Brief Definitive Report

Humoral and Cell-mediated Autoimmunity in Allergy to *Aspergillus fumigatus*

By Reto Crameri,* Alexander Faith,* Stefanie Hemmann,* Rolf Jaussi,‡ Chaim Ismail,§ Günter Menz,§ and Kurt Blaser*

*From the *Swiss Institute of Allergy and Asthma Research (SIAF), CH-7270 Davos, Switzerland; ‡Institut für Radiobiologie, Paul Scherrer Institut, CH-5232 Villigen-PSI, Switzerland; and §Hochgebirgsklinik, CH-7275 Davos-Wolfgang, Switzerland*

Summary

A cDNA encoding an allergenic protein was isolated from an *Aspergillus fumigatus* (*A. fumigatus*) cDNA library displayed on the surface of filamentous phage. Serum immunoglobulin E (IgE) from *A. fumigatus*–sensitized individuals was used to enrich phage-expressing gene products binding to IgE. One of the cDNAs encoded a 26.7-kD protein that was identified as a manganese superoxide dismutase (MnSOD) sharing 51.5% identity and 67.2% homology to the corresponding human enzyme. Both human and *A. fumigatus* MnSOD coding sequences were expressed in *Escherichia coli* as $[His]_6$-tagged fusion proteins and purified by Ni^{2+}-chelate affinity chromatography. The two recombinant MnSODs were both recognized by IgE antibodies from subjects allergic to the *A. fumigatus* MnSOD and elicited specific immediate type allergic skin reactions in these individuals. Moreover, both human and *A. fumigatus* MnSOD induced proliferation in peripheral blood mononuclear cells of *A. fumigatus*–allergic subjects who showed specific IgE responses and reacted in skin tests to MnSOD. These observations provide evidence for autoreactivity to the human MnSOD in allergic persons sensitized to an environmental allergen from *A. fumigatus* who share a high degree of sequence homology to the corresponding human enzyme.

Members of the genus *Aspergillus* are ubiquitously distributed fungi that are considered opportunistic pathogens. They are associated with a wide spectrum of diseases in humans and animals, ranging from benign colonization of the lung and allergy to life-threatening diseases such as invasive aspergillosis or allergic broncho-pulmonary aspergillosis (1). *Aspergillus fumigatus* is the etiologic agent identified in 80% of *Aspergillus*-related human diseases and is of particular clinical relevance. A variety of underlying conditions, including impaired immune status, contribute to development of aspergillosis (2). However, the pathophysiologic mechanisms leading to *Aspergillus*-related pulmonary complications are largely unknown. A better understanding of the mechanisms leading to fungal allergy requires more information about structure and function of the antigens/allergens involved. Cloning and sequencing of allergen-encoding cDNAs have permitted the functional characterization of some allergens by sequence comparison with those of known proteins. The spectrum ranges from enzymes such as proteases (3) or phospholipases (4) to pathogenesis-related or structural plant proteins (5). From *A. fumigatus*, the 18-kD protein Asp f1, a member of the ribotoxins (6), has been cloned (7), produced, and shown to be a major allergen suitable for in vivo and in vitro diagnosis of sensitization to *A. fumigatus* (8, 9).

We cloned cDNAs encoding allergenic *A. fumigatus* proteins distinct from Asp f1 using a new technology based on display of cDNA libraries on the surface of filamentous phage (10, 11). Here, we describe the sequence and properties of one of these allergens, identified as an *A. fumigatus* superoxide dismutase (SOD; E.C. 1.15.1.1.), by sequence homology and enzymatic activity (12). The protein is recognized by IgE antibodies of individuals allergic to *A. fumigatus*, shows strong humoral cross-reactivity to human manganese superoxide dismutase (MnSOD), and both human and fungal MnSODs induce strong responses in PBMC.

