

Farmer's lung: Patients' IgG₂ antibodies specifically recognize *Saccharopolyspora rectivirgula* proteins and carbohydrate structures

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Farmer's lung is a frequent form of extrinsic allergic alveolitis. In Europe and Northern America the main source of the antigenic components that induce farmer's lung is the bacterium Saccharopolyspora rectivirgula (Micropolyspora faeni). It remains unclear, however, which S. rectivirgula components are responsible for the disease. We approached these problems by investigating the serologic reaction of patients with farmer's lung and demonstrated specific binding of patients' IgG₂ to S. rectivirgula antigens. No such antibodies were found in exposed, unaffected subjects. Thus IgG₂ antibodies reacting with S. rectivirgula antigens are useful for the serologic diagnosis of patients with farmer's lung and for the isolation of disease-causing antigens. After separation of S. rectivirgula extract on concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden), we found that approximately one third of the patients' IgG₂ reactivity occurred with nonglycosylated proteins. Among these, we characterized two major acidic proteins with molecular weights of 12 and 30 kd, respectively, and with identical N-terminal sequences. Approximately two thirds of the patients' IgG₂ reactivity was observed against concanavalin A-binding glycoproteins that contained mainly glucose, mannose, and galactose residues. Deglycosylation of the concanavalin A-bound fraction indicated that most of the IgG₂ reactivity occurred with the carbohydrate components. (J Allergy Clin Immunol 1996;98:441-50.)

Key words: Farmer's lung, extrinsic allergic alveolitis, *Saccharopolyspora rectivirgula* (*Micropolyspora faeni*), IgG₂, carbohydrate antigens, protein antigens, concanavalin A

Farmer's lung belongs to a group of immunologic lung disorders called *extrinsic allergic alveolitis* or *hypersensitivity pneumonitis*.¹⁻³ Extrinsic allergic alveolitis may occur as a result of repeated and extensive inhalation of organic dusts. The disease is a predominantly mononuclear inflammation of the lung parenchyma in the terminal bronchioles and alveoli. It often leads to emphysema and may progress to fibrosis. The main symptoms are coughing, dyspnea, malaise, fever, and rales. Farmer's lung is reported to be the consequence of a

Abbreviations used

ACA:	<i>Amaranthus caudatus</i> agglutinin
ConA:	Concanavalin A
IEF:	Isoelectric focusing
MW:	Molecular weight
pI:	Isoelectric point
pK:	Dissociation constant
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TFMS:	Trifluoromethanesulfonic acid
WGA:	Wheat germ (<i>Triticum vulgare</i>) agglutinin

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type III hypersensitivity reaction in combination with a type IV allergy.^{4,5} The formation of immune complexes seems to be the initiating mechanism of this disease.⁶⁻⁸ These immune complexes activate the complement cascade, resulting in a release of vasoactive amines (histamine, 5-hydroxytrypta-

mine) and leading to a higher permeability of the blood vessels. Initiation of the alternative complement cascade, indicated by the release of C3a and C5a, has also been described.⁹⁻¹² C5a attracts polymorphonuclear granulocytes, which release lysosomal enzymes, causing damage to the lung tissue. Additionally, accumulated lymphocytes activate macrophages, which in the case of this disease fail to phagocytose the immune complexes, resulting in epithelial granulomas and interstitial inflammation.

Saccharopolyspora rectivirgula, formerly called *Micropolyspora faeni*,¹³ is the main source of antigens that lead to the formation of immune complexes in farmer's lung. This gram-positive, thermophilic actinomycete has been found to be associated with disease in Europe, North America, and India.¹⁴⁻¹⁸ *S. rectivirgula* grows best in moist grain and hay. Thus farmer's lung is mainly a problem in regions with high rainfall, such as Great Britain, Northern Germany, and the West Coast of Canada.

Many investigators have detected precipitin formation associated with *S. rectivirgula* antigens in the sera of patients with farmer's lung¹⁹⁻²² and have shown that these precipitins are composed of IgG and bacterial components.²³⁻²⁵ Measurements of total serum IgG concentrations in patients with farmer's lung have demonstrated an elevation of IgG molecules compared with blood donors not exposed to antigen. However, comparison of IgG serum levels in patients with farmer's lung and unaffected but exposed subjects frequently failed to show significant elevation of patients' IgG. Precipitins of *S. rectivirgula* antigens with total IgG in serum of exposed individuals without symptoms of farmer's lung have also been detected.^{23, 26, 27} Consequently, the measurement of total serum IgG cannot serve as a specific serologic marker to distinguish between patients with farmer's lung and exposed farmers without clinical evidence of disease. Recent investigations of the different IgG subclasses in sera taken from patients with farmer's lung by Reese et al.²⁸ demonstrated that the IgG₂ subclass was the principal component. These specific reactivities could be used to isolate components of *S. rectivirgula* associated with farmer's lung.

