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Tumour necrosis factor-α expression and cell recruitment in Sephadex particle-induced lung inflammation: effects of dexamethasone and cyclosporin A

*Cara M.M. Williams, ‡Lance Smith, †Brian F. Flanagan, ‡L. Steve Clegg & *,[1]John W. Coleman

Departments of *Pharmacology and Therapeutics and †Immunology, University of Liverpool and ‡Research Department, Knoll Pharmaceuticals, Nottingham

1 Tumour necrosis factor-α (TNF-α) is a cytokine with diverse properties consistent with a possible role in inflammatory disease. We investigated whether TNF-α is induced during the progression of lung inflammation elicited by a particulate non-antigenic stimulus, and whether pharmacological control of TNF-α expression influences recruitment of specific inflammatory cell types.

2 A single intravenous injection of Sephadex particles into rats led to extensive granulomatous inflammation in lung alveolar and bronchial tissue that peaked in intensity after 24–72 h. Mononuclear cells were the principal component of granulomas, but neutrophils and eosinophils were also abundant. Numbers of mononuclear cells, neutrophils and eosinophils recovered by bronchoalveolar lavage (BAL) peaked at 72 h, 48 h and 72 h, respectively.

3 Messenger RNA encoding TNF-α was induced in lung epithelial cells, lung granulomas and BAL cells 6 h after Sephadex administration and remained elevated for 72 h before declining to baseline by 7 days. In BAL cell populations TNF-α protein was localized to mononuclear cells at all times points pre- and post-Sephadex administration.

4 Treatment of rats with dexamethasone significantly reduced the Sephadex-induced recruitment of mononuclear cells, neutrophils and eosinophils into the bronchoalveolar cavity, and significantly reduced TNF-α mRNA expression by BAL cells.

5 Treatment of rats with cyclosporin A was without effect on Sephadex-induced elevations of mononuclear cell numbers and expression of TNF-α, but did reduce significantly recruitment of neutrophils and eosinophils to BAL cell populations.

6 These results show that a sequential asthma-like recruitment of neutrophils, eosinophils and mononuclear cells into lung tissue can be induced by single exposure to a non-antigenic stimulus. Pharmacological and histological studies reveal that mononuclear cell mobilization relates closely to induced TNF-α expression, whereas mobilization of neutrophils and eosinophils appears secondary to expression of the cytokine.

Keywords: Cyclosporin A; cytokine expression; dexamethasone; eosinophils; lung inflammation; neutrophils; Sephadex particles; tumour necrosis factor-α (TNF-α)

Introduction

Inflammation of the lung, seen for example in asthma or alveolitis, is multi-causative and undoubtedly involves the interplay of diverse chemical mediators. The identification of these mediators, definition of their roles and an understanding of how their synthesis and actions can be pharmacologically controlled, are important research objectives. In allergen-driven lung disease, immunological activation of mast cells and/or T lymphocytes leads to tissue infiltration by the major inflammatory cell types, namely neutrophils, eosinophils and mononuclear cells, and these cells together are believed to be responsible for disease progression and airway debilitation (DeMonchy *et al*., 1985; Djukanovic *et al*., 1990; Wasserman, 1994). The sequence of events involved in non-allergen-driven inflammatory lung disease is less well understood, but there are almost certainly points of convergence with immunological mechanisms, probably involving induction of common chemical mediators important in inflammatory cell recruitment. Inducible cell-derived proteins, cytokines and chemokines, may be important in this respect. One such candidate cytokine is tumour necrosis factor-α (TNF-α), a 17 kDa protein derived from many cell types, including monocytes/macrophages, T cells, neutrophils, mast cells and epithelial cells. TNF-α upregulates adhesion molecules on endothelial cells to initiate migration of inflammatory cells from blood to lung (Gamble *et al*., 1985; Cavender *et al*., 1987; Thornhill *et al*., 1991; Walsh *et al*., 1991), is chemoattractant for neutrophils and monocytes (Ming *et al*., 1987), enhances the cytotoxic capability of eosinophils leading to damage of the endothelium (Slungaard *et al*., 1990) and increases airway hyperresponsiveness in rats, sheep and man (Wheeler *et al*., 1990; Kips *et al*., 1992; Yates *et al*., 1993). TNF-α is elevated in sputum of patients with acute attacks of asthma and TNF-α levels are up to 20 times higher in bronchoalveolar fluid from symptomatic compared to asymptomatic asthma patients (Ying *et al*., 1991). Furthermore, blocking of TNF-α activity *in vivo* with a TNF receptor fusion protein inhibits influx of granulocytes into BAL fluid in animal models of lung inflammation (Renzetti *et al*., 1996; Gater *et al*., 1996).

To identify any molecule as a mediator of disease it is necessary to show that it has the requisite biological activities, that it is elevated during disease progression and that inhibition of its production or activity alleviates disease symptoms or pathological correlates. Although TNF-α possesses many of the requisite biological activities to be expected of a mediator of asthma and is elevated in asthmatic disease, no previously cited work has demonstrated directly TNF-α induction in lung tissue following exposure of man or experimental animals to allergen or any other agent (Kips *et al*., 1993; Shah *et al*.,

[1]Author for correspondence at: Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX.

1995). Furthermore it is not known how lung TNF-α expression may be controlled pharmacologically, nor whether inhibition of TNF-α expression might influence inflammatory changes in lung, particularly cell recruitment. To answer these questions we have utilized the rat model of Sephadex-induced lung inflammation, in which particles become lodged in the capillaries of the lung and the resulting trauma leads to cell infiltration and airway hyperresponsiveness (Laycock *et al*., 1986; Cook, 1990; Spicer *et al*., 1990; Asano *et al*., 1992; Gater *et al*., 1996). We have studied the sequence of migration of neutrophils, eosinophils and mononuclear cells into the lung in this model, its relation to expression of TNF-α mRNA, and the pharmacological control of these processes.

