ORIGINAL ARTICLE

Virginie Dangles · Ilan Halberstam · Antonio Scardino
Jeannine Choppin · Mireille Wertheimer · Sophie Richon
Erwann Quelvennec · Romain Moirand
Jean-Gérard Guillet · Kostas Kosmatopoulos
Dominique Bellet · Dominique Zeliszewski

Tumor-associated antigen human chorionic gonadotropin beta contains numerous antigenic determinants recognized by in vitro-induced CD8+ and CD4+ T lymphocytes

Abstract The beta subunit of human chorionic gonadotropin (hCGβ) is markedly overexpressed by neoplastic cells of differing histological origin including those present in colon, breast, prostate and bladder tumors. We have previously shown that some patients with hCGβ-producing urothelial tumors have circulating T cells that proliferate in response to hCGβ. To make a comprehensive study of hCGβ as a potential target for cancer immunotherapy, we investigated whether hCGβ peptides could induce CD4+ or CD8+ T-cell responses in vitro. By stimulating peripheral blood mononuclear cells (PBMCs) from three donors with mixtures of overlapping 16-mer synthetic peptides analogous to portions of either the hCGβ20–71 or the hCGβ102–129 region, we established six CD4+ T-cell lines that proliferated specifically in response to five distinct determinants located within these two hCGβ regions. Three antigenic determinants (hCGβ52–67, 106–121 and 114–125) were presented by HLA-DR molecules, while the two other antigenic determinants (hCGβ48–63 and 56–67) were presented by HLA-DQ molecules. Interestingly, one T-cell line specific for peptide hCGβ106–121 recognized hCGβ peptides comprising, at position 117, either an alanine or an aspartic acid residue, with the latter residue being present within the protein expressed by some tumor cells. In addition, three other hCGβ-derived peptides that exhibited HLA-A*0201 binding ability were able to stimulate CD8+ cytotoxic T cells from two HLA-A*0201 donors. These three immunogenic peptides corresponded to regions hCGβ40–48, hCGβ44–52 and hCGβ75–84. Our results indicate that the tumor-associated antigen hCGβ possesses numerous antigenic determinants liable to stimulate CD4+ and CD8+ T lymphocytes, and might thus be an effective target antigen for the immunotherapy of hCGβ-producing tumors.

V. Dangles · I. Halberstam · M. Wertheimer · S. Richon
D. Bellet · D. Zeliszewski (✉)
Université René Descartes-Paris 5,
Laboratoire d'Immunologie des Tumeurs,
ESA 8067 CNRS,
Faculté des Sciences Pharmaceutiques et Biologiques de Paris,
4 Avenue de l'Observatoire,
75006 Paris, France
E-mail: zeliszewski@pharmacie.univ-paris5.fr
Fax: +33-1-43297912 or +33-1-42115266

A. Scardino · K. Kosmatopoulos
Laboratoire INSERM U487,
Institut Gustave Roussy, Villejuif, France

J. Choppin · J.-G. Guillet
Laboratoire INSERM U445, ICGM,
Hôpital Cochin, Paris, France

E. Quelvennec
Laboratoire d'Histocompatibilité et d'Immunogénétique,
Etablissement Français du Sang de Bretagne,
Rennes, France

R. Moirand
Clinique de Maladies du Foie, Hôpital Pontchaillou,
Rennes, France

Introduction

Specific cancer immunotherapy is largely based on the identification of tumor antigens recognized by T cells [21, 33]. Many of the tumor-associated antigens (TAA) so far characterized are encoded by non-mutated genes and can be considered as self-antigens [3, 33]. CD8+ CTL (cytotoxic T-lymphocyte) responses to these TAA have been

extensively studied for many years, since CTLs have been regarded as the main antitumoral effectors and are implicated in the direct destruction of tumor cells [3, 4, 11, 12, 33]. However, it is now accepted that CD4 + T cells also play an important role in antitumor immunity through either direct or indirect mechanisms [9, 19, 27, 28, 34]. Growing evidence suggests that antitumoral CD4 + T cells might be implicated in the generation and/ or in the maintenance of potent long-term antitumor responses [18, 34]. The use of T helper (Th) epitopes in addition to CTL epitopes might enhance the therapeutic effectiveness of TAA-based tumor vaccines [19]. Thus, self-proteins overexpressed in some human tumors, and which are able to elicit a CTL and a Th response in vitro, are attractive targets for the induction of an immune response directed at these tumors in vivo. In this regard, both CTL and Th epitopes have been defined for HER-2/neu [6, 13] and p53 proteins [7, 22, 26], which are overexpressed in many tumor cells.

