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Vaccination against encapsulated bacteria in hereditary C2 deficiency results in antibody response and opsonisation due to antibody-dependent complement activation

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Abbreviations used in this paper: C2D, hereditary C2 deficiency or C2-deficient, C4b2a, classical pathway C3 convertase, GMC, geometric mean concentration, Hib, Haemophilus influenzae type b, MBL, mannan-binding lectin or mannose-binding lectin, Neisseria meningitidis, N. meningitidis, Streptococcus pneumoniae, S. pneumoniae.
Abstract

Hereditary C2 deficiency (C2D) is an important susceptibility factor for invasive infections caused by encapsulated bacteria such as pneumococci and *Haemophilus influenzae* type b. The infections are mostly seen in childhood indicating that antibody-mediated acquired immunity is affected. C2D persons and healthy controls were vaccinated with ActHIB® and Pneumo23®. Analysis of specific antibodies to pneumococci serotype 6B, 7F, and 23F, and Hib was performed. Post-vaccination IgG antibodies against pneumococci serotype 6B and 23F at a concentration ≥1.0 mg/L was found in similar frequency in C2D persons and controls. Post-vaccination sera from C2D persons showed poor complement-mediated opsonization and phagocytosis of pneumococci by granulocytes when depending on classical and lectin pathway activation only, but increased (p=0.007) and equaled that of the normal controls when also alternative pathway activation was allowed due to antibody-dependent C2 bypass activation. In conclusion, the C2D persons benefited from the vaccination and achieve an increased phagocytic capacity.

Keywords

The complement system, C2 deficiency, Invasive infection, Vaccination, Opsonization, Phagocytosis
1. Introduction

Complement supports many immunological functions that contribute to protection from disease as well as to expression of disease manifestations. The complement system can be activated mainly through three pathways: the classical (C1qr2s2, C4 and C2), the alternative (C3, factor B, factor D and properdin), and the lectin pathway (MBL or ficolins /MASPs, C4 and C2) [1]. Each of the three activation pathways leads to the formation of a C3 convertase, C4b2a for the classical and the lectin pathway and C3bBb for the alternative pathway. The C3 convertases cleave C3 which leads to formation of the principal opsonins C3b and iC3b that stimulate phagocytosis. Complement activation subsequently continues with the terminal complement components (C5-C9) that assemble to form a cell lysing membrane attack complex which may kill gram-negative bacteria such as Neisseria (N.) meningitidis and Haemophilus influenzae type b (Hib). However, gram-positive bacteria as for example Streptococcus (S.) pneumoniae resist the bactericidal action of C5-C9.

Hereditary deficiency of the second component of complement (C2D) is one of the most common complement deficiency states in populations of Western descent and has an estimated prevalence of 1 in 20,000 [2, 3]. Two principal variants of C2D have been described [4, 5]. The predominant variant of C2D is type I (90%), which is caused by homozygosity for a 28-base pair deletion in the C2 gene resulting in a complete lack of C2 synthesis. This C2 deficiency gene is usually part of the major histocompatibility complex (MHC) haplotype HLA-B18, S042, DR2 [4, 5, 6]. C2D is associated with autoimmune diseases such as systemic lupus erythematosus (SLE) and with an increased susceptibility to infections caused by encapsulated bacteria such as S. pneumoniae and Hib [2, 3, 7, 8, 9]. C2D
may also be a risk factor for development of atherosclerosis [7]. However, many persons with C2D are apparently healthy [2, 7, 8].

Vaccination may be beneficial in complement-deficient patients [9]. For instance, favorable in vitro responses to tetravalent meningococcal vaccination have been demonstrated in properdin deficiency and in late-complement component deficiencies [10, 11, 12]. Vaccination of C2D patients has been considered to be advisable despite a lack of supportive data [2, 13]. In C2D, generation of C3 fragments by action of the classical pathway C3 convertase C4b2a, which is an important mechanism for recruitment of complement-mediated defense by specific antibodies, will not work [14, 15]. Also, the classical pathway is known to promote antibody responses to thymus-dependent antigens [16] and might influence responses to thymus-independent antigens [17] such as polysaccharides [18]. These circumstances suggest that vaccination responses in C2D are uncertain particularly against polysaccharide antigens. Nevertheless, we have reported for two C2D patients that anticapsular antibodies supported serum bactericidal reactions against *N. meningitidis* and Hib [19]. This raises the question if immunization with pneumococcal polysaccharides would promote opsonophagocytic killing of *S. pneumoniae* in C2D.

We have described a cohort of 40 C2D patients with a high frequency of severe infections (57%) mainly caused by encapsulated bacteria [7]. In a follow-up study of 44 C2D persons, we found that the G2M*n/*G2M*n genotype was associated with protection against severe infections suggesting the involvement of an immunoglobulin (Ig)-dependent defense mechanism [20].
In the present study we have measured antibody responses following vaccination with the 23-valent pneumococcal vaccine Pneumo23® and Hib conjugate vaccine ActHIB® in C2D. The antibody responses were compared with those in a control group. We also investigated if immunization could promote opsonophagocytosis of S. pneumoniae in C2D. Issues regarding the clinical effect of vaccination were addressed in the C2D patients through review of medical records and a mailed questionnaire.

2. Materials and Methods

2.1. Patients and controls

Between 1977 and 2007, 49 persons with C2D were identified in clinical routine analysis at the Clinical Immunology unit, University Hospital of Lund, Sweden. Since the initiation of the present study in 1993, 25 C2D persons were enrolled and a written informed consent was obtained from each person. None of the C2D persons or controls was vaccinated with any pneumococcal vaccine or Hib vaccine before inclusion in the present study. Demographics and clinical manifestations of the vaccinated persons are shown in table 1. The distribution of gender was equal between the C2D persons (F: M, 16:9) and controls (F: M, 39:12). However, the C2D persons (median 41 years, range 2-63 years) were older than the control group (median 27 years, 16-61 years, $p=0.02$, Mann-Whitney U test).

