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Detection of immunoglobulins G and A to *Aspergillus fumigatus* by immunoblot analysis for monitoring Aspergillus-induced lung diseases

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ABSTRACT: The aim of the study was to investigate whether patients with Aspergillus-induced lung disease can be monitored by immunoblot analysis to detect antibodies to *Aspergillus fumigatus* (Af).

Immunoblotting was performed by incubating 57 longitudinally collected sera from 13 patients on nitrocellulose sheets, blotted with Af antigen, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Bound antibodies were demonstrated by peroxidase-labelled antihuman immunoglobulins (Ig)G and IgA antiserum and diaminobenzidine plus H_2O_2 as substrate. The immunoblot patterns were related to the patients' clinical status and time.

Each patient had a characteristic immunoblot pattern that varied with time. There was a relationship between disease activity or clinical response and changes in immunoblot antibody patterns: a rise in anti-Af IgG and IgA antibodies was seen in sera collected during active disease, compared with before active disease, and a significant decline in anti-Af IgG and IgA was demonstrated in sera collected during recovery, compared with during active disease. Only in the acute stage of allergic bronchopulmonary aspergillosis were IgA antibodies against Af antigens of <20,000 Da demonstrated.

Immunoblot analysis can be used to monitor the disease activity and the responses to treatment of patients with Aspergillus-induced lung diseases. Changes in specific immunoglobulin A may be more informative than specific immunoglobulin G.
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Aspergillus can induce a spectrum of lung diseases, which require aggressive treatment in order to prevent irreversible lung damage. Although patients with Aspergillus-induced lung diseases have to be monitored carefully, no single tool is adequate to evaluate the clinical response to treatment. Since the presence of antibodies to Aspergillus is either a feature of or one of the criteria for the diagnosis of Aspergillus-induced lung diseases [1], changes in the levels of specific antibodies could be used to monitor disease activity. Immunodiffusion and enzyme-linked immunosorbent assay (ELISA) are the most widely used methods to detect antibodies to *Aspergillus fumigatus* (Af). However, immunoblotting has the advantage of revealing additional information about the relevant reactive antigens [2, 3] and can, for instance, be used to demonstrate the presence of antibodies to specific Af antigens [3–9]. In general, the antibody response is determined by several factors, such as genetic background, dose and nature of antigens, and route of entry. For example, immunization of animals by the nasal (mucosal) route results in high amounts of specific immunoglobulin (Ig)A antibodies in saliva and bronchial secretions and in specific IgG antibodies in the blood [10, 11]. Inhalation of Aspergillus can result in similar antibody responses. Allergic bronchopulmonary aspergillosis (ABPA) is suggested to be more of a mucosal disease, whereas Aspergillus infection is more of a parenchymal disease, and thus the antibody response is different [1]. Assessment of total IgE and specific IgE antibodies has been successfully used to monitor patients with ABPA [12, 13]. In general, no or less specific IgE antibodies are detected in patients with aspergilloma and Aspergillus infection.

To investigate whether there is a relationship between disease activity and changes in immunoblot antibody patterns, IgG and IgA anti-Af antibodies were analysed in consecutively obtained sera from patients with Aspergillus-induced lung diseases.

Materials and methods

Patients and materials

Sera (n=57) from 13 patients with Aspergillus-induced lung diseases were analysed. Sera were collected at different time intervals and stored at -70°C, until immunoblot analysis was performed. Sera were taken from five patients with ABPA, four patients with aspergilloma, and four

patients with Aspergillus infection and/or invasive aspergillosis. The relevant medical history of the patients is given in table 1, while clinical and laboratory data are presented in table 2.

Patients were considered to have ABPA if the following criteria were met at longitudinal evaluation: asthma, peripheral blood eosinophilia, infiltrates on chest radiography, immediate cutaneous reactivity to Af, serum precipitins to Af, elevated total serum IgE, proximal bronchiectasis, and elevated serum IgE and IgG antibodies to Af [12, 13]. The diagnosis of aspergilloma was confirmed by typical radiographic findings and either positive precipitins against Af or culture of Af from sputum or tissue obtained by biopsy from the suspected lesion [14]. Patients were considered to be infected with Aspergillus if there was culture and histological evidence of Aspergillus in deep-organ biopsy specimens taken while the patient was alive [9].