The beta subunit of human chorionic gonadotropin (hCGβ) is overexpressed in a high proportion of trophoblastic and non-trophoblastic tumors, as assessed by the presence of hCGβ protein in the serum of numerous cancer patients [10, 15, 16]. In urothelial cancers, hCGβ serum levels are highly correlated with the clinical stage of the disease [17]. Moreover, some normal tissues have been found to differ from their tumor counterparts in both the pattern and the level of expression of CGβ genes encoding the hCGβ subunit [1]. Indeed, this glycoprotein is encoded by a cluster of genes: CGβ6 and its allele CGβ7, and CGβ5, CGβ8, and CGβ3 and its allele CGβ9 [14, 20]. CGβ6 and CGβ7 alleles (type I CGβ genes) can be distinguished from CGβ8, CGβ5 and CGβ3/β9 alleles (type II CGβ genes) by one substitution at codon 117: type I CGβ genes code for an alanine (A) at position 117, whereas type II genes code for an aspartic acid (D) at that position [1, 14]. In normal trophoblasts, hCGβ is encoded by type II CGβ genes, while normal non-trophoblastic tissues of differing histological origin including the breast, prostate, colon, uterus and bladder tissue express low amounts of CGβ mRNA transcribed exclusively from type I CGβ genes. Interestingly, malignant transformation of the latter tissues is characterized by the transcription of type II CGβ genes in addition to that of type I genes [1]. In bladder carcinomas, increased CGβ mRNA levels are correlated with tumor progression. Moreover, the emergence of type II CGβ transcripts in breast carcinoma may have a prognostic value for determining high-risk breast cancer patients [2].

We have previously reported that some patients with hCGβ-producing bladder or testicular tumors displayed a specific peripheral proliferative response to hCGβ, while pregnant women and healthy controls did not [8]. Moreover, the carboxyl-terminal portion (CTP) 109–145 of the hCGβ subunit is currently used to treat patients with non-trophoblastic advanced cancers. Vaccination of these patients with synthetic peptide hCGβ109–145 conjugated with diphtheria toxoid (CTP37) results in a humoral anti-hCGβ response [30, 31], as well as in a slight increase in the peripheral proliferative response against the CTP37 peptide or the hCGβ protein [31]. Taken together, these data indicate that immune tolerance to this self-protein can be broken, and that hCGβ might induce both humoral and cellular responses in vivo.

In an attempt to obtain additional information on the capacity of hCGβ to elicit an anti-tumor immune response, we studied both HLA class I- and HLA class II-restricted epitopes derived from the hCGβ sequence, and determined whether the cellular immune response to this glycoprotein could be preferentially elicited against the hCGβ subunit expressed by tumor cells. Our results show that a large number of determinants within hCGβ are presented by HLA class I and HLA class II molecules, and are recognized by in vitro-induced T-cell lines.

Material and methods

Donors

Peripheral blood mononuclear cells (PBMCs) were collected from blood donors and from patients with hemochromatosis after obtaining informed consent. HLA class I and class II genomic typing of all donors was carried out by polymerase chain reaction using sequence-specific primers (PCR-SSP technique) or the reverse-dot technique. Four donors expressing frequent HLA class II molecules were selected for in vitro Th induction. One DR15 donor did not respond to any peptide. The three other donors were MAC (DRB1*0101 DQB1*0501/DRB1*0301 DQB1*02), BEL (DRB1*0103 DQB1*0501/DRB1*0401 DQB1*0301) and CHR (DRB1*0103 DQB1*0501/DRB1*0401 DQB1*0301). To study CTL responses, eight HLA-A2 donors were selected by flow cytometry using the anti-HLA-A2 monoclonal antibody (mAb) BB7.2, and the HLA-A*0201 subtype was confirmed by genomic typing. The two donors shown in the present study were DOR (A*0201 B*4001/A*03 B*07) and ROU (A*0201 B*51/A*6801 B*4012).

Cell lines

The T2 cell line (HLA-A*0201 TAP-deficient hybrid between T and B lymphomas) and the DEU B-lymphoblastoid cell line (B-LCL expressing DRB1*0401, DQB1*0301) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.04 mM 2β-mercapto-ethanol, penicillin and streptomycin.

Monoclonal antibodies

HLA-specific mAbs were used as ascitic fluid at the dilutions indicated in the figure and table legends. mAbs W6.32 (anti-HLA-class I), BB7.2 (anti-HLA-A2 and A28) and IVA12 (anti-HLA class II) were provided by F. Triebel (Département de Biologie Clinique, Institut Gustave Roussy, Villejuif, France); mAbs B7.21.1 (anti-DP) and L2 (anti-DQ) were produced by the Laboratoire d'Histocompatibilité et d'Immunogénétique (Etablissement Français du Sang, Rennes, France); and mAb L243 (anti-DR) was provided by S. Chouaib (U487 INSERM, Institut Gustave Roussy, Villejuif, France).

Synthetic peptides

For the identification of HLA class-II-restricted Th epitopes, 18 overlapping 16-mer peptides covering hCGβ regions 20–71 and

102–129 were purchased from Mimotopes (Paris, France). These synthetic peptides are offset along the protein sequence by four residues. Of note, two series of four homologous peptides spanning the hCGβ102–129 region and with either a D or an A at position 117 were synthesized. These peptides have an acetylated amino-terminus and an amide carboxyl-terminus.

For the identification of HLA-A2-restricted CTL epitopes, 13 peptides analogous to various portions of hCGβ were constructed by a solid-phase method using an automatic model 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.), and purified as previously described [23]. The identity of the peptides was checked by amino-acid composition analysis (Alpha LKB Analyzer, LKB, Rockville, Md.) and by sequencing on an automated protein sequencer (Applied Biosystems). These peptides comprised 9 or 10 amino acids.