In this report we demonstrate the successful use of disease-associated IgG₂ of patients with farmer's lung to isolate their corresponding antigens. In particular, we report the isolation and characterization of two nonglycosylated proteins and several glycoproteins that react with such antibodies and demonstrate that the carbohydrate moieties of the

glycoproteins are the principal antigenic components.

METHODS

Preparation of bacterial extract

S. rectivirgula (ATCC 15437) was cultured in a synthetic medium, according to the method of Kurup and Fink,²⁹ in shaking cultures for 3 days at 55° C. For the preparation of extracts, the supernatant was filtered through 0.45 µm membranes and concentrated by ultrafiltration (molecular weight [MW] cutoff, 10 kd) in an Amicon stirred cell (Amicon, Beverly, Mass.). Afterward, the extract was dialyzed three times against distilled water. The lyophilized extract was stored at -20° C.

Collection of patients' sera

Serum samples from 35 patients with farmer's lung were investigated. Diagnosis was confirmed by history, clinical symptoms, bronchoalveolar lavage, X-ray examinations, lung function analysis, positive double immunodiffusion test results, and provocation tests. Serum samples were collected before and after provocation, and the samples showed no difference in reactivity. Sera of 15 antigen-exposed family members without symptoms and sera of 20 blood donors served as controls. The latter were subjects who did not live in agricultural areas and had no obvious exposure to *S. rectivirgula* antigens.

Measurement of IgG₂ reactivities

Western and dot blots of the whole extract and extract components were incubated with patients' sera overnight, diluted 1:100 in washing buffer (Tris-buffered saline-Tween: 100 mmol/L Tris-HCl, 100 mmol/L NaCl, 2.5 mmol/L MgCl₂, 0.025% vol/vol Tween-20, pH 7.4). Human IgG₂ was detected by a subclass-specific monoclonal antibody (GOM 1, 1:2000; Unipath, Manchester, U.K.) and by alkaline phosphatase-conjugated goat-anti-mouse IgG (heavy and light chain specific) antibody (Dianova, Hamburg, Germany). The alkaline phosphatase-enzyme reaction was performed at 37° C with a substrate-chromogen mixture. The latter was prepared by mixing 30 ml of 0.033% wt/vol nitroblue tetrazolium chloride (Serva, Heidelberg, Germany) in 100 mmol/L Tris-HCl (100 mmol/L NaCl, 5 mmol/L MgCl₂, pH 9.5 with 1 ml of 0.5% wt/vol 5-bromo-4-chloro-indolyl-phosphate disodium salt (Serva) solubilized in distilled water.³⁰

Preparative isoelectric focusing (IEF)

Preparative IEF was performed in a Rotofor Cell (Bio-Rad, Hercules, Calif.). Twenty milligrams of the lyophilized bacterial extract was diluted in a buffer containing 6 mol/L urea and 2% ampholytes, pH 3 to 6, (Serva) to a final volume of 55 ml and focused for 4 to 6 hours (2000 V, 12 W, 30 mA). The anolyte consisted of 0.1 mol/L H₃PO₄, and the catholyte was 0.1 mol/L NaOH.³¹

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The bacterial extract was separated by SDS-PAGE with the use of 10 mm high stacking gels totally composed of 5% acrylamide and bis-acrylamide (T) with a cross-linking of 0.75% (C); (C, percentage ratio of bis-acrylamide to T) and 60 mm high gradient running gels (T, 7.5% to 17%; C, 0.75%) in the buffer system according to the Laemmli method.³² The sample buffer contained 54.1 mmol/L Tris-H₂SO₄ (pH 6.14), 5 mmol/L ethylenediaminetetraacetic acid, 12.5% wt/vol glycerol, 1% wt/vol SDS, and 0.01% wt/vol bromophenol blue.³³ The lyophilized bacterial extract was diluted in aqua bidest to a final concentration of 1 mg/ml and was mixed with sample buffer in a ratio of 1:1. Samples were incubated at 95° C for 5 minutes before application, and 1 ml of the sample was loaded on a 10 cm wide gel. Electrophoresis was performed at 100 V for 30 minutes and increased to 175 V for 1 hour.

Dot blotting

Glycan hydrazone samples were diluted with aqua bidest to a final volume of 5 µl and dotted under vacuum onto CNBr-activated nitrocellulose membranes. The following blocking of the membrane was done in Tris-buffered saline-Tween (pH 7.40) 0.025% vol/vol Tween-20.

