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Pneumococcal Polysaccharides Complexed with C3d Bind to Human B Lymphocytes via Complement Receptor Type 2

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The immunoregulatory function of the complement system has been the focus of many investigations. In particular, fragments of complement factor C3 have been shown to play a role in B-lymphocyte activation and proliferation, lymphokine production, and the generation of in vitro antibody production. Purified pneumococcal polysaccharides (PS) can induce direct activation of C3 via the alternative pathway. Using sera of C1q-deficient patients and healthy subjects, we demonstrated that C3d, a split product of C3 that is generated after degradation of iC3b, can be bound to PS antigens. The binding of C3d to PS can occur in the absence of specific antibodies. Subsequently, we showed that PS complexed with C3d can be recognized by complement receptor type 2 that is expressed on B cells. Treatment of B cells with a monoclonal antibody recognizing the C3d-binding site of complement receptor type 2 reduces the binding of PS-C3d to the cells. In addition, we showed that PS4 complexed with C3d exerted an increased immunogenicity compared with free PS4. Our results show that the complement system plays a role in the activation of PS-specific B cells, carrying membrane receptors for C3d. Consequently, the complement system plays a regulatory role in the antibody response to T-cell-independent type 2 antigens such as PS.

In recent years, complement fragments have been found to play a role in B-cell activation (24). This regulatory role of complement (5, 18, 20, 25) is mediated by complement receptors expressed on B cells (4, 7); C3d, the ligand of complement receptor type 2 (CR2; 17) is described to be a growth factor for murine B cells (24). Furthermore, Carter et al. (2, 3) showed synergistic signaling of murine B cells after ligation of both CR2 and membrane immunoglobulin M (IgM), as measured by intracellular calcium mobilization. In view of our studies on B-cell activation by pneumococcal polysaccharide (PS) antigens, a culture system was developed to study the in vitro antibody response to PS by human B lymphocytes (11a, 32). This culture system, a model system to study the antibody response to T-cell-independent type 2 (TI-2) antigens, is used to study the role of complement and complement receptors in the B-cell response to these antigens. A finding which might bear relevance to the role of CR2 in B-cell activation by PS antigens is the observation that marginal-zone B cells in the neonatal spleen show a reduced expression of CR2 compared with adult spleen B cells (38). We have also demonstrated this decreased expression on neonatal blood B cells (11a). During ontogeny, acquisition of adult levels of CR2 expression on B cells coincides with onset of responsiveness to TI-2 antigens (38). In the model system that is used for study of neonatal unresponsiveness to TI-2 antigens, that using CBA/N mice, there is also reduced CR2 expression (22). In order to examine the role of CR2 and its ligands in an antibody response to PS antigens, we first studied the ability of different soluble purified PS to activate complement via the alternative route. We also ascertained whether PS can bind the degradation fragment C3d and whether the resultant complex can bind to CR2 on B cells. We suggest that the synergistic signaling after ligation of CR2 and secretory IgM (sIgM) as described by Carter et al. (2, 3) is a phenomenon that can take place in B-cell recognition of PS bound to C3d. This is demonstrated by the fact that PS type 4 (PS4)-C3d complexes, better than free PS4, can stimulate B cells to respond to PS4.

MATERIALS AND METHODS

Donors, sera, and reagents. For this study, blood was obtained by venipuncture from healthy adult donors. To discriminate between alternative and classical pathway complement activation, sera of two C1q-deficient patients (patients 1 and 2) were used (13). Furthermore, serum of a patient with severe combined immunodeficiency (SCID) lacking serum Ig and complement factor C1q (patient 3) was used. In this serum, normal alternative hemolytic complement activity was present while there was no classical pathway hemolytic activity detectable. Normal human serum (NHS) of normal healthy donors was used for the complement studies. Blood withdrawn for complement studies was allowed to clot for 2 h. Clotting was allowed to take place at 0°C to minimize complement activation. After centrifugation at 1,000 × *g* for 10 min at 4°C, serum was stored at −70°C in small aliquots until use.

Purified PS from different serotypes (types 1, 3, 4, and 14, Danish nomenclature, referred to as PS1, PS3, PS4, and PS14) were obtained from the American Type Culture Collection (Rockville, Md.). PSpool refers to a mixture of eight different PS (types 1, 3, 4, 8, 9, 12, 14, and 19; American Type Culture Collection). Rabbit antibodies specific for human C3d were obtained from Dakopatts (Glostrup, Denmark). A rabbit antiserum against *Streptococcus pneumoniae* type 4 capsular polysaccharide was obtained from the State Serum Institute (Copenhagen, Denmark). Rabbit anti-PS14 antiserum was a kind gift of H. Snippe (Department of Microbiology, State University Utrecht, Utrecht, The Netherlands).

