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Anti-inflammatory activity of c(ILDV-NH$(CH_2)_5$CO), a novel, selective, cyclic peptide inhibitor of VLA-4-mediated cell adhesion

*,[1]Duncan Haworth, [1]Amanda Rees, [1]Peter J. Alcock, [1]Linda J. Wood, [1]Anand S. Dutta, [1]James J. Gormley, [2]Huw B. Jones, [1]Alec Jamieson & [1]Christopher F. Reilly

[1]CM&M Research Department, Zeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG and [2]Safety of Medicines Department, Zeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG

1 Small, N- to C-terminal cyclized peptides containing the leucyl-aspartyl-valine (LDV) motif from fibronectin connecting segment-1 (CS-1) have been investigated for their effects on the adhesion of human T-lymphoblastic leukaemia cells (MOLT-4) to human plasma fibronectin *in vitro* mediated by the integrin Very Late Antigen (VLA)-4 ($\alpha_4\beta_1$, CD49d/CD29).

2 Cyclo(-isoleucyl-leucyl-aspartyl-valyl-aminohexanoyl-) (c(ILDV-NH$(CH_2)_5$CO)) was approximately 5 fold more potent (IC_{50} 3.6 ± 0.44 μM) than the 25-amino acid linear CS-1 peptide. Cyclic peptides containing two more or one less methylene groups had similar potency to c(ILDV-NH$(CH_2)_5$CO) while a compound containing three less methylene groups, c(ILDV-NH$(CH_2)_2$CO), was inactive at 100 μM.

3 c(ILDV-NH$(CH_2)_5$CO) had little effect on cell adhesion mediated by two other integrins, VLA-5 (α_5,β_1, CD49e/CD29) (K562 cell adhesion to fibronectin) or Leukocyte Function Associated molecule-1 (LFA-1, $\alpha_L\beta_2$, CD11a/CD18) (U937 cell adhesion to Chinese hamster ovary cells transfected with intercellular adhesion molecule-1) at concentrations up to 300 μM.

4 c(ILDV-NH$(CH_2)_5$CO) inhibited ovalbumin delayed-type hypersensitivity or oxazolone contact hypersensitivity in Balb/c mice when dosed continuously from subcutaneous osmotic mini-pumps ($0.1-10$ mg kg^{-1} day^{-1}). Maximum inhibition (approximately 40%) was similar to that caused by the monoclonal antibody PS/2 (7.5 mg kg^{-1} i.v.) directed against the α_4 integrin subunit.

5 c(ILDV-NH$(CH_2)_5$CO) also inhibited oxazolone contact hypersensitivity when dosed intravenously 20 h after oxazolone challenge ($1-10$ mg kg^{-1}). Ear swelling was reduced at 3 h and 4 h but not at 1 h and 2 h post-dose (10 mg kg^{-1}).

6 Small molecule VLA-4 inhibitors derived from c(ILDV-NH$(CH_2)_5$CO) may be useful as anti-inflammatory agents.

Keywords: VLA-4; $\alpha_4\beta_1$ integrin; cell adhesion; leukocyte recruitment; delayed-type hypersensitivity; inflammation

Abbreviations: BCECF-AM, 2′, 7′-bis(2-carboxyethyl)-5/6-carboxyfluorescein acetoxymethyl ether; c(ILDV-NH$(CH_2)_5$CO), cyclo(-isoleucyl-leucyl-aspartyl-valyl-aminohexanoyl-); c(ILDV-NH$(CH_2)_2$CO), cyclo(-isoleucyl-leucyl-aspartyl-valyl-aminopropionyl-); CHO, Chinese hamster ovary; CS-1, connecting segment-1; DMEM, Dulbecco's modified Eagles Medium; DTH, delayed-type hypersensitivity; FCS, foetal calf serum; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; ICAM-1, intercellular adhesion molecule-1; LDV, leucyl-aspartyl-valine; LFA-1, leukocyte function associated molecule-1 (CD11a/CD18); MAdCAM-1, mucosal addressin adhesion molecule-1; PBS, Dulbecco's phosphate buffered saline; PMA, phorbol myristate acetate; RFU, relative fluorescence units; RGD, arginyl-glycyl-aspartic acid; VCAM-1, vascular cell adhesion molecule-1; VLA, very late antigen

Introduction

The extravasation of circulating leukocytes is dependent on the interaction between adhesion molecules on the leukocyte surface and specific counter receptors on vascular endothelial cells (Sharar *et al*., 1995). The integrin Very Late Antigen-4 (VLA-4, $\alpha_4\beta_1$ CD49d/CD29) is expressed on human lymphocytes, monocytes (Hemler *et al*., 1987) and eosinophils (Bochner *et al*., 1991) and may have an important role in the recruitment of these cells during inflammation. VLA-4 is a receptor for vascular cell adhesion molecule-1 (VCAM-1) (Elices *et al*., 1990) and the alternatively-spliced type III connecting segment region of fibronectin containing the 25-amino acid connecting segment-1 (CS-1) (Wayner *et al*., 1989). VCAM-1 expression is upregulated on endothelial cells *in vitro* by inflammatory cytokines (Osborn *et al*., 1989) and in human inflammatory diseases such as rheumatoid arthritis (Morales-Ducret *et al*., 1992), multiple sclerosis (Cannella & Raine, 1995) and allergic asthma (Fukuda *et al*., 1996). CS-1 expression on microvasculature has also been shown to be upregulated in rheumatoid arthritis (Elices *et al*., 1994). Monoclonal antibodies directed against the α_4 integrin subunit have been shown to be effective in animal models of autoimmune or allergic diseases such as experimental autoimmune encephalomyelitis (Kent *et al*., 1995), collagen-induced arthritis (Zeidler *et al*., 1995) and antigen-induced bronchial hyperreactivity and eosinophil recruitment (Pretolani *et al*., 1994).

Integrins recognize short peptide motifs in their ligands. The minimal VLA-4 binding epitope in CS-1 is the tripeptide leucyl-aspartyl-valine (LDV) (Komoriya *et al*., 1991) while VCAM-1 contains the similar sequence isoleucyl-aspartyl-

*Author for correspondence.

serine (Clements *et al*., 1994). The 25-amino acid CS-1 peptide (DELPQLVTLPHPNLHGPEILDVPST) is a competitive inhibitor of VLA-4 binding to VCAM-1 (Makarem *et al*., 1994). Small molecular linear (Molossi *et al*., 1995) and cyclic peptide (Doyle *et al*, 1996; Vanderslice *et al*., 1997; Viles *et al*., 1996) VLA-4 inhibitors based on the LDV sequence in CS-1 have been described. In the present study we have investigated the effects of small, N- to C-terminal cyclized, LDV peptides on VLA-4 mediated cell adhesion *in vitro*. One of these compounds, cyclo(-isoleucyl-leucyl-aspartyl-valyl-aminohexanoyl-) (c(ILDV-NH$(CH_2)_5$CO)), has been tested further for selectivity against other integrins and for its anti-inflammatory activity in mouse models of T-cell-dependent inflammation, ovalbumin-induced delayed-type hypersensitivity (DTH) and oxazolone-induced contact hypersensitivity.

