

Immunological and Structural Analysis of IgE-Mediated Cross-Reactivity between Manganese Superoxide Dismutases

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Key Words

Allergy · Autoreactivity · Manganese superoxide dismutase · Molecular modelling

Abstract

Background: Allergy results from inappropriate immune responses to normally innocuous proteins. More than 300 IgE-binding proteins have been cloned and shown to cover structurally and functionally heterogeneous protein families including enzymes, backbone and storage proteins as well as proteins with unknown function. Structurally related cross-reactive allergens are involved in the pathogenesis of important clinical syndromes. We found that phylogenetically highly conserved proteins, including human manganese superoxide dismutase (MnSOD), are involved in IgE-mediated autoreactivity. **Methods:** MnSOD cloned from different phylogenetically distant species were produced as recombinant proteins in *Escherichia coli* and used to study IgE-mediated cross-reactivity and proliferative responses in peripheral blood mononuclear cells (PBMC) of individuals sensitised to *Aspergillus fumigatus* MnSOD. Homology models of the three-dimensional structures of MnSOD from *A. fumigatus*, *Drosophila melanogaster* and *Saccharomyces cerevisiae* using the human MnSOD structure refined at 2.2 Å as template were constructed to identify conserved ami-

no acid residues exposed to the solvent. **Results:** Cross-reactivity between the MnSOD at B and T cell level was demonstrated by inhibition experiments showing shared B cell epitopes and by the capability of the different MnSODs to induce proliferative responses in PBMCs of sensitised individuals. Structural modelling allowed to identify conserved residues exposed to the solvent. The identified residues are scattered over the sequence of the enzyme indicating putative conformational IgE-binding epitopes. **Conclusions:** The results obtained corroborate molecular mimicry as a plausible mechanism to explain autoreactivity to human MnSOD. Moreover, homology modelling provides a rational tool to identify conserved residues involved in defining cross-reactive B cell epitopes.

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Introduction

Superoxide dismutases (SODs, EC 1.15.1.1) are critical enzymes in the physiologic responses to oxygen toxicity [1] and potentially involved in pathologic conditions related to oxidative stress [2], lung and tissue damage [3, 4] and acute and chronic inflammation [5]. SODs belong to the metalloenzyme superfamily and act to dismutate toxic superoxide radicals to oxygen and hydrogen perox-

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ide [1]. Among the three forms of SOD which differ in the catalytic metal co-factor, manganese superoxide dismutase (MnSOD) is of primary biological interest as it is involved in defence mechanisms to protect mitochondrial DNA against oxidative damage [6]. In contrast to Cu, ZnSOD and FeSOD which are constitutively produced in vivo, MnSOD is an inducible enzyme [7, 8] as observed following treatment with X-radiation [9], interleukin-1 [10] and tumour necrosis factor [11]. Thus increased levels of MnSOD provide protection during periods of oxidative stress and, therefore, MnSOD has been considered for medical uses [12]. The general distribution pattern of the SODs indicates that the Cu,ZnSOD is essentially an eukaryotic and FeSOD a prokaryotic enzyme, whereas MnSOD is present in both, prokaryotes and eukaryotes [1]. SODs are considered as the first line of defence against the toxicity of oxygen radicals and, therefore, essential for all organisms depending on oxygen. Structurally FeSOD and MnSODs share a similar α/β fold [13] and are unrelated to the Greek key β -barrel fold of Cu,ZnSOD [14]. Comparison of complete amino acid sequence data for MnSODs demonstrates that the MnSODs in eukaryotic mitochondria and the MnSODs of prokaryotes have significant homology [15]. Apparently, MnSOD displays a high degree of sequence identity in a wide variety of phylogenetically distant organisms. This observation supports the endosymbiotic hypothesis of mitochondrial origin [16]. Moreover, X-ray crystallographic data for tetrameric human MnSOD [17], tetrameric MnSOD of *Thermus thermophilus* [18] and dimeric MnSOD of *Bacillus stearothermophilus* [19] show that these proteins are structurally related. Each subunit of these bacterial MnSODs, like those of human MnSOD, is divided into N- and C-terminal domains which are structurally similar [17]. In all known MnSOD structures, the metal ion is bound by four invariant protein ligands: one aspartate and three histidines [20]. This conserved motif is also found in MnSOD isolated from the pathogenic mould *Aspergillus fumigatus* [21], the etiologic agent identified in 80% of *Aspergillus*-related diseases [22]. The ubiquitous mould is considered as an opportunistic pathogen associated with an impressive list of pulmonary and allergic complications in humans and animals [22]. Although the pathophysiologic mechanisms leading to *Aspergillus*-related complications are largely unknown, cloning systems based on phage surface display technology [23, 24] allowed isolation and characterisation of a large panel of IgE-binding molecules from the fungus [24, 25]. The availability of pure *A. fumigatus* allergens allowed to dissect the complex polyclonal IgE-mediated

immune responses to the fungus at the molecular level and led to the discovery of disease-specific allergens [26]. Among these, MnSOD represents a specific marker for allergic bronchopulmonary aspergillosis (ABPA), an intense inflammatory response to *Aspergillus* in the lung [21, 26]. *A. fumigatus* MnSOD shows a high degree of T and B cell-mediated cross-reactivity with the human enzyme, both in vitro and in vivo [21]. IgE-mediated reactions against autoantigens seem to be restricted to patients suffering from severe chronic forms of allergic diseases [21, 27, 28] and to involve mainly phylogenetically highly conserved proteins. Therefore, molecular mimicry between conserved T and B cell epitopes [29] seems to be a reasonable mechanism to explain these adverse reactions. In the present work we have investigated the IgE-mediated cross-reactive responses to human MnSOD and to the homologous enzymes from phylogenetically distant organisms. Structural relationships between the different MnSODs were obtained by modelling the three-dimensional structures of the enzymes based on the known crystal structure of the human MnSOD as a first step to define conserved putative residues involved in the binding of cross-reactive IgE antibodies.

