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Review

Peptides as tools and drugs for immunotherapies‡

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Abstract: Peptides are essential tools for discovery and pre-clinical and pharmaceutical development of viral and cancer vaccines ('active immunotherapies') as well as for therapeutic antibodies ('passive immunotherapies'). They help to trigger and analyze immune responses at a molecular level (B-cell, T-helper and CTL epitopes). They contribute largely to the design of new vaccine candidates and to the generation of monoclonal antibodies. They are also valuable analytical reference compounds for the structural characterisation by liquid chromatography and mass spectrometry of recombinant proteins used as biopharmaceuticals. As for other therapeutic applications, formulation, solubilisation, batch consistency and stability, issues have to be addressed to allow the pre-clinical and clinical development of this class of compounds as immunotherapeutic drugs. In the present review, three case studies dealing with (i) the design and the characterisation of Respiratory Syntycial Virus subunit vaccines, (ii) peptide-based melanoma vaccines, and (iii) therapeutic monoclonal antibodies, all investigated in clinical trials, are reported and discussed. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide mapping; glycopeptide mapping; epitope mapping; mass spectrometry; subunit vaccines; monoclonal antibodies

INTRODUCTION

The humoral and cellular arms of the adapted immune response, mediated by B- and T-lymphocytes respectively, differ in the way in which they recognise antigens that are present in the extra-cellular fluid within the cells. B-cells as well as antibodies are able to recognise conformational and linear epitopes; T-cells are specialised in the recognition of peptides derived from intracellular fragmentation of larger antigens or of synthetic origin and administrated as vaccines (Figure 1). These peptides are bound by major histocompatibility complex (MHC) molecules present at the surface of antigen-presenting cells (APC) and are recognised in this context by T-cell receptors (TCRs).

B-cell Epitopes

In linear binding sites, the key amino acid residues that mediate the contacts to the antibody are located within one part of the primary structure. Such continuous epitopes, usually not exceeding 15 amino acids in length, may be identified by scanning with sets of synthetic overlapping peptides, spanning a whole antigen sequence (e.g. PEPSCAN [1] or SPOTSCAN technologies [2]). In discontinuous binding sites, also known as conformational epitopes, two or more binding regions are not contiguous in the primary structure. In this case, peptides that cover only one binding region have low affinity, which often cannot be measured by ELISA or surface plasmon resonance (SPR), and are much more difficult to delineate.

Cytotoxic T-Lymphocyte Epitopes

Foreign proteins (i.e. viral, microbial...) are processed though MHC class-I restricted peptides (8–12 amino acids 'CTL' epitopes). Antigens are degraded by the proteasome machinery in the cytosol, transported into the endoplasmic reticulum lumen by transporters and assembled with nascent MHC class-I molecules. The resulting peptide – class-I complexes are transported to the cell surface and CD8+ T-cells recognised peptides in the context of class-I MHC molecules. CD8+ cytotoxic T-lymphocytes play a pivotal role in killing virus-infected and malignant transformed cells [3,4].

T-Lymphocyte Epitopes

MHC class-II molecules present antigenic peptides (12–25 amino acid residues) to CD4+ T Helper-cells

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BIOGRAPHY

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Dr. Beck completed a PhD in Bioorganic Chemistry at the University Louis Pasteur in Strasbourg, France (1989). He went then to McGill University, Montreal, Canada as a Post-doc (Merck-Frost fellow, 1989–1990). Back to France, he became the first employee of MEDAFOR (1990–1993). In 1994 he joined Pierre Fabre Médicament at the Center of Immunology Pierre Fabre (CIPF), a biopharmaceutical R&D center focused on Therapeutic Antibodies. He first held a position of Head of Analytical Research and QC and became one year later, Head of Physico-Chemistry Department and member of CIPF's board of Directors. As a multi-activity project-team member, he contributed to the R&D of several recombinant proteins and synthetic peptides, in the field of cancer and infectious diseases, from discovery to human clinical trials. He acts also as temporary advisor for the WHO (Geneva) in the field of molecular characterization of vaccines & biologicals. He is co-inventor of 15 patents, author and co-author of more than 50 research publications and 80 communications in scientific meetings.

(Th).These peptides result from the internalisation and processing of antigens in the endocytic pathway of APCs, where they meet class-II molecules transported to the cell surface. T helper cells also play a critical role in regulating immune responses to intracellular pathogens. MHC class-II-restricted CD4+ cells expressing TCRs can be divided into TH1 and TH2 subsets according to their cytokine secretion pattern. Th1 cells produce IL-2, IFN-γ and TNF-β, whereas TH2 cells secrete IL-4, IL-5, IL-10 and IL-13. In many cases, the dominance of either Th1 or Th2 response correlates with the outcome and the severity of the disease [5].

In the case of therapeutic monoclonal antibodies generated by molecular engineering, the identification and removal of Th epitopes may help to design low immunogenic protein to limit human anti-mouse (HAMA), anti-chimeric (HACA) or anti-human antibody (HAHA) responses [6].

In the present account, three case studies are reviewed illustrating (i) the characterisation of human respiratory syncytial virus (hRSV) subunit vaccine prototypes, including primary structure, disulfide bridges assignment and B and T-cell epitopes mapping; (ii) CTL epitope-based melanoma vaccines stability and the management of their solubility, and (iii) therapeutic monoclonal antibodies primary structure assessment and post-translational modifications identification.

HUMAN RESPIRATORY SYNCYTIAL VIRUS: RECOMBINANT AND PEPTIDE VACCINE CANDIDATES

hRSV is one of the most common causes of respiratory infection in infants and in young children and also causes serious disease in immuno-compromised individuals and aged people [7–9]. Previous attempts to vaccinate children failed. In addition, a severe to fatal pulmonary disease, characterised in part by eosinophilia, was induced after RSV infection of children previously vaccinated with a formalin-inactivated whole respiratory syncytial virus (FI-RSV) preparation [10,11]. RSV encodes two major surface glycoproteins, designed G and F (Figure 2(A)). Both

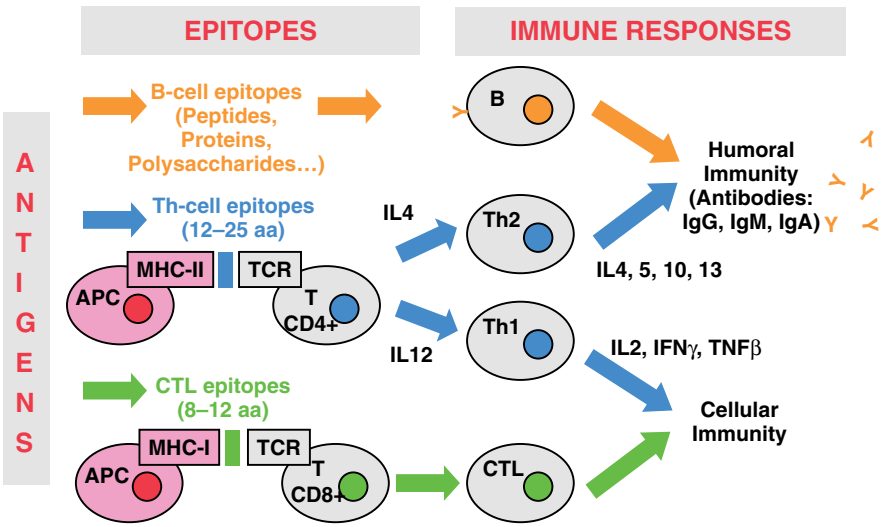

Figure 1 Epitopes and immune responses. B-cell, Cytotoxic T-Lymphocytes and T-helper epitopes.

