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Critical Role for T Cells in Sephadex-Induced Airway Inflammation: Pharmacological and Immunological Characterization and Molecular Biomarker Identification

El-Bdaoui Haddad,*† Stephen L. Underwood,*† Dominika Dabrowski,* Mark A. Birrell,‡ Kerryn McCluskie,‡ Cliff H. Battram,¶ Michaela Pecoraro,* Martyn L. Foster,§ and Maria G. Belvisi†‡

Intratracheal instillation of Sephadex particles is a convenient model for assessing the impact of potential anti-inflammatory compounds on lung eosinophilia thought to be a key feature in asthma pathophysiology. However, the underlying cellular and molecular mechanisms involved are poorly understood. We have studied the time course of Sephadex-induced lung eosinophilia, changes in pulmonary T cell numbers, and gene and protein expression as well as the immunological and pharmacological modulation of these inflammatory indices in the Sprague Dawley rat. Sephadex increased T cell numbers (including CD4+ T cells) and evoked a pulmonary eosinophilia that was associated with an increase in gene/protein expression of the Th2-type cytokines IL-4, IL-5, and IL-13 and eotaxin in lung tissue. Sephadex instillation also induced airway hyperreactivity to acetylcholine and evoked a pulmonary eosinophilia that was associated with an increase in gene/protein expression of the Th2-type cytokines IL-4, IL-5, and IL-13 and eotaxin in lung tissue. Sephadex-induced eosinophilia and Th2 cytokine gene and/or protein expression were accompanied by a significant inhibition of IL-4, IL-13 and eotaxin gene expression together with suppression (65% inhibition) of eosinophils in lung tissue 24 h after Sephadex treatment. Sephadex-induced eosinophilia and Th2 cytokine gene and/or protein expression were sensitive to cyclosporin A and budesonide, compounds that inhibit T cell function, suggesting a pivotal role for T cells in orchestrating Sephadex-induced inflammation in this model. The Journal of Immunology, 2002, 168: 3004–3016.

Asthma is a complex chronic inflammatory disease of the airways that involves the activation of many inflammatory and structural cells, all of which release inflammatory mediators that result in the typical pathophysiological changes of asthma (1, 2). It is now recognized that chronic inflammation is an important aspect of asthma (3). This chronic inflammation may result in structural changes in the airway, such as fibrosis (particularly under the epithelium), increased thickness of the airway smooth muscle layer (hyperplasia and hypertrophy), hyperplasia of mucus-secreting cells, and new vessel formation (angiogenesis). Some of these changes may be irreversible, leading to fixed narrowing of the airways. Cytokines play an integral role in the coordination and persistence of the inflammatory process in the chronic inflammation of the airways in asthma. They are capable of inducing many of the proinflammatory effects characteristic of this disease and are being increasingly recognized as important targets for treatment (2, 4).

Experimental animal models are important tools for enhancing our understanding of the pathophysiology of airway diseases. Models of allergic pulmonary eosinophilia in the rat and mouse have been widely used, because Ag challenge in these species leads to increased Th2-type cytokine gene expression in lung tissue, eosinophilia, and airway hyperreactivity (5–7). Airway eosinophilia can also be induced by nonallergic stimuli such as i.v. or intratracheally (i.t.)2 administered Sephadex (particles of cross-linked dextran) (8, 9). Rats have an endogenous hypersensitivity to dextrins such as Sephadex, which results in an eosinophilic inflammatory response (10). In several laboratory animals, it has been shown that i.t. or i.v. Sephadex administration induced blood and lung eosinophilia (11–15). The increase in eosinophil numbers is accompanied by the development of pulmonary granulomas. Lung histology showed massive perialveolar and peribronchial edema and granulomatous infiltrates, primarily with eosinophils, after i.t. application of Sephadex (9). In rodent models, Sephadex produces pulmonary inflammation that may be associated with bronchial hyperresponsiveness (16–18). This model is extremely rapid compared with other, more classical models, of lung eosinophilia and airway hyperresponsiveness (AHR) in that no prior sensitization to the foreign agent is required as in the traditional OVA-sensitized and challenged animals used in most models. This model is amenable for investigating the effects of small molecule inhibitors and biotherapeutics on airway inflammation; however, the limitations and usefulness of this model are unclear because there is limited information regarding the cellular and molecular mechanisms underlying the inflammatory response in this model.

In this study, we have performed extensive kinetic studies to determine the relationship of lung cell recruitment to proinflammatory cytokine and chemokine gene and/or protein expression. Because T cells are generally held to promote airway eosinophilia in asthma, we have investigated the contribution of T cells as well as mast cells to the inflammatory responses induced by Sephadex particles through both pharmacological and immunological modulation.

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2 Abbreviations used in this paper: i.t., intratracheally; AHR, airway hyperresponsiveness; ACh, acetylcholine; p.o., orally.
Materials and Methods

Animals
Male Sprague Dawley rats (280–320 g) were purchased from Harlan-Olac (Bicester, U.K.) and housed for 5 days before being used for any experimental procedures. Food and water were supplied ad libitum. United Kingdom Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed.

