

An annexin 1 (ANXA1)-derived peptide inhibits prototype antigen-driven human T cell Th1 and Th2 responses *in vitro*

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Summary

Background Annexin-1 (ANXA1, lipocortin 1) is a pleiotrophic protein produced by many cell types including peripheral blood leucocytes. Although it has been shown to inhibit 'macroscopic' inflammatory processes in animal models, its direct effects on antigen-activated human T cells have not been studied.

Objective To test the hypothesis that ANXA1-derived peptides inhibit antigen-driven prototype Th1 and Th2-type human T cell responses of clinical relevance and lectin-driven responses *in vitro*.

Methods Peripheral blood mononuclear cells (PBMC) were isolated from 14 atopic subjects sensitized to house dust mite allergen (*Dermatophagoides pteronyssinus*, Der p) and purified protein derivative (PPD) of *Mycobacterium tuberculosis*. PBMC (1×10^6 /mL) were cultured with phytohaemagglutinin (PHA; 5 µg/mL; 4 days), Der p (25 µg/mL; 6 days), PPD (10 µg/mL, 6 days) or medium control. Two ANXA1-derived peptides, Ac2–26 and AF-2 (5–500 µM), were assessed for possible inhibition of PHA- and antigen-induced T cell proliferation (measured by ^{3} H-thymidine uptake), while Ac2–26 was assessed for inhibition of Der p-induced interleukin (IL)–5 release and PPD-induced interferon-γ (IFN-γ) release (measured by ELISA). Comparison was made with dexamethasone as an established inhibitory control. Endogenous production by PBMC of cell surface-associated and intracellular ANXA1 in response to PHA, Der p and PPD in the presence and absence of dexamethasone was measured by specific ELISA.

Results Both PHA- and antigen-induced T cellular proliferation were inhibited by dexamethasone. Although neither ANXA1-derived peptide significantly altered PHA-induced proliferation, both effected concentration-dependent reductions in antigen-induced proliferation, Ac2–26 being the more potent. Peptides of identical amino acid composition to Ac2–26 and AF-2, but of random sequence, were ineffective at equivalent concentrations. In addition, Ac2–26 and dexamethasone inhibited Der p-induced IL-5 release and PPD-induced IFN-γ release in a concentration-dependent fashion. Endogenous ANXA1 was detectable in PBMC, but at concentrations approximately 10^4 -fold lower, in molar terms, than the effective concentrations of the exogenously added, ANXA1-derived inhibitory peptides. Endogenous production was not significantly altered by any of the T cell stimuli employed in this study, in the presence or absence of dexamethasone.

Conclusion In prototype Th1 and Th2-type human T cell responses, ANXA1-derived peptides can inhibit antigen-driven cellular proliferation and cytokine production.

Keywords: Ac2–26, AF-2, antigen, annexin 1, interleukin-5, interferon-γ, lipocortin 1, T cell

Clinical and Experimental Allergy, Vol. 31, pp. 1116–1125. Submitted 19 January 2000; revised 3 January 2001; accepted 5 February 2001.

Introduction

The purpose of this study was to investigate whether annexin 1 (ANXA1; lipocortin 1)-derived peptides modulate human T lymphocyte activation in response to clinically relevant antigens. Annexins are members of a super-family of proteins found throughout the animal kingdom, at least 10 which are found in vertebrates. They share the ability to bind calcium and phospholipid [1,2] through the possession of a common core of four highly conserved structural domains, but each has a unique N-terminal sequence which is thought to confer specificity of function. Each annexin has a specific cellular distribution. Cellular sources of ANXA1 in the respiratory tract include stromal cells, such as epithelial and tracheal gland cells as well as infiltrating leucocytes [3–9].

ANXA1 is a pleiotropic molecule with a number of anti-inflammatory actions of relevance to the regulation of neutrophil-driven inflammatory processes (10–13, reviewed in 14). In addition, and of potential relevance to the regulation of immune function, ANXA1 and peptides derived from its primary sequence have been shown to exert suppressive effects on mitogen-stimulated murine thymocytes [15], rodent T cell lines [16] and an immortalized T cell line [17]. Although preliminary experiments suggest possible effects of ANXA1 on mitogen-activated T cells in humans [18,19], the effects of ANXA1 on antigen-driven human T cell proliferation and cytokine production have never been examined.

The mechanism by which ANXA1 exerts these effects is not fully understood. It appears to depend on externalization of the protein and its interaction with one or more binding proteins [20–22], which in some cells also bind FMLP [23]. Expression and externalization of ANXA1 are induced in some, but not all cell types by glucocorticoids [14,24–29], and there is evidence that it may mediate certain of the anti-inflammatory effects of glucocorticoids in some animal models [14,30–32]. In addition, other stimuli, including IL-6 [33] and lipopolysaccharide [34] may induce ANXA1 expression and externalization. To date, nothing is known about its synthesis and externalization during antigen-mediated human T cell activation in the presence of glucocorticoids or otherwise. Nevertheless, the studies on mitogen-stimulated cells suggest that ANXA1 could act as an autocrine or paracrine regulator of immune responses both in the absence and presence of exogenous glucocorticoid.

Because the native ANXA1 protein is readily denatured *in vitro* and since recombinant ANXA1 from many sources is biologically inactive, stable peptides derived from its primary amino acid sequence have been identified and are often used for *in vitro* studies. The peptide Ac2–26 shares many of the anti-inflammatory activities of ANXA1 and, as

the name suggests, comprises the acetylated N-terminal sequence of ANXA1 [35]. Its effects on T cell proliferation have not been examined. A second peptide, antiinflammin-2 (AF-2), comprising amino acids 246–254 of ANXA1, is also anti-inflammatory in rodents [36] and also suppresses proliferation of an immortalized T-cell line [17].

