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Tumour-associated antigen (TAA)-specific cytotoxic T cell (CTL) response *in vitro* and in a mouse model, induced by TAA-plasmids delivered by influenza virosomes

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Abstract

We investigated influenza virosomes as a TAA-gene delivery system for use in TAA-directed anti-cancer vaccine therapy. An engineered plasmid (GC90) expressing the parathyroid hormone-related peptide (PTH-rP), a protein secreted by prostate and lung carcinoma cells, was included in influenza virosomes (GC90V). The ability of GC90V to elicit a PTH-rP-specific cytotoxic T cell (CTL) response was demonstrated in BALB/c mice immunised with intranasal (i.n.) GC90V±adjuvant subcutaneous (s.c.) interleukin-2 (IL-2). A PTH-rP-specific CTL response with antitumour activity was also demonstrated in human peripheral blood mononuclear cells (PBMC) stimulated *in vitro* with GC90V infected autologous dendritic cells (DC). These results provide a rationale for investigating GC90V in clinical trials of anticancer vaccine therapy.

Keywords: Anti-cancer immunotherapy; Tumour-associated antigen-gene; Parathyroid hormone-related protein; Influenza virosomes; CTL response

1. Introduction

Active tumour-associated antigen (TAA)-specific immunotherapy is a promising strategy for the treatment of human cancer [1]. We investigated the possibility of using intranasally (i.n.) administered influenza virosomes as a delivery system for human TAA genes in order to elicit a cytotoxic T cell (CTL)-mediated immune reaction with antitumour activity. Parathyroid hormone-related peptide (PTH-rP) [2] was chosen as a target antigen because it is produced in small amounts in normal adult tissue [3], but is expressed in 90% of prostate and spino-cellular lung cell carcinomas, and 95% of all epithelial cancer cells from bone metastases [4]. PTH-rP is a secreted protein that seems to be indispensable for tumour cell metastasis insofar as it stimulates the osteoclast production of growth factors and cytokines in bone tissue, which promotes tumour cell growth and makes bone a feasible micro-environment for their survival [5]. It is also known that the administration of PTH-rP blocking mAbs in nude mice protects against the occurrence of bone metastases from prostate or breast cancer cells inoculated by intravenous (i.v.) injection [6].

I.n. administered influenza virosomes are a good means of delivering antigens to first respiratory tract antigen-presenting cells (APC), which stimulate a specific humoral and particularly cell response [7–9]. We first tested the immunogenic potential of influenza virosomes including *PTH-rP* gene plasmids in *in vivo* models by investigating the occurrence of an antigen-specific CTL response in mice immunised with i.n. administered GC90 virosomes±adjuvant cytokine. The immunogenic potential was then further evaluated in human models by investigating the possibility of eliciting a PTH-rP-specific CTL response by stimulating *in vitro* human peripheral blood monouclear cells

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(PBMCs) with autologous dendritic cells (DC) infected with GC90 virosomes.

2. Materials and methods

2.1. Cell cultures

The LNCaP and DU-145 prostate carcinoma cell lines, the SW1463 colon carcinoma cell line and the DH5 and VERO cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured as suggested by the provider in complete medium Roswell Park Memorial Institute (RPMI)-1640 with 10% fetal bovine serum (FBS), 2 mM L-glutamine (all purchased by Gibco Corp., New Island, NY, USA). Dr Jeffry Schlom (National Cancer Institute, National Institutes of Health, Bethesda, MD, USA) kindly provided the CIR-A2 cell line, and Dr A. Castrucci (National Institute of Health, Rome, Italy) the P815 murine mastocytic leukaemia cell line. Both cell lines were cultured in the same medium described above.