Primary induction of hCGβ peptide-specific CD4+ T lymphocytes

Peptide-specific CD4+ T-lymphocyte lines were induced by repeated stimulations of T cells from healthy donors with PBMCs loaded with hCGβ peptide mixtures (referred to as pools A, D and P). A total of 20×10^6 PBMCs were distributed at 2×10^6 cells/well in 24-well plates, or 2×10^5 cells/well in 96-well flat-bottomed plates, and peptide pools were added at a concentration of 1 µg/ml (for each peptide). Cultures were carried out in RPMI 1640 medium supplemented with 10% heat-inactivated AB human serum (Etablissement Français du Sang, Bobigny, France), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.04 mM 2β-mercapto-ethanol, penicillin and streptomycin (T-cell medium) for 10 to 14 days. Recombinant interleukin-2 (IL-2; Valbiotech, Paris, France; also kindly donated by Chiron Technologies, Clayton, Victoria, Australia) was added at 10 IU/ml on day 7. T cells were then stimulated every week with autologous irradiated (400 Gy) PBMCs pulsed with the same peptide mixtures at responder:stimulator ratios of 1:2 or 1:3. IL-2 was added every 2 days at 20 IU/ml. Growing T cells were tested for peptide-specific proliferative activity after the third stimulation.

HLA-A2.1 binding affinity of synthetic peptides

Peptide binding to HLA-A2.1 molecules was studied by flow cytometric assay based on the use of TAP-deficient T2 cells, as described previously [29]. Briefly, T2 cells (3×10^5/ml) were incubated at 37°C for 16 h with various concentrations of peptides in serum-free medium supplemented with 100 ng/ml of human β_2-microglobulin (β_2m; Calbiochem, Meudon, France). A high-affinity peptide, HIVpol 589 (IVGAETFYV), was used in parallel as the reference peptide. Cells were then washed twice, and the expression of HLA-A2 molecules was revealed by indirect immunofluorescence using BB7.2 mAb. The relative affinity (RA) was calculated by the ratio of the concentration of each test peptide to the concentration of the reference peptide that induced 20% of HLA-A2.1 expression (100% corresponds to the expression observed with 100 µM of the reference peptide). The mean RA for each peptide was determined from three independent experiments. In all experiments, 20% of HLA-A2.1 expression induced by the reference peptide was obtained with 1–3 µM of the latter peptide.

Peptide/HLA-A2.1 complex stability determination

T2 cells (10^6/ml) were incubated overnight with 100 µM of peptides in serum-free medium supplemented with β_2m at 37°C, and washed four times. The cells were incubated with brefeldin A (10 µg/ml) for 1 h in order to block cell surface expression of newly synthesized HLA-A2 molecules, washed and incubated at 37°C for various time periods, i.e. 0, 2, 4, 6 or 8 h. Then BB7.2 mAb staining was performed. For each time point, peptide-induced HLA-A2.1 expression was evaluated by the formula: "mean fluorescence of T2 cells incubated with peptide" minus "mean fluorescence of T2 cells treated in similar conditions in the absence of peptide" [29].

Primary in vitro induction of peptide-specific CTLs

Primary induction of CTLs was performed according to two protocols: the first was modified from Cerny et al. [5]. Briefly, 40×10^6 PBMCs from HLA-A*0201 donors were stimulated in 10 wells of a 24-well culture plate, with each peptide and with tetanus toxoid (TT) at a final concentration of 1 µg/ml in 2 ml of T-cell medium. TT was added as a Th-cell stimulus. HLA-A*0201 donors were tested for their peripheral proliferative response to TT. In the second induction protocol, autologous dendritic cells pulsed with mixtures of peptides were used as stimulator cells. Dendritic cells were prepared from 50×10^6 PBMCs as previously described [24]. At day 7, they were incubated for 2 h with peptide mixtures (10 µg/ml of each peptide), washed and seeded into 24-well plates at 5×10^4 cells/well together with 10^6 PBMCs/well.

In both protocols, interleukin-7 (IL-7) was added at 50 IU/ml (Roche Diagnostics, Meylan, France) after 3 days. On day 7, cells from each well were stimulated with 10^6 autologous PBMCs which had been incubated with 10 µg/ml of peptide(s) for 2 h at 37°C, washed and irradiated. On days 1 and 4 after stimulation, 50 IU/ml IL-7 and 10 IU/ml IL-2 were added to the cultures. T cells were restimulated weekly and tested for CTL activity after the third stimulation. After four stimulations, the concentration of IL-2 was increased to 50 IU/ml. From the 7th to the 10th stimulation, cultures from donor ROU against hCGβ75–84 were set up in 96-well plates to maintain growth: each well contained 10^5 T-cell responders and 10^5 stimulators.

Proliferation assays

In vitro-induced CD4+ T cells were seeded into 96-well round-bottomed plates at 10^4 cells/well in T-cell medium. Autologous or allogeneic PBMCs pulsed or not with peptides and irradiated were added at 10^5 cells/well. For inhibition experiments with anti-HLA mAbs, the latter were added at the beginning of the assay. After 2 days of culture, tritiated thymidine (NEN Life Science Products, Paris, France) was added for 18 h at 29.6 kBq/well. Cells were then harvested, and thymidine incorporation was determined in a Microbeta 1450 counter (Wallac, Perkin-Elmer Instruments).

