SPANDIDOS PUBLICATIONS

Induction of multiple CD8+ T cell responses against the inducible Hsp70 employing an Hsp70 oligoepitope peptide

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Abstract. The inducible heat shock protein Hsp70 has been described as a tumour antigen being frequently overexpressed in tumours of various histologic origins, with a role in tumourigenicity, as a critical event in tumour progression. A strategy to enhance the immune response to an antigen is the identification of multiple epitopes and the induction of a polyspecific response. Applied to tumour vaccination, such a polyspecific response should lead to a more robust antitumour efficacy. The long peptide $Hsp70_{380-402}$ encompasses three nonamer peptides with a high affinity for HLA-A*0201. In a previous paper, we have shown that two of these nonamer peptides, p391 and p393, can raise CTL to recognize tumour cells overexpressing Hsp70. In the present paper, we demonstrate that the third nonamer peptide, p380, is a new epitope efficient in raising an antitumour immune response. The p380-402 polypeptide was able to induce an immune response against each of the three constituent epitopes both *in vivo* in HLA-A*0201 transgenic mice and *in vitro* with human PBMC. This polypeptide therefore constitutes an interesting candidate for the induction of multiple HLA-A*0201-restricted anti-Hsp70 antitumour CTL responses.

Introduction

The major stress-inducible heat shock protein Hsp70 is overexpressed in a majority of tumours and more particularly in breast (40-90%), lung (50-90%), colorectal (80%), and cervical carcinomas (50-75%) and osteosarcoma (40%) (1-10). Hsp70 overexpression seems to be a critical step in tumourigenicity; it protects cells from a wide range of apoptotic, necrotic and hypoxic stimuli, thus conferring a survival advantage to tumour cells (11-13). We have previously identified two HLA-A*0201 restricted epitopes derived from Hsp70, p391 and p393, which are able to raise an immune response targeting tumour cells overexpressing Hsp70, thus demonstrating the role of Hsp70 as a universal tumour antigen (14).

The lack of consistency of response to a tumour epitope among patients dampens the efficacy of vaccination strategies (15-21). However, such variability can be compensated by simultaneous vaccination against multiple determinants from the same antigen. Indeed, it ensures a more consistent immunogenicity at the global level of the antigen (22,23). A polyspecific immune response resulting from a multi-epitopic vaccination strategy can prove more efficient than the immune response triggered by a single epitope, even immunodominant (24,25). An enhancement of the response can be caused by a synergistic effect of multiple CD8 epitope-specific responses (26) or by the presence of a CD4 helper epitope when using linear polyepitopes (22,23,27). Therefore, it is advantageous for antitumour immunotherapy to use a polypeptide comprising multiple epitopes derived from the same antigen, as it generates a more consistent immune response in heterogeneously responding patients, enhances the breadth of the priming, and can lead to a stronger anti-tumour immunity.

The 22 amino-acid region from the sequence of the inducible Hsp70 spanning from amino acids 380 to 402 contains three nonamer peptides with a high affinity for HLA-A*0201. In a previous paper, we have shown that two of these nonamer peptides, p391 and p393, can stimulate CTL able to recognize tumour cells overexpressing Hsp70 (14). In the present paper, we demonstrate that the third nonamer peptide with high affinity for HLA-A*0201, p380, is a new epitope efficient in inducing an antitumour immune response in mice. We further show that the p380-402 polypeptide stimulated CTL against each of the three constituent nonamer epitopes in vaccinated HLA-A*0201 transgenic mice and from human PBMC.

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Materials and methods

Animals. HLA-A*0201 transgenic HHD mice were previously described (28).

Cells. Murine RMA-S HHD cells were previously described (28). The HLA-A*0201-expressing human tumour cells were T2 (TAP1/2 deficient), SAOS (sarcoma), MCF-7 (breast cancer); M44 and M113 (melanoma), kindly provided by Professor F. Jotereau (INSERM U463, Nantes, France); SEG (bladder carcinoma), kindly provided by Dr D. Zeliszewsli (CNRS, Paris, France). Cells were grown in RPMI-1640 or DMEM medium supplemented with 10% fetal calf serum (FCS).

Plasmids and peptides. Peptides were synthesized by Synt:em (Nîmes, France). The plasmid pCMVHsp70 containing the cDNA coding for Hsp70 was kindly provided by Dr M. Ladjimi (CNRS) (29). The HHD construct was previously described (28).

