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A comparison of allergen and polycation induced cutaneous responses in the rabbit

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1 Allergic inflammatory responses contribute to the symptoms of a number of diseases including atopic dermatitis, asthma and rhinitis. Cationic proteins are released from inflammatory cells and levels are known to be raised in disease states.

2 Using an *in vivo* model of acute inflammation, we investigated the characteristics of cutaneous responses to antigen (*Alternaria tenuis*, AT) and poly-L-lysine (PLL, used as a paradigm for cationic proteins). We aimed to compare the inflammatory profile of cationic polypeptides and the allergic response and to identify similarities and differences between these responses.

3 Responses to intradermal injection of the polycation, PLL and antigen were compared using radiolabelled protein (^{125}I-bovine serum albumin, BSA) and cells (^{111}In-neutrophils, PMN) to study plasma exudation (PE) and PMN accumulation (PMNA) in the skin of AT sensitized rabbits.

4 Both PLL and antigen caused dose-related increases in PE and PMNA. PE (and PMNA) responses to PLL were prolonged (up to 3 h), as were those to antigen. This is in contrast to PE responses to fMLP which were maximal at 45 min.

5 In immunized animals, treated with colchicine (1 mg kg^{-1} i.v.), PE responses to the directly acting mediator, bradykinin (BK), were not affected, whereas PE responses to the neutrophil dependent mediator, f-met-leu-phe (fMLP), were significantly ($P<0.01$) reduced. Antigen-induced PE responses were significantly (50, 500 ($P<0.05$); 200 ($P<0.01$) p.n.u. site^{-1}) inhibited by colchicine, but PLL-induced responses were not significantly affected.

6 We conclude that although PLL-induced responses had a similar time course to those of antigen, some differences were observed between responses, which indicate that although polycations may contribute to allergic responses, these two responses are produced by distinct mechanisms.
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Keywords: Antigen; polycation; plasma exudation; leukocyte accumulation; colchicine

Abbreviations: ACA, active cutaneous anaphylaxis; AHR, airway hyperresponsiveness; AT, *Alternaria tenuis*; BALF, bronchoalveolar lavage fluid; BSA, bovine serum albumin; ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; EPO, eosinophil peroxidase; fMLP, f-methionyl-leucyl-phenylalanine; HA, histamine; ^{125}I, ^{125}Iodine; ^{111}In, ^{111}Indium; ICAM-1, intercellular cell adhesion molecule-1; Ig, immunoglobulin; MBP, major basic protein; PCA, passive cutaneous anaphylaxis; PE, plasma exudation; PG, prostaglandin; PLL, poly-L-lysine; PMNA, polymorphonuclear leukocyte accumulation.

Introduction

The major characteristics of inflammation are redness, swelling, pain, heat and, sometimes, loss of function. Of these, two key factors are polymorphonuclear leukocyte (PMN) extravasation and associated plasma exudation (PE). Neutrophils are the body's first line of defence against invading microorganisms. Recruited by chemotaxis, they engulf pathogens *via* a phagocytotic process and digest them using enzymes and antimicrobial proteins which are exocytosed either into the phagocytotic vacuole or the surrounding medium. Of neutrophilic granules, primary granules contain cationic polypeptides and proteins such as bactericidal permeability increasing protein, myeloperoxidase, proteases and defensins. Secondary granules contain soluble proteins such as lactoferrin.

Allergic inflammatory responses are the cause of the symptoms of many types of disease including atopic dermatitis and some exacerbations of asthma and rhinitis. It is well documented that the eosinophil is of prime importance in allergic responses (Gleich, 1990), but the role of the neutrophil is less well defined. However, neutrophils have been shown to be present in immunoglobulin (Ig) E mediated reactions in the nose (Bascom *et al*., 1988), in the skin (Charlesworth *et al*., 1991) and the lung (Dupuis *et al*., 1992). In particular, neutrophils have been shown to be the predominant infiltrating cell in the airspaces of tissues from patients who died from sudden onset fatal asthma (Sur *et al*., 1993).

A number of cationic proteins are released from cells in inflammatory disease and stress and it is now widely accepted that such cationic proteins contribute to the pathogenesis of these diseases. There are four main cationic proteins released from eosinophils, namely, major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPO) (Gleich & Adolphson, 1986). ECP levels are raised in allergic rhinitis

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(Fergusson *et al*., 1995) and cystic fibrosis (Robinson *et al*., 1995). MBP is known to elicit altered airway responses in experimental animals (Coyle *et al*., 1993) and has been implicated in the induction of airways hyperresponsiveness (AHR), as exposure to MBP or synthetic polycations causes damage to the airway epithelium (Motojima *et al*., 1989; Frigas *et al*., 1980). Also, MBP causes upregulation of intercellular cell adhesion molecule-1 (ICAM-1) on cultured epithelial cells (Altman *et al*., 1993) and can cause further recruitment of eosinophils through causing their degranulation and release of the chemokine, interleukin (IL)-8 (Kita *et al*., 1995). Further support is derived from experiments showing AHR to be correlated with peripheral blood eosinophilia in patients with atopic or non atopic asthma (Gleich & Adolphson, 1998) and with the percentage of eosinophils in bronchoalveolar lavage fluid (BALF) from patients with bronchial asthma (Wardlaw *et al*., 1988). Induction of eosinophilia by inhalation of *Ascaris suum* antigen by cynomolgus monkeys, resulted in a correlation of eosinophil numbers and MBP levels in BALF with methacholine PC_{100} values. Also, MBP and EPO have been shown to cause an increase in respiratory resistance in cynomolgus monkeys although only MBP caused AHR (Gundel *et al*., 1991). Coyle *et al*. (1994), using PLL to mimick the effects of MBP, found that activation of C fibres was largely involved in this response. Furthermore, it has been suggested that bradykinin (BK) plays a role in MBP- or poly-L-lysine-induced AHR (Coyle *et al*., 1995). Eosinophils localize around nerves (Costello *et al*., 1997) and, thus, MBP is released at nerve endings. MBP has been shown to be an allosteric antagonist at the M_2 receptor (Jacoby *et al*., 1993) and therefore increases vagal responsiveness through inhibition of the effect of acetylcholine on prejunctional M_2 receptors. Heparin, which binds MBP, has been shown to restore M_2 receptor function (Fryer & Jacoby, 1992). In addition, an antibody to guinea-pig MBP prevented M_2 receptor dysfunction and AHR (Lefort *et al*., 1996; Evans *et al*., 1997).

In vivo, synthetic polyamino acids cause increased permeability and leukocyte infiltration when injected either intraperitoneally or intradermally in rats (Stein *et al*., 1956). PLL has been reported to cause release of prostacyclin and cytoplasmic purine from pig aorta and human umbilical vein endothelial cells and, in addition, local oedema formation after intradermal injection in the rabbit (Needham *et al*., 1988). One possible mechanism by which polycations effect increases in permeability is through electrostatic interaction with anionic sites on the luminal surface of vascular endothelium. Most of these anionic sites are sulphated glycoproteins and glycosaminoglycans (Simionescu *et al*., 1981; Chang & Voelkel, 1991). Upon interaction of polycations with endothelial cells these anionic sites cluster together. This process, leaving much of the cell surface without a negatively charged barrier, may cause the increase in permeability (Pelikan *et al*., 1979; Skutelsky & Danon, 1976) and contribute to inflammatory cell recruitment. It has been shown that polycations, such as poly-L-arginine and poly-L-lysine produce PE in rabbit skin (Needham *et al*., 1988) and oedema and neutrophil (PMN) infiltration in rat lung (Santana *et al*., 1993). Furthermore, the PLL-induced PE response was shown to persist for at least 2 h in rabbit skin (Sasaki *et al*., 1991).