Activation of the complement system by PS. To study complement activation by soluble PS, serum samples were

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incubated for 60 min at 37°C either with 50 μg of PS per ml, diluted from a stock solution of 1 mg of PS per ml of saline, or with saline alone. Samples were tested in the complement assays as indicated.

Complement assays. Hemolytic complement activity (CH_{50}) was determined by a microadaptation of the method described by Mayer (23). Sheep erythrocytes were collected in Alsever solution from adult sheep and coated with Amboceptor (National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands). The hemolytic activity of the alternative pathway was measured after incubation of 10^7 rabbit erythrocytes with various dilutions of sera in half-isotonic Veronal buffer containing gelatin and glucose in the presence of 5 mM EDTA in 0.2 ml for 60 min at 37°C. Concentrations of C3d were determined by rocket immunoelectrophoresis as described by Laurell (21). Briefly, serum samples were precipitated with a polyethylene glycol (PEG) 6000 solution (1:1 [vol/vol]) (Merck, Darmstadt, Germany; 22% in borate buffer–10 mM EDTA [pH 8.3]). After an incubation of 1 h on ice, serum samples were centrifuged at 7,000 × *g* for 2 min. Supernatants were collected and loaded on a 1% agarose gel containing 70 μg of rabbit anti-human C3d per ml. After a running time of 3 h at 20 V, gels were stained with Coomassie brilliant blue for 30 min, destained, and dried. Calibration was performed using samples with known C3d concentrations (12, 35, 110, 310, and 610 U/ml) (Immuntech, Odense, Denmark). In some experiments serum was used in which C3d was generated artificially by incubation with IgG bound to protein A-Sepharose CL-4B beads (2.9 mg of beads per ml of serum; Pharmacia, Uppsala, Sweden).

Anti-C3d ELISAs. To detect binding of C3d to PS, two different approaches were used. A sandwich enzyme-linked immunosorbent assay (ELISA) was performed, using anti-PS antisera as a coating on 96-well ELISA plates (overnight; rabbit anti-PS4, 1:1,000; rabbit anti-PS14, 5 μg/ml). Wells, washed extensively, were incubated for 2 h at 37°C with the (PS-treated) serum samples. After the plate was washed, rabbit anti-human C3d was added (Dakopatts; final dilution, 1:2,000) and incubated for 3 h at 37°C. Subsequently, the wells were washed and were allowed to react with goat anti-rabbit Ig conjugated to peroxidase (1:2,000; Dakopatts). The wells were then allowed to react with 0.1 ml of 5.5 mM *O*-phenylenediamine–0.015% H_2O_2 in citrate phosphate buffer (pH 5). The yellow-brown reaction product was measured after 10 to 15 min at 450 nm with an ELISA reader.

Binding of C3d to PS was also demonstrated by coating PS directly on the ELISA plates. 96-well ELISA plates were coated overnight at 37°C with 10 μg of PS per ml in 0.9% NaCl. The wells were subsequently allowed to react with serum samples from patients with either high or low C3d concentrations in serum. The ELISA was performed by subsequent incubation with the rabbit anti-human C3d, and processing was done as indicated for the C3d sandwich ELISA.

Anti-PS ELISA. The PS ELISAs were carried out as described previously (31). In short, ELISA plates were coated with PS as described for the anti-C3d ELISA. A reference serum with known anti-PS antibody concentrations was used for calibration (36). This hyperimmune serum contained 2,224 ng of anti-PS1 immunoglobulin per ml, 2,648 ng of anti-PS3 per ml, 1,196 ng of anti-PS4 per ml, and 1,778 ng of anti-PS14 per ml. To determine the anti-PS4 concentrations in a given sample, serial dilutions were titrated into the plate. Adsorption with cell wall PS was carried out by incubating serum samples (dilution, 1:50) overnight at 4°C with 50 μg of cell wall PS (State Serum Institute) per ml. The ELISA was processed by adding peroxidase-conjugated goat anti-human Ig (Tago, Burlingame, Calif.) and incubating for 3 h at 37°C. Subsequently, treatment with *O*-phenylenediamine was performed as indicated for the C3d ELISA.

Immunofluorescence. Two-color immunofluorescence utilizing direct phycoerythrin (PE)-conjugated antibodies and indirect fluorescein isothiocyanate (FITC)-conjugated reagents required four separate incubations of 30 min each. Peripheral blood mononuclear cells were isolated by Ficoll Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation of heparinized blood from healthy adult volunteers. After treatment with C3d-coated PS4 or PS14, cells were incubated with an unconjugated rabbit anti-PS4 serum (1:1,000 final dilution; State Serum Institute) or an anti-PS14 serum (5 μg/ml) and then with FITC-conjugated goat anti-rabbit Ig (1:10; Nordic, Tilburg, The Netherlands). The final incubation was performed with the PE-conjugated CD20 monoclonal antibody (Leu-16-PE; Becton Dickinson, Mountain View, Calif.). In some experiments the FITC staining was blocked by previous incubation of cells with monoclonal antibodies OKB7 (anti-CR2; 20 μg/ml; Ortho Diagnostic Systems, Raritan, N.J.), HB5 (anti-CR2; 20 μg/ml; Becton Dickinson), anti-CR1 (20 μg/ml; Becton Dickinson), and Leu-12 (CD19; 20 μg/ml; Becton Dickinson). Stained cells were analyzed on a FACS Analyzer with C30 software (Becton Dickinson).

