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Expression, purification and characterization of a mouse–human chimeric antibody and chimeric Fab′ fragment

David J. KING,* John R. ADAIR, Sarojani ANGAL, Donald C. LOW, Karen A. PROUDFOOT, J. Christopher LLOYD, Mark W. BODMER and Geoffrey T. YARRANTON
Celltech Ltd., 216 Bath Road, Slough, Berks. SL1 4EN, U.K.

B72.3 is a mouse monoclonal antibody against a tumour-associated antigen, TAG72, which recognizes breast, ovarian and colorectal tumour tissue. A mouse–human chimeric version of B72.3 has been expressed in Chinese-hamster ovary cells. This molecule has the binding specificity of B72.3 and constant regions from human IgG4. The chimeric B72.3 assembles to intact IgG and recognizes TAG72 as well as B72.3 in competitive binding assays. A proportion of the chimeric B72.3 (approx. 10%) does not form inter-heavy-chain disulphide bonds but still assembles into the IgG tetramer. This appears to be a general property of human IgG4 molecules. Co-expression of the chimeric light chain with a chimeric Fd′ gene resulted in the expression of functional Fab′. Very little $F(ab')_2$ is produced, although the Fab′ can be oxidized to the dimeric $F(ab')_2$ *in vitro*. The production of Fab′ and $F(ab')_2$ by this method is an attractive alternative to proteolytic digestion of IgG. The ability to produce these molecules in large quantities will allow the production and testing of a range of anti-tumour antibody and antibody fragment conjugates.

INTRODUCTION

The use of antibodies for imaging and therapy of human tumours has been a subject of considerable interest for many years. Research has concentrated on several aspects, including the development of suitable antibodies and the attachment of diagnostic or toxic agents to the antibody to permit tumour imaging and therapy [1]. In recent years many different monoclonal antibodies have been raised and shown to detect human tumours *in vivo* [2]. However, a number of problems remain that limit their effective clinical use.

One problem is the patient's generation of an immune response to the administered mouse monoclonal antibody [3]. This human anti-(mouse antibody) response results in the rapid clearance of the administered antibody such that the dose is drastically diminished and may not be effective. Multiple-dose therapy is probably required for effective antibody therapy, and the human anti-(mouse antibody) response prevents this [4]. One approach to this problem is the generation of human monoclonal antibodies, but the technical difficulties involved in generating human antibodies are considerable, and it is not feasible to raise high-affinity human monoclonal antibodies at present [5]. An alternative approach is the construction of mouse–human hybrid molecules by recombinant DNA techniques. A number of laboratories have now produced chimeric antibodies in which the mouse variable domains are fused to human constant regions at the gene level [6–10]. Expression of these chimeric antibodies results in production of antibody molecules that retain the specificity of the original mouse antibody but take on the effector functions of the human constant regions. These molecules would be expected to be less immunogenic in man than the original antibody, and some preliminary clinical data suggest that this is the case [11].

Antibody fragments have been widely studied as potential reagents for tumour imaging and therapy [12]. Antigen-binding fragments such as Fab and $F(ab')_2$ bind to the tumour efficiently, and serum concentrations are rapidly cleared [13]. This results in a higher signal-to-noise ratio (tumour/blood), often better tumour images and more favourable dosimetry for therapy. In addition, fragments may allow for more effective penetration into the tumour mass and improve tumour-cell killing [13]. These fragments are normally prepared by proteolytic digestion of the antibody molecule, with for example pepsin or papain. An alternative approach that might lead to a more homogeneous product is to produce an Fab′ molecule by direct expression of the light chain with a truncated heavy chain (Fd′).

The mouse monoclonal antibody B72.3 was raised against tissue from a metastatic breast carcinoma and it recognizes a large mucin-like tumour-associated glycoprotein termed TAG72 [14]. B72.3 binds to approx. 90% of colorectal carcinomas and 95% of ovarian carcinomas [15]. B72.3 has already been used in several clinical studies and shows considerable potential for the detection and future therapy of colorectal, breast and lung carcinoma [16,17]. The cloning of B72.3 heavy-chain and light-chain genes and the construction of a mouse–human chimeric molecule (chimeric B72.3) has been described [18]. This molecule is capable of localization to human tumours in a mouse xenograft model system in a similar fashion to the original mouse antibody [19]. In the present paper we describe the detailed characterization of the chimeric antibody and the direct expression, purification and characterization of an Fab′ fragment of chimeric B72.3.

MATERIALS AND METHODS

Construction of Fd′ heavy-chain gene

pJA79 is an M13tg130 vector that contains the human IgG4 heavy-chain gene modified so that the sequence from the first nucleotide after the last codon of the hinge exon to the last nucleotide of the CH3 domain inclusive has been removed by oligonucleotide-directed site-specific deletion. The hinge and 3′ untranslated region and part of the M13 sequence was isolated as a 1.1 kbp *Bgl*II fragment. This fragment was used to replace the analogous fragment in a full-length B72.3–IgG4 chimeric heavy-chain gene clone [18] to give plasmid JA94, which therefore contains a chimeric gene potentially capable of being expressed to produce a B72.3–IgG4 chimeric Fd′ protein. This plasmid was then used to recover the Fd′ gene as an *Eco*RI–*Bam*HI fragment. This fragment was cloned into the unique *Eco*RI site of the

Abbreviation used: CHO cells, Chinese-hamster ovary cells.