Methods

Monoclonal antibodies

The following monoclonal antibodies directed against integrin subunits were used: anti-human α_4 (HP2/1) and anti-human α_5 (SAM-1), from Serotec (Oxford, U.K.), anti-human β_1 (mAb 13) from Becton Dickinson (Oxford, U.K.) and anti-human α_L (clone 38) from R&D Systems (Abingdon, U.K.). Anti-mouse α_4 (PS/2) was purified from rat hybridoma supernatant on a Protein G Sepharose affinity column (Amersham Pharmacia Biotech, Little Chalfont, U.K.) by Derek Barratt, Zeneca Pharmaceuticals. The purified antibody was dialyzed into 20 mM sodium phosphate, 150 mM sodium chloride pH 7.4, snap frozen and stored at -80 °C. The concentration of PS/2 was determined by amino acid analysis.

Peptides

Cyclic peptides and the linear peptide CS-1 were obtained by the solid phase peptide synthesis methods using commercially available Wang and 2-chlorotritylchloride resins and various coupling and deblocking reagents. The synthesis of c(ILDV-NH$(CH_2)_5$CO) is shown as an example in Figure 1. The crude deprotected cyclic peptides were extensively purified by preparative reverse phase high performance liquid chromatography (HPLC) on a Vydac 218TP1022 column using a gradient of acetonitrile-water containing 0.1% (v v^{-1}) trifluoroacetic acid at a flow rate of 10 ml min^{-1}. The homogeneity of the peptides was checked by analytical HPLC before characterization by amino acid analysis and mass spectroscopy. For the synthesis of the 25-amino acid peptide CS1, the C-terminal threonine residue was attached to the Wang resin and the chain assembly was continued by using N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) protected amino acid derivatives. Standard deblocking and coupling procedures were employed. The peptide resin was then treated with trifluoroacetic acid to remove the side chain protecting groups and to cleave the peptide from the resin. The Arg-Gly-Asp (RGD)-containing peptide, echistatin was purchased from Sigma. For *in vitro* studies, peptides were dissolved in dimethyl sulphoxide and diluted in cell culture medium to the concentrations required. For *in vivo* studies, peptides were dissolved in sterile saline and the pH adjusted to neutral with sodium hydroxide.

Cell lines

MOLT-4 cells (human T-lymphoblastic leukaemia) and U937 cells (human monoblastic leukaemia) were obtained from the European Collection of Animal Cell Cultures (Porton Down, U.K.). Chinese hamster ovary cells (CHO DG44) were obtained from Professor L. Chasin (Columbia University, New York, U.S.A.) and K562 cells (human erythroleukaemia) from the Christie Hospital (Manchester, U.K.). MOLT-4, K562 and U937 cells were maintained in RPMI-1640 medium with 2 mM L-glutamine (Life Technologies, Paisley, U.K.) and 10% foetal calf serum (FCS, Advanced Protein Products, Brierley Hill, U.K.) under a humidified 5% (v v^{-1}) CO_2 atmosphere at 37°C. CHO cells were transfected with human ICAM-1 cDNA cloned into a pCDM8 expression vector (R&D Systems) by calcium phosphate precipitation (Georgia Cerillo, Zeneca Pharmaceuticals). An ICAM-1 expressing clone was maintained in Dulbecco's modified Eagles Medium (DMEM) containing 2 mM L-glutamine, 1% penicillin/streptomycin, non-essential amino acids (Life Technologies) and 10% FCS. The rat hybridoma PS/2 (Miyake *et al*., 1991) was obtained from the American Type Culture Collection (Rockville, U.S.A.).

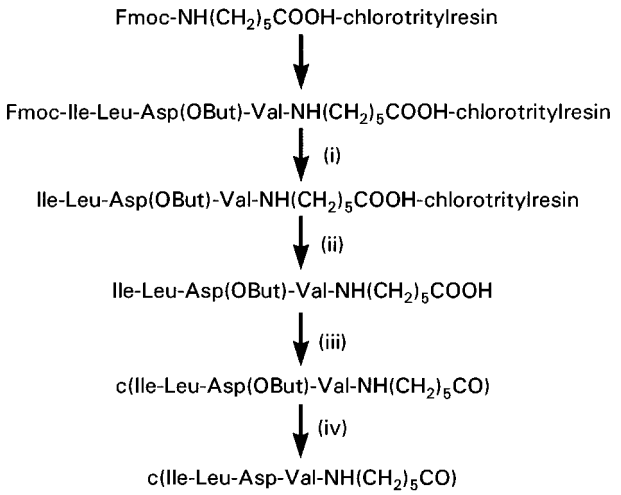

Figure 1 Procedure for the synthesis of c(ILDV-NH$(CH_2)_5$CO). The same procedure was used for the synthesis of other cyclic peptides using the appropriate aminoalkanoic acid. Reagents used at each step were: (i) piperidine; (ii) acetic acid-trifluoroethanol-dichloromethane; (iii) *O*-(benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole and diisopropylethylamine and (iv) trifluoroacetic acid-water-triisopropylsilane. Fmoc is 9-fluorenylmethoxycarbonyl.

Treatment of U937 cells with PMA

U937 cells were suspended at 1×10^5 ml^{-1} in RPMI-1640 containing 10% FCS and 2 mM L-glutamine and incubated overnight with 0.1 μM phorbol myristate acetate (PMA, Sigma) at 37°C in a humidified 5% (v v^{-1}) CO_2 atmosphere. After aspiration of non-adherent cells, the adherent cells were detached with trypsin/EDTA and washed twice in RPMI-1640 containing 5% FCS and 2 mM L-glutamate.

Measurement of cell surface integrin expression

Integrin expression by the cell lines used was analysed by flow cytometry using a FACScan (Becton Dickinson). Cells were washed and resuspended in Hanks' Balanced Salts Solution (Life Technologies) containing 5% heat inactivated (65°C for 1 h) FCS and incubated with antibodies to integrin subunits or isotype controls (Becton Dickinson) for 30 min at 4°C as follows: anti-α_4 and IgG_1 control (1 μg ml^{-1}), anti-α_5 and

IgG_{2b} control (5 μg ml^{-1}), anti-β_1 and IgG_{2a} control (10 μg ml^{-1}) and anti-α_L and IgG_{2a} control (0.5 μg ml^{-1}). Cells were then washed with DMEM containing 2% (v v^{-1}) heat inactivated FCS and 0.1% (w v^{-1}) sodium azide (Sigma) and incubated with $F(ab')_2$ rabbit anti-mouse IgG-fluorescein isothiocyanate conjugate (Serotec) (10 μg ml^{-1}) for 30 min at 4°C in the dark. Samples were washed in DMEM containing 2% heat inactivated FCS and 0.1% sodium azide and resuspended in 200 μl 1% (w v^{-1}) propidium iodide in the same medium before analysis.

Fluorescent labelling of K562 and U937 cells

K562 and PMA-treated U937 cells were labelled with the fluorescent dye 2′, 7′-bis(2-carboxyethyl)-5/6-carboxyfluorescein acetoxymethyl ether (BCECF-AM, Molecular Probes, Eugene, U.S.A.). Cells (3×10^6 ml^{-1}) were resuspended in 100 μM BCECF-AM dissolved in RPMI-1640 with 5% FCS and 2 mM L-glutamine and incubated for 1 h at room temperature in the dark. BCECF-labelled cells were washed three times and resuspended in RPMI-1640 with 5% foetal calf serum and 2 mM L-glutamine before use in an adhesion assay. Fluorescence of BCECF-labelled cells (excitation 485 nm, emission 538 nm) was linearly-related to cell number confirming published observations (Ross *et al*., 1992).

