Protein D, the Glycerophosphodiester Phosphodiesterase from *Haemophilus influenzae* with Affinity for Human Immunoglobulin D, Influences Virulence in a Rat Otitis Model

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Received 21 March 1994/Returned for modification 7 July 1994/Accepted 19 August 1994

A mutant lacking the ability to express the surface-exposed lipoprotein protein D was constructed by linker insertion and deletion mutagenesis of a cloned DNA insert containing the protein D structural gene from a nontypeable *Haemophilus influenzae* strain (NTHi). An isogenic NTHi mutant was isolated after transformation of genetically competent bacteria. The transformant was unreactive to a protein D-specific monoclonal antibody in a colony immunoassay. In addition, the mutant lacked the ability to synthesize detectable levels of protein D by protein staining, immunoblot methods, glycerophosphodiester phosphodiesterase activity, and binding studies of radiolabelled immunoglobulin D. The isogenic protein D-deficient mutant was compared with its parental strain for its ability to induce experimental otitis media in rats challenged with bacteria. An approximately 100-times-higher concentration of the mutant compared with that of the wild-type strain was required in order to cause otitis among all rats challenged with that given dose. The protein D mutant exhibited a generation time that was equal to that of the wild-type strain in complex broth medium. No difference in lipopolysaccharide expression was found between the mutant and the parental strain. These results suggest that protein D may influence the pathogenesis of NTHi in the upper respiratory tract.

*Haemophilus influenzae* is an important pathogen in the human respiratory tract and a frequent cause of severe diseases of childhood, including septicemia and meningitis. The molecular basis for the pathogenesis of these invasive diseases, which most frequently are caused by *H. influenzae* type b (Hib), has revealed that the capsular polysaccharide of this organism is an important virulence factor (29, 47). Another important virulence determinant is the lipopolysaccharide (LPS) of the Hib outer membrane since alterations in LPS expression have been shown to reduce the virulence potential of this bacterium (20, 21, 50). No protein has been shown to be essential for the virulence of Hib, but at least a 28-kDa lipoprotein has been suggested to aid transepithelial invasion (8).

Nontypeable *H. influenzae* (NTHi) is a frequent colonizer of the upper respiratory tract and a leading cause of human otitis media and respiratory infections, including pneumonia and sinusitis (31, 34, 44). The pathogenesis of NTHi disease is believed to begin with colonization of the nasopharynx. Subsequently, there is a contiguous spread within the respiratory tract to the middle ear, the sinuses, the conjunctiva, or the lungs. *H. influenzae* expresses adhesive pili that are likely to be involved in colonization of the host (3, 19, 45). However, nonpiliated NTHi organisms are also capable of adhering to eukaryotic cells via high-molecular-weight nonpilus adhesins (41).

Colonization of mucosal membranes by *H. influenzae* may also involve interaction with the local mucosal immune system. *H. influenzae* is one of a group of pathogenic microorganisms that produce an extracellular immunoglobulin A1 (IgA1) protease that specifically cleaves the heavy chain of human IgA1 (35). Another interesting immunological interaction is the IgD binding properties of *H. influenzae* and Moraxella (*Branhamella*) catarrhalis (13). A 42-kDa lipoprotein, named protein D, which is exposed on the surfaces of all *H. influenzae* strains, was first identified as the protein responsible for this interaction (2, 16, 36). However, it was later discovered that protein D did not bind to the majority of IgD myelomas tested and that Hib strains express an additional IgD-binding factor or cofactor that is lacking in NTHi (37). Despite this finding, protein D is still an interesting molecule since it is highly conserved among all *H. influenzae* strains (2, 18), a feature which is relatively rarely found among NTHi outer membrane proteins (4, 32, 33). A possible explanation for this conservation might be that protein D has an important enzyme activity to fulfill, since it was recently shown that protein D is 67% identical to the periplasmic glycerophosphodiester phosphodiesterase of *Escherichia coli* (30). Extracts of *E. coli* expressing recombinant protein D had increased specific activity of glycerophosphodiester phosphodiesterase compared with that of extracts of *E. coli* not expressing protein D (30).

