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Objective - To characterize the relation between bronchoalveolar and blood eosinophil numbers, serum total IgE concentration, and non specific airway reactivity in healthy dogs.
Animals - 26 healthy Beagles.
Procedure - Prior to measurement of non specific airway responsiveness, dogs were anesthetized and bronchoscopy was performed to recover bronchoalveolar lavage (BAL) fluid. Repeated measurements were made in 6 dogs.
Results - The percentage of blood eosinophils varied between 0 and 13 (mean ± SD, 5.6 ± 3.6) %, the percentage of eosinophils in BAL fluid ranged between 0 and 63.5 (8.8 ± 12.9) %, and total serum IgE concentration was 0.1 to 107.5 (23.40 ± 29.11) U/ml. A strong association was evident between numbers of blood eosinophils and total serum IgE concentration (R^2 = 0.413, P<0.001), and a trend toward an association between numbers of blood eosinophils and numbers of eosinophils in BAL fluid was apparent (R^2 = 0.110, P=0.053). There were no significant associations between any other aspects of the blood and BAL fluid cell profiles and total serum IgE concentration or airway reactivity. Serum total IgE concentration was not associated with airway reactivity. Further, in dogs examined on repeated occasions, variation in BAL fluid eosinophil numbers was not associated with any change in serum total IgE concentration or airway reactivity.
Conclusions - Neither numbers of bronchoalveolar or blood eosinophils nor serum total IgE concentration have a significant role in determining airway reactivity in healthy dogs.

Increases in serum IgE concentration [1,2] and numbers of blood and bronchoalveolar eosinophils [3-6] are important features of asthma in human beings and are thought to be implicated in the pathogenesis of the airway hyperresponsiveness that characterizes this condition. The airway and blood eosinophilia that characterize asthma in human beings correlates with the severity of symptoms and the degree of non specific airway hyperreactivity that develop.[3] Further, degranulated eosinophils are found beneath the bronchial basement membrane and among epithelial cells in asthmatics, and their presence is related to the extent of epithelial damage.[6] Eosinophils are, therefore, thought to have a principal role in the pathogenesis of bronchial asthma, and mediation of their pathogenic effects is thought to be related to the release of cytotoxic proteins, such as major basic protein and eosinophil cationic protein.[7] These proteins are reported to damage and desquamate bronchial epithelium, resulting in an increase of bronchial responsiveness.[8-10] There is a strong relation between serum total IgE concentration and airway responsiveness in children with asthma and in apparently normal children.[2]

Although dogs develop spontaneous allergic disease, predominantly characterized by atopic dermatitis,[11] and lower respiratory tract diseases characterized by eosinophilic infiltrates,[12,13] clinical signs of wheezing similar to those in human asthmatics are seldom recognized in the former. Despite this, dogs have been extensively used as experimental models of human asthma, manifesting, alone or in combination, the features of airway hyperresponsiveness, airway eosinophilia, and increased serum IgE concentration.[14-17,a,b] This apparent dichotomy may arise as a consequence of intense sensitization or lung challenge exposure procedures, or both, used in experimental models. In healthy dogs with airway eosinophilia or increased total serum IgE concentration, or both, lung physiologic and anatomic factors, such as wide airways [18,19] and excellent collateral ventilation, [20] may prevent the clinical

manifestation of wheezing, although airways are indeed hyperreactive. We sought to determine whether bronchoalveolar or blood eosinophilia and total serum IgE concentration are associated with airway hyperreactivity in healthy dogs.

Experimental animals - Twenty-six Beagles, 18 females and 8 males, (age, 2.72 to 8.69 years; bodyweight, 5.8 to 15 kg) from a closed colony at the Institute were selected for study on the basis of representing a range of bronchoalveolar eosinophilia. All dogs were clinically normal at the beginning and throughout the study. They lived in kennel buildings with indoor and outdoor runs. Each dog was fed dry dog food once a day and water was available at all times. Food was withheld for 18 hours prior to administration of the anesthetic agent. At 72 hours prior to measurement of non specific airway responsiveness, dogs were anesthetized, and bronchoscopy was performed to recover bronchoalveolar lavage fluid. As part of a related study examining the seasonal variation in bronchoalveolar eosinophilia, repeated measurements were made in 6 dogs 69 to 216 days after the initial assessment.

