

A comparative study on cockroach and ovalbumin sensitizations and challenge responses in Hartley guinea-pigs

Gang Chen ^a, Daohong Zhou ^b, Bann C. Kang ^{b,*}

^a Jack Bell Research Center, Vancouver, BC, Canada

^b Division of Allergy, Immunology & Rheumatology, Department of Internal Medicine, University of Kentucky, K528 Kentucky Clinic, 800 Rose Street, Lexington, KY 40536, USA

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Abstract

The role of allergens in asthmatic inflammation is not clearly understood. To elucidate the mechanism of cockroach allergen (CRa)-induced airway disease, we studied three groups of Hartley guinea-pigs sensitized to control, ovalbumin (OA) or CRa. Parameters measured were anaphylactic antibodies by allergy skin test (AST), PCA assay and Western blot, changes in specific airway resistance (SRaw), analysis of bronchoalveolar lavage (BAL) and contracture responses of tracheal muscle (TSM) to non-specific and specific stimuli, in vitro. Both OA and CRa animals showed a similar allergic sensitization (AST and PCA), while Western blot identified several reaginic bands in CRa group compared to a single band in OA group. SRaw illustrated that CRa induce dual-asthmatic responses (4/6) in the CRa group, whereas OA induce only an early asthmatic response (3/6) in the OA group ($P < 0.01$). The average total leukocytes in BALF of the CRa were 27.0×10^6 , mostly neutrophils and eosinophils, while those of the OA showed 3.5×10^6 , mostly eosinophils, respectively ($P < 0.0001$). TSM responses to non-specific stimuli were similar in both groups ($P > 0.1$), while the antigen-specific TSM contractions were more brisk in the OA group than those of CRa group ($P < 0.001$). Thus, the study indicates that both CRa and OA sensitize guinea-pigs, yet CRa induces more severe and persistent late-phase inflammation than OA. This appears to be related to an influx of neutrophils rather than anaphylactic bronchospasm. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The role of indoor allergens in the pathogenesis of asthma is well recognized and often linked with the recent trend of increasing morbidity and mortality of asthma worldwide (Sporik et al., 1990; Woolcock and Peat, 1997). Among indoor allergens, cockroach allergen (CRa) has long been

Abbreviations: Ach, acetylcholine; BAL, bronchoalveolar lavage; CRa, cockroach allergen; EFS, electric field stimulation; LPR, late-phase asthmatic reaction; OA, ovalbumin; PCA, passive cutaneous anaphylaxis; SRaw, specific airway resistance; TSM, tracheal smooth muscle ring.

* Corresponding author. Tel.: +1-859-3235199; fax: +1-859-3231197.

recognized as a major allergen, together with dust mites and domestic pets (Bernton and Brown, 1967; Mendoza and Snyder, 1970; Pollart et al., 1989; Platts-Mills et al., 1991; Call et al., 1992; Gelber et al., 1993). The importance of CRa sensitivity in particular lies in the fact that it is highly prevalent among severe asthmatics of inner-city populations, among people who require frequent emergency-room and hospital visits (Kang et al., 1979, 1992; Sperber et al., 1993). Studies from our laboratory and others have indicated that the presence of CRa at home and other confined environment sensitizes people and causes asthma (Steinberg et al., 1987; Kang et al., 1989; Sarpong et al., 1997). Moreover, it has been proven that inhalation of CRa produces asthmatic responses in the cockroach sensitive subjects with asthma. The cockroach-specific asthmatic responses appear at various time points after inhalation challenge exhibiting immediate, late, dual or persistent pattern along with variable degrees of peripheral eosinophilia (Kang, 1976; Kang et al., 1979, 1992). The significance of CRa in the asthmatic inflammation has been reinforced by the recent National Cooperative Inner-City Asthma Study (NCICAS), in which high levels of CRa in the bedroom is directly linked to the severity of asthma of the children (Rosenstreich et al., 1997). Yet, the study could not show a similar causal relationship with other indoor allergens. The reason(s) is not clear as to why cockroach hypersensitivity is particularly correlated to the severity of asthma in the afflicted. Could it be that CRa is more asthmatogenic than other allergens? Could it be a host factor, i.e. genetically severe asthmatic subjects get CRa sensitization? It is plausible that CRa may possess a certain characteristic that is prone to induce asthmatic airway inflammation to the host.