*Measurement of peptide relative affinity for HLA-A*0201*. The protocol used to measure relative affinity has been described previously (30). Briefly, T2 cells were incubated with various concentrations of peptides (0.1-100 μM) for 16 h and then stained with the mAb BB7.2 to quantify the surface expression of HLA-A*0201. For each peptide concentration, the HLA-A*0201-specific staining was calculated as the percentage of the staining obtained with 100 μM of the reference peptide $HIVpol_{589}$ (IVGAETFYV). The relative affinity (RA) is determined as: concentration of each peptide/concentration of the reference peptide that induces 20% of HLA-A*0201 expression.

Generation of CTL in HHD mice. HHD mice were injected subcutaneously with equimolarity of peptides (100 μg for nonamer peptides, and 240 μg for the p380-402 polypeptide) emulsified in incomplete Freund's adjuvant (IFA) in the presence of 150 μg of the I-Ab restricted HBVcore128 T-helper epitope. To establish long-term T cell lines, spleen cells ($5x10^7$ cells in 10 ml) at day 11 were stimulated *in vitro* with peptide (10 μM) in RPMI-1640 + 10% FCS. The CTL lines were established by weekly re-stimulation *in vitro* with irradiated spleen cells in the presence of decreasing doses of peptide (1 to 0.1 μM) and 50 U/ml IL-2 (Proleukin, Chiron Corp., Emeryville, CA, USA).

Cytotoxic assay. Murine RMA-S HHD cells or human T2 cells were used as targets for cytotoxicity as described (28). Briefly, $2.5x10^3$ ^{51}Cr-labeled targets were pulsed with peptides at 37˚C for 60 min. Effector cells (10^5) in 100 μl were then added and incubated at 37˚C for 4 h. After incubation, 100 μl of supernatant was collected and radioactivity was measured in a γ-counter. Percentage of specific lysis was determined as: Lysis = (experimental release - spontaneous release) / (maximal release - spontaneous release).

Peptide processing assay on COS-transfected cells. Simian COS-7 cells ($2.2x10^4$) were plated in flat-bottomed 96-well plates in DMEM + 10% FCS, in triplicate for each condition. Cells were transfected with 100 ng of each DNA plasmid with DEAE Dextran 18 h later. After 4 h, PBS + 10% DMSO was added for 2 min. Transfected COS cells were incubated in DMEM + 10% FCS for 40 h and then used to stimulate murine CTL in a TNFα secretion assay.

TNFα secretion assay. Transfected COS-7 cells at day 4 and human tumour cells (10^5 cells/well) suspended in 50 μl of RPMI + 10% FCS were used as stimulating cells. When necessary, they were incubated with 10 μM peptide for 2 h. T cells ($5x10^4$) were then added in 50 μl RPMI 10% FCS and incubated for 6 h. Each condition was tested in triplicate. Of the supernatant, 50 μl was collected to measure TNFα as previously described (31).

Western blot analysis of inducible Hsp70 expression by tumour cells. Cellular samples were rinsed in PBS, and then lyzed for 30 min at 4˚C in 125 mM Tris/HCl pH 6.8 containing 3 mM EDTA, 10 mM NaF and 0.1% sulfobetain 14. After centrifugation (13000 rpm, 10 min, 4˚C), supernatants were quantified for their protein content using BCA assay (Pierce, Rockford, IL, USA). Of proteins, 30 μg were analyzed using anti-inducible Hsp70 mAb (clone SPA-810, dilution 1:1000; Stressgen, Victoria, Canada), anti-actin mAb (dilution 1:2000; Chemicon, Temecula, CA, USA), a peroxidase-conjugated anti-mouse Ig (Sigma, Oakville, Canada), and the ECL kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Relative protein expression was quantitated with the Photo-Capt software (Vilber-Lourmat, Marne-la-Vallée, France).