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proteins induce neutralising antibodies and protective immunity in animal models.

Synagis/palivizumab, a humanised neutralizing monoclonal antibody directed against the F protein of RSV, was approved in 1998 for prevention of severe RSV infection of high-risk children and a higher-affinity improved antibody is in its late clinical trials [8,9]. Protection of mice has also been recently reported for a human chimeric IgG to the RSV G protein [12]. But no prophylactic vaccine is available to date, despite tremendous efforts.

BBG2Na, a recombinant subunit vaccine candidate, elicits protective immunity against RSV in rodents [13–16] in combination with different adjuvants [17,18] and by different routes of administration [19]. BBG2Na (MW: 38 672 Da) is a well defined chimeric protein composed of G2Na, the central domain of hRSV-A G protein, which contains four conserved cysteines, and BB, an albumin binding domain of the streptococcal protein G. BB was used as the carrier protein and was mapped for B and T-cell epitopes [20]. BBG2Na was produced in *E. coli* under cGMP conditions and was evaluated in clinical trials [7,21]; quantification of residual process-related impurities like *E. coli* proteins [22,23] and DNA [24,25] were performed using specific and validated methods. BBG2Na was extensively characterised both from an immuno- and a physico-chemical point of view, in large part with the help of peptides.

Physico-Chemical Characterisation: BBG2Na Primary Structure Confirmation and Disulfide Bridges determination by peptide mapping

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) analysis of a trypsin digested BBG2Na was used for identification and primary structure checking (Figure 2(B)) [26]. Trypsin cleaves the proteins at the *C*-terminal side of the basic residue lysine (except for lysine-proline motifs) and arginine. The fingerprint from a sample of each batch (Figure 2(C)) was compared with the map of the reference protein digested and analysed during the same run. Most of the peptides obtained after trypsin digestion were characterised by mass spectrometry, liquid chromatography coupled to mass spectrometry (LC-MS) and/or microsequencing by Edman degradation. Two peptides of the map were of particular interest for the quality control of BBG2Na obtained upon *E. coli* expression: peptide T37, which contains the four cysteines and which was used for disulfide bond localisation and peptide T47, the protein *C*-terminal peptide, which was isolated and used to confirm *C*-terminal integrity by Edman degradation (Figure 2(B) and (C)). Due to the tandem repeat structure of BB, several tryptic fragments were obtained two or three times. T37, the 36 amino acid residue peptides containing the four cysteines was identified (calculated mass = 4150.72 Da; measured mass = 4150.00 Da ± 0.14) on the RP-HPLC chromatogram and manually collected for further disulfide assignment studies (Figure 3(A)).

No cleavage site for traditionally used enzymes (trypsin, endoproteases Lys-C, Glu-C, Asp-N) was available in the four cysteine-containing fragment (PCSICSNNPTCWAICK). T37 was submitted to thermolysin sub-digestion. This enzyme cleaves non-specifically peptide bonds involving generally hydrophobic acids. The identified cystine pairing was in agreement with 1–4/2–3 native types of connections (Figure 3(B)). In order to confirm this result obtained for the recombinant protein-derived peptide, the three possible oxidised isomers derived from the T37 sequence were chemically synthesised in order to have analytical standards for RP-HPLC [27]. The three synthetic T37 peaks were separated by RP-HPLC and analysed by ES-MS and LC-MS after thermolysin digestion: the sT37-ox1 peptide map was in agreement with 1–3/2–4 connections, sT37-ox2 with 1–4/2–3 and sT37-ox3 with 1–2/3–4. A mixture of the three synthetic isomers was analysed using the same RP-HPLC conditions as for trypsin-digested BBG2Na and showed that the chosen gradient was able to resolve the three different oxidised isomers. This second method also confirmed that recombinant protein BBG2Na produced in *E. coli* as inclusion bodies was correctly refolded and that the two disulfide bridges corresponded only to the native 1–4/2–3 form. The three-dimensional structure of the 1–4/2–3 native cystine pattern was also confirmed by Nuclear Magnetic Resonance (NMR) with a synthetic 16 amino acid residues model peptide (Figure 2(D)). To complete the primary structure analysis based on the trypsin map, a second overlapping LC-MS map was also performed, using staphylococcal protease V8, which cleaves specifically glutamyl peptide bonds. Both maps allowed a 100% confirmation of the primary amino acid sequence deduced from the nucleic acid sequence and were used routinely during process development and scaling-up as well as during the cGMP manufacturing of BBG2Na clinical batches.

Immuno-Chemical Characterization of G2Na: B and T-cell Epitope Mapping

Immuno-chemical characterisation, including B and T-cell epitope mapping, was performed using a panel of monoclonal antibodies, several sets of overlapping synthetic peptides of different lengths and peptides conjugated to different carrier proteins. Ninety overlapping 12-mer peptides spanning residues 130–230 (G2Na) of the human RSV-A G protein were synthesised on non-cleavable derived rods for B cell epitope mapping (Figure 4(A)). The peptides were tested for their binding ability with Monoclonal Antibodies (MAbs)

