Pergamon

European Journal of Cancer, Vol. 34, No. 7, pp. 968–976, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(98)00035-5

Special Paper

Exemplifying Guidelines for Preparation of Recombinant DNA Products in Phase I Trials in Cancer: Preparation of a Genetically Engineered Anti-CEA Single Chain Fv Antibody

A. Mayer, K.A. Chester, J. Bhatia, R.B. Pedley, D.A. Read, G.M. Boxer and R.H.J. Begent

Cancer Research Campaign Targeting and Imaging Group, Department of Clinical Oncology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

Products of recombinant DNA technology have potential for the diagnosis or treatment of cancer. There is a need to investigate whether they function by the intended mechanism in small phase I clinical trials before their suitability for more extensive studies can be assessed. Quality and safety of these products should be assured prior to their use in humans in a way which is appropriate to the preliminary nature of the trials but not inhibitory to progress. The Cancer Research Campaign control recommendations for products derived from recombinant DNA technology (Begent RHJ and associates. *Eur J Cancer* 1993, 29A, 13, 1907–1910) provide guidelines for the production of new biotechnology products in academic research units within a relatively short time, while ensuring appropriate quality and safety. The practical application of the guidelines requires that solutions are found for the quality and safety issues during the production of recombinant products. We describe an approach to the relevant quality and safety issues during and after the production and purification of a genetically engineered anti-carcinoembryonic antigen (CEA) single chain Fv (scFv) antibody for a phase I trial of radioimmunoguided surgery with the intention of providing a model for other products.

Key words: quality, safety, control recommendations, biotechnology, single chain Fv
Eur J Cancer, Vol. 34, No. 7, pp. 968–976, 1998

1. INTRODUCTION

RECOMBINANT PEPTIDES and proteins have become a relevant part of cancer treatment during the last two decades. The manufacturing process of recombinant proteins consists of the proliferation of cells that produce the protein followed by expression of the protein and isolation of the final product. The second phase, 'downstream processing' includes purification steps to convert the original crude protein into a clinically useful product [2]. Prior to clinical use, these products need to be tested for their quality, safety and efficacy. Animal models are an essential part of the testing of an anticancer drug, but have been proven to reflect the human situation only partly, because of the unique mechanisms of tumour growth and host response in the human organism. The lack of reliable animal models results in the need for human trials, especially small mechanistic trials to investigate the function of the substance in man and to assess safety.

Correspondence to K.A. Chester.
Received 3 Jul. 1997; revised 19 Nov. 1997; accepted 2 Dec. 1997.

Guidelines for quality and safety in the form of an operation manual for products derived from recombinant DNA technology prepared for investigational administration in patients with cancer in phase I trials have been developed by the Cancer Research Campaign, the National Institute for Biological Standards and Control and the European Organization for Research and Treatment of Cancer [1]. The guidelines have been designed for academic research units and aim to ensure appropriate purity and biological activity whilst minimising costs and time for toxicology, which may delay the clinical use and potential benefit of a substance.

Application of these guidelines has not been described in the context of a specific product and solutions to issues raised in the guidelines need to be found. Here we describe such an application using an example of an engineered single chain Fv (scFv).

ScFvs are antibody fragments, which consist of the variable heavy and variable light chain region tethered by a flexible linker and are, as such, the smallest anybody moiety (27 kD) to retain full binding capacity, although monovalent. Their

low molecular weight allows rapid blood clearance and good tumour penetration. ScFvs have the potential for wide clinical use and are, therefore, a good example for application of the guidelines. The guidelines cover the areas of product development, establishment of the seed lot and production, with emphasis on the characterisation of the final product. Our aim with this document has been to interpret each section of the guidelines using this specific example and to give a brief description of the protocol development for the phase I trial.

MFE-23-his, the scFv described here, is derived from a bacteriophage library and was cloned and expressed in *E. coli* [3]. MFE-23-his is an scFv antibody with high affinity and specifity for carcinoembryonic antigen (CEA) and was purified by immobilised metal affinity chromatography (IMAC) by virtue of an engineered hexa-histidine tag and by size exclusion chromatography [4]. It has been successfully used for radioimmunoimaging in patients with CEA-expressing breast and bowel tumours [5]. The current clinical batch was prepared for a phase I trial of radioimmunoguided surgery (RIGS) in primary and recurrent colorectal carcinomas. For clarity, documentation in this manuscript and in the manual, which includes all relevant information concerning production, safety and quality for review by the Cancer Research Campaign Drug Development Office, is in the same sequence as in the guidelines.

2. PRODUCT DEVELOPMENT

2.1 *Name of product*

The product was named MFE-23-his 6J605. The ending 'his' indicates the hexa-histidine tag and 6J605 is the batch number allocated by the pharmacy. This name ensures that the antigen and the production history can be unambiguously identified.

2.2 *Expression system*

MFE-23-his was expressed from the pUC 119 plasmid vector [6] in *E. coli* TG1 cells (Medical Research Council, Cambridge, U.K.). TG1 is a well characterised cell line, which neither modifies nor restricts transfected DNA [6]. TG1 also has an F-pilus which overexpresses the *lacI*^{q} gene whose product represses transcription of cloned proteins until specific induction. Expression of the product in bacteria instead of eukaryotic cells avoids the problem of contamination with pathogenic viruses.

Documentation of the plasmid map of pUC 119, the genotype of TG1 [6] and the method of insertion of the vector into the host cell (electroporation) is available.

2.3 *Sequence of the cloned gene*

The gene for MFE-23-his consists of the information for the variable heavy and variable light chain, the hexa-histidine tag, a linker sequence of 15 amino acids ([Gly 4 Ser]×3) and the *pelB* signal, which directs antibody fragments into the bacterial periplasm where they are folded into antigen binding conformation. The complete DNA sequence was recorded. The gene encodes a protein of 251 amino acids with a molecular weight of 27 kD.