Increased vascular permeability and inflammatory cell infiltration are also known to be associated with Type 1 hypersensitivity reactions and such changes have been seen in both the skin and the lungs of allergic animals (Behrens *et al*., 1987). *Alternaria tenuis* (AT) is a saprophytic mould, which has been shown to be a potent inducer of immediate and late cutaneous and airways responses in humans (Umemoto *et al*., 1976; Cockcroft *et al*., 1979). We have used an animal model of allergic skin responses where animals are immunized to *Alternaria tenuis* extract since Behrens *et al*. (1987) reported that, 30 min after intradermal injection of *Alternaria tenuis* extract, profound inflammatory changes were observed.

In the present study, we have compared the inflammatory responses induced by an extract of *Alternaria tenuis* with those produced by a synthetic polycation, used as a model of polycations released by activated granulocytes, as measured by ^{111}Indium-labelled PMN leukocyte accumulation (^{111}In-PMNA) and ^{125}Iodine-labelled bovine serum albumin (^{125}I-BSA) exudation in rabbit skin. In addition, we have included cutaneous PE responses to formyl methionyl leucyl phenylalanine (fMLP) and leukotriene (LT) B_4 which are dependent on circulating neutrophils (Hellewell *et al*., 1989) and histamine (HA) and bradykinin (BK) which elicit plasma leakage by a direct action on endothelial cells via H_1 (Asako *et al*., 1994) or B_2 (Whalley, 1987) receptors respectively. fMLP, LTB_4, HA and BK were injected as a mixture with prostaglandin (PG) E_2, in accordance with the 'two mediator hypothesis' (Williams & Peck, 1977; Williams *et al*., 1983), which describes the quantity of plasma protein leakage to be dependent on both the extent of venular permeability and the magnitude of arteriolar dilatation (provided by the vasodilator prostaglandin). Colchicine treatment has also been used to assess the neutrophil dependency of the PE response to *Alternaria tenuis* in comparison to that produced by PLL, with PE responses to fMLP and BK as neutrophil dependent and independent controls.

Methods

Animals

All experiments were carried out on New Zealand White (NZW) rabbits. Animals were fed a normal diet and received water *ad libitum*. Blood for isolation of neutrophils was taken from males weighing 3.0–3.5 kg. Skin tests were carried out in rabbits within the weight range of 2.3–3.5 kg. In experiments on naïve rabbits, all those used were male. Sensitized rabbits were used litter by litter, thus both males and females were used in these experiments.

Sensitization protocol

The immunization of NZW rabbits was carried out over a thirteen week period using a protocol similar to that reported by Shampain *et al*. (1982). Rabbits were injected intraperitonally on the day of birth with 0.5 ml of a mixture of *Alternaria tenuis* extract (40,000 PNU ml^{-1}) and aluminium hydroxide ($Al(OH)_3$) moist gel diluted 1 : 1 with 0.9% sterile saline, in the ratio 2 : 1 : 1 by volume. Intraperitoneal allergen injection was repeated weekly for the first month and then bi-weekly for the next two months.

Isolation of rabbit neutrophils

New Zealand White (NZW) rabbits (2.3–3.0 kg) were anaesthetized with 30 mg kg^{-1} i.v. sodium pentobarbitone, the neck shaved and 3 ml lignocaine dihydrochloride (Lignavet) injected subcutaneously. The carotid artery was exposed and cannulated and blood was collected into 8 ml of acid citrate dextrose (ACD; a filtered solution of citric acid, D-glucose and tri-sodium citrate) in sterile 50 ml tubes. 15 ml of whole blood was taken into ACD and centrifuged at 1550 *g* (×2) to obtain platelet poor plasma (PPP). The remainder (~110 ml) was mixed in a 1:1 ratio with 6% hydroxyethylcellulose (Hespan) and left to sediment for 30–45 min.

The leukocyte rich plasma was removed and centrifuged (400 *g*, 10 min, 19°C). Any red blood cells remaining in the pellet were removed by hypotonic lysis (using lysing buffer consisting of a filtered solution of 1 mM trizma base and 2 mM EDTA). Osmolality was resumed after 20 s by addition of 10×HANKS solution. 15 ml Ficoll was placed underneath the suspension and the tubes centrifuged again (500 g, 20 min, 19°C). The pellets were combined and resuspended in 2 ml PPP, and 10 μl taken for counting.

Viability of PMN preparation

Ten μl of the neutrophil suspension was diluted with 240 μl HANKS solution and 250 μl Trypan blue solution and the number of viable and activated/dead neutrophils was evaluated microscopically under×40 magnification. The number of viable neutrophils per ml of suspension was calculated as a percentage of the total (viable+activated neutrophils) number of cells. Cell samples were >95% viable.

Purity of PMN preparation

One drop of the neutrophil suspension was removed using a Pasteur pipette, and diluted with 9 drops of 0.9% sterile saline and 5 drops of this suspension were cytospun onto microscope slides at 1300 r.p.m. for 1 min. The slides were removed, air dried and then stained using the haematoxylin/eosin method. Differential cell counts were carried out for four fields of view per slide, and the number of neutrophils expressed as a percentage of the total cell count. The neutrophil samples were >95% pure.

Labelling of isolated rabbit neutrophils

Mercaptopyridine was added to the cell suspension to give a final concentration of 10 μg ml^{-1}. The cells were incubated with the mercaptopyridine for 5 min followed by addition of 11.1 MBq ^{111}Indium chloride (^{111}In) for 30 min. At the end of this period, 3×10 μl samples were removed for gamma counting to obtain the total radioactivity. The cells were washed to remove excess Indium and after the final wash, were resuspended in 2 ml PPP, and 10 μl removed for cell counting. Neutrophils were injected at a concentration of between 0.7 and 1×10^7 cells kg^{-1}, depending on the number of cells available and the size of the recipient rabbits.

Preparation of agents for injection

All agents were diluted to the desired concentration in 0.9% sterile, pyrogen free saline immediately before use. FMLP, LTB_4, histamine and bradykinin were mixed with the vasodilator prostaglandin, PGE_2, as described by the two mediator hypothesis (Williams & Peck, 1977; Williams *et al*., 1983). A dose response curve to PLL was constructed and, in subsequent experiments, PLL was used at a dose of 100 μg $site^{-1}$. It was not necessary to mix PLL with PGE_2 because at this dose, addition of this prostanoid did not potentiate this response. Antigen was used at a dose of 200 p.n.u. $site^{-1}$ (also determined from a dose response curve) and was not mixed with PGE_2.

Skin tests

Recipient NZW rabbits (2.3–3.0 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and the dorsal and lateral fur shaved at least 1 h before intradermal injections. The neutrophil suspension was divided in order that each animal received the same concentration per kg. The labelled cells were injected and, 1 h later, 0.18 MBq of ^{125}I BSA mixed with ~2 ml (25 mg ml^{-1} in 0.9% sterile NaCl) Evans Blue solution was injected intravenously into each animal. Mediators were injected into marked sites on the skin (0.1 ml $site^{-1}$) using 27G needles according to a block design which allowed for inter site variation. Saline controls were included in each experiment.