Previously, we have reported a guinea-pig asthma model sensitized with aerosolized CRa develops asthma-like inflammation, for which the characteristics largely resemble human cockroach asthma (Kang et al., 1995; Zhou et al., 1998). The sensitized animals show antigen-specific airway obstructions at various time points together with an abundance of inflammatory cells in the bronchoalveolar lavage fluid (Chen et al., 1998; Zhou

et al., 1998). Using this asthma model, we aimed to study the difference(s) in asthmatic inflammations induced by CRa as compared to ovalbumin (OA)-induced asthma. The OA was chosen as it is the most commonly used allergen in experimental asthma studies.

2. Materials and methods

2.1. Animals

Male Hartley guinea-pigs (Hilltop Laboratories, Scotsdale, PA), weighing 200–250 g, were randomly assigned to three groups: Saline (control), OA, and CRa. The experiment was carried out with the approval of the Institutional Animal Care and Use Committee at the University of Kentucky.

2.2. Allergen sensitization

Sensitization to either allergens, CRa or OA, was carried out following the method previously described (Chen et al., 1998; Zhou et al., 1998). Briefly, a DeVilbiss ultrasonic nebulizer (Model 100, Somerset, PA) was used to generate and deliver the aerosols of saline, (10 ml), OA (5 mg in 10 ml saline, from Sigma Chemical Co., St. Louis, MO), or CRa (5 mg of lyophilized whole body extract of German cockroach in 10 ml saline, from Greer Laboratories, Inc., Lenior, NC) to a plastic chamber that holds guinea-pigs. The aerosol exposure was carried out for a duration of 45/min, twice a day, 5 days a week, for period of 4 weeks. The development of anaphylactic sensitivity, to either CRa or OA, was measured by both an allergic skin test and passive cutaneous anaphylaxis assay (PCA) for IgG1 and IgE antibodies by the method described (Chen et al., 1998; Zhou et al., 1998). In addition, the anaphylactic antibodies were further analyzed by Western blot.

2.3. Western blot

Allergens, CRa and OA, were separated by SDS-PAGE following Laemmli's method (Laemmli, 1970). Five, 10 and 20 µg of protein of

CRA or OA were separated on a 12% separating gel and then stained with Coomassie Blue R250. In another experiment, 20 µg of protein of CRA or OA was separated and then transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). After blocking with 5% milk in TTBS (1 × TBS with 0.05% Tween-20) overnight at 4°C, the membrane was cut into strips and incubated with 1:500 diluted sera from each group of the guinea-pigs. After thoroughly washing, the strips were incubated with a horse-radish peroxidase (HRP)-conjugated goat antibody against guinea-pig IgG1 (1:5000, Bethy Laboratories, Inc.). The proteins on the membrane were then detected by the ECL system (Amersham, Arlington Heights, IL).

2.4. Measurement of lung function

The changes in lung function of the guinea-pigs were measured in a conscious state using a double chamber-plethysmograph (Buxco Electronics, Inc., Troy, NY) following the method described previously from our laboratory (Zhou et al., 1998).

2.5. Bronchoalveolar lavage (BAL)

At 24 h after antigen challenge in vivo, BAL was performed following the method described previously (Zhou et al., 1998). Hank's balanced salt solution was used instead of normal saline. Approximately 84% of the BAL fluid was retrieved.

2.6. Contractile responses of tracheal rings

After BAL, the trachea smooth muscle (TSM) was prepared and the contractile responses of the TSM to electric field stimulation (EFS), acetylcholine (ACh), and to allergens were measured as the method described previously (Chen et al., 1998). The allergen-induced tracheal contraction was measured as a cumulative dose–response curve of either OA or CRA from 0.01 to 100 µg/ml (final bath concentration) with a 0.5 log unit increment. The time course of the specific allergen-induced tracheal contraction was also determined in a separate experiment by adding a

single dose of OA or CRA (100 µg/ml) to the TSM of OA or CRA group and measuring the contraction continuously up to 15 min post-antigen treatment. The antigen-induced contraction was expressed as a percentage of the maximal contraction induced by ACh (3×10^{-5} M) in the same TSM. The viability of the TSM was confirmed by the response to ACh challenge at the end of each experiment.