The participants received the 23-valent pneumococcal vaccine, which contains 25 μg of the following type-specific capsular polysaccharide: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F (Pneumo23®, Sanofi Pasteur MSD,
S.N.C., Paris, France). The C2D persons were also vaccinated with Haemophilus type b conjugate vaccine, tetanus toxoid conjugate (ActHIB®, Sanofi Pasteur MSD). In 4 of the C2D persons vaccinated with Haemophilus type b conjugate vaccine, the pre- or post-vaccination blood samples were not technically handled in accord with the study protocol and therefore excluded from further analysis. A control group consisting of 51 healthy persons was also vaccinated with Pneumo23® and ActHIB®. The investigation was approved by the Lund University Ethics Committee (protocol LU 350-93).

2.2. Follow-up of adverse reactions and side effects to vaccination

Adverse reactions were followed among the C2D persons and controls. All vaccinated persons were asked to inform about any complaints by phone or by visiting the clinic during the first week after vaccination. A standardized form was filled out at three follow-up visits to the clinic (one month, 6 months and one year, respectively). No short-term adverse reactions as well as late adverse reactions and side effect were documented in the C2D persons. No SLE flare was triggered by the vaccination. Among controls, side effects were observed in one of the vaccinated. The person that experienced side effects to Pneumo23® in the control group was recorded for fever, swelling and redness at the site of vaccination. All symptoms resolved completely after three days.

2.3. Assessment of invasive infections caused by pneumococci and Hib

Medical records were reviewed and discussed with patient’s physicians. The investigation was supplemented with a mailed questionnaire. The following questions were addressed: Since the inclusion in the present study have you had any severe infection? If applicable, what type of infection/s were you treated for? Which years and where were you treated for the
infection/s? After inclusion in the present study have you had any additional vaccination? Year and name of the vaccine, alternatively what the vaccine should protect against? The questionnaire was given to 24 of the 25 C2D persons included in the study and they all responded. One C2D person died during the investigated period. The questionnaire was addressed to C2D persons parents if they were <18 years.

2. 4. Serum samples

Venous blood samples were collected from all subjects before vaccination, and at 4 to 6 weeks after vaccine administration. In accord with a standardized protocol, serum samples were without delay stored in aliquots at −80°C until they were analyzed or used in the experiments. In 4 C2D persons serum samples were collected over 4-6 years for long-term follow-up after vaccination.

Serum samples from 12 of the C2D persons, selected to cover the range of specific anti-pneumococcal antibodies were used in complement deposition and phagocytosis assays. An equally sized control group was used with sera matched according to antibody response.

2. 5. Antibodies to pneumococcal and Hib capsular polysaccharides

Specific IgG, IgA, IgM, IgG1 and IgG2 to capsular polysaccharides of S. pneumoniae (serotypes 6B, 7F, and 23F) and Hib were determined by ELISA [21, 22, 23, ]. Hib was measured by antibodies to the capsular polysaccharide in human sera using an antigen composed of Haemophilus type b oligosaccharides conjugated to human serum albumin kindly provided by Dr. Moon H. Nahn (Departments of Microbiology and Pathology University of Alabama at Birmingham 845 19th Street South, BBRB614 Birmingham,
Purified pneumococcal capsular polysaccharides were provided by Pasteur Mérieux Connaught (Marcy-l’Etoile, France). Pneumococcal C-polysaccharide for preabsorption of serum samples was purchased from Statens Serum Institut (Copenhagen, Denmark). In the assay, bound IgG, IgA and IgM was detected with corresponding goat anti-human IgG, IgA and IgM alkaline phosphatase conjugate (γ-, α-, and μ-chain specific F(ab’)_2 fragment, product no. A-3312 (IgG), A-3062 (IgA) and A-1067 (IgM) (Sigma Biosciences, St. Louis, MO, USA). Monoclonal anti-human IgG1 (NL-16) and anti-human IgG2 (HP6014) were from Skibio (Bedfordshire, UK). For detection of the monoclonal antibodies alkaline phosphatase conjugated goat anti-mouse IgG antibodies (Dako, Glostrup, Denmark) were used. The color reaction was developed with p-nitrophenylphosphate (1 mg/mL) in diethanolamine, pH 9.8, for one hour at room temperature. Absorbance was measured at 405 nm in a Multiscan Plus photometer (Labsystems Ltd., Helsinki, Finland). Values obtained were mean absorbance values from two coated wells with subtraction of background absorbance in the uncoated well. For expression of antibody concentrations in mg/L, calibration of a local reference serum was made against an international calibrator provided by Dr C Frasch, Bethesda, MD, USA (anti-pneumococcal antibodies lot 89 SF and anti-Hib antibodies serum pool lot 1983). The anti-Hib pool contained: IgG 60.9 mg/L, IgA 5.6 mg/L, IgM 3.5 mg/L, IgG1 30.9 mg/L, and IgG2 16.1 mg/L. The detection limits for the ELISA tests were calculated to be between 0.01 µg/L and 0.03 mg/L when the limit was defined as the lowest point on the dilution curve significantly higher than baseline (>+2 SD). Post-vaccination concentrations of specific IgG antibodies ≥1 mg/L were considered as long-term protective against infections caused by S. pneumoniae and Hib [24, 25, 26, 27]. All data concerning pre- and post-vaccination antibody concentrations are shown in the supplementary figures s1-s5.
2. 6. **Immunoglobulins and complement proteins**

IgG, IgA, IgM, C3 and C4 were determined by turbidometry (Cobas Mira, Roche Diagnostic, Basel, Switzerland). Age-related immunoglobulin reference intervals were used [28, 29]. Concentrations of the IgG subclasses IgG1, IgG2 and IgG3 were determined by single immunodiffusion and 2.5-97.5 percentiles age-related reference intervals were used [30]. IgG4 levels were measured with a commercial ELISA (Bindazyme, The Binding Site Ltd, Birmingham, UK). Screening for detection of complement deficiency was mainly performed with hemolytic gel assays [31]. C3 and C4 were determined by turbidimetry (Cobas Mira, Roche Diagnostic). C2 concentrations were measured by electroimmunoassay and given in mg/L assuming a normal concentration at 26 mg/L [32]. C2D was defined as serum C2 concentration <0.5 mg/L.

2. 7. **Preparation of S. pneumoniae serotype 23F**

Pneumococci serotype 23F, obtained from Statens Serum Institut, was stored on glass beads at -70°C. The pneumococci were cultured from the glass beads over night at 37°C anaerobically on blood agar and subcultured once before use. After inoculation into Todd-Hewitt broth, the bacteria were grown at 37°C until the absorbance was 0.4 at OD₆₀₀. The bacteria were washed in 0.15 M NaCl and killed by addition of 0.8% glutaraldehyde in 0.15 M NaCl for 20 min. After additional washes the bacteria absorption was adjusted to 1.0 at OD₆₀₀ and the bacteria were frozen in small aliquots at -80°C.

