

Covalent binding of proteins to grafted plastic surfaces suitable for immunoassays. II. Picograms of IgE detected in BAL fluid in sarcoidosis

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Received 12 June 1997; revised 1 September 1997; accepted 17 October 1997

Abstract

An ELISA is described for the quantitation of minute amounts of human IgE using microtiter strips which were surface modified according to a procedure previously described. This ELISA used an IgG fraction of a rabbit polyclonal anti-IgE antibody coupled to the carboxylated surface, which prior to coupling had been preactivated with a water soluble carbodiimide. A biotinylated affinity purified rabbit anti-IgE antibody was used together with streptavidin conjugated alkaline phosphatase and biotinylated enzyme for the detection of bound analyte. Sarcoidosis is a systemic disorder of as yet unknown cause with epithelioid granuloma formation in the lung as a prominent feature. With the bronchoalveolar lavage technique, epithelial lining fluid was obtained and analyzed for IgE content since differences of immunoglobulin concentration in bronchoalveolar lavage fluid (BALF) may reflect disease activity. Our modified ELISA system was used for measuring IgE in BALF from healthy controls and sarcoid patients. The assay using modified strips demonstrated a considerably improved sensitivity compared to the use of physical adsorption of first antibody to untreated strips. The detection limit of the improved assay was approximately 3 pg IgE/sample (100 μ l). Quantitative recovery of IgE was demonstrated within the measuring range (51.2–12 500 pg IgE/ml) and more than 40% (23/54) of the sarcoid patients showed values above the lowest standard concentration (51.2 pg/ml). In contrast, none of the healthy controls (0/22) had detectable IgE as defined by the detection limit of the assay. © 1997 Elsevier Science B.V.

Keywords: Solid phase; Covalent; Crotonic acid; Grafting; ELISA; IgE; Bronchoalveolar lavage fluid; Sarcoidosis

Abbreviations: PS, polystyrene; CA, crotonic acid; EDAC, 1-ethyl-3(dimethylamino-propyl)carbodiimide HCl; PBS, phosphate buffered saline; SA, streptavidin; AH-BNHS, amino-hexanoyl-biotin-N-hydroxy-succinimide ester; ALP, alkaline phosphatase; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; NRG, normal rabbit IgG

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1. Introduction

In two previous papers (Larsson et al., 1987, 1989) a simple method was described for the chemical modification of commercially available plastic materials commonly used as solid phases in immunoassays. This method, referred to as grafting, was used to prepare polystyrene surfaces with carboxylic groups, resulting from a free radical driven reaction between the surface and an unsaturated carboxylic acid (crotonic acid). The reaction was initiated and mediated by ^{60}Co -irradiation. The chemistry of protein/peptide linking to matrix carboxylic groups is well documented in the literature and we have chosen a simple approach to achieve covalent protein coupling using a water soluble carbodiimide. In this paper we describe the use of our surface modification protocol to achieve a sensitive and reproducible IgE ELISA.

Sarcoidosis is a multisystem disorder of as yet unknown cause. In its initial stage it contains the features of a local inflammatory process characterized by the presence of inflammatory activated lymphocytes and macrophages within the alveolar space (Crystal et al., 1981) and formation of non-caseating epithelioid cell granulomas (James et al., 1976). T cells bearing the CD4 marker can be seen closely associated with epithelioid cells in the granuloma center, while both CD4 and CD8 are observed at the periphery (Cambell et al., 1985). One typical immunological abnormality in peripheral blood and bronchoalveolar lavage fluid (BALF) from sarcoidosis patients is hyperglobulinemia (Hunninghake and Crystal, 1981). The numbers of cells secreting IgG/IgM recovered in BALF from patients with pulmonary sarcoidosis do not differ from controls despite the elevated Ig BALF levels. This finding suggests a localized interstitial Ig synthesis (Hance et al., 1988).