Coating of 96-well plates with fibronectin

Polystyrene 96-well plates (Costar, High Wycombe, U.K.) were coated overnight at 4°C with human plasma fibronectin (Bioproducts Laboratory, Elstree, U.K.), 100 μl of 20 μg ml^{-1} in Dulbecco's phosphate buffered saline (PBS, Life Technologies). Non-specific adhesion sites were blocked by adding 100 μl bovine serum albumin, fraction V (ICN, Thame, U.K.), 20 mg ml^{-1} in PBS. After incubating for 1 h at room temperature, the solutions were aspirated.

MOLT-4 cell adhesion to fibronectin

MOLT-4 cells suspended in serum-free RPMI-1640 at 2×10^6 ml^{-1} (50 μl) and peptide or antibody solutions diluted in the same medium (50 μl) were added to wells of a fibronectin-coated 96-well plate in triplicate. After incubation for 2 h at 37°C in a humidified atmosphere of 5% (v v^{-1}) CO_2, non-adherent cells were removed by gentle shaking followed by aspiration. Adherent cells were quantified by a colorimetric phosphatase assay (Prater *et al*., 1991). To each well was added 100 μl p-nitrophenyl phosphate (Boehringer Mannheim, Lewes, U.K.), 6 mg ml^{-1} in 50 mM sodium acetate, pH 5.0 containing 1% Triton X-100 (Sigma, Poole, U.K.). After incubation for 1 h at 37°C, 50 μl sodium hydroxide (1 M) was added to each well and the absorbance 405 nm measured using a Titertek Multiscan Plus (Flow Laboratories, Irvine, U.K.). Absorbance was linearly-related to cell number up to 10^5 cells, confirming published results (Prater *et al*., 1991).

K562 cell adhesion to fibronectin

BCECF-labelled K562 cells resuspended at 2×10^6 ml^{-1} (50 μl) and peptide or antibody solutions diluted in RPMI containing 5% FCS and 2 mM glutamine (50 μl) were added to wells of a fibronectin-coated plate in triplicate. After incubation for 30 min at 37°C in 5% (v v^{-1}) CO_2 the plates were washed three times with assay medium and 2% (w v^{-1}) Triton X-100, pre-warmed to 37°C, (100 μl) was added to each well. Fluorescence (excitation 485 nm, emission 538 nm) was measured using a Fluoroskan II (Labsystems, Cambridge, U.K.).

U937 cell adhesion to ICAM-1 transfected CHO cells

ICAM-1 CHO cells were added to 96-well plates at 5×10^4 per well in assay medium (DMEM with 5% FCS and 2 mM glutamine) and incubated overnight at 37°C in a humidified atmosphere of 5% (v v^{-1}) CO_2. Plates containing ICAM-1 CHO cells were inspected under a microscope to confirm that they had grown to confluence and then were washed twice and incubated with 100 μl assay medium containing test compounds in triplicate for 45 min at 37°C in 5% (v v^{-1}) CO_2. BCECF-labelled U937 cells resuspended at 1×10^6 ml^{-1} (100 μl) were added to each well, the plates incubated at 37°C for a further 30 min. The fluorescence of adherent cells was measured as described for K562 cells.

Animals

Balb/c mice (20–25 g) were obtained from the Zeneca Pharmaceuticals Barriered Breeding Unit (Alderley Park, U.K.) and housed for at least 1 week before use. Food and water were supplied *ad libitum*. Animal welfare and experimental procedures were carried out strictly in accordance with the Animals (Scientific Procedures) Act, 1986 and the Zeneca International Policy on the Use of Animals.

Ovalbumin delayed type hypersensitivity

Female mice were immunized on the flank (0.1 ml s.c.) with a 1 : 1 emulsion of 2 mg ml^{-1} ovalbumin (Sigma) in saline and complete Freund's adjuvant (Life Technologies). Seven days later mice were challenged in the right hind foot pad with a subplantar injection of 1% (w v^{-1}) heat-aggregated ovalbumin in saline (30 μl). After 24 h, the thickness of both hind feet was measured with a micrometer. The heat-aggregated ovalbumin was prepared by heating a 5% solution of ovalbumin in saline for 2 h at 70°C. After cooling and centrifugation, the pellet was washed twice with saline, resuspended at 2% in saline and aliquots stored at −20°C. Before injection, the heat-aggregated ovalbumin was diluted with an equal volume of saline and sonicated.

Oxazolone contact hypersensitivity

Oxazolone contact hypersensitivity was induced by a modification of published methods (Chisholm *et al*., 1993; Elices *et al*., 1993). Male mice were sensitized with oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma) by topical application of 50 μl of a 0.24% solution in acetone/olive oil (4 : 1) to a shaved area of skin on the back under halothane anaesthesia (4% in O_2). Seven days later the mice were challenged by topical application of a 0.25% (w v^{-1}) solution of oxazolone in acetone/olive oil (25 μl) to both ears. Ear thickness was measured with a micrometer before and at 24 h after oxazolone application.

Dosing of peptides and antibody

Peptides were dosed by continuous infusion from 3 day osmotic mini-pumps (Alzet, Paolo Alto, U.S.A.) implanted subcutaneously under halothane anaesthesia (4% in O_2) on the day before antigen challenge. The anti-α_4 monoclonal antibody or isotype control (purified rat $IgG_{2b\kappa}$, Serotec) were dosed by tail vein injection 4 h before antigen challenge. In some

experiments, peptides and antibody were dosed intravenously 20 h after oxazolone challenge.

Histology

Ovalbumin-inflamed and control feet were removed *post mortem* from a group of six mice 24 h after subplantar injection and fixed in 10% formalin buffered saline. After decalcification, the samples were embedded in paraffin, sectioned and stained with haematoxylin and eosin.

Data analysis

IC_{50} values for peptides in the cell adhesion assays were estimated by sigmoid curve fitting of triplicate absorbance or fluorescence data using Origin (Microcal Software, Northampton, U.S.A.). Ear swelling in oxazolone contact hypersensitivity was expressed as the percentage increase in ear thickness at 24 h over the thickness before topical application of the antigen. Foot swelling in ovalbumin delayed-type hypersensitivity was expressed as the percentage increase of the thickness of the ovalbumin-injected foot over the uninjected foot at 24 h. Results are expressed as mean ± s.e.mean. The effect of peptides or antibody was expressed as percentage inhibition of the inflammation in vehicle-dosed control groups. Statistical significance of the difference between group means was determined using Student's *t*-test (two-tailed) with $P<0.05$ being treated as significant. The dose of c(ILDV-NH$(CH_2)_5$CO) giving half the inhibition by the anti-α_4 monoclonal antibody was estimated by sigmoid curve fitting of the foot swelling of individual mice in each dose group.