2.7. Statistic analysis

Data are expressed as the mean ± S.E. A statistical analysis was performed using analysis of variance (ANOVA), corrected with repeated measurements when appropriate. The main effect was considered significant at $P < 0.05$. A further post-hoc analysis was conducted using the Student–Newman–Keuls test. Student's *t*-test was used for a simple mean comparison between two groups. The difference between means was significant at $P < 0.05$.

3. Results

3.1. Anaphylactic antibodies

As shown in Table 1, the saline sensitized guinea-pigs showed a negative reaction to CRA and OA by both the skin test and the PCA assay. The CRA and OA sensitized showed very similar levels of “wheal-flare” reactions to their respective allergens. There was no cross-reactivity between the two allergens.

Western blot analysis revealed that CRA contained several distinctive allergens, at least seven allergen bands (approximately 203, 75, 70, 60, 55, 45 and 40 kDa) from 27 different protein bands ranging from 200 to 7 kDa identified by the pool of serum of CRA sensitized guinea-pig (Fig. 1). The major CRA allergen(s) was located around 60–70 kDa (Fig. 2, lane 3), whereas the OA contained a major protein band at about 40–50 kDa. (Fig. 1), which appears to be the major allergen (Fig. 2, lane 2). There was no cross reacting allergen between OA (lane 1) and CRA (lane 4).

Table 1

Results of anaphylactic antibodies measured by the skin test and PCA assay in the saline-, CRa- and OA-sensitized guinea-pigs (mean $\pm$ S.E.)^a

Animals sensitized by	Skin test ($n = 6$)					PCA assay ($n = 5$)	
	Saline	Histamine	Allergen (OA or CRa)			IgG1	IgE
			0.5 μ g	1.0 μ g	5.0 μ g		
Saline	0	12.7 $\pm$ 0.3	0	0	0	0	0
CRa	0	13.1 $\pm$ 0.3	9.2 $\pm$ 0.3	11.6 $\pm$ 0.2	12.5 $\pm$ 0.2	70 $\pm$ 29.7	0
OA	0	12.5 $\pm$ 0.2	10.7 $\pm$ 0.4	12.1 $\pm$ 0.3	12.8 $\pm$ 0.4	128 $\pm$ 19.6	0

^a Skin-test results are presented as diameters of wheals (mm) at 60 min post-saline, histamine or CRa injection. PCA results are presented as titers of PCA.

3.2. Asthmatic responses of guinea-pigs to allergen challenges

Fig. 3 shows that there are increased specific airway resistances (SRaw) noted in both CRa-sensitized guinea-pigs and OA-sensitized guinea-pigs following their respective antigen challenges when compared to the control group. Using a criterion of changes in SRaw equal to or greater than 150% of the baseline, both CRa and OA groups showed airway obstructions in the early phase of the observation. However, the increased SRaw of the CRa group persisted during the next 8 h, while that of the OA group returned to baseline ($P < 0.01$). In the CRa group, four out of six animals that showed early increases in SRaw continued to have mild and sustained increases in SRaw in the late phase. The increases were peaked at a period between 4 and 7 h and lasted up to 24 h post-CRa challenges in one half of the CRa sensitized animals. One of the CRa sensitized guinea-pigs had an isolated early phase response. In the OA group, four out of six OA-sensitized animals showed an isolated increase in SRaw in early phase, and one guinea-pig showed an isolated increase in SRaw in the late phase without early phase.

3.3. Inflammatory cellular responses in the BALF

Analyses of bronchoalveolar lavage fluid revealed that CRa challenges induced a marked influx of inflammatory cells in the CRa sensitized

animals as compared to those of OA challenges in the OA sensitized animals ($P < 0.001$, Table 2). The increases were due mostly to neutrophils and eosinophils in the CRa group. Whereas, OA challenges in the OA sensitized animals induced significant increases in the numbers of eosinophils, yet total leucocytes in BALF were not significantly different from those of the saline group. Differences in inflammatory cells noted in BALF between CRa and OA-sensitized and -challenged guinea-pigs showed the CRa to have an overwhelming presence of neutrophils.