Pulse experiments

Animals were prepared for skin tests as described above. Intradermal injections were made 2.5, 1.5 and 0.5 h before intravenous administration of ^{125}I-BSA. A further set of intradermal injections were performed immediately after this i.v. injection. Accumulation of ^{125}I-BSA was measured over a 30 min period. At the end of this time, animals were then sacrificed by sodium pentobarbitone overdose.

Preparation of blood and plasma samples

At the end of the allocated time period animals were anaesthetized with a further i.v. injection of 30 mg kg^{-1} pentobarbitone, 10 ml blood collected by cardiac puncture into heparinized tubes and the animals then sacrificed by sodium pentobarbitone overdose.

Preparation of skin samples

The skin was removed and the treatment sites excised with a 17 mm diameter punch. Skin sites were counted along with cell, blood and plasma samples in an automated gamma spectrometer (LKB, Wallac 1282 Compugamma, CS) with windows set for ^{111}In and ^{125}I. Correction for the spillover (2%) of radioisotope emissions from the ^{111}In channel to the ^{125}I channel was performed on the data.

Calculation of results

PE was expressed as equivalent μl plasma $site^{-1}$ as calculated by skin sample ^{125}I counts / ^{125}I counts in 1 μl plasma.

Neutrophil accumulation was expressed as the number of cells site^{-1} as calculated by corrected ^{111}In counts in skin site / ^{111}In counts per neutrophil. Blood and plasma samples were also used to determine the level of free ^{111}In in plasma, which was below 15% in all cases, this value being used to correct for plasma ^{111}In in skin sites.

Statistical analysis

All data are presented as mean ± s.e.mean for the indicated number of experiments. Data were analysed by two-way analysis of variance (ANOVA) and significant differences were determined by *t*-test.

Reagents

Poly-L-lysine hydrobromide (150–300 kD), LTB_4, fMLP, PGE_2, Evans Blue dye, sodium pentobarbitone, Ficoll Histopaque-1077, sterile water, 10 × HANKS solution, mercaptopyridine and sodium pentobarbitone were purchased from Sigma Chemicals, Poole, Dorset, UK. Hespan (6% hydroxyethylcellulose) was purchased from Dupont Pharmaceuticals, Letchworth, Herts. U.K. Lignavet (lignocaine hydrochloride) was purchased from Veterinary Sciences, U.K. Sterile saline was purchased from Baxter Healthcare Ltd., Thetford, Norfolk, U.K. ^{111}Indium chloride, was purchased from Amersham International plc, Bucks., U.K. ^{125}I-bovine serum albumin was purchased from ICN Pharmaceuticals, Thame, UK.

Results

Neutrophil preparation

Neutrophils were isolated with a purity of 97.6 ± 0.2% and a viability, after labelling, of 98.2 ± 0.05% (data from 28 experiments). From 19 experiments in pentobarbitone anaesthetized animals, the percentage of ^{111}In free in plasma 2 h after injection of ^{111}In-PMNs was determined to be 5.3 ± 0.5%, with a range of 2.6–11.2%.

Responses to directly acting and neutrophil dependent inducers of PE

Intradermal injection of fMLP (5×10^{-11} moles site^{-1}), HA (4.5×10^{-9} moles site^{-1}), LTB_4 (5×10^{-10} moles site^{-1}) or BK (4.7×10^{-10} moles site^{-1}), all mixed with PGE_2 (3×10^{-10} moles site^{-1}), caused significant increases in PE ($P<0.001$, $P<0.001$, $P<0.01$, $P<0.001$, respectively) compared to that caused by PGE_2 alone (Figure 1(a)). Figure 1(b) shows that only fMLP ($P<0.001$) and LTB_4 ($P<0.01$) caused significant increases in PMNA which were approximately 40 fold compared to accumulation in PGE_2 injected sites.

Dose response effects of poly-L-lysine (PLL)

Intradermal injection of PLL caused dose-related PE and PMNA over a 3 h period (Figure 2), which was significantly greater than that in saline-injected sites at 30 ($P<0.05$) and 100 ($P<0.001$) μg site^{-1}.

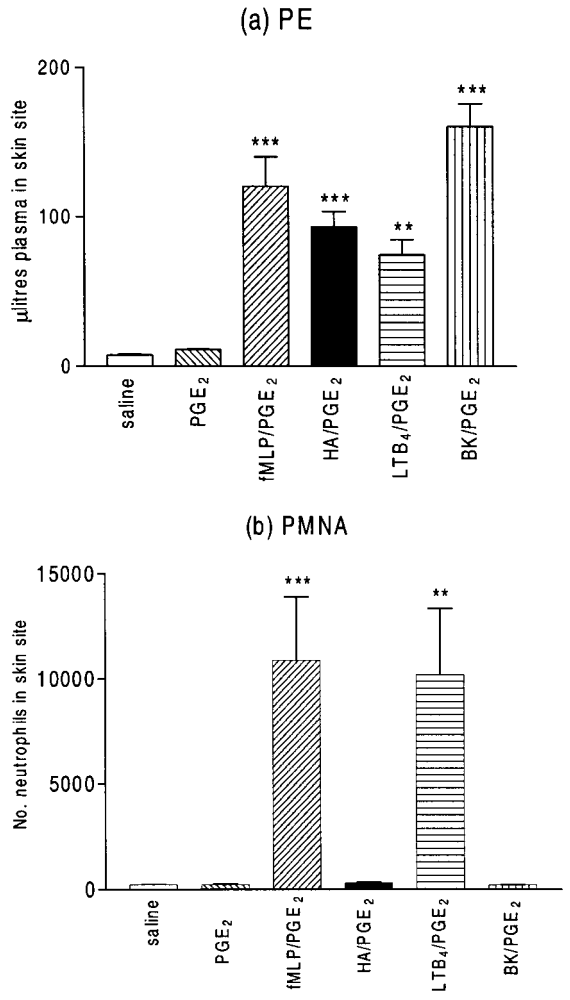


Figure 1 (a) Plasma exudation (PE) and (b) ^{111}In-labelled PMN accumulation (PMNA) in rabbit skin sites, to a range of stimuli, namely; the directly acting mediators histamine (HA, 4.5×10^{-9} moles site^{-1}) and bradykinin (BK, 4.7×10^{-10} moles site^{-1}) and the neutrophil dependent mediators f-met-leu-phe (fMLP, 5×10^{-11} moles site^{-1}) and leukotriene B_4 (LTB_4, 5×10^{-10} moles site^{-1}) all mixed with the vasodilator prostaglndin E_2 (PGE_2, 3×10^{-10} moles site^{-1}) measured at 45 min. Mean ± s.e.mean values ($n=5$) are shown $^{**}P<0.01$, $^{***}P<0.001$.

Dose response effects of antigen

I.d. injection of the antigenic extract *Aternaria tenuis* (AT) also caused dose-related increases in PE and PMNA over a 3 h period (Figure 3). PE responses were significantly increased compared to saline injected sites at doses of 100 ($P<0.01$) and 500 ($P<0.001$) p.n.u. site^{-1}. PMNA was also significantly increased at these doses ($P<0.05$, $P<0.001$).