Bronchoalveolar lavage - Anesthesia was induced in dogs with isoflurane, and an endotracheal tube was inserted. A flexible fiberoptic bronchoscope (5.5mm OD)[c] was advanced to the right cardiac lobe and wedged in a segmental bronchus. The subtended segment was lavaged by installation and withdrawal of five, 10-ml aliquots of normal saline (0.9% NaCl) solution. Lavage fluid from each lung lobe was separated into supernatant and cells by centrifugation at 200 x g for 10 minutes at 4C. The cell pellets were washed twice in phosphate-buffered saline solution (PBSS), and cells were counted.[d]. Cytocentrifuge slides were prepared and stained, using a modified Wright-Giemsa stain [e] for differential counting of 200 cells. Cells were

classified as neutrophils, macrophages, eosinophils, or lymphocytes, according to standard morphologic criteria.[21] Cells not classified in the aforementioned groups were recorded as 'other' cells. Blood smears were prepared from heparinized jugular vein blood samples drawn immediately prior to lavage and were stained as described previously. Serum was frozen and stored for subsequent assessment of total IgE concentration.

Measurement of pulmonary mechanics - Anesthesia was induced (7mg/kg) and maintained (0.6 to 0.8mg/kg/min) by IV infusion of propofol.[f] After anesthesia induction, an endotracheal tube was placed and connected to a small animal ventilator .[g] Ventilation was maintained with room air at a tidal volume of 15 ml/kg of body weight. Respiratory rate was adjusted [h] to maintain end-tidal CO_2 within the range of 4.5 to 5.5%. An esophageal balloon catheter was positioned in the caudal thoracic portion of the esophagus at a point where fluctuations in esophageal pressure were maximal, and transpulmonary pressure was measured by connecting one side of the esophageal catheter to a differential pressure transducer [i] and the other side to a port at the proximal end of the endotracheal tube. Respiratory flow was measured, using a pneumotachograph (Fleisch No. 1) connected to the endotracheal tube. Outputs from flow and transpulmonary pressure transducers were conditioned, amplified as necessary, and converted from analog to digital form, using appropriate hardware.[j] Custom-designed computer software [k] was used to facilitate integration of the flow signal to yield volume and to derive the pulmonary mechanical indices of resistance (RL) and dynamic compliance (Cdyn). These indices were calculated, using the method of least squares linear regression.[22,23]

Measurement of airway responsiveness - After stabilization of mechanical indices, airway responsiveness was assessed by obtaining dose-response curves to saline and histamine solutions. Solutions of histamine diphosphate[l] were prepared according to accepted guidelines [24] at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, 3, 10, 30, and 100 mg/ml. Aerosols were generated by use of a jet nebulizer[m] connected directly to the endotracheal tube. Five breaths of saline or histamine solution, standardized to an end-inspiratory transpulmonary pressure of 15 cm of H_20, were allowed, and the response was assessed at 2 minutes after the cessation of aerosolization. Values of RL and Cdyn were allowed to recover to within 10% of baseline values prior to subsequent challenge. The concentration of the histamine solution was steadily increased until RL had increased to at least 150% or Cdyn had decreased to below 75% of baseline values or both. The response was quantified by interpolation or extrapolation of the dose-response curves to yield the provocative concentration (PC) of histamine that would be required to induce a 50% increase in RL (PC 150% RL) and a 25% decrease in Cdyn (PC 75% Cdyn) relative to those observed after saline aerosolization. Data were normalized by transforming PC 150% RL and PC 75% Cdyn to Log_{10} PC 150% RL and Log_{10} PC 75% Cdyn, respectively.

Measurement of serum IgE concentration - A direct ELISA was used to measure total serum IgE concentration. Modified flat-bottom 96-well polystyrene microtitration plates[n] were coated with 100 µl of dog serum diluted 1:1,000 in 0.1*M* sodium carbonate buffer (pH 9.6) and were incubated for 16 hours at 4C. After 5 manual wash cycles with 0.15*M* NaCl-0.05% Tween-20, the remaining binding sites in each well were blocked by adding 200 µl of 0.4% bovine serum albumin in PBSS. After incubation at 20C (room temperature) for 2 hours, the plates were again washed 5 times, and 100 µl of mouse anti-dog IgE monoclonal antibody (antibody D9)[25] was

added to each well (at a dilution of 1:2,000) followed by incubation for 2 hours at 37C. After a third wash cycle, 100 µl of donkey anti-mouse IgG horseradish peroxidase-conjugated antibody [o] was added to each well (at a dilution of 1:1,000), and the plates were incubated for 1 hour at 37C. After washing 5 times, 100 µl of 2.2'-azino-di(3-ethyl-benzthiazoline sulfonate) at a concentration of 0.6 g/L in glycine buffer plus 0.02% H_2O_2 in citric acid buffer [p] was added to each well, and the plates were incubated for 60 minutes at 37C. Color development was stopped by adding 100 µl of 5% sodium dodecyl sulfate in water to each well. Plates were read at optical density of 410 nm by use of an automated microtitration plate reader,[q] with subtraction of blank values.

