The Role of Properdin in Zymosan- and *Escherichia coli-*Induced Complement Activation

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Abstract

Properdin is well-known as enhancer of the alternative complement amplification loop when C3 is activated, whereas its role as recognition molecule of exogenous pathogen-associated molecular patterns and initiator of complement activation is less understood. We therefore studied the role of properdin in activation of complement in normal human serum (NHS) by zymosan and various *Escherichia coli* strains. In ELISA, microtiter plates coated with zymosan induced efficient complement activation with deposition of C4b and TCC on the solid phase. Virtually no deposition of C4b or TCC was observed with MBL deficient serum. Reconstitution with purified MBL showed distinct activation in both readouts. In ELISA, NHS-induced deposition of properdin by zymosan was abolished by the C3 inhibiting peptide compstatin. Flow cytometry was used to further explore whether properdin acts as initial recognition molecule reacting directly with zymosan and three *E. coli* strains. Experiments reported by other authors were made with EGTA Mg^{++} buffer permitting auto-activation of C3. We found inhibition by compstatin on these substrates indicating that properdin deposition depended on initial C3b deposition followed by properdin in a second step. Properdin released from human polymorphonuclear cells (PMN) stimulated with phorbol-myristate acetate did not bind to zymosan or *E. coli*, but when incubated in properdin-depleded serum this form of properdin bound efficiently to both substrates in a strictly C3-dependent manner, since the binding was abolished by compstatin. Collectively, these data indicate that properdin in serum as well as PMN-released properdin is unable to bind and initiate direct alternative pathway activation on these substrates.

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Introduction

Zymosan which is a carbohydrate complex derived from yeast cell walls, is an essential reagent in studies of complement activation. Pillemer and his collaborators first described properdin providing evidence that zymosan-properdin complexes induced antibody-independent complement activation (1,2). Reaction between properdin and various bacterial polysaccharides was also reported. The findings were controversial at that time, difficult to reproduce, and with Pillemer's death (3–5) properdin was largely dismissed by the scientific community. The properdin system was reborn as the alternative pathway (AP) more than 20 years later (2,4). Properdin is now denoted factor P (fP), and the current description of two different functions of fP (2,6–9) has been a valuable basis for further studies and development in this area.

In the long-standing model, AP is slowly auto-activated by hydrolysis of C3 with exposure of the internal thioester bond, leading to an initial AP convertase which cleaves C3, and C3b is subsequently covalently attached to nearby targets. Bound C3b then binds to fB, which is cleaved by fD. The Ba fragment is released into the fluid phase. Bb remains as part of the C3bBb complex, the potent AP C3 convertase, which splits more C3 resulting in self-amplification and generation of the C5 convertase C3bBbC3b. The C3bBb complex is relatively unstable with a half-life of 90 seconds under physiological conditions (10,11). fP associates with C3bBb, forming a more stable C3bBbP complex (12,13) with longer lasting enzyme activity essential for effective AP amplification. Notably, fP is the only known regulator of complement which enhances the activation, whereas all other regulatory proteins have inhibitory properties.

Evidence for the role of fP as a recognition molecule in AP started with experiments showing that purified fP bound to a biosensor surface could serve as a platform for *in situ* assembly of AP C3 convertases (9,14). Extensive experiments indicating that AP is initiated by non-covalent attachment of fP to a target surface (2) were, however, performed in EGTA Mg^{++} buffer in which the auto-activation ("tick-over") of C3 and the AP pathway amplification is intact. Thus, this system would permit continuous C3b deposition, making it difficult to demonstrate whether fP reacts as an initial recognition molecule of zymosan or subsequently binds after C3b deposition. Different reaction patterns of isolated fP, fP in serum, and fP released from activated, degranulated neutrophils are essential in studies of this matter as pointed out previously (15,16) and further described in our Results section.

In addition to mannose-binding lectin (MBL), ficolin-1 (M), ficolin-2 (L) and ficolin-3 (H) are recognition units in the lectin pathway (LP) (17–19). Like MBL, the ficolins react with N-acetylglucosamine, but the fine carbohydrate binding specificity differs from MBL and they do not react with mannan (19,20).

Compared with mannan with an extensive literature on reactivity and induction of LP activation, in particular initiated by MBL, less has been reported concerning the mechanisms involved in zymosan induced LP activation. An early study indicated interaction with proteins later identified as essential constituents of LP (21), while formal identification of LP activation by zymosan was first provided by Brouwer et al. (22).

Specific activation of the classical pathway (CP) by heat aggregated human IgG (HAIGG) has been extensively studied, while development of systems for specific activation of LP in close to physiologic conditions has encountered considerable difficulties. However, we have recently developed a system for specific activation of LP without involvement of the classical pathway (CP) and with no direct, initial AP activation, using mannan coating on the solid phase of ELISA plates and normal human serum (NHS) at high concentration (diluted 1:2) (20). Three main observations indicated LP specificity of this system: i)

Mannan on the solid phase induced activation of NHS but not of MBL deficient serum, showing that MBL is required for the activation. ii) After reconstitution of MBL deficient serum with purified MBL, activation was obtained, showing that MBL is responsible for activation. iii) Monoclonal anti-MBL antibody 3F8, documented to inhibit MBL function (23), abolished the activation of NHS, showing that activation depends exclusively on MBL.

We have reported that AP amplification contributed to more than 80 % of the final C5a generation after initial HAIGG induced activation highly specific for CP (24). In mannan induced LP activation AP amplification has been described by various authors (21,22). In the system with highly specific LP activation of human whole serum (20), we also found that AP amplification is crucial for the downstream effect of initial LP activation, being responsible for more than 80 % of TCC release into the fluid phase (25).

The purpose of the present study was to apply similar technology to characterize complement activation induced by zymosan and to explore whether fP in normal human serum as well as fP released from phorbol-myristate acetate (PMA) stimulated polymorphonuclear cells (PMN) act as initial recognition molecule reacting directly with zymosan or whole *E. coli*, thereby starting AP activation by itself. To obtain conditions with control of initial C3 activation, we studied the effect of the C3 inhibiting peptide compstatin (26–29) on fP deposition on zymosan and three different strains of "properdin-reactive" *E. coli* strains (2) by flow cytometry. The findings demonstrate that this peptide served to distinguish between initial direct fP-binding (30) and secondary fP-binding to C3b.

Materials and Methods

Monoclonal antibodies

Mouse anti-MBL mAbs 3F8 (IgG1κ) and 1C10 (IgG2b) were raised by immunization with purified human MBL, reacting with MBL with high affinity. 3F8 reacts with a conformation dependent epitope in the hinge within the carbohydrate recognition domain (CRD) of MBL and inhibits MBL-dependent C3 deposition on mannan-coated plates completely at 10 μg/ml NHS (23), while 1C10 reacts with another epitope being non-inhibiting (31). Mouse anti-human fD (clone 166-32, IgG1κ) (32) and its isotype-matched control mAb (clone G3-519, anti-HIV gp120, IgG1κ) were kindly provided by Michael Fung. Inhibiting mouse anti-human fP, clone A233 (IgG1κ) raised by immunization with purified human fP was obtained from Quidel Corporation, San Diego, CA. Clone IS7 (IgG1κ anti-human CD22) from Diatec Monoclonals AS (Oslo, Norway) was used as its isotype control. mAb aE11 reacting with a neoepitope exposed when C9 is incorporated in the terminal complement complex (TCC) (33) was obtained from Diatec Monoclonals. Clone 5A7 from Diatec Monoclonals was used as its IgG2a isotype control.