Methods

Animals and procedures

Male Wistar rats (150–180 g), were purchased from Charles River, housed two per cage and fed *ad libitum* on a standard diet. Animals were anaesthetized by intraperitoneal injection of Brietal (5 mg per 100 g body weight) before the administration of 1.0 ml of Sephadex G200 suspension (0.5 mg ml^{-1}) or 1.0 ml of isotonic saline (controls) intravenously via the tail vein. Sephadex G200 (Pharmacia, Uppsala, Sweden) was prepared by suspension in isotonic saline (0.5 mg ml^{-1}), autoclaved for 20 min and stored at 4°C for a minimum of 48 h before use. In some experiments, rats were administered either cyclosporin A (10 or 30 mg kg^{-1} in olive oil), dexamethasone (0.1 or 0.3 mg kg^{-1} in Cellosize (Sigma, Poole, U.K.)) or vehicle alone intraperitoneally 24 h and 90 min before the administration of Sephadex.

Isolation of bronchoalveolar lavage cells and lung tissue

At various times (6, 24, 48, 72 h and 7 days) after Sephadex administration rats were killed, the trachea cannulated and 5 ml of heparin-treated saline (5 u ml^{-1}) were administered by syringe into the airway. After two min, the lavage fluid was recovered by gentle aspiration. Three more lavages were performed and the four fluid collections were combined and stored on ice before sedimentation of the cells. A small piece of lower right lung was removed, fixed in 10% buffered formalin for 24 h and processed to wax for histological and *in situ* hybridization analysis. Bronchoalveolar lavage (BAL) cells were pelleted at 150 *g* at 4°C for 10 min, the supernatant fraction discarded and the cell pellet resuspended in 1 ml of heparin-treated saline. Total white cells were counted by Coulter Counter (Coulter Electronics). Differential leukocyte counts were made on cytocentrifuged preparations stained with 'Diff-Quik' (Merz and Dade AG). A minimum of 200 cells were counted on each slide. To eliminate bias, cytospin slides were coded by an independent observer and cells were counted blindly. Statistical analysis of differences in BAL cell numbers was determined by two-tailed Mann Whitney U test at the 95% confidence interval.

Extraction of RNA from bronchoalveolar lavage cells

BAL cells, pooled from paired rats in each treatment group, were sedimented at 150 *g* for 5 min and 1.0 ml of TRIzol, a monophasic solution of phenol and guanidinium isothiocyanate (GIBCO, Uxbridge, U.K.) was added to the cell pellets for 5 min at room temperature. Chloroform (0.2 ml per 1.0 ml of TRIzol) was then added and the samples capped and shaken by hand for 15 s. Following a further incubation for 2 min at room temperature, the samples were centrifuged at 12,000 *g* for 15 min at 4°C and the upper aqueous phase containing the RNA was removed into a clean tube and precipitated in 0.5 ml of isopropyl alcohol for 10 min at room temperature. The RNA was then pelleted at 12,000 *g* for 10 min at 4°C, the supernatant fraction removed and the RNA pellet washed once in 1.0 ml of 70% ethanol. The RNA was centrifuged at 7,500 *g* for 5 min at 4°C, the pellet air dried and dissolved in 20 μl of RNase-free water and incubated for 10 min at 60°C. RNA yields were measured by absorbance at 260 nm.

Reverse transcription-polymerase chain reaction (RT-PCR)

Three micrograms of RNA from each cell extract were reverse transcribed by use of an oligodeoxythymidine primer of 20 nucleotides and 45 u of AMV reverse transcriptase (Promega, Southampton, U.K.). Preliminary RT-PCR experiments with RNA concentrations from 100 ng to 1 μg revealed that within this range the product band intensity was linearly related to the amount of starting RNA. Consequently PCR were performed with 8 μl of cDNA equivalent to 400 ng of starting RNA in 50 μl of 10 × PCR buffer (50 mM Tris-HCl, pH 8.0; 50 mM KCl: Roche Molecular Systems, Branchburg, New Jersey U.S.A.) containing 5 μg ml^{-1} of each primer, 1 μl of 10 mM dNTPs, 1.2 u Taq DNA polymerase (Roche), $MgCl_2$ (1.5 mM final concentration) and tetramethylammonium chloride (Sigma) at a final concentration of 10^{-5} M. Intron spanning TNF-α primers were designed from the rat TNF-α cDNA sequence