Since antigen-induced T cell activation and cytokine production are frequently involved in responses to external antigens, we wished to examine the effects of ANXA1-derived peptides on these processes using human T cells and clinically relevant antigens. We also took the opportunity to examine the effects of exposure to these antigens, as well as dexamethasone, on the expression and release of endogenous ANXA1. We chose to investigate PPD-induced T cellular proliferation and IFN- γ production as an example of a prototypical Th1 type response, and allergen-induced T cellular proliferation and IL-5 production as an example of a Th2-type response. Our aims were to investigate the effects of the peptides Ac2–26 and AF-2 (alone and in combination) on antigen and lectin-induced T cell proliferation and antigen-induced cytokine release.

Methods

Subjects

A total of 14 subjects (median age 27, range 21–57 years, six male) were used for these experiments, although not all of the experiments were conducted on each subject. All subjects were sensitized to house dust mite allergen (*Dermatophagoides pteronyssinus*, Der p) as shown by a positive skin prick test ('Soluprick', ALK, Hørsholm, Denmark) and/or RAST ('Phadebas', Pharmacia, Uppsala, Sweden) > 0.7 IU/mL and a positive Heaf test (1 : 1000). The subjects were otherwise healthy and taking no medication. All subjects gave informed consent to participate in the study, which was approved by the Riverside Ethical Committee.

Materials

Lyophilized *Dermatophagoides pteronyssinus* (Der p) allergen and *Mycobacterium tuberculosis* PPD were purchased from ALK and Farollin, Romford, Essex, UK, respectively. Dexamethasone was obtained as the soluble sodium phosphate salt from David Bull Laboratories, Warwick, UK. Histopaque, phytohaemagglutinin (PHA), RPMI-1640 culture medium, human AB + serum, glutamine and antibiotics were from Sigma (Poole, Dorset, UK). Tritiated thymidine (approximately 1TBq/mmol) was obtained from Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK. ELISA kits for quantification of

IL-5 and IFN- γ were purchased from R & D Systems (Abingdon, UK).

Four peptides were employed in this study. Ac2–26 represents the N-terminal sequence of human ANXA1 (N-acetyl-AMVSEFLKQAWFIENEEQEYVQTVK) and anti-flammin-2 (AF-2) represents amino acids 246–254 of human ANXA-1 (HDMNKVLDL). Scrambled Ac2–26 (AcS) contained the same amino acids as Ac2–26 in random order (N-acetyl-KIQLYEWNTEAQMEVFVEK VAFQES), while scrambled AF-2 (AFS) contained the same amino acids as AF-2, in random order (KLH MLNDVD). All the peptides used in this study were synthesized in the Advanced Biotechnology Centre at Charing Cross Hospital. They were purified to >90% purity by reverse phase HPLC and their identities confirmed by mass spectrometry, amino-acid analysis and capillary electrophoresis.

Isolation and culture of peripheral blood mononuclear cells

Peripheral venous blood (60 mL) was collected into a heparinized syringe, diluted with an equal volume of RPMI-1640 containing glutamine (2 mM), penicillin, streptomycin and amphotericin and layered over Histopaque. Following density gradient centrifugation at 350 g for 20 min at 20 °C, the peripheral blood mononuclear cells (PBMC) were harvested from the plasma/Histopaque interface, washed twice in RPMI and a sample removed, stained with Kimura stain and counted using a Neubauer haemocytometer. The remaining cells were then resuspended at a concentration of 2×10^6 /mL in RPMI supplemented with 2% human AB⁺ serum, glutamine and antibiotics. Cells were cultured in round bottomed 96-well plates at a final concentration of 1×10^6 /mL in the presence of PHA (5 μ g/mL), Der p (25 μ g/mL), PPD (10 μ g/mL) or medium control in a final volume of 200 μ L/well. Culture conditions for these assays have previously been characterized [37]. In various experiments, triplicate aliquots of PBMC cultured with PHA, Der p or PPD were additionally cultured in the presence of (a) 5–500 μ M AF-2; (b) 5–500 μ M Ac2–26; (c) both peptides; or (d) dexamethasone (10–100 nM). In some studies AcS and AFS (500 μ M) were included as a negative control. All the peptides, dexamethasone, Der p, PPD and PHA were made up as 10-fold concentrated stock solutions in RPMI containing 2% human AB⁺ serum, glutamine and antibiotics prior to addition to the cultures. PBMC were incubated for 6 days (4 days with PHA) at 37 °C in 95% air and 5% CO₂, after which ³H-thymidine diluted in culture medium (25 kBq/10 μ L/well) was added to the wells and the cells incubated for a further 16 h. A portion (150 μ L) of the culture supernatant was removed from each well and stored at –20 °C until assayed for cytokines. The cells were stored frozen in the plates at –20 °C for measurement of

³H-thymidine uptake or prepared for assay of ANXA1 as described below.