Material and Methods

Patients, Control Subjects and Routine Assessments

Sera from groups of patients sensitised to *A. fumigatus* suffering from ABPA or severe atopic dermatitis (AD) were analysed. Sensitisation to *A. fumigatus* was determined according to clinical history and immediate skin reactivity to *A. fumigatus* extracts. Patients suffering from ABPA fulfilled at least seven of the eight diagnostic criteria proposed by Greenberger and Patterson [30]. Patients suffering from AD were diagnosed according to standard procedures [31]. Patient characteristics including age, sex, total IgE level, RAST class to *A. fumigatus* and IgE responses to *A. fumigatus* MnSOD are summarised in table 1. Total serum IgE and allergen-specific IgE levels were determined with the Pharmacia CAP System (Pharmacia, Uppsala, Sweden). Skin prick tests were performed with commercial preparations from Allergopharma (Hamburg, Germany) and Ben-card (SmithKline Beecham, Neuss, Germany) using standard techniques [32]. Intradermal skin tests with recombinant MnSODs were performed as described [21]. For control purposes sera from healthy, non-atopic individuals without history of allergy or asthma and normal IgE levels were included. The study was carried out according to a clinical protocol approved by the local ethics committee.

Cloning, Production and Purification of MnSODs

A. fumigatus MnSOD was originally isolated from a cDNA library displayed on phage surface using serum IgE from individuals sensitised to the mould [21]. Human and *Drosophila melanogaster* MnSODs were isolated from commercially available cDNA libraries by PCR as described [33]. *Saccharomyces cerevisiae* MnSOD was amplified by PCR from genomic DNA preparations [33]. The re-

Table 1. Clinical data of *A. fumigatus*-sensitised patients with ABPA or AD and healthy controls

Patient	Diagnosis	Age	Sex	Total IgE kU/l ^a	RAST ^{a, b}	MnSOD-specific IgE, EU/ml ^c
1	ABPA	63	m	3,555	6	132
2	ABPA	53	m	808	4	85
3	ABPA	49	f	1,228	5	78
4	ABPA	60	m	263	3	49
5	ABPA	48	m	746	4	111
6	AD	35	m	15,000	4	98
7	AD	27	f	5,225	4	126
8	AD	26	m	4,870	1	31
9	ABPA	64	f	3,887	5	148
10	AD	36	f	6,470	4	72
11	ABPA	31	f	538	3	2
12	ABPA	52	f	7,862	6	4
13	AD	19	m	2,337	1	2
14	AD	37	f	3,436	3	3
15	AD	32	f	1,520	2	1
16	healthy	31	f	16	0	0
17	healthy	32	f	36	0	0
18	healthy	48	m	48	0	0
19	healthy	27	m	22	0	0
20	healthy	33	m	14	0	0
21	healthy	48	m	123	0	0

^a Determined with a UniCAP device (Pharmacia, Uppsala, Sweden).

^b Against *A. fumigatus* extract.

^c Arbitrary enzymatic units per millilitre (EU/ml) determined according to Cramer et al. [26]. Values below 7 EU/ml are considered as negative.

gions coding for the mature proteins were subcloned into the pQE 30 high-level expression vector (Qiagen, Hilden, Germany), constructs verified by nucleotide sequence determinations and used to produce hexahistidine-tagged recombinant proteins in *Escherichia coli* M15 strain [21]. The fusion proteins were purified in a single step by Ni²⁺-chelate affinity chromatography (Qiagen) under denaturing conditions and refolded by dialysis against deionised water containing 0.1 mM MnCl₂. Enzymatic activity of the recombinant proteins was determined with a specific assay calibrated with human erythrocyte SOD [1, 21]. Purity and molecular size of the proteins were analysed by polyacrylamide gradient gels (4–20%, Novex, San Diego, Calif., USA) under denaturing and reducing conditions using standard procedures [21]. Samples of 1 mg of the pure proteins were lyophilised for long-term storage.

IgE Binding and Inhibition Experiments

The specific binding properties of serum IgE from *A. fumigatus*-sensitised individuals to the recombinant MnSODs were analysed by an allergen-specific ELISA [32]. Inhibition ELISAs were performed to demonstrate that the enzymes share common IgE epitopes. For IgE dot blot analysis, proteins were spotted onto Hybond-ECL nitrocellulose (Amersham, Arlington Heights, Ill., USA), incubated with patients' sera diluted 1:10 as 'first antibody' followed by incubation with mouse anti-human IgE mAb TN-142 as a second antibody [21]. Dot blots were developed with the ECL system (Amersham) using horseradish peroxidase-conjugated goat anti-mouse IgG (Dako, Bur-

lingame, Calif., USA) as the detection system. Cross-reactivity between the different MnSODs was also demonstrated using mouse monoclonal antibodies raised against the *A. fumigatus* MnSOD.

Proliferative Responses of Peripheral Blood Mononuclear Cells from Individuals Sensitised to MnSOD

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised peripheral venous blood samples from individuals sensitised to MnSOD by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation, washed 3 times in phosphate-buffered saline and resuspended in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mM β-MET, 1% MEM non-essential amino acids and vitamins, 100 µg/ml streptomycin, 1,000 U/ml penicillin (all from Life Technologies, Basel, Switzerland), and 10% heat-inactivated fetal calf serum (Sera-Lab, Sussex, UK). Aliquots thereof (10⁶ cells/ml) were stimulated with different concentrations of the recombinant MnSOD preparations in triplicate for 7 days in humidified atmosphere with 5% CO₂. Proliferation was measured as incorporation of tritiated thymidine (DuPont/New England Nuclear, Boston, Mass., USA) during the final 16 h of culture [21]. A stimulation index >3 was considered as positive.

Sequence Analysis and Structural Modelling

The sequences of *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD were downloaded from the Swiss-Prot data bank [34]. By means of the alignment program ALIGN, the sequences were com-

Table 2. Sequence similarity and identity (in parentheses) in percent between the studied MnSODs

	<i>A. fumigatus</i>	<i>S. cerevisiae</i>	<i>H. sapiens</i> (1ABM)	<i>T. aquaticus</i> (1MNG)	<i>E. coli</i> (1VEW)
<i>Saccharomyces cerevisiae</i>	73.3 (43.9)				
<i>Homo sapiens</i> (1ABM)	75.1 (45.7)	73.8 (43.8)			
<i>Thermus aquaticus</i> (1MNG)	68.8 (42.5)	64.4 (39.3)	69.8 (45.7)		
<i>Escherichia coli</i> (1VEW)	63.8 (41.1)	63.5 (35.7)	67.6 (39.7)	75.9 (53.1)	
<i>Drosophila melanogaster</i>	71.5 (44.5)	67 (41.4)	82.4 (57.3)	69 (41.2)	73.2 (43.5)

The highest similarity values of the studied MnSODs compared to the human MnSOD are shown in italics.

pared to the functionally analogous MnSODs of human, *E. coli* and *T. thermophilus* whose structures have been solved and deposited in the PDB data bank with the codes 1ABM [17], 1VEW [35] and 1MNG [18], respectively (table 2). The initial three-dimensional models of *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD were constructed by homology modelling using the human MnSOD structure refined at 2.2 Å resolution (1ABM) as template. The Sybyl 6.4 molecular modelling package (Trypos, USA) was used to construct the model. 169 crystallographic water molecules situated either in the active site or in protein cavities were taken into account. All hydrogen atoms were added and the resulting protein was solvated into a 7.5 Å tick shell of TIP3P water molecules [36]. No position constraints were placed on the solvent molecules. All water molecules which had an atom closer than 1.75 Å to any atom of a solute molecule were discarded. A dielectric constant of 1 was used. Non-bonded interactions were calculated within a residue-based cut-off of 10 Å and a secondary cut-off of 12 Å. Based on the structural information indicating that the metal binding sites of MnSODs of different species are identical, positional constraints were applied to the metal binding site involving one Mn²⁺ ion, three histidines and one aspartate. The solvated models were then submitted to a two-step protocol. The first step consisted in an energy minimisation followed by 150-ps molecular dynamics (MD) to explore conformational space followed by a second energy minimisation of 30-ps time-averaged structures. In the second step the minimised 30-ps time-averaged structures were subjected to the same 150-ps MD at 297 K as in step one to assess the stability of the model. The quality of the geometry of the minimised mean structures was analysed by means of PROCHECK [37]. The minimised mean structure with the best geometry and conformational stability over time was chosen as the final three-dimensional model of the MnSOD of *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* that were used to study IgE-mediated cross-reactivity at humoral and cellular level. Energy minimisation and MD were performed using the force field of AMBER 5 [44] with the standard parameter set parm96 for the all-atoms mode. Both energy minimisations were carried out using 1,000 steps of steepest descent followed by a conjugate gradient until the root mean square gradient of the potential energy was less than 0.15 kcal/mol Å. The coordinates resulting from the first minimisation were then used as starting point for a 150-ps MD simulation at 297 K. Initial velocities were chosen from a Maxwellian distribution at 50 K. The system was progressively heated from 50 to 297 K for 1 ps and thereafter kept at 297 K through coupling to a heat bath with a 0.2-ps constant. The SHAKE

algorithms [39] were used, allowing a 2-fs integration step. Coordinates, energies and velocities were saved every 0.2 ps. The MD was equilibrated after 30 ps. To assess the quality of the protocol used, the template structure of the human MnSOD was submitted to the same energy minimisation and dynamics. The minimisation as well as the simulation were analysed using the CARNAL module implemented in AMBER 5. All calculations were run on a CRAY J90. Graphic display and analysis were achieved with the SYBYL 6.4 molecular modelling package.

Fractional Solvent-Accessible Surface Area

Solvent-accessible surface areas (SASAs) were calculated on the final three-dimensional model of the MnSOD with the program NACCESS [40], an implementation of the Lee and Richards solvent accessibility algorithm [41], using a probe radius of 1.4 Å and a slice width of 0.05 Å. The fractional SASA (FSASA) represents the ratio between the SASA of a given amino acid in the protein and its standard SASA calculated with a 1.4-Å probe radius and included in the NACCESS program.

Results

Production and Characterisation of MnSODs

MnSOD from a wide variety of phylogenetically distant organisms displays a high degree of sequence identity as shown in figure 1 for the MnSOD of *A. fumigatus*, *Homo sapiens*, *D. melanogaster* and *S. cerevisiae*. The complete cDNAs encoding these four MnSODs were subcloned into the high-level expression vector pQE 30 and constructs verified by sequence determination. Vectors carrying the correct inserts were used to express [His]₆-N-terminal fusion proteins in *E. coli*. Recombinant inclusion body proteins were solubilised in 6 M guanidinium chloride and purified under denaturing conditions by Ni²⁺-chelate affinity chromatography. The recombinant MnSODs were virtually pure when analysed by SDS-PAGE and Coomassie blue staining and the estimated molecular masses were in agreement with the calculated

<i>A. fumigatus</i>	10 20 30 40 50 60	QYTLPLPYPYDALQPYISQIMELHHKHHQTYVNGLNAALEAQKKAEEATDVPKLVSV
<i>H. sapiens</i>	10 20 30 40 50 60	KHSLPDLPYDYGALPHINAQIMQLHHSKHHAAVNNLNVTEEEKYQEAALAKGDTVTAQIAL
<i>A. fumigatus</i>	70 80 90 100 110 120	QQAIFNGGGHINHSLFWKNLAPEKSGGGKIDQAPVLKAAIEQRWGSFDFKFDAFNTTLL
<i>H. sapiens</i>	70 80 90 100 110	QPALKFNGGGHINHSIFWTNLS--NGGGE-PKGELLEAIKRDFGSFDFKFEKLTAAASV
<i>A. fumigatus</i>	130 140 150 160 170	GIQSGSGWGLVTDGPKGKLDITTTHTDQDPV---TGAAPVFGVDMWEHAYYLQYLNKASY
<i>H. sapiens</i>	120 130 140 150 160 170	GVQSGSGWGLGFNKERGHLQIAACPNDPLQGTGLIPLLGIDVWEHAYYLQYKNVRPDY
<i>A. fumigatus</i>	180 190 200	AKGIWNVINWAEAEENRYIAGDKGGHPFMKL
<i>H. sapiens</i>	180 190	LKAIWNVINWENVTERYMACCK-----
<i>D. melanogaster</i>	10 20 30 40 50 60	KHTLPKLKYDYAALEPIICREIMELHHQHHQTYVNNLNAAEEQLEEAKS KSDTKL IQL
<i>H. sapiens</i>	10 20 30 40 50 60	KHSLPDLPYDYGALPHINAQIMQLHHSKHHAAVNNLNVTEEEKYQEAALAKGDTVTAQIAL
<i>D. melanogaster</i>	70 80 90 100 110 119	APALRFNGGGHINHTIFWQNLS PNKT-QPSDDLKKAIESQWKSLEEFKKELTTLTVAVQG
<i>H. sapiens</i>	70 80 90 100 110 120	QPALKFNGGGHINHSIFWTNLS PNGGEPKGELLEAIKRDFGSFDFKFEKLTAAASVGVQG
<i>D. melanogaster</i>	130 140 150 160 170 179	SGWGLGFNKKSGKLQLAALPNQDPLEASTGLIPLFGIDVWEHAYYLQYKNVRPSYVEAI
<i>H. sapiens</i>	130 140 150 160 170 180	SGWGLGFNKERGHLQIAACPNDPLQGTGLIPLLGIDVWEHAYYLQYKNVRPDYLKAI
<i>D. melanogaster</i>	190 200	WDIANWDDISCRFQEA K LGC
<i>H. sapiens</i>	190	WNVINWENVTERYMACCK---
<i>S. cerevisiae</i>	10 20 30 40 50 60	KVTLPDLKWDGALPEYISGQINELHYTKHHQTYVNGFNTAVDQFQELSDLLAKEPSPAN
<i>H. sapiens</i>	10 20 30 40 50	KHSLPDLPYDYGALPHINAQIMQLHHSKHHAAVNNLNVTEEEKYQEA---LAK---GD
<i>S. cerevisiae</i>	70 80 90 100 110 120	ARKMIAIQNIKFHGGGFTNHCLFWENLAPESQGGGEPPTGALAKAIDEQFGLDELICKL
<i>H. sapiens</i>	60 70 80 90 100 110	VTAQIALQPALKFNGGGHINHSIFWTNLS PN--GGGEPK-GELLEAIKRDFGSFDFKFEK
<i>S. cerevisiae</i>	130 140 150 160 170	TNTKLAGVQSGWAFIVKNLSNGKLDVVQTYNQDTVTPGP--LVPLVAIDAWEHAYYLQY
<i>H. sapiens</i>	120 130 140 150 160 169	LTAASVGVQSGWGLGFN-KERGHLQIAACPNDPLQGTGLIPLLGIDVWEHAYYLQY
<i>S. cerevisiae</i>	180 190 200	QNKKADYFKAIWNVINWKEASRRFDAGKI
<i>H. sapiens</i>	180 190	KNVRPDYLKAIWNVINWENVTERYMACCK

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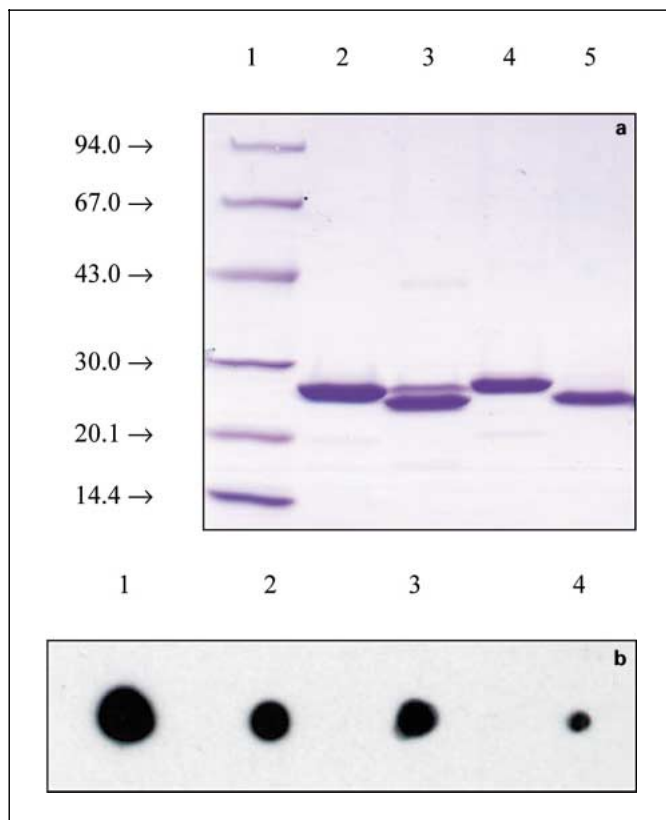


Fig. 2. a Recombinant proteins (3 µg) were separated on an SDS-polyacrylamide gradient gel (4–20%) and stained with Coomassie blue. Molecular mass standards (in kD) are indicated on the left side. Lane 1 = Marker proteins; lane 2: = *A. fumigatus* MnSOD; lane 3 = *H. sapiens* MnSOD; lane 4 = *D. melanogaster* MnSOD; lane 5 = *S. cerevisiae* MnSOD. b Dot blot analysis of IgE binding. 2 µg of each purified MnSOD protein (1 = *A. fumigatus*, 2 = *H. sapiens*, 3 = *D. melanogaster*, 4 = *S. cerevisiae*) were dotted onto a nitrocellulose membrane and blocked. The membrane was incubated with serum from *A. fumigatus*-allergic patients and bound IgE was identified by enhanced chemiluminescence detection.

Fig. 1. Alignment of the deduced amino acid sequences of *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD with the sequence of the homologous human MnSOD. Identical amino acids are shown in red, deletions, insertions and amino acids involved in manganese binding in green, magenta and blue, respectively. Identical amino acids exposed to the solvent (FSASA >0.5) are depicted in bold red italic letters.

values for the His-tagged proteins (fig. 2a). The upper band on lane 3 of figure 2a is likely to correspond to co-purified *E. coli* MnSOD because the proteins do not contain a [His]₆-fusion tag (data not shown). Production yields varied between a minimum of 40 mg/l culture for *S. cerevisiae* and a maximum of 180 mg/l for human MnSOD. The proteins, refolded by dialysis against deionised water containing 0.1 mM MnCl₂, were soluble at concentrations up to 2 mg/ml. The recombinant MnSOD preparations were demonstrated to be enzymatically active reaching specific activities varying from 1.8 to 2.7 arbitrary units/µg protein, depending on the batch. These activities were comparable to the enzymatic activity of human erythrocyte SOD used as a standard [21]. Enzymatic activity was regarded as indication for native-like refolding of the proteins after complete denaturation, resulting from the extraction and purification procedure.

Allergenic Properties of the MnSODs

The IgE-binding capacity of the purified recombinant MnSODs was first assessed by dot blot analysis using the pure recombinant proteins. Sera from patients sensitised to *A. fumigatus* MnSOD were tested for IgE binding to the structurally homologous enzymes. Specific IgE raised against all MnSODs could be detected; however, the affinity for IgE binding was strongest for *A. fumigatus* MnSOD and decreasing for *H. sapiens*, *D. melanogaster* and *S. cerevisiae* MnSOD (fig. 2b). Moreover, two monoclonal antibodies raised against the *A. fumigatus* MnSOD were able to recognise all the recombinant MnSODs used in this study (data not shown). These results show that MnSOD can act as pan-allergen in individuals sensitised to the fungal MnSOD. IgE binding to *A. fumigatus* MnSOD could be inhibited by pre-incubation of sera from patients sensitised to the fungal MnSOD with increasing amounts of the different MnSODs demonstrating that the enzymes share IgE-binding epitopes (fig. 3).

Primary Proliferative Responses of PBMCs from *A. fumigatus*-Sensitised Individuals

Representative dose responses of freshly isolated PBMCs to the four recombinant MnSOD preparations are shown in figure 4. Stimulation indices to optimal concentration of the MnSOD antigens for 10 patients with high levels of *A. fumigatus*-specific IgE, 5 *A. fumigatus*-sensitised individuals without IgE against MnSOD and 6 control subjects are reported in table 3. Individuals sensitised to the fungal MnSOD showed positive responses to fungal extract and to all recombinant enzymes, whereas the *A. fumigatus*-sensitised persons without serum IgE

Fig. 3. Competitive inhibition of IgE binding to solid-phase coated *A. fumigatus* recombinant MnSOD. Serum from *A. fumigatus*-sensitised patients was pre-incubated with different amounts of recombinant *A. fumigatus* (●), *H. sapiens* (■), *D. melanogaster* (+) and *S. cerevisiae* MnSOD (▲) or with rAsp f 4 protein (○) as negative control. Pre-incubated serum samples were transferred to wells coated with *A. fumigatus* MnSOD, and bound IgE was analysed by antigen-specific ELISA.