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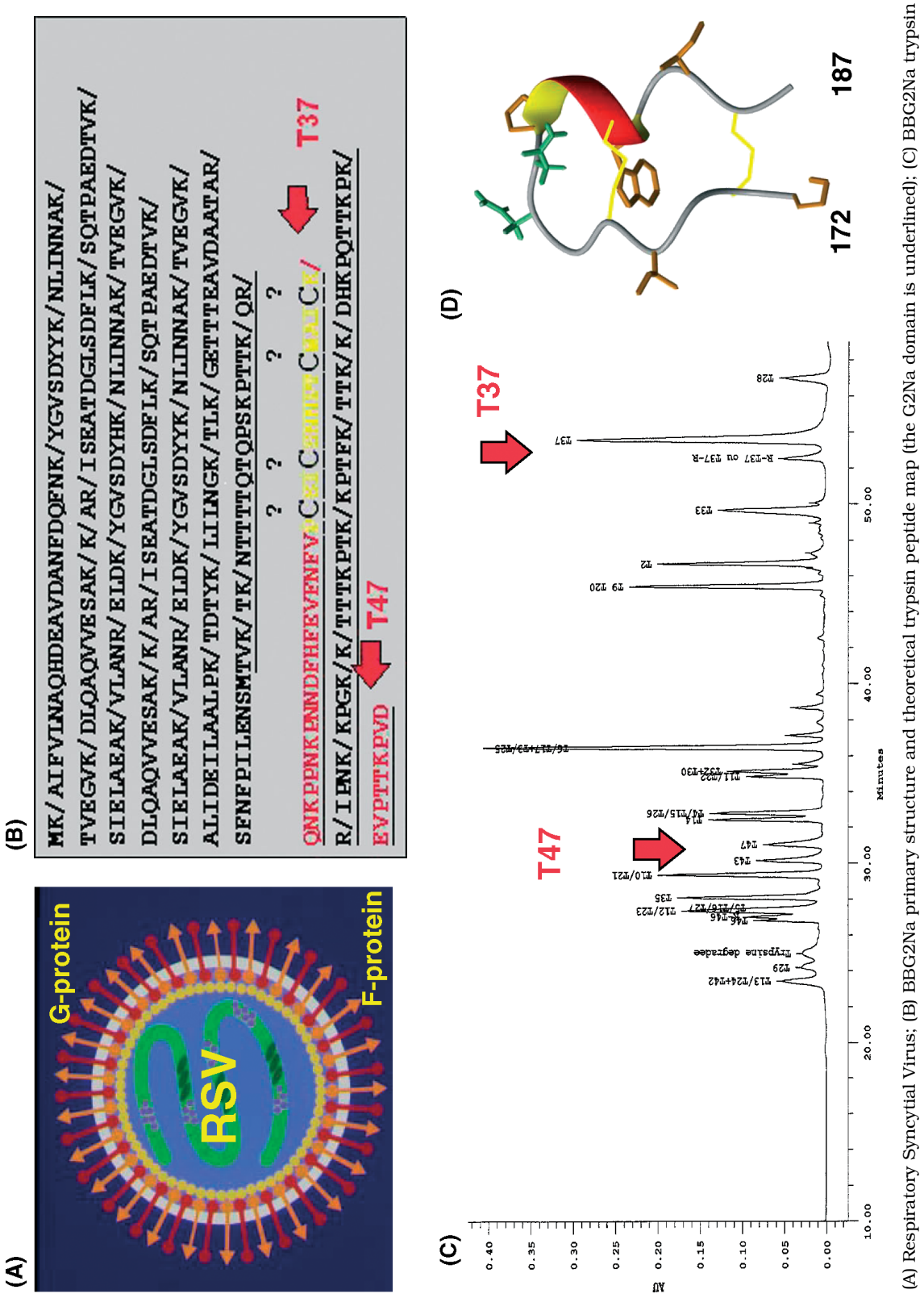

Figure 2 (A) Respiratory Syncytial Virus; (B) BBG2Na primary structure and theoretical trypsin peptide map (the G2Na domain is underlined); (C) BBG2Na trypsin RP-HPLC map and, (D) Immunodominant peptide G4 cystine nose 3-D structure.

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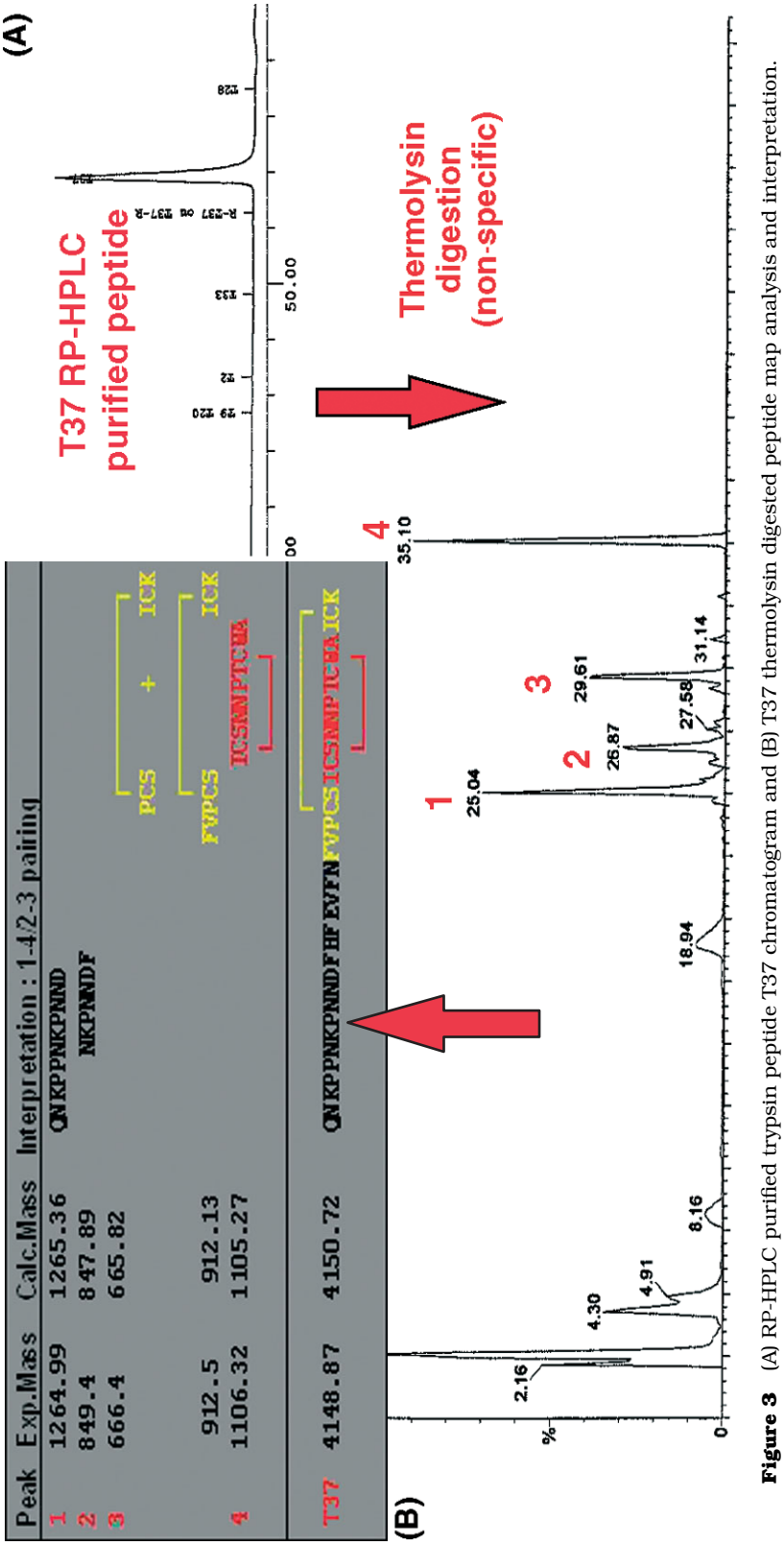

Figure 3 (A) RP-HPLC purified trypsin peptide T37 chromatogram and (B) T37 thermolysin digested peptide map analysis and interpretation.