2. 8. **Analysis of opsonization of pneumococci by complement fragments**
Experiments were initially performed with both live and killed bacteria and different serotypes (23F, 19F and 6B) in complement opsonisation and phagocytosis assays giving similar results (Selander B. et al, unpublished data). All experiments reported here were performed with killed *S.pneumoniae* serotype 23F and thereby a standardized number of bacteria. Bacteria (2.5 μL suspension) were incubated with 100 μL serum prediluted 1/10, in veronal buffered saline with 0.15 mM Ca$^{2+}$ and 0.5 mM Mg$^{2+}$ (VBS CaMg) for 30 min at 37°C with continues agitation. The incubation was stopped by addition of 500 μL ice-cold VBS CaMg. The bacteria were then spun down at 405 x g for 5 min and 100 μL was added of anti-C3c or anti-C4c antibodies (Quidel, San Diego, CA, USA) prediluted 1/100 in VBS CaMg followed by incubation for 30 min at 4°C. The bacteria were washed twice and 100 μL fluorescein isothiocyanate (FITC) conjugated antibody (Dako) prediluted 1/20 in VBS CaMg were added and further incubated for 30 min at 4°C. After the incubation the bacteria were washed once, resuspended in 500 μL VBS CaMg and then analyzed by flow cytometry (Epics XL-MCL Beckman-Coulter, FL, USA). The results are presented as mean fluorescence index (MFI) ratio defined as the MFI value for the serum sample divided by the MFI value for heat-inactivated serum.

**2.9. Reconstitution of complement components to depleted serum**

In order to clarify the need of the various complement proteins for the activation leading to deposition of C3 fragments a serum deficient of MBL and C2 was depleted of C1q, factor D and properdin. This serum contained antibodies against 23F at a concentration of 4.3 mg/L. The serum reagent was reconstituted regarding one or several of the lacking complement proteins using purified complement proteins as previously described by Selander et al., 2006 [33]. The serum reagents were diluted 1/5 to ascertain conditions allowing alternative pathway activation. The various serum reagents were then used to investigate C3 deposition.
as described above. To assess the importance of antibodies, the serum reagent was incubated with recombinant EndoS, a bacterial endoglycosidase which hydrolyzes glycans in the effector part of the IgG molecule, at a concentration of 0.25 mg/mL for 20 hours at 37°C. EndoS has been shown to prevent complement activation and IgG interaction with Fcγ receptors [34].

2. 10. Hemolysis of red blood cells

Sheep red blood cells (RBCs) (Håtunalab AB, Håtunaholm, Sweden) were washed three times in VBS CaMg (1125 x g, 5 min) and resuspended to 1x10^8 cells/mL. Rabbit anti-sheep RBC IgG antibodies were purified from Amboceptor (SPL, Stockholm, Sweden) by gel filtration. Rabbit anti-sheep IgG (1/10) was incubated with RBCs at a total volume of 200 μL for 60 min at room temperature with continuous agitation. Serum was added, diluted 1/5 in 200 μL VBS CaMg, and incubated at 37°C with continuous agitation for 90 min. The reaction was ended by addition of 1 mL ice-cold VB-EDTA. The RBCs were pelleted by centrifugation (1125 x g, 5 min) and the absorbance of the supernatant measured at 412 nm. A negative control (RBCs treated with VBS CaMg) was used in all experiments and the absorbance value of this control was subtracted from all sample values.

2. 11. Analysis of phagocytosis of pneumococci by granulocytes

FITC-labelled *S. pneumoniae* serotype 23F diluted 1/40 in VBS CaMg, were incubated with serum diluted 1/10 in VBS CaMg for 30 min at 37°C with continuous agitation. Polymorphonuclear neutrophils (PMNs) from healthy donors were obtained by Polymorph
Prep (Axis-Shield PoC AS, Oslo, Norway) density-gradient centrifugation according to standard procedures. The opsonized bacteria were added to the PMNs (2x10^5 cells/mL) and incubated for 30 min at 37°C with continuous agitation before flow cytometry analysis. Results are given as MFI ratio.

2. 12. Statistical analysis

Wilcoxon signed rank test was used for analysis of vaccination responses observed in C2D persons and controls by comparing pre- and post-vaccination IgG, IgA and IgM antibody concentrations to the investigated antigens, respectively. Fisher’s exact test was used to determine if the number of responders to the investigated antigens at a level of IgG ≥1 mg/L was different between C2D persons and controls. Mann-Whitney U test was used for analysis of differences between C2D persons and controls regarding concentrations of antigen-specific pre- and post-vaccination IgG, IgA and IgM antibodies. Furthermore, quotients of pre- and post-vaccination concentrations (fold increase) in the C2D persons and controls were compared using the Mann-Whitney U test. This test was also used to analyze differences between pre- and post-vaccination antibody concentrations in the complement fragment deposition and phagocytosis experiments. Correlations between C3 and C4 deposition and IgG antibody concentration were determined by the Spearman rank correlation test. If a pre- or post-vaccination antibody concentration was not measurable, the result was set to be at the detection limit. All p-values were two-tailed and considered significant at p<0.05.
3. Results

3.1. Antibody responses to vaccination with 23-valent pneumococcal polysaccharide vaccine

No difference was demonstrated between the C2D persons and controls for any of the serotypes investigated in IgG pre-vaccination antibody concentrations and in general, the C2D persons (n=25) responded well to immunization with the 23-valent pneumococcal polysaccharide vaccine (Table 2). However, there was evidence of decreased levels of post-vaccination antibodies against serotype 7F as compared to healthy controls but no such difference could be seen regarding antibodies against serotype 6B and 23F (Table 2). Twenty-four of the C2D persons (96%) responded with a 2-fold or higher increase or had a post-vaccination concentration of specific IgG ≥1.0 mg/L to at least one serotype. Twenty C2D persons (80%) obtained this kind of response to all three serotypes. The corresponding rates for controls were 98% (50/51) and 84% (43/51), respectively. However, there was a lower response rate to serotype 7F in the C2D persons than in controls (fold increase \( p<0.0001 \), post-vaccination concentration \( p=0.0006 \), Mann-Whitney \( U \) test). The number of responders to serotype 7F at a level of IgG ≥1 mg/L was also shown to be lower among the C2D persons (19/25, 76%) compared to controls (50/51, 98%, \( p=0.004 \), relative risk 0.8, 95% CI 0.6-1.0, Fisher’s exact test). Most of the IgG anti-pneumococcal antibodies in the C2D persons and also in the controls were of the IgG2 subclass.

