

A canine model of cutaneous late-phase reactions: prednisolone inhibition of cellular and cytokine responses

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Summary

Immunoglobulin E (IgE)-mediated late-phase reactions can be induced in atopic humans by intradermal injection of relevant allergens or anti-IgE antibodies. The histology of these reactions resembles that of naturally occurring atopic dermatitis. Strikingly similar responses can be induced in dogs, suggesting that a canine model could prove valuable for preclinical investigation of drugs targeting late-phase reactions. This study was designed to characterize the cellular, cytokine and chemokine responses after intradermal anti-IgE injection in untreated and prednisolone-treated dogs. Normal beagles were untreated or treated with prednisolone before intradermal injection of polyclonal rabbit anti-canine IgE or normal rabbit IgG. Biopsies were taken before injection and 6, 24 and 48 hr after injection. Samples were evaluated by histological and immunohistochemical staining, as well as by real-time quantitative polymerase chain reaction analysis. Dermal eosinophil and neutrophil numbers increased dramatically within 6 hr after injection of rabbit anti-canine IgE, and remained moderately elevated at 48 hr. The numbers of CD1c⁺ and CD3⁺ mononuclear cells were also increased at 6 hr. The real-time quantitative polymerase chain reaction demonstrated marked increases in mRNA expression for interleukin-13 (IL-13), CCL2, CCL5 and CCL17. Levels of mRNA for IL-2, IL-4, IL-6 and IFN- γ did not change within the limits of detection. Prednisolone administration suppressed the influx of neutrophils, eosinophils, CD1c⁺ and CD3⁺ cells, as well as expression of IL-13, CCL2, CCL5 and CCL17. These data document the cytokine and chemokine responses to anti-IgE injection in canine skin, and they demonstrate the ability of the model to characterize the anti-inflammatory effects of a known therapeutic agent.

Keywords: animal models/studies: cows/pigs/horses/cats/dogs; allergy; atopy; basophils/mast cells; skin (dermatology) immunology/disease

Introduction

Atopic dermatitis (AD) is a common inflammatory skin disorder of humans that is showing increasing prevalence.¹ Similarly, dogs have been shown to manifest spontaneous atopic dermatitis.^{2,3} In humans and dogs, the disorder is often characterized by genetic predisposition,

early age of onset, dermatitis and pruritus (predominantly on flexural surfaces), elevated immunoglobulin E (IgE) antibodies to environmental allergens, and a predisposition to secondary infections with bacteria and yeast.³

Research into the pathogenesis of this disorder and the development of new therapies has been hampered by the lack of a suitable animal model. Most research on

Abbreviations: AD, atopic dermatitis; IFN- γ , interferon- γ ; IgE, immunoglobulin E; IL-13, interleukin-13; LPR, late-phase reaction; MCP-1, monocyte chemotactic protein-1 (CCL2); mRNA, messenger RNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RANTES, Regulated upon Activation, Normal T-cell-Expressed and Secreted (CCL5); TARC, Thymus and Activation Regulated Chemokine (CCL17); TNF- α , tumour necrosis factor- α .

the pathogenesis and treatment of AD has been performed in mouse models, such as spontaneously allergic NC/Nga or passively sensitized BALB/c mice.^{4,5} However, it must be considered that information derived from groups of inbred, genetically identical and/or artificially or passively sensitized subjects may not necessarily be suitable for extrapolation to spontaneous disease in genetically predisposed humans, which entails complex interactions between genetic predisposition and host environment, skin barrier defects, microbial skin infections and other immunological factors.¹ The risk of over-extrapolation from mouse to man is further highlighted by the existence of subtle but important differences in the cellular distribution and function of several receptors and mediators critical to the development and maintenance of cutaneous hypersensitivity, such as the high- and low-affinity receptors for IgE^{6,7} and IgG.^{8–10}

In the dog, surface expression of IgE has been detected *in situ* on epidermal Langerhans cells, dermal dendritic cells and dermal mast cells¹¹ as well as on circulating B cells, CD14⁺ mononuclear cells and CD1c⁺ dendritic cells.¹² Aggregation of this IgE by the intradermal injection of cross-linking anti-canine IgE antibodies has recently been demonstrated to produce immediate reactions and late-phase reactions (LPR) in the skin of both normal dogs and dogs with naturally occurring AD.¹³ These reactions grossly and microscopically resemble those generated by the intradermal injection of allergen or anti-IgE in humans with atopic disorders, including AD.^{13–17} These similarities suggest a need for further investigation into the utility of this model for the study of the development of LPR and for use in preclinical studies of new treatments targeted to the LPR.

The specific aims of this study were two-fold. Our first objective was to expand upon previous work describing canine anti-IgE-induced LPR by extending the study observation period and by determining the cytokine expression profile of these reactions. This objective was achieved by obtaining biopsies of normal dog skin, before and 6, 24 and 48 hr after intradermal injection of anti-IgE. These samples were submitted for routine histology, immunohistochemistry and quantitative messenger RNA (mRNA) analysis.

Our second objective was to investigate the potential utility of this model for the evaluation of the effects of pharmacological therapy upon the LPR. This was achieved by administering prednisolone before and during the experimental period.

Normal dogs (rather than dogs with AD) were used in this study to maximize and further evaluate the practicality of this model. Although AD is common in dogs, it would be difficult, expensive and impractical for most research laboratories to maintain a dedicated colony of dogs with AD. In contrast, a model designed for use in normal dogs could be instituted at any research laborat-

ory. Similar studies using intradermal injection of anti-IgE in healthy, non-allergic humans have been used to evaluate the efficacy of a variety of drugs upon immediate wheal and flare reactions and LPR.^{18–20}

Materials and methods

Subjects

Thirteen normal, sexually intact, male and female beagles (mean age 32 months) were used for this study. These dogs were chosen based upon lack of clinical or historical evidence of allergic skin disease, cutaneous bacterial infections or systemic disease. Dogs that had received medication in the 14 days prior to the study were excluded. Physical examinations were performed on all dogs 1 day prior to the start of the study. Housing and experimental samplings were in accordance with the National Research Council's 1996 *Guide for Care and Use of Laboratory Animals*. All experimental protocols were approved by an Institutional Animal Care and Use Committee.

