoresed for a further 5 min. The solution from the cavity was collected and the procedure was repeated until all the DNA had been isolated. The solution containing the DNA was then passed through the ELUTIP-d column and washed with low salt solution. Bound DNA was eluted from the column in 0.4 ml high salt solution as recommended by the manufacturer. Precipitation of the DNA was accomplished by addition of 2 volumes of absolute ethanol and the mixture was incubated at -20°C overnight. The DNA was then centrifuged in a microcentrifuge at 12,000*g* for 15 min at 4°C. The pellet was rinsed with 75% ethanol, centrifuged at 12,000*g* for 5 min at

4°C and vacuum dried. Precipitated DNA was resuspended in sterile water.

2.2.8 PURIFICATION OF PLASMID DNA USING GENECLEAN

After digestion with RE, plasmid DNA was purified and isolated using the GENECLEAN protocol. A GENECLEAN kit was obtained from Stratagene, La Jolla, CA. All procedures were carried out according to the instructions supplied by the manufaturer. However, it was found that higher recoveries were obtained if the plasmid DNA was incubated with the silica beads for 2-8 hr at room temperature on a rotating stand. The silica beads were then washed and the DNA eluted as recommended.

2.2.9 LIGATION OF DNA INSERT AND THE VECTOR

1. Ligation using compatible RE sites

Prior to ligation the DNA insert and linearised vector were purified using one of the methods described above. In general a vector:DNA insert ratio of 1:1 to 1:10 was used in the ligation depending on the vector used. The amount of insert required was calculated using the following formula from the pCR-ScriptTM SK(+) cloning kit (Stratagene, La Jolla, CA).

$$\frac{\text{ng vector} \times \text{kb size of DNA insert}}{\text{kb size of vector}} \times \frac{\text{molar ratio of insert}}{\text{vector}} = \text{ng insert}$$

A ligation mixture was prepared with the required amount of vector, DNA insert, 5x Ligation buffer (BRL, Gaithersburg, USA), 1 unit T4 DNA Ligase (BRL, Gaithersburg, USA) and sterile water to give a final volume of 20 µl. The mixture was incubated overnight at 14°C. On the following day the ligation mixture was either transformed directly into competent *E.coli* cells or ethanol precipitated before transformation.

2. Ligation of PCR product using T-tailed Bluescript

Blunt end cloning of PCR products is inefficient due to the addition of dATP at the 3' end of the fragment by the template-independent terminal transferase activity of *Taq* polymerase. Addition of a single thymidine to the 3' end of the cloning vector enables direct cloning of the PCR product into the T-tailed plasmid. A T-vector was created using the vector Bluescript (Stratagene, La Jolla, CA), according to the method of Marchuk *et al.* (1990) to enable direct cloning of the purified PCR

product. Approximately 1-2 ng of T-vector was mixed with 1-5 ng of purified DNA insert, 5x ligase buffer (BRL, Gaithersburg, USA) and 4 units of T4 DNA ligase (BRL) in a total volume of 30 µl. The ligation mixture was incubated at 14°C overnight. After incubation the reaction mixture was DNA precipitated with 2 volumes absolute ethanol, centrifuged, washed with 75% ethanol, dried and resuspended in sterile water. The ligation mixture was then transformed into *E.coli* cells.

In some cases a pCR-ScriptTM SK(+) cloning kit was used and the protocol for ligation was carried out as recommended by the manufacturers (Stratagene, La Jolla, CA).

2.2.10 TRANSFORMATION OF *E.coli* CELLS WITH THE LIGATION MIXTURE

1. Transformation using $CaCl_2$ competent cells

Before transformation of the ligation mixture into *E.coli* cells, they were made competent using the $CaCl_2$ method (Sambrook *et al.*, 1989). Briefly, a single colony of the *E.coli* strain of choice was removed from a LB plate (appendix) and used to inoculate 40 ml LB medium (Appendix). Cells were grown at 37°C until an A_{600} of 0.2-0.5 was reached. The culture was then centrifuged at 2,500*g* for 5 min at room temperature. The supernatant was discarded and the cell pellet was gently resuspended in 2 ml of 50 mM $CaCl_2$. The volume of the cell suspension was increased to 20 ml with 50 mM $CaCl_2$ and placed on ice for 30 min. The cells were centrifuged at 2,500*g* for 5 min at 4°C and then resuspended in 4 ml ice cold 50 mM $CaCl_2$. Cells were then stored at 4°C overnight before transformation. The genotypes for *E.coli* strains used for transformation are shown in the Appendix and includes competent cells obtained from Stratagene, La Jolla, CA.

Transformation was carried out by adding 40 µl of cells into a pre-chilled 15 ml Falcon 2059 polypropylene tube. An aliquot (0.7 µl) of 1.44 M ß-mercaptoethanol was added to the cells, the mixture was swirled gently and placed on ice for 10 min. An aliquot of the ligation mixture was added to the tube and the solution was gently swirled. Cells were placed on ice for 30 min and then heat pulsed at 42°C for exactly 45 s. The transformation mixture was placed on ice for 2 min and then 0.45 ml of 2x YT medium or SOC medium (appendix) which was previously warmed to 42°C was added. Cells were placed at 37°C and

grown for 1hr in an orbital shaker at 200-250 rpm. Aliquots (100 µl and 200 µl) of the transformation mixture were then plated out onto LB+amp plates (appendix) and grown overnight at 30°C for pPOW or 37°C for other vectors.

2. Transformation of *E.coli* cells using electroporation

Electroporation using a BIO RAD Gene Pulser (BIO RAD, Richmond, CA) was used to transform electrocompetent *E.coli* cells with the ligation mixture. The cells were made electrocompetent according to the procedure outlined in the manual. An aliquot of cells was thawed on ice and 50 µl was added to 3 µl of precipitated, resuspended ligation mixture. Transformation was carried out at 1.8 kvolts, 25 uFD and 200 Ohms using a path length of 0.1 cm according to the recommended protocol. Cells were then added to 1 ml SOC medium and placed at 37°C for 1 hr. Finally, aliquots (100 µl and 200 µl) were plated out on LB+amp plates and incubated overnight at 37°C.

Both the Bluescript and pCR-Script^{TM}SK(+) vectors enable alpha complementation with the appropriate substrate. Thus, transformed cells using these vectors were plated on LB+amp plates containing 20 µl of 10% x-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) and 20 µl of 0.2 M isopropylthiogalactoside (IPTG). After overnight growth at 37°C clones containing DNA insert were identified by white/blue selection.

2.2.11 IDENTIFICATION OF POSITIVE CLONES

1. Hybridisation using a radioactive probe

After ligation and transformation into *E.coli* cells, colonies containing the V_H insert were identified by hybridisation with a radioactive PCR probe. Individual colonies were removed from plates with toothpicks and spotted onto nitrocellulose filters (Schleicher & Schuell, Dassel, West Germany). A negative control of vector without insert was included as was a positive control of the V_H PCR product. Filters were treated with 1.5 M NaCl, 0.5 M NaOH for 7min and then transferred to a solution of 1.5 M NaCl, 0.5 M Tris-HCl pH 7.2 and 1 mM EDTA for 3.5 min. These steps were repeated with fresh solutions before rinsing with 2x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) blotted dry with filter paper and wrapped in plastic film wrap (Glad Wrap). Finally the membrane was exposed to UV light in order to fix the DNA.

A radioactive probe was prepared using the USB Random Primed DNA Labelling Kit (United States Biochemical, Cleveland, USA) with approximately 30 ng of purified V_H PCR product as template and [a-^{32}P] dATP (50 µCi; 3000 Ci/mmole from Amersham, UK). The radiolabelled probe was prepared according to instructions recommended by the manufacturer.

Membranes were placed in Hybaid bottles and rinsed with 2x SSC. This was replaced with 10 ml pre-hybridisation mix (6x SSC containing 0.1% BSA) and the filter was incubated at 68°C for 1hr. Previously prepared radioactive probe was denatured for 5 min at 100°C for 5 min and added to 6x SSC, 0.5% SDS and 100 µg/ml salmon sperm DNA (Stratagene, La Jolla, CA). The probe was incubated with the filter overnight at 68°C in a Hybaid oven. After incubation the membranes were rinsed with 2x SSC and washed 3 times in 2x SSC with 0.1% SDS for 15 min. Finally the filter was air dried and developed with X-Ray film (Kodak, USA) overnight. Clones containing the DNA insert were identified and individual colonies were re-plated on LB+amp plates for further analysis.

2. PCR screening of *E.coli* colonies to identify the DNA insert

After transformation, plating and overnight incubation, positive clones were identified by PCR screening. Briefly, individual colonies were removed with a toothpick and used as PCR template by dipping the cells into a PCR tube containing 25 pmol of forward primer, 25 pmol backward primer and PCR reagents (as described in section 2.2.4). The samples were subjected to 25 cycles of PCR using the conditions outlined previously. The toothpicks containing the stab cultures were then dropped into 2x YT medium and grown overnight in a shaking incubator at 37°C. Products from the amplification mixture (14 µl) were visualised on a 1.0% agarose gel.

2.2.12 DNA SEQUENCING OF PLASMID MINIPREPS

After growth of the transformation mixture on LB+amp plates, single colonies were removed and used to inoculate 10 ml of 2x YT medium. Cultures were grown overnight and then plasmid DNA was prepared using the Alkaline Lysis method described in Sambrook *et al.*, (1989). The purified plasmid DNA was resuspended in 50 µl TE pH 8.0 (10 mM

Tris and 1 mM EDTA) and treated with 1 µg/ml DNAase free RNAase (Stratagene, La Jolla, CA).

Purified plasmid DNA was sequenced using the Sequenase version 2.0 sequencing kit from Stratagene, La Jolla, CA. Briefly, 2-4 µg plasmid DNA was denatured in the presence of approximately 4 pmol of either forward or backward sequencing primers. Subsequent annealling and termination reactions were performed as described in the kit protocol using [a-^{35}S]-dATP. The reactions were resolved on a 6% polyacrylamide gel containing 0.315 g/ml urea and TBE pH 8.0 and then electrophoresed using a LKB Base Runner System. The gel was fixed in 10% acetic acid and 10% methanol for 30 min, washed for 30 min in water and air dried before leaving overnight on an X-ray film (Kodak, USA). After 48 hr the film was developed and the sequence analysed.

Sequencing primers for the vector pPOW are described in the Appendix. Bluescript sequencing primers, Reverse primer and SK primer, were obtained from Stratagene, La Jolla, CA. The sequencing primers T3 and T7 were used to sequence the pCR-Script™ SK(+) vector and were obtained from Stratagene, La Jolla, CA.

2.3 *RESULTS*

2.3.1 RNA

Total RNA extracted from K-1-21 hybridoma cells was resolved on a 1% denaturing agarose gel and the results are shown in Fig 2.1. Discrete bands representing the major ribosomal RNA fractions within the cell, 18S and 28S, were observed. From the OD_{260} the concentration of RNA was estimated to be 4.5 µg/µl. Approximately 500 µg of total RNA was used in the isolation and concentration of mRNA before first strand synthesis of cDNA (as described in methods). The concentration of cDNA was estimated to be 1.4 ng/µl by calculating the amount of [α-^{32}P] dATP incorporated.

2.3.2 CONSTRUCTION OF THE V_H-FLAG GENE

As mentioned previously the V_H gene was amplified using PCR primers complementary to the 5' and 3' ends of the V_H gene. A schematic outline of the PCR procedure used to isolate the V_H gene and subsequent cloning of the V_H DNA insert into the vector pPOW is shown in Fig 2.2.

Primers designed to bind to the 5' region of the V_H gene.

A degenerate primer designed by Ward *et al.*, (1989) was used to isolate the V_H gene from cDNA obtained from K-1-21 cells as described in section 2.2.3. This primer was complementary to the conserved nucleotides in the first framework region of the rearranged Ig gene. A Pst 1 restriction site was incorporated in the oligonucleotide sequence to enable directional cloning of the PCR amplified gene and is underlined.

VH1 for (sense)

5'-AGG TSM ARC TGC AGS AGT CWGG -3'

S = C or G M = A or C R = A or G W = A or T

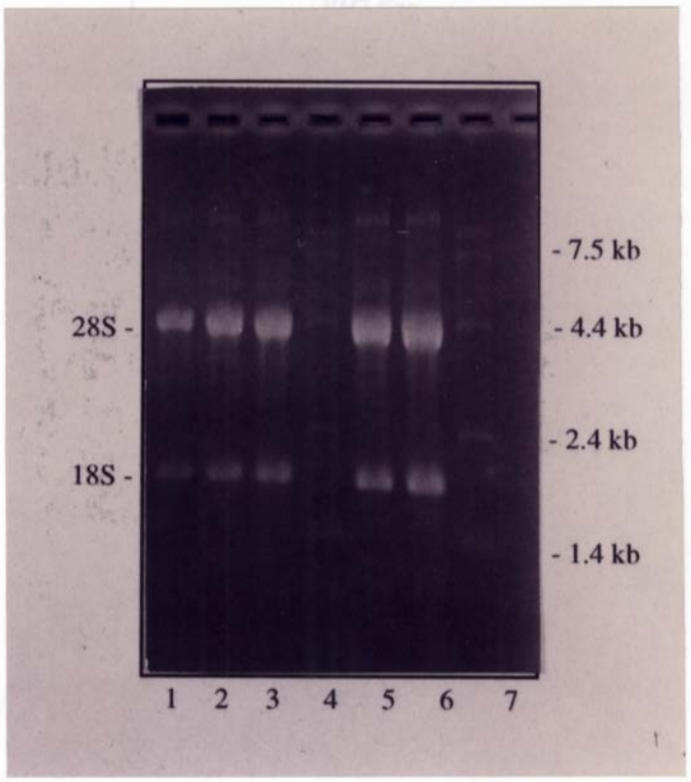


Fig 2.1 ELECTROPHORESIS OF ISOLATED TOTAL RNA
Increasing amounts of total RNA isolated from K-1-21 hybridoma cells were resolved on a 1% denaturing agarose gel. The gel was stained with ethidium bromide (Eth.Br) and visualised under ultra violet (UV) light. Lane 1, 1 µg RNA ; lane 2 and 3, 2 µg RNA ; lane 4 and 7,; 0.24-9.5 kb RNA molecular weight standards ; lane 5 and 6, 4 µg RNA.

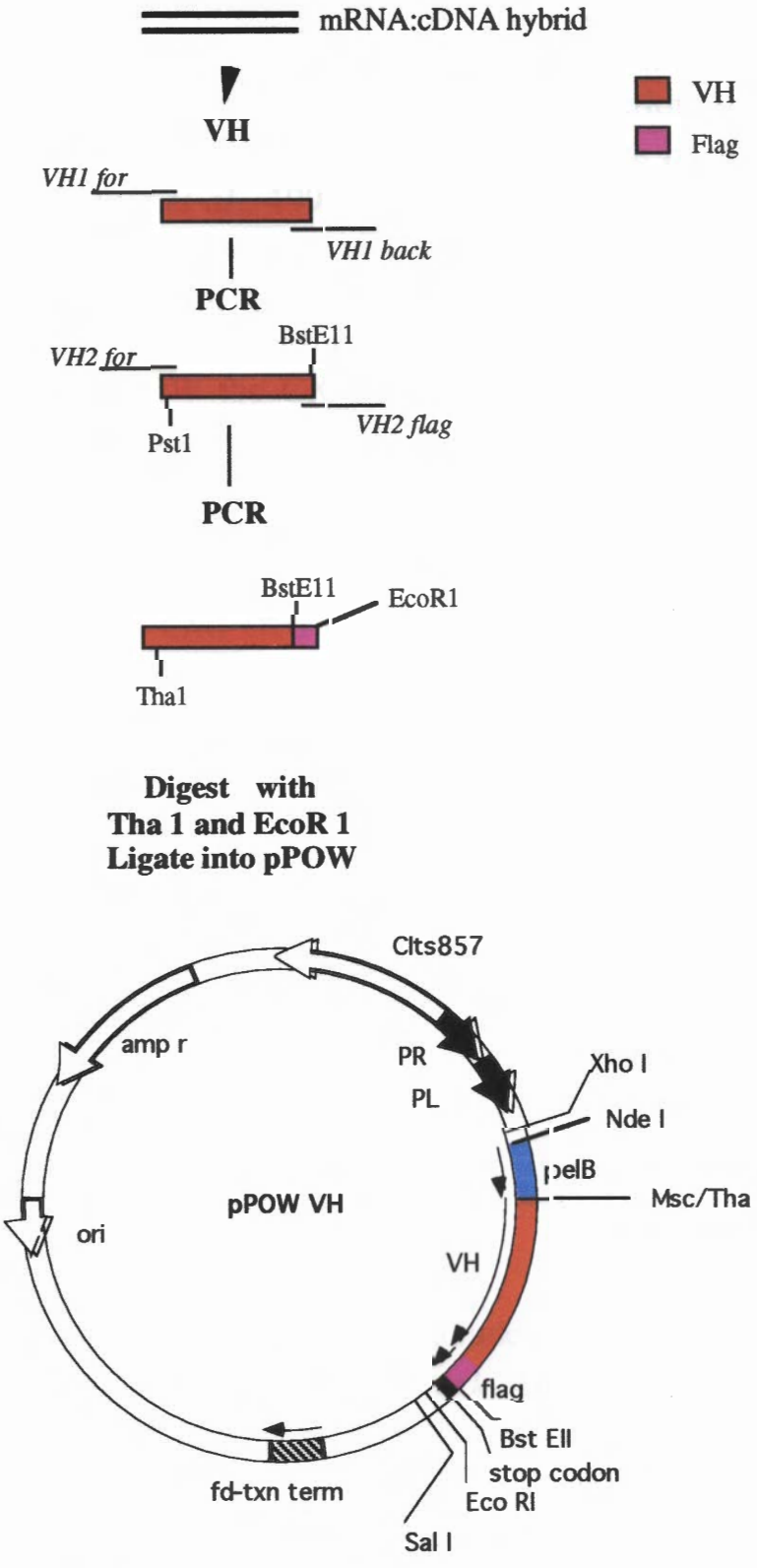


Fig 2.2 SCHEMATIC DIAGRAM SHOWING PCR ISOLATION AND CLONING OF K-1-21 V_H GENE

Primers *VH1 for* and *VH1 back* were used to isolate the V_H gene from K-1-21 cDNA. RE sites Tha 1 and EcoR 1 were incorporated in the second set of primers *VH2 for* and *VH2 flag*. The sequence encoding the flag octapeptide was added using the primer *VH2 flag*. After the second PCR amplification the V_H-flag DNA was digested with enzymes Tha1 and EcoR1 and ligated into pPOW restricted with the same enzymes.

A second V_H forward primer, *VH2 for*, had the same sequence as one of the combinations from *VH1 for* and was used to introduce a Tha 1 restriction site into the isolated V_H gene. The restriction enzyme site is underlined and enabled cloning into a compatible site in the expression vector pPOW (Power *et al.*, 1992). Digestion with Tha 1 results in the last 2 nucleotides CX (where X is any of the four nucleotides), as shown below in bold type. Using this primer, digestion of the amplified DNA with Tha 1 creates a blunt end at the 5' end of the gene to be cloned. Once ligated into pPOW the CX becomes GCX and recreates the last amino acid codon (alanine) of the *pelB* signal, keeping the gene in the correct reading frame for expression.

VH2 for (sense)

5'- T ATC GC**G** CAG GTG CAG CTG CAG -3'

Complementary primers designed to bind to the 3' region of the V_H gene sequence.

The backward V_H primer is a consensus primer complementary to the conserved nucleotides of the J-region of rearranged V_H genes. This primer incorporates a BstE 11 restriction enzyme site which is underlined. Although this primer was used to amplify the original V_H gene it was superseded by *VH2 flag* in all pPOW constructs.

VH1 back (anti-sense)

5'- TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CCAG -3'

The second V_H back primer, *VH2 flag*, includes the flag sequence and the 3' end is similar to the 3' end of *VH1 back*. This primer introduced an EcoR 1 restriction site which was used for directional cloning of the V_H gene into the expression vector pPOW. A nucleotide sequence encoding an octapeptide, which is recognised by the monoclonal antibody anti-FLAG M2, was included at the 5' end of the primer. The expressed protein would include the octapeptide at the carboxy terminus end which should facilitate monitoring and isolation of the expressed product. In addition, a BstE 11 restriction site was introduced immediately after the nucleotide sequence encoding the J_H region at the 3' end to enable directional cloning of the linker-V_L gene and to allow construction of a

scFv gene fragment. The restriction enzyme sites are underlined and the nucleotide sequence for the flag peptide is in bold print.

VH2 flag (anti-sense)

5'- ATG ATG GAA TTC TTA TTA **TTT ATC ATC ATC ATC TTT ATA ATC TGA** GGA GAC GGT GAC CGT GGT CCC TTG GCC CCA -3'

Amplification of the V_H gene

PCR amplification using the cDNA/mRNA hybrid as template with the heavy chain V-region primers, *VH1 for* and *VH1 back* was carried out as described in section 2.2.4. A DNA band of approximately 350 bp was produced which is the expected size of the V_H gene (Fig 2.3).

A preparative PCR was carried out using optimised conditions for V_H gene amplification. The amplified reaction products were pooled, extracted and the purified DNA was resuspended in water to a concentration of 200 ng/µl. Approximately 600 ng was subjected to digestion with a restriction enzyme relevant to directional cloning of the DNA fragment. This was to ascertain whether internal restriction sites were present in the amplified V_H DNA. Digestions using Tha 1, Pst 1, BstE 11 and EcoR 1 were carried out and a negative control consisted of DNA in the absence of RE. The results of digestion of the purified V_H PCR product with these restriction enzymes are shown in Fig 2.4. Amplified V_H DNA digested with Pst 1 shows two bands which have a smaller molecular size than the 350 bp undigested sample. In the presence of the other enzymes, Thal, EcoR 1 and BstE 11, amplified DNA was present as a single band of 350 bp. From these results it was apparent that the PCR primer *VH1 for* could not be used to amplify the V_H gene because digestion with Pst 1 for directional cloning would cut the gene.

Addition of the flag sequence to V_H

The PCR was carried out as described in section 2.2.4 using 1 µl of purified V_H template and primers *VH2 for* and *VH2 back*. After

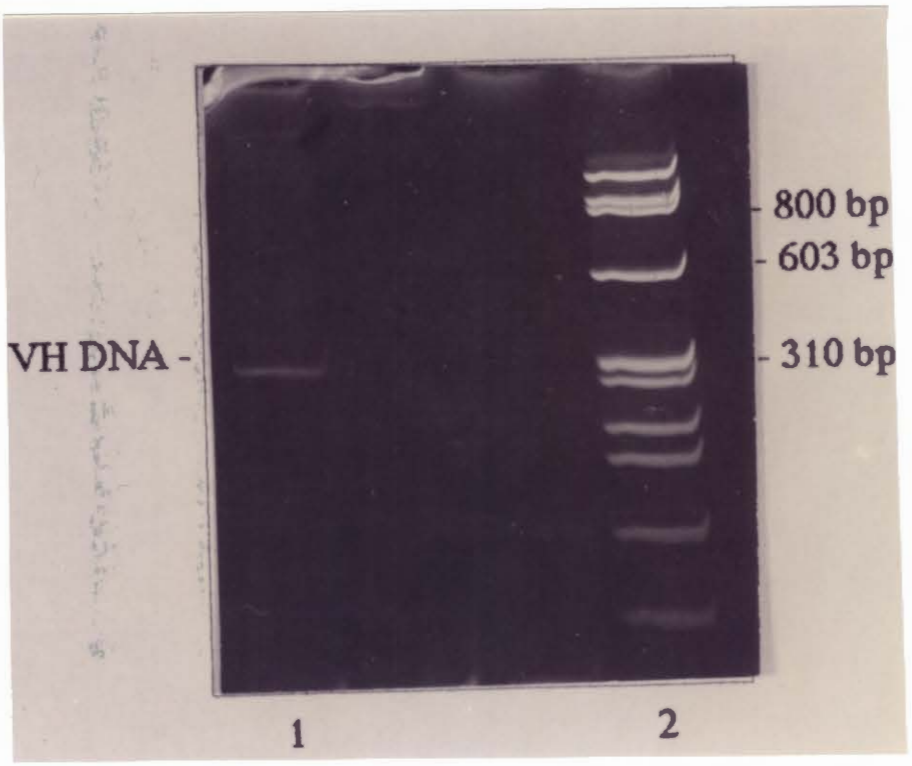


Fig 2.3 AMPLIFIED V_H GENE USING PRIMERS *VH1 for* AND *VH1 back.*

The K-1-21 V_H gene was amplified using PCR primers *VH1 for* and *VH1 back* as described in section 2.2.2. An aliquot (16 µl) of the PCR product was resolved by PAGE on a 10% acrylamide gel and stained with Eth.Br. Lane 1, V_H PCR product; lane 2, 1 µl Øx174 Hae digested DNA markers 72-1353 bp (USB, Cleveland, Ohio).

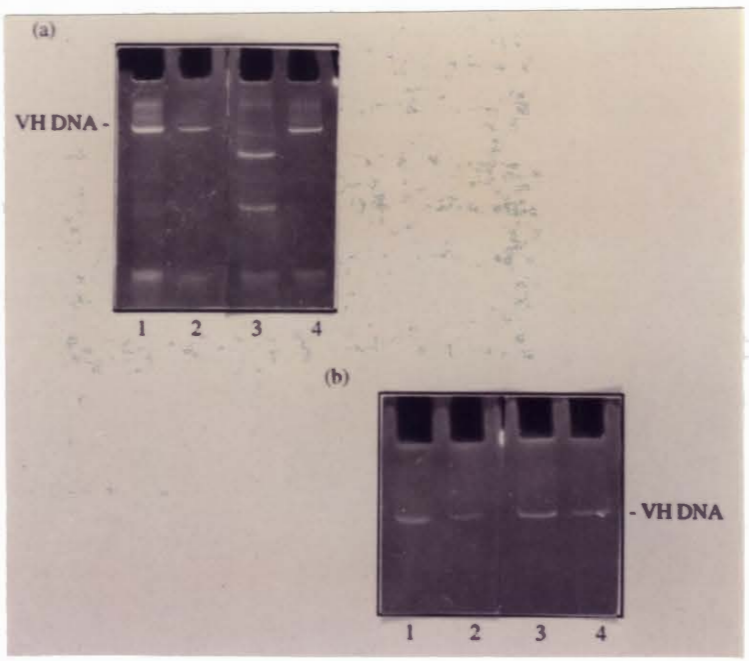


Fig 2.4 RESTRICTION ENZYME ANALYSIS OF THE V_H GENE
Aliquots (600 ng) of isolated PCR product representing the V_H gene (350 bp) were digested with restriction enzymes Tha 1, Pst 1, BstE 11 and EcoR 1. The resulting digests were resolved by PAGE on a 10% acrylamide gel and stained with Eth.Br. In: (a) lane 1, V_H DNA digested with Tha 1 ; lane 2, no enzyme ; lane 3, V_H DNA digested with Pst 1 ; lane 4, no enzyme. (b) lane 1, V_H DNA digested with BstE 11 ; lane 2, no enzyme ; lane 3, V_H DNA digested with EcoR 1 ; lane 4, no enzyme.

amplification the aqueous layer was extracted with Phe/Chl, precipitated and dried. The pellet was resuspended in 40 µL sterile water and electrophoresed on a 1.5% agarose gel. A DNA band of about 350 bp representing the expected size of V_H was isolated and purified using a NA-45 membrane. The precipitated V_H-flag DNA was resuspended in 30 µl sterile water and then digested with RE, Tha 1 and EcoR 1, for directional cloning into pPOW.

Cloning of V_H-flag in pPOW

Approximately 300 ng of purified V_H-flag was digested in a total volume of 50 µl with Tha 1. Digested DNA was then ethanol precipitated and re-digested with EcoR 1 in a total volume of 50 µl. The digested DNA was purified by Phe/Chl extraction and resuspended in 10 µl water. A 2 µl aliquot of the digested, purified V_H-flag DNA was resolved on a 1.5% agarose gel and the results are shown in Fig 2.5. The vector pPOW was digested with Msc 1 to create a compatible blunt end for the Tha 1 digested DNA and re-create the last codon for the PelB signal. Afterwards, pPOW was digested with EcoR 1 to produce compatible cloning sites for the digested V_H-flag gene. Linearized vector was purified on a 1% agarose gel and concentrated using NA-45 membranes with subsequent purification.

A ligation reaction was carried out using RE digested pPOW (20 ng) and V_H-flag (10 ng) as described in section 2.2.9. Aliquots of the ligation mixture (2 µl and 8 µl) were transformed directly into the *E.coli* strain, TG1, and 50 µl, 100 µl and 200 µl aliquots of the transformation mixture were plated out on LB+amp plates and grown at 30°C overnight.

Identification of V_H-flag recombinants

Following ligation of V_H-flag DNA insert into pPOW and transformation into TG1 cells, single colonies from the LB+amp plates were removed by toothpick and spotted onto nitrocellulose membranes. The membranes were hybridised with a V_H-PCR probe as described in section 2.2.11. All clones, including the positive and negative controls, were spotted onto the membrane in duplicate and the results are shown in Fig 2.6. The grids were numbered in duplicate from 2-34 and the positive control, which consisted of V_H PCR product, was spotted on

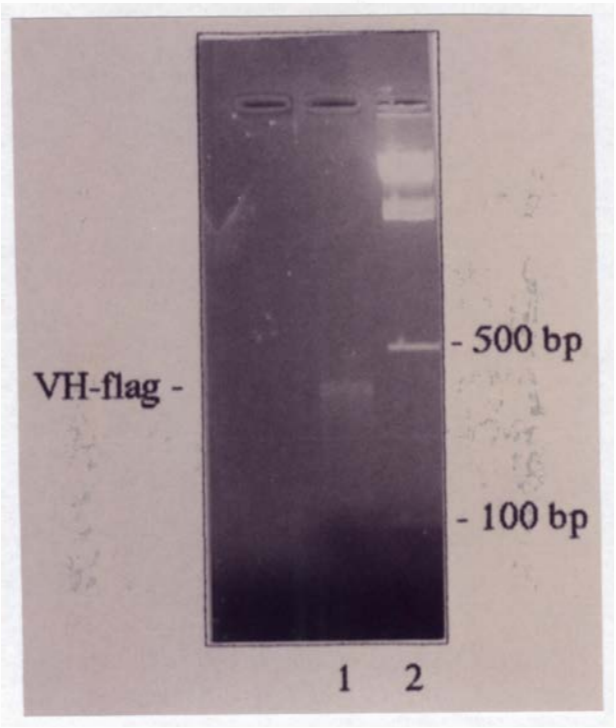


Fig 2.5 PURIFIED V_H-flag DNA FROM THE PCR USING PRIMERS *VH2 for* AND *VH2 flag*

The Tha 1 and EcoR 1 digested V_H gene was purified using agarose gel electrophoresis and then collected onto NA-45 membranes. After purification the DNA was resuspended in 10 µl water and an aliquot of 2 µl was resolved on a 1.5% agarose gel. The DNA was stained with Eth.Br. and visualised under UV light. Lane 1, 2 µl of purified, digested V_H DNA ; lane 2, DNA molecular weight standards (Hind 111 digested lambda DNA, 0.2-2.5kb ; Promega Corporation, Madison, USA).

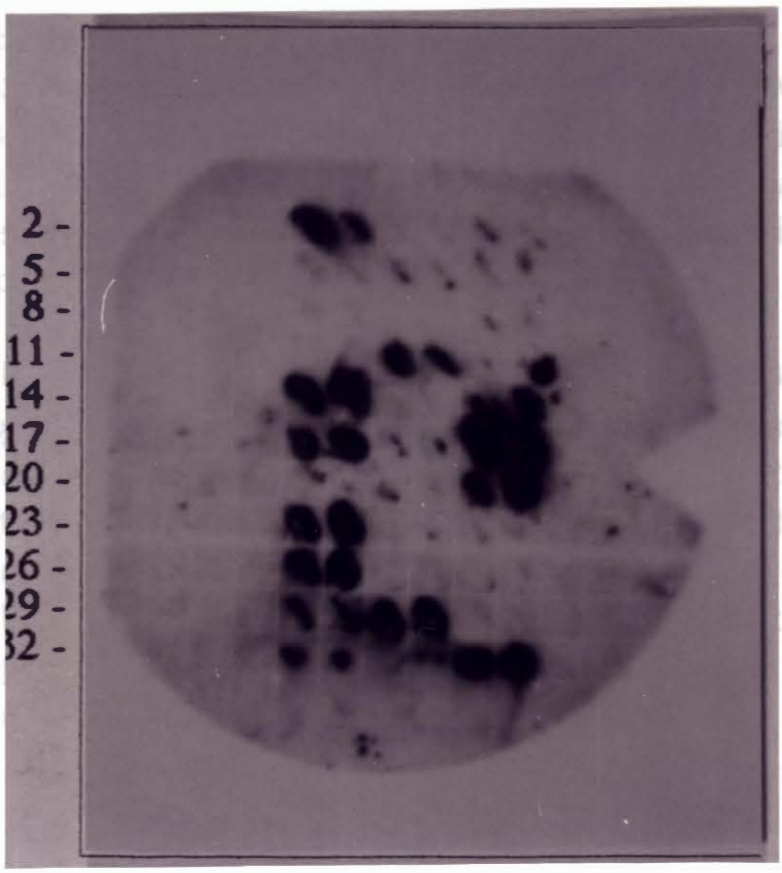


Fig 2.6 IDENTIFICATION OF V_H RECOMBINANTS USING A RADIOLABELLED PCR PROBE

Colonies were spotted onto the nitrocellulose membrane and hybridised with the radioactive V_H PCR probe as described in section 2.1.11. A photograph of the results is shown. Each colony was spotted in duplicate from left to right. Numbers on the left hand side correspond to the first colony in that row and each row depicts three duplicate samples. A negative control, which contained pPOW without insert, is shown in duplicate in number 33. Positive controls consisting of purified V_H PCR product are shown in duplicate in number 32 (1 µl) and 34 (2 µl). Colonies 12, 14, 17, 19 and 30 were sequenced as detailed in the text.

grids 32 (1 μl) and 34 (2 μl). A negative control using pPOW without insert was spotted in position 33. Altogether five clones were sequenced and these corresponded to numbers 12,14,17,19 and 30 from the membrane grid.

V_H nucleotide sequence

Single colonies identified as containing the V_H-flag gene were removed from LB+amp plates and grown overnight in 2x YT medium. Plasmid DNA mini-preparations were carried out and the DNA was sequenced using the Sequenase Version 2.0 Kit with pPOW sequencing primers as described in section 2.2.12.

The nucleotide sequence for the K-1-21 V_H gene is shown in Fig 2.7. This sequence was confirmed from five different clones which were sequenced in both the forward and reverse direction. Sequence representing CDR1, CDR2 and CDR3 was identified according to Kabat (1989) and is underlined. Part of the 3' region of the forward PCR primer, *VH2 for*, is shown by a single broken line. The reverse primer *VH2 flag* is indicated by double broken lines. Restriction enzyme sites, BstE 11 and EcoR 1, are shown in italics.

2.3.3 CLONING OF THE V_L GENE

A schematic outline of the PCR primers used to isolate the V_L gene and then add the linker sequence is presented in Fig 2.8. Cloning of the linker-V_L gene into the vector to create the recombinant pPOW scFv construct is also shown.

Primers designed to bind to the 5' region of the V_L gene

Initially a consensus primer designed by Ward *et. al.* (1989) was used to amplify the V_L gene, however, this approach was unsuccessful. For this reason partial amino acid sequence of the light chain was determined using an Applied Biosystems 475A Protein Sequencer. A degenerative primer was designed based on the first four amino acids at the amino terminal end of the light chain. These are aspartic acid (D), isoleucine (I), valine (V) and methionine (M).

