Cytokine profiling of pulmonary aspergillosis

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Summary

Aspergillus fumigatus is ubiquitous and yet causes invasive, chronic and allergic disease of the lung. Chronic cavitary pulmonary aspergillosis (CCPA) is a slowly destructive form of pulmonary aspergillosis, without immunocompromise. We hypothesized that CCPA cytokine gene polymorphisms would differ from patients with allergic bronchopulmonary aspergillosis (ABPA) and uninfected controls.

We have profiled functional cytokine gene polymorphisms for interleukin (IL)-10, IL-15, transforming growth factors (TGF)-β1, tumour necrosis factor (TNF)-α and interferon (IFN)-γ in patients with CCPA (n = 24) who were compared with other forms of aspergillosis (mostly ABPA) (n = 15) and with ethnically matched controls (n = 65–330). Results are described with reference to the high-producing genotype in each case.

Susceptibility to aspergillosis (all patients compared with normal controls) was associated with higher frequency of the IL-15 +13689*A allele (OR = 2.37, P = 0.0028) and A/A genotype (χ^2 = 10.31, P < 0.001), with a lower frequency of the TNF-α −308*A/A genotype (χ^2 = 11.05, P < 0.01). Within the aspergillosis patients, CCPA is associated with lower frequency of the IL-10 −1082*G allele (OR = 0.38, P = 0.0006) and G/G genotype (χ^2 = 22.45, P < 0.001) and with a lower frequency of the TGF-β1 +869 *T allele (OR +0.42, P < 0.0029) and T/T genotype (χ^2 = 17.82, P < 0.001) compared with non-CCPA patients and normal controls.

Patients infected with *Aspergillus* appear to be higher producers of IL-15, a Th2-promoting cytokine, and lower producers of TNF-α, a cytokine central in protective responses. CCPA occurs in patients who are genetically lower producers of both IL-10 and TGF-β1. As these cytokines are regulatory and anti-inflammatory, CCPA may be a consequence of poor inflammatory response control in the lung.

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Introduction

Aspergillus fumigatus is unique among microorganisms in causing severe invasive infection in those with immuno-compromised states, allergy in atopic patients and indolent progressive infection in those without immunodeficiency but prior pulmonary insults. This spectrum of disease offers a special opportunity to understand the relationship of the host and pathogen. Chronic pulmonary aspergillosis comprises chronic cavitary (CCPA) and chronic fibrosing pulmonary aspergillosis (CFPA) as well as simple aspergilloma (Denning *et al*., 2003). The background cause of CCPA and CFPA has been little investigated. We have previously documented defects in innate immunity (mannose-binding protein and more recently surfactant) in these patients (Crosdale *et al*., 2001; Vaid *et al*., unpublished data). Allergic bronchopulmonary aspergillosis, allergic fungal sinusitis and the recently coined term severe asthma with fungal sensitisation (SAFS) (Denning *et al*., 2006) comprise the main allergic diseases associated with exposure to *A. fumigatus*. We care for many of these patients in our clinic and so we took the opportunity to compare their genetic backgrounds with respect to candidate pro- and anti-inflammatory cytokines that might influence the development and outcome of CCPA.

Macrophages have been shown to participate in the early phase of defence against *Aspergillus conidia* (Waldorf *et al*., 1984; de Repentigny *et al*., 1993; Rhodes, 1993; Cenci *et al*., 1998; Duong *et al*., 1998), whereas neutrophils and circulating monocytes cause damage to escaping hyphae (late phase of defence) by secreting microbicidal oxidative metabolites and non-oxidative compounds (Schaffner *et al*., 1982; Levitz *et al*., 1986; Roilides *et al*., 1996). However, the role of cytokines in the modulation of responses of circulating or tissue phagocytes to *Aspergillus* has only recently begun to be elucidated. There are experimental data suggesting the protective role of Th1 CD4+ predominance against invasive aspergillosis in the imbalance of Th1/Th2 response (Cenci *et al*., 1997, 1998, 2000). In experimental invasive aspergillosis, certain inbred mouse stains are substantially more susceptible (notably DBA/2(H2^d)) compared with others, such as BALB/c (H-2^d) and (57BL/6 (H-2^d). The difference in susceptibility appears to be determined primarily by the balance between CD4+, Th1 and Th2 responses (Cenci *et al*., 1997, 1998). Th1 cell responses are associated with resistance and onset of protective immunity, whereas Th2 responses are associated with progressive disease, more tissue damage