The C2D persons achieved a good IgA response to serotype 6B and 23F, but not to serotype 7F (Table 2). Pre-vaccination concentrations of IgA antibodies to serotype 6B and 7F were found to be the same as compared with controls. The C2D persons had a higher pre- and post-
vaccination concentration of 23F anti-IgA antibodies than the controls (\(p=0.02\) and \(p=0.05\), respectively, Mann-Whitney \(U\) test). The IgM pre- and post-vaccination antibody concentrations for all serotypes showed no difference between C2D persons and controls (Table 2).

3.2. Antibody responses to Hib vaccination

The antibody responses of the 21 C2D persons immunized with the Hib conjugate vaccine are presented in Table 2. The vaccination gave a mean 82-fold increase (range 0.6-686, \(p<0.0001\), Wilcoxon signed rank test) in the C2D persons and in controls a mean 278-fold increase (range 1.2-1400, \(p<0.0001\), Wilcoxon signed rank test) of IgG anti-Hib antibodies. Pre-vaccination concentrations of anti-Hib IgG and IgA showed no difference between C2D persons and controls (data not shown). Interestingly, the pre-vaccination concentrations of anti-Hib IgM were found to be significantly higher in C2D than compared with controls (\(p=0.006\), Mann-Whitney \(U\) test).

After vaccination against Hib 19 (90%) C2D persons and 51 (100%) of controls reached a level of specific IgG \(\geq 1.0\) mg/L (\(p=0.08\), Fisher’s exact test). Post-vaccination geometric mean concentration (GMC) increased to 9.4 mg/L in the C2D group (range 0.9-48.0 mg/L) and in the controls to 35.2 mg/L (range 1.5-77.0 mg/L). The C2D persons also showed an increase in IgA and IgM antibody levels (both \(p<0.001\), Wilcoxon signed rank test, Table 2). Both the C2D persons and controls showed an IgG2 subclass predominance of antibodies to Hib (both had 62.5% of IgG2 antibodies after vaccination).
3.3. Investigations of antibody responses over time

Four C2D persons were followed over 4-6 years with measurements of IgG anti-pneumococcal antibodies (Figure 1A-1D). The two C2D patients shown in figure 1A and B were siblings and both had SLE but still showed different responses, which may partly be explained by differences in disease severity and treatment as the nonresponder (figure 1A) was treated with corticosteroids at high dose (prednisolone >15 mg/day). Other reasons for the differences in vaccination responses may be previously acquired anti-pneumococcal antibodies or, in one case presence of a MHC haplotype not previously described in relation to the C2 null gene [7]. Thus, the results indicate that C2D per se does not severely limit responses to vaccination with polysaccharide antigens and specific antibodies may remain at a high level for all three serotypes for a duration of at least 5 years.

3.4. Analysis of immunoglobulin concentrations

All 25 vaccinated C2D persons had normal levels of IgG, IgA, and IgM. With few exceptions also the IgG1 and IgG2 concentrations were within normal ranges. One adult C2D person had an increased IgG1 concentration of (13.1 g/L, reference interval 4.2-10.7 g/L) and three had low IgG2 concentrations (1.2-1.3 g/L, reference interval 1.7-6.1 g/L). One of five C2D children had a low IgG2 concentration (0.39 g/L, age-related reference 0.43-2.54 g/L). Among the 51 healthy controls, we found no abnormal immunoglobulin concentrations.

3.5. Relationship between susceptibility to infection and specific antibodies

The C2D persons were stratified into two groups in accord to severity of infections in order to facilitate further analysis of different vaccination responses in relation to documented
infections (Table 3). Group I had only minor infections while group II had in varying degree more severe infections as described in table 1. About 65% of all episodes with invasive infections (meningitis and septicemia) occurred before the age of 13.

Interestingly, the C2D patients recorded for severe infections (groups II) had a lower GMC of anti-pneumococcal IgG antibodies and a higher GMC of anti-pneumococcal IgM antibodies before vaccination than the patients in group I (Table 3). After vaccination the IgG and IgM antibody GMC increased in both groups, but higher IgG levels were still found in group I and higher IgM levels in groups II.

Patients with rheumatologic manifestations were fairly evenly distributed among the patient groups (Table 1). The C2D SLE patients (n=7) showed the same vaccination responses as the other C2D persons in the cohort (p>0.1, Mann-Whitney U test).
3.6. Follow-up of invasive infection with pneumococci or Hib in vaccinated C2D persons

Medical records were reviewed covering a total of 1,121 person-years of which 221 person-years were after vaccination. Data from the medical records were supplemented with a mailed questionnaire. The incidence of invasive infection was 10 cases per 1,000 person-years before vaccination and after immunization the incidence decreased to 4.5 cases per 1,000 person-years. At the time of analysis the median age in the C2D cohort was 41 years and the age related incidence in the general Swedish population of invasive pneumococcal disease was about 0.1 cases per 1,000 (Data from the Swedish Institute for Infectious Disease Control, Stockholm, Sweden). The five children (2-15 years) included in the study were not recorded for any new severe infection during the following 31 person-years after vaccination. The findings suggest a decrease in incidence of invasive pneumococcal or Hib infection in vaccinated C2D persons.