2.2. Generation of a PTH-rP plasmid and influenza virosomes

The *PTH-rP* gene was amplified from the DU-145 prostate carcinoma cell line by means of reverse transcriptase-polymerase chain reaction (RT-PCR) [10] starting from the specific mRNA by using the sense primer 5′TTGGATCCATGCAGCGGAGACTGGTT3′ and the antisense primer 5′CCGAATTCTCAATGCCTCCGTGAATCGA3′, and cloned in *BamHI-EcoRI* sites of the pcDNA3 expression vector (InVitrogen) in order to obtain the recombinant plasmid GC90. The construct was grown in DH5 cells. Plasmid DNA was purified using the Qiagen Endo Free plasmid kit (QIAGEN) as described by the manufacturer. The influenza virosomes were prepared as described elsewhere [7]. Non-encapsulated plasmids were separated by 0.1 gel filtration on a High Load Superdex 200 column (Pharmacia) equilibrated with sterile phosphate-buffered solution (PBS). The void volume fractions containing the virosomes and encapsulated plasmids were eluted with PBS and collected.

2.3. Cell transfection

Approximately 10^5 target cells (Vero, P815 or CIR-A2 cells) were grown in six-well microplates at 37 °C and infected with 0.3 μg of DNA virosomes or transfected with 1 μg of plasmid DNA using the Effectene Transfection reagent (QIAGEN) as described by the manufacturer. After 2 days, PTH-rP antigen expression was analysed by evaluating the presence of specific mRNA by means of RT-PCR and immunofluorescence. Briefly, the cells were washed twice with PBS, fixed with cold methanol/acetone and treated with a rabbit anti-PTH-rP serum (Calbiochem) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1/100) (DBA Italia SRL, Milan, Italy). The coverslips were mounted on slides and examined using a Diaplan microscope (Leitz).

2.4. Generation of DC and T cell lines

PBMC were isolated from heparinised blood derived from an HLA-$A2^+$ male normal volunteer using a lymphocyte separation gradient medium (Organon Tecknika, NC, USA) as previously described [11].

2.4.1. Dendritic cells

DC enrichment was performed using PBMCs as previously described [12]. After 7 days culture in a medium containing 25 ng/ml of granulocyte macrophage-colony stimulating factor (GM-CSF) (Schering-Plough Corp., New Jersey, NJ, USA) and 5 ng/ml of interleukin-4 (IL-4) (R&D Corp., Minneapolis, MN, USA), direct immunofluorescence flow cytometry revealed a DC phenotype with the expression of CD1a, human leukocyte antigen class I (HLA-I), human leukocyte antigen DR (HLA-DR), CD11c, CD40, CD80, CD83 and CD86.

2.4.2. T cell lines

The PBMCs for CTL primary cultures were suspended in AIM-V medium (Life Technologies, Inc., Milan, Italy) supplemented with 5% pooled human AB serum (Valley Biomedical, Winchester, VA, USA), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (Gibco). Each well of a 96-well microplate (Corning, Costar Corp. Cambridge, MA, USA) was seeded with 2×10^5 cells in a volume of 100 μl. The autologous DCs were first infected with 300 ng of GC90 virosomes and, after 48 h of culture at 37 °C and 5% CO_2, they were irradiated (5000 R) and added to the lymphocyte cultures at a final ratio of 1:5. One *in vitro* stimulation cycle (IVS) was designed as a 5-day period of cell incubation with antigen-loaded DC plus a 10-day period of cell stimulation with 50 IU of IL-2 (Cetus Corp., Emeryville, CA, USA). The medium was replaced by cytokine-containing fresh complete medium every 48 h. On the 16th day, the T cell cultures were re-stimulated with autologous irradiated DCs infected with the GC90 virosomes used as antigen-presenting cells.