B-cell activation. Peripheral blood mononuclear cells were separated into T cells and non-T cells by rosetting with 2-amino-ethylisothiouronium bromide-treated sheep erythrocytes. Purified B cells were obtained by removing excess numbers of monocytes from non-T cells by iron carbonyl treatment. These cell preparations consisted in general of 45 to 60% $CD20^+$ B cells, less than 10% monocytes, and 3% $CD3^+$ T cells. All cell cultures were carried out in RPMI 1640 medium supplemented with antibiotics, glutamine (2 mM), and 10% fetal calf serum (Flow, Irvine, United Kingdom) and incubated at 37°C and 100% relative humidity with 5% CO_2. Cultures were done in Falcon tissue culture tubes for 6 days with 0.5×10^6 purified B cells, 10% irradiated (2,000 rads) autologous T cells, and 25% (vol/vol) T-cell replacing factor. T-cell replacing factor is defined as the supernatant of a 24-h culture of irradiated T cells supplemented with 5% monocytes in the presence of PWM (37). The PWM was coupled to agarose to obtain mitogen-free supernatant. Cell-free T-cell replacing factor-containing culture supernatants were passed through a 45-μm-pore-size Millipore filter and stored at −70°C until use. PS4 or PS2 (American Type Culture Collection) at a concentration of 10^{-6} μg/ml was added at the initiation of the culture. The specificity of the in vitro-induced anti-PS4 antibody response was tested in B-cell cultures with PS2 or tetanus toxoid (5 limiting flocculations [lf]/ml; National Institute of Public Health and Environmental Protection).

SFC assay. Cultured cells were assayed for the presence of specific antibody-secreting cells in the spot-forming cell (SFC) assay (35). The culture system, as described above, allows generation of only IgM anti-PS4-secreting cells. IgG or IgA anti-PS4-secreting cells were generally not induced or were induced at very low levels. In some experiments the number of total IgM-secreting cells was determined. Flexible 96-well ELISA plates (Titertek; Flow) were coated overnight at 37°C either with 1 μg of rabbit anti-human IgM (Tago) per ml in carbonate buffer (pH 9.6) or with 10 μg of PS4 per ml in 0.9% NaCl. Serial dilutions of cultured cells

were added to the wells and incubated overnight at 37°C and 100% relative humidity with 5% CO_2. After removal of the cells, the wells were sequentially incubated with alkaline phosphatase-conjugated goat anti-human IgM (1:1,000 final dilution; Dako) and 5-bromo-4-chloro-3-indolylphosphate (1 mg/ml in 1 M 2-amino-2-methyl-1-propanol-containing buffer [pH 10.2]) in gelling agarose (0.3% [vol/vol]). After an overnight incubation, spots were enumerated with an inverted microscope. The specificity of the test was determined by the addition of free PS4 (1 mg/ml) during the incubation with cultured cells. This resulted in an 80% inhibition of the number of anti-PS4 spots. B cells cultured with PS2 or without antigen did not result in anti-PS4 spots above background levels. The Student *t* test was applied to determine the statistical significance of observed differences.

RESULTS

Ability of soluble purified PS to induce activation of C3. It has been shown (39) that soluble capsular PS can activate the alternative complement pathway. We were also able to detect a decrease in hemolytic complement activity (CH_{50} and AP_{50}) and an increase in C3d concentrations in NHS after incubation in the presence of different PS. The observed reduction in complement activity may be caused by immune complexes formed by the interaction between PS and anti-PS antibodies which are present in NHS and which could lead to activation of the classical pathway. Therefore, sera of two C1q-deficient patients were evaluated for their ability to generate C3d following treatment with PS. IgG and IgA anti-PS antibody levels in these sera were generally below 10% of that observed in normal human serum. IgM anti-PS antibodies were present at normal or elevated levels (IgM anti-PS1 was 379 and 199% of that in hyperimmune serum [36] with known anti-PS titers [see Materials and Methods], IgM anti-PS3 was 374 and 256%, IgM anti-PS4 was 500 and 206%, and IgM anti-PS14 was 157 and 97% for patients 1 and 2, respectively). Figure 1 shows the percent increase in C3d concentrations after incubation of these sera with different PS types, compared with the addition of saline alone. Since C1q is structurally defective in these patients (13), classical pathway activation is impaired, implying that the alternative pathway activation of C3 is responsible for C3d generation. It is obvious from Fig. 1 that the level of C3d generation is not the same for every PS type. The magnitude of anti-PS titers in the patients' sera does not correlate with the level of C3d generated. These results support the hypothesis that various PS types differ in their ability to activate complement (11) and that the C3d generation measured can take place independent of classical pathway activation and hence independent of the presence of specific antibodies. The latter was confirmed by using serum of a SCID patient in which, in agreement with the presence of low serum IgG levels (less than 0.1 g/liter), extremely low levels of anti-PS-specific antibodies were present. In this serum, PS were also able to induce C3d generation (Fig. 1, patient 3).