Table 1. – Medical history of the patients

Patient No.	Relevant medical history and clinical features
1	Allergic asthma
2	Allergic asthma, restrictive prednisone management because of tuberculosis with resection
3	Allergic asthma
4	Allergic asthma, later bullous deformation of the lungs. Diagnosis M. Bechterew
5	Allergic asthma, tuberculosis, later bullous deformation of the lungs
6	Tuberculosis at the age of 24 yrs, treated by resection. Later recurrence of tuberculosis with cavity. Also diagnosis of benign paraproteinaemia
7	Presentation with pulmonary nodule in left upper lobe. Peroperative diagnosis of aspergilloma, some bullous deformation of the lung. No history of tuberculosis
8	Tuberculosis at the age of 15 yrs. First aspergilloma at the age of 45 yrs with successful resection of left upper lobe. Later second aspergilloma with left-sided pneumonectomy
9	Tuberculosis with many cavities
10	Tuberculosis and COPD-emphysema
11	Bullous emphysema with many courses of prednisone therapy
12	COPD-emphysema, complicated pneumonia with pneumothorax
13	Complicated pneumothorax, later pleurectomy

COPD: chronic obstructive pulmonary disease.

Methods

Aspergillus fumigatus antigen. A purified, standardized antigen was provided by ALK/Diephuis Laboratories (Groningen, the Netherlands). Af was cultured on a semisynthetic liquid medium and Aspergillus was harvested, concentrated, dialysed (molecular weight cut-off 10,000 Da) and freeze-dried, as described by KAUFFMAN and DE VRIES [15].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous buffer system was performed, as described by LAEMMLI [16]. A vertical slab gel electrophoresis unit was used (LKB-2001; Pharmacia LKB Biotechnology, Uppsala, Sweden). The stacking gel (top) was 3% acrylamide, pH 6.8, and the separating gel was 12% acrylamide, pH 8.8. The gels in a glass sandwich were positioned in a tank with buffer (Tris 25 mM, glycine 192 mM, pH 7.35, with 0.5% SDS (w/v)). The antigen was dissolved in 1.00 mL demineralized water and 1.50 mL buffer (0.06 M Tris, 10% glycerol, 2% SDS, 0.001% bromophenol blue; (w/v) Merck, Darmstadt, Germany) was added. For later IgG immunoblot purposes 2.5 mg antigen was dissolved; for IgA immunoblot purposes 10.0 mg antigen was dissolved. This solution was heated at 90°C for 10 min, cooled and centrifuged at 5,500×g. Then, 1 mL of supernatant was distributed on the polymerized stacking gel (width of well 12.0 cm). One separate small well was filled with molecular marker (14,000–97,400 Da; BioRad Laboratories, Hercules, CA, USA). The gels were run at 100 mA at 8°C, using an electrophoresis power supply (LKB-2197; Pharmacia).

Immunoblot analysis. The method of electrophoretic transfer on to nitrocellulose was based on the technique des-

Table 2. – Clinical and laboratory data of the patients

Patient No.	Age yrs	Diagnosis	Year of diagnosis	Sera*	Serum precipitins to Af	Blood eosinophils mm^{-3}†	Total IgE‡ $IU \cdot mL^{-1}$	RAST#	Immediate skin-test Af	Asp§	Radiograph¶
1	51	ABPA	1979	4	pos	2176	6700	4	pos	pos	inf
2	73	ABPA	1991	3	pos	1721	1060	3	pos	pos	inf
3	27	ABPA	1975	5	pos	2300	8000	4	pos	pos	inf
4	48	ABPA	1989	4	pos	300	3000	3	pos	neg	inf
5	63	ABPA	1980	7	pos	600	13000	4	pos	pos	inf
6	51	asp-oma	1989	3	pos	NE	NE	0	NP	neg	asp-oma
7	73	asp-oma	1989	4	pos	NE	NE	0	NP	pos**	nodule
8	53	asp-oma	1990	5	neg	NE	NE	0	pos	pos	asp-oma
9	49	asp-oma	1991	3	pos	NE	NE	0	pos	pos	asp-oma
10	67	asp-inf	1989	4	pos	600	NE	0	pos	pos	inf
11	56	asp-inf	1989	4	pos	NE	1720	2	NP	pos	inf
12	67	asp-inf	1991	4	neg	NE	1900	0	neg	pos	inf
13	43	asp-inf	1992	7	pos	900	390	0	NP	pos	inf