VL1 for (sense)

5'- CCC GCC AGA CGT/C GAT/C ATT/C GTG/C ATG -3'

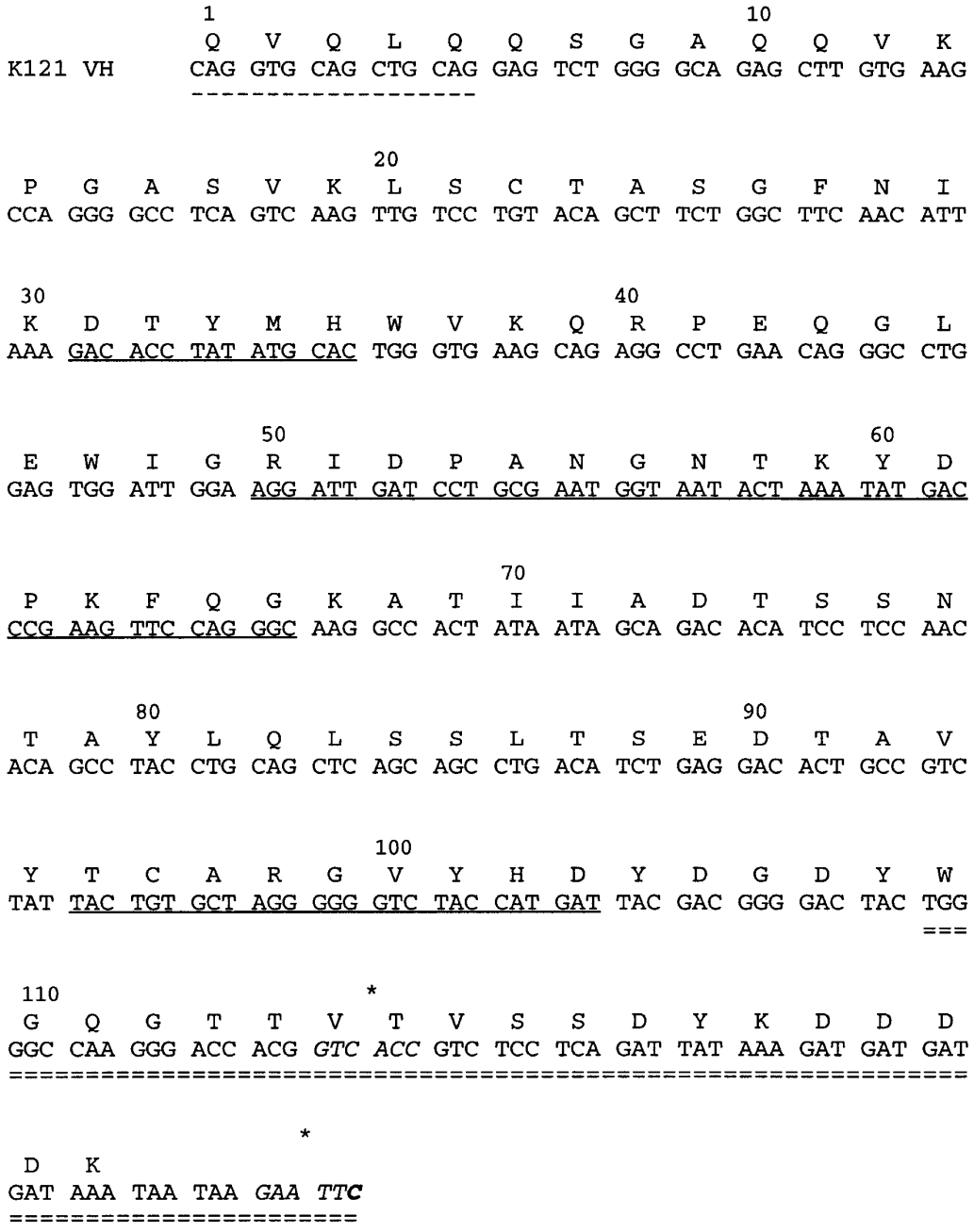


Fig 2.7 V_H DNA SEQUENCE

The nucleotide sequence for the V_H gene was obtained from cDNA as described in the text. The 3' end of the primer *VH2 for* is indicated by single broken lines and the complete reverse primer *VH2 flag* is shown by double broken lines. The complementarity determining regions CDR1, CDR2 and CDR3 are underlined and were identified according to Kabat (1989). Derived amino acid sequence is shown using the single letter notation. Restriction enzyme sites are in italics with an asterisk above the codons.

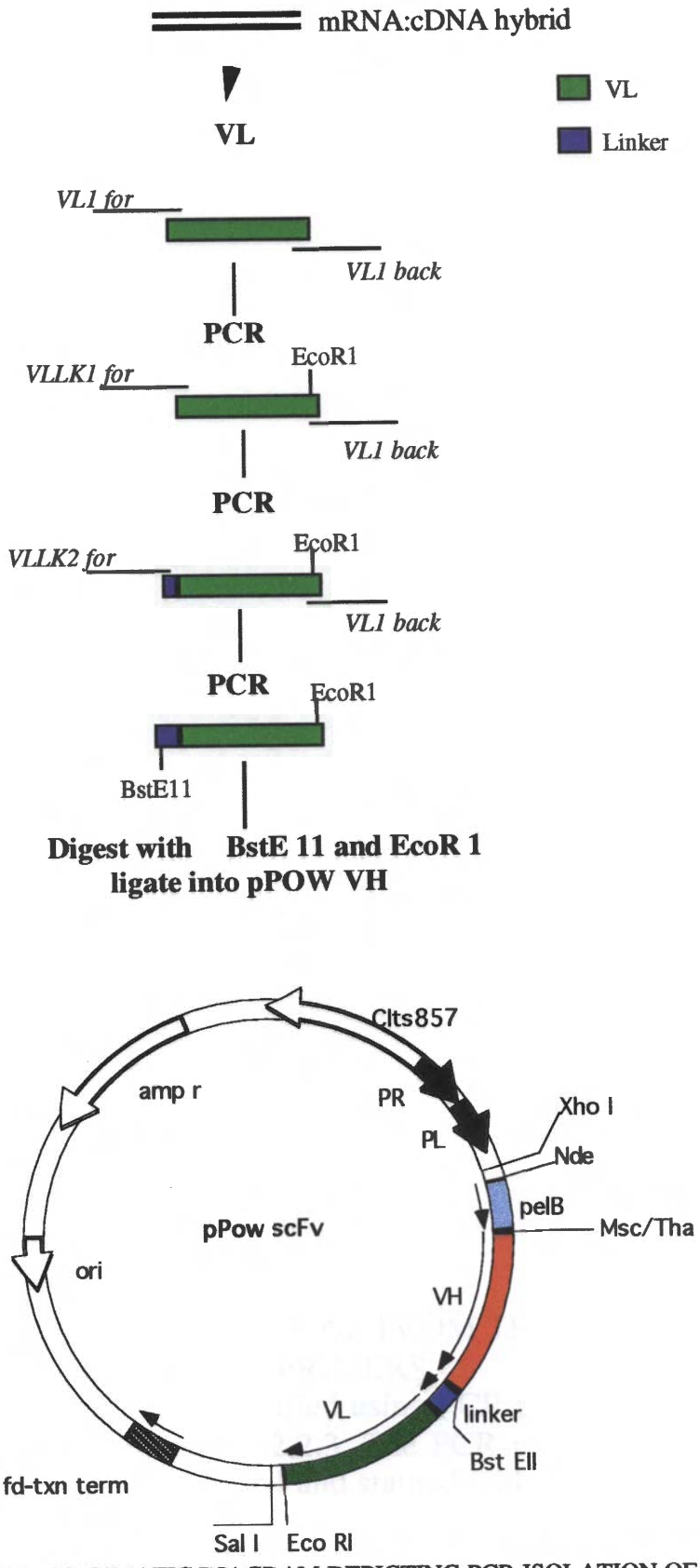


Fig 2.8 SCHEMATIC DIAGRAM DEPICTING PCR ISOLATION OF THE K-1-21 V_L GENE AND CONSTRUCTION OF THE scFv FUSION GENE

The V_L gene was amplified from K-1-21 cDNA using primers *VL1 for* and *VL1 back*, the latter included the RE site, EcoR 1. This V_L gene was used as template to extend the 5' end with partial linker sequence using *VLLK1 for*. The complete linker sequence (54 bases) was added to the 5' end of the V_L gene using the primer *VLLK2 for*. This primer included the RE site, BstE 11. After digestion with BstE 11 and EcoR 1 the PCR product was cloned into pPOW VH to create the scFv gene fragment.

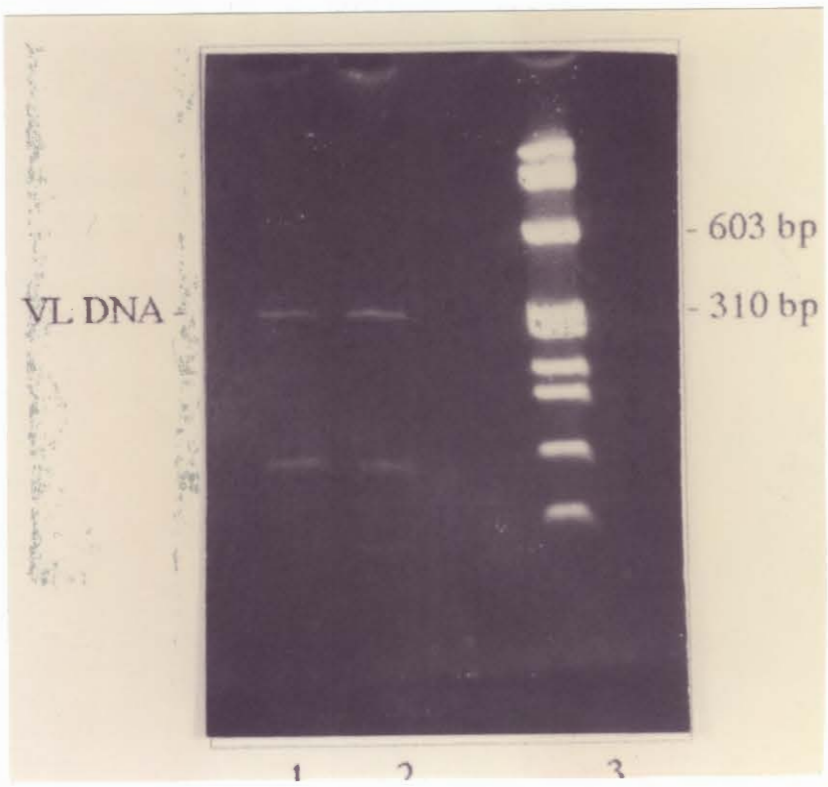


Fig 2.9 THE AMPLIFIED V_L GENE FROM THE PCR USING *VL1 for* AND *VL1 back* PRIMERS

The K-1-21 V_L gene was amplified using PCR primers *VL1 for* and *VL1 back* as described in section 2.2.3. The PCR product was subjected to PAGE on a 10% acrylamide gel and stained with Eth.Br. Lane 1 and 2, a 16 µl aliquot of the V_L PCR product; lane 3, 1 µl Øx174 Hae digested DNA markers 72-1353 bp (USB, Cleveland, Ohio).

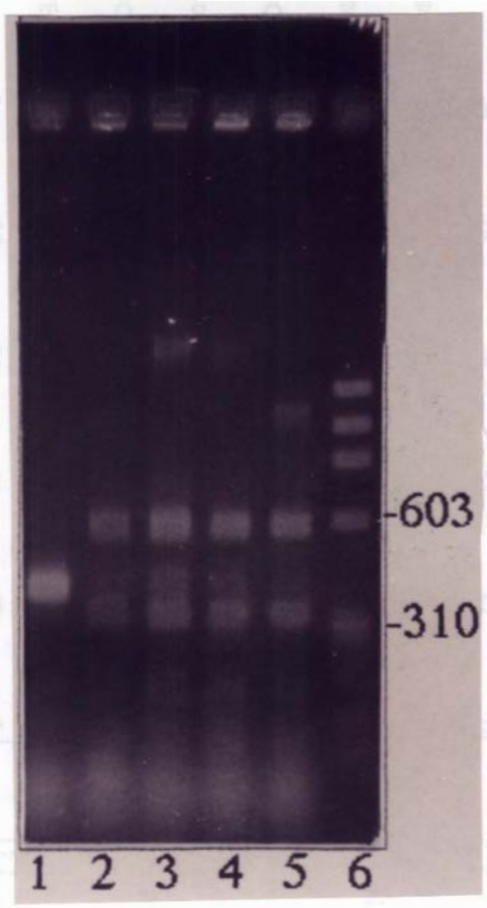


Fig 2.10 PCR SCREENING OF COLONIES WITH V_L INSERT
Individual colonies were removed with a toothpick, placed in PCR tubes containing PCR reagents and reactions were cycled as described in section 2.1.11. Aliquots (16 µl) of each reaction mixture were resolved by agarose gel electrophoresis on a 1.2% gel and stained with Eth.Br. Lanes 1-5, are the PCR reaction products ; lane 6, 1 µl DNA molecular weight standards 72-1353 bp (Øx174 Hae digest).

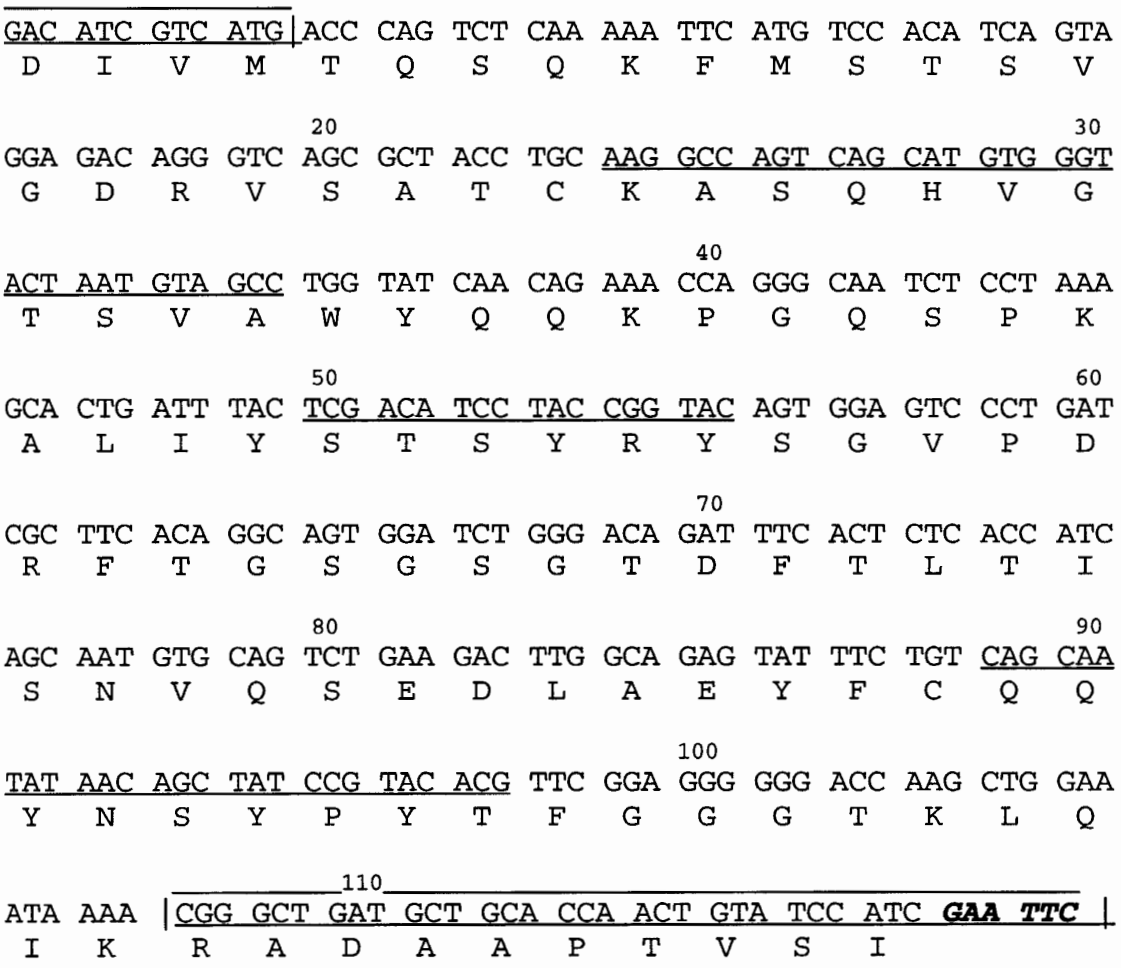


Fig 2.11 V_L DNA SEQUENCE

The nucleotide sequence for the V_L gene was determined from two clones. Derived amino acid sequence is shown using single letter notation. The regions corresponding to CDR1, CDR2 and CDR3 are underlined and were identified from Kabat (1989). The last four codons at the 3' end of the forward primer *VL1 for* and the complete reverse primer *VL2 back* are boxed. Restriction enzyme sites are shown in bold italics.

Primers complementary to the 3' region of the V_L gene sequence.

A primer complementary to the constant region of murine V *kappa* light chain (codons 109-117) was used to amplify the 3' region of the rearranged V_L gene (Chiang *et al.*, 1989). This primer contained an EcoR 1 restriction site to enable directional cloning into pPOW.

VL1 back (anti-sense)

5'- CC GAA TTC GAT GGA TAC AGT TGG TGC AGC ATC AGC CCG -3'

Amplification using primers *VL1 for* and *VL1 back* to isolate the V_L gene from K-1-21 cDNA was carried out using the PCR kit as described in section 2.2.4. The reaction was cycled as described and the PCR products were resolved by PAGE on a 10% acrylamide gel. Amplification of the V_L gene using these primers resulted in a product of about 350 bp (Fig 2.9). The PCR product was purified by treating the aqueous layer with Proteinase K and then purifying the DNA as described in section 2.2.5.

Ligation and transformation of V_L

The isolated DNA (1-5 ng) was ligated into T-tailed Bluescript (1-2 ng) as described in section 2.2.9. In order to remove the buffer salts the ligation mixture was precipitated with ethanol, washed, dried and resuspended in 10 µl of sterile water. All the purified ligation mixture was used to transform electrocompetent Sure cells by electroporation as detailed in section 2.2.10. Single colonies were removed from LB+amp plates and screened by PCR using the primers *VL1 for* and *VL1 back*. Products from the PCR screening mixture were identified after electrophoresis on a 1.0% agarose gel. Four clones with insert of approximately 350 bp, which is the expected size for the V_L gene, were observed (Fig 2.10).

V_L nucleotide sequence

Four colonies were identified as containing an insert of the expected size for the V_L gene and two of these were used to prepare plasmid DNA for DNA sequencing. Bluescript sequencing primers, Reverse primer and SK primer were used in the reactions. Two of the clones from Fig 2.10,

lanes 3 and 4, were sequenced in both the forward and reverse directions. The results of DNA sequencing and the derived amino acid sequence are presented in Fig 2.11. Both clones gave identical nucleotide sequence. Sequence for part of 3' region of the forward primer, *VL1 for*, and the complete anti-sense primer, *VL1 back*, are boxed. The positions of CDR1, CDR2 and CDR3 were determined from Kabat (1989) and are underlined. The restriction enzyme site, EcoR 1, is in italics.

2.3.4 CONSTRUCTION OF THE scFv FUSION GENE

The strategy for constructing the scFv fusion gene is shown in Fig 2.8. As outlined in the schematic diagram the linker sequence was added to the isolated V_L gene by two consecutive PCR steps. The primer *VLLK1 for* used for partial extension of the linker sequence was designed to bind to the 5' end of the isolated V_L gene.

The PCR primers designed to extend the 5' region of the VL gene and encode the linker sequence

The first four codons at the 3' end of the *VLLK1 for* linker primer are complementary to 5' end of the V_L gene. The remaining eleven codons, shown in bold type, encode part of the linker sequence consisting of two blocks of one serine and four glycines, $2(S,G_4)$.

VLLK1 for (sense)

5'- **TCA GGA GGA GGA GGT TCG GGT GGT GGT GGT TCG**
GAC ATC GTC ATG - 3'

After extension at the 5' region of the V_L gene a second linker primer, *VLLK2 for*, was used to lengthen the sequence encoding the joining peptide. The nucleotide sequence at the 3' end of this primer was complementary to the 5' end of *VLLK1 for* (shown above in bold) and would thus extend the linker sequence at the 5' end of the first primer. The final extension sequence will encode the full 15 amino acid linker peptide, $3(S,G_4)$. A BstE 11 site is present at the 5' end of the *VLLK2 for* primer to allow cloning of the linker-V_L gene into a compatible site in pPOW VH to create the gene construct for a single chain Fv (pPOW scFv). The restriction site is underlined.

VLLK2 for (sense)

5'- CCC ACG GTC ACC GTC GCC TCC GGT GGT GGT GGT
TCA GGA GGA GGA GGT - 3'

A PCR reaction using amplified V_L DNA as template and primers *VLLK1 for* and *VL1 back* was carried out with the heat stable DNA polymerase, *Pfu* (Section 2.2.4). Amplified DNA from this PCR should contain the V_L gene which has been extended at the 5' end with primer *VLLK1 for* to give partial linker-V_L. This fragment was isolated by agarose gel electro-elution, purified and concentrated using an ELUTIP-d column (Section 2.2.7).

Isolated V_L DNA containing the partial linker sequence was used as template to extend the linker further with the primer *VLLK2 for*. A PCR reaction with *Pfu* DNA polymerase was carried out as before using primers *VLLK2 for* and *VL1 back* with partial linker-V_L as template. In this case the DNA in the reaction mixture was denatured at 94°C for 3 min then annealled at 65°C for 3 min and cycled 35 times at 72°C for 1 min, 94°C for 30 s and 65°C for 30 s. A product of the estimated molecular size (420 bp) for the linker-V_L gene was isolated and purified using agarose gel electro-elution and an ELUTIP-d column.

The gel photograph in Fig 2.12 shows the purified PCR product of the V_L gene in lane 1. Amplified V_L DNA after addition of 33 nucleotides using *VLLK1 for*, which constitutes part of the linker sequence, in lane 2 (partial linker-V_L) and V_L DNA including the complete linker sequence consisting of 54 bases in lane 3. The purified linker-V_L gene shown in lane 3 was ligated into T-tailed Bluescript and then transformed into $CaCl_2$ competent NM522 cells. Positive clones were selected by white/blue colour selection. Altogether 28 colonies were selected for PCR screening with primers *VL1 for* and *VL1 back*. The results of the PCR screening are shown in Fig 2.13. A positive control which consisted of purified PCR product was run in track 15 (b) and a negative control which was Bluescript without insert was run in track 16 (b). Molecular weight standards were present in tracks 8 (a) and 9 (b). From the PCR screening results it can be seen that 20 out of 28 colonies produce PCR

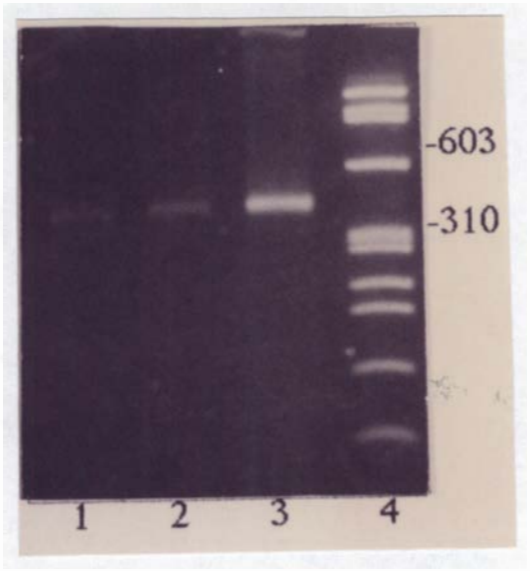


Fig 2.12 ADDITION OF THE LINKER SEQUENCE TO V_L USING PCR

The linker sequence was added to the 5' end of the V_L gene by two consecutive PCR experiments as detailed in section 2.2.4. Purified PCR products were then resolved by PAGE on a 10% acrylamide gel and stained with Eth.Br. In lane 1, 5 µl of purified V_L using primers *VL1 for* and *VL1 back* ; lane 2, 5 µl of purified V_L PCR product using primers *VLLK1 for* and *VL1 back* ; lane 3, 10 µl of purified V_L PCR product using primers *VLLK2 for* and *VL1 back* ; lane 4, Øx174 Hae digested DNA standards 72-1353 bp.

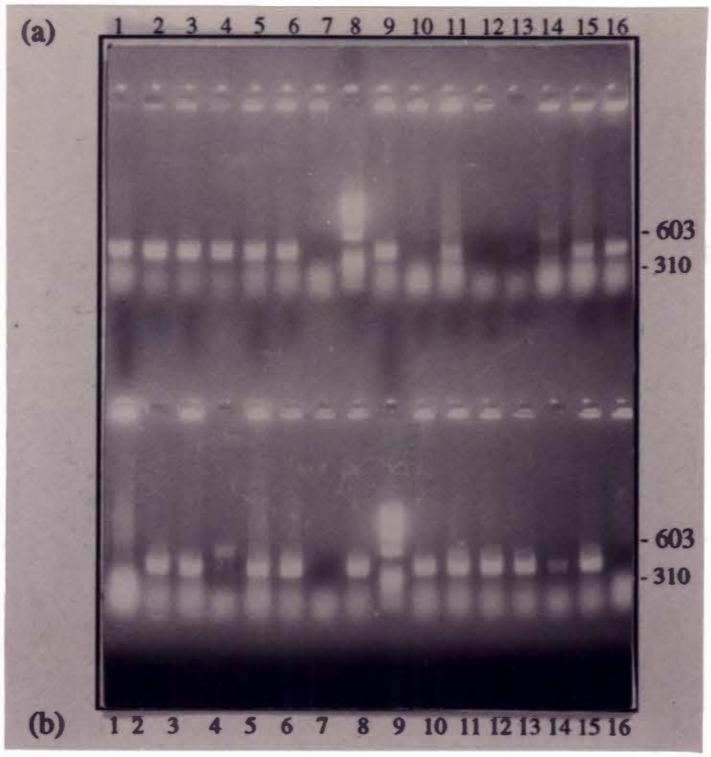


Fig 2.13 PCR SCREENING OF COLONIES CONTAINING THE LINKER-V_L SEQUENCE

Colonies were removed from the plate with a toothpick and placed in PCR tubes containing PCR reagents. The reactions were cycled as described in section 2.1.11. The aqueous layer was removed and 16 µl aliquots of the products were subjected to electrophoresis on a 1% agarose gel and stained with Eth.Br. Lanes 8a and 9b, contained molecular weight standards (Øx174 Hae digest, 72-1353 bp) ; lane 15 b, is a positive control and consisted of PCR product ; lane 16 b, is a negative control and represents Bluescript vector without insert. All other lanes contain DNA from selected colonies some of which appear to contain insert of the expected size (350 bp). Clones from lanes 2a and 3a were sequenced as described in section 2.1.12.

product of 350 bp which is the correct molecular size for the V_L gene. Two clones (from lanes 2a and 3a) which had DNA insert of the expected size were sequenced in both directions. The linker sequence which consists of 18 codons and the first 45 bases of the V_L gene are presented in Fig 2.14

Ligation of linker V_L in pPOW VH

Purified linker-V_L DNA (500 ng) was digested with EcoR 1 in a total volume of 50 µl. After digestion the DNA was extracted with Phe/Chl as described in section 2.2.5. The precipitate was then resuspended in 38 µl of water and digested with BstE 11 in a total volume of 50 µl. After separation by electrophoresis on a 1.5% agarose gel the digested DNA was collected on a NA-45 membrane, eluted and precipitated. A gel photograph is shown in Fig 2.15 (a). The results show the PCR product of the linker-V_L fusion gene (lane 2) before purification. A 5 µl aliquot of purified, enzyme digested PCR product is shown in lane 3. The linker-V_L DNA shown in lane 3 was used for ligation into restricted pPOW VH.

Digestion of pPOW VH was carried out in parallel using approximately 20 µg of plasmid DNA. Firstly, plasmid DNA was digested with EcoR 1 and then extracted with Phe/Chl as detailed in section 2.2.5. The purified DNA was then resuspended and incubated with BstE 11 in a total volume of 50 µl. After digestion the DNA was again extracted and precipitated as before. Linearised plasmid was separated on a 1% agarose gel, collected on a NA-45 membrane, eluted and precipitated. Resultant linearised pPOW VH DNA is shown in Fig 2.15 (b), lane 3.

Directional cloning of the digested, purified linker-V_L PCR product was carried out with the digested pPOW VH. A ligation mixture, consisting of 10 ng digested vector and 5-10 ng linker-V_L PCR product, was incubated as previously described in section 2.2.9. Aliquots (5 µl and 10 µl) of the ligated products were transformed into $CaCl_2$ competent *E.coli* cells, LE 392. Transformed cells (100 µl and 200 µl) were plated out on LB+amp plates and grown overnight at 30°C. Several colonies were selected, transferred to 2x YT medium and grown overnight in an orbital shaker at 30°C. Purified plasmid DNA (7 µl) obtained from the selected clones was digested with the restriction enzymes EcoR 1 and Nde 1. Digested products were separated by PAGE on a 10% acrylamide gel. Results of the digestion are shown in Fig 2.16. There were seven

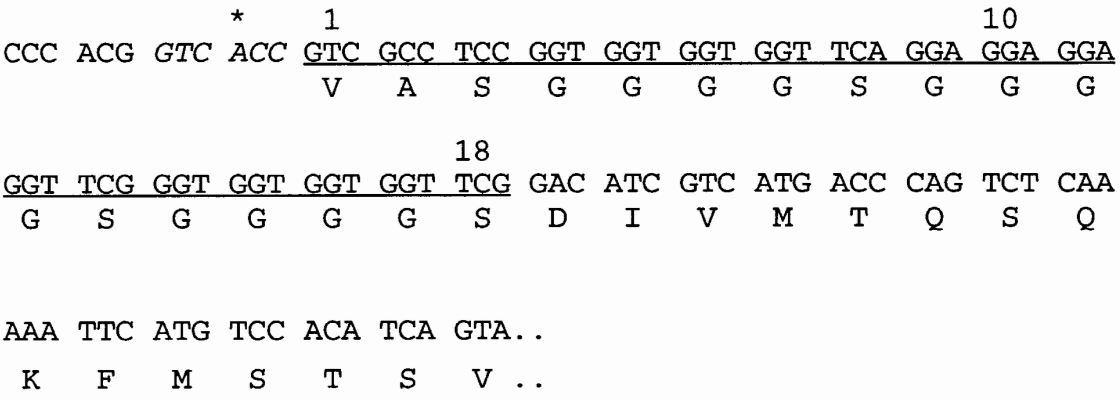


Fig 2.14. DNA SEQUENCE OF THE 5' REGION OF THE LINKER-V_L GENE

Addition of the linker sequence to the V_L gene using PCR primers *VLLK1 for* and *VLLK2 for* with the reverse primer *VL1 back* is described in detail in section 2.2.2. The linker-V_L fragment was then cloned and sequenced in Bluescript vector. The nucleotide sequence for the linker region is shown above as codons 1-18 and is underlined. The first fifteen codons of the 5' region of V_L gene are also given. Derived amino acid sequence is presented using single letter notation below the nucleotide sequence. A restriction enzyme site for BstE 11 is shown in italics with an asterisk above the codons.

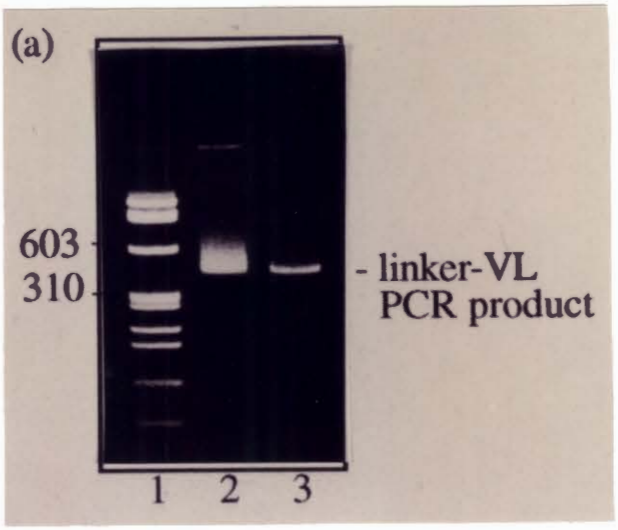


Fig 2.15 (a) LINKER-V_L PCR PRODUCTS USED TO CONSTRUCT THE scFv FRAGMENT

The PCR product, linker-V_L obtained from using primers *VLLK2 for* and *VL1 back*, was digested with enzymes BstE 11 and EcoR 1. After purification the DNA fragment was ligated into the restricted vector pPOW V_H. Amplified products were electrophoresed on a 10% acrylamide gel and stained with Eth.Br. Lane 1, Øx174 Hae digest, molecular weight standards 72-1353 bp.; lane 2, 16 µl PCR product linker-V_L ; lane 3, a 5 µl aliquot of digested, purified linker-V_L product.

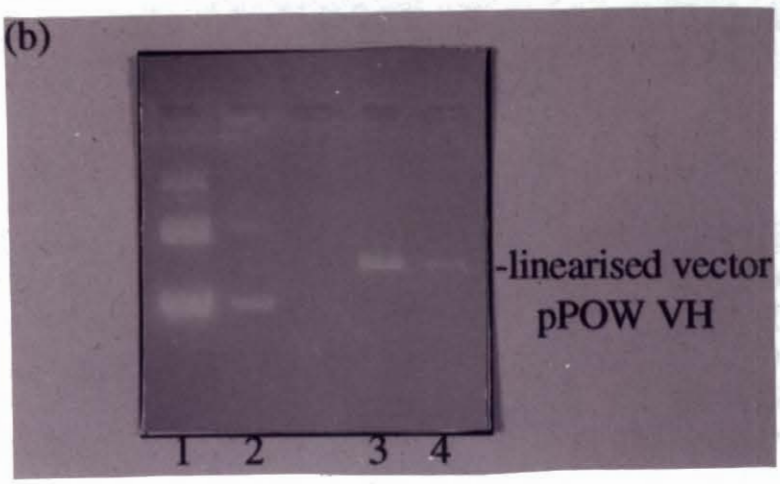


Fig 2.15 (b) DIGESTION AND PURIFICATION OF pPOW V_H

Plasmid DNA, pPOW V_H, was digested with restriction enzymes BstE 11 and EcoR 1. The restricted plasmid DNA was isolated, purified and resuspended in 50 µl water. Linearised plasmid was then visualised on a 1% agarose gel by staining with Eth.Br. Lane 1 and 2, 5 µg and 2 µg respectively of undigested pPOW V_H ; lane 3, 5 µl of purified, digested pPOW V_H ; lane 4, 2 µl of purified, digested pPOW V_H.

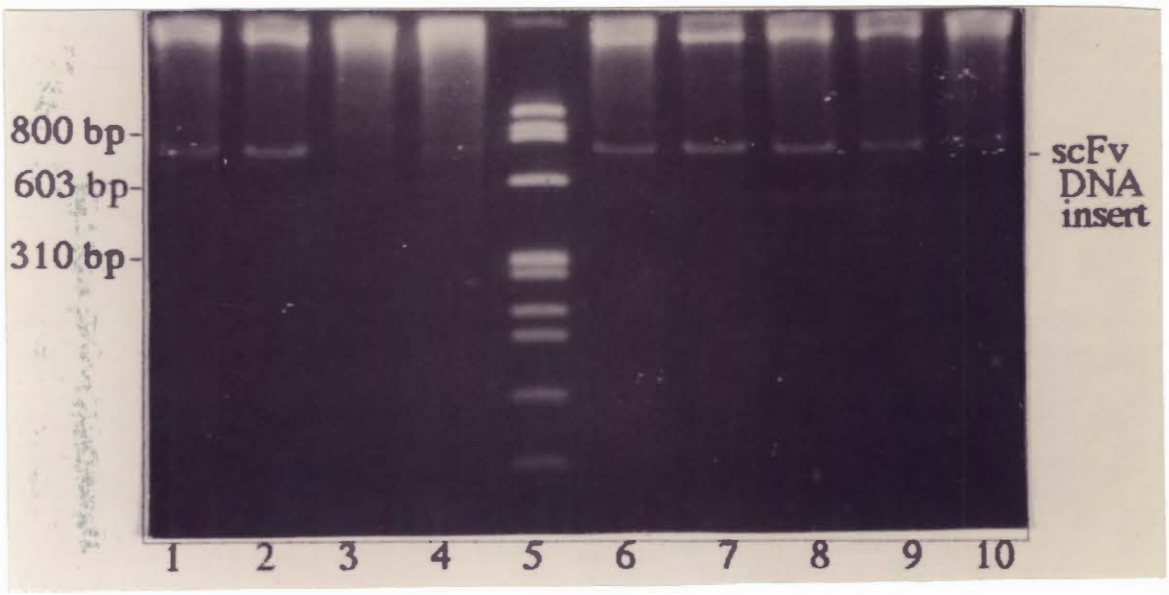


Fig 2.16 CLONES CONTAINING THE scFv FUSION GENE
Ligation of linker-V_L DNA with pPOW V_H to produce the construct pPOW scFv resulted in several positive clones. Plasmid DNA mini-preparations (7 µl) from 9 colonies were digested with enzymes Nde 1 and EcoR 1 and then separated by PAGE on a 10% acrylamide gel. The resulting fragments were observed after staining with Eth.Br. Lane 1-4, digested plasmid DNA ; lane 5, DNA molecular weight standards Øx174 Hae digest 72- 1353 bp ; lane 6-10 digested plasmid DNA.

positive clones containing DNA insert of 750 bp which is the estimated size for the scFv fragment. Two clones containing pPOW scFv (lanes 6 and 7) were sequenced as previously described and the DNA sequence is shown in Fig 2.17.

2.3.5 CONSTRUCTION OF THE LINKER-MELITTIN-FLAG FUSION GENE

In order to add the sequence encoding the linker and the flag peptide to the melittin gene a PCR reaction was carried out using the melittin oligonucleotide as template and the PCR primers *MELLK for* and *MEL flag* . A schematic diagram of the PCR amplification and extension of the melittin oligonucleotide and subsequent cloning into pPOW scFv is shown in Fig 2.18.