Two-dimensional electrophoresis

First dimension. An IEF polyacrylamide gel (T = 4%, C = 4%) with an immobilized pH gradient from 3.5 to 5.0 was prepared on Gelbond film (Pharmacia, Uppsala, Sweden). The pH gradient was prepared with Immobiline (Pharmacia) as follows. For the acidic solution, Immobilines with a dissociation constant (pK) of 3.6 (199 µl), 4.6 (149 µl), and 6.2 (105 µl) per 11 ml were applied. For the basic solution, Immobilines with a pK of 3.6 (141 µl), 4.6 (207 µl), and 6.2 (310 µl) per 11 ml were used. The polymerized gel was washed five times for 10 minutes in 200 ml of distilled water and dried in a microwave oven. This gel was cut into strips, and *S. rectivirgula* extracts (800 µg per strip) were separated by IEF (3000 V, 2 mA for 8 hours). The strips of this first dimension were equilibrated in a buffer containing 6 mol/L urea, 0.5 mol/L Tris, 2% wt/vol SDS, 30% vol/vol glycerol, 1% wt/vol dithiothreitol, and 260 mmol/L iodoacetamide.^{34, 35}

Second dimension. The extracts carrying IEF strips were applied vertically to a polyacrylamide gel with a gradient of 8% to 13% T and 4% C. For the stacking gel, 5% T and 4% C were applied. The electrophoresis was done at 30 mA, 30 W, and 200 V for the stacking gel and increased to 800 V for the separating gel. Run time for the second dimension was 3 to 4 hours.

Western blotting

After SDS-PAGE or two-dimensional electrophoresis was performed, the proteins were blotted onto nitrocel-

lulose membranes electrophoretically (Schleicher and Schüll, Dassel, Germany) or Immobilon membranes (Millipore Corp., Bedford, Mass.) by the semi-dry-blotting method as described by Khyse-Anderson.³⁶

Lectin detection of glycoconjugates

Digoxigenin-labeled lectins (Boehringer, Mannheim, Germany) were incubated with Western blot strips and with Western blots of the two-dimensional electrophoresis of *S. rectivirgula* extracts according to the manufacturer's instructions. Detection with an anti-digoxigenin alkaline phosphatase-conjugated monoclonal antibody (Boehringer), diluted 1:5000, was visualized enzymatically with nitroblue tetrazolium-5-bromo-4-chloro-indolyl-phosphate as described above.³⁷

Deglycosylation

Deglycosylation by trifluoromethanesulfonic acid (TMSF) (Oxford-Glyco-System, Oxford, U.K.) was carried out according to the manufacturer's instructions. Briefly, 600 µg of lyophilized bacterial extract was diluted in 50 µl toluene-TMSF (stock solution was composed of 60 µl toluene and 250 µl TMSF) and incubated for 4 hours at -20° C. Afterward, the reaction mixture was neutralized with 150 µl pyridine. Subsequently, the sample was put on dry ice for 5 minutes and on ice for 15 minutes before the addition of 400 µl of 0.5% ammonium bicarbonate. The deglycosylated proteins were dialyzed against 0.01% ammonium bicarbonate (pH 7.0) for 8 hours, lyophilized, and stored at -20° C.³⁸⁻⁴¹

Hydrazinolysis

Five milligrams of lyophilized extract was incubated with 200 µl hydrazine at 95° C for 4 hours. Released glycan hydrazones were separated from excess hydrazine and amino acid hydrazides by chromatography. We modified the standard protocol, according to Oxford-Glyco-System (Oxford, U.K.), by leaving the hydrazone group connected to the carbohydrate chains, obtaining glyco-hydrazone chains. This procedure offered the ability to fix glyco-hydrazone chains onto CNBr-activated nitrocellulose membranes. Glycan hydrazones were then detected by dot blot assay.^{42, 43}

Concanavalin A (ConA) affinity chromatography

Five milliliters of ConA-Sepharose beads (Pharmacia) was equilibrated twice with 40 ml of 20 mmol/L Tris and 0.5 mol/L NaCl (pH 7.4) for 30 minutes. Forty to fifty milligrams of lyophilized *S. rectivirgula* extract was diluted in 30 ml of water and bound to ConA-Sepharose beads for 6 hours during agitation (50 rpm). The supernatant and the Sepharose were separated by centrifugation at 1000 g for 5 minutes. The supernatant, which contained unbound proteins, was collected and dialyzed against distilled water for 16 hours, lyophilized, and stored at -20° C. Bound glycoproteins were eluted with 1 mol/L methyl- α -D-mannopyranoside, the complemen-