Cytolysis assays

Cytolytic activity was tested using a standard 4-h ^{51}Cr-release assay. The target cells were labeled with 3.7 MBq $Na_2^{51}CrO_4$ (NEN Life Science Products). Cells were washed twice and ^{51}Cr-labeled target cells were plated in 96-well conical-bottomed plates (2,000 to 5,000 cells/well). Peptides were added and incubated for 30 min at room temperature with the targets. The MHC-dependence of the lysis was controlled by inhibition experiments with anti-HLA mAbs. These mAbs were added to plated targets for 30 min at room temperature before adding the effectors at various effector:target ratios.

Flow cytometry analysis

The phenotypes of T-lymphocyte populations were analyzed by double staining with FITC- or phycoerythrin-conjugated mAbs directed against CD3, CD4, CD8 or CD56 (Coulter, Margency, France). Flow cytometry analysis was performed on an EPICS XL cytometer (Coultronics, Hialeah, Fla., USA). Selectively-gated total events corrected for non-specific binding by isotype-matched controls were considered.

Results

Identification of hCGβ-derived determinants recognized by CD4+ T cells

As hCGβ-specific proliferative responses can be observed in PBMCs from some cancer patients [8], and since both antibody and cellular responses can be induced by the vaccination of patients with a synthetic peptide analogous to the CTP of hCGβ, designated CTP37 [30, 31], we were interested in identifying hCGβ-derived determinants recognized by CD4+ Th cells.

Two hCGβ regions were chosen for this study. First, region hCGβ20–71 was investigated on the basis of our previous study of peripheral proliferative responses against hCGβ in cancer patients [8]. Indeed, using long peptides representing the entire protein we had previously shown that the proliferative response of most patients studied was directed to determinants located between residues 20 and 65. Thus 10 overlapping 16-mer peptides, offset along the sequence by four residues, enabled coverage of this region. These 10 peptides were pooled to induce peptide-specific T-cell lines from the PBMCs of three healthy donors. As shown in Fig. 1, T cells from two donors (MAC and BEL) responded to this peptide mixture (pool P) and three independent T-cell lines were established. These three T-cell lines were almost exclusively composed of CD3/CD4+ cells (data not shown). By testing the proliferative response of each T-cell line against individual peptides, it was found that the pool-P-specific T-cell line issued from PBMC MAC (line MAC-H) recognized a determinant included within the hCGβ52–67 portion. The other two T-cell lines were issued from two independent culture wells of PBMCs from the same donor (BEL): the BEL606 T-cell line recognized peptide hCGβ48–63, and the BEL642 T cell line recognized a determinant common to the hCGβ52–67 and 56–71 portions (Fig. 1).

The second hCGβ region studied here included the residue at position 117. Indeed, cancer cells may express an hCGβ subunit having a D at position 117 in addition to the hCGβ subunit found in some normal cells, and having an A at position 117, and we investigated whether CD4+ T cells could specifically recognize this amino-acid change. Thus, two mixtures of homologous peptides covering sequence hCGβ102–129 were studied: a mixture of four overlapping 16-mer peptides with an A residue at position 117 (pool A) and a mixture of four homologous peptides with a D residue at the same position (pool D). They were tested for in vitro T-cell induction from PBMCs of three healthy donors. Three CD4+ T-cell lines specific for peptides from pool A were established from PBMCs of two donors (MAC and CHR). Stimulation of PBMCs from the same donors with pool D did not enable the induction of CD4+ T-cell lines. As shown in Fig. 2, both T-cell lines MAC344 and CHR741 recognized a peptide sequence common to peptides hCGβ110–125 and hCGβ114–129. Consequently, these two T-cell lines were specific for a determinant located between residues 114 and 125. In contrast, the CD4+ T-cell line MAC304 recognized a determinant included only within peptide hCGβ106–121 (Fig. 2). We then tested whether T-cell lines MAC304, MAC344 and CHR741 distinguished between epitopes comprising either an A or a D residue at position 117. As shown in Table 1, neither the CHR741 nor the MAC344 cell line recognized peptides comprising a D at position 117, whereas, in striking contrast, MAC304 proliferated in response to homologous hCGβ106–121 peptides whatever the nature of the residue at position 117.

Identification of HLA molecules involved in the presentation of hCGβ peptides to CD4+ T-cell lines

The HLA class II molecule presenting peptides to the six CD4+ T-cell lines was first studied by blocking experiments using mAbs directed to various HLA isotypes. Table 2 shows that the proliferative activity of four T-cell lines (CHR741, MAC-H, MAC344 and MAC304) was specifically blocked by the two mAbs recognizing DR molecules, while the activity of T-cell lines BEL606 and BEL642 was blocked by the anti-DQ L2 mAb. mAb IVA12, which recognizes some – but not all – HLA-DQ molecules, partially inhibited the proliferative activity of BEL642 but did not inhibit that of BEL606.