* To whom correspondence should be addressed.

expression vector pEE6.HCMV [20] with the use of a *Bam*HI-to-*Eco*RI oligonucleotide adaptor to give pJA97. Expression of this construct was examined in COS cells in conjunction with the chimeric light-chain expression plasmid [18]. SDS/PAGE analysis of the expression products and subsequent examination of the DNA sequence of the CH1-hinge intron suggested that splicing out of the intron was not occurring correctly, leading to the production of an aberrant heavy-chain polypeptide. Thus this intron was removed as follows. The chimeric Fd′ heavy-chain gene from JA94 was isolated as an *Eco*RI fragment and recloned into M13tg130. A *Sal*I site was introduced towards the 3′-end of the CH1 domain by oligonucleotide-directed site-specific mutagenesis to give pJA108. The introduced *Sal*I site-codes for the fifth- and fourth-from-last amino acid residues of the CH1 domain. To reconstruct the hinge on to the end of the CH1 domain four oligonucleotides were made that together code for the remainder of the CH1 domain, the hinge sequence, two in-frame stop codons and an *Eco*RI site. The oligonucleotides were assembled and cloned into M13mp11 between the *Sal*I and *Eco*RI sites in the polylinker, sequenced, re-isolated and ligated to the gene containing the *Eco*RI–*Sal*I 700 bp fragment from pJA108 to reconstruct the Fd′ gene. This Fd′ gene was then cloned into the *Eco*RI vector fragment of pJA96 to give pJA114. The genes were then tested in COS cells as before, and non-reducing SDS/PAGE analysis revealed the production of apparently assembled Fab′.

Development of stable cell lines in CHO cells

Chinese-hamster ovary (CHO-K1) cell lines expressing chimeric B72.3 light chain and co-expressing chimeric light chains and heavy chains have been constructed [19]. A stable CHO line producing cB72.3 Fab′ was produced by introducing pJA114 into the light-chain-producing cell line cL18 in the same way as the heavy-chain gene was introduced into the cB72.3 line previously described [19]. Cell lines resistant to mycophenolic acid and expressing functional Fab′ were identified by screening cell culture supernatants in an antigen-binding e.l.i.s.a. format assay (see below). Cell lines expressing between 0.1 and 20 μg of Fab′/ml were isolated. One cell line, FB9, was used for further studies.

Purification methods

Chimeric B72.3 was purified from culture supernatant by affinity chromatography on Protein A–Sepharose followed by ion-exchange chromatography as previously described [19]. cB72.3 Fab′ was purified by two methods, immunopurification and ion-exchange purification.

Immunopurification was achieved with the use of the antibody NH3/41, which has specificity for human κ chain. NH3/41 was purified from ascites fluid (kindly provided by Dr. Gordon Koch, M.R.C. Laboratory of Molecular Biology, Cambridge, U.K.) by Protein A–Sepharose affinity purification and the purified antibody was coupled to CNBr-activated Sepharose (at 5 mg/ml of Sepharose) by standard techniques. This material was then packed into a column (1.0 cm × 10 cm) and equilibrated with phosphate-buffered saline (0.15 M-NaCl/50 mM-sodium phosphate buffer, pH 7.4). CHO-cell-culture supernatant from the FB9 cell line was then applied to the column and washed with phosphate-buffered saline. Elution of Fab′ was then achieved with 4.5 M-$MgCl_2$ followed by 6 M-guanidinium chloride. Fractions containing Fab′ were then dialysed extensively into phosphate-buffered saline and concentrated by ultrafiltration.

Alternative purification of cB72.3 Fab′ was achieved by first concentrating culture supernatant 4-fold by ultrafiltration and then either diluting 4-fold with 10 mM-sodium phosphate buffer, pH 8, or dialysing into 50 mM-sodium phosphate buffer, pH 8. The sample was then loaded on to a DEAE-Sepharose ion-exchange column (2.5 cm × 30 cm) that had been pre-equilibrated with 50 mM-sodium phosphate buffer, pH 8. This column acts as a negative purification, and as such the Fab′ does not bind to the column but is present in the flow-through. The flow-through was collected, buffer-exchanged with 10 mM-sodium citrate/phosphate buffer, pH 4.5, and loaded on to an S-Sepharose column (1.0 cm × 15 cm) that had been pre-equilibrated with 10 mM-sodium citrate/phosphate buffer, pH 4.5. After washing the column with the equilibration buffer the Fab′ was eluted with a linear gradient of 0–1 M-NaCl in 10 mM-sodium citrate/phosphate buffer, pH 4.5. The pH of the eluate was adjusted to 6 and the Fab′ was concentrated by ultrafiltration. This sample was then compared with immunopurified cB72.3 Fab′.