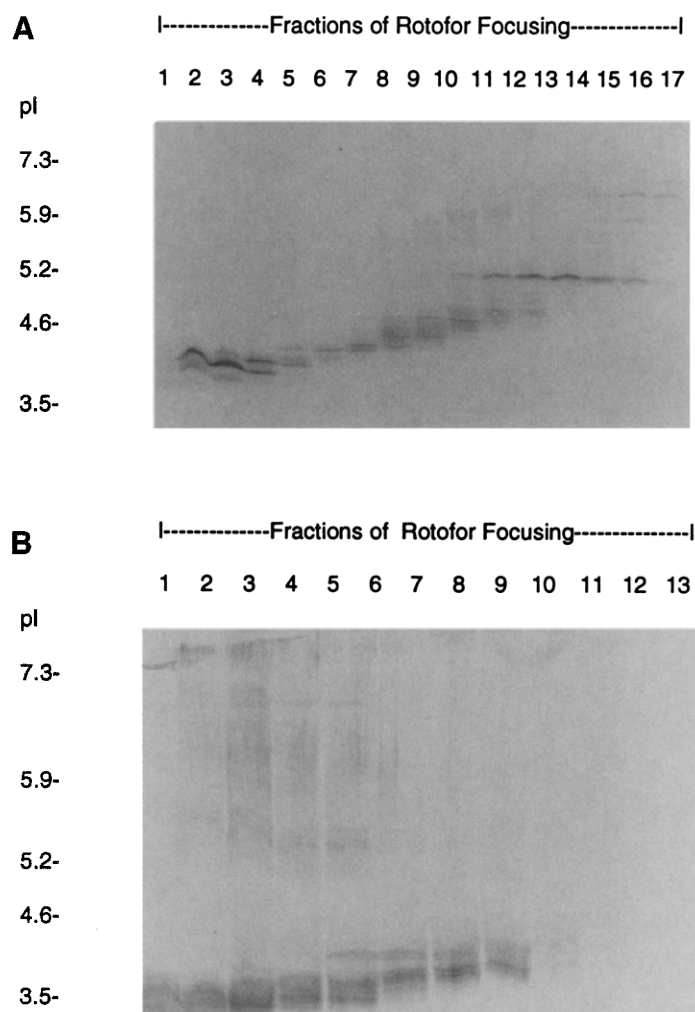


FIG. 1. IEF of total *S. rectivirgula* extract in a Rotofor cell. Electrophoresis was performed with a pH gradient of 3 to 10. **A**, Coomassie brilliant blue (Serva, Heidelberg, Germany) staining of extract. **B**, IgG₂ binding of patients' pooled sera to the total extract. Lanes 1 to 17, Rotofor fractions.

tary sugar, and treated as described for the supernatant.^{40, 41}

N-terminal protein microsequencing

Sequencing was performed with a 473A Protein Sequencer with an online PTH amino acid analyzer (Applied Biosystems, Division of Perkin-Elmer Corp., Foster City, Calif.). Both protein spots removed from Western blots and lyophilized protein samples resuspended in distilled water and dotted on a polyvinylidene difluoride membrane were used for microsequencing.

RESULTS

Characteristics of IgG₂ binding to the *S. rectivirgula* bacterial extract

Preparative IEF of the *S. rectivirgula* extract revealed that 85% of the components focused in a pH range of 3.5 to 6.0. Patients' IgG₂ reactivity

with the bacterial components was found exclusively in a pH range of 3.5 to 5.0 (Fig. 1).

Western blots of the complete extract demonstrated that patients' IgG₂ reacted with numerous proteins with MWs in the 10 to 100 kd range (Fig. 2). Approximately 65% of our examined patients showed very similar IgG₂-binding patterns, differing mostly in the intensity of the antibody reaction. The IgG₂ fraction from the remainder reacted only with a few proteins in the *S. rectivirgula* extract. The binding patterns of the latter were individual, and no attempt was made to cluster them in subgroups. In addition to the binding of IgG₂ to distinct proteins, we also observed that in about 70% of reacting sera a diffuse smear was revealed by the procedure (Fig. 2), suggesting that the proteins are glycosylated.

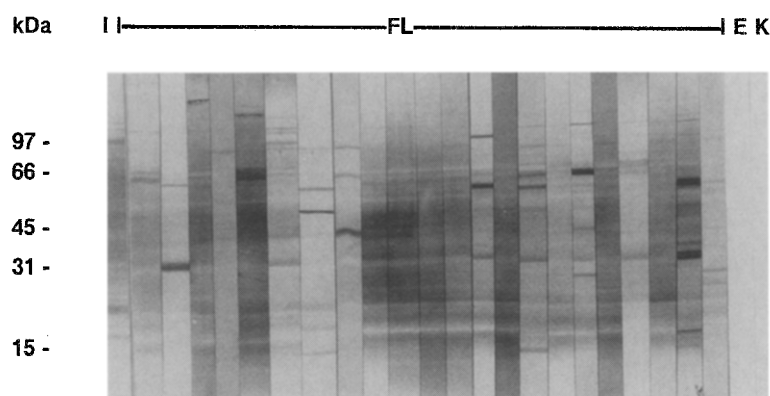


FIG. 2. Western blot of *S. rectivirgula* extract binding to patients' IgG₂. *I*, India ink staining; *FL*, patients' IgG₂ binding (*n* = 22); *E*, IgG₂ reactivity with an exposed person's pooled serum; *K*, antibody control; *kDa*, MW in kilodaltons.