Allogeneic PBMCs or B-LCLs sharing some DR and/or DQ molecules with the donors were then used as antigen-presenting cells (APCs) to further identify the

Fig. 1 Peptide specificity of the three T-cell lines induced by peptide pool P. Proliferative responses of pool P-specific T-cell lines MAC-H, BEL606 and BEL642 were tested in the presence of autologous irradiated PBMCs and of each individual peptide contained in pool P (10 μg/ml)
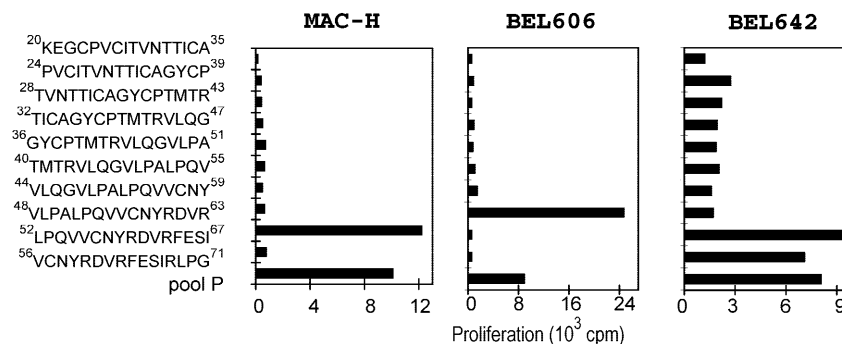
[20]KEGCPVCITVNTTICA[35]
[24]PVCITVNTTICAGYCP[39]
[28]TVNTTICAGYCPTMTR[43]
[32]TICAGYCPTMTRVLQG[47]
[36]GYCPTMTRVLQGVLPA[51]
[40]TMTRVLQGVLPALPQV[55]
[44]VLQGVLPALPQVVCNY[59]
[48]VLPALPQVVCNYRDVR[63]
[52]LPQVVCNYRDVRFESI[67]
[56]VCNYRDVRFESIRLPG[71]
pool P

MAC-H   BEL606   BEL642

Proliferation (10^3 cpm)

Fig. 2 Peptide specificity of the three T-cell lines induced by peptide pool A. Proliferative responses of pool A-specific T-cell lines CHR741, MAC344 and MAC304 were tested in the presence of autologous irradiated PBMCs and of each individual peptide contained in pool A (10 μg/ml)
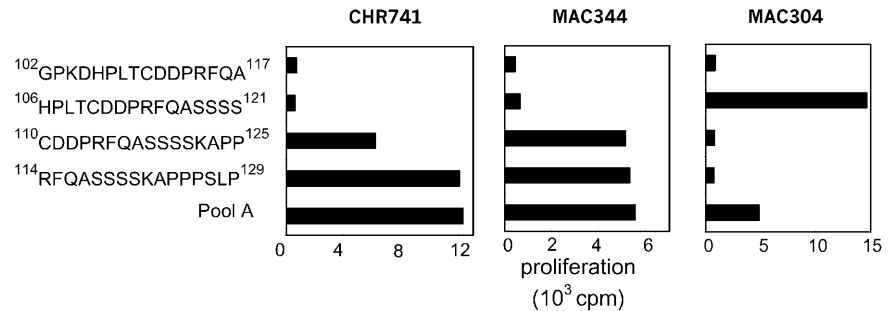
restriction molecules. The T-cell lines CHR741, BEL606 and MAC-H exhibited cytotoxic activity and could thus be tested in cytolytic assays using homozygous B-LCLs as targets. Figure 3 shows as a representative example that peptide-loaded B-LCL DEU (DRB1[*]0401, DRB4[*]0101, DQB1[*]0301) was lyzed by T-cell lines CHR741 and BEL606 in a dose-dependent manner. This result, as well as those of proliferation assays using allogeneic donors (data not shown), allowed us to conclude that T-cell line CHR741 recognized its relevant peptide in the context of a DR molecule encoded by allele DRB1[*]0401 or DRB4[*]0101, whereas BEL606 recognized its relevant peptide in the context of a DQ molecule of DQB1[*]0301 specificity. Similarly, we determined that MAC-H recognized peptide hCGβ52–67 in the context of a DRB1[*]0301- or DRB3[*]0101-encoded molecule (data not shown).

Identification of hCGβ-derived determinants recognized by HLA-A2-restricted CTLs

In order to perform a comprehensive analysis of T-cell epitopes present on the hCGβ protein and that might be implicated in antitumoral responses, it was important to study the CD8+ T-cell response to this protein in addition to the CD4+ T-cell response. To conduct this investigation, the hCGβ protein sequence was analyzed for consensus motifs found in HLA-A2.1 binding peptides. Thirteen appropriate peptides were chosen for synthesis (Table 3). Their binding affinity and the half-lives of HLA-peptide complexes were determined using a flow cytometric assay on T2 cells [29]. Indeed, the immunogenicity of HLA class-I-restricted peptides is influenced both by their binding affinity and by the stability of the HLA-peptide complex. T2 cells express only low amounts of unstable HLA-A2 molecules, and the binding of an exogenous peptide to these HLA molecules results in the upregulation of surface HLA-A2 expression that can be quantified by flow cytometric analysis. Table 3 shows that three peptides were able to combine with the HLA-A2 molecule with high affinity, namely hCGβ40–48, 44–52 and 47–55. These three peptides also formed stable peptide-HLA-A2 complexes (dissociation complex 50 [DC$_{50}$] > 2 h). Significant binding to the HLA-A2 molecule was also observed with peptides hCGβ48–56, 75–84, 78–86 and, to a lesser