The oligonucleotide encoding the sequence for melittin.

A nucleotide sequence for melittin (*MEL*) was derived from the known amino acid sequence which has previously been published (Habermann and Jentsch, 1967). Before synthesis the sequence was checked using a MacGeneSearch software programme to ensure that no restriction enzyme sites used for subsequent cloning of the fragment were present in the derived sequence.

MEL

5'- GGC ATT GGA GCT GTG CTA AAA GTC CTC ACC ACA GGT CTT CCA GCA TTG ATA TCC TGG ATC AAG CGT AAA CGG CAG CAG - 3'

The forward primer, *MELLK for*, used in the amplification of *MEL* encoded a 15 amino acid linker composed of serine and 4 glycines in 3 blocks, $3(S,G_4)$. This primer was also complementary to the first five codons at the 5' region of the melittin oligonucleotide and is shown in bold type. An EcoR 1 restriction site was included at the 5' end of the primer to allow directional cloning of the amplified product into pPOW scFV.

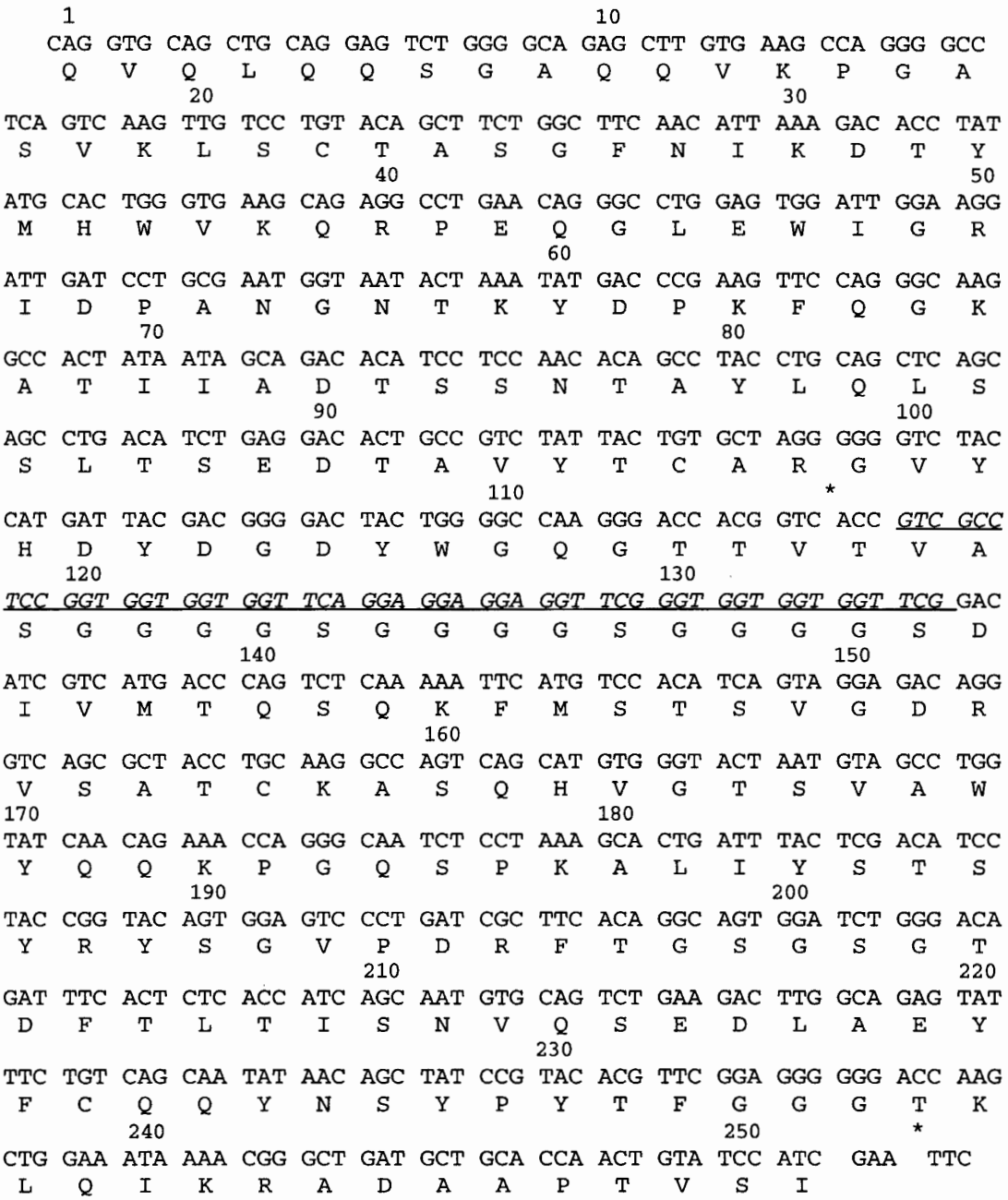


Fig 2.17 THE DNA SEQUENCE FOR K-1-21 scFv AND DERIVED AMINO ACID SEQUENCE

The DNA sequence of scFv is shown above and includes 759 bases of the V_H-linker-V_L fusion gene up to the EcoR 1 restriction site. The linker sequence is shown in italics and is underlined. Restriction enzyme sites BstE 11 and EcoR 1 are indicated by an asterisk above the codons.

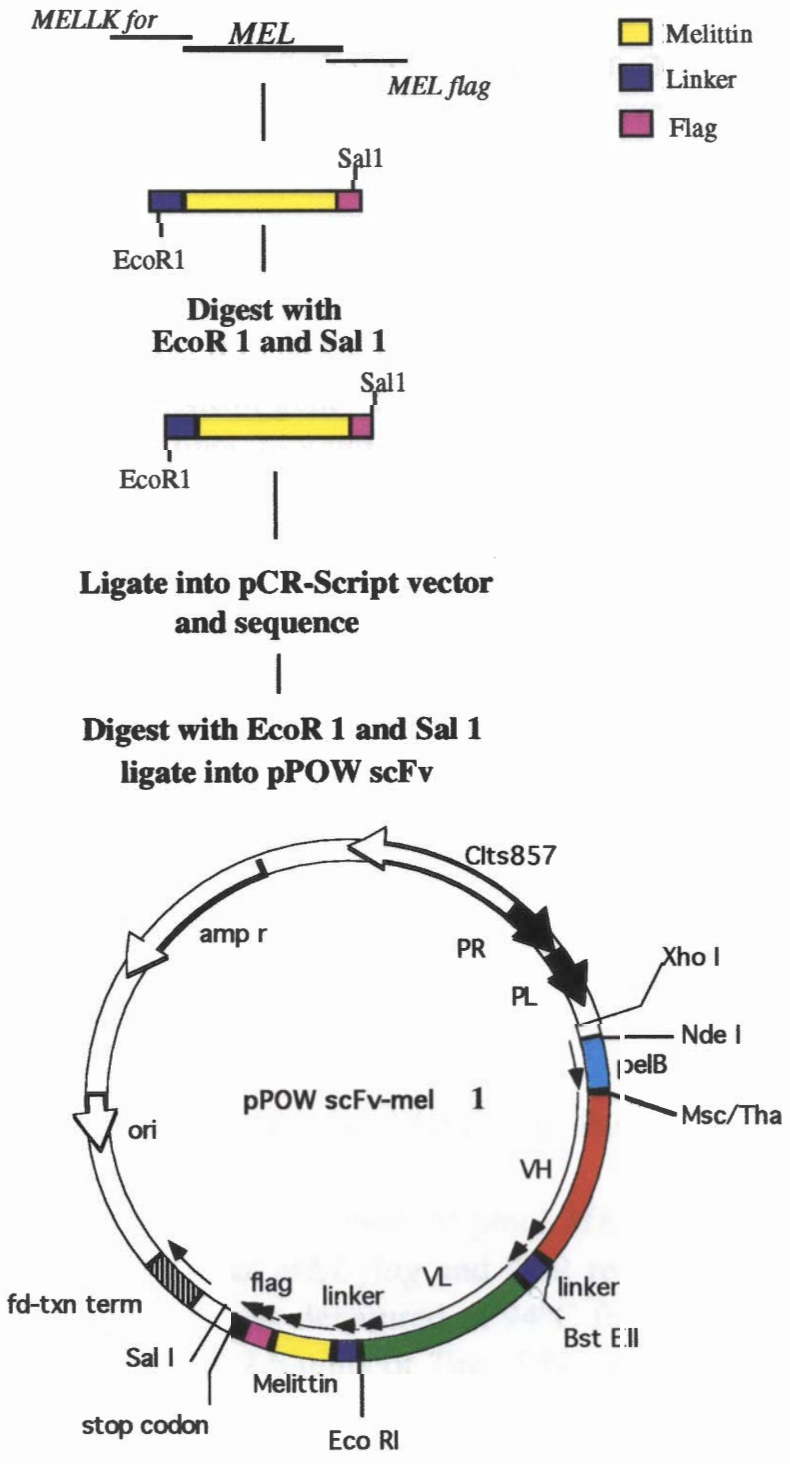


FIG 2.18 PCR CONSTRUCTION OF THE LINKER-MELITTIN-FLAG GENE AND CONSTRUCTION OF THE pPOW scFv RECOMBINANT

The melittin oligonucleotide was extended at the 5' end using the linker primer *MELLK1 for*, which also incorporates the EcoR 1 site. Sequence encoding the flag peptide was added to the 3' end with the primer *MEL flag* and the Sal 1 site was included at the 5' end of the primer. The resulting PCR fragment was cloned into pCR-Script and sequenced. This vector was digested with EcoR 1 and Sal 1 and the melittin fusion fragment was isolated. The isolated fragment was directionally cloned into pPOW scFv at compatible sites, to give pPOW scFv-mel 1

MELLK for (sense)

5'- ATG ATG GAA TTC TCC GGA GGC GGT GGC TCG GGC GGT GGC GGC TCG GGT GGC GGC GGC TCT **GGC ATT GGA GCT GTG** - 3'

In order to monitor the production of the recombinant immunotoxin during subsequent expression experiments a primer encoding the flag octapeptide, previously described in section 2.3.2, was used to amplify the 3' region of the melittin gene. The nucleotide sequence encoding the flag peptide is in bold type. In addition, the last three codons at the 3' end of the primer are complementary to the last three codons at the 3' of the melittin oligonucleotide. A Sal 1 restriction site (underlined) was incorporated at the 5' end of the primer for directional cloning of the amplified product into pPOW scFV.

MELflag back (anti-sense)

5'- ATG ATG GTC GAC TTA TTA **TTT ATC ATC ATC ATC TTT ATA ATC TGA** GGA GAC CTG CTG CCG -3'

Primers described above were used in the PCR to create a linker-melittin-flag fusion gene sequence for cloning into the expression vector pPOW scFv.

Construction of the linker-melittin-flag fusion gene by PCR amplification

The PCR reaction mixture contained 40 pmol *MEL* oligonucleotide, 50 pmol *MEL LK for*, 50 pmol *MEL flag* and PCR reagents as described in section 2.2.5. The DNA was denatured at 94°C for 5 min. At this stage the reaction was held and 2.5 units of *Taq* DNA polymerase was added to each tube. An annealling step was carried out at 45°C for 5 min and then the reaction was cycled at 72°C for 1 min, 94°C for 30 s and 45°C for 30 s, for 35 cycles. Products from the amplification reaction were observed after PAGE on a 10% acrylamide gel (Fig 2.19). An intense DNA band of approximately 200 bp, which corresponds with the estimated size of the fusion gene product, was observed. All the PCR product was separated on a 1.8% agarose gel and the 200 bp band was collected on NA-45 membranes, eluted and precipitated.

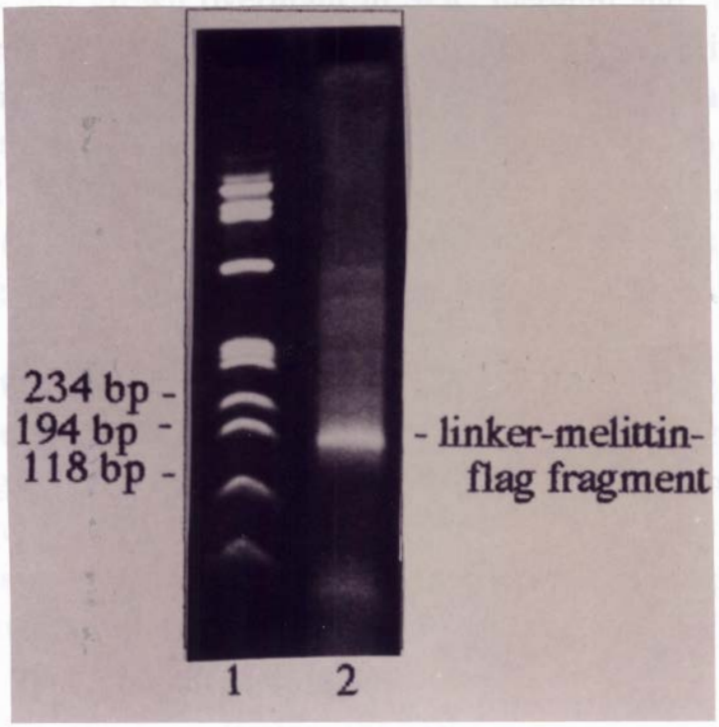


Fig 2.19 PCR EXTENSION OF THE MELITTIN OLIGONUCLEOTIDE TO CREATE THE LINKER-MELITTIN-FLAG FRAGMENT

PCR primers, *MEL for* and *MEL flag* back, were used to extend the melittin oligonucleotide and to create restriction enzyme sites for directional cloning of the fragment into pPOW scFv. Details of the PCR are given in section 2.2.5. An aliquot (16 µl) of the PCR product was resolved by PAGE on a 10% gel and stained with Eth.Br. Lane 1, Øx174 Hae digest 72-1353 bp ; lane 2, a 16 µl aliquot of the linker-melittin-flag PCR fragment.

Ligation of the linker-melittin-flag fusion gene

A 5 µl aliquot of purified PCR product was ligated into pCR-Script™ SK(+) vector. Approximately 60 ng of linker-melittin-flag PCR product was used in the ligation reaction with 10 ng of vector. All procedures were carried out as described in section 2.2.9. After ligation the mixture was transformed into XL-1 Blue competent cells, which were then plated out on LB+amp plates and grown overnight at 37°C. White colonies were selected and grown overnight in SOC medium and then plasmid DNA was prepared from 8 colonies. Aliquots (7µl) of purified plasmid DNA were digested with restriction enzymes EcoR 1 and Sal 1 and the products were separated on a 10% acrylamide gel using PAGE. Resulting plasmid DNA digestions with EcoR 1 and Sal 1 are shown in Fig 2.20. DNA bands which correspond to the approximate size of the melittin fusion gene fragment (200 bp) were observed in four colonies.

Nucleotide sequence of the linker-melittin-flag fusion gene

A single clone containing insert of the estimated size (200 bp) for the fusion gene was sequenced in the forward and reverse directions using the Sequenase Version 2.0 kit and sequencing primers T3 and T7. The nucleotide sequence obtained from the pCR-Script™SK(+) vector containing the linker-melittin-flag insert is shown in Fig 2.21.

Ligation of the linker-melittin-flag fusion gene into the pPOW scFv construct

To construct the scFv-linker-melittin-flag fusion gene the vector pPOW scFv was used to directionally clone in the melittin fusion gene. This approach involved digestion of both the vector pPOW scFv and the pCR-Script™ SK(+) vector containing the linker-melittin-flag fusion gene sequence, with EcoR 1 and Sal 1. The vector pPOW scFv was digested with EcoR 1 and Sal 1 in a total volume of 50 µl. The restricted vector was purified using the GENECLEAN protocol. Likewise, the pCR-Script™ SK(+) vector containing the melittin fusion gene was digested with EcoR 1 and Sal 1. Afterwards, the digested melittin fusion fragment was separated from plasmid DNA by electrophoresis on a 1.5% agarose gel and then collected by electro-elution. The eluted DNA was purified and concentrated using an ELUTIP-d column as described previously. Purified linker-melittin-flag insert is shown in Fig 2.22 (a) and isolated restricted pPOW scFv is shown in Fig 2.22 (b).

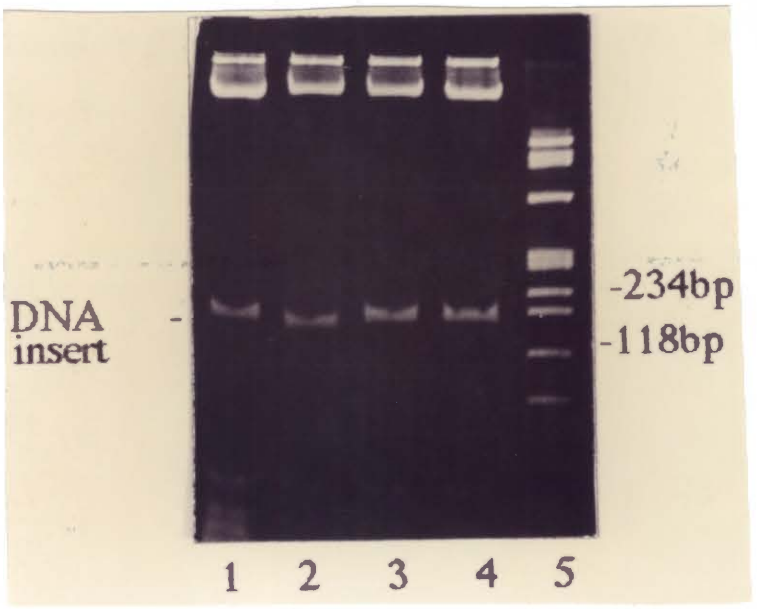


Fig 2.20 LINKER-MELITTIN-FLAG FRAGMENTS CLONED INTO pCR-SCRIPT^{TM}SK(+) VECTOR

Portions (7 µl) of purified plasmid DNA were digested with restriction enzymes EcoR 1 and Sal 1. The digests were subjected to PAGE on a 10% acrylamide gel and stained with Eth.Br. Lanes 1-4, digested pCR-Script^{TM}SK(+) vector DNA from 4 clones ; lane 5, molecular weight standards Øx174 Hae digest 72- 1353 bp.

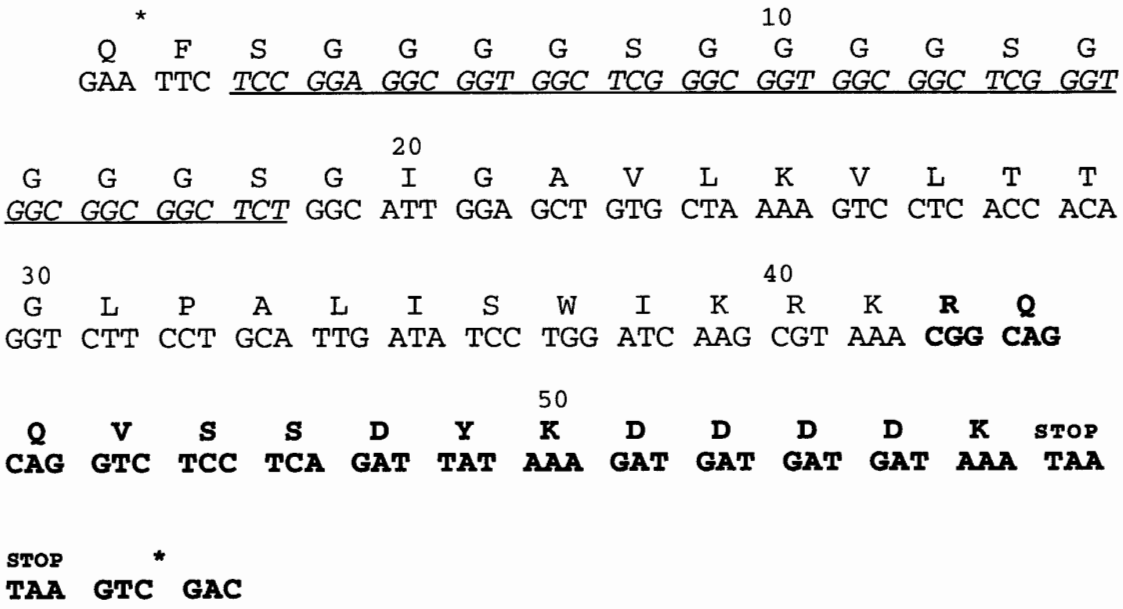


Fig 2.21. DNA SEQUENCE OF THE LINKER-MELITTIN-FLAG FUSION GENE

Nucleotide sequence obtained from the linker-melittin-flag PCR fragment ligated into the vector, pCR-Script^{TM}SK(+). The linker sequence is in italics and underlined. The *MEL flag* PCR primer is shown in bold type. Restriction enzyme sites are indicated by an asterisk above the relevant codons. The nucleotide sequence which encodes melittin is from codon 20 to codon 44. Derived amino acid sequence is shown in single letter notation.

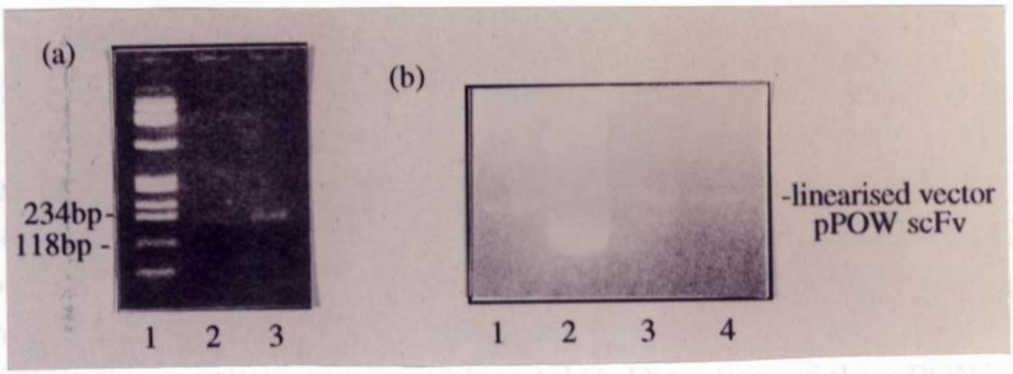


Fig 2.22 CLONING OF THE LINKER-MELITTIN-FLAG FRAGMENT INTO pPOW scFv

The linker-melittin-flag fusion fragment (L-M-F) was digested out of pCR-Sript™ SK(+) vector with restriction enzymes EcoR 1 and Sal 1. Likewise, the vector pPOW scFv was digested with the same enzymes to create compatible ligation sites. In; (a) lane 1, molecular weight standards Øx174 Hae digest 72-1353 bp ; lane 2, a 1 µl aliquot of digested and purified L-M-F ; lane 3, a 3 µl aliquot of digested and purified L-M-F. (b) lanes 1 and 3, pPOW scFv digested and purified (5 µl) ; lane 2, uncut pPOW scFv vector ; lane 4, a 2 µl aliquot of the cut pPOW scFv.

Ligation of digested pPOW scFv and the melittin fusion gene fragment was carried out using 10 ng pPOW vector and 60 ng of the fusion gene fragment. Afterwards, aliquots of the ligation mixture were transformed into XL1-Blue competent cells, plated out (100 µl and 200 µl) on LB+amp plates and colonies were grown overnight at 30°C. Plasmid DNA (7 ul) from selected colonies was digested with enzymes Xho 1 and Sal 1. The digested products were separated by PAGE on 10% polyacrylamide gels and the results are shown in Fig 2.23. A number of different size fragments of approximately 900-1000 bp were observed. A single clone which corresponded to the insert shown in lane 6 was sequenced only in the reverse direction. The resulting nucleotide sequence is presented in Fig 2.24. It appears that the fusion gene has been cut with EcoR 1 and has been cloned into the same site in pPOW scFv. This has resulted in additional nucleotide sequence from the pCR-Script^{TM}SK(+) vector.

2.3.6 CONSTRUCTION OF pPOW scFv-mel 2

As mentioned earlier the construction of a second scFv-mel fusion gene was necessary because it was subsequently found that only one recombinant pPOW VH expressed the antibody fragment (details of protein expression will be presented in Chapter 3). In addition, the fusion gene cloned into pPOW scFv-mel 1 contained additional sequence from the pCR-Script^{TM}SK(+) vector (Fig 2.24). Digestion of the pPOW scFv-mel 1 construct with BstE 11 and Sal 1 could result in a gene fragment which no longer incorporated this additional sequence. The strategy used for cloning is shown in Fig 2.25.

Ligation of the linker-V_L-linker-melittin-flag fusion gene into pPOW VH

Vector containing the appropriate V_H-flag sequence (pPOW VH) was digested with restriction enzymes BstE 11 and Sal 1. The cut pPOW VH plasmid was purified using GENECLEAN. Purified, restricted pPOW VH is shown in Fig 2.26 (a). Likewise, pPOW containing the scFv-mel 1 fusion gene was digested with the same enzymes. In the latter, the resulting gene fragment consisted of the linker-V_L-linker-melittin-flag (L-V_L-L-MEL-F) gene sequence. A fragment corresponding to the

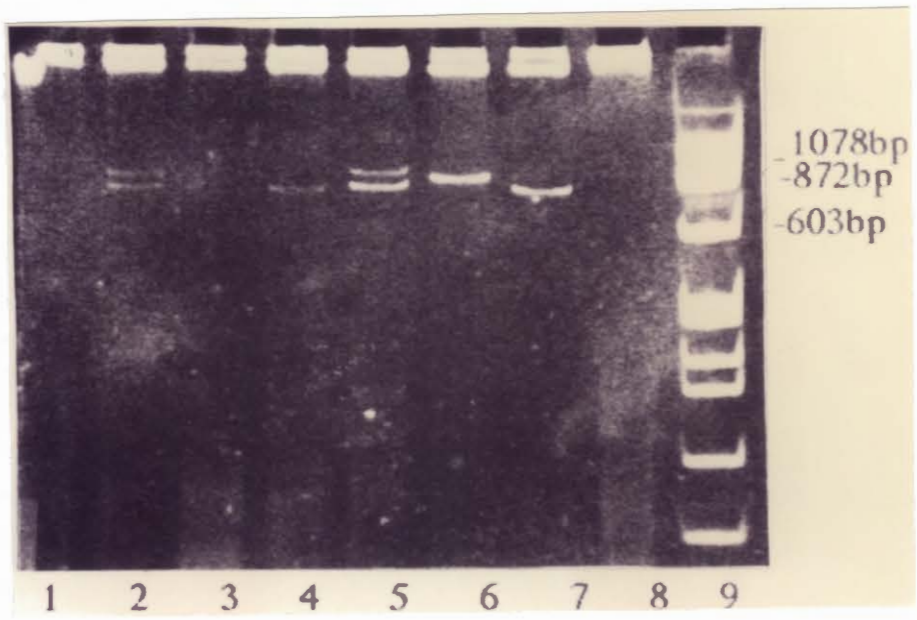


Fig 2.23 DIGESTED PRODUCTS FROM pPOW scFv-mel 1 CLONES
The linker-melittin-flag fragment was cut out of pCR-Script™ SK(+) with EcoR 1 and Sal 1 and ligated into restricted pPOW scFv which had compatible sites. Plasmid DNA (7 µl) from resulting colonies were digested with Xho 1 and Sal 1. Digests were resolved by PAGE on a 10% acrylamide gel and stained with Eth.Br. Lanes 1-8, aliquots of digested pPOW scFv-mel 1. DNA fragments of the approximate size (918 bp) for scFv-mel are present in lanes 2, 4, 5, 6 and 7. Lane 9 contains molecular weight standards Øx174 Hae digest 72-1353 bp.

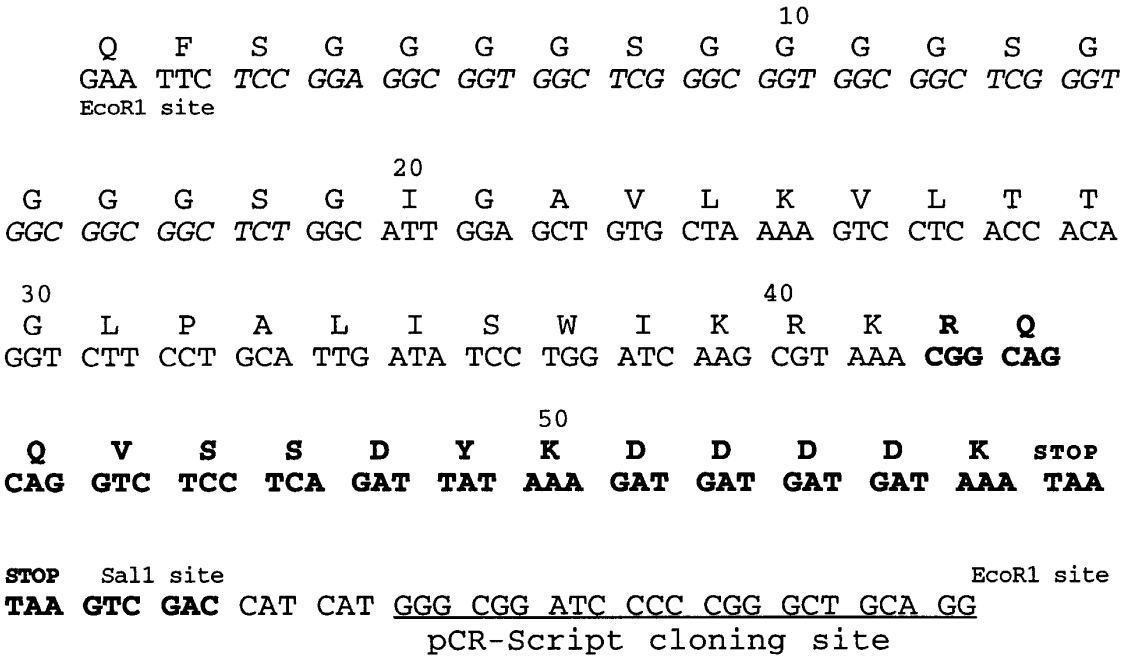


Fig 2.24. DNA SEQUENCE AT THE 3' END OF THE LINKER-MELITTIN-FLAG FUSION GENE CLONED INTO pPOW scFv

The nucleotide sequence of the linker-melittin-flag gene fragment which was cloned into pPOW scFv is shown above. Linker sequence is in italics and the *MEL flag* PCR primer is in bold type. Restriction enzyme sites are noted above the nucleotide sequence and the additional sequence from the vector pCR-Script^{TM}SK(+) is underlined. The sequence encoding melittin is from codon 20 to codon 44.

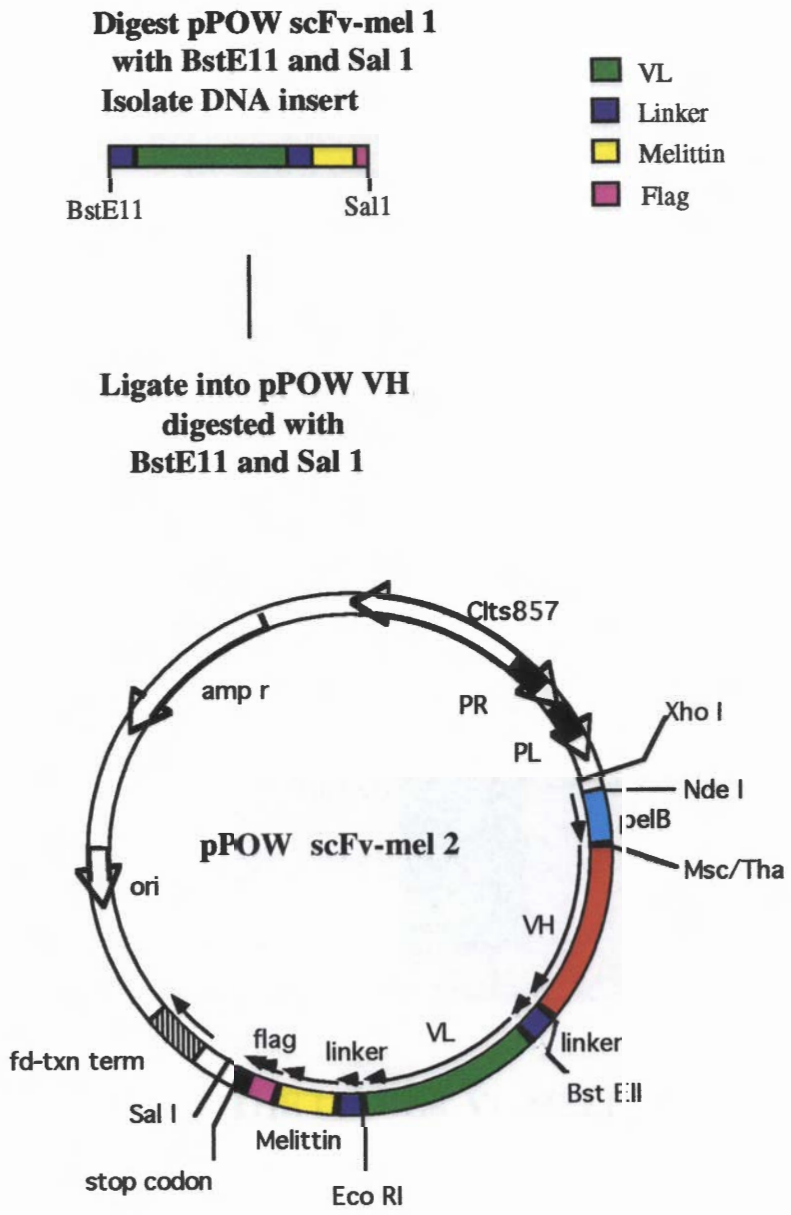


Fig 2.25 ISOLATION OF THE LINKER-VL-LINKER-MELITTIN-FLAG FUSION GENE AND CLONING OF THE DNA FRAGMENT IN pPOW VH

The vector pPOW scFv-mel 1 was digested with BstE 11 and Sal 1 and the insert containing the fusion gene, L-V_L-L-MEL-F, was isolated. After agarose gel purification the insert was cloned into pPOW VH. This particular pPOW VH was previously shown to express the single domain antibody, K-1-21 VH. The final construct for the recombinant immunotoxin, K-1-21(scFv)-mel, in pPOW is shown in the diagram as pPOW scFv-mel 2.

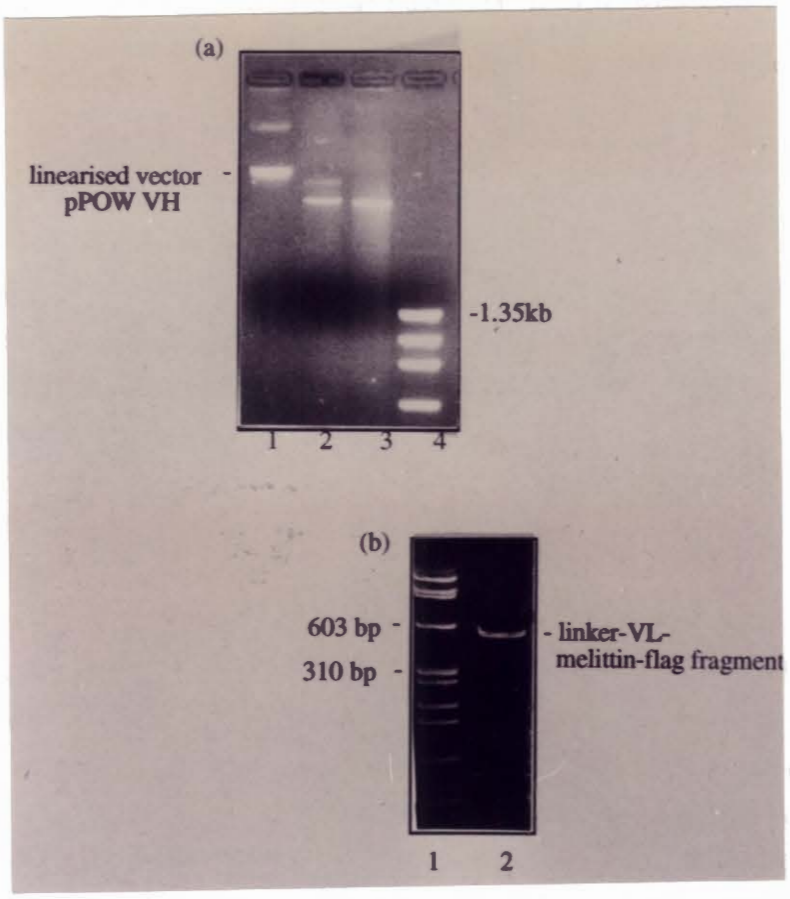


Fig 2.26 CLONING OF THE LINKER-V_L-MELITTIN-FLAG FRAGMENT INTO pPOW V_H

The linker-V_L-linker-melittin-flag fragment (L-V_L-L-MEL-F) was cut out of pPOW scFv-mel 1 construct with enymes BstE 11 and Sal 1. Similarly the vector pPOW V_H was digested with the same enzymes. Shown in (a), 10 µl of purified linearized pPOW V_H which was resolved on a 1% agarose gel and stained with Eth.Br. Lane 1, linearized pPOW V_H ; lane 2 and 3, aliquots (1 µl and 2 µl respectively) of uncut pPOW V_H plasmid DNA ; lane 4, molecular weight standards Øx174 Hae digest 72- 1353 bp. In (b), purified L-V_L-L-MEL-F was resolved by PAGE on a 10% acrylamide gel and stained with Eth.Br. Lane 1, molecular weight standards Øx174 Hae digest 72- 1353 bp ; lane 2, a 10 µl aliquot of the L-V_L-L-MEL-F fragment after purification.

theoretical size of this fragment (640 bp) was separated from plasmid DNA on a 1% agarose gel and electro-eluted. DNA from the eluate was purified and concentrated using an ELUTIP-d column. The isolated gene fragment, L-V_L-L-MEL-F, is shown in Fig 2.26 (b)

A ligation reaction consisting of 5 ng pPOW VH and 100 ng L-V_L-L-MEL-F gene fragment was carried out as described in section 2.2.9. The DNA in the ligation mixture was ethanol precipitated and then resuspended in 5 µl of sterile water. The resuspended mixture was transformed into competent XL-1 Blue cells and plated out on LB+amp plates. A total of 30 colonies were selected and the plasmid DNA (7 µl) was digested with Xho 1 and Sal 1.

