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Immunoglobulin allotypes and IgG subclass antibody response to *Aspergillus fumigatus* in cystic fibrosis patients

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Abstract

Background: A majority of patients with cystic fibrosis (CF) become colonised with *Aspergillus fumigatus* (*Af.*), but only a minority develops allergic bronchopulmonary aspergillosis (ABPA). ABPA is associated with increased levels of specific immunoglobulin G (IgG) anti-*Af.* antibodies with a characteristic IgG subclass distribution. We examined whether this characteristic immune response was under the influence of GM and KM allotypes, which are genetic markers (antigenic determinants) on gamma- and kappa-light chains, respectively. **Methods:** Sera from 233 CF patients were typed for seven GM determinants and two KM determinants. The types were correlated to IgG subclass anti-*Af.* antibody levels and to the presence or absence of *Af.* colonisation as well as ABPA. **Results:** The IgG2 antibody level was significantly higher in heterozygous GM (1,2,17 23 5,21 and 1,3,17 23 5,21) compared to homozygous GM allotypes ($p = 0.02$). Patients with the same allotypes tended to have higher IgG1 ($p = 0.051$). In patients with ABPA, being heterozygous for G1M and G3M was linked to higher IgG4 and lower IgG3 as compared to the other genotypes. The KM markers did not influence the antibody levels. The allotype GM(3 23 5), associated with atopic bronchial asthma, tended to make a relatively larger group in ABPA patients compared to non-ABPA and patients not colonised with *Af.* ($p = 0.09$). **Conclusions:** An influence of the GM allotypes on the immune response to *Af.* and on the development of ABPA in patients with CF is suggested.

Keywords: Allergic bronchopulmonary aspergillosis (ABPA); GM phenotype; IgG subclass antibodies; KM type

1. Introduction

Patients with cystic fibrosis (CF) are frequently colonised in the airways with *Aspergillus fumigatus* (*Af.*) with a cumulative incidence around 57% [1] but with a wide range from under 5% to almost 100% in different reports [2–4]. Differences in the frequency of sampling and in culture techniques are variables that may explain this wide range. A minority of patients, ranging from 1% to 14% in various studies [4–7], develops a complicating hypersensitivity reaction termed allergic bronchopulmonary aspergillosis (ABPA), which is characterised by a dual type immunoglobulin G (IgG) and IgE humoral antibody response [1,8,9]. The diagnosis is sometimes difficult and rests upon a set of generally accepted criteria [4,10,11].

The four human immunoglobulin G (IgG) subclasses (IgG1–4) differ in their biological properties and have, except for IgG4, their own constellation of hereditary antigenic determinants, called GM allotypes, located on the C region of 1,2 and 3 [12]. KM allotypes are hereditary antigenic determinants of the K-type light chains. Various studies have demonstrated that the specific antibody response to different infectious agents is in part subclass-specific. IgG1 and IgG3 primarily represent responses to viral proteins [13], IgG2 dominate in response to bacterial polysaccharides [14] and IgG4 to human filariasis [15]. In patients with CF and complicating ABPA, the specific IgG anti-*Af.* antibodies are predominantly found in the IgG1, IgG2 and IgG4 subclasses [16]. In chronic *Pseudomonas aeruginosa* (PA) infection in patients with CF, a specific

Abbreviations: Af., *Aspergillus fumigatus*; ABPA, allergic bronchopulmonary aspergillosis; Asp., Aspergillus; CF, cystic fibrosis; PA, *Pseudomonas aeruginosa*.

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IgG subclass pattern to PA with high IgG2 and IgG3 is correlated to poor lung function and poor clinical condition [17]. Furthermore, IgG3 antibody levels are significantly increased in sera homozygous for GM (3 5), lower in heterozygous sera and significantly lower in sera homozygous for GM (1,2,17 21) [18]. As certain GM and KM allotypes associate with the immune responsiveness to particular infectious pathogens [19], we wanted to examine whether there is a similar link between certain GM allotypes, the levels of IgG subclasses and the presence of ABPA in patients with CF.