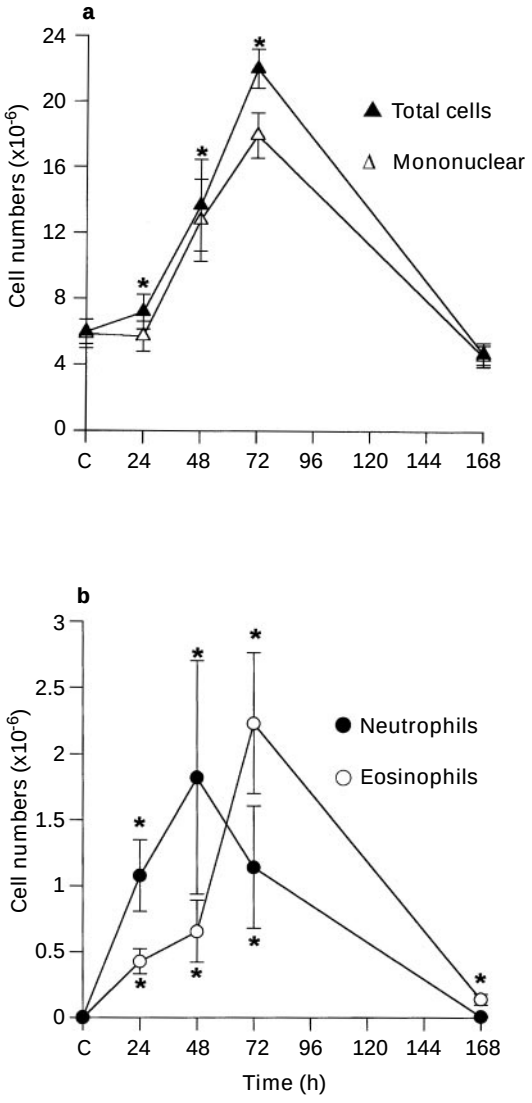

Figure 1 (a) Numbers of total cells and mononuclear cells and (b) neutrophils and eosinophils in BAL fluid isolated from rats at various times following intravenous injection of Sephadex particles. Results are means for 10 rats; vertical lines show s.e.mean. $*P<0.05$ for comparisons with control (C) rats, by two-tailed Mann Whitney U test.

(Shirai *et al*., 1989). Their sequences were 5′-CCA-CGT-CGT-AGC-AAA-CCA-CCA-AG-3′ and 5′-CAG-GTA-CAT-GGG-CTC-ATA-CC-3′ amplifying a 316 base pair (bp) fragment. G3PDH primers were designed from the rat cDNA sequence (Fort *et al*., 1985). Their sequences were 5′-CTC-AAG-ATT-GTC-AGC-AAT-GC-3′ and 5′-CAG-GAT-GCC-CTT-TAG-TGG-GC-3′ which amplify a fragment of 404 bp. Reaction mixtures were overlaid with mineral oil and 38 PCR cycles were performed. Reactants were cycled at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min for 37 cycles followed by a final cycle of 94°C for 1 min, 60°C for 1 min and 72°C for 5 min. Aliquots (10 μl) of the reaction products were run on 2% agarose gels containing ethidium bromide (0.5 μg ml^{-1}) in Tris-acetate-EDTA-buffer. DNA molecular weight markers (1 μg, Promega) were run in parallel. The gels were visualized and photographed under ultraviolet light. Negatives were scanned by laser densitometry (LKB Ultroscan XL Pharmacia, Uppsala, Sweden) and the area under the curve of band peaks calculated (GelScan XL 2.1 software, Pharmacia). Differences between band intensities of PCR products generated from the BAL cells of control rats or those from Sephadex treated rats were analysed statistically by Mann Whitney U test at the 95% confidence interval. The identity of the TNF-α RT-PCR product (316 bp) was confirmed by digestion with the restriction enzyme Sau3A giving predicted fragment sizes of 84 bp and 232 bp.

Immunohistochemistry

BAL cell cytospins were fixed in 10% buffered formalin for 5 min and stained with a rabbit polyclonal anti-mouse (rat cross-reactive) TNF-α antibody (Genzyme) followed by conventional alkaline phosphatase-labelled antibody detection system (ZYMED, Carlton Court South, San Francisco, CA, U.S.A.). Normal rabbit serum was used in place of the primary antibody as negative control.

Histological analysis of lung tissue

Paraffin wax embedded lung tissue was cut into 4–5 μm sections and mounted on microscope slides. Serial sections were either stained for mast cells and neutrophils with naphthol chloroacetate (Vince *et al*., 1991) (Sigma kit) or for eosinophils by use of conventional haemotoxylin and eosin.