Soluble PS bind C3d. To test the possibility that PS can bind C3d, PS were added to PEG-treated NHS in which C3d was generated by use of IgG-coated protein A beads (see Materials and Methods). Binding of C3d to PS may lead to an altered electrophoretic mobility and hence to an altered C3d rocket height. Figure 2 shows that all types of PS tested inhibit C3d rocket formation. Nonspecific interference of PS in the electrophoresis as such could be excluded since these PS types were not able to affect the magnitude of tetanus

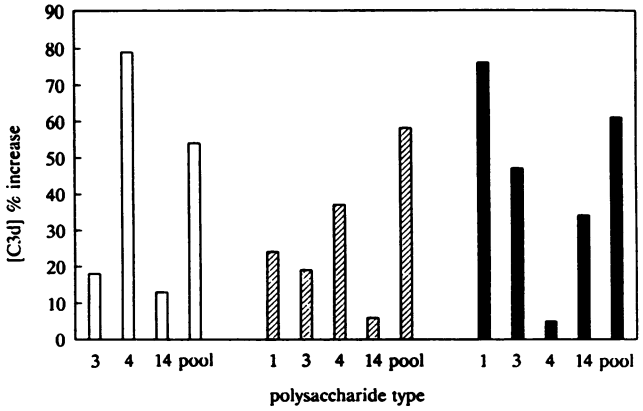

FIG. 1. PS-induced rise of C3d concentration. Shown are relative elevations of C3d levels by addition of 50 μg of PS per ml, as measured by rocket electrophoresis, compared with addition of medium (one representative experiment out of three) in sera of two patients with a C1q deficiency (patient 1, open bars; patient 2, hatched bars) and one with SCID (patient 3, closed bars). The C3d concentrations in untreated sera of patients 1, 2, and 3 were 123, 170, and 150 U of C3d per ml, respectively. IgG anti-PS1 was 3.0, 4.0, and 20.1% (as percentages of that in NHS), IgG anti-PS3 was 11.0, 28.5, and 29.1%, IgG anti-PS4 was 9.2, 5.6, and 31.4%, and IgG anti-PS14 was 2.0, 6.0, and 8.6% for patients 1, 2, and 3, respectively.

toxoid rocket electrophoresis (data not shown). It is also shown in Fig. 2 that neither a protein (tetanus toxoid) at the same concentration nor comparable concentrations of a monosaccharide (glucose) or disaccharide (sucrose) diminished the C3d concentration in a serum sample.

In order to demonstrate C3d binding to PS, 96-well tissue culture plates were coated with PS and subsequently incubated with serum samples with different C3d levels. Figure 3 shows that C3d could be detected on the PS coating, which indicates binding of C3d to PS. To discriminate between the possibilities that either C3d binds directly to PS or C3d is associated with PS immune complexes, experiments were performed using NHS and serum of a SCID patient (patient 3) containing very low anti-PS titers. These sera were incubated for 1 h at 37°C in the presence of 50 μg of PS4 per

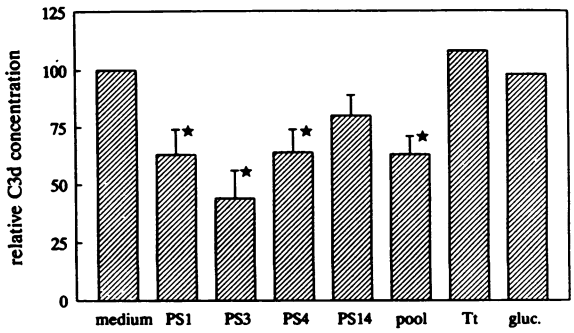

FIG. 2. Inhibition of C3d rocket electrophoresis by PS. Different pneumococcal serotype PS were added at a concentration of 50 μg/ml to the rocket electrophoresis samples. Serum in which C3d was artificially generated (from 35 to 232 U/ml) by incubation with IgG bound to protein A beads was used (see Materials and Methods). Data shown are mean values of three independent experiments (±1 standard deviation); the stars indicate a significant ($P < 0.05$) difference from the medium control.