2. Patients and methods

2.1. CF patients

The diagnosis of CF was established on the basis of abnormal sweat electrolytes, characteristic clinical features and CF genotype. All patients are seen on a regular monthly basis in the outpatient clinic. Lower respiratory tract secretions are obtained at each visit by expectoration or nasolaryngeal suction and microbiological studies include plating on Sabaroud maltose agar (pH = 4) to detect fungal growth. Biochemical markers of ABPA including specific anti-*Af*. IgG and IgE antibodies, eosinophil leukocyte counts, and total serum IgE are determined at least once every year. Because the diagnostic parameters of ABPA often fluctuate over time in individual patients according to disease activity, we purposely pooled data from a prolonged observational period of 5 years to allocate patients into three groups. GM allotypes do not change over time, and misclassification of ABPA was minimised.

(a) ABPA-group: Patients with positive culture for *Af*. (positive Aspergillus cultures for at least 6 months per year) as well as increased levels of both precipitating antibodies to *Af*. and specific anti-*Af*. IgE antibodies were included [median age 16.5 years (9–37), $n = 26$]. These patients fulfilled the commonly accepted criteria for ABPA [1,3,4] [mean 6.4 positive criteria (range 6–8) at least once within the observation period]. The most recent accepted criteria for ABPA include five positive criteria to confirm the diagnosis [11].
(b) Non-ABPA group: Patients with positive sputum cultures (positive Aspergillus cultures for at least 6 months per year) but without increased levels of the diagnostic markers were included (median age 19 years (9–44), $n = 35$). Patients in groups a and b had a similar distribution of chronically and intermittently PA infected patients as well as distribution of males/females.
(c) Non-Aspergillus (Asp.) group: Patients with no or only rare [< 10% (median) of total] positive cultures and without specific antibodies were included [median age 18 years (1–45), $n = 172$]. Some patients in this group were born within the study period and therefore had an observation period below 5 years.

There was no age difference within the three groups (Kruskal–Wallis test, $p < 0.05$). The study population represents 88% of the total population of patients with CF in the centre (in 30 patients, measurements of either IgG subclasses or GM allotypes had not been performed).

2.2. Follow-up

Within the period 1995–2001, additional 18 patients were diagnosed with ABPA. In three of these patients, GM haplotypes have not been determined.

2.3. Determination of total IgG and IgG subclass antibodies to A. fumigatus in sera

Total IgG and the subclasses IgG1, IgG2, IgG3 and IgG4 to *Af*. were measured by ELISA as previously described and published [16,17]. The serum samples were taken "at random" within 6 months independently of disease activity. Following the disintegration of mycelial mats of *Af*. strain ATCC 42202 in an X-press operated at a maximal force of 200 MPa at −30 °C, preparation of water-soluble somatic hyphal (WSSH) antigens was prepared as described elsewhere [20]. The values were determined in relation to a logarithmic curve drawn from results of a standard serum pool and expressed in ELISA units (EU) [16].

2.4. Determination of allotypes

The GM allotypes are expressed as amino acid sequences on the constant region of the heavy chains (chromosome 14, band q 32) and KM allotypes on the kappa light chains (chromosome 2, band p 12). They are inherited in a codominant Mendelian way, in fixed combinations called haplotypes. GM allotypes are restricted to one subclass and located on the heavy chains of IgG1, IgG2 or IgG3. Immunoglobulin allotypes were determined by haemagglutination inhibition using specific antibodies against GM and KM markers (epitopes) [21]. In Caucasians, the G1M(1) and G1M(3) are alternative alleles for IgG1, G3M(5) and G3M(21) are alternative alleles for IgG3, whereas there is only one marker G2M(23) for IgG2. For the kappa light chains, KM(3) and KM(1) are alternative alleles. The serum samples were types for G1M(1) and G1M(3), G2M(23), G3M(5) and G3M(21), as well as for KM(1) and KM(3). They were also tested for two additional IgG1 markers: G1M(2) and G1M(17), which are variably expressed on IgG1 molecules and therefore do not mutually exclude each other or G1M(1) and G1M(3). For some of the present analysis, the patients were divided into groups according to the most frequent phenotypes based upon the mutually exclusive markers on IgG1 and IgG3—the presence or absence of the IgG2 marker—and the two KM markers.

2.5. Statistical analysis

The Mann–Whitney test was used for nonparametric unpaired data where two groups were compared. Single- or two-factor analysis of variance (ANOVA) and Kruskal–Wallis test for nonparametric data were used when more than two groups were compared. Calculations were performed using StatView 4.5 as software. For the ANOVA tests, data were log transformed before calculations. Their level of significance was 5% for two-tailed comparison.

