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Properdin Plays a Protective Role in Polymicrobial Septic Peritonitis

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Properdin is a positive regulator of complement activation so far known to be instrumental in the survival of infections with certain serotypes of Neisseria meningitidis. We have generated a fully backcrossed properdin-deficient mouse line by conventional genome-specific targeting. In vitro, properdin-deficient serum is impaired in alternative pathway-dependent generation of complement fragment C3b when activated by Escherichia coli DH5a. Properdin-deficient mice and wild-type littermates compare in their levels of C3 and IgM. In an in vivo model of polymicrobial septic peritonitis induced by sublethal cecal ligation and puncture, properdin-deficient mice appear immunocompromised, because they are significantly impaired in their survival compared with wild-type littermates. We further show that properdin localizes to mast cells and that properdin has the ability to directly associate with E. coli DH5a. We conclude that properdin plays a significant role in the outcome of polymicrobial sepsis. The Journal of Immunology, 2008, 180: 3313–3318.

Cecal ligation and puncture (CLP) is a model for acute polymicrobial septic peritonitis following perforated appendicitis or diverticulitis. The severity of this model for the animal varies with length of the ligated cecum, needle size, and number of perforations (1). Complement activation, mast cells, and neutrophils are some of the outcome-determining factors of the host. Blockade of C5a or its receptor C5aR is beneficial for survival of the acute model of CLP in mice (2, 3) by suppression of C5a-mediated functions of peritoneal neutrophils (migration, oxidative burst, phagocytic response) (4). In the subacute model of CLP, however, neutrophil recruitment to the peritoneal cavity (5) and IL-12-mediated, IFN-γ-driven neutrophil phagocytosis (6) are important for survival. During acute CLP, neutrophil migration is effected by TNF, which is produced locally by peritoneal mast cells, which, thereby, are significant players in disease outcome (7). Mast cell degranulation, TNF release, and neutrophil infiltration are significantly impaired in C3-deficient mice during acute CLP, accounting for their high mortality compared with wild-type (WT) mice (8). Properdin is an oligomeric serum protein that amplifies ongoing complement activation. By virtue of each of its monomers to bind to C3b, it acts to extend the half-lives of the C3-converting and C5-converting enzyme complexes (C3bBb and C3b,Bb), respectively (9–11). These are generated after activation of the alternative pathway of complement via deposition of C3b to target surfaces by so-called spontaneous tickover of fluid phase C3 or by activation of classical or lectin pathways (via recognition of immune complexes or carbohydrate moieties on microorganisms, respectively). C1q-deficient mice (deficient of the recognition molecule of the classical pathway) and mice with a dual deficiency of factor B and C2 (affecting activities for lectin, classical and alternative pathways) (all on Sv129 background) have previously been studied in sublethal CLP (12). The study concluded that intact lectin and alternative pathways are important for survival of sublethal CLP; however, it did not allow quantification of the separate contribution of the alternative pathway amplification to this effect. Therefore, the sublethal CLP model seemed a highly suitable model to characterize the phenotype of a properdin-deficient mouse line, which we have developed and describe herewith for the first time. So far, properdin deficiency is known in humans and shows variable penetrance with predilection to succumb to fatal meningococcicol disease when infected with uncommon serotypes Y and W-135 (13).

Materials and Methods

Generation of a properdin-deficient mouse line

The mouse properdin gene was isolated from a mouse (129Sv) genomic bacteriophage library (A2001) using mouse properdin cDNA (European Molecular Biological Laboratory/GenBank accession number X12905) as a probe. A DNA of three hybridizing clones was purified using a Nucleobond Λ phage purification kit (Clontech) (14). BamHI digest of two overlapping clones yielded 7.6-, 3.4-, and 0.5-kb fragments (corresponding to bp position 10,309–21,407 of the mouse BAC clone, European Molecular Biological Laboratory/GenBank accession number AL671853.7) that were subcloned in pBSKS+ and sequenced. A targeting construct was generated in pKO-NTKV 1901 (Stratagene) designed to contain the 5′ part of the properdin gene (promoter region, exon for thrombospondin repeat (TSR) 1) and the 3′ part of the gene (comprising exons for TSR 5, 6a, and 6b) flanking a positive selection marker gene (neo+). Embryonic stem cells derived from male 129/Ola mice (E14.1a) were transfected with the linearized construct by electroporation. Clones were grown in G418 (positive selection for the presence of neo+) and gancyclovir (negative selection against random integration, which produces thymidine kinase activity). Homologous recombination of this vector with the WT gene results in a replacement of exons 3–7 (1.6 kb) coding for the TSR 2–4 modules by the
neor gene (1.6 kb) of the targeting vector. Positive clones were identified by Southern blotting through the presence of a 6-kb band in HindIII-
digested genomic DNA (an additional HindIII site is introduced during targeting) using a probe external to the targeted region. One of the 170 embryonic stem cell clones screened contained the disrupted allele of the targeted gene and was microinjected into C57BL/6 blastocysts which were implanted in pseudopregnant mice. Two male chimeras were derived. Germline transmission of the targeted allele was obtained upon mating to C57BL/6 mice (agouti offspring). As expected, all male offspring were WT and all female offspring were heterozygous for the targeting event. Target-specific PCR with restriction mapping of this fragment with BstXI showed correct replacement in these heterozygotes. Intercrossing resulted in hemizygous males, heterozygous females, and WT offspring, as identified by PCR, at the expected ratio. The properdin-deficient mice appear normal in weight and fertility and are maintained in a barrier unit according to institutional guidelines. The mice used in these experiments were backcrossed onto the C57BL/6 background for 9 and 10 generations. WT littermates were controls.