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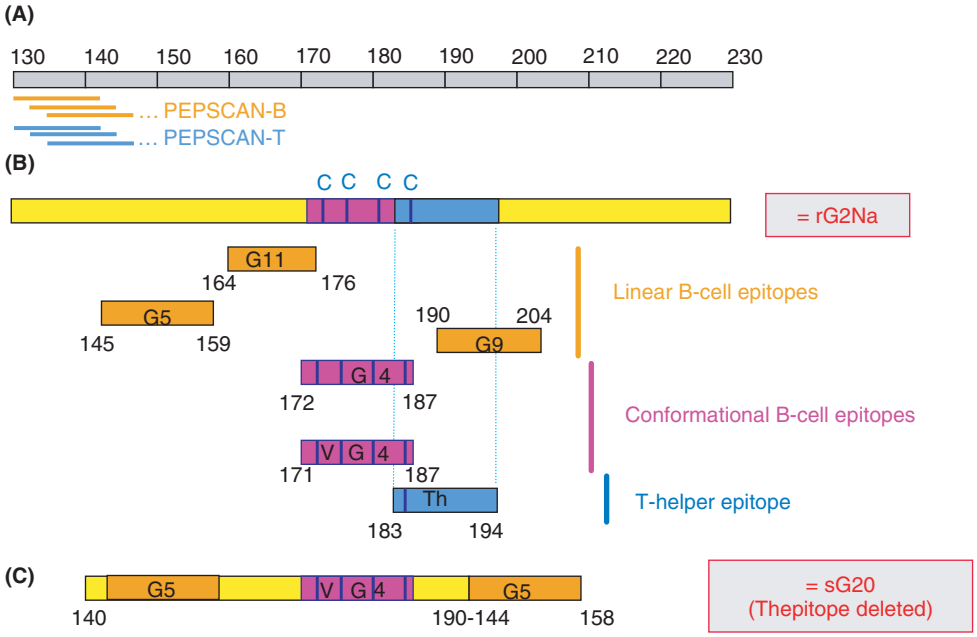

Figure 4 (A) RSV G2Na PEPSCAN-B and PEPSCAN-T (12-mer) mapping; (B) G2Na linear B protective epitopes G5, G9, G11; conformational B immunodominant epitopes G4 and VG4; T-Helper (C) Synthetic 69 amino acid peptide G20 Th epitope-delated.

or animal and patient sera by ELISA [28,29]. Moreover, all three possible oxidised isomers of the G4a hexadecamer (PCSICSNNPTCWAICK) were obtained by solid-phase synthesis of the respective reduced derivative further treated under various oxidation conditions [27]. The dimethylsulfoxide (DMSO) oxidation conditions resulted in three main peaks in a 34/42/24 ratio easily separated by preparative RP-HPLC. ES-MS of each isolated peak showed a mass in agreement with the oxidised versions of G4a (minus 4 Da). Using thermolysin as for peptide T37, new fragments were obtained for each previously isolated isomer. These fragments were analysed by LC-MS and G4a-ox1 peptide was identified as G4a(1–3/2–4) isomer, G4a-ox2 as G4a(1–4/2–3) and G4a-ox3 as G4a(1–2/3–4) [26]. Finally, the G4a (1–4)/(2–3) three-dimensional structure was investigated by NMR spectroscopy [30]. The disulfide bridges were assigned as Cys^{173}-Cys^{186} and Cys^{176}-Cys^{183} on the basis of long range nuclear Overhauser effects (NOEs), probably forming a surface exposed loop [31]. The three-dimensional solution structure of these immunodominant central conserved region of the G protein of human RSV-A was composed by a compact assembly forming a cystine nose (Figure 2(D)) similar to those of the homologous protein of the bovine RSV that may be important in receptor binding specificity [32].

Specific MAbs were also generated by immunising BALB/c mice with BBG2Na or synthetic peptides from it, conjugated to keyhole limpet haemocyanin (KLH), P40 or BB carrier proteins with two different coupling agents (glutaraldehyde or *N*-hydroxysuccimidyl bromoacetate) as previously described [33,34]. Peptide G5a(144–159) was immunogenic and protected the mice from RSV-A challenge. However, these properties were entirely dependent on the orientation of the covalent peptide coupling to the carrier proteins. Mice immunised twice with 20 µg of P40-$_{G144-159}$Cys (*C*-terminal coupling) developed moderate RSV-A serum antibody titers and their lungs were protected from RSV-A challenge. In contrast two doses of 20 µg of P40-$Cys_{G144-159}$ (*N*-terminal coupling) were poorly immunogenic and no lung protection was observed. Similar results were obtained when BB was used for conjugation, indicating that the results were independent of the carrier protein [28]. Using this panel of monoclonal antibodies and synthetic peptides, the structural elements of G2Na implicated in protective efficacy were mapped and five different B-cell epitopes (145–159, 164–176, 171–187, 172–187 and 190–204) were identified (Figure 4(B)), covering the highly conserved cystine nose domain [28,29]. Using the PEPSCAN technology, there was a noticeable absence of reactivity with the 12-mer peptides spanning the cysteine-rich region but the corresponding epitope was unambiguously identified using the correctly folded synthetic G4 hexadecapeptide.

T-cell determinant regions on an antigen were experimentally defined by testing overlapping peptides [1]. Previous studies demonstrated that a T-cell epitope was located on G2Na that was responsible for the generation of G2Na-specific T-cell clones [10]. In order to localise T-cell determinants, a set of 90 overlapping dodecapeptides offset by one residue and covering the whole G2Na sequence was synthesised on diketopiperazine (DKP) cleavable peptides [1].

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Proliferation of inguinal lymph nodes from either naive or BBG2Na-immunised mice was investigated. After activation of these cells by each peptide, proliferation of cells from BBG2Na-immunised mice was only observed with peptides covering the region 183–194 of G2Na corresponding to the sequence WAICKRIPNKKP. The cell proliferation was dose-dependent with a maximal effect for 10 μg/ml of peptide. These results indicated that a unique T-cell epitope was located on the portion 130–230 of the G protein of RSV-A. Previous experiments demonstrated that depletion in CD4-positive cells abolished T-cell proliferation observed after G2Na activation [10]. Similarly, proliferation of cells from BBG2Na-immunised mice in response to the peptides located in the region 183–194 was completely abrogated after depletion in CD4-positive cells, confirming that the proliferation observed was CD4-mediated.

Design, synthesis and characterisation of a RSV peptide vaccine candidate for the newborn

The BALB/c mouse model of RSV infection has proved to be a valuable model since a pulmonary pathology similar to that observed in the human infant treated with formalin-inactivated RSV vaccines, is induced in these mice following priming with FI-RSV or a recombinant vaccinia virus expressing RSV G protein and RSV challenge [10,35]. Attempts to elucidate the immunologic mechanisms responsible for the pulmonary pathology supported the notion that type 2 helper T (Th2) cells are involved. In addition, an association was made between the pulmonary pathology and the priming of CD4+ T-cells recognizing the T-helper peptide 183–194 of RSV G protein (Figure 4(B)).

Peptide G20 (MEF G140–190 G144–158) is a peptide of 69 amino acid residues with two disulfide bridges, which comprises multiple protective B-cell epitopes including the conformational immunodominant cystine nose [36] (Figure 4(C)). It was deleted from the T-helper cell epitope 183–194, which was found to induce pulmonary pathology after RSV challenge in mice. Interestingly, G20 was able to generate a highly protective antibody response against a RSV challenge in mice. Furthermore from a practical point of view, the synthesis, refolding and purification of this 69 amino acid residues long peptide was successfully achieved more easily than the production of the recombinant equivalent in *E. coli*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a single band for the synthetic crude sG20 and sG23 peptides. In contrast, the recombinant rG20 produced in *E. coli*, was expressed only at a low level and embedded in hundreds of host-cell proteins difficult to remove (Figure 5). Fermentation and cell culture are more suitable production systems for large protein than for peptides. This was also observed for several HIV Tat protein variants up to 100 amino acid residues long, showing that chemical synthesis may be a good alternative to recombinant *E. coli* proteins during the discovery stage of vaccines or to obtain high-quality material for structure-activity relationship studies [37]. However, for later pharmaceutical development when larger batches are needed, full protein synthesis (>100 amino-acid residues) remains too expensive when compared to cell-based production.