Generation of IgE-mediated LPR

All dogs were shaved on their lateral thorax on study Day 0. On Day 3, each dog was sedated with medetomidine (Domitor®, Pfizer, Exton, PA) injected intravenously. Five dogs received no medication before the study, and were injected intradermally (0.05 ml each injection) at two sites with phosphate-buffered saline (PBS) and at eight sites with protein G affinity-purified rabbit polyclonal IgG specific for canine IgE (anti-canine IgE)²¹ diluted to 0.08 mg/ml in PBS. Three dogs were injected at eight sites with 0.05 ml of normal rabbit IgG (Jackson ImmunoResearch Laboratories, Westgrove, PA) diluted to 0.08 mg/ml in PBS, instead of anti-canine IgE. The optimal injection volume and concentration had been previously determined by serial titration.

Determination of the effect of prednisolone upon IgE-mediated LPR

Five remaining dogs were given a moderate anti-inflammatory dose (0.5 mg/kg) of prednisolone orally twice daily, starting on study Day 0, and continuing through the study. On Day 3, these dogs were sedated, injected at two sites with PBS and at eight sites with anti-IgE and biopsied as described below.

Grading of macroscopic reactions

Injected skin sites were examined at 20 min and at 6, 24 and 48 hr after injection. The diameter of the cutaneous reactions was measured in two perpendicular directions and used to determine the reaction area.

Specimen collection and processing

Two 8-mm punch biopsies of normal skin on the lateral thorax were collected before injection. Similar paired samples were collected at injection sites 6, 24 and 48 h after injection with anti-canine IgE or normal rabbit IgG. One sample from each pair was immediately placed into a cryotube, snap-frozen in liquid nitrogen and stored at -70° for gene expression analysis. The other sample was bisected immediately after collection. One half was placed in 10% neutral buffered formalin for routine processing in paraffin. The other half was placed in Optimal Cutting Temperature medium (OCT Tissue Tek, Baxter Diagnostics Inc., McGaw Park, IL), immersed in isopentane cooled in liquid nitrogen and stored at -70° until cryosectioning.

Histological staining

Five-micrometre sections were cut from paraffin blocks and stained with haematoxylin & eosin for examination and pattern analysis. Eosinophils were visualized and counted using Luna's stain for eosinophils.²² A low-pH (1.5) toluidine blue stain^{23,24} was used to facilitate evaluation of dermal mast cells.

Immunophenotyping of cutaneous mononuclear cells

A three-step labelled streptavidin method modified from Affolter and Moore²⁵ was used to characterize the mononuclear cell infiltrate. Briefly, 6- μ m cryosections were fixed by immersion in acetone. Endogenous peroxidase activity was quenched by immersion in hydrogen peroxide (diluted to 0.3% in PBS) containing sodium azide (0.01%), then blocked with 1% fetal calf serum in PBS. Monoclonal antibodies specific for canine CD1c, CD3, CD4 and CD8 (courtesy of Dr Peter F. Moore, University of California, Davis, CA) were used as cell culture supernatant diluted 1 : 10 in PBS. Biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) was diluted 1 : 400 in PBS, followed by horseradish peroxidase-conjugated streptavidin (Zymed, San Francisco, CA), diluted 1 : 400 in PBS. Amino-9-ethyl-carbazole (AEC, AEC Substrate Kit, Biogenix, San Ramon, CA) was applied as a chromogen, followed by a haematoxylin counterstain (Sigma-Aldrich, St Louis, MO).

Enumeration of dermal cells

The number of total nucleated cells/mm² in the dermis was obtained by counting 51 consecutive 0.14 mm $\times$ 0.14-mm fields of superficial dermis (excluding endothelial cells and adnexae). Neutrophils, mast cells and eosinophils, as well as CD1c⁺ and CD3⁺ mononuclear cells/mm² of dermis were determined by counting

13 consecutive 0.24 mm $\times$ 0.32-mm fields of superficial dermis.

RNA expression analysis

For real-time quantitative polymerase chain reaction (PCR) analysis, the Biopulverizer (BioSpec #59013, Biospec Inc., Bartlesville, OK) was used to crush the skin biopsies on dry ice and total RNA was isolated using Qiagen RNeasy Midi kit according to the manufacturer's instructions (Qiagen RNeasy Midi handbook, second edition pp. 41–47 and Appendix E pp. 91–92, respectively; Qiagen Inc., Valencia, CA). Total RNA (5 μ g) was subjected to further treatment with DNase (Ambion Inc., Austin, TX) according to the manufacturer's instructions to eliminate possible genomic DNA contamination.

Real-time quantitative PCR for gene expression

DNase-treated total RNA was reverse-transcribed using Superscript II (Gibco/BRL, Carlsbad, CA) according to the manufacturer's instructions. Primers were designed using PRIMER EXPRESS (PE Biosystems, Foster City, CA). Real-time quantitative PCR on 10 ng cDNA from each sample was performed using two gene-specific unlabelled primers at 400 nm in a Perkin Elmer SYBR green real-time quantitative PCR assay utilizing an ABI 5700 or 7900 instrument. The absence of genomic DNA contamination was confirmed using primers that recognize the genomic region of the canine MMP-9 promoter. Ubiquitin levels were measured in a separate reaction and used to normalize the data by the $\delta\delta$ Ct method. [Using the mean cycle threshold (Ct) value for ubiquitin and the gene of interests for each sample, the equation: $1.8^{\text{Ct ubiquitin} - \text{Ct gene of interest}} \times 10^4$ was used to obtain the normalized values.]