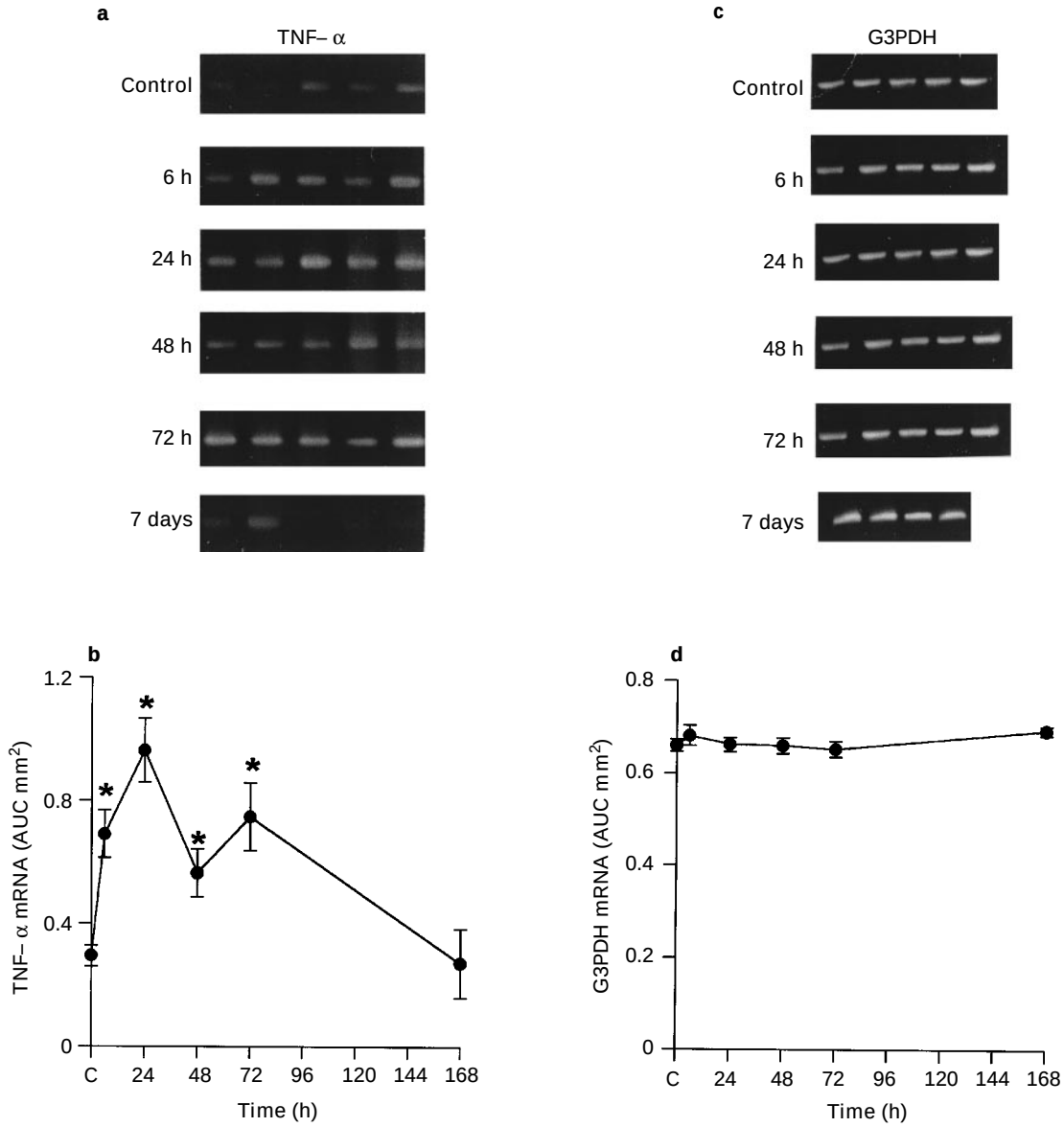

Figure 2 RT-PCR analysis of mRNA for TNF-α (a,b) and G3PDH (c,d) in BAL cells isolated from rats at various times following intravenous administration of Sephadex. Control (C) refers to saline-injected rats. Each band in (a) and (c) represents RT-PCR product from BAL cells pooled from two rats. Gels were scanned by laser densitometry and the intensity of RT-PCR products calculated as area-under-the curve (AUC) of densitometric traces (b,d). Results are means for five experiments, each with BAL cells pooled from two rats; vertical lines show s.e.mean. $*P<0.05$ for comparisons with control rats by two-tailed Mann-Whitney U test.

In situ *hybridization*

Digoxigenin labelled RNA probes were generated by insertion of TNF-α cDNA into the TA Cloning vector (Invitrogen, R & D Systems, Abingdon, Oxon, U.K.). The plasmid was linearized with Xba 1 or Bam H1 to produce antisense (having a complimentary sequence to TNF-α mRNA) or sense (having identical sequence to TNF-α mRNA) probes, respectively. Labelled antisense (cRNA) or sense (mRNA) transcripts of TNF-α mRNA were synthesized in the presence of adenosine-5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), creatine 5′-triphosphate (CTP), uridine 5′-triphosphate (UTP) digoxigenin 11 UTP and SP6 polymerase or T7 polymerase to generate antisense or sense probes respectively (reagents from Boehringer Mannheim, Mannheim, Germany). For *in situ* hybridization, paraffin embedded sections (4–5 μm) were mounted on 3-aminopropyl triethoxysaline coated slides, wax was removed (xylene, 2 × 5 min) and sections were rehydrated through decreasing concentrations of ethanol. Sections were then incubated in 2 × standard saline citrate buffer (SSC) for 10 min at 60°C and rinsed in diethylpyrocarbonate (DEPC) treated water. The preparations were washed in 50 mM Tris (pH 7.6) for 5 min at room temperature before permeabilization with proteinase K (25 μg ml^{-1}, Sigma) in 50 mM Tris (pH 7.6) for 30 min at 37°C. The reaction was terminated by immersion of the preparations in 4% paraformaldehyde for 20 min at 4°C. Sections were then washed with PBS before hybridization. For hybridization, 200 μl of either sense or antisense RNA probe (500 ng ml^{-1}) diluted in hybridization buffer (5 ml formamide, 2 ml 50% dextran sulphate, 3 ml 20 × SSC) were applied to each section. Sections were covered with H_2O_2 treated coverslips and hybridization was performed in a humid chamber for 18 h at 42°C. Post hybridization washing was performed in a decreasing concentration of SSC (2 × SSC for 10 min at room temperature followed by 0.1 × SSC/50% formamide at 42°C for 1 h). Preparations were then washed in modified Tris (50 mM Tris, pH 7.6, containing 150 mM NaCl, 2 mM $MgCl_2$, 0.1% bovine serum albumin and 0.1% Triton X-100) for 15 min at room temperature. Excess buffer was removed carefully and 300 μl of alkaline phosphatase-labelled sheep anti-digoxigenin Fab fragment (Boehringer Mannheim) at a dilution of 1:500 in modified Tris was applied to each section. Sections were incubated at room temperature for 30 min, washed in fresh modified Tris and rinsed in DEPC water before the application of 300 μl of revealing agent (2.4 ml of 0.125 M Tris pH 9.5, 300 μl of 1 M NaCl, 300 μl of 0.5 M $MgCl_2$, 13 μl of 4-nitro blue tetrazolium chloride (Boehringer Mannheim), 10 μl of 5-bromo-4-chloro-3-indoyl-phosphate 4-toluidine salt (Boehringer Mannheim) and 60 μl of 1 M levamisole). Coverslips were applied to each section and preparations were incubated at room temperature in the dark for 6 h. Subsequently, sections were washed in water for 5 min and mounted.