During the last decades bronchoalveolar lavage (BAL) has frequently been used to study pathogenic mechanisms of interstitial lung disorders. The BAL fluid is a very diluted sample and the analysis of non-cellular constituents requires the use of very sensitive assays. In allergic asthma, cytophilic IgE in the lung plays a central role as an inflammatory initiator. Although no causal extrinsic factor(s) is established for sarcoidosis we sought to investigate

whether IgE in BALF could be demonstrated and quantitated in patients with sarcoidosis. Using our ELISA, IgE could be detected in BALF in more than 40% of patients with sarcoidosis (23/54) but was not detected in the healthy controls (0/22).

2. Materials and methods

2.1. Solid phases

Nunc-Immuno Maxisorp PS modules (Nunc, Roskilde) were used for grafting or were left untreated as controls. These modules will be referred to as strips.

2.2. Reagents, buffers and protein preparations

Graft monomer: 10% (w/w) CA (trans-2-butenic acid, purum, Fluka, Buchs) in methanol/water 1:1 (w/w). Carbodiimide coupling reagent: EDAC (1-ethyl-3(dimethylamino-propyl)carbodiimide HCl, Bio-Rad, Richmond, CA). Carbodiimide coupling buffer: 3 mM potassium phosphate pH 6.3. Passive adsorption buffer: 0.1 M sodium carbonate/bicarbonate pH 9.6. Washing buffer: 2.5 mM sodium phosphate, 0.15 M NaCl, 0.02% NaN_3 and 0.1% Tween 20 (Kebo Lab, Stockholm) pH 7.4. Quenching buffer: PBS, 0.1% NaN_3 and 0.2% casein (Sigma Chemical, St. Louis, MO) pH 7.4. Assay buffer: identical to quenching buffer but also containing 0.1% Tween 20. ALP-substrate and buffer: 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical, St. Louis, MO) in 1 M diethanolamine pH 9.8. The following biological reagents were used: casein from bovine milk (Sigma Chemical, St Louis, MO), human IgG (Kabi, Stockholm), rabbit anti-human IgG (Fc_γ , Dakopatts, Glostrup), rabbit anti-human IgE (Fc_ϵ , specific and non-reactive against other isotypes as measured by immunoelectrophoresis) and normal rabbit IgG which were in-house preparations of Na_2SO_4 -precipitated and subsequently DEAE Sepharose fractionated sera. Affinity purified rabbit anti-human IgE ($\text{D}_{\epsilon 2}$ -specificity) was the kind gift of

Assistant Professor T. Karlsson, Pharmacia (Uppsala). Alkaline phosphatase (calf intestine, enzyme immunoassay quality) was purchased from Boehringer Mannheim (Mannheim). Amino-hexanoyl-biotin-*N*-hydroxy-succinimide ester (AH-BNHS), streptavidin conjugated alkaline phosphatase and biotinylated alkaline phosphatase were all purchased from Zymed Labs (San Francisco, CA).

2.3. Subjects

Fifty-four patients (14 smokers; 18 females) with untreated, histologically proven sarcoidosis, median age 35 (range 24–64) years and 22 healthy volunteers (10 smokers; 10 females), median age 26 (range 20–43) years participated in the study. Chest radiographic stages of sarcoidosis patients were: stage 0 ($n = 4$), stage I ($n = 13$), stage II ($n = 16$) and stage III ($n = 21$). The study had the approval of the local Ethics Committee. All subjects gave their informed consent.

2.4. Bronchoalveolar lavage (BAL) and handling of BAL-fluid

BAL was performed as described previously (Eklund and Blaschke, 1986). Briefly, a flexible fiberoptic bronchoscope was wedged in a middle lobe bronchus under local anesthesia with 2% lignocaine after premedication with morphine and scopolamine. Sterile saline solution (5×50 ml) at 37°C was instilled and gently suctioned back after each instillation. The recovered fluid was collected in a siliconized plastic bottle kept on ice. The fluid was centrifuged at $400 \times g$ for 5 min at 4°C. Then the supernatant was decanted and stored at -70°C until further analyzed.