Statistical analysis

Unless otherwise specified, all statistical analyses were performed using SAS Software, Version 8.2 of the SAS system for Windows (SAS institute, Cary, NC, USA). Statistical analysis of dermal cell counts or mRNA expression within groups was performed by evaluating differences of least squared means using a PROC MIXED analysis of log-transformed raw data. Comparisons between groups were performed by evaluating the differences of least squared means/PROC MIXED analyses of fold changes from baseline. Comparisons of injection site areas were performed using two-tailed paired and unpaired *t*-tests, and correlation analyses were performed using a two-tailed Spearman correlation test, both calculated with GRAPHPAD PRISM software, version 3.03 for Windows.

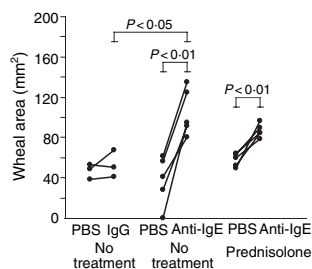


Figure 1. Intradermal injection of anti-canine IgE (but not normal rabbit IgG) induces the formation of an immediate wheal and flare response that is not inhibited by prednisolone. Normal beagles (treated or not with prednisolone, 0.5 mg bid) were injected intradermally with PBS and either normal rabbit IgG or polyclonal rabbit IgG anti-canine IgE. Wheal dimensions were measured 20 min after injection.

Results

Intradermal injection of anti-IgE produces macroscopic immediate and late-phase reactions

Intradermal injection of anti-IgE produced mildly to moderately erythematous and indurated focal urticarial reactions within 20 min (Fig. 1). These areas were significantly ($P < 0.01$) larger than paired PBS injection sites (mean 105.3 mm² and 37.5 mm², respectively). By 6 hr, mild erythema and/or induration remained at anti-IgE injection sites in three dogs. Macroscopic LPR were not seen at 24 and 48 hr.

In contrast, sites injected with normal rabbit IgG did not differ significantly in area or appearance from paired PBS injection sites, and they were significantly smaller than anti-IgE-injected sites (mean 67.3 mm² and 105.3 mm², respectively; $P < 0.05$).

Intradermal injection of anti-canine IgE induces degranulation of dermal mast cells

Low pH toluidine blue staining revealed large, granular, oval cells throughout the dermis and clustered around blood vessels, hair follicles and adnexae. Although toluidine blue stains cytoplasmic granules in both canine basophils and mast cells, basophils are rarely found in the skin of either normal dogs or dogs with AD.¹³ For this reason, positively staining cells were considered to be mast cells. Injection of anti-IgE resulted in an almost nine-fold decrease in intact mast cells by 6 hr ($P < 0.01$) and a 10-fold decrease by 24 hr ($P < 0.001$), followed by a return to near preinjection levels by 48 hr (Fig. 2a). Faintly stained cells and cells with dermal granule dispersion were frequently seen. In contrast, sites injected with normal rabbit IgG exhibited a 2.3-fold decrease in intact mast cells compared to baseline, and did not show an increase in visibly degranulated mast cells (data not shown).

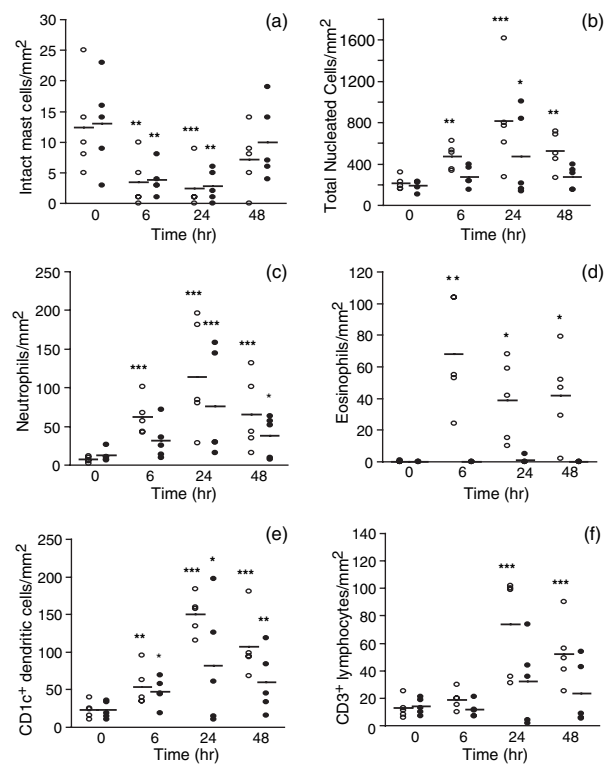


Figure 2. Intradermal injection of anti-IgE produces mast cell degranulation and a biphasic cellular response. Skin biopsies were taken before and after anti-IgE injection in untreated (□) or prednisolone-treated (■) dogs. (a) Intact mast cells, (b) total nucleated cells, (c) neutrophils, (d) eosinophils, (e) CD1c⁺ dendritic cells, (f) CD3⁺ lymphocytes. Data represent dermal cell counts/mm² from individual dogs and group means. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to baseline.

