

Conjugates of ovalbumin and monomethoxypolyethylene glycol abolish late allergic responses and decrease IL-4 and IL-5 mRNA expression in the rat

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Abstract

The purpose of this study was to test the therapeutic potential of monomethoxypolyethylene glycol (mPEG) conjugated-allergen using a rodent model of allergic asthma. Previously, this conjugate has been shown to possess the dual capacity of inducing long-term ovalbumin (OA)-specific suppression of the antibody response and inactivating rat mast cells that have been sensitized with murine IgE to OA.

Ovalbumin sensitized and challenged Brown Norway rats were studied. Fourteen days after sensitization, a test group of six rats received mPEG–OA solution intratracheally and were challenged 30 min later with aerosolized OA. Another group of seven sensitized rats was similarly challenged with OA 30 min after intratracheal administration of normal saline. A group of six sensitized rats received mPEG–OA solution intratracheally but were challenged with normal saline. Another group of seven sensitized rats received mPEG–BSA solution intratracheally and were challenged 30 min later with aerosolized OA. A final group of five unsensitized rats were neither challenged nor medicated intratracheally. Pulmonary resistance was measured before and for 8 h following inhalation challenge. mPEG–OA treatment had an inhibitory effect on the allergic late airway response, but the early response was not significantly altered. Both mPEG–OA and mPEG–BSA reduced the total cells, eosinophils and neutrophils, in bronchoalveolar lavage and decreased the expression of IL-4, IL-5 and IFN- γ mRNA.

In conclusion, mPEG–OA can prevent the development of allergen-induced late airway responses and reduce airway Th2-type cytokine expression whereas mPEG conjugated to an irrelevant antigen (BSA) is anti-inflammatory but does not affect the late response.

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1. Introduction

Conjugates—consisting of protein antigens (Ag) onto which an appropriate number (n) of the biocompatible and amphiphilic polymer, monomethoxypolyethylene glycol (mPEG), are coupled, i.e. Ag(mPEG) _{n} —are not only nonallergenic [1] but also immunosuppressive [2]. Thus, these conjugates induce immunological tolerance to specific proteins, which is attributed, at least in part, to the activation of antigen-specific CD8 + suppressor T cells [3–5].

Moreover, Ag-specific suppressive factors produced by CD8 + T cells (Ts factors) downregulate antibody responses to an unrelated Ag (Ag*), when the latter is presented in the form of covalently linked conjugate, i.e. Ag–Ag*, Ag representing the antigen incorporated in the tolerogenic Ag(mPEG) _{n} [6]. From the results of clinical trials with close to 300 allergic patients, it was concluded that mPEG-conjugates might be useful for hyposensitization therapy [7].

Few studies have addressed the potential utility of mPEG-conjugates for the inhibition of allergen-induced airway responses. However, mPEG-conjugates have been shown to prevent mast cell degranulation both in vivo and in

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vitro [8]. Such an effect would be expected to alter the early airway response (EAR) which is an IgE-mast cell dependent transient bronchoconstriction that occurs within minutes of airway challenge and resolves within an hour or so. Inhibition of the EAR by mPEG conjugates pre-treatments has been confirmed in dogs [9]. However, whether the second phase of airway narrowing, namely the late airway response (LAR) that occurs a few hours after initial challenge, could also be inhibited by OAmPEG conjugates has not been examined. The LAR is of particular importance to the pathophysiology of asthma because of its association with airway inflammation and airway hyperresponsiveness, two of the defining characteristics of asthma. Although the LAR usually follows EAR it appears to be independent of IgE dependent mechanisms. The LAR is a T cell dependent reaction as demonstrated by its transfer by antigen primed CD4 + T cells harvested from sensitized donors to naïve recipients [10,11] and is accompanied by the expression of T cell cytokines of the Th2 type [12,13]. The proposed impairment of T cell activation by mPEG-conjugates supports the plausibility of an inhibitory action of these substances on the LAR also.

To test the efficacy of mPEG-conjugates as inhibitors of allergic airway responses we examined the effects of intratracheal administration of OA(mPEG)₁₁ on OA-induced bronchoconstriction and airway inflammation in presensitized Brown Norway (BN) rats, a well characterized model of allergic asthma [14]. Both EAR and LAR and the magnitude of inflammation as reflected in cell numbers in bronchoalveolar lavage (BAL) fluid were assessed. In addition we evaluated the role of the mast cell by the measurement of histamine in the BAL and the T cell through assessment of a number of T cell cytokines, namely interleukin (IL)-4, IL-5 and interferon (INF)- γ . We particularly focused on these cytokines because of their potential role in the regulation of allergic inflammation and eosinophilia [12,15,16].

2. Methods

2.1. Preparation of mPEG–OA

A solution of OVA (grade V, Sigma Chemicals, St-Louis, MO) in borate buffer (0.05 M, pH 9.2) at a concentration of 50 mg/ml was de-aggregated by passage through a Superdex 60/600 gel filtration column, which had been equilibrated in the same buffer. A portion (1.2 g) of the freshly de-aggregated OA, at a concentration of 10 mg/ml, was added with constant stirring to a solution containing 12.8 g of the *p*-nitrophenyl carbonate (PNC) derivative of mPEG dissolved in 120 ml of double distilled water (DDW). The PNC-mPEG derivative had been custom-synthesized by Shearwater Polymers, Inc. (Huntsville, AL), utilizing a highly purified preparation of mPEG (average MW: 3200 Da), containing only a few percentages of diols.