3.7. Increased C3 and C4 deposition after vaccination

Since C2D individuals got increased antibody titers after vaccination we wanted to investigate if post-vaccination C2D sera could support an increased opsonization of S. pneumoniae serotype 23F. Post-vaccination C2D sera supported an increased deposition of both C3 and C4 fragments ($p=0.007$ and $p=0.02$, respectively, Wilcoxon signed rank test, Figure 2A-B). Furthermore, post-vaccination sera from the control group supported similar C3 fragment deposition as the C2D post-vaccination sera ($p=0.16$, Mann Whitney U test). Post-vaccination C2D sera did also support a higher deposition of C4 fragments than sera from the control group ($p=0.002$, Mann Whitney U test). Thus, C2D post-vaccination sera supported C3 and C4 fragment deposition resulting in equal or higher deposition than the control sera.
3.8. Identification of a C2-bypass pathway operating in C2D

To clarify the mechanism resulting in C3 deposition in the absence of C2, experiments were performed with serum reagents lacking selected complement proteins (MBL, C2, C1q, factor D and properdin). Addition of MBL, C2 or C1q alone did not support complement activation but when combining C1q and C2 the classical complement pathway got activated with increased C3 fragment deposition on the bacteria (Figure 3A). Restitution of serum reagent with C1q supported an increased C4 fragment deposition on the bacteria (Figure 3B). Upon addition of both C1q and C2, the C4 fragment deposition was diminished, possibly due to a reduced binding of the antibody to C4 once the C4 molecule had formed complex with C2 (Figure 3B). Furthermore, activation of the lectin pathway appeared not to be of importance in the system (Figure 3A and B). Reconstitution of the alternative pathway supported C3 fragment deposition in the absence of C1q and C2, but with addition of C1q the deposition was equal to fully reconstituted serum (Figure 3C) demonstrating a C1q-dependent, C2-independent activation of the alternative pathway (Figure 3D). Treatment with EndoS, which inhibits antibody-induced classical pathway activation, also reduced the C3 deposition demonstrating that the C2-independent C1q-dependent activation of the alternative pathway is antibody-dependent (Figure 3C).

3.9. C2-independent hemolysis of antibody-coated sheep red blood cells

C2D was for long identified by the inability of C2D serum to lyse Ig-coated sheep red blood cells (SRBCs). However, Knutzen et al reported in 1989 that increased amount of Ig on the target cells could overcome this inability through a C2-bypass mechanism [35]. Therefore, we created heavily IgG-opsonized SRBCs and incubated them with different serum reagents to investigate if the C2-bypass mechanism was operating in this system. When using a low
concentration of IgG, the C2D serum was not able to lyse the SRBCs (data not shown). However, when using a higher IgG concentration the C2D serum had a hemolytic ability of about 50% of a normal human serum which is in line with previous findings [35]. To investigate which complement components that were responsible for this C2-independent hemolysis we used a serum reagent lacking MBL, C1q, C2, factor D and properdin, and used heat-inactivated serum as a negative control. Neither heat-inactivated serum nor the serum reagent itself could induce any hemolysis of the SRBCs (Figure 4). Furthermore, reconstitution of the alternative pathway could not improve the hemolytic ability of the serum reagent. However, when reconstituting the alternative pathway together with C1q we observed a 50% hemolytic ability (Figure 4). In summary, under these conditions we could demonstrate that the C1q-dependent C2-independent complement activation pathway is operating in a hemolytic system.

3.10. Post-vaccination C2D sera support increased phagocytosis

Since post-vaccination sera supported increased opsonization of bacteria compared to pre-vaccination sera we investigated if vaccination also influenced phagocytosis measured as the uptake of FITC-labeled bacteria of granulocytes. C2D post-vaccination sera as well as the controls supported an increased phagocytosis (p=0.007 and p=0.03, respectively, Wilcoxon signed rank test, Figure 5) and no differences could be seen between C2D persons and controls (p=0.89, Mann Whitney U test) resembling the previous C3 opsonization results. There was also a strong correlation between the ability of sera from C2D persons to support phagocytosis and C3 fragment deposition (p<0.0001, r=0.69, Spearman rank correlation) demonstrating the necessity of C3 deposition in the phagocytosis assay. Thus, C2D post-
vaccination sera support an increased phagocytosis which correlated well to the opsonization level.

4. Discussion

Deficiency of the second component of complement is a strong susceptibility factor for development of severe invasive infections caused by encapsulated bacteria [2, 7]. Here we have clearly demonstrated that in a large C2D cohort gathered at a single center, vaccination of C2D persons against pneumococci and HIB gives rise to an antibody response and increased opsonization. Our data also indicate that vaccination diminishes the risk for infection caused by encapsulated bacteria.

The C2D persons were able to develop a good antibody response both to the 23-valent pneumococcal polysaccharide vaccine and to the Haemophilus b conjugate vaccine. Both vaccines were found to be beneficial to a majority of the C2D persons in the sense that a concentration of ≥1 mg/L of specific IgG was achieved, which is considered to be protective against invasive pneumococcal and Hib infection in normal persons [24, 25, 26, 27]. Among the vaccinated C2D persons, the anticapsular antibodies were primarily of the IgG2 subclass. The IgA and IgM response patterns of the C2D patients to the investigated antigens were similar to that of IgG with a lower response to Hib and pneumococcal serotype 7F than to serotype 6B and 23F antigens for which the response was equal to that found in healthy controls. The reasons for the lower responses may be several. The polysaccharide capsule mediates virulence of both *S. pneumoniae* and Hib, and determines the serotype. The antigenic variation of the serotypes is due to the heterogeneous structural and chemical composition of the capsular polysaccharides [36]. For instance, the pneumococcal serotype 7F
is structurally characterized by a branched molecular form while 6B and 23F display at least one side with a straight repeated polysaccharide structure [37]. This structural difference might affect the functions of pattern recognition in innate immunity and thereby influence the adaptive immunity responses.

Results from studies in experimental animal models using C4-deficient and C3-depleted guinea pigs predict that pneumococcal vaccination of patients with classical pathway deficiencies should be of limited value [38]. Impaired function of the classical pathway may limit antibody production due to lack of the adjuvant effect of C3d fragments on the immune response [16, 39]. However, in humans a normal or impaired vaccination response to capsular polysaccharides have been reported in C3 deficiency [10, 40, 41], implying that an impaired response to such antigens should not necessarily be expected in C2D. Furthermore, susceptibility to pneumococcal infection in C2D is most pronounced during childhood [2, 7, 13], suggesting that acquisition of antibody-mediated immunity is operative.