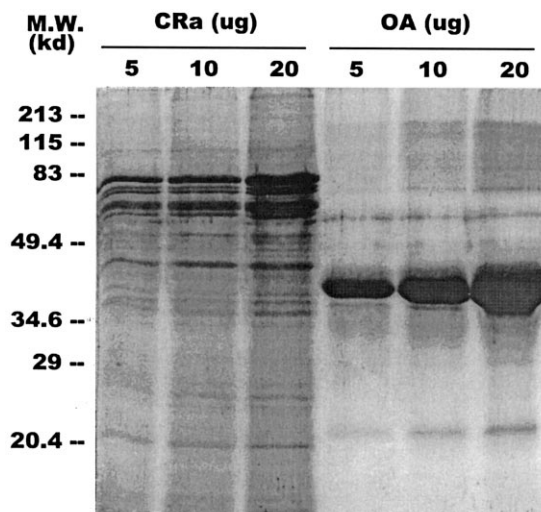


Fig. 1. Allergens (CRa and OA) separated by SDS-PAGE. Five, 10 and 20 μ g of proteins per lane of CRa or OA were used on a 12% separating gel under reducing condition and then stained with Coomassie Blue R250.

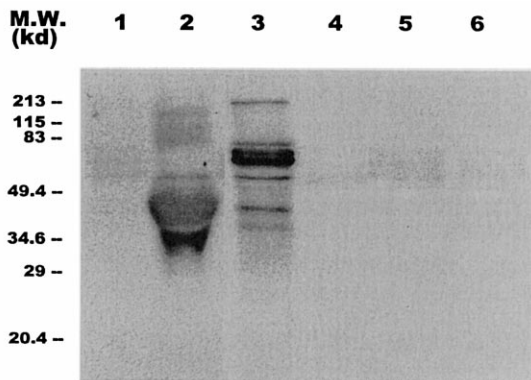


Fig. 2. Western blot analysis of CRa and OA reactive IgG1 antibodies from three groups of guinea-pigs. SDS-PAGE separated CRa (lane 1, 3, 5) and OA (lane 2, 4, 6) were immunoblotted with sera from OA- (lane 1, 2), CRa- (lane 3, 4) or saline- (lane 5, 6) sensitized guinea-pigs.

3.4. Contractile responses of tracheal smooth muscle *in vitro*

The contractile responses of the tracheal smooth muscle ring (TSM) to EFS (data not shown) and to ACh (Fig. 4) were similar in both CRa- and OA-sensitized groups as well as those in saline-sensitized group. The TSMs of CRa or OA sensitized guinea-pigs, however, responded with dose-dependent contractions to the additions of

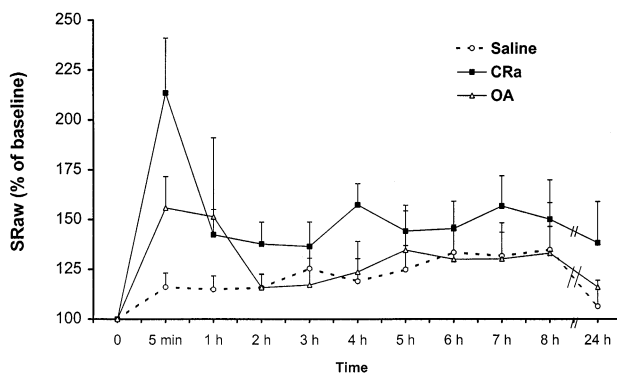


Fig. 3. Changes in specific airway resistance (SRaw) following saline, CRa or OA challenges in saline-, CRa- and OA-sensitized guinea-pigs. The SRaw data were expressed as a percentage of baseline. Vertical bars represent the standard error of the mean. Repeated-measurement ANOVA revealed significant differences among all the groups: $P = 0.0006$ (CRa vs. OA), $P = 0.0001$ (CRa vs. saline), $P = 0.0293$ (OA vs. saline).