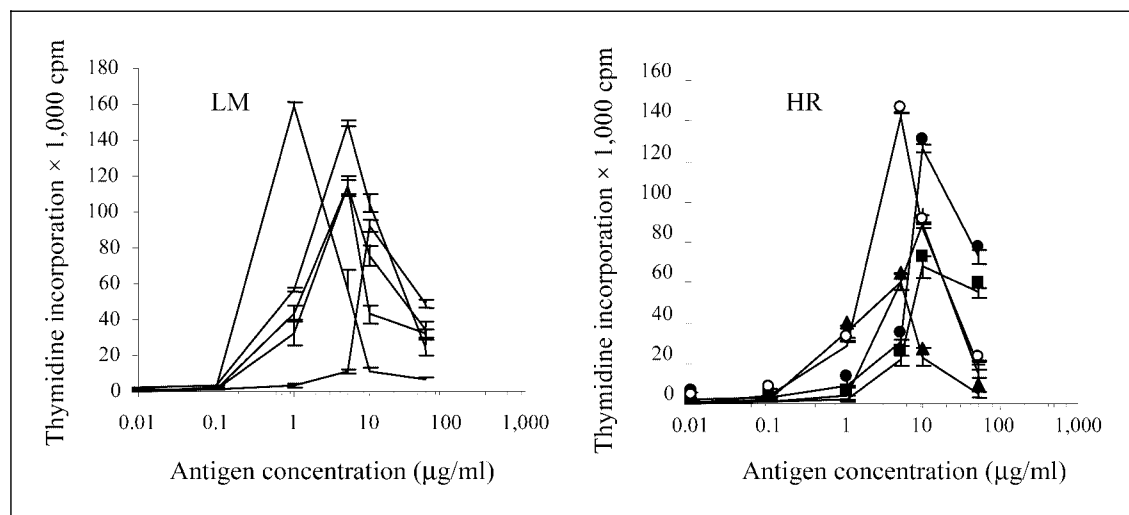
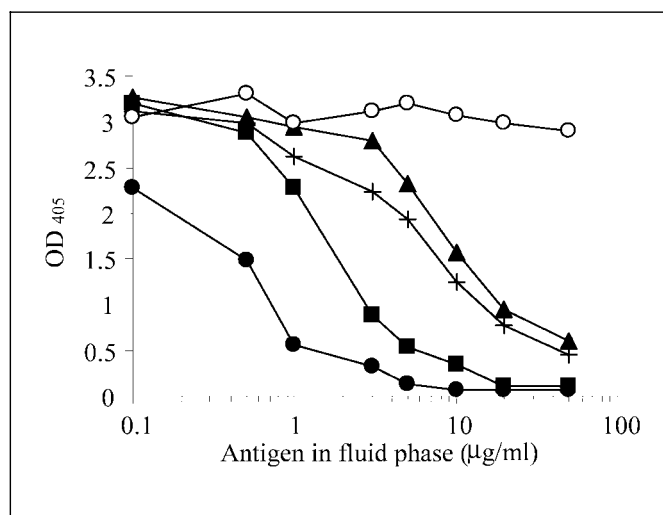


Fig. 4. Representative proliferative dose responses of PBMC from 2 patients sensitised to *A. fumigatus* MnSOD (LM and HR, table 1) to different MnSODs and to fungal extract. MnSOD from *A. fumigatus* (●), *H. sapiens* (■), *D. melanogaster* (+), *S. cerevisiae* (▲) and *A. fumigatus* extract (○) was used.

against MnSOD responded to the fungal extract only, indicating that the recombinant antigens did not induce non-specific effects. Healthy individuals did not respond to any of the antigen preparations (table 3). Comparison of the values by the rank sum test indicates highly significant differences ($p < 0.01$). The proliferative responses induced by human MnSOD in individuals sensitised to the *A. fumigatus* MnSOD indicate a pathogenesis related to autoreactive T cells resulting from molecular mimicry

between homologous peptide sequences present in the human and structurally homologous MnSODs [29].

Structural Modelling of *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD

Cross-reactivity is determined by structural aspects, and proteins are only cross-reactive if they share structural features [42]. To provide first structural insights into the IgE-mediated cross-reactivity among the MnSOD

Table 3. Stimulation indices of PBMCs of MnSOD-sensitised and control individuals after stimulation with different MnSODs

Patient	<i>A. fumigatus</i> extract	<i>A. fumigatus</i> MnSOD	<i>H. sapiens</i> MnSOD	<i>D. melanogaster</i> MnSOD	<i>S. cerevisiae</i> MnSOD
<i>A. fumigatus</i> - and MnSOD-sensitised individuals					
1	8.5	5.9	4.6	4.5	3.9
2	8.2	2.3	7.7	7.7	8.9
3	13.6	26.4	23.1	18.9	29.9
4	22.4	10.1	7.7	6.8	4.4
5	13.0	10.9	5.7	9.2	9.1
6	11.6	4.7	3.7	4.0	4.5
7	10.2	4.2	3.2	3.4	4.0
8	11.3	7.0	4.6	3.7	2.7
9	13.2	9.6	8.8	5.7	4.9
10	9.6	12.5	8.8	4.5	6.5
<i>A. fumigatus</i> -sensitised individuals without MnSOD sensitisation					
11	8.5	1.2	1.6	2.2	1.7
12	11.9	1.4	1.9	1.3	1.7
13	33.4	1.1	1.2	1.5	1.1
14	11.9	1.5	1.3	0.7	0.8
15	14.9	1.5	1.2	0.8	1.3
Healthy controls					
16	0.9	0.9	1.0	0.5	0.4
17	1.1	0.6	0.3	0.4	0.4
18	0.6	0.8	0.6	0.8	1.1
19	0.4	1.1	0.9	0.7	0.9
20	0.9	0.7	1.1	1.3	0.4
21	1.0	1.0	1.0	1.1	1.4

family, we have modelled the structures of *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD (fig. 5) based on the solved crystal structure of the human MnSOD [17]. The energy minimisation and MD protocol were assessed using the human MnSOD as template. The MD equilibrated after 30 ps and remained stable over the simulated time. The low root mean square deviation (r.m.s.d.) values of 2 Å for the human MnSOD suggest that the starting conformation, corresponding to the minimised X-ray structure, was reproduced by the MD protocol used. This indicates that the MD protocol is suitable for sampling the conformational space and for assessing the stability of the structural models. Conformational space sampling was performed resulting in the construction of several minimised 30-ps time-averaged structures based on the r.m.s.d. of the MD. The minimised mean structures of MnSOD of *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* with the best geometry show a geometrical quality corresponding to that of the template [17]. The dynamic stability of the time-averaged structures was assessed and the analysis of the r.m.s.d. yielded values around 2 Å indicating that the models of the three MnSODs are dynam-

cally stable and, therefore, reliable three-dimensional models.