Reference serum obtained as a pool from a number of parasitized dogs with flea infestation or heartworm infection [26] was assigned a value of 100 IgE U/ml and was used to generate a standard curve, from which absorbance values of unknown samples were obtained. To assess the agreement between the aforementioned direct ELISA and a previously reported capture ELISA,[26] 97 serum samples with serum total IgE values ranging from 0 to 132 U/ml were examined by use of both methods.

Analysis of data - Results obtained by the 2 methods were strongly associated ($R^2 = 0.92$, $P<0.0001$). Agreement between the direct and capture ELISAs was assessed using appropriate methods.[27] A plot of the difference versus the mean for the 2 methods indicated that they were in excellent agreement and that, relative to the capture ELISA, the direct method underestimated serum total IgE concentration by 1.24 U/ml (95% CI = -0.26 to 2.73 U/ml). The limits of

agreement (± 2 SD) were from -13.6 to 16.1, and the differences did not vary in any systematic way over the range of measurement (R^2 = 0.004, P = 0.541).

The percentage of blood eosinophils varied between 0 and 13 (mean ± SD, 5.6 ± 3.6)%, and the percentage of eosinophils in BAL fluid varied between 0 and 63.5 (8.8 ± 12.9)%. Total serum IgE concentration varied between 0.1 and 107.5 (23.40 ± 29.11)U/ml. Baseline values for RL and Cdyn were 2.04 ± 0.59 cm of H_20/L/s and 62.2 ± 20.8 ml/cm of H_20, respectively, and the geometric mean(± SD) PC 150% RL and PC 75% Cdyn values were 1.500 ± 2.664 and 1.027 ± 2.368 mg/ml, respectively.

The relation between airway and blood, cellular, and humoral characteristics, and airway reactivity were determined (Table 1). A nonsignificant trend toward an association between numbers of blood and bronchoalveolar eosinophils was apparent (R^2 = 0.11, P=0.053), and a stronger association was evident between numbers of blood eosinophils and total serum IgE concentration (R^2 = 0.413, P<0.001, Fig 1). There were no significant associations between any other aspects of the blood and BAL cell profiles and total serum IgE values or airway reactivity. Serum total IgE concentration was not associated with airway reactivity.

Analysis of results for the 6 dogs was repeated (Fig 2). Although increases in BAL eosinophil numbers in all 6 dogs and less marked increases in blood eosinophil numbers in 5 of the 6 dogs were obvious, there were no concomitant changes in airway reactivity or serum total IgE concentration as assessed visually and through calculation of the Spearman rank correlation coefficient.

Discussion - We examined, in a population of healthy dogs, whether bronchoalveolar or blood eosinophilia and total serum IgE concentration are associated with airway hyperreactivity. The results of this study indicate that the expression of airway hyperreactivity is independent of each of these variables.

Although the percentage of eosinophils in BAL fluid from clinically normal dogs is generally <5%,[21,28] variation in this value may be anticipated in dogs with experimentally induced asthma. Chung *et al,*[14] using a dog model of asthma involving sensitization concomitant with immunization with live attenuated distemper virus,[17] reported eosinophil values of approximately 10 and 1.5% at 2 and 6 hours after allergen exposure, respectively. In another model of asthma in which dogs received repeated intraperitoneal injections of ragweed and aluminum hydroxide at a young age, Becker *et al,*[15] and Baldwin and Becker [29] reported eosinophil values of approximately 11, and 14.3±3.7%, respectively. In dogs naturally sensitized to *Ascaris suum*, Woolley *et al* [30] reported a baseline eosinophil value of 4.6±1.9% for those that developed non specific airway reactivity after inhalation of *Ascaris* antigen. In this study, over half the BAL fluid samples had >5% eosinophils, with over a third of them exceeding 15% eosinophils; thus, the range of bronchoalveolar eosinophilia is at least comparable to that seen after experimental manipulation and should be adequate to investigate the putative role of eosinophils in the pathogenesis of non specific airway reactivity. Further, the concentration of total serum IgE concentration in this population of dogs bears comparison with that reported by Hill *et al.*[31] in a study of immunoglobulin concentrations in healthy, atopic, and parasitized dogs (because those authors used the capture ELISA alluded to previously,[26] their results are directly comparable to those obtained by us). Although those authors found no significant difference in IgE concentration between the groups, the overall range of values (<1 to 105.9 U/ml) indicated