Af: *Aspergillus fumigatus*; IgE: immunoglobulin E. *: Number of sera used for longitudinal evaluation; †: normal range: 40–420 $\cdot mm^{-3}$; ‡: normal value: <100 $IU \cdot mL^{-1}$; #: Af-IgE: radioallergosorbent-test (0: <0.35; 1: 0.35–0.70; 2: 0.70–3.50; 3: 3.50–17.5; 4: >17.5 RAST units $\cdot L^{-1}$); §: culture of Af or histological demonstration of Af hyphae; ¶: radiographical demonstration. pos: positive; neg: negative; NE: not elevated; NP: not performed; ABPA: allergic bronchopulmonary aspergillosis; asp-oma: aspergillosis; asp-inf: aspergillus infection; inf: infiltrate. **: after surgery histological analysis of lung tissue revealed in aspergilloma.

cribed by TOWBIN *et al*. [17]. Blotting was performed in a semidry blot system (LKB-2117; Pharmacia). Blotting time was 1 h. Nitrocellulose sheets (Sleicher and Schuell, Dassel, Germany) were cut into 5 mm wide strips in the direction of electrophoresis. To determine the transfer of the separated proteins, one slice of nitrocellulose was stained with silver stain (Amersham International, Amersham, UK). Protein bands were detected with molecular weights ranging from 90,000 to 10,000 Da. Nitrocellulose strips were blocked with 5% milk powder in phosphate-buffered saline (PBS)-Tween 20 0.5% for 1 h at room temperature. Then the strips were incubated with patients' sera, diluted 1:100 in dilution buffer (2% (v/v) normal rabbit serum, 0.5% milk powder in PBS-Tween 20 0.5% (v/v)) for 2.5 h at room temperature. The strips were washed in washing buffer and PBS-Tween 20. Next, the nitrocellulose strips were incubated with peroxidase-labelled rabbit antihuman Ig (Dako, Glostrup, Denmark) (α-IgG diluted 1:3,000; α-IgA diluted 1:1,000) in dilution buffer (2% (v/v) normal rabbit serum in PBS-Tween 20 0.5% (v/v)) for 90 min at 37°C. After washing the strips, bound antibodies were visualized by incubating the strips with diaminobenzidine 0.05% + H_2O_2 0.01% in substrate buffer for 30 min at room temperature. Colour development was stopped by washing the strips twice with demineralized water. The strips were air dried.

The optimal dilution of serum and conjugate for the detection of antibodies against Af was determined in pilot studies. Positive and negative controls were included, which indicated that only interactions between the specific bound conjugate and the substrate resulted in stained bands.

Analysis of results

Qualitative analysis of immunoblot patterns. The number of visible bands in the immunoblots was counted and molecular weights were estimated relative to those of the molecular markers used as reference.

Quantitative analysis of band intensity. A band-intensity rating scale was used to evaluate semiquantitatively the different antibody responses to Af [18]. The scale ranged from 0 (no visible band) to +4 (dark, well-demarcated band). Two investigators separately interpreted the immunoblot patterns. If bands were scored differently, the investigators re-evaluated the bands to reach agreement. Tandem schematic and semiquantitative drawings of the IgG and IgA immunoblot patterns of individual patients were made. For illustration purposes, four gradations of grey to black were chosen, with light grey corresponding to an intensity of +1 and black corresponding to +4. Total counts of intensities of blots are reported in the top of the drawing (fig. 2).

Statistical analysis

Where available, immunoblot patterns at three stages for each patient were taken into consideration for statistical analysis. Stage 1: 4 months before active disease (preactive); stage 2: during active disease or at diagnosis (active); and stage 3: at least 4 months after active disease, when the patient showed a clinical response to treatment (recovering). In order to investigate whether significant differences were present between preactive and active moments, and between active and recovering moments, for each patient one IgG and one IgA immunoblot at the three defined stages were used to count the intensities of all bands in each immunoblot. Data are expressed as means. Variables that were not normally distributed were compared by means of the centred Signed Rank test, as provided by the SAS univariate procedure (SAS Institute, Cary, NC, USA). Changes are presented as median differences with 95% confidence intervals (CI).