Results

Histological changes in rat lung tissue

Twenty four hours after a single i.v. injection of Sephadex particles, granuloma formation was observed in alveolar tissue

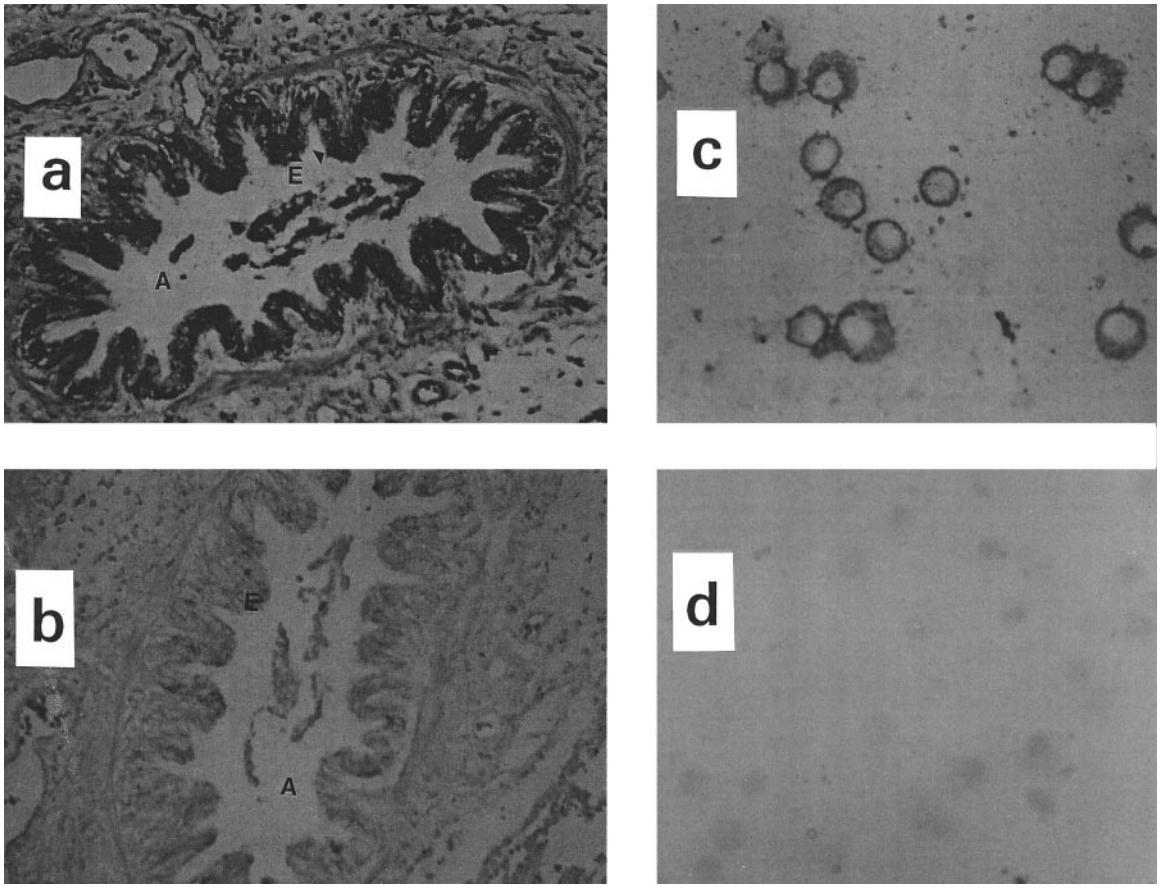

Figure 3 Localization of TNF-α mRNA in lung sections and TNF-α protein in BAL cell preparations. (a) *In situ* hybridization analysis of mRNA expression for TNF-α in rat lung tissue 72 h after injection of Sephadex particles with a TNF-α antisense RNA hybridization probe – positive cells stained black; (b) negative control with a TNF-α sense RNA hybridization probe (A = airway; E = airway epithelial cells); (c) immunohistochemical analysis of BAL cells isolated 24 h after Sephadex administration and stained with anti-TNF-α antibody (TNF-α positive cells stained dark); (d) negative control stained BAL cells. Original magnification × 200 (a,b), × 1000 (c,d).

and around bronchioles. Granulomas increased in size up to 72 h but by 7 days had declined markedly although were still evident. Mononuclear cells were the principal component of granulomas at all time points. Granulomas contained appreciable eosinophils at 24 h and eosinophil numbers increased further to reach maximal levels after 72 h, thereafter declining to negligible levels by day 7. Neutrophils were also observed in granulomas: their intensity peaked between 24 and 48 h but declined markedly by 72 h.

Cellular changes in bronchoalveolar lavage fluid

BAL fluid from control rats contained an average of approximately 6 million cells, comprising almost exclusively (>99%) mononuclear cells. Exposure of rats to Sephadex produced a substantial increase (approximately 3 fold) in numbers of total and mononuclear cells in BAL fluid. Total and mononuclear cell numbers peaked after 72 h and declined to baseline levels by 7 days (Figure 1a). A significant infiltrate of neutrophils into BAL fluid was observed 24 to 72 h after exposure to Sephadex but by 7 days neutrophils were undetectable (Figure 1b). Eosinophils appeared in BAL fluid at significant levels 24 h after exposure and continued to rise to peak at 72 h, thereafter declining substantially although still present at significant levels after 7 days (Figure 1b). Numbers of eosinophils and neutrophils in BAL fluid peaked at an average of approximately 2 million of each cell type per rat (Figure 1b).