that the population of dogs used in this study had IgE concentration that spanned those anticipated in dogs with atopic or parasitic disease, thus again indicating that the search for a putative association between non specific airway reactivity and total serum IgE concentration should not be inhibited by a narrow range of the latter.

The significant association between numbers of blood eosinophils and total serum IgE concentration indicates the possibility that control of both of these elements is linked. Indeed, in atopic allergy and helminthic infections Th2 lymphocytes preferentially transcribe and translate mRNA for interleukins 4 and 5 [32]; the former is required for the induction of IgE synthesis in B cells, and the latter governs eosinophilic inflammation. There is, therefore, the possibility that the dogs with large numbers of blood eosinophils and high total serum IgE concentration are mounting a Th2-type immune response that is either parasitic or allergic in origin. The association of the blood and bronchoalveolar eosinophils tempts us to speculate that the lung is the primary focus of this response. In this regard, the possibility that parasites, such as the lungworm *Filaroides hirthii,*[33] may have influenced serum IgE concentration and numbers of eosinophils in these dogs cannot be excluded. However, these dogs were obtained from a closed colony where appropriate anthelminthic controls are used and where stringent clinical and pathologic monitoring processes consistently fail to indicate appreciable parasite burden within the population. Equally, the possibility exists that exposure to environmental aeroallergens may have influenced eosinophil numbers and total serum IgE concentration in the study population.

Pulmonary responsiveness ranged from 0.61 to 6.36 mg/ml of histamine for PC Cdyn 75%, and from 0.61 to 17.70 mg/ml of histamine for PC RL 150%. The large between-dog variation in pulmonary responsiveness has been documented [34] and presumably, as it does in human beings,[35] reflects polygenetic control and environmental influence. In this study,

environmental influences, although not controlled, were similar for all dogs, and therefore, the potential influence of this variable on pulmonary hyperresponsiveness should have been minimized. However, the magnitude of genetic variability in pulmonary responsiveness may have precluded, within the larger group, detection of an association between this variable and either eosinophils or IgE. To address this issue, airway reactivity was measured repeatedly in dogs with variability in bronchoalveolar eosinophilia, pulmonary responsiveness being reproducible within dogs over time.[34] However, even within the same dog, there was no correlation between non specific airway reactivity and either total serum IgE concentration or numbers of blood or bronchoalveolar eosinophils.

A number of studies in experimental animals and humans beings confirm that bronchoalveolar eosinophilia is not necessarily accompanied by airway hyperreactivity. Human beings with eosinophilic bronchitis [36] have levels of eosinophil numbers in sputum similar to those in asthmatic patients, yet do not have airway hyperresponsiveness. Further, allergic guinea pigs have bronchoalveolar eosinophilia in the absence of hyperresponsiveness,[r] and airway eosinophilia does not appear to be related to responsiveness in sensitized rats [37] and monkeys [38] exposed to chronic antigen inhalation.

In *Ascaris suum*-sensitized dogs, the number of bronchoalveolar eosinophils has a role in determining the degree of non specific airway hyperresponsiveness [30,39] that is induced after airway challenge exposure with this allergen (ie., an additional component relating to local challenge exposure with the specific allergen is required for induction of airway hyperresponsiveness). It remains to be elucidated whether, in this species, allergen challenge exposure results in eosinophil activation and release of granule proteins with their anticipated effects on bronchial epithelium.[8-10] However, there is much evidence from studies in other

species to suggest that this is the case, and it is the intensity of activation of the airway eosinophils, rather than their numbers, that has a role in the development of airway hyperresponsiveness.[40-42] One can, therefore, speculate that, in this study, absence of airway hyperreactivity in the dogs with bronchoalveolar eosinophilia was a consequence of insufficient eosinophil activation.