Results

Effect of compounds on MOLT-4 cell adhesion to fibronectin

MOLT-4 cells were used to assess the effects of compounds on VLA-4-mediated adhesion. Flow cytometric analysis showed that the MOLT-4 cells expressed the α_4 and β_1 subunits of VLA-4 but not the α_5 subunit of VLA-5 (Table 1). MOLT-4 cells also expressed the α_L subunit of Leukocyte Function Associated molecule-1 (LFA-1, $\alpha_L\beta_2$, CD11a/CD18) (Table 1). In control wells, MOLT-4 cell adhesion to immobilized human plasma fibronectin was 1.4 ± 0.065 absorbance units (approximately 100% of added cells) while non-specific adhesion to wells not coated with fibronectin was 0.053 ± 0.0069 absorbance units ($n=40$). Monoclonal antibodies to the α_4 and β_1 integrin subunits of VLA-4 (0.5 μg ml^{-1}) inhibited MOLT-4 cell adhesion by $95\pm1.2\%$ ($n=15$) and $96\pm2.7\%$ ($n=8$) respectively while an antibody to the α_5 subunit of VLA-5 ($\alpha_5\beta_1$, CD49e/CD29) (1 μg ml^{-1}) had no effect ($-0.71\pm0.40\%$ inhibition, $n=5$). The cyclic compound c(ILDV-NH$(CH_2)_5$CO) was a 5 fold more potent inhibitor of MOLT-4 cell adhesion than the 25 amino acid CS-1 linear peptide (Table 2). A dose response to c(ILDV-NH$(CH_2)_5$CO) is shown in Figure 2. Cyclic compounds with one less or two more methylene groups had similar potency to c(ILDV-NH$(CH_2)_5$CO) (Table 2). The cyclic compound with three less methylene groups was inactive ($8.6\pm4.3\%$ inhibition, $n=3$) at 100 μM (Table 2).

Table 1 Expression of integrin subunits by cell lines used in adhesion assays

	Integrin subunit			
Cell line	α_4	α_5	β_1	α_L
MOLT-4	+	−	+	+
K562	−	+	+	not measured
PMA-treated U937	+	+	+	+

Integrin subunit expression was determined by FACS analysis using mouse monoclonal antibodies directed against each integrin subunit and an FITC-conjugated (Fab')$_2$ anti-mouse IgG. Cells were incubated with an appropriate isotype control to determine background fluorescence. + Increased relative fluorescence intensity compared with the isotype control. − No difference in relative fluorescence intensity compared with the isoptype control.

Effect of c(ILDV-NH$(CH_2)_5$CO) on K562 cell adhesion to fibronectin

K562 cells were used to assess the effect of c(ILDV-NH$(CH_2)_5$CO) on VLA-5-mediated adhesion. Flow cytometric analysis showed that K562 cells expressed the α_5 and β_1 subunits of VLA-5 but not the α_4 subunit of VLA-4 (Table 1). Control K562 cell adhesion to immobilized human plasma fibronectin was 25 ± 3.5 ($n=14$) relative fluorescence units (RFU) while non-specific adhesion to wells not coated with fibronectin was 2.7 ± 0.12 RFU ($n=14$). Monoclonal antibodies to the α_5 and β_1 integrin subunits of VLA-5 (1 μg ml^{-1}) inhibited K562 cell adhesion by $100\pm0.32\%$ ($n=10$) and $96\pm2.3\%$ ($n=4$) respectively while an antibody to the α_4 subunit of VLA-4 (1 μg ml^{-1}) had no effect ($-5.2\pm2.8\%$ inhibition, $n=13$). The Arg-Gly-Asp containing peptide echistatin (0.1 μM) also inhibited K562 cell adhesion by $93\pm5.4\%$ ($n=4$). c(ILDV-NH$(CH_2)_5$CO) had no effect on K562 cell adhesion at concentrations up to 300 μM (Figure 2).

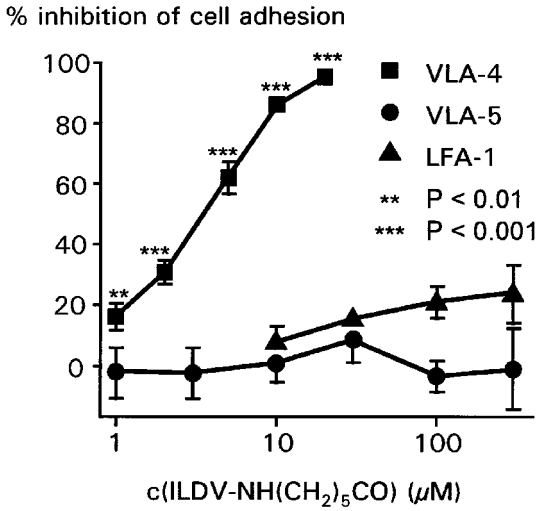

Figure 2 The effect of c(ILDV-NH$(CH_2)_5$CO) on MOLT-4 cell adhesion to immobilized human plasma fibronectin (VLA-4-mediated, $n=9$), K562 cell adhesion to immobilized human plasma fibronectin (VLA-5-mediated, $n=3$) and PMA-treated U937 cell adhesion to ICAM-1 transfected CHO cells (LFA-1-mediated, $n=5$). Values are shown as mean ± s.e.mean. Statistical significance of the inhibition of VLA-4-mediated cell adhesion was determined by a paired *t*-test comparing adhesion in control and compound-treated samples.

Effect of c(ILDV-NH(CH$_2$)$_5$CO) on U937 cell adhesion to ICAM-1 transfected CHO cells

The effect of c(ILDV-NH(CH$_2$)$_5$CO) on LFA-1-mediated cell adhesion was assessed using PMA-treated U937 cells and ICAM-1 transfected CHO cells. PMA-treated U937 cells were shown to express the α_L subunit of LFA-1 by flow cytometry (Table 1). Adhesion of PMA-treated U937 cells to ICAM-1 transfected CHO cells in the absence of c(ILDV-NH(CH$_2$)$_5$CO) was 28 ± 3.3 RFU ($n=7$) while non-specific adhesion on the same plates was 2.8 ± 0.078 RFU ($n=7$). A monoclonal antibody against the α_L integrin subunit of LFA-1 (5 μg ml^{-1}) inhibited U937 cell adhesion by $89 \pm 1.0\%$ ($n=5$) while an antibody to the α_4 subunit of VLA-4 (1 μg ml^{-1}) had no effect ($9.5 \pm 5.2\%$ inhibition, $n=6$). c(ILDV-NH(CH$_2$)$_5$CO) had little effect on U937 cell adhesion at concentrations up to 300 μM (Figure 2).