Materials and Methods

Construction of a cDNA Library Displayed on Phage Surface and Biopanning. An *A. fumigatus* cDNA library was constructed in phagemide pJuFo (10) and displayed on the surface of filamentous phage M13 as described (11). The library was selectively enriched for phage displaying IgE-binding proteins by biopanning in microtiter plates coated with serum IgE from individuals allergic to *A. fumigatus*. Serum donors were selected according to case history, specific IgE to *A. fumigatus* determined by radioallergosor-

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H.sapiens     KHSLPDLPYD YGALEPHINA QIMQLHHSKH HAAYVNNLNV TEEKYQEALA .......KGD VTAQIALQPA LKFNGGGHIN HSIFWTNLSP NGGGEPKGE. ...LLEAIKR
A.fumigatus   QYTLPPLPYP YDALQPYISQ QIMELHHKKH HQTYVNGLNA ALEAQKKAAE .......ATD VPKLVSVQQA IKFNGGGHIN HSLFWKNLAP EKSGGGKIDQ APVLKAAIEQ
S.cerevisiae  KVTLPDLKWD FGALEPYISG QINELHYTKH HQTYVNGFNT AVDQFQELSD LLAKEPSPAN ARKMIAIQQN IKFHGGGFTN HCLFWENLAP ESQGGGE.PP TGALAKAIDE

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H.sapiens     DFGSFDKFKE KLTAASVGVQ GSGWGWLG.F NKERGHLQIA ACPNQDPLQG TTGLIPLLGI DVWEHAYYLQ YKNVRPDYLK AIWNVINWEN VTERYMACKK
A.fumigatus   RWGSFDKFKD AFNTTLLGIQ GSGWGWLV.T DGPKGKLDIT TTHDQDPVTG ...AAPVFGV DMWEHAYYLQ YLNDKASYAK GIWNVINWAE AENRYIAGDK GGHPFMKL
S.cerevisiae  QFGSLDELIK LTNTKLAGVQ GSGWAFIVKN LSNGGKLDVV QTYNQDTVTG ..PLVPLVAI DAWEHAYYLQ YQNKKADYFK AIWNVVNWKE ASRRFDAGKI
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Figure 1. Amino acid sequences of human (21) and yeast (25) MnSOD aligned with the *A. fumigatus* MnSOD sequence. Identical amino acid residues are marked by vertical lines. Gaps are indicated by dots. Sequence identity between *A. fumigatus* and human MnSOD is 51.5% and between *A. fumigatus* and yeast MnSOD 50.75%, whereas sequence identity with the MnSOD from other species varied between 30 and 49%. Numbers above the sequence indicate the residue number, including gaps, starting at the NH_2-terminal lysine of the mature human enzyme.

bent test (RAST), and skin reactivity to commercial *A. fumigatus* extracts (8). A wide variety of phage binding to human IgE was isolated (11).

Identification of a Clone Encoding A. fumigatus MnSOD. Selected clones differing in the length of the inserts were sequenced using the dideoxynucleotide chain termination method (13). Both DNA strands were sequenced using vector-derived primers. Homology searches were performed with BLAST and the Genetics Computer Group program FASTA (14, 15). Clone 19 revealed strong homology with nucleotide sequences encoding superoxide dismutases (Fig. 1).

Cloning of the Human MnSOD, Production, and Characterization of Recombinant Proteins. The cDNA encoding human MnSOD was amplified by standard PCR (16) from a commercial human lymphoma U937 lung cDNA library (Stratagene, La Jolla, CA) using the following primers: 5′-primer 5′-GGAAGATCTAAGCACAGCCTCCCCGACCTGCCC-3′; 3′-primer 5′-CCCAAGCTTTTACTTTTTGCAAGCCATGTATCTTTC-3′. The complete cDNA encoding the putative *A. fumigatus* MnSOD was amplified from the original clone by PCR using the following primers: 5′-primer 5′-GGAAGATCTCAATACACGCTCCCACCCCTCC-3′, 3′-primer 5′-CCCAAGCTTTCACAGCTTCATGAATGGG-3′. PCR cycling conditions were 94°C for 60 s, 53°C for 60 s, and 72°C for 60 s for 30 cycles, followed by a terminal extension cycle at 72°C for 10 min. The amplification products were purified over QIAquick spin columns (Quiagen Inc., Chatsworth, CA), digested with BglII and HindIII, ligated to BamHI/HindIII–restricted p(His)$_6$-DHFR vector (17) and transformed into *E. coli* strain M15 (7) by electroporation (18). Transformants were grown in liquid culture to verify the nucleotide sequence (13) and used to produce hexahistidine-tagged recombinant proteins as described (7, 17). The fusion proteins were purified in a single step by Ni^{2+}-chelate affinity chromatography (17) to obtain recombinant proteins of high purity. Purity and molecular size of the proteins were analyzed by polyacrylamide gradient gels (12.5–20%) using standard procedures (19), and were shown to be biologically active by determination of the enzymatic activity of the preparations (12).