Expression of mRNA for TNF-α in bronchoalveolar lavage cells

Messenger RNA encoding TNF-α was detectable in BAL cells from control rats (Figure 2a). Following the intravenous injection of Sephadex, levels of TNF-α mRNA were elevated at various stages during the inflammatory response (Figure 2a). Analysis of band intensities by laser densitometry revealed that the Sephadex-induced increase in TNF-α mRNA reached statistical significance after 6 h (Figure 2b). TNF-α mRNA levels peaked 24 h after the Sephadex injection and remained significantly elevated up to 72 h but then returned to baseline by 7 days (Figure 2b). Throughout these experiments mRNA expression for the housekeeping gene G3PDH was not altered by injection of the Sephadex particles at any time point (Figure 2c, d).

Localization of TNF-α mRNA in rat lung tissue and TNF-α protein in BAL cells

TNF-α mRNA was localized by *in situ* hybridization to epithelial cells lining large airways but not elsewhere in lung sections from 4/5 saline-injected control rats. Six hours after Sephadex injection, mRNA expression of TNF-α had increased dramatically in epithelial cells and appeared strongly in infiltrating inflammatory cells in all (5/5) rats. Intensity of expression continued to rise in both epithelial cells and granulomas up to 24 h and thereafter (48 and 72 h) declined in

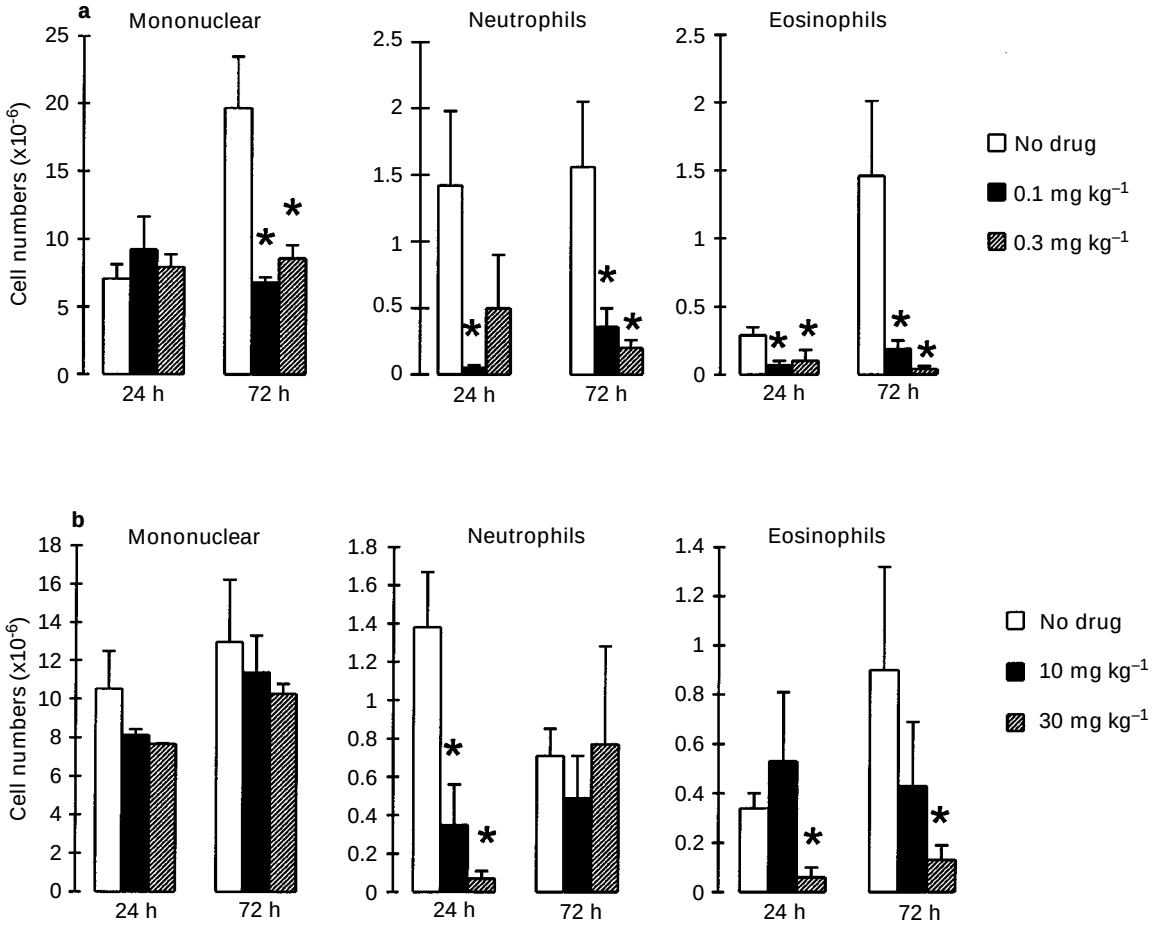

Figure 4 The effects of dexamethasone (a) or cyclosporin A (b) on numbers of mononuclear cells, neutrophils and eosinophils in BAL fluid isolated 24 and 72 h after injection of Sephadex particles. Rats were treated with 0.1 or 0.3 mg kg^{-1} dexamethasone, or 10 or 30 mg kg^{-1} cyclosporin A or vehicle alone 1.5 and 24 h before the Sephadex injection. For (a) results are means ± s.e.mean for 10 rats; for (b) results are means ± s.e.mean for 5 rats. *$P<0.05$ by comparison with no drug controls as determined by two-tailed Mann Whitney U test.

inflammatory granulomas but not in epithelial cells lining airways. Figure 3a shows typical localization of TNF-α mRNA to epithelial and inflammatory cells 72 h after Sephadex administration. Control staining of lung sections with mRNA sense *in situ* hybridization probes was consistently negative (Figure 3b).