Time course of poly-L-lysine-induced responses

The time courses of PE and PMNA, in response to injection of PLL, are shown in Figure 4. PE is noticeable at 30 min after injection and is significantly greater than saline control at all post-injection times ($P<0.001$). PMNA, however, lags behind PE, being significantly higher than the saline control only at 2 ($P<0.01$) and 3 ($P<0.001$) h post injection. This

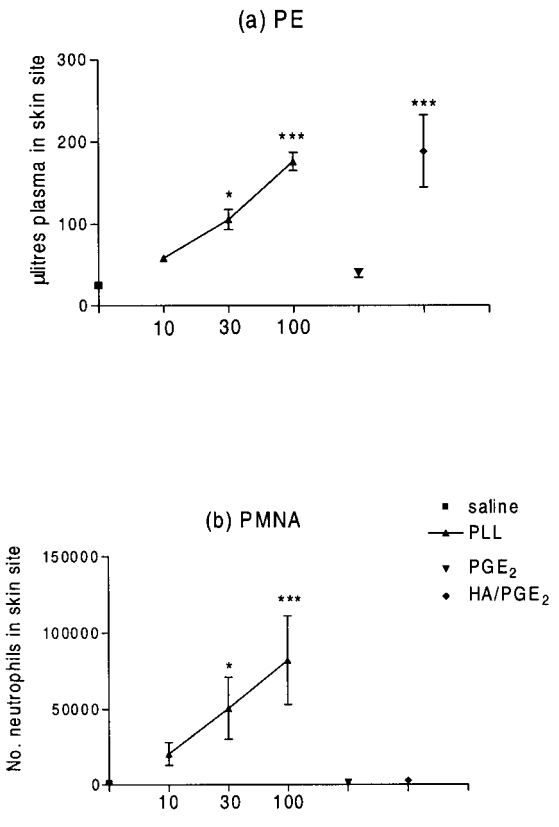


Figure 2 (a) Plasma exudation (PE) and (b) ^{111}In-labelled PMN accumulation (PMNA) in rabbit skin sites measured at 3 h. Dose-response effect of poly-L-lysine (PLL, 10–100 μg site^{-1}). Responses to histamine (HA, 4.5×10^{-9} moles site^{-1}) mixed with prostaglandin E_2 (PGE_2, 3×10^{-10} moles site^{-1}) and PGE_2 alone (3×10^{-10} moles site^{-1}) are shown for comparison. Mean$\pm$s.e.mean values ($n=4$) are shown. *$P<0.05$, ***$P<0.001$.

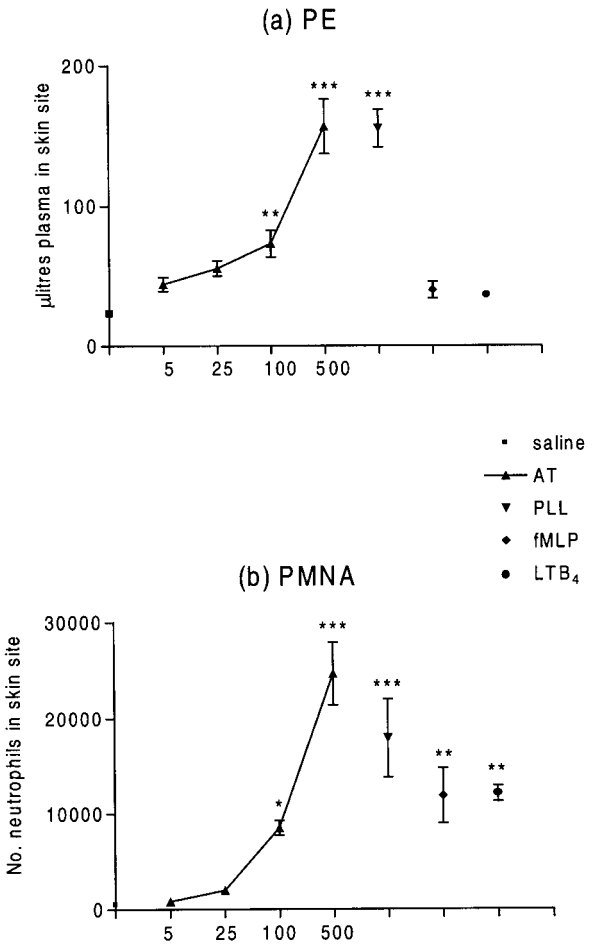


Figure 3 (a) Plasma exudation (PE) and (b) ^{111}In-labelled PMN accumulation (PMNA) in rabbit skin sites measured at 3 h. Dose-response effect of antigen (extract of *Alternaria tenuis*; 5–500 p.n.u. site^{-1}). Poly-L-lysine (PLL, 100 μg site^{-1}), f-met-leu-phe (fMLP, 10^{-11} moles site^{-1}) and leukotriene B_4 (LTB_4, 10^{-10} moles site^{-1}) are shown for comparison. Mean$\pm$s.e.mean values ($n=5$) are shown. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

figure also shows that mixture of PLL with PGE_2 does not potentiate its ability to cause PE and PMNA.

Pulse experiments – PLL-induced PE

The PE elicited by PLL in the time intervals 0–0.5, 0.5–1.0, 1.5–2.0 ($P<0.01$) and 2.5–3.0 ($P<0.05$) h following intradermal injection were all significantly greater than that in saline sites at the same time intervals (Figure 5).

Time course of Alternaria tenuis- *and fMLP-induced responses*

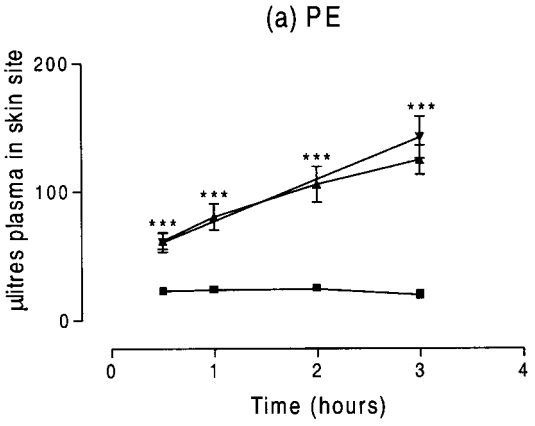
The time courses of responses to *Alternaria tenuis* were measured, along with those for fMLP, in the presence and absence of PGE_2 (Figure 6). PE responses to fMLP/PGE_2 were maximal at 45 min and were significantly greater than that in corresponding saline injected sites at all time points ($P<0.001$), but the response to *Alternaria tenuis*, although rapid in onset, was still increasing at 3 h (and was significantly greater than PE in saline injected sites at 90 and 180 min ($P<0.001$). PMNA induced by fMLP (in the presence of PGE_2) was also still increasing at 3 h and was significantly greater than that in saline injected sites all time points ($P<0.001$). Also, the increasing PMNA response to fMLP in the absence of PGE_2 persisted and was significantly greater than corresponding saline controls at 180 min ($P<0.001$).

Pulse – Alternaria tenuis-*induced PE*

The PE elicited by *Alternaria tenuis* in the time intervals 0.5–1.0, 1.5–2.0 and 2.5–3.0 h following intradermal injection were all significantly greater (0.5–1.0 h $P<0.01$, 1.5–2.0 and 2.5–3.0 h $P<0.05$) than that in saline sites (Figure 7).

Effect of i.v. pretreatment with colchicine on PE responses

Figure 8 shows that the PMN-dependent PE induced by fMLP is significantly ($P<0.01$) decreased when neutrophils are inactivated, whereas responses to BK are unaffected by this treatment. PE induced by *Alternaria tenuis* extract was partially reduced by colchicine treatment (50 p.n.u. site^{-1}, $P<0.05$, 200 p.n.u. site^{-1}, $P<0.01$), whereas the equivalent response to PLL was not significantly affected.