Analysis of purified chimeric B72.3 and Fab′

Purified chimeric antibody and Fab′ were analysed on SDS/PAGE under reducing and non-reducing conditions by using the method of Laemmli [21]. Low-M_r markers were obtained from Pharmacia. Native PAGE was carried out at pH 8.9 with a stacking gel at pH 6.9 as described by Hames [22]. Gel-filtration analysis was performed on a Hewlett–Packard 1090 h.p.l.c. system with a Zorbax GF-250 column (DuPont) with a mobile phase of 0.2 M-sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min.

Antigen-binding ability was determined in an e.l.i.s.a. format assay. Micro-titre plates were coated with 0.25 μg of antigen TAG-72/well, purified according to the method of Johnson *et al.* [14] from tumour xenografts grown in nude mice. Purified chimeric B72.3 or Fab′ was diluted appropriately in sample conjugate buffer containing 0.1 M-Tris/HCl buffer, pH 7.0, 0.1 M-NaCl, 0.02% (v/v) Tween 20 and 0.2% (w/v) casein. A 100 μl portion of diluted sample was added to each well and incubated for 1 h at room temperature with gentle agitation. Following six washes of phosphate-buffered saline containing 0.2% (v/v) Tween 20, pH 7.2, a 100 μl portion of a 1/5000 dilution of standard horseradish peroxidase conjugated to goat anti-[human IgG $F(ab')_2$] antibody was added to each well and incubated for a further 1 h at room temperature. The plates were then washed six times as above, and 100 μl of substrate buffer containing 0.1 mg of tetramethylbenzidine/ml, 0.1 M-sodium citrate buffer, pH 6.0, and 0.005% (v/v) H_2O_2 was added to each well to generate a colour change. The reaction was terminated after 30 min by adjusting the solution to 2.5 M-H_2SO_4 and the absorbance was determined at 450 nm in a micro-titre-plate reader.

A competitive e.l.i.s.a. was also used to determine the ability of cB72.3 to compete with the mouse antibody. Micro-titre plates were coated with TAG-72 as above, and purified cB72.3 was serially diluted in sample conjugate buffer in a volume of 50 μl. B72.3 was biotinylated by the succinimide ester method at a molar ratio of 10:1 biotin/antibody. Biotinylated B72.3 was then diluted into sample conjugate buffer, and 50 μl portions were added to all sample, standard and zero wells. After incubation for 2 h at 37 °C the plates were washed and bound biotinylated antibody was detected by the addition of 100 μl of streptavidin–horseradish peroxidase conjugate (diluted 1/1000 in sample conjugate buffer). After 1 h incubation at room temperature the plates were washed again and horseradish peroxidase activity was detected as before.

RESULTS

Purification and characterization of chimeric B72.3

Chimeric B72.3 was purified by Protein A–Sepharose affinity chromatography as described previously [19]. Typical yields of

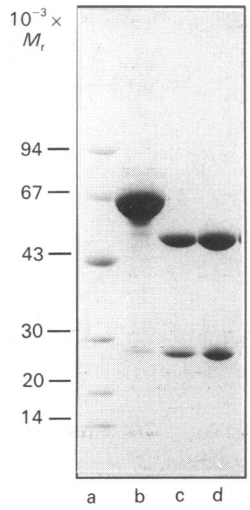

Fig. 1. SDS/PAGE of chimeric B72.3 purification

SDS/PAGE was carried out on 12% gels under reducing conditions. Lane a, marker proteins (M_r indicated); lane b, cell-culture supernatant; lane c, Protein A–Sepharose-purified chimeric B72.3; lane d, DEAE-Sepharose/Protein A–Sepharose-purified chimeric B72.3.

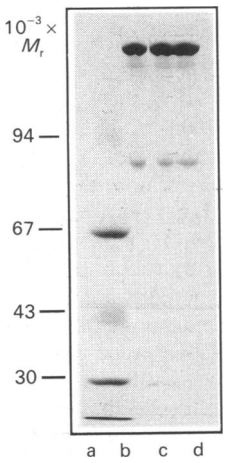

Fig. 2. Non-reducing SDS/PAGE of chimeric B72.3

SDS/PAGE was carried out on 8% gels under non-reducing conditions. Lane a, marker proteins (M_r indicated); lanes b, c and d, chimeric B72.3 from three different preparations.

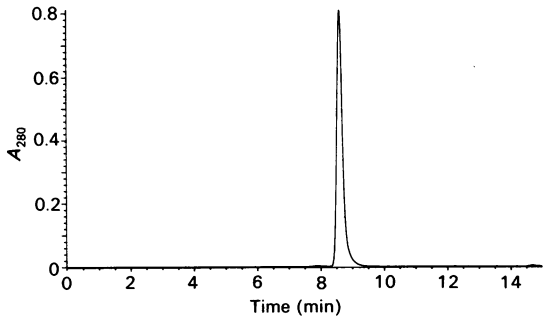

Fig. 3. Gel-filtration h.p.l.c. of chimeric B72.3

DuPont Zorbax GF-250 h.p.l.c. was run as described in the text.