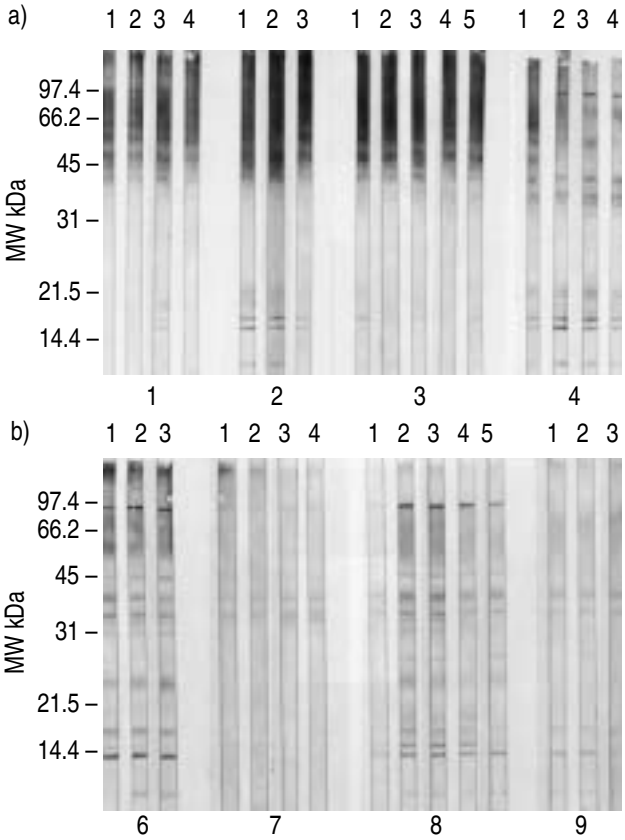

Fig. 1. – Immunoglobulin G immunoblot patterns of a) patients 1–4 and b) patients 6–9. a) Patient 1: serum 1: June 1987 ABPA remission; 2: April 1989 remission; 3: December 1989 exacerbation; 4: April 1991 remission. Patient 2: serum 1: January 1991 ABPA acute; 2: February 1991 ABPA acute, during treatment; 3: September 1992 remission. Patient 3: serum 1: February 1989 ABPA exacerbation; 2: June 1989 remission; 3: October 1989 exacerbation; 4: November 1991 remission; 5: March 1992 remission. Patient 4: serum 1: July 1989 ABPA-acute; 2: September 1989 ABPA acute, during treatment; 3: January 1990 remission; 4: September 1992 remission. b) Patient 6: serum 1: April 1989 aspergilloma, no surgical treatment; 2: June 1991 aspergilloma, embolization; 3: April 1992 aspergilloma, stable disease. Patient 7: serum 1: September 1989 aspergilloma, preoperative; 2: December 1989 aspergilloma, postoperative; 3: June 1991 aspergilloma, postoperative; 4: March 1992 aspergilloma, postoperative. Patient 8: serum 1: May 1987 aspergilloma, postoperative 4 years; 2: May 1990, recurrent aspergilloma; 3: October 1990 recurrent aspergilloma, postoperative; 4: April 1991 aspergilloma, postoperative; 5: August 1992 aspergilloma, postoperative. Patient 9: serum 1: March 1991 aspergilloma, peroperative, amphotericin B; 2: April 1991 aspergilloma, postoperative, itraconazole; 3: May 1991 aspergilloma, postoperative, itraconazole. MW: molecular weight.