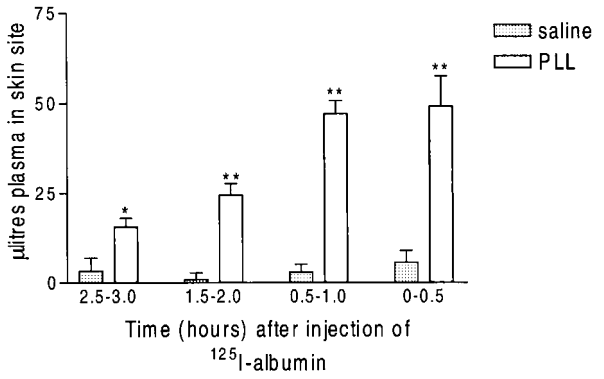


Figure 5 Plasma exudation (PE) responses induced by i.d. injection of poly-L-lysine (PLL, 100 μg site^{-1}) at different time intervals. PE is measured as accumulation of i.v. injection of ^{125}Ialbumin, over 30 min. Values have been corrected by subtracting for appropriate uninjected values. Mean$\pm$s.e.m. values ($n=5$) are shown. *$P<0.05$, **$P<0.01$.

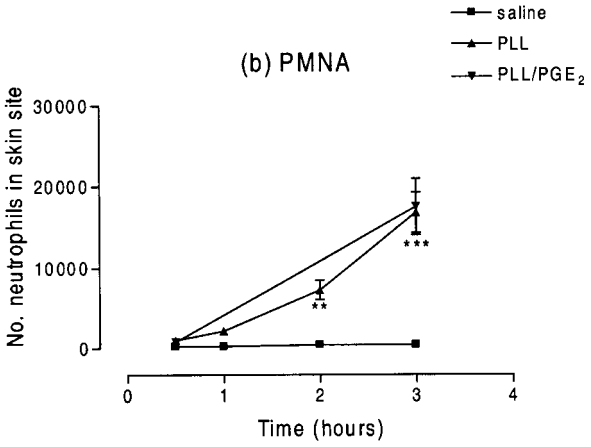
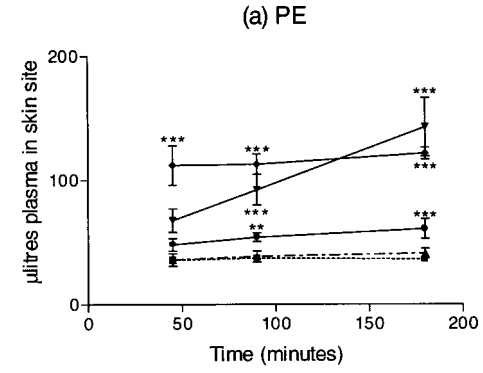


Figure 4 (a) Plasma exudation (PE) and (b) ^{111}In-labelled PMN accumulation (PMNA) in rabbit skin sites measured at 3 h. Time-course for poly-L-lysine-induced responses (PLL, MW 300,000, 100 μg site^{-1}). Responses for PLL mixed with prostaglandin E_2 (PGE_2, 3×10^{-10} moles site^{-1}) are shown for 0.5 h and 3 h time points after injection of ^{111}In-PMNs/ ^{125}I-BSA. Saline controls are also included. Mean$\pm$s.e.mean values ($n=5$) are shown. **$P<0.01$, ***$P<0.001$.



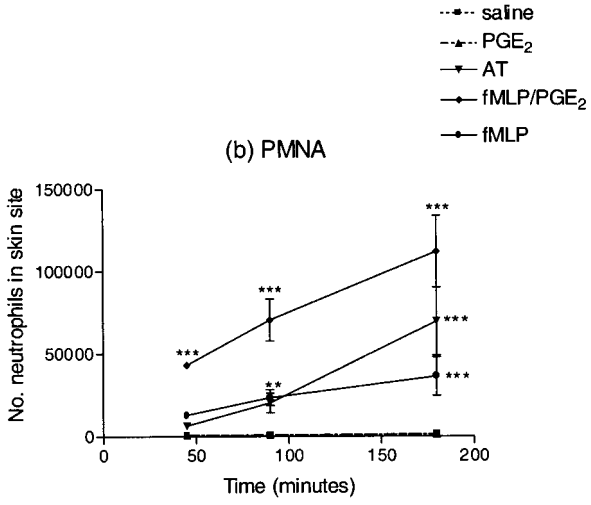


Figure 6 (a) Plasma exudation (PE) and (b) ^{111}In-labelled PMN accumulation (PMNA) in rabbit skin sites measured at 3 h. Time course for antigen-induced responses (AT, 200 p.n.u. site^{-1}) and f-met-leu-phe (fMLP, 10^{-11} moles site^{-1}) with and without PGE_2 (3×10^{-10} moles site^{-1}). Saline and PGE_2 (3×10^{-10} moles site^{-1}) are shown for comparison. Mean$\pm$s.e.mean values are shown (AT, $n=7$; fMLP, without PGE_2 $n=4$, with PGE_2 $n=3$). **$P<0.01$, ***$P<0.001$.

Discussion

We have compared plasma exudation and PMN accumulation in response to intradermal injection of poly-L-lysine (used as a paradigm for the polycationic proteins released from activated leukocytes) and antigen.

Histological examination of tissues has shown that both neutrophils and eosinophils contribute to the cutaneous late phase reaction in response to intradermal injection of allergen or anti-IgE (Dolovich *et al*., 1973; Solley *et al*., 1976). Of direct relevance to the studies described in this paper, Behrens *et al*. (1987) examined cutaneous histopathology in *Alternaria tenuis* sensitized rabbits at 1/2, 6, 24 and 48 h after a single antigen challenge. They reported the presence of interstitial oedema and vessel dilatation at 30 min after injection, with a moderate mixed leukocyte infiltrate at 6 h with residual oedema. At 24 and 48 h there was a mixed

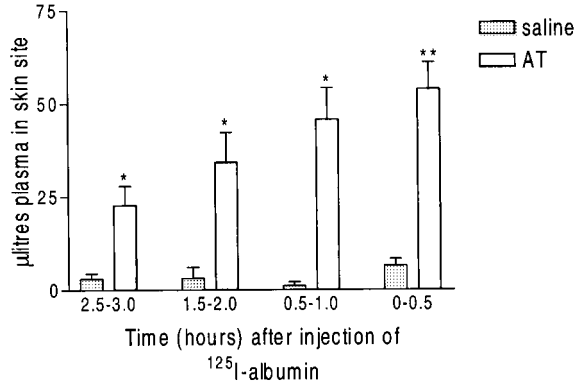


Figure 7 Plasma exudation (PE) responses induced by i.d. injection of antigen (AT, 200 p.n.u. site^{-1}) at different time intervals. PE is measured as accumulation of i.v. injection of ^{125}I-albumin, over 30 min. Values have been corrected by subtracting for appropriate blank values. Mean±s.e.mean values ($n=4$) are shown. $^{*}P<0.05$, $^{**}P<0.01$.