Materials
Sephadex G-200, budesonide, Wright-Giemsa stain, reagents for RNA extraction and ethidium bromide (Sigma, Poole, U.K.); cyclosporin A (singly purchased from Aventis Pharma, Dagenham Research Center, Dagenham, U.K.); mAbs against rat αβ-TCR (clone R73) and Abs for flow cytometry (Seropec, Oxford, U.K.); sodium pentobartibonate (Sagatal) and halothane (both from Rhône Mérieux, Harlow, U.K.); RPMI 1640 and FBS (Life Technologies, Paisley, U.K.); collagenase and DNase (Boehringer Mannheim, Lewes, U.K.); Immunoprep leukocyte preparation reagents (Coulter, Luton, U.K.); reagents for RT-PCR (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) except Tris-acetate-EDTA buffer (Life Technologies).

Primers were synthesized from published sequences (Table I) by Life Technologies (Bicester, U.K.) and housed for 5 days before being used for any experimental procedures. Food and water were supplied ad libitum. United Kingdom Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed.

Experimental protocols

Time course of Sephadex-induced pulmonary inflammation.
Rats were given vehicle (saline, 1 ml/kg) or Sephadex G-200 (5 mg/kg) i.e.t. (19). Sephadex was prepared as a suspension by soaking in sterile saline for 3 days. This study also included a group of naive animals. Animals were sacrificed (sodium pentobartibonate, 200 mg/kg i.p.) at various times (2, 4, 6, 12, 24, 48, and 72 h) after saline or Sephadex challenge. Cell populations and gene expression in lung tissue were determined as described below. The group size was 6–8.

Quantification of lung tissue cellularity.
At the indicated time point after saline or Sephadex instillation, animals were euthanized (sodium pentobarbitone, 200 mg/kg i.p.). The thoracic cavity was exposed, and the lung was recovered from lung tissue using enzymatic digestion (7). Total cell count required for cytokine protein and gene expression analyses. Cells were recovered from lung tissue using enzymatic digestion (7). Total cell count was performed on samples recovered from lung tissue using an automated cell counter (Cobas Argos; Roche ABX Hematologie, Montpellier, France). For differential cell count, cytopsins of these samples were prepared. Slides were fixed and stained on a Hema-tek 2000 (Ames, Elkhart, IN) with modified Wright-Giemsa stain. Four-part differential counts on 200 cells/slide were performed by light microscopy following standard morphological criteria, and the percentages of eosiophils, macrophages/monocytes, lymphocytes, and neutrophils were determined.

T lymphocytes recovered from lung tissue were labeled with fluorophore-labeled mAbs and counted by flow cytometry. Cells (1 × 10<sup>6</sup>) were incubated (30 min, 4 °C) with saturating concentrations of a FITC-labeled Ab against CD2 (MRC OX34), together with a PE-labeled Ab against either CD4 (W3/25) or CD8 (MRC OX8). Alternatively, cells were similarly incubated with a FITC-labeled Ab against αβ-TCR. After the incubation, commercial reagents (ImmuNoPrep, Coulter) were used to lyse erythrocytes and to stabilize and fix the remaining cells. To control for autofluorescence and nonspecific binding, respectively, unlabeled cells and fluorophore-labeled IgG isotype control Abs were used. Flow cytometry was performed using an EPICS XL flow cytometer and software (Coulter Electronics, Luton, U.K.). To define the total lymphocyte population, preliminary gating was achieved with forward and side scatter characteristics. T lymphocytes were first defined as CD2<sup>+</sup> cells. Subsequently, the numbers of these cells that were also CD4<sup>+</sup> and CD8<sup>+</sup> were calculated from two-color plots of FITC fluorescence against PC fluorescein.

Lung cytokine protein expression.
Approximately 200 mg of lung tissue were homogenized in saline using an Ultraturrax T25 homogenizer (BDH, Lutterworth, U.K.). The samples were then spun (13,000 × g, 20 min, 4°C), and the supernatants were stored for further analysis. Lung cytokine levels were determined by ELISA using commercially available kits according to the manufacturer’s instructions.

Lung cytokine gene expression (RT-PCR).

RNA extraction.
Total cellular RNA from rat lung recovered at various time points after saline or Sephadex instillation was isolated by guanidinium thiocyanate-phenol-chloroform extraction according to the method described by Chomczynski and Sacchi (20). Purity and integrity of the RNA samples were assessed by OD<sub>260</sub>/OD<sub>280</sub> spectrophotometric measurements. RNA was stored at −80°C until used for reverse transcription and PCR.

Reverse transcription.
A 1-μg portion of total RNA was subjected to first strand cDNA synthesis in a 25-μl reaction mixture containing avian myeloblastosis virus reverse transcriptase (10 U), dNTP (2 mM concentrations of each dNTP), oligo(dT) <sub>12-18</sub> primers (10 μM), and reaction buffer as supplied with the enzyme (50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, and 10 mM DTT). The samples were incubated in a PerkinElmer thermal cycle (PerkinElmer, Wellesley, MA) at 42°C for 60 min followed by enzyme denaturation step at 94°C for 2 min. The reverse transcription mixture was diluted with 25 μl of RNase-free water and stored at −80°C for use in PCR. All the reagents were obtained from Promega (Southampton, U.K.).