Normal human serum, plasma, and purified protein and cells

Normal human serum (NHS) was collected from nine healthy volunteers, all showing normal CP, LP and AP activity in the Wielisa total complement system screen assay (34). Prior to pooling, the sera were tested for LP activation after exposure to mannan on the solid phase (0.5 μg/well) using solid phase TCC deposition as readout. Activation was obtained and TCC deposition was inhibited completely by 3F8 mAb in all nine sera, excluding interference by CP activation due to complement fixing anti-mannan antibodies occurring in occasional sera (20,35). Subsequently the sera were pooled and stored as aliquots at –70°C. The MBL concentration of the pool was 740 μg/l. To approach physiological conditions in activation assays and to ensure a fully active AP NHS was used at a final dilution of 1:2, which was the lowest possible dilution to obtain a constant final concentration of serum after addition of buffer and mAbs. Normal human plasma (NHP) was prepared as follows: blood

from a healthy volunteer, drawn into an evacuated sterile NUNC plastic tube containing lepirudin (Refludan, Pharmion, Copenhagen, Denmark) to provide 50 µg lepirudin/ml blood. After mixing, the tube was centrifuged for 15 min at 1400 g at 4°C. The supernatant plasma was aliquoted and then frozen at −70°C for later flow cytometry analysis.

Factor P-Depleted Serum (A339 Lot # 8a) and purified factor P (A139 Lot # 21a) were obtained from Complement Technology Inc, Tyler, TX. Factor P was quantified by the Human Properdin ELISA Kit (Cat. Nr. HK334) from Hycult biotech, Uden, The Netherlands, according to the instructions of the manufacturer. Purified plasma-derived MBL was obtained from Statens Serum Institut, Copenhagen, Denmark (36).

Polymorphonuclear (PMN) cells were isolated by density gradient centrifugation on Polymorphprep (Axis-Shield, Medinor, Oslo, Norway) according to the instructions of the manufacturer, and the cells (a total of 1×10^8 cells in a buffer volume of 2.6 ml) were activated by Phorbol-myristate acetate (PMA) (Cat.no. 79346, Sigma-Aldrich, St. Louis, MO) at final concentration 500 ng/ml in BSA-coated tubes for 37°C for 30 min on a cell roller. The fP concentration in the supernatant after stimulation was quantified in fP ELISA and found to be 6 µg/ml.

Bacteria

E. coli strain D31m3, D31m4 and ED3869 were obtained from the *E. coli* Genetic Stock Center, Yale university, New Haven, CT and cultured on tryptose blood agar base medium (Oxoid, Basingstoke, UK) with 2% lactose and 0.5 % NaCl, subsequently subcultured in Heart infusion broth (Becton Dickinson, Sparks, MD) and harvested during exponential growth before flow cytometry experiments.

MBL deficient serum

MBL deficient (MBLd) serum was selected from an MBL *D/D* (codon 52 variant in the *MBL2* gene) homozygous 35 year old healthy male with no anti-mannan antibodies. The serum had normal CP and AP activity in the Wielisa assay (34) and standard hemolytic assays. It contains dysfunctional low molecular weight MBL which cannot bind ligands nor activate the MBL complement system efficiently under physiological conditions (37). Reconstituting the serum with recombinant wild type MBL has previously shown that the capability of MASP-1/3 and MASP-2 to interact with MBL is normal as well as the capacity to deposit C4 (38).

Zymosan induced activation of serum

Zymosan A from *Saccharomyces cerevisiae* (Cat.no. Z4250 Sigma-Aldrich, Steinheim, Germany) in a 100 µl volume was coated on Costar 3590 flat-bottomed polystyrene 96-well plates (Corning Inc, Corning, NY) in 50 mM Na-carbonate buffer pH 9.6 overnight at room temperature and the fluid was removed. After washing, the remaining binding sites in the wells were saturated with a blocking buffer, PBS pH 7.4 containing 1 % BSA (Cat.no. A8327, Sigma-Aldrich, St. Louis, MO) and 0.1 % Tween 20 (Cat.no. P1379, Sigma-Aldrich, St. Louis, MO), for 1 h at 37°C. After three times washing with PBS containing 0.1 % Tween 20, 50 µl NHS diluted 1:2 in veronal buffer pH 7.5 containing 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05 % Tween 20 and 0.1 % gelatin (GVB++ buffer) was added to each well for complement activation for 30 min at 37°C. To stop LP activation and to ensure complete inhibition of continuing background AP amplification the microtiter plate was incubated on ice and 10 µl EDTA was added immediately to each well to a final concentration of 20 mM before assay for activation products on the solid phase and in the supernatant (fluid phase). Coating with mannan on the solid phase for activation was done in the same way, as reported previously (20).

Readouts for activation products on the solid phase

Deposition of MBL on the solid phase following activation was demonstrated in ELISA using polyclonal rabbit anti-human MBL2 antibody 1:50 (Atlas Antibodies AB, Stockholm, Sweden) obtained by immunization with PrEST technology (39,40) followed by HRP conjugated donkey anti-rabbit Ig 1:1,000 (Amersham Biosciences, Little Chalfont, UK) with ABTS as substrate. The recombinant peptide used for immunization covered amino acids 102–240 of the MBL sequence (www.atlasantibodies.com), and antigenicity plot analysis (41) indicated a series of antibody-reactive epitopes in this area.

Deposition of C4b and C3b on the solid phase was demonstrated with polyclonal rabbit anti-human C4 (OSAO 194 1:10,000) and anti-human C3 (OSAP 192 1:40,000) antibodies (Dade Behring, Marburg, Germany), HRP conjugated donkey anti-rabbit Ig 1:2,000 and ABTS as substrate.

Deposition of fP on the solid phase in ELISA was with monoclonal anti-fP (Quidel A233 1:1000) with HRP conjugated F(ab′)$_2$ anti-mouse IgG (GE Healthcare UK Ltd, Little Chalfont, UK, 1:1000) with ABTS as substrate. In inhibition experiments with mAbs, deposition of fP was demonstrated with polyclonal goat anti-human fP (A239 45 mg/ml 1:1,000) (Complement Technology, Tyler, TX), HRP conjugated mouse anti-goat Ig 1:1,000 (Jackson ImmunoResearch Europe Ltd, Newmarket, Suffolk, UK) with ABTS as substrate.

Deposition of the terminal complement complex (TCC) was based on reaction with purified mAb aE11 (42)(1 mg/ml 1:6,000 in PBS with 0.2 % Tween 20) followed by biotin-conjugated rat anti-mouse IgG2a mAb 1:1,000 (BD Biosciences Pharmingen, Erembodegem, Belgium) and HRP conjugated streptavidin 1:2,000 (Amersham) with ABTS as substrate, using OD values at 405 nm to represent the relative amount of deposited TCC in the wells.