Table 2

Inflammatory cells (total and differential leukocyte counts) in bronchoalveolar lavage fluid at 24 h post-allergen challenge in saline-, CRa- and OA-sensitized guinea-pigs^a

	Saline	CRa	OA
Total leukocyte ($\times 10^6$)	3.4 ± 0.6	$27.0 \pm 2.8^{***}$	3.5 ± 0.5 # # #
Neutrophil (%)	7.5 ± 2.5	$62.6 \pm 8.9^{***}$	13.3 ± 4.0 # # #
Lymphocyte (%)	12.7 ± 4.7	4.1 ± 1.5	12.8 ± 5.9
Macrophage (%)	74.7 ± 6.3	$16.0 \pm 3.2^{***}$	$41.8 \pm 5.8^{**}$ # #
Eosinophil (%)	4.8 ± 1.4	$17.3 \pm 5.2^*$	$32.2 \pm 5.0^{***}$ #

^a The BALF were obtained from saline-, CRa- and OA-sensitized guinea-pigs. The data are mean $\pm$ S.E. ($n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. saline group. # $P < 0.05$, # # $P < 0.01$ and # # # $P < 0.001$ vs. CRa group.

their respective allergens. There was no cross-reactive contraction noted. The allergen-specific contractile responses were significantly different ($P = 0.0001$) between the CRa-sensitive and OA-sensitive TSMs (Fig. 5); The OA group started with a brisk contraction to OA at the minimal concentration ($0.01 \mu\text{g/ml}$), contracted progressively more strongly to the additions of higher

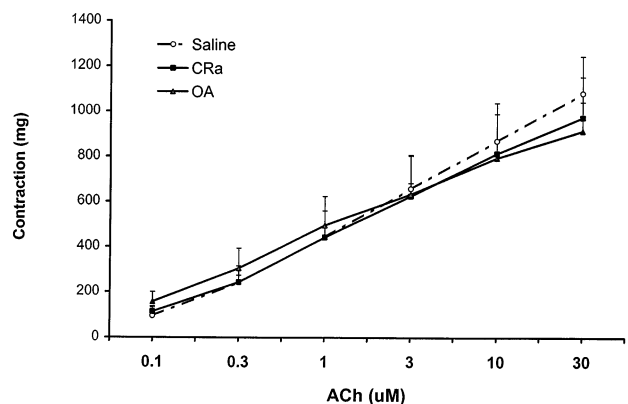


Fig. 4. Cumulative dose-response curves of tracheal smooth muscles (TSM) to acetylcholine. The TSMs were isolated from saline-, CRa- and OA-sensitized and challenged guinea-pigs. Individual TSM was stimulated with cumulative doses of ACh *in vitro*. The data are expressed as isometric tension. Vertical bars represent the standard error of the mean.

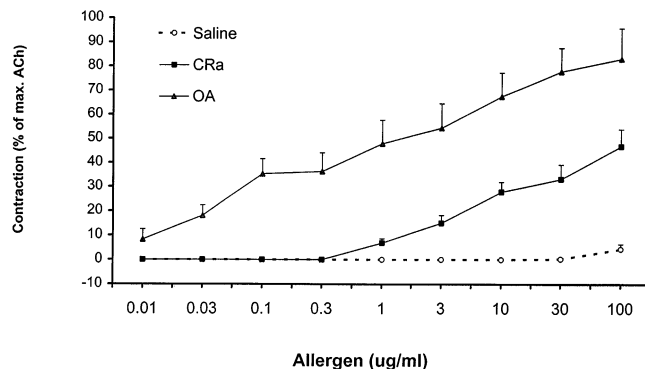


Fig. 5. Cumulative dose–response curves of tracheal smooth muscles (TSM) to CRa or OA challenge. The TSMs were isolated from CRa- or OA-sensitized guinea-pigs 24 h after the respective allergen challenge. The data are expressed as a percentage of the maximal contraction of ACh (3×10^{-5} M). Vertical bars represent the standard error of the mean. Repeated-measurement ANOVA revealed significant differences ($P < 0.0001$) among all the groups.

concentrations of the antigen, OA, 100 $\mu\text{g/ml}$, and induced a peak TSM contraction that was 83% of the maximal ACh-induced contraction, whereas, the TSM of CRa group contracted mildly and slowly to the additions of CRa, started at 1 $\mu\text{g/ml}$ or more, and reached 47% of the maximal ACh-induced contraction at the highest level of the CRa (100 $\mu\text{g/ml}$). A similar phenomenon was noted in the single contraction studies as well: the TSM of the OA-sensitized guinea-pigs showed a more robust and prolonged contraction as compared to the response noted on the TSM of the CRa-sensitized guinea-pigs (Fig. 6).