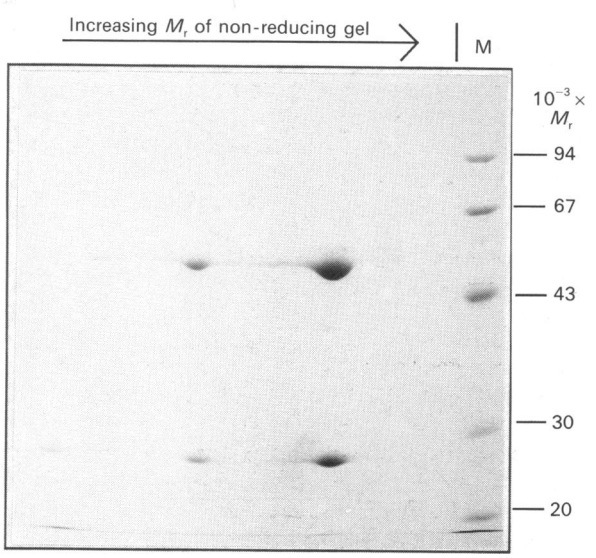

Fig. 4. Non-reducing–reducing two-dimensional SDS/PAGE of chimeric B72.3

A 30 μg portion of purified chimeric B72.3 was applied to an 8% rod gel under non-reducing conditions. After running the gel was equilibrated in 60 mM-Tris/HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (w/v) glycerol and 5% (v/v) 2-mercaptoethanol for 30 min. The rod gel was then applied to a 12% slab gel in the orientation indicated and run at 30 mA. Marker proteins (M_r indicated) were also applied to the 12% gel as indicated.

purified antibody were 20–40 mg/litre of cell-culture supernatant. This yield was consistent with the amount of antibody present in the supernatant as determined from e.l.i.s.a. assay. Reducing SDS/PAGE analysis of material purified on Protein A–Sepharose revealed a purity of approx. 90% as determined by densitometric scanning of gels stained with Coomassie Blue (Fig. 1). The expected heavy chains and light chains of the antibody were seen at apparent M_r values of 55000 and 28000 respectively, consistent with the size of the protein bands seen on expression in COS cells [18]. The antibody was further purified by ion-exchange chromatography on DEAE-Sepharose to a purity of over 95% (Fig. 1). Assembly to a 150000-M_r intact IgG molecule was demonstrated by non-reducing SDS/PAGE (Fig. 2) and gel-filtration h.p.l.c. (Fig. 3). Gel-filtration h.p.l.c. revealed a single peak with a retention time of 8.7 min, corresponding to the expected M_r value. An identical retention time was seen for chimeric B72.3 and the original mouse antibody.

On non-reducing SDS/PAGE two bands are seen for chimeric B72.3, the major band with an apparent M_r of 150000 and also a minor band at M_r approx. 80000 (Fig. 2). The minor band co-purified with the chimeric B72.3 150000-M_r species, but was not seen with mouse B72.3 either hybridoma-derived or produced as a recombinant antibody by CHO cells. An 80000-M_r species could not be seen on gel-filtration h.p.l.c. (Fig. 3) under conditions that resolve 150000-M_r and 80000-M_r proteins easily, even at loadings up to 250 μg. The proportion of the 80000-M_r band remained constant on storage and is similar in all preparations of chimeric B72.3. Western blotting revealed that the 80000-M_r band was recognized by antisera to both human γ4 chain and human κ chain. The 80000-M_r band was then shown to consist of both intact heavy chain and intact light chain of chimeric B72.3 by non-reducing/reducing two-dimensional SDS/PAGE (Fig. 4). A non-reducing rod gel of 8% acrylamide was run and it was then equilibrated in reducing buffer and run on reducing SDS/PAGE as described. Fig. 4 clearly shows that both the 150000-M_r and 80000-M_r bands seen on non-reducing

Fig. 5. Native PAGE of chimeric B72.3

Native PAGE was carried out on an 8% gel as described in the text. Lane a, chimeric B72.3; lane b, mouse B72.3.

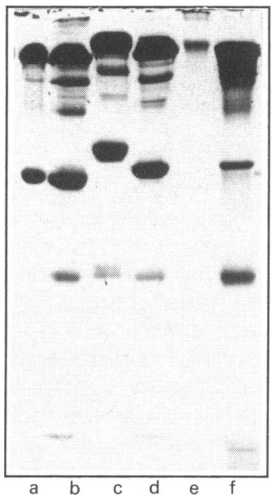

Fig. 6. SDS/PAGE of human IgG4 antibodies

SDS/PAGE was carried out on a 10% gel under non-reducing conditions. Lane a, chimeric B72.3 batch 1; lane b, chimeric B72.3 batch 2; lane c, IgG4 from human myeloma serum (from ICN Biochemicals) purified on Protein A–Sepharose; lane d, IgG4 from human myeloma serum (from Cambio) purified on Protein A–Sepharose; lane e, human IgG4 from Calbiochem; lane f, chimeric anti-4-hydroxy-3-nitrophenacetyl with human IgG4 constant regions (courtesy of Dr. M. Neuberger, M.R.C. Laboratory of Molecular Biology, Cambridge, U.K.).