The LPR induced by anti-IgE is characterized by a rapid influx of neutrophils and eosinophils, followed by CD1c⁺ dendritic cells and CD3⁺ lymphocytes. Sites injected with anti-IgE had significant ($P < 0.001$) increases in total dermal nucleated cell numbers after injection (Fig. 2b, Table 1). These cells were increased by more than two-fold relative to baseline by 6 hr, with cell counts continuing to increase at 24 hr, and remaining elevated 48 hr after injection. The 6-hr influx was dominated by a significant increase in eosinophils ($P < 0.01$) and neutrophils ($P < 0.001$), with peak accumulations at 6 and 24 hr, respectively (Figs 2c,d and 3a,b, Table 1). All 6-hr and 24-hr samples contained eosinophils exhibiting granule aggregation or dispersion consistent with degranulation. Both CD1c⁺ dendritic cells and CD3⁺ lymphocytes were increased as early as 6 hr after injection, and peaked at 24 hr (Figs 2e,f and 3c,d). These increases became significant at 6 hr for dendritic cells ($P < 0.01$) and at 24 hr for lymphocytes ($P < 0.001$).

Compared to sites injected with anti-IgE, intradermal injection with normal rabbit IgG produced considerably

Table 1. Dermal cellular infiltrate before and after intradermal injection of polyclonal rabbit IgG anti-IgE or normal rabbit IgG

	Normal rabbit IgG (<i>n</i> =3)				Anti-IgE, no treatment (<i>n</i> =5)				Anti-IgE, prednisolone-treated (<i>n</i> =5)			
	Baseline	6hr	24hr	48hr	Baseline	6hr	24hr	48hr	Baseline	6hr	24hr	48hr
Total nucleated cells												
Mean	309.3	474.3	362.0	366	212.4	471**	815.6***	527**	193.8	276.4	471*	273
(SE)	(44.4)	(65.2)	(60.8)	(39.7)	(30.6)	(55.3)	(221.4)	(82.5)	(23.5)	(46.3)	(186.7)	(49.8)
Median	345	535	347	351	187	514	774	503	223	234	215	314
Fold change		1.6	1.2	1.2		2.2	2.6	2.7		1.6	2.0	1.4
Intact mast cells												
Mean	23.2	18.3	15.3	12.7	12.4	3.4**	2.4***	7.2	13	3.8**	2.8**	10
(SE)	(2.9)	(9.4)	(5.4)	(5.2)	(3.5)	(1.9)	(1.7)	(2.3)	(3.4)	(1.2)	(1.2)	(2.8)
Median	24	10	11	8	10	1	1	8	14	3	2	7
Fold change		-1.1	-1.8†	-2.3		-8.6	-10.6	-2.3		-4.1	-9.0	-0.7
Eosinophils												
Mean	2.0	19.7	8.7	5.7	0.2	68**	38.8*	41.8*	0	0	1	0
(SE)	(1.5)	(5.5)	(5.4)	(4.3)	(0.2)	(15.7)	(11.6)	(12.8)	(0)	(0)	(1.0)	(0)
Median	1	23	6	3	0	55	42	47	0	0	0	0
Fold change		N/A	N/A	N/A		N/A	N/A	N/A		N/A	N/A	N/A
Neutrophils												
Mean	8.3	95.7***	55.3**	22	7	62.2***	114***	65.4***	12.2	31.4	75.6***	37.8*
(SE)	(2.3)	(26.7)	(20.5)	(4.6)	(1.8)	(10.8)	(32.0)	(21.9)	(3.6)	(11.1)	(31.0)	(12.1)
Median	10	101	40	19	6	57	84	43	11	25	30	52
Fold change		16.4	12.2	2.9		12.9	21.9	17.6		2.2	7.3	3.1
CD1c⁺												
Mean	82.7	45	117	73.7	23	53.8**	150.4***	107.2***	23	47*	81.8*	59.6**
(SE)	(9.8)	(5.2)	(17.3)	(18.2)	(5.1)	(11.8)	(11.7)	(19.2)	(5.1)	(8.7)	(35.6)	(18.6)
Median	76	45	117	76	24	40	158	95	19	45	61	45
Fold change		-1.9†	1.6†††	-0.6††		2.8	8.4	6.1		2.6	2.8††	2.5†
CD3⁺												
Mean	35.7	25	88.7	50	12.6	19	73.6***	52.2***	14	11.6	32	23.4
(SE)	(6.4)	(9.9)	(14.3)	(18.9)	(3.3)	(3.3)	(16.4)	(10.8)	(2.7)	(2.6)	(13.4)	(10.4)
Median	40	20	96	38	11	20	99	49	13	11	36	9
Fold change		-1.4	3.3†	0.7†		1.7	7.8	4.9		-0.1	0.4††	0.1†

****P*<0.001, ***P*<0.01, **P*<0.05, relative to baseline cell numbers. †††*P*<0.001, ††*P*<0.01, †*P*<0.05, decreased fold change compared to that seen in time-matched, anti-IgE-injected skin in untreated dogs.

fewer infiltrates, with notable exceptions. Dermal neutrophils were significantly increased relative to baseline both 6 and 24 hr after injection of IgG (Table 1, *P* < 0.001 and *P* < 0.01, respectively). Total nucleated cells were moderately increased at 6 hr, but had returned to near baseline by 24 hr (Table 1).

Injection of normal rabbit IgG produced minimal influx of other inflammatory cells. A small increase in eosinophils was noted 6 hr after injection (Table 1), but this increase was not statistically significant. CD1c⁺ and CD3⁺ cells increased minimally at 24 hr (1.6-fold and 3.3-fold, respectively), and had returned to baseline levels by 48 hr (Table 1). Fold increases in CD1c⁺ cells after normal rabbit IgG injection were significantly smaller at all time-points compared to those seen in dogs injected with anti-IgE (*P* < 0.001–0.05; Table 1). Fold increases in

CD3⁺ cells at 24 and 48 hr were also significantly lower than those seen in dogs injected with anti-IgE (*P* < 0.05).