CTL-EPITOPE BASED TUMOUR VACCINES IN COMBINATION WITH KPOMPA ADJUVANT PROTEIN

Dozens of 9- or 10-mer peptides corresponding to cytotoxic T-cell epitopes (CTLs) have been identified and investigated in clinical trials as anti-cancer or anti-infectious vaccine candidates [3,4,38]. Furthermore, peptide-binding assays and HLA class-II transgenic mice can be used to select Th epitopes [39]. Design of peptide-based vaccines was recently extensively reviewed and discussed by Pietersz *et al.* [40].

One interesting melanoma vaccine candidate is the HLA-A2.1 restricted decapeptide ELA (ELAGIGILTV) used on its own or in combination with other tumour-associated antigens. ELA is a Melan-A/MART-1_{26-35}E27 immunodominant peptide analogue, which was shown to be more immunogenic than the parent EAA peptide (EAAGIGILTV) [41]. The differentiation antigen Melan-A/MART1 is a 118 amino acid residues membrane protein expressed in melanocytes and in melanomas [42]. Melan A/MART1 is poorly processed by melanoma cells expressing immunoproteasomes *in vitro* [43].

ELA peptide is better recognised than the parental Melan/MART-1_{26-35} peptide by tumour-infiltratring

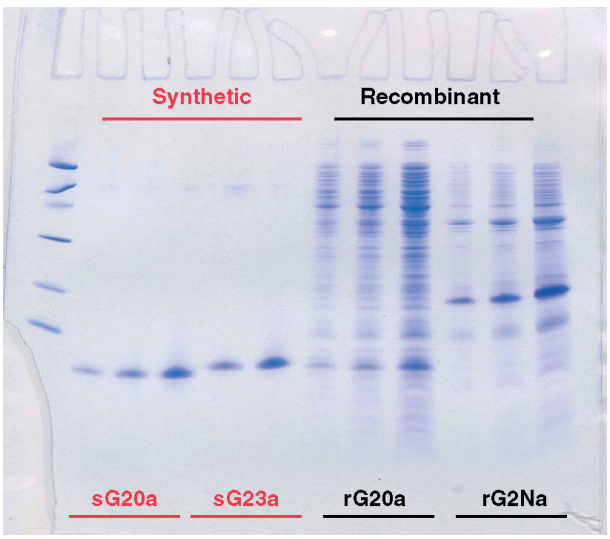

Figure 5 (A) Comparative SDS-PAGE gel stained with Coomassie blue of two crude synthetic peptides (sG20 and sG23) and to recombinant proteins (rG20 and rG2Na).

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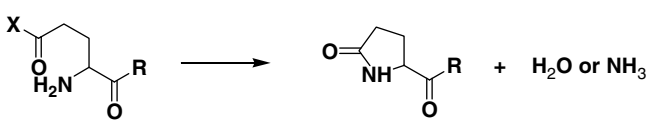
lymphocytes (TILs) as well as by Melan-A-specific CTL clones derived from melanoma patients [41]. Nevertheless, peptides administered alone are not immunogenic enough to be used as vaccines and must be combined with an adjuvant [44].

rP40, a recombinant form of *Klebsiella pneumoniae* outer membrane protein A (KpOmpA), was shown to have good antigen carrier properties for covalently-coupled viral or tumour-associated peptides [45–47] as well as for bacterial outer membrane polysaccharides [48]. Interestingly, the ELA decapeptide, mixed or chemically conjugated to rP40, has been shown to induce a strong CTL response, which made the ELA/rP40 combination a promising candidate for further clinical development [49]. As part of the preclinical development of an ELA/rP40 melanoma vaccine candidate, the stability and CTL activity studies of ELA and two peptide derivatives (PyrELA and AcELA), as well as three different ELA salt formulations (trifluoroacetate, acetate, hydrochloride) were investigated in normal and accelerated degradation conditions.

Peptide ELA Pharmaceutical Development

ELA is a peptide with an *N*-terminal glutamic acid. It has been reported that the amino group and the γ-carboxylic group of glutamic acids as well as the amino group and the γ-carboxamide group of glutamines, easily condense to form pyroglutamic derivatives (Figure 6(A)). To overcome this stability problem, several peptides of pharmaceutical interest have been developed with a pyroglutamic acid instead of *N*-terminal glutamine or glutamic acid, without loss of pharmacological properties. Unfortunately compared to ELA, the pyroglutamic acid derivative (PyrELA) as well as the *N*-terminal acetyl capped derivative (AcELA) failed to elicit CTL activity [50]. Despite the apparent minor modifications introduced in PyrELA and AcELA, these two derivatives have probably lower affinities than ELA for the specific class-I major histocompatibility complex (MHC). This was clearly illustrated by the binding of the peptides to the HLA-A2.1 molecules present on Tap negative T2 cells: ELA showed a much higher MHC binding stability than the PyrELA and the AcELA derivatives and was also confirmed by molecular modelling [49]. Taken together, all the available data demonstrated that the formation of PyrELA had to be avoided in order to preserve ELA full CTL activity. Furthermore, this stability problem was even worse in the case of clinical grade ELA, formulated as acetate salt, like most of the pharmaceutical grade peptides. Fortunately the hydrochloride salt formulation showed a higher stability (>5 fold at 37°C after 3 months) than the acetate salt, and was suitable for use in humans, and finally adopted for the cGMP batches production. Similar stability data were also obtained for MAGE-3 (EVDPIGHLY), another *N*-terminal glutamic acid containing CTL peptide in clinical development, leading us to suggest that all *N*-terminal glutamic acid and probably glutamine containing CTL peptide epitopes may be stabilised as hydrochloride salts rather than acetates.

(A)



Glutamic acid (E) : X = OH
Glutamine (Q) : X = NH2

Pyroglutamic acid (<E)

(B)

Peptide	Sequence	Mass (Da)	ES-MS (Da)	Hydro	pI	Solub. (mg/ml)
ELA	ELAGIGILTV	985.19	985.01 ± 0.06	–3.94	6.1	< 2
PyrELA	PyrELAGIGILTV	967.01	967.21 ± 0.37	–3.94	6.1	< 2
AcELA	AcELAGIGILTV	1027.20	1026.80 ± 0.06	–3.94	6.1	< 2
Flu-M1	GILGFVFTL	966.19	966.14 ± 0.12	–10.85	8.2	< 2
K-ELA	KELAGIGILTV	1113.36	1112.96 ± 0.22	–2.01	8.2	< 2
2K-ELA	KKELAGIGILTV	1241.45	1241.34 ± 0.10	–0.07	10.2	< 2
3K-ELA	KKKELAGIGILTV	1369.61	1369.56 ± 0.05	1.87	10.7	6.6
4K-ELA	KKKKELAGIGILTV	1497.78	1497.71 ± 0.08	3.81	10.9	11.5
ELA-4K	ELAGIGILTVKKKK	1497.78	1497.89 ± 0.07	3.81	10.9	10.9

Figure 6 (A) Pyroglutamic acid formation from *N*-terminal glutamic acid containing peptides or proteins; (B) ELA melanoma-associated CTL peptide and derivatives. Structure-activity relationship studies for stability, solubility, MHC class-I binding and CTL activity in a HLA-A2/kb transgenic mice model.

