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A 16-mer peptide (RQIKIWFQNRRMKWKK) from antennapedia preferentially targets the Class I pathway

Geoffrey A. Pietersz [a,*], Wenjun Li [a], Vasso Apostolopoulos [b]

[a] *The Austin Research Institute, Studley Road, Heidelberg, 3084 Victoria, Australia*
[b] *Department of Molecular Biology, Scripps Research Institute, 10550 North Torrey Pines Road (BCC-206), La Jolla, CA 92037, USA*

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Abstract

Translocation of antigenic peptides into the cytosol of antigen presenting cells facilitates proteosomal processing and loading into Class I molecules for MHC presentation on the cell surface. The DNA binding domain of the *Drosophila* transcription factor (Antennapedia), a 60 amino acid protein, is rapidly taken up by cells and has been fused to selected antigens to enhance their immunogenicity. We now demonstrate that a 16 amino acid peptide from antennapedia can facilitate the cytoplasmic uptake of CTL epitope 9-mer peptides. Synthetic peptides were made containing the 16-mer antennapedia peptide linked in tandem to the ovalbumin SIINFEKL CTL peptide. The peptide complex was shown to rapidly internalise into APCs by confocal microscopy. This peptide induced CTL in C57BL/6 mice and protected them against growth of an ovalbumin expressing tumour cell line (E.G7-OVA). The ability of the hybrid peptide to be processed and presented by APCs was similar, whether the SIINFEKL sequence was appended at the C-terminus or N-terminus of the Antennapedia peptide. The production of synthetic peptides containing other CTL peptide epitopes may be useful for priming CTLs in vitro and in vivo © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Peptide; Cytotoxic T lymphocyte; Disease

1. Introduction

Cytotoxic T lymphocyte (CTL) responses to infectious diseases or cancer are generally associated with protective immunity in animals [1]. While whole proteins can be used for CTL production, peptides are now being examined [2]. These antigens have been selected based on their unique expression in the parasite or virus or, in the case of cancer, overexpression in malignant cells. Numerous antigens are overexpressed in cancer including Her2/neu, MUC1 and p53, and are potential T cell targets as there is little or no expression in normal tissue [3–5]. However, administering peptides does not usually lead to CTL production unless the peptides enter the cytoplasmic compartment for processing and loading into the class I molecules [6]. Exogenous antigens are generally internalised by receptor mediated endocytosis and presented by the class II pathway to stimulate helper T cells, although exceptions exist and a number of approaches have been used to introduce proteins or CTL epitope peptides into the cytoplasm for class I presentation [2,7]. A recent approach is the use of the DNA binding domain (homeodomain) of the *Drosophila* transcription factor Antennapedia, which is spontaneously internalised by all cells by a non-receptor dependent mechanism [8,9]. The Antennapedia DNA binding domain contains 60 amino acids and consists of 3 α-helices, the region responsible for internalisation having been mapped to a 16 amino acid peptide (RQIKIWFQNRRMKWKK) within the third [10]. Antennapedia protein/peptides have been used in several studies. Firstly, a recombinant fusion protein consisting of the 60 amino acid homeodomain and a T cell epitope of the influenza nucleoprotein (residues 147–156) was used to facilitate cytoplasmic import and class I presentation to CTL [11]. Secondly, a synthetic peptide incorporating the 16 amino acid segment of the antennapedia homeodomain

* Corresponding author. Tel.: +1-613-92870666; fax: +1-613-92870600.
E-mail address: g.pietersz@ari.unimelb.edu.au (G.A. Pietersz).

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linked to a peptide sequence capable of binding to Grb-2 was used for inhibition of intracellular signalling [12] or linked to the fibroblast growth factor (FGF) receptor high affinity binding site for phospholipase Cγ (PLCγ) to inhibit activation of PLCγ by FGF [13]. We now report the synthesis of short peptides incorporating the 16-mer sequence to import CTL epitopes into the cytoplasm for class I presentation. A peptide containing the 16 amino acid peptide of the third helix domain and the ovalbumin CTL epitope peptide SIINFEKL (OVA-K^b) was successful in priming CTL in vivo.

2. Materials and methods

2.1. Mice, tumour cells and antibodies

C57BL/6 mice were produced at the Biological Research Laboratories, Austin and Repatriation Medical Centre, Heidelberg, Australia. E.G7-OVA is the EL4 tumour cell line (C57BL/6 derived, H-2^b) stably transfected with ovalbumin cDNA [14]. The ovalbumin specific B3Z H-$2K^b$ restricted T cell hybrid cell line was obtained from Dr Jim McCluskey, Department of Microbiology, University of Melbourne [15]. The anti-MUC1 antibody, 13.14 (used as supernatant), that recognises the APPAH sequence of the Mucin 1 HLA-A2 epitope, STAPPAHGV, was obtained from Dr Xing at The Austin Research Institute.