Immunostaining of BAL cell cytospin preparations revealed that TNF-α was localized to mononuclear cells at all time points. Figure 3c and d show typical positive and negative control staining, respectively, for TNF-α in BAL mononuclear cells 24 h after Sephadex administration.

Effects of dexamethasone or cyclosporin A on Sephadex-induced cell infiltration into the bronchoalveolar lumen and TNF-α expression by BAL cells

Rats were treated with dexamethasone (0.1 or 0.3 mg kg^{-1}), cyclosporin A (10 or 30 mg kg^{-1}) or vehicle at 24 and 1.5 h before a single intravenous injection of Sephadex particles. Bronchoalveolar lavage was performed either 24 or 72 h after the Sephadex injection: these times were chosen since earlier experiments had shown that after 24 h neutrophils and eosinophils but not mononuclear cells are elevated whereas at 72 h numbers of all three cell types are elevated (Figure 1). The isolated cells were prepared for histology and mRNA analysis.

Dexamethasone was without effect on mononuclear cell numbers at 24 h, consistent with there being no significant increase in this cell type at this time point. However, both doses of dexamethasone completely blocked the Sephadex-induced infiltration by mononuclear cells at 72 h (Figure 4a). Dexamethasone also inhibited significantly the infiltration by neutrophils and eosinophils observed 24 and 72 h after the Sephadex injection (Figure 4a). Thus dexamethasone effectively blocked the infiltration into the bronchalveolar lumen of all three inflammatory cell types.

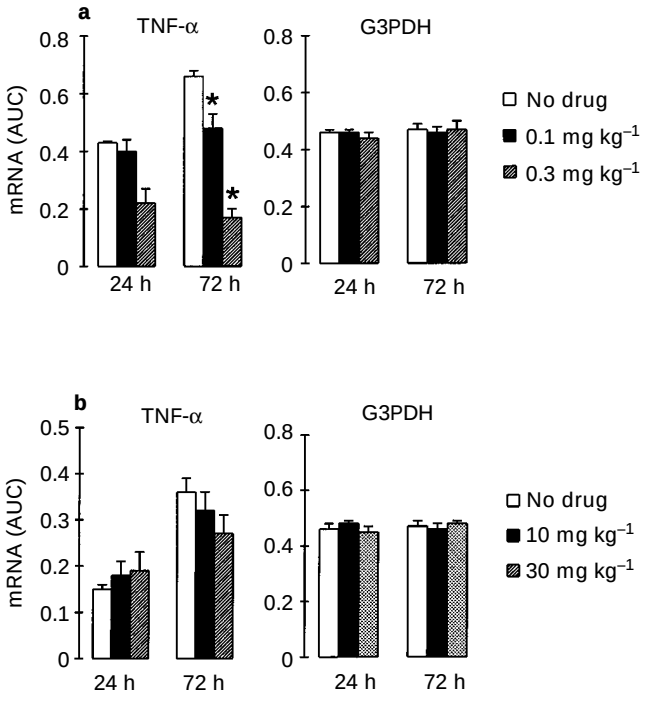

Figure 5 The effects of dexamethasone (a) or cyclosporin A (b) on levels of TNF-α mRNA and G3PDH mRNA extracted from rat BAL cells 24 or 72 h after Sephadex administration. Further experimental details as for Figure 4. Results are means of PCR product band intensities ± s.e.mean for at least 4 experiments, each with cells from two rats. Band intensity was measured as area-under-the curve by laser densitometry. $*P<0.05$ by comparison with no drug controls as determined by the two-tailed Mann Whitney U test.

Cyclosporin A was without effect on mononuclear cell numbers in the bronchoalveolar lumen at both time points (Figure 4b). The higher dose of cyclosporin A inhibited infiltration by neutrophils and eosinophils at 24 h whereas at 72 h it inhibited only eosinophil migration (Figure 4b). The lower dose of cyclosporin A produced a significant inhibition of neutrophil but not eosinophil infiltration at 24 h but was without effect at 72 h. Thus cyclosporin A inhibited migration of neutrophils and eosinophils (most markedly at 24 h) but was without effect on mononuclear cell migration.

Dexamethasone at both doses significantly reduced TNF-α mRNA levels in BAL cells at 72 h but not 24 h after Sephadex administration (Figure 5a), whereas cyclosporin A was without effect on TNF-α mRNA levels at both doses and both time points (Figure 5b). G3PDH mRNA levels were constant across all groups (Figure 5a,b).