3. Results

3.1. GM phenotype distribution

The frequencies of GM phenotypes in the 233 CF patients were comparable to the distribution observed in groups of healthy Caucasian controls ($n=86$) [22] and ($n=430$) [23].

3.2. GM allotypes and IgG subclass antibodies to A. fumigatus

The entire patient population represented 15 different phenotypes. Table 1 gives the median levels of specific anti-*Af.* antibodies for each of the four IgG subclasses as well as for total IgG for each GM phenotype. Five of the phenotypes including only one or two patients were excluded from the statistical calculations ($n=7$). No significant difference in IgG subclass (1–4) antibody levels according to the remaining eight GM phenotypes was found (Table 1).

Following exclusion of the five rare phenotypes, the rest of the patients ($n=226$) could be allocated to one of three groups according to the IgG1 and IgG3 markers: homozygous for G1M(3) and G3M(5) [$n=94$], homozygous for G1M (1) and G3M(21) [$n=30$] and heterozygous for G1M(1/3) and G3M(5/21) [$n=102$; Table 2]. The 226 patients were also divided in two groups according to the presence or absence of the IgG2 marker G2M(23) [Table 2] and in two groups according to the KM markers (Table 2). The IgG2 antibody level was highest in the patients heterozygous for the IgG1 and IgG3 markers ($p=0.02$) as compared to the patients homozygous for these markers (Table 2). The heterozygous patients also tended to have higher IgG1 ($p=0.051$) and IgG4 ($p=0.11$) than the two homozygous groups (Table 2), whereas no difference was observed between homozygous and heterozygous for IgG3 and IgG4. The presence or absence of the IgG2 marker G2M(23) could not be linked to the total or IgG subclass antibody levels (Table 2), nor could the two KM markers. Homozygous KM(1/1) is very rare and was not found in our patients.

Table 1
The distribution of GM phenotypes and IgG subclass antibodies to *Aspergillus fumigatus* in 233 patients with cystic fibrosis

GM phenotype	Number of patients	IgG	IgG1	IgG2	IgG3	IgG4
1,17 21	10	14.5	9.3	19.5	13.4	14.5
1,17 23 21	2	46	109	110	16.5	15.3
1,17 23 5,21	1[a]	14	4.8	22	9	15
1,2,17 21	17	18	9	17	12	16
1,2,17 23 21	1	14	8.4	17	14.4	16
1,2,17 23 5,21	2[a]	21	23.7	34.5	10.3	10
1,2,3,17 5,21	15	30	30	30	11.6	16
1,2,3,17 23 5	1[a]	36	5.6	54	26	14
1,2,3,17 23 5,21	19	31	11	64	14.4	26
1,3,17 23 21	1[a]	36	124	42	15	128
1,3,17 21	2[a]	14.2	101	125	18.5	36
1,3,17 5,21	34	25.5	11.5	28	11.5	16
1,3,17 23 5,21	34	30	11.9	22	12.4	15
3 5	23	20	10	19	13.2	16
3 23 5	71	19	7.6	20	12.8	15

The antibody level is given as ELISA units (EU).
[a] Indicates patients left out of the calculations.

Table 2
GM and KM types according to IgG and IgG subclass antibodies to *Aspergillus fumigatus* in 233 patients with cystic fibrosis

	Number	IgG	IgG1	IgG2	IgG3	IgG4
Homozygote for G1M(3) and G3M(5)						
3 5						
3 23 5	94	19.5	8	20	12.8	15
Homozygote for G1M(1) and G3M(21)						
1,2,17 21						
1,17 21	30	18	9.1	19.5	13.3	15
Heterozygote for G1M and G3M						
1,2,3,17 5,21						
1,2,3,17 23 5,21						
1,3,17 5,21						
1,3,17 23 5,21	102	28	12.6	25.5	12.4	16
GM 23						
positive	127	23	10	22	12.8	15
negative	99	20	10.4	22	12	16
KM						
1,3	31	18	10	24	13.2	15
3	195	24	10	22	12.8	16

Values are medians of the groups given as ELISA units (EU).

3.3. GM allotypes and A. fumigatus status

The levels of total IgG and IgG subclass antibodies against *Af.* according to GM and KM allotypes in the three patient groups, ABPA, non-ABPA and non-Asp., are shown in Table 3. All levels were in general increased in non-ABPA as compared to non-Asp., and even more in the ABPA group. The association of increased IgG2 levels and being heterozygous for the GM markers on IgG1 and IgG3 found in the entire population (Table 2) did not reach statistical significance when the patients were split into the three groups which could be due to smaller patient sample groups.