The LPR induced by anti-IgE is characterized by increased expression of mRNA for IL-13, IL-5, CCL2, CCL5 and CCL17

Significant increases in expression of mRNA for interleukin-13 (IL-13; *P* < 0.001), IL-5 (*P* < 0.01), CCL2 (monocyte chemotactic protein-1, MCP-1; *P* < 0.001) CCL5 (Regulated Upon Activation, Normal T-cell-Expressed and Secreted, RANTES; *P* < 0.01) and CCL17 (thymus and activation regulated chemokine, TARC; *P* < 0.001) were noted 6 hr after injection of anti-IgE (Figs 4A–D). However, mRNA expression of all three mediators rapidly decreased by 24 hr.

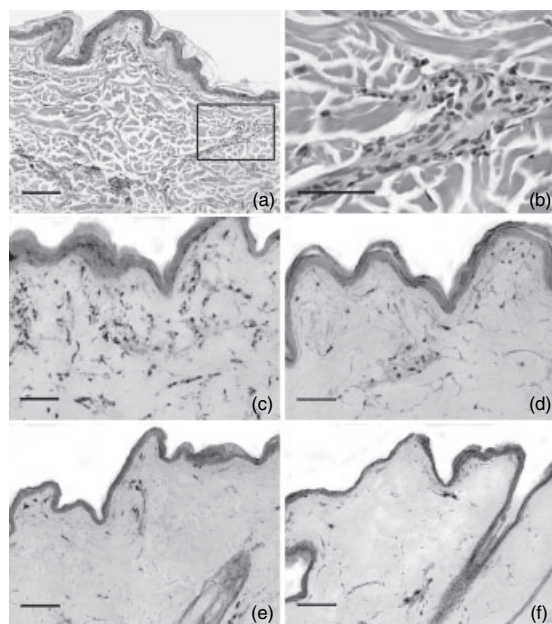


Figure 3. Intradermal injection of anti-IgE produces a rapid cellular influx that is markedly inhibited by prednisolone. (a) Perivascular accumulation of neutrophils and eosinophils 6 hr after injection in untreated dog skin, haematoxylin & eosin stain, bar = 100 µm. (b) Higher magnification of area outlined in (a), bar = 50 µm. (c–f) Immunohistochemical staining of biopsies taken 48 hr after injection in untreated dogs demonstrates aggregates of CD1c⁺ dendritic cells (c) and CD3⁺ lymphocytes (d). In contrast, biopsies taken 48 hr after injection in prednisolone-treated dogs exhibit few CD1c⁺ dendritic cells (e) or CD3⁺ lymphocytes (f). Amino-9-ethyl-carbazole chromogen with haematoxylin counterstain, bar = 100 µm.

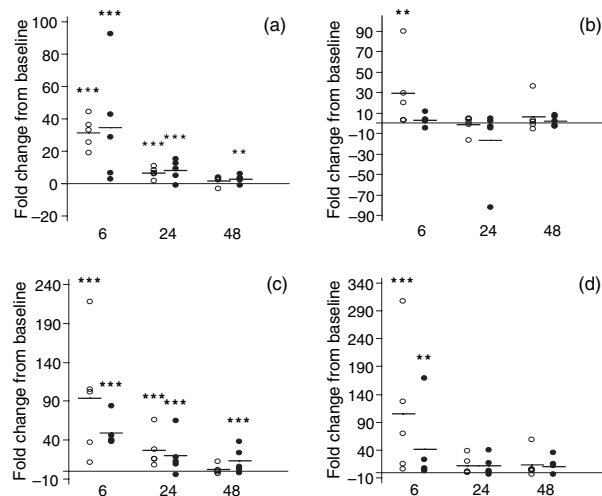


Figure 4. Intradermal injection of anti-IgE produces rapid increases in mRNA expression for IL-13, CCL2, CCL5 and CCL17. Fold changes (relative to baseline levels) were calculated for mRNA expression of IL-13 (a), CCL5 (b), CCL17 (c) and CCL2 (d) after intradermal injection of anti-IgE. Data represent group means and individual changes in untreated (□) or prednisolone-treated (■) dogs. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to baseline.

Minor increases were noted in mRNA for tumour necrosis factor- α (TNF- α) and IL-10, while expression of mRNA for IL-2, IL-4, IL-6 and interferon- γ (IFN- γ) remained negligible at all times.

At sites injected with normal rabbit IgG, 6-hr elevations were also seen in expression of mRNA for IL-13, CCL2, CCL5 and CCL17, as well as for TNF- α . These increases were not significant for CCL5 and CCL17. However, the increases in mRNA for IL-13, CCL2 and TNF- α were more robust, and did reach statistical significance ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively). Expression of mRNA for IL-2, IL-4 and IL-6 remained negligible, and small, non-statistically significant increases in mRNA expression for IL-5, IFN- γ and IL-10 were seen (Table 2).

Treatment with prednisolone has no significant effect on anti-IgE-induced immediate wheal and flare reactions, but abrogates macroscopic LPR

In prednisolone-treated dogs, the intradermal injection of anti-IgE produced wheal and flare reactions within 20 min that were similar in appearance and area to those seen in untreated dogs injected with anti-IgE (Fig. 1). However, unlike anti-IgE-injected untreated dogs, prednisolone-treated dogs did not have macroscopically evident LPR at any time after injection of anti-IgE.