We sought to investigate whether protein D is involved in the pathogenesis of NTHi. An isogenic mutant lacking the ability to express protein D was constructed. The mutant was compared with the wild-type strain for its ability to cause infection in the middle ears of rats in an otitis model originally developed for pneumococcal otitis (15). This rat model was recently modified for studies of otitis media caused by *H. influenzae* (27).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *E. coli* JM83 (49) was used as a recipient for plasmids pUC4K (Pharmacia Biotech, Sollentuna, Sweden) and pHIC348 (17) and derivatives thereof. *H. influenzae* 772, a nontypeable clinical isolate of biotype II, has been described previously (16–18, 36). *H. influenzae* strains were grown on chocolate agar.
plates, in brain heart infusion broth (Difco Lab., Inc., Detroit, Mich.) supplemented with NAD and hemin (Sigma Chemical Co., St. Louis, Mo.) each at 10 µg/ml (sBHI), or on sBHI plates. *E. coli* JM83 was grown in LB broth (24) supplemented where necessary with ampicillin (100 µg/ml) or kanamycin (50 µg/ml). Antibiotics were purchased from Sigma. LB agar also contained 1.5% (wt/vol) agar. All cultures were initially inoculated from stocks stored in glycerol broth at −70°C onto solid media and grown at 37°C. For comparison of mutant NTHi and wild-type NTHi, we used glycerol broth stocks of bacteria isolated from the blood of mice that had received high doses of NTHi intraperitoneally. This was done to avoid the loss of virulence after repeated subcultivation in vitro (26).

**Antibodies.** Monoclonal antibody (MAb) 19B4 is reactive against protein D from all 127 *H. influenzae* strains tested by Western blot (immunoblot) analysis (2). A panel of MAb's recognizing different epitopes of *H. influenzae* LPS were kindly provided by Alf Lindberg. MAb's were used in the form of cell culture supernatants. The raising and purification of polyclonal anti-protein D immune serum will be described in detail elsewhere (1). Briefly, a nonacetylated, recombinant form of protein D (16) was purified from periplasmic extracts of *E. coli* expressing this protein. Rabbits were immunized with the purified protein in order to raise polyclonal antiseraum against protein D. Immune serum was passed through a column with protein D coupled with CNBr-activated Sepharose 4B. Bound materials, containing anti-protein D antibodies, were eluted from the column after being washed, and eluted material was dialyzed against phosphate-balanced saline (PBS).

**Construction of protein D mutants.** The 1.9-kbp *H. influenzae* insert from pHIC348 (17) encoding protein D was excised from the pUC18 vector (49) by BamHI and HindIII digestion (see Fig. 1). Bands corresponding to the vector and insert were excised from a preparative agarose gel and extracted with GeneClean (Bio 101, Inc., La Jolla, Calif.). The 1.9-kbp insert was digested with *Hae*III and then with *Nla*IV to cleave a 534-bp internal *Hae*III fragment in half. An 816-bp *BamH*I-*Hae*III fragment and a 544-bp *Hind*III-*Hae*III fragment were excised from an agarose gel after electrophoresis. The isolated 816- and 544-bp fragments were dephosphorylated with calf intestine alkaline phosphatase (24). A corresponding amount of pUC4K was digested with *Hin*ClI, and the 1.3-kbp band corresponding to the kanamycin resistance cassette was excised from gel and extracted with GeneClean. DNA fragments were ligated to the 2.7-kbp *BamH*I-*Hind*III fragment from pHIC348, i.e., the pUC18 vector, and transformed into *E. coli* JM83 (28). Kanamycin-resistant transformants were picked and checked for lack of protein D expression in a colony immunoassay with MAb 19B4 (17, 36). Plasmids from protein D-negative transformants were prepared with Magic Miniprep (Promega Corp., Madison, Wis.) and analyzed on 0.8% agarose gels after restriction endonuclease digestions. One plasmid (pHIK3) was chosen for further experiments. All enzymes were from Boehringer Mannheim GmbH, Mannheim, Germany.

**Genetic transformation of *H. influenzae*.** Cells made competent by the aerobic-anoxic incubation procedure of Goodgal and Herriot (14) as modified by Cameron (5) were transformed with pHIK3 DNA, linearized with *BamH*I. Transformants were selected on sBHI plates containing 15 µg of kanamycin per ml. Transformants were checked for a lack of reactivity to MAb 19B4 in a colony immunoassay (17, 36).