Table 3
GM genotypes in 226 patients with cystic fibrosis distributed in groups of ABPA, non-ABPA and non-Aspergillus patients

Genotypes	ABPA ($n=24$)					non-ABPA ($n=31$)					non-Aspergillus ($n=171$)				
	IgG					IgG					IgG				
	number	1	2	3	4	number	1	2	3	4	number	1	2	3	4
3 5	13	72	120	17	52	8	10.2	50	20.5	17	73	7	17	11	15
1,2,3,17 5,21	3	210	110	17	84	9	43	58	19	66	22	9.5	25	11.1	16
1,3,17 5,21	7	110	180	15	88	8	22.5	24.5	11.3	15.5	53	10	22	12	15
1,17 21	1	560	340	34	74	1	180	140	20	18	10	9.3	19.5	12.9	13.5
1,2,17 21	0					5	100	28	12	25	13	8.2	16	13.6	10
GM 23															
positive	16	78	115	16.5	56	16	17	29.5	17	18.5	95	8	19	12	14
negative	8	172	260	14.5	82	15	48	68	15	19	76	8.6	18.5	11.3	15
KM															
1,3	4	215	598	23	90	7	24	132	12	66	20	7.3	20	11.3	13
3	20	87	143	16	75	24	19.5	38	17	18.5	151	8.4	19	12	15

Seven patients who did not belong to any of the three groups were left out of calculations. Values are medians given as ELISA units (EU).

When comparisons are made between the ABPA group and the non-ABPA group, Table 3 shows that levels of IgG1, IgG2 and IgG4—but not IgG3—increased in the ABPA group. The increase was seen in all GM and KM groups, but when the levels in the two KM groups in the ABPA patients were compared, it appears that levels of all four IgG subclasses were higher in the heterozygous KM(1/3) than in the homozygous KM(3/3) patients (Table 3). Similarly, in the ABPA patients, the levels of IgG1, IgG2 and IgG4 were higher in the G2M(23)-negative patients than in the G2M(23)-positive patients (Table 3). Finally, the number of patients in the three groups was not randomly distributed. Table 3 shows that 54% of the ABPA patients were homozygous for G1M(3) and G3M(5) versus only 26% in the non-ABPA patients ($p=0.09$). Also, 67% of the ABPA patients were positive for G2M(23) versus 52% of the non-ABPA patients.

Patients who developed ABPA within the period 1995–2001 had a similar distribution of GM haplotypes, except that a few more were heterozygous for G1M and G3M. Eight of fifteen patients were heterozygous for G1M and G3M, six patients were homozygous for GM (3 5) and only one patient was homozygous for GM(1,17 21). GM(23) positivity was found in 8 of 15 patients, 2 patients were KM(1,3) and 13 patients were KM(3).

4. Discussion

4.1. Association between GM and KM allotypes and specific anti-Af. antibody levels

We found the frequency of GM allotypes to be the same as in the normal Caucasian background population [22]. Indeed, it would not be expected that CFTR mutations should be linked to genes coding for immunoglobulin. It appears that polymorphisms at the level of immunoglobulin genes are not related to survival in patients with CF because this would have created a selection bias and a distribution of GM allotypes differing from the normal population.

The results of determination of IgG and IgG subclass antibodies against *Af.* in the total patient population did not differ among the 15 GM allotypes detected. Seven of the phenotypes were however represented by only one or two patients (Table 1). After exclusion of these patients, the remaining 226 could be pooled to form three groups: two homozygous for IgG1 and IgG3 markers (3 5 and 1 21) and one heterozygous (Table 2). We now found that the heterozygous patients had the highest levels of specific anti-*Af.* antibodies in subclasses IgG1, IgG2 and IgG4, although only reaching significance for IgG2 (Table 2). Looking only at the ABPA patients versus the non-ABPA patients, the latter being equally exposed to *Af.* as the ABPA patients, the heterozygous patients had higher levels of specific IgG4 antibodies than the two homozygous groups (Table 3). The levels of IgG1 and IgG2—but not IgG3—antibodies were also higher in the heterozygous group compared to the G1M(3) and G3M(5) homozygous groups. Only one ABPA patient was homozygous for G1M(1) and G3M(21), and levels cannot be properly evaluated. IgG subclass antibody levels were significantly higher in ABPA patients as compared to non-ABPA patients although the ABPA population was a pool of all ABPA patients irrespective of ABPA activity. We purposely chose not to split up in subgroups due to the limited number of patients and because GM haplotypes do not change over time. Furthermore, the treatment of ABPA within the study period tended to be a longterm treatment, i.e., years, which may confound the actual disease activity.