In the present investigation we found a great individual variation in antibody responses and obviously the strength of the response could not be ascribed to C2D alone. One explanation to the variation may be difference in IgG heavy chain allotypes (GM) as suggested by our previous findings that absence of infections in C2D is found in carriers of the G2M*n allele [20], which is known to promote antibody responses to polysaccharide antigens. Other factors may be involvement of other antibodies or recognition molecules such as MBL that contributes to complement activation also involving amplification by the alternative pathway. An MBL-dependent C2 bypass mechanism for alternative pathway-mediated C3 activation has been demonstrated and is operating in C2D [33, 42, 43]. However, in our experimental
setting we did not observe any activation of the lectin pathway (Figure 3) which is in line with
the previously demonstrated inability of MBL to bind to \textit{S. pneumoniae} [44]. Thus, the MBL-
dependent C2 bypass pathway is not operating in our system. Another C1q-dependent C2
bypass mechanism for complement activation which is operating in C2D has been observed
when IgG antibodies are bound at high density as described already in 1989 by Knutzen et al
[35]. Furthermore, it has been shown in an animal model that IgG-induced complement-
mediated tissue damage may occur in the absence of C2 [45]. Anti-C1q antibodies are
frequently found in C2D [46] and may increase the efficiency of this mechanism. Thus, C2
collapse mechanisms are operating resulting in C3 deposition also in the absence of an intact
classical pathway, see figure 3. C3d formed as a result of complement activation will, in
complex with the antigen by binding to the CR2 on B cells, function as an adjuvant enhancing
the antibody production [39].

As shown in this study, the C2D persons possess another advantage as compared to those with
C4 deficiency or C1q deficiency in the ability to deposit C4b on a target which in turn can
interact with the C3b/C4b receptor (CR1). Triggering of this receptor on B cells positively
regulates the specific antibody response to low doses of antigen by enhancing B cell
differentiation whether T cell help is provided by intact T cells or by T cell-derived
differentiation factors [47].

The classical pathway induces complement-mediated phagocytosis of \textit{S. pneumoniae} and this
is considered to be an explanation to the increased susceptibility to \textit{S. pneumoniae} infections
in C2D [48]. However, in C1, C4 and C2 deficiency, there is evidence that anticapsular
antibodies can activate the alternative pathway and facilitate the killing of \textit{S. pneumoniae} [49,
An important novel finding in our study was that the immunization contributed to a higher degree of opsonization and phagocytosis of *S. pneumoniae*. A positive correlation between IgG antibody responses and opsonization was demonstrated. This effect was apparently dependent on amplification of complement activation involving the alternative pathway, since when only classical pathway activation was allowed the C2D sera did not support phagocytosis. Thus, C2D post-vaccination sera supported an increased C3 and C4 fragment deposition under conditions similar to the *in vivo* situation when all complement pathways are operating. The effect we have observed fits perfectly with the described C2 bypass activation mechanism [35]. It is also in accordance with the results from a C2D guinea pig model reported by Wagner et al [45]. Our results clearly demonstrated that C2D post-vaccination sera support an increased phagocytosis which correlated well with the degree of opsonization. Thus, specific antibodies can in fact activate complement even in the absence of an intact classical pathway (Figure 3). In this context it should be pointed out that the prevalence of C2D [8] implies that the majority of C2D persons do not have any proneness for infection which leads to the diagnosis of C2D. They may have antibodies that induce complement-mediated defense and this is likely of major importance.

Interestingly, the C2D persons without documented invasive pneumococcal infections had acquired a higher concentration of specific IgG antibodies and had a lower concentration of specific IgM before vaccination than the C2D persons with a history of invasive infections. After vaccination, the C2D persons that were recorded for invasive pneumococcal infections improved in their IgG antibody concentration. However, the response resembled a primary antibody response with an aberrant high IgM concentration indicating a difficulty to switch from IgM to IgG. These findings are in line with previous vaccine responses found in humans and in animal models with early complement deficiency states [16]. In a previous study [20],
we found that almost all of the C2D persons without previously documented infections also carried the favorable G2M(n) allotype. The possibility that the \( GM^{*b,f,n} \) haplotype contributes to early antibody diversification in CD27\(^+\) memory B cells might be considered [52]. The inability in some C2D persons to switch from IgM to IgG may be attributed to a failure to acquire adequate B cell memory to relatively low stimulating antigens due to genetic background.

Findings regarding the duration of protection after a first dose of unconjugated polysaccharide vaccines are limited to observational studies with the clinical endpoint of pneumococcal bacteraemia [53, 54]. Furthermore, the exact protective concentration of specific anti-pneumococcal antibodies remains to be determined although some experts have estimated it to be \( \text{IgG} \geq 1 \text{ mg/L} \) [24, 25, 26, 27]. Levels of antibodies to most pneumococcal vaccine antigens remain elevated for at least 5 years in healthy adults. In some persons, antibody concentrations decrease to pre-vaccination levels after 10 years [55, 56]. In the present study, the clinical efficacy of pneumococcal vaccination in C2D was difficult to determine. The main reason for this was a lack of power due to a low number of participants. Although we could not provide statistical evidence of a reduced number of cases with severe pneumococcal disease, a decline in the incidence after vaccination was observed.

5. Conclusions

In summary, vaccination against pneumococci and \( H. \text{influenzae} \) type b gave rise to antibody responses in C2D persons with or without rheumatic disease that varied from normal to impaired, partly depending on the antigen. We could demonstrate that antibodies promoted
opsonization by deposition of complement fragments on pneumococci by C1q-dependent C2 bypass mechanisms. The findings clearly indicate a protective effect of vaccination against invasive infections by these bacteria in C2D and should encourage further investigation of immune responses in C2D. Our results might also contribute to the establishment of future rationales for the use of vaccines against pneumococcal disease in C2D and should be considered in vaccine development.
Acknowledgments

We would like to express the deepest gratitude to our mentor, the late Dr Anders G Sjöholm, who initiated the present study in 1993. Without his lifetime achievement it would not have been possible to obtain the present results. We are indebted to our research nurse Ann Åkesson for administering the vaccines and excellent logistic work concerning the immunized persons and to Dr. Mattias Collin for his gift of recombinant EndoS. We would also like to express our gratitude to the staff at the Department of Infectious Diseases, the University Hospital of Lund, Sweden, for participating in the present investigation.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.
References


**Figure legends**

**Figure 1.** Antibody response after vaccination against pneumococci over time. Four C2D persons (A – D) were followed over 4-6 years with measurements of IgG anti-pneumococcal antibodies and the great variation in response is illustrated. The patients are numbered as in table 1 where the individual clinical data are given. Arrows indicate time of vaccination with the 23-valent pneumococcal vaccine Pneumo23®.