2.5. PTR-2 and PTR-4 peptides

The PTR-2 and PTR-4 peptides (whose amino acid sequences are FLHHLIAEIH and TSTTSLELD, respectively) were synthesised using a solid phase automatic peptide synthesiser (Model Syto, MultiSyntech,

Witten, Germany) and the fluorenylmethoxycarbonyl (Fmoc)/diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBT) strategy. The peptides were cleaved from the resins and defracted by treatment with trifluoroacetic acid containing ethandiethiol, water tris-buthyl silone and anisole (93/2.5/2/1.5/1). The crude peptides were purified by means of high performance liquid chromatography (HPLC) using a Vydac C18 column (25×1 cm, 10 μm). The products were dissolved in bi-distilled water, sterile filtered and frozen at −70 °C at a concentration of 2 mg/ml. HPLC showed that the purity of the peptides was more than 90%. The carcinoembryonic antigen peptide carcino embryonic antigen ((CAP)-1) control peptide was kindly donated by Dr J. Schlom (EOS, NCI, Bethesda, MD, USA).

2.6. Cytotoxic assays

Various target cells were labelled with 50 μCi of [^{51}Cr]-isoquinoline (Medi Physics Inc., Arlington, IL, USA) for 60 min at room temperature. The target cells (0.5×10^4) were added to each of the wells of 96-well microplates in 100 μl of complete RPMI-1640. The labelled targets were incubated at 37 °C in 5% CO_2 before adding effector cells at different E:T ratios. The T cells were then suspended in 100 μl of AIM-V medium and added to the target cells. The plates were incubated at 37 °C for 6 h, and 100 μl of the supernatant of each sample was harvested for γ-counting. The determinations were carried out in triplicate and standard deviations were calculated. All of the experiments were repeated at least three times. Specific lysis was calculated as it follows:

$$\text{Specific lysis } \% = \frac{\text{Observed release (cpm)} - \text{spontaneous release (cpm)}}{\text{Total release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

Spontaneous release was determined from wells to which 100 μl of complete medium were added instead of effector cells. Total releasable radioactivity was measured after treating the target with 2.5% Triton X-100.

For HLA blocking experiments, UPC-10 (Cappel/Organon Technique Corp., West Chester, PA, USA) control mAb or anti-HLA-A2 (A2.69, #189HA-1; One Lambda, inc., Canoga Park, CA, USA), or anti-mouse-CD8, or anti-mouse-CD19, or anti-mouse-H2^{kb} mAb (Cappel/Organon Technique Corp., West Chester, PA, USA) were added to the [^{51}]Cr loaded target cells and incubated for 1 h prior to the cytotoxic assay.

2.7. Flow cytometry

The procedure for single-colour flow cytometric analysis has been previously described [13], and is the same as that used for dual-colour flow cytometry. The cells were analysed using a Becton Dickinson fluorescent activated cell sorter (FAC)Scan equipped with a blue laser with excitation of 15 nW at 488 nm. The data gathered from 10 000 live cells were used to evaluate the results. The PBMCs were HLA phenotyped by the Blood Bank of the Azienda Ospedaliera Senese, Policlinico "Le Scotte", Siena, Italy, using a standard antibody-dependent micro-cytotoxicity assay and a defined panel of anti-HLA antisera for HLA class I determinations.

2.8. Statistical analysis

The differences between the means were statistically analysed using Stat View statistical software (Abacus Concepts, Berkeley, CA, USA). The results are expressed as the mean values of four determinations from three different experiments ± standard deviation. The differences between the means were determined using the two-tailed Student *t*-test for paired samples, and considered statistically significant at a P value of <0.05.

2.9. Mouse immunisation

Four-week-old female BALB/c mice (Charles River) were anaesthetised with ketamine–xylazine and immunised by means of the intranasal i.n. of 5 μg of DNA associated with influenza virosomes/mouse in a volume of 20 μl, thus ensuring deposition of the inoculum throughout the respiratory tract. Six mice in each group were immunised with the GC90–virosome complex (group A), pcDNA3 virosomes (group B), GC90 virosomes plus the subcutaneous (s.c.) administration of IL-2 100 IU/day for 5 days a week (group C), or pcDNA3 virosomes plus IL-2 (group D). Boosters were given 3 and 5 weeks after primary immunisation. The mice were sacrificed by cervical dislocation under anaesthetic 10 days after the last immunisation, their spleens were harvested, and the spleen cells cultured in the presence of 100 IU of IL-2 for 7 days before being examined for PTH-rP specific CTL activity. Each experiment was repeated three times to ensure the reproducibility of the results.