SDS/PAGE are made up of the same-size heavy chains and light chains. These results suggest that the 80000-M_r band is a heterodimer consisting of one heavy chain and one light chain, i.e. a half-antibody.

Fig. 5 shows native (non-denaturing non-reducing) PAGE of chimeric B72.3. Only one band is seen, and this co-migrates with a mouse B72.3 standard. This, together with the evidence from gel filtration, suggests that the 80000-M_r protein is not present under native conditions. The 80000-M_r band may represent a proportion of chimeric B72.3 that fails to form an inter-heavy-chain disulphide bond, but under native conditions the molecule is held together as a tetramer by other (non-covalent) inter-

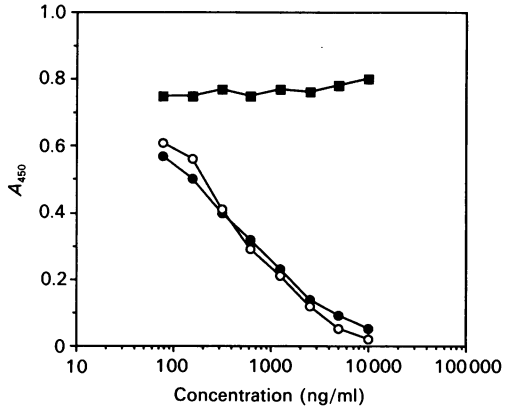

Fig. 7. Antigen-binding e.l.i.s.a. (competitive format) of chimeric B72.3

Chimeric B72.3 (○) was compared with the mouse B72.3 standard (●) and a non-specific control antibody, MOPC21 (■), by using the competitive e.l.i.s.a. described in the text.

heavy-chain interactions. It is only when these other interactions are disrupted, for example by running on non-reducing SDS/PAGE, that the two halves of the tetramer separate and are revealed with an apparent M_r of 80000.

The presence of an 80000-M_r band in other human IgG4 antibodies was also investigated. Non-reducing SDS/PAGE of several human IgG4 antibodies was carried out (Fig. 6). These include chimeric B72.3 from two different CHO cell lines, two human IgG4 myeloma patients and a chimeric anti-4-hydroxy-3-nitrophenacetyl IgG4. All of these preparations contain a similar proportion of the 80000-M_r band, which appears to exhibit similar properties to that derived from chimeric B72.3. The presence of this band is not an artifact of the purification method, as myeloma IgG4 purified by $(NH_4)_2SO_4$ precipitation also contains the 80000-M_r band (results not shown). Human IgG4 purified from serum (obtained from Calbiochem) was also examined, but this material was found to be highly aggregated and most of the protein did not enter the gel, making it impossible to determine whether this preparation contained an 80000-M_r band. We have examined antibodies of all other human subclasses including chimeric antibodies but have not yet found a preparation that gives rise to an 80000-M_r band on non-reducing SDS/PAGE (results not shown). From these results it is possible that the presence of an 80000-M_r band on non-reducing SDS/PAGE is a general property of human IgG4 molecules.

The amino acid sequence of chimeric B72.3 light chain was examined by *N*-terminal sequencing. The amino acid sequence obtained corresponded exactly to that expected from the DNA sequence. The heavy chain was found to be *N*-terminally blocked and not amenable to *N*-terminal sequencing.

The ability of chimeric B72.3 to bind the antigen was demonstrated by using an e.l.i.s.a. based on the TAG72 antigen. An identical specific activity to that of mouse B72.3 was demonstrated in a competitive antigen-binding assay. Both B72.3 and chimeric B72.3 displaced biotinylated B72.3 equally well (Fig. 7).

Purification and characterization of chimeric B72.3 Fab′

Chimeric Fab′ was expressed by CHO cells to a level of 10–20 mg/litre of supernatant in roller-bottle cultures. The chimeric Fab′ was purified by both immunopurification and an ion-exchange method. The chimeric Fab′ bound very tightly to the immunoaffinity column because of the high affinity of the antibody used. Therefore the conditions required to elute the chimeric Fab′ from the column were harsh. $MgCl_2$ (4.5 M) eluted

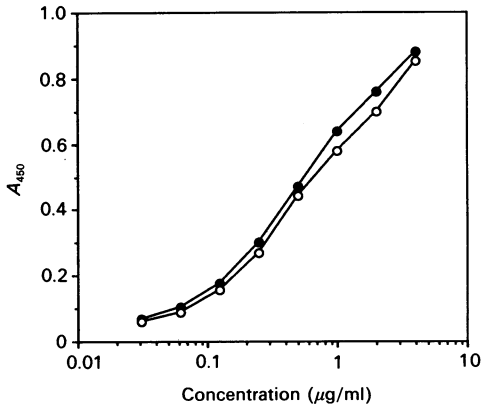

Fig. 8. Antigen-binding e.l.i.s.a. of chimeric B72.3 Fab'

Ion-exchange purified Fab' (●) was compared with immunopurified Fab' (○) by using the direct antigen-binding e.l.i.s.a. described in the text.