Table 1 Influence of substitution of residue 117 (A to D) on T-cell recognition of hCGβ peptides. Proliferation of each T-cell line was tested in the presence of autologous PBMCs and various peptides at 10 μg/ml. Results are expressed as cpm ± SD of triplicate cultures. *ND* Not determined

Peptide tested	Proliferative activity of T-cell lines		
	CHR741	MAC344	MAC304
None	128 ± 21	432 ± 26	1,987 ± 396
110–125 (A)	7,433 ± 130	7,700 ± 2324	ND
110–125 (D)	508 ± 486	701 ± 85	ND
114–129 (A)	11,534 ± 1024	7,953 ± 1097	ND
114–129 (D)	198 ± 45	717 ± 44	ND
106–121 (A)	ND	ND	9,632 ± 287
106–121 (D)	ND	ND	7,483 ± 1060

extent, with hCGβ17–25. Finally, six peptides exhibited very weak or no affinity (RA > 20) for the HLA-A2 molecule (Table 3).

Taking into account both binding affinity and stabilizing ability, we selected seven out of 13 peptides (hCGβ40–48, 44–52, 47–55, 48–56, 75–84, 78–86 and 17–25) which were then tested for their immunogenicity. We screened these peptides for their ability to generate specific CTLs from PBMCs of naive blood donors, using two different protocols based on primary stimulation with either autologous peptide-loaded PBMCs (protocol 1) or autologous peptide mixture-pulsed dendritic cells (protocol 2). In both protocols, T cells were restimulated weekly with autologous peptide-loaded PBMCs.

By repeated stimulations of T cells from donors DOR and ROU according to protocol 1, two peptides (hCGβ40–48 and hCGβ75–84) gave rise to peptide-specific CTL lines. In addition, PBMCs from donors DOR and ROU were also stimulated according to protocol 2: dendritic cells used for induction were pulsed with either a mixture of high-affinity peptides (hCGβ40–48, 44–52, 47–55) or a mixture of intermediate-affinity peptides (hCGβ75–84, 48–56, 78–86, 17–25). Using the latter approach, peptide hCGβ44–52 was able to stimulate T cells from donor DOR, allowing the development of a third peptide-specific CTL line designated DOR44–52.

The three anti-hCGβ peptide CTL lines, mainly composed of CD3/CD8+ T cells (data not shown), were investigated for their peptide specificity and HLA restriction. As shown in Fig. 4A, these T-cell lines were

Table 2 Determination of the HLA molecule involved in the presentation of hCGβ peptides to CD4+ Th lines. Proliferative responses were tested using as APCs autologous PBMCs previously incubated with 10 μg/ml of various peptides. Specific proliferations in the absence of mAb ranged from 3,465 cpm to 16,328 cpm. L243 mAb was used at a final dilution of 1/2,700 for MAC-H or 1/8,000 for other T-cell lines. All other mAbs were tested at a final dilution of 1/1,000. Results in *bold* indicate significant inhibition (>50%). *ND* Not determined

mAb added	% Inhibition of proliferative response of T-cell lines[a]					
	CHR741	MAC-H	MAC304	MAC344	BEL606	BEL642
IVA12	**74**	**99**	**85**	**80**	0	**59**
L243	**92**	**100**	**79**	**86**	0	19
L2	0	0	14	0	**93**	**97**
B7.21.1	0	38	1	0	0	0
W6.32	34	0	7	12	ND	ND

[a]Percentages of functional inhibition were calculated according to the following formula: $\frac{cpm\,with\,peptide\,and\,mAb - cpm\,without\,peptide}{cpm\,with\,peptide\,without\,mAb - cpm\,without\,peptide}$
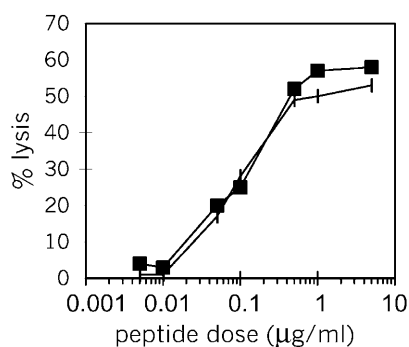
Fig. 3 Dose-dependent cytolytic activity of CHR741 and BEL606 T cell lines against a DR4.1 target. Cytolytic activity of CHR741 (†) and of BEL606 (■) T cells was tested using B-LCL DEU as the target, in the presence of titrated amounts of peptide hCGβ110–125 (CHR741) or hCGβ48–63 (BEL606). The effector:target ratio was 10:1

Table 3 Sequence, relative affinity and stabilization ability of synthetic hCGβ peptides exhibiting HLA-A*0201-specific motifs. (*RA* relative affinity compared to a reference high-affinity peptide (HIV pol 589), as described in Material and methods; DC_{50} dissociation complex 50: time required for the loss of 50% of the mean fluorescence intensity observed at 0 h)