Confirmation that the appropriate chromosomal rearrangement had taken place was obtained by Southern hybridization experiments. A 403-bp probe for the detection of protein D-specific sequences was constructed by using oligonucleotides 5'-GTCGCCCTTGTGGTTAAC-3' and 5'-GTAAAGTCGATGACATAGTA-3' (−68 to −55 and 315 to 334 bp from the translational start of the protein gene [hpd]) for PCR with digoxigenin-labelled dATP (Boehringer Mannheim) included in the reaction mixture (10). A 32P-labelled 1.3-kbp *Pst*I fragment corresponding to the kanamycin linker of pUC4K was used for the detection of DNA fragments hybridizing to this fragment. Hybridization conditions were described previously (18).

**Triton X-114 extraction of bacteria.** *H. influenzae* 772 and a selected protein D-negative mutant designated 772Δhpd1, grown to mid-logarithmic phase, were extracted with Triton X-114 as previously described (16, 42). Triton X-114-insoluble residue was suspended in 20 mM Tris-HCl (pH 8.0)–10 mM EDTA–1% sodium dodecyl sulfate (SDS) and boiled for 5 min. Proteins were precipitated from lysates or Triton X-114 extracts by the addition of 10 volumes of acetone at −20°C.

**LPS preparation and analysis.** LPS was purified from *H. influenzae* strains grown overnight on chocolate agar plates by a small-scale modification (12) of the hot phenol-water extraction method (48).

A whole-cell enzyme-linked immunosorbent assay (ELISA) was used for the determination of immunological properties of LPS from *H. influenzae* 772 and the protein D-deficient mutant. Bacteria grown overnight were harvested from chocolate agar plates and suspended in PBS, pH 7.2, at an optical density at 600 nm of 0.2. Flat-bottom microtiter plates (Nunc) were coated with 0.1 ml of this suspension per well at 4°C overnight. Residual protein binding sites were blocked for 2 h at room temperature with 0.1 ml of 1.5% ovalbumin in PBS per well. Plates were washed four times with PBS containing 0.05% Tween 20 before 0.1 ml of MAb culture supernatant, diluted 1:10 (vol/vol) in PBS containing 1.5% ovalbumin, was added. Plates were incubated for 2 h at room temperature and washed as described above. Wells were incubated with 0.1 ml of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako a/s, Glostrup, Denmark), diluted 1:1,000 (vol/vol) in PBS–1.5% ovalbumin, for 2 h at room temperature. After a final wash, 0.1 ml of chromogenic substrate (tetramethylbenzidine) was added to each well. Absorbance at 450 nm was read after 30 min and the addition of 50 µl of 1 M H2SO4 per well.

**SDS-PAGE and Western blot analysis.** SDS-polyacrylamide gel electrophoresis (PAGE) of protein extracts on 10.6% modified Laemmli gels and the subsequent transfer of proteins to Immobilon polyvinylidine difluoride membranes (Millipore, Bedford, Mass.) were performed as described previously (16). LPS analysis was performed with SDS–10% polyacrylamide gels by using the Tricine buffer system (38).

**Direct binding of radiolabelled IgD to intact bacteria.** The binding of 125I-labelled human IgD myeloma 4490 to intact bacteria was performed as described earlier (2).

**Enzyme assay.** Sonic extracts of *H. influenzae* grown to mid-logarithmic phase in 35 ml of sBHI broth were prepared as described previously (30) except that cells were not frozen prior to sonication. The glycophosphodiester phosphodiesterase activities of sonicate supernatants were determined by a coupled spectrophotometric assay using sn-glycerol-3-phosphate dehydrogenase and NAD with glycophosphocholine as the substrate (7, 22, 23).

**Experimental rat otitis model.** Bacteria were inoculated into broth and allowed to grow to a density of approximately 2 × 108 CFU per ml. Suspensions were then diluted with sBHI or concentrated by centrifugation for 10 min at 4,000 × g at 4°C, suspended in broth, and kept on ice until used (0.5 to 1.5 h). In some experiments, bacteria were heat inactivated at 100°C for
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FIG. 1. Insertion and deletion mutagenesis of pHIC348 for the construction of a protein D-deficient mutant. The restriction endonuclease sites used in the construction of the mutant and Southern blot analysis of the resultant H. influenzae mutant are indicated. Vector DNA sequences are represented by thicker lines. The kanamycin resistance gene is indicated by a gray box. The direction of the hpd open reading frame (open box) is indicated by an arrow.