PCR.
PCR was conducted to amplify target cDNA fragments for IL-4, IL-5, IL-13, eotaxin, RANTES, IFN-γ, and the housekeeping gene GAPDH used as an internal standard. PCR was performed on 4 μl of reverse transcriptase product using Ready-To-Go PCR beads (Amersham Pharmacia Biotech), containing Taq DNA polymerase, dNTP, buffer, and 0.5 μM of each gene-specific forward and reverse primers (obtained from Life Technologies, Paisley, U.K.) in a total volume of 25 μl. Gene-specific oligonucleotide primers, listed in Table I, were designed from published rat sequences. The PCR was conducted in a Perkin-Elmer GeneAmp PCR system 9700. After an initial denaturation at 95°C for 5 min, amplification was conducted through 25–35 cycles of denaturation at 94°C for 30 s; annealing at 55°C (GAPDH), 64°C (IL-4), or 60°C (for all other transcripts) for 30 s; and extension at 72°C for 45 s. Final extension was at 72°C for 7 min followed by a final hold at 4°C. Negative controls (PCR mixture without cDNA) and positive controls (PCR mixture with a standard cDNA sample) were included in preliminary PCR runs. Initial experiments were conducted to determine the optimal annealing temperature for each set of gene-specific primers and also the numbers of cycles necessary for optimal amplification.

Table I. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5′→3′)</th>
<th>Product (bp)</th>
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<tr>
<td>IL-4</td>
<td>Sense: ACGTGGTCTGCCCAGTTCCTG</td>
<td>352</td>
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<tr>
<td></td>
<td>Antisense: GTGGTCTGACCTGAGTTCAG</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>Sense: TTCTGCTTGTCTGCGGTAATT</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td>Antisense: TGCTTGTGGTCTGACCTGAA</td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>Sense: TCAGCTTGGCTGTCGTTC</td>
<td>311</td>
</tr>
<tr>
<td></td>
<td>Antisense: CTGGGGTACTTGTGTGTC</td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>Sense: TCAACGGCTCATCTGGCTTCA</td>
<td>279</td>
</tr>
<tr>
<td></td>
<td>Antisense: ACATCTGTCCGAGTCTTCCA</td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>Sense: AACCTGCTTGGTTCCTGACCT</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>Antisense: TTTTGAGTTGGTGTGGT</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Sense: ATTCCTTCATTGGTCACACTCTTCACT</td>
<td>432</td>
</tr>
<tr>
<td></td>
<td>Antisense: CACATCTATTGGAAAGGCTCC</td>
<td>983</td>
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<tr>
<td>GAPDH</td>
<td>Sense: TGAAAGGCTGCGTTCACAGAGTCGGTCCACC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: CATGTAGGCACATGAGTCGGTCCACC</td>
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to ensure linear amplification of each target fragment (data not shown). The cycle numbers used were 28 for GAPDH, and 35 for all other fragments.

PCR products together with molecular size markers were separated by electrophoresis using 2% agarose gels stained with ethidium bromide to visualize cDNA products. Bands of each target transcript were visualized by ultraviolet transillumination and captured using a digital camera. Optical densities for each band were quantified by image analysis software (Phoretix 1D Quantifier, version 4.01; Phoretix International, Newcastle upon Tyne, U.K.). The level of gene expression of each transcript was normalized to that of the housekeeping gene GAPDH.

Effect of Sephadex instillation on airway responsiveness to spasmodgens.

The rats were dosed i.t. with vehicle (saline) or Sephadex (5 mg/kg) in a dose volume of 1 ml/kg under halothane anesthesia (4% in oxygen for 3 min). Twenty-four hours after Sephadex instillation, the animals were anesthetized with pentobarbitone (90 mg/kg i.p.) and connected to a Fleisch tube via a tracheal cannula to facilitate measurement of airflow. Water filled, esophageal cannula was placed such that transmucosal pressure could be recorded. The animals were then connected to a Ugo-Basile (Comerio-Varese, Italy) respiration pump set at 90 ppm, and tidal volume adjusted to 3.5 ml. Airways resistance was computed on a breath-by-breath basis on the Buxco LS-20 system (Buxco Electronics, Peterfield, U.K.). Acetylcholine (ACh; 10, 20, 40, 100, and 200 mM aerosolized for 5 s) or bradykinin (1 mM, aerosolized for 30 s) was administered by a nebulizer connected in line and reactivity assessed. For flow-induced responses, U.A.S.E.S. (Buxco Electronics, Peterfield, U.K.) was used to aerosolize water and bradykinin (1 mM, aerosolized for 30 s). The level of gene expression of each transcript was normalized to that of the housekeeping gene GAPDH.

Role of mast cells in Sephadex-induced airway inflammation.

To assess the contribution of mast cells in this model, we have depleted mast cells from preformed mediators using the mast cell secretagogue compound 48/80 (0.6 mg/kg i.p.) injected each morning and evening for 4 days (21). On the fifth day, rats received the final i.p. injection of 1.2 mg/kg compound 48/80 4 h before saline or Sephadex instillation. This protocol has been shown to deplete mast cells in previous experiments. Control rats received saline vehicle (i.p.). In another set of experiments, rats were dosed with the mast cell stabilizer cromoglycate (100 mg/kg i.p., a dose previously shown to be effective (22), 1 h before saline or Sephadex instillation. Inflammatory indices were assessed 24 h after saline or Sephadex challenge.

Effects of T cell depletion on Sephadex-induced pulmonary inflammation.

To investigate the role of T cells in Sephadex-induced airway inflammation, we used the Ab R73 against the αβ-TCR (1 mg/kg i.v.) delivered into the tail vein to deplete T cells. In a satellite group, we confirmed the depleting effect of R73. Rats received Sephadex or vehicle i.t. or act as untreated controls (as described above). Twenty-four hours beforehand, rats received Ab R73 (1 mg/kg i.v.) or an isotype-matched control Ab (mouse IgG, 1 mg/kg i.v.) or no treatment. Cell populations and cytokine gene expression in lung tissue were determined 24 h after Sephadex or saline administration.

Effects of budesonide and cyclosporin A on Sephadex-induced pulmonary eosinophilia.

Rats received Sephadex or saline (i.t.) or acted as untreated controls (as described above). Twenty-four and two hours and beforehand, rats were dosed orally with budesonide A (500 μg/kg), budesonide B (30 mg/kg), or vehicle or received no treatment. Doses of compound were selected from previous studies where these doses were appropriate for inhibiting Sephadex-induced inflammatory responses (23) and allergen-induced eosinophilia (24) in rats. Compounds were prepared at a dose volume of 1 ml/kg as suspensions in 1% methylcellulose in water. Cell populations and cytokine gene expression in lung tissue were determined 24 h after Sephadex or saline administration. The group size was 12.

Effect of Sephadex on cultured rat T cell IL-2 production.

T cells were collected and cultured as in the method of Hofstra et al. (25). Briefly, SD rats were euthanized with an overdose of pentobarbitone (200 mg/kg i.p.), the spleens were removed and pushed through a microsieve. The T cells were counted and diluted in RPMI, and 2 × 10^6 were added to round bottom 96-well plates. The cells were then cultured in the presence of saline, Sephadex (0.01, 0.03, 0.1, 0.3, 1.0 μg/ml), or the positive control, Con A (6.5 μg/ml), for 24 h. The supernatant was then taken off and assessed for IL-2 by ELISA using commercially available kits according to manufacturer’s instructions.

Data analysis

Values are expressed as mean ± SEM of n independent observations. Statistical comparisons were made using the Kruskal-Wallis test followed by Dunn’s post-test. All treatments were compared with vehicle control values: ∗p < 0.05; ∗∗p < 0.01.

Results

Time course of Sephadex-induced pulmonary inflammation

Cell accumulation

Data pooled from all time points showed that cells isolated from the lung tissue of untreated Sprague Dawley rats contained 1% eosinophils, 29% neutrophils, 20% CD2^+ cells (total T cells), and 49% other mononuclear cells. Of the CD2^+ cell population, 55% were CD4^+ and 17% were CD8^- . Sephadex (5 mg/kg i.t.), but not vehicle, caused significant accumulations of neutrophils and eosinophils that were apparent 6 and 12 h after treatment respectively (Fig. 1). Significant eosinophilia was still apparent 72 h after treatment, whereas neutrophil numbers were no longer significantly elevated at this time point. There was no significant increase in the total number of mononuclear cells (Fig. 1). Sephadex also caused an increase in the number of CD2^+ cells (total T cells) in the lung tissue. The number of these cells was significantly increased 48 h after treatment and remained elevated 72 h after treatment. This increase included significant accumulations of both CD4^+ and CD8^- cells (Fig. 1).

Cytokine gene expression

A representative PCR gel showing the expression of IL-4, IL-5, IL-13, eotaxin, and the housekeeping gene GAPDH 24 h post-treatment is depicted in Fig. 2. Sephadex instillation induced a significant increase in mRNA expression for the Th2-type cytokines IL-4, IL-5, and IL-13 as well as for the C-C chemokine eotaxin when compared with saline-treated animals (Fig. 2). However, the level of the CC chemokine RANTES and the Th1-type cytokine IFN-γ expression are unaffected by Sephadex treatment (Fig. 2). The effect of Sephadex on IL-4, IL-5, IL-13, and eotaxin mRNA expression was apparent at 6–24 h post-treatment and had generally declined toward basal levels by 48–72 h (Fig. 2). Saline instillation also induced a significant increase in IL-5, IL-13, and eotaxin expression when compared with naive animals. The overall kinetic profiles were similar for each induced cytokine when the levels were normalized to those obtained in saline-treated animals (data not shown). The kinetics of cytokine induction either preceded (IL-13 and eotaxin) or was concomitant to the eosinophil influx into the lung, suggesting a causal relationship.