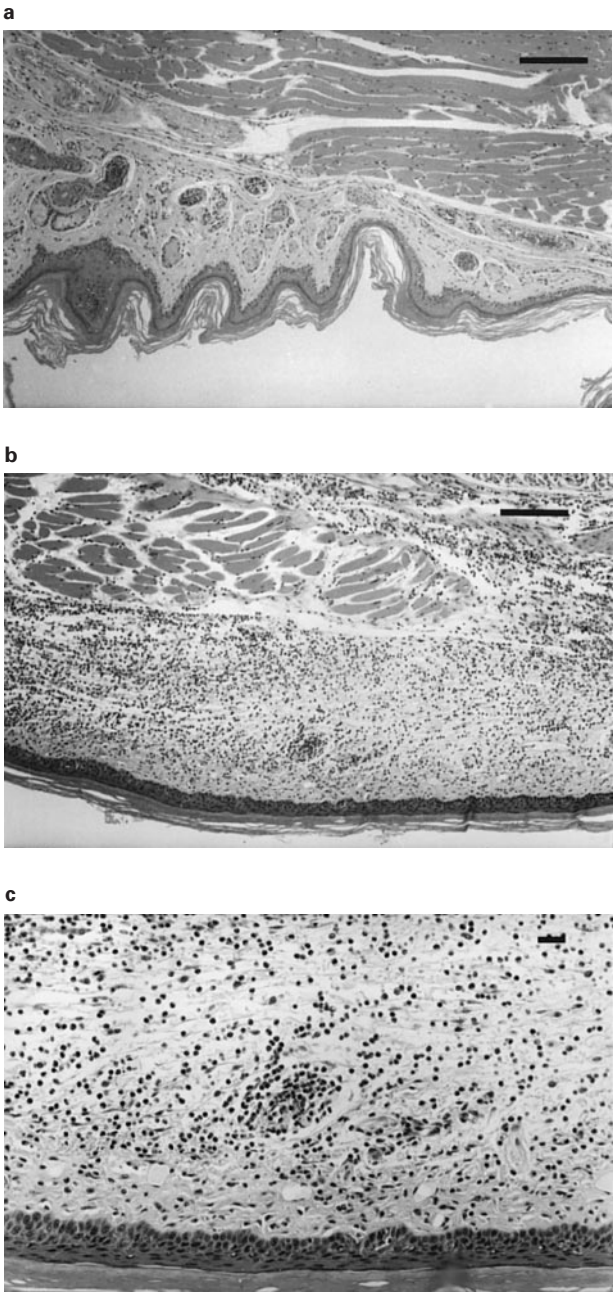

Figure 3 Representative photomicrographs of mouse foot sections stained with haematoxylin and eosin. (a) Normal appearance of the plantar surface of a saline-injected foot. Bar = 125 μm. (b) Appearance of an ovalbumin-injected foot 24 h following induction of ovalbumin delayed-type hypersensitivity. The tissues of the plantar surface of the foot show substantial oedema of the dermis and adjacent muscle (separation of muscle fibres) and marked infiltration of acute inflammatory cells (predominantly neutrophils). Bar = 125 μm. (c) Increased magnification of part of the ovalbumin-injected foot section showing the composition of the inflammatory cell infiltrate as predominantly neutrophils and monocytes/macrophages. Bar = 20 μm.

Effect of c(ILDV-NH(CH$_2$)$_5$CO) on mouse ovalbumin delayed-type hypersensitivity

The anti-inflammatory effect of c(ILDV-NH(CH$_2$)$_5$CO) was assessed in mouse ovalbumin-induced DTH, a model of T-cell-dependent inflammation, and compared with the effect of the anti-α_4 monoclonal antibody PS/2. The T-cell dependence of the inflammation was confirmed by comparing the inflammatory response in immunized mice with that in mice which had not been immunized with ovalbumin. In immunized mice, foot swelling 24 h after ovalbumin injection was $56 \pm 1.1\%$ ($n=13$) while in non-immunized mice the swelling was only $14 \pm 2.0\%$ ($n=5$). Histological assessment of sections from swollen feet of immunized mice 24 h after ovalbumin injection showed oedema and substantial infiltrates of acute inflammatory cells into the subplantar tissues (Figure 3). The oedema was associated with the inflammatory cell infiltrate in the dermis but occasionally, also extended between and separated the fibres of adjacent deep-lying skeletal muscle. The inflammatory cell infiltrate consisted predominantly of neutrophils and few monocytes or macrophages. Very few lymphocytes could be seen in the 24 h sections. In saline-injected control feet, the histological appearance of the plantar and subplantar hind foot tissues was normal (Figure 3).

In mice immunized with ovalbumin, PS/2 inhibited ovalbumin-induced foot swelling dose-dependently and maximum inhibition ($41 \pm 1.5\%$, $n=16$) was obtained at a dose of 7.5 mg kg^{-1} (Table 3). PS/2 had no effect on the small foot swelling induced by ovalbumin in non-immunized mice ($-0.09 \pm 9.4\%$ inhibition, $n=8$). c(ILDV-NH(CH$_2$)$_5$CO), when dosed continuously from a subcutaneous osmotic minipump (0.1 – 10 mg kg^{-1} day^{-1}), also inhibited foot swelling in a dose-dependent manner (Figure 4). The inhibition of foot swelling by the cyclic peptide at 10 mg kg^{-1} day^{-1} ($42 \pm 2.9\%$, $n=3$) was similar to that of the anti-α_4 monoclonal antibody PS/2 dosed at 7.5 mg kg^{-1} i.v. ($39 \pm 3.7\%$, $n=6$) and greater than that of the 25-amino acid linear CS-1 peptide at 10 mg kg^{-1} day^{-1} ($24 \pm 4.6\%$, $n=5$) (Figure 4). c(ILDV-

Table 2 The effects of CS-1 peptide and cyclic compounds on MOLT-4 cell adhesion to fibronectin

Compound	n	*Concentration range tested* (μM)	*Number of data points*	*IC$_{50}$* (μM)
CS-1	10	5 – 200	5 – 6	19 ± 2.3
c(ILDV-NH(CH$_2$)$_5$CO)	9	1 – 20	5 – 6	3.6 ± 0.44
c(ILDV-NH(CH$_2$)$_4$CO)	4	0.3 – 100	6	2.3 ± 0.58
c(ILDV-NH(CH$_2$)$_7$CO)	4	0.3 – 200	6 – 8	7.0 ± 1.2
c(ILDV-NH(CH$_2$)$_2$CO)	3	100	1	>100

MOLT-4 cells were incubated with compounds for 2 h in 96-well plates coated with human plasma fibronectin and the adherent cells were measured using an acid phosphate assay. IC$_{50}$ values, estimated by sigmoid curve fitting of triplicate absorbance data using Microcal Origin, are shown as mean $\pm$ s.e.mean for n separate experiments.

$NH(CH_2)_2CO$), which was inactive *in vitro*, did not inhibit foot swelling (Figure 4). The dose of c(ILDV-$NH(CH_2)_5CO$) required to cause half the inhibition by PS/2 was 0.7 mg kg^{-1} day^{-1}.

Effect of c(ILDV-$NH(CH_2)_5CO$) on the development of mouse oxazolone contact hypersensitivity

The anti-inflammatory effect of c(ILDV-$NH(CH_2)_5CO$) was also assessed in mouse oxazolone contact hypersensitivity, another model of T-cell-dependent inflammation. In control mice implanted with saline-filled osmotic mini-pumps, 24 h ear swelling was $112 \pm 4.1\%$ ($n=10$). Inhibition of ear swelling by c(ILDV-$NH(CH_2)_5CO$) was dose-dependent (1–10 mg kg^{-1} day^{-1} s.c.) and the inhibition at 10 mg kg^{-1} day^{-1} was similar to that by the anti-α_4 monoclonal antibody, PS/2 (Table 4). CS-1 was inactive when dosed at 10 mg kg^{-1} day^{-1} (Table 4). The dose of c(ILDV-$NH(CH_2)_5CO$) required to cause half the inhibition by PS/2 was 2 mg kg^{-1} day^{-1}.

Effect of c(ILDV-$NH(CH_2)_5CO$) on established inflammation

Ear swelling 24 h after oxazolone challenge in control mice dosed intravenously with saline was $106 \pm 2.1\%$ ($n=15$). After intravenous dosing 20 h after oxazolone challenge, c(ILDV-$NH(CH_2)_5CO$) inhibited ear swelling dose-dependently (1–10 mg kg^{-1}) and the effect at 10 mg kg^{-1} was similar to that of PS/2 (Table 5). CS-1 was inactive at 3 mg kg^{-1} i.v. (Table 5). The dose of c(ILDV-$NH(CH_2)_5CO$) required to cause half the inhibition by PS/2 was 1 mg kg^{-1} i.v. Ear swelling reduced gradually over 1–4 h following intravenous injection of saline 20 h after oxazolone challenge (Figure 5) and the difference between 20 and 24 h ear swelling was statistically significant ($P<0.05$). When mice were dosed with c(ILDV-$NH(CH_2)_5CO$) (10 mg kg^{-1} i.v.) ear swelling decreased to a greater extent than controls and was significantly different from controls at 3 and 4 h post-dose (Figure 5).