The reaction mixture was transferred to dialysis tubing (Spectra/Por #4; MWCO 12–14 kDa) and constantly stirred in 4 l of borate buffer (0.05 M, pH 9.2) for 1 h at room temperature. Thereafter it was stirred and maintained at 8 °C overnight. The large volume of buffer outside the dialysis bag served as a pH-stat. The product from the reaction was isolated by passage through a Superdex column, which had been equilibrated with DDW. The conjugate emerged in the void volume and was followed by hydrolyzed mPEG-PNC and finally by *p*-nitrophenol (which had released in the coupling reaction and by the buffer salts). The OA–mPEG conjugate in DDW was lyophilized and stored at –20 °C.

2.2. Preparation of mPEG–BSA

The procedure employed to couple mPEG to BSA was essentially identical to that used to couple mPEG to OA. The only change was in the weight of mPEG-PNC intermediate used: 9 g of PNC intermediate instead of 12.8 g.

2.3. Sensitization to ovalbumin

Twenty-six rats were actively sensitized to OA (grade V, Sigma Chemicals, St-Louis, MO, USA) on day 0 with a 1 ml subcutaneous injection of a solution containing 1 mg OA, 4.28 mg Al(OH)₃ (Anachemia Chemicals, Montreal, Quebec, Canada) and isotonic saline. In addition, a 0.5 ml of *Bordetella pertussis* vaccine (Institut Armand Frappier; Laval, Quebec, Canada) containing 10⁹ heat-killed bacilli was injected.

2.4. Measurement of pulmonary resistance

Rats (180–220 g) were anesthetized by an intraperitoneal injection of urethane of 1 g/kg^{–1} (Sigma Chemicals, St-Louis, MO) and were intubated with 6 cm of PE-240 polyethylene tubing (Becton Dickinson Diagnostics, Sparks, MD) using transluminal illumination [17]. Animals were placed on a heating pad and rectal temperature was continuously monitored with an electric thermometer (Telethermometer; Yellow Spring Instrument Co., Yellow Springs, OH). Animals were kept in lateral recumbency. The tip of the tracheal tube was connected to a plastic box (volume 175 ml; Commercial Plastics, Montreal, Quebec, Canada). For measuring airflow ($\dot{V}$), a pneumotachograph (Fleisch 00; Bionetics Limited, Quebec, Canada) coupled to a piezoresistive differential transducer (Micro-Switch 163PC01D36; Honeywell, Scarborough, ON, Canada) was connected to the opposite side of the box. Transpulmonary pressure (P_{pt}) was measured using a water-filled polyethylene catheter (PE-160, Becton Dickinson Diagnostics, Sparks, MD) placed in the lower third of the esophagus and connected to the port of a differential pressure transducer (Transpac II disposable transducer, Sorenson, Salt Lake City, UT). The other port of the transducer was connected to the plastic box. The pressure and flow signals

were amplified and recorded with a 12-bit analog-to-digital converter at a rate of 200 Hz. All signals were recorded at 10 s intervals, stored on a computer and analyzed using a commercially available software package (RHT Infodat Inc, Montreal, Quebec, Canada). Pulmonary resistance (R_L) was calculated by multiple linear regression for solving the following equation for motion of the lung

$$P_{pt} = E_L V + R_L \dot{V} + K$$

where V is the volume obtained by the integration of $\dot{V}$, R_L is resistance, E_L is elastance, and K is a constant.

2.5. Bronchoalveolar lavage

BAL was performed 8 h after OA challenge through the endotracheal tube. Isotonic saline of 37 °C was infused (25 ml) into the lungs in 5 ml aliquots and then aspirated after gentle massage of the lungs. BAL fluid was centrifuged (1500 rpm, 5 min, 5 °C), and pellets were resuspended in 5 ml of RPMI 1640 culture medium. The total volume of BAL fluid recovered was over 90% in the five groups. The total cell counts were determined on a fresh specimen of lavage fluid using a hemacytometer and viability was assessed by the Trypan blue dye exclusion test. The differential cell counts were determined on a cytopsin slide that was prepared with a Cytospin model II (Shandon, Pittsburgh, PA) and stained with a Wright Giemsa stain. At least two hundred cells in each specimen were counted under oil-immersion microscopy.

2.6. Measurement of BAL histamine levels

Before performing the histamine assay, the proteins contained in the BAL fluid were removed to avoid the occurrence of the Pauli reaction with the imidazole group contained in histidine. Briefly, 125 µl of HCl 1.4N were added to 500 µl of BAL fluid and incubated for 2 min at 20 °C. Histamine levels in the BAL fluid were measured in selected groups using a modified quantitative colorimetric assay based on the Pauli reaction and specific for the imidazole group for the determination of histamine in buffer solutions initially described by Marwaha et al. [18] and modified by Hojo et al. [19]. The precipitated proteins were centrifuged (600g, 5 min, 20 °C) and 500 µl of the supernatant were collected. This volume was mixed with 100 µl of 1% sulfanilic acid (in 1.4N HCl) and 100 µl of 5% sodium nitrite solution in borosilicate-glass tubes. After 10 min of incubation at room temperature, 1.3 ml of 5% sodium carbonate solution was added and 2 min later 1 ml of 75% ethanol was added. Two hundred microliters of the medium were put in a 96-well microplates and the absorbance was determined at 530 nm using an ELISA plate reader (400 ATC, STL Labinstruments, Austria). The blank was obtained by subjecting 500 µl of PBS to the same

procedure and a standard curve was generated using different concentrations of histamine (10–40 mg ml⁻¹). The results were expressed as milligrams of histamine contained in the volume of BAL fluid recovered after administration of the first 5 ml of saline.