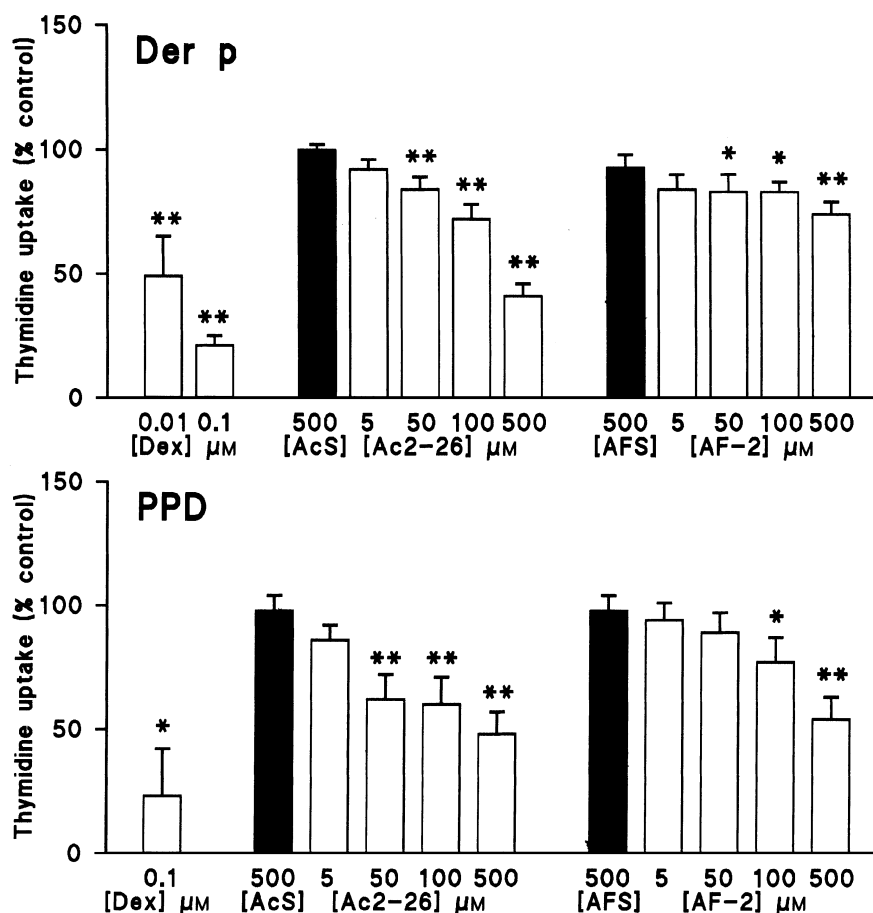
Extraction of intracellular and cell surface-associated ANXA1

To measure expression of intracellular and cell surface-associated ANXA1, additional aliquots of cells were cultured in duplicate as described above, but in 24-well plates in a total volume of 1 mL in the presence of PHA, Der p, PPD or medium control with and without additional dexamethasone (100 nM). Cultures were terminated by gently resuspending the contents of each well with a pipette. After pelleting the cells by centrifugation (300 g, 10 min, 4 °C), the supernatants were carefully aspirated. Cell surface-associated ANXA1 was recovered by resuspending the pellets in 100 μ L of 0.1 M PBS containing 1 mM EDTA and the protease inhibitors PMSF (1 mM), leupeptin (1 μ g/mL), pepstatin (1 μ g/mL) and aprotinin (1 μ g/mL). After incubating for 10 min at 4 °C, the cells were pelleted again and the supernatants retained for measurement of surface-bound ANXA1. The cell pellets were then lysed in lysis solution (2% Nonidet P-40, 10 mM EDTA and 1 mM PMSF prepared in Ca²⁺- and Mg²⁺-free Hank's balanced salt solution) and the lysates retained for measurement of total intracellular ANXA1.

Quantification of ANXA1 by ELISA

ANXA1 was measured by means of a sandwich ELISA developed in-house. MaxiSorp ELISA plates (Nunc) were coated with 75 μ g/well of murine monoclonal anti-ANXA1 capture antibody 1-B (a gift from Dr J Browning, Biogen, Cambridge, MA, USA) in carbonate/bicarbonate buffer, pH 9.6 for 18 h at 4 °C. Plates were washed 3 times in carbonate/bicarbonate buffer then blocked with 10% goat serum in 0.1 M PBS, pH 7.4 for 1 h at 37 °C. The blocking buffer was removed and replaced by undiluted samples (50 μ L lysate/well; 100 μ L EDTA wash/well) or ANXA1 standards (2.5 pM–25 nM) prepared in either PBS/1 mM EDTA (for surface ANXA1 samples) or lysis solution (for lysates). Biologically inactive, recombinant human ANXA1 isolated from *Schizosaccharomyces pombe* (a generous gift of Dr Nicholas Goulding, William Harvey Research Institute) was used as a standard. Standards and samples were incubated for 1.5 h at 37 °C then the samples discarded and the plates washed 5 times in PBS/0.05% Tween 20 (PBS-T) to remove unbound ANXA1. To complete the sandwich, 100 μ L of an IgG-fractionated rabbit anti-ANXA1 polyclonal antibody (raised in-house to full length recombinant human ANXA1 and diluted 1 : 250 in PBS-T) were added to each well and incubated at 37 °C for 1 h. Plates were washed 5 times in PBS-T and immune

Fig. 1. Tritiated thymidine incorporation (expressed as percentages of the positive control) by PBMC of atopic subjects cultured for 6 days with Der p (25 µg/mL) or PPD (10 µg/mL) and in the presence of the ANXA1-derived peptides Ac2-26 (Der p, $n = 14$; PPD, $n = 8$) or AF-2 (Der p, $n = 9$; PPD, $n = 6$) at each of the concentrations shown. In each experiment, dexamethasone (Dex) was employed as an established inhibitory control, while in four experiments with each stimulus comparison was also made with the scrambled peptides AcS and AFS (both 500 µM) (filled bars). Data are expressed as the mean and SEM. Absolute thymidine uptake was 8459 ± 2269 and 619 ± 105 cpm in the positive (Der p only) and corresponding negative (medium only) control wells, respectively, and $15\,663 \pm 2668$ and 973 ± 432 cpm in the positive (PPD only) and corresponding negative (medium only) control wells, respectively. Ac2-26/Der p $P < 0.0001$; Ac2-26/PPD $P = 0.032$; AF-2/Der p $P < 0.001$; AF-2/PPD $P = 0.01$ (Kruskal-Wallis analysis of variance). * $P < 0.05$, ** $P < 0.01$ compared with antigen alone (Mann-Whitney test).