2.2. Synthetic peptides

Peptides (Table 1) were synthesised at the ARI by Mr J. Karkaloutsos; the purity of the peptides (> 95%) was determined by mass spectrometry. OVA-K^b is the ovalbumin H-$2K^b$ CTL epitope 9-mer peptide, SIINFEKL [16]. IntSIN and SINInt are peptides incorporating the 16 amino acid antennapedia peptide and the ovalbumin CTL peptide. OVA-24 is a peptide incorporating the ovalbumin H-$2K^b$ CTL epitope and the preceeding 16 amino acids in the ovalbumin protein sequence. MUC1-A2 is the HLA-A*0201 CTL epitope peptide, STAPPAHGV from the human mucin 1 [17]. IntMUC1 is a peptide containing the antennapedia peptide and the human MUC1 HLA-A*0201 CTL peptide. The peptide p1-30 corresponds to a region in the repeat of the human mucin1 sequence [18].

Table 1
Synthetic peptides used in the study

Name	Amino acid sequence
OVA-K^b	SIINFEKL
IntSIN	RQIKIWFQNRRMKWKKSIINFEKL
SINInt	SIINFEKLRQIKIWFQNRRMKWKK
OVA-24	MLVLLPDEVSGLEQLESIINFEKL
MUC1-A2	STAPPAHGV
IntMUC1	RQIKIWFQNRRMKWKKSTAPPAHGV
p1-30	PDTRPAPGSTAPPAHGVTSAPDTRPAPGST

2.3. ELISA

The IntSIN, IntMUC1 and p1-30 peptides were coated on a polyvinyl chloride (PVC) microtiter plate at 10 μg/ml in buffer (0.2 M $NaHCO_3$ buffer, pH 9.6) overnight at 4°C and non-specific binding was blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature. After washing (0.05%Tween 20/phosphate buffered saline (PBS)), serial dilutions of the anti-MUC1 antibody were added and incubated for a further 1 h at room temperature. The plates were washed and bound antibody was detected using HRP-conjugated sheep anti-mouse antibody (Amersham, UK) and the chromogenic substrate 2,2″-azino-di(3-ethylbenzthiazoline) sulphonate (ABTS) (Amersham, UK). Absorption at 405 nm was recorded using a microplate reader. For inhibition ELISA, serial dilutions of the STAPPAHGV (MUC1-A2) peptide were mixed with anti-MUC1 antibody (1/50) and added to a IntMUC1-coated microtitre plate. The plate was incubated at room temperature for 2 h and bound antibody was detected as above.

2.4. Confocal microscopy

Peritoneal exudate cells (PEC) from C57BL/6 mice and J774 cells in 500 μl aliquots of 2×10^6 were cultured to adhere in 1 cm^2 multichamber slides (Nalge Nunc International, Naperville, IL) overnight, the medium was removed and each were incubated with peptide in medium (200 μl, 50 μg/ml) for various times (1 min–2 h) at 37°C and washed three times with medium. Paraformaldehyde (500 μl, 4%) was then added at room temperature for 5 min. Ethanol/acetic acid (95/5) was added to the drained chambers and kept at −20°C for 5 min followed by washing three times at room temperature with PBS [10]. The anti-MUC1 antibody was added and incubated for a further 30 min at room temperature. The wells were washed (× 3) and stained with FITC-labelled sheep anti-mouse immunoglobulin (1/50) and examined using confocal microscopy.

2.5. Stimulation of lacZ inducible ovalbumin-specific T cell hybrid

The B3Z T cell hybrid cell line contains a gene construct of *E. coli* lacZ reporter gene linked to the nuclear factor of activated T cells (NFAT). Recognition of OVA-K^b peptide in the context of Class I by the TCR results in activation of the enzyme and conversion

of the chromogenic substrate that can be measured by absorbance spectrophotometry [19]. A total of 1 ml of various cells (EL4, C57BL/6 splenocytes), 1×10^6, were incubated with 3 μg/ml peptide (OVA-K^b, IntSIN, SIN-Int, OVA24, IntMUC1) for 2 h, irradiated and washed and 100 μl (1×10^5) were added to 100 μl (1×10^6) B3Z cells in a microtitre plate. After overnight incubation at 37°C, 6% CO_2, the cells were lysed and incubated with chloro red β-galactoside (Calbiochem, San Diego, CA) for a further 4 h and optical density ratio was measured at 540 nm/630 nm using a microplate reader.

2.6. Cytotoxicity assay

Seven to 10 days after immunisation, spleen cells were harvested, restimulated in culture for 7 days, and lytic activity was determined. Splenocytes (4×10^7) in 20 ml medium were cultured in upright flasks with 10 μg/ml OVA-K^b or 1×10^7 irradiated OVA-loaded splenocytes [20] for 6 days. The effector cells were harvested and a standard 4-h ^{51}Cr-release assay was performed in round bottomed 96 well plates using ^{51}Cr labelled OVA-K^b peptide-pulsed EL4 cells, E.G7-OVA cells or parent EL4 cells (2 h, 37°C). Radioactivity of the supernatant was measured in a γ-counter. For inhibition assays, target cells were incubated for 45 min with inhibitors [1 μg/ml brefeldin A (Sigma, St Louis, MO); 20 mM chloroquine (Sigma); 50 μM lactacystin (Calbiochem-Novabiochem, Australia) followed by incubation with OVA-K^b. Results were expressed as % Specific lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was determined by incubating target cells in medium and maximum release by incubating in 1% SDS. All assays were performed in triplicate and errors did not exceed $> 5\%$ of mean value.