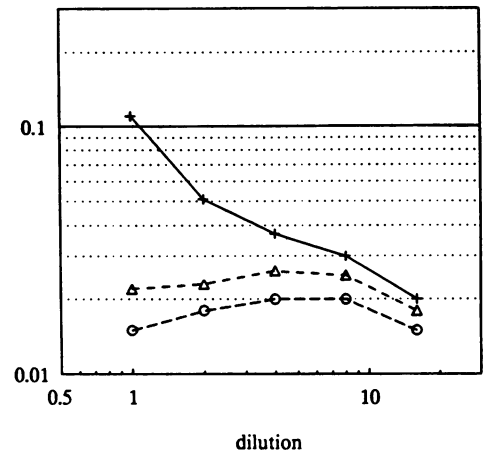

FIG. 3. Binding of C3d to PS4 can be detected by ELISA. Sera of two patients differing in C3d concentrations (+, 300 U of C3d per ml; △, <12 U of C3d per ml) were incubated (after removal of high-molecular-weight fragments of C3 by PEG precipitation) on a PS4-coated plate (data for one representative experiment out of three are shown). Bound C3d could be detected if serum with high C3d levels was used. Control values (○) were obtained by incubation with medium alone.

ml. A sandwich ELISA was performed by coating a 96-well microtiter plate with anti-PS4 antibodies. The assay was performed by incubation of the PS4-treated serum and detection of C3d with anti-C3d antibodies. Figure 4 demonstrates that C3d could be detected on PS4 incubated in NHS as well as in serum of patient 3. Since in both cases, C3d binding to PS occurred, this indicates that C3d binding is directly to PS and is independent of specific antibodies. This experiment was also performed using PS14 and anti-PS14 antibodies. Binding of C3d to PS14 could also be demonstrated in both NHS and serum of patient 3 (data not shown).

PS-bound C3d is recognized by CR2 on B cells. To test the hypothesis that formation of PS-C3d complexes could be relevant in activation of PS-specific B cells, we tested whether B cells are able to bind C3d that is bound to PS. Peripheral blood mononuclear cells were incubated with serum of patient 3 treated with 50 μg of PS4 per ml and hence containing PS4-C3d complexes. Leu-16-counterstained B cells appeared to be positive for PS4 in indirect immunofluorescence analysis with rabbit anti-PS4 antiserum (Fig. 5a). No fluorescence was observed after incubation of B cells in either PS4 (Fig. 5b) or serum of patient 3 incubated with medium alone (Fig. 5c). It was demonstrated that binding of PS4-C3d complexes is mediated via CR2 on B cells, since it could be blocked by OKB7, an anti-CR2 monoclonal antibody that recognizes the C3d binding site of CR2 (26). Treatment of B cells with OKB7 prior to incubation with PS4-C3d complexes resulted in a 50 to 70% reduction of fluorescence intensity (Fig. 5d), while treatment with anti-CD19 antibodies (Fig. 5e) or anti-CR1 antibodies (Fig. 5f) did not reduce fluorescence intensity. These experiments were also performed with PS14. Binding of PS14-C3d complexes on B cells could also be demonstrated and could be blocked by OKB7 antibodies (data not shown).

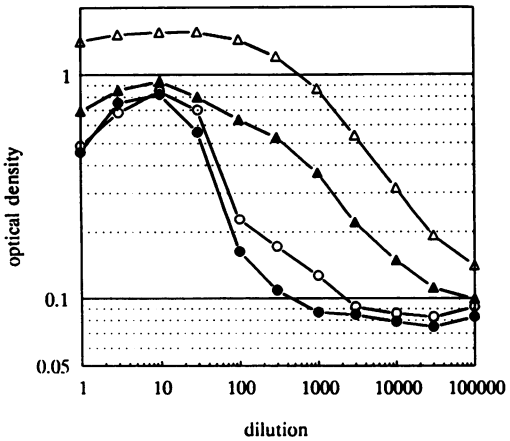

FIG. 4. Detection of C3d binding to PS4 by using a sandwich ELISA. Sera incubated with either PS4 (triangles) or medium alone (circles) were tested on an anti-PS4-coated plate. C3d bound to PS4 could be detected by using anti-C3d antibodies (open symbols represent NHS, and closed symbols represent serum from patient 3). Results are given for one representative experiment out of three.

PS4-C3d complexes show increased immunogenicity compared with free PS4. Data shown above indicate that C3d complexed to PS4 can bind to B cells via CR2. To test whether this leads to increased immunogenicity of PS4, B cells were cultured with PS4 incubated in serum (see above) and subsequently assayed in vitro for induction of anti-PS4 antibody production (32). Figure 6 shows that PS4 complexed with C3d is immunogenic at concentrations at which free PS4, or comparable amounts of serum alone, are not able to induce antibody formation. Furthermore, the magnitude of the in vitro anti-PS4 response, in terms of absolute numbers of PS4-specific SFC, is higher when PS4 complexed to C3d is used as the immunogen.