Rabbit RBC lysis

Target cells were prepared as follows. Rabbit blood (Biomedical Services, University of Leicester, Leicester, U.K.) was collected in Alsever’s solution (114 mM sodium citrate, 27 mM glucose, and 72 mM sodium chloride; pH 6.1), spun down at 200 × g, washed twice in PBS, and the pellet was resuspended in 1× VBS (727 mM sodium chloride, 16 mM 5,5-diethylbarbituric acid, and 9 mM 5,5-diethylbarbituric acid sodium salt)-20 mM MgCl2-8 mM EGTA (pH 7.4) to where lysis of 50 μl of RBC in 200 μl of water gave an absorption at 413 nm of 1.0–1.2 (positive control). The assay was set up in duplicate, diluting 100 μl of mouse serum (sample and heat-inactivated control) with 100 μl of VBS-MgCl2-EGTA, then adding 50 μl of adjusted rabbit RBC. The cells in 200 μl of VBS-MgCl2-EGTA were the negative control. After 2 h at 37°C, absorptions were read and hemolysis
was expressed as a percentage against the controls (relative hemolysis = [(mean OD sample – mean OD heat inactivated mouse serum)/(mean OD water – mean OD buffer)] × 100%) (15).

**C3 activation of WT and properdin-deficient sera**

Properdin-deficient or WT mouse sera were diluted 1/20 in either PBS+ (with Ca2+ and Mg2+), GVB+, GVB/Mg2++EGTA, or GVB+−EDTA buffer and incubated at 37°C with 1/10th ml formalin-fixed (10% formal saline) E. coli DH5α. (washed in the respective buffers). After 30 min, the supernatant, that is, activated serum, was removed and separated under reducing conditions (2-ME, boiling) on 10% SDS-polyacrylamide gels. After electrophoretic transfer using cellulose membrane 2micron (Bio-Rad Laboratories), the membrane was incubated with monoclonal biotin-coupled rat anti-mouse C3 (Cedarlane Laboratories), detected with a streptavidin-HRP ABC kit (DakoCytomation), and developed using ECL reagent (Pierce).

**IgM and C3 levels in mouse sera**

Serum was prepared from mouse blood obtained via the tail vein and frozen in aliquots. Serum levels for IgM and C3 were determined by sandwich ELISAs according to the manufacturers’ manuals (Bethyl Laboratories, Universal Biologicals and Immunology Laboratory, Immune Systems) and analyzed using a five-parameter logistics curve.

**Sublethal CLP**

Mice were anesthetized by i.p. injection of 75 mg/kg Ketanest (Parke-Davis) and 16 mg/kg Rompun (Bayer). The cecum was exteriorized and the distal ileum (second to third sections) was ligated and punctured once with a needle (0.4-mm diameter, 27-gauge) to achieve a sublethal CLP as previously described (16). Mice were observed for 2 wk. Kaplan-Meier survival curves were compared using the log rank test. The experiments were performed in compliance with federal guidelines for animal experimentation (State of Bavaria, Germany).