Nucleotide sequence of the recombinant immunotoxin, scFv-mel

Ligation of the DNA fragment into pPOW VH produced a single clone with an insert of 918 bp, which is the expected size for the scFv-mel fusion gene (Fig 2.27). Plasmid DNA containing this insert was sequenced in both the forward and reverse directions on the automated sequencer (Applied Biosystems model 373A). The complete nucleotide sequence of the recombinant immunotoxin gene construct is given in Fig 2.28. Sequence encoding the 3 CDRs for the V_H gene and the 3 CDRs for the V_L gene are underlined. The linker sequence joining the V_H and V_L gene is shown in italics. Likewise the linker sequence between the carboxy end of the V_L gene and melittin is in italics. Restriction endonuclease sites are illustrated by an asterisk above the relevant codons.

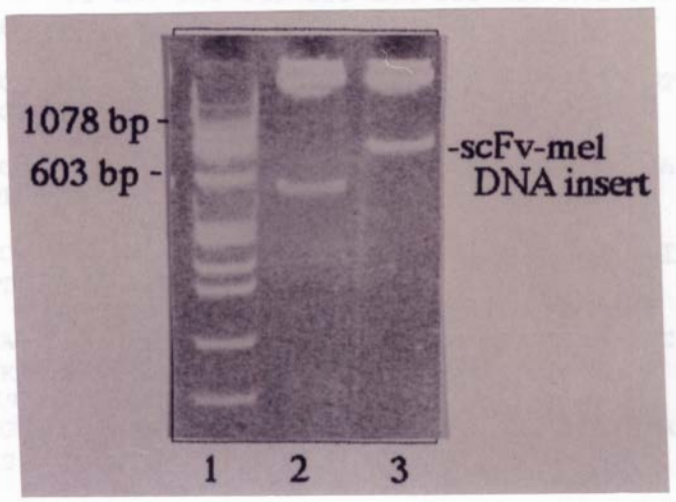


Fig 2.27 DIGESTED FRAGMENTS FROM THE pPOW scFv-mel 2 CONSTRUCT

After ligation of L-V_L-L-MEL-F into pPOW V_H plasmid DNA was prepared from selected colonies. Aliquots (7 µl) were digested with restriction enzymes Xho 1 and Sal 1 and the digests were resolved by PAGE on a 10% acrylamide gel and stained with Eth.Br. Lane 1, molecular weight standards Øx174 Hae digest 72-1353 bp ; lane 2, digested vector pPOW V_H containing insert of approximately 500 bp, lane 3, digested pPOW scFv-mel 2 containing insert of approximately 1000 bp.

```
    1                                                  10
   CAG GTG CAG CTG CAG GAG TCT GGG GCA GAG CTT GTG AAG CCA GGG GCC
    Q   V   Q   L   Q   Q   S   G   A   Q   Q   V   K   P   G   A
            20                                          30
TCA GTC AAG TTG TCC TGT ACA GCT TCT GGC TTC AAC ATT AAA GAC ACC TAT
 S   V   K   L   S   C   T   A   S   G   F   N   I   K   D   T   Y
                        40                                      50
ATG CAC TGG GTG AAG CAG AGG CCT GAA CAG GGC CTG GAG TGG ATT GGA AGG
 M   H   W   V   K   Q   R   P   E   Q   G   L   E   W   I   G   R
                                    60
ATT GAT CCT GCG AAT GGT AAT ACT AAA TAT GAC CCG AAG TTC CAG GGC AAG
 I   D   P   A   N   G   N   T   K   Y   D   P   K   F   Q   G   K
        70                                      80
GCC ACT ATA ATA GCA GAC ACA TCC TCC AAC ACA GCC TAC CTG CAG CTC AGC
 A   T   I   I   A   D   T   S   S   N   T   A   Y   L   Q   L   S
                    90                                      100
AGC CTG ACA TCT GAG GAC ACT GCC GTC TAT TAC TGT GCT AGG GGG GTC TAC
 S   L   T   S   E   D   T   A   V   Y   T   C   A   R   G   V   Y
                                110
CAT GAT TAC GAC GGG GAC TAC TGG GGC CAA GGG ACC ACG GTC*ACC GTC GCC
 H   D   Y   D   G   D   Y   W   G   Q   G   T   T   V   T   V   A
    120                                     130
TCC GGT GGT GGT GGT TCA GGA GGA GGA GGT TCG GGT GGT GGT GGT TCG GAC
 S   G   G   G   G   S   G   G   G   G   S   G   G   G   G   S   D
                140                                     150
ATC GTC ATG ACC CAG TCT CAA AAA TTC ATG TCC ACA TCA GTA GGA GAC AGG
 I   V   M   T   Q   S   Q   K   F   M   S   T   S   V   G   D   R
                            160
GTC AGC GCT ACC TGC AAG GCC AGT CAG CAT GTG GGT ACT AAT GTA GCC TGG
 V   S   A   T   C   K   A   S   Q   H   V   G   T   S   V   A   W
170                                     180
TAT CAA CAG AAA CCA GGG CAA TCT CCT AAA GCA CTG ATT TAC TCG ACA TCC
 Y   Q   Q   K   P   G   Q   S   P   K   A   L   I   Y   S   T   S
            190                                     200
TAC CGG TAC AGT GGA GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA
 Y   R   Y   S   G   V   P   D   R   F   T   G   S   G   S   G   T
                        210                                     220
GAT TTC ACT CTC ACC ATC AGC AAT GTG CAG TCT GAA GAC TTG GCA GAG TAT
 D   F   T   L   T   I   S   N   V   Q   S   E   D   L   A   E   Y
                                    230
TTC TGT CAG CAA TAT AAC AGC TAT CCG TAC ACG TTC GGA GGG GGG ACC AAG
 F   C   Q   Q   Y   N   S   Y   P   Y   T   F   G   G   G   T   K
        240                                     250
CTG GAA ATA AAA CGG GCT GAT GCT GCA CCA ACT GTA TCC ATC GAA*TTC TCC
 L   Q   I   K   R   A   D   A   A   P   T   V   S   I   Q   F   S
                    260                                     270
GGA GGC GGT GGC TCG GGC GGT GGC GGC TCG GGT GGC GGC GGC TCT GGC ATT
 G   G   G   G   S   G   G   G   G   S   G   G   G   G   S   G   I
                                280
GGA GCT GTG CTA AAA GTC CTC ACC ACA GGT CTT CCT GCA TTG ATA TCC TGG
 G   A   V   L   K   V   L   T   T   G   L   P   A   L   I   S   W
    290                                     300
ATC AAG CGT AAA CGG CAG CAG GTC TCC TCA GAT TAT AAA GAT GAT GAT GAT
 I   K   R   K   R   Q   Q   V   S   S   D   Y   K   D   D   D   D
AAA TAA TAA GTC*GAC
 K  STOP STOP
```

Fig 2.28 THE DNA SEQUENCE FOR THE RECOMBINANT IMMUNOTOXIN, scFv-mel, AND THE DERIVED AMINO ACID SEQUENCE

The nucleotide sequence for scFv-mel is given above. The derived amino acid sequence is shown as single letter notation. Sequence encoding the two linkers is in italics and restriction sites have an asterisk above the relevant codons.The 6 CDRs are underlined.

2.4 *DISCUSSION*

K-1-21 V_H GENE

The isolation of mRNA from total RNA (Fig 2.1) and subsequent synthesis of cDNA produced about 1.4 ng/µl of cDNA. Approximately 14 ng of cDNA, used for PCR amplification of the V_H gene, produced a band of 350 bp (Fig 2.3) which is the estimated size of the gene fragment. Digestion of the PCR fragment with restriction enzymes (Fig 2.4) showed that the V_H gene has an internal Pst 1 site and therefore this enzyme could not be used for directional cloning of the gene and it's subsequent expression. Addition of the flag sequence to the V_H gene produced a DNA fragment which corresponded to the expected size of the gene (Fig 2.5). Hybridisation of radio-labelled V_H PCR product to isolated clones (Fig 2.6) indicates that 66% of the clones tested contain V_H DNA insert. DNA sequence obtained from 5 of these clones was identical (Fig 2.7) and analysis of the data using Genbank identified the insert as a rearranged murine immunoglobulin V_H gene.

V_H NUCLEOTIDE SEQUENCE

Comparison of K-1-21 V_H nucleotide sequence with the Genbank data base shows that this sequence has a 92% homology with two murine hybridoma V genes (Fig 2.29). The V_H gene G8Ca1.7 was obtained from an anti-GAT monoclonal antibody (Schiff *et al*, 1983) and C16-15F6 was isolated from a monoclonal antibody to (T,G)-A-L (Borriero *et al.*, 1990). There is a 91% homology between K-1-21 V_H and the V gene of 17.2.25 which was derived from a monoclonal antibody to the hapten 4-(hydroxy-3-nitrophenyl) acetyl, (anti-NP), expressing the NP^a idiotypic marker (Boersch-Supan *et al*, 1985)

Altogether there are 4 nucleotide differences between the K-1-21 V_H gene and G8Ca1.7 and C16-15F6. These changes occur at codons 1, 2, 22 and 71. Only one of these changes results in an amino acid substitution in K-1-21 and this is a T to I residue at codon 71. There are 7 nucleotide differences between the V gene of K-1-21 and 17.2.25. These include the four base changes of the other two V genes as well as substitutions at codons 13, 23 and 76. Resulting amino acid changes between the V genes of 17.2.25 and K-1-21 are R to K (codon 13) and T to S (codon 76).

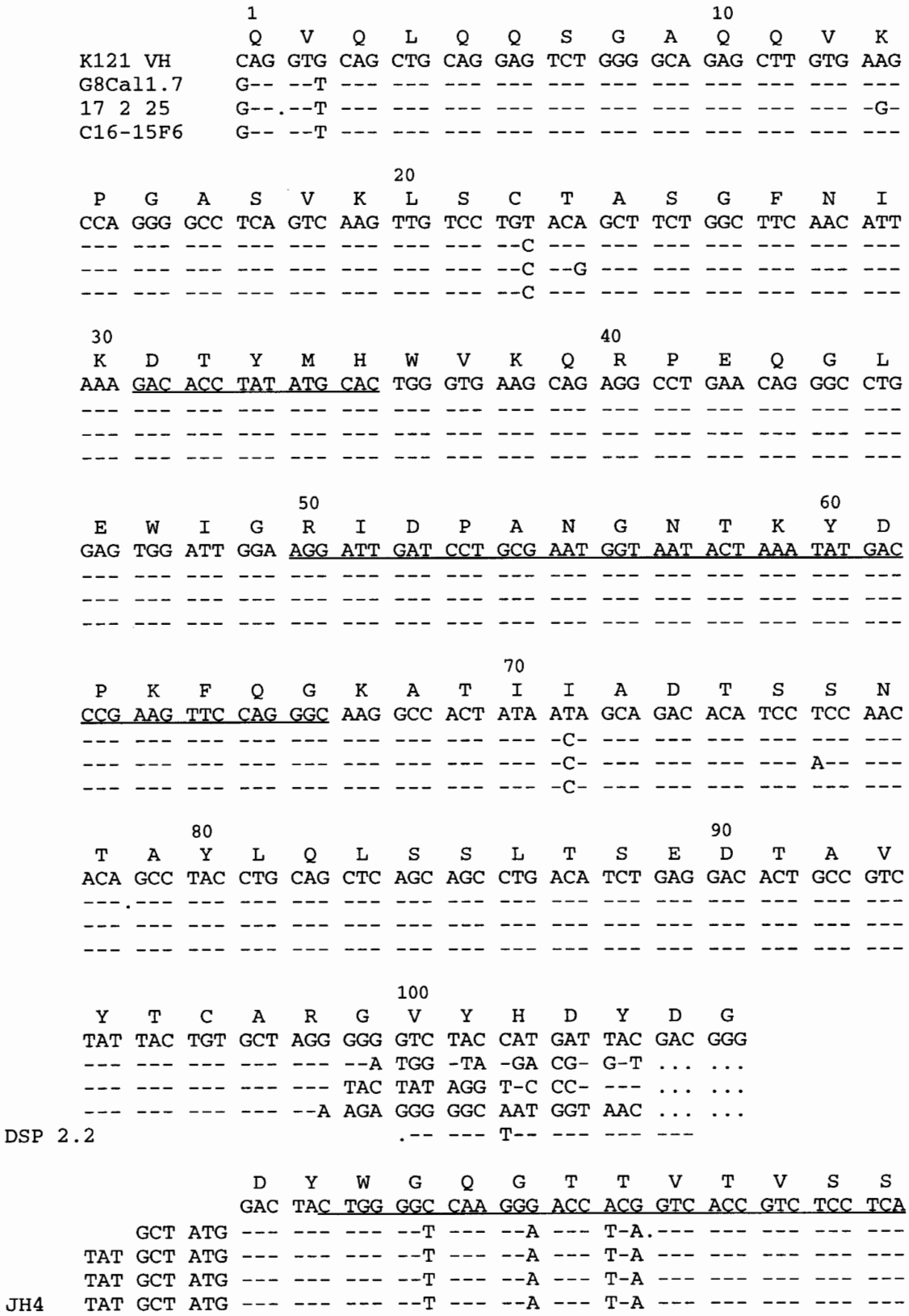


Fig 2.29 A COMPARISON OF K-1-21 V_H WITH THREE mAB V-GENES

The nucleotide sequence of K-1-21 V_H is compared to V_H genes G8Cal1.7 (anti-GAT mAb), 17.2.25 (anti-NPa mAb) and C16-15F6 (anti-[T,G]-A-L mAb). Dashes depict similar bases and dots are used to align the sequence. Single letter notation is used for amino acids.

None of these base changes occur within the CDR1 or CDR2 coding regions. In fact, the two base substitutions at codons 1 and 2 are possibly a result of the highly degenerative primer, *VH1 for*, which was used in the initial PCR reaction (section 2.3.2).

The nucleotide sequence from the cloned, genomic rearranged V gene obtained from the hybridoma C16-15F6 was compared with the nucleotide sequence of the V_H J558 family germ-line gene H10 (Borriero *et al.*, 1990). Sequence analysis showed that nucleotides encoding the leader sequence and the V gene sequence of H10 and V_H 15F6 were identical, as were their restriction sites. The authors concluded that the V_H 15F6 gene is derived from the H10 germ-line gene. In addition, the anti-NP^a hybridoma 17.2.25 also appears to use the H10 germ-line gene (Boersch-Supan *et al.*, 1985). A comparison between the K-1-21 V gene sequence, the germ-line gene H10 and VH 15F6 is given in Fig 2.30. The V gene from the hybridoma C16-15F6 appears to be unmutated which is a characteristic of the early primary response to many antigens including (T,G)-A-L (Borriero *et al.*, 1990) and NP (Cumano and Rajewsky, 1985). Thus the five nucleotide changes observed between H10 and K-1-21 are exactly the same as the differences between K-1-21 and C16-15F6. Once again it is possible that the use of a degenerate PCR primer (*VH1 for*) has resulted in the nucleotide changes in codons 1 and 2, at positions 1 and 6 respectively. The nucleotide change at codon 22 does not result in an amino acid substitution, whereas the change at codon 71 (position 2) becomes I for K-1-21. These base changes are probably due to somatic mutation of the K-1-21 V_H gene associated with the secondary response to the antigen, human *kappa* Bence Jones proteins (BJP). The K-1-21 hybridoma was derived from a hyperimmunised mouse.

COMPARISON OF THE V_H-D-J_H JOINING REGION

A comparison of the heavy chain joining regions (J_H) between K-1-21 and the three hybridomas G8Cal.7, 17.2.25 and C16-15F6 shows that all four V_H genes combine with the same joining segment gene, J_{H4} (Fig 2.29). The sequence is aligned to show the homology with the J_{H4} gene. The reverse PCR primer which was used to isolate the K-1-21 V_H gene, *VH1 back*, was derived from a consensus primer which used the most common base at that position in all J_H genes (Ward *et al.*,1989). The sequence representing the *VH1 back* primer is underlined and it can be

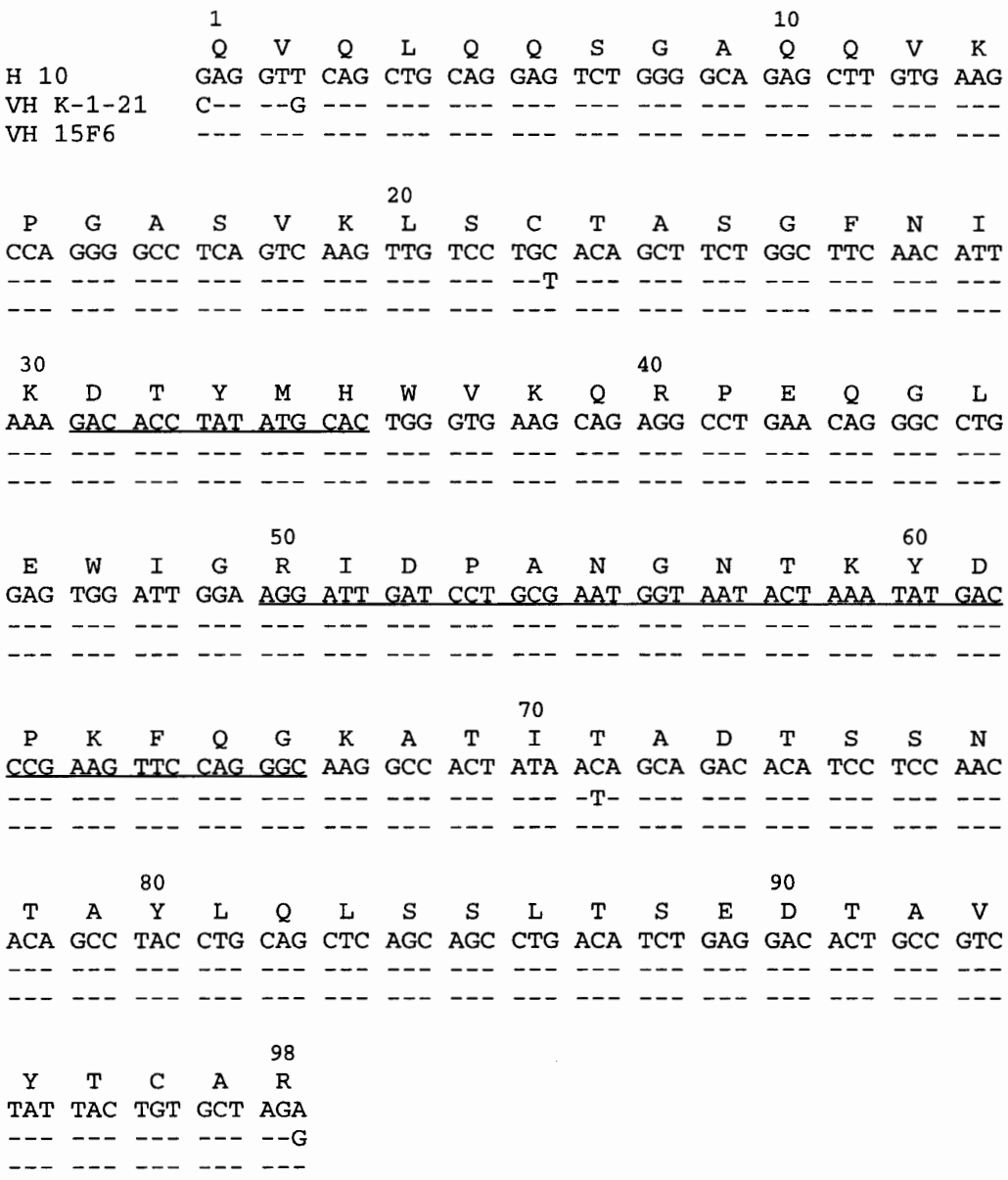


Fig 2.30 COMPARISON OF K-1-21 V_H WITH A GERMLINE GENE
A comparison of K-1-21 V_H gene with the germ-line gene H10 from the J558 family and the V_H gene from the anti-(T,G)-A-L mAb, C16-15F6. Dashes represent similar bases and CDR1 and CDR2 are underlined. The amino acid sequence of H10 is given using single letter notation.

seen that there are four bases in the J_H region of K-1-21 which differ from the J_{H4} nucleotide sequence. These base differences are a result of the consensus J region primer used for PCR. However, these base changes result in only one amino acid change from S to T (codon 114) in K-1-21.

In contrast, the D segments of the hybridomas are very different. The diversity genes used by hybridomas C16-15F6 and G8Ca1.7 were not assigned, whereas partial homology with the D germline gene DSP2.3/4 was suggested for the hybridoma 17.2.25 (Boersch-Supan *et al*, 1985). There appears to be a fairly good homology between the D segment of K-1-21 V_H gene and the germ-line D gene DSP2.2 (Fig 2.29) and therefore a tentative assignment can be made to this D segment. The bases at codon 107 appear to be non-coded (N) nucleotides as they are not present the germline gene.

Despite the differences in D segments there is one consensus feature amongst the four hybridomas and that is the common V-D joining codon 98, which is AGX. This characteristic has previously been noted for V_H genes from anti-NP hybridomas (Boersch-Supan *et al.*, 1985) and anti-GAT hybridomas (Rocca-Serra *et al.*, 1983) but it is not found in all the H chains from anti-(T,G)-A-L antibodies (Borriero *et al.*, 1990). Although the monoclonal antibodies K-1-21, G8Ca1.7, 17.2.25 and C16-15F6 share similar V_H and J_H sequences their D segments are extremely diverse and therefore the amino acids which define their CDR3 structure would be unique. As the CDR3 plays a major role in antigen binding this probably accounts for their different specificities.

K-1-21 V_L GENE

Isolation of the V_L gene using PCR primers *VL1 for* and *VL1 back* produced a band of 350 bp which is the correct size for a V_L gene.(Fig 2.9). Subsequent cloning of the PCR product resulted in several clones containing DNA insert of 350 bp (Fig 2.10). The nucleotide sequence derived from isolated plasmid DNA from two different clones was homologous to rearranged murine immunoglobulin *kappa* light chain, Vκ (Fig 2.11)

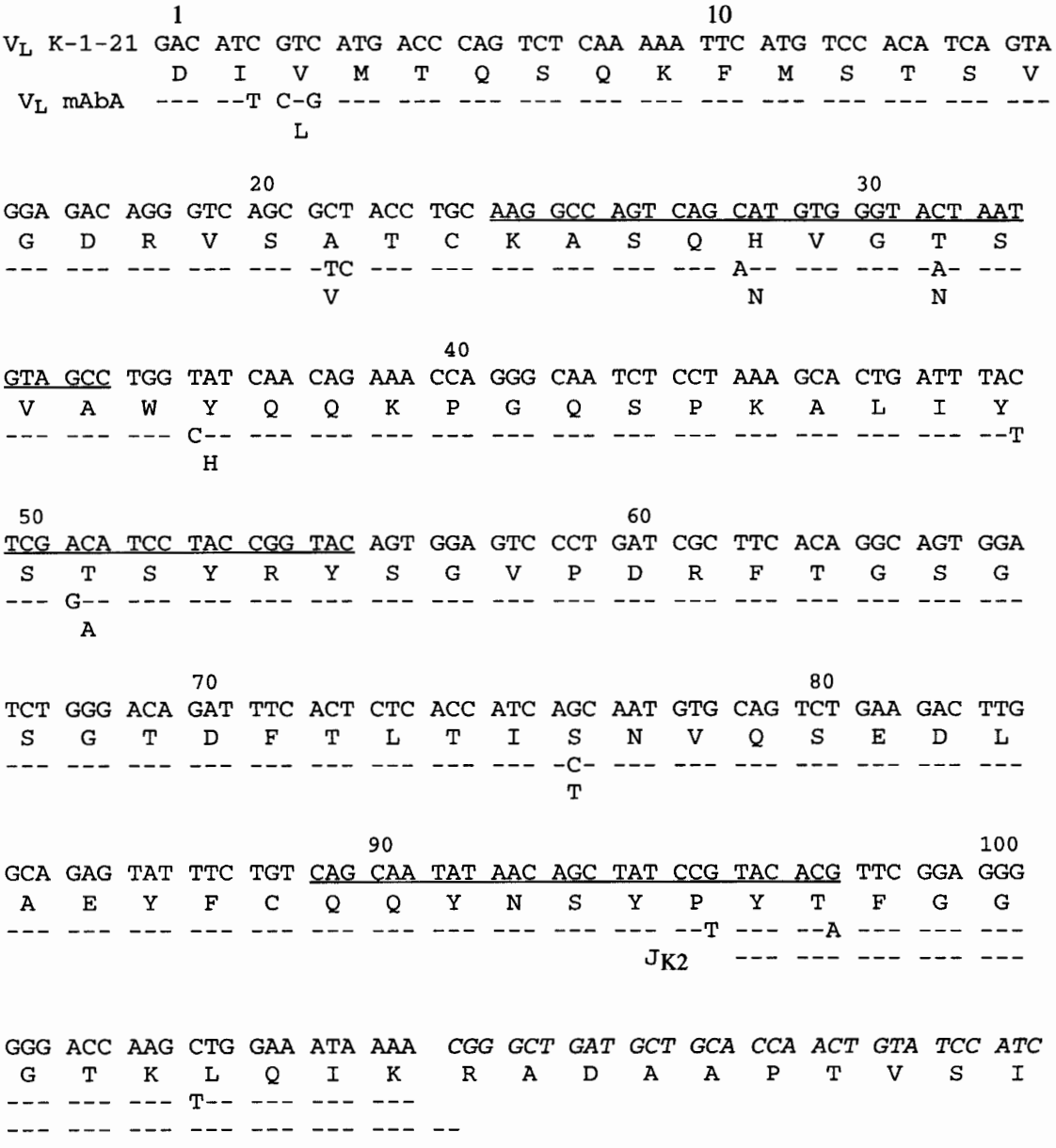


Fig 2.31 COMPARISON OF K-1-21 V_L WITH A REARRANGED MURINE V_K LIGHT CHAIN SEQUENCE

The nucleotide sequence for K-1-21 V_L gene is compared to the V_L gene derived from an anti-haloperidol mAb (V_L mAbA). Identical nucleotides are represented by dashes. Similarities between V_L K-1-21 and the J_{K2} germ-line gene are shown from codon 96 onwards. Complementarity determining regions CDR1, CDR2 and CDR3 are underlined and the PCR primer *VL1 back* is in italics.

The nucleotide sequence of K-1-21 V_L was analysed using the Genbank data base and a 90% homology at the nucleotide level was found with a rearranged Vκ gene from an anti-haloperidol monoclonal antibody previously described by Sherman *et al.*, (1988). A comparison with this V_L gene (mAbA) shows that there are fourteen base differences throughout the K-1-21 V_L gene and the joining (J) gene (Fig 2.31). Four of these base differences (codons 2, 49, 95 and 104) do not result in amino acid changes. In fact, the base changes at position 6 in codon 2 and position 9 in codon 3 might be a result of the degenerate primer, *VL1 for*, used to isolate K-1-21 V_L. The other ten base changes are responsible for a total of seven amino acid differences between K-1-21 V_L and the mAbA V_L. These occur at codons 3 (V to L), 21 (A to V), 28 (H to N), 31 (T to N), 36 (Y to H) 51 (T to A) and 76 (S to T). Two amino acid differences at positions 28 and 31 are within the CDR1 region and a third amino acid change at position 51 is in the CDR2 region. Thus the differences in nucleotide sequence are probably a result of somatic mutation occurring in both genes. Both Vκ genes use the joining gene $J_{\kappa 2}$ which is shown from codon 96 in Fig 2.31. These light chain V genes, K-1-21 and mAbA, are homologous to the germ-line gene Vκ19 which lacks the Kabat residues 27A-27F and gives rise to a rather shallow binding pocket or groove (Sherman and Bolger, 1987).

CONSTRUCTION OF THE scFv FRAGMENT

Addition of the linker fragment 3(S, G_4) to the V_L gene using PCR was successful (Fig 2.12) and the sequence obtained from the two clones confirmed that nucleotides encoding the complete linker gene had been fused to the 5' end of the V_L region.(Fig 2.14). From the nucleotide sequence data and derived amino acid sequence the scFv gene construct is in the correct reading frame for expression of the foreign protein. The linker peptide 3(S G_4) was initially designed by Huston *et al.* (1988) to maintain the close proximity between the VH and VL domains without disrupting the folding of the two domains. Using X-ray co-ordinates for Fab fragments these authors determined that the distance between the carboxyl terminus of VH and the amino terminus of VL was approximately 3.5 nm. A 15 amino acid peptide was required to span this distance and the linker should be flexible without any ordered secondary structure which might interfere with the association of the domains. The serine residues of the peptide confer hydrophilicity whereas the glycine residues maintain flexibility of the linker. A scFv derived from the anti-

digoxin monoclonal antibody, 26-10, was constructed from synthetic oligonucleotides and incorporated this linker gene. Subsequent cloning and expression of the 26-10 scFv resulted in an antibody fragment which retained specificity for digoxin. Other linker peptides designed by Bird *et al.* (1988) were less flexible and were obtained using computer graphics to superimpose potential peptides on the MCPC603 variable region structure. In this case the linker for the scFv fragments from the anti-bovine growth hormone mAb (3C2-scFv) and anti-fluorescein mAb (18-2-3-scFv) was derived from a segment of the protein, carbonic anhydrase, and consisted of an 18 amino acid sequence (KESGSVSSEQLAQFRSLD). This peptide was chosen as it could span the distance between the domains and would not interfere with the Fv structure. A second linker peptide (EGKSSGSGSESKST) was designed to fit into the groove on the back of the Fv structure and was used to link the VL and the VH domains of the anti-fluorescein antibody, 18-2-3. In all scFv constructs using the latter mAb, the carboxy terminus of the V_L gene was linked to the amino terminus of the V_H gene. After cloning and expression the purified, renatured scFv fragments retained both specificity and affinity for their relevant antigens. These studies have shown that at least three different linker peptides can be used to join the VL and VH domains of a Fv fragment without altering their specificity and they can be joined through either domain (reviewed in Sandhu, 1992). In fact further studies by Batra *et al.* (1990 b), have shown that linking the carboxy terminus of VH to the amino terminus of VL, and vice versa, in the recombinant immunotoxin, anti-Tac scFv-PE40, does not influence the activity of the recombinant protein.

CREATION OF THE RECOMBINANT IMMUNOTOXIN, scFv-mel

The different size bands cloned into pPOW scFv (Fig 2.23) were a result of digestion of melittin out of pCR-Script^{TM}SK(+) with enzymes EcoR 1 and Sal 1. Both these sites are downstream of the melittin DNA insert in the latter vector and incomplete digestion of these sites in the multiple cloning region (MCR) has resulted in fragments of different sizes being cloned into pPOW scFv. For example, the doublets in lane 2 and 5 probably result from insert which has been cut with Sal 1 in the MCR of the vector and the isolated insert has been sub-cloned into pPOW scFv through the EcoR 1 site of the insert and the Sal 1 site of the pCR-Script^{TM}SK(+) vector. The DNA sequence in Fig 2.24 shows that in this recombinant the enzyme, EcoR 1, has restricted the pCR-Script^{TM}SK(+)

vector at the site downstream of the melittin insert in the MCR resulting in additional sequence from the vector. The melittin fusion gene insert was sub-cloned through the EcoR 1 site at the 5' end and the Sal 1 restriction site at the 3' end remains intact. It appears that the melittin fusion gene has been cloned into pPOW scFv which was only linearised with EcoR 1 and was not digested successfully with Sal 1. Although there is a Sal 1 site in pPOW scFv-mel 1 downstream of the DNA insert, complete digestion of the vector with BstE 11 and Sal 1 has resulted in isolation of the correct gene fragment, L-V_L-L-MEL-F. Ligation of this BstE 11 and Sal 1 restricted fragment (Fig 2.26 b) into digested pPOW VH (Fig 2.26 a) resulted in a clone with DNA insert of approximately 918 bp which is the estimated size for the scFv-mel fusion gene construct (Fig 2.27). Derived amino acid sequence from the nucleotide sequence data confirmed that the fusion product had combined in the correct manner and was in the correct reading frame for expression of the recombinant immunotoxin, scFv-mel (Fig 2.28).

As discussed in Chapter 1, several recombinant immunotoxins have been produced by fusing the scFv fragment from a variety of clinically relevant antibodies to the genes encoding either PE or DT. Geneticelly engineered mutants of these toxins have also been produced. Both toxins inhibit protein synthesis by ADP ribosylation of elongation factor 2 therefore they need to be internalised by the cell to exert their toxic effects (reviewed in Pastan and Fitzgerald, 1991). One example of a genetically engineered DT immunotoxin is the DT388-anti-Tac(scFv) produced by Chaudhary *et al.* (1990b). This recombinant immunotoxin consists of the VH and VL domains of the anti-IL-2 receptor mAb, anti-Tac, linked together by a 15 amino acid peptide. The gene construct for the scFv was created using PCR. The toxic protein DT388 consists of the first 388 amino acids of DT including the portions of the molecule required for translocation and toxicity. However, it lacks the last 150 amino acids at the carboxy terminus which are responsible for its cell binding properties. The gene for this truncated toxin was linked to the amino terminus of the V_H gene by addition of two extra amino acids (H and M) at the 3' end of the DT388 gene. Similarly, DT388-anti-TFR(scFv), an anti-transferrin receptor recombinant immunotoxin was produced by fusion of the 3' region of DT388 gene to the 5' end of the V_H gene (Batra *et al.*,1991). The expressed, purified DT388-anti-Tac(scFv) and DT388-anti-TFR(scFv) proteins were cytotoxic to cell

lines bearing the relevant target antigens. An intersting feature of the anti-Tac DT and PE recombinant immunotoxins is that although they are linked to different ends of the scFv they are equally cytotoxic and therefore the orientation of the toxin does not affect the antigen binding site (Chaudhary *et al.*, 1990b).

Other examples of recombinant immunotoxins consist of scFv fragments linked to the truncated PE40 toxin. For instance the anti-Tac(scFv)-PE40 lacks the cell binding domain (23 kDa, domain I) of PE and therefore specificity is conferred by the antigen binding site of anti-IL-2(scFv) (Chaudhary *et al.*, 1989). In the construction of this fusion gene, codon 107 (K) of the Vκ gene was fused to codon 253 (G) of the PE domain II using the amino acid seqence KAFGG which incorporates a Hind 111 recognition site. This linker sequence was present in all PE40 recombinant immunotoxins cloned into the expression vector, pVC38. The expression vector contains the bacteriophage T7 promoter and expression was carried out in *E.coli*, BL21 (λ DE3) cells. This cell line incorporates the gene encoding the phage T7 polymerase which is inserted next to a *lac* promoter and can be induced with IPTG. Examples of recombinant immunotoxins expressed in this system include the OVB3(scFv)-PE which reacts with ovarian cancer cells (Chaudhary *et al.*, 1990a), anti-TFR(scFv)-PE40 which binds to cells expressing the transferrin receptor (Batra *et al.*, 1991), BR96(scFv)-PE40 which binds to the carbohydrate receptor composed in part by the Lewis-Y antigen (Friedman *et al.*, 1993), Mik-*β* 1(scFv)-PE40 which binds to the beta subunit of the IL-2 receptor (Kreitman *et al.*, 1992) and e23(scFv)-PE40 which reacts with the human erbB2 product expressed in many cancers including lung, breast, ovary and stomach adeno-carcinomas (Batra *et al.*, 1992).

Although the small linker peptide between the scFv and PE or DT does not appear to effect the cytotoxicity of the immunotoxin it does play a role in the solubility of the protein. It was subsequently shown that the presence of a more flexible linker (KASGG) between the scFv structure and a truncated form of PE improved the yield of active immunotoxin due to decreased aggregation and improved rate of formation (Brinkmann *et al.*, 1992). Thus, the linker gene can play an important role in the production of the recombinant protein.