DISCUSSION

Previous studies in our laboratory on the regulation of the antibody response to PS in humans showed the role of T cells in the human in vitro antibody response to PS4 (11a). In a separate line of research we found that neonatal B cells which are unresponsive to PS antigens have decreased cell surface expression of CR2 (11b). Therefore our hypothesis is that complement and complement receptors play a role in the B-cell response to PS. In our studies that address the role of complement receptors in the antibody response to PS, we have demonstrated that CR2 is not only involved in B-cell activation as such but also plays a role in the in vitro antibody response to PS4. The results presented in this article provide evidence that PS, as present in the capsule of *S. pneumoniae*, are able to activate complement via the alternative pathway. It can be concluded from the results that even in soluble form, these molecules are able to activate C3 and induce generation of split products such as C3d. Earlier studies have already indicated that intact pneumococci (9, 11, 15) as well as the cell wall components teichoic acid and peptidoglycan (40) are able to activate complement.

A drawback of using complete serum to study complement activation initiated through the alternative pathway is interference of anti-PS antibodies. Observed decreases in CH_{50} and AP_{50} may reflect complement consumption by classical pathway activation. Furthermore, the decrease in AP_{50} values might be due to the formation of Ig immune complexes which are reported to be able to activate the alternative route of complement (1, 8, 12). Therefore, these experiments were repeated using sera from C1q-deficient patients