Peptide hCGβ	Sequence	RA	DC$_{50}$ (h)
44–52	VLQVGLPAL	0.4	>6
47–55	GVLPALPQV	0.6	5
40–48	TMTRVLQGV	1.3	2–4
75–84	GVNPVVSYAV	6	2–4
78–86	PVVSYAVAL	3	2
48–56	VLPALPQVV	6	0–2
17–25	AVEKEGCPV	14	2
4–12	PLRPRCRPI	24	2–4
32–41	TICAGYCPTM	>50	2
54–62	QVVCNYRDV	>50	2
61–69	DVRFESIRL	>50	2
108–117D	LTCDDPRFQD	>20	0–2
108–117A	LTCDDPRFQA	>20	0

highly specific for their respective relevant peptide since the CTLs lyzed only T2 cells pulsed with the immunogen but did not lyze T2 cells pulsed with a strong binder-irrelevant hCGβ peptide, even though the latter was used at high concentrations. Titration experiments determined the peptide concentration needed to induce half-maximal lysis by T cells. As shown in Fig. 4A, half-maximal lysis by DOR40–48 CTL was observed with about 0.5 ng/ml (0.5 nM) of peptide. In comparison to HLA-peptide avidity of CTLs directed against viral antigens or against melanoma-associated antigens [25, 32], this CTL line exhibited high avidity for the hCGβ40–48-HLA complex. The ROU75–84 CTL line displayed lower affinity for the hCGβ75–84-HLA complex, as the half-maximal lysis of peptide-loaded T2 cells was reached at a peptide concentration of about 100 ng/ml (Fig. 4A). Finally, the DOR44–52 CTL line exhibited very strong avidity for the hCGβ44–52-HLA complex, as half-maximal lysis was observed with a peptide concentration of about 0.02 ng/ml (20 pM). Moreover, prior incubation of targets with either the anti-class I W6.32 mAb or the anti-HLA-A2 BB7.2 mAb strongly inhibited the cytotoxic activity of the three CTL lines, confirming that the differing hCGβ peptides were recognized in the context of HLA-A2.1 (Fig. 4B).

Discussion

In the present study, we identified for the first time differing peptide epitopes derived from the tumor-associated hCGβ protein and capable of inducing both CD4+ and CD8+ human cellular immune responses. We selected hCGβ as a candidate target for the antitumoral immune response on the basis of several observations. First, the hCGβ protein is overexpressed by cancer cells of differing histological types. Interestingly, the detection of the free hCGβ subunit in the serum of cancer patients is highly diagnostic of aggressive non-gonadal and non-trophoblastic malignancies [17]. Second, tumor progression is associated with changes in the pattern of transcription of the CGβ genes [1, 15], leading to the expression of an hCGβ protein with an aspartic acid at position 117 in addition to the protein with an alanine at that position. Moreover, the 109–145 carboxyl-terminal portion of the hCGβ subunit has been shown to induce humoral responses after immunization of some patients with advanced cancers [30, 31]. Finally, we have previously shown that hCGβ is capable of inducing a

proliferative peripheral cell-mediated response in some patients with hCGβ-producing tumors [8].

Several CD4+ T-cell lines were induced against synthetic peptides mimicking various portions of hCGβ and located within either the 20–71 central region or the 102–129 carboxyl-terminal region (Fig. 5). It is noteworthy that: (a) these T cell lines were elicited from PBMCs of three out of four donors; (b) two donors responded to at least two different peptides; and (c) one donor responded to peptides located in both the 20–71 and the 102–129 regions of hCGβ. Within the hCGβ20–71 region, the three antigenic determinants were localized between residues at positions 48 and 67 of the protein. Within the hCGβ102–129 region, at least two antigenic determinants were identified. Interestingly, the same short hCGβ sequence, 114–125, was recognized by CD4+ T cells that are issued from two donors expressing different DR molecules.

Taken together, these results, which are in line with the proliferative responses previously observed in cancer patients [8], strongly suggest that the tumor-associated antigen hCGβ is capable of inducing a CD4+ T-cell response directed against multiple epitopes. Moreover, it is noteworthy that at least one peptide epitope recognized by CD4+ T cells from two donors is located within the hCGβ109–145 portion that elicits humoral responses in vaccinated patients [31]. In this context, it was of interest to determine whether the residue at position 117 of hCGβ could play a role in T-cell recognition. It is noteworthy that one in vitro-induced CD4+ T-cell line was able to recognize hCGβ peptides comprising either an alanine or an aspartic acid residue at position 117 (Table 1). This observation should be put in the context of data demonstrating that cancer cells may express an hCGβ protein displaying an aspartic acid residue at position 117 [1]. Collectively, these observations might have important implications for the choice of epitopes to be included in peptide-based cancer vaccines directed to hCGβ-producing tumors.