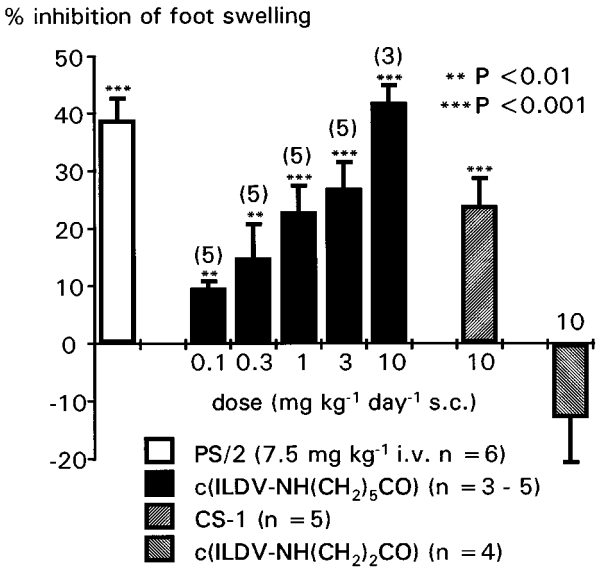

Figure 4 Effect of c(ILDV-$NH(CH_2)_5CO$), c(ILDV-$NH(CH_2)_2CO$), linear CS-1 peptide and an anti-mouse α_4 integrin subunit monoclonal antibody (PS/2) on ovalbumin-induced delayed-type hypersensitivity in female Balb/c mice. The inflammatory response was induced by subplantar injection of heat-aggregated ovalbumin into the feet of mice which had been sensitized to ovalbumin 1 week previously. Mice were dosed continuously with peptides from subcutaneous osmotic mini-pumps implanted the day before ovalbumin injection. PS/2 was dosed intravenously (7.5 mg kg^{-1}) 4 h before ovalbumin injection. Foot swelling was measured 24 h after ovalbumin injection. Data are expressed as mean ± s.e.mean ($n=3-6$). For statistical analysis, each dose group was compared with a vehicle-dosed control group. Foot swelling in control mice was $58 \pm 1.6\%$ ($n=25$).

Discussion

Inhibition of the interaction between vascular endothelial cell adhesion molecules and counter receptors expressed on the surface of leukocytes is of considerable interest as an approach to developing new anti-inflammatory therapies (Sharar *et al*., 1995). VLA-4 inhibition is a particularly attractive target because it is expected to reduce human lymphocyte, monocyte and eosinophil recruitment without affecting the recruitment of human neutrophils which do not express VLA-4 in peripheral blood (Bochner *et al*., 1991). In contrast, therapies directed towards β_2 integrins may also inhibit neutrophil recruitment and affect the ability to combat bacterial infection as seen in patients deficient in β_2 integrins (Anderson *et al*., 1985).

Table 3 The effect of the anti-α_4 monoclonal antibody PS/2 on ovalbumin-induced delayed-type hypersensitivity in mice

Treatment	*Dose* (mg kg^{-1} i.v.)	n	*% Inhibition*	P
PS/2	2.5	4	6.5 ± 5.9	n.s.
	3.75	4	19 ± 5.5	<0.05
	5.0	4	36 ± 2.7	<0.001
	7.5	16	41 ± 1.5	<0.001
	15	4	33 ± 3.5	<0.001
Isotype control	7.5	4	-7.1 ± 1.8	n.s.

The delayed-type hypersensitivity response was induced by subplantar injection of 1% (w v^{-1}) heat-aggregated ovalbumin in saline (30 μl) into the right hind foot pad of female Balb/c mice which had been immunized with a 1 : 1 emulsion of 2 mg ml^{-1} ovalbumin in saline and complete Freund's adjuvant 1 week previously. PS/2 or the isotype control (purified rat $IgG_{2b\kappa}$) were dosed intravenously 4 h before ovalbumin injection. Foot swelling was measured 24 h after ovalbumin injection. Data are expressed as mean ± s.e.mean. For statistical analysis each dose group was compared with a vehicle-dosed group. Foot swelling in the control mice was $53 \pm 0.77\%$ ($n=48$).

Table 4 The effects of VLA-4 inhibitor peptides, dosed from subcutaneous osmotic mini-pumps, and antibody on oxazolone contact hypersensitivity in mice

Inhibitor	*Dose*	*% Inhibition*	P
c(ILDV-$NH(CH_2)_5CO$)	1	7.2 ± 1.4	n.s.
(mg kg^{-1} day^{-1} s.c.)	3	27 ± 2.5	<0.001
	10	38 ± 2.9	<0.001
CS-1 (mg kg^{-1} day^{-1} s.c.)	10	7.1 ± 6.6	n.s.
PS/2 (mg kg^{-1} i.v.)	7.5	36 ± 3.7	<0.001

Oxazolone contact hypersensitivity was induced by topical application of oxazolone in acetone/olive oil onto the ears of male Balb/c mice which had been sensitized to oxazolone 1 week previously. Mice were dosed continuously with peptides from subcutaneous osmotic mini-pumps implanted the day before oxazolone injection. PS/2 was dosed intravenously 4 h before oxazolone challenge. Ear swelling was measured 24 h after oxazolone challenge. Data are expressed as mean ± s.e.mean ($n=5$). For ststistical analysis, each group was compared with a vehicle-dosed control group.

Table 5 The effects of VLA-4 inhibitor peptides and antibody, dosed intravenously, on oxazolone contact hypersensitivity in mice

Inhibitor	*Dose*	n	*% Inhibition*	P
c(ILDV-NH(CH_2)$_5$CO)	1	4	15±5.1	n.s.
	3	4	35±6.8	<0.01
	10	4	39±2.0	<0.001
CS-1	3	5	−1.6±2.3	n.s.
PS/2	7.5	4	35±1.4	<0.001

Oxazolone contact hypersensitivity was induced by topical application of oxazolone in acetone/olive oil onto the ears of male Balb/c mice which had been sensitized to oxazolone 1 week previously. Mice were dosed intravenously 20 h after oxazolone challenge with peptides or the anti-α_4 monoclonal antibody PS/2. Ear swelling was measured 4 h post-dose. Data are expressed as mean±s.e.mean. For statistical analysis, each dose group was compared with a vehicle-dosed control group.