ELISA and IgE Immunoblots. The specific binding of human serum IgE from individuals sensitized to *A. fumigatus* to recombinant human and *A. fumigatus* MnSOD was analyzed by an antigen-specific ELISA as described previously (8). Inhibition ELISAs were performed to demonstrate that the enzymes share common epitopes. For IgE immunoblot analysis, proteins were separated on an SDS-polyacrylamide gradient gel (12.5–20%), transferred to Hybond-ELC nitrocellulose (Amersham, Arlington Heights, IL), incubated with patient sera diluted 1:10 as "first antibody," followed by incubation with mouse anti–human IgE mAb TN-142 (8) as second antibody. Western blots were developed with the ECL system (Amersham) using horseradish peroxidase–conjugated goat anti–mouse Ig (Tago, Inc., Burlingame, CA) as a detection system.

Proliferative Responses of PBMC from Individuals Sensitized to A. fumigatus MnSOD. PBMC aliquots (10^6 cells/ml) isolated from heparinized blood samples from individuals sensitized to *A. fumigatus* MnSOD were stimulated with different concentrations of *A. fumigatus* extract, recombinant human, or *A. fumigatus* MnSOD for 7 d. Proliferation was measured as incorporation of tritiated thymidine during the final 16 h of culture. A stimulation index >3 was considered as positive.

Skin Tests with Recombinant MnSOD. Lyophilized recombinant protein (1 mg) was dissolved in 1 ml 0.9% saline and diluted at serial 10-fold dilutions. For intradermal skin tests, 100 μl of the solutions were injected with a syringe starting from a concentration of 10^{-5} to 1 μg ml^{-1}. 0.01% histamine dihydrochloride and 0.9% saline were used as positive and negative controls, respectively. The skin reactions were recorded after 15 min and re-

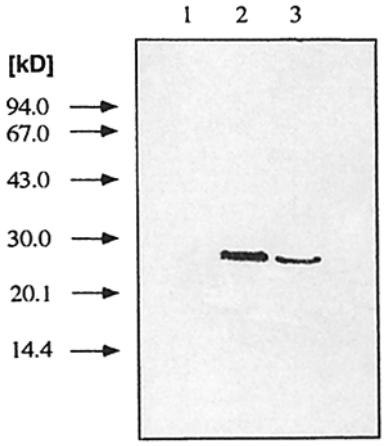

Figure 2. Autoradiography of IgE immunoblots: molecular mass standards (in kilodaltons) are indicated at the left side. Each recombinant protein (1 μg) was separated on an SDS-polyacrylamide gradient gel (12.5–20%) and blotted onto a nitrocellulose membrane. The membrane was incubated with serum from *A. fumigatus*–allergic patients diluted 1:10 in 50 mM sodium phosphate, pH 7.4, 5% Tween 20, 2% BSA. Bound IgE was identified by enhanced chemiluminescence detection (8). Lane *1*, marker proteins as negative control; lane *2*, recombinant *A. fumigatus* MnSOD; lane *3*, recombinant human MnSOD.

garded positive if the wheal was at least half the size of the skin reaction induced by the positive histamine control (8). An ethical approval for skin testing human subjects with recombinant proteins was provided via the local ethics committee. A full explanation of the procedure was given to all participants, and their written consent was obtained before testing.

Results and Discussion

Isolation of cDNA Clones and Sequence of the A. fumigatus MnSOD. Cloning of cDNA libraries on phagemid pJuFo (10) and display of the expression products on the surface of filamentous phage (11, 20) allow efficient isolation of cDNAs that encode proteins for which a ligand is available. The procedure circumvents immobilization of the libraries on solid-phase supports that hamper selective enrichment of clones expressing the desired protein. We have generated a cDNA library from *A. fumigatus* displayed on the surface of the filamentous phage M13, which was screened for displayed gene products binding to human serum IgE from patients allergic to the fungus (11). The large phage population obtained after selective enrichment by ligand–product interaction contained phage encoding different allergenic proteins as demonstrated by sequence determinations