Murine IFN-γ Elispot. Multiscreen nitrocellulose 96-well plates (Millipore, Bedford, MA, USA) were coated with 10 μg/ml of monoclonal antibody (mAb) specific for murine IFN-γ (BD, Franklin Lakes, NJ, USA) for 1 h at 37˚C. After saturating the wells with RPMI supplemented with 10% FCS (1 h, 37˚C), $2x10^6$ splenocytes/well were seeded in triplicate and stimulated with peptide. Concanavalin-A (Sigma) added at 10 μg/ml was used as a positive control for stimulation. Plates were incubated for 20 h at 37˚C in 5% CO_2, washed, and then incubated with biotinylated anti-IFN-γ mAb (BD). After 2h of incubation, the plates were washed, stained for 1 h with a Vectastain Elite kit (Ab Cys, Paris, France), and revealed with aminoethyl carbazol at 1 mg/ml in 50 mM acetate buffer with 0.015% H_2O_2 (all from Sigma). Counting of spot-forming cells was performed using a computer-assisted microscope (Carl Zeiss, Le Pecq, France). CD8 staining (PharMingen, Mississauga, Canada) of splenocytes enabled us to calculate the frequency of IFN-γ secreting cells for 10^5 $CD8^+$ cells. Specific wells were considered positive when their count was greater than the background count determined with the irrelevant peptide $HIVpol_{589}$ plus three times standard deviation.

Generation of CTL from human peripheral blood mononuclear cells (PBMC). PBMC were collected by leukapheresis from healthy HLA-A*0201 volunteers. Dendritic cells (DC) were produced from adherent cells cultured for seven days ($2x10^6$ cells/ml) in the presence of 500 IU/ml GM-CSF (Leucomax®, Schering-Plough, Kenilworth, NJ, USA) and 500 IU/ml IL-4 (R&D Systems, Minneapolis, MN, USA) in complete medium (RPMI-1640 supplemented with 10% heat inactivated human

ffinity of Hsp70-derived peptides for HLA-A*0201.

Peptide	Sequence	RA[a]
Polypeptide p380-402	LMGDKSENVQDLLLLDVAPLSL	
p380	LMGDKSENV	1.7
p391	LLLLDVAPL	0.5
p393	LLDVAPLSL	0.5

[a]Relative affinity: concentration of each peptide/concentration of the reference peptide that induce 20% of HLA-A*0201 expression obtained by 100 μM of the reference peptide. Result of three or more independent experiments.

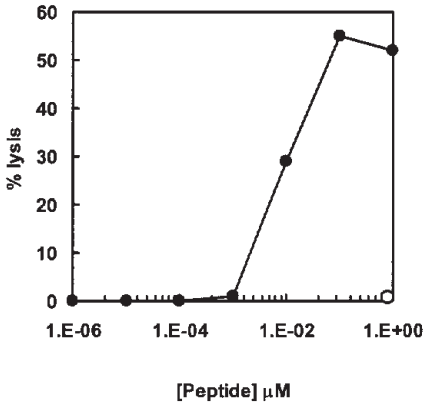

Figure 1. p380 recognition by mCTL380 cell line. The mCTL380 cell line was established as described in Materials and methods. It was tested against RMA-S HHD targets in the presence of various concentrations of the p380 peptide (•) or with 1 μM of the irrelevant HIVpol$_{589}$ (○) at an E/T ratio of 40/1.

AB serum, 2 μM L-Glutamine and antibiotics). On day seven, DC were pulsed with 10 μM polypeptide for 2 h; maturation agents Poly I:C (Sigma) at 100 ng/ml and anti-CD40 mAb (clone G28-5; ATCC, Manassas, VA, USA) at 2 μg/ml were added in the culture and DC were incubated at 37˚C overnight or for up to 48 h. Mature DC were then irradiated (3500 rads). CD8$^+$ cells were purified by positive selection with CD8 MicroBeads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. CD8$^+$ cells (2x10^5) + CD8$^-$ cells (6x10^4) were stimulated with 2x10^4 peptide pulsed DC in complete culture medium supplemented with 1000 IU/ml IL-6 and 5 IU/ml IL-12 (R&D Systems) in round-bottomed 96-well plates. From day seven, cultures were weekly re-stimulated with peptide-loaded DC in the presence of 20 IU/ml IL-2 (Proleukin) and 10 ng/ml IL-7 (R&D Systems). After the third *in vitro* restimulation, CD8 cells from 6 wells were pooled and tested in an IFN-γ release assay.