2.4 *Expression*

pUC 119 contains the gene for ampicillin resistance, which allowed selective pressure for cells containing the plasmid by addition of ampicillin to the growth media. Although ampicillin is thought to be removed during the purification procedure, patients allergic to ampicillin are excluded from the protocol with regard to safety in man. The *lacZ* site, which is adjacent to MFE-23-his contains information for the first 146 amino acids of the β-galactosidase gene. Transcription of the β-galactosidase gene is induced by lactose or lactose analogues and repressed in the presence of glucose, which allows the use of the lactose analogue isopropyl β-D-thiogalactoside (IPTG) (Boehringer Mannheim, Lewes, East Sussex, U.K.) for the induction of expression of MFE-23-his. IPTG is expected to be removed by size exclusion gel filtration and by dialysis due to its small size of 238.3 Dalton. No known health hazard associated with IPTG was found either in the Guy's Hospital Poison Unit data base or in the American Poisons Unit data base. The manufacturer confirmed that it was free of dioxane. Expression was performed according to SOP 2.2 (Appendix 1).

3. SEED LOT

3.1 *Description*

The seed lot, which consists of a homogenous suspension of *E. coli* transformed by the expression vector containing the genetic information for MFE-23-his, was prepared according to SOP 2.1 (Appendix 1).

3.2 *Control of seed lot*

The seed lot was tested by DNA sequencing for homogeneity and contamination. Single stranded DNA was prepared using a 1/100 dilution of the seed lot and a partial sequence was obtained including 281 base pairs of the light chain. Sequencing was performed by the dideoxy chain termination method using reagents and protocol from the Sequenase version 2.0 sequencing kit (United States Biochemical Corporation, Cleveland, Ohio, U.S.A.). Plasmid stability was confirmed after overnight incubation of a 1/100 dilution of seed lot in 2TY media with 100 μg/ml ampicillin and 1% glucose. This was followed by restriction of the MFE-23 gene and electrophoresis on 1% agarose to reveal one band corresponding to plasmid DNA and one corresponding to the excised gene. Restriction was performed with Nco I and Not I enzymes releasing a fragment of 750 base pairs.

CEA-binding of the seed lot clones was tested after inducing 300 individual colonies to express MFE-23. Seed lot culture supernatants were applied to CEA coated wells (2 μg/ml) prior to application of ^{125}I labelled purified MFE-23 (166 KBq/μg). After every 15 samples a negative control (single chain antibody B1.8 anti-NIP hapten undiluted culture supernatant) was included and a positive control consisting of neat culture supernatant (MFE-23-his) was used on each plate. This competition method was chosen as no anti-MFE antibody suitable for an enzyme linked immunosorbent assay (ELISA) was available at that time. Results showed that wells incubated with ^{125}I MFE alone gave binding of 2446.94 cpm (standard deviation: 611.05, $n = 17$) and wells incubated with B1.8 control scFv gave binding of 2945.36 cpm (standard deviation: 624.57, $n = 10$), i.e. showed no inhibition of ^{125}I MFE. Wells preincubated with the seed lot supernatants showed binding of 359.83 cpm (standard deviation: 143.96, $n = 300$) with a range of 76.3–933.8 cpm which represented an inhibition of up to 50%. Since none of the control scFv wells showed inhibition of ^{125}I MFE binding all of the test samples were considered positive. The variations in effectiveness of inhibition were considered acceptable as variation in induction of MFE-23 expression from well to well would be expected. If any of the 300

colonies did not express anti-CEA their plasmid would require DNA sequence characterisation to establish whether the seed lot was contaminated and, therefore, of unacceptable standard.

4. PRODUCTION

4.1 *General principles*

MFE-23-his for this clinical trial was produced according to SOP 2.2 (Appendix 1). Purification was carried out by IMAC and size exclusion chromatography as described in section 4.5. Fourteen aliquots from one seed lot were used and pooled after the grow-up resulting in 12 litres culture broth (25 mg/l MFE-23-his). As there was no separate room available to be reserved exclusively for preparation of clinical products, an SOP was established to standardise a cleaning procedure (SOP 2.0. Appendix 1). Contamination of the material with impure intermediates from earlier stages was prevented by using new containers and equipment and moving to different areas within the laboratory for each step. Filter sterilisation was performed using 0.8 μm. 0.45 μm and 0.2 μm filter units (Nalgene, Techmate, Milton Keynes, U.K.) in order to prevent bacterial growth after separation of the bacterial supernatant from *E. coli*, after concentration, after purification by IMAC and after purification by size exclusion chromatography. Filtering and processing the material as quickly as possible allowed maintenance of sterility throughout the production process without addition of toxic preservative, although the material was handled at room temperature at various stages.

4.2 *Cross-contamination*

The risk of cross-contamination was reduced as no other bacteria were propagated in the area dedicated to the clinical batch or by the person responsible for the clinical product. This issue is also addressed in SOP 2.2.

4.3 *Production at finite passage (single harvest)*

The production was based on single harvest. Details of cultivation are described in SOP 2.2.

4.4 *Continuous culture production (multiple harvest)*

This section is not applicable due to the above mentioned production at finite passage.

4.5 *Method of purification*

An Amicon CH2 ultrafiltration system (Amicon, Stonehouse, Gloucestershire, U.K.) was used for concentration of the 12 litres of bacterial supernatant to 800 ml, which were subsequently purified. The Amicon CH2 ultrafiltration system was equipped with new tubing and a new spiral cartridge. It was cleaned with 0.1 M NaOH for 15 minutes prior to flushing with 4 l distilled water. The purification was carried out by IMAC (40 ml chelating sepharose fast flow gel, Pharmacia, St Albans, Hertfordshire, U.K.) and size exclusion chromatography (400 ml Sephacryl S-100 HR, Pharmacia) in order to remove undesired proteins, process additives and contaminants of the host cell [4]. IMAC is based on binding of the hexa-histidine tag to metal ions. Imidazole (250 ml 200 mM concentration) is used for elution of the bound antibody, while subsequent size exclusion chromatography removes any aggregates.