Cytokine protein expression

In agreement with the gene expression data, IL-13 and eotaxin protein expression was also induced in lung tissue homogenates after Sephadex instillation (Fig. 3). The kinetics of IL-13 and eotaxin protein expression were similar to the data obtained for gene expression. There was no specific rat ELISA kit to quantify IL-5 protein expression. However, protein levels for the pleiotropic and proinflammatory cytokines IL-1β and TNF-α were markedly up-regulated by Sephadex treatment with a delayed kinetics profiles compared with those of eotaxin and IL-13 (Fig. 3). RANTES and IFN-γ protein production was not altered after Sephadex challenge in agreement with the mRNA data (Fig. 3).

Effect of Sephadex instillation on airway responsiveness to spasmodgens.

Sephadex treatment significantly increased basal resistance when compared with naive and saline treated animals (0.156 ± 0.007, 0.172 ± 0.007, and 0.274 ± 0.03 cm H₂O/ml/s for naive, saline-treated, and Sephadex-treated groups, respectively, p <
Saline administration had no impact on reactivity to inhaled ACh or bradykinin; however, reactivity in the Sephadex-treated group was significantly increased (Fig. 4).

Contribution of mast cells to Sephadex-induced airway inflammation

To delineate the contribution of mast cells to Sephadex-induced airway eosinophilia, we have depleted mast cells from neo- and preformed mediators using the mast cell secretagogue, compound 48/80 dosed twice a day for 4 days (0.6 mg/kg i.p.). In this study, Sephadex administration significantly increased the eosinophils in the lung tissue compared with saline-treated vehicle control rats ($1.27 \pm 0.28$ cells $\times 10^6$/mg of tissue compared with $0.24 \pm 0.06$ cells $\times 10^6$/mg of tissue in saline-treated control rats, $p < 0.05$). Using this well-characterized protocol, there was no significant protection from Sephadex-induced airway eosinophilia after mast cell depletion ($1.91 \pm 0.38$ cells $\times 10^6$/mg of tissue). Furthermore, we have confirmed this hypothesis using the mast cell degranulation inhibitor, sodium cromoglycate (100 mg/kg i.p., 1 h before Sephadex or saline treatment), which again did not significantly change the eosinophil burden in the lung tissue of Sephadex-treated rats ($0.97 \pm 0.13$ cells $\times 10^6$/mg of tissue).

FIGURE 1. Time course of Sephadex-induced eosinophil (A), neutrophil (B), mononuclear cell (C), CD2$^+$CD4$^+$ (D), and CD2$^+$CD8$^+$ (E) T cell accumulation in rat lung tissue. Rats were untreated or received saline (1 ml/kg i.t.) or Sephadex (5 mg/kg i.t.). Cells were recovered from lung tissue at various time points after challenge by enzymatic digestion as described in Materials and Methods. Cell number was determined in tissue digest by differential cell counting and flow cytometry. Results are expressed as the mean $\pm$ SEM of six to eight animals. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett’s post-test (*, $p < 0.05$, compared with saline-treated groups; †, $p < 0.05$, saline-treated groups compared with untreated groups).
0.18 cells × 10^5/mg of tissue). These data appear to rule out the contribution of mast cells to Sephadex-induced airway inflammation.

Contribution of T cells to Sephadex-induced airway inflammation

Because of the pattern of cytokine expression after Sephadex instillation resembles a Th2 profile and due to the documented involvement of T cell in allergic asthma and eosinophilic inflammation, we have investigated T cell requirement in this model using both immunological (using Ab against the αβ-TCR) and pharmacological (using drugs that suppress T cell function such as cyclosporin A and budesonide) approaches.

Study to confirm the depleting effect of Ab R73

There was no increase in T cell numbers in lung tissue 24 h after administration of Sephadex. However, in Sephadex-treated rats that had been pretreated with Ab R73 (1 mg/kg i.v.), the numbers of αβ-TCR^+ and CD2^-CD4^- cells were each reduced by ~74% (Fig. 5), and the number of total CD2^+ cells was reduced by ~54% (data not shown) as measured by flow cytometry 24 h later. The number of CD2^-CD8^- cells was also lower, but the difference did not achieve significance (data not shown).

Effects of T cell depletion on Sephadex-induced pulmonary inflammation

In rats pretreated with Ab R73, the reduction in T cell numbers was accompanied by a significant suppression (~65% inhibition) of eosinophil accumulation (Fig. 6, top). The numbers of neutrophils (Fig. 6, top) and total mononuclear cells were not affected by either Sephadex or Ab R73 treatments. Sephadex also caused a significant increase in mRNA for IL-4, IL-13, and eotaxin (but not IL-5 mRNA expression in this particular experiment) in lung tissue that was prevented in rats pretreated with Ab R73 (Fig. 6, bottom).
Effects of budesonide and cyclosporin A against Sephadex-induced airway inflammation in Sprague Dawley rats

To further investigate the sensitivity of this model to compounds known to inhibit of T lymphocyte function, we have studied the protective effects of the immunosuppressant drug cyclosporin A and the glucocorticoid budesonide.