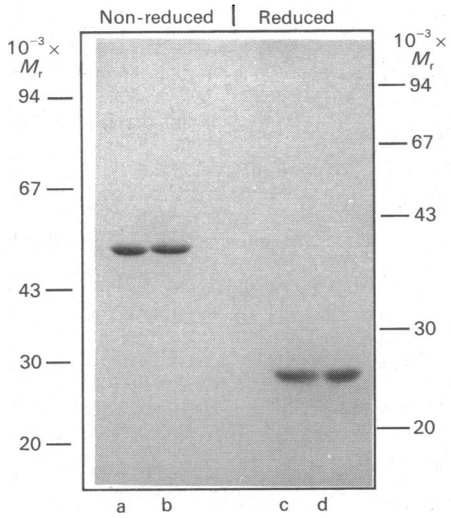

Fig. 9. SDS/PAGE of chimeric B72.3 Fab'

SDS/PAGE was carried out on a 10% gel as described in the text. Lanes a and b, immunopurified Fab' under non-reducing conditions; lanes c and d, immunopurified Fab' under reducing conditions. Positions of M_r-marker proteins are indicated.

70% of the bound material from the column, and the remaining 30% could be eluted with 6 M-guanidinium chloride. Chimeric Fab' purified by ion-exchange chromatography under mild conditions was compared with immunopurified chimeric Fab' that had been dialysed into a neutral buffer by using the TAG72-antigen-binding e.l.i.s.a. (Fig. 8). These chimeric Fab' preparations had identical activity. On SDS/PAGE analysis the immunopurified material was greater than 95% pure as judged by densitometric scanning of Coomassie Blue-stained gels (Fig. 9). Under non-reducing conditions the material was found to be predominantly assembled Fab' with an M_r of 50000, with approx. 10% of $F(ab')_2$ also seen. Under reducing conditions on a 10% acrylamide gel the light chain and truncated heavy chain of the Fab' (Fd') ran together at an M_r of 25000. Material in the supernatant before purification was also found to be mostly Fab' with a small proportion of $F(ab')_2$ by Western-blot analysis. Gel-filtration h.p.l.c. also confirmed that the material ran at an M_r of approx. 50000, and that it was co-eluted with a mouse B72.3 Fab' standard (results not shown).

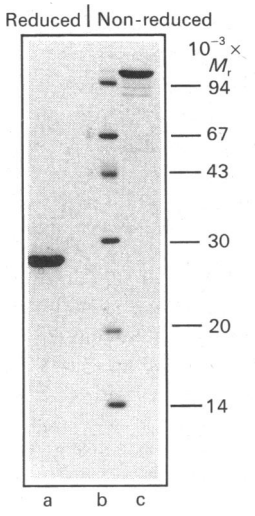

Fig. 10. SDS/PAGE of Fab' oxidized to $F(ab')_2$

SDS/PAGE was carried out on a 12% gel. Chimeric B72.3 Fab' was oxidized to $F(ab')_2$ by first partial reduction to liberate an active thiol group and then re-oxidation in the presence of dithiodipyridine to promote disulphide interchange. Fab' was treated with 50 mM-2-mercaptoethylamine for 30 min at 37 °C (in 50 mM-sodium citrate/phosphate buffer, pH 6) followed by removal of the reducing agent on a Sephadex G-25 PD10 column (Pharmacia). The partially reduced material was then incubated overnight at 37 °C with a 2-fold molar excess of 2,2'-dimercaptobipyridyl. Lane a, re-oxidized $F(ab')_2$ under reducing conditions; lane b, marker proteins (M_r indicated); lane c, re-oxidized $F(ab')_2$ under non-reducing conditions.

As only a small proportion of the purified protein was recovered as $F(ab')_2$, the state of the hinge thiol groups was tested by titration with dithiodipyridine. No reactivity was found and thus the hinge thiol groups appeared to be blocked. A gentle reduction with 2-mercaptoethylamine could regenerate active thiol groups, and this partially reduced material could be re-oxidized to form $F(ab')_2$ (Fig. 10). This suggests that thiol-group blocking takes place by the formation of a disulphide bond to an unidentified blocking agent during expression, during secretion or during the recovery process. A similar effect has also been observed during the production of antibody molecules with introduced surface cysteine residues produced in the same system [23].

DISCUSSION

The production of functional chimeric antibodies has been reported by a number of workers [6–10]. These reports have described the construction of chimeric genes and expression in a number of different myeloma cell types. We have now demonstrated high-level expression of chimeric B72.3 in CHO cells and the large-scale production of a chimeric antibody. This has enabled a more detailed biochemical characterization of the antibody than has been possible previously.