10 min prior to the operation. The middle ears of male Sprague-Dawley rats were injected with approximately 50 μl of bacterial suspension exactly as previously described (27). Operations and inspections were carried out under a conventional otomicroscope by two members blinded to the identity of each animal and each inoculum. The animals were observed for signs of infection on days 4 and 8 after the operation.

RESULTS

Construction of protein D-negative mutant. To construct a mutant deficient in the expression of protein D, we began with plasmid pHIC348 (17), which contains two HaeIII sites within the protein D-encoding gene. The internal 534-bp HaeIII site was replaced with a kanamycin cassette isolated on a 1.3-kbp HincII fragment from pUC4K (Fig. 1). Protein D-negative transformants were identified by a colony immunoassay with MAb 19B4. Plasmid DNA was prepared from a selected protein D-negative transformant. Restriction endonuclease analysis of this plasmid, designated pHIK3, showed that the kanamycin cassette from pUC4K had been inserted into the expected site of the gene encoding protein D. In addition, protein D was not detectable in Western blots of whole-cell lysates of this E. coli transformant when probed with protein D-directed MAb 19B4 (data not shown).

pHIK3 was linearized with BamHI and transformed into NTHi 772. Kanamycin-resistant transformants lacking reactivity to MAb 19B4 were identified by a colony immunoassay. Polyclonal antiserum directed against protein D was used in Western blot analysis to exclude the possibility that the lack of reactivity to MAb 19B4 was due to a loss of the epitope to which it binds. Triton X-114 extracts of one selected MAb 19B4-unreactive transformant (772Δhpd1) and the wild-type parent strain (772) were subjected to SDS-PAGE analysis and then Coomassie blue staining or Western blot analysis (Fig. 2). The solubilized protein D from H. influenzae 772 partitioned to the detergent-phase fraction, consistent with previous results (16). A considerable amount of protein D was also found in Triton X-114-insoluble material. The MAb 19B4-unreactive transformant did not express any immunologically detectable levels of protein D when probed with the affinity-purified antiserum raised against purified protein D (Fig. 2). No differences in protein profiles except for protein D expression were observed for extracts from the mutant and the parental wild-type strain. Southern blot analysis with the protein D gene probe described above demonstrated an increase in the size of the 15-kbp EcoRI fragment containing the 5' part of hpd (18) to approximately 16 kbp in strain 772Δhpd1. In contrast, the 1.9-kbp PstI fragment containing the gene encoding protein D demonstrated a decrease in size to 0.7 kbp (Fig. 3A). To confirm that the differences in sizes for strain 772Δhpd1 were due to the insertion of the kanamycin cassette and deletion of the 534-bp HaeIII fragment, a probe corresponding to the

FIG. 2. Triton X-114 extraction and phase partitioning of proteins from H. influenzae 772 and H. influenzae 772Δhpd1. Solubilized proteins were acetone precipitated prior to SDS-PAGE through 10.6% T-3.3% C polyacrylamide gels. Triton X-114-insoluble residue and precipitated proteins were suspended in sample buffer and boiled for 5 min under reducing conditions prior to loading. (1) Coomassie brilliant blue staining; (2) Western blot with affinity-purified polyclonal rabbit anti-protein D antibodies. Lanes I, Triton X-114-insoluble proteins; lanes A, aqueous-phase fractions; lanes D, detergent-phase fractions. Molecular sizes (in kilodaltons) are given on the left.

FIG. 3. Southern blot analysis of chromosomal DNA from H. influenzae 772 and H. influenzae 772Δhpd1. Restriction fragments of chromosomal DNA hybridizing with a digoxigenin-labelled hpd-specific probe (A) or a 32P-labelled kanamycin linker-specific probe (B). Lanes 1 and 2, EcoRI-digested DNA from H. influenzae 772 and H. influenzae 772Δhpd1, respectively. Lanes 3 and 4, PstI-digested DNA from H. influenzae 772 and H. influenzae 772Δhpd1, respectively. Molecular sizes (in kilobase pairs) are indicated on the left. The sizes of fragments larger than 8 kbp are approximations as a result of the technique's low resolution of large DNA fragments.
kanamycin linker of pUC4K was prepared. This probe hybridized to a 16-kbp EcoRI fragment and a 1.3-kbp PstI fragment of DNA from the mutated strain (Fig. 3B). The latter fragment corresponds to the size of the kanamycin cassette since it contains PstI sites flanking the kanamycin resistance gene (Fig. 1). DNA from *H. influenzae* 772 did not hybridize with the kanamycin probe. From these data, it is concluded that strain 772Δhpd1 is an isogenic mutant of NTHi strain 772 that no longer expresses detectable levels of protein D.