In the present study the K-1-21 scFv fragment incorporates the same linker peptide, 3(S, G_4), as the examples cited from the literature. However the toxin, melittin, is a membrane lytic peptide and would require a joining peptide which is long enough to allow association of the toxin with the cell membrane. This peptide should span the distance of the scFv fragment bound to antigen which would be at least 3.5 nm. Therefore, a linker peptide of 15 amino acids was incorporated at the carboxy terminus of K-1-21 V_L gene and the amino terminus of the melittin gene. It has been suggested that lipid bound melittin has a conformation similar to the tetrameric form in aqueous solution and this formation is responsible for perturbation of the membrane resulting in holes which allow leakage of cellular contents (Dempsey, 1990). In order to allow self association of melittin molecules in the membrane the linker peptide between the scFv structure and melittin should be flexible. It should not have a propensity for secondary structure, which might interfere with the lytic action of melittin, and it should be hydrophilic to retain solubility of the recombinant protein. All these features are present in the linker peptide which was used to join the VH and VL domains of K-1-21 scFv. For this reason, sequence encoding the same peptide was chosen to link the carboxy terminus of the V_L gene to the amino terminus of the melittin gene to produce a single chain Fv-melittin fusion gene (scFv-mel).

Expression of scFv-mel was carried out in the vector pPOW (Power *et al.*, 1993) which contains the powerful lambda left and right promoters in tandem and can result in over-expression. The vector incorporates the temperature sensitive repressor gene, *cI* 857, which requires induction at high temperatures but allows a wide choice of host cells. It also contains the gene encoding the signal peptide, *pelB* , which facilitates transport of the fusion protein from the cytoplasm to the periplasm (Lei *et al.*, 1987). Antibody fragments expressed in the periplasmic space tend to fold correctly producing functional proteins (Skerra, 1993). Furthermore, it has been shown that a periplasmic protein, DsbA, is required for disulphide bond formation in *E.coli* (Bardwell *et al.*, 1993). This is an important consideration for expression of antibody fragments as they contain intramolecular disulphide bonds which are responsible for the formation of the two domains, VH and VL. Results of the expression of the two gene constructs K-1-21 VH and K-1-21 scFv-mel will be discussed in detail in Chapter 3.

3.1 *INTRODUCTION*

As discussed in Chapter 1, a variety of antibody fragments have been expressed in prokaryotic cells. There are a number of different expression vectors available and several promoter systems have been used to produce foreign proteins. An overview of the published data suggests that successful expression of eukaryotic proteins in *E.coli* is related to moderate induction of the maximum number of cells at temperatures below 30°C. The secretion strategy using bacterial secretion peptides has also resulted in the production of soluble, correctly folded protein in the periplasm of bacterial cells. Alternatively, antibody fragments have been expressed as insoluble inclusion bodies with subsequent renaturation and refolding to produce active protein. Although the conditions of expression can be optimised in some situations, the solubility of foreign proteins is ultimately dependent on their primary amino acid sequence.

Recombinant immunotoxins have also been expressed in prokaryotic cells. They have generally been derived from PE and DT which are linked to stabilised antibody fragments of varied specificity. In most situations the expressed immunotoxin has been isolated from insoluble inclusion bodies with subsequent refolding and renaturing. The secretion strategy for expression of a single chain recombinant immunotoxin as a soluble protein in the periplasm of *E.coli* cells has not been described previously.

In this study the gene constructs for the single chain recombinant immunotoxin, K-1-21(scFv)-mel, and the single domain antibody (VH) will be cloned into the expression vector pPOW and then expressed in *E.coli*. The expressed fragments will be directed to the periplasmic space using the bacterial secretion peptide *pelB*. Production of the foreign protein will be monitored by western blot analysis using the monoclonal antibody anti-FLAG M2 and isolation of soluble protein from the periplasmic or soluble whole cell fraction will be carried out using affinity chromatography.

Chapter 3

EXPRESSION, PURIFICATION AND BINDING OF THE RECOMBINANT IMMUNOTOXIN, K-1-21(scFv)-mel, AND THE SINGLE DOMAIN ANTIBODY FRAGMENT K-1-21 VH

After purification and concentration of the antibody fragments they will be analysed by functional assays to determine whether the targeting molecule has retained its binding specificity. An ELISA using immobilised antigen, BJP, will be carried out to determine whether the K-1-21 VH and scFv-mel are capable of binding soluble antigen. In addition, binding of K-1-21(scFv)-mel to the human cancer cell-lines, HMy2 and K562, will be assessed by immunofluorescence staining. Binding to the cells will also be determined after pre-incubation of the recombinant immunotoxin with BJP to determine whether the cell association is occurring via the antigen binding site, or via direct association of melittin with the cells. This is an important consideration as the charged carboxyl terminal residues of melittin which are responsible for cell binding will be present in the immunotoxin. Specific cytotoxicity of the recombinant immunotoxin will be determined using a human cancer cell line, HMy2, which expresses the carcinoma antigen (KMA) and compared to a second cell line, K562, which does not express KMA.

3.2 *METHODS*

3.2.1 HOST CELLS

Both the K-1-21 VH and scFv-mel gene constructs cloned into the expression vector pPOW were previously described in Chapter 2. The VH fragment was expressed in *E.coli* strains TG1 and TOPP2, whereas the scFv-mel fragment was expressed in TOPP2, LE 392, DH5a and TG1 (Appendix). About 10-100 ng of purified plasmid DNA isolated from pPOW VH and pPOW scFv-mel 2 gene constructs was used to transform *E.coli* cells according to the transformation protocols recommended by the suppliers. A transformation control using pUC 18 (1-10 ng) was always included. Transformed cells were grown overnight on LB+amp plates at 30°C. A recombinant anti-neuraminidase single chain Fv (NC10 scFv), cloned into pPOW and transformed into the *E.coli* strain TG1, was produced by Malby *et al.* (1993) and was used as a positive control in most of the protein expression experiments.

3.2.2 EXPRESSION OF K-1-21 VH, scFv-mel and NC10 scFv.

1. Primary expression of the protein fragments

Initially the following procedure was carried out on whole cell cultures containing pPOW VH and pPOW scFv-mel to determine whether the protein was expressed in the host cell line. A loop of culture was removed from the plate and placed in 1 ml of Superbroth (Appendix) in a 15 ml Falcon 2059 tube. The culture was grown overnight at 30°C. The following day an aliquot of the culture was used to inoculate 1 ml of superbroth in a 15 ml Falcon tube to give an A_{600} of 0.2-0.5. Cultures were grown at 30°C in an orbital shaker at 150-200 rpm and samples (10 µl) were removed at hourly intervals and diluted 1:10 to determine the rate of cell growth by measuring the absorbance at 600 nm. In some cases a graph of A_{600} versus time was plotted and just before the cultures reached the end of log phase (approximately 4 hr) a zero time sample (50 µl) of uninduced culture was removed and added to 5x SDS loading buffer (SDS LB : Appendix) and boiled for 5 min. Protein expression was then induced by placing cultures in a shaking water bath at 42°C for 15 min. After induction the cultures were returned to the orbital shaker and grown for a further 4 hr at 37°C or 30°C, or overnight at 39°C to establish the optimal temperature, which

was then used for later experiments. After growth at a particular temperature a 50 µl aliquot of induced whole cell culture was removed and added to 5x SDS LB. In addition, a portion of the culture was removed (0.2 ml) and was centrifuged in an Eppendorf 5415C microcentrifuge at 12,000*g*. An aliquot (50 µl) of the supernatant was added to 5x SDS LB and stored at 4°C. The SDS samples were then loaded onto a 10% or 12% SDS polyacrylamide gel (Appendix) and resolved by SDS-PAGE at 150-200 volts (Laemmli, 1970). The gel was then electroblotted across to nitrocellulose paper for Western antibody probing (Towbin *et al.*, 1979) using the monoclonal antibody anti-FLAG M2 which binds to the flag octapeptide.

2. Determination of the cellular location of expressed antibody fragments K-1-21 VH and NC10 scFv by cell fractionation

Originally the periplasmic fraction was isolated by treating bacterial cells with lysozyme in the presence of high concentrations of sucrose (20% w/v) to prevent osmotic swelling after the cell wall is removed. The protoplast, consisting of the bacterial cell surrounded by the cell membrane, was then ruptured to release the cytoplasmic proteins. This method was followed according to the procedure described in Sambrook *et al.* (1989). Briefly, 1 ml of induced and uninduced TG1 cultures containing pPOW VH and NC10 scFv were placed in 1.5 ml microfuge tubes and centrifuged in a microcentrifuge at 12,000*g* to pellet the cells. The cells were then resuspended in 100 µl of freshly prepared lysozyme (1 mg/ml), 20% w/v sucrose, 30 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0) and placed on ice for 10 min. Afterwards, the cells were pelleted at 12,000*g* and the supernatant containing the periplasmic fraction was removed. A 50 µl aliquot of the supernatant was added to 5x SDS LB, boiled and stored at 4°C for subsequent Western blot analysis. Remaining cells were resuspended in 100 µl of 0.1 M Tris (pH 8.0) and placed on dry ice for 10 min then thawed at 37°C for 10 min. This step was repeated twice. The suspension was then centrifuged at 12,000*g* for 5 min at 4°C. Cytoplasmic proteins are present in the supernatant and an aliquot was removed and added to 5x SDS LB. The pellet consists of cell membranes and insoluble inclusion bodies. These were solubilized by treating the pellet with 100 µl of 1% Triton X-100 for 10 min at 4°C.

An aliquot of the resulting solution was added to 5x SDS LB, boiled and stored.

All samples taken for further analysis were loaded onto a 10% or 12% SDS polyacrylamide gel, electrophoresed at 100-150 volts and then blotted across to nitrocellulose paper. The membrane was probed with anti-FLAG M2 antibody. This method of cell fractionation was first used for small batch analysis of expression of VH and NC10 scFv antibody fragments. However, the high sucrose content made it difficult to filter the periplasmic fraction. It was later found that the fractionation procedure recommended for the anti-FLAG M2 affinity column (IBI, New Haven, CA) was better suited to large scale fractionation. For this reason, and also to standardise the isolation procedure, the analytical fractionation of some VH and NC10 scFv protein fragments was by the IBI protocol, whereas all the expressed scFv-mel antibody was fractionated by the analytical method described below.

3. Analytical fractionation of expressed proteins

Once the expression of antibody fragments, VH, NC10 scFv and scFv-mel, had been determined in whole cells they were fractionated to establish whether the protein was present in the periplasmic, soluble or insoluble whole cell fraction. Cellular fractionation was carried out according to the protocol supplied with the anti-FLAG M2 affinity column (IBI, New Haven, CA). Briefly, 10 ml of SOC+trace elements medium (Appendix) was inoculated with cells and grown overnight at 30°C. The cultures were diluted to an A_{600} of 0.2-0.5 in 100 ml of SOC+trace elements or Superbroth+trace eelements (in a 500 ml flat bottomed flask) and then grown at 30°C. A zero time sample (50 µl) was removed, added to 5x SDS LB, boiled and stored at 4°C. After induction cultures were grown for a further 4 hr at 37°C or 30°C. The culture was then divided into two and centrifuged at 5000*g* for 10 min at 10°C.

(A) Periplasmic fraction.

The pellet was warmed to room temperature and resuspended in 40 ml 0.5 M Sucrose, 0.03 M Tris, 1 mM EDTA pH 8.0 at a final concentration of 40 ml/g of cells. The cell suspension was centrifuged at

3,500*g* for 10 min at 10°C. Afterwards the cell pellet was subjected to osmotic shock by resuspending at a concentration of 25 ml/g of cells in ice-cold, sterile water . This was followed by centrifugation at 3,500*g* for 10 min at 4°C. After osmotic shock the supernatant was collected and 50 µl was added to 5x SDS LB. Protease inhibitors, Aprotinin (2 µg/ml), AEBSF (10 µg/ml) and sodium azide (50 µg/ml, NaN_3) were added to the remaining periplasmic fraction which was stored at -20°C for further purification.

(B) Whole cell extract: soluble and insoluble fractions.
The second pellet was extracted at the same time by resuspending in 5 ml of Extraction Buffer A (50 mM Tris pH 8.0, 5 mM EDTA, 0.25 mg/ml lysozyme and 50 µg/ml NaN_3). The solution was incubated at room temperature for 5 min or until it became viscous, indicating cell lysis. At this stage 0.5 ml of Extraction Buffer B (1.5 M NaCl, 0.1 M Ca Cl_2 , 0.1 M $MgCl_2$, 0.02 mg/ml DNAse1, 2 µg/ml Aprotinin and 10 µg/ml AEBSF) was added and the solution was incubated for a further 5 min. The sample was then centrifuged at 18,000*g* for 1hr at 4°C. Thereafter the supernatant containing the soluble cell fraction was collected. A 50 µl aliquot of the soluble fraction was added to 5x SDS LB and the remaining solution was frozen at -20°C. Insoluble whole cell material remained in the pellet which was dissolved in 5 ml of Extraction Buffer A. An aliquot (50 µl) of this insoluble material was added to 5x SDS LB and the remainder was frozen at -20°C. Samples added to 5x SDS LB were subjected to SDS PAGE on 10% polyacrylamide gels, blotted across onto nitrocellulose paper and analysed by Western blot using anti-FLAG M2 monoclonal antibody.

4. Preparative isolation of expressed antibody fragments

Analytical fractionation showed that some protein was expressed in the periplasmic fraction as well as the soluble whole cell fraction Preparative isolation from the soluble whole cell fraction would therefore also contain periplasmic protein. For this reason, expressed antibody fragments were isolated from both cell fractions for comparative studies. Preparative isolation was carried out in the same way as analytical fractionation except a total of 200-400 ml of culture was extracted. The final volumes from a 100 ml cell culture were 20-25

ml of periplasmic fraction, 10-15 ml soluble whole cell fraction and 5-10 ml insoluble whole cell fraction.

3.2.3 ISOLATION OF EXPRESSED PROTEIN FROM THE PERIPLASMIC AND SOLUBLE FRACTIONS.

Both the periplasmic and soluble whole cell fractions were purified using the anti-FLAG M2 Affinity Gel supplied by IBI (New Haven, CT). A chromatography column containing 2 ml of affinity gel was washed and equilibrated with TBS pH 7.4 (50 mM Tris and 150 mM NaCl) according to the recommended protocol. The bacterial cell fractions were filtered using Whatman No 1 filter paper and then applied to the column. The unbound fraction was collected and re-applied to the column a total of three times at a flow rate of 0.5-1 ml/min. The column was then washed three times with 12 ml aliquots of TBS. Bound protein was eluted in 0.1 M Glycine pH 3.0, with 6x1 ml fractions collected in microfuge tubes containing 25-30 µl of 1 M Tris base at pH 8.0.

Samples (30 µl) of the eluate were added to 5x SDS LB, boiled and resolved by SDS PAGE. The resolved samples were then electroblotted across to nitrocellulose paper and probed with the anti-FLAG M2 antibody. Fractions containing the eluted protein (either VH, NC10 scFv or scFv-mel) were pooled and dialysed against PBS-az. Afterwards the samples were concentrated by dialysis against PEG 6000 in Spectra/Por Membrane, MWCO 1,000 dialysis tubing (Spectrum, Houston, USA). An alternative procedure to concentrate the expressed K-1-21 scFv-mel fragment was carried out using centrifugation on Centricon 10,000 membranes (Amicon). The membrane was first blocked with 1 ml of 0.1% BSA in PBS-az and centrifuged at 4,000*g* for 30 min at 10°C. The Centricon membrane was then washed 3 times with 2 ml PBS-az to remove unbound BSA. The pooled fractions containing the scFv-mel protein were added to the Centricon and centrifuged at 4,000*g* and 10°C until concentrated to a volume of approximately 0.5 ml. The membrane was then washed three times with 2 ml aliquots of PBS-az or RPMI until the final volume was 0.5-1 ml. The solution containing the protein was mixed by gentle agitation of the tube and then removed and stored at -20°C.

3.2.4 WESTERN BLOT ANALYSIS

Expressed protein samples were loaded onto a 10% or 12% SDS gel and were electrophoresed by SDS-PAGE (Laemmli, 1970). The protein bands were then electroblotted onto nitrocellulose membranes (Towbin *et al,* 1979). Thereafter, the nitrocellulose paper was blocked with 3% BSA in TBS-az for 30-60 min at room temperature or overnight at 4°C and then washed three times with TBS-Tween, pH 7.4 (50 mM Tris, 150 mM NaCl and 0.05% v/v Tween 20). The primary antibody, anti-FLAG M2 (IBI, New Haven, CT), was used at a concentration of 10 µg/ml in TBS (pH7.4) and incubated with the membrane for 1-2 hr at room temperature. Afterwards, the membrane was washed three times with TBS-Tween and then incubated with alkaline phosphotase conjugated anti-mouse Ig (1 mg/ml ; Promega, Madison, USA) at a 1:7500 dilution in TBS-tween for 30-60 min. After washing three times with TBS-Tween and once with TBS, the colour was developed with 50 mg/ml nitroblue tetrazolium (NBT) and 50 mg/ml bromochloroindolylphosphate (BCIP) in dimethylsulphoxide (DMSO) for 15-30 min at room temperature. Colour development was stopped by rinsing the nitrocellulose membrane in water before air drying.

3.2.5 QUANTITATION OF PROTEIN BANDS FROM WESTERN BLOTS USING A DENSITOMETER

As the affinity purified scFv-mel fragment was concentrated in the presence of BSA it was difficult to estimate the relative protein concentration using conventional assays. For this reason a Western blot was carried out using aliquots of purified, concentrated K-1-21 fragments VH, scFv-mel and also the NC10 scFv positive control. Concentration controls included a series of dilutions (50-600 ng) of the FLAG-BAP positive control protein (IBI, New Haven,CT). After developing and drying the membrane it was analysed using a PDI desktop scanning densitometer (Daintree Industries, Victoria, Australia). The area of each band representing either a protein fragment or the control protein was estimated and a standard curve was plotted. From a plot of the standard FLAG-BAP protein concentrations versus OD x

mm^2 it was possible to estimate the relative protein concentrations of the fragments.

3.2.6 PREPARATION OF THE K-1-21 FAB FRAGMENT

An Fab fragment of K-1-21 was prepared by digestion of the antibody with papain according to the method of Johnstone and Thorpe (1982). Briefly, 2 mg/ml monoclonal antibody (K-1-21) in 100 mM sodium acetate pH 5.5, 50 mM cysteine and 1mM EDTA was incubated with 80 µg papain at 37°C for 8 hr. The reaction was stopped by the addition of 75 mM iodoacetamide. After digestion the product was dialysed against PBS-az and then purified on a BJP affinity column. Eluted fractions (1 ml) were collected and subjected to SDS PAGE on a 10% polyacrylamide gel and stained with Coomassie dye. Fractions containing the Fab fragment were pooled and dialysed against PBS-az. The protein concentration was determined by measuring the absorption at 280 nm, using an absorption coefficient for Fab of $E_{280} = 15.3$ for a 10 mg/ml protein solution.

3.2.7 ANTIGEN BINDING OF EXPRESSED, PURIFIED ANTIBODY FRAGMENTS

1 ELISA

As previously mentioned the mAb K-1-21 binds to human free *kappa* light chains (Bence-Jones Protein, BJP). An ELISA was carried out by adding 50 µl aliquots of the antigen (50 µg/ml, in PBS-az) to the wells and incubating at 37°C for 1 hr. The plate was then washed three times with PBS-az and blocked with BSA overnight at 4°C. Afterwards, the plate was washed three times in PBS-az and then dilutions of the isolated K-1-21 fragments and NC10 scFv were added to the relevant wells and incubated at 37°C for 1 hr. After washing three times, 25 µl of anti-FLAG M2 antibody (3 µg/ml) was added to the wells and incubated at 37°C for 1 hr. Once again the plate was washed three times and then 25 µl of a 1:7500 dilution of biotinylated sheep anti-mouse Ig (1 mg/ml Promega, Madison, USA) was added to the wells and incubated at 37°C for 1 hr. The incubation was followed by washing 3 times and then 25 µl of alkaline phosphatase conjugated avidin (avidin-AP) was added to the wells and incubated at 37°C. Finally the plate was washed three times

with PBS-az and once with Carbonate buffer, pH 9.6 (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3) before developing the colour with 50 µl of 1 mg/ml p-nitrophenol phosphate for 15-20 min at 37°C. Absorbance at 405 nm was then read in a microelisa plate reader.

2. Analysis of binding of K-1-21 monoclonal antibody and the Fab fragment to HMy2 cells using flow cytometry

HMy2 cells were suspended in cold buffered saline/bovine serum albumin (PBS/BSA ; 0.1% BSA in PBS-az, pH 7.4) at a concentration of $2x10^6$ cells/ml. Aliquots (0.5 ml) were placed in microfuge tubes and centrifuged for 4-6 s at room temperature. The supernatant was aspirated and the cells were gently resuspended in 50 µl of either K-1-21 or the Fab fragment. Afterwards cells were incubated at 4°C for 30 min with gentle agitation after 15 min. Following incubation the cells were washed once with 0.5 ml cold PBS/BSA and incubated with 25 µl of 10, 100 or 1000 µg/ml sheep anti-mouse Ig labelled with fluorescein isothiocyanate (FITC). The cells were incubated at 4°C for 30 min and then washed three times with 0.5 ml cold PBS/BSA. Finally the cells were fixed with 0.5 ml 1% paraformaldehyde in PBS-az and analysed by flow cytometry. Fluorescein stained cells were analysed on a single-laser FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). FITC was excited by using the 488 nm line of an argon ion laser and fluorescent emissions were selectively collected by using a 535 ± 15nm bandpass filter. A total of 10^4 cells were analysed per sample and fluorescence was measured on a logarithmic scale.

3. Binding of scFv-mel and NC10 scfv antibody fragments to human lymphoma cell lines by flow cytometry analysis

Two human cancer cell-lines, HMy2 and K562, were used to determine scFv-mel and NC10 scFv antibody binding to the cell surface. The cell line HMy2 expresses the surface antigen, KMA, whereas K562 cells do not. Cells were suspended in cold PBS/BSA at a cell density of $1x10^7$/ml. Aliquots containing 10^5 cells were placed in microfuge tubes and 30 µl of purified NC10 scFv or scFv-mel was added to triplicate samples. A negative control consisting of PBS was also added to triplicate cell samples. Cells were then incubated at 4°C for 30 min with gentle agitation after 15 min. Following incubation the cells were

washed once with 0.5 ml cold PBS/BSA and incubated with 30 µl of anti-FLAG M2 (7.5 µg/ml) at 4°C for 30 min with agitation as above. The cells were then washed once with 0.5 ml cold PBS/BSA and resuspended with 25 µl of 100 µg/ml FITC labelled sheep anti-mouse Ig. Once again the cells were incubated at 4°C for 30 min and then washed three times with 0.5 ml PBS/BSA. At this stage the cells were fixed with 0.5 ml 1% paraformaldehyde in PBS-az and analysed by flow cytometry as described above. A positive control, using the parent molecule K-1-21 (20 µg/ml) as the first antibody and FITC labelled sheep anti-mouse Ig (100 µg/ml) as the second antibody, was carried out using the same cells to confirm the presence of the antigen on the cell surface.

3.2.8 LYSIS OF HMy2 AND K562 CELLS BY scFv-mel AND MELITTIN

Both HMy2 and K562 cells ($1x10^5$) were incubated with NC10 scFv and scFv-mel (30 µl) in triplicate for 2 hr at 24°C or 18 hr at 37°C in a humidified incubator. At the same time, cells were incubated with increasing concentrations of melittin (Sigma Chemical, St. Louis, MO) using the same conditions. After incubation, cell viability was determined by the addition of ethidium bromide (10 µg/ml) to the cell suspension. Immediately after addition of ethidium bromide cells were analysed by flow cytometry to determine the number of viable cells. Ethidium bromide fluoresces with a peak intensity of approximately 595 nm when excited with a wavelength of 488 nm. Cell death was estimated by determining the percentage of cells incorporating ethidium bromide.

3.3 *RESULTS*

3.3.1 EXPRESSION OF VH

In Chapter 2 it was shown that five clones containing the VH gene produced DNA of identical sequence (Fig 2.7). Plasmid DNA (pPOW VH) from these clones had been transformed into the *E.coli* cell-line TG1 which was shown to be a suitable host for expression of antibody fragments (Malby *et al.*, 1993). In the initial expression experiments involving the K-1-21 VH fragment, the positive control NC10 scFv was also expressed using the same conditions. Primary expression of all five VH clones was performed as descibed in section 3.2.2 (1). Samples of the whole cell culture after expression were resolved by SDS PAGE on a 12% polyacrylamide gel. It was found that only one clone expressed the VH domain (Fig 3.1, lane 3). The size of the expressed protein was estimated to be about 15 kDa which corresponds with the expected molecular size of the VH fragment. This positive clone was used in all further expression experiments of the VH domain. Expression of NC10 scFv produced a band at approximately 32 kDa and two bands of lower MWt which also reacted with the anti-FLAG M2 antibody.

1. Analytical fractionation

The expression of VH and NC10 scFv before induction (zero time) and in the culture supernatant was compared to whole cell expression after overnight expression at 39°C (Fig 3.2). No expressed product was found in the supernatant or uninduced whole cell samples of both VH and NC10 scFv. Analytical fractionation of VH and NC10 scFv was carried out as described in section 3.2.2 (2). The resulting fractions were analysed by Western blot. The three fractions periplasmic, cytoplasmic and insoluble membranes are shown in Fig 3.3 (a). Expressed fragments were observed in all three fractions and the high background was due to a high concentration of the second antibody. As all three fractions are the same volume (100 µl) it can be seen that most of the VH antibody fragment appeared in the periplasm and this fraction was used for preparative isolation of the protein. In all three fractions NC10 scFv showed the same pattern of a band at 32 kDa and two lower MWt bands which bind anti-FLAG M2. Most of the expressed NC10 scFv protein

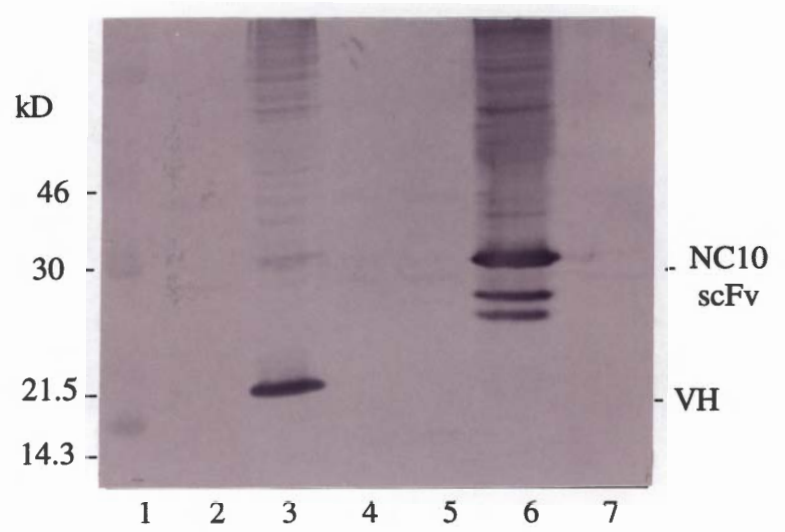


Fig 3.1 EXPRESSION OF K-1-21 VH AND NC10 scFv FRAGMENTS
The constructs pPOW VH and pPOW NC10 scFv were expressed in the host cell-line TG1. Induction was performed at 42°C and then the cell cultures were grown at 39°C overnight. Aliquots of 25 µl from whole cell expression culture were resolved by SDS PAGE on a 12% polyacrylamide gel. Proteins were then transferred by electroblotting to a nitrocellulose membrane and probed with the anti-FLAG M2 antibody. Results of the Western blot are show, lane 1, Rainbow protein molecular weight markers (Amersham, Buckinghamshire, UK); lanes 2-5 and lane 7, 25 µl samples from VH primary whole cell expression culture; lane 6, a 25 µl sample from whole cell expression of the positive control NC10 scFv.

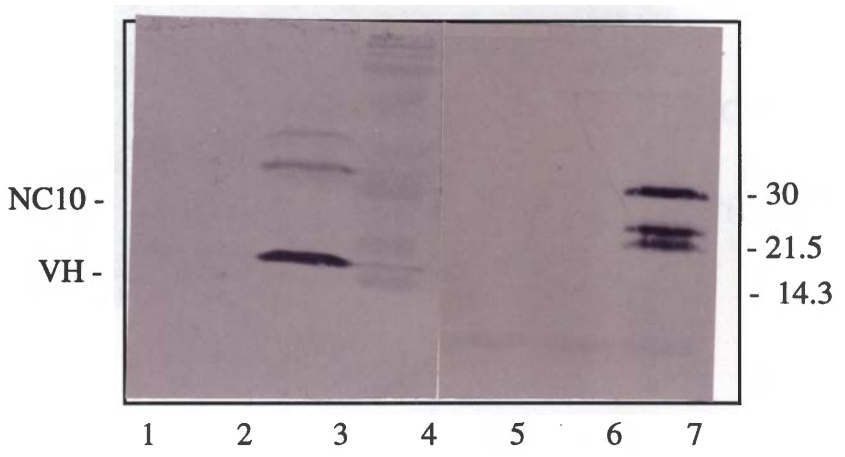


Fig 3.2 WESTERN BLOT OF UNINDUCED, SUPERNATANT AND WHOLE CELL EXPRESSION OF THE VH AND NC10 scFv ANTIBODY FRAGMENTS

Samples were taken from VH and NC10 scFv expression cultures immediately before induction and from the culture supernatant or whole cell culture after overnight growth at 39°C. An aliquot (25 µl) from each sample was subjected to SDS PAGE on a 10% polyacrylamide gel and then analysed by Western blot using the anti-FLAG M2 antibody. Lane 1, uninduced VH cell culture ; lane 2, VH culture supernatant ; lane 3, whole cell VH expression culture ; lane 4, Rainbow protein molecular weight markers ; lane 5, uninduced NC10 scFv whole cell culture ; lane 6, culture supernatant from NC10 scFv culture ; lane 7, induced NC10 scFv cell culture.

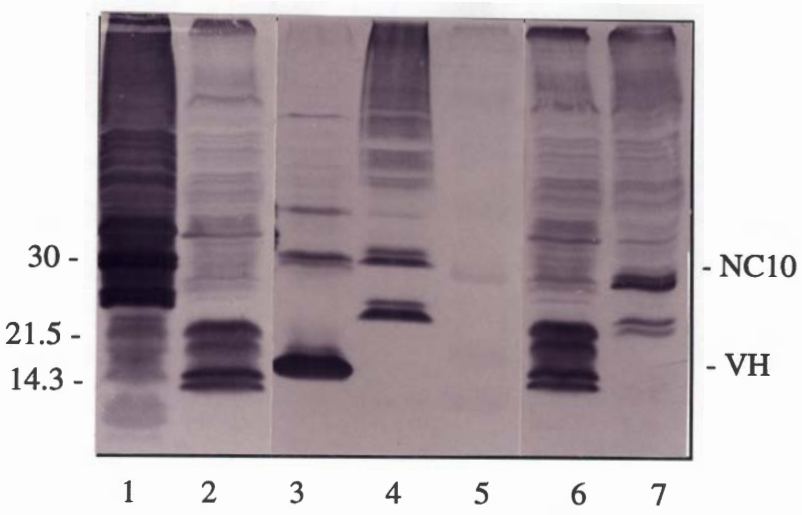


Fig 3.3 (a) ANALYTICAL FRACTIONATION OF EXPRESSED VH AND NC10 scFv ANTIBODY FRAGMENTS AT 37^{o}C

Cell cultures (1 ml) containing expressed VH and NC10 scFv antibody fragments were fractionated into the periplasmic, cytoplasmic and membrane proteins. Fractionation was carried out using the method described in section 3.2.2 (2). Aliquots of 25 µl from each fraction were resolved by SDS PAGE on a 10% polyacrylamide gel and then analysed by Western blot using the anti-FLAG M2 antibody. Results of the Western blot show; lane 1, NC10 membrane fraction ; lane 2, VH membrane fraction ; lane 3, VH periplasmic ; lane 4, NC10 periplasmic; lane 5, VH cytoplasmic ; lane 6, NC10 cytoplasmic.

was found in the insoluble membrane fraction. Although expression of NC10 scFv in the periplasm was relatively low compared to VH this fraction was also used in subsequent preparative isolation of the protein.

2. Preparative fractionation

A preparative fractionation of VH and NC10 scFv was carried out and in this case 2 x 100 ml of each culture was grown. After induction at 42°C, cells were grown for a further 4 hr at 37°C and then the periplasmic fraction was isolated (Fig 3.3, b). The final volume of each periplasmic fraction was approximately 40 ml. The periplasmic isolates were passed directly over the affinity column and eluted fractions were collected and analysed by Western blot (Fig 3.4). Fractions from lanes 2 and 3 containing the VH fragment were pooled and dialysed against PBS-az. The protein concentration was determined by A_{280} using the calculated extinction coefficient $E^{0.1\%}$= 15.3. Results showing purified VH protein after SDS PAGE and Coomassie staining are presented in Fig 3.5.

Similarly the isolated NC10 scFv periplasmic fraction was dialysed against PBS-az, affinity purified and eluted protein was identified by Western blot (Fig 3.6). The fractions from lanes 3 and 4 were pooled, dialysed against PBS-az and the protein concentration determined by A_{280} using the calculated extinction coefficient $E^{0.1\%}$= 15.3. The protein concentration of VH was calculated as 1.12 mg/ml in a final volume of 1 ml and NC10 scFv was 98 µg/ml in a final volume of 1 ml.

3. Comparison of VH expression at 30°C and 37°C

Cultures containing pPOW VH were split into equal volumes of exactly 100 ml, grown at 30°C for 4 hr and then induced at 42°C for 15 min. They were then grown for a further 4 hr at either 30°C or 37°C. Cells were fractionated using the analytical procedure (section 3.2.2, 3) and the periplasmic, soluble and insoluble cell fractions were isolated. Samples from each of these isolates were subjected to SDS PAGE on a 10% polyacrylamide gel and then analysed by Western blot using anti-FLAG M2 antibody. The expression results for VH are shown in Fig 3.7 (a) and (b). The prominent band at about 15 kDa represents the

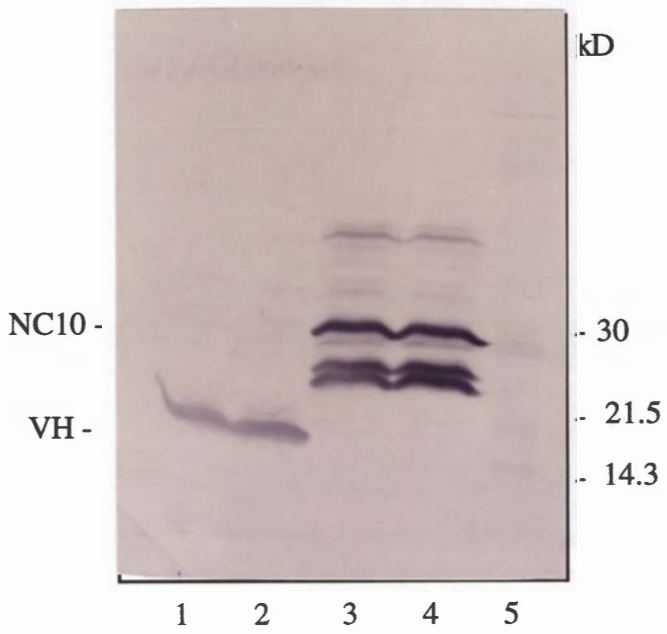


Fig 3.3 (b) PERIPLASMIC FRACTIONS FROM PREPARATIVE CELL FRACTIONATION OF VH AND NC10 scFv

The periplasmic fraction from VH and NC10 scFv cultures was obtained as described in section 3.2.2 (2). A total of 40 ml of periplasmic fraction was isolated for each fragment. Samples (25 µl) from each periplasmic isolate before they were pooled were added to 5x SDS LB, resolved by SDS PAGE and analysed by Western blot. Duplicate samples are shown in; lanes 1 and 2, VH periplasmic fraction ; lanes 3 and 4, NC10 scFv periplasmic fraction ; lane 5, Rainbow protein molecular weight markers.