Although CD4+ Th cells are thought to play an important role in the antitumoral immune network system [9, 27, 34], it was important to study CD8+ CTL

Fig. 4A, B Functional characteristics of the three in vitro-induced CTL lines. **A** Peptide specificity was investigated in chromium release tests against T2 targets which were incubated with titrated amounts of either hCGβ40–48 (■), hCGβ47–55 (Δ), hCGβ75–84 (□) or hCGβ44–52 (*). Effector:target ratios were 2:1 for DOR40–48, 20:1 for ROU75–84 and 5:1 for DOR44–52. **B** The HLA-A×0201 dependence of the lysis was evidenced by inhibition experiments with W6.32 and BB7.2 mAbs. The reagents (peptide and/or mAbs) added to the wells are indicated *on the left* of the figure. Effector:target ratios were the same as in **A**. Peptides hCGβ40–48 and hCGβ44–52 were used at 1 ng/ml; hCGβ75–84 was used at 1 μg/ml. The mAbs were used at a final dilution of 1/100
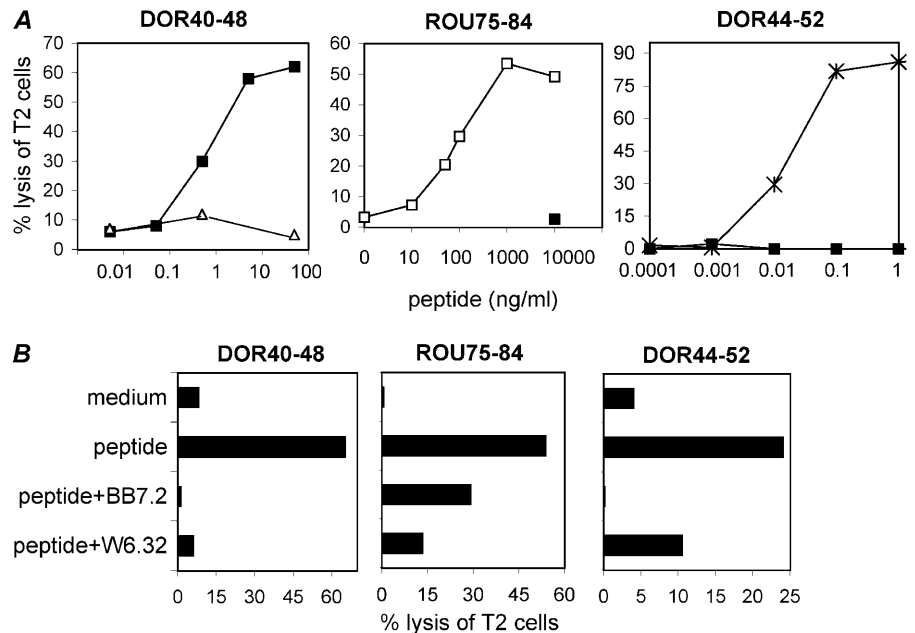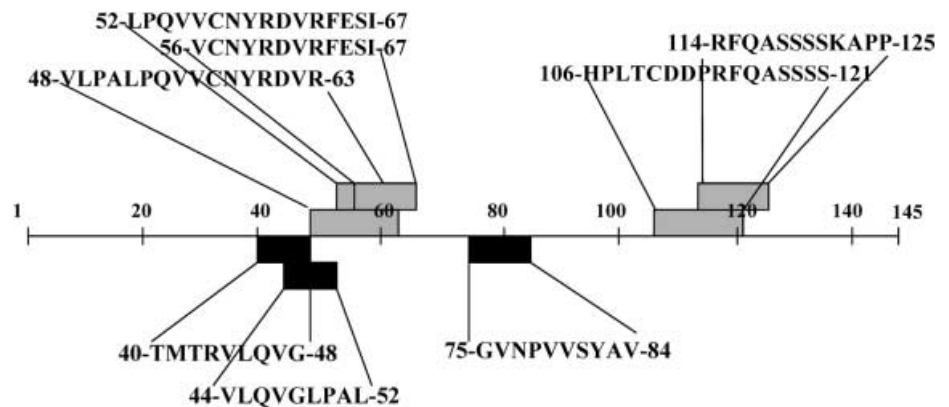

Fig. 5 Recapitulation of hCGβ-derived antigenic determinants identified in this study. *Grey boxes* represent hCGβ sequences that contain determinants recognized by CD4+ T-cell lines. *Dark boxes* represent determinants recognized by CD8+ CTL lines

responses against hCGβ peptides as these effectors can act directly against tumor cells overexpressing this protein. By repeated stimulations of PBMCs from two donors with peptides analogous to distinct regions of hCGβ, CTL responses were induced in vitro towards three hCGβ peptides presented by the HLA-A2 molecule (Fig. 5). These three peptides were among the peptides which bound to HLA-A2 with the highest affinity and/or which led to the highest stability of HLA-peptide complexes (Table 3). The two CTL lines that were induced against peptides hCGβ40–48 and hCGβ44–52 exhibited high avidities for the relevant HLA-peptide complexes, as half-maximal activities were observed with about 500 pg/ml and 20 pg/ml of peptide, respectively. Of note, the hCGβ40–67 region was particularly immunogenic and liable to induce both CD4+ and CD8+ T cell responses (Fig. 5).

The observation that at least three CTL epitopes and five Th epitopes are present in distinct regions of hCGβ strongly suggests that this tumor-associated protein might be immunogenic in cancer patients. In such patients, we and others have described the existence of hCGβ-specific proliferative and humoral responses [8, 31]. These data taken together indicate that this protein is likely to stimulate several types of immune effectors, and is thus a promising target for cancer immunotherapy. Since the hCGβ protein is overexpressed by a significant number of cancer cells of differing histological origin, the advantage of the T-cell epitopes identified on hCGβ would be that these epitopes may be shared by numerous tumors and, in particular, by non-trophoblastic tumors with more aggressive behavior [15].

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