complexes detected by incubating with 100 µL of a goat anti-rabbit-IgG-alkaline phosphatase conjugate (Sigma) diluted 1 : 1000 in PBS-T for 1 h at 37 °C. Following 5 washes in PBS-T, 100 µL of p-nitrophenyl phosphate (1 mg/mL) in carbonate/bicarbonate buffer were added to all wells and colour development monitored for 20–30 min before determination of absorbance at 405 nm in a ThermoMax plate reader (Molecular Devices).

Detection and characterization of ANXA1 by Western blotting

All samples were assayed for total protein concentrations using the bicinchoninic acid reagent (Pierce, Chester, UK) according to the manufacturer's instructions, then diluted to equivalent total protein concentrations and subjected to SDS-PAGE on 15% polyacrylamide gels (BioRad MiniProtein System). Proteins were then electro-transferred onto PVDF Immobilon membranes (Millipore, Watford, Herts, UK) according to manufacturer's guidelines. PVDF blots were blocked in bovine serum albumin in PBS (10 mg/mL) for 1 h at room temperature, then incubated with an IgG anti-ANXA1 murine monoclonal antibody 1-B (Biogen) diluted at 1 : 1000

in wash buffer (50 mM Tris-HCl, 500 mM NaCl, 1 mg/mL BSA, 0.1% Tween-20, pH 7.4) for 18 h at 4 °C with constant agitation. Blots were washed in wash buffer (3 × 5 min) then incubated with a polyclonal sheep anti-mouse IgG conjugated to horseradish peroxidase (DAKO, Ely, Cambridgeshire, UK) diluted at 1 : 1000 in wash buffer for 1 h at room temperature. Following 3 × 10 min washes in wash buffer and 2 × 10 min rinses Tris-buffered saline, pH 7.4, protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). ANXA1-positive bands were confirmed by comparison with the same recombinant standard used for the ELISA and with prestained molecular weight markers (New England Biolabs, Hitchin, Herts, UK).

Quantification of cellular proliferation and cytokine production

The insoluble fraction of the thawed cell lysates was harvested onto glass fibre filters using a semiautomatic cell harvester (Packard, Pangbourne, UK) according to the manufacturer's instructions. The incorporation of ^3H -thymidine into the insoluble fraction was determined by counting the ^3H deposited on the filters using a β -spectrophotometer

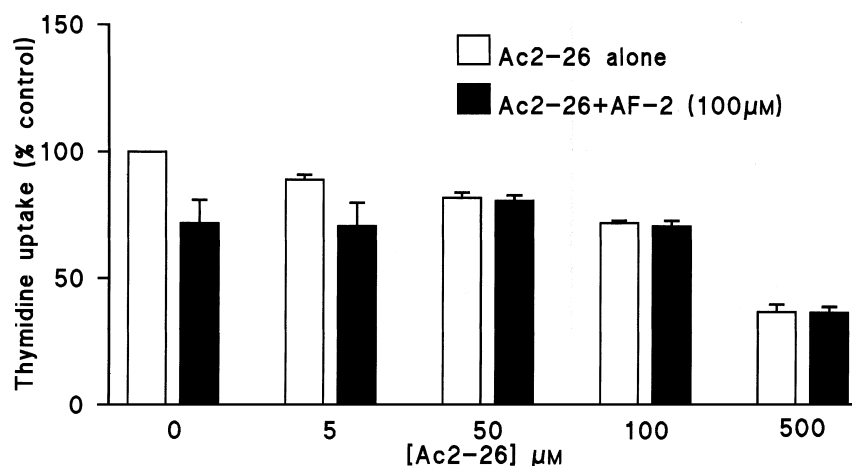


Fig. 2. Tritiated thymidine incorporation (expressed as percentages of the positive control) by PBMC of atopic subjects cultured for 6 days with Der p (25 μg/mL) and in the presence of the ANXA1-derived peptide Ac2-26 at each of the concentrations shown without (open bars) or with (filled bars) additional 100 μM AF-2 ($n = 14$) or corresponding medium control. Data are expressed as the mean and SEM. Absolute thymidine uptake was 8459 ± 2269 and 619 ± 105 cpm in the positive (Der p only) and corresponding negative (medium only) control wells, respectively.