Prednisolone completely inhibits eosinophil influx and greatly decreases recruitment of dermal neutrophils, dendritic cells and lymphocytes in anti-IgE-induced LPR

Although treatment with prednisolone completely inhibited macroscopic LPR, treated dogs did not appreciably differ from untreated dogs with regards to postinjection changes in intact and visibly degranulated mast cells (Fig. 2a, Table 1). Despite this, prednisolone treatment greatly inhibited the infiltration of most cell types. Total nucleated cell counts were elevated at 24 hr after injection, but the relative increase was still markedly lower than that seen in untreated dogs at the same time-point (Fig. 2b; Table 1).

Prednisolone also inhibited infiltration of neutrophils, CD1c⁺ dendritic cells and CD3⁺ lymphocytes (Fig. 3e,f). Most dramatically, administration of prednisolone was associated with an almost complete abrogation of the eosinophil response (Fig. 2c–f).

Prednisolone inhibits expression of mRNA for IL-13, CCL2, CCL5 and CCL17 in LPR

Although prednisolone-treated dogs did have 6-hr increases in mRNA expression for IL-13, CCL2, CCL5 and CCL17 (Fig. 4a–d), only IL-13, CCL2 and CCL17 increases reached significance. Furthermore, the relative increase

Table 2. Changes in cytokine and chemokine mRNA expression after intradermal injection of polyclonal rabbit IgG anti-IgE or normal rabbit IgG

	Messenger RNA expression (in units relative to Ubiquitin)											
	Normal rabbit IgG (<i>n</i> = 3)				Anti-IgE, no treatment (<i>n</i> = 5)				Anti-IgE, prednisolone-treated (<i>n</i> = 5)			
	Baseline	6 hr	24 hr	48 hr	Baseline	6 hr	24 hr	48 hr	Baseline	6 hr	24 hr	48 hr
CCL2 (MCP-1)												
Mean	3.4	90.7**	26.7	9.1	2.9	109.0***	4.8	13.5	2.2	15.2**	5.0	7.2
(SE)	(0.8)	(10.2)	(7.2)	(1.5)	(1.1)	(67.3)	(1.3)	(6.2)	(1.1)	(1.2)	(2.0)	(3.7)
Median	3.1	90.4	20.6	7.8	4.1	41.6	5.7	12.6	1.8	16.4	3.9	3.8
Fold change		28.2	7.8	3.0		104.7	12.0	13.9		41.6	11.3	9.5
CCL5 (RANTES)												
Mean	1.1	3.9	3.7	3.0	1.2	6.3**	1.6	3.8	0.9	1.7	0.8	1.6
(SE)	(0.1)	(1.2)	(0.6)	(1.0)	(1.0)	(2.4)	(1.2)	(2.5)	(0.6)	(0.8)	(0.4)	(0.6)
Median	1.1	3.1	3.8	2.4	0.2	4.3	0.7	0.9	0.4	0.9	0.7	1.0
Fold change		3.9	3.6	2.7		28.9	-1.6	6.0		2.9	-16.7	2.3
CCL17 (TARC)												
Mean	0.6	1.4	0.8	0.5	0.2	13.7***	3.8***	0.3	0.0	1.3***	0.7***	0.4***
(SE)	(0.3)	(0.6)	(0.2)	(0.2)	(0.1)	(6.7)	(1.4)	(0.1)	(0.0)	(0.6)	(0.5)	(0.3)
Median	0.4	1.2	0.8	0.3	0.2	10.2	3.0	0.2	0.0	0.8	0.3	0.0
Fold change		2.9†††	1.2	-0.6		94.3	26.7	2.0		49.5†	19.8	13.5
IL-13												
Mean	0.5	3.6**	1.0	0.7	2.0	62.7***	11.1***	4.0	1.6	26.2***	13.9***	3.6**
(SE)	(0.1)	(0.8)	(0.3)	(0.2)	(0.4)	(13.0)	(1.8)	(0.8)	(0.6)	(8.8)	(6.1)	(1.0)
Median	0.6	4.3	1.2	0.6	1.8	80.3	10.6	4.8	0.8	19.8	10.0	2.8
Fold change		7.7	1.5	0.8		31.6	6.6	1.8		34.6	8.1	2.8
IL-5												
Mean	0.6	0.8	0.4	0.6	2.9	8.8**	2.8	2.1	3.9	3.6	1.9	3.2
(SE)	(0.1)	(0.2)	(0.1)	(0.2)	(0.1)	(3.8)	(0.6)	(0.2)	(1.7)	(0.5)	(0.5)	(0.3)
Median	0.5	0.7	0.3	0.7	3.0	5.2	2.8	1.9	2.3	3.5	2.0	3.2
Fold change		0.8	-1.3	0.4		3.0	-0.1	-1.5		0.6†	-1.7	0.6
TNF-α												
Mean	12.2	28.0*	26.0*	11.6	16.8	22.4	18.0	13.0	8.2	16.4**	13.7	9.0
(SE)	(4.0)	(9.8)	(5.1)	(1.8)	(4.5)	(4.6)	(1.9)	(1.7)	(1.6)	(1.8)	(5.2)	(1.8)
Median	8.8	27.1	21.1	10.1	13.5	20.3	15.7	13.5	10.6	17.1	8.3	8.2
Fold change		2.4	2.3	0.3		1.2	0.8	-0.4		2.4	0.7	0.4
IL-10												
Mean	0.6	1.1	1.2	0.8	11.9	17.4	11.6	18.9	11.7	17.5	10.6	16.0
(SE)	(0.1)	(0.2)	(0.3)	(0.1)	(1.2)	(2.3)	(2.5)	(3.1)	(3.0)	(3.1)	(2.3)	(2.6)
Median	0.7	1.2	1.3	0.7	11.8	20.2	10.8	20.2	7.7	19.9	11.1	16.9
Fold change		1.9	1.9	0.7		1.5	-0.4	1.2		1.1	-0.3	1.6

****P* < 0.001, ***P* < 0.01, **P* < 0.05, relative to baseline mRNA expression. †††*P* < 0.001, ††*P* < 0.01, †*P* < 0.05, decreased fold change compared to that seen in time-matched, anti-IgE injected skin in untreated dogs.

in CCL17 was significantly (*P* < 0.05) decreased compared to that seen in untreated dogs. Prednisolone had no appreciable inhibitory effect upon mRNA expression of IL-10 and TNF-α, but did significantly inhibit post-injection expression of IL-5 (*P* < 0.05).