Evidence is accumulating to suggest that IgE may have a direct role in the induction of airway hyperresponsiveness. Sensitizing dogs at an early age by intraperitoneal injections of ragweed results in high serum total IgE concentration.[43] Airway hyperresponsiveness is apparent prior to airway exposure to the allergen,[a] and *in vitro* studies have indicated increased shortening velocity and capacity of bronchial smooth muscle from these dogs.[44] Further studies using this model have established an increase in myosin light chain kinase content and resultant ATPase activity in airway smooth muscle cells from sensitized dogs.[45,46] There is some evidence to suggest that the IgE *per se* is capable of conferring an inherent change in the contractility of airway smooth muscle,[47, s] and that this direct effect contributes to the association between serum total IgE concentration and airway reactivity in human beings.[2]

The population of dogs in this study had IgE concentrations that span those anticipated in dogs with atopic or parasitic disease. An increase in an unspecified antigen-specific clone or a polyclonal activation of B lymphocytes producing IgE may contribute to the increased values observed. Despite the abundance of IgE in some dogs, association was not observed between serum total IgE concentration and airway reactivity. Our findings bear comparison with those of a study [48] where, in dogs actively sensitized to ragweed, significant relationship could not be documented between non specific airway reactivity and antigen-specific serum IgE.

It is possible that the potential role of IgE in this regard is influenced by structural and functional heterogeneity of the molecule itself. Alternatively spliced forms of human IgE have been identified and characterized,[49] and additional studies confirm presence of these isoforms in human beings with atopic dermatitis and the hyper-IgE syndrome.[50,51] Different conformation and glycosylation patterns between isoforms will influence binding to low- and high-affinity IgE receptors [49] and possibly activate different signal transduction mechanisms leading to functional heterogeneity. Such heterogeneity may contribute to the failure to observe any association between serum total IgE concentration and airway reactivity in this study.

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Footnotes

a. Lemen R, Kunke K, Bice D, et al. Airway eosinophilia and hyperresponsiveness to ragweed (RW) or histamine (H) bronchoprovocation in RW sensitized beagle puppies. *Am J Respir Crit Care Med* 1995;151:A438(Abstract)

b. Woolley MJ, Lane CG, Ellis R, et al. Airway cellular composition prior to allergen inhalation influences the development of airway responsiveness in dogs. *Am Rev Respir Dis* 1993;147:A857(Abstract)

c. Model BF-4B2, Olympus Corp, Lake Success, NY.

d. Model ZBI cell counter, Coulter Electronics, Hialeah, Fla.

e. Diff-Quik; Baxter Healthcare, McGraw Park, Ill.

f. Diprivan; Stuart Pharmaceuticals, Wilmington, Del.

g. Model 613, Harvard Apparatus, South Natick, Mass.

h. LB2, Medical Gas Analyser, Beckman Instruments, Palo Alto, Calif.

i. Model MP45, Validyne, Northridge, Calif.

j. AT-MIO-16, National Instruments, Austin, Tex.

k. LabView 3.0.1, National Instruments, Austin, Tex.

l. Sigma Chemical Co, St Louis, Mo.

m. No. 950, Hospitak, Lindenhurst, NJ.

n. Easy Wash, Corning Inc, Corning, NY.

o. Jackson ImmunoResearch Laboratories Inc, West Grove, Pa.

p. ABTS Peroxidase Substrate System, Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md.

q. MR700, Dynatech Laboratories Inc, Chantilly, Va.

r. Aoki S, Boubekeur K, Kristersson A, et al. Is allergic airway hyperreactivity of the guinea-pig dependent on eosinophil accumulation in the lung?. *Br J Pharm* 1988;94:365P(Abstract)

s. Rabe KF, Morton B, Dent G, et al. Increased responsiveness to histamine, anti-IgE and allergen after passive sensitization of human airways *in vitro*. *Eur Respir J* 1993;6:481s(Abstract)

Table 1 - Association between serum total IgE concentration (U/ml), blood and bronchoalveolar eosinophils (total cell numbers), and airway reactivity as assessed by $\log_{10}$ PC 75% Cdyn and $\log_{10}$ PC 150% RL.