4. Discussion

The present study demonstrates that CRa and OA produce different types of asthma-like airway obstructions in their respectively sensitized guinea-pigs irrespective of their similar levels of anaphylactic sensitizations. CRa induces exuberant airway inflammation that corresponds with either dual-phase- or late-phase airway obstruction (LPR), while OA induces mild airway inflammation with early-phase bronchospasm. This is in spite of the findings of anaphylactic sensitization, a high titer of anti OA-IgG1 antibodies and brisk OA-specific contractions of TSMs noted in the OA guinea-pigs. The persistently increased SRaw, a sign of airway obstruction with its correspond-

ing leucocytosis in BALF in the CRa group is similar to the data previously reported from our laboratory (Zhou et al., 1998). Evidence of anaphylactic sensitizations, the findings in vitro; CRa-specific IgG1 antibodies and TSM responses to CRa, is also similar to those reported (Chen et al., 1998; Zhou et al., 1998). However, the OA-sensitive animals sensitized in the same method show an absence of LPR. It is unclear as to why there are LPRs in the CRa group or as to why

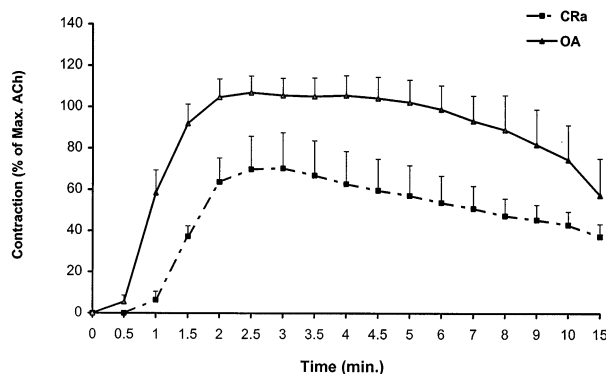


Fig. 6. Tracheal smooth muscle (TSM) contraction curves to a single-dose CRa or OA stimulation. The TSMs were isolated from CRa- and OA-sensitized, and challenged guinea-pigs ($n = 6$) at 24 h post-allergen challenge were stimulated with a single dose of CRa or OA (100 $\mu\text{g/ml}$). The data are expressed as a percentage of the maximal contraction of ACh (3×10^{-5} M). Vertical bars represent the standard error of the mean. Repeated-measurement ANOVA revealed significant differences ($P < 0.001$) between the two groups.

OA animals lack LPR at present. It is plausible, however, that a relatively small amount of OA used and/or not using an adjuvant during the sensitization period might be related to a mild airway response in the OA. Yet, the fact that the OA-sensitized animals show very strong signs of adequate anaphylactic sensitization *in vitro* negates the argument (see Table 1 and Figs. 4 and 5).

Antigen-dependent differences have been noted in the asthma-like airway obstructions in various experimental asthma. There are also reports of similar absences of late-phase airway obstruction in the OA-aerosol-sensitized guinea-pigs (Iijima et al., 1987; Underwood et al., 1992). Underwood et al. (1992) studied the effects of three allergens (OA, house dust and *Ascaris suum*) in guinea-pigs and noted that OA induced only an early asthmatic response, while house dust induced no bronchospasm. In the same model, *Ascaris suum*-sensitized guinea-pigs show a mild persistent airway obstruction in a few animals. The discrepancy in the airway obstructions, however, could not be accounted for by the influx of eosinophils in BAL as all three antigen challenged animals exhibited a similar influx of inflammatory cells. Arakawa et al. (1995) also reported that dust mite (Df) and TMA-sensitized guinea-pigs showed slowly progressing bronchoconstrictions to both antigen challenges, while OA challenge induced only brisk bronchoconstriction in the early phase. Dual-phase asthmatic responses, however, have also been reported in the OA-aerosol-sensitized guinea-pigs (Larsen et al., 1984; Hutson et al., 1988; Sagara et al., 1997). Such a response is often associated with eosinophilia in BALF (Larsen et al., 1984; Boushey and Holtzman, 1985; Gundel et al., 1990; Sagara et al., 1997).