Analysis of the *S. rectivirgula* components recognized by patients' IgG₂

In the first instance we investigated the binding characteristics of different lectins to complete bacterial extract.³⁷ Western blots of the extract showed reactivity with the following lectins: ConA, wheat germ agglutinin (WGA), and *Amaranthus caudatus* agglutinin (ACA), indicating the presence of carbohydrate chains containing mannose, glucose, and galactose units (Fig. 3).

In further experiments the extract was subjected to hydrazinolysis to separate intact N- and O-linked glycoconjugates from the protein cores.^{42, 43} Subsequently, these carbohydrate structures were fixed as glyco-hydrazone chains onto CNBr-activated nitrocellulose membranes. Subsequent incubation with the patients' pooled sera revealed extensive IgG₂ reactivity. This signal was obtained with the amount of carbohydrate structures cleaved from 10 µg of complete bacterial extract (Fig. 4).

No proteins and peptides could be detected in the carbohydrate fraction (data not shown). Control reactions with exposed farmers' pooled sera did not show reactivities with the glycosides of *S. rectivirgula* (Fig. 4). This result indicated that the pure carbohydrate structure of *S. rectivirgula* is antigenic for patients' IgG₂.

Protein deglycosylation with TFMS destroys the carbohydrates during the reaction but leaves the protein core essentially intact.³⁹⁻⁴¹ TFMS-treated *S. rectivirgula* extract was separated two-dimensionally and blotted onto a nitrocellulose membrane. Because of the IEF results described above, the two-dimensional electrophoresis was performed in a pH gradient of 3.5 to 5.0 and in an MW range of 10 to 100 kd. Two-dimensional

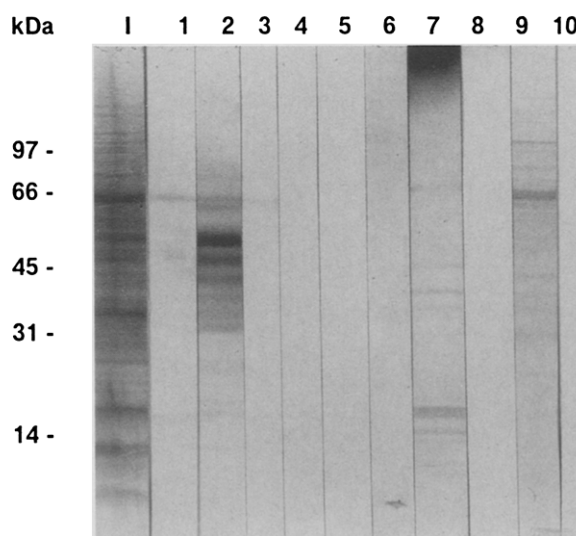


FIG. 3. Lectin-binding pattern of *S. rectivirgula* extract. Lanes 1 to 9, Lectin names and their specificity. Lane 1, *Ricinus communis* agglutinin, α-D+β-D-galactose; lane 2, *Canavalia ensiformis* agglutinin, α-D-mannose, α-D-glucose, α-D-N-acetylglucosamine; lane 3, *Galanthus nivalis* agglutinin, α-D-mannose; lane 4, *Maackia amurensis* agglutinin, α-D-N-acetylneuraminic acid; lane 5, *Arachis hypogaea* agglutinin, β-D-galactose; lane 6, *Aleuria aurantia* agglutinin, α-L-fucose; lane 7, *Triticum vulgaris* agglutinin, β-D-N-acetylglucosamine; lane 8, *Datura stramonium* agglutinin, β-1-4-N-acetylglucosamine, N-acetylglucosamine-Ser/Thr; lane 9, *Amaranthus caudatus* agglutinin, β-D-galactose-1-3-α-D-N-acetylglucosamine-Ser/Thr; lane 10, control. *kDa*, MW in kilodaltons.