and poor survival. Th1-produced cytokines [including interferon (IFN)-γ, interleukin (IL)-6, IL-12, tumour necrosis factor (TNF)-α, IL-1] activate neutrophils and pulmonary macrophages, the key effector cells in invasive aspergillosis, whereas Th2 cytokines, notably IL-4 and IL-10, are associated with reduced IL-12 and TNF-α and worse outcome. IL-10-knock-out mice survive an otherwise lethal challenge (Clemons *et al.*, 2000) and IL-4–knock-out mice show deficient Th2 responses and are more resistant to infection (Cenci *et al.*, 1999). Additional work supports this hypothesis as antagonism of TNF-α resulted in increased death in experimentally infected mice and more fungal burden in the lungs, whereas a TNF-α agonist peptide improved survival (Mehrad *et al.*, 1999). Chemokines appear to enhance the recruitment and effector functions of lymphocytes and macrophages and contribute to granuloma formation and fibrosis. It is hypothesized that a Th1/Th2 dysregulation and a switch to a Th2 immune response may contribute to development and unfavourable outcome of invasive aspergillosis in humans. Investigating this in patients with acute invasive aspergillosis is challenging, because there are multiple other confounding factors (such as temporary neutropenia and variable corticosteroid doses).

Patients, material and methods

We have investigated the functional polymorphisms in certain cytokines in patients with CCPA (*n* = 24) and those were compared with other forms of aspergillosis [mostly allergic bronchopulmonary aspergillosis (ABPA) (*n* = 9), SAFS (*n* = 2) (1), allergic fungal sinusitis (*n* = 1) and others (*n* = 3)] and with normal ethnically matched controls (*n* = 65–330). The duration of the disease in patients with CCPA ranged from several months to > 12 years. The clinical, radiological characteristics and the outcome for a proportion of the patients who were recruited in the present study have been described in a comprehensive recent article (Denning *et al.*, 2003). The control population consisted of British Caucasoid individuals who were typed for the polymorphism using amplification refractory mutation system–polymerase chain reaction (ARMS–PCR) as previously described (Perrey *et al.*, 1998). British Caucasoid controls were unrelated, healthy volunteers with an age range of 20–60 years. They were from the north-west region of England. For IL-10, 330 controls, for IL-15 65, for IFN-γ 164, for transforming growth factors (TGF)-β 107 and for TNF-α 106 controls were compared with patients.

After the informed written consent was obtained from the patients to use their DNA for enhancing scientific knowledge of their disease, peripheral blood was drawn and DNA was extracted from EDTA blood samples and typed for polymorphisms in genes encoding IL-10, IL-15, TGF-β1, TNF-α and IFN-γ using sequence-specific primers (SSP)–PCR methodology. Genomic DNA from whole frozen EDTA-containing samples was obtained by phenol extraction and ethanol precipitation following proteinase K (Boeringer Mannheim, Lewes, UK) digestion. Commer-

cially available DNA extraction kits have been used successfully in other studies. Polymorphisms were identified using SSP–PCR methodology, as previously described (Perrey *et al.*, 1998), using allele-specific primers to identify the high and low producer alleles of each biallelic polymorphic site for IL-10 gene (−1082*G/A and −592*C/A, respectively), TNF-α −308*G/A, IL-15 (−80*T/C and +13689*T/A), IFN-γ (+874*T/A), TGF-β1 (+869*T/C, codon 10).

The protocol for the PTC-100 PCR machine (MJ Research Inc, Waltham, MAA) was as follows: 95 °C for 1 min; then 10 cycles at 95 °C for 15 s, 65 °C for 50 s and 72 °C for 40 s; followed by 20 cycles at 95 °C for 20 s, 59 °C for 50 s, and 72 °C for 50 s. DNA products were then analysed using electrophoresis on 2% agarose gel. IFN-γ assay has been described elsewhere (Pravica *et al.*, 2000). Following electrophoresis, gels were stained with ethidium bromide (10 mg L^{-1}), visualized with UV light on a transilluminator and photographed for the definition and identification of alleles.

Odds ratios (OR) with 95% confidence intervals were calculated to compare allele frequencies and genotype frequencies, after transformation into percentages, using the χ^2 test.

Results

Genotypes of 24 CCPA patients were compared with 15 patients with allergic forms of aspergillosis. The results of the distribution of genotypes and alleles are shown in Tables 1–3.

Susceptibility to aspergillosis

Aspergillosis is associated with higher frequency of the IL-15 +13689*A allele [OR = 2.37 (1.34–4.20), *P* = 0.0028] and A/A genotype (χ^2 = 10.31, *P* < 0.001) compared with normal controls, but not the IL-15−80 polymorphism. Aspergillosis is also associated with a lower frequency of the TNF-α −308*A/A genotype (χ^2 = 11.05, *P* < 0.01), and a higher frequency of IFN-γ (+874) T/T genotype (χ^2 = 8.16, *P* < 0.02) but no particular allele on its own with either gene. There was no association with IL-10 or TGF-β1 genotype or allele.

CCPA vs. allergic aspergillosis

Among the aspergillosis patients, CCPA is associated with lower frequency of the IL-10 −1082*G allele [OR = 0.38 (0.21–0.67), *P* = 0.0006] and G/G genotype (χ^2 = 22.45, *P* < 0.001) compared with those with allergic disease, but not the IL-10 −592 polymorphism. In addition, CCPA was associated with a lower frequency of the TGF-β1 +869 *T allele [OR +0.42 (0.24–0.75), *P* < 0.0029] and T/T genotype (χ^2 = 17.82, *P* < 0.001) compared with non-CCPA patients and normal controls [OR +0.32 (0.15–0.64), *P* = 0.001 (T allele) and χ^2 = 19.26, *P* > 0.001 (T/T genotype)]. There was no association with IL-15, IFN-γ or TNF-α genotype or allele.

Table 1. Frequencies (%) of IL-10 (at position –1082 and –592) alleles and genotypes

Groups	IL-10 (–1082) allele		IL-10 (–1082) genotype			IL-10 (–592) allele		IL-10 (–592) genotype		
	G	A	G/G*	G/A	A/A	C	A	C/C*	C/A	A/A
1. CCPA	39	61	17	43	39	75	25	56	38	6
2. Other aspergillosis groups	63	37	40	47	13	73	27	54	38	8
3. All aspergillosis combined	49	51	26	45	29	74	26	55	38	7
4. Normal controls	51	49	30	42	28	77	23	59	34	8
Comparisons	1 vs. 2; OR = 0.38 (0.21–0.67) P = 0.0006		1 vs. 2; χ^2 = 22.45, P < 0.001; 3 vs. 4 no differences			ns	ns			

* High producer genotype.

Table 2. Frequencies (%) of IL-15 (at positions –80 and +13689) alleles and genotypes

Groups	IL-15 (–80) alleles		IL-15 (–80) genotypes			IL-15 (+13689) alleles		IL-15 (+13689) genotypes		
	T	C	T/T*	C/T	C/C	A	T	A/A*	T/A	T/T
1. CCPA	21	79	0	42	58	63	37	39	48	13
2. Other aspergillosis groups	30	70	0	60	40	70	30	47	47	7
3. All aspergillosis combined	24	76	0	49	51	66	34	42	47	11
4. Normal controls	31	69	5	34	61	45	55	28	44	28
Comparisons	ns	ns				3 vs. 4; OR = 2.37 (1.34–4.2) P = 0.0028 All others ns		3 vs. 4; χ^2 = 10.31, P < 0.01		

* High producer genotype.

Table 3. Frequencies (%) of TNF-α (–308), IFN-gamma (+874), TGF-β (+869) alleles and genotypes

Groups	TNF-α –308 alleles		TNF-α –308 genotypes			IFN-γ (+874) alleles		IFN–γ (+874) genotypes			TGF-β (+869) alleles		TGF-β (+869)		
	A	G	A/A*	G/A	G/G	T	A	T/T*	T/A	A/A	T	C	T/T*	C/T	C/C
1. CCPA	26	74	4	43	52	59	41	39	39	22	43	57	18	50	32
2. Other aspergillosis groups	10	90	0	20	80	60	40	40	40	20	64	36	43	43	14
3. All aspergillosis combined	20	80	3	34	63	59	41	39	39	21	51	49	28	47	25
4. Normal controls	27	73	16	23	61	48	52	21	54	25	65	35	41	48	11
Comparisons	ns		3 vs. 4; χ^2 = 11.05, P < 0.01			ns		3 vs. 4; χ^2 = 8.16, P < 0.02			1 vs. 2; OR = 0.42 (0.24–0.75) P = 0.0029 1 vs. 4; OR = 0.32 (0.15–0.64) P = 0.001 3 vs. 4; OR = 0.51 (0.29–0.89) P < 0.016		1 vs. 2; χ^2 = 17.82, P < 0.001 1 vs. 4; χ^2 = 19.26, P < 0.01 2 vs. 4 not significant		

* High producer genotype.