2.7. Cytologic preparations for immunocytochemistry and in situ hybridization

Cytospin slides were prepared on poly-L-lysine-coated glass slides, fixed in 4% paraformaldehyde for 30 min and washed with PBS (twice for 5 min each time) prior to processing. BAL cells were immunostained with BMK13 mAb (BMK 13 was kindly provided by Dr Redwan Moqbel, University of Alberta, Alberta, Canada), a mouse anti-major basic protein (MBP) mAb, using the alkaline phosphatase anti-alkaline phosphatase method. MBP positive cells were quantified by microscopy by an investigator blinded to group status. A minimum of 500 BAL cells were counted and the percentage of cells expressing MBP immunoreactivity were evaluated.

In situ hybridization was performed as previously described [20,21]. Briefly, antisense and sense riboprobes were prepared from cDNAs coding for rat IL-4, IL-5, and IFN-γ mRNA. cDNAs were first inserted into a pGEM vector and linearized with appropriate enzymes. In vitro transcription was carried out in the presence of ³⁵S-UTP and the T7 or SP6 RNA polymerases. For detection of cytokine mRNAs, cytopsin preparations obtained from BAL were permeabilized with Triton X-100 and proteinase K (1 mg ml⁻¹) in 0.1 M Tris containing 50 mM EDTA for 20 min at 37 °C. To prevent nonspecific binding of ³⁵S-labeled RNA probes, the preparations were incubated with 10 mM *N*-ethyl maleimide and 10 mM iodoacetamide for 30 min at 37 °C and followed by incubation in 0.5% acetic anhydride and 0.1 M triethanolamine for 10 min at 37 °C. Prehybridization was performed with 50% formamide and 2 × standard saline citrate for 15 min at 37 °C. For hybridization, antisense or sense probes (10⁶ cpm/section) were diluted in hybridization buffer. Dithiothreitol (100 mM) was present in the hybridization mixture to ensure blocking of any nonspecific binding of the ³⁵S-labeled probes. Posthybridization washing was performed in decreasing concentrations of standard saline citrate (× 4 SSC to 0.5 SSC) at 40 °C. Unhybridized single-strand RNA was removed by RNase A (20 mg/ml⁻¹). After dehydration, the slides were immersed in NBT2 emulsion and exposed for 10 days. The autoradiographs were developed in Kodak D-19, fixed and counterstained with hematoxylin.

Slides were coded and positive cells were counted blindly using × 40 magnification with an eyepiece graticule. For negative controls cytopsins were hybridized with sense probes or pre-treated with RNase prior to the application of probes.

2.8. Experimental protocol

Thirty-one male BN SSn rats (Harlan, England) 8–11 weeks of age were studied. Rats were randomly divided into five groups. Fourteen days after sensitization, a test group of six rats (mPEG–OA/OA group) received mPEG–OA solution (1.73 mg of mPEG–OA diluted in 100 μ l of normal saline) intratracheally through a tracheal tube. They were challenged 30 min later with aerosolized OA (5%, w/v in saline) using a Hudson nebulizer (Model 1880; Hudson, Temecula, CA) with an airflow of 10 l min⁻¹ for 5 min. Another group of sensitized rats (Saline/OA group; $n = 7$) was similarly challenged with OA 30 min after intratracheal administration of normal saline (100 μ l). A group of sensitized rats (mPEG–OA/Saline group; $n = 6$) received mPEG–OA solution intratracheally but were challenged with normal saline. Another group of sensitized rats (mPEG–BSA/OA group; $n = 7$) received mPEG–BSA solution (1.73 mg of mPEG–BSA diluted in 100 μ l of normal saline) intratracheally and were challenged 30 min later with aerosolized OA. R_L was measured in all rats before saline or mPEG–OA intratracheal administration (pre-treatment), following inhalational challenge and at 5 min intervals for 30 min and at 15 min intervals for a total of 8 h. A final group of unsensitized rats (unsensitized/unchallenged group; $n = 5$) was not challenged nor medicated intratracheally to provide baseline data on parameters of airway inflammation.

Rats were anesthetized, as previously described, prior to intubation and drug administration. Intraperitoneal injections of urethane (0.25 g kg⁻¹) were repeated as required to maintain anesthesia of rats during the 8 h period. To evaluate the possibility of airway obstruction attributable to intratracheal administrations, respiratory mechanics were measured in six rats before and 30 min after mPEG–OA administration.

2.9. Data analysis

EAR was derived from the highest R_L measurements recorded ≤ 30 min after OA challenge and expressed as a percentage of baseline. LAR was measured as the area under the curve for R_L versus time above the pre-treatment R_L between 3 and 8 h after challenge.

Differences among groups were determined using analysis of variance. When significant differences were found, data were further analyzed using Fisher's PLSD test. When there was inequality of variances between groups, values were compared using unpaired Student *t*-test. Values within groups were compared with a paired Student *t*-test. Differences were considered significant when *P* values were less than 0.05. Data are expressed as mean $\pm$ 1 standard error (s.e.mean).

3. Results

3.1. Allergic airway responses

Results of R_L measurements before and 30 min following mPEG–OA intratracheal administration support the view that mPEG–OA does not significantly modify R_L (baseline, 0.135 ± 0.01 cm H₂O ml⁻¹/s; 30 min after mPEG–OA administration, 0.131 ± 0.009 H₂O ml⁻¹/s). The changes of R_L in rats following challenge are shown over the 8 h time course (Fig. 1) and are expressed as a percentage to correct for small but significant inter-animal differences in baseline values. The changes in R_L in the saline/OA group were characteristic of those usually observed in sensitized BN rat following OA challenge. A brisk rise in R_L following challenge faded out by 30 min. There was a subsequent secondary rise in R_L starting approximately 2 h later, which reached a maximum between 6 and 8 h. On the contrary, the response following OA challenge was blunted by the administration of mPEG–OA conjugates (mPEG–OA/OA group).