SDS-PAGE blots of the deglycosylated proteins were incubated with the patients' pooled sera, and the IgG₂-binding pattern was analyzed (Fig. 5, A). Between 12 and 15 proteins were detected, reacting specifically with patients' IgG₂ but not with sera of exposed persons or blood donors. TFMS-degly-

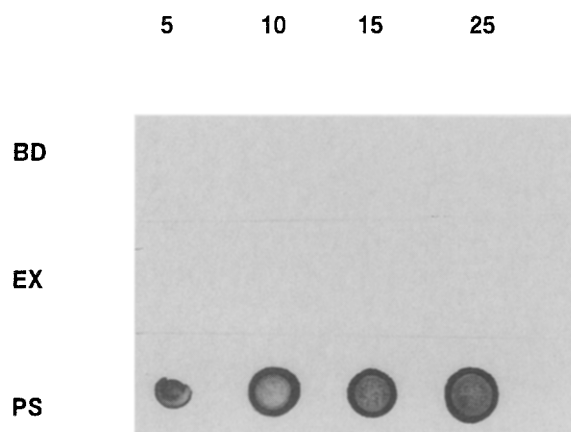


FIG. 4. Dot blot analysis of *S. rectivirgula* carbohydrate residues produced by hydrazinolysis. IgG₂ reactivity of patients' sera (PS), exposed persons' sera (EX), and blood donors' sera (BD). 5, 10, 15, 25, Micrograms of bacterial extract used for hydrazinolysis.

cosylated bacterial extract did not show binding with the lectins ConA, WGA, or ACA anymore. These results indicated the complete removal of the carbohydrate chains.

Thus by performing the described experiments we were able to show that IgG₂ antibodies from patients with farmer's lung bind to both the carbohydrate and protein portions of the IgG₂-reactive glycoproteins of *S. rectivirgula*.

ConA affinity chromatography

The experiments described above with TFMS could have caused degradation of the N- and C-terminal ends of the proteins. To obtain unmodified antigenic proteins, we performed ConA affinity chromatography, because the lectin ConA bound most of the IgG₂-reactive antigenic carbohydrate structures.^{44,45}

As determined by ConA affinity chromatography, the majority of IgG₂-binding glycoproteins were separated from nonglycosylated proteins, which were enriched in the flow-through fraction. This flow-through fraction was analyzed in two-dimensional SDS-PAGE blots.

We could identify nonglycosylated proteins that reacted with patients' IgG₂ (Fig. 5, B) but not with exposed subjects' and blood donors' IgG₂. To exclude IgG₂ reactions with glycoproteins, the blots were incubated analogously with the lectins ConA, WGA, and ACA, which act as markers for the IgG₂-specific carbohydrates. These control incubations showed no binding reactivities.

ConA-Sepharose-linked carbohydrate structures were released from the matrix by the com-

petitive sugar methyl- α -D-mannopyranoside. Analysis with this fraction was performed as described for the non-ConA-binding proteins. Patients' IgG₂ antibodies reacted with carbohydrate structures (Fig. 5, C), whereas exposed farmers' and blood donors' IgG₂ did not. A further incubation of this carbohydrate fraction with TFMS led only to a few protein cores reacting with patients' IgG₂ (data not shown).

Using ConA affinity chromatography, we developed a strategy to isolate nonglycosylated and unmodified proteins specifically binding to patients' IgG₂. Two of these proteins were analyzed further: Sac r 1 and Sac r 2 (Fig 5, B). Sac r 1 is a 12 kd protein with an isoelectric point (pI) of 3.7, and Sac r 2 is a 30 kd protein with a pI of 4.2. The determined N-terminal sequences of both antigens were identical: ADXDLLAQE. Analyses of the amino acid composition of both proteins resulted in a closely related distribution pattern (Table I). We found high amounts (14.4%/22%) of acidic amino acids, namely aspartic acid and glutamic acid, probably responsible for the low pI of these antigens.

Using ConA affinity chromatography, we were able to isolate the IgG₂-reactive antigenic proteins in the microgram range. The isolated antigens, Sac r 1 and Sac r 2, were cleaved with trypsin; and the fragments were separated by high-performance liquid chromatography (reverse-phase columns). Subsequently, the N-terminal sequences of these fragments of Sac r 1 and Sac r 2 were determined. These sequences of five fragments from Sac r 1 and six fragments from Sac r 2 were all different. The identical N-terminal sequences of Sac r 1 and Sac r 2 and the nonidentical internal sequences were compared with the European Molecular Biology Laboratory and Switzerland-Protein databases by using Basic Local Alignment Search Tool.⁴⁶

DISCUSSION

S. rectivirgula is accepted as the main antigen source in farmer's lung.^{14-18, 47-53} In previous investigations we were able to show a serologic difference between patients with farmer's lung and exposed persons with regard to specific IgG₂ binding to acidic components of *S. rectivirgula* in an IEF immunoprint (Fig. 1).²⁸

In this article we report the isolation and characterization of *S. rectivirgula* components specifically recognized by patients' IgG₂.