**Immunohistochemical and immunofluorescent analysis of mouse mast cells**

Four-micrometer sections were cut from paraffin-embedded connective tissue obtained from the abdominal wall, incubated with proteinase K (2 mg/ml), 3% H2O2, and, after blocking with 5% BSA and application of SeroBlock FcR (BUF041A; Serotec) were incubated with goat anti-human properdin Fab Igs (1/100; Nordic Immunology), or as control, goat anti-rabbit IgG (P0448; DakoCytomation), and rabbit anti-goat IgG, HRP-conjugated (1/400, A5420; Sigma-Aldrich). After development with tetrahydrochloride (D5637; Sigma-Aldrich), sections were counterstained with toluidine blue. For immunofluorescent analysis, sections were processed as above, incubated with FITC-coupled goat anti-human properdin IgG (1/200; Nordic Immunology) or rabbit anti-human mast cell tryptase Ab (1/400; sc-32889; Santa Cruz Biotechnology) and goat-anti-rabbit IgG, F(ab')2-TRITC (1/400, sc-3841; Santa Cruz Biotechnology). Fluorescence signals were analyzed using a Nikon TE300 wide-field epifluorescence microscope.

**Binding of properdin to E. coli DH5α**

Formalin-fixed E. coli DH5α (1 × 107/ml) were incubated with human sera (diluted 1/20 in PBS/Mg2++5% BSA) of known concentration of properdin (determined using a properdin ELISA kit obtained from Antibodyshop). After washing with PBS, monoclonal anti-human properdin Ab (HyB 039-06, diluted 1/1000; AntibodyShop) was added for 1 h and, after another washing step with PBS, FITC-coupled anti-mouse Fab Igs (Sigma-Aldrich) were added (1/4000). Fluorescence signals were analyzed as above. In parallel, an equal concentration of purified properdin (purity >98%; Europa Bioproducts) was added (diluted in PBS/Mg2++5% BSA to the predetermined level for the respective sera), and slides were processed as above. Part of this latter incubation, along with a control (E. coli without properdin), was used for immunoprecipitation studies, in which the microbes are washed, then lysed in radioimmunoprecipitation assay buffer with protease inhibitors (P8340; Sigma-Aldrich), sheared, precleared with protein G-Sepharose (F3296; Sigma-Aldrich), and incubated with Sepharose-coupled monoclonal anti-human properdin Ab (HyB 039-06; AntibodyShop). The immunoprecipitate was run on a 10% SDS-polyacrylamide gel, blotted, and probed with rabbit anti-human properdin Ab.

**Results**

**Generation of properdin-deficient mice**

A properdin-deficient mouse line was generated by specific targeting and disruption of the murine properdin gene, which is located on chromosome X (17). The strategy was to delete those exons coding for thrombospondin repeats (TSR) with importance in binding to C3b and thereby stabilization of the C3bBb complex or for oligomerization of the protein (18) (Fig. 1A). Homologous recombination of mouse properdin DNA contained in the targeting vector and segments of the properdin gene in 129/Ola embryonic stem cells was confirmed in 1 of 170 clones (Fig. 1B). This clone was microinjected in C57BL/6 blastocysts and two chimeric mice were derived. Both showed germline transmission and produced heterozygous female and WT male offspring. These were intercrossed, obtaining genotypes at the expected Mendelian ratio and X chromosome linkage. Heterozygous mice were backcrossed to C57BL/6 to obtain male WT and properdin-deficient littermates (Fig. 1C). The hemizygous properdin gene-targeted mice lack properdin protein in their sera (Fig. 1D) and they are grossly impaired in the alternative pathway-dependent diagnostic test of lysing rabbit RBC (19) (Fig. 1E). Heterozygous mice showed an intermediate phenotype (data not shown).

**C3 activation in sera of WT and properdin-deficient mice**

Western blot analysis was performed for different WT and properdin-deficient sera activated by E. coli DH5α in four different
Alternative pathway (Fig. 2). In conditions favoring activation of complement via the inhibiting assembly of all recognition molecules to surfaces (GVB⁺⁺/GVB⁺⁺EGTA), but not in the most physiologic of these conditions (PBS⁺⁺).

Serum C3 levels of the sera thus analyzed were found to be comparable, as was the range of levels determined for larger numbers of the two groups (properdin-deficient, n = 14: 1.19 ± 0.39 mg/ml, and WT, n = 17: 0.99 ± 0.30 mg/ml).

Survival of WT and properdin-deficient mice in CLP

The severity of the CLP model was chosen to produce sublethal and prolonged inflammation. In the first 7 days of the observation period, 14 of 16 WT mice survived, whereas only 6 of 16 properdin-deficient mice survived (Fig. 3).

Properdin is produced by mast cells

Sections of connective tissue mast cells of unchallenged WT mice were analyzed for reactivity of properdin by standard immunohistochemistry. Representative figures from two independent experiments clearly demonstrate properdin reactivity localizing to the granules of mast cells (Fig. 4A), which compares to the granule-specific metachromatic stain produced by toluidine blue on the control section (Fig. 4B). Subsequent immunofluorