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A Monoclonal Antibody That Specifically Binds Chitosan *In Vitro* and *In Situ* on Fungal Cell Walls

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We report the generation of the first monoclonal antibody that specifically binds to the polysaccharide chitosan. Mice were immunized with a mixture of chitosans, and hybridoma clones were screened for specific binders, resulting in the isolation of a single clone secreting a chitosan-specific IgM, mAbG7. In ELISAs, the antibody could bind to chitosans of varying composition, but demonstrated the highest affinity for chitosans with lower degrees of acetylation (DA) and very poor binding to chitin. We tested the ability of the antibody to bind to chitosan *in situ*, using preparations of fungal cell walls. Immunofluorescence microscopy confirmed that the antibody bound strongly to the cell walls of fungi with high levels of chitosan, whereas poor staining was observed in those species with cell walls of predominantly chitin or cellulose. The potential use of this antibody for the detection of fungal contamination and the protection of plants against fungal pathogens is discussed.

Keywords: Degree of acetylation (DA), D-glucosamine, IgM, *N*-acetyl-D-glucosamine, polysaccharides

Chitin, the second most abundant naturally occurring polysaccharide in the world after cellulose, is embedded as building substance into the exoskeletons of insects, crustacean shells, nematodes, arthropod cuticles [26], and vertebrates [2]. It is also a major component of the fungal cell wall, where it helps to maintain the cell's shape, strength, and integrity [23]. Chitin is not found in higher plants or prokaryotes [6].

Chitin and its more soluble derivative chitosan are linear polysaccharides comprising β-1,4-linked *N*-acetyl-D-glucosamine (GlcNac) and D-glucosamine (GlcN) residues. Chitin and chitosan differ in the degree of acetylation (DA) at the C2 atom of the glucosamine moiety. Chitin has a high DA (≥50%) and is insoluble in aqueous and organic solvents, whereas chitosan has a low DA (<50%) and is soluble in dilute acid because of the protonation of the amino group [17]. The unique properties of chitosan make it useful in medicine, nutrition, and industrial chemistry [20, 35]. Anti-chitosan antibodies would be valuable for quality control during the industrial synthesis of the polymer, as well as for the detection of chitosan in food and feed (which would indicate fungal contamination) [21]. Beyond mere detection, anti-chitosan antibodies could be used to develop strategies for the protection of plants against fungal pathogens, for example by coupling the antibodies to lytic enzymes that target the fungal cell wall. A similar strategy has been used to generate *Fusarium*-resistant plants [24, 29].

Polyclonal antibodies against chitosan have been generated by several groups, using chitosans with different properties such as DA, molecular weight (MW), and degree of polymerization (DP_w) [10, 12, 18, 32, 34, 36]. However, monoclonal antibodies (mAbs) recognizing chitosan and the monomer GlcN have not been reported thus far, although mAbs against GlcNac are available [8, 33]. Monoclonal antibodies generated by hybridoma technology are advantageous because they bind specifically to a single antigen and can provide stable quality in unlimited amounts. To the best of our knowledge, we are the first to report the generation of an anti-chitosan mAb that binds specifically to chitosan in ELISAs and detects chitosan *in situ* in fungal cell walls.

Materials and Methods

Generation of Anti-Chitosan Monoclonal Antibodies

Six to eight weeks old BALB/c mice were immunized with 100 μg of a mixture of natural chitosan polymers (equal parts 0%, 10%, and

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70% DA) [22] as well as with 100 µg of commercial chitosan (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) that was pretreated with Wch1 wheat endochitinase [25]. After seven rounds of immunization, the mice were sacrificed, spleens were isolated, and hybridoma cells were generated as previously described [7, 37]. Two rounds of limiting dilution were carried out prior to testing by ELISA, and positive monoclonal cells were selected. After the second round, antibody-producing hybridoma cells were expanded from 96-well microtiter plates into suspension mass cultures. Antibody production, reactivity, isotype, and specificity were characterized as previously described [13]. Cultures of mAbG7, an IgM/kappa isotype, were settled in FCS-free medium Panserin H5000 (PAN-Biotech, Aidenbach, Germany). The purity and conformation of the mAbG7 preparation were confirmed by SDS–PAGE and immunoblot analysis.

Fungal Isolates and Media

Pure cultures of *Aspergillus niger* van Tieghem and *Botrytis cinerea* Persoon: Fries (Fraunhofer IME, Aachen, Germany), *Penicillium hirsutum* Dierckx and *Fusarium culmorum* (W.G. Smith) Saccardo (University Hospital Aachen, Germany), *Fusarium graminearum* BBA72297 and *Phytophthora infestans* (Mont.) de Bary (Biologische Bundesanstalt für Land-und Forstwirtschaft, Braunschweig, Germany), and *Rhizoctonia solani* Kühn (International Rice Research Institute, Manila, The Philippines) were cultivated on potato dextrose agar (PDA; Merck, Darmstadt, Germany), tomato agar [25% (v/v) tomato juice, 3 g/l $CaCO_3$, 15 g/l agar], or liquid potato dextrose broth medium (PDB; Merck). Carboxymethylcellulose medium (CM) [9] was used to isolate *Fusarium* sp. spores. For spore germination, commercial tomato juice was centrifuged (4,000 ×*g*, 4°C, 5 min), and the clear supernatant was diluted 1:1 with water, filtered through 0.45-µm and 0.22-µm membranes, and then used.

Isolation and Germination of Spores

Cultures of *A. niger*, *B. cinerea*, and *P. hirsutum* were cultivated on PDA for 3–7 days. Spores were isolated by washing them from mycelia of the overgrown plate with sterile water and scraping the plate with a sterile spatula. The supernatant was filtered through three layers of Miracloth (Calbiochem) and washed three times with sterile PBS by centrifugation (4,000 ×*g*, 10 min, room temperature). *Fusarium* sp. spores were enriched by cultivating a piece of *Fusarium*-overgrown in 300 ml of CM medium (28°C, 160 rpm, 3–10 days). At a spore density of 10^7/ml, the spores were filtered and washed as described above. *P. infestans* (oomycota) was cultivated on tomato agar for 5–8 days at 15°C in the dark, and spores were isolated from the plate as described above. A piece of overgrown PDA plate of basidiomycete *R. solani* was used to inoculate 50 ml of PDB medium and cultivated at 28°C, 160 rpm for 3 days. Because *R. solani* does not produce spores, mycelial fragments in the supernatant were used for microscopy. For all other fungi, the spores were germinated in tomato medium and incubated for 8–20 h at room temperature.

ELISA

The concentration of murine anti-chitosan IgM antibodies from hybridoma supernatants was determined by sandwich ELISA using a commercial IgM (M3795; Sigma-Aldrich, Steinheim, Germany) to generate a calibration curve. A high-binding microtiter plate (Greiner Bio-One GmbH, Frickenhausen, Germany) was coated with goat-Fab anti-mouse Fab (600 ng/ml in PBS) (Jackson ImmunoResearch, Suffolk, U.K.) overnight at 4°C. All further incubation steps were performed for 1.5 h at room temperature, unless otherwise stated. Nonspecific binding was blocked with 3% (w/v) skimmed milk in PBS containing 0.05% (v/v) Tween-20 (PBST) for 1 h. Several dilutions of mAbG7, ranging from 1:800 to 1:256,000 and including a PBS blank, were loaded onto the ELISA plate next to the calibration curve of the standard IgM antibody ranging from 10 to 40 ng/ml. Binding was detected using 200 ng/ml goat anti-mouse IgM Fcγ antibody labeled with horseradish peroxidase (HRPO) (Millipore, Schwalbach, Germany) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the substrate. After 30 min incubation at room temperature, the plates were read at 405 nm. After each incubation step, the microtiter plate was washed three times with PBST and all measurements were taken in triplicate. To measure the specificity of mAbG7 binding to chitosans, standard preparations of chitosans (DA 30%, 40%, 50%, and 70%) were dissolved in PBS acidified with HCl to pH 5.5 and filtered through a 0.22-µm membrane. Then, 100-µl aliquots were used to coat high-binding microtiter plates (5 µg/ml) overnight at 4°C. After blocking as described above, several concentrations of mAbG7, ranging from 0.01–4 µg/ml, were applied as primary antibody, followed by detection as described above.

Immunofluorescence Microscopy

Immunofluorescence microscopy was carried out to check the binding of mAbG7 to chitosan present in the fungal cell wall. Round glass cover slips were treated with 0.01% (v/v) poly-L-lysine solution (Sigma-Aldrich) and deposited in pre-blocked 12-well tissue culture plates for antigen coating (Greiner Bio-One GmbH). A 1-ml aliquot of the prepared mycelia or germinated spore solution was transferred onto the cover slips and the culture plate was centrifuged (2,000 ×*g*, 15 min, room temperature) to ensure tight coating. Then, 250 µl of mAbG7 (40 µg/ml) from the culture supernatant was added to the wells, and binding was detected using 250 µl (5 µg/ml) of Alexa Fluor 568-labeled goat anti-mouse Fc_μ (Invitrogen, Leek, The Netherlands). All incubation steps were performed at room temperature for 1.5 h, and cover slips were carefully washed six times with PBST after each incubation step. The prepared cover slips were transferred upside down onto a glass slide and sealed to prevent the samples drying out. A Leica DMR fluorescence microscope, fitted with an oil immersion objective (HCX PL APO 100×/1.40 oil PH 3 CS) and connected to a Leica DFC320 camera (Leica Microsystems Heidelberg GmbH, Mannheim, Germany), was used to record the results.

RESULTS

Production and Characterization of Anti-Chitosan Antibodies

To identify hybridomas secreting chitosan-specific monoclonal antibodies, the culture supernatant was tested by ELISA using microtiter plates that were coated with chitosan (DA 70%). After two rounds of selection using limiting dilutions, two cell lines producing chitosan-specific mAbs were established and the cells were settled into serum- and protein-free medium. mAbG7 showed the best binding

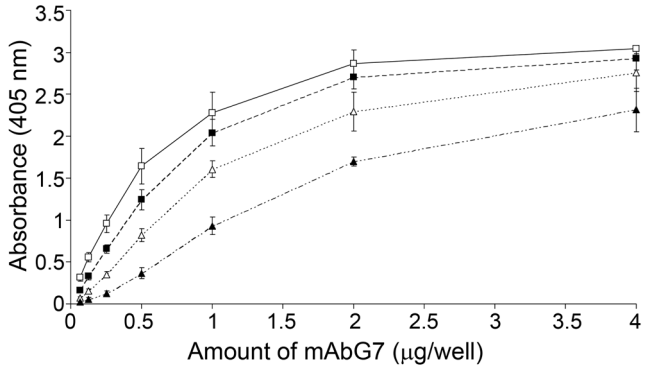

Fig. 1. Variation in absorbance as a function of antibody quantity for chitosans with different DA [DA 30% (—□—), DA 40% (– ■ –), DA 50% (· · ▵ · ·), and DA 70% (– ▲ –·)] as observed by ELISA.
Data represent means and standard deviations of three independent biological replicates.

against coated chitosan in ELISA (data not shown) and was therefore used for further experiments. The concentration of mAbG7 in the cell line was 50 µg/ml culture medium as determined by ELISA.

Binding Study of mAbG7

The specificity of mAbG7 for four different chitosans was tested by ELISA. The antibody clearly bound to all four preparations, but the specificity was lowest for high-DA chitosans and increased as the DA declined (Fig. 1). No signal was detected in negative controls with no antibody or commercial IgM antibody unrelated to chitosan. For each chitosan preparation, there was a linear relationship between antibody binding and concentration up to 1 µg/ml, when saturation effects set in. Importantly, mAbG7 did not bind to other polysaccharides such as starch, dextran, cellulose, or carboxymethylcellulose (data not shown), confirming its specificity for chitosan.

Detection of Fungal Cell Walls

Immunofluorescence microscopy was carried out to determine whether mAbG7 was able to detect chitosan *in*

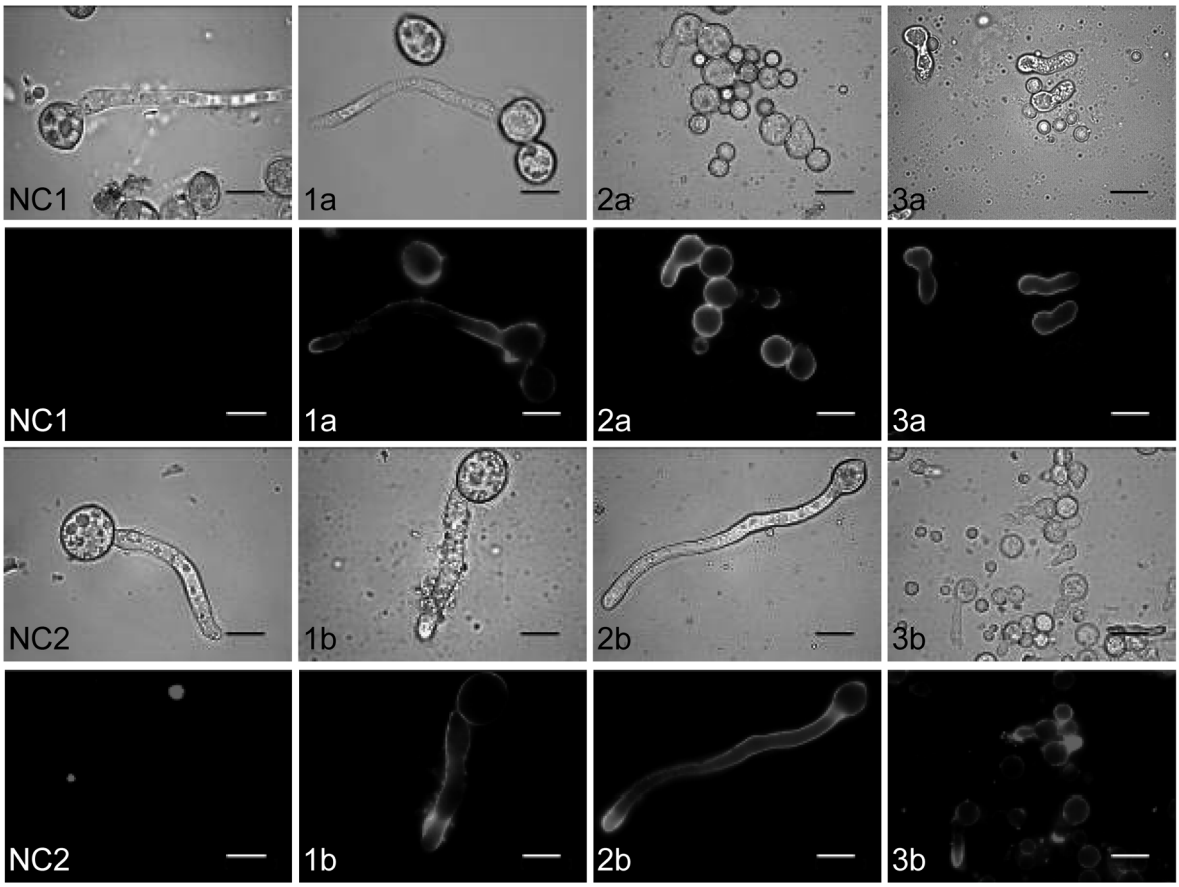

Fig. 2. Immunofluorescence of fungal spores and germination tubes using mAbG7 from hybridoma supernatant.
Bright light and corresponding fluorescent images are presented for *B. cinerea* (1a,b), *A. niger* (2a,b), and *P. hirsutum* (3a,b). Germinated spores of all fungi were incubated only with the detection antibody (NC1) and an unrelated commercial IgM (NC2) to evaluate the degree of nonspecific binding to the fungal cell wall. These controls are here depicted for *B. cinerea* only, as representative for all controls. Scale bars=5 µm.

situ in the fungal cell wall. As shown in Fig. 2, mAbG7 bound strongly to the cell walls of the ascomycetes *A. niger*, *B. cinerea*, and *P. hirsutum*, whereas weaker binding was observed in samples of *F. culmorum* and *F. graminearum*. No binding was observed when we tested the basidiomycete *R. solani* and the oomycete *P. infestans* (data not shown). The strongest signals were observed on round conidiospores, at the tips of germination tubes, and at the point where the germination tube grows out of the spore. Confocal images stacked to a layer showed that the staining surrounded the outer surface of the spore (data not shown).

Discussion

Previous reports have described polyclonal antibodies from rabbit raised against chitin and chitosan and their use to detect these polysaccharides in fungal cell walls when the fungi are either free or in the process of invading plants. Chitosan, like other carbohydrate-based polymers, has a low immunogenicity [27], so it is necessary to couple chitosan electrostatically or covalently to carrier proteins or to use impure chitosan to achieve a sufficient humoral immune response [1]. Sorlier *et al*. [34] used haptenized chitosan (DA 5.2%, DP_w 2,261) to generate polyclonal anti-chitosan antibodies, and they showed by ELISA that the binding specificity increased as the DA declined, with the highest-affinity binding observed at a DA of 15% [34].

We have produced a monoclonal antibody against chitosan, although we followed a similar strategy to Sorlier and colleagues to achieve a sufficient immune response. We used impure, enzymatically prepared chitosan, and performed seven boosts compared with the three boosts described by Sorlier *et al*. [34]. Our results concur with the earlier study, with the highest-affinity binding observed when the DA was low (in this case, 30%). Our monoclonal antibody is highly specific, and does not bind other polysaccharides such as starch, dextran, cellulose, and carboxymethylcellulose. The preference for low-DA chitosan indicates that the epitope recognized by mAbG7 is probably not the *N*-acetyl group, or binding follows a defined sequence of *N*-acetylated and charged deacetylated spots at the chitosan polymer, although further experiments are required to define the epitope precisely.

Immunofluorescence microscopy revealed that the antibody stains the conidiospores of the ascomycetes *A. niger, B. cinerea*, and *P. hirsutum* strongly and uniformly, although there is more intense staining at the tip of the germination tube and the site at which the germination tube emerges. The mycelia of all ascomycota were stained weakly (data not shown). These findings agree with previous studies [10, 12]. Although the fungal cell wall is a very dynamic structure whose chemical composition changes during growth and stress, the shifting distribution of chitin and chitosan in the fungal cell wall has been studied in detail [23]. In the early spore stages, completely deacetylated chitosan is found on the outermost layer of the fungal spore, whereas the inner layers predominantly comprise chitin [10]. Therefore, chitosan is present in germinated fungal spores on the outer edge of the infecting conidiospore and on the tips of germinated spores [12]. Fungal ascospores contain up to 4.5-fold more chitosan than mycelia [4].

The relative amounts of chitin and chitosan in different fungi have been reported [3, 19, 31, 38], although the values are influenced by cultivation methods, strain properties, and the extraction procedure [15], and so must be treated with caution. We observed the most intense staining in *A. niger*, perhaps reflecting the high content of chitin (31.2%) [39] and chitosan (11%) [31], and the low DA (15.8%) [16]. The low DA value, in particular, correlated with the higher staining intensity, with the stronger affinity of mAbG7 for chitosan seen with a low DA, as shown by ELISA. Other fungi contain no chitin, even though in some cases they may be closely related to another species in which the polymer is abundant [30]. For example, filamentous protists (*e.g*., oomycota) have cell walls that mainly comprise cellulose rather than chitin [11], correlating with the absence of staining we observed in spores and germination tubes of *P. infestans*. The ascomycete *F. oxysporum* and the basidiomycete *R. solani* contain chitin but no chitosan [5], so this explains why mAbG7 bound only weakly to germinated *F. culmorum* and *F. graminearum* spores and not at all to *R. solani* (data not shown).

In summary, we have generated (to the best of our knowledge) the first monoclonal antibody against chitosan. Monoclonal antibodies are versatile and specific, and are beginning to replace classical chemical dyes such as Calcofluor white or FITC-conjugated Concanavalin A, because they are more sensitive and can distinguish between closely related substances [14, 28]. This antibody, mAbG7, has the potential to be developed into a quality control assay, a contamination detection assay, a means to distinguish between fungal species, or a tool to analyze the structure of fungal cell walls. In the future, its ability to target specific fungi could lead to applications in crop protection, and the containment of fungal disease outbreaks.

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