2.7. Tumour protection

Groups of C57BL/6 mice ($n = 6$) were immunised intraperitoneally with PBS, or three different doses (5, 10 or 50 μg) of IntSIN or OVA-K^b in PBS on days 0, 7 and 14. Eight days later, mice were challenged with a subcutaneous dose (1×10^7) E.G7-OVA cells. The growth of the tumour was monitored by measuring the two perpendicular diameters using a caliper square and expressing the results as the product of the two perpendicular diameters.

3. Results

3.1. Cellular import of fusion peptide

The internalisation of the peptides was studied using the IntMUC1 peptide (Table 1), chosen because of the availability of an anti-MUC1 antibody. An ELISA assay was performed to ascertain the reactivity of the antibody with the IntMUC1 peptide. As shown in Fig. 1(a), the anti-MUC1 antibody reacted equally well with the internalising peptide IntMUC1 and the p1-30 peptide while no reaction was seen with the IntSIN control peptide. Specificity was also demonstrated using an inhibition assay where free MUC1-A2 peptide inhibited the binding of the anti-MUC1 antibody to IntMUC1 (Fig. 1(b)). Therefore, the recognition of the APPAH sequence by the anti-MUC1 antibody was not affected when included in a longer peptide.

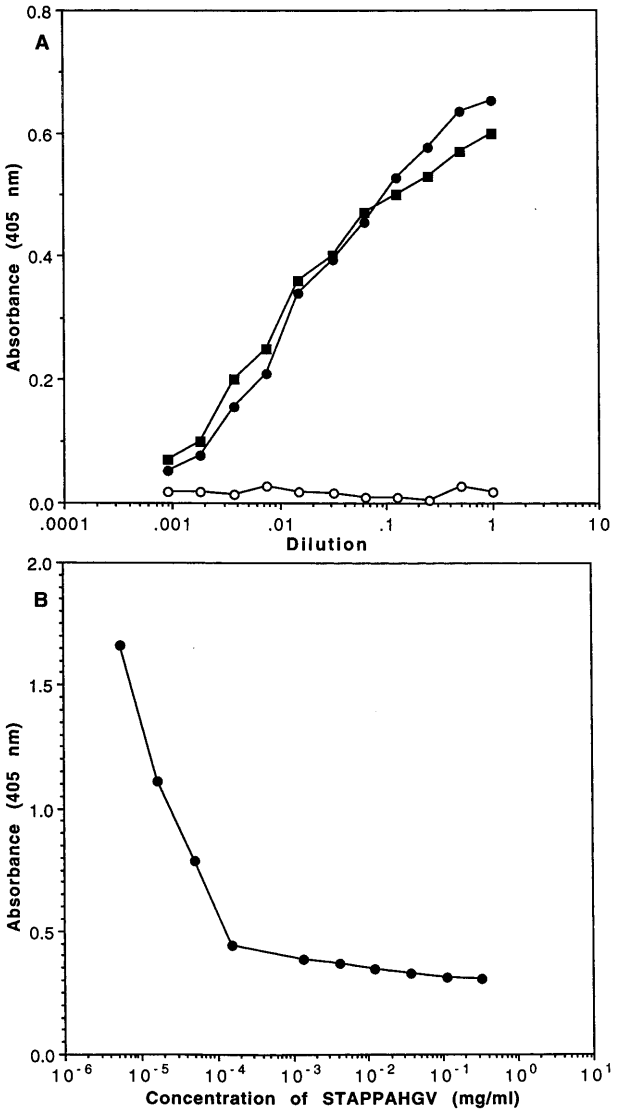

Fig. 1. Binding of anti-MUC1 antibody to IntMUC1-A2 peptide detected by an ELISA assay. (a) A microtitre plate was coated with IntMUC1-A2 peptide (●), MUC1 peptide p1-30 (■) and IntSIN peptide (○) and detected with the anti-MUC1 antibody 13.14 that reacts with the APPAH epitope. (b) Inhibition of binding of anti-MUC1 antibody to plate-bound IntMUC1 by free MUC1-A2 peptide STAPPAHGV (●).