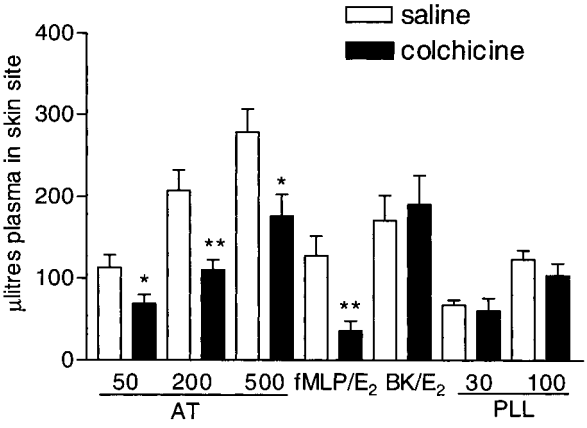


Figure 8 Effect of a 10 min intravenous preinjection of colchicine (1 mg kg^{1}) on plasma exudation responses in rabbit skin to antigen (AT 50–250 p.n.u. site^{-1}), poly-L-lysine (PLL, 30–100 μg site^{-1}) and also, f-met-leu-phe (fMLP, 10^{-11} mol kg^{-1}) and bradykinin (BK, 4.7×10^{-10} moles site^{-1}), both mixed with PGE_2 (3×10^{-10} moles site^{-1}) measured at 3 h. Mean±s.e.mean values ($n=5$) are shown. $^{*}P<0.05$, $^{**}P<0.01$.

cellular infiltrate and little or no oedema. Measurements of antigen-induced ^{111}In-PMN and ^{125}I-BSA accumulation over 3 h, in this model, reflect this histological assessment made by Behrens *et al.* (1987). With respect to substances released from activated leukocytes, Fujisawa *et al.* (1990) reported neutrophil infiltration and extracellular elastase deposition at 1 h and 8 h after challenge, in addition to eosinophils and MBP deposition, suggesting that both neutrophils and eosinophils play a role in cutaneous allergic inflammation. For example, antibodies to MBP have been used to determine the presence of degranulated eosinophils in tissues from patients with atopic dermatitis (Leiferman *et al.*, 1985) and urticaria (Peters *et al.*, 1983). In addition, these proteins are known to cause direct damage to the cells with which they come into contact (Gleich *et al.*, 1979). It is of interest here to note results published by Sur *et al.* (1998) who, through the use of indirect immunofluorescence, showed that neutrophils contain substances which are either identical to, or cross react with EDN and ECP, indicating that neutrophils may cause damage in the way that eosinophils are known to. However, the authors reported that MBP was not detected in neutrophils.

Intradermal injection of the cationic polypeptide, PLL, has been shown previously to elicit a PE response in rabbit skin, and heparin has been shown to inhibit this response (Sasaki *et al.*, 1991). The results presented here demonstrate that, as well as producing PE responses, which were significantly greater than saline controls at all time points in the 3 h experiment, PLL induced PMN accumulation at 2 and 3 h post injection which was significantly greater than that in corresponding saline-injected sites. PLL-induced PMN accumulation lagged behind the PE, indicating that the mechanism of action by which polycations induce inflammatory responses is different to that by which fMLP and LTB_4 do so as, upon injection of these mediators, PMN accumulation parallels PE.

Needham *et al.* (1988) reported that, at low doses, PLL-induced responses were potentiated by PGE_2 but, as the dose of PLL was increased, the potentiating effect of PGE_2 was reduced. They concluded that, at increased doses, PLL, as well as producing an increase in microvascular permeability, also increased microvascular blood flow. Our results are in agreement with this. Neither PLL- (at 100 μg site^{-1}) induced PE or the PMN accumulation response was potentiated (at either 30 min or 3 h after injection) by mixing the polycation with PGE_2. Thus, in subsequent experiments, where PLL was used at a dose of 100 μg site^{-1}, it was not mixed with PGE_2.

Similarly to that for poly-L-lysine, the time course of antigen-induced responses was also prolonged. PMN accumulation persisted at 3 h post-injection. More surprisingly, these experiments also revealed that the antigen-induced PE response persists over the 3 h period that was measured, whereas the PE response to fMLP was maximal at 45 min. In addition, pulse experiments showed that in the period 2.5–3.0 h after intradermal injection, plasma leakage was still occurring. Injection of antigen into the immunized rabbit should cause a Type 1 immune reaction involving histamine release from mast cells and wheal and flare as a result, which occurs in minutes. In order that the PE response can persist over 3 h, other mediators must be released, which prolong this inflammatory response. Indeed, the fact that PMN accumulation also, is still increasing at 3 h post-injection, makes it feasible that mediators released from activated leukocytes may cause further cell accumulation and thus contribute to the maintenance of the PE response. For example, pharmacological concentrations of MBP have been shown to upregulate ICAM-1 on cultured human epithelial cells (Altman *et al.*, 1993) and also, eosinophil supernatants increased expression of ICAM-1, E-selectin and VCAM-1 on human umbilical vein endothelial cells and caused further accumulation of this cell type (Molet *et al.*, 1998). In addition, MBP has been reported to cause eosinophil degranulation and the production by these cells, of the chemoattractant, interleukin-8 (Kita *et al.*, 1995), and also, histamine release from rodent mast cells and human basophils (O'Donnell *et al.*, 1983; Zheutlin *et al.*, 1984), indicating degranulation and thus, release of other leukocyte chemoattractants such as leukotrienes. Also, there could be a neural component of the PE response, occurring *via* a

mechanism similar to that reported in the airways by Coyle *et al*. (1995) whereby PLL was found to activate C fibres.

The long duration of the PE response in this active cutaneous response (ACA) is in contrast to that for the passive cutaneous anaphylactic (PCA) response, which, when measured over 30 min was reported to have a half-life of 15 min and to be almost complete by 60 min (Hellewell *et al*., 1992). This PCA response was also found to be dependent on prostaglandins (as determined by the use of indomethacin), but was not affected by H_1 receptor antagonists, platelet activating factor antagonists, 5-lipoxygenase inhibitors, kallikrein inhibitors, bradykinin antagonists or an anti-C5a antibody. A neutrophil-dependent mediator was thought to be involved as the microtubule inhibitor, colchicine was an effective inhibitor of this response. Like PLL-induced responses, PE and PMN accumulation responses to intradermal injection of antigen were of long duration. Superficially, it therefore appears that, in the rabbit, the ACA response shares some characteristics with PLL-induced responses and thus, it is possible that release of polycations may be a component of the antigen-induced response. However, the time courses of antigen-induced PE and PMN accumulation differ to those for PLL-induced responses, in that PMN accumulation did not appear to greatly lag behind PE, as was observed for the polycation-induced responses. The difference in the PE response to these two stimuli was further investigated using the microtubule inhibitor colchicine, which inactivated neutrophils. Hellewell *et al*. (1992) showed that intravenous injection of colchicine caused a slow decline in the total number of circulating leukocytes and in neutrophil number although colchicine causes a rapid inactivation of neutrophil chemotaxis through inhibition of microtubule activity. A 10 min intravenous pretreatment with colchicine (1 mg kg^{-1}, as described by Hellewell *et al*., 1992), significantly inhibited PE responses to the neutrophil dependent mediator, fMLP, but had no effect on PE induced by the directly acting mediator, BK. Antigen-induced responses were significantly inhibited by colchicine, whereas PLL-induced responses were unaffected. Such results suggest differences in the mechanisms of antigen and PLL-induced responses. In contrast to the results obtained with colchicine, that suggest differences in leukocyte dependency of these stimuli, we did not see any significant accumulation of platelets in response to poly-L-lysine or antigen (data not shown), suggesting that neither the PE or PMN accumulation was likely to be secondary to platelet activation.