3. Results

3.1. Generation of a PTH-rP DNA plasmid virosome

A PTH-rP DNA plasmid virosome (GC90V) was generated and characterised in our laboratory. Its ability to infect human (CIR- A2) and murine target cells (P815) by inducing the *in vitro* expression of PTH-rP was demonstrated by means of immunoradiometric assay, the RT-PCR detection of specific mRNA, and immunofluorescence using a rabbit anti-PTH-rP serum (data not shown in the figure). The same techniques

Table 1
HLA-A2.1 molecule expression and PTH-rP production in lymphocyte target cells

Target cells	HLA-A2.1 expression (%)[a,c]	PTH-rP production (pg/ml×10^6 cells)[b,c]
P815 transfected with pcDNA3	Not detectable	Not detectable
P815-transfected with GC90	Not detectable	10.56 (3.6) (39% of positive cells)
CIR-A2	98.5 (2.2)	Not detectable
CIR-A2 pc-DNA3	96.3 (3.2)	Not detectable
CIR-A2 GC90	99.9 (1.23)	11.56 (45% of positive cells) (2.2)
LNCaP	29.8 (3.7)	15.2 (5.5)
SW1463	90.5 (3.6)	Not detectable

PTH-rP, parathyroid hormone-related peptide.

[a] HLA-A2.1 expression was evaluated by indirect immunofluorescence using an anti-HLA-A2.1 mAb (A2.69) and a FITC-conjugated goat-anti-mouse. Results are expressed as a percentage of fluorescent cells. Marker expression was considered negative when lower than 4%. Results are expressed as percentage of each cell sample reactive with mAb. Routinely, 2–4% cells are stained when treated either with no priming mAb or an isotype-related control mAb.

[b] PTH-rP was evaluated by a sandwich immunoradiometric assay (IRMA); values lower than 1.5 pg were considered negative. The percentage of cells positive for the expression of PTH-rP was evaluated by immunofluorescence.

[c] Numbers in parentheses represent standard deviations (S.D.).

were used to demonstrate the ability of the GC90 plasmid used for target cell transfection to induce the transient expression of PTH-rP in the CTL target cells in the cytotoxic assays (Table 1).

3.2. Immunological and toxicological effects of GC90V in a mouse model

The immunological and toxicological effects of i.n. instilled GC90V in BALB/c mice was tested in the presence or absence of IL-2. The mice were divided into four groups of six animals each as described in the Methods.

Group A received GC90-virosomes complex, group B pcDNA3-virosomes, group C GC90-virosomes plus sc. administration of IL-2 (100 IU/day for 5 days a week) and group D pcDNA3-virosomes plus IL-2.

Ten days after the last immunisation, mice were sacrificed and the spleens harvested. Spleen cells were then cultured in presence of (100 IU) IL-2 for 7 days before being examined for PTH-rP-specific CTL activity.

Cytotoxic assays based on 6-h [^{51}]Cr release revealed significant cytotoxic activity against P815 target cells transfected with PTH-rP plasmids in the spleen cells from the mice of groups A and C (Fig. 1a). Target cell lysis was major histocompatibility complex (MHC)-class I restricted, since CTL activity was abrogated by the addition of anti-H2^{kb} (Fig. 1b). The finding that group C spleen cells had the most efficient cytotoxic activity suggests that IL-2 treatment enhances the immunological activity of GC90V. PTH-rP-specific cytotoxic activity in our experiments was mediated by the CD8+ cell population because the addition of an anti-mouse mAb against CD8 almost completely abrogated spleen cell cytotoxic activity against PTH-rP-transfected P815 target cells (Fig. 1d), but the addition of an anti-CD19 negative control antibody did not affect CTL activity (data not shown). No cytotoxic activity was detected against untransfected parental P815 cells (data not shown) or the cells transfected with the plasmid backbone pcDNA3 (Fig. 1c). The spleen cells from groups B and D did not lyse any of the target cells (Fig. 1).