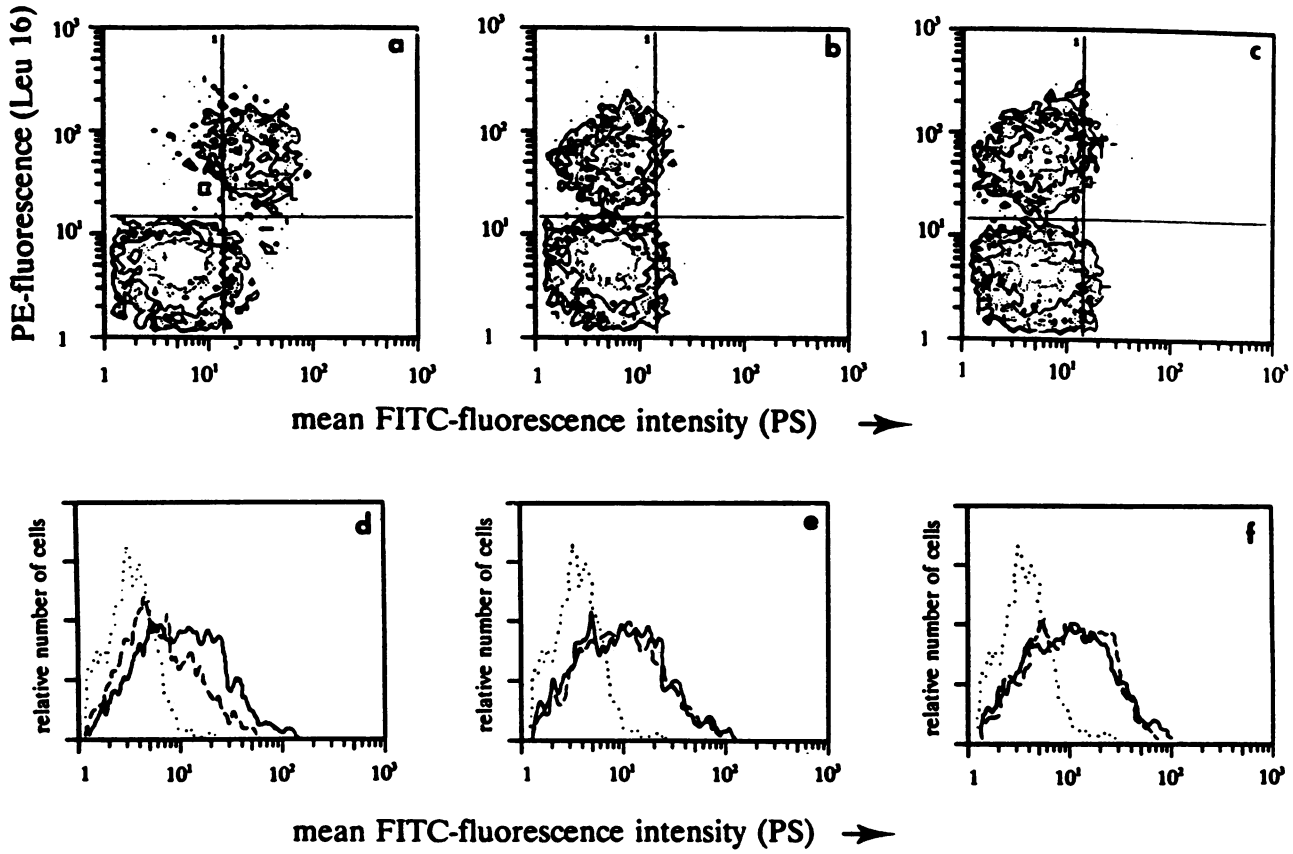

FIG. 5. C3d complexed to PS4 is bound by CR2 on B cells. Serum of patient 3 was incubated with 50 μg of PS4 per ml for 1 h at 37°C. Peripheral blood non-T cells were incubated in PS4-treated serum and subsequently with rabbit anti-PS4 followed by FITC-conjugated goat anti-rabbit Ig. Next, cells were stained with PE-conjugated Leu-16 (CD20) (a). Cells which were incubated in the first step with 50 μg of PS4 per ml or untreated serum only are shown in panels b and c, respectively. Data are presented in contour plot format with log FITC fluorescence on the *x* axis and log PE fluorescence on the *y* axis. Markers were set so that >95% of nonstained cells fell in the lower left quadrant. (d) Mean FITC fluorescence (PS) of $CD20^+$ B cells treated with PS4-containing serum (solid line), incubated with OKB7 prior to treatment with PS4-containing serum (dashed line), and treated with serum alone (dotted line). (e and f) Dashed lines represent CD35 and CD19 incubations, respectively, prior to treatment with PS4-containing serum. The solid and dotted lines are the same as for panel d.

(patients 1 and 2). In these sera, C3d can be generated only via the alternative pathway since there is a defective classical route (13). One could speculate that the relatively high IgM anti-PS levels in the sera might be responsible for the activation of the alternative route of complement. This is very unlikely because there is a considerable difference in these IgM titers between the two patients (see Results), while there is only a moderate difference in C3d formation upon activation with different PS (Fig. 1). On the basis of these data it cannot be excluded that complement activation via the alternative pathway is independent of PS-specific antibodies. However, data obtained from experiments performed with serum from a SCID patient indicated that C3d generation can take place independently of the presence of PS-specific antibodies.

It has been reported (29, 39) that complement consumption takes place after addition of different PS to mouse and guinea pig sera. Our experiments confirmed these studies using NHS. PS14 is a poor activator of complement in both guinea pig serum and human serum (39; Fig. 2). In contrast, PS3, which readily binds human C3d, is not able to activate guinea pig C3 (39). We have no adequate explanation for this difference other than differences in the methods used; in our laboratory, binding of, for example, PS1 to human C3d takes place at a PS concentration of 50 μg/ml while 9- to 18-fold higher concentrations are required before significant consumption of guinea pig C3 is observed (39).

Subsequent results indicate that PS are able to bind the C3 fragment C3d. This finding could be of major importance for activation of PS-specific B cells. PS coated with C3d could exert augmented immunogenicity compared with free PS antigens, rendering PS stimulatory at concentrations that normally would be subimmunogenic. Binding of C3d to PS was demonstrated in an ELISA using PEG-purified C3d preparations of two sera that differ from each other only in C3d concentration. The specificity of the anti-C3d reagents used is of critical importance in these studies. The rabbit anti-human C3d antibody used only recognizes determinants on C3d after the cleavage of C3 (14). Moreover, high-molecular-weight C3 fragments were removed by PEG precipitation prior to rocket electrophoresis. Treated serum samples revealed only a single rocket upon electrophoresis in anti-C3d containing gels (31a). The experiments for which results are shown in Fig. 2 and 3 showed binding of already generated C3d to PS. This is not the physiological mechanism by which C3d is reported to bind to biological substrates. This binding originates from esterification of the internal thiolester of metastable C3b and further degradation