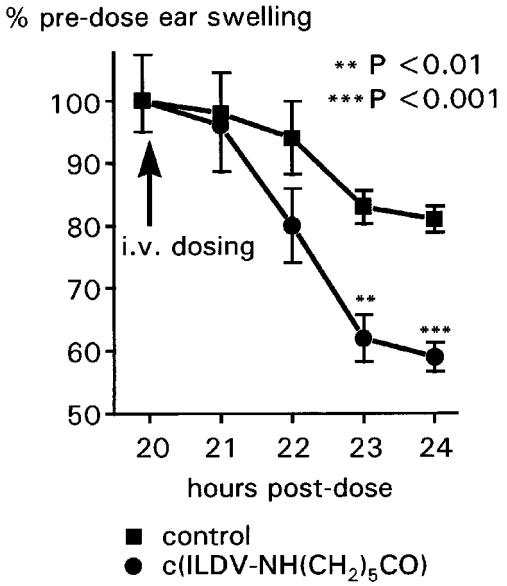

Figure 5 Effect of c(ILDV-NH(CH_2)$_5$CO) on an established oxazolone-induced contact hypersensitivity response in male Balb/c mice. The inflammatory response was induced by topical application of oxazolone in acetone/olive oil onto the ears of mice which had been sensitized to oxazolone 1 week previously. Mice were dosed intravenously with either the cyclic peptide (10 mg kg^{-1}) or saline 20 h after oxazolone challenge. Ear swelling was measured immediately before and at 1, 2, 3 and 4 h after intravenous dosing. Pre-dose ear swelling was not significantly different between the control and peptide-dosed groups (135±10% and 111±5.6% respectively). Data are expressed as mean±s.e.mean ($n=5$). For statistical analysis, foot swelling in the group dosed with c(ILDV-NH(CH_2)$_5$CO) was compared with that in the vehicle dosed group at each time point.

To assess the *in vitro* activity of VLA-4 inhibitors, we measured adhesion of the human T-lymphoblastic leukaemia cell line, MOLT-4 to immobilized human plasma fibronectin. MOLT-4 cells have been reported to express VLA-4 but not VLA-5 (Wayner *et al*., 1989) and we confirmed this by flow cytometric analysis. Additionally, MOLT-4 cells do not express the integrin $\alpha_4\beta_7$ (G. Seddon, Manchester University, M.Sc. Thesis, 1997) which binds to Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1) and is also reported to bind VCAM-1 and fibronectin (Postigo *et al*., 1993). MOLT-4 cells were also shown to express LFA-1, confirming published results (Ross *et al*., 1992), but this integrin does not bind to fibronectin. Using monoclonal antibodies directed against the α_4, α_5 and β_1 integrin subunits we confirmed that MOLT-4 cell adhesion to fibronectin is dependent on VLA-4.

In our search for novel small molecule VLA-4 inhibitors, we first made a selection of small linear fragments of 25-amino acid CS-1. We found that these were considerably less potent inhibitors of MOLT-4 cell adhesion to fibronectin (unpublished observations) and reasoned that this was because they could not adopt the active conformation of the parent molecule. We hoped that cyclization would constrain the ILDV sequence in a bioactive conformation and synthesized cyclic compounds containing the ILDV sequence from CS-1 and aminoalkanoic acids of different lengths to get a range of ring sizes. Peptides cyclized with aminopentanoic, aminohexanoic and aminooctanoic acids had similar potency *in vitro* and were more potent than CS-1. Cyclization was important in these molecules as the corresponding linear peptides containing aminohexanoic and aminoctanoic acids were at least 10 fold less potent (unpublished results). The peptide cyclized with aminopropionic acid was inactive indicating that the size of the ring is too small for the LDV motif to achieve the bioactive conformation.

c(ILDV(CH_2)$_5$CO) was tested for selectivity against two other integrins, VLA-5 and LFA-1. VLA-5 is a β_1 integrin which is widely expressed in many cell types and is a receptor for fibronectin, recognizing an argininyl-glycyl-aspartic acid (RGD) motif (Ruoslahti, 1996). K562 cells have been reported to express VLA-5 but not VLA-4 (Hemler *et al*., 1987; Wayner *et al*., 1989) and this was confirmed by flow cytometry. K562 cells have been used by others to assess VLA-5-mediated cell adhesion (Vanderslice *et al*., 1997; Zheng *et al*., 1994) and our results with monoclonal antibodies confirmed that adhesion of K562 cells to fibronectin is mediated by VLA-5 but not VLA-4 and is inhibited by the RGD peptide echistatin. A disulphide cyclic peptide containing the LDV sequence has been shown not to affect VLA-5-mediated cell adhesion (Vanderslice *et al*., 1997) while a cyclic RGD peptide inhibited both VLA-4 and VLA-5-mediated cell adhesion (Cardarelli *et al*., 1994). LFA-1, a β_2 integrin which recognizes an isoleucyl-glutamyl-threonine motif in ICAM-1, is expressed on many leukocytes and is involved in neutrophil adhesion and migration (Smith *et al*., 1989). PMA-treated U937 cells expressed the α_L subunit of LFA-1 and also VLA-4 and VLA-5 which do not bind to ICAM-1. Adhesion of PMA-treated U937 cells to ICAM-1 CHO cells was shown to be LFA-1-dependent using monoclonal antibodies. c(ILDV(CH_2)$_5$CO) had little effect on cell adhesion mediated by VLA-5 or LFA-1 at concentrations up to 300 μM giving confidence that the compound is unlikely to have effects on these integrins at doses that inhibit VLA-4 *in vivo*. However, effects on $\alpha_4\beta_7$ cannot be excluded as cyclic LDV peptides have also been shown to affect this integrin (Vanderslice *et al*, 1997) and preliminary results (S. Higgs, Manchester University) indicate that c(ILDV(CH_2)$_5$CO) inhibits $\alpha_4\beta_7$-mediated cell adhesion to MAdCAM-1 with an IC_{50} of approximately 50 μM while c(ILDV(CH_2)$_2$CO) is inactive.

DTH responses in mice are classic models of cell-mediated immunity. They are initiated by antigen-induced activation of specific memory T-lymphocytes in animals that have been immunized with the antigen, leading to an inflammatory response at 24–48 h post-exposure. In human DTH, the cellular infiltrate is predominantly mononuclear but in mice most cells recruited to the site of inflammation at 24 h are neutrophils with few mononuclear cells (Chisholm *et al*., 1993; Elices *et al*., 1993).

We have used ovalbumin-induced DTH to measure the effect of c(ILDV$(CH_2)_5$CO) on T-cell-dependent inflammation in mice. In this model, mice are immunized with the protein antigen ovalbumin in Complete Freund's Adjuvant and 7 days later injected with ovalbumin into the footpad to induce an inflammatory response which is measured 24 h later. Histology of sections of inflamed feet at 24 h showed that the inflammation was associated with a cell infiltrate consisting predominantly of neutrophils and few monocytes or macrophages. Very few lymphocytes could be seen in these sections. These results confirmed the published histology of the DTH response to topical application of oxazolone (Elices *et al*., 1993). The requirement for T-cell priming was confirmed by showing that injection of ovalbumin into the feet of mice that had not been immunized with ovalbumin 7 days earlier induced a much smaller 24 h foot swelling than in immunized mice. Additionally, the ovalbumin DTH response could be transferred to naive mice by injecting T-cells harvested from lymph nodes of immunized mice and inhibited by cyclosporin A, an immunosuppressive agent that interferes with T-cell signalling (unpublished observations).

The ovalbumin DTH response was inhibited by the anti-α_4 monoclonal antibody, PS/2, and maximum inhibition of approximately 40% was obtained at an intravenous dose of 7.5 mg kg^{-1}. This confirmed published observations that DTH responses are only partially dependent on VLA-4 (Chisholm *et al*., 1993; Tamraz *et al*., 1995). At this dose, the PS/2 binding sites on mouse peripheral blood lymphocytes have been shown to be saturated *in vivo* (Chisholm *et al*., 1993). The remaining portion of the response to ovalbumin is probably due to cell recruitment *via* other adhesion pathways, possibly LFA-1 (Issekutz, 1993), and the small inflammatory response seen at 24 h in unprimed mice which is unaffected by PS/2.