For internalisation studies, adherent peritoneal exudate cells were incubated for various times with the IntMUC1 peptide, fixed and incubated with the anti-MUC1 antibody followed by detection with a FITC-labelled sheep anti-mouse antibody and confocal microscopy. Cells exposed to the IntMUC1 peptide showed cytoplasmic staining and nuclear staining (Fig. 2). The staining was specific as no intracellular fluorescence was seen in cells exposed to buffer or the irrelevant IntSIN peptide (Fig. 2, Panel F). The p1-30 peptide that is recognised by the anti-MUC1 antibody but is devoid of the internalising sequence only showed weak staining (Fig. 2, Panel E). Similar results were seen with the J774 macrophage cell line (not shown). Intracellular staining was also apparent when incubation was carried out at 4°C, suggesting energy and temperature independent uptake of peptide by forming an inverted micelle (Fig. 2, Panel A). Furthermore there was no difference in fluorescence between $T = 1$, 30, 120 min indicating rapid uptake of peptide (Fig. 2, Panels B–D). Therefore, the attachment of the MUC1-A2 CTL epitope peptide to the homeodomain peptide did not interfere with its internalisation and a MUC1 peptide without an internalising sequence was not efficiently internalised.

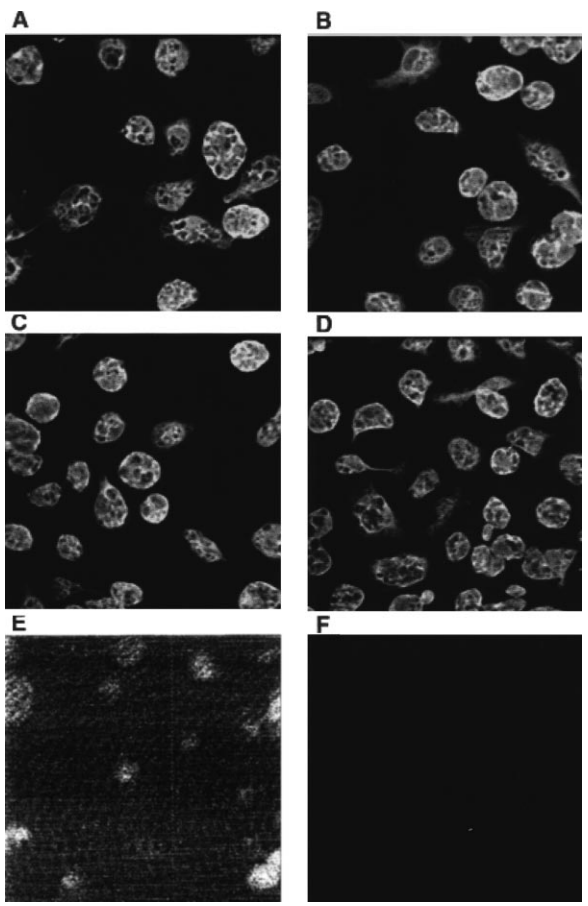

Fig. 2. Uptake of IntMUC1 into peritoneal exudate cells. PEC were incubated with IntMUC1 at 4°C for 1 h (Panel A), at 37°C for 1 min (Panel B), 30 min (Panel C), 2 h (Panel D), p1-30 at 37°C for 1 h (Panel E) or media only (Panel F) fixed and stained with anti-MUC1 antibody 13.14 followed by FITC-labelled sheep anti-mouse antibody and examined by confocal microscopy. Photomultiplier gain and power was identical for all samples.

3.2. Stimulation of ovalbumin-specific T cell hybrid cell line

When OVA-K^b pulsed splenocytes were exposed to the B3Z T cell hybrid, a 7 fold increase in chromogenic response in relation to the medium control was seen (Fig. 3(a)). IntSIN and SINInt pulsed splenocytes showed a 5.2- and 4.8-fold increase in absorbance compared to the medium control, indicating that both peptides were processed similarly to be correctly presented to the TCR of the B3Z (Fig. 3(a)). However, a longer ovalbumin peptide, OVA-24 incorporating the OVA-K^b peptide showed a 2-fold increase in absorbance compared to the medium control. EL4 cells were also pulsed with OVA-K^b, IntSIN or SINInt and incubated with B3Z cells generating absorbances, respectively, of 4.3, 4.3 and 5.6 times controls demonstrating that SINInt and IntSIN are both presented by EL4 cells, irrespective of whether linked to the internalising peptide by the C- or N-terminal of OVA-K^b (Fig. 3(b)). The irrelevant peptide IntMUC1 had no effect on the B3Z cell line. The efficiency of OVA-K^b presentation by Class I was measured by incubating splenocytes with various concentrations of either IntSIN or OVA-K^b and it was shown that OVA-K^b was 13 times more efficient than IntSIN in stimulating the B3Z cell line, which could be due to direct loading of the 9-mer peptide onto surface class I molecules (Fig. 3(c)).