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Peptide ELA Aqueous Soluble Derivatives

Low aqueous solubility is a general issue for most of the HLA-A2 binding epitopes like ELA or Flu-M1 (58–66) influenza A matrix protein derived 9-mer [51] which are all highly hydrophobic. They need frequent addition of organic solvents like DMSO, unsuitable for pharmaceutical formulations. To overcome this point, derivatives of ELA and Flu-M1 peptides were designed and synthesised and their solubility investigated in phosphate buffer saline (PBS) by RP-HPLC [52]. *In vitro* binding to T2-cells was evaluated, as well as their capacity to induce CTL responses in HLA-A2/Kb transgenic mice. The peptides were mixed with KpOmpA adjuvant and carrier protein and used in different formulations [53]. Nearly a hundred derivatives of ELA and Flu-M1 peptides were synthesised and their solubility in PBS determined by RP-HPLC. The ELA and FluM1 derivatives consisted in addition of one to five times the same amino acid residue (Asp, Glu, Ser, Thr, Asn, Gln, Lys, His and Arg) either at N- or C-terminus of the peptides [52]. The peptides were characterised by RP-HPLC and by mass spectrometry (ES-MS). The HPLC peak areas of peptides in PBS (10 mg/ml) were calculated and compared to those obtained with totally soluble peptides in DMSO. This allowed the quantification of soluble peptide in PBS. Addition of at least three lysines or arginine either at *N*- or *C*-terminus of the peptides increased their solubility in PBS from 0.5 upto 10 mg/ml and from 1.7 up to 10 mg/ml for FluM1 and ELA, respectively. None of the other amino acid residues added at *N*- or *C*-terminus of FluM1 or ELA were able to improve their aqueous solubility. Evaluation of the ability of peptides to stabilise HLA-A2 molecules at the surface of T2-cells was carried out by FACS analysis (T2-cells (ATCC CRL-1992) are lymphoblasts that do not express HLA DR and that are class-II MHC antigen negative). All the peptide derivatives soluble in PBS (Figure 6(B)) were able to bind to T2-cells and were assayed for CTL activity. All ELA and FluM1 peptide lysine- or arginine-derivatives were as potent as parent peptides to induce a CTL response in HLA-A2/Kb transgenic mice [52]. These results are striking, considering that apparent minor modifications like acetylation or pyroglutamate of ELA resulted in derivatives unable to bind T2-cells and which failed to induce a CTL response in HLA-A2/Kb transgenic mice. Furthermore, the recognition of melanoma cells expressing an *N*-terminal elongated ELAGIGILTV by the natural sequence was less efficient than for the parent ELA decapeptide [54].

In summary, elongation of HLA-A2 peptide epitopes either by arginine or lysine allows both aqueous solubilisation and conservation of the specific cytotoxic potency of the parent hydrophobic peptides. These properties make these new hydrophilic HLA-A2 pro-epitopes more suitable for a pharmaceutical development as vaccine candidates. Moreover, the extension by basic amino acid residues may confer cell-penetrating properties to these ELA and FluM1 derivatives like Penetratins, HIV–Tat(48–60), Transportan and other derivatives [55]. A nice example of an effective *in vivo* inhibition of a Tat-based Erb2-targeting signal transducter was recently described in a breast cancer model [56].

THERAPEUTIC MONOCLONAL ANTIBODIES

The potential for disease-specific targeting and low toxicity profiles have made MAbs attractive and successful drug candidates not only in oncology but also in several other areas like transplantation rejection, inflammatory and autoimmune diseases, asthma and allergic disorders and against RSV. More then 20 therapeutic antibody-based products are currently approved worldwide by health authorities [57].

Many tumour antigens that are recognised by monoclonal antibodies are over-expressed on malignant cells. The binding of MAbs to growth-factor receptors on tumour cells competes for the binding of endogenous ligands and leads to the inhibition of phosphorylation of receptor-protein tyrosine kinases and inhibition of downstream signalling events. Herceptin/trastuzumab, which targets HER2 receptor [58], Erbitux/cetuximab, which targets EGFR receptor [59] and Avastin/bevacizumab, which is specific for VEGF, an angiogenesis growth factor [60], act through this mechanism. Furthermore, tumour-cells may also be killed by activation of the immune system; the crystallisable-fragment (Fc) of antibodies containing an Asn-Xxx-Thr consensus *N*-glycosylation site, is in that case crucial for antibody-dependent cell-mediated cytotoxicity (ADCC) [61,62].

IGF-1R MAbs generation and characterisation

Insulin-like growth factor type 1 receptor (IGF-1R), another tyrosine kinase receptor, has been shown to be involved in tumourigenesis and several studies indicate that a number of tumours like breast, colon and osteosarcoma, over-express this receptor [63]. The potential of selective blocking of this new target with an immunoglobulin (IgG) was demonstrated in our laboratory [64] and by other groups [63,65]. Murine antibodies were first generated in BALB/c mice by injecting a recombinant form of human IGF-1R extra-cellular domain. After various screens, 7C10 monoclonal antibody was identified and humanised by complementary determining regions (CDRs) grafting on human IgG1κ frameworks [64]. During the pre-clinical development of the recombinant humanised antibodies, Chinese Hamster Ovary (CHO) and mouse myeloma NS0 cell lines producing 7H2HM and A2CHM MAbs respectively,