The differences of values of the groups A (GC90V) and C (GC90V + IL-2) compared with those of the groups B (pcDNA-3) and D (pcDNA-3 + IL-2), respectively, were statistically significant ($P < 0.05$) at E:T ratios of 6.25, 12.5 and 25:1.

Autoptic pathological examination of the mice who had received influenza virosomes including PTH-rP plasmids ±IL-2 did not reveal the occurrence of any toxic or auto-immune reactions (data not shown). The organs and tissues in which parathyroid hormone (parathyroid glands) or low levels of PTH-rP (skin, breast, brain, the first tract of the airway-digestive mucosa) can be detected [14] were examined for the presence of lymphocyte infiltration, necrosis or apoptosis, but no differences were found in comparison with those taken from the control mice. PTH and PTH-rP target tissues (bone and kidneys) were also investigated and showed no anomalies. Since PTH and PTH-rP act on calcium phosphate turnover, ionised calcium and phosphate levels were investigated in all of the sera samples, but were in the normal ranges during treatment (Table 2). On the basis of these results, it can be concluded that the influenza virosome delivery system is safe and induces a good antigen-specific cellular response in animal models, especially when combined with IL-2. It also has the additional advantage of using very small amounts (5 μg) of TAA gene-specific DNA that can be administered i.n.

3.3. Generation of a human PTH-rP-specific CTL response in PBMCs stimulated in vitro with GC90V infected autologous dendritic cells

The system was also tested in human models *in vitro* with the aim of eliciting a PTH-rP-specific CTL