Readout for TCC in the fluid phase

TCC in the fluid phase was measured by a double-antibody assay described in detail previously (33,43). Briefly, mAb aE11 was used as catching antibody, biotinylated anti-C6 mAb 9C4 (43) as detection antibody, HRP-labeled streptavidin (Amersham), and ABTS as substrate.

Inhibition assays

mAbs were added to serum followed by incubation for 5 min at room temperature prior to activation and assay. Optimal concentration was determined in pilot experiments, control antibody being used in the same amount. The amount of purified mAb/well is recorded as transformed to amount/ml of undiluted serum, anti-MBL 3F8 at 20 µg/ml, anti-MBL 1C10 at 20 µg/ml, anti-fD 166-32 at 10 µg/ml, and anti-fP A233 at 20 µg/ml.

The compstatin analogue (Ac-I[CV(1MeW)QDWGAHRC]T) with increased C3 inhibiting activity produced as previously described (27,29) was used at 50 µM.

Flow cytometry

A FACSCalibur instrument, (Becton Dickinson, Franklin Lakes, NJ), was used for flow cytometry with FlowJo analysis software version 7.6.4 (Tree Star Inc., Ashland, OR). Zymosan A (Sigma Z-4250, Steinheim, Germany) was suspended in PBS with 1 % BSA. Blocking was with 1 % BSA in PBS pH 7.4 with 1 % Tween 20 for 1 h at 37°C. Subsequent washing at this and all later steps was in PBS with 0.1 % BSA. The zymosan was resuspended in GVB++ buffer. Incubation with NHS 1:2 was at 37°C for 20 min in GVB++ buffer after 5 min preincubation with compstatin at a final concentration of 50µM. To

demonstrate binding of fP, the zymosan was incubated with monoclonal anti-fP (Cat. No. A233 Quidel) diluted 1:50 for 15 min at room temperature. The samples were washed twice, and further incubated with anti-mouse IgG (Fc specific) FITC conjugated F(ab′)$_2$ (Sigma F2772, Steinheim) diluted 1:50 for 15 min at room temp in the dark. The samples were washed twice, resuspended in PBS with 1 % BSA, and put on ice until analysis in the flow cytometer. Gating was based on forward- and side scatter and 20,000 events acquired. For analysis of *E. coli*, the bacteria were washed and suspended in PBS with 0.1 % BSA. Blocking was with 1 % BSA in PBS without Tween for 1 h at 20°C. Subsequent washing at this and all later steps was in buffer without Tween to avoid interaction between Tween and lipids in the bacterial surface layer. Incubation with NHS 1:2 was in GVB++ buffer at 37°C for 20 min after 5 min preincubation with compstatin at final concentration 50μM. For analysis of TCC binding to *E. coli* the procedures were adjusted to obtain optimal conditions based on the experiments in ELISA. Gating was based on forward- and side scatter and 20,000 events were acquired.

Statistics

GraphPad Prism version 5 (GraphPad Software, San Diego, CA) was used for statistical analyses. Data were examined with analysis of variance (ANOVA), by one way ANOVA followed by the Bonferroni post-test, ANOVA with repeated measurements followed by Dunnett's multiple comparison test, and the student t-test as appropriate for the different experiments. A *p*-value of 0.05 was considered statistically significant.

Ethics

The study was approved by the Norwegian Government Regional Committee for Medical Research Ethics.

Results

C4b and TCC deposition on zymosan and mannan by NHS and MBL deficient serum

NHS induced efficient deposition of C4b and TCC on the solid phase in microtiter wells coated with zymosan or with mannan for comparison (Fig. 1A and B), consistent with activation of the whole cascade on both coatings. By contrast, virtually no deposition of C4b or TCC was seen with the MBL deficient (MBLd) serum, with a similar result obtained for zymosan and mannan. Reconstitution with purified MBL showed distinct activation with OD values in both readouts comparable to the values for NHS, after addition of 10 μg MBL/ml MBLd serum. C4b-values with MBLd was significantly lower than with NHS and MBLd +MBL (p = 0.0013 and 0.004, respectively) (Fig. 1A). TCC-values with MBLd was significantly lower than with NHS and MBLd+MBL (p = 0.004 and 0.0016, respectively) (Fig 1.B). In all sets the difference of NHS vs MBLd+MBL was not significant. Similar observations were made regarding deposition of C3b on the solid phase and release of TCC into the fluid phase (data not shown).

A dose-dependent deposition of C4b on the solid phase was observed after activation of NHS by different amounts of zymosan on the solid phase (Fig. 2A). Addition of the inhibiting anti-MBL mAb 3F8 abolished C4b deposition at all zymosan concentrations, showing OD values similar to the negative buffer control, indicating that no direct, MBL-independent activation leading to C4b deposition occurred in this system. Interference by ficolin 1, 2 and 3 is not expected from recent information on their recognition specificity (19,44–46). Addition of the non-inhibiting mAb 1C10 gave OD values similar to those obtained with NHS alone. The difference between NHS and NHS + 3F8 was statistically significant (p < 0.05) while the difference between NHS and NHS + 1C10 was not significant.

Deposition of MBL on the solid phase after zymosan induced activation was also completely inhibited with anti-MBL mAb 3F8 (Fig. 2B). The findings concerning statistics were the same as for Fig. 2A. Control experiments with mannan induced activation (0.5 μg/w) showed the same reaction pattern (in black).

C3b deposition and TCC formation after zymosan induced activation and inhibition with anti-MBL

The dose-dependent NHS mediated C3b deposition on zymosan (Fig. 3A, open circles) was substantially, but not completely, inhibited by anti-MBL mAb 3F8 (Fig. 3A, open squares), similar to the results observed with MBLd serum (Fig. 3A, closed diamonds), whereas the control anti-MBL mAb 1C10 values (Fig. 3A, open triangles) were similar to NHS. There was a highly significant difference between the curves of Fig. 3A ($p < 0.0001$). Thus, the difference between NHS and NHS+3F8, and between NHS and MBLd were both significant ($p < 0.05$), whereas the difference between NHS and NHS+1C10 was not significant. Notably, in a control with mannan on the coat, known to activate only LP in this system (20), the anti-MBL mAb 3F8 completely inhibited C3b deposition (Fig. 3A, closed square), indicating some MBL-independent activation of AP, as judged by C3b deposition, by zymosan but not by mannan.

Data on deposition of TCC on the solid phase (Fig. 3B) showed similarity to deposition of C3b, but with less inhibition by anti-MBL mAb 3F8 on the zymosan coat, whereas the inhibition with anti-MBL mAb 3F8 completely inhibited the activation on mannan, again indicating additional, MBL-independent activation of AP by zymosan. There was a higly significant difference between the curves of Fig. 3B ($p = 0.0035$), MBLd being significantly different from NHS ($p < 0.05$). Release of TCC into the fluid phase is showed inhibition by MBLd only ($p<0.05$) (Fig. 3C).