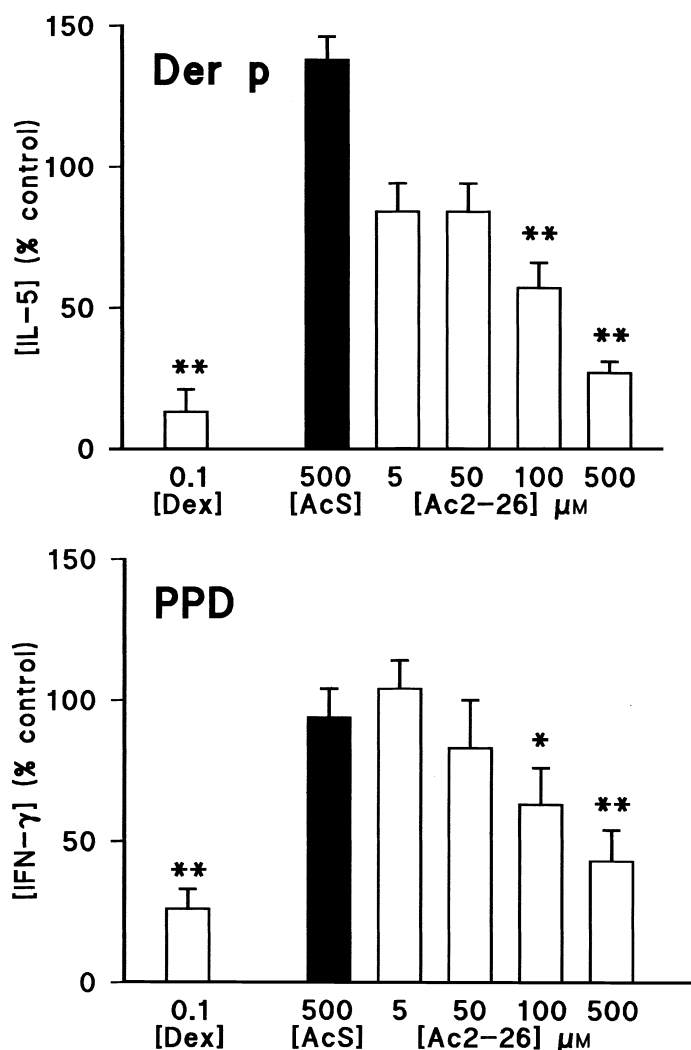


Fig. 3. Concentrations of IL-5 and IFN-γ (expressed as percentages of the positive control) in culture supernatants of PBMC of atopic subjects cultured for 6 days with Der p (25 μg/mL) or PPD (10 μg/mL) alone and in the presence of the ANXA1-derived peptide Ac2-26 (Der p, $n = 8$; PPD, $n = 6$) at each of the concentrations shown. In each experiment, dexamethasone (Dex) was employed as an established inhibitory control, while in four experiments with each stimulus comparison was also made with the scrambled peptide AcS (500 μM) (filled bars). Data are expressed as the mean and SEM. Absolute IL-5 concentrations were 68 ± 19 and 6 ± 2 pg/mL in the positive (Der p only) and corresponding negative (medium only) control wells, respectively, and IFN-γ concentrations were 606 ± 273 and 8 ± 3 pg/mL in the positive (PPD only) and corresponding negative (medium only) control wells, respectively. Ac2-26/Der p $P < 0.01$; Ac2-26/PPD $P = 0.01$ (Kruskal-Wallis analysis of variance). * $P < 0.05$, ** $P < 0.01$ compared with antigen alone (Mann-Whitney test).

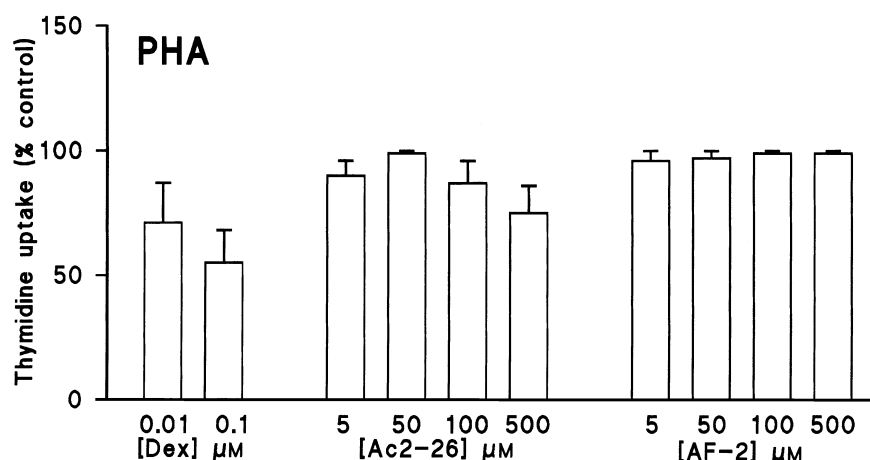


Fig. 4. Tritiated thymidine incorporation (expressed as percentages of the positive control) by PBMC of atopic subjects cultured for 4 days with PHA (5 $\mu\text{g}/\text{mL}$) and in the presence of the ANXA1-derived peptides Ac2-26 ($n = 10$) or AF-2 ($n = 5$) at each of the concentrations shown. In each experiment, dexamethasone (Dex) at the concentrations shown was used as an established inhibitory control. Data are expressed as the mean and SEM. Absolute thymidine uptake was $94\,302 \pm 15\,496$ and 1238 ± 345 cpm in the positive (PHA only) and corresponding negative (medium only) control wells, respectively. Dex $P = 0.004$; Ac2-26 $P = 0.344$; AF-2 $P = 0.644$ (Kruskal-Wallis analysis of variance).

('Top Count', Packard) as a measure of lymphocyte proliferation. Geometric means of triplicate counts were calculated then, where appropriate, expressed as percentages of the relevant positive control.

The concentrations of IL-5 and IFN- γ in the conditioned culture media from pooled triplicate wells were measured in duplicate using commercial ELISA calibrated with recombinant human cytokines according to the manufacturer's instructions. Supernatant samples from the same individual were assayed on the same plate. Arithmetic means of duplicate concentrations were calculated then expressed where appropriate as percentages of the relevant positive control.