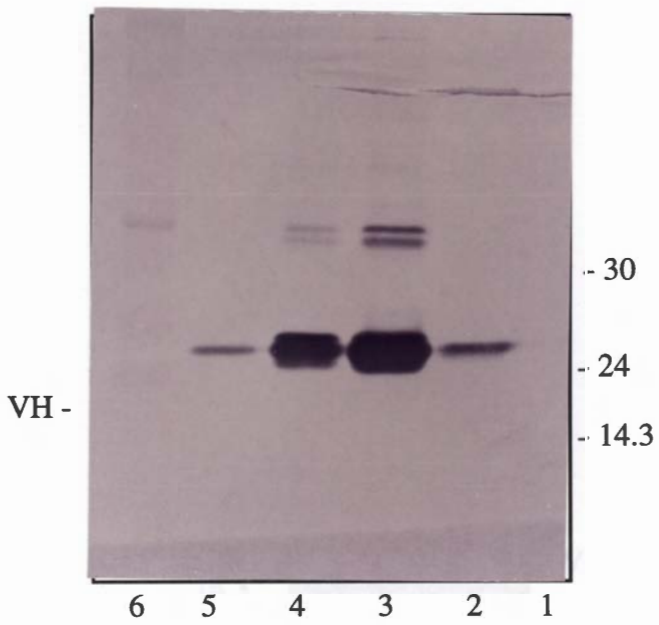


Fig 3.4 PURIFICATION OF VH ANTIBODY FRAGMENT ON THE ANTI-FLAG M2 AFFINITY COLUMN

The periplasmic fraction containing the expressed VH antibody fragment from Fig 3.3 (b) was applied to the anti-FLAG M2 affinity column and the bound protein was eluted using 0.1 M Glycine pH 3.0. Fractions (1 ml) were collected in 25-30 µl of 1 M Tris base pH 8.0 and aliquots (25 µl) were subjected to SDS PAGE on a 10% polyacrylamide gel. After electrophoresis the proteins were electroblotted onto a nitrocellulose membrane and probed with the anti-FLAG M2 antibody. The results show; lanes 1-5, eluted VH fragment ; lane 6, Rainbow protein molecular weight markers.

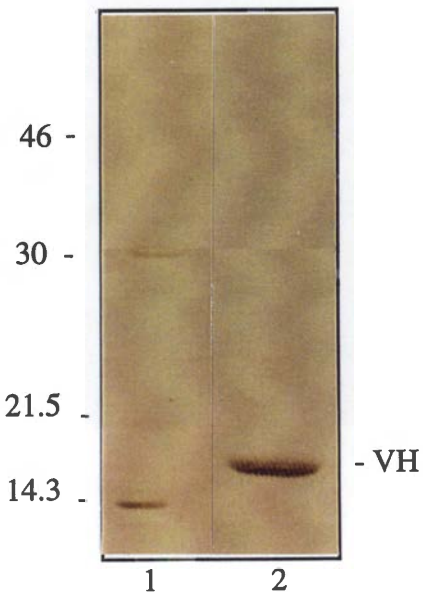


Fig 3.5 PURIFIED K-1-21 VH ANTIBODY FRAGMENT ON SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

An aliquot (25 µl) of the purified fragment VH which had been dialysed against PBS-az was subjected to SDS PAGE on a 10% polyacrylamide gel. The gel was stained with Coomassie dye and then dried. The gel shows ; lane 1, Rainbow protein molecular weight markers ; lane 2, purified VH fragment.

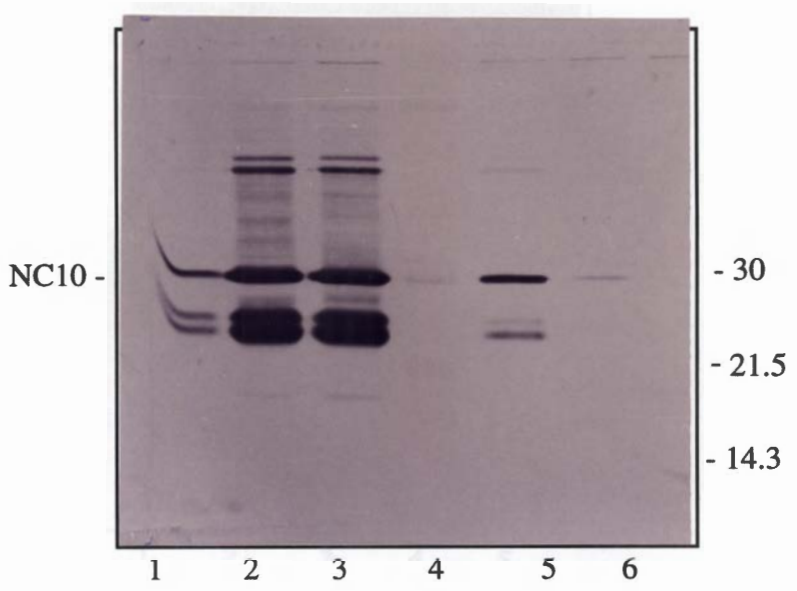


Fig 3.6 PURIFICATION OF EXPRESSED NC10 scFv FRAGMENT ON THE ANTI-FLAG M2 AFFINITY GEL

After expression of the NC10 scFv fragment the periplasmic fraction (Fig 3.3, b) was applied to the anti-FLAG M2 affinity column. Bound protein was eluted and 1 ml fractions were collected. Samples of the eluted fractions (25 µl) were run on a 10% SDS polyacrylamide gel and then analysed by Western blot. The results show; lanes 1-3 and 5-6, eluted NC10 scFv fragments ; lane 4, Rainbow protein molecular weight markers.

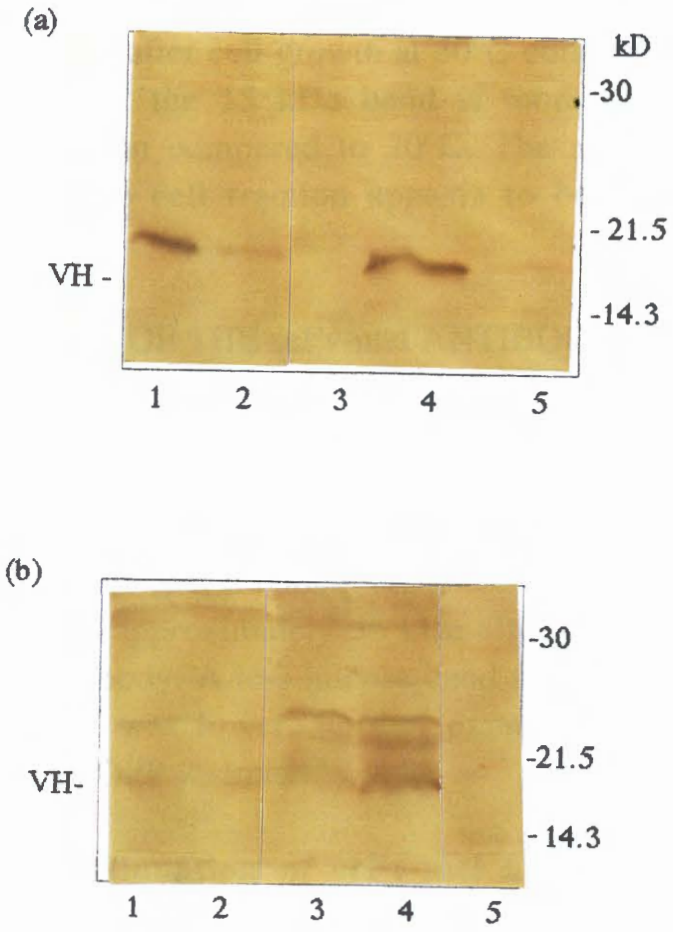


Fig 3.7 COMPARISON OF VH EXPRESSION AT 30°C AND 37°C AFTER INDUCTION AT 42°C

Two flasks of exactly 100 mls from the same VH cell culture were grown at 30°C and then induced at 42°C for 15 mins. Thereafter, one flask of culture was grown at 30°C and the other at 37°C for 4 hrs. Cells were then fractionated and the periplasmic, soluble whole cell and insoluble whole cell fractions were isolated. Aliquots of 20 µl were resolved by SDS PAGE on a 10% polyacrylamide gel and analysed by Western blot with the anti-FLAG M2 antibody. In (a) expressed VH fragment ; lane 1, periplasmic fraction at 30°C ; lane 2, periplasmic fraction at 37°C ; lane 3, Rainbow protein molecular weight markers ; lane 4, soluble fraction at 30°C ; lane 5, soluble fraction at 37°C. In (b) expressed VH fragment ; lane 1, whole cell culture at 30°C ; lane 2, whole cell culture at 37°C ; lane 3, insoluble cell membrane fraction at 30°C ; lane 4, insoluble cell membrane fraction at 37°C ; lane 5, Rainbow protein molecular weight markers.

expressed VH fragment. This band is more intense in the periplasmic and soluble fractions after cell growth at 30°C compared to 37°C. After cell growth at 37°C the 15 kDa band is more pronounced in the insoluble cell fraction compared to 30°C. The amount of expressed protein in the whole cell fraction appears to be the same for both temperatures.

3.3.2 EXPRESSION OF THE scFv-mel ANTIBODY FRAGMENT

1. Host cell lines

The scFv-mel pPOW gene construct was transformed into host cell-lines TOPP 2, TG1, LE392 and DH5α (Appendix). Results of primary expression of the scFv-mel fragment are shown in Fig 3.8. All cell lines produced a band of approximately 36 kDa after induction which binds anti-FLAG M2 antibody. A less intense band was observed with DH5α as the cell density was lower. Further expression of scFv-mel was carried out in the TOPP 2 strain.

2. Analytical fractionation of scFv-mel and NC10 scFv

Analytical extractions of both scFv-mel and NC10 scFv antibody fragments were carried out using the method described in section 3.2.2. (3). A growth curve for the expression of both fragments is shown in Fig 3.9. After 5 hr growth at 30°C cell cultures containing scFv-mel reached an A_{600} of 3.8 and NC10 scFv was 4.1 before induction . The cells were fractionated into periplasmic, whole cell soluble and insoluble fractions. Aliquots from each isolate were analysed by Western blot using the anti-FLAG M2 antibody. Results of the Western blot are given in Fig 3.10. Samples containing scFv-mel (36 kDa) and NC10 scFv (32 kDa) show bands representing expressed protein in the whole cell culture, periplasmic, soluble and insoluble whole cell fractions. Although there is expression of scFv-mel in the periplasmic and soluble cell fractions a substantial amount of the protein appears in the insoluble cell membranes. The NC10 scFv fragment is produced mainly in the periplasmic and soluble cell fractions.

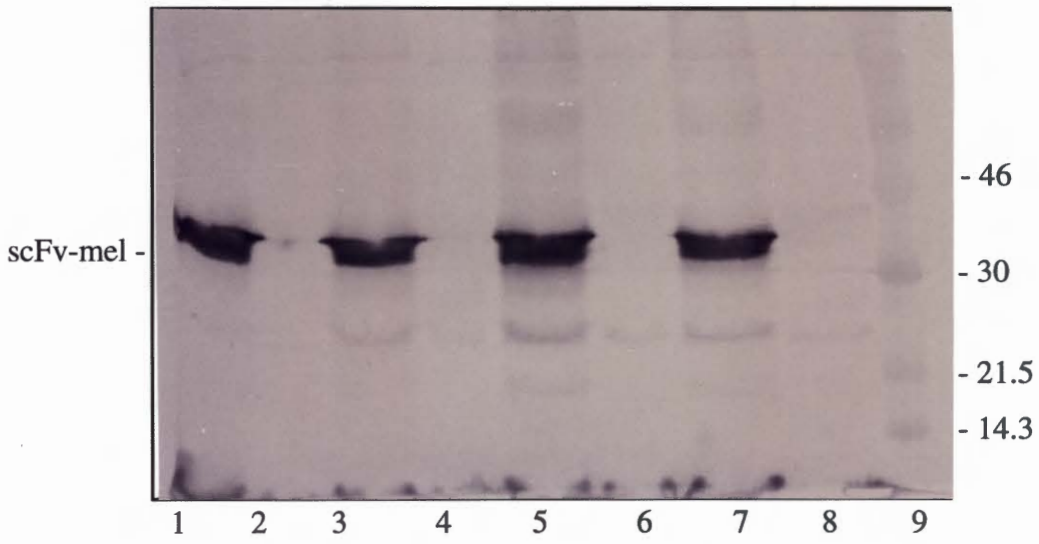


Fig 3.8 EXPRESSION OF scFv-mel FRAGMENT IN DIFFERENT HOST CELLS

The gene construct scFv-mel in pPOW was expressed in host cell-lines TOPP2, LE392, TG1 AND DH5α. Samples (25 µl) of induced and uninduced whole cell cultures (1 ml) from a primary expression experiment were subjected to SDS PAGE on a 10% polyacrylamide gel. The resolved proteins were then electroblotted onto a nitrocellulose membrane which was probed with the antibody, anti-FLAG M2. In (a), lanes 1 and 2, TG1 induced and uninduced; lanes 3 and 4, LE392 induced and uninduced; lanes 5 and 6, TOPP2 induced and uninduced; lanes 7and 8 DH 5α induced and uninduced; lane 9, Rainbow protein markers.

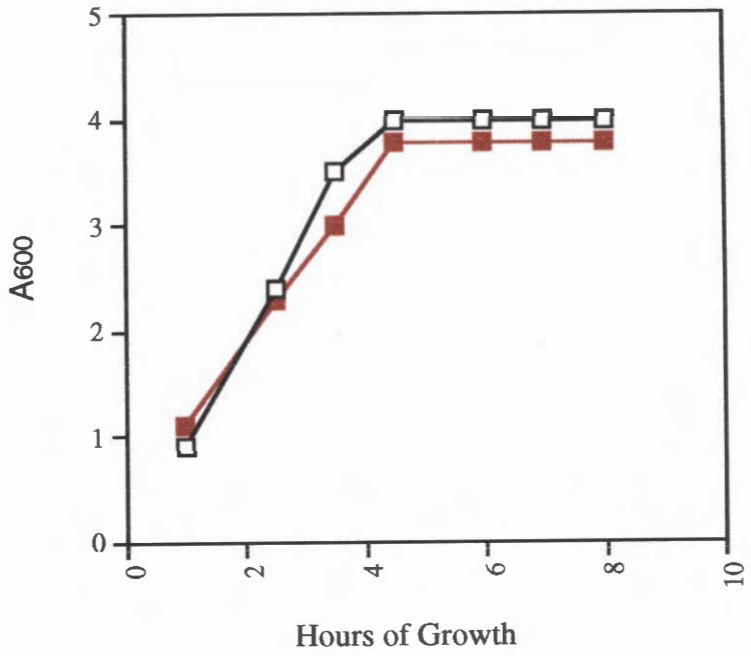


Fig 3.9 GROWTH CURVE OF scFv-mel AND NC10 scFv CELL CULTURES

Overnight cell cultures containing scFv-mel and NC10 scFV were diluted to give an A_{600} of 0.5 in 400 ml for each culture and then grown in volumes of 4x100 ml at 30°C. Once cell growth began to plateau the cultures were induced by placing the flasks in a shaking water bath at 42°C. The cultures were subsequently grown for a further 5 hr at 30°C. Samples were removed at various intervals and the A_{600} was determined. A graph of time versus A_{600} shows cell density for svFv-mel (red) and NC10 scFv (black).

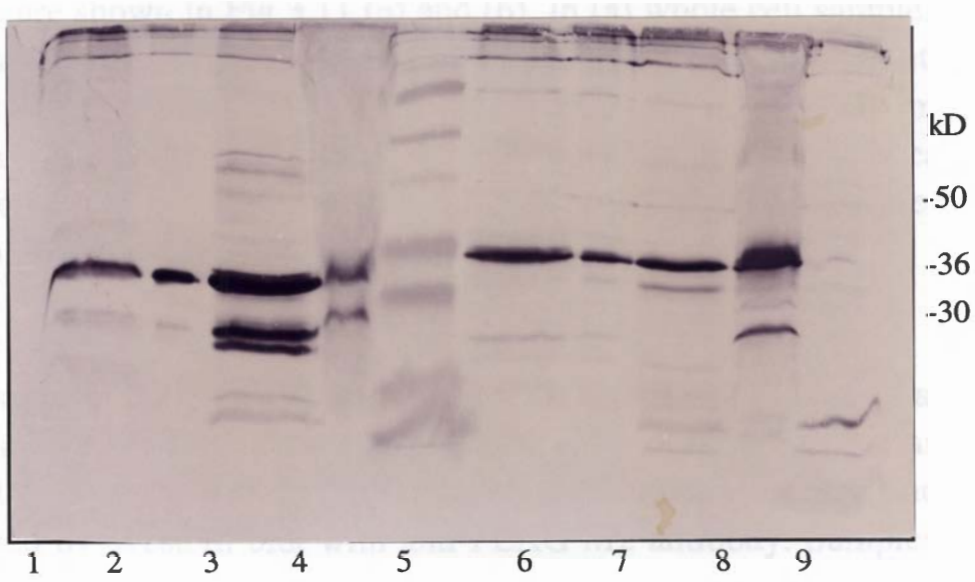


Fig 3.10 WESTERN BLOT OF ANALYTICAL CELL FRACTIONATION OF scFv-mel AND NC10 scFv

Cells were grown in 2x100 ml batches at 30°C for 4 hrs until an A_{600} of 3.5 for scFv-mel and 3.8 for NC10 cultures. Expression of these cultures was induced at 42°C for 15 mins and then grown for a further 5 hrs at 30°C. Cells were fractionated into periplasmic, soluble and insoluble membrane fractions. Samles (25 µl) were resolved by SDS PAGE on a 10% gel and analysed by Western blot with the anti-FLAG M2 antibody. The total volume of each fraction is shown in brackets. Expressed NC10 scFv fragments from fractions ; lane 1, whole cell culture (200 mL) ; lane 2, periplasm (20 mL) ; lane 3, soluble whole cell fraction (15 mL) ; lane 4, insoluble membranes (6mL); lane 5, SEE BLUE protein molecular weight standards (Amersham, Buckinghamshire, UK). Samples of expressed scFv-mel fragments ; lane 6, whole cell culture (200 mL) ; lane 7, periplasm (20 mL) ; lane 8, soluble whole cell fraction (15 mL); lane 9, insoluble membranes (6 mL).

3. Preparative expression of scFv-mel

Large scale (4x100 ml) cell cultures containing pPOW scFv-mel were grown and induced. Concurrently, 50 µl aliquots were removed from the cultures immediately before induction, immediately after induction and at 1, 2 and 3 hourly intervals after induction for Western analysis. After growth at 30°C for 2 hr, the cell culture was fractionated and only the whole cell soluble fraction was isolated as previously described. The results are shown in Fig 3.11 (a) and (b). In (a) whole cell samples were analysed before induction of protein expression and at 1 hr intervals after induction. Samples of duplicate soluble cell fractions from cells grown for 2 hr after induction are presented in (b). In both cases a prominent protein band which reacts with anti-FLAG M2 is present and has an approximate molecular weight of 36 kDa.

3.3.3 AFFINITY PURIFICATION OF scFv-mel

The soluble fraction (70 ml) of scFv-mel from the preparative expression was applied to the anti-FLAG M2 affinity column and the bound protein was eluted. Fractions (1 ml) were collected and then analysed by Western blot with anti-FLAG M2 antibody. Samples from the eluant are shown in Fig 3.12. Fractions 3-6 were pooled and concentrated. A 50 µl aliquot of purified scFv-mel was analysed by Western blot. Samples of previously purified VH and NC10 scFv antibody fragments and FLAG BAP positive control protein were analysed at the same time. The results of the Western analysis are shown in Fig 3.13. The protein bands were quantitated as described in section 3.2.5. These results are shown in Table 4. The concentration of isolated expressed protein from the soluble fractions was determined from starting culture and was estimated as 5 µg/l for scFv-mel and 100 µg/l for NC10 scFv. The purified scFv-mel and NC10 scFv samples were resolved on a 12% SDS acrylamide gel and then stained with silver stain (Fig 3.24). The gel scan shows a distinct protein band of 36 kDa for concentrated scFv-mel (lane 4). The control sample NC10 scFv shows a single protein band at 30 kDa (lane 1). A number of large molecular weight bands (>60 kDa) are present in the affinity purified scFv-mel sample and they are concentrated along with the immunotoxin. The band at 69 kDa in lane 4 is probably BSA which has leaked off the centricon membrane. These high molecular weight bands are not

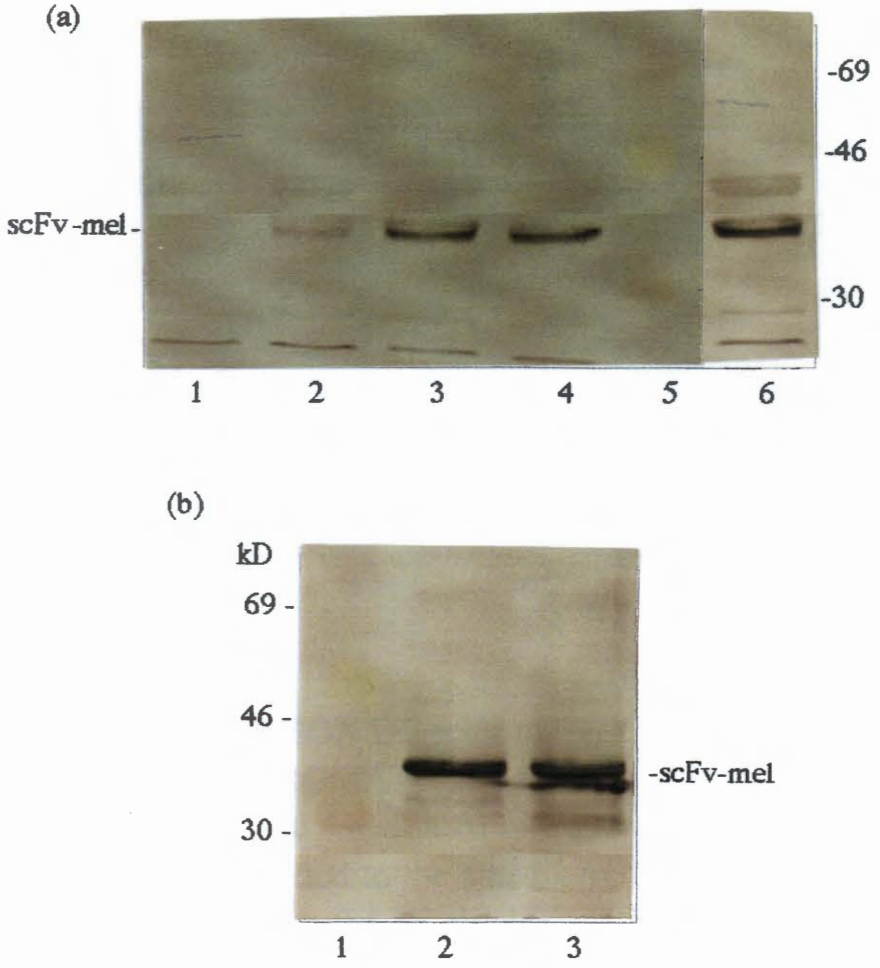


Fig 3.11 PREPARATIVE FRACTIONATION OF scFv-mel
A preparative fractionation of expressed scFv-mel fragment was carried out as described in the text. Samples (25 µl) of the expressed fragments were removed at intervals throughout the experiment and analysed by SDS PAGE on a 10% polyacrylamide gel and then a Western blot using the anti-FLAG M2 antibody. In (a) lane 1, uninduced scFv-mel ; lane 2, scFv-mel immediately after induction ; lane 3, scFv-mel 1 hr after induction ; lane 4, scFv-mel 2 hr after induction ; lane 5, Rainbow protein molecular weight markers ; lane 6, scFv-mel 3 hr after induction. In (b) lane 1, Rainbow protein molecular weight proteins ; lane 2 and 3, soluble whole cell fractions of scFv-mel 2 hr after induction.

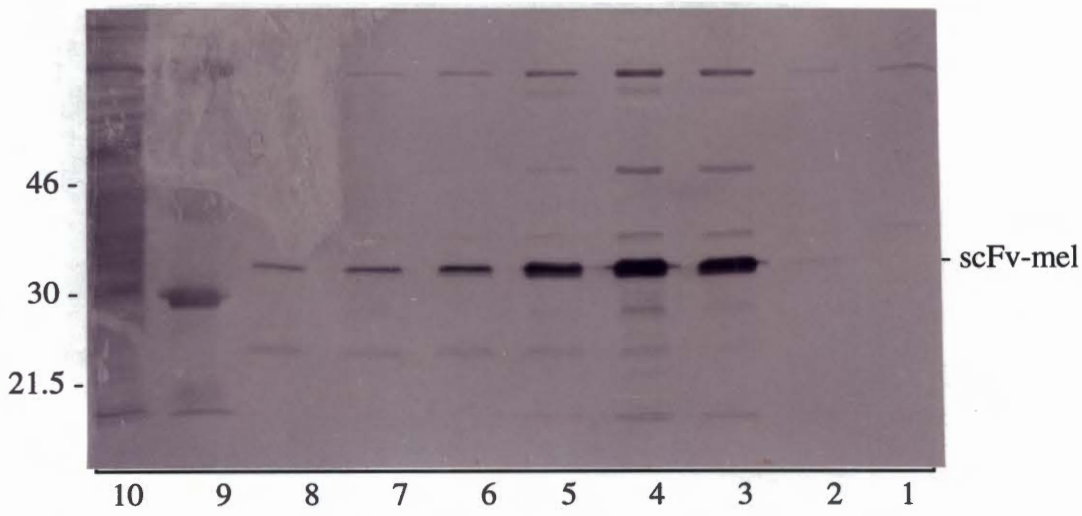


Fig 3.12 PURIFICATION OF scFv-mel BY ANTI-FLAG M2 AFFINITY CHROMATOGRAPHY

The soluble whole cell fraction from a preparative expression of scFv-mel fragment was applied to the anti-FLAG M2 affinity column. Bound protein was eluted with 0.1 M glycine pH 3.0 and 1 ml fractions were collected. Aliquots of 20 µl were subjected to SDS PAGE on a 10% polyacrylamide gel and analysed by Western blot with the anti-FLAG M2 antibody. Lanes 1-8, eluted scFv-mel fragment ; lane 9, Rainbow molecular weight markers ; lane 10, soluble fraction collected after 3 passes through the affinity gel.

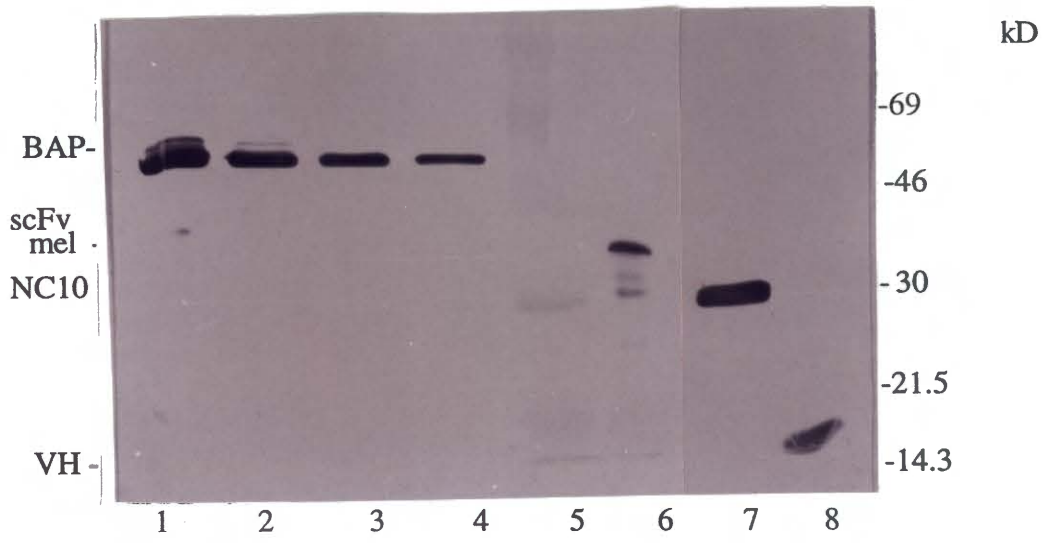


Fig 3.13 QUANTITATION OF EXPRESSED VH, NC10 scFv AND scFv-mel ANTIBODY FRAGMENTS BY WESTERN BLOT ANALYSIS

The affinity purified scFv-mel antibody fragment was concentrated by centrifugation on a centricon 10,000 filter as detailed in section 3.2.5. A final volume of 1 ml in PBS-az was achieved for the scFv-mel fragment. Aliquots (25 µl) of purified, concentrated VH, NC10 scFv and scFv-mel were resolved by SDS PAGE on a 10% polyacrylamide gel and then analysed by Western blot. Similarly, samples containing 600 ng, 450 ng, 225 ng, and 113 ng of FLAG-BAP positive control protein were loaded onto the same gel. Concentrations of the FLAG-BAP positive control protein are ; lane 1, 600 ng ; lane 2, 450 ng ; lane 3, 225 ng ; lane 4, 113 ng ; lane 5, Rainbow protein molecular weight markers ; lane 6, scFv-mel ; lane 7, NC10 scFv ; lane 8, VH

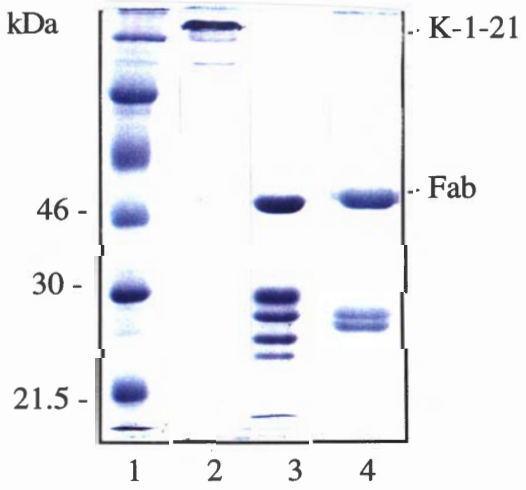


Fig 3.14 ISOLATION OF A K-1-21 Fab FRAGMENT
The monoclonal antibody K-1-21 was digested with papain as detailed in section 3.2.6. The digested product was passed through an affinity column in which human free *kappa* light chains or Bence-Jones proteins (BJP) were immobilized on CNBr-activated sepharose 4B. Eluted fractions were collected and samples containing the purified Fab were pooled and dialysed against PBS-az. Samples (25 µl) containing K-1-21 antibody, unpurified digested Fab and purified Fab were resolved by SDS PAGE on a non-reducing 10% polyacrylamide gel and then stained with Coomassie. Lane 1, Rainbow protein standards ; lane 2, K-1-21 prior to digestion ; lane 3, K-1-21 after digestion ; lane 4, the Fab fragment after affinity gel purification.

Table 4 QUANTITATION OF PROTEIN BANDS FROM Fig 3.13 USING DENSITOMETRY

FLAG BAP	OD X MM^2	AMOUNT. ng	nM
lane 2	1.790	450	
lane 3	1.292	225	
lane 4	0.609	113	
scFv-mel	0.181	35	50
NC10 scFv	0.904	170	280
VH	0.179	35	143

present in the control sample NC10 scFv. They do not bind to anti-FLAG M2 on a Western blot but appear to be released from the affinity gel at low pH. A possible explanation is that after several passages the affinity gel is slowly degenerating and the anti-FLAG M2 antibody is coming off the gel.

3.3.4 BINDING OF K-1-21 AND THE FAB FRAGMENT TO HMy2 CELLS USING FLOW CYTOMETRY

A K-1-21 Fab fragment was produced as previously described (section 3.2.6). Samples from before and after affinity purification were subjected to SDS PAGE on a 10 % polyacrylamide gel and the results are shown in Fig 3.14. From the gel, purified K-1-21 Fab has a MWt of about 50 kDa. The lower MWt products in the unreduced sample may be due to dissociation of the VL and Fd antibody fragments. The concentration of Fab using the extinction coefficient $E^{0.1\%}$=15.3 gave a concentration of 8 mg/ml. Binding of the parent antibody K-1-21 and the Fab fragment to HMy2 cells was determined using immunofluorescence staining as detailed in section 3.2.7 (2). An aliquot (50 µl) of K-1-21 at a concentration of 50 nM was incubated with 10^6 HMy2 cells. Similarly, a 50 nM concentration of Fab was incubated with HMy2 cells. The second antibody, FITC-sheep anti-mouse Ig, was 20 µg/ml. These results are shown in Fig 3.15. The binding of native K-1-21 was 2 fold higher than the binding of the same concentration (50 nM) of Fab.

3.3.5 ANTIGEN BINDING PROPERTIES OF scFv-mel

1. Comparative ELISA

The ELISA was performed in triplicate as described in section 3.2.7 (1). The primary antibodies consisted of purified, concentrated samples of VH (143 nM), NC10 scFv (280 nM) and scFv-mel (50 nM) which were serially diluted. A graph showing the results of the ELISA is presented in Fig 3.16. The molar concentrations were calculated using MWt values of the antibody fragments estimated from SDS PAGE, as described in Table 4. In all cases the binding to antigen was titrated back to zero. Binding of scFv-mel to antigen is greatly increased compared to the same molar concentrations of both VH and NC10 scFv.

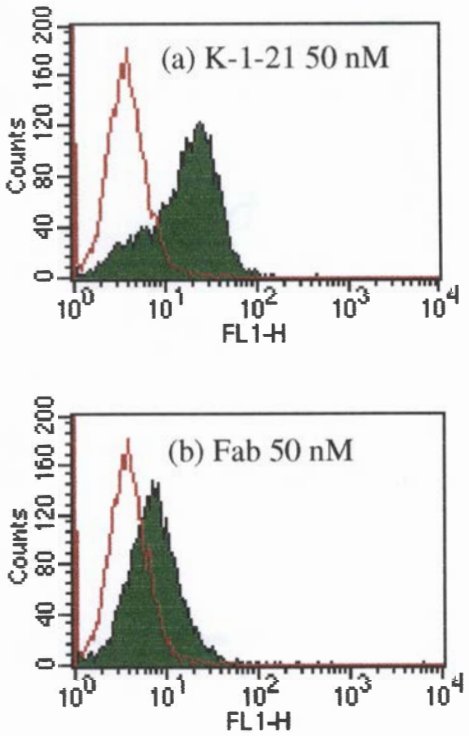


Fig 3.15 BINDING OF MURINE MONOCLONAL ANTIBODY K-1-21 AND THE Fab FRAGMENT TO HMY2 CELLS

Cells (10^6) were incubated with 50 µl of either K-1-21 (50 nM) or Fab (50 nM and 16 µM). The cells were then washed and the second antibody FITC-sheep anti-mouse Ig was added. After incubation the cells were washed and then analysed for cell surface immunofluorescence by flow cytometry. Background fluorescence of cells incubated with PBS followed by the second antibody is shown as the white histogram. Fluorescence of cells incubated mAb K-1-21 is shown as a solid green histogram in (a). Fluorescent staining of cells incubated with Fab is shown in (b) 50 nM. The scale on the x axis shows the log fluorescence intensity and the y axis shows cell number.

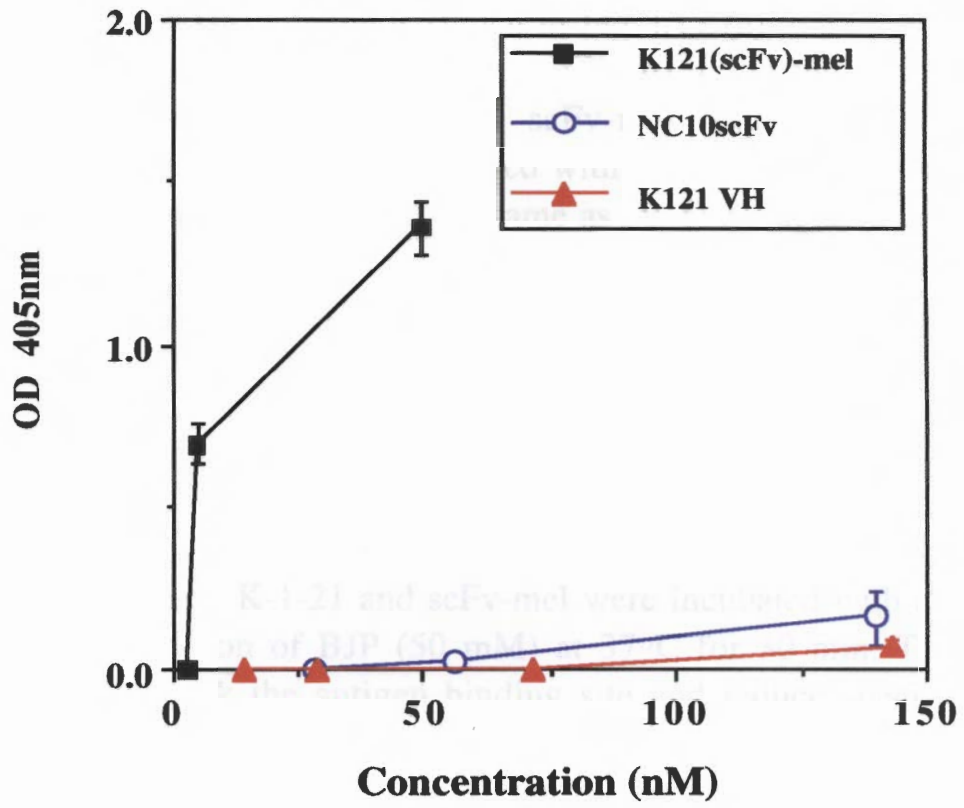


Fig 3.16 BINDING OF VH, NC10 scFv AND scFv-mel ANTIBODY FRAGMENTS TO HUMAN FREE *kappa* LIGHT CHAINS

The ELISA was carried out as detailed in section 3.2.7 (1). Briefly, wells were coated with human free *kappa* light chains (BJP), blocked with 3% BSA, washed and then 50 µl of dilutions from K1-21 VH (143 nM), scFv-mel (50 nM) and NC10 scFv (280 nM) was added to the wells. After washing 50 µl of anti-FLAG M2 antibody (3 µg/ml) was added and then biotinylated sheep anti-mouse Ig. Finally avidin-AP was added to the wells and the colour was developed. The black squares represent scFv-mel, the open blue circles represent NC10 scFv and the red triangles represent K-1-21 VH.