In conclusion, both antigen and PLL were able to elicit both PE and PMN accumulation responses, with the antigen-induced responses, but not PLL-induced responses, being sensitive to PMN inactivation. Our results suggest that leukocyte derived cationic proteins may contribute to allergic reactions in the skin as has been suggested to be the case in the lung (Madison & Schramm, 2000; Gleich, 2000), but that the mechanisms of these responses are likely to be distinct from one another.

References

ALTMAN, L.V., AYARS, G.H., BAKER, C.& LUCHTEL, D.L. (1993). Cytokines and eosinophil-derived cationic proteins upregulate intercellular cell adhesion molecule-1 on human nasal epithelial cells. *J. Allergy Clin. Immunol.,* **92,** 527 – 536.

ASAKO, H., KUROSE, I., WOLF, R., DEFREES, S., ZHENG, Z.-L., PHILLIPS, M.L., PAULSON, J.C. & GRANGER, D.N. (1994). Role of H_1 receptors and P-selectin in histamine-induced leukocyte rolling and adhesion in postcapillary venules. *J. Clin. Invest.,* **93,** 1508 – 1515.

BASCOM, R., PIPKORN, L.L., LICHTENSTEIN, L.M. & NACLERIO, R.M. (1988). The influx of inflammatory cells into nasal washings during the late response to antigen challenge. Effect of systemic steroid treatment. *Am. Rev. Respir. Dis.,* **138,** 406 – 412.

BEHRENS, B.L., CLARK, R.A.F., PRESLEY, D.M., GRAVES, J.P., FELDSIEN, D.C. & LARSEN, G.L. (1987). Comparison of the evolving histopathology of early and late cutaneous and asthmatic responses in rabbits after a single antigen challenge. *Lab Invest.,* **56,** 101 – 113.

CHANG, S.-W. & VOELKEL, N.F. (1991). Charge related lung microvascular injury. *Am. Rev. Respir. Dis.,* **139,** 534 – 545.

CHARLESWORTH, E.N., KAGEY-SOBOTKA, A., SCHLEIMER, R.P., NORMAN, P.S. & LICHTENSTEIN, L.M. (1991). Prednisolone inhibits the appearance of inflammatory mediators and the influx of eosinophils and basophils associated with the cutaneous late phase response to allergen. *J. Immunol.,* **146,** 671 – 676.

COCKCROFT, D.W., RUFFIN, R.E. & FRITH, P.A. (1979). Determinants of allergen-induced asthma: dose of allergen, circulating IgE antibody concentration and bronchial responsiveness to inhaled histamine. *Am. Rev. Respir. Dis.,* **120,** 1053 – 1058.

COSTELLO, R.W., SCHOFIELD, B.H., KEPHART, G.M., GLEICH, G.J., JACOBY, D.B. & FRYER, A.D. (1997). Localization of eosinophils to airway nerves and effect on neuronal M_2 muscarininc receptor function. *Am. J. Physiol.,* **273,** L93 – L103.

COYLE, A.J., ACKERMAN, S.J., BURCH, R., PROUD, D. & IRVIN, C.G. (1995). Human eosinophil major basic protein and synthetic polycations induce airway responsiveness in vivo dependent upon bradykinin generation. *J. Clin. Invest.,* **95,** 1735 – 1740.

COYLE, A.J., ACKERMAN, S.J. & IRVIN, C.G. (1993). Cationic proteins induced airway hyperresponsiveness dependent on charge interactions. *Am. Rev. Respir. Dis.,* 1993, **147,** 896 – 900.

COYLE, A.J., PERETTI, F., MANZINI, S. & IRVIN, C.G. (1994). Cationic protein-induced sensory nerve activation: role of substance P in airway hyperresponsiveness and plasma protein extravasation. *J. Clin. Invest.,* **94,** 2301 – 2306.

DOLOVICH, J., HARGREAVE, F.E., CHALMERS, R., SHIER, K.J., GAULDIE, J. & BIENENSTOCK, J. (1973). Late cutaneous allergic responses in isolated IgE dependent reactions. *J. Allergy Clin. Immunol.,* **52,** 38 – 46.

DUPUIS, I.R., COLLINS, D.S., KOH, Y.Y., POLLICE, M., ALBERTINE, K.H., FISH, J.E. & PETERS, S.P. (1992). Effect of antigen dose on the recruitment of inflammatory cells to the lung by segmental antigen challenge. *J. Allergy Clin. Immunol.,* **89,** 1609 – 1619.

EVANS, C.M., JACOBY, D.B., GLEICH, G.J., FRYER, A.D. & COSTELLO, R.W. (1997). Pretreatment with antibody to eosinophil major basic protein prevents hyperresponsiveness by protecting neuronal m2 receptor function in antigen-challenged guinea pigs. *J. Clin. Invest.,* **110,** 2254 – 2262.

FERGUSSON, A.C., VAUGHAN, R., BROWN, H. & CURTIS, C. (1995). Evaluation of eosinophilic cationic protein as a marker of disease activity in chronic asthma. *J. Allergy Clin. Immunol.,* **95,** 23 – 28.

FRIGAS, E., LOEGERING, D.A. & GLEICH, G.J. (1980). Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium. *Lab. Invest.,* **42,** 35 – 43.

FRYER, A.D. & JACOBY, D.B. (1992). Function of pulmonary M_2 muscarinic receptors in antigen-challenged guinea-pigs is restored by heparin and poly-L-glutamate. *J. Clin. Invest.,* **90,** 2292 – 2298.

FUJISAWA, T., KEPHART, G.M., GRAY, B.H. & GLEICH, G.J. (1990). The neutrophil and chronic allergic inflammation. Immunochemical localization of neutrophil elastase. *Am. Rev. Respir. Dis.,* **141,** 689 – 697.

GLEICH, G.J. (1990). The eosinophil and bronchial asthma: current understanding. *J. Allergy. Clin. Immunol.,* **85,** 422 – 436.

GLEICH, G.J. (2000). Mechanisms of eosinophil-associated allergy. *J. Allergy Clin. Immunol.,* **105,** 651–653.

GLEICH, G.J. & ADOLPHSON, C.R. (1986). The eosinophilic leukocyte: structure and function. *Adv. Immunol.,* **39,** 177–253.

GLEICH, G.J. & ADOLPHSON, C.R. (1998). The eosinophil and bronchial asthma: evidence for a critical role of eosinophils in pathophysiology. In *Interleukin-5: from molecule to drug target for asthma*. ed. Sanderson C.J. **125,** pp 1–37. New York: Marcel Dekker.

GLEICH, G.J., FRIGAS, E., LOEGERING, D.A., WASSOM, D.L. & STEINMULLER, D. (1979). Cytotoxic properties of the eosinophil major basic protein. *J. Immunol.,* **123,** 2925–2927.

GUNDEL, R.H., LETTS, L.G. & GLEICH, G.J. (1991). Human eosinophil major basic protein induces airway constriction and airway hyperresponsiveness in primates. *J. Clin. Invest.,* **87,** 1470–1473.

HELLEWELL, P.G., JOSE, P.J. & WILLIAMS, T.J. (1992). Inflammatory mechanisms in the passive cutaneous anaphylactic reaction in the rabbit: evidence that novel mediators are involved. *Br. J. Pharmacol.,* **107,** 1163–1172.