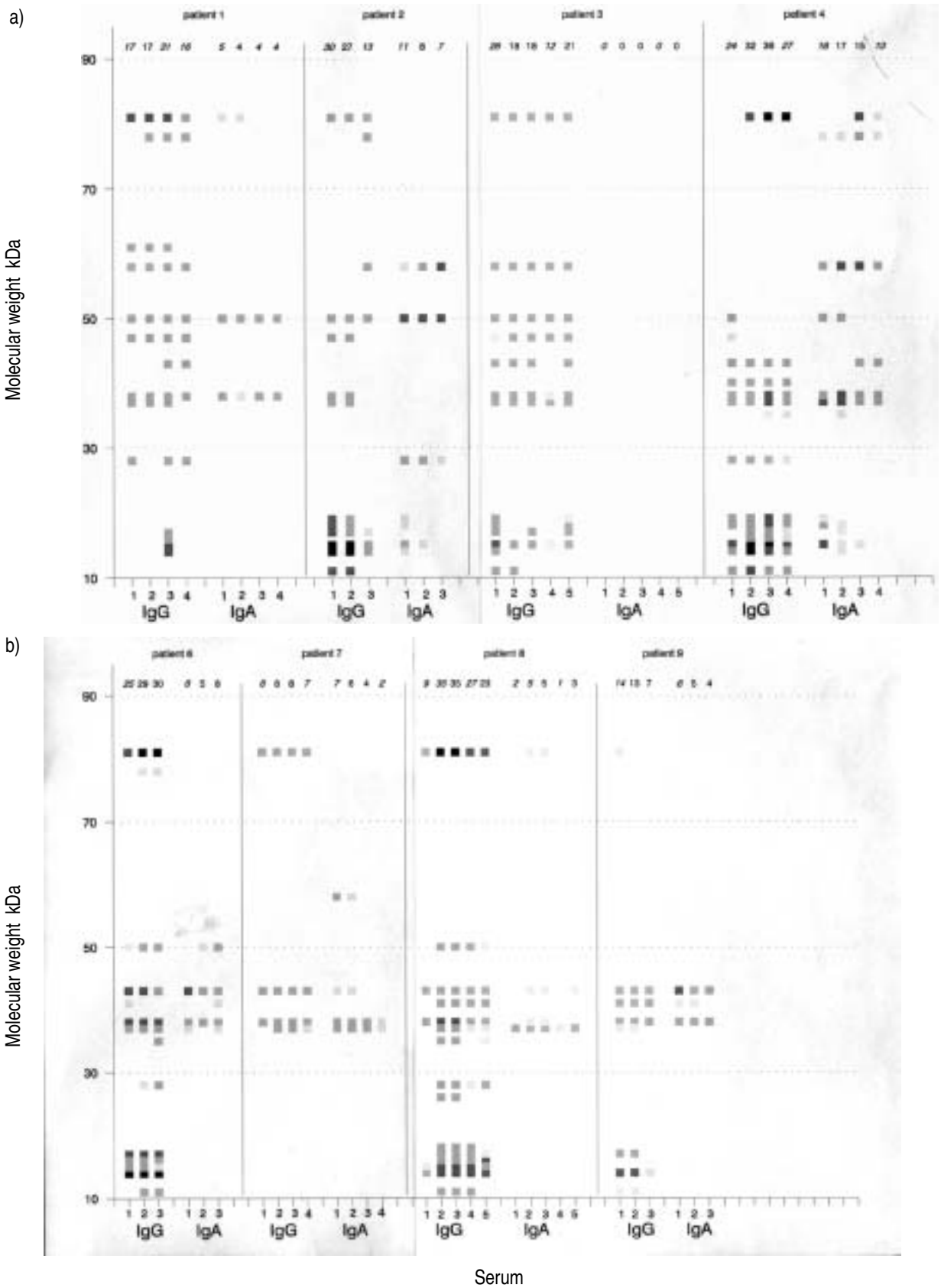

Fig. 2. – Number and intensity of classified bands of the consequent immunoglobulin (Ig)G and IgA immunoblots of individual patients with a) allergic bronchopulmonary aspergillosis (ABPA) and b) aspergilloma. Total intensities are given at the top of each immunoblot. Italic counts represent defined moments and were used for statistical analysis.

Results

The sera of all patients showed individual immunoblot patterns. Patients' sera reacted with 21 different Af antigens, but there were specific individual patterns. Figure 1 shows the IgG immunoblot patterns of four ABPA patients and four aspergilloma patients, with corresponding clinical data. Figure 2 shows the tandem graphic presentation of the IgG and IgA immunoblots of these patients, using the intensity rating scale.

During acute and exacerbation stages of ABPA, the immunoblots contained more bands and more intensely staining bands than during a remission phase. In the acute stage of ABPA (cases 2 and 4) IgA antibodies to Af antigens <20,000 Da were detected. Although patient 3 had an exacerbation of ABPA, no specific IgA could be detected. The beginning of exacerbation of ABPA in patient 5 was recognized retrospectively by an increase in IgG antibodies against Af antigens of <20,000 Da and by an increase in total IgE. Treatment of precipitin-positive ABPA patients led to a reverted precipitin test, but new disease activity was not necessarily associated with positive precipitins.