Alternatively, PBMC of HLA-A*0201$^+$ volunteers were differentiated into DC in the presence of IL-13 and GM-CSF with the VacCell processor (IDM S.A., Paris, France) as previously described (32), and pulsed in AIM-V medium (Gibco, Paisley, UK) overnight with 20 μM of polypeptide. They were washed twice and put in contact with CD8$^+$ cells at a ratio of 5:1 (1.5x10^5 CD8$^+$ cells + 3x10^4 peptide-pulsed DC + 5x10^4 CD8$^-$ cells) in Iscove MDM (BioWhittaker Europe, Verviers, Belgium) + 10% autologous serum in the presence of a maturation cocktail composed of a bacterial extract (FMKp, Pierre Fabre Médicaments, St-Julien-en-Genevois, France) at 1 μg/ml and recombinant human IFN-γ (Imukin, Boehringer, Ingelheim, France) at 500 IU/ml and anti-CD40 mAb (G28-5 hybridoma; ATCC) at 2 μg/ml. The specific immune responses induced were monitored after 4 weekly stimulations in an IFN-γ release assay.

Intracellular IFN-γ staining. T cells (10^5) were incubated with 2x10^5 stimulating peptide loaded T2 cells in the presence of 20 μg/ml brefeldin-A (Sigma). Six hours later, they were washed, stained with r-phycoerythrin-conjugated anti-CD8 antibody (Caltag Laboratories, Burlingame, CA, USA) in PBS for 25 min at 4˚C, washed and fixed with 4% PFA. Then, cells were permeabilized with PBS 0.5% BSA 0.2% saponin (Sigma), and stained with allophycocyanin-conjugated anti-IFNγ mAb (PharMingen) for 25 min at 4˚C. Cells were analyzed on FACScalibur™ (Becton Dickinson, Mountain View, CA, USA).

Results

*Identification of a new HLA-A*0201-restricted epitope derived from the 380-402 region of Hsp70*. We have previously identified two overlapping HLA-A*0201 epitopes derived from Hsp70, p391 and p393, and shown that they were efficient in inducing murine CTL *in vivo* and human CTL *in vitro*, able to recognize the Hsp70 epitope at the surface of tumour cells naturally overexpressing Hsp70. This demonstrated that Hsp70 is an interesting tumour antigen to target in cancer immunotherapy because of its broad overexpression in tumour cells. The extensive screening of the Hsp70 sequence for peptides with a high affinity for HLA-A*0201 revealed the existence of a new nonamer peptide, p380, which exhibited a high relative affinity (RA) and was proximal to the p391 and p393 epitopes (Table I). Interestingly, like p391 and p393, p380 was not shared with the highly homologous constitutive Hsc70 protein which is likely to be involved in tolerization of Hsp70-specific T cell repertoires.

We assessed the capacity of p380 to trigger CTL in HHD mice. Peptide-specific CTL were generated in two out of five mice immunized with p380, as demonstrated by specific lytic activity of splenocytes in culture against p380-pulsed target cells. A CTL line, hereafter referred to as mCTL380, was obtained from spleen cells of one responder mouse after multiple *in vitro* restimulations with decreasing doses of peptide. mCTL380 lysed RMA-S HHD targets pulsed with p380, but not target cells pulsed with 1 μM of the irrelevant peptide HIVpol$_{589}$ (Fig. 1). mCTL380 exhibited a high functional T cell avidity since half maximal lysis of RMA-S HHD targets was obtained when pulsed with <10 nM of peptide. mCTL380 was characterized as being composed phenotypically of ~90% of CD8$^+$ cells (data not shown).

We next addressed whether p380 is naturally processed by cells expressing Hsp70 endogenously. We first stimulated