Effect of various complement inhibitors on factor P deposition on solid-phase zymosan by NHS

NHS induced a dose-dependent fP deposition on zymosan (Fig 4A, open circles), which was identical to that seen with the non-inhibitory anti-MBL mAb 1C10 (Fig 4A, open triangles), whereas anti-MBL mAb 3F8 inhibited the deposition incompletely (Fig 4A, open squares), similar to what was obtained with anti-fD (open diamonds). By contrast, anti-fP A233 completely abrogated fP deposition (Fig. 4A, inverted open triangles). There was a highly significant difference between the curves of Fig. 4A ($p < 0.0001$), reflected by significances between NHS and NHS+3F8 and between NHS and MBLd serum, and between NHS+anti-fD and NHS+anti-fP. Notably, in the control with mannan induced activation specifically activating LP in this system (Fig. 4A, black symbols), both anti-MBL mAb 3F8 (black square) and anti-fP (black inverted triangle) completely inhibited deposition of fP.

In ELISA, deposition of fP on solid-phase zymosan by NHS was completely inhibited by the C3 inhibiting peptide compstatin ($p=0.013$) (Fig. 4B) in marked contrast to the fully retained deposition of C4b generated earlier in the complement activation cascade ($p=0.95$) (Fig. 4C).

In separate ELISA experiments, deposition of C3b on solid phase zymosan by NHS was inhibited significantly ($p<0.05$) by anti-MBL mAb 3F8, but not by the control anti-MBL mAb 1C10 nor by the inhibiting anti-fP mAb A233 or its isotype control (Suppl. Fig. 1). These findings correspond to observations in an earlier study (6) indicating that fP is not essential for zymosan-induced AP complement activation.

Factor P deposition on zymosan by plasma and factor P depleted serum

To supplement the ELISA experiments on fP deposition on the solid phase after zymosan induced activation of NHS (Fig. 4A and B), similar experiments were made with fP depleted (fPd) serum. With NHS high OD values for fP deposition were obtained and low values with fPd serum, increasing again after reconstitution with fP (Fig. 5A). The OD value of fPd serum was statistically different (p < 0.05) from NHS and from fP reconstituted fPd serum (n=6).

To study the reactivity of zymosan in as close to physiological conditions as possible, interaction with lepirudin plasma (NHP) was studied in flow cytometry using deposition of fP and TCC as readouts. The recombinant hirudin analogue lepirudin is a highly specific thrombin inhibitor and a suitable anticoagulant without adverse effects on complement in studies of the inflammatory response in whole blood and plasma (47–49). Deposition of fP on zymosan was substantial with mean fluorescence intensity (MFI) of 42 after activation of NHP (Fig. 5B, dark red curve). Compstatin inhibited the signal to MFI values identical to the isotype control (Fig. 5B, light red curve). The inhibition of fP deposition by compstatin was statistically significant (p<0.0001) (n=6). fP deposition by fPd was identical to the isotype control and increased after reconstitution with fP (Figure 5, panel C). The individual MFI data are given in Supplementary Table I.

Deposition of TCC on zymosan after interaction with NHP was substantial with MFI 336 (Suppl. Fig 2, dark green curve). Compstatin inhibited this binding to MFI 6.5 (Suppl. Fig. 2, light green curve). The corresponding MFI values of the isotype control was 3.9 (Suppl. Fig. 2, black stipled line), confirming C3 dependence of TCC deposition. Similar experiments with NHS gave results corresponding fully to those obtained with NHP.

Factor P deposition on E. coli by flow cytometry

Incubation of *E. coli* strain D31m4 with NHS induced deposition of fP on the bacteria (Fig. 6A, dark red curve), which was blocked by compstatin (light red curve) giving MFI values similar to the isotype control (Supplementary Table I). The inhibition of fP deposition by compstatin was statistically significant (p<0.0001) (n=6). Incubation of *E. coli* strain D31m4 with fPd serum gave an MFI value similar to the isotype control and increased after reconstitution with fP (Fig. 6B and Supplementary Table I). Similar results were found in two other *E. coli* strains (D31m3 and ED3869).

Deposition of factor P released from human blood PMN cells on zymosan and E. coli

Deposition of fP released from PMA activated PMN cells (PMNfP) on zymosan was studied in ELISA (Fig. 7A). No deposition of PMNfP alone was found, since the OD values were identical to the isotype control antibody. By contrast, MPNfP from this supernatant showed significant (p<0.0001) deposition when incubated in fPd serum. In turn, addition of compstatin inhibited this PMNfP deposition completely (Fig. 7A, Supplementary Table I), analogous to what was repeatedly observed in experiments with normal serum. Identical observations were made in flow cytometry regarding deposition of PMNfP on *E. coli* (Fig. 7B, C).

Discussion

In the present study we found that inhibition of fP binding in NHS and lepirudin plasma to zymosan and to *E. coli* by compstatin served to distinguish between primary binding of fP, consistent with fP as a recognition molecule, and secondary binding to C3b initially formed in the tick-over reaction, consistent with fP as an amplification molecule. In our experiments we consistently found that fP both from human serum and released from activated PMN

cells did not bind directly to zymosan or *E. coli*, but that binding of fP was dependent on initial C3b binding.

The amplification function of fP is well established, whereas a direct activation of AP by fP recognition is a current topic of investigation. The amplification function is associated with the unstable C3bBb complex in AP (10,11) interacting with fP to form a more stable C3bBbP complex (12) with longer lasting enzyme activity essential for effective AP amplification. The dependency of CP and LP on AP amplification is similar. Thus, in our earlier study, anti-fD mAb inhibited more than 80 % of C5a and TCC release into the fluid phase after HAIGG induced activation of CP (24) similar to data later found for LP (25) and corresponding to recent detailed structural studies showing analogous interactions in the initiating complexes of CP and LP (50).

Evidence for the role of fP as a recognition molecule in AP started with experiments showing that purified fP bound to a biosensor surface could serve as a platform for *in situ* assembly of AP C3 convertases (9,14). Extensive further experiments indicating that AP is initiated by non-covalent attachment of fP to a target surface (2) were performed in EGTA Mg^{++} buffer. In this system, however, the auto-activation ("tick-over") of C3 and the AP pathway amplification is intact (51–54) and would permit slow continuous C3b deposition, making it difficult to demonstrate whether fP reacts as an initial pattern recognition molecule or binds secondarily after initial C3b deposition on a reactive surface.

The authors of the studies describing properdin as recognition molecule (2,14) noted that purified properdin may react differently from properdin in serum due to the "tick-over" reaction. Properdin released from activated, degranulated neutrophils may also react differently from properdin in serum (15). Our data clearly indicate that plasma and serum fP and fP released from PMN cells behave similarly with respect to binding to zymosan and *E. coli*. Notably, PMN-released fP did not bind unless incubated in fPd serum. Then efficient binding took place, but was completely abolished by compstatin, exactly as seem for serum fP.