2.5. Grafting procedure

The PS strips were carboxylated using the grafting protocol as described by Larsson et al. (1987) with some slight modifications. Essentially, the strips were immersed in oxygen depleted CA solution in a plastic container attached to the ^{60}Co reactor. The container was sealed under N_2 and then irradiated. The received dosage of radiation was approximately

3 Mrad. Thereafter, the strips were extensively washed with distilled water and also stored in water at 4°C until used.

2.6. Preparation of ELISA conjugates

Human IgG was conjugated to alkaline phosphatase according to the one step glutaraldehyde method described by Avrameas (1969). Rabbit anti- $\text{D}_{\text{E}2}$ was biotinylated according to the protocol accompanying the AH-BNHS product.

2.7. Functional test of grafted and control strips

Evaluation of the coupling time and retained functional activity of the immobilized antibody was achieved as follows: one set of grafted strips was preactivated with EDAC (10 mg/ml) in coupling buffer (100 μl /well) for 60 min at ambient temperature. The strips were thereafter washed 3 times with distilled water and incubated with 100 μl /well of rabbit anti-human IgG at 10 μg /ml in coupling buffer at ambient temperature. The same amount of antibody in coupling buffer containing EDAC at 5 mg/ml was also added to another set of grafted strips. Passive adsorption on control strips was run in parallel. The incubation was terminated after 10, 15, 20, 25, 30, 45, 60 min and 2, 3, 4, 5, 6, 7, 8 and 24 h by washing 3 times and adding 100 μl /well of quenching buffer. All assay points were run in quadruplicate. All strips up to 8 h were stored with quenching buffer at 4°C overnight. The following day the 24 h strips were incubated with quenching buffer for 60 min at ambient temperature. After additional washing 100 μl /well of human IgG-ALP, appropriately diluted in assay buffer, was added to all strips which were then incubated for 60 min at ambient temperature. All strips were then washed and subsequently 100 μl /well of substrate was added. After approximately 30 min absorbance values at 405 nm (using 620 nm as reference wavelength) were measured using an SLT 210.1 ELISA reader (SLT Labinstruments, Grödig).

2.8. Modified IgE ELISA

To grafted strips, preactivated with EDAC as mentioned above, 100 μl /well of the IgG fraction

of rabbit anti-human IgE (10 $\mu\text{g/ml}$), in coupling buffer, was added. Passive adsorption to control strips was run in parallel. The coupling reaction was allowed to proceed overnight at 4°C. All washes were performed 3 times and all incubations were done at 37°C for 60 min. The sample volume used was 100 μl /well. As standards appropriate dilutions of the reference IgE from the standard curve of Phadebas IgE RIA (Pharmacia, Uppsala) was used. This was also used for preparing the low, intermediate and high controls. All necessary dilutions were made in assay buffer. The following day the strips were washed and incubated with quenching buffer. After washing, the strips were incubated with standards, controls and samples. Thereafter, the strips were washed and incubated with the appropriately diluted biotinylated affinity purified anti-IgE. In two subsequent separate steps appropriately diluted SA-ALP and biotinylated ALP were added. The colorimetric reactions were developed and readings were performed as above with the exception that a PC-interfaced Anthos 2001 reader (Ekoscann, Upplands Väsby) was used. For quantitative evaluation we used a 4-parameter curve fit supplied in the ELISA + program (Meddata, New York, NY) that was used for the on-line collection and processing of raw data. Seven of the patient samples with IgE values significantly above the lowest standard point were tested for the presence of heterophilic antibodies with anti-rabbit activity, which in our system could produce falsely elevated values. Those samples were diluted in assay buffer with or without the addition of 0.1% NRG and then reassayed.

2.9. Unmodified IgE ELISA

The unmodified ELISA was performed on untreated microtiter strips. The methodology was identical to that of the modified assay except that the coating step was done using the carbonate/bicarbonate buffer.