Discussion

The study of the delayed inflammatory response exhibited by some eczema patients after cutaneous challenge with

allergens is not a new discipline. Indeed, the suggestion that observation of cutaneous LPR could provide information regarding the nature and aetiology of AD was published in the scientific literature almost 100 years ago.²⁶ Great progress has been made since that time, both in the understanding of the phenomena that comprise the LPR, and in the development of safer and more efficient ways to study those phenomena. Many excellent studies have been performed to evaluate the inflammatory response in allergic and normal human volunteers.

However, most LPR research has been performed in mice, using actively or passively sensitized subjects. The ready availability of murine subjects meeting exact genetic and phenotypic specifications (and of appropriate research reagents for the study of these mice) has made the mouse an invaluable asset to the study of AD.

However, mouse models also have their disadvantages. A number of significant differences exist between the cutaneous immune system of the mouse and that of humans. Perhaps the most conspicuous of these differences is the lack of the high-affinity receptor for IgE on murine Langerhans cells and dendritic cells,^{7,9,27} as well as on circulating and tissue-resident monocytes, macrophages and eosinophils.^{6,9,27} In humans, cross-linking of surface-bound IgE can induce full activation of Langerhans cells and dendritic cells.^{28,29} These activated cells are then able to elaborate a number of inflammatory and chemotactic mediators (such as IL-16) that recruit cells important to hypersensitivity responses, such as CD4⁺ T cells and eosinophils.^{30,31} Langerhans cells and dendritic cells activated in this manner are able to efficiently prime naive T cells.^{30,32} This priming is not restricted to T cells specific for the IgE-cross-linking allergen, and is believed to play a major role in the sensitization to new allergens ('epitope spreading') as well as the perpetuation of existing hypersensitivity. The absence of the high-affinity IgE receptors on LC and DC in mouse skin suggests the likelihood of significant differences between mice and humans in the phenomena resulting in sensitization and perpetuation of AD. Indeed, further evidence of significant interspecies differences is provided by the fact that mouse IgG subclasses (especially IgG1) are able to mediate many of the immune phenomena typically associated with IgE responses in humans, such as cutaneous and/or systemic anaphylaxis.^{9,10,33} For this reason, murine models of AD that are designed solely to investigate IgE-related responses (such as IgE passive sensitization models) may be of limited utility for extrapolation to human disease. Clearly, some form of alternative model is needed to complement the results obtained from murine studies.

One candidate for such a complementary model is the dog, a species in which spontaneous allergic diseases are common and in which artificial sensitization is both possible and commonly performed. Historically, research in this species has been limited by the lack of canine-specific reagents. However, recent advances in the development of monoclonal and polyclonal antibodies and primer sequences for use in the dog now allows research to be performed at a high level of sophistication.

The canine cutaneous immune system shares many features with that of humans, which are not shared by the mouse. For example, surface-bound IgE has been detected on canine Langerhans cells and dendritic cells¹¹ as well as circulating monocytes, dendritic cells and B cells.¹² The distribution of these IgE-bearing cells is similar to that in

human skin, and appears to be up-regulated in the face of active atopic disease in a comparable fashion.¹¹ Furthermore, canine skin has been demonstrated to display more 'typical' IgE-mediated, mast-cell-driven inflammatory responses as well, such as the development of immediate and late-phase reactions after cutaneous challenge with relevant allergens or cross-linking anti-canine IgE antibodies.¹³ These facts, when considered together, suggest the likelihood of significant similarities in the pathogenesis and perpetuation of AD between dogs and humans.

The current study expands upon previous work by our laboratory and others in the characterization of the LPR in canine skin.^{13,14,34} Specifically, our study provides new information regarding the cellular and cytokine/chemokine response in LPR following intradermal injection of anti-IgE in dogs.^{13,14,34} We have confirmed that the anti-IgE-induced LPR in healthy research dogs are grossly and microscopically similar to allergen-induced LPR in dogs with AD¹³ as well as to allergen and anti-IgE-induced LPR in humans.^{16,35–37} We have shown that anti-IgE-induced canine LPR exhibit increases in the mRNA transcription of several mediators typical of Th2-dominated responses, notably IL-13, IL-5, CCL5 and CCL17. It was also demonstrated that these cellular and mediator responses can be inhibited by prednisolone therapy, in a manner similar to that seen in allergen-induced LPR in humans.^{38–41} Finally, our isotype-matched controls produced new information that underscores the importance of this control in interpreting the results of anti-IgE-induced LPR.