In contrast to our findings with exogenous ligands (zymosan and *E. coli*), compelling evidence for C3 independent direct fP binding to endogenous ligands, i.e. late apoptotic or necrotic cells (55), was based on a set of different, independent criteria: binding was demonstrated after incubation of the cells with purified human fP in serum-free RPMI culture medium, purified fP bound to necrotic splenocytes of C3 knockout ($C3^{-/-}$) mice like to cells from wild type mice, and purified fP bound to necrotic cells in C3-deficient serum in EDTA containing medium, like binding in NHS. The ligand involved in fP binding was studied by confocal microscopy confirming that fP was co localized exclusively with fragmented DNA exposed on late apoptotic cells. This is direct evidence for fP reactivity as recognition molecule of endogenous ligands under certain conditions.

Purified fP often forms a series of oligomers of a ~53,000 *Mr* subunit, assembling into dimers (P_2), trimers (P_3), tetramers (P_4) and higher forms (P_n) which can be separated by cation exchange chromatography and gel filtration(16). In this study, unseparated purified fP and P_n was found to bind to live Raji and Jurkat cells while physiological forms of fP (P_2, P_3, and P_4) only bound to necrotic and Jurkat cells. The live and necrotic cells were incubated with different forms of fP in Hank's buffered saline solution for 1 h at 37°C, washed, and analyzed by FACS with an anti-fP mAb or an IgG1 mAb isotype control, followed by an FITC-conjugated anti-mouse IgG antibody. This procedure clearly indicates C3-independence of the physiological forms of fP binding to necrotic Raji and Jurkat cells.

To explore whether fP bound initially as a recognition molecule with initial activating function or participated as amplifier of AP after initial C3b deposition, we performed

inhibition experiments with the C3 reactive peptide compstatin. Compstatin was the first non-host-derived complement inhibitor shown to block the CP, LP and AP activation pathways (26). This 13-residue cyclic peptide was discovered by a phage-display, random peptide library search. Analogues with markedly increased potency (V4W, H9A) were later identified (27). These compounds bind C3, $C3(H_2O)$, C3b, and C3c with high specificity, inhibiting complement activation (56). The crystal structure of compstatin in complex with C3c has been presented (28). The compstatin binding site is formed by the macroglobulin (MG) domains 4 and 5. A model was proposed in which compstatin sterically hinders the access of substrate C3 to the convertase complexes thus blocking complement activation and amplification (28). In the present experiments, compstatin completely inhibited fP binding to zymosan in ELISA, while deposition of C4b, occurring earlier in the cascade, was not inhibited. By contrast, C4b deposition was increased in the presence of compstatin, most likely due to increased availability of acceptor sites for covalent binding of C4b in the absence of C3b.

Inhibition by anti-fD was incomplete, while anti-fP inhibited fP deposition completely at all zymosan concentrations tested (Fig. 4A). To explain the latter distinction, an interaction of fP with C3b independent of fD would need to occur. In Surface Plasmon Resonance (SPR) spectroscopy, fP has been shown to bind strongly to C3b and C3bB without further action of fD (14) explaining the finding of complete inhibition by anti-fP.

The importance of fP reactivity *in vivo* has been studied in murine autoimmune models by using gene-targeted fP deficient mice in studies of zymosan-induced arthritis (57). Further, fP deficiency rescued mice from AP complement-mediated embryonic lethality cased by deficiency of the membrane complement regulator *Crry* and markedly reduced disease severity in the K/BxN model of arthritis (58). In the present *in vitro* studies fP reactivity with the exogenous antigens zymosan and *E. coli* C3b dependence was demonstrated, while C3b independence and direct fP activity as recognition molecule was seen with selected endogenous antigens, such as necrotic and late apoptotic cells (16,55), whereas the paper by Ferreira et al.(16) showed that fP bound to zymosan as well.

The reaction pattern of purified fP and fP in serum has revealed some striking differences, e.g. in reaction with apoptotic T-cells (15). Purified fP bound to apoptotic T-cells while similar binding of fP in serum to the apoptotic cells was C3 dependent. Additional experiments indicated that fP freshly released from activated, degranulating neutrophils bound to apoptotic T-cells while endogenous fP in serum is strictly regulated. These observations appear directly related to complement involvement in neutrophil-mediated diseases (59).

In our experiments we included both serum and lepirudin plasma, the latter to get as close to physiological conditions as possible. All results were comparable. Thus we conclude that binding of fP in human serum and plasma, as well as fP released from PMN cells (59,60), to zymosan and *E. coli* was C3-dependent, indicating an initial binding of C3b followed by secondary binding of fP. Similar observations were made in another just published study showing that fP in serum bound to glycan particles and zymosan "only in the presence of active C3" (61). In this study the authors showed binding of fP in flow cytometry after incubation of glycan particles and zymosan in NHS in EGTA Mg^{++} buffer. Binding of fP was not obtained in C3-depleted serum, but in the C3-depleted serum reconstituted with purified C3. Another recent study showed that the tissue damage in an elastase-induced mouse model of abdominal aortic aneurysm depended on AP activation, being C3-dependent (62). Direct binding of purified fP as recognition molecule has been demonstrated in other circumstances (16,55). Our study indicates that inhibition experiments with