**Phenotypic characterization of the protein D-deficient mutant.** The protein D-negative mutant was compared with wild-type strain 772 in various experiments before it was tested in an animal model. The inability to express protein D would lead to a deficiency in catalyzing the reaction from glucophosphodiester to glycerol-3-phosphate. The lack of protein D would also result in the inability to bind to human IgD myeloma 4490. To test these hypotheses, we performed enzyme activity and IgD binding experiments. The glucophosphodiester phosphodiesterase activity in a sonic extract of strain 772Δhpd1 was less than 4% of the activity in the sonicate supernatant of strain 772. In binding experiments with radiolabelled human IgD myeloma 4490, the protein D-deficient mutant bound 8% of the added IgD in comparison with 59% by the parental strain, in agreement with earlier results (17).

The growth characteristics of the NTHi mutant lacking protein D were compared with those of the wild-type parent strain, 772. The experiment was repeated three times. No significant difference in cell density or generation time was found between the two strains (Fig. 4).

Characterization of the LPS of the mutant strain in this study involved both antigenic and physicochemical analyses of LPS molecules. The protein D-negative mutant and the parental strain showed identical binding patterns to the six MAbs recognizing different epitopes in the oligosaccharide portion of *H. influenzae* LPS in a whole-cell ELISA. SDS-PAGE analysis and silver staining of purified LPS were used to study LPS expression from these two strains. No difference in the amount of LPS expressed or in electrophoretic mobility was found between LPS from these NTHi strains (Fig. 5).

**Virulence in an experimental rat otitis model.** To investigate the role of protein D in the pathogenesis of the upper respiratory tract, we used an otitis media model that was originally designed for experimental pneumococcal otitis media (15). This model was recently used for studying the course of experimental otitis media caused by *H. influenzae* (27). *H. influenzae* 772 and 772Δhpd1, grown to mid-logarithmic phase, were used to inoculate the middle ears of the animals. Equal numbers of animals were inoculated with each strain at each of the three operation occasions. The animals were examined for signs of infection by otomicroscopy on days 4 and 8 after the operation. The diagnosis of acute otitis media required direct visualization of opaque fluid behind the tympanic membrane, and a clear effusion was diagnosed as a reaction. Figure 6 reports the diagnoses of animals that were injected with various amounts of *H. influenzae* 772 or *H. influenzae* 772Δhpd1. The minimal concentration of NTHi 772 required to cause otitis media among all operated rats was 10^7 CFU/ml (Fig. 6A), whereas a 100-times-higher concentration of the protein D-deficient isogenic mutant 772Δhpd1 was required to cause otitis media among eight of nine animals challenged (Fig. 6B). Heat-killed bacteria, given in high doses, did not induce otitis to the same degree as viable bacteria (Fig. 6).

**DISCUSSION**

NTHi has become well established as an important human pathogen in the past several years. Clear distinctions between NTHi strains and encapsulated strains of serotype b concerning patient populations, types of infections caused, differences in surface antigens presented to the host, and genetical differences have been established (31). The pathogenesis of NTHi differs from that of Hib strains in that NTHi strains often establish an infection locally in the nasopharynx, from which there is a contiguous spread to other parts of the upper respiratory tract. Encapsulated strains are often more invasive and enter the bloodstream or cerebrospinal fluid. The capsule is believed to be the most important virulence factor of Hib. NTHi has developed adhesins in order for it to persist on the mucosa, but the mechanism behind its ability to cause disease is still unknown. LPS of *H. influenzae* demonstrates biologic...
activities characteristic of endotoxins (11) and is an important virulence factor, but its role in the pathogenesis of NTHi is not fully understood.