IEF experiments showed that IgG₂-reactive and disease-associated antigens were acidic proteins

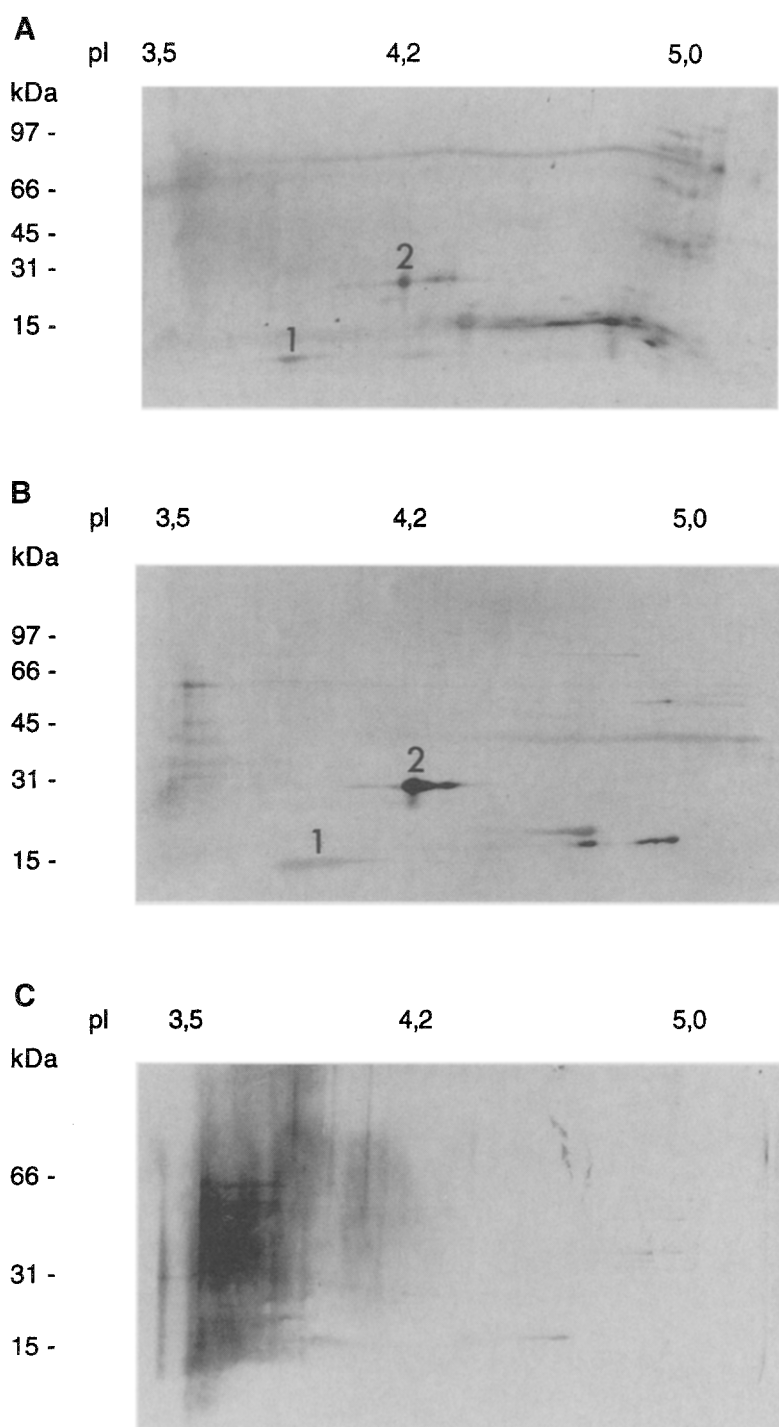


FIG. 5. IgG₂ reactivity of a patients' pooled sera with two-dimensionally separated modified *S. rectivirgula* extract after TFMS treatment (**A**), with non-ConA-binding *S. rectivirgula* proteins (**B**), and with ConA-binding *S. rectivirgula* structures (**C**). *kDa*, MW in kilodaltons; 1, antigen Sac r 1; 2, antigen Sac r 2.

(pH 3.5 to 5.0, Fig. 1). When determined by Western blot, the MWs of such antigens ranged between 10 and 100 kD. In addition, in approximately 70% of the patients a continuous smear of IgG₂ reactivity was present (Fig. 2). Because it is

known that IgG₂ antibodies tend to react with carbohydrate antigens, we decided to determine, by analysis, whether this smear was caused by carbohydrate epitopes.