compstatin are valuable to identify additional settings in which fP reacts as recognition molecule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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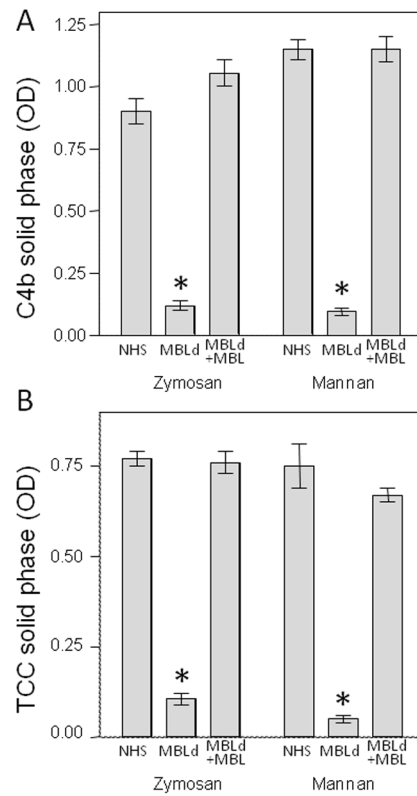
FIGURE 1.
Reaction pattern of normal human serum (NHS) compared with MBL deficient (MBLd) serum after activation with zymosan on the solid phase, 0.5 µg/well. Reconstitution with 10 µg purified MBL/ml MBLd serum showed distinct activation with increasing OD values for deposition of C4b and TCC on the solid phase. Data are presented as mean values and range from two independent experiments. At the right mannan coating on the solid phase (0.5 µg/ w) for comparison. Typical finding in four experiments. * = p < 0.05.
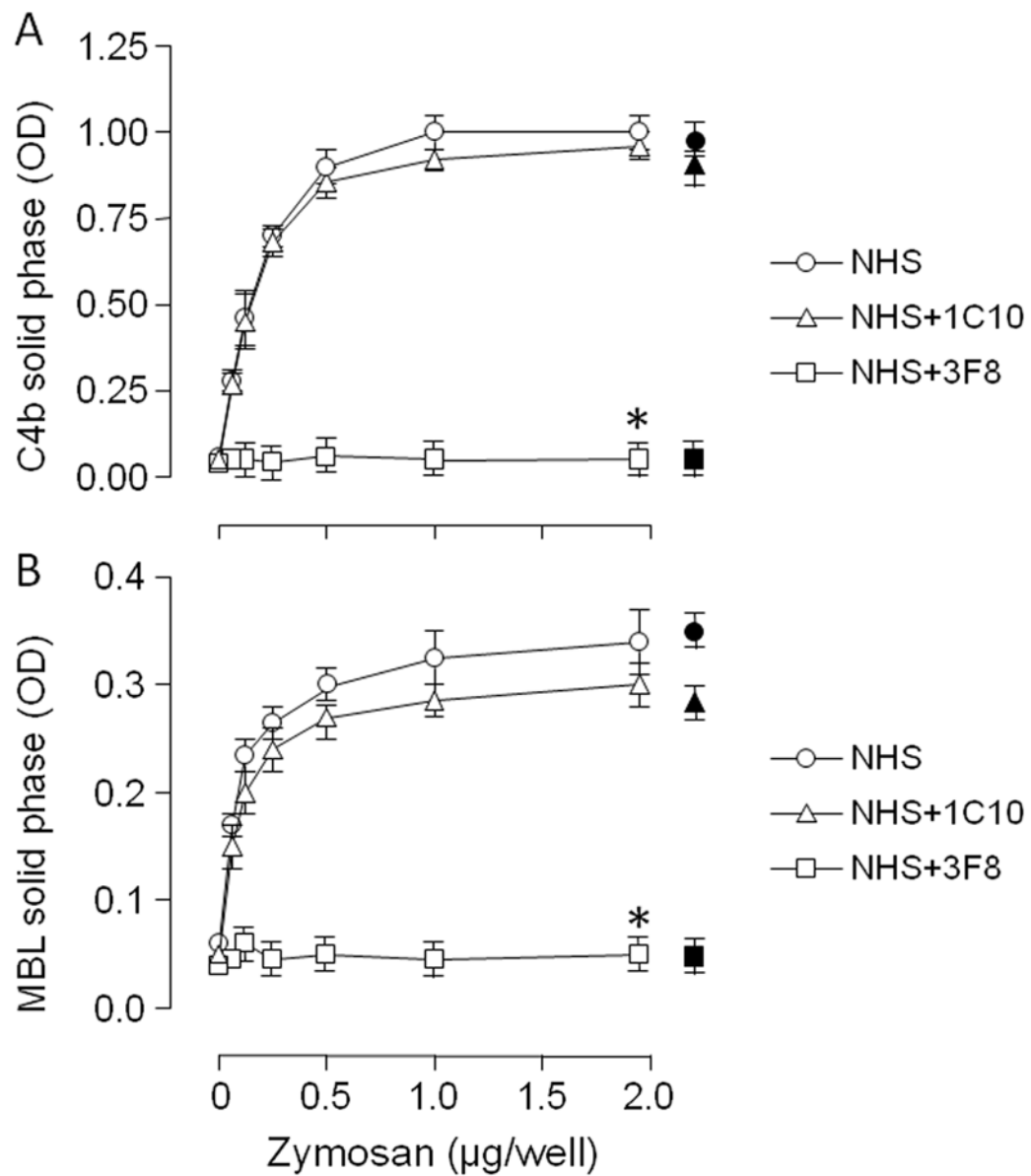
FIGURE 2.
Deposition of C4b (A) and MBL (B) on the solid phase after activation of NHS by different amounts of zymosan on the solid phase. 3F8 and 1C10 = anti-MBL inhibiting and non-inhibiting monoclonal antibodies respectively. At the right, in black, mannan coating (0.5 μg/w) for comparison. Typical finding in four experiments. * = $p < 0.05$.