We sought to investigate what role the surface-exposed lipoprotein protein D has in the pathogenesis of NTHi in the middle ear. Protein D binds to immunoglobulin D, an antibody that is present only in trace amounts in serum (9). A substantial local synthesis of IgD in the nasopharynx and middle ear cavity has been observed (39, 40); therefore, it is tempting to speculate that the IgD binding of protein D is important for the virulence of NTHi in the upper respiratory tract. It was recently shown that protein D does not bind the majority of human IgD myelomas tested (37). However, it was not shown that unreactive IgD myelomas are typical of the IgD present in the upper respiratory tract. There is a possibility that protein D only binds to IgD of a certain configuration or in association with some factor found only in the upper respiratory tract. We are currently analyzing differences in the structures of IgDs and the role that structure has in the binding of IgD to protein D.

In this study, we have shown that the expression of protein D renders an NTHi more than 100 times more virulent in an experimental rat otitis model than an isogenic protein D-deficient mutant. It is not possible to draw any conclusions about the mechanism behind this difference in virulence from our data, since we have no evidence that protein D interacts with rat IgD. The difference in virulence is probably not due to differences in LPS expression or some cell wall structure, since heat-killed bacteria at high concentrations were less pathogenic to rats than viable bacteria (Fig. 6). The bacteria used for the inoculation of the middle ears of rats were passaged through mice and obtained from the animals' blood prior to injection into rats since it was observed in another study that prolonged passage in vitro made bacteria less virulent (26). The LPS expression of Hib can vary, depending upon the site in the body from which bacteria are isolated and/or culture conditions (20). The expression of pili also varies in a similar manner. Hib strains isolated from mucosal sites are often pilated, whereas bacteria from systemic sites do not express pili (25). We were unable to detect any variation in electrophoretic mobility or antigenicity of LPS between animal-passaged and non-animal-passaged strains, and we used bacteria isolated from the blood of mice in our studies. It is, therefore, less likely that the alteration in virulence observed in our experiment was due to changes in the phenotypic expression of LPS or differences in the degree of pilation. The insertion of an antibiotic resistance gene into the gene encoding protein D might have polar effects on possible virulence genes situated immediately downstream of hpd. This risk is, however, relatively small since we have recently identified the 5' end of an open reading frame approximately 220 bp downstream of hpd (1) which is homologous to the glycerol diffusion facilitator protein (GlpF) of E. coli (46). The downstream open reading frame is preceded by a putative promoter; therefore, it probably belongs to some operon other than hpd.

It was recently discovered that protein D is homologous to the periplasmic glycerophosphodiester phosphodiesterase (GlpQ) of E. coli (30, 43). The lack of an enzyme involved in the metabolism of an important carbon source might lead to impaired growth of a mutant lacking this enzyme. Our protein D-negative mutant of H. influenzae 772 grew as well as the wild-type strain in a complex broth medium. However, the enzyme might be more essential to the organism under the growth conditions of the host; consequently, it cannot be excluded that the observed difference in virulence is a result of growth differences in vivo. E. coli possesses two enzymes with glycerophosphodiester phosphodiesterase activity, cytoplasmic UgQ (6) and periplasmic GlpQ (22). We were unable to detect any glycerophosphodiester phosphodiesterase activity in sonicate extracts of NTHi 772Δhpdi. This result does not, however, show that only one enzyme with this activity is present in H. influenzae 772, since no UgQ activity has been detected in cellular extracts of E. coli glpQ mutants (6). It seems that UgQ can only hydrolyze diesters that are transported by the ugp-encoded transport system of E. coli (6). An important difference between protein D and GlpQ of E. coli K-12 is that protein D is a surface-exposed lipoprotein (16) while GlpQ is a soluble, periplasmic protein (23). We have no explanation for why protein D is localized on the surface of H. influenzae, but if it is involved in the virulence of NTHi, its surface localization would not be unnatural. There is a possibility that protein D might have a broader substrate specificity than GlpQ of E. coli (22) and act as a phospholipase. We are, therefore, currently analyzing the substrate specificity of protein D.

ACKNOWLEDGMENTS

We thank Anders Grubb for sharing his limited amount of IgD with us and Gertrud Hansson for skillful technical assistance.
This work was supported by grants from the Swedish Medical Research Council (projects 03163 and 05196), the Medical Faculty of Lund, the Alfred Osterlund Foundation, the Crafoordska Foundation, the G. and J. Kock Foundation, and the Malmö Medical Service Foundation.

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