				Eosinophils	
Variable	**Total IgE (U/ml)**	**$\log_{10}$ PC 75% Cdyn**	**$\log_{10}$ PC 150% RL**	**Bronchoalveolar**	**Blood**
Total IgE (U/ml)	-	-	-	-	-
$\log_{10}$ PC 75% Cdyn	$R^2 = 0.003$ $P = 0.767$	-	-	-	-
$\log_{10}$ PC 150% RRL	$R^2 = 0.018$ $P = 0.461$	$R^2 = 0.51$ $P < 0.001$	-	-	-
Eosinophils					
Bronchoalveolar	$R^2 = 0.040$ $P = 0.242$	$R^2 = 0.00$ $P = 0.979$	$R^2 = 0.01$ $P = 0.524$	-	-
Blood	$R^2 = 0.413$ $P < 0.001$	$R^2 = 0.03$ $P = 0.369$	$R^2 = 0.02$ $P = 0.472$	$R^2 = 0.11$ $P = 0.053$	-

Pearson's product moment correlation coefficient was calculated for each relation; R^2 - coefficient of determination, P = probability.
Log_{10} PC 75% Cdyn = $\log_{10}$ concentration of histamine aerosol required to reduce lung dynamic compliance to 75% of the value obtained after saline aerosol administration.
Log_{10} PC 150% RL = $\log_{10}$ concentration of histamine aerosol required to increase lung resistance to 150% of the value obtained after saline aerosol administration.

Figure Legends

Figure 1 - Association between serum total IgE concentration (U/ml) and blood eosinophils (EOS, total cells/ml). The solid circles represent individual measurements made in each of 20 dogs. The 12 open circles represent repeated measurements for 6 dogs. The solid line represents the line of least squares linear regression The positive association between the variables is strongly significant ($P < 0.001$).

Figure 2a-e: Dot plots representing the concomitant variation in bronchoalveolar lavage (BAL) fluid and blood eosinophils, serum total IgE concentration, log PC 75% Cdyn, and log PC 150% RL in 6 dogs in which repeated measurements were made as part of a related study examining seasonal variation in bronchoalveolar eosinophilia. Values were assigned to either low (Lo) or high (Hi) groups on the basis of the subsequent change in bronchoalveolar eosinophilia seen between measurements. There are no concomitant changes in airway reactivity or serum total IgE concentration as assessed visually. Spearman rank correlation coefficients confirm the lack of significant association between these variables. See Table 1 for key to variables.