2.10. Statistical analyses

Standard descriptive statistics and linear regression analysis was used for evaluation and comparisons of collected data. The Mann–Whitney non-

parametric ranking test was used for the analysis of the samples tested for the presence of heterophilic antibodies. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Functional characteristics of modified versus control strips

Grafted strips either preactivated or used with EDAC present during the coupling reaction both displayed faster binding kinetics as compared to the passive adsorption controls (Fig. 1). Approximately 80–90% of the rabbit anti-human IgG functional activity was bound within 10 min to the modified strips. For the passive adsorption controls this required approximately 30 min. However, there was also a clear discrepancy between the overall results of the two different coupling protocols used for the grafted strips. The preactivation procedure retained an overall higher level of functional activity, whereas when EDAC was present in solution functional activity decreased with time. On the basis of this observation the preactivation protocol was chosen as the more appropriate one for further studies of solid phase immobilization of antibodies.

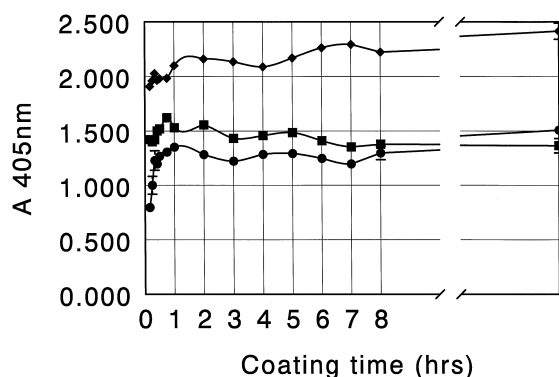


Fig. 1. Functional coupling capacity and dependency of coating time of grafted versus control strips. Detection of bound rabbit anti-human IgG with human IgG-ALP. Grafted strips preactivated with EDAC (♦); grafted strips with EDAC present in coupling solution (■); untreated strips using passive adsorption immobilization of rabbit antibody (●). Standard deviation of quadruplicate samples is less than symbol size.

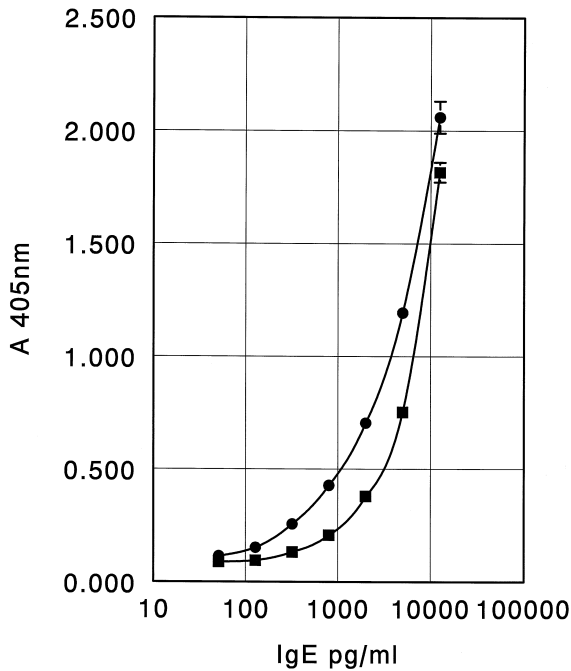


Fig. 2. Comparison of standard curves of IgE ELISA. Unmodified ELISA was performed on untreated (■) strips and modified ELISA on grafted strips (●). Symbols and vertical bars indicate standard points and their standard deviation respectively.

3.2. Modified IgE ELISA specifications

Generally, we obtained an up to 5-fold increase of sensitivity using EDAC preactivated modified strips

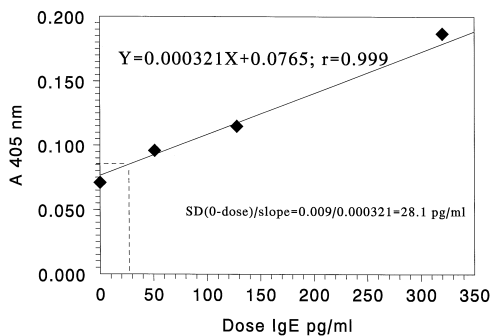


Fig. 3. Determination of detection limit of the modified IgE-ELISA. Zero dose (blank) and absorbance values for the 3 lowest standard IgE concentrations are plotted as dose versus absorbance in a linear regression plot for 10 assays. The detection limit is given by the SD (zero dose) divided by the slope of the regression line.