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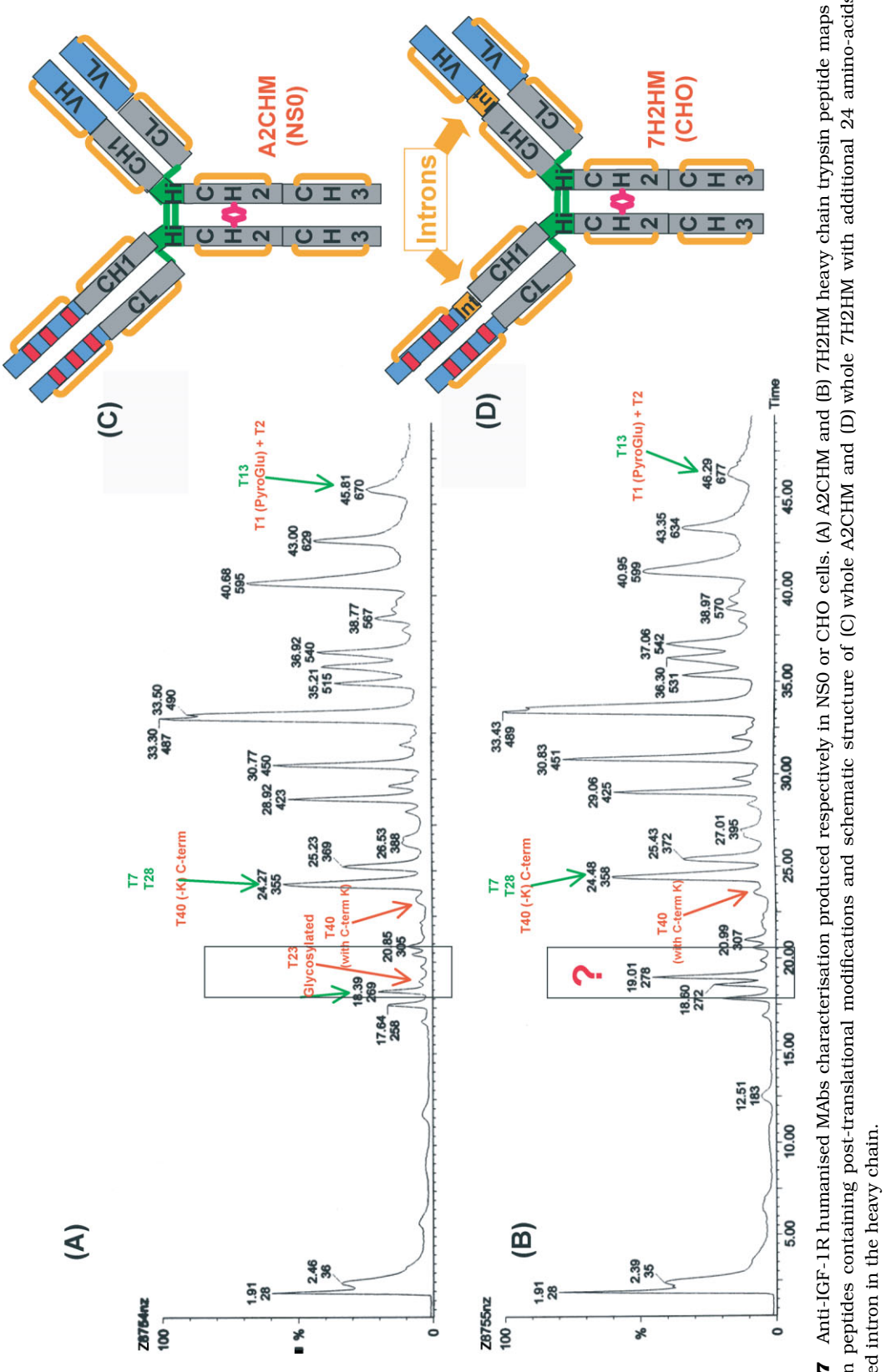

Figure 7 Anti-IGF-1R humanised MAbs characterisation produced respectively in NS0 or CHO cells. (A) A2CHM and (B) 7H2HM heavy chain trypsin peptide maps showing the main peptides containing post-translational modifications and schematic structure of (C) whole A2CHM and (D) whole 7H2HM with additional 24 amino-acids from a translated intron in the heavy chain.

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were generated [66]. 7H2HM and A2CHM were investigated for micro-heterogeneities usually described for similar IgG like *N*-terminal pyroglutamate formation as for ELA peptide (Figure 6(A)); *C*-terminal Lys clipping and Asn-linked carbohydrates [67–70]. Analytical methods based on differences in masses (SDS-PAGE and SEC) gave similar homogeneous pictures for both whole antibodies and after reduction, for both light and heavy chains. Size-Exclusion Chromatography was the method of choice to demonstrate the removal of dimers and aggregates during the purification process (in-process controls after each chromatography steps) and during long-term storage of drug substance and drug product. Isoelectric focusing (IEF) was used to determine 7H2HM and A2CHM isoelectric points (pI) and to analyse the isoform profiles. A2CHM displayed a main band with an isoelectric point of 8.7, which is a value close to pIs reported for other humanised IgG1s. As a quality control method, IEF is primarily used to ensure the identity, consistency and stability of a protein when compared to reference material. The use of IEF stained with Coomassie blue and scanned by densitometry provided a semi-quantitative approach for monitoring charged isoforms. The different patterns observed by IEF were first attributed to differences in glycosylation that may occur when two different expression systems are used. Later, this hypothesis was not confirmed by whole antibody PNGase digestion (N-Glycosidase F that cleaves oligosaccharides from N-linked glycoproteins) and IEF analysis. RP-HPLC based on differences of hydrophobicity and cationic-exchange chromatography (C-IEX) based on differences of charge, were investigated as complementary analytical methods. They clearly showed a difference of homogeneity between both antibodies. RP-HPLC was particularly useful to resolve and quantify three families of peaks for 7H2HM, which were isolated and submitted to mass spectrometry. Direct MALDI-TOF analysis, as well as ES-MS, confirmed that A2CHM yielded an experimental mass in agreement with the mass predicted from translation of the nucleotide sequence and that 7H2HM was a mixture of three families of IgGs having either the expected mass or an excess of around 3000 and 6000 Da, respectively. Mass spectrometry was also useful to demonstrate that this excess of mass was not located on the light chains but exclusively on the heavy chains: one-third of them had the expected mass and two thirds had around 3000 Da excess of mass. Trypsin map analysis of the heavy chain of 7H2HM alone was not sufficient to identify the presence of an additional peptide, which was later shown to co-elute with a mixture of Asn^{297} containing glycopeptides. Furthermore, the comparative analysis of trypsin digested RP-HPLC maps of 7H2HM and A2CHM did not show an obvious difference. Conversely, LC-MS with Total Ion Content (TIC) detection showed the presence of an important additional peak (Figure 7(A) and (B)). This peptide was isolated, submitted to mass measurement and micro-sequenced, leading to the identification of a part of a fragment of an translated intron located between heavy chain variable and constant domain (Figure 7(C) and (D)). This 24 amino-acids intron should be spliced out (high score of donor-acceptor splice site junctions) but in case of the CHO cell line and vector combination for 7H2HM expression [CHO Dux B11 (dhfr −/+) cells], correct splicing only occurred for around 33% of the heavy chains. The calculated mass of this 24-mer reduced-alkylated peptide was 2713 Da close to the around 3000 Da excess of mass measured for the main part of 7H2HM heavy chain by two mass spectrometry methods. The second part of the intron (GEWILCAWAQLCPTPR) was not detected on the trypsin map. This may be explained by the exceptionally high hydrophobic character of this trypsin-digested 30-mer peptide, in which half of the amino acid residues are hydrophobic (VFFDYWGQGTLVTVSS-GEWILCAWAQLCPT). That probably makes it difficult to be eluted from the RP-HPLC column. The retrospective interpretation of the analytical data was in agreement with the presence of this translated intron in 7H2HM compared to A2CHM, which had the expected structure. SDS-PAGE and SEC of whole antibodies, which are very large proteins, were not able to resolve a difference of mass of 4%. The ES-MS/MaxEnt spectrum of 7H2HM was interpreted as a superposition of three families of antibodies with 0, 1 and 2 translated introns, overlapping isoforms with 0, 1 and 2 lysines as for A2CHM and other IgG1. Glycosylation of Asn^{297} in both mammalian cell production systems was addressed by MALDI-TOF and LC-MS analyses after trypsin digestion. *N*-linked glycans of human IgG and of other mammalian species are mainly composed of complex biantenary structures with core fucose and often terminated with sialic acid residues [71]. Five types of monosaccharide units are usually present in human and hamster *N*-glycoproteins: galactose and mannose (= hexoses; 162.0 Da), fucose (= deoxyhexose; 146.1 Da), *N*-acetylglucosamine (= HexNac; 203.1 Da), N-acetylneuraminic Acid [= NeuAc; NANA; sialic acid; 291.1 Da] and another sialic acid in mice, *N*-glycolylneuraminic Acid [= NeuGc; NGNA; 307.1 Da]. The only position showing a consensus sequence for *N*-glycosylation (Asn-X-Ser/Thr, where X = any amino acid except Pro) in 7H2HM and A2CHM is asparagine 297 as in other IgGs. Glycopeptides containing the glycosylated Asn^{297} were generated and analysed after trypsin digestion (-R/EEQYN^{297}STYR/V-; 9-mer) by direct LC-TOF analysis of after-peak isolation by RP-HPLC and MALDI-TOF analysis. The three observed main peaks were interpreted as the so-called 'G0' ($HexNAc_4Hex_3DeoxyHex_1$), 'G1' ($HexNAc_4Hex_4DeoxyHex_1$) and 'G2' forms ($HexNAc_4Hex_5DeoxyHex_1$) (Figure 8). They were present in both antibodies but in different ratios: (61/36/3) for 7H2HM and (15/52/25) for A2CHM. Two additional galactose