Aspergilloma patients, in particular, had a marked specific IgG response, which decreased after treatment. The IgA response to Af was moderate in these patients but, if present, showed a relation to disease activity. In one patient (case 8) who had been treated in the past for aspergilloma, recurrence of aspergilloma was accompanied by an intensification of the immunoblot pattern. Three out of four aspergilloma patients were treated successfully with surgery and postoperative antifungal treatment. In two of these patients, who were monitored for a longer time, the IgG and IgA antibodies to Af decreased after surgery. However, the immunoblot patterns did not become negative, even though there was no evidence of recurrence of the aspergilloma. In the third surgically treated aspergilloma patient, whose serological follow-up was shorter, there was a similar tendency towards a decreased specific antibody response. No such changes were found in the fourth aspergilloma patient (case 6), who was not treated. This patient showed an unaltered antibody pattern, indicating the persistence of antibodies to Af. Figure 2b shows the schematic and semiquantitative drawings of the immunoblot patterns of individual aspergilloma patients.

The antibody patterns of the Aspergillus-infected patients were very diverse (in number and intensity of bands) and were not always directly related to disease activity, but when clinical improvement occurred the immunoblot patterns became first more intense and then weaker. Most Aspergillus-infected patients had a modest IgA immunoblot. Patient 10 showed a clear specific IgA response at the time of disease diagnosis, which decreased after treatment.

Figure 3 shows the total intensity of the IgG and IgA bands on immunoblot patterns at three defined stages. The immunoblots of the defined stages "preactive" and "active" were studied in three patients. In two patients there was an increase in IgA antibodies to Af and in three patients there was an increase in specific IgG. Statistical analysis was not possible because there were not enough data. The difference between the active and recovering stage was studied in 10 patients. Immunoblot analysis showed a significant difference in specific IgG and IgA antibodies between the active and the recovering stage. The total intensity of IgG-reactive bands decreased by 7.7, with a median difference of 8.5 (95 % CI -2–15, p<0.03), and the total intensity of IgA-reactive bands decreased by 3.7, with a median difference of 3.5 (95% CI 0–6, p<0.01).

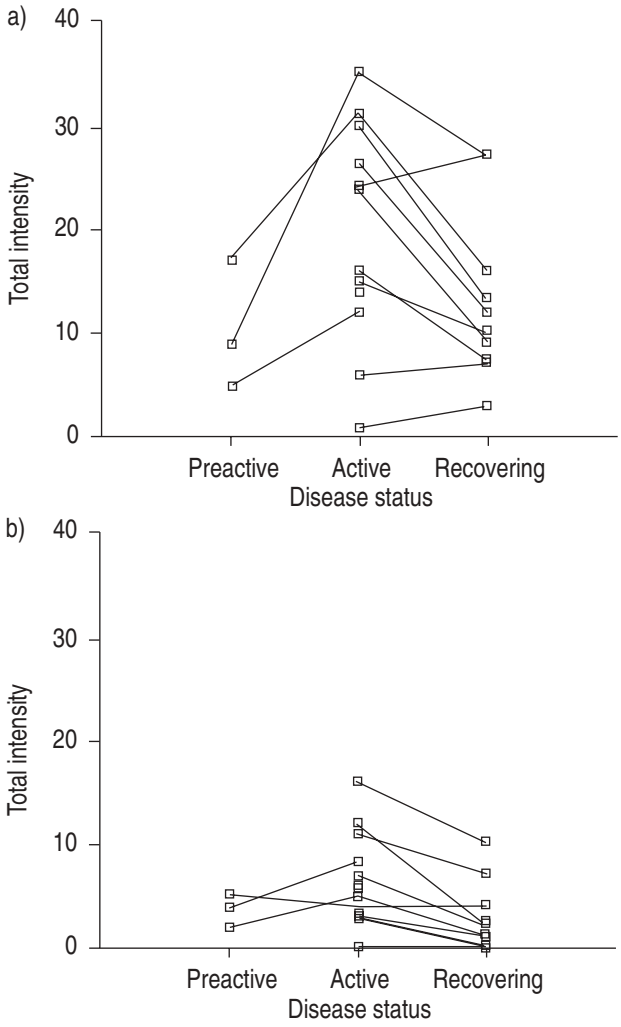

Fig. 3. – Comparison of total intensity of bands at least 4 months before (preactive) (n=3) and during active disease (active) (n=13), and at least 4 months after treatment with clinical improvement (recovering) (n=10) for a) immunoglobulin (Ig)G and b) IgA immunoblot analysis. For each time event one serum sample per patient was taken for analysis if available.