As shown in the in pair alignment of the amino acid sequences (fig. 1), 102, 124 and 98 identical amino acids are shared between the primary structure of the *A. fumigatus*, *D. melanogaster*, *S. cerevisiae* and human MnSOD, respectively. The conserved amino acids include the three histidine residues at position 26, 74 and 163 and the aspartate at position 159 on the human MnSOD, which are involved in binding of the Mn²⁺ metal [17]. However, as shown in the structural models depicted in figure 5, only 20, 23 and 24 of the pairwise conserved residues between human and *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD, respectively, shown in bold red colour and italics in figure 1, are exposed to the solvent and therefore accessible for antigen-antibody interactions. The conserved residues are scattered over the surface of the MnSOD molecules (fig. 5), corroborating the conformational nature of cross-reactive epitopes, and could account for the accommodation of many epitopes within the tetrameric structure of the antigens [43]. However, there are only five identical solvent-exposed amino

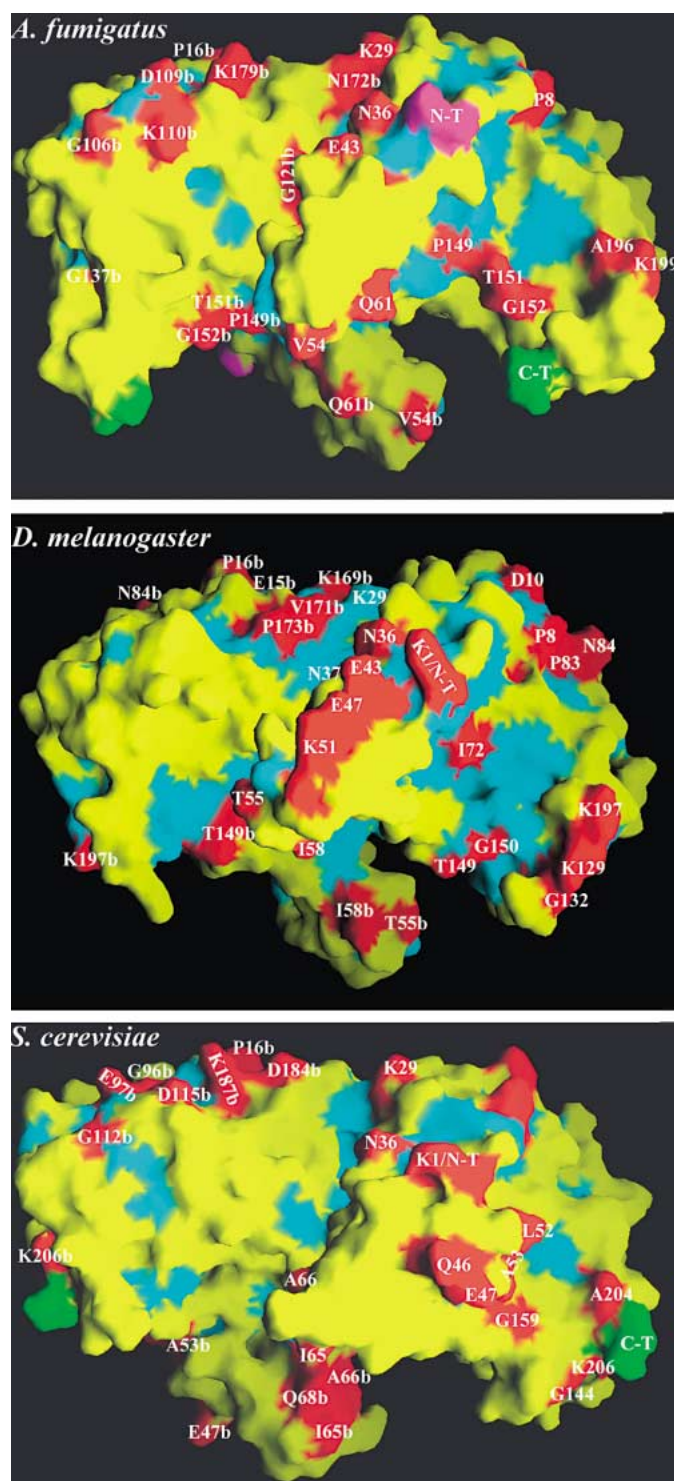


Fig. 5. Surface representation of the structural models of the *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD dimers. N- and C-terminal amino acids are shown in green. Solvent-exposed amino acid residues that are identical to those of the human MnSOD with FSASA >0.5 are shown in red, those with FSASA <0.5 in blue. The surfaces resulting from residues that are not identical to residues of the human enzyme are coloured in yellow.

acids (FSASA >50%) shared between the four MnSODs, corresponding to positions Pro16, Lys29, Asn36, Gly133 and Lys198 of the human MnSOD (fig. 1). From the surface analysis three of them, Lys29 and Asn36 of one monomer and Pro16 of the second monomer, are located close together in the three-dimensional space. The distances measured between Pro16-Lys29 (23.6–25 Å), Pro16-Asn36 (30.4–32 Å) and Lys29-Asn36 (10.4–11.5 Å) throughout the set of MnSODs studied are well conserved. These residues could define a shared conformational epitope. Because eukaryotic MnSODs are assembled from four identical subunits [1], each tetramer would contain at least two epitopes satisfying the minimal requirement needed to induce cross-linking of allergen-specific IgE bound to the surface of effector cells.