Figure 1

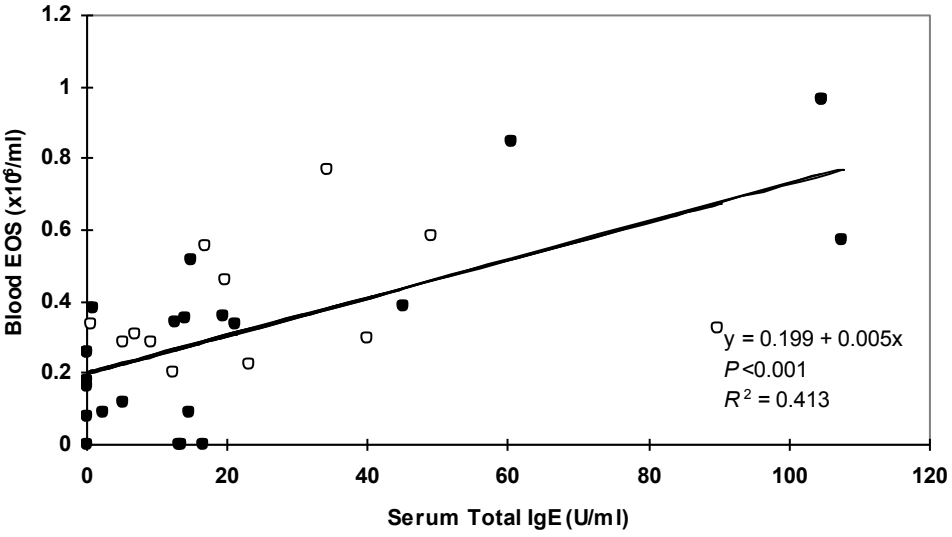
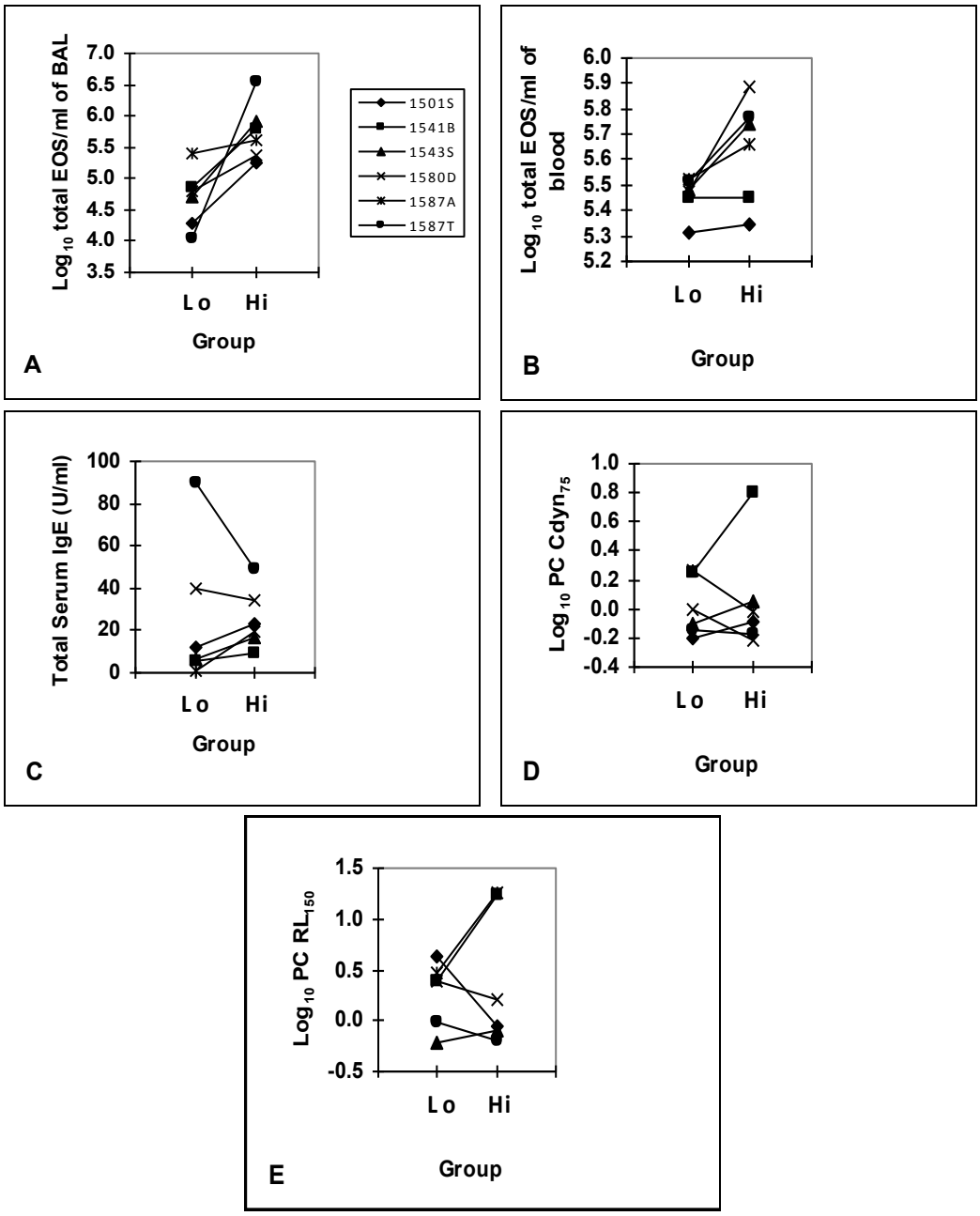

1.2
1
0.8
0.6
0.4
0.2
0
Blood EOS (x10⁶/ml)
0
20
40
60
80
100
120
Serum Total IgE (U/ml)
y = 0.199 + 0.005x
P<0.001
R² = 0.413

Figure 2a-e


Log10 total EOS/ml of BAL
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
Lo
Hi
Group
A
1501S
1541B
1543S
1580D
1587A
1587T
Log10 total EOS/ml of blood
6.0
5.9
5.8
5.7
5.6
5.5
5.4
5.3
5.2
Lo
Hi
Group
B
Total Serum IgE (U/ml)
100
80
60
40
20
0
Lo
Hi
Group
C
Log10 PC Cdyn75
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
Lo
Hi
Group
D
Log10 PC RL150
1.5
1.0
0.5
0.0
-0.5
Lo
Hi
Group
E

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