An association between heterozygosity for IgG1 and IgG3 markers and significantly increased total IgG4 antibodies has been reported in normal healthy individuals [24]. Increased levels of IgG4 have been found in filarial infections [15,25]. Eosinophilia is characteristic of filarial infec-

tion, of atopic diseases and of CF patients exposed to *Af*., and all three conditions are associated with increased IgG4 levels [15,16,26]. We have previously shown that IgG4 antibodies against *Af*. have the highest diagnostic specificity of the four subclasses in ABPA [16]. There are no allotypic markers for IgG4, but it has been suggested that the GM(3 23 5) exerts a positive effect on IgG4 levels in normal sera [27]. The present study failed to confirm this effect. Some studies have reported an association between patients being homozygous for GM(3 5) and having high levels of IgG3 [18,28–31], which could not be confirmed by Morell et al. [24] in healthy control donors nor in the present study.

The presence of the IgG2 G2M(23) marker has been associated to high total IgG2 antibodies [24,28,29] but was not found in the present study, as well as we, in concordance with most other studies [24,28–30], neither found an association between the presence of G2M(23) and increased IgG4.

The linkage between KM allotypes and IgG subclasses appear to vary. Patients with CF and complicating chronic *Pseudomonas aeruginosa* infection who were homozygous for KM(3) had increased anti-PA IgG2 antibodies [18] as well as increased IgG1 [31]. Using *Af*.-specific antigens in the selected group of ABPA patients KM markers did not influence the antibody levels (Table 3). KM allotypes are located on the light chains and are not restricted to one class or subclass of immunoglobulins [21], which may in part result in different associations.

4.2. Association between GM and KM allotypes and the presence of ABPA

It appears from the data shown in Table 3 that the different haplotypes were not always randomly distributed among the three groups of patients: ABPA, non-ABPA and non-Asp. However, the rather small sample sizes have to be taken into account. Fifty-four percent of the ABPA patients were homozygous for G1M(3) and G3M(5) versus 26% of the non-ABPA patients. If the ABPA patients in the follow-up group ($n = 15$) were included, the percentage of patients homozygous for G1M(3) and G3M(5) was 49%. The follow-up group has a relatively larger group of patients being heterozygous, i.e., in total 46% of the ABPA patients. An association between the phenotype GM(3 23 5) and atopic asthma has been reported by Oxelius et al. [26], and it is therefore noteworthy that in the relatively large group of GM(3 5) in the ABPA patients, the majority (11/13) was GM(3 23 5) and only two were GM(3 5). Children with atopic bronchial asthma have been shown to have an imbalanced class switch in the rearrangement of the genes for IgG [32].

It has been suggested that GM and KM allotypes themselves may not influence disease susceptibility, but the associations may reflect linkage disequilibrium with other polymorphisms of the constant region genes or with specific variable region genes [33]. Isotype switching has been suggested for the mechanisms involved in GM allotype effect on the IgG subclass concentrations [34]. The amino acid and codon substitutions correlating with allotypes are cloned in several instances. Allotyping at the genomic level by PCR may increase the capacity to precisely relate polymorphic variants to particular functions and pathophysiological events [35,36].

We have shown that ABPA associates to a specific IgG subclass pattern and to a specific TH-2 cytokine profile with significantly increased IL-4 and IL-5 in ABPA patients as compared to non-ABPA patients [37]. IL-4 is a critical mediator of B-cell switching to IgE as well as IgG4 synthesis. Markers around the IL-4 locus on chromosome 5q31.1 linked to a gene controlling IgE levels, GM allotypes on chromosome 14q32 and six HLA-DR2/5 subtypes on chromosome 6 reported to be of pathophysiological importance to ABPA [38] indicate a polygenic inheritance of the immune response.

The linkage between being heterozygous for G1M and G3M, having increased IgG1, IgG2 and IgG4 subclasses as well as G2M(23) and having ABPA, suggests a GM allotypic influence on the immune response. GM allotyping may contribute to the identification of CF patients at risk of ABPA.

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