Intradermal injection of anti-IgE in normal dogs induced the immediate development of erythematous and indurated urticarial plaques, which were indistinguishable from the wheal and flare reactions seen after intradermal injection of allergen extracts in dogs with AD¹³ as well as those described after intradermal injection of allergen or anti-IgE in humans.^{18,41} These reactions were followed by macroscopic erythema and induration at 6 hr but not at later times. These areas were similar to the LPR following injection of allergen or anti-IgE in humans or dogs with AD.^{13,16,18,41}

Biopsies of injected sites exhibited a clear decrease in the number of intact mast cells and an increase in the number of visibly degranulated mast cells. These changes were seen not only in samples from macroscopic LPR, but also in 24- and 48-hr samples. Similar changes have been reported in biopsies taken after intradermal injection of allergen in humans.^{35,42,43}

Injection of anti-IgE resulted in a rapid influx of inflammatory cells. The observed biphasic increase in granulocytes followed by mononuclear cells (including both CD1c⁺ dendritic cells and CD3⁺ lymphocytes) is typical of those described in LPR after injection of allergen or anti-IgE in both dogs¹³ and humans.^{16,35,41,43}

Our results indicated that IgE-mediated canine LPR are characterized by rapid but transient increases in mRNA transcription for IL-13, IL-5, CCL2, CCL5 and CCL17. Spearman analysis demonstrated that increases in mRNA expression for IL-13, CCL2 and CCL17 correlated significantly with increases in tissue eosinophil numbers ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively) in untreated dogs injected with anti-IgE. Expression of mRNA for IL-10 was also detected, but increased minimally postinjection. Remarkably, only negligible levels of mRNA for IFN- γ , IL-2, IL-4 and IL-6 were found at any time. Our first samples were taken 6 hr after injection, which corresponded to the acme of the clinical LPR, but may have been too late to detect transient increases.

In this study, administration of prednisolone for 3 days prior to injection of anti-IgE had no appreciable effect upon the appearance of immediate reactions, nor upon the numbers and appearance of intact and degranulated mast cells. Corticosteroid therapy has been reported to have varying effects upon allergen or anti-IgE-induced wheal and flare reactions, such that, although topical application of either a 0.015% triamcinolone solution³⁴ or a 1% hydrocortisone conditioner¹⁴ before anti-IgE injection decreased wheal diameter in two studies, oral administration of prednisolone at varying doses (40 mg, single dose;³⁸ 60 mg daily for 3 days;⁴¹ or 20 mg daily for 5 days) before injection of allergen did not. Despite this, glucocorticoids have been almost universally reported to significantly inhibit LPR following the injection of allergen in humans.^{39–41} In our study, prednisolone-treated dogs did not develop visible LPR at any point after injection.

Prednisolone sharply decreased cellular infiltration at all time-points, resulting in marked decreases in neutrophils, CD1c⁺ dendritic cells and CD3⁺ lymphocytes, compared to untreated dogs. However, prednisolone had the most marked effect upon eosinophil response, which was inhibited greater than 97% relative to that seen in untreated dogs. Similar degrees of inhibition have been seen in human allergen-induced LPR, in which eosinophil influx was inhibited 70% after a single dose of prednisolone⁴⁰ and completely inhibited after 3 days of prednisolone administration.⁴¹ Cytokine and chemokine responses were also inhibited at 6 hr by 58%, 59%, 86%, 73% and 90.8% (for IL-13, IL-5, CCL2, CCL5 and CCL17, respectively) in prednisolone-treated dogs as compared to placebo-treated dogs. Glucocorticoid treatment has been noted to decrease transcription of all of these mediators both *in vitro*^{44–48} and/or *in vivo*.^{49–51}

Finally, although our results demonstrate many similarities between anti-IgE-induced LPR in normal dog skin and allergen-induced LPR in allergic dogs and humans, they also highlight the requirement for appropriate control samples. Intradermal injection of normal rabbit IgG produced neither visible wheal and flare

responses nor macroscopic LPR, failed to induce significant increases in dermal infiltration of eosinophils, CD1c⁺ and CD3⁺ cells and failed to significantly increase transcription of IL-5, CCL5 and CCL17. However, it did produce a significant increase in neutrophils and in transcription of TNF- α , IL-13 and CCL2. Although marked neutrophilia has been reported as a characteristic of both allergen-induced and anti-IgE-induced LPR in humans and dogs^{13,15,16,41,52} it is not a typical feature of uninfected AD skin in either species.^{11,35,53,54} However, the significance of this response can be difficult to evaluate when 'irrelevant' antigen challenges have not been included in experimental protocols. Our results suggest that the contribution of non-specific inflammatory responses within the LPR may be underestimated if only diluent controls are used.

In summary, our study has expanded upon previous work investigating the use of a canine model of IgE-mediated inflammation for the study of the pathogenesis and treatment of AD. We have confirmed that intradermal injection of anti-IgE produces an inflammatory response essentially identical to the LPR seen after intradermal injection of allergen. We have shown that this response is present even in normal, outbred research dogs, thus eliminating several potential problems associated with the study of artificially sensitized or inbred subjects. Furthermore, we have demonstrated that this response is diminished by prednisolone in a fashion similar to that seen in human allergen-mediated LPR, thus supporting the potential utility of this model in studying therapies targeted to the LPR. We have also provided evidence that some of the inflammatory response seen in experimentally induced LPR may in fact be artefactual, and suggest that the ready availability of isotype-matched control antibodies provides the anti-IgE model of LPR with a clear advantage over traditional allergen-injection models.

In closing, we feel that our results indicate great promise for this model in the evaluation of the pathogenesis of LPR, and in the development of drugs designed to target LPR. It should be pointed out that this model is most reflective of the inflammatory response associated with acute onset or exacerbation of atopic dermatitis. Chronically active AD is associated with the development of a number of cutaneous alterations (such as dermal fibrosis, epidermal hyperplasia and hyperkeratosis, and up-regulation of IgE expression on Langerhans cells and dendritic cells), which would not be well represented by this model as it is currently designed. It is likely that the continued use of allergen-sensitized models will be necessary for the evaluation of events and therapies relating to chronic inflammation associated with AD. Nonetheless, the current model can be easily modified to accommodate sensitized subjects as well, which will allow evaluation of IgE-mediated

exacerbations of chronic disease. In this fashion, the majority of AD-associated inflammatory responses can be efficiently modelled.

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