New columns were prepared for IMAC and size exclusion chromatography. The IMAC column was sanitised with 250 ml 0.5 M NaOH prior to washing with distilled water until the pH was neutral. The size exclusion chromatography column was cleaned with 0.5 M NaOH at a flow rate of 2 ml/min for 60 min. After washing with 1 M acetic acid/1 M ethanol for 60 min the column was equilibrated with two column volumes of PBS.

Loss of the desired product was minimised by avoiding repeated concentration as this has previously resulted in considerable protein loss and the risk of bacterial contamination. Careful analysis of the material to ensure that only eluant of the IMAC column containing high concentration of scFv was pooled and applied to the size exclusion chromatography column, resulted in reduction of the volume of the fraction containing MFE-23-his from 250 ml to 60 ml. 55 mg monomer MFE-23-his (concentration 0.7 mg/ml) were obtained after size exclusion chromatography.

Samples were analysed for pyrogen contamination by the *Limulus amebocyte* gel clot assay (Charles River Endosafe, Bognor Regis, West Sussex U.K.) according to the manufacturer's instruction as endotoxins are not entirely removed by filtering. A polymyxin column (10 ml Detoxi-gel, Pierce & Warriner, Chester, U.K.) was prepared for the clinical batch and used to remove endotoxins if the sample was contaminated. Approximately 10 ml sample was loaded onto the 10 ml column at a time and was allowed to bind to the gel for at least 10 min. The process was repeated until the total volume of the contaminated sample (35 ml) had been passed through the column. Samples were only further processed if the *Limulus amebocyte* gel clot assay showed freedom from pyrogen contamination (sensitivity 0.25 EU/ml).

Samples were aliquoted in a sterile cabinet in pharmacy using 0.2 μm filters. Filter integrity was checked by the bubble point test after the procedure. The final product was allocated a batch number in the pharmacy, where it was stored at +4°C. The temperature of the refrigerator was continuously monitored. The label included the name of the product, batch number, the concentration and the amount in the vial. Removal of any sample was recorded in the pharmacy.

4.6 *Reproducibility of the purification procedure*

Each purification step was documented in a laboratory book including records of sodium-dodecyl-sulphate polyacrylamide gel (SDS-PAGE) electrophoresis, ELISAs, Western blots, isoelectric focusing (IEF) and chart records of chromatography procedures. After the final purification step, i.e. size exclusion chromatography, monomer MFE-23-his was consistently found in fractions 24-31 (17 ml sample applied, flow rate 2 ml/min, AUFS 0.2, void volume 180 ml, fraction size 4 ml), which was confirmed by SDS gel electrophoresis showing a single band at 27 kD. Approximately 20% of the amount of MFE-23 in the bacterial supernatant remained after purification. A brief summary of all relevant testing procedures is given in Table 1. Consistency was confirmed by comparison with previous documentation.

5. FINAL PROCESSED PRODUCT

5.1 *Characterisation of the purified active substance*

The required tests, which were performed for the characterisation of the purified active substance, included SDS-PAGE electrophoresis on a 12% SDS gel and Coomassie blue staining for the size of the product, IEF on an Ampholine PAG plate pI 3.5–9.5 (Pharmacia) stained with Coomassie blue and Crocein scarlet for the isoelectric point (pI)

Table 1. *Summary of testing procedures*

Purification stage	Analysis
Clone MFE-23-his gene into pUC 119	DNA sequencing
Transfer TG1 cells into seed lot	Plasmid stability
	CEA reactivity
Grow seed lot	CEA reactivity (ELISA)
Concentration	
Filter sterilisation	
IMAC	Purity (SDS-PAGE)
	Identity (Western blot)
Size exclusion chromatography	Purity (SDS-PAGE)
	Pyrogenicity (*Limulus amoebocyte* gel clot assay)
Polymyxin column	Pyrogenicity (*Limulus amoebocyte* gel clot assay)
Aliquoting in pharmacy	Sterility (culture)
	Pyrogenicity (rabbit pyrogen test)
	Bacterial DNA testing (Digoxigenin DNA labelling and detection kit)
	Copper content (flame photometry)
	Purity and identity (SDS-PAGE, IEF, FPLC, Western blot)
	CEA reactivity (ELISA)
	In vivo localisation/potency
	Tissue distribution (immunohistochemistry)
	Toxicity

and an ELISA in order to determine the CEA-binding of the product. SDS–PAGE showed one single band at a molecular weight of 27 kD (Figure 1) and IEF 2 major bands at pI 6–6.5 as expected. CEA-binding was confirmed by ELISA (Figure 2) using CEA coated wells (2 μg/ml) and a polyclonal rabbit anti-MFE antibody (Cancer Research Campaign Laboratories, Department of Clinical Oncology, Royal Free Hospital, London, U.K.).

5.2 *Purity*

Possible contaminants in the final processed product include bacterial DNA and pyrogens, process additives such as copper,

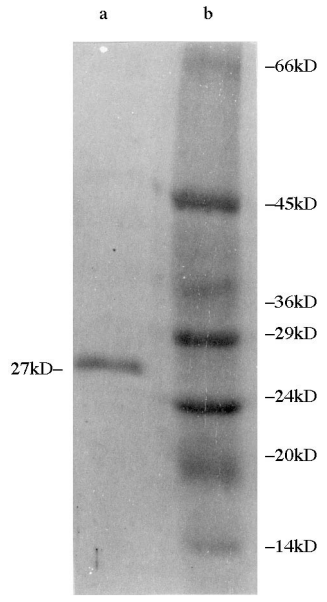

Figure 1. The purified MFE-23-his appearing as a single band with a molecular weight of 27 kD on 12% SDS PAGE. (a) MFE-23-his 6J605; (b) Molecular weight markers (Bio-Rad).