When the immediate effects of airway challenge on lung function were evaluated in individual groups, there were significant increases in R_L in the mPEG–OA/Saline group, in the Saline/OA group and in the mPEG–BSA/OA within 30 min of challenge (Fig. 2). The increase in R_L after challenge in the mPEG–OA/OA group did not reach statistical significance ($P = 0.1$). However, an ANOVA failed to reveal a significant difference in EAR among groups. There was no significant difference in histamine levels assayed in the BAL fluid between the mPEG–OA/OA group (31.7 ± 3.4 mg/ml⁻¹) and the Saline/OA group (34.9 ± 9.5 mg/ml⁻¹), consistent with the lack of effect of mPEG–OA treatment on the EAR.

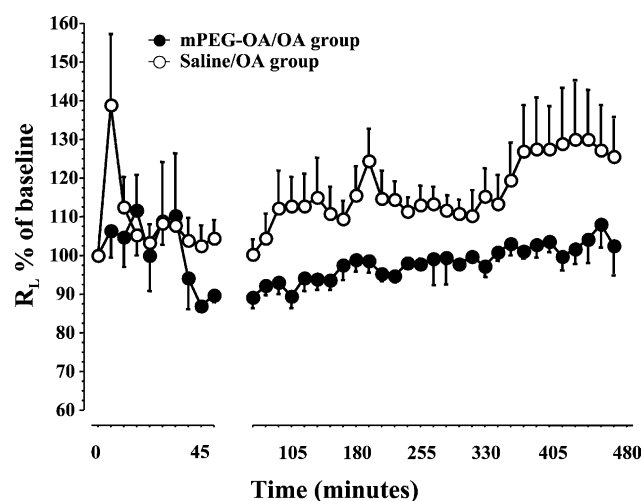


Fig. 1. Lung resistance measurements (R_L), expressed as a percentage of the baseline value, from 0 to 8 h after ovalbumin inhalational challenge. Rats were administered mPEG–OA intratracheally (mPEG–OA/OA) or saline (Saline/OA) 30 min prior to challenge. The mean ($\pm$ s.e.mean) values are shown.

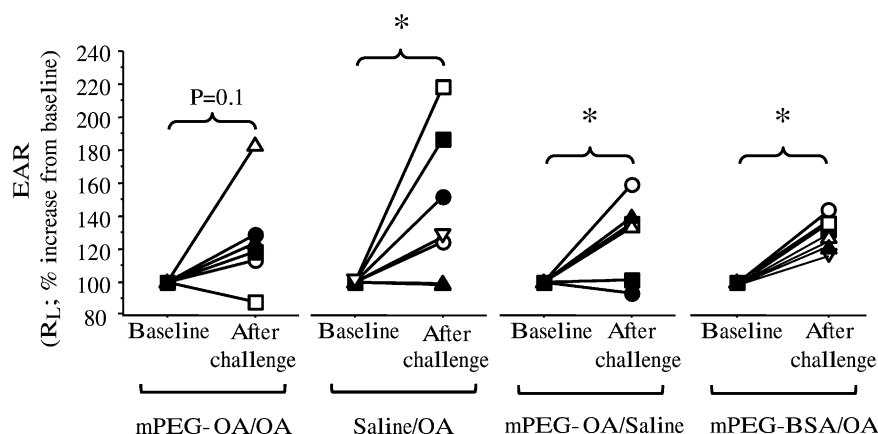


Fig. 2. Early airway response (EAR) to inhaled ovalbumin (mPEG–OA/OA; Saline/OA; mPEG–BSA/OA) or saline (mPEG–OA/Saline), expressed as a percentage of the baseline values of R_L . Rats were administered mPEG–OA intratracheally (mPEG–OA/OA; mPEG–OA/Saline), mPEG–BSA (mPEG–BSA/OA) or saline (Saline/OA) 30 min prior to challenge. *Significant differences before and after challenges ($P < 0.05$).

In contrast to the minor and insignificant changes in R_L immediately after OA challenge, there were substantial differences in R_L during the period of the late response to challenge. The LAR was virtually abolished in the mPEG–OA/OA group compared to the Saline/OA group ($P = 0.02$). However, the LAR was not significantly suppressed in mPEG–BSA treated animals. The mean values of the area under the curve of R_L versus time were 1.3 ± 0.6 , 3.8 ± 1.4 , 6.7 ± 3.4 , and 9.8 ± 3.1 cm H_2O ml $^{-1}$ /s in the mPEG–OA/OA group, mPEG–OA/Saline group, mPEG–BSA/OA group, and Saline/OA group, respectively (Fig. 3).

3.2. Bronchoalveolar lavage leukocytes

mPEG–OA administration prevented the increase in total cell count (Fig. 4), eosinophilia, expressed as MBP positive cells (Fig. 5), and neutrophilia in BAL fluid observed following OA challenge. Lymphocyte counts were lower in the mPEG–OA/OA group, the Saline/OA group and mPEG–OA treated group than in the mPEG–OA/Saline group. There were no significant differences in macrophage counts among the groups.

3.3. Cytokine mRNA expression

Positive hybridization for IL-4, IL-5 and IFN- γ mRNA was observed in the BAL cells of all animals (Fig. 6a–c). mPEG–OA and mPEG–BSA/OA administration prevented the increase in cells which expressed IL-4 and IL-5 mRNA following OA challenge. The number of mRNA positive cells for IFN- γ was significantly higher in the Saline/OA group compared to the mPEG–OA/OA and the mPEG–BSA/OA groups. No hybridization was present in the specimens that were incubated with sense probes or in the specimens that were pre-treated with RNAase.

4. Discussion

OA challenge triggered a typical delayed onset of bronchoconstriction or LAR in sensitized animals that was suppressed by the administration of mPEG–OA conjugates intratracheally prior to challenge. There was also a significant modification of the inflammatory response to OA challenge by mPEG conjugates which was manifested as a reduction in eosinophilia and neutrophilia in BAL. mPEG conjugates pre-treatment also affected events upstream in the cascade of inflammation leading to inhibition of IL-4 and IL-5 mRNA expression by BAL cells.