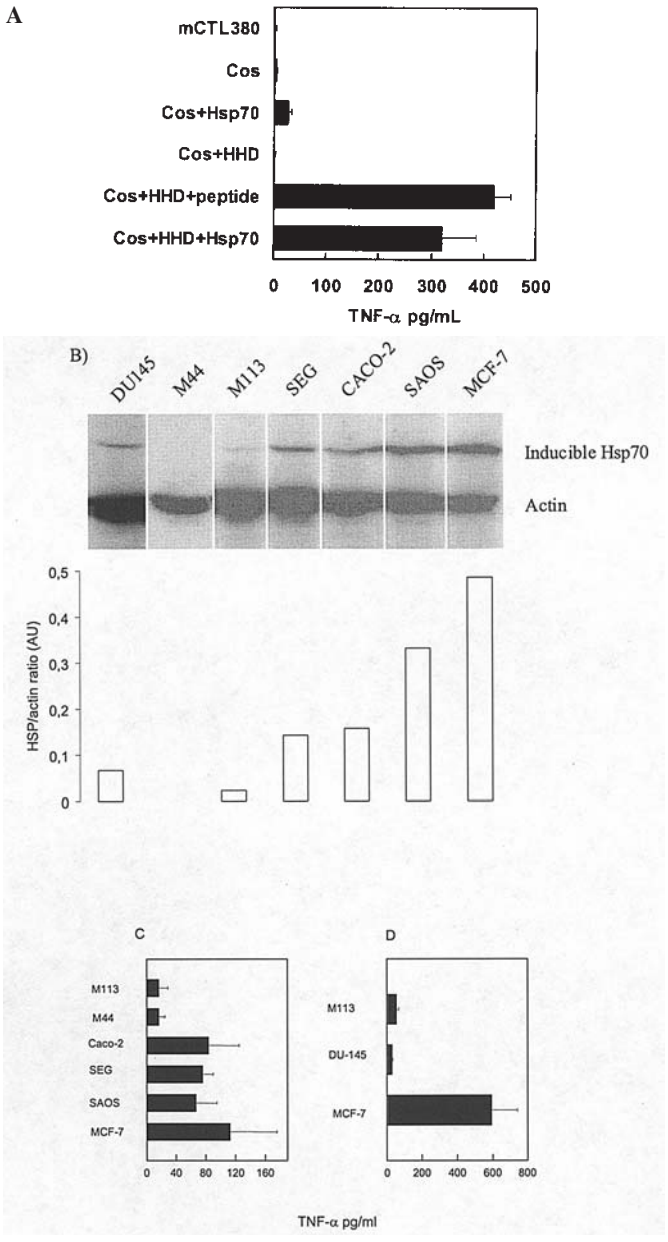

Figure 2. Stimulation of mCTL380 by tumour cells expressing endogenous Hsp70. (A) mCTL380 cells were stimulated with COS-7 cells expressing HHD and/or Hsp70 as indicated. In a positive control, mCTL380 cells were stimulated with HHD expressing COS-7 cells incubated with the peptide (10 μM). mCTL380 activation was evaluated by measurement of secreted TNF-α using the TNF-α-sensitive WEHI cell line. Results represent the mean ± SD of triplicates and were confirmed in three independent experiments. (B) Inducible Hsp70 expression by human tumour cells assessed by Western blotting as described in Materials and methods. (C) mCTL380 cells were stimulated with the Hsp70$^+$ SEG, Caco-2, SAOS and MCF-7, and the Hsp70$^-$ M113 and M44 cells. mCTL activation was evaluated by measurement of secreted TNF-α as described in A. Results were confirmed in three independent experiments. (D) mCTL380 were tested against the Hsp70$^+$ MCF-7 cells, the Hsp70$^-$ M113 cells, and the HLA-A*0201$^-$ DU145 cells.

mCTL380 with COS cells co-transfected with the HHD (HLA-A*0201 transgene) and Hsp70 expression plasmids, or with COS cells transfected with either the HHD or the Hsp70 plasmids. mCTL380 activation was evaluated by TNF-α secretion. mCTL380 responded to COS cells co-transfected with both HHD and Hsp70 plasmids but not to COS cells transfected only with either the HHD or the Hsp70 plasmids

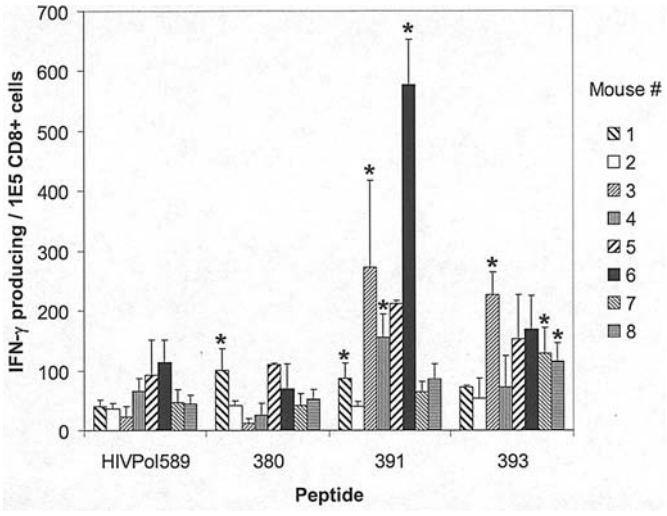

Figure 3. Immune responses targeting the various Hsp70 epitopes triggered by vaccination of HHD mice with the p380-402 polypeptide. Eight mice were vaccinated with the p380-402 polypeptide and the frequency of CD8$^+$ cells specific for each Hsp70 epitope was determined in splenocytes of each individual mouse by *ex vivo* IFN-γ Elispot. The histogram represents the number of IFN-γ producing cells/10^5 CD8$^+$ cells in each mouse when stimulated by each of the individual Hsp70 epitopes or the negative control peptide HIV Pol_{589}. *Frequencies of Hsp70 peptide-specific CD8$^+$ T cells superior to the mean of control peptide + 3 x SD.