The late phase-asthmatic response is well known to accompany influx of inflammatory cells, neutrophils, eosinophils and mononuclear cells into the airways in experimental asthma, although degrees of cellular responses vary widely in different reports. Iijima et al. (1987) reported a similar leucocytosis in BALF in the *Ascaris suum*-sensitized guinea-pigs, which showed a persistent airway obstruction. Even in some studies, OA

sensitization with high concentrations, there have been appreciable degrees of neutrophilia and marked eosinophilia noted in BALF at 24 h when those animals showed dual-phase airway obstruction (Boushey and Holtzman, 1985). Hsiue et al. (1997) reported a similarly intense influx of both eosinophils and neutrophils in BALF by crude dust-mite extract, a natural allergen, and in those of the OA guinea-pigs. In this study, however, the inflammatory cellular responses noted between CRA and OA groups are strikingly different (see Table 2). The noted leucocytosis with marked neutrophilia and eosinophilia noted in the CRA-sensitized guinea-pigs appears to be a CRA-dependent phenomenon, and it correlates well with the LPR noted in the CRA animals. By contrast, the OA-sensitized guinea-pigs failed to elicit LPR, although they had moderate eosinophilia. The neutrophil-rich leucocytosis has been observed as early as 6 h after CRA challenge in the CRA-sensitized guinea-pigs and has been found to last longer than 48 h (Zhou et al., 1998). The phenomenon is also dependent on the amount of CRA used during the sensitization period and/or amount of CRA used in the challenge phase (Kang, Zhou and Chen, unpublished). The noted association between neutrophil influx and the late phase airway obstruction suggests that the presence of neutrophils in the airways is likely responsible for the appearance of LPR in the CRA-sensitized animals, and the lack of neutrophils for the lack of LPR in the OA-sensitized guinea-pigs.

Infiltration of neutrophils, along with eosinophils, in the airway wall and in BALF has been frequently associated with LPR to allergen provocation (Boushey and Holtzman, 1985; Wilson et al., 1986). The neutrophilia associated with the late-phase airway obstruction is largely an endothelial ELAM-1-dependent process (Gundel et al., 1991). Neutrophils in the endothelial layer are capable of releasing preformed mediators from their granules, such as neutrophil elastase and metalloproteinases, as well as de-novo synthesized oxidative products and eicosanoids (Wilson et al., 1986; Gundel et al., 1991). Consequently, the neutrophil products contribute to allergen-induced asthmatic inflammation and

have a deleterious influence on airway function of the sensitive hosts (Weiss, 1989).

The clear mechanism(s) underlying the observed differences between two antigen-induced experimental asthma models is not clear. Yet, the complexity of the allergen through multiple antibodies produced in the host may be responsible; OA-sensitized guinea-pigs developed a high titer of IgG₁ antibody against a major allergenic band, while the CRa-sensitized guinea-pigs carried multiple IgG₁ antibodies to several similarly strong allergenic bands (see Fig. 2). Several different anaphylactic antibodies, with perhaps low affinities, against several allergenic bands of a given complex allergen such as CRa or *Ascaris suum* may induce variable intensities of low grade allergen-antibody reactions. Consequently, they could create sequential and/or synergistic reactions in vivo where an influx of inflammatory cells is readily available. In other words, a sum of cumulative anaphylactic inflammations may result in the form of LPR, and this comes from the heterogeneity of the antibodies rather than the level of an antibody. However, we cannot rule out the fact that the neutrophils are attracted by a contaminant substance such as polysaccharides in the CRa exerting added inflammation directly to the sensitized animals. A further investigation is warranted.

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