Previous studies of mPEG conjugates suggest multiple possible sites of interference with the expression of allergic airway responses in sensitized subjects following challenge. We had anticipated that IgE mediated mast cell degranulation might be inhibited by mPEG–OA conjugates [7]. A prior study demonstrated the efficacy of mPEG–OA in preventing the development of the EAR in sensitized dogs

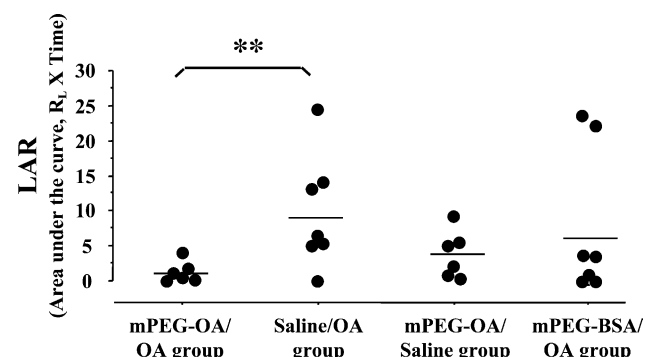


Fig. 3. Late airway responses (LAR) to ovalbumin (mPEG–OA/OA; Saline/OA; mPEG–BSA/OA) or saline (mPEG–OA/Saline), expressed as the area under the curve of R_L against time from 3 to 8 h. Rats were administered mPEG–OA intratracheally (mPEG–OA/OA; mPEG–OA/Saline), mPEG–BSA (mPEG–BSA/OA) or saline (Saline/OA) 30 min prior to challenge. Individual data points and the group means (horizontal lines) are shown. **The LAR is abolished in the mPEG–OA treated animals ($P = 0.02$).

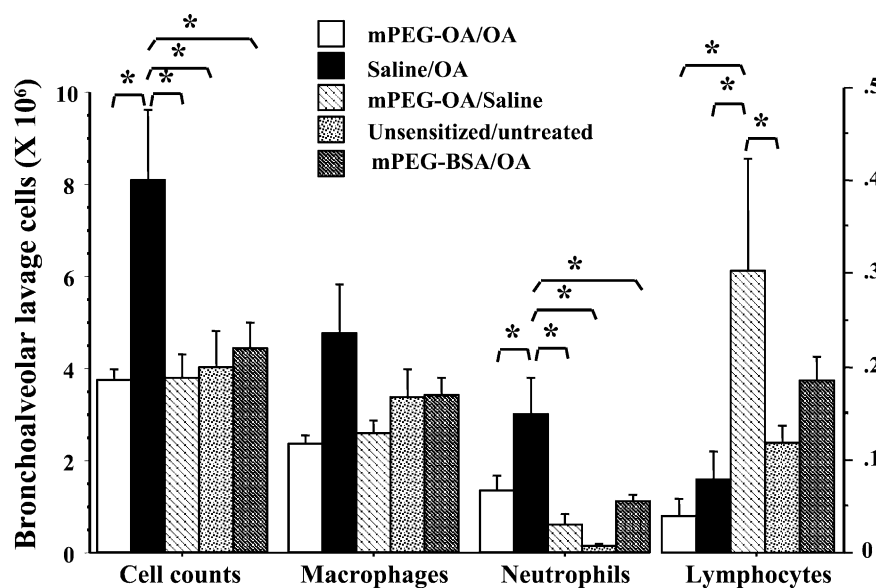


Fig. 4. Bronchoalveolar total cell count and differential cell counts 8 h after ovalbumin inhalational challenge (mPEG–OA/OA; Saline/OA; mPEG–BSA/OA) or saline inhalation (mPEG–OA/Saline). Rats were administered mPEG–OA intratracheally (mPEG–OA/OA; mPEG–OA/Saline), mPEG–BSA (mPEG–BSA/OA group), or saline (Saline/OA) 30 min prior to challenge. Rats from the unsensitized/untreated group were neither sensitized nor subjected to inhalation challenge. The increase in BAL cell and in neutrophils in sensitized rats following inhalational challenge with OA was prevented by mPEG–OA administration. *Significant differences between groups ($P < 0.05$).

[9] when administered 2 h prior to challenge. The postulated explanation for this effect was an inhibition of mast cell degranulation caused by the internalized mPEG molecule [9]. The magnitude of the EAR in the present study was small and variable. Although the degree of inhibition of the EAR by the mPEG conjugates was insufficient to reach statistical significance the power of the ANOVA to detect differences was low. However, the similarity in BAL fluid histamine levels, a marker of mast cell activation, between mPEG treated and OA challenged animals and the saline treated and OA challenged animals is also suggestive of a lack of effect of mPEG–OA conjugates on the EAR.

Although the timing of administration of mPEG conjugates in the present study would not have led to suppression of antibodies, administration of mPEG conjugates to naïve mice has resulted in profound suppression of IgE and IgG antibody production on subsequent injections of the corresponding intact antigen [2,7,22]. However, when mPEG conjugates are administered after sensitization, only a moderate suppression of IgE occurs [7].