2. Immunofluorescent staining of scFv-mel binding

A comparative binding assay was carried out with scFv-mel (50 nM) and K-1-21 (50 nM) using HMy2 cells and K562 cells. After immunofluorescence staining the cells were analysed by flow cytometry. Binding of K-1-21 to HMy2 cells is shown in Fig 3.17 (a) and binding of scFv-mel (50 nM) to HMy2 cells is shown in (b). From the shift in the histogram it can be seen that the scFv-mel fragment shows significant binding to HMy2 cells compared with K-1-21. This binding is only 2 fold lower than K-1-21 and the same as the Fab fragment (Fig 3.15) Binding of scFv-mel to K562 cells does not result in a shift compared to background. After a ten fold decrease in scFv-mel concentration, binding to the cells is no longer observed (Fig 3.18, b). In addition, binding of NC10 scFv (280 nM) to HMy2 cells does not result in a shift above background levels.

3. Inhibition of scFv-mel binding

In a similar experiment, K-1-21 and scFv-mel were incubated with a 3 fold excess concentration of BJP (50 mM) at 37°C for 30 min. This should effectively block the antigen binding site and reduce specific binding to the KMA on the surface of HMy2 cells. The same experiment was undertaken using K562 cells as a control. Immunofluorescence staining was then carried out and the results of binding inhibition on HMy2 cells are shown in Fig 3.19. The histogram shows K-1-21 binding to HMy2 cells in the absence (a) and in the presence (b) of an excess of soluble antigen and demonstrates specific inhibition of this binding. Similarly, binding of scFV-mel to HMy2 cells is presented in the absence (c) and in the presence (d) of soluble antigen. Pre-incubation of scFv-mel with BJP results in complete inhibition of binding to target cells. Inhibition of binding to K562 cells is shown in Fig 3.20. The results in (a) show that the native antibody, K-1-21, does not bind to K562 cells and pre-incubation with BJP does not affect the binding. Similarly, scFv-mel does not bind to K562 cells (c) and there is no change in the presence of BJP (d).

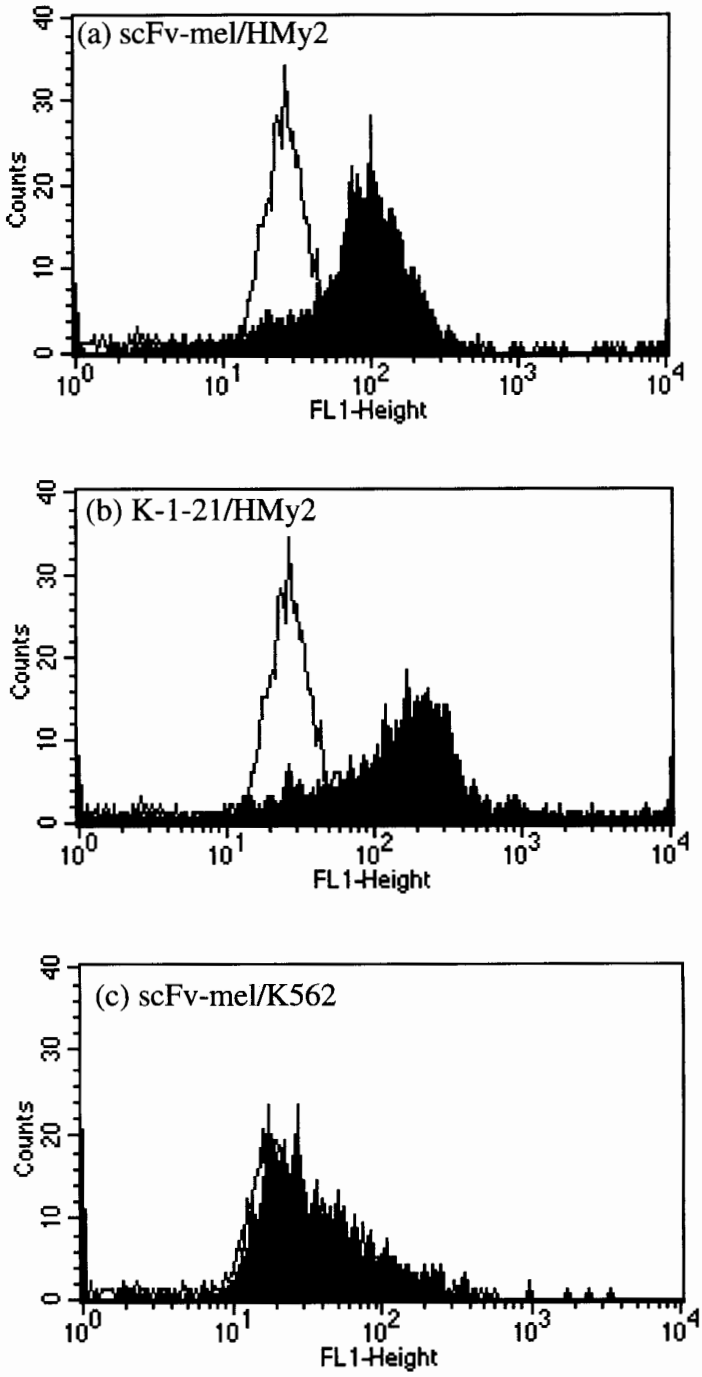


Fig 3.17 IMMUNOFLUORESCENCE STAINING OF HMy2 AND K562 CELLS WITH scFv-mel

Both HMy2 and K562 cells (10^5) were incubated with 30 µl scFv-mel or K-1-21 and then 2 µg/ml anti-FLAG M2 antibody. The cells were then stained with 100 µg/ml FITC-sheep anti mouse Ig and analysed by flow cytometry. The x-axis shows fluorescence intensity and the y-axis shows cell number. Fluorescence of cells incubated with scFv-mel or K-1-21 is shown in black and the white histogram represents cells incubated with PBS-az followed by second antibody. In (a) HMy2 cells incubated with 30 µl of 50 nM K-1-21 ; (b) HMy2 cells with 30 µl of 50 nM scFv-mel ; (c) K562 cells with 30 µl of 50 nM scFv-mel.

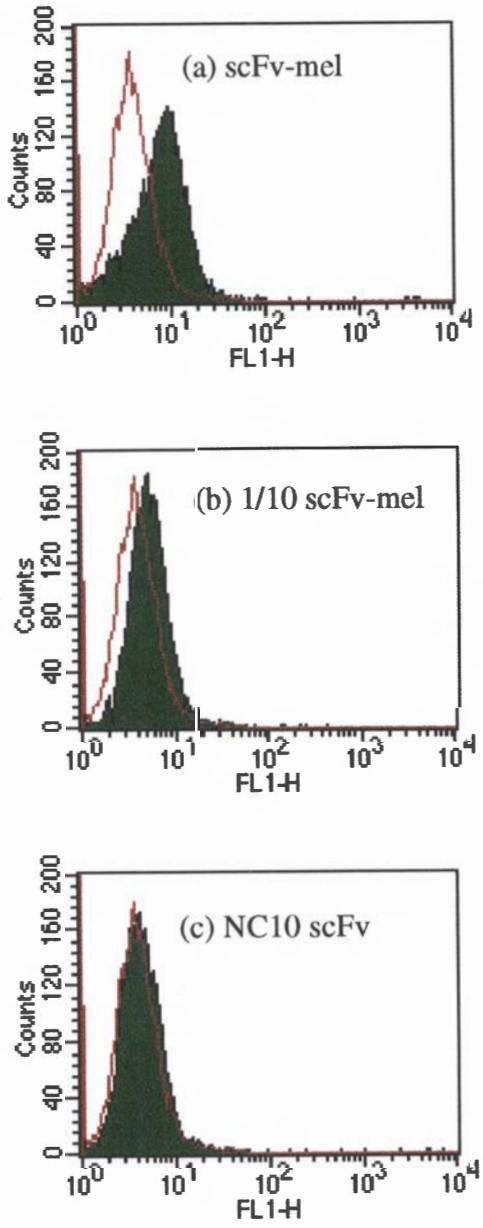


Fig 3.18 BINDING OF scFv-mel AND NC10 scFv TO HMY2 CELLS
HMy2 cells were incubated with 50 nM scFv-mel, 5 nM scFv-mel and 280 nMNC10 scFv. The binding assay was carried out as described in section 3.2.7, (3). The histogram outlined in red represents cells incubated in PBS-az followed by the second antibody and the FITC-sheep anti-mouse Ig. The green histogram in (a) shows binding of 50 nM scFv-mel, (b) shows a 1/10 dilution of scFv-mel (5 nM) and (c) shows binding of NC10 scFv.

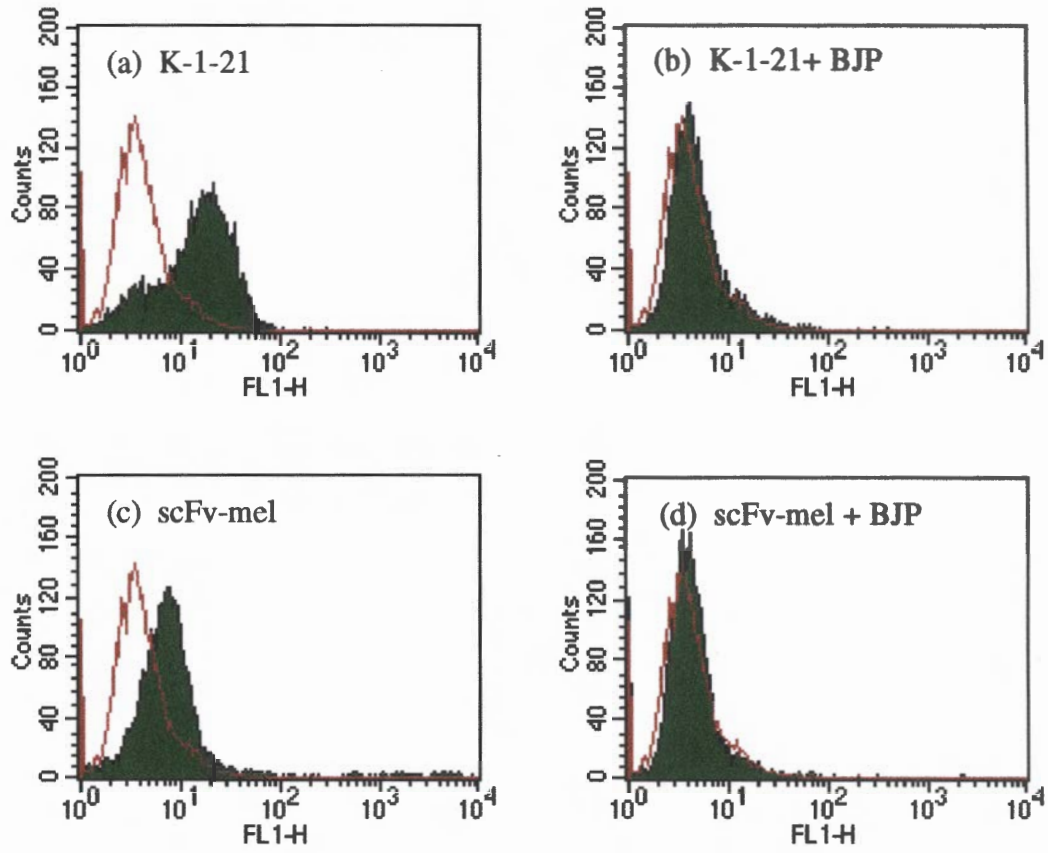


Fig 3.19 INHIBITION OF scFv-mel BINDING TO HMy2 CELLS AFTER INCUBATION WITH SOLUBLE ANTIGEN

Purified scFv-mel (30 nM) and mAb K-1-21 (50 nM) were pre-incubated with BJP (50 μM) at 37°C for 30 min. They were then added to HMy2 cells (10^5) and binding in the presence or absence of BJP was determined by immunofluorescence staining as described in section 3.2.7 (3). The histogram outlined in red represents cells incubated with PBS-az followed by FITC anti-mouse Ig. The solid green histogram in (a) shows K-1-21 binding to cells in the absence of BJP and in (b) in the presence of BJP. In (c) the solid green histogram shows scFv-mel binding to cells in the absence of BJP and (d) after incubation in the presence of BJP.

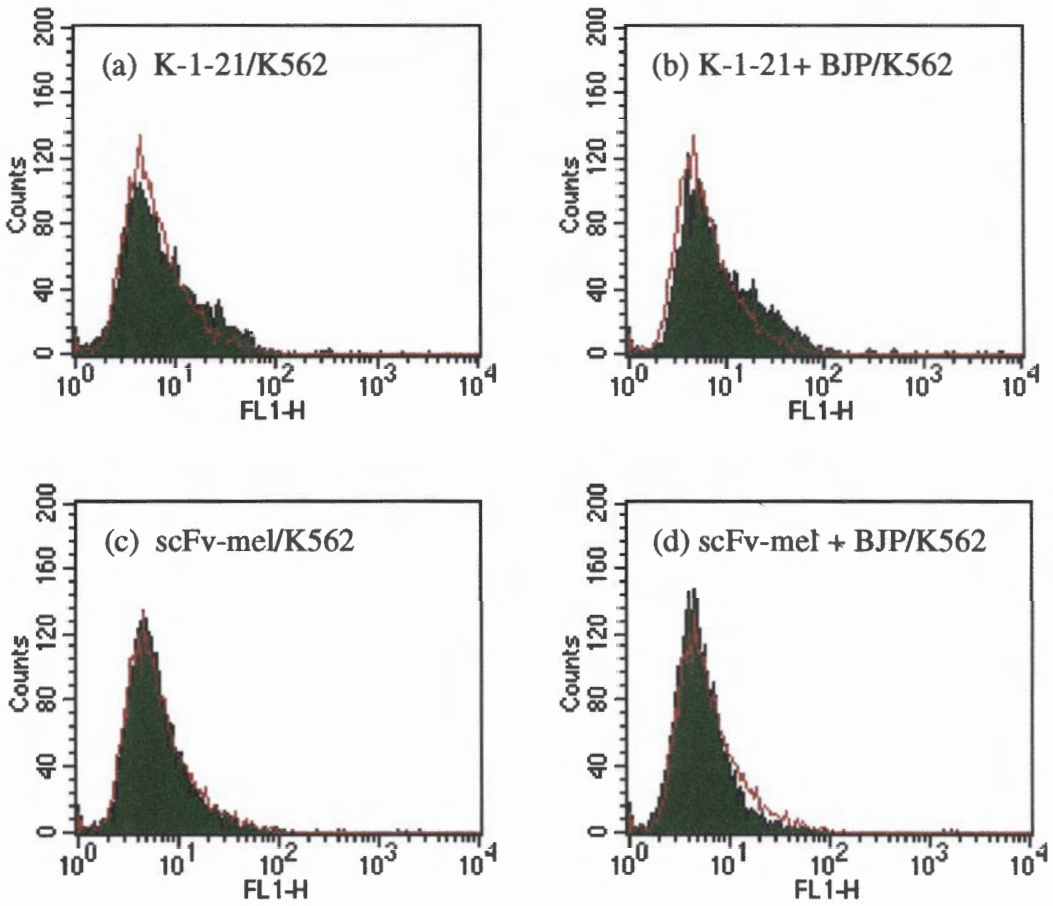


Fig 3.20 BINDING OF scFv-mel AND K-1-21 TO K562 CELLS IN THE PRESENCE OR ABSENCE OF SOLUBLE ANTIGEN, BJP

Both scFv-mel (50 nM) and K-1-21 (50 nM) were incubated with soluble antigen, BJP (1.25 mg/ml), at 37°C for 30 min. K562 cells (10^5) were then incubated with scFv-mel and K-1-21 which had previously been bound to BJP. Binding to the cells was determined by immunofluorescence staining as described in section 3.2.7 (3). The histogram outlined in red represents cells incubated with PBS-az followed by FITC anti-mouse Ig. The results show; (a) K-1-21 binding to K562 cells in the absence BJP and (b) in the presence of BJP. The histogram in (c) shows binding of scFv-mel to K562 cells in the absence of BJP and (d) in the presence of BJP.

4. Comparison of binding of scFv-mel from the periplasmic and soluble fractions

Expressed scFv-mel product was isolated and purified from both the periplasmic and soluble fractions. The concentration of both scFv-mel preparations was estimated to be the same by densitometry analysis. A total of 30 µl of each was incubated with HMy2 cells as described in section 3.2.7 (3). Immunofluorescence staining (Fig 3.21) shows that purified scFv-mel from both fractions binds to HMy2 cells and the histograms are superimposable.

3.3.6 DETERMINATION OF SPECIFIC LYSIS BY scFv-mel USING HMy2 AND K562 CELLS

Both HMy2 and K562 cells (10^5) were incubated with increasing concentrations of melittin (8.8 - 0.55 µM) in 1% BSA in sterile PBS for 18 hr at 37°C. After incubation, ethidium bromide was added and the percentage cell death was determined immediately as described in section 3.2.8. A graph of cell death with increasing melittin concentrations is shown in Fig 3.22. Incubation with melittin results in cell death in both HMy2 and K562 cell lines. At a melittin concentration of 8.8 µM none of the cells are viable. Incubation of cells without melittin but in the presence of the 1% BSA in PBS, results in 4.5% ± 0.6 cell death after 18 hr. From the graph, 50% cell death (ID_{50}) occurs at a melittin concentration of 2.5-3.0 µM for both cell lines. The concentration of melittin which results in 67% cell death of HMy2 cells is approximately 3.5 µM.

At the same time separate aliquots of of both cell lines were incubated with scFv-mel which was obtained from two seperate expression experiments of scFv-mel. The expressed products had been isolated by affinity chromatography and then concentrated. The results Fig 3.23 refer to immunotoxin 1 (A) and immunotoxin 2 (B). When incubated with HMy2 and K562 cells the cell binding profiles for these two preparations are shown in Fig 3.17 a (IT 1) and Fig 3.19 c (IT 2). In Fig 3.23 (A), HMy2 cells incubated with 50 nM of scFv-mel resulted in approximately 67% cell death, whilst the lower concentration of immunotoxin (5 nM) only showed background levels of cell lysis. By contrast, neither concentration of scFv-mel was cytotoxic to K562 cells

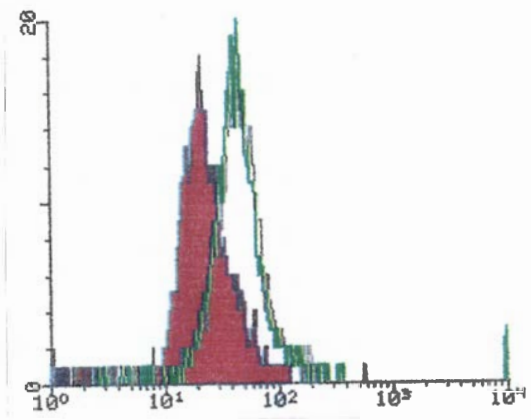


Fig 3.21 BINDING OF scFv-mel ISOLATED FROM THE PERIPLASMIC AND SOLUBLE WHOLE CELL FRACTIONS

Equal quantities of scFv-mel isolated from the periplasmic and soluble fractions were incubated with HMy2 cells (10^5) and then binding was determined by immunofluorescece staining. The solid red histogram represents the control in which cells are incubated with PBS-az followed by anti-FLAG M2 and then FITC anti-mouse Ig. The histogram with the green outline shows binding to HMy2 cells of scFv-mel from the whole cell soluble fraction. The histogram with the black outline shows binding to cells of scFv-mel isolated from the periplasmic fraction.

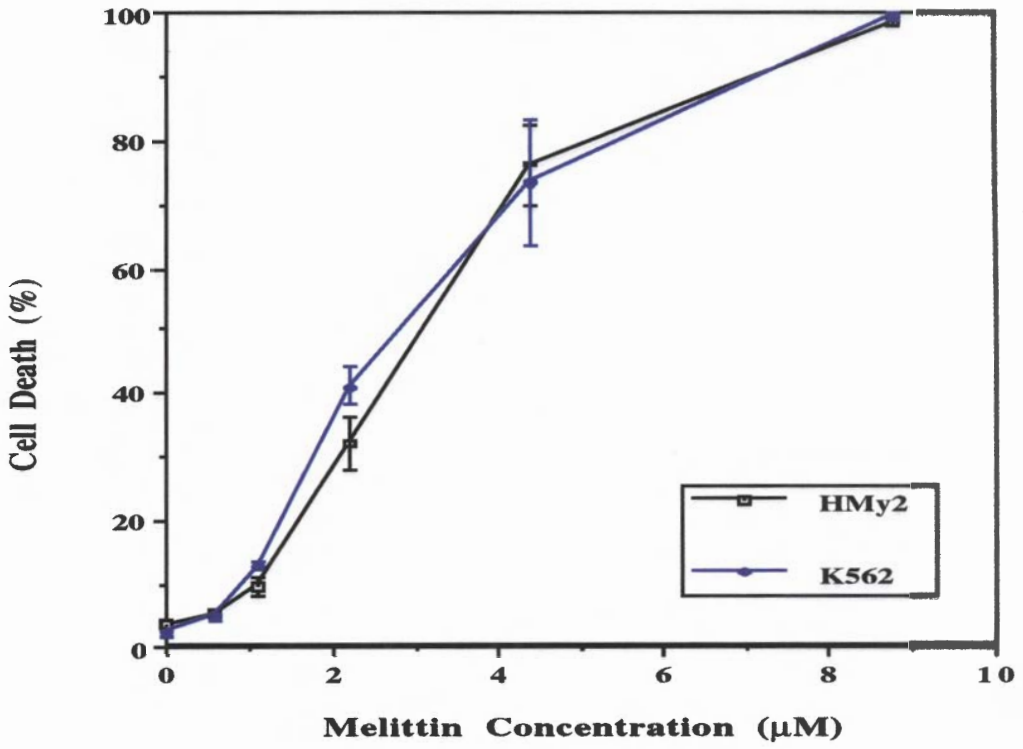


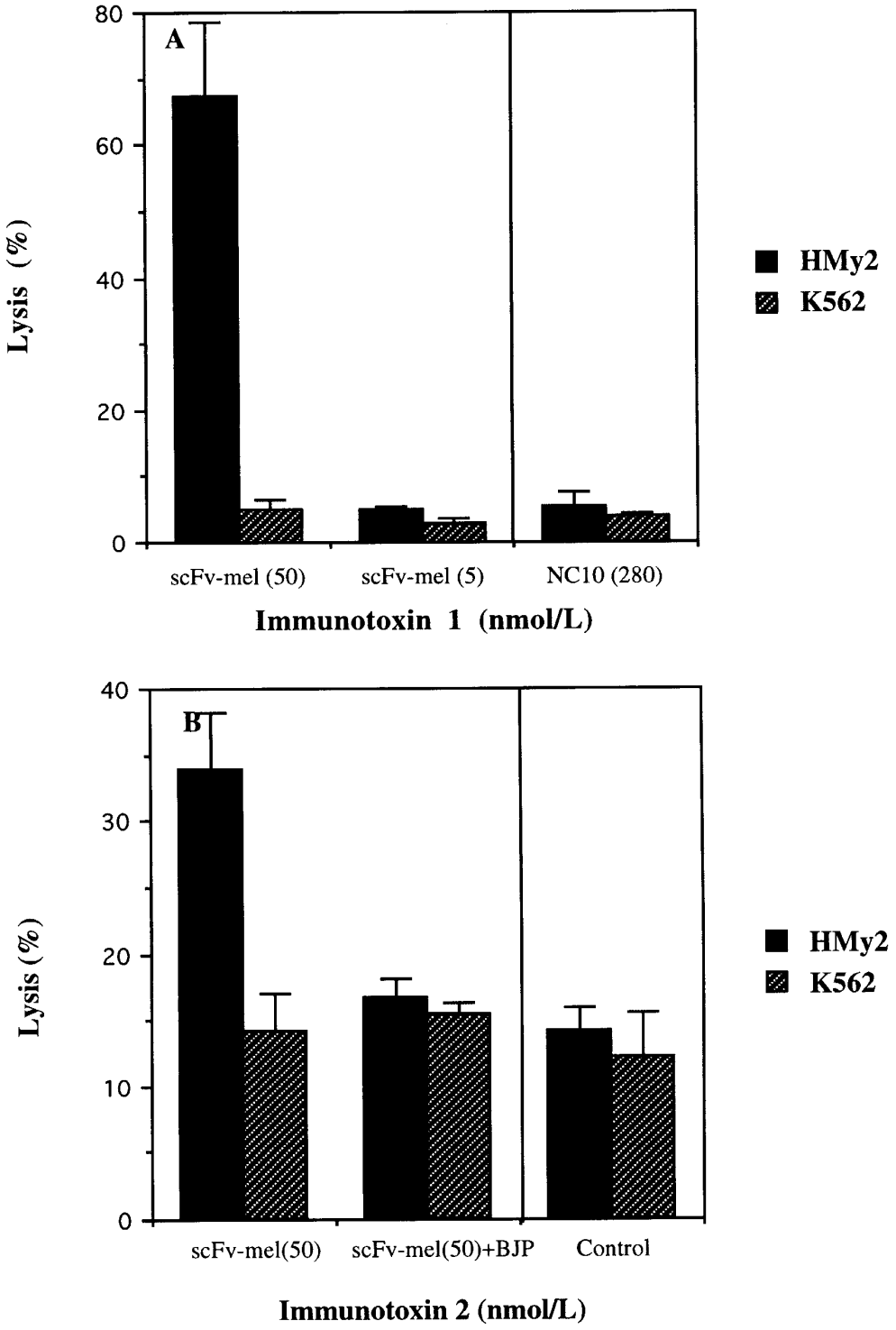
Fig 3.22 DETERMINATION OF CELL DEATH IN HMy2 AND K562 CELLS WITH INCREASING CONCENTRATIONS OF MELITTIN

HMy2 and K562 (10^5) cells were incubated with increasing concentrations of melittin (0.55-8.8 μM) in 1% PBS/BSA for 18 hr at 37°C in a humidified incubator. Percentage cell death was determined by measuring cell uptake of ethidium bromide using flow cytometry as described in section 3.1. A graph of cell death versus μM melittin was plotted for HMy2 and K562 cells.

and cell viability was the same as background levels. The control antibody fragment, NC10 scFv, did not cause cell death under these experimental conditions. In Fig 3.23 (B) immunotoxin 2 at the same concentration as immunotoxin 1 only showed 20% cell death above the background levels. However, this cytotoxicity was inhibited after incubation of scFv-mel with an excess of soluble BJP. Incubation of K562 cells with scFv-mel in both the presence and absence of BJP, resulted in background cytotoxicity. Background cytotoxicity was incubation of both cell lines for 18 hr in the presence of RPMI.

Fig 3.23 CYTOTOXICITY OF scFv-mel ON HMy2 AND K562 CELLS (Following page)

Both HMy2 and K562 cells were incubated with scFv-mel for 18 hr as described in section 3.2.8. The results are presented graphically with the % cell lysis on the y axis and the concentration of immunotoxin on the x axis. In A (Immunotoxin 1), the cells were incubated with 50 nM scFv-mel and 5 nM scFv-mel. The control consisted of cells incubated in the presence of 280 nM NC10 scFv. Graph B represents results using a second batch of scFv-mel (Immunotoxin 2) incubated with HMy2 and K562 cells in the presence or absence of 50 mM BJP.In this experiment the control cells were incubated in the absence of scFv-mel.


A
80
60
40
20
0
Lysis (%)
scFv-mel (50)
scFv-mel (5)
NC10 (280)
Immunotoxin 1 (nmol/L)
HMy2
K562
B
40
30
20
10
0
Lysis (%)
scFv-mel(50)
scFv-mel(50)+BJP
Control
Immunotoxin 2 (nmol/L)
HMy2
K562

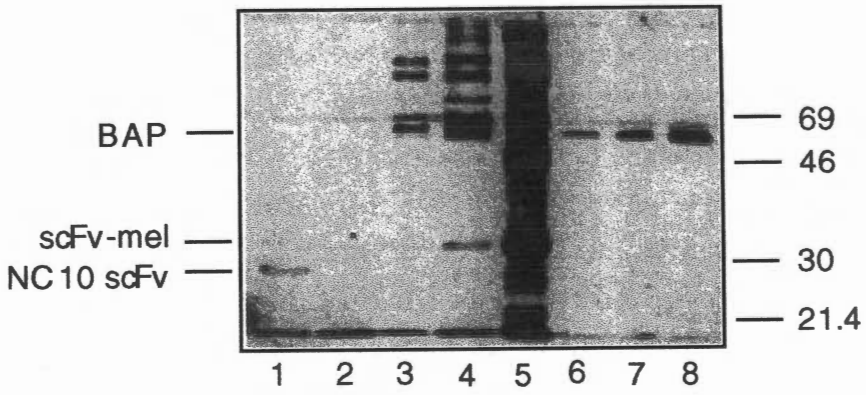


Fig 3.24 SDS POLYACRYLAMIDE GEL OF scFv-mel and NC10 scFv
Affinity purified and concentrated samples (20 υl) of scFv-mel and NC10 scFv were run on a 12% SDS acrylamide gel and then silver stained. Control protein, BAP, was resolved on the same gel. The gel was then scanned using the Adobe software. The samples shown are: lane 1, NC10 scFv ; lane 2,, K-1-21 VH ; lane 3, unconcentrated scFv-mel ; lane 4, concentrated scFv-mel ; lane 5, rainbow protein MWt markers ; lane 6, 56.5 ng BAP protein ; lane 7, 113 ng BAP protein ; lane 7, 225 ng BAP protein.

3.4 *DISCUSSION*

3.4.1. EXPRESSION OF K-1-21 VH AND NC10 scFv

A possible explanation for expression of only one VH fragment (Fig 3.1), out of 5 positive clones with the same DNA sequence, is that the flag sequence may be incorrectly coded due to the poor fidelity of Taq polymerase (Dunning *et al.*, 1988; Krawczak *et al.*, 1989). As the reverse sequencing primer for pPOW is only 8 bp from the multiple cloning site (Power *et al.*, 1992), the complete sequence at the 5' end of the reverse PCR primer (VH2 *flag*) was not obtained. If a base change resulted in an alteration in the amino acid sequence the expressed product would not be recognised by the anti-FLAG M2 antibody and the protein would not be detected. As SDS PAGE analysis was not performed on the expressed VH domains it was not possible to confirm this interpretation. Alternatively, the flag peptide could have been degraded after expression (Ward *et al.*, 1989). However, it is unlikely that this would affect some and not all of the clones expressing the same gene product.

It has been suggested that expression of antibody fragments at high temperatures and for prolonged periods results in secretion into the culture supernatant as a result of increased membrane permeability of *E.coli* (Takkinen *et al.*,1991; Kelley *et al.*,1992; Hoogenboom *et al.*, 1991). However, this interpretation has been challenged by Darveau *et al.* (1992) who suggest that the appearance of expressed fragments in the supernatant coincides with cell death and lysis. Neither the K-1-21 VH nor NC10 scFv fragments (Fig 3.2) are secreted into the supernatant after overnight expression at 39°C.

In general it was found that K-1-21 VH was expressed mainly in the periplasmic fraction although some product was also present in the cytoplasmic and membrane fractions (Fig 3.3). These results suggest that the bacterial secretion peptide, *pelB*, does direct transport of the K-1-21 VH antibody fragment to the periplasm. Although some expression of NC10 scFv was present in the periplasm the product consisted of a protein band of about 32 kDa and lower molecular weight fragments which bind anti-FLAG M2 and probably represent degraded expressed

protein. The amount of lower molecular weight product in the periplasmic fraction was higher after expression at 37°C (Fig 3.3 b) compared to 30°C (Fig 3.10). Heat induced expression may result in the production of bacterial heat shock proteins some of which are proteases. In some cases the expressed protein may be susceptible to the actions of these enzymes. Perhaps anti-neuraminidase scFv is degraded by the prokaryotic proteases produced at higher temperatures. However, the presence of a protein band representing NC10 scFv in the periplasmic fraction after cell growth at both 30°C and 37°C implies that the *pelB* signal has exported some of the expressed protein to the periplasmic space and it is soluble.

The formation of disulphide bonds in the bacterial periplasm is important for the production of correctly folded, disulphide linked antibody domains (Proba *et al.*, 1995). Previous studies using the secretion signal peptide, *pelB*, have demonstrated expression of anti-lysozyme and anti-keyhole-limpet haemocyanin VH fragments in the culture supernatant after prolonged expression (Ward *et al.*,1989). In addition, using the bacterial secretion peptide with co-expression of VH and VL antibody domains, a number of Fv fragments have been expressed in the periplasm. These include the phosphocoline binding IgA McPC603 Fv fragment (Skerra and Pluckthun, 1988), the anti-digoxin antibody 26-10 Fv fragment (Constantine *et al.*,1992) and anti-lysozyme Fv fragments for hen and pheasant (Eisele *et al.*, 1992). Using the secretion signal strategy a wide variety of scFv fragments have also been expressed as soluble, correctly folded proteins in the periplasm (reviewed in Skerra, 1993 : Lilley *et al.*, 1994).

By contrast, Power *et al.* (1992) used the vector pPOW for expression of a murine anti neuraminidase VH antibody fragment and found most of the expressed fragment associated with the cell membrane fraction. Likewise, the anti-neuraminidase scFv fragment (NC10 scFv) expressed in pPOW (Malby *et al.*, 1993) with the *pelB* secretion signal was associated with the insoluble cell membrane fraction. The authors demonstrated that the secretion signal had been removed from the amino terminus of the expressed protein. The resulting protein was present as insoluble aggregates in the periplasmic space or "periplasmic inclusion bodies". Other studies using temperature induction and a secretion

peptide have also demonstrated prokaryotic expression of antibody fragments as "periplasmic inclusion bodies" (Pantoliano *et al.*, 1991; Denzin and Voss, 1992). These results suggest that heat induction and overexpression may result in insoluble product in the periplasm. Another explanation for periplasmic inclusion bodies is that variations in amino acid sequence may influence the folding and intermolecular interactions with bacterial periplasmic proteins (Darveau *et al.*, 1992) These authors also suggest that folding and refolding events in the periplasm may be a problem for particular antibody specificities and for specificity/leader combinations. They have also shown that a scFv derived from an anti-human carcinoma antibody is not folded correctly in the periplasm in the absence of a bacterial secretion peptide.

In general, previous studies suggest that although bacterial secretion signals are necessary for expression of correctly folded proteins in the periplasm, the solubility of the expressed proteins is determined by the primary amino acid sequence. Indeed, Mitraki and King (1989) have demonstrated that a single amino acid mutation in tailspike endorhamnosidase of phage P22 can prevent aggregation of the expressed protein. Recently, Knappick *et al.* (1994) have shown that a single amino acid change at position H64 on the heavy chain of a scFv derived from the monoclonal antibody McPC603 results in increased solubility of the expressed scFv. These authors suggested that the solubility of the scFv was determined by the aspartic acid protruding from the tertiary amino acid structure and not collapsing into the hydrophobic core of the protein.

In comparison to previous studies (Malby *et al.*, 1993) the results presented here show that NC10 scFv can be expressed at low levels as soluble protein in the periplasm (Fig 3.3, b). Expression in the soluble and periplasmic cell fractions was higher than that found in the insoluble membrane fraction after growth at low temperatures following induction (Fig 3.10). The increase in solubility is probably related to the different expression regime used in this study. However, expression at the lower temperature resulted in decreased yields of NC10 scFv as the overall yield of expressed NC10 scFv reported by Malby *et al.*, (1993) was 3 mg/l whereas it was estimated as 0.1 mg/l from soluble isolates in this study.

Comparitive expression of K-1-21 VH at 30°C and 37°C, after induction at 42°C, shows that the amount of product in the periplasmic and soluble cell fraction is increased at the lower temperature (Fig 3.7). Although samples from the whole cell fraction of VH suggest that the amount of expressed protein is similar at both temperatures the amount of insoluble membrane associated fragment is increased at 37°C compared to 30°C.