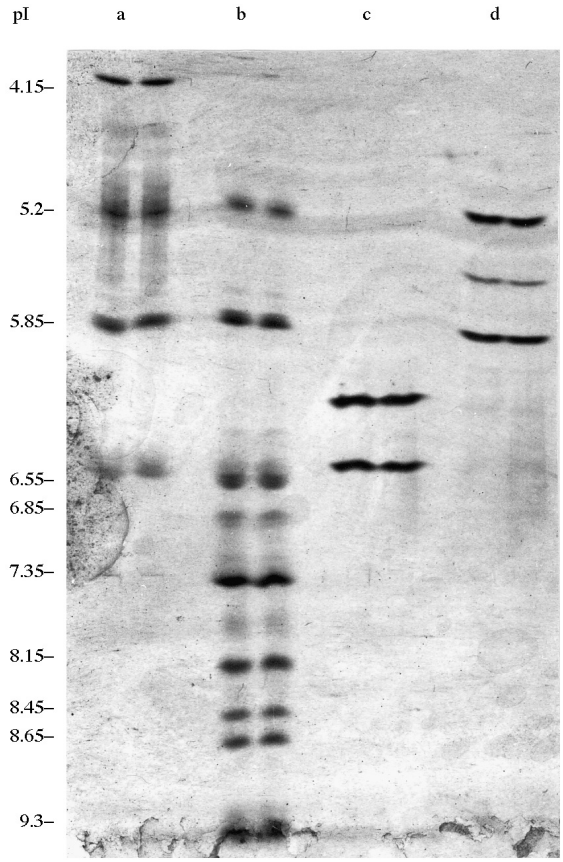

Figure 2. IEF performed on an Ampholine PAG plate pI 3.5–9.5 comparing MFE-23-his 6J605 with a previous batch of MFE-23-his stored for 2 years at +4°C. (a) pI markers (Pharmacia); (b) pI markers (Pharmacia); (c) MFE-23-his 6J605; (d) MFE-23-his stored for 2 years at +4°C. The initial concentration of both samples was 4.9 μg/ml, 1/2 dilutions were used for the following points.

which may have leached from the IMAC column, proteins and growing microorganisms, such as bacteria and fungi.

A Digoxigenin DNA labelling and detection kit (Boehringer Mannheim) was used for the exclusion of contamination with bacterial DNA. A probe consisting of a mixture of equal proportions of MFE-23-his and total bacterial DNA was constructed, which was able to detect 1.2 ng of DNA added to 2.5 μg of MFE-23-his (0.05%) and confirmed freedom from bacterial DNA.

The rabbit pyrogen test (Safepharm Laboratories, Derby, U.K.) was used to exclude pyrogenic contamination. Three times the patients dose was injected (weight/weight) into rabbits, the summed rise was found to be 0.4°C which is within the allowed range of 1.1°C.

Flame photometry (Trace Element Laboratory, University of Guildford, Guildford, Surrey, U.K.) was performed to check the copper content. It was found to be 0.5 μmol/l resulting in an actual dose of less than 1 nmol, which is much less than the recommended daily intake for patients receiving parenteral nutrition (5–20 μmol/day).

Protein contamination was excluded by electrophoresis (section 5.1) and freedom from aggregation determined by fast performance liquid chromatography (FPLC) using a Superose 12HR/50 column (Pharmacia), revealing a single peak consistent with a monomer scFv with a molecular weight of 27 kD.

Absence of bacterial and fungal at growth was confirmed by culturing of plates inoculated with the product (Microbiology Department, Royal Free Hospital, London, U.K.).

5.3 *Potency*

The potency of the current clinical batch of MFE-23-his was assessed in terms of its ability to localise colorectal tumours *in vivo* in comparison with MFE-23 purified using affinity chromatography on CEA-Sepharose 2B, which was shown to have a dissociation constant of 2.5 nM by fluorescence quench [3, 7]. The results for the affinity purified MFE-23 were 0.5% injected activity/gram tumour and a mean tumour to blood ratio of 9:1 after 24 h. Results for the current clinical batch are described in section 6.2 and indicate that it is at least as potent in terms of tumour localisation as the product with a defined affinity for CEA.

6. FINISHED PRODUCT

6.1 *Specification and reference*

Evidence of purity and potency have been described in section 5.1 and 5.2. Identity was confirmed by Western blotting using the above mentioned polyclonal rabbit anti-MFE antibody.

Stability data for MFE-23-his including long term storage were obtained using one aliquot prepared for a previous radioimmunoscintigraphy trial [5], which had been stored for more than 2 years at + 4°C.

Purity and freedom from aggregation of this product were confirmed by SDS-PAGE electrophoresis and FPLC. IEF showed a shift of the pI from 6–6.5 as described immediately after purification to 5.2–5.8 (Figure 3). A Western blot using a monoclonal anti-histidine antibody (Dianova, The Binding Site, Birmingham, U.K.) as primary antibody showed the absence of the hexa-histidine tag on the product stored for 2 years at + 4°C. The positive charge of histidine is in agreement with this pI shift. CEA binding was not affected by storage as determined by ELISA (Figure 2). In the meantime the current clinical batch has been repeatedly tested by Western blotting for the hexa-histidine tag, which was still detectable after 7 months of storage.

6.2 *Biological characterisation*

Reactivity of the final product with human tissues was determined by immunohistochemistry. MFE-23-his was applied to cryostat sections (5 μm) of a range of neoplastic and normal tissues including peripheral blood, bone marrow, brain, large and small intestine, kidney, liver, lung, lymph node, pancreas, prostate, spleen, stomach, testis, thyroid and trophoblast. A peroxidase-labelled monoclonal mouse anti-histidine antibody (Dianova) was used for detection. MFE-23-his was found to bind selectively to colorectal cancer, while its distribution of binding to normal tissue was confined mainly to the in crypt epithelium of large and small intestine.

MFE-23-his was radiolabelled with ^{125}I (Amersham, Little Chalfont, Buckinghamshire, U.K.) according to the Iodogen method (SOP 6.10, Appendix 1) to give a specific activity of 1.3 MBq/5 μg antibody for an *in vivo* experiment in mice bearing LS174T xenografts [8]. The radiolabelled product was analysed by thin layer chromatography for protein incorporation [4], which was found to be 99%. CEA-binding capacity was tested on a CEA coupled Sepharose 4B column [4]. 79% of radioactivity was bound in the fraction representing MFE-23-his, which was bound to CEA. A Sephacryl S-300 column (Pharmacia) was used to determine that MFE-23-his remained a monomer after radiolabelling.

Eight nude mice bearing LS174T colorectal xenografts were injected via the tail vein when tumours were approximately 0.5 g in weight. Mice were sacrificed at 24 and 48 h time points. Tumour and a range of normal tissues were removed and assessed for activity using a 1470 Wizard counter (Wallac, Milton Keynes, U.K.). 1.7% injected activity/gram of tumour was observed after 24 h and 0.7% after 48 h, mean tumour to blood ratios were 52:1 and 89:1, respectively (Figure 4).

We further refer to section 5.1 concerning the biological characterisation by ELISA. The dilutions of the above mentioned ELISA should be used as standard for further batches.

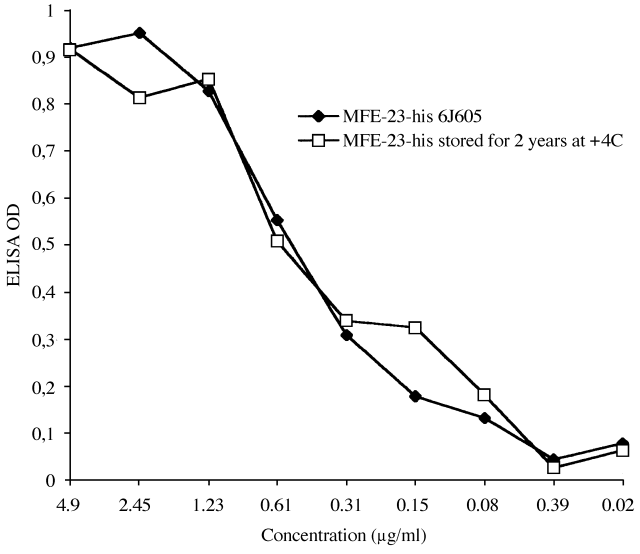

Figure 3. ELISA comparing MFE-23-his 6J605 with a previous batch of MFE-23-his stored for 2 years at +4°C.

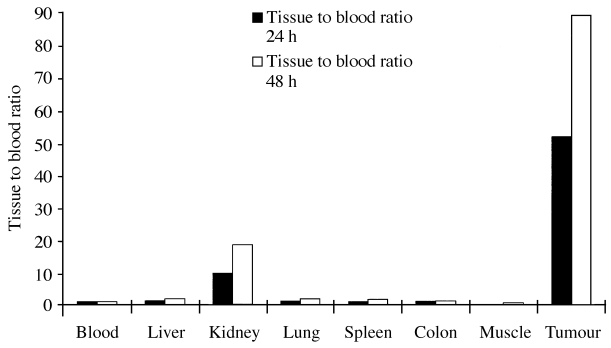

Figure 4. Tumour/blood ratio of ^{125}I labelled MFE-23-his 6J605 in nude mice bearing xenografts of human colon carcinoma (LS174T) 24 and 48 h after injection (4 mice per time point).

6.3 *Toxicity*

Ten times the patient dose unlabelled MFE-23-his was given to 6 guinea pigs (weight/weight: 56 μg/guinea pigs were kept as controls. There were no signs of disease or fever during an observation period of approximately 28 days after which the animals were bled and sacrificed. There was no evidence of abnormality or toxicity in macroscopic investigation of all relevant tissues and, therefore, no microscopic investigation was carried out. The red cell, white cell and platelet counts and the differential assessment of white cells fell within the range of the control animals.

PROTOCOL DEVELOPMENT

The protocol was written according to the minimal guidelines for early clinical trials [9]. Since this is the first trial using a scFv for RIGS, data of the previous radioimmunoscintigraphy trial [5] were used for a computer model (Neoprobe Cooperation, Dublin, Ohio, U.S.A.) to determine the optimal time between administration of the radiolabelled antibody and surgery in order to order to discriminate tumour and normal tissue. The results indicated that 185 MBq ^{125}I should allow intervals of 24, 48 and 72 h between administration and surgery.

The Estimated Dose Equivalent (EDE) for ^{125}I (185 MBq) labelled MFE-23-his was determined in order to obtain a license for the administration of radioactive substances (ARSAC). The calculation of the Estimated Dose Equivalent (EDE) was based on the residence time in each organ [10]. Data of the previous radioimmunoscintigraphy trial were used to determine the integral of the time activity curve using the trapezoidal rule assuming organ activity equals zero at time zero and a bi-exponential model was used to determine the clearance beyond the last measurement of activity. The residence time was used to calculate the absorbed dose to the target organs [10]; these doses were combined to give the EDE dose [11] 4.4×10^{-3} mSv/MBq.

The protocol was reviewed by three independent reviewers and the Cancer Research Campaign Drug Development Office. A Doctors and Dentists Exemption (DDX) was obtained from the Medicines Control Agency at the U.K. Department of Health and the study was approved by the local Ethical Practices Sub-Committee.

DISCUSSION

The control recommendations for products derived from biotechnology were developed to allow the investigation of new drugs prepared in academic research units for phase I/II trials. They intend to assure appropriate quality and safety while keeping costs and time to a minimum. The example given here illustrates how the control recommendations may be met, but the wide range of recombinant products which may be produced will require diverse solutions to the issues of quality and safety. The method described here illustrates how a clinical product can be developed in which the known potential hazards are effectively excluded. This task is eased by the small scale of production which, in contrast with industrial scale production, limits the potential for a contaminating or mutant organism to proliferate. Nevertheless the integrity of the product and freedom from contamination are demonstrated.