Statistical analysis

The statistical significance of differences between individual treatment groups and corresponding positive controls was determined using the Mann-Whitney test for paired data. In Figs 1 and 3-6, significant overall variability, or otherwise, was assessed using non-parametric analysis of variance (Kruskal-Wallis test). Despite the non-parametric analysis, for clarity, data are presented as the mean and standard error of the mean (SEM).

Results

Effect of peptides and dexamethasone on antigen-induced T cell proliferation

The ANXA1-derived peptide Ac2-26 caused a concentration-dependent inhibition of both Der p- and PPD-induced

T cellular proliferation, which was statistically significant at the $P < 0.01$ level at or above a concentration of 50 μM (Fig. 1). The scrambled control peptide, AcS, showed no significant inhibitory effect on Der p- or PPD-induced T cellular proliferation at a concentration (500 μM) corresponding to the highest concentration of Ac2-26 employed (Fig. 1). The peptide AF-2 was also efficacious, but somewhat less potent, in inhibiting both Der p- and PPD-induced T cellular proliferation (Fig. 1). As with AcS, the corresponding control peptide, AFS, was entirely ineffective in this regard at a concentration of 500 μM (Fig. 1). In the same experiments, dexamethasone significantly inhibited Der p-induced T cellular proliferation in a concentration-dependent fashion and also inhibited PPD-induced proliferation at the single concentration utilized (Fig. 1).

Addition of AF-2 at a concentration suboptimal for inhibition of Der p-induced T cell proliferation (100 μM) did not significantly alter the inhibitory activity of Ac2-26 over the range of concentrations tested (Fig. 2). Mixtures of the scrambled peptides had no significant effect on allergen-induced proliferation (data not shown).

Effect of Ac2-26 on antigen-induced T cell cytokine release

Since, over the range of concentrations tested, Ac2-26 appeared more potent than AF-2 in inhibiting both Der p- and PPD-induced T cellular proliferation, we tested only the effects of Ac2-26 on Der p-induced production of IL-5 and PPD-induced production of IFN- γ .

Ac2-26 effected concentration-dependent reductions in both Der p-induced IL-5 release and PPD-induced IFN- γ

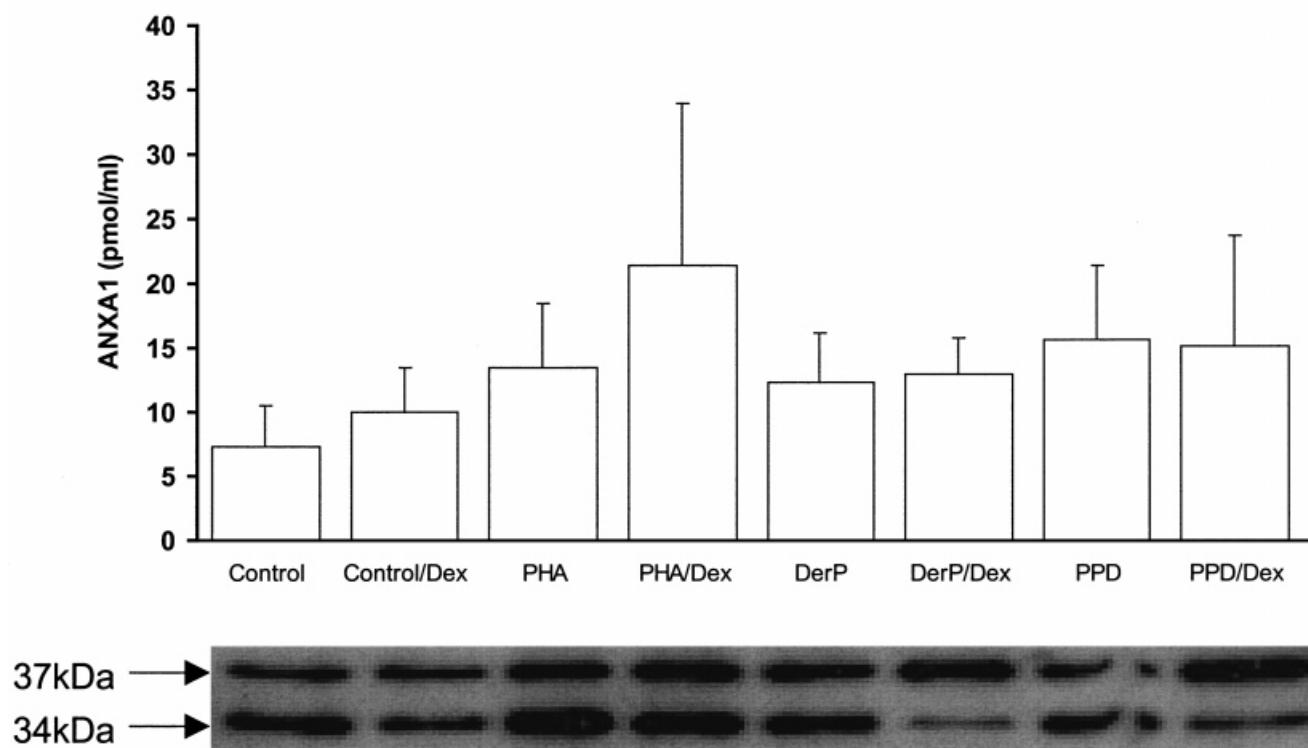


Fig. 5. *Top panel:* Concentrations (by specific ELISA) of extracellular ANXA1 in PBMC incubated with medium control, 6 days (Control), dexamethasone 100 nM, 6 days (Control/Dex), PHA 5 μ g/mL, 4 days (PHA), PHA 5 μ g/mL and dexamethasone 100 nM 4 days (PHA/Dex), Der p 25 μ g/mL, 6 days (DerP), Der p 25 μ g/mL and dexamethasone 100 nM, 6 days (DerP/Dex), PPD 10 μ g/mL, 6 days (PPD), PPD 10 μ g/mL and dexamethasone 100 nM, 6 days (PPD/Dex). Data are expressed as the mean and SEM ($n = 5$). $P = 0.178$ (Kruskal-Wallis analysis of variance). *Bottom panel:* Western blot of membrane-associated ANXA1 showing 37 kDa species corresponding to the molecular size of ANXA1 and a second cleavage product of size 34 kDa which is also recognized by the anti-ANXA1 monoclonal antibody used in this blot as well as in the ELISA assays.