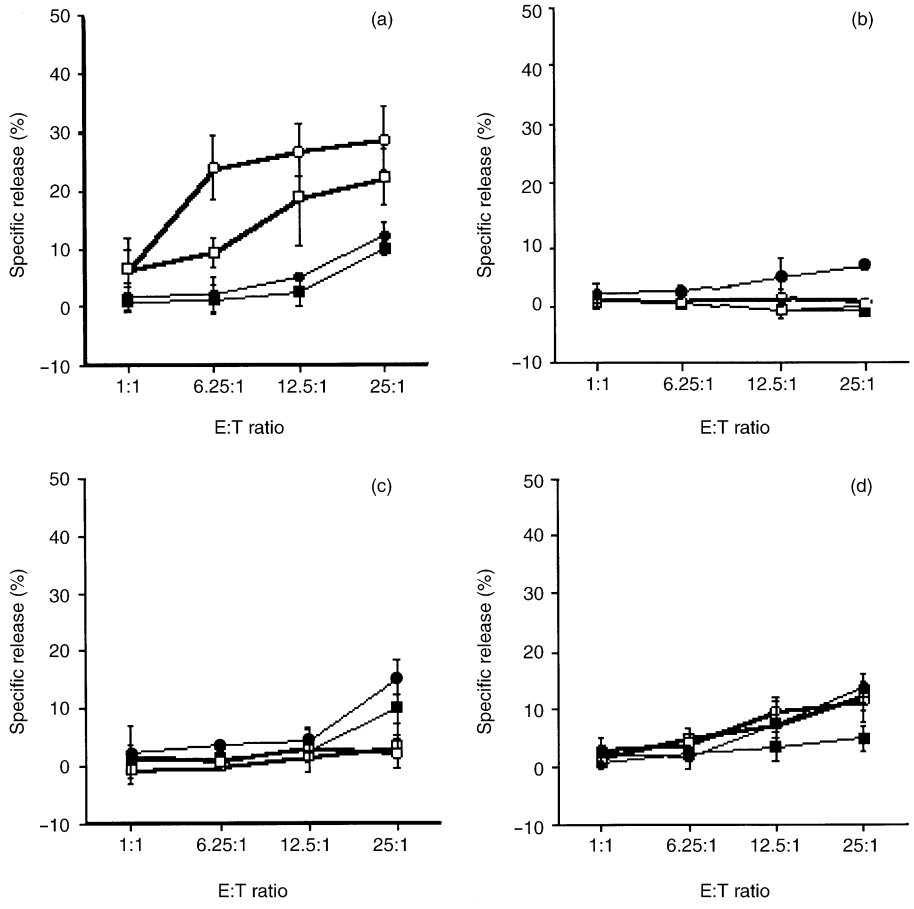

Fig. 1. Six-hour [^{51}Cr] release cytotoxic assay. Parathyroid hormone related peptide (PTH-rP) specific cytotoxic activity of mouse spleens pooled from different mouse groups against P815 target cells transfected with the *PTH-rP* gene (a), P815 target cells transfected with the *PTH-rP* gene in the presence of anti-H2^{kb} monoclonal Ab mAb (b), P815 transfected with pcDNA3 (c), P815 target cells transfected with *PTH-rP* gene (d) in presence of anti-CD8 mAb. Group A (-□-) was immunised with intranasal (i.n.) GC90 virosome, group B (-■-) with i.n. pcDNA3-virosome, group C (-○-) with i.n. GC90 virosome + daily subcutaneous (s.c.) interleukin-2 (IL-2) for five days a week, and group D (-●-) with i.n. pcDNA3 virosome + daily sc IL-2 for five days a week. The data are from three different experiments with similar results. The differences of groups A and C respectively, in comparison with those of groups B and D, were statistically significant (a) ($P < 0.05$).

response because our preliminary laboratory data had shown that GC90V can infect human DCs *in vitro* by inducing PTH-rP production.

The DCs used in this study were generated from PBMCs isolated from an HLA-A2.1$^+$ healthy donor, and cultured in the presence of GM-CSF and IL-4 as described by other authors [12]. Direct immunofluorescence flow cytometry revealed that they express CD1a (20%), CD40 (31.95%), CD11c (87.25%), CD80 (22%), CD83 (35%), CD86 (99.59%), HLA-class IA, B, C (99.65%), and HLA-Dr (99.8%). A human PTH-rP-specific CTL line could be generated *in vitro* by means of the cyclical stimulation of normal HLA-A2.1$^+$ donor PBMCs with GC90 virosome-infected autologous DCs.

After four *in vitro* stimulations (2 months of culture), the cell line showed a CD3$^+$ (95%), CD4$^-$/CD8$^+$ (75%), CD56$^-$ (5.8%) phenotype. Six-hour cytotoxic assays revealed that the T cell line was cytotoxic to class I matching (HLA-A2.1$^+$) target cells (CIR-A2) transfected with GC90 plasmid (Fig. 2). Cytotoxic T lymphocytes recognise protein antigens as 9–10 amino acid peptides derived from the antigen proteolysis of proteasomes in the cell cytoplasm, and bound to HLA molecules on the target cell membrane (data not shown). The peptide binding to specific HLA isotypes is endorsed by the presence of specific amino acid sequences (*HLA-binding amino acid consensus motifs*) ([15]; data not shown), the most common of which (including HLA-A2.1) have been published in the literature [15]. Four PTH-rP-derived peptides capable of binding HLA-A2.1 molecules have recently been described and characterised by our group (PTR-1, 2, 3 and 4), and used to generate human CTL lines capable of the *in vitro* killing of HLA-A2.1$^+$ human prostate carcinoma and breast carcinoma cell lines producing PTH-rP [16].