The method for production is relevant for possible viral contamination. Viral contamination of biological derived products like blood products or vaccines has been a problem in the past due to the difficulty in detecting single infectious virus particles, the rapid amplification and the great diversity of viruses [2]. Production in mammalian cells bears the risk of contamination with potentially pathogenic viruses which results in the necessity of efficient virus inactivation or removal [12], but only acceptable limits of the likelihood of freedom from viral contamination can be determined. The use of bacteria for production avoids contamination with viruses pathogenic to humans, which contributes to the safety of products derived from bacteriophage libraries and makes inactivation or removal of viruses redundant. The guidelines also cover steps necessary for ensuring appropriate safety of products made in eukaryotic cells, which are not exemplified here.

Although the guidelines cover the areas of production and purification, their emphasis is on the final product testing in order to show the integrity, purity and biological activity of the product, which allows the administration of a safe product with a defined activity. The restriction of toxicology to testing in mice and guinea pigs limits the expense and time taken for this aspect. This has been found safe for a range of drugs in phase I studies in patients with cancer under the auspices of the CRC phase I/II trials committee [5, 13].

A final judgement about the safety of a drug will only be possible after the whole batch has been used without any complication. However, standardisation of requirements for the safety and quality of those products based on scientific knowledge protects the patient and allows the clinician to give a drug with known likely effects on normal and cancer tissues.

1. Begent RHJ, Chester KA. Connors F, *et al.* Cancer Research Campaign operation manual for control recommendations for products derived from recombinant DNA technology prepared for administration to patients with cancer in phase I trials. *Eur J Cancer* 1993, **29A**, 13, 1907–1010.
2. Berthold W, Waiter J. Protein purification: aspects of processes for pharmaceutical products. *Biologicals* 1994, **22**, 135–150.
3. Chester KA, Begent RHJ, Robson L, *et al.* Phage libraries for generation of clinically useful antibodies. *Lancet* 1994, **343**, 8895, 455–456.
4. Casey JL, Keep PA, Chester KA, Robson L, Hawkins RE, Begent RHJ. Purification of bacterially expressed single chain Fv antibodies for clinical applications using metal chelate chromatography. *J Immunol Methods* 1995, **179**, 105–116.
5. Begent RHJ, Verhaar MJ, Chester KA, *et al.* Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. *Nature Med* 1996, **2**, 979–984.

6. Maniatis T, Fritsch EF, Sambrook J. *Molecular cloning: a laboratory manual*. New York, Cold Spring Harbour Laboratory, 1982.
7. Verhaar MJ, Chester KA. Keep PA, *et al.* A single chain Fv derived from a filamentous phage library has distinct tumour targeting advantages over one derived from hybridoma. *Int J Cancer* 1995, **61**, 497–501.
8. Pedley RB, Boden JA, Boden R, *et al.* Ablation of colorectal xenografts with combined radioimmunotherapy and tumor blood flow-modifying agents. *Cancer Res* 1996, **56**, 3293–300.
9. Arrigo C, Koier IJ, McVie JG, Marsoni S, Newlands E, Schwartsmann G. Minimal guidelines for the monitoring of early clinical trials (phase I–II) in Europe under CRC/EORTC/NCI joint agreement. *Eur J Cancer* 1992, **28A**, 1289–1292.
10. Loevinger R, Budinger TF, Watson EE. *MIRD Primer for Absorbed Dose Calculations*. New York, The Society of Nuclear Medicine, 1991.
11. International commission on radiological protection. 1990 *Recommendations of the International Commission on Radiological Protection*. Oxford, ICRP publication 60, Pergamon Press, 1990.
12. The working party on clinical use of antibodies. Operation manual for control of production, preclinical toxicology and phase I trials of anti-tumour antibodies and drug antibody conjugates. *Br J Cancer* 1986, **54**, 557–568.
13. Lane DM, Eagle KF, Begent RHJ, *et al.* Radioimmunotherapy of metastatic colorectal tumours with iodine-131-labelled antibody to carcinoembryonic antigen: phase I/II study with comparative biodistribution of intact and F(ab')$_2$ antibodies. *Br J Cancer* 1994, **70**, 521–525.

Acknowledgements—The authors would like to thank Dr P. Minor at the National Institute for Biological Standards and Control and Dr S. Burtles at the Cancer Research Campaign Drug Development Office for reading the manuscript. We are grateful to Dr J.L. Casey for performing the isoelectric focusing, Dr A. Green, Dr M.P. Napier, J.A. Boden, R. Boden and L. Robson at the Cancer Research Campaign Laboratories at the Royal Free Hospital School of Medicine; Dr M.S. Cooper and B. Chauhan at the pharmacy at the Royal Free Hospital for their advice and technical assistance. This work was supported by the Cancer Research Campaign, the European Society for Medical Oncology and the Ronald Raven Chair in Clinical Oncology Trust.

APPENDIX: STANDARD OPERATING PROCEDURES (SOPs)

2.0 THE PREPARATION OF ROOM ON 16 FOR THE PRODUCTION OF *E. COLI* SEED LOT

1. Turn off air conditioning in the room.
2. Clear the bench top of all items between the centrifuge and the vortex generator.
3. Remove all dirty glassware.
4. Clear the New Brunswick rotary incubator.
5. Clear the 37°C incubator
6. Remove any bacterial plates/tubes from the incubator.
7. Empty the waste bag.
8. Autoclave a rack to hold 30 ml Universal tubes.
9. Prepare sterile glycerol.
10. Label screw cap cryovials.
11. Spray the interior of the New Brunswick rotary incubator with 70% ethanol and allow it to evaporate.
12. Run rotary incubator at 65–70°C for 3 h.
13. Seal the bacterial plate refrigerator with adhesive tape.
14. Spray all work surfaces in the room with 70% ethanol.
15. Spray the floor of the room with 70% ethanol.
16. Tape autoclave bags over the shelving above the working bench.
17. Tape an autoclave bag over the water bath.
18. Spray the 37°C incubator with 70% ethanol—leave open for 10 minutes.
19. Close the door of the 37°C incubator.
20. Turn off the New Brunswick rotary incubator.
21. Mark the outline of the clean area around the bunsen burner (a 2 foot length of the bench).
22. Respray the marked area with 70% ethanol.
23. Close the room door until room is required for seed lot production (< 24 h).