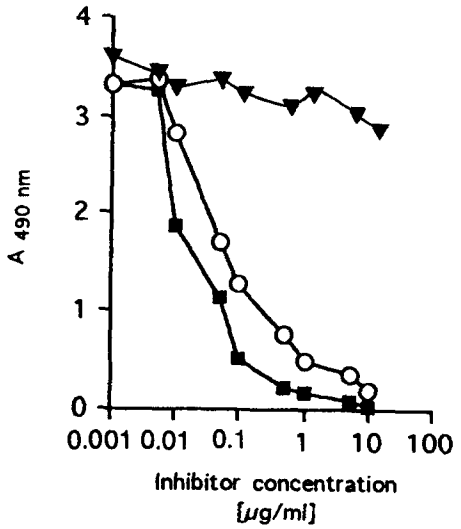

Figure 3. Competitive inhibition of IgE binding to solid-phase–coated *A. fumigatus* recombinant MnSOD. Serum from *A. fumigatus*–sensitized patients was preincubated with different amounts of recombinant *A. fumigatus* (■) MnSOD, human (○) MnSOD, or Asp f1 protein as negative control (▼). Preincubated serum samples were transferred to wells coated with *A. fumigatus* MnSOD, and IgE bound was analyzed by antigen-specific ELISA (8).

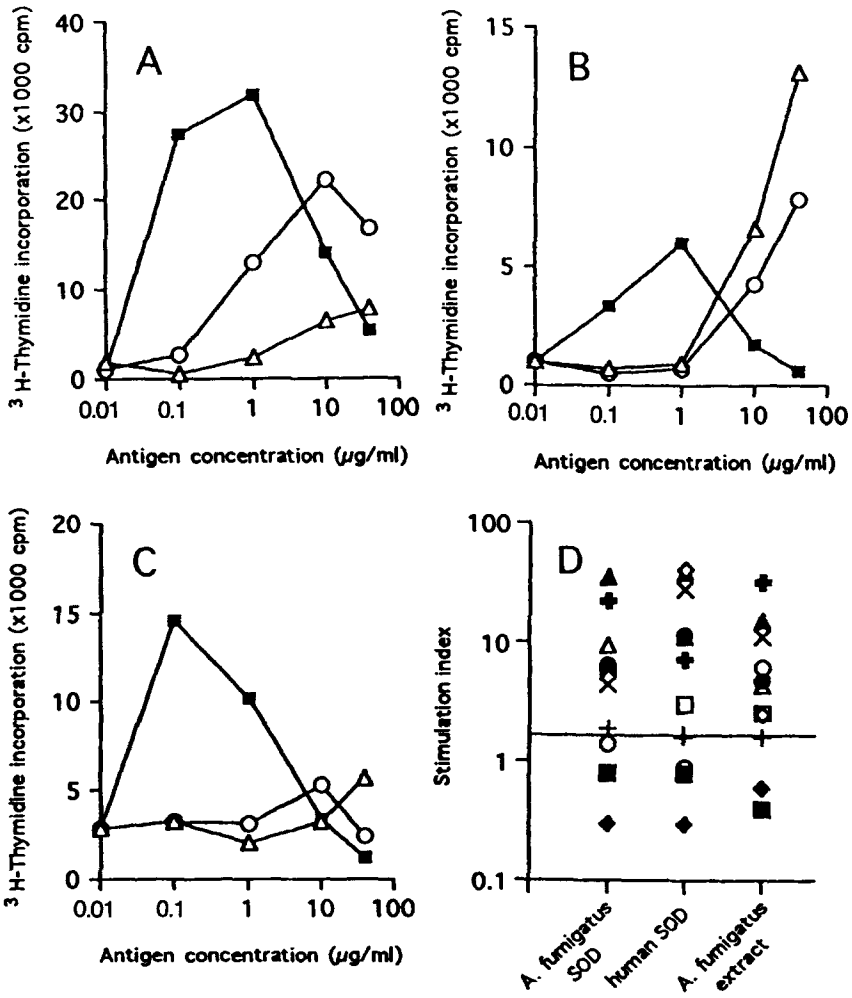

Figure 4. Proliferative responses of PBMC from patients sensitized to *A. fumigatus* to recombinant *A. fumigatus* MnSOD (○), recombinant human MnSOD (Δ), and *A. fumigatus* extract (■). Representative dose responses to each of the antigens in PBMC from patients sensitized to the fungal MnSOD are shown in *A–C*. Stimulation indices to optimal concentration of the antigens for seven patients responding to both recombinant *A. fumigatus* and human MnSOD (◇ ▲ □ ● Δ + X), one patient without detectable IgE against *A. fumigatus* MnSOD responding to the *A. fumigatus* extract alone (○), and three control subjects (◆ ■ +) are given in *D*.