(Fig. 2A). This demonstrates that p380 is processed from the endogenous Hsp70 protein and presented by HLA-A*0201.

We further studied whether the natural overexpression of Hsp70 in human tumour cell lines results in efficient presentation of p380. We used previously described tumour cell lines (14) [various HLA-A*0201$^+$ tumour cell lines expressing Hsp70 (MCF-7, SAOS, Caco-2, and SEG)], at least 7 to 25 times more than two other HLA-A*0201$^+$ tumour cell lines, M44 and M113, and an Hsp70$^+$ HLA-A*0201$^-$ tumour cell line, DU-145 (Fig. 2B). mCTL380 was activated by the different HLA-A*0201$^+$ tumour cells overexpressing Hsp70, such as Caco-2, SEG, SAOS, and MCF-7, but not by cells expressing very low levels of Hsp70, namely M113 and M44 (Fig. 2C). mCTL380 was not activated by the HLA-A*0201$^-$ Hsp70$^+$ DU-145 cells. This demonstrated that mCTL380 recognized Hsp70-expressing tumours in an HLA-A*0201 restricted manner (Fig. 2D).

Human CD8$^+$ cells were stimulated with dendritic cells pulsed with p380, and tested for their specificity by cytotoxicity and IFNγ secretion upon encounter with peptide pulsed targets. In four donors tested, no significant number of CD8 cells specific for p380 was observed (data not shown). The lower efficiency of p380 to induce human CTL compared to the previously described Hsp70 peptides p391 and p393 could be attributed to a slightly lower affinity for HLA-A*0201 (Table I).

Collectively, these data establish that p380 is efficiently presented by Hsp70 overexpressing tumours and, therefore, is a target of antitumour CTL immune responses, similarly to p391 and p393.

Induction of CD8 responses targeting each of the epitopes included in p380-402. We then investigated if the p380-402

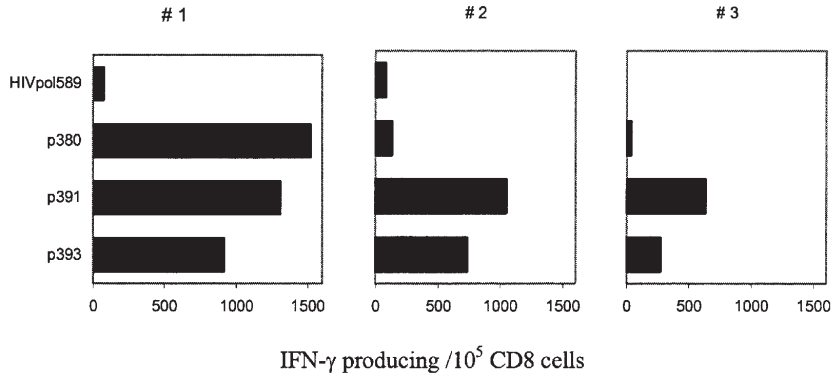

Figure 4. Capacity of the p380-402 polypeptide to induce human CD8 immune responses against its three constituent HLA-A*0201 epitopes *in vitro*. $CD8^+$ cells of healthy donors were stimulated by dendritic cells pulsed with the p380-402 polypeptide. Pulsing of the dendritic cells was performed in the presence (donors #1 and #2) or absence (donor #3) of serum. Following three stimulations, $CD8^+$ cells were tested for their recognition of individual epitopes by intracellular IFN-γ secretion when placed in the presence of T2 cells pulsed with individual peptides or the $HIVpol_{589}$ irrelevant peptide. For each donor the number of induced cells specific for each epitope is represented.

polypeptide, which contains three HLA-A*0201-restricted epitopes, can advantageously be used to induce an immune response against each of the composing epitopes.