2.1 THE LAYING DOWN OF A SEED LOT—*E. COLI*

1. Prepare room and incubators for seed lot production (SOP 2.0)
2. Set rotary incubator to 37°C and 250 rpm.
3. Wipe clean the working area around the bunsen using 70% ethanol.
4. Make 100 ml fresh 2TY/Amp/glu (SOP 9.2).
5. Remove colony plate from refrigerator.
6. Fire loop in bunsen flame.
7. Select single colony and transfer this from the plate to 2 ml 2TY/Amp/glu in a 30 ml Sterilin tube.
8. Return colony plate to refrigerator.
9. Make a control Sterilin tube with 2 ml medium—no bacteria.
10. Incubate both tubes for 5 h at 37°C, 250 rpm in rotary incubator.
11. Remove both tubes from incubator.
12. Add 2 ml 2TY/Amp/glu to both tubes.
13. Return tubes to incubator and continue incubation for a further 2 h.
14. Remove both tubes from incubator and check for growth in colony tube—should be visibly dense with bacteria.
15. Check control tube—should be clear.
16. Add 700 µl sterile glycerol to bacterial tube using sterile plugged micropipette tip.
17. Dispense into 45×100 µl aliquots in sterile screw-top microcentrifuge tubes.
18. Plunge tubes into liquid nitrogen for 10 min.
19. Store in labelled box in − 70°C freezer.

2.2 THE PRODUCTION OF ENGINEERED ANTIBODY FROM AN *E. COLI* SEED LOT

1. Thaw a single aliquot of the seed lot.
2. Transfer thawed seed lot to 10 ml 2TY/Amp/glu (SOP 9.2) in a 30 ml Sterilin Universal tube.
3. Incubate overnight at 37°C and 250 rpm in rotary incubator.
4. Add 5 ml from the overnight culture to 500 ml 2TY/Amp/low glu (SOP 9.10) in each of two 2 litre conical flasks.
5. Incubate at 37°C and 250 rpm in rotary incubator until cells reach maximum density − OD_{600} 0.9 (approx. 3 h).
6. Using a sterile pipette, add 250 mM isopropyl beta-D-Thiogalactoside (IPTG) (Boehringer Mannheim) solution (SOP 9.11) to each flask.
7. Incubate overnight at 30°C at 250 rpm to express product.
8. Pour the culture from each flask into a 500 ml Beckman centrifuge bottle and pellet the cells by centrifugation at 10.000 rpm, 4°C for 60 min.
9. Pour the supernatants into autoclaved sterile 1 litre glass bottles.
10. Filter product by using 0.8 µm, 0.45 µm and 0.2 µm filter-units (Nalgene).
11. Store at 4°C.

ALL CONTAINERS AND REAGENTS WERE STERILE. DURING PREPARATION NO OTHER BACTERIA WERE PROPAGATED IN THE AREA DEDICATED TO THE CLINICAL BATCH.

6.2 PRIMING OF THE PD10 COLUMN

Priming is performed in a sterile cabinet.

1. Clean surface with 70% ethanol.
2. Place the PD10 column in a holder, remove the cap and start the flow.

3. Add 1 ml of 3% HSA to the column using a sterile syringe. Allow the albumin solution to wash into the top of the gel bed.
4. Wash the column with 40 ml of 0.05 M sodium phosphate buffer ph 7.4.
5. Replace the column cap, leaving at least 1 ml of sodium phosphate buffer ph 7.4 above the gel bed. Replace the column stopper.

6.10 IODOGEN RADIOLABELLING METHOD

1. Make sure that gauntlets with surgical gloves are fixed over the portholes of the labelling cabinet.
2. Monitor the cabinet for radioactivity (sink, clamp, walls, floor) and note the readings on a sheet provided in the labelling suite. Monitor the floor around the cabinet and note the reading. Note the isotope batch on the sheet provided.
3. Label one bijou capacity 7 ml (Sterilin) for each of: void, antibody, iodine, filtered antibody skin test.
4. Cover the working area in the cabinet with Benchkote, shiny side down. Turn on the fan.
5. Clamp the PD10 column in the cabinet. Place the rack for collecting the column fractions under the column.
6. Spray the inside of the cabinet with 70% ethanol.
7. Place inside the cabinet the following: Hamilton syringe, sterile syringes capacity 1 ml, 2 ml, 5 ml and 10 ml, syringe needles no. 21 G, one sterile filter 0.2 μm, 3 dual end stoppers, 5 bijous labelled as mentioned above, one 250 ml Sterilin container for waste sharps, plastic bag for solid waste, sterile saline vial, sterile water vial, ethanol vial, 0.05 M phosphate buffer, steret swab.
8. Place the antibody vial and the Iodogen vial inside the cabinet.
9. Place the bijous labelled 'void', 'antibody', 'iodine' in the rack in line under the PD10 column. Remove the bijou caps.
10. Remove buffer from the top of the column bed, remove column stopper.
11. Wash the Hamilton syringe twice with ethanol followed by five washes with pyrogen-free water.
12. Swab the top of the vial containing the antibody and the top of the vial containing ^{125}iodine with a steret swab.
13. Draw up 1.5 ml of antibody in a syringe and 296 MBq ^{125}iodine in the Hamilton syringe, add both to the Iodogen vial.
14. Let react for 20 min with shaking the Iodogen vial every 5 min.
15. Transfer the reaction mix on the PD10 column using a 2 ml syringe.
16. Rinse the reaction vial with 1 ml 0.05 M phosphate buffer and add this to the column.
17. Collect the eluate in the bijou marked 'void'.
18. Add 3.5 ml of 0.05 M phosphate buffer pH 7.4 to the column and collect this eluate in the bijou marked 'antibody'.
19. Add 3.5 ml of 0.05 M phosphate buffer pH 7.4 to the column and collect this eluate in the bijou marked 'iodine'.
20. Replace the column cap and stopper.
21. Cap the bijous containing the void, antibody and iodine fractions. Count the radioactivity in each.
22. Place 0.4 ml sterile saline in a bijou, capacity 7 ml marked 'skin test'.
23. Draw up 2 ml sterile saline in a 5 ml capacity syringe and pass through a 0.2 μm filter. Take up the antibody fraction and pass through the 0.2 μm filter into the bijou labelled 'filtered antibody'.
24. Take 0.1 ml of the filtered antibody in a sterile syringe and add to skin test vial. Shake gently to mix, then draw up 0.2 ml of this solution in a 1 ml capacity syringe. Place a dual end stopper on the end of the syringe. Label the syringe 'skin test'.
25. Take up 0.2 ml of sterile saline in a 1 ml capacity syringe and place a dual end stopper on the end of the syringe. Label the syringe 'saline control'.
26. Take up the remaining filtered antibody in a sterile syringe capacity 10 ml, place a dual end stopper on the syringe. Count the radioactivity. Attach a label to the syringe with patient's name, date antibody, volume, amount of protein, isotope and radioactivity count (MBq) and operator's initials.
27. Place the syringes containing the saline control, skin test and radiolabelled antibody in a lead box carrier.
28. Clean the Hamilton syringe with ethanol and water as before and replace in its box.
29. Place all used syringe needles and sharps in a Sterilin container and seal the top with radioactive tape. Place all other waste in the designated waste bag and estimate the solid and liquid waste. Remove all waste from the cabinet. Clean the cabinet and spray with alcohol.
30. Monitor the cabinet for contamination and note the readings on the provided sheet. Monitor yourself (hands, arms, coat. shoes).
31. Monitor the thyroid the following day.