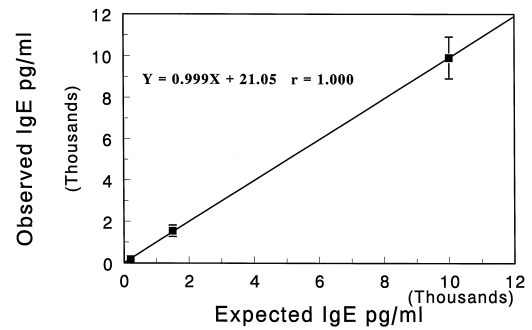


Fig. 4. Precision profile of the modified ELISA ($n = 7$ assays). Reference concentrations of IgE were 200, 1500 and 10000 pg/ml.

versus passive adsorption controls (Fig. 2). The detection limit of the assay was approximately 30 pg IgE/ml (3 pg/100 μ l sample) as defined by the standard deviation of dose measurements at zero dose (Ekins et al., 1989). The value of 30 pg/ml is given by the standard deviation ($n = 10$ assays) of the signal response divided by the dose–response curve slope at zero dose (Fig. 3). The highest standard 12500 pg/ml was always within the exponential part of the standard curve. Typically, the intra-assay coefficient of variation (CV%) was 5% (5 experiments) and the interassay variation (CV%) did not exceed 12% within the 200–10000 pg/ml range (Fig. 4). Recovery within the 300–11250 pg/ml range was 101–107% (Fig. 5).

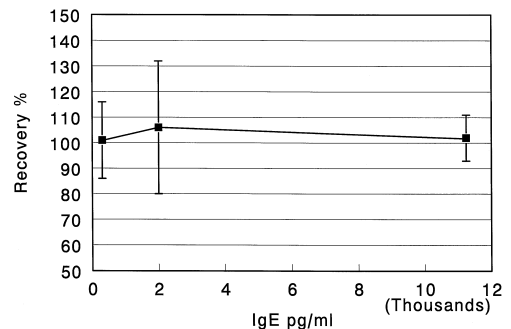


Fig. 5. Recovery of IgE in the modified ELISA ($n = 7$ assays) at 3 reference IgE concentrations of 300, 2000 and 11250 pg/ml. Vertical bars indicate standard deviation.

3.3. Performance of IgE-ELISA and measurements of BALF

The described assay model was used for measuring IgE levels in BALF from healthy controls and sarcoidosis patients respectively. Of the 22 controls tested none was found to have IgE values above the detection level of the assay. However, 23 out of 54 patients (42.6%) exhibited values above the lowest standard point (Fig. 6). The arithmetic mean of these was 227 pg/ml and the median was 113 pg/ml (range: 52.2–936.6 pg/ml). As these findings could be influenced by the presence of heterophilic antibodies to rabbit IgG in the patient samples, seven of those showing the highest values were tested for such antibodies. There was good agreement between sample values obtained with or without the addition of normal rabbit IgG to the sample diluent ($r = 0.98$). A nonparametric ranking test (Mann–Whitney) indicated no difference between the values ($p = 0.655$).