Discussion

Allergens represent a group of structurally and functionally heterogeneous proteins [43] characterised by their property to elicit hypersensitivity reactions through cross-linking of effector cell-bound IgE antibodies [44]. The ability of allergens to provoke cross-linking requires that allergenic proteins contain more than one B cell epitope. In spite of the fact that the primary structure of a large number of different allergens has been elucidated [45], no common structural or functional features allowing the prediction of whether a given protein possesses an intrinsic property to behave as an allergen have been found [43]. In contrast to the molecular features determining allergenicity which are not yet understood, cross-reactivity is largely determined by structural aspects and has been described to play a role in many clinically relevant allergy syndromes [46, 47]. Generally, cross-reactivity is rare between proteins sharing less than 40% sequence identity, but very common between proteins with higher amino acid sequence identity [43]. Striking examples of IgE-mediated cross-reactivity induced by environmental allergens are cellular and humoral autoreactivities elicited by allergen-homologous human proteins [21, 27, 28, 48]. Normally it would be expected that the presence of a human homologue accessible to the immune system is likely to induce immune tolerance to the common epitopes. However, in some instances tolerance to the human proteins appears to be absent, resulting in autoreactive IgE antibodies. A clear demonstration that human proteins can be involved in humoral and cell-mediated auto-reactivity in vivo has been reported for human MnSOD [21] and acidic ribosomal P₂ protein [28]. In both cases

the *A. fumigatus* allergens as well as the structurally homologous human proteins were recognised by IgE antibodies from allergic individuals sensitised to these *A. fumigatus* allergens, induced proliferative responses in PBMC and elicited strong type 1-specific skin reactions in these individuals [21, 28, 48]. In this study we show that recombinant MnSODs derived from *A. fumigatus*, *H. sapiens*, *D. melanogaster* and *S. cerevisiae* are able to bind IgE from sera of patients suffering from long-lasting chronic allergy to *A. fumigatus* (fig. 2). As shown in figure 3, all MnSODs are able to inhibit IgE binding to *A. fumigatus* MnSOD coated in the solid phase in a concentration-dependent manner if added to the serum in the fluid phase, demonstrating that the phylogenetically conserved MnSODs share IgE-binding epitopes. Moreover, as shown in figure 4 and table 3, all MnSODs were able to induce proliferative responses in PBMCs from individuals sensitised to *A. fumigatus* MnSOD, whereas *A. fumigatus*-sensitised persons lacking IgE antibodies against the fungal MnSOD responded to the fungal extract only. As allergen-specific IgE production is dependent on T cell help, proliferative responses indicate a T cell-mediated pathogenesis. Although sensitisation to human and *S. cerevisiae* MnSOD as a direct consequence of exposure cannot be excluded, it is very unlikely that patients become naturally exposed to the MnSOD of *D. melanogaster*. The fact that humoral and cell-mediated immune responses to *D. melanogaster* MnSOD are comparable to those of the other MnSODs investigated supports molecular mimicry between conserved T and B cell epitopes as a plausible mechanism to explain the observed reactions. In many infectious diseases, antibody responses are directed towards microbial antigens with a high degree of homology to self proteins [49], and molecular mimicry at the T cell level between homologous peptide sequences from human and microbial agents has been demonstrated in several autoimmune diseases [50, 51]. The availability of the crystal structure of the human MnSOD [17] and of the deduced amino acid sequences of the investigated MnSODs (fig. 1) allowed to define in more details the possible residues likely to be involved in IgE-mediated cross-reactivity by molecular modelling. The three-dimensional structures of *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD were modelled using homology-based approaches (fig. 5). Obviously, only identical amino acids of the different MnSODs exposed to the solvent are expected to play a key role for binding of cross-reactive IgE antibodies. From the approximately 100 identical amino acids deduced from the pairwise alignment of the sequences (fig. 1), only 20–24% are solvent-exposed (fig. 5).

These amino acids are scattered over the MnSOD sequence in agreement with experimental results obtained by co-crystallisation of Fab fragments with their protein antigens, which demonstrate that antibodies elicited by natural exposure to native protein antigens recognise discontinuous epitopes [42, 49, 50]. Comparisons of alignments and structure models revealed, unexpectedly, that between the four MnSODs only five identical amino acids were solvent-exposed. These residues are likely to define a cross-reactive conformational B cell epitope shared between all MnSODs investigated. However, pairwise comparisons of the modelled structures with the solved human MnSOD structure (fig. 5) show 20, 23 and 24 identical, solvent-exposed residues for *A. fumigatus*, *D. melanogaster* and *S. cerevisiae* MnSOD, respectively (fig. 1). These residues, scattered over the whole surfaces (fig. 5), would allow to accommodate more than four antibodies assuming a surface area of 38,000 Å² for the tetrameric MnSOD structure [17] and a buried surface of 540–900 Å² for the immune complex with intact protein antigens [42, 51]. This conservative estimation, which does not take into account possible contributions from conserved residues with FSASA <0.5 and from homologous residues spatially located in the same environment within the structures, indicates that cross-reactivity between conserved proteins represents a complex phenomenon. Although some specificities may be immunodominant and therefore more accessible to experimental verification by site-directed mutagenesis, it has to be pointed out that most of the protein surface is antigenic in polyclonal responses [52]. Since these responses are not restricted to a small number of epitopes [52], many antibody specificities can be expected in response to natural exposure. Depending on their specificity, subsets of antibodies derived from different memory cells may be able to react with a given MnSOD pair failing to recognise the other closely related proteins. More experimental work will be required to estimate the number of epitopes involved in cross-reactivity among MnSODs.

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