6.11 PREPARATION OF IODOGEN TUBES

Preparation is done in a sterile hood.

1. Clean surface with 70% ethanol.
2. Weigh 10 mg Iodogen (Pierce & Warriner) into a 10 ml volumetric flask.
3. Using a glass pipette, add dichloromethane up to the 10 ml graduation mark, and dissolve Iodogen.
4. Transfer 2 ml×the dissolved Iodogen to a 100 ml glass bottle using a glass pipette. Add 100 ml dichloromethane using a glass measuring cylinder. This is the working Iodogen solution.
5. Pipette 1.5 ml working Iodogen solution into each glass bijous.
6. Allow the dichloromethane solvent to evaporate overnight.
7. Seal the bijous and store, at + 4°C.

9.2 THE PREPARATION OF 2TY/AMP/GLU MEDIUM

This method generates 1 litre of medium for the culture of *E. coli*.

1. Weigh, and dispense to a 2 litre conical flask, the following components:
   16 g bacto tryptone (Difco, West Molsay, Surrey, U.K.)
   10 g yeast extract (Difco)
   5 g NaCl (Sigma, Poole, Dorset, U.K.)
2. Add 1 litre pyrogen free sterile water.
3. Swirl the flask and allow the components to dissolve.
4. Dispense the medium into clean glass 'Duran' bottles.
5. Sterilise by autoclaving.
6. Stand bottles at room temperature and allow to cool.
7. Add, using a sterile pipette, 50 ml stock 20% glucose.
8. Add, using a sterile pipette, 4 ml stock 25 mg/ml ampicillin.

9.4 THE PREPARATION OF STOCK 20% GLUCOSE

This method is for the preparation of 200 ml 20% glucose (Sigma) in water, used to supplement bacterial growth media.

1. Weigh and dispense 40 g d-glucose into a 250 ml Sterilin container.
2. Add 200 ml sterile, pyrogen-free water.
3. Mix by inversion of the container.
4. Allow the glucose to dissolve.
5. Filter the glucose solution using a 0.2 μm disposable vacuum filter (Nalgene).
6. Discard the filter chamber and close the receiver bottle using the sterile storage cap.
7. Store at 4°C.

9.5 THE PREPARATION OF STOCK AMPICILLIN

This method is for the preparation of 20 ml, 25 mg/ml solution Ampicillin (Boehringer Mannheim) used to supplement bacterial growth media.

1. Weigh and dispense 500 mg ampicillin (sodium salt) into a 30 ml Sterilin Universal tube.
2. Add 20 ml sterile, pyrogen-free water.

3. Allow the ampicillin to dissolve.
4. Withdraw the ampicillin solution into a 20 ml sterile disposable syringe.
5. Pass the solution through a 0.22 μm sterile syringe filter into a 30 ml Sterilin Universal tube.

9.10 THE PREPARATION OF 2TY/AMP/LOW GLU MEDIUM

This method generates 1 litre of medium for the culture of *E. Coli*.

1. Weigh, and dispense to a 2 litre conical flask, the following components:
   16 g bacto tryptone (Difco)
   10 g yeast extract (Difco)
   5 g NaCl (Sigma)
2. Add 1 litre pyrogen-free sterile water.
3. Swirl the flask and allow the components to dissolve.
4. Dispense the medium into 2× clean 2 litre glass flasks (500 ml/flask).
5. Cover the neck with a sponge and with aluminium foil.
6. Sterilise by autoclaving.
7. Stand bottles at room temperature and allow to cool.
8. Immediately before culturing *E. coli*, add to each flask, using a sterile pipette, 2.5 ml stock 20% stock glucose (SOP 9.4)
9. Add to each flask, using a sterile pipette, 2 ml stock (25 mg/ml) ampicillin (SOP 9.5).

9.11 THE PREPARATION OF 250 MM IPTG SOLUTION

This method generates IPTG solution sufficient for the induction of product expression in 1 litre *E. coli* culture.

1. Weigh, and dispense 238 mg IPTG into a 30 ml Sterilin Universal tube.
2. Add 4 ml sterile, pyrogen-free water.
3. After dissolving the IPTG in the water, pass the solution through a 0.22 μm sterile syringe filter into a 30 ml Sterilin Universal tube.