23 patients were found to have detectable IgE levels, using the modified ELISA, but in approximately half of the patients IgE could not be detected

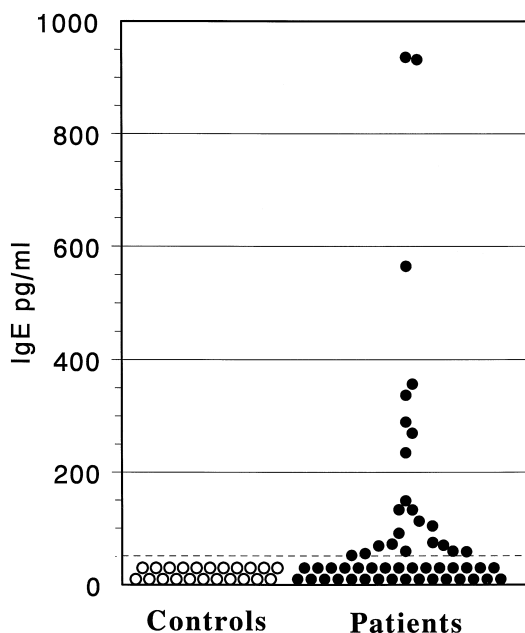


Fig. 6. Results of IgE quantitation in bronchoalveolar lavage fluids from healthy controls (○) ($n = 22$) and sarcoidosis patients (●) ($n = 54$) using the modified ELISA.

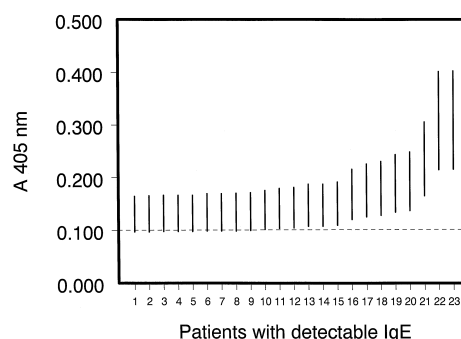


Fig. 7. Hi-low absorbance plot of 23 sarcoidosis patients with detectable IgE as defined by the modified ELISA. Lower end of bars correspond to absorbance values as read by the unmodified ELISA. Higher end of bars correspond to absorbance values as read by the modified ELISA. Broken line defines the absorbance detection limit of the unmodified ELISA using the same cut off criteria as for the modified assay.

using the less sensitive unmodified assay (Fig. 7). This protocol only permitted accurate quantitation of BALF IgE from a lowest standard concentration of 320 pg/ml, rendering 5 out of the 54 patients 'positive'. These 5 values were in close agreement with the corresponding values obtained using the modified assay ($r = 0.99$, data not shown).

4. Discussion

Using a sensitive ELISA measurable levels of IgE were demonstrated in 23 out of 54 BALF samples obtained from patients with sarcoidosis compared to nondetectable levels in all 22 healthy controls. We have used the grafting method to prepare crotonic acid modified microtiter strips and compared them to untreated strips in terms of coating efficiency and functional capacity. Modified strips were then used/tested for specific functional performance in the IgE ELISA.

IgE-measuring assays may often differ in protocol and reagent composition and this may affect both reliability and sensitivity especially when minute amounts of the analyte is measured (Helm et al., 1986; Massicot and Ishizaka, 1986). Several approaches have been published with the aim of improving the detection system in order to increase the

sensitivity of ELISA type assays. One of the most interesting examples is the enzyme amplification immunoassay described by Stanley et al. (1985). This assay protocol has also been described as 'enhanced ELISA' by Macy et al. (1988). However, it is our belief that every component in a high sensitivity ELISA system should be considered, including the quality and performance of the solid phase. By introducing carboxylic groups on a polystyrene surface its innate hydrophobic properties are changed and it becomes hydrophilic. This can be measured in a simple way by determination of the contact angle as described by Dahlgren and Sundqvist (1981). A hydrophilic surface will generally be less prone to non-specific interactions/binding of components through physical adsorption. Using covalent immobilization of ligands to such a surface will generally yield a better signal/noise ratio. By employing this strategy we have improved the sensitivity of our 'in-house' IgE ELISA and thus been able to accurately quantitate low amounts of the analyte.