FIGURE 3.
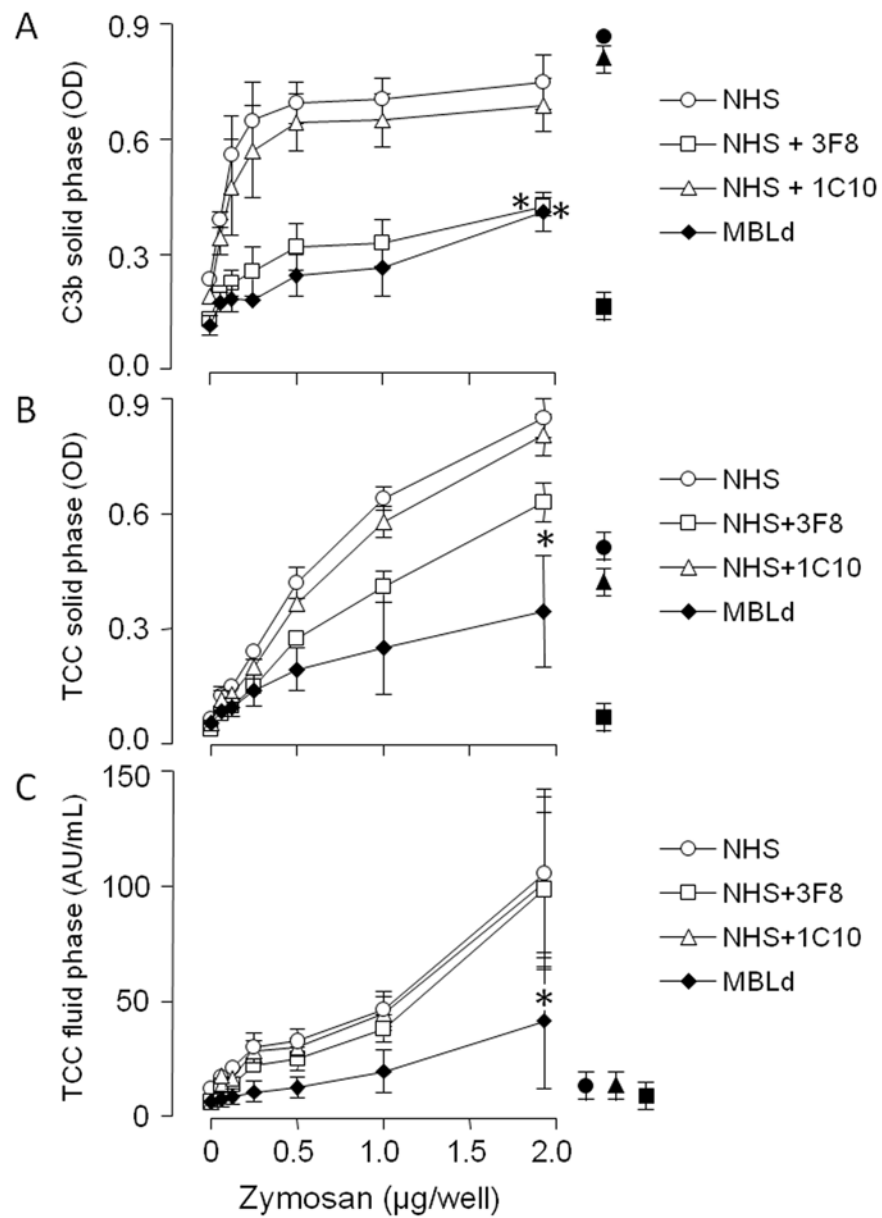Deposition of C3b (A) and TCC (B) on the solid phase and release of TCC into the fluid phase (C) after activation of NHS by different amounts of zymosan on the solid phase. Description and data presentation as in Fig. 2. Typical finding in four experiments. * = p < 0.05.
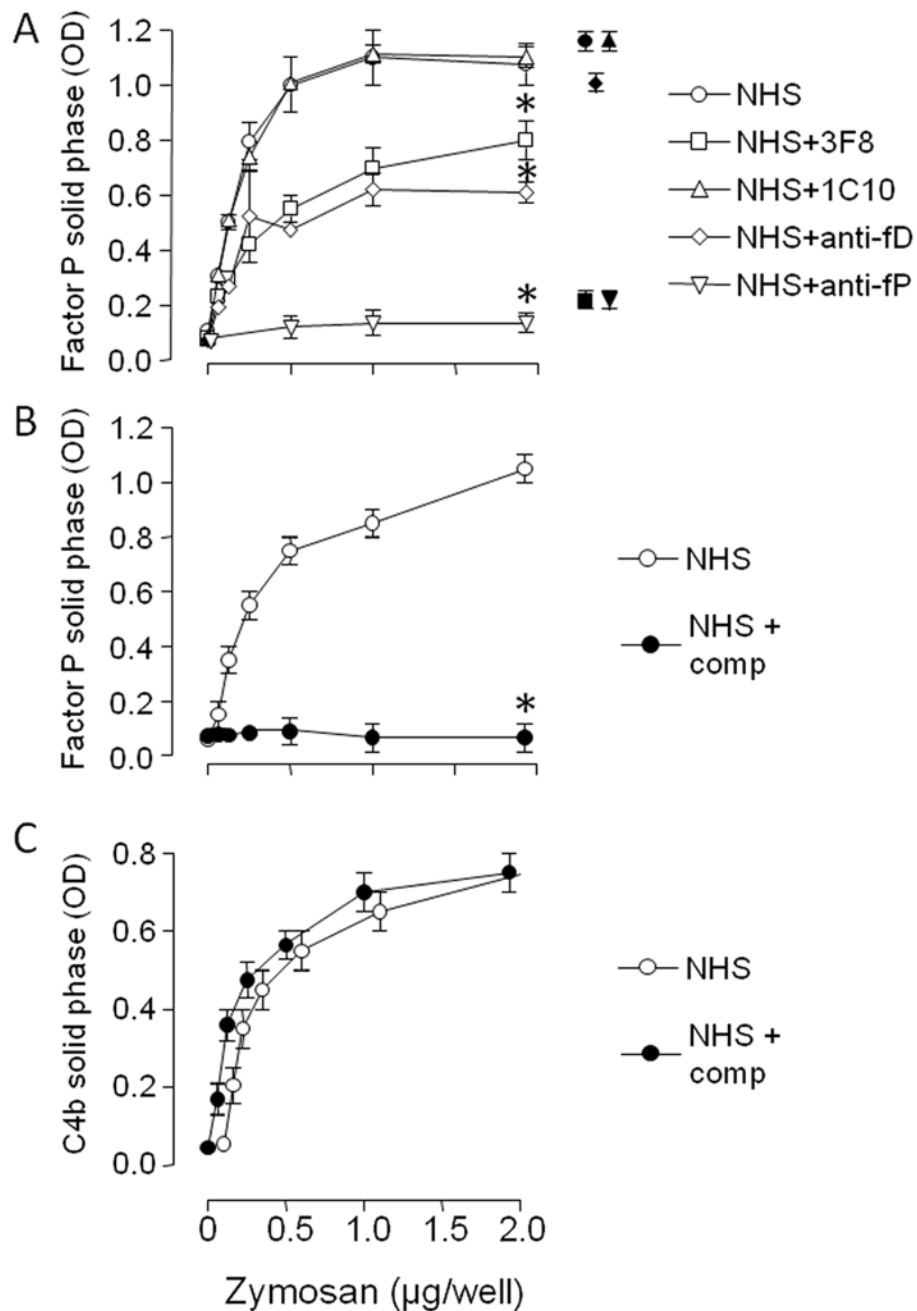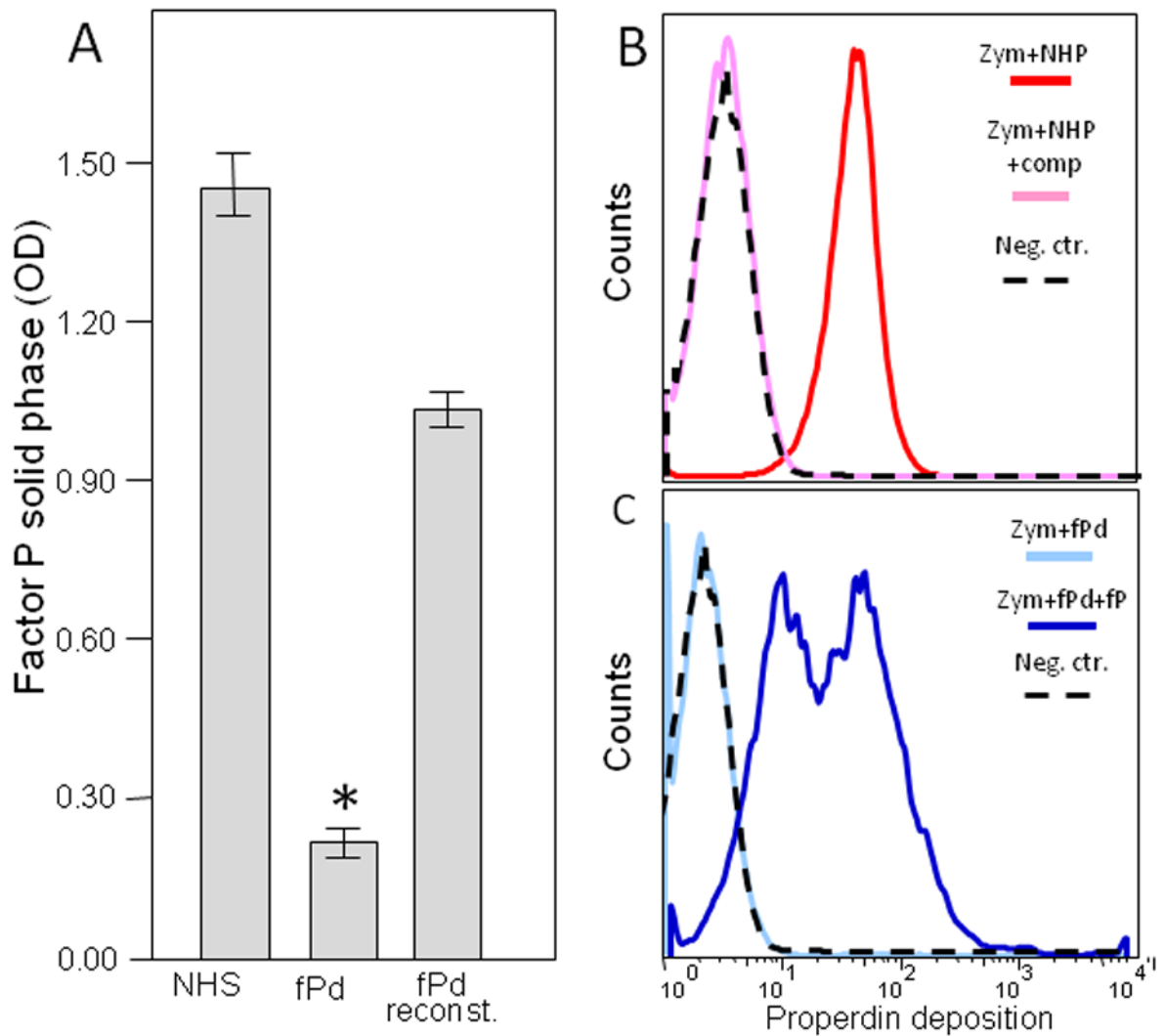
FIGURE 4.
Inhibition of deposition of fP on the solid phase by anti-MBL, anti-fD (a-fD) and anti-fP (a-fP) monoclonal antibodies after activation of NHS by different amounts of zymosan on the solid phase (A). Description and data presentation as in Fig. 2. Effect of adding compstatin to serum (50 μM/ml) on deposition of fP (B) and C4b (C) on the solid phase after activation of NHS by different amounts of zymosan on the solid phase. Typical finding in four experiments. * = p < 0.05.