The human T cells stimulated with GC90V-infected DCs recognised multiple PTH-rP epitopes as they lysed the CIR-A2 target cells pulsed with PTR-2 or with PTR-4 (Fig. 2); the same cell line was also cytotoxic to HLA-A2.1$^+$ prostate carcinoma (LNCaP) cells, which

Table 2
Serum [Ca^{++}] levels in BALB/c mice during the immunological treatment

Reagent administered[a]	Group	[Ca^{++}] Concentration (mmol/l)							
		1st administration[b]				2nd administration[c]			
		1	2	3	Mean[d]	1	2	3	Mean[d]
GC90V	A	1.12	1.25	1.30	1.22	1.50	1.58	1.29	1.46
PcDNA3	B	1.51	1.14	1.30	1.32	1.61	1.69	1.67	1.66
GC90V + IL-2	C	1.21	1.19	1.24	1.21	1.44	1.58	1.65	1.56
IL2	D	1.25	1.19	1.40	1.28	1.55	1.58	1.39	1.51
No virosome	E	1.11	1.09	1.13	1.11	1.35	1.51	1.55	1.47

IL-2, interleukin-2.
[a] Sequence homology between human and murine PTH-rP protein sequences was >90%.
[b] Blood sample drawn 10 days after the first administration in three different animals.
[c] Blood sample drawn 10 days after reboost in the same animals.
[d] Differences among groups A, B, C, D and E were found statistically to be non-significant.

produce large amounts of PTH-rP (Fig. 3). The CTL cytotoxic activity was HLA-class I (HLA-A2.1) restricted because it was abrogated by the addition of the A2.69 mAb to the HLA-A2.1 molecules (Fig. 3). However, the cytotoxic T cells could not lyse peptide unpulsed CIR-A2 target cells, CIR-A2 cells transfected with pcDNA3 plasmid, CIR-A2 cells pulsed with PTH-rP-unrelated peptides with HLA-A2.1 binding amino acid consensus motifs (data not shown) or SW1463 HLA-A2.1$^+$ colon carcinoma cells unable to produce PTH-rP (Fig. 3). Before each cytotoxic experiment, PTH-rP production in the target cells was demonstrated by RT-PCR and radioimmunometric assays, and HLA-A2.1 expression by means of indirect immunofluorescence flow cytometry (Table 1).

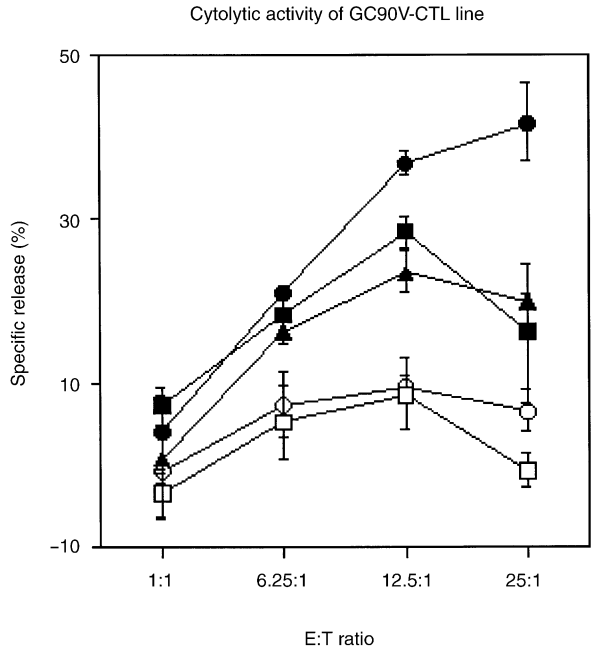

Fig. 2. Six-hour [^{51}Cr] release cytotoxic assay. PTH-rP-specific HLA-A2.1 restricted the cytolytic activity of a human cytotoxic T cell line generated *in vitro* with low dose IL-2 and GC90-virosome infected dendritic cells against different target cells. The figure shows CTL activity against HLA-A2.1$^+$ CIR-A2 cells (○), CIR-A2 cells pulsed with 25 μg/ml of PTH-rP (PTR-4) peptide (▲), CIR-A2 cells pulsed with 25 μg/ml of PTH-rP (PTR-2) peptide (■), CIR-A2 cells transfected with pcDNA3 (□), and CIR-A2 cells transfected with PTH-rP plasmid (GC90) (●). Data are from three different experiments with similar results. The differences between the values of the PTH-rP peptide pulsed- and PTH-rP transfected-CIR-A2 cells compared with the values of the unpulsed CIR-A2 and pcDNA3-transfected CIR-A2 cells respectively were statistically significant ($P<0.05$).