The administration of mPEG–OA conjugates in the present study completely abolished the LAR, which suggests independent regulation of EAR and LAR. The notion that the EAR is not a necessary precursor to the LAR is supported by a number of observations. Using the technique of adoptive transfer, it has been possible to show that antigen-primed CD4 + T cells administered to an unsensitized recipient rat results in a LAR following antigen challenge [10,23]. Furthermore, the LAR and the accompanying eosinophilia appear to be independent of IgE-mediated mechanisms [10]. This raises the possibility that mPEG conjugates may inhibit the LAR through a

modulation of T cell function. Indeed, evidence for interference with T cell reactions to antigen has been obtained and appears to reflect an indirect effect of mPEG conjugates through blocking antigen presentation by antigen-presenting cells [24]. It has been postulated that mPEG increases the resistance of the conjugates to proteolytic degradation in antigen-presenting cells, resulting in the failure to generate epitopes for T cell recognition [25].

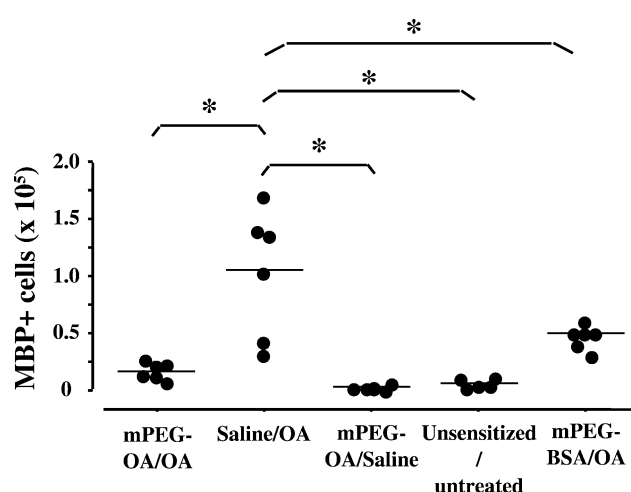


Fig. 5. Bronchoalveolar eosinophil counts (expressed as MBP positive cells by immunocytochemistry) 8 h after ovalbumin inhalational challenge (mPEG–OA/OA; Saline/OA; mPEG–BSA/OA) or saline inhalation (mPEG–OA/Saline). Rats were administered mPEG–OA intratracheally (mPEG–OA/OA; mPEG–OA/Saline), mPEG–BSA (mPEG–BSA/OA), or saline (Saline/OA) 30 min prior to challenge. Rats from the unsensitized/untreated group were neither sensitized nor subjected to inhalation challenge. *Significant differences between groups ($P < 0.05$).

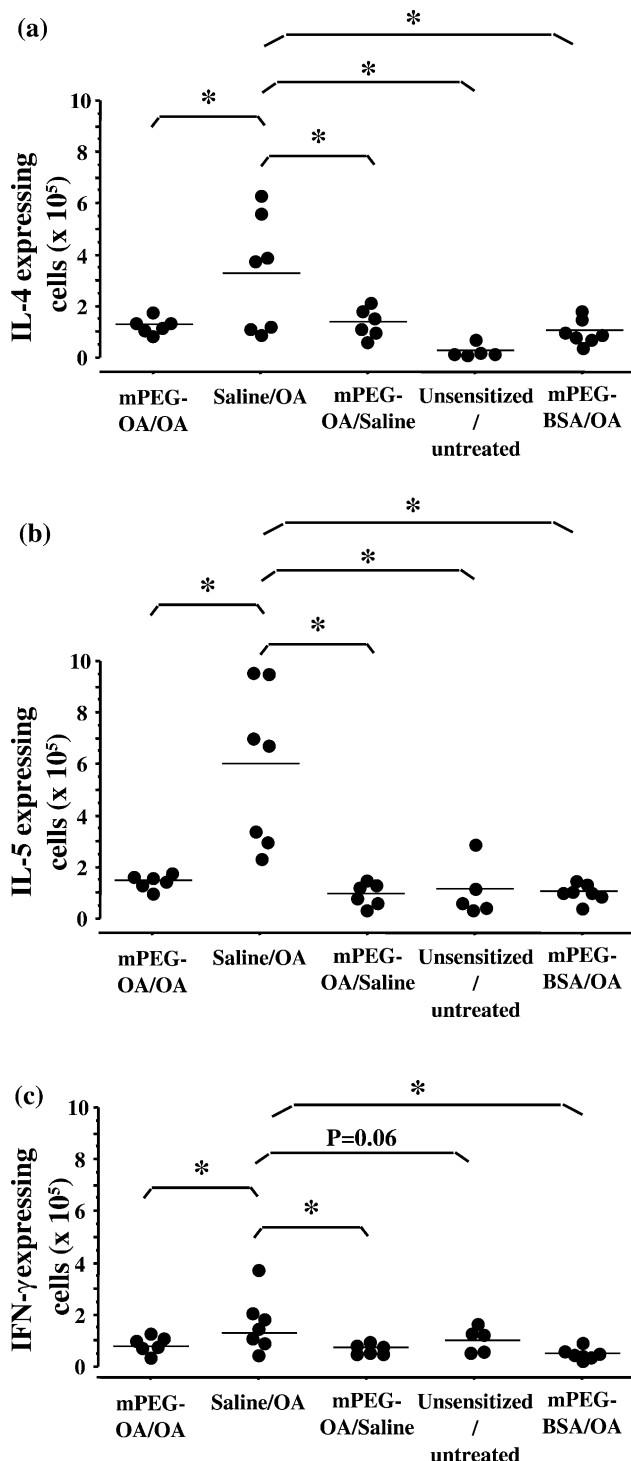


Fig. 6. Quantification of cytokine mRNA expression for interleukin IL-4 (a), IL-5 (b) and IFN- γ (c) in BAL fluid cells by in situ hybridization. The results of the number of total cells expressing each of the cytokines for each animal are shown as well as the mean values (horizontal lines). *Significant differences between groups ($P < 0.05$).

Recently, the important place of the dendritic cells, the professional antigen presenting cells of the airway, in allergic inflammation has been demonstrated [26], enhancing the plausibility of the above mechanism.