FIGURE 5.
Deposition of fP on the solid phase in ELISA after incubation of zymosan with NHS compared with fPd serum and fPd serum reconstituted with purified fP (A). Flow cytometry showing deposition of fP on zymosan after activation of NHP (dark red curve) and inhibition by compstatin (light red curve) with isotype control in the black stippled curve (B). Similar experiment to compare zymosan reactivity with fPd serum and fPd serum reconstituted with purified fP (C). $*$ = $p < 0.05$.
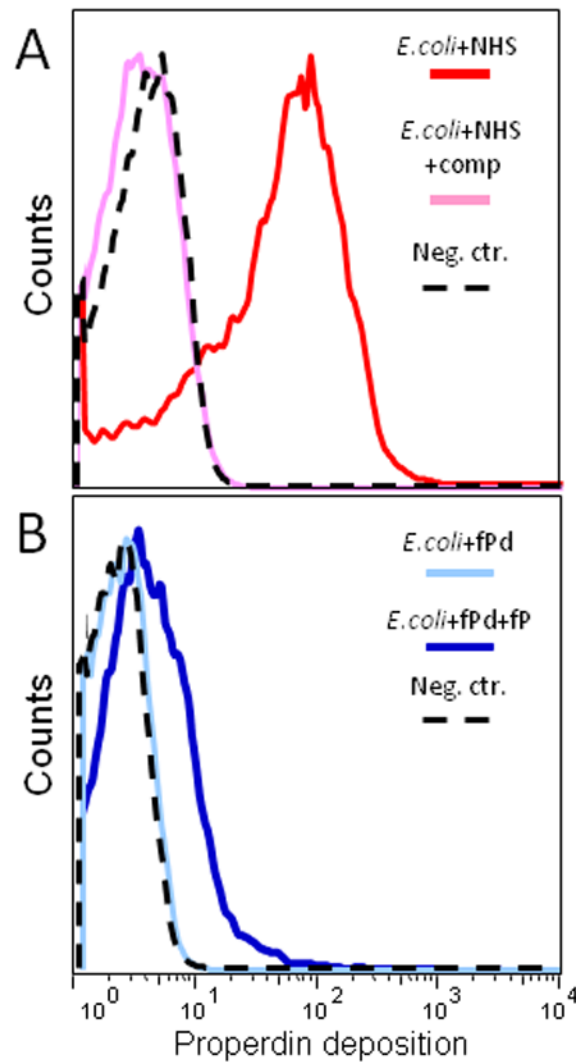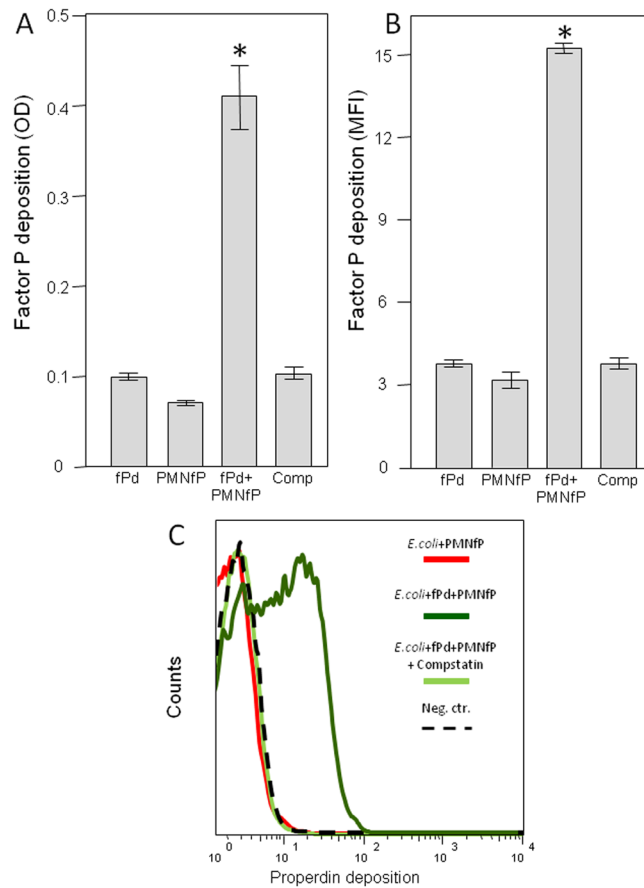
FIGURE 6.
Flow cytometry to show deposition of fP in normal human serum 1:2 on *E. coli* (dark red curve) and effect of compstatin (light red curve). Deposition of fP was detected by reaction with anti-fP mAb and FITC-conjugated anti-mouse IgG. Control replacing anti-fP mAb with its isotype control (stippled black curve) (A). The same layout with fPd serum in light blue and reconstitution with fP in dark blue (B). Similar findings in six experiments on three *E. coli* strains.

FIGURE 7.
Deposition of PMN released fP (PMNfP) on the solid phase in ELISA after incubation of zymosan with fPd serum, PMNfP, PMNfP incubated in fPd serum, and the latter inhibited with compstatin (Comp) (A). Flow cytometry showing PMNfP deposition on *E. coli* bacteria with same experimental design as described in A (B). Histogram from one representative experiment described in B, showing PMNfP (red curve), PMNfP incubated in fPd serum (dark green curve), and the latter inhibited with compstatin (light green curve). The isotype control (black stippled curve) (C). * = $p < 0.05$.