Discussion

This study was performed to determine whether changes in immunoblot patterns in patients with Aspergillus-induced lung diseases are related to the course of the disease. In most patients, disease activity was related to a higher serum concentration of IgG and IgA antibodies against Af, reflected by more and more intensely staining bands on immunoblots. Therefore, serial determination of these antibodies by immunoblot analysis can be used for monitoring.

Several other authors have used immunoblot analysis to detect antibodies to Aspergillus antigens in ABPA patients [18–20]. To investigate the antibody response of ABPA patients in relation to the clinical stages, Leser *et al.* [19]

used serum from 43 patients with ABPA categorized according to the clinical stage. In active disease (*i.e.* stages I and III according to PATTERSON *et al.* [12]), they also observed more and more intense staining of bands of low molecular weight Af antigens in IgG blots. They distinguished only three serological stages or phases of ABPA, namely active, intermediate and remission. In contrast to the study of LESER *et al.* [19], the present study involved analysis of several sera from ABPA patients at different stages of their disease. The results suggest that the acute stage (stage I) of ABPA can be identified serologically and distinguish-ed from the exacerbation stage (stage III), but only by the presence of IgA antibodies to low molecular weight (<20,000 Da) antigens. The pathogenesis of lung injury and destruction in ABPA is still not understood, but previous results [21–23] and the results of this study suggest that IgA plays a contributory role in these processes. It is possible that IgA antibodies have a protective role during the development of ABPA. Immunoblotting showed more (illustrated by more bands and more intensely staining bands) specific IgA in ABPA patients than in aspergilloma and Aspergillus-infected patients, supporting the suggestion that ABPA is a mucosal-type disease. If specific IgA is present in sufficient quantity in aspergillosis, changes in IgA immunoblot patterns are often more informative than IgG. Further investigations into the role of specific IgA antibodies in aspergillosis are needed. The local antibody response to Af can be investigated by using the same me-thod of antibody detection and other materials, such as bronchoalveolar lavage fluid [23].

Aspergillus infection is most commonly associated with neutropenia or a phagocytic dysfunction. Many Aspergillus-infected patients are immunocompromised as result of underlying disease or immunosuppressive therapy. The patients studied with Aspergillus infection were prone to invasive disease because of their altered lung architec-ture and/or because they were receiving intermittent predni-sone therapy. None of them was or had been neutropenic. In these patients very diverse immunoblot patterns were seen. Remarkably, in two patients a more intense immunoblot pattern was initially seen after the start of antifungal therapy. This may reflect the increased antigen load as a result of increased shedding of Af antigens following fungal killing [24]. Taking this phenomenon into account, the changes in immunoblot patterns were related to the clinical status, reaction to therapy and time. The value of monitoring Aspergillus-infected patients by measuring antibody responses is still under discussion [1, 5, 25]. In general, to evaluate antibody responses as a parameter of disease, patients need to have a functioning humoral im-mune system, *i.e.* the capacity to respond with an antibody reaction to the invasive organism. One should always be aware of the limitations of this parameter for diagnosis as well as for monitoring disease activity. Changes in immunoblot pattern may reflect a change in disease activity, an altered function of the immune system, or both, as a result of treatment.

The use of immunoblot analysis to evaluate disease activity in patients with Aspergillus-induced lung disease also has methodological limitations. It should be emphasized that the immunoblot analysis was performed in one session under standard conditions. In practice, different batches of antigen can slightly affect the results of analysis, owing to minor batch-to-batch variation. If this technique is used to monitor the patients serologically, it is advisable to analyse all sera in the same test session. Therefore serum should be collected at diagnosis and when treatment is instigated or changed, and stored for later paired analysis.

In conclusion, within the general limitations of measuring antibody responses, immunoblot analysis of immunoglobulin G and A antibodies against *Aspergillus fumigatus* antigens can be used to monitor the clinical responses of patients with Aspergillus-induced lung diseases, and in particular allergic bronchopulmonary aspergillosis and as-pergilloma. If specific immunoglobulin A is present in suf-ficient quantity, changes in this immunoglobulin are often more informative than changes in specific immunoglobulin G in monitoring disease.

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