**Figure 2.** C2D and control sera in dilution 1:10 were incubated with *S. pneumoniae* serotype 23F and (A) C3 and (B) C4 deposition were analyzed by flow cytometry using anti-C3c and anti-C4c antibodies, respectively. The values are reported as the mean fluorescence index (MFI) ratios of at least two experiments. Horizontal lines show medians.

**Figure 3.** Requirement of complement factors for deposition of C3 and C4 fragments on pneumococci. Experiments were performed with a serum reagent (R) lacking MBL, C1q, C2, factor D (D) and properdin (P). The serum reagent was reconstituted as indicated and then used to analyze C3 and C4 deposition on *S. pneumoniae* serotype 23F by flow cytometry analysis. (A) C3 deposition is shown with serum reagents allowing classical and lectin pathway activation. (B) C4 and (C), C3 deposition is shown with serum reagents allowing activation also by the alternative pathway. In C also results with reagents treated with rEndoS are shown. Efficient C3 deposition was obtained with C1q present and the alternative pathway reconstituted and this is dependent on IgG as shown by rEndoS treatment. (D) Illustration of C2 bypass pathways operating in C2 deficiency. Activation of the classical pathway (CP) and lectin pathway (LP) is hindered since only C4b and no complete convertase C4b2a is formed, but C3 may still be cleaved by the alternative pathway (AP) convertase C3bBb. In addition to activation by the alternative pathway only, two C2 bypass mechanism may result in increased
C3 cleavage, the C1q-dependent C2 bypass activation initiated by antibodies at high density described by Knutzen Steuer et al., 1989 [35], also shown in this study and the MBL-dependent activation recently described by Selander et al., 2006 [33].

**Figure 4.** C2-independent hemolysis of sheep red blood cells (SRBCs). Heavily IgG-opsonized SRBCs were incubated with a serum reagent (R) lacking MBL, C1q, C2, factor D (D) and properdin (P) restituted as indicated and hemolysis was measured. The values reported are the mean and standard deviation of the hemolytic activity compared to normal human serum. As negative control heat-inactivated normal serum (IA) was used. Values from two or more independent experiments are given.

**Figure 5.** Phagocytosis of *S. pneumoniae* serotype 23F. The ability of pre- and post-vaccination sera (dilution 1:10) to support phagocytosis was investigated for 12 C2D persons and 12 healthy controls. The MFI ratios are the mean of two or more experiments.
Figure 1.
Figure 2
Figure 3
Figure 4

Figure 5
Table 1. Clinical manifestations in the vaccinated C2-deficient persons (n=25) stratified into groups according to documented infection.

<table>
<thead>
<tr>
<th>Group / Patient No.</th>
<th>Gender</th>
<th>Age at vaccination</th>
<th>Received vaccines</th>
<th>Documented infections</th>
<th>Isolated bacteria (blood or liquor)</th>
<th>Other disease manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>41</td>
<td>Pnc/Hib</td>
<td>Minor infections</td>
<td>SLE, Asthma.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>39</td>
<td>Pnc/Hib</td>
<td>Minor infections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>27</td>
<td>Pnc/Hib</td>
<td>Minor infections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>48</td>
<td>Pnc/Hib</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>40</td>
<td>Pnc/Hib</td>
<td>Minor infections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>F</td>
<td>51</td>
<td>Pnc/Hib</td>
<td></td>
<td>UCTD, Lung emphysema, Chronic bronchitis, Pneumothorax.</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>M</td>
<td>50</td>
<td>Pnc/Hib</td>
<td></td>
<td>SLE, AMI x 2. Prostate cancer.</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>M</td>
<td>63</td>
<td>Pnc/Hib</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group IIA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>46</td>
<td>Pnc</td>
<td>Pneumonia x 2</td>
<td>SLE, Pancreatitis, Depression.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>43</td>
<td>Pnc/Hib</td>
<td>Pneumonia x 2</td>
<td>SLE, AMI, Sjögren syndrome, Pernicious anaemia.</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>50</td>
<td>Pnc/Hib</td>
<td>Pneumonia x 2</td>
<td>SLE</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>M</td>
<td>42</td>
<td>Pnc/Hib</td>
<td>Pneumonia x 4</td>
<td>UCTD</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>F</td>
<td>11</td>
<td>Pnc/Hib</td>
<td>Pneumonia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Epiglottitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group IIB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>51</td>
<td>Pnc/Hib</td>
<td>Septicaemia, Pneumonia x 2</td>
<td>Hypertension.</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>37</td>
<td>Pnc/Hib</td>
<td>Meningitis x 2</td>
<td>UCTD, Pustulosis palmaris et plantaris.</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>F</td>
<td>54</td>
<td>Pnc</td>
<td>Septicaemia, Pneumonia x 2</td>
<td>Enterococcal species SLE, Pancreatitis.</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>F</td>
<td>48</td>
<td>Pnc/Hib</td>
<td>Septicaemia x 2</td>
<td>S. aureus, SLE, AV-block II-III. Parotid gland tumour.</td>
<td></td>
</tr>
<tr>
<td><strong>Group IIC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>20</td>
<td>Pnc/Hib</td>
<td>Septicaemia x 2</td>
<td>Streptococcal species, Mnc</td>
<td>Appendicitis.</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>10</td>
<td>Pnc</td>
<td>Septicaemia x 2</td>
<td>Pnc all 3 times</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>15</td>
<td>Pnc/Hib</td>
<td>Meningitis x 2</td>
<td>Pnc 2 times, S. agalatiae, Mnc</td>
<td>Eczema</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>24</td>
<td>Pnc/Hib</td>
<td>Septicaemia, Meningitis.</td>
<td></td>
<td>Epilepsy</td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>12</td>
<td>Pnc/Hib</td>
<td>Epiglottitis with septicaemia x 2</td>
<td>Hib and K. kingae, S. agalatiae</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>42</td>
<td>Pnc/Hib</td>
<td>Septicaemia, Pneumonia x 3</td>
<td>Vasculitis, Chronic bronchitis.</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>M</td>
<td>2</td>
<td>Pnc</td>
<td>Septicaemia x 3, Pneumonia x 3</td>
<td>Pnc</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>38</td>
<td>Pnc/Hib</td>
<td>Pyelonephritis x 2</td>
<td>UCTD, Anorexia nervosa, Preeclampsia, Membranous glomerulonephritis (WHO, class V).</td>
<td></td>
</tr>
</tbody>
</table>
a Group I only minor infections, group II minor infections and at least one documented
episode of pneumonia (IIA), one invasive infection combined with pneumonia and other
infections (IIB) or at least two invasive infections (IIC). The patient numbers are in accord
with our previous published investigations on C2D [7, 19, 41]. Abbreviations: AMI, acute
myocardial infarction, Hib, *Hemophilus influenzae* type b, *K. kingae*, *Kingella kingae*, Mnc,
agalatiae, *Streptococcus agalatiae*, SLE, systemic lupus erythematosus, UCTD,
undifferentiated connective tissue disease.
Table 2. Pre-and post-vaccination geometric mean concentrations (GMC) of specific antibodies in C2-deficient persons (n=25) and controls (n=51).