3.3. Cytotoxic T cell responses In IntSIN immunised mice

To determine whether CTL are generated in vivo in response to immunisation of mice with the IntSIN peptide, groups of C57BL/6 mice were injected intraperitoneally on days 0, 7 and 14 with 10, 50 or100 μg IntSIN or OVA-K^b. The splenocytes of immunised mice were assayed for CTL activity after an in vitro stimulation of 6 days. Two different restimulation assay protocols involving the use of OVA-K^b peptide and ovalbumin-loaded splenocytes were compared. The same lytic activity was detected with immunising doses of 10, 50 and 100 μg, therefore data for the 100 μg dose are shown. Splenocytes from mice injected with 100 μg IntSIN that were restimulated with OVA-K^b showed a cytolytic response of 70% lysis at E:T ratio of 25:1 with OVA-K^b pulsed EL4 targets (Fig. 4(a)) but not EL4 cells. Similar lytic activity on OVA-K^b loaded targets and E.G7-OVA was also seen when splenocytes from IntSIN immunised mice were stimulated with OVA

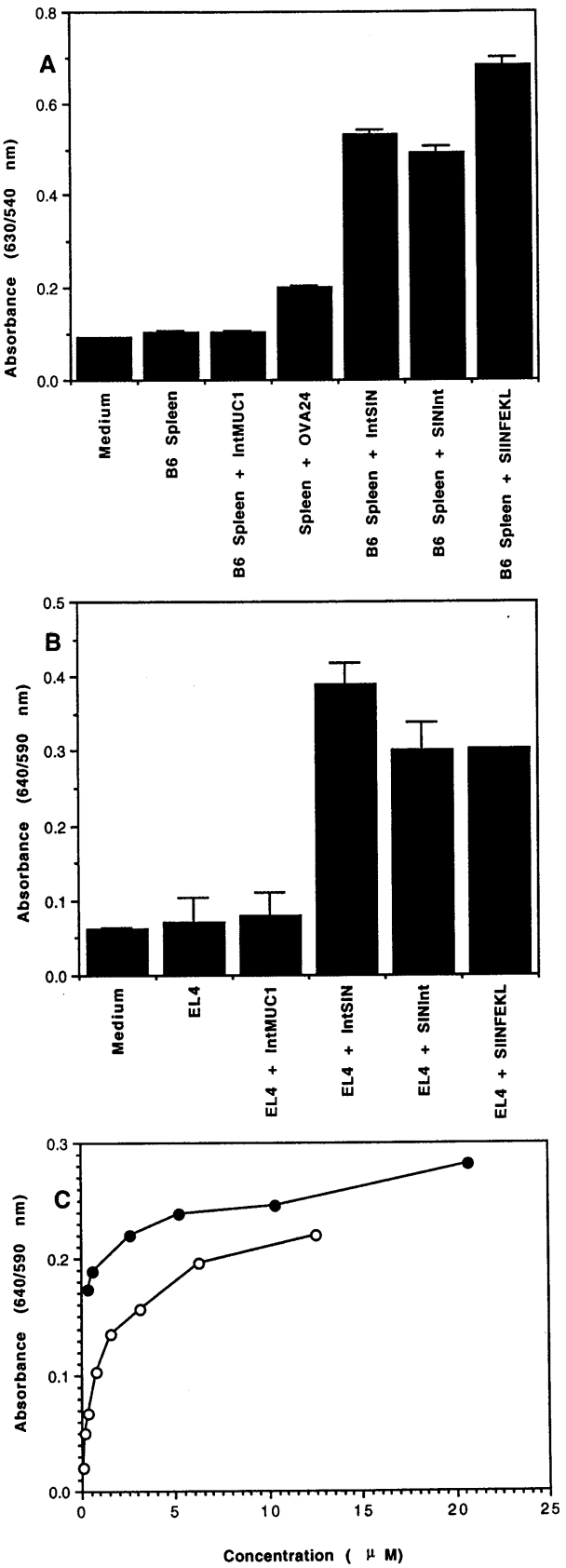

Fig. 3.

loaded splenocytes with lysis of 50% at E:T ratios of 60:1 for both (Fig. 4(b)), indicating that the CTL can recognise native ovalbumin. Non specific lysis was $<$ 15%. No CTL were detected in any of the mice immunised with OVA-K^b (Fig. 4(c)). Therefore, mice immunised with IntSIN generate CTL specific for the OVA-K^b peptide.

3.4. Effect of inhibitors on antigen presentation

To further demonstrate that the IntSIN peptide is processed via the class I presentation pathway the effects of brefeldin A, lactacystin and chloroquine were examined. Lysis of EL4 target cells incubated with IntSIN in the presence of either brefeldin A or lactacystin was inhibited by 64 and 97%, respectively (Fig. 4(d)). This indicates that IntSIN utilises the secretory pathway for class I presentation and that proteosomal processing is required. In contrast, the lysis was not inhibited by chloroquine that interferes with vesicular acidification (Fig. 4(d)). Brefeldin A, lactacystin and chloroquine did not significantly inhibit lysis of EL4 cells pulsed with OVA-K^b (not shown). Thus, these results confirm that IntSIN is processed by the class I presentation pathway.

3.5. In vivo protection assay

We next investigated the ability of IntSIN immunised mice to be protected from a challenge of ovalbumin expressing E.G7-OVA tumour cells. Groups of mice ($n = 6$) were injected intraperitoneally on days 0, 7 and 14 with 5, 10 or 50 μg of IntSIN, OVA-K^b or PBS. Eight days after immunisation mice were challenged with 1×10^7 E.G7-OVA tumour cells. In mice treated with PBS, tumours grew to an average size of 4.5 cm^2 in less than 4 weeks and were culled (Fig. 5(a)). By day 26, the tumours in two out of six mice treated with 5 μg OVA-K^b had disappeared and three of the tumours in mice treated with 10 μg OVA-K^b showed reduced tumour growth (Fig. 5(a–b)), while tumours in mice treated with 50 μg OVA-K^b were not significantly different to the controls (Fig. 5(c)). The growth of E.G7-OVA tumours was greatly reduced when mice were treated with IntSIN. In mice receiving 5, 10 or 50 μg doses, 3 of 5, 4 of 6 and 2 of 5 tumours, respectively,

Fig. 3. Stimulation of the B3Z T cell hybrid by various peptides. C57BL/6 splenocytes (a) or EL4 cells (b) were incubated with medium, IntSIN, SINInt, OVA-24 or OVA-K^b and subsequently incubated with B3Z cells. The cells were lysed and after incubation with substrate, colour was measured using a microtitre plate reader. (c) Various concentrations of either IntSIN (○) or OVA-K^b (●)were incubated separately with C57BL/6 splenocytes and activation of B3Z cells measured colorimetrically. Data are means of three replicate wells ($\pm$ SD)

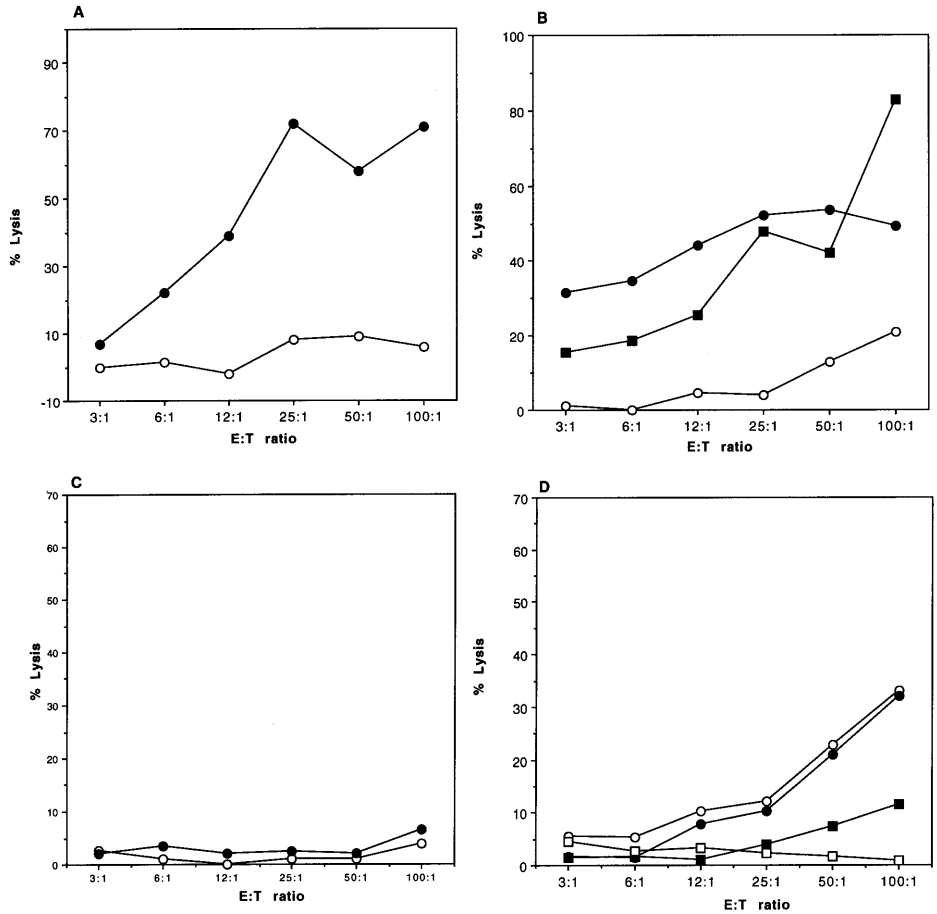

Fig. 4. CTL activity of splenocytes from mice immunised with IntSIN peptide. Splenocytes were restimulated with (a) OVA-K^b or (b) Ovalbumin-loaded splenocytes and their cytolytic activity was measured on ^{51}Cr-labelled (a) OVA-K^b pulsed EL4 (●) or EL4 cells (○); or (b) EL4 (○), OVA-K^b pulsed EL4 (●) or E.G7-OVA cells (■). (c) CTL activity of splenocytes from mice immunised with OVA-K^b. Splenocytes were restimulated with OVA-K^b and their cytolytic activity was measured with ^{51}Cr-labelled OVA-Kb pulsed EL4 (●) or EL4 (○)cells. (d) Effect of inhibitors on target cell lysis by CTL from IntSIN immunised mice. EL4 cells were incubated in media alone (●), brefeldin A (■), lactacystin (□) or chloroquine (○) for 45 min followed by OVA-K^b for 2 h and lysis measured in a ^{51}Cr-release assay.

were eradicated and the mice remained tumour free for greater than 100 days. Therefore, immunisation with IntSIN incorporating the OVA-K^b CTL epitope and the antennapedia internalising peptide protected mice from a tumour challenge without the need for adjuvant and was more effective than the OVA-K^b 9-mer epitope alone.