release, both of which were significant at Ac 2–26 concentrations of 100 μ M or greater (Fig. 3). In contrast, the scrambled control peptide, AcS, did not significantly alter IL-5 or IFN- γ production at a concentration corresponding to the highest concentration of Ac2–26 employed (500 μ M, Fig. 3). In the same experiments, dexamethasone also significantly inhibited IL-5 and IFN- γ production (Fig. 3).

Effect of ANXA1-derived peptides and dexamethasone on PHA-induced T cell proliferation

In contrast to antigen-induced T cellular proliferation, neither Ac2–26 nor AF-2 showed any significant effect on PHA-induced T cellular proliferation over the concentration range tested (5–500 μ M; Fig. 4). The peptides were not therefore tested in combination. In the same experiments, dexamethasone significantly inhibited PHA-induced proliferation in a concentration-dependent fashion (Fig. 4).

Effects of antigen, lectin and dexamethasone exposure on expression of surface-bound and intracellular ANXA1 protein by PBMC

Intracellular and cell surface-associated ANXA1 were detectable in the cultured PBMC (Figs 5 and 6). Both membrane-bound and intracellular ANXA1 were detectable by ELISA at concentrations of the order of 10^4 -fold lower, on a molar basis, than the effective concentrations of the ANXA1-derived inhibitory peptides. Concentrations of ANXA1 were not significantly altered by culture of the PBMC with PHA, Der p and PPD with or without additional dexamethasone as compared with culture in medium alone (Figs 5 and 6). Concomitant Western blotting of washed and lysed PBMC supernatants demonstrated that the anti-ANXA1 capture antibody, which was the same as that employed in the specific ELISA, recognized a protein of the appropriate molecular size (37 kDa) which comigrated with the recombinant ANXA1 standard. In addition, Western blotting demonstrated the

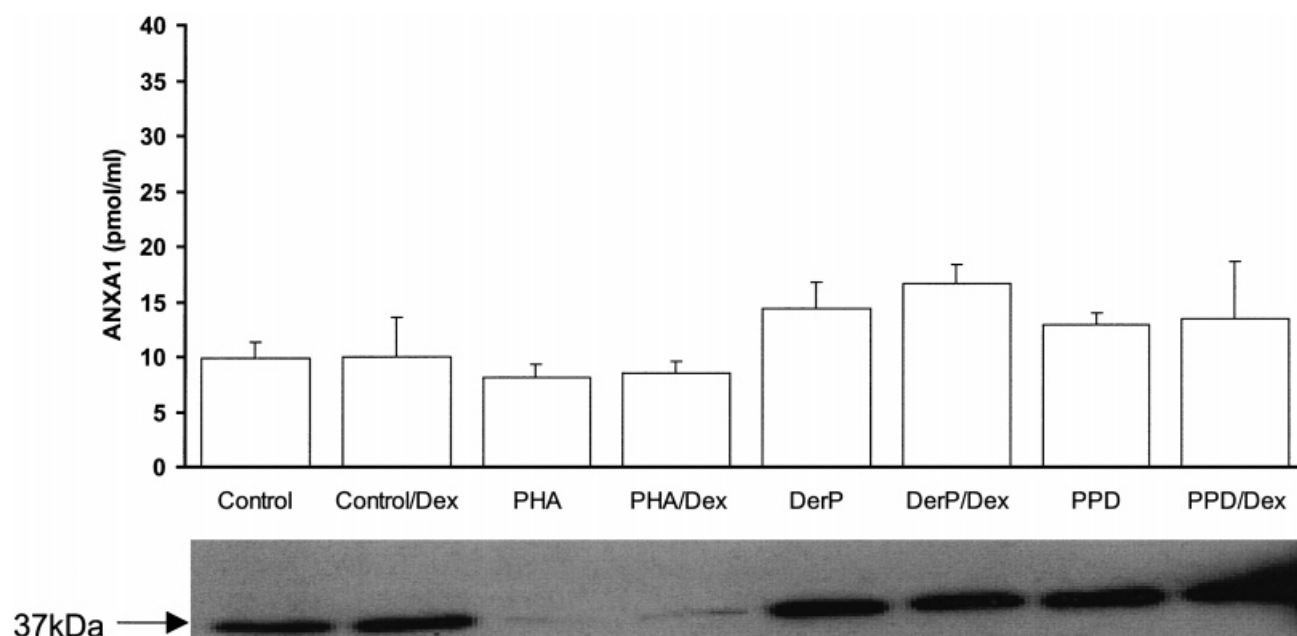


Fig. 6. *Top panel:* Concentrations (by specific ELISA) of intracellular ANXA1 in PBMC incubated as in Fig. 5. Data are expressed as the mean and SEM ($n = 3$). $P = 0.856$ (Kruskal-Wallis analysis of variance). *Bottom panel:* Western blot of membrane-associated ANXA1 showing 37 kDa species corresponding to the molecular size of ANXA1. In this extract the 34 kDa cleavage product (see Fig. 5) is absent.

presence in all cell surface samples of an additional 34 kDa species, which is a known extracellular proteolytic breakdown product of ANXA1 [38].