Lectin binding analyses demonstrated that car-

TABLE I. Amino acid composition of the antigens Sac r 1 and Sac r 2

Amino acid	Sac r 1 (pI = 3.7)	Sac r 2 (pI = 4.2)
Aspartic acid (D)	9.6%	6.6%
Glutamic acid (E)	12.5%	7.8%
Serine (S)	9.2%	13.0%
Glycine (G)	15.3%	30.8%
Histidine (H)	2.5%	0.4%
Arginine (R)	4.5%	3.6%
Threonine (T)	5.6%	4.4%
Alanine (A)	10.5%	9.9%
Proline (P)	12.7%	7.8%
Tyrosine (Y)	2.8%	1.6%
Valine (V)	6.3%	3.6%
Methionine (M)	1.8%	0.0%
Isoleucine (I)	1.2%	3.8%
Leucine (L)	3.8%	5.4%
Phenylalanine (F)	1.7%	2.0%
Lysine (K)	0.0%	0.0%

bohydrate structures, potentially carrying N- and O-linked glycoconjugates, were antigenic. On the basis of these examinations, we attempted to determine the location of the IgG₂-binding epitopes. For instance, were they located on the carbohydrate residues or on the protein core of the glycoproteins? The carbohydrate residues could be obtained by hydrazinolysis, a method that has become popular in recent years, especially in the field of eucaryotic glycostructure analysis.^{42, 43, 54} Carbohydrate chains composed of glucose, mannose, and galactose units of *S. rectivirgula* proved to be the components associated with causation of farmer's lung. Kaukonen et al.²³ also described mannose and mannoproteins as farmer's lung-associated antigens. These antigens were isolated from *Aspergillus umbrosus*, the main antigen source of this disease in Finland. The investigations of Kaukonen et al.²³ were performed by using patients' total IgG and not IgG₂, but it is known that total IgG levels are not significantly different in patients with farmer's lung and exposed healthy individuals.²³⁻²⁵

Deglycosylated protein cores of *S. rectivirgula* antigens recognized by patients' IgG₂ could be isolated by TFMS treatment of the bacterial extract. This method offered the possibility of determining whether the IgG₂-binding epitopes were localized exclusively on the carbohydrate chains or could be found on the protein cores of the glycoproteins. It could be demonstrated that the carbohydrate chains and the cores of the

glycoproteins were both recognized by patients' IgG₂.

By means of ConA affinity chromatography we were able to isolate, from *S. rectivirgula* extracts, ConA-binding glycoproteins under native conditions. Subsequent cleavage of ConA-binding glycoproteins with TFMS showed the carbohydrate residues to be the main IgG₂-reacting antigenic component of this fraction. Only a few remaining protein cores preserved reactivity with patients' IgG₂.

The nonglycosylated proteins that failed to bind to ConA were further analyzed. The N-terminal sequences of two antigenic proteins, Sac r 1 and Sac r 2, were determined and showed that the first nine amino acids of both proteins had the identical sequence, ADXDLLAQE, raising the possibility that Sac r 1 and Sac r 2 may be related proteins (isoforms?). Analysis of the amino acid composition of both proteins was also similar (Table I).

TFMS treatment released two proteins reacting with patients' IgG₂, which had the same biochemical characteristics as Sac r 1 (pI, 3.7; MW, 12 kd) and Sac r 2 (pI, 4.2; MW, 30 kd) (Fig. 5, A). This is strong evidence that these antigens are identical and that these proteins are not the product of de novo glycosylation. In support of this is the observation that the glycosylated ConA-binding fraction did not include the antigens Sac r 1 and Sac r 2 after TFMS treatment. However, our studies do not confirm the existence of glycosylated and nonglycosylated forms of these two antigens.

The data presented in this article suggest that nonglycosylated proteins, as well as the carbohydrate side chain of glycoproteins derived from *S. rectivirgula*, act as major antigenic determinants in the etiology of farmer's lung.

In general, farmer's lung develops after extensive and repeated inhalation of antigen over a considerable length of time. We questioned whether the antigens involved are somehow able to avoid removal by the immune system. Further molecular analysis of the characterized antigens might be one way to answer this question.

Use of the isolated antigens in a mouse or guinea pig model could be one way to identify the mediators involved in this special type of inflammation. One approach is to elucidate the ability of the immune complexes (IgG₂ proteins and/or IgG₂ carbohydrates) to activate complement and macrophages. It is reported that IgG₂ immunoglobulins are not able to activate the complement cascade or to bind the Fcγ-receptor on macrophages.⁵⁵⁻⁵⁸ A low

clearance rate of these immune complexes, resulting in their residing in lung tissue, may explain the specific role of *S. rectivirgula* antigens in the pathogenesis of farmer's lung.

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