(our unpublished results). Clone 19 containing an insert of 751 bp consists of a coding region of 630 bp (bases 34–633) and revealed strong homology with nucleotide sequences encoding superoxide dismutases. The 3′-noncoding region has a polyadenylated tail of 24 bp. The cDNA and deduced amino acids sequence (210 residues) were submitted to GeneBank/EMBL/DDBJ (accession number U53561). The deduced amino acid sequence of this cDNA clone (Fig. 1) was homologous to MnSOD, showing the highest sequence identity of 48–52% to the human (21), bovine (22) fruit fly (23), gum tree (24), yeast (*Saccharomyces cerevisae*; 25), and *Escherichia coli* (26) enzyme. Apparently, the *A. fumigatus* MnSOD displays a similar degree of sequence identity to MnSOD from a wide variety of phylogenetically distant organisms.

Manganese SOD is one of three enzyme forms that catalyze the dismutation of superoxide radicals. The SODs are considered as the first line of defence against the toxicity of oxygen-related radicals and are widespread in all organisms that depend on oxygen (12). Reactive oxygen species have been postulated to underlie the pathogenesis of various inflammatory diseases and to play important roles in cellular defence including bactericidal action of phagocytes (27, 28).

Production and Characterization of Recombinant A. fumigatus and Human MnSOD. The complete cDNA encoding the putative *A. fumigatus* MnSOD was expressed in *E. coli* as a hexahistidine-tagged fusion protein using the p$(His)_6$-DHFR vector and purified by nickel-chelate affinity chromatography (17). The cDNA encoding human MnSOD was isolated by PCR, subcloned into p$(His)_6$-DHFR, expressed, and affinity purified. Both recombinant MnSODs were virtually pure when analyzed by SDS-PAGE and Coomassie blue staining and the estimated molecular mass was in agreement with the calculated values of 26.713 and 25.725 kD for the *A. fumigatus* and human MnSOD, respectively (data not shown). The recombinant proteins were demonstrated to be enzymatically active and reached a specific activity varying from 2.32 to 2.61 U/mg, depending on the batch, which is comparable to the enzymatic activity of human erythrocyte SOD used as a standard (2.45×10^3 U/mg; reference 29).

Allergenic Properties of the MnSODs. The *A. fumigatus* MnSOD was isolated as an IgE binding protein displayed on phage surface and thus expected to be an allergen. However, both *A. fumigatus* and human recombinant MnSODs were identified as relevant allergens by IgE-immunoblots (Fig. 2) and by ELISA assays with sera from 60 individuals suffering from *A. fumigatus*-related complications (data not shown). The binding of serum IgE from patients allergic to *A. fumigatus* to solid-phase–bound *A. fumigatus* MnSOD was inhibited by increasing amounts of recombinant human MnSOD added to the fluid phase (Fig. 3), demonstrating that the proteins share common IgE-binding epitopes. Although both proteins are able to fully inhibit the binding of IgE to *A. fumigatus* MnSOD, ~10 times more of human MnSOD was required to achieve the same inhibitory effect, indicating that serum IgE antibodies bind to human MnSOD with lower affinity.

Primary Proliferative Responses of PBMC from A. fumigatus Sensitized Individuals. As allergen-specific IgE production is dependent on T cell help, we measured the proliferative responses of mononuclear cells from individuals sensitized to *A. fumigatus* to fungal extract, recombinant *A. fumigatus* MnSOD, and recombinant human MnSOD. Individuals sensitized to the fungal MnSOD had positive responses to both recombinant enzymes, whereas an *A. fumigatus*–sensitized person lacking IgE antibodies against the fungal MnSOD responded to the fungal extract only. This also indicates that the recombinant antigens did not induce nonspecific effects. Three individuals with high level background proliferation did not respond to any of the antigen preparations (Fig. 4). In additional experiments, the mean proliferative responses of six control individuals to *A. fumigatus* extract, fungal MnSOD, and human MnSOD were 968, 1,388, and 1,336 cpm, respectively. The mean proliferative responses of six individuals sensitized to the fungal MnSOD to the same antigens were 6,981, 7,282, and 12,300