Effect on cell accumulation

Twenty-four hours after intratracheal instillation of Sephadex particles there was a significant influx of eosinophils, but not neutrophils or mononuclear cells. Pretreatment with cyclosporin A (50 mg/kg p.o.) or budesonide (30 mg/kg p.o.) completely abrogated lung eosinophil accumulation (Fig. 7).

Effect on cytokine gene expression

Instillation of Sephadex particles i.t. was also associated with increased gene expression for Th2 cytokines IL-5, IL-13, and eotaxin, and this effect was significantly inhibited by cyclosporin A and budesonide treatment (Fig. 8). There was a tendency toward an increase in IL-4 levels in the Sephadex-treated group, but this did not
reach significance. Similarly, there was a tendency toward a reduced IL-4 mRNA levels by cyclosporin A and budesonide (Fig. 8).

**Effect on cytokine protein expression**

Similar to the Th2-type cytokine mRNA expression data, there was an increase in IL-13 and eotaxin protein levels after Sephadex instillation (Fig. 9). Consistent with the Th2-type cytokine expression, there was an increase in the proinflammatory cytokine TNF-α and IL-1β production after Sephadex instillation (Fig. 9). Cyclosporin A and budesonide pretreatment markedly inhibited the increase in TNF-α, IL-1β, IL-13, and eotaxin production (Fig. 9). IL-4 levels in lung tissue could not were not increased after Sephadex instillation in this particular experiment.

**Effect of Sephadex on cultured rat T cell IL-2 production**

Sephadex treatment did not alter the amount of IL-2 released from cultured rat T cells. However, in parallel experiments, the positive control Con A did induce significant IL-2 release (0 pg/ml to 651.8 ± 104.0 pg/ml, p < 0.05) from these cells.

**Discussion**

In this study, we have shown that instillation of Sephadex particles i.t. in rats induced a significant eosinophil recruitment into the lung confirming previously published data. We have now extended these findings to describe molecular biomarker expression and the role played by T cells and mast cells in this model. We showed that Sephadex instillation is associated with increased gene and/or protein expression of key inflammatory cytokines and chemokines including IL-4, IL-5, IL-13, and eotaxin. We have also shown that T cells but not mast cells orchestrate the pulmonary eosinophilia induced by Sephadex.

The results of this study show that Sephadex instillation induced increased eosinophil recruitment to lung tissue. The increase in eosinophil influx to lung tissue was apparent 6 h after challenge and remained elevated at least 72 h after treatment in confirming previous reports (16, 18, 26, 27). Our data also demonstrate an accumulation of T cells, including CD4+ (helper) T cells, in lung tissue. This accumulation may have been a consequence of cell recruitment or proliferation of resident cells. Although the increase in T cell numbers occurred after eosinophil numbers were already significantly increased, this finding does not preclude a role for T cells in this model because eosinophilia could be orchestrated by activated resident T cells. To further elucidate the cellular and molecular processes that could orchestrate eosinophil recruitment, we first performed extensive kinetic studies to determine the relationship of lung cell recruitment to proinflammatory cytokine and chemokine gene and/or protein expression. Many studies have highlighted the crucial role played by the putative Th2-type cytokines such as IL-4, IL-5, and IL-13 in eosinophil inflammation and subsequent development of airway hyperreactivity. Therefore, we
FIGURE 6. Effects of the Ab R73 against αβ-TCR on eosinophil and neutrophil number and cytokine gene expression in lung tissue. Rats were given saline or Sephadex (i.t.) or remained untreated. Twenty-four hours before Sephadex instillation, rats were treated with Ab R73 (1 mg/kg i.v.) or an isotype-matched control Ab or received no treatment. Lung tissue was dissected out 24 h after challenge, and cells were recovered and counted (top). For cytokine gene expression analyses, RT-PCR was conducted. Cytokine mRNA signals were expressed as a ratio to GAPDH mRNA as measured by densitometric scanning of the agarose gels (bottom). Group size was six to eight animals in each experimental group. Results represent mean ± SEM. *, p < 0.05 compared with saline-treated animals; †, p < 0.05 compared with Sephadex vehicle-treated groups.
investigated the gene and protein expression of these and other candidate cytokines in this model. RT-PCR analysis showed that Sephadex instillation induced a marked increase in the gene expression of these cytokines. This effect was apparent 6–24 h post-treatment and declined toward basal levels by 48–72 h postchallenge. Besides Th2 cytokines, the CC chemokine eotaxin was also increased after challenge with a similar kinetic profile. We have also extended these observations to the pleiotropic cytokines TNF-α and IL-1β. However, the effect of Sephadex on cytokine gene expression is not a general feature, because the other CC chemokine RANTES and the Th1-type cytokine IFN-γ expression were unaffected by Sephadex treatment. In agreement with the gene expression data, eotaxin and IL-13 protein expression were also induced in lung tissue homogenates after Sephadex instillation, whereas those of RANTES and IFN-γ were not.