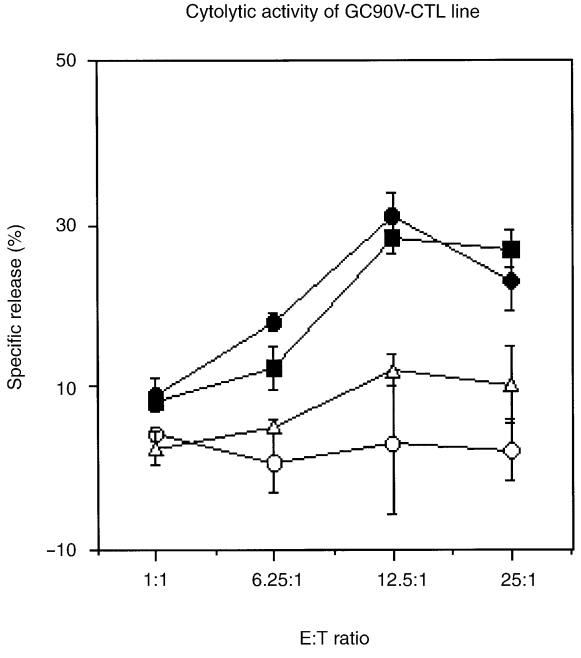

Fig. 3. A six-hour [^{51}]Cr release cytotoxic assay. The figure shows cytotoxic T cell (CTL) activity against HLA-A2.1$^+$/PTH-rP$^+$ prostate carcinoma LNCaP cells (●), LNCaP cells in the presence of anti-HLA-A2.1 mAb (dilution 1:100) (△), LNCaP cells in the presence of a control isotype mAb (UPC-10)(■), and HLA-A2.1$^+$/PTH-rP$^-$ colon carcinoma SW-1463 cells (○). The data are from three different experiments with similar results. The difference resulted statistically significant.

4. Discussion

Results of this study suggest that influenza virosomes including PTH-rP plasmids elicit a multi-epitopic CTL-mediated immune response with cytotoxic activity to class I matching cancer cells that produce PTH-rP.

The search for new vectors capable of directly transferring TAA proteins or genes to antigen-presenting cells in order to obtain a more efficient immune response is still open and investigated worldwide [17,18]. In an attempt to improve the antigen presentation process, some researchers have investigated the possibility of using antigenic peptides or genes loaded with autologous DCs because DCs are ultimately responsible for starting an efficient TAA-specific immune response. New protocols now make it possible to culture large quantities of these cells easily *ex vivo* and to re-inject them loaded with peptide antigens or after they have been engineered to express the target antigen gene. The results of our study show that i.n. administered influenza virosomes provide a good delivery system for inducing MHC-class I restricted effectors capable of recognising the antigen *ex vivo*. We are currently undertaking further studies designed to investigate their *in vivo* ability to migrate in tumour tissue where they may have an antitumour effect.

Our system can also efficiently infect human DCs *in vitro* and, after infection, these retain their ability to present tumour antigen epitope peptides to CTL precursors, thus initiating an *in vitro* TAA-specific immune response. PTH-rP plasmids included in influenza virosomes seem to be an effective tool for targeting and gene delivery and may be clinically investigated in the active specific immunotherapy of patients with prostate and lung carcinoma and those with bone metastases from epithelial malignancies.

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