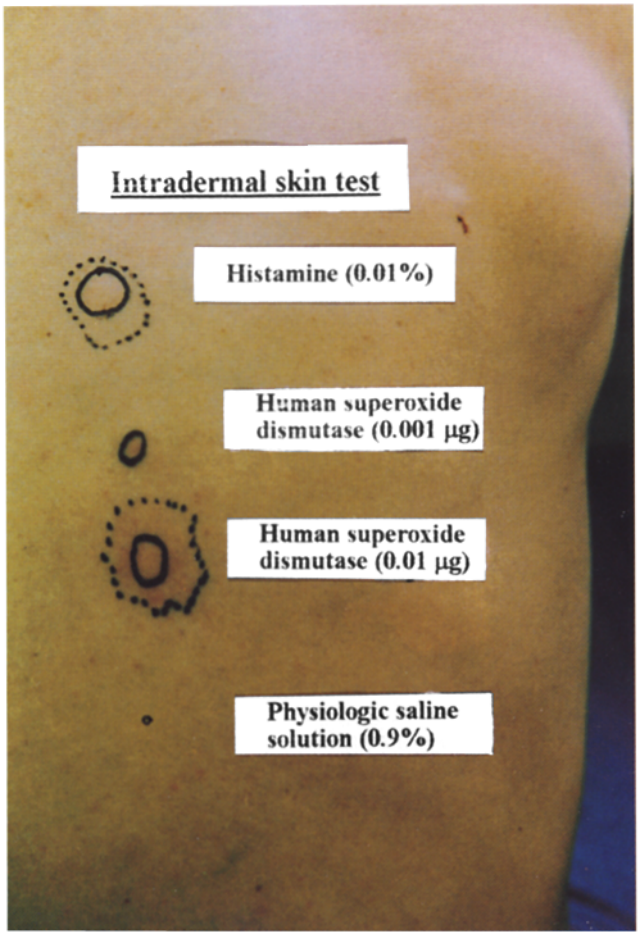

Figure 5. Skin test reactivity to human recombinant MnSOD. For intradermal skin tests, 100 μl of the solutions were injected with a syringe starting from a concentration of 10^{-4} μg ml^{-1}. 0.01% histamine dihydrochloride and 0.9% saline were used as positive and negative controls, respectively. The reactions show that 0.01 μg human MnSOD are able to elicit a wheal that is comparable with the size of the skin reaction induced by the positive histamine control.

cpm, respectively. Comparison of the values by the rank sum test indicates highly significant differences ($P < 0.01$). These responses to a self antigen in individuals sensitized to the *A. fumigatus* MnSOD indicate an autoantigenic T cell–mediated pathogenesis. In many infectious diseases, dominant antibody responses are directed towards microbial antigens with a high degree of homology to self proteins (30). Molecular mimicry at the T cell level to homologous peptide sequences from human and microbial antigens has been demonstrated in several autoimmune diseases (30, 31).

In Vivo Relevance of the Recombinant MnSODs. The final demonstration that a protein acts as an allergen is its ability to elicit type I skin reactions in allergic individuals. Therefore, we investigated whether the cross-reactivity of the anti-MnSOD IgE antibodies is sufficient to provoke allergic reactions in vivo by skin tests (8). Four individuals allergic to *A. fumigatus* MnSOD, two individuals allergic to *A. fumigatus* proteins other than MnSOD, and two nonallergic control persons were tested for their ability to respond to intradermal challenge with recombinant *A. fumigatus* and human MnSOD. A positive skin reaction to *A. fumigatus* MnSOD was observed only in individuals who had detectable IgE antibodies to *A. fumigatus* MnSOD. These individuals also showed strong skin reaction to human MnSOD challenge (Fig. 5). The immediate skin reaction depends on mast cells that are rapidly degranulated and release mediators, particularly histamine, which is triggered through antigen-dependent IgE cross-linking to specific receptors on the mast cell membrane (32). These results show that human MnSOD is able to elicit IgE cross-linking on mast cells in vivo, and they suggest a humoral autoimmune response in some of the patients suffering from *A. fumigatus* allergy.

In summary, these data provide strong evidence for in vitro and in vivo humoral and cell-mediated autoimmune reactivity to human MnSOD in patients allergic to *A. fumigatus*. Whether the cause of these autoimmune reactions is caused by molecular mimicry between conserved T and B cell epitopes present on the fungal and on the structurally related human enzyme or by sensitization to human MnSOD due to an inflammatory process remains to be elucidated.

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Address correspondence to Reto Crameri, Ph.D., Swiss Institute of Allergy and Asthma Research, Obere Strasse 22, CH-7270 Davos Platz, Switzerland.

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