There is a large body of data supporting a key role for eotaxin and Th2-type cytokines in the orchestration of airway inflammation and AHR in allergic asthma. Using animal models of allergic airway inflammation, there is now considerable evidence to support the hypothesis that eotaxin plays an important role in eosinophil homing and tissue recruitment (28–30). Indeed, eotaxin mRNA and/or protein expression is up-regulated in guinea pig and rodent models of allergic asthma that parallel eosinophil accumulation (31–33). It was further shown that a neutralizing Ab to eotaxin induced a significant inhibition of eosinophil infiltration following Ag challenge (32) and eotaxin knockout mice exhibited a significantly reduced eosinophil recruitment to the lung 18 h after allergen challenge (34, 35). In our model, the basal expression of eotaxin mRNA and/or protein was up-regulated after Sephadex instillation in agreement with published data (36, 37). Furthermore, it has been shown that i.v. injection of blocking Ab to eotaxin significantly decreased eosinophil infiltration induced by i.t. instillation of Sephadex beads (37).

Sephadex instillation was also associated with increased expression of the proeosinophilic cytokine IL-5 that parallels eosinophil load in lung tissue. These data argue for a role for IL-5 in this model. Evidence for a role for IL-5 has emanated from in vivo work in animal models of allergic inflammation. Thus, transgenic mice overexpressing the IL-5 gene exhibit evidence of airway remodeling and the induction of AHR (38) and administration of neutralizing anti-IL-5 mAbs has been demonstrated to inhibit the eosinophilia induced by nematode infection or Ag exposure in sensitized animals (39–41). Likewise, exposure of IL-5 gene knockout mice to aerosolized Ags caused an ablated eosinophil recruitment into the lungs (42). The results from both in vitro and in vivo investigations also suggest that eotaxin and IL-5 may act cooperatively and synergistically to promote the recruitment of eosinophils into tissues (29, 30, 43, 44). In mice, eotaxin-induced recruitment of eosinophils to the lung and skin was only consistently observed in IL-5-transgenic mice, which have elevated levels of IL-5 and a pronounced basal blood eosinophilia (29, 30). Thus, during the inflammatory response, IL-5 may provide the signal for the release of a pool of eosinophils from the bone marrow, whereas eotaxin may elicit the signal for eosinophil localization to the site of inflammation by up-regulating integrins and stimulating chemotaxis. In our model, both eotaxin and IL-5 are increased after Sephadex instillation. This result suggests that these cytokines may act in synergy to recruit eosinophils. In support for a role of IL-5 in this model, it was shown that airway eosinophilia induced by i.v. administration of Sephadex particles to guinea pigs was totally inhibited by an anti-IL-5 mAb (45).

In addition to IL-5, IL-4 and IL-13 production, activated CD4+ Th2 lymphocytes may also play a major role in orchestrating airway inflammation, including eosinophilia, in allergic asthma (46–49). Specifically, AHR, eosinophilic inflammation, and IgE production, normally seen after local Ag challenge, do not develop in mice in which the gene for IL-4 or IL-13 is disrupted or in animals in which the cytokine or its receptor are blocked in vivo (40, 50–58). Likewise, it was recently shown in a pulmonary granuloma model, induced with *Schistosoma mansoni* eggs, that although eosinophil infiltration, IgE, and IL-5 production are reduced in the IL-4-deficient mice and IL-13-deficient mice, they are abolished only in the combined absence of both cytokines (51). It was shown recently that IL-13-transgenic mice with targeted pulmonary over-expression of IL-13 have significant numbers of eosinophils in the airways, associated with epithelial cell hypertrophy, mucous cell metaplasia, subepithelial airway fibrosis, airways obstruction, and nonspecific AHR (59). In our model, all of the Th2 cytokines and the pleiotropic cytokines IL-1β and TNF-α were markedly induced after Sephadex instillation with kinetics mirroring eosinophil recruitment in lung tissue, which may suggest a causal relationship.