4. Discussion

For generation of effector CTL, exogenous antigens have to be processed in the cytoplasm of cells and presented to CD8 T cells in the context of Class I molecules [21]. Exogenous antigens are normally processed in the lysosomes and presented by Class II molecules [21]. For the therapeutic generation of CTL to exogenous antigens, it is important to study novel ways of introducing proteins or peptides into the class I processing pathway. In this report we demonstrate that a synthetic peptide incorporating an internalising sequence from the antennapedia homeodomain linked to the ovalbumin CTL epitope peptide is rapidly and efficiently processed and presented to ovalbumin specific T cell hybrids. The processing and presentation was similar regardless of whether the OVA-K^b peptide was linked to either the C-terminus or N-terminus of the antennapedia peptide. However, the OVA-K^b 9-mer peptide was presented by spleen cells more efficiently than IntSIN, which could be due to the direct loading of peptide into Class I molecules on the surface [22]. However, IntSIN was presented more effectively than a 24 amino acid peptide of similar length incorporating the OVA-K^b CTL peptide, indicating the advantage of having an internalising sequence.

Confocal studies demonstrated very rapid uptake of the IntMUC1 peptide into the cytoplasm and nucleus of peritoneal exudate cells, similar to that described earlier for the antennapedia peptide [9]. A control peptide without the internalising sequence was not efficiently taken up by PECs as demonstrated by weak staining, which also demonstrated that uptake was not

due to non-specific phagocytosis of the peptide. Once peptides enter the cytoplasm they will be degraded by the proteosomes and gain entry to the Class I loading compartment. Recognition of OVA-K^b was inhibited by incubation with brefeldin A and lactacystin but not by chloroquine, suggesting utilisation of the secretory pathway for class I presentation. These findings also indicates that the internalising peptides are not taken up by the pathway described by Day et al. which bypasses the cytoplasm and Golgi complex [23]. Previous studies with the whole 60 amino acid antennapedia homeodomain fused to the influenza nuclear protein T cell epitope peptide indicated a brefeldin A sensitive pathway [11], showing dependence of intracellular processing.

Mice immunised with IntSIN produced CTL that were specific for ovalbumin and were able to lyse E.G7-OVA target cells pulsed with OVA-K^b and mice immunised with the IntSIN peptide were also protected from the growth of ovalbumin expressing tumour cells. In the dose range tested of 5, 10 and 50 μg, IntSIN was more effective in stimulating effective anti-tumour immunity than the corresponding dose of OVA-K^b. Schutze-Redelmeier et al., utilised an expression vector containing the whole 60 amino acid homeodomain to fuse to the 170–179 HLA-Cw3-derived peptide and 147-156 influenza nucleoprotein peptide [11]. These recombinant fusion proteins were successful in priming a