The airway inflammation evoked by OA challenge of the sensitized BN rat is similar to that observed in human asthmatic allergic subjects. The eosinophilic airway inflammation is accompanied by an increase in the expression of Th2 cytokines [12,21,27]. The mPEG conjugates to OA or BSA exerted an 'anti-inflammatory' effect, causing an inhibition of both pulmonary eosinophilia and neutrophilia. Infiltration of the airways by these cells is, at least in part, T cell dependent [28,29]. The observed changes in the expression of IL-4 and IL-5 mRNA in BAL cells by mPEG conjugates is in agreement with the finding of suppression of Th2-type response by mPEG conjugated proteins [30] and is suggestive evidence of altered T cell recruitment or activation. Although the cell source of the cytokines was not determined in the current study, previous experiments have confirmed the predominant co-localization of IL-5 to CD3 + cells in the BN rat following OA challenge [31]. The mechanisms involved in eosinophil recruitment have been clearly linked to the Th2 cytokine, IL-5 [32,33] and the reduced expression of this cytokine is a potential explanation for the lesser degree of eosinophilia.

There was clear evidence of an effect on allergic inflammation by mPEG-BSA, as there was inhibition of both Th1 and Th2 cytokines as well as a reduction of BAL leukocytes. Despite this effect, the LAR was not significantly inhibited. These findings indicate a dissociation between eosinophilic inflammation and the LAR which has been previously reported in this model [34]. mPEG conjugated to an unrelated antigen may induce a nonantigen specific attenuation of T cell function, thereby explaining the observed effects of mPEG-BSA on inflammation. While mPEG alone does not alter the immune response [25], a possible explanation may be that mPEG is required to be covalently linked to a protein to abrogate the capacity of antigen presenting cells to appropriately interact and activate T cells. Support for this hypothesis is the finding that pegylation of cells prevent the CD28-CD80 co-stimulatory pathway which is required for the T cell activation by antigen presenting cells [35]. The capacity of mPEG conjugates to reduce T cell function does not appear to be related to T cell mediated toxicity [36].

The anti-inflammatory effect of mPEG-OA conjugates and the inhibitory effects on LARs suggest the possibility that conjugates of this type may have therapeutic potential. Although the OA sensitized rat model provides proof of concept for this therapeutic strategy, the postulation of a plausible mechanism underlying this phenomenon requires further experimentation. Whether the same mechanism is implicated in the inhibition by mPEG-OA conjugates of the LAR and pulmonary eosinophilia remains to be established. The inhibition of inflammation and T cell cytokine expression by mPEG-BSA treatments indicates a non-specific anti-inflammatory response. The dissociation between these effects and the lack of suppression of the LAR indicates that OAmPEG must have had additional

effects so that to inhibit the LAR. The nature of these effects requires clarification. Nevertheless, the current observations support the hypothesis that mPEG conjugates of other more pertinent allergens may prove to be of therapeutic benefit in the treatment of allergic asthma, in particular if the disease is caused predominantly by a single antigen.