Human IgG4 molecules contain two inter-heavy-chain disulphide bonds at the hinge region. Detailed analysis of chimeric B72.3 has revealed that, although this is the case for the majority of molecules produced, a proportion do not have these bonds. This proportion is constant in different preparations of chimeric B72.3 from CHO cells, and also when expressed in a transient COS-cell system [18]. As other human IgG4 molecules from a variety of sources also contain a similar proportion of the 80000-M_r band it appears that this phenomenon is not specific to one

IgG4 molecule or one type of cell system, but appears to be a general property of human IgG4. The reason for the formation of this sub-population of human IgG4 molecules is unknown. So far we have been unable to separate this from the bulk of the chimeric B72.3, and therefore attempts to measure the functionality of the hinge thiol groups have been difficult to quantify. It appears that non-covalent Fc interactions are sufficient to maintain a tetrameric structure in solution, and it is only when these interactions are disrupted that half-molecules are formed. Other workers have also noted the appearance of an 80000-M_r band in a human IgG4 molecule [24]. The presence of this band was clearly related to the hinge region, as substitution of the IgG4 hinge sequence by that from a human IgG3 antibody resulted in the removal of the 80000-M_r band. These workers also suggested that half-molecules were not present under native conditions. Other groups have also provided data showing the presence of this band in human IgG4, but without comment [25]. Human myeloma IgG half-molecules have also been reported [26]. In this case it was thought that a deletion in the Fc region of the heavy chain resulted in a lack of non-covalent Fc interactions, leading to a decreased ability to form the normal tetrameric molecule. These studies emphasize the importance of non-covalent Fc interactions in the formation of a normal IgG tetramer.

In studies on the assembly of human IgG chains *in vitro* it has been shown that human IgG1 and IgG4 heavy chains associate by different mechanisms [27]. The re-association of IgG1 chains to a tetrameric molecule was relatively fast with a half-time of less than 70 min, and the reaction proceeded via a series of different intermediates including H_2, HL and H_2L (where H represents heavy chain and L represents light chain). For IgG4 the assembly was much slower, with a half-time of 960 min, and the only apparent intermediate was the HL half-molecule. These different kinetics and mechanisms, even though IgG1 and IgG4 have identical numbers of disulphide bonds, may be reflected in the production of incompletely disulphide-bonded molecules in the case reported here.

Antigen-binding assays of chimeric B72.3 revealed that a fully functional molecule was produced that appears to have an identical antigen-binding ability to that of B72.3 itself. Thus the chimerization of the molecule has had no detrimental effect on antigen-binding ability. Therefore we have produced a chimeric B72.3 molecule that has potential for development as a reagent for tumour imaging and therapy. Animal model studies suggest that chimeric B72.3 behaves in a similar way to B72.3 *in vivo*, showing good tumour localization [19].

The direct expression of fully functional Fab′ fragments has revealed that this approach can remove the need to produce these fragments by proteolytic digestion of the intact IgG. Proteolytic digestion is not always a straightforward process, and often the production of immunoreactive fragments is difficult [28]. Traces of enzymes may be difficult to remove owing to the formation of covalent adducts with the antibody [29], and scale-up can be difficult and may result in a heterogeneous preparation containing ragged *C*-terminal ends of the fragment. The production of chimeric Fab′ fragments by direct expression might be expected to produce a more defined protein than proteolytic digestion and certainly allows a simplified production method.

Recently bacterial systems have been developed for the expression and secretion of antibody fragments [30,31]. Expression of small Fv fragments (M_r 25000) appears to be efficient, whereas Fab fragments are produced in lower yields [32]. It should be possible to apply this technology to the expression of Fab′ fragments, although this remains to be demonstrated.

The chimeric B72.3 Fab′ produced in this study is immunoreactive and is capable of re-oxidation to $F(ab')_2$ after a gentle reduction step. The production of the molecule as the monomeric Fab′ fragment with blocked hinge thiol groups may be due to blocking of these groups during secretion of the molecule. A recent report has suggested that blocking of free thiol groups is required for efficient secretion of immunoglobulin [33].

B72.3 has been used for the detection of human colorectal, ovarian, breast and lung carcinoma [17]. The use of a chimeric molecule might allow the development of reagents that can be administered several times without losing effectiveness. The ability to produce antibody fragments and alter pharmacokinetics and the stable attachment of radioisotopes [34] or drugs to the protein in a site-specific manner [23] will be important in defining a clinically important molecule.