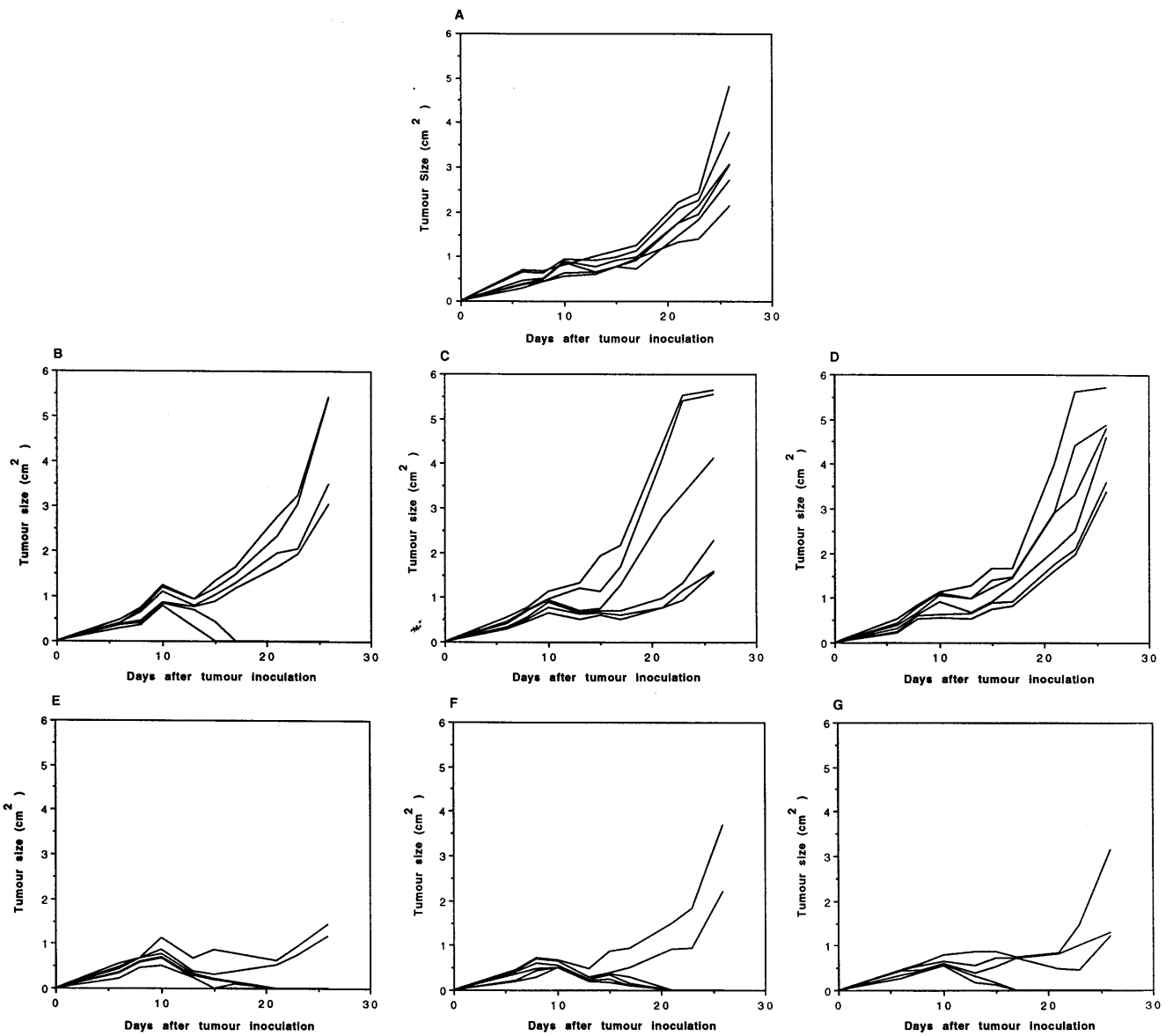

Fig. 5. Inhibition of tumour growth by IntSIN. Groups of 6 C57Bl/6 mice were immunised with PBS (a) or 5 μg (b), 10 μg (c) or 50 μg (d) of OVA-K^b or IntSIN (e–g). After subcutaneous challenge subcutaneously with 1×10^7 cells, tumour growth was monitored. Data show tumour growth for each animal for each group.

CTL response in vivo but only when used with SDS as an adjuvant while IFA, saponin and aluminium hydroxide were without effect. It is interesting that in our study, in vivo priming was successful without the need for any adjuvants. The mice immunised with the 5 μg dose of OVA-K^b showed some antitumour effects but this was not as effective as the mice treated with IntSIN and none of the mice treated with the 9-mer OVA-K^b peptide had detectable CTL.

It is evident from our data that CTL epitope peptides tandemly linked to the antennapedia peptide rapidly access the cytoplasm of cells and are subsequently processed and presented by Class I molecules in vitro. In vivo, the IntSIN peptide was more effective than the OVA-K^b peptide. We cannot rule out other factors such as stability, bioavailability and the presence of helper epitopes that could be contributing to the increased efficacy in vivo, and we are currently investigating these aspects.

Several other proteins are highly efficient at entering the cytoplasm via the endosomal membrane. Bacterial toxins are internalised via a cell surface receptor and the toxin escapes from the endosome into the cytosol. *Pseudomonas* exotoxin A [24], diphtheria toxin [25], shiga toxin [26] and anthrax toxin [27] have all been used successfully to deliver T cell epitopes into the cytoplasm. Thus, microbial toxins can be readily utilised to translocate peptides or proteins into the cytosol of cells, however the value of such constructs in humans remains to be seen, due to the presence of immunity to some toxins. In a recent study, the HIV *tat* peptide was chemically linked to ovalbumin and used to incorporate ovalbumin into dendritic cells for priming CTL in vivo [28].

Despite success in a recent report describing the use of synthetic peptides alone as a vaccine [29], peptides are generally ineffective as vaccines without an adjuvant. Several strategies have been used to overcome the limitations of peptides which are rapid clearance, small size and instability, however there are several advantages for using synthetic peptide constructs [2] similar to that described in this paper. There are very few innocuous adjuvants that invoke cellular responses in humans. It will be preferable to use synthetic peptides incorporating the antennapedia internalising sequence linked to specific CTL epitopes that are efficiently taken up by all types of cells and are effective in vitro and in vivo in stimulating specific cellular responses.

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