Discussion

Despite sequencing the genome (Nierman *et al.*, 2005), the intrinsic reason for the remarkable pathogenicity and allergenicity of *A. fumigatus* compared with any other member of the *Aspergillus* genus is not yet well understood. Gene polymorphisms of specific host immune defence appear to be of major importance in the risk and manifestation of infectious disease. Allelic variants of cytokine genes are associated with higher or lower production of cytokines both *in vitro* and *in vivo*. All steps of host response to infection may be affected by genetic factors. Some examples of genetic defects associated with high rates and predilection for infections that include Fc-γ receptor, Toll-like receptor or mannose-binding protein mutations have been shown to modify outcome of severe pneumococcal infections, Gram-negative septic shock, and meningococcal disease, respectively. Moreover, TNF polymorphisms have been associated with susceptibility to malaria and other infections; chemokine receptor polymorphisms with susceptibility to HIV; natural resistance-associated macrophage protein 1 with tuberculosis; and surfactant proteins with respiratory syncytial virus in infants (Kwiatkowski, 2000; McNicholl *et al.*, 2000; Charpentier & Mira, 2001; Jack *et al.*, 2001; Lofgren *et al.*, 2002). Susceptibility to both chronic pulmonary aspergillosis and ABPA is linked with mannose-binding protein and surfactant A2 defects in some patients (Crosdale *et al.*, 2001; Madan *et al.*, 2005; Vaid *et al.*, unpublished data), and ABPA also with an increased IL-10-producing genotype (Brouard *et al.*, 2005), but these clearly do not explain the disease phenotypes alone.

Both innate and adaptive [T helper (Th)] immunity play a central role in fungal infection. A bi-directional function exists between the two arms of the immune response, mainly through cytokine production. Some cytokines may have beneficial or deleterious effects on infection, depending on the dose and timing of endogenous production or exogenous administration (Mencacci *et al.*, 2000).

This pilot study investigated candidate cytokine genes that could contribute to the risk of developing CCPA. Specifically, the study examined genes involved in Th1 and Th2 pathways of the immune response that are critical for effective antifungal activity. The dominance of either Th1 or Th2 pathways directly correlates with the outcome and severity of infection. However, studying of a single cytokine gene, for the mapping of complex diseases, in several published genetic case-control association studies, is not a realistic and informative approach, as the cytokine network is highly flexible and complex, containing interactive cascades of gene activation and suppression, and a considerable overlap between the function of individual cytokines exists. This fact complicates efforts to analyse both the function of individual cytokines and the impact of cytokine gene polymorphism on gene expression and disease, studying each cytokine in isolation.

A novel finding of the present study is that cytokines' genes polymorphisms are associated with the development of CCPA and could explain the devastating consequences of the disease in otherwise healthy individuals without any obvious immune defect.

The IL-15 +13689 A allele and A/A genotype were significantly more common than in controls. The A allele appears to be associated with higher IL-15 production by LPS-stimulated peripheral blood mononuclear cells, while the T allele appears to be associated with lower IL-15 production, but this remains to be confirmed. IL-15 is supposed to play a role in the activation of Th2 responses. In a previous study, the +13689*A allele was more frequent in atopic individuals compared with the same control group as that used in this study (I Pravica and IV Hutchinson, unpublished data). The odds ratio was 0.6 (0.3–0.9), $P = 0.02$, similar to this study. There were no significant associations of aspergillosis with IL-15 −80 alleles or genotypes. One possible interpretation is that high IL-15 producers are predisposed to aspergillosis because patients make a Th2 response which is not protective. However, in an *ex vivo* study, IL-15 augmented neutrophil damage of *A. fumigatus* hyphae (Winn *et al.*, 2003), illustrating the complexity of cytokine action.

With respect to TNF-α, the high producer −308 A/A genotype is less frequent in patients with any form of aspergillosis compared with healthy controls. TNF-α is a pro-inflammatory cytokine, important to the host response to infectious challenge, including fungal pathogens. TNF-α is secreted very early during infection. *In vitro* studies have demonstrated that TNF-α enhances specific phagocytic activity against conidia by pulmonary alveolar macrophages (as an early event) and augments the neutrophil damage of *Aspergillus* hyphae (as a late event) (Roilides *et al.*, 1998). The protective role of TNF-α has also been documented in murine models with invasive aspergillosis (IA): exogenous administration of TNF-α protected animals from IA and lowered mortality as well as the number of organs infected by *Aspergillus* (Nagai *et al.*, 1995). The development of IA in individuals with autoimmune diseases as an adverse event soon after therapy with anti-TNF-α immunomodulatory treatment has also been reported (De Rosa *et al.*, 2003).