Discussion

Our data clearly show that the ANXA1-derived peptide Ac2–26 can inhibit antigen-induced proliferation and cytokine production in peripheral blood T cells from sensitized human subjects. The second ANXA1-derived peptide, AF-2, was also inhibitory, but less potent than Ac2–26. To our knowledge, this is the first demonstration that ANXA1-derived peptides can inhibit prototype, antigen-driven human Th1, as well as Th2 T cell responses *in vitro*.

The observation that the control peptides AcS and AFS, of identical amino acid composition to the corresponding active compounds but in random order, were ineffective suggests that this is a stereospecific effect dependent on the three-dimensional structure of the peptides. This in turn implies the existence of one or more cellular surface ANXA1 receptors. Our finding that the effects of Ac2–26 and AF-2 were not synergistic reduces the probability that they are acting on different receptors. Previously published binding studies with ANXA1 similarly suggest the existence of specific surface receptors on peripheral blood leucocytes [20,21,28], while a more recent study implicates a member of the FMLP receptor family as a candidate

receptor for Ac2–26 [23]. The observation that the anti-inflammatory activities of ANXA1 can be abrogated both *in vitro* and *in vivo* by anti-ANXA1 neutralizing antibodies [30–32] further suggests the existence of one or more cell surface receptors for ANXA1. Together, these observations support the hypothesis that ANXA1 acts in an autocrine or paracrine fashion on cells such as monocytes, which may both secrete ANXA1 and bind it extracellularly. Other investigators [33] have gone as far as to nominate ANXA1 as an “acute phase” protein which may act systemically.

Although Ac2–26 and AF-2 inhibited T cell proliferation, high concentrations were needed to produce an effect. This is in accord with published studies [10,16,17,35] showing that, in a variety of systems *in vitro*, ANXA1-derived peptides are less potent than intact ANXA1 and may act as partial agonists. Given the modest size of the peptides compared to the intact protein, this could reflect the absence of critical structural features needed to increase affinity for the binding site and/or to evoke a signalling response. In the present study, we were not able to compare the activities of the peptides with that of intact ANXA1 directly since purified, intact, biologically active ANXA1 was not available to us. Another possible explanation for the low activity of the peptides is that they may have been degraded in culture (Western blotting showed that endogenous ANXA1 was subject to limited proteolytic degradation once externalized (Fig. 5)). We are confident that this was not the case since, in separate experiments (not

shown), we replenished the peptides throughout the culture period, rather than administering them once at the beginning of the incubation and found no change in efficacy of either peptide. Finally, the apparent potencies of ANXA1-derived peptides may also be influenced by variability in the numbers of receptors on target cells, as well as by possible physiological antagonism by pro-inflammatory mediators such as cytokines.

We were able to detect endogenous ANXA1 in our culture system. Culture of the PBMC under conditions identical to those employed in the peptide studies was associated with intra- and extracellular concentrations of ANXA1 approximately 10^4 -fold lower, in molar terms, than the effective inhibitory concentrations of the ANXA1-derived peptides. Endogenous ANXA1 production was not significantly altered in the presence of Der p, PPD or PHA with or without additional dexamethasone (100 nM), as compared with culture in medium alone. It seems clear that this production did not significantly influence the effects of the ANXA1-derived peptides, since if this had been the case, the inhibitory concentration/response curves of the peptides, as well as the differential effects of the stereospecific and scrambled peptides, would have been obliterated. The lack of significant enhancement by dexamethasone of either intracellular or cell surface ANXA1 in our system highlights the probability that ANXA induction by glucocorticoids is likely cell- and system-specific. For example, in a previous study [18], which did demonstrate an increase in ANXA1 expression by PBMC following exposure to glucocorticoid, such an increase was apparent only if the cells were treated with PMA prior to dexamethasone treatment. Other studies [4,39] have differed with regard to the requirement for phorbol ester priming for up-regulation and externalization of ANXA1 by dexamethasone *in vitro*.

Our observation that both ANXA1-derived peptides inhibited antigen-, but not lectin-induced T cell proliferation suggests their predominant effect is on T cell activation mechanisms dependent on antigen presentation. Other studies are in line with this: for example, activation of rat myelin basic protein-specific T cell lines by autologous antigen-presenting cells was inhibited by prior incubation of the latter cells with ANXA1, whereas ANXA1 was ineffective at inhibiting IL-2-driven proliferation of these T cell lines subsequent to antigen-specific activation [16]. In addition, ANXA1 binding sites are more abundant on monocytes compared with lymphocytes [20,21]. One caveat to this conclusion relates to the lower efficacy of the ANXA1-derived peptides compared with ANXA1 itself, since the latter has been reported in a single study to inhibit lectin-induced T cell proliferation [19].

In summary, we have shown that ANXA1-derived

peptides can inhibit antigen-driven human T cellular proliferation and cytokine production, and have provided preliminary data that clinically relevant antigens do not modulate expression and externalization of the endogenous protein by PBMC, at least *in vitro*. This does not exclude the possibility of paracrine effects of ANXA1 on T cell function *in vivo*.

Acknowledgements

SFS and CJC thank the Wellcome Trust (project grant number: 053525) and the Special Trustees of Charing Cross Hospital for supporting this project. AMK thanks the Wellcome Trust for personal support and MdeSW thanks the Wellcome Trust for the award of a Vacation Scholarship, during which pilot experiments for this study were undertaken.

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