<table>
<thead>
<tr>
<th>Specific immunoglobulin isotype and antigen</th>
<th>C2-deficient persons</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GMC (mg/L)</td>
<td>Post-vaccination GMC (mg/L)</td>
</tr>
<tr>
<td>IgG Pnc 6B</td>
<td>2.6</td>
<td>5.4</td>
</tr>
<tr>
<td>IgG Pnc 7F</td>
<td>1.7</td>
<td>3.0</td>
</tr>
<tr>
<td>IgG Pnc 23F</td>
<td>1.2</td>
<td>3.3</td>
</tr>
<tr>
<td>IgG Hib</td>
<td>0.9</td>
<td>9.4</td>
</tr>
<tr>
<td>IgG2 Pnc 6B</td>
<td>2.4</td>
<td>4.5</td>
</tr>
<tr>
<td>IgG2 Pnc 7F</td>
<td>2.1</td>
<td>3.8</td>
</tr>
<tr>
<td>IgG2 Pnc 23F</td>
<td>1.5</td>
<td>2.9</td>
</tr>
<tr>
<td>IgG2 Hib</td>
<td>0.6</td>
<td>5.9</td>
</tr>
<tr>
<td>IgA Pnc 6B</td>
<td>0.2</td>
<td>0.7</td>
</tr>
<tr>
<td>IgA Pnc 7F</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>IgA Pnc 23F</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>IgA Hib</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>IgM Pnc 6B</td>
<td>1.8</td>
<td>3.4</td>
</tr>
<tr>
<td>IgM Pnc 7F</td>
<td>0.9</td>
<td>2.2</td>
</tr>
<tr>
<td>IgM Pnc 23F</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>IgM Hib</td>
<td>0.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

- Statistical differences between pre- and post-vaccination levels were calculated by Wilcoxon signed rank test.
- The Mann-Whitney U test was used for comparisons of post-vaccination levels between C2-deficient persons and controls.
Table 3. Pre-and post-vaccination geometric mean concentration (GMC) of specific antibodies in C2-deficient persons (n=25) in relation to severity of infection.

<table>
<thead>
<tr>
<th>Specific immunoglobulin isotype and antigen</th>
<th>Pre-vaccination</th>
<th>Post-vaccination</th>
<th>p-value b</th>
<th>Pre-vaccination</th>
<th>Post-vaccination</th>
<th>p-value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG Pnc 6B</td>
<td>9.4</td>
<td>1.6</td>
<td>&lt;0.05</td>
<td>12.9</td>
<td>3.7</td>
<td>0.2</td>
</tr>
<tr>
<td>IgG Pnc 7F</td>
<td>3.9</td>
<td>1.2</td>
<td>0.08</td>
<td>5.7</td>
<td>2.3</td>
<td>0.1</td>
</tr>
<tr>
<td>IgG Pnc 23F</td>
<td>3.5</td>
<td>0.8</td>
<td>&lt;0.05</td>
<td>8.4</td>
<td>2.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IgG Hib</td>
<td>0.7</td>
<td>1.1</td>
<td>0.5</td>
<td>10.6</td>
<td>8.8</td>
<td>1.0</td>
</tr>
<tr>
<td>IgG2 Pnc 6B</td>
<td>7.2</td>
<td>1.5</td>
<td>&lt;0.05</td>
<td>11.2</td>
<td>2.9</td>
<td>0.07</td>
</tr>
<tr>
<td>IgG2 Pnc 7F</td>
<td>4.9</td>
<td>1.4</td>
<td>0.07</td>
<td>8.1</td>
<td>2.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IgG2 Pnc 23F</td>
<td>3.6</td>
<td>1.0</td>
<td>&lt;0.05</td>
<td>7.0</td>
<td>1.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IgG2 Hib</td>
<td>0.9</td>
<td>0.5</td>
<td>0.7</td>
<td>9.2</td>
<td>4.6</td>
<td>0.2</td>
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<tr>
<td>IgA Pnc 6B</td>
<td>0.2</td>
<td>0.1</td>
<td>0.6</td>
<td>0.4</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>IgA Pnc 7F</td>
<td>0.1</td>
<td>0.1</td>
<td>0.6</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>IgA Pnc 23F</td>
<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
<td>0.9</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>IgA Hib</td>
<td>0.1</td>
<td>0.1</td>
<td>0.8</td>
<td>0.4</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>IgM Pnc 6B</td>
<td>0.9</td>
<td>2.5</td>
<td>&lt;0.05</td>
<td>1.7</td>
<td>4.7</td>
<td>0.1</td>
</tr>
<tr>
<td>IgM Pnc 7F</td>
<td>0.5</td>
<td>1.2</td>
<td>0.1</td>
<td>2.0</td>
<td>2.3</td>
<td>1.0</td>
</tr>
<tr>
<td>IgM Pnc 23F</td>
<td>0.3</td>
<td>0.7</td>
<td>0.07</td>
<td>0.3</td>
<td>1.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IgM Hib</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>2.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

a C2D persons were divided into two groups according to the severity of infections (see Table 1).

b Comparison between group I and group II of pre- and post-vaccination concentrations. All p-values were calculated with the Mann-Whitney U test. Abbreviations: Pnc, Streptococcus pneumoniae, Hib, Hemophilus influenzae type b.