We hypothesize that the improved performance of our modified assay may basically relies on the fact that the coating antibody/ligand is irreversibly attached to the solid phase. The multilayer detection system (streptavidin-enzyme + biotin-enzyme) used may lead to shedding of bound immune complexes from the plastic surface if noncovalent coating has been employed (unpublished findings). A functional monolayer of immobilized IgG antibody amounts to approximately 400 ng/cm². That amount is reached for both types of coating procedures (adsorption/covalent). The issue at stake would then be the stability of the coated solid phase (Lehtonen and Viljanen, 1980) and we believe that we have improved that aspect.

Grafting techniques and the necessary equipment should generally be available in most larger research departments. Hospitals may use commercially available ⁶⁰Co-reactors for large scale sterilization of instruments etc. In later studies (unpublished) we have found that microtiter plates and strips grafted according to our protocol can be washed and stored dry at room temperature for at least 18 months. This was established by binding experiments and monitoring the contact angle (Larsson et al., 1987). Different commercially available grafted/surface modified microtiter plastics include the following: Nunc (Co-

vaLink™), Pierce Chemical (Reacti-Bind™) and Corning Costar (Covalent Assay Plates).

Alveolitis is a prominent feature in various interstitial disorders. Sarcoid alveolitis is characterized mainly by the presence of activated lymphocytes and macrophages (Crystal et al., 1981). Lymphocytes have been reported as predominantly T helper cells of the Th1-type (Walker et al., 1994). According to other investigators an accumulation of Th1 cells and intermediate Th0–Th1 cell types occurs at the site of granuloma formation, whereas in the alveolar lumen high numbers of both Th1 and Th2 cells can be found (Bäumer et al., 1997). However, the lymphocytic alveolitis in sarcoidosis seems not to be dominated by T-cells of the Th2-type which is normally associated with stimulation of IgE production. It was recently reported that the imbalance in Th1- and Th2-like activity in the sarcoid lung can be restored by treatment with corticosteroids (Milburn et al., 1997).

It has been demonstrated that mast cells produce cytokines with a Th2 like profile (Bradding et al., 1992). There is thus a possibility that mast cells have contributed to the stimulation of IgE production in sarcoidosis. This hypothesis is partly supported by the finding of an increased number of mast cells in BAL fluid in this disease (Bjermer et al., 1987). In the present study serum/plasma levels of IgE were not measured. Results from Walker et al. (1994) indicate that the IgE plasma concentration is normal in sarcoidosis and others have found that the skin response to anti-IgE is normal in these patients (Stålenheim et al., 1996). This may suggest that the increased IgE in BAL fluid from the sarcoidosis patients is locally produced. We therefore hypothesize that the increased levels of BALF IgE found in sarcoidosis is stimulated by mast cells located in the airways and alveolar space. Significant histamine release from the pulmonary mast cells of sarcoidosis patients has been demonstrated, suggesting a role in the pathogenesis of the disease (Gaik et al., 1992).

Crimi et al. (1983) demonstrated a correlation between total IgE in BALF and sera from asthmatics and healthy controls. This was interpreted as reflecting a simple exchange of immunoglobulins over the alveolar capillary membrane. Whether this is caused by a non-specific stimulation of alveolar cells or generated by a more specific stimulus in these pa-

tients remains unclear. Possibly the increase could be caused by a leakage of IgE from blood to alveoli. It has also been reported that mast cells present in BALF from sarcoidosis patients are significantly correlated with the presence of CD3+ and CD4+ lymphocytes, thus suggesting an ongoing immune reaction at the alveolar level (Rottoli et al., 1988). It is possible that the IgE measured in solution derives from mast cells and therefore may actually reflect the intensity of the ongoing disease. If that is the case, it may imply that our assay is of therapeutic value. However, further and expanded studies would be needed to validate that hypothesis. The clinical relevance of our findings in this paper remains to be further elucidated.

Acknowledgements

This study was supported by grants from the Swedish Medical Research Council (no. 16X-105), the Swedish Work Environment Fund (84/1302), the Swedish Heart–Lung Association and internal grants from the Karolinska Institute. The authors also wish to express their appreciation of the excellent technical support from Mrs. Helene Bröchner.

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