Inheritance of the IL-10 −1082*A allele is associated with the development of CCPA in the present study. The A allele is associated with lower IL-10 production by lymphocytes. Because IL-10 is anti-inflammatory, such patients may be more prone to necrotic sequelae. In contrast, non-CCPA aspergillosis is associated with the G allele (high producer), in accordance with the published data. However, the numbers are too small to see a difference in either group compared with normal controls. A doubling of the numbers in the CCPA group would achieve significance. Interestingly, there were no significant associations of aspergillosis with −592 (monocyte production) alleles or genotypes.

Interleukin-10 exerts inhibitory effects on immune responses, including the functional suppression of phagocytic cells against *A. fumigatus*. IL-10 is constitutively produced by bronchial epithelial cells and potentially inhibits cytokine production by cultured alveolar macrophages and lung dendritic cells. Therefore, the potential role of IL-10 in regulating pulmonary immune responses

and in suppressing acute inflammation seems to be crucial. Data from studies using murine models have indicated that IL-10 is an important regulator of the T-cell-mediated immune response to *A. fumigatus* (Kurup & Grunig 2002; Grunig *et al.*, 1997). Studies in murine models of mice deficient in IL-10 revealed increased resistance to *A. fumigatus* infection, whereas mice expressing high serum IL-10 levels show decreased resistance to aspergillosis and were protected by neutralization of this cytokine (Cenci *et al.*, 1998; Clemons *et al.*, 2000; Centeno-Lima *et al.*, 2002).

In accordance with experimental studies, high IL-10 concentrations in clinical studies have been reported to be associated with severe outcomes in bacterial infections and development of invasive aspergillosis and a possible correlation with unfavourable outcome in non-neutropenic immunocompromised patients (Roilides *et al.*, 2001). In a recent article (Brouard *et al.*, 2005), a significant relationship was found between the −1082*G/G genotype (high producers) and both colonization with *A. fumigatus* and allergic bronchopulmonary aspergillosis (ABPA) in patients with cystic fibrosis. Moreover, measurements of serum IL-10 levels in the same study revealed higher serum IL-10 in patients with *A. fumigatus*-related pulmonary disease.

We found that CCPA occurs in patients who are genetically lower producers of TGF-β1, whereas in ABPA, the allele and genotype frequency was consistent with the general population. TGF-β1 is produced by several cells and promotes proliferation of fibroblasts and the deposition of collagen. Production of TGF-β1 varies between individuals and partly depends on polymorphisms in the TGF-β1 gene at positions +869 and +915 encoding codons 10 and 25. As TGF-β1 is an anti-inflammatory cytokine, the development of CCPA may be a consequence of failing to control the inflammatory response. Polymorphisms in TGF-β1 are associated with variations in cytokine levels and are linked to fibrosis in a number of tissues. No previous work has addressed any fungal infection and TGF-β1 polymorphism.

We found a just significant effect of the high-producing IFN-γ T/T (+874) genotype, although not with the T allele and all forms of aspergillosis. This cytokine is known to potentiate the antifungal activity of human phagocytic cells. In an experimental mice model with invasive aspergillosis, the development of protective acquired immunity was associated with the presence of CD4+ cells producing IFN-γ and macrophages producing IL-12, whereas impaired neutrophil antifungal activity, observed in susceptible mice, was concomitant with a predominanct production of IL-4 by CD4+ splenocytes (Cenci *et al.*, 1997). The protective role of this TH-1 cytokine has also been documented in clinical studies (Hebart *et al.*, 2002). This association needs further confirmation and could be artefactual.

Genetic susceptibility factors have been suggested in the case of ABPA by the occasional familial occurrence of the disease. However, in the case of CCPA, this is the first study highlighting the importance of a panel of cytokine polymorphisms in the pathogenesis of the disease and the profound role that genetic factors can play in CCPA.

A caveat of the present study, although cases and controls were adequately matched, is the low number of CCPA patients, due to the rarity of the disease, which lacks the statistical power to detect small or moderate gene effects. Moreover, we did not examine the impact of cytokine genetic polymorphisms in each clinical phenotype of CCPA patients (Denning *et al.*, 2003). Future multicenter studies including a larger cohort of CCPA patients are required and the genetic investigation of each group separately, according to the recent classification, in order to explain on genetic grounds the clinical, radiological, histological and outcome differences among patients with CCPA. However, the commonality of some genetic defects between two manifestations of disease caused by the same organism, and the contrast between other cytokine genetic profiles (notably IL-10 and TGF-β1) and different diseases is striking, and of general significance in understanding the pulmonary responses to ubiquitous airborne microorganisms.

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