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(A)

Fragment	Sequence	Calculated Mass	Exp. Mass (CHO) 7H2HM	Exp. Mass (NS0) A2CHM
HT1.2 (1–44)	Q[1]VQLQESGPGLVKPSETLSLTC[22]TVSGYSITGGYLWNWIR.QPPGK	4835.5	4817.5	4817.4
...				
HT10(102–121)	VFFDYWGQGTLVTVSSASTK	2192.1	2192.0	2192.1
Intron-fragment 1	*VFFDYWGQGTLVTVSSGEWILCAWAQLCPTPR*	*3718.3*	*n.d.**	/
Intron-fragment 2	*SHGTTSLAASTK*	*1160.3*	*1159.61*	/
HT11(122–123)	GPSVFPLAPSSK	1185.6	1185.7	1185.7
...				
HT23(293–301)	EEQYN[297]STYR	1181.3	n.d.	n.d.
	HT23+Gal0Fuc-NAcGlc	2430.2	2431.3	2431.2
	HT23+Gal1Fuc-NAcGlc	2592.2	2593.4	2593.3
	HT23+Gal0Fuc (= G0F)	2633.2	2634.4	2634.7
	HT23+Gal1Fuc (= G1F)	2795.2	2796.5	2796.6
	HT23+Gal2Fuc (= G2F)	2957.2	2958.5	2958.6
	HT23+Gal2Fuc+ α1,3–Gal	3119.2	/	3121.7
	HT23+Gal2Fuc+2 α1,3–Gal	3281.2	/	n.d.
...				
HT40(440–447)	SLSLSPGK	787.4	787.0	787.0
HT40-K (440-446)	SLSLSPG(-K[447])	659.4	659.4	659.4

(B) Glycoforms

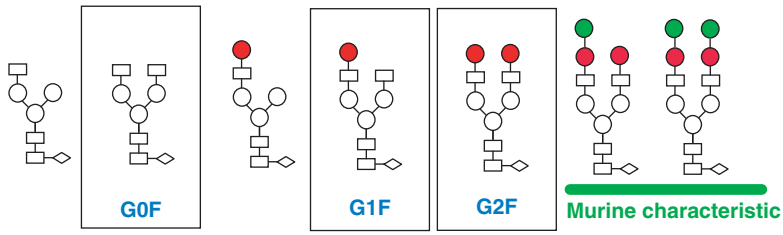

Figure 8 (A) A2CHM and 7H2HM heavy chains LC-MS peptide mapping after trypsin digestion. Post-translational modifications included *N*-terminal pyroglutamic acid cyclisation, Asn[297] glycosylation, *C*-terminal Lys clipping and an unexpected intron translation between the variable and the constant domains; (B) Structure of 7 glycoforms in agreement with experimental mass-spectrometry data, the so-called G0F, G1F and G2F are the main isoforms observed in both production systems.

α(1–3) linked glycoforms ($HexNAc_4Hex_6DeoxyHex_1$) and ($HexNAc_4Hex_7DeoxyHex_1$) not present in humans and hamsters, were also identified for A2CHM with a 4 and 2% ratio, respectively, as previously reported for antibodies produced in NS0 cells [66]. The glycopeptide LC-MS maps were specific for each protein expression system (mouse or hamster) and could be used as fingerprints to assess the NS0 or CHO origin of the humanised MAbs. The micro-heterogeneity of IgG glycans affects biological functions such as complement dependent cytotoxicity (CDC), ADCC [62], binding to various Fc receptors, binding to C1q protein and plasmatic half-life [72]. It is therefore crucial to determine precisely these post-translational modification events for full characterisation of a MAb molecule.

OVERALL SUMMARY AND CONCLUSIONS

Altogether these three case studies show that peptides are useful screening tools in the field of immunotherapeutic drugs discovery and pre-clinical and clinical development. They contribute to the design of new prophylactic and therapeutic vaccine candidates as well as to the generation of monoclonal antibodies. They are also valuable analytical reference compounds for the structural characterisation of recombinant proteins and for Quality Control during biomanufacturing and pharmaceutical development.

Fine definition of linear B-cell epitopes and T-helper was successfully achieved with PEPSCAN-B and PEPSCAN-T overlapping peptide syntheses for two RSV vaccine candidates. During the clinical trials of BBG2Na vaccine, antigen-specific serum IgG levels were determined by ELISA based on these epitopes, showing their potential as diagnostic tools. Furthermore, knowledge of the T-cell epitope leads to the design of a new vaccine candidate tailored for newborns. Disulfide pairing assignments of the immunodominant cystine nose was performed by two successive peptide maps involving trypsin and the currently less used thermolysin and LC-MS analysis, as well as with synthetic model peptides of all three possible isoforms. The 3D-structure was confirmed by molecular modelling and by NMR structure determination in solution.

CTL peptide epitopes used as vaccines and containing *N*-terminal glutamic acids or glutamine should be stabilised to allow pharmaceutical development and conservation of the MHC-class-I binding properties. Moreover, many CTL epitopes and in particular HLA-A2 are very hydrophobic. A valuable alternative for the use of solvents like DMSO consists of the *N* or *C*-terminal derivation by three to five basic amino

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acids like lysines or arginines, which greatly improves aqueous solubility and retains *in vitro* and *in vivo* CTL activity when combined for example with *K. pneumoniae* OmpA adjuvant protein or anionic adjuvant like CpG type nucleic acids.

During the generation of recombinant humanised IGF-1R MAbs, the first-line analytical methods like SDS-PAGE, SEC and IEF were not stringent enough to demonstrate the occurrence of an unexpected translational event in the case of a CHO clone. Conversely, a combination of MS and the less currently used RP-HPLC of large proteins (IgGs MW = 150 000 Da) (but routinely used by peptide chemists), helped to clearly highlight this event. This emphasises that it is essential to use orthogonal methods based on different physical and chemical separation properties, even at the early discovery stage. The elucidation of the unknown structure was greatly facilitated by the availability of a well-characterised antibody exhibiting the correct sequence (NS0 clone) and a comparison of both peptide maps by liquid chromatography and mass spectrometry detection.

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