An LDV-containing linear peptide (Ferguson *et al*., 1991) and a small molecule VLA-4 inhibitor (Tamraz *et al*., 1995) have been shown to be active in mouse DTH models. Our results show that c(ILDV$(CH_2)_5$CO), when dosed continuously from subcutaneous osmotic mini-pumps, inhibits mouse ovalbumin DTH responses and the maximum inhibition is similar to that obtained with PS/2. c(ILDV$(CH_2)_2$CO), which did not inhibit VLA-4 mediated cell adhesion *in vitro*, had no effect on the ovalbumin DTH response indicating that the inhibition by c(ILDV$(CH_2)_5$CO) was likely to be due to its integrin inhibitory activity. CS-1 was active but less effective in ovalbumin DTH reflecting the lower potency of this linear peptide *in vitro*.

The anti-inflammatory activity of c(ILDV$(CH_2)_5$CO) was confirmed in another mouse DTH model, oxazolone contact hypersensitivity, which has previously been used to show the inhibitory effects of anti-α_4 monoclonal antibodies and a small molecule VLA-4 inhibitor (Chisholm *et al*., 1993; Elices *et al*., 1993; Tamraz *et al*., 1995). In this model the inflammatory response is induced in the skin by topical application of the hapten oxazolone in previously sensitized mice. Published data reflect our findings with ovalbumin DTH that there is only a small inflammatory response at 24 h in unsensitized mice (Tarayre *et al*., 1990) and that the response to antigen can be transferred by injecting naive mice with T-cells from sensitized mice (Elices *et al*., 1993) and is inhibited by cyclosporin A (Tarayre *et al*., 1990). The maximum inhibition of the oxazolone response by c(ILDV$(CH_2)_5$CO), when dosed from subcutaneous mini-pumps, was again similar to that of PS/2. c(ILDV$(CH_2)_5$CO) was slightly less potent in the oxazolone model than in ovalbumin DTH and CS-1 was inactive. The lower potency of c(ILDV$(CH_2)_5$CO) and CS-1 in oxazolone contact hypersensitivity may reflect differences in bioavailability and/or pharmacokinetics in male mice compared with the female mice used for ovalbumin DTH. Alternatively, there may be differences in the activation of VLA-4 or upregulation of VLA-4 ligands between oxazolone-induced skin inflammation and ovalbumin-induced foot swelling which may affect the potency of VLA-4 inhibitors.

When dosed by subcutaneous osmotic mini-pump, c(ILDV$(CH_2)_5$CO) inhibited the development of the inflammatory response as the mini-pumps were implanted the day before antigen challenge. However, c(ILDV$(CH_2)_5$CO) also suppressed existing inflammation since when it was dosed intravenously 20 h after antigen challenge, when the ear swelling was maximal, the ongoing inflammatory response was significantly reduced. The inhibitory effect was not instantaneous with significant differences between dosed and control groups seen at 3–4 h post-injection. These results suggest that there may be constant turnover of inflammatory cells in the lesion and that continued recruitment of blood leukocytes is required to maintain the inflammatory response as was observed with an anti-α_4 monoclonal antibody in guinea-pig experimental autoimmune enchephalomyelitis (Kent *et al*., 1995).

In addition to a role in leukocyte adhesion and extravasation, VLA-4 has been implicated in cell activation. Ligand binding by VLA-4 can provide a costimulatory signal required for T-lymphocyte activation and a cyclic LDV peptide has been shown to inhibit T-lymphocyte proliferation *in vitro* (McIntyre *et al*., 1997). Recent work with a murine asthma model indicates that the effects of PS/2 on bronchial hyperresponsiveness may be due to inhibition of cell activation rather than on eosinophil recruitment (Henderson *et al*., 1997). It is possible that at least part of the mechanism of inhibition of the DTH responses by c(ILDV$(CH_2)_5$CO) observed in this study could be due to effects on cell activation and cytokine release rather than a direct effect on cell adhesion and recruitment.

The discovery that monoclonal antibodies to the α_4 subunit of VLA-4 were effective in animal models of inflammation indicated that blocking VLA-4 may be a useful therapy for inflammatory diseases. Although a humanized monoclonal antibody is in clinical development, attention has focused on developing small molecule VLA-4 inhibitors. Peptide and peptide mimetic inhibitors based on the integrin binding motifs in fibronectin have been reported. Based on reports of the effects of these agents on VLA-4-mediated cell adhesion *in vitro*, c(ILDV$(CH_2)_5$CO) is more potent than a non-peptide LDV mimetic and a linear LDV octapeptide (Greenspoon *et al*., 1994), a cyclic LDV hexapeptide containing a β-turn mimetic (Doyle *et al*., 1996) and a cyclic LDV pentapeptide (Viles *et al*., 1996). c(ILDV$(CH_2)_5$CO) has similar potency to two small molecule β-turn mimetics (Souers *et al*., 1998) and two cyclic peptides derived from the RGD tripeptide (Cardarelli *et al*., 1994; Nowlin *et al*., 1993) although the RGD-derived peptides are not selective for VLA-4. c(ILDV$(CH_2)_5$CO) is less potent *in vitro* than a cyclic LDV hexapeptide cyclized with a disulphide bond (Vanderslice *et al*., 1997) and the most potent of a series of cyclic penta and hexapeptides based on the arginyl-cysteinyl-aspartic acid tripeptide (Jackson *et al*., 1997).

There is little published information on the activity of other small molecule VLA-4 inhibitors in DTH models. c(ILDV$(CH_2)_5$CO) had similar *in vivo* potency to a linear peptidomimetic based on the LDV tripeptide tested in an adoptive transfer oxazolone contact hypersensitivity mouse model (Tamraz *et al*., 1995). Maximal inhibition by both

c(ILDV(CH_2)$_5$CO) and the linear peptidomimetic was similar to that induced by the anti-α_4 monoclonal antibody PS/2. It has been reported that intravenous dosing of PS/2 induces an increase in circulating leukocytes, primarily due to lymphocytosis, at doses that inhibit DTH responses, possibly because of blockade of lymphocyte recirculation to gut-associated lymphoid tissue or release of cells from the bone marrow (Chisholm *et al*., 1993). Circulating leukocytes were not measured in the DTH experiments with c(ILDV(CH_2)$_5$CO) but we have shown that other cyclic peptide VLA-4 inhibitors have no significant effect on circulating lymphocyte or monocyte counts in mice when dosed at 10 mg kg^{-1} day^{-1} from subcutaneous osmotic mini-pumps.

In conclusion, we have shown that small N- to C-terminal cyclic peptides containing the ILDV sequence are more potent inhibitors of VLA-4 mediated cell adhesion to fibronectin *in vitro* than the 25-amino acid CS-1 linear peptide. c(ILDV(CH_2)$_5$CO) was selective for VLA-4 over VLA-5 and LFA-1-mediated cell adhesion *in vitro*. c(ILDV(CH_2)$_5$CO) inhibited DTH responses induced by ovalbumin or oxazolone in mice when dosed subcutaneously from osmotic mini-pumps. c(ILDV(CH_2)$_5$CO) also reduced established oxazolone-induced inflammation when dosed intravenously. Small molecule VLA-4 inhibitors derived from c(ILDV(CH_2)$_5$CO) may have utility as anti-inflammatory agents.

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