Eight HHD mice were vaccinated with the polypeptide, and the peptide specificity of the immune responses triggered were analysed *ex vivo* by IFN-γ Elispot. The polypeptide was immunogenic, as six out of eight mice responded to at least one Hsp70 HLA-A*0201 restricted epitope (Fig. 3). The p380-402 polypeptide was able to induce a T cell response against each of its three constituent epitopes, as demonstrated by the recognition of each individual Hsp70 nonamer peptide in at least one of the eight mice (Fig. 3).

In order to investigate if the p380-402 polypeptide can induce an immune response against each of its three constituent epitopes in human PBMCs, we stimulated human $CD8^+$ cells from four HLA-A*0201 healthy donors with polypeptide-loaded autologous DC. The generation of Hsp70 epitope-specific CTL was evaluated by intracellular IFN-γ staining upon stimulation with individual nonamer peptide-loaded T2 cells. IFN-γ producing cells to Hsp70 epitopes were detected in cultures of three out of four donors (Fig. 4). More importantly, the polypeptide induced responses against all three constituent epitopes: out of three responding donors, one donor (#1) responded against all three epitopes, and two donors (#2 and #3) responded against two epitopes. Thus, the p380-402 polypeptide was able to induce CD8 immune responses against its three constituent HLA-A*0201 epitopes.

Discussion

The inducible heat shock protein Hsp70 constitutes an interesting universal tumour antigen for the induction of immune responses with broad application to tumours. The identification of multiple epitopes efficient to raise an anti-tumour immune response makes it possible to induce poly-specific responses, with a more robust and efficient profile than monospecific responses. In this paper, we describe a new HLA-A*0201-restricted epitope, p380, which is located close to the previously described p391 and p393 epitopes. p380 displayed a high affinity for the HLA-A*0201 molecule, was immunogenic *in vivo* in HLA-A*0201 transgenic HHD mice and specific murine CTL recognized Hsp70 over-expressing tumour cells of various histological origins. The 22 amino-acid polypeptide comprising the Hsp70-derived epitopes p380, p391 and p393, displayed an interesting potency to induce multiple immune responses against each of these epitopes *in vivo* in HLA-A*0201 transgenic HHD mice and *in vitro* in a human setting. This polypeptide therefore constitutes an interesting candidate for anti-Hsp70 anti-tumour vaccination. The safety of anti-Hsp70 vaccination was suggested by our observation that mice vaccinated with the various Hsp70 peptide epitopes did not show any detectable sign of long-term autoimmune lesions to major organs (our unpublished observation).

Vaccination with several epitopes is highly desirable for the enhancement of the breadth and efficiency of the generated immune response (24-26). Also, using multiple epitopes derived from the same antigen enables a more frequent and consistent response in heterogenous individuals (22,23). A multitude of immune responses targeting multiple epitopes can be induced by different modalities, among which is the delivery of a mix of the peptides of interest, or of a polypeptide containing the different peptides. The polyepitope mode of delivery conveys several advantages.

First, the juxtaposition of several nonamer peptides to form a longer polypeptide can create a CD4 epitope in the polypeptide, which enhances the breadth of the immune response against the CD8 epitopes by the action of T helper cells. In this line, Zwaveling *et al* have used a long human papillomavirus-derived peptide able to raise a CD4 reponse which potentiates the CD8 response against an encompassed CTL epitope (27). Also, the use of long peptides derived from the tumour antigen Her-2/neu was shown to trigger a CD4 response accompanied by remarkably frequent and long-lasting CD8 responses against CTL epitopes contained within the long peptides (22).

Second, a sustained TCR signal is required for efficient priming of naive CTLs (33,34). But peptide/MHC class I display a high turnover rate at the surface of APCs, and therefore when an exogenous source of peptide is used, the number of peptide/MHC class I complexes decreases at a fast rate (35). Such a fast half-life could seriously dampen their

efficacy in priming CTLs. By contrast, longer peptides which require cross-presentation by professional APCs could in turn constitute an endogenous source of peptide to be presented in much slower and sustained kinetics. This long peptide strategy has been shown to result in an enhanced immunogenicity over the short peptide form (27).

Our results showing the capacity of the p380-402 polypeptide to induce CD8 immune responses against its three constituent HLA-A*0201 epitopes demonstrate that this immunogenic polypeptide is an interesting candidate to induce multiple anti-Hsp70 antitumour CTL responses.

Acknowledgments

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