Discussion

In this study we utilized the Sephadex model of rat lung inflammation to address the following questions: whether sequential recruitment of inflammatory cells, as seen in antigen-driven disease, could be driven by a physical particulate stimulus; whether this type of inflammation was associated with induction of the cytokine TNF-α; and whether recruitment of specific cell types and TNF-α expression may be controlled by dexamethasone and cyclosporin A. Intravenous administration of Sephadex particles led to the sequential appearance over 24–72 h of neutrophils and eosinophils in lung granulomas and BAL cell populations, against a backgound of increased mononuclear cell numbers. Alongside and preceding cell recruitment, mRNA encoding TNF-α was detected in lung epithelial tissue, granulomas and BAL cells, and was elevated in these sites as early as 6 h following Sephadex administration. In BAL cell populations the cytokine was localized to mononuclear cells by immunostaining. These results are consistent with induction of TNF-α being an important early event in the induction of lung inflammation. Although it is known that TNF-α possesses diverse pro-inflammatory properties (see Introduction and reviews by Kips *et al*., 1993; and Shah *et al*., 1995) and blockade of the actions of TNF-α abrogates granulocyte mobilization into lung (Gater *et al*., 1996; Renzetti *et al*., 1996), this is the first description of induction of TNF-α expression in lung tissue following an inflammatory stimulus. These observations raise the question as to whether TNF-α expression simply reflects mononuclear cell numbers or whether the cytokine has an active role to play in chemotaxis of these cells. Certainly, the early expression of TNF-α mRNA by BAL cells and in lung tissue is consistent with an inductive role.

Our pharmacological studies provide further information regarding the relationship between TNF-α expression and the mobilization of different cell types. We found that the glucocorticoid dexamethasone and the immunosuppressive agent cyclosporin A exerted quite distinct effects. Treatment of rats with dexamethasone inhibited Sephadex-induced TNF-α expression and also inhibited infiltration of the bronchoalveolar lumen by all three inflammatory cell types (neutrophils, eosinophils and mononuclear cells). On the other hand, cyclosporin A did not reduce TNF-α expression but did inhibit influx of neutrophils and eosinophils without an effect on mononuclear cell numbers. These results are consistent with TNF-α playing a role in mononuclear cell mobilization but not a direct role in mobilization of granulocytes. However, because inhibition of TNF-α by dexamethasone is associated with inhibition of influx all three cell types, we suggest that TNF-α induces secondary factors responsible for neutrophil and eosinophil mobilization, for example eotaxin (Jose *et al*., 1994; Rothenberg *et al*., 1995) and MIP-2 (Feng *et al*., 1995) and synthesis of these factors, but not TNF-α, is inhibited by cyclosporin A.

Further light on the role of TNF-α in lung inflammation is shed by the studies of Gater and colleagues (Gater *et al*., 1996;

Renzetti *et al*., 1996) who showed that a TNF receptor fusion protein blocked granulocyte infiltration into rat lung tissue in both Sephadex- and antigen-induced inflammation. These findings are consistent with our own conclusions that induction of TNF-α expression is important in cell mobilization even though we believe, on the basis of our findings with cyclosporin A, that the effects of TNF-α to mobilize neutrophils and eosinophils are indirect and involve induction of other factors. Thus inhibition of TNF-α expression by dexamethasone, or of TNF-α activity by soluble receptor protein, blocks mobilization of all three cell types, whereas inhibition of synthesis of TNF-α-induced factors for example by cyclosporin A leads to inhibition of neutrophil and eosinophil influx in the absence of inhibition of TNF-α expression.

The present findings are consistent with previous studies showing that TNF-α mRNA expression by mononuclear cells is resistant to the inhibitory effects of cyclosporin A (Cockfield *et al*., 1993). Furthermore, because T cells rather than macrophages/monocytes are the primary target for the action of cyclosporin A (Granelli-Piperno *et al*., 1988), we conclude that T cells may be the major source of cyclosporin-inhibitable genes that encode cytokines/chemokines responsible for chemotaxis of neutrophils and eosinophils. As mentioned above, candidates for these roles are the chemokines eotaxin (Jose *et al*., 1994; Rothenberg *et al*., 1995) and MIP-2 (Feng *et al*., 1995) that have eosinophil and neutrophil recruiting properties, respectively.

We have shown previously that expression of rat mast cell TNF-α is inhibited *in vitro* by cyclosporin A and dexamethasone (Williams & Coleman, 1995) and others have found that these agents can significantly suppress the tissue swelling and leukocyte infiltration associated with mast cell and TNF-α-dependent inflammation in rat skin *in vivo* (Wershil *et al*., 1995). Since the TNF-α expression in the present study was resistant to cyclosporin A, we conclude that mast cells are not the source of this cytokine in Sephadex-induced lung inflammation.

The Sephadex-induced sequential mobilization of neutrophils and eosinophils against a background of mononuclear cell mobilization is similar to that in allergen-driven asthma in animals and man (DeMonchy *et al*., 1985; Djukanovic *et al*., 1990; Elwood *et al*., 1991; Kips *et al*., 1993; Wasserman, 1994; Barnes, 1994; Rothenberg *et al*., 1995) and points to similarities and convergence between antigen-driven and non-antigen-driven forms of lung inflammation. Importantly, the present results show that infiltration of lung by the three inflammatory cell types can occur independently of immunological sensitization of either mast cells or T cells that depend on T and/or B cell clonal selection and proliferation, events that are too slow to contribute to inflammation that occurs within 24 h of primary exposure. Since elicitation of lung inflammation by allergen and by non-antigen stimulation (Sephadex particles) results in the same sequence of cellular events, some early point of convergence between these two activation pathways must be postulated. Subsequent to the activation of this common step, induction of neutrophil, eosinophil and mononuclear cell infiltration must follow regardless of the nature of the initial stimulus, i.e. whether immunological or non-immunological. The identity of this point of convergence is not known, although the present findings are consistent with TNF-α being important in this respect.

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