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References

- [1] Lee WY, Sehon AH. Abrogation of reaginic antibodies with modified allergens. *Nature* 1977;267:618–9.
- [2] Sehon AH. Suppression of antibody responses by chemically modified antigens. *Int Arch Allergy Appl Immunol* 1991;94:11–20.
- [3] Lee WY, Sehon AH, Åkerblom E. Suppression of reaginic antibodies with modified allergens. IV. Induction of suppressor T cells by conjugates of polyethylene glycol (PEG) and monomethoxy PEG with ovalbumin. *Int Arch Allergy Appl Immunol* 1981;64:100–14.
- [4] Takata M, Maiti PK, Kubo RT, Chen Y, Holford-Strevens V, Rector ES, et al. Cloned suppressor T cells derived from mice tolerized with conjugates of antigen and monomethoxypolyethylene glycol. *J Immunol* 1990;145:2846–53.
- [5] Chen Y, Takata M, Maiti PK, Rector ES, Sehon AH. Characterization of suppressor T cell clones derived from a mouse tolerized with conjugates of ovalbumin and monomethoxypolyethylene glycol. *Cell Immunol* 1992;142:16–27.
- [6] Bitoh S, Takata M, Maiti PK, Holford-Strevens V, Kiersek-Jaszczuk D, Sehon AH. Antigen-specific suppressor factors of noncytotoxic CD8 + suppressor T cells downregulate antibody responses also to unrelated antigens when the latter are presented as covalently linked adducts with the specific antigen. *Cell Immunol* 1993;150:168–93.
- [7] Dreborg S, Åkerblom EB. Immunotherapy with monomethoxypolyethylene glycol modified allergens. *Crit Rev Ther Drug Carrier Syst* 1990;6:315–65.
- [8] Bitoh S, Wakefield R, Lang GM, Sehon AH. Inhibition of the effector phase of IgE-mediated allergies by tolerogenic conjugates of allergens and monomethoxypolyethylene glycol. *Int Arch Allergy Immunol* 1995;107:316–8.
- [9] Lang GM, Bitoh S, Becker AB, Sehon AH. Potential therapeutic efficacy of allergen-monomethoxypolyethylene glycol conjugates for in vivo inactivation of sensitized mast cells responsible for common allergies and asthma. *Int Arch Allergy Immunol* 1997;113:58–60.
- [10] Watanabe A, Mishima H, Renzi PM, Xu LJ, Hamid Q, Martin JG. Transfer of allergic airway responses with antigen-primed CD4 + but not CD8 + T cells in Brown Norway rats. *J Clin Invest* 1995;96:1303–10.
- [11] Haczku A, Macary P, Huang TJ, Tsukagoshi H, Barnes PJ, Kay AB, et al. Adoptive transfer of allergen-specific CD4 + T cells induces airway inflammation and hyperresponsiveness in brown-Norway rats. *Immunology* 1997;91:176–85.
- [12] Renzi PM, al Assaad AS, Yang J, Yasrael Z, Hamid Q. Cytokine expression in the presence or absence of late airway responses after antigen challenge of sensitized rats. *Am J Respir Cell Mol Biol* 1996;15:367–73.
- [13] van der Veen MJ, Van Neerven RJ, De Jong EC, Aalberse RC, Jansen HM, van der Zee JS. The late asthmatic response is associated with baseline allergen-specific proliferative responsiveness of peripheral T lymphocytes in vitro and serum interleukin-5. *Clin Exp Allergy* 1999;29:217–27.
- [14] Turner DJ, Martin JG. Animal models. In: Woolcock AJ, editor. *Asthma*. Philadelphia: Lippincott–Raven; 1997. p. 261–74.
- [15] Del Prete G. Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. *Allergy* 1992;47:450–5.
- [16] Ohkawara Y, Lei X-F, Stämpfli MR, Marshall JS, Xing Z, Jordana M. Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* 1997;16:510–20.
- [17] Turner DJ, Myron P, Powell WS, Martin JG. The role of endogenous corticosterone in the late-phase response to allergen challenge in the Brown Norway rat. *Am J Respir Crit Care Med* 1996;153:545–50.
- [18] Marwaha RK, Jonhson BF, Wright GE. Simple stability-indicating assay for histamine solutions. *Am J Hosp Pharmacy* 1985;42:1568–71.
- [19] Hojo M, Maghni K, Issekutz TB, Martin JG. Involvement of alpha-4 integrins in allergic airway responses and mast cell degranulation in vivo. *Am J Respir Crit Care Med* 1998;158:1127–33.
- [20] Kay AB, Ying S, Varney V, Gage M, Durham SR, Moqbel R, et al. Messenger mRNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5 and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous response in atopic subjects. *J Exp Med* 1991;173:775–8.
- [21] Watanabe A, Mishima H, Kotsimbos TC, Hojo M, Renzi PM, Martin JG, et al. Adoptively transferred late allergic airway responses are associated with Th2-type cytokines in the rat. *Am J Respir Cell Mol Biol* 1997;16:69–74.
- [22] Sehon AH, Lee WY. Specific downregulation of IgE antibodies by tolerogenic conjugates of haptens and allergens with synthetic polymers. *Int Arch Allergy Appl Immunol* 1981;66(Suppl 1):39–42.
- [23] Olivenstein R, Renzi PM, Yang JP, Rossi P, Laberge S, Wasserman S, et al. Depletion of OX-8 lymphocytes from the blood and airways using monoclonal antibodies enhances the late airway response in rats. *J Clin Invest* 1993;92:1477–82.
- [24] Holford-Strevens V, Jackson C-JC, Charlton J, Akiyama KA, Lang GM, Carter BG, et al. Induction of in vivo helper activity for murine antibody responses by macrophages pulsed with ovalbumin–monomethoxypolyethylene glycol (OA–mPEG) conjugates. *Cell Immunol* 1987;104:245–54.
- [25] So T, Ito HO, Koga T, Ueda T, Imoto T. Reduced immunogenicity of monomethoxypolyethylene glycol-modified lysozyme for activation of T cells. *Immunol Lett* 1996;49:91–7.
- [26] Stumbles PA, Thomas JA, Pimm CL, Lee PT, Venaille TJ, Proksch S, et al. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. *J Exp Med* 1998;188:2019–31.
- [27] Robinson DS, Ying S, Bentley AM, Meng Q, North J, Durham SR, et al. Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *J Allergy Clin Immunol* 1993;397–403.
- [28] Robinson DS, Hamid Q, Ying S, Bentley AM, Assoufi B, Durham SR, et al. Prednisolone treatment in asthma is associated with modulation of bronchoalveolar lavage cell interleukin-4, interleukin-5, and interferon-gamma cytokine gene expression. *Am Rev Respir Dis* 1993;148:401–6.
- [29] Renzi PM, Sapienza S, Wasserman S, Du T, Olivenstein R, Wang NS, et al. Effect of interleukin-2 on the airway response to antigen in the rat. *Am Rev Respir Dis* 1992;146:163–9.
- [30] Ito HO, So T, Hirata M, Koga T, Ueda T, Imoto T. Tolerogenic activity of polyethylene glycol-conjugated lysozyme distinct from that of the native counterpart. *Immunology* 1998;93:200–7.
- [31] Ihaku D, Suzuki M, Martin JG, Hamid QA. Montelukast, a selective leukotriene antagonist, inhibits antigen-induced bronchoconstriction in Brown Norway rats. *J Allergy Clin Immunol* 1999;103:1147–54.

- [32] Eidelman DH, Minshall E, Dandurand RJ, Schotman E, Song YL, Yasrael Z, et al. Evidence for major basic protein immunoreactivity and interleukin 5 gene activation during the late phase response in explanted airways. *Am J Respir Cell Mol Biol* 1996;15:582–9.
- [33] Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airway hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996;183:195–201.
- [34] Suzuki M, Maghni K, Molet S, Shimbara A, Hamid QA, Martin JG. Interferon-gamma secretion by CD8 + T cells inhibits allergen-induced airway eosinophilia but not late airway responses. *J Allergy Clin Immunol* 2002;109(5):803–9.
- [35] Murad KL, Gosselin EJ, Eaton JW, Scott MD. Stealth cells: prevention of major histocompatibility complex class II-mediated T-cell activation by cell surface modification. *Blood* 1999;94: 2135–41.
- [36] Chen AM, Scott MD. Current and future applications of immunological attenuation via pegylation of cells and tissue. *BioDrugs* 2001; 15:833–47.