HELLEWELL, P.G., YARWOOD, H. & WILLIAMS, T.J. (1989). Characteristics of oedema formation induced by N-formyl-methonyl-leucyl-phenylalanine in rabbit skin. *Br. J. Pharmacol.,* **97,** 181–189.

JACOBY, D.B., GLEICH, G.J. & FRYER, A.D. (1993). Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M_2 receptor. *J. Clin. Invest.,* **91,** 1314–1318.

KITA, H., ABU-GHAZALEH, I., SUR, S. & GLEICH, G.J. (1995). Eosinophil major basic protein induces degranulation and IL-8 production by human eosinophils. *J. Immunol.,* **154,** 4749–4758.

LEFORT, J., NAHORI, M.A., RUFFIE, C., VARGAFTIG, B.B. & PRETOLANI, M. (1996). In vivo neutralization of eosinophil-derived major basic protein inhibits antigen-induced bronchial hyperreactivity in sensitized guinea-pigs. *J. Clin. Invest.,* **97,** 1117–1121.

LEIFERMAN, K.M., ACKERMAN, S.J., SAMPSON, H.A., HAUGEN, H.S., VENENCIE, P.Y. & GLEICH, G.J. (1985). Dermal deposition of eosinophil granule major basic protein in atopic dermatitis. Comparison with onchocerciasis. *N. Engl. J. Med.,* **313,** 282–285.

MADISON, J.M. & SCHRAMM, C.M. (2000). Cationic proteins and bronchial hyperresponsiveness. *Am. J. Respir. Cell. Mol. Biol.,* **22,** 513–516.

MOLET, S., COSSET, P., VANHEE, D., TILLIE-LEBLOND, I., WALLAERT, B., CAPRON, M. & TONNEL, A.-B. (1998). Modulation of cell adhesion molecules on human endothelial cells by eosinophil derived mediators. *J. Leukoc. Biol.,* **63,** 351–358.

MOTOJIMA, S., FRIGAS, E., LOEGERING, D.A. & GLEICH, G.J. (1989). Toxicity of eosinophil cationic proteins from guinea pig tracheal epithelium in vitro. *Am. Rev. Respir. Dis.,* **139,** 801–805.

NEEDHAM, L., HELLEWELL, P.G., WILLIAMS, T.J. & GORDON, J.L. (1988). Endothelial functional responses and increased vascular permeability induced by polycations. *Lab. Invest.,* **59,** 538–548.

O'DONNELL, M.C., ACKERMAN, S.J., GLEICH, G.J. & THOMAS, L.L. (1983). Activation of basophil and mast cell histamine release by eosinophil granule major basic protein. *J. Exp. Med.,* **157,** 1981–1991.

PELIKAN, P., GIMBRONE, Jr M.A. & COTRAN, R.S. (1979). Distribution and movement of anionic cell surface sites in cultured human vascular endothelial cells. *Atherosclerosis,* **32,** 69–80.

PETERS, M.S., SCHROETER, A.L., KEPHART, G.M. & GLEICH, G.J. (1983). Localisation of eosinophil granule major basic protein in chronic urticaria. *J. Invest. Dermatol,* **81,** 39–43.

ROBINSON, D.S., ASSOUFI, B., DURHAM, S.R. & KAY, A.B. (1995). Eosinophil cationic protein (ECP) and eosinophil protein X (EPX) concentrations in serum and bronchial lavage fluid in asthma. Effect of prednisolone. *Clin. Exp. Allergy,* **25,** 1118–1127.

SANTANA, A., HYSLOP, S., ANTUNES, E., MARIANO, M., BAKHLE, Y.S. & DE NUCCI, G. (1993). Inflammatory responses induced by poly-L-arginine in rat lungs in vivo. *Agents Actions,* **39,** 104–110.

SASAKI, M., PAUL, W., DOUGLAS, G.J. & PAGE, C.P. (1991). Cutaneous responses to poly-L-lysine in the rabbit. *Br. J. Pharmacol.,* **104,** 444P.

SHAMPAIN, M.P., BEHRENS, B.L., LARSEN, G.L. & HENSON, P.M. (1982). An animal model of late pulmonary responses to *Alternaria* chalenge. *Am. Rev. Respir. Dis.,* **126,** 493–498.

SIMIONESCU, M., SIMIONESCU, N., SILBERT, J.E. & PALADE, J.E. (1981). Differentiated microdomains on the luminal surface of the capillary endothelium. II. Partial characterization of their anionic sites. *J. Cell Biol.,* **90,** 614–621.

SKUTELSKY, E. & DANON, D. (1976). Redistribution of surface anionic sites on the luminal front of blood vessel endothelium after interaction with polycation ligand. *J. Cell Biol.,* **71,** 232–241.

SOLLEY, G.O., GLEICH, G.J., JORDON, R.E. & SCHROETER, A.L. (1976). The late phase of the immediate wheal and flare skin reaction. Its dependence upon IgE antibodies. *J. Clin. Invest.,* **58,** 408–420.

STEIN O., DE VRIES A., & KATCHALSKI, E. (1956). The effect of polyamino acids on the blood vessels of the rat. *Arch. Int. Pharmacodyn.,* **107,** 243–253.

SUR, S., CROTTY, T.B., KEPHANT, G.M., HYMA, B.A., COLBY, T.V., REED, C.E., HUNT, L.W. & GLEICH, G.J. (1993). Sudden onset fatal asthma: A distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? *Am. Rev. Respir. Dis.,* **148,** 713–719.

SUR, S., GLITZ, D.G., KITA, H., KUJAWA, S.M., PETERSON, E.A., WEILER, D.A., KEPHART, G.M., WEGNER, J.M., GEORGE, T.J., GLEICH, G.J. & LEIFERMAN, K.M. (1998). Localisation of eosinophil-derived neurotoxin and eosinophil cationic protein in neutrophilic leukocytes. *J. Leukoc Biol.,* **63,** 715–722.

UMEMOTO, L., POOTHULLIL, J., DOLOVICH, J. & HARGREAVE, F.E. (1976). Factors which influence late cutaneous allergic responses. *J. Allergy Clin. Immunol.,* **58,** 60–68.

WARDLAW, A.J., DUNNETTE, S., GLEICH, G.J., COLLINS, J.V. & KAY, A.B. (1988). Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma: relationship to bronchial hyperreactivity. *Am. Rev. Respir. Dis.,* **137,** 62–69.

WHALLEY, E.T. (1987). Receptors mediating the increase in vascular permeability to kinins: comparative studies in rat, guinea-pig and rabbit. *Naunyn Schmiedebergs Arch. Pharmacol.,* **336,** 99–104.

WILLIAMS, T.J., JOSE, P.J., WEDMORE, C.V., PECK, M.J. & FORREST, M.J. (1983). Mechanisms underlying inflammatory edema: the importance of synergism between prostaglandins, leukotrienes, and complement derived peptides. *Adv. Prostalgan. Thrombox. Leukot. Res.,* **11,** 33–37.

WILLIAMS, T.J. & PECK, M.J. (1977). Role of prostaglandin-mediated vasodilatation in inflammation. *Nature,* **270,** 530–532.

ZHEUTLIN, L.M., ACKERMAN, S.J., GLEICH, G.J. & THOMAS, L.L. (1984). Stimulation of basophil and rat mast cell histamine release by eosinophil granule-derived cationic proteins. *J. Immunol.,* **133,** 2180–2185.

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