The temporal association among the increased IL-4, IL-5, and IL-13 gene and/or protein expression in lung tissue, with no concomitant increase in IFN-γ gene expression, is consistent with selective secretion of Th2 cytokines from T cells. This pattern is similar to that observed in human asthma and in animal models of allergic asthma. This profile of cytokine expression prompted us to investigate the involvement of T cells in this model using the mAb R73, which is directed against the rat αβ-TCR. This Ab recognizes 97% of rat peripheral T cells, including all CD4+ T cells (60). It depletes or prevents their activation and suppresses a number of immune diseases in the rat including arthritis induced by Ag, collagen, and bacterial cell wall preparations (61, 62), experimental immune neuritis (63), and experimental allergic encephalomyelitis (64). The mechanism of T cell depletion has not been elucidated but is likely to involve opsonization (61). The Ab treatment depleted CD4+ T cells by ~74%, but even this partial depletion was
able to markedly suppress Sephadex-induced eosinophil accumulation by ~65%. IL-4, IL-13, and eotaxin mRNA were also significantly reduced. These data demonstrate that T cells do play a key role in orchestrating key inflammatory indices in this model. The mechanism that leads to T cell involvement in this model is unknown. Conventionally naive T lymphocytes travel to T cell areas
FIGURE 9. Effects of cyclosporin A and budesonide on Sephadex-induced cytokine protein production in rat lung. Rats were treated with Sephadex (i.t.) or saline (i.t.) or received no treatment. The Sephadex-treated rats were dosed p.o. with cyclosporin A (50 mg/kg), budesonide (30 mg/kg), or drug vehicle (vehicle, 1% methylcellulose) or received no treatment 24 and 2 h before Sephadex instillation. **Top**, IL-4 (A), IL-13 (B), and eotaxin (C) protein levels; **bottom**, IL-1β (A) and TNF-α (B) protein levels which were determined by ELISA. Results are presented as mean ± SEM of six to nine animals. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett’s post-test (*, p < 0.05 compared with saline group; ††, p < 0.01 compared with Sephadex vehicle group).
of secondary lymphoid organs in search of Ags presented by dendritic cells. On encountering specific Ag, T helper naive precursor cells become activated, an event that is regulated not only by engagement of the TCR with peptide presented in the context of MHC class II molecules but also by a number of costimulatory signals (65). Because Ag presentation is not involved, Sephadex presumably could not activate T cells conventionally through the TCR. Activation might occur directly through other pathways or indirectly through activation of other cell types that then secrete T cell-activating or -proliferating mediators. The results from the in vitro experiments performed suggest that Sephadex does not directly activate T cells. However, these experiments are not conclusive because this effect may be dependent on the specific population of T cells studied; i.e., we used T cells isolated from the spleen (major source of T cells), which may behave differently from the more mature resident cells found in the lung, which may be more readily stimulated.

Indeed, it has been hypothesized that the dextran constituent of Sephadex might initiate an inflammatory response by eliciting mast cell degranulation (15). In allergic inflammation, mast cells are one of the main effector cells through the release of a myriad of pre- and co-modifiers and cytokines that are potentially important in the allergic response. In this model, however, we could not demonstrate any role for mast cells using pharmacological tools. Indeed, there was no significant protection from Sephadex-induced airway inflammation in rats depleted of mast cell mediators by pretreatment with the mast cell secretagogue, compound 48/80, or pretreated with the mast cell degranulation inhibitor, sodium cromoglycate. These data aside, there are some concerns about the validity of these tools and their effectiveness in depleting mast cells. However, the data implicating a role for the T cell in Sephadex-induced pathology are far more convincing, and as such the mast cell would appear to make a minimal contribution to Sephadex-induced airway inflammation.

To investigate further the role of T cells in this model, we studied the protective effects of cyclosporin A and budesonide. These agents suppress T cell function and have antiasthma activity. Cyclosporin A is a potent immunosuppressant that inhibits signal transduction pathways in activated T cells that control transcription of a number of genes including those for key proinflammatory cytokines. Glucocorticoids such as budesonide are currently the most effective agents available to treat asthmatic airway inflammation. These compounds reduce the number of T cells (66, 67) and the number of cells expressing mRNA for IL-4 and IL-5 (68, 69) in asthmatic airways. Much of their protective activity can be explained by their suppressive effects against the processing of genes for key proinflammatory cytokines such as IL-4 and IL-5 by T cells (70). Although both cyclosporin A and budesonide are likely to have suppressive effects on other cell types, our finding that these agents completely suppress Sephadex-induced eosinophilia supports our hypothesis of a key role for T cells in this model. Although our study suggests a key role for T cells and Th2-type cytokines in promoting eosinophilia, further studies are needed to dissect out the relative contribution of each cytokine and also delineate the contributions of other cell types such as the macrophage in this model.

In conclusion, we have shown that i.t. instillation of Sephadex particles caused a sustained airway eosinophilia in Sprague Dawley rats, which was associated with increased gene and/or protein expression for TNF-α; IL-1β; the CC chemokine eotaxin; the Th-2 cytokines IL-4, IL-5 and IL-13; and airway hyperreactivity. We have also demonstrated that T cells but not mast cells are involved in eosinophilic inflammation as well as cytokine expression observed in this model. Furthermore, we have shown that drugs, which inhibit T cell function, are efficacious in this model. Collectively, our findings argue for a key role for T cells in orchestrating Sephadex-induced pulmonary inflammation. These data, which characterize the molecular mechanisms of action involved in the airway inflammatory response generated in this model, should allow investigators to use this rapid, simple methodology for profiling novel therapeutic agents in development for the treatment of asthma. Furthermore, the identification of compound-sensitive molecular biomarkers may be useful as early indicators of drug efficacy in the clinic and for determining the molecular mechanisms involved in the generation of eosinophilia.

References