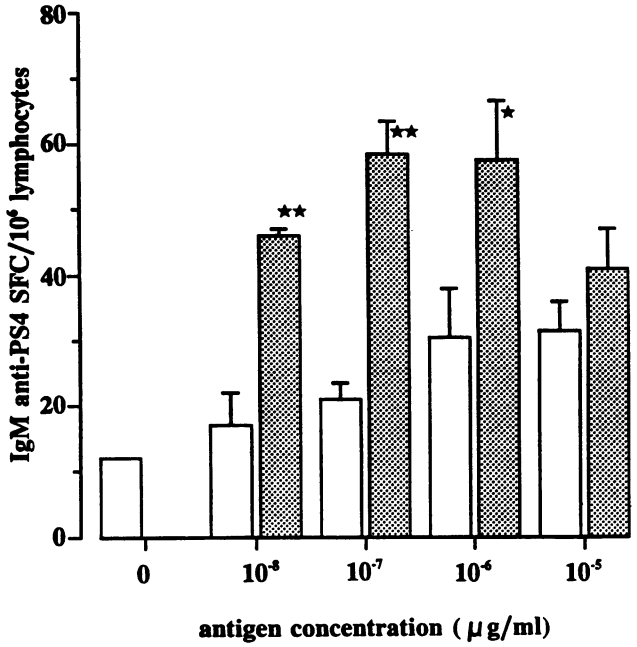

FIG. 6. In vitro B-cell response to PS4 after activation of B cells with either free PS4 (open bars) or PS4 incubated in serum of patient 3 (stippled bars). Addition of equivalent amounts of serum alone (1:5 × 10^7) did not result in induction of PS4-specific SFC. The number of IgM-secreting SFC generated in these cultures (70 × $10^3/10^6$ cultured B cells) was not affected by addition of PS4 in either the native or C3d-complexed form. Results (means ± standard errors of the means of IgM anti-PS4 SFC per 10^6 cultured B cells for duplicate cultures) of one representative experiment out of three independent experiments are shown. Bars representing serum-treated PS4 and identified with stars are significantly different (★, $P < 0.1$; ★★, $P < 0.02$) from bars representing equivalent amounts of free PS4.

of C3b (16, 19, 27). We do not know whether this physiological binding of C3d is required to mediate its biological functions. Therefore, the experiments for which results are shown in Fig. 4 were performed, which allowed physiological binding of C3d to PS. The results (Fig. 4) indicate that after treatment of serum with PS, C3d can be detected on the two PS tested (PS4 and PS14) by using the sandwich ELISA as described in Materials and Methods. It is known that CR1 acts as a cofactor for degradation of iC3b to C3d (34). However, the experiments described in this report were carried out with sera. Apparently, complexes can be formed without addition of CR1 (or CR1-expressing erythrocytes). It can be concluded that this binding is independent of antibodies, since similar results were obtained with agammaglobulinemic serum.

To investigate the physiological relevance of C3d binding to PS, experiments were set up to determine whether C3d complexed to PS can be recognized by CR2 on peripheral blood B cells. It turned out to be possible to detect binding of PS-C3d complexes to all $CD20^+$ B cells (Fig. 5a). This experiment was carried out with serum of patient 3 to exclude formation of immune complexes which are reported to bind C3d. The binding of PS-C3d complexes could be blocked by anti-CR2 monoclonal antibodies (Fig. 5d), indicating an interaction with the receptor for C3d, CR2. It was also demonstrated that complex binding could not be inhibited by prior incubation with anti-CR1 monoclonal antibody, indicating that complexes are not bound to B cells by interaction of C3b with its receptor. This observation is important since CR1 is also reported to mediate B-cell activation (6, 9, 10).

It has been reported that CR2 synergizes with sIgM in the activation of B cells (3). We have also demonstrated that cross-linking of CR2 on human B cells augments the calcium mobilization response to otherwise nonstimulatory doses of anti-IgM antibodies (11a). Therefore, it is tempting to suggest that fixation of PS-C3d complexes to the surface of B cells leads to a facilitated activation of PS-specific B cells. We realize that detection of PS-C3d complexes bound to the surface of B cells is possible only because in peripheral blood all B cells express the CR2 molecule.

To investigate the functional relevance of this phenomenon, the in vitro immunogenicity of PS4 complexed to C3d was studied. Optimal in vitro anti-PS4 antibody responses of blood B cells from normal healthy adults (32) were observed at lower concentrations with PS4-C3d than with untreated PS4. It was also shown that the absolute number of anti-PS4 antibody-secreting cells is higher under these conditions. These results suggest that the cross-linking of CR2 to sIgM is responsible for the increased immunogenicity of PS4 complexed to C3d.

The relevance of complement in the generation of an antibody response was already demonstrated in the classical studies of Pepys (28). He initially showed that C3 regulates the antibody response to T-cell-dependent antigens by demonstrating that cobra venom factor administration in vivo impairs the antibody responses to T-cell-dependent but not TI antigens. These results led to the hypothesis that C3 (or C3 fragments) is involved in T-B lymphocyte cooperation (29). The same investigator also showed that the antibody response to polyvinylpyrrolidone, a TI antigen, is not affected by cobra venom factor administration. There are, however, several observations which indicate that the antibody response to TI antigens is influenced by C3. These studies describe a partial abrogation of the anti-PS3 antibody response by a prolonged C3 depletion and an impaired response at low antigen concentrations only (30). Preliminary data indicate that naturally acquired IgG anti-PS antibodies are low in both C1q-deficient patients and C3-deficient patients (33).

Increased susceptibility of C3-deficient patients to infections by encapsulated bacteria therefore appears to be the net result of the central role of C3 both in the induction of an anti-PS antibody response and in opsonophagocytosis.

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