Earlier studies have shown that expression of eukaryotic proteins in *E.coli* grown at temperatures below 30°C result in active soluble protein whereas the same cultures grown at 37°C produce insoluble protein (reviewed in, Schein, 1989). Observations by Mitraki and King (1989) suggest that insoluble products or "inclusion bodies" result from the incorrect association of partially folded thermolabile intermediates. These authors demonstrated that proteins synthesized at higher temperatures can result in active, functional product if the temperature is lowered immediately after translation. A possible explanation for this is that partial ß-sheet conformations, which melt at high temperatures form incorrect intermolecular interactions resulting in protein aggregation. If the heat sensitive intermediates are allowed to fold at a lower temperature this aggregation can be prevented. In another study, it was demonstrated that functional, active Fab fragments were produced in the cytoplasmic fraction by growing *E.coli* at suboptimal temperature (Cabilly, 1989). In addition, Glockshuber *et al.* (1990) have isolated functional Fv, dsFv and scFv from the soluble periplasmic cell fraction after expression at 20°C in *E.coli.* Thus a number of studies have shown that the expression of soluble protein can be achieved if the culture is maintained at a lower temperature after induction. Although the amount of expressed product may not be very high, the combination of lower temperatures and bacterial secretion signals appears to favour soluble, correctly folded expressed products in the periplasm.

In this study we have used the expression vector pPOW which incorporates a number of features found in the vector pJLA501 (Schauder *et al.*, 1987). In these vectors the λ*c I*857 gene product provides tight control over transcriptional initiation at 28-30°C. Full induction is achieved by increasing the temperature to 42°C. In order to

prevent aggregation of the newly translated K-1-21 VH and NC10 scFv antibody fragments the temperature was reduced to 30°C after induction. Although this would result in repression of transcription it was predicted that a high cell density at induction would provide reasonable amounts of correctly folded expressed product. From the results presented here it would appear that the amount of soluble K-1-21 VH and NC10 scFv in the periplasm is enhanced when cells are grown at 30°C after induction. However, the ELISA results show that the VH domain did not bind to BJP which suggests that the antibody fragment may not be functional. A more likely explanation is that the monomeric single VH domain does not bind to antigen with the same avidity as bivalent Ig molecules (Pack and Pluckthun, 1992).

3.4.2 PURIFICATION OF ANTIBODY FRAGMENTS

Although earlier studies required the use of immobilized antigen affinity chromatography for purification of the expressed protein there have recently been a number of improvements in isolation techniques. It has been shown that incorporation of a carboxyl terminal peptide tag can assist in isolating VH antibody fragments expressed in prokaryotic cells (Ward *et al.*, 1989; Power *et al.*, 1992). This approach is particularly useful for VH single domain fragments which generally do not bind antigen as efficiently as the native antibody (Skerra, 1993). The results shown here demonstrate that the carboxyl terminal flag peptide can be used successfully to isolate two antibody fragments with diverse specificity. This is particularly useful for comparative studies using different antibody fragments such as NC10 scFv, K-1-21 VH and scFv-mel.

A more recent technique allows recovery of active Fv fragments by incorporating carboxyl terminal peptide sequences which bind streptavidin and allow elution by competition with natural ligands of streptavidin (Schmidt and Skerra, 1993). Another approach is the development of a carboxyl terminal oligo-histidine peptide tag which enabled single-step purification of the McPC603 scFv fragment from the total cell lysate and the periplasmic cell fraction by metal chelate affinity chromatography (Skerra *et al.*, 1991). These techniques are particularly useful for monovalent antibody fragments which have a lower avidity

than the parent antibody (Adair *et al.*, 1993 ; Pack and Pluckthun, 1992). This indirect approach to isolation could result in better recovery of an expressed monovalent fragment.

3. EXPRESSION OF K-1-21(scFv)-mel

All cell lines tested produced a 36 kDa protein after induction (Fig 3.8). The expressed product reacted with anti-FLAG M2 on a Western blot suggesting that this was the K-1-21(scFv)-mel fragment. Total cell expression appeared to be the same in all *E.coli* strains except for DH5a which had a lower cell density resulting in decreased amounts of expressed product. The TOPP2 strain was used for further expression experiments of the recombinant immunotoxin. A comparison of expressed fragments shows that most of the expressed NC10 scFv is found in the soluble cell fraction, whereas most of the scFv-mel fragment is present in the insoluble membrane fraction (fig 3.10). Thus although the amount of expressed K-1-21(scFv)-mel is relatively low compared to NC10 scFv, some protein has been transported to the periplasmic space where it is present as soluble material. Considering the total volume of the different fractions, a significant amount of recombinant immunotoxin protein (scFv-mel) was present in the periplasmic as well as the whole cell soluble fraction and the latter was used for affinity purification.

Isolation of the scFv-mel fragment from the whole cell soluble fraction will include periplasmic protein and cytoplasmic protein. The periplasmic fraction should contain the fragment with the correct disulphide-bond formation to produce functional protein and the signal peptide should be removed. However, the cytoplasmic protein may not have the correct tertiary conformation as the bacterial cytoplasm does not contain the pathways for disulphide bond formation (Proba *et al.*, 1995). In addition, it will contain expressed protein with the secretion signal peptide still present at the amino terminus. Unfortunately, the relatively low yields of expressed protein in the periplasm made it impossible to isolate substantial amounts of the fragment for functional assays. By isolating the fragment from the whole cell soluble fraction the yields of total protein improved (Fig 3.11). Despite the possibility of

of incorrectly folded scFv-mel in the fraction isolated by anti-FLAG M2 affinity chromatography it was later shown to contain functional material. This activity was probably due to the presence of some periplasmic protein in the isolated sample. In support of this strategy a previous report has shown that an Fab fragment expressed at low temperatures in the cytoplasm of *E.coli* retained significant antigen binding activity (Cabilly, 1989).

There are several examples of recombinant immunotoxins expressed in *E.coli.* As discussed in section 1.4, the nucleotide sequence encoding scFv fragments from a number of different monoclonal antibodies directed against cell surface tumour antigens have been linked to the sequence encoding either PE or DT. Generally the expression of recombinant immunotoxins such as (scFv)-PE and DT is not directed to the periplasm using bacterial signal peptides (Pastan and Fitzgerald., 1991 ; Chaudhary *et al.*,1990a and 1990b ; Kreitman *et al.*,1990). One example of expression in *E.coli* is a scFv derived from a monoclonal antibody, BR96, which reacts with tumour antigens on the surface of human lung, breast and colon cancer cell lines (Friedman *et al.*, 1993). The scFv gene construct was fused to the gene encoding PE40 (BR96 scFv-PE40) and then expressed in *E.coli.* The authors reported that the expressed immunotoxin was found in inclusion bodies from which the fusion protein was extracted and purified. A large proportion of the isolated protein was present as aggregates which did not bind to antigen. However, approximately 3% of the total inclusion protein was present as correctly folded monomer which was functional. Another example is the anti-Tac(scFv)-PE38KDEL construct which was expressed in *E.coli.* (Spence *et al.*, 1993). The expressed recombinant immunotoxin was found in insoluble inclusion bodies and was renatured after extraction with 6 M guanidine hydrochloride. However, inactive aggregates of the immunotoxin were formed during dialysis and concentration of the isolated protein.

A different construct was produced by Reiter *et al.* (1994b) in which disulphide bonds were engineered into conserved framework regions of Fv fragments (dsFv) and either the VH or VL gene was linked to the toxin gene for PE38KDEL to produce a recombinant immunotoxin. Three different immunotoxins were produced including, e23(dsFv)

which binds to the erbB2 carcinoma related antigen, anti-Tac(dsFv) which binds to the p55 subunit of the IL-2 receptor and B3(dsFv) which binds to a carbohydrate antigen present on many human tumours. All three recombinant immunotoxins were expressed as inclusion bodies. It was found that during renaturation VH-toxin molecules formed aggregates and these could easily be removed by chromatography leaving the active VL+VH-toxin combination. The properly folded isolated dsFv fragments resulted in improved yields of up to 70 mg/l of bacterial culture because of improved stability, decreased aggregation and better refolding of the dsFvs compared to scFvs.

From published data it appears that scFv and dsFv recombinant immunotoxins linked to PE and DT are expressed as insoluble "inclusion bodies" which can then be renatured to give active cytotoxic protein fragments. In contrast the K-1-21(scFv)-mel recombinant immunotoxin is directed to the periplasmic compartment of prokaryotic cells by the signal peptide, *pelB*. This is the first demonstration of an active single-chain recombinant immunotoxin isolated from the periplasmic and soluble fraction of *E.coli.*

Recently it has been shown that functional McPC603 scFv molecules with a non-toxic amphipathic tail (miniantibodies) can be expressed in the periplasm of *E coli* (Pack and Pluckthun, 1992). The amphipathic helix was chosen as it tends to form a four helix bundle. As mentioned previously in section 1.3.2 (4), melittin is an amphipathic helix which tends to form a tetrameric structure under certain conditions. A superficial comparison of the "miniantibodies" described by Pluckthun with scFv-mel reveals that in both cases the scFv is linked by a flexible, hydrophilic peptide to an amphipathic helix. These authors used the soluble fraction from whole cell lysates for preparative affinity isolation of the product. Unfortunately, there was no direct comparison of the relative expression of scFv-amphipathic-helix in the periplasmic and soluble cell fractions. Nevertheless, the periplasmic expression of a scFv-amphipathic helix in both studies suggests that this protein structure can be expressed as a soluble protein in prokaryotic cells. One advantage of this is that correctly folded material can be isolated directly from the soluble fraction without the tedious denaturing and

refolding procedure which tends to increase the possibility of aggregation and precipitation.

Purification of K-1-21 VH, NC10 scFv and scFv-mel using anti-FLAG affinity chromatography produced concentrated material of the expected molecular size for each fragment (Fig 3.13). In comparison to the other fragments the yield of purified scFv-mel was poor.

Electrophoresis of the isolated fragments on a 10% SDS polyacrylamide gel and subsequent staining with coomassie, revealed that a large amount of BSA had leaked off the membrane and was present in the concentrated scFv-mel sample. This prevents accurate quantitation by measuring Absorption at 280 nm or the use of other established protein methods. For this reason the protein concentrations of the expressed fragments were determined by comparative densitometric analysis of the Western membrane using a FLAG-BAP protein of known concentration (Fig 3.13). The results estimated in this way are probably not as accurate as more specific methods and, as the detection system uses anti-FLAG M2, it does not distinguish between functional and non-functional material. However, the protein concentrations using this method were similar to those determined by A_{280} for K-1-21 VH and NC10 scFv.

3.4.4 BINDING OF scFv-mel

In the ELISA (Fig 3.16) the binding of scFv-mel to immobilised BJP was significantly higher compared to the control NC10 scFv and VH. These results imply that the scFv-mel is correctly folded and functional. A possible explanation for the relatively high binding of scFv-mel is non-specific binding of the melittin tail to protein. However, a similar ELISA using 1% BSA instead of BJP did not show binding above background levels for any of the isolated fragments. Another possible explanation for the increased binding of scFv-mel is that two or more molecules of melittin have associated to form multimers of the scFv-mel fragment. As mentioned previously, Pack and Pluckthun (1992) have designed and expressed a bivalent scFv fragment by attaching the linker sequence encoding the hydrophilic hinge region from IgG3 and an amphipathic helix to the carboxyl terminal of the scFv gene. This helix is known to associate as anti-parallel dimers to form a four helix bundle

similar to the tetramer formed by melittin. Although a tail section encoding a cysteine residue was engineered to enable subsequent chemically induced disulphide-bond formation, it was found that the scFv fragments with the attached amphipathic helix spontaneously formed bivalent molecules including disulphide-bond formation in the periplasm. The fragments without the tail also formed non covalently associated dimers in the periplasm and in solution. The antigen binding properties of the bivalent scFv fragments were similar to the parent antibody whereas binding of the monovalent scFv fragment was 2 orders of magnitude lower. This augmented binding to antigen is a direct result of the increase in "avidity" due to the bivalency of scFv fragments as the thermodynamic binding affinity of monovalent and bivalent antibody fragments was identical (Skerra *et al.*,1990). Although there is no direct evidence that the linker-melittin polypeptide at the carboxyl terminal of the K-1-21 scFv-mel fragment forms a bivalent scFv fragment, it is a similar construct which exhibits strong binding and therefore the possibility of bivalency does exist.

Immunofluorescence staining shows that both the K-1-21 monoclonal antibody and the Fab fragment bind to HMy2 cells (Fig 3.15). Using these conditions binding of the parent antibody to cells is approximately 2 fold higher than the Fab fragment. This has been found in a number of studies and is a result of the decreased avidity due to monovalency of the Fab fragment (Adair *et al.*, 1993; Skerra, 1993; Bird *et al.*,1988). These results also indicate that the cells are expressing the *kappa* myeloma antigen (KMA) to which the antibody binds.

Binding of 50 nM scFv-mel to HMy2 cells shows a shift in the fluorescence compared to the control (Fig 3.17, a). Although the binding of scFv-mel and native K-1-21 to HMy2 cells is not directly comparable, due to detection of the former using anti-FLAG M2 as the second antibody, it appears that the immunotoxin binding is only 2 fold lower than K-1-21 (Fig 3.17, b). The specificity of scFv-mel for HMy2 cells is demonstrated by the fact that it does not bind to K562 cells which do not express KMA (Fig 3.17, c). Furthermore, a 10 fold decrease in the amount of scFv-mel results in a loss of binding to HMy2 cells (Fig 3.18, b). The NC10 scFv fragment (Fig 3.18, c) which has a different specificity but which also contains the flag peptide does not

bind to HMy2 cells. Thus the flag peptide does not account for binding to the cells. This result suggests that the antigen binding site of the scFv-mel fragment has retained binding specificity.

Pre-incubation of K-1-21 and scFv-mel with BJP completely inhibits binding of both antibodies to HMy2 cells (Fig 3.19). Once again binding of K-1-21 and scFv-mel was not detected on K562 cells and as expected pre-incubation with BJP had no effect on the results (Fig 3.20). As pre-incubation with BJP would block the antigen binding site the loss of binding implies that the binding of scFv-mel to target cells occurs via the antigen binding site. Thus the recombinant immunotoxin has retained binding specificity to the target cell.

As mentioned earlier, the isolation of scFv-mel from the soluble whole cell fraction may result in the purification of a heterogeneous group of products which bind to the anti-FLAG M2 affinity column. It is expected that scFv-mel fragments from the cytoplasm will retain the *pelB* secretion peptide. As this peptide is only 22 amino acids it is too small to detect differences in MWt on a 10% polyacrylamide gel. In addition, antibody fragments which have not formed disulphide bonds and therefore are not correctly folded will be present. A comparison of binding to HMy2 cells using scFv-mel isolated from the periplasm and from the soluble whole cell fraction shows similar binding (Fig 3.21). This indicates that both fractions contain the same amount of active protein. Quantitation of the scFv-mel bands by densitometry showed that the same amount of protein was present in both fractions. This may be a reflection of the inefficient method used for quantitation as a higher protein concentration was expected for the soluble fraction containing heterogeneous products. Another possibility is that all the scFv-mel isolated from the soluble whole cell fraction is functional. However, in a repeat experiment in which the amount (50 nM) of isolated, purified scFv-mel from the soluble fraction (Immunotoxin 2) was estimated to be the same as the previous experiment (Immunotoxin 1), binding to HMy2 cells was 3 fold lower than K-1-21 binding (Fig 3.19) compared to a 2 fold decrease previously observed (Fig 3.17). These results indicate that there may be some variation in the amount of functional protein expressedfrom batch to batch and Immunotoxin 2 may contain less active material than Immunotoxin 1. Nevertheless, this discrepancy

between the amount of binding and the protein concentration could be related to inaccurate quantitation of scFv-mel.

3.4.5 CYTOTOXICITY OF scFv-mel

Confirmation of scFv-mel binding activity and specificity to KMA was obtained by analysis of the cytotoxic effects on target cells. Incubation of both cell lines with melittin shows that they are equally susceptible to cell lysis by this peptide (Fig 3.22). However, K-1-21 scFv linked to melittin results in specifically directed cytotoxicity as only the target cells (HMy2) are lysed (Fig 3.23). Concentrations of 50 nM recombinant immunotoxin result in approximately 67% cell death after an 18 hr incubation. Incubation of K562 cells with scFv-mel using the same conditions does not result in cell death. This experiment was repeated using a different batch of scFv-mel (Immunotoxin 2) which showed a 3 fold decrease in binding to HMy2 cells compared to K-1-21 (Fig 3.19). Incubation of Immunotoxin 2 with HMy2 cells resulted in lower cytotoxicity (Fig 3.24) as only 20% of HMy2 cells were lysed after 18 hr. This cytotoxicity to target cells was completely inhibited by pre-incubation of scFv-mel with BJP. Incubation of K562 cells with scFv-mel did not result in cell death above background levels. The lower cytotoxicity of IT 2 is probably related to the observed decrease in scFv-mel binding to HMy2 cells, indicating that a minimum number of sites need to be bound before lysis can occur. As melittin kills cells by disrupting the membrane it would require a significant number of molecules to produce enough pores to cause leakage of cell contents and cell death. It may be inferred that cell death is probably related to the number of molecules bound per cell.

Studies using PE and DT recombinant immunotoxins have determined cytotoxicity of the molecule by measuring inhibition of protein synthesis by 50% (ID_{50}) after 16-24 hr incubation with target cells (Chaudhary *et al.*, 1989). A comparative study of an anti-Tac(scFv)-PE40 recombinant immunotoxin with chimaeric toxins and chemically conjugated immunotoxins has shown that the recombinant immunotoxin is the most toxic at pM concentrations (Kreitman *et al.*, 1990). Another derivative, anti-Tac(scFv)-C3-PE38KDEL, which has increased cytoplasmic delivery and hence toxic activity gave an ID_{50} of 0.8 pM when incubated

with HUT 102 cells. A similar construct but with a different specificity, e23(scFv)PE38KDEL, gave an ID_{50} of 0.18 ng/ml compared to e23(scFv)PE40 of 3 ng/ml when incubated with human breast cancer cells BT474 (Batra *et al.*, 1992). A different recombinant immunotoxin, BR96scFv-PE40, which is cytotoxic to the breast carcinoma cell line MCF-7, inhibited protein synthesis by 50% at a concentration of 5 pM (Friedman *et al.*, 1993). In general, the published data shows that recombinant immunotoxins using PE have an ID_{50} range of 1-250 pM when tested against their specific target cells. By comparison the amount of scFv-mel required to kill 67% of target cells was in the nM range. However, the higher concentration of scFv-mel needed for cytotoxicity is probably due to the different method of measuring cell death. Recently, Siegall *et al.*, (1994), compared the H3396 cell killing activity of BR96scFv-PE40 with the ability to inhibit 50% protein synthesis. Cell killing was estimated by measuring the amount of calcein fluorescence released by intracellular esterase hydrolysis of the membrane permeable substrate calcein-AM (the substrate is not fluorescent until hydrolized). The authors found that the recombinant immunotoxin inhibited protein synthesis by 50% at a concentration of 42 pM after 24 hr, whereas 50% cell death occurred at 310 pM after 24 hr and 50 pM after 48 hr. These results suggest that inhibition of protein synthesis occurs at least 24 hr prior to cell death and is therefore not an accurate estimate of cytotoxicity. It is also apparent that the longer incubation time results in a lower ID_{50} for cell killing. This is probably due to the gradual depletion of protein stores within the cell as *de novo* synthesis is inhibited by PE. The mechanism of cell killing by melittin is primarily due to disruption of the cell membrane (Terwilliger and Eisenberg, 1982). However, it also exhibits a number of other pharmacological properties including activation of tissue phospholipase A2 and phospholipase C, opening of Ca^{2+} release channels and alteration of the Ca^{2+}+Mg^{2+}-ATPase in cardiac and skeletal muscle (Fletcher and Jiang, 1993). Thus prolonged incubation of target cells with scFv-mel might result in increased cytotoxicity due to the secondary effects of melittin.

Melittin itself is not a highly toxic peptide as studies of its membrane action demonstrated that 50% lipid hydrolysis occurred at a concentration of approximately 10^{-6} M (Shipolini, 1984). In the present

investigation, an 18 hr incubation of native melittin with HMy2 and K562 cells gave an ID_{50} of 2.5-3.0 μM. Using the same conditions it was found that 67% cell death occurred with 50 nM of K-1-21(scFv)-mel and the same percentage cell death was observed with a melittin concentration of 3.5 μM. Using Avagadros constant (N) the number of molecules of melittin in K-1-21(scFv)-mel at a 50 nM concentration is $3.01x10^{16}$ and the number of molecules of melittin in 3.5 μM is $2.1x10^{18}$. Thus the recombinant immunotoxin is at least 100 fold more cytotoxic than melittin itself. There are no receptors on the cell surface which specifically bind melittin; rather melittin binds to the cell surface via charge interactions. Thus, the increased cytotoxicity of the recombinant immunotoxin could be explained by attachment of K-1-21(scFv)-mel to a defined receptor molecule on the surface of the cell. Binding of melittin to the cell membrane is then determined by the number of antigen binding sites and the affinity of the scFv fragment for antigen. There are approximately 10,000 K-1-21 binding sites on HMy2 cells and the affinity constant for binding of K-1-21 to KMA is approximately 10^{-8} M (Goodnow, Hons Thesis, 1983). Possibly, the K-1-21(scFv)-mel has a higher affinity for KMA than the affinity of melittin ($2x10^{-7}$) attaching non-specifically to the cell membrane. As the KMA is not internalised by antibody binding (Boux *et al.*, 1985) the concentration of melittin molecules bound to the cell membrane at any one time, by the higher affinity scFv binding, might exceed the number of melittin molecules bound non-specifically on their own. This would account for increased cytotoxicity of the recombinant immunotoxin. As mentioned previously cytotoxicity appears to be related to the amount scFv-mel bound to cell surface antigen. It would be interesting to analyse cell surface immunofluorescence staining and correlate the percentage cell death on cells which express different amounts of KMA. In this way it would be possible to relate the cytotoxicity of K-1-21(scFv)-mel to antigen binding.

Another consideration is the effect of the flag peptide on the cytotoxicity of K-1-21(scFv)-mel. It is interesting that scFv-mel is cytotoxic despite the presence of the flag peptide at the carboxyl terminal of the molecule. The presence of the highly negatively charged flag peptide might augment the effects of the positively charged melittin tail. Another possibility is that the increased charge may result in electrostatic

repulsion and inhibit the activity of melittin to some extent. Further studies of the scFv-mel immunotoxin without the flag peptide are required to assess these possibilities.

An additional factor which might influence cell toxicity is the relative stability of scFv-mel and melittin under physiological conditions. Perhaps the melittin molecule is degraded more rapidly than the melittin attached to the scFv fragment. The scFv itself has a variable half life under physiological conditions. For example it has been reported that an Fv had a half life of 1.3 hr, scFv was 15 hr and the two dsFv fragments were 88 and 70 hr respectively (Glockshuber *et al.*, 1990). In a similar study Reiter *et al.*, (1994a) demonstrated that a renatured recombinant immunotoxin B3(scFv)-PE38KDEL was stable for 1-2 hr whereas the disulphide linked B3(dsFv)-PE38KDEL protein was stable for longer than 48 hr. Obviously a recombinant immunotoxin with greater stability and increased binding to target cells would be more advantageous for clinical applications. Thus a comparative study between recombinant immunotoxins, scFv-mel and a dsFv-mel, would provide useful information about their relative stability and toxicity, which is an important consideration for further applications.

One of the major problems in this study has been the poor yields of recombinant immunotoxin in the periplasm. A recent observation suggests that a significant amount of product which does not bind anti-FLAG M2 is expressed in the periplasm (results not shown). This fragment was observed by crossreaction with a particular anti-mouse Ig-AP (Sigma Corporation) and it appeared as a fairly diffuse band below the scFv-mel band on a Western blot. Most of this fragment was present in the periplasm and soluble fractions of cells expressing scFv-mel. Although it did not bind to the anti-FLAG M2 affinity gel it did bind to BJP affinity gel. This implies that a significant amount of functional scFv-mel is being expressed in the periplasm but during transport across the cell membrane the flag peptide is being cleaved off. At present it is not known whether the melittin peptide is still attached or whether this has also been removed. As the product appears as a broad band it probably consists of the scFv with varying lengths of the melittin peptide. Accumulation of the flag peptide and possibly melittin peptide in the bacterial cell membrane during expression of the foreign protein

might be cytotoxic to the cells. This would account for both the large amount of anti-FLAG M2 reactive protein in the insoluble membrane fraction and the observed decrease in expression of scFv-mel. In view of these observations it might be worth changing the expression strategy from one of secretion to cytoplasmic expression with subsequent renaturing and refolding of the recombinant immunotoxin to increase the overall yield of functional product.

Conclusions

5.1 *CONCLUSION*

Recent developments in antibody engineering techniques have resulted in a wide variety of genetically manipulated therapeutic antibodies. For example a number of strategies have been designed to reduce the immunogenicity of murine monoclonal antibodies which recognise specific cancer antigens on the cell surface. One approach has been to fuse the variable region genes from a mouse mAb to the genes encoding a human constant region with subsequent expression of the chimaeric antibody (Morrison, 1992). An alternative approach has been to "humanise" the murine mAb by fusing the genes encoding the CDR regions with the genes from human framework regions. This "humanised" antibody was then expressed in mammalian cells (Foote and Winter, 1992). Potential manipulation of these gene constructs to improve antigen binding and reduce immunogenicity offers great promise for future therapeutic applications of chimaeric antibodies. A novel approach to isolation and manipulation of human antibodies which bind human antigens is the *in vitro* selection system. The variable region genes have been isolated from large human antibody repertoires and then expressed as either scFv or Fab fragments on the surface of filamentous phage fd. A number of antibody fragments which bind to human antigens have been produced using this technique (Griffiths and Hoogenboom, 1993). The inherent advantage of this technology to produce and select human antibodies to self antigen suggests that it could replace hybridoma technology in the future. The use of recombinant DNA technology has also led to the production of antibody fragments which retain binding activity and specificity resulting in the production of immunotoxins using a variety of antibody specificities and toxic agents (Blakey, 1992). The use of recombinant immunotoxins in cancer therapy is still in the early stages of development. However, preliminary clinical trials using both chemically conjugated immunotoxins and recombinant immunotoxins have shown promising results (Ghetie and Vitetta, 1994 ; Strom *et al.*, 1993).

In previous studies recombinant immunotoxins have been produced from DT and PE which are both inhibitors of protein synthesis and are highly toxic agents. Genetic manipulation of these toxins has resulted in

improved toxicity and decreased non-specific binding of recombinant immunotoxins. Analysis of *in vitro* cytotoxicity and specifity suggests that these immunotoxins have great potential in cancer therapy. However, a general feature of toxins such as ricin, PE and DT is the apparent side effects reported in phase 1 clinical trials. Clinical observations suggest that the toxins are responsible for vascular leak syndrome, myalgias, neutropenia and occasional neuropathies. Furthermore, PE and native ricin immunotoxins result in hepatotoxicity whereas the less toxic proteins such as RTA, SAP, PAP and DT do not (Ghetie and Vitetta, 1994). It has also been shown that both ricin and DT containing immunotoxins are highly immunogenic when administered to patients. These findings have led to the suggestion that less toxic human proteins might be more useful in therapeutic immunotoxins as they would result in fewer side effects and decreased immunogenicity. Other toxins which have potential as therapeutic immunotoxins are the pore forming proteins or PFPs. Melittin is an example of a PFP and the mechanism of cell lysis is the formation of pores in the cell membrane resulting in leakage of cellular contents and eventual cell death. The advantage of these toxins is that they act at the cell surface and as they are small peptides they may be less immunogenic.

In the study described in this thesis a recombinant immunotoxin using the membrane lytic peptide melittin was produced and shown to bind specifically to target cells. Although binding of the recombinant immunotoxin was 2-3 fold lower than the intact Ig molecule it was approximately the same as the Fab fragment. In addition, immunotoxin binding was inhibited in the presence of excess soluble antigen indicating that the antigen binding site is functional and is responsible for recognition of the cell surface antigen. It was found that the immunotoxin was cytotoxic to target cells and did not kill cells which do not express the surface antigen. Furthermore, blocking the antigen binding site of the immunotoxin with soluble antigen inhibited lysis of the target cells. Thus, it has been shown that the scFv-mel immunotoxin is functional and retained specifity. As the mechanism of action of melittin is disruption of the cell membrane with subsequent cell death it would probably require a large number of molecules on the cell surface to result in cell lysis. This appears to be the case as a comparison of

published data using PE and DT recombinant immunotoxins with the present data shows that on a molar level scFv-mel was approximately 3 orders of magnitude less toxic towards target cells. The lower toxicity may be explained by the presence of the flag peptide which is still attached to the carboxy terminus of melittin in the scFv-mel fragment. The presence of heterogeneous immunotoxin molecules which may be incorrectly folded or still have the *pelB* signal attached to the amino terminus may result in innaccurate quantitation of the active product. Despite these technical problems the preliminary data is promising and suggests that a single-chain recombinant immunotoxin using melittin can be produced in *E.coli*. The expressed, isolated product is specific and cytotoxic towards target cells.

There are at least two possible approaches to improve the cytotoxicity of scFv-mel using genetic manipulation. Firstly, the antigen binding site could be changed by mutagenising the VH CDR3 to produce a scFv with increased affinity for the target cells (Denzin and Voss, 1992 ; Routledge *et al.*, 1993)). The binding site could also be stabilised by genetically engineering disulphide bonds within the framework regions (Reiter *et al.*, 1994b). Another possibility is the manipulation of the linker peptide between VH and VL. A shorter linker has been shown to favour association of VH and VL domains on opposite chains of the scFv resulting in bivalent antibodies (Holliger *et al.*, 1993). This would result in a bivalent scFv-mel in which there is not only increased avidity but two melittin molecules would be closely associated. Together these features might result in increased cytotoxicity.

The second possibility is the manipulation of the melittin gene to improve cytotoxicity. For instance it was previously shown that peptide I has a similar structure to melittin but it was 2.5 times more lytic (Degrado *et al.*, 1990). The longer hydrophobic region at the amino terminus was responsible for this increased cytotoxicity. Therefore, by increasing the hydrophobicity at the amino terminus of melittin it might be possible to improve cytotoxicity. Finally, the feasibility of linking two melittin molecules at the end of a scFv should be explored. Although this strategy might result in a more toxic immunotoxin it would probably be difficult to express this protein in *E.coli* using the secretion strategy. It is likely that a tail consisting of two melittin

molecules will become imbedded in the cell membrane during transportation, resulting in poor yields of intact protein.

The lower toxicity of the scFv-mel fragment may prove to be an advantage in immunotoxin therapy. As mentioned earlier administration of the less toxic, deglycosylated immunotoxins to human subjects does not result in hepatotoxicity and as melittin is less toxic and is not glycosylated it may not exhibit this side effect. Another common feature of the highly toxic agents used in therapy is the production in patients of neutralising antibodies to these toxins. As melittin is a small peptide it could easily be manipulated at the gene level to produce a molecule which is still cytotoxic but is less immunogenic.

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APPENDIX

E.coli STRAINS AND THEIR GENOTYPE.

NM522	*supE, thi-1, Δ(lac-proAB), Δ(hsdMS-mcrB)5 (r_{k-}, m_{k-}), [F', proAB lacI^q ZΔM15]*
LE392	*supE44, supF58, hsdR514, galK2, galT22, metB1, trpR55, lacY1*
TG1	*supE, hsdΔ5, thi, Δ(lac-proAB), F' [traD36 proAB lacI^q ZΔM15]*

Competent *E.coli* strains were obtained from Stratagene, La Jolla, CA. The strains and their genotype are listed below.

XL-1 BLUE:	*recA1, endA1, gyrA96, thi-1,hsdR17, supE44, relA1, lac, [F' proAB, lacI^q ZΔM15,Tn10 (tet^r)]*
Sure:	*e14-(mcrA), Δ(mcrCB-hsdSMR-mrr)171, supE44, thi-1gyrA96, end A1, relA1, lac, recB, recJ, sbcC, umuC:: Tn5 (kan^r), uvrC, [F', proAB, lacI^q ZΔM15,Tn10 (tet^r)]*
TOPP2:	*rif^r [F', proAB lacI^q ZΔM15,Tn10 (tet^r)]*

SUPERBROTH MEDIUM (SB)

Bacto-tryptone	10 g/L
Yeast extract	10 g/L
Sodium chloride	5 g/L
K_2HPO_4	2.5 g/L
$MgSO_4.7H_2O$	1 g/L

Autoclave, cool and then add filtered stocks of:

Biotin (0.1 mg/ml)	1 ml/L
Thiamine (1 mg/ml)	1 ml/L
Ampicilin (100 mg/L)	1 ml/L

Trace element solution	3 ml/L

Trace element solution (100 ml)

$FeCl_3$	1.6 g
$ZnCl_2.4H_2O$	0.2 g
$CoCl_2.6H_2O$	0.2 g
$Na_2MoO_4.2H_2O$	0.2 g
$CaCl_2.2H_2O$	0.1 g
$CuCl_2$	0.1 g
H_3BO_4	0.05 g
HCL (10 mol/L)	10 ml
H_2O	to 100 ml

SOC MEDIUM

Bacto-Tryptone	20 g/L
Yeast extract	5 g/L
NaCl	0.5 g/L
H_2O	to 900 ml

Autoclave and cool.

Mix the following seperately and make up to 100 ml with H_2O:

$MgCl_2$	2.03 g
$MgSO_4$	1.20 g
Glucose	3.60 g

Filter sterilise and add to 900 ml of autoclaved SOC.

LB+amp PLATES

Bacto-tryptone	10 g/L
Yeast extract	5 g/L
Sodium cloride	10 g/L
Bacto agar	15 g/l

Autoclave, cool to 50oC and then add Ampicillin to 100 μg/L.

RNA AGAROSE GEL

A 1-2% denaturing RNA agarose gel contained:
1 g of RNA grade agarose in 100 ml 1x MOPS buffer.

10x MOPS [3-(N-morpholino)-propanesulphonic acid} buffer
To 800 ml DEPC-treated Milli Q water add 41.8 g MOPS. Adjust to pH 7.0 with 10 M NaOH and then add 16.6 ml 3 M sodium acetate. Add 20 ml 0.5 M EDTA. Bring to 1L final volume with DEPC-treated Milli Q water.

RNA loading buffer:

bromophenol blue	25 µl
xylene blue	25 µl
glycerol	5 ml
EDTA (pH 8.0)	0.37 g

Add water to 10 ml.

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Acrylamide/Bis 30%:

Acrylamide	29.2 g/100 ml
Bis (N N-methylene-bis-acrylamide)	0.8 g/100 ml

Filter and store in the dark at 4°C.

Resolving gel buffer, pH 8.8

Tris-HCL	1.5 mol/L

Stacking gel buffer, pH 6.8

Tris-HCL	0.5 mol/L

Running buffer, pH 8.3 (10x)

Tris base	30 g/L
Glycine	144 g/L
SDS	10 g/L

5x SDS loading buffer, pH 6.8 (5x SDS LB)

Tris-HCL (0.5 mol/L)	1.0 ml
Glycerol	0.8 ml
SDS (10% w/v)	1.6 ml
Bromophenol blue (1% w/v)	0.5 ml
H_2O	4.0 ml

SDS polyacrylamide resolving gel:

	10%	12%
Acrylamide/Bis (30%)	1.7 ml	2.0 ml
Resolving gel buffer, pH 8.8	1.25 ml	1.25 ml
H_2O	1.3 ml	1.0 ml
Glycerol	0.67 ml	0.67 ml
SDS (10% w/v)	50 µl	50 µl
$(NH_4)_2S_2O_8$ (10% w/v)	25 µl	25 µl
TEMED (N,N,N,N tetramethyl-ethylenediamine)	5 µl	5 µl

SDS polyacrylamide stacking gel (4%):

Acrylamide/Bis (30%)	325 µl
Resolving gel buffer pH 8.8	650 µl
H_2O	1.5 ml
SDS (10% w/v)	25 µl
$(NH_4)_2S_2O_8$ (10% w/v)	12.5 µl
TEMED	5 µl

WESTERN BLOT

Transfer buffer:

Tris base	0.025 mol/L
Glycine	0.192 mol/L
Methanol	20% v/v

Soak the SDS acrylamide gel and the nitrocellulose membrane in transfer buffer and then electro-blot at 100 volts for 1 hr using the BioRad chamber.

Tris buffered saline pH 7.4 and Tween 100 (TBS-Tween):

Tris-HCl	50 mmol/L
NaCl	150 mmol/L
Tween 100	0.05% (v/v)

Alkaline Phosphatase buffer, pH9.5 (AP):

Tris-HCl	100 mmol/L
NaCl	100 mmol/L
$MgCl_2$	5 mmol/L

SEQUENCING PRIMERS FOR pPOW

N1019 pPOW for sequencing primer

5'-GCTCGAGTAATTTACCAACACTACTAC-3'

N697 pPOW back sequencing primer

5'-CTGGGTTGAAGGCTCTCAAGGG-3'