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REFERENCES

1. Britton, K. & Granowska, M. (1987) Cancer Surveys **6**, 247–267
2. Davis, F. M. & Rao, P. N. (1984) in Novel Approaches to Cancer Chemotherapy (Sunkara, P. S., ed.), pp. 23–92, Academic Press, New York
3. Seccamani, E., Tattenelli, M. & Mariani, M. (1989) Nucl. Med. Biol. **16**, 167–172
4. Reynolds, J. C., Del Vecchio, S. & Sakahara, H. (1989) Nucl. Med. Biol. **16**, 121–128
5. Carson, D. A. & Freimark, B. D. (1986) Adv. Immunol. **38**, 275–311
6. Sahagan, B. G., Dorai, H., Saltzgaber-Muller, J., Toneguzzo, F., Guindon, C. A., Lilly, S. P., McDonald, K. W., Morrisey, D., Stone, B. A., Davis, G. L., McIntosh, P. K. & Moore, G. P. (1986) J. Immunol. **137**, 1066–1074
7. Sun, L. K., Curtis, P., Radowicz-Szulczynska, E., Ghrayeb, J., Chang, N., Morrison, S. L. & Koprowski, H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 214–218
8. Nishimura, Y., Yokoyama, M., Araki, K., Ueda, R., Kudo, A. & Watanabe, T. (1987) Cancer Res. **47**, 999–1005
9. Liu, A. Y., Robinson, R. R., Hellström, K. E., Murray, E. D., Chang, C. P. & Hellström, I. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3439–3443
10. Beidler, C. B., Ludwig, J. R., Cardenas, J., Phelps, J., Papworth, C. G., Melcher, E., Sierzega, M., Myers, L. J., Under, B. W., Fisher, M., David, G. S. & Johnson, M. J. (1988) J. Immunol. **141**, 4053–4060
11. LoBuglio, A. F., Wheeler, R. H., Trang, J., Haynes, A., Rogers, K., Harvey, E. B., Sun, L., Ghrayeb, J. & Khazaeli, M. B. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 4220–4224
12. Larson, S. M., Carrasquilo, J. A. & Krohn, K. A. (1983) J. Clin. Invest. **72**, 2101–2114
13. Wahl, R. L., Parker, C. W. & Philpott, G. W. (1983) J. Nucl. Med. **24**, 316–325
14. Johnson, V. G., Schlom, J., Paterson, A. J., Bennet, D., Magnani, J. L. & Colcher, D. (1986) Cancer Res. **46**, 850–857
15. Stramigoni, D., Bowen, R., Atkinson, B. & Schlom, J. (1983) Int. J. Cancer **31**, 543–552
16. Sheer, D. G., Schlom, J. & Cooper, H. L. (1988) Cancer Res. **48**, 6811–6818
17. Thor, A., Ohuchi, N., Szpak, C. A., Johnston, W. W. & Schlom, J. (1986) Cancer Res. **46**, 3118–3124
18. Whittle, N., Adair, J., Lloyd, C., Jenkins, L., Devine, J., Schlom, J., Raubitshek, A., Colcher, D. & Bodmer, M. (1987) Protein Eng. **1**, 499–505
19. Colcher, D., Milenic, D., Roselli, M., Raubitshek, A., Yarranton, G., King, D., Adair, J., Whittle, N., Bodmer, M. & Schlom, J. (1989) Cancer Res. **49**, 1738–1745
20. Stephens, P. & Cockett, M. (1989) Nucleic Acids Res. **17**, 7110
21. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
22. Hames, B. D. (1981) in Gel Electrophoresis of Proteins (Hames, B. D. & Rickwood, D., eds.), pp. 1–91, IRL Press, Oxford
23. Lyons, A., King, D. J., Owens, R. J., Yarranton, G. T., Millican, A., Whittle, N. R. & Adair, J. (1990) Protein Eng. **3**, 703–708
24. Tan, L. K., Shopes, R. J., Oi, V. T. & Morrison, S. L. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 162–166

25. Fasler, S., Skarvill, F. & Lutz, H. U. (1983) Anal. Biochem. **174**, 593–600
26. Spiegelberg, H. L., Heath, V. C. & Lang, J. E. (1975) Biochemistry **14**, 2157–2163
27. Petersen, J. G. L. & Dorrington, K. J. (1974) J. Biol. Chem. **249**, 5633–5641
28. Milenic, D. E., Esteban, J. M. & Colcher, D. (1989) J. Immunol. Methods **120**, 71–83
29. Boguslawski, S. J., Ledden, D. J. & Fredrickson, R. A. (1989) J. Immunol. Methods **120**, 51–56
30. Skerra, A. & Pluckthun, A. (1988) Science **240**, 1038–1041
31. Better, M., Chang, C. P., Robinson, R. R. & Horwitz, A. H. (1988) Science **240**, 1041–1043
32. Pluckthun, A. (1990) Nature (London) **347**, 497–498
33. Alberini, C. M., Bet, P., Milstein, C. & Sitia, R. (1990) Nature (London) **347**, 485–487
34. Craig, A. S., Helps, I. M., Jankowski, K. J., Parker, D., Beeley, N. R. A., Boyce, B., Eaton, M. A. W., Millican, A. T., Millar, K., Phipps, A., Rhind, S. K., Harrison, A. & Walker, C. (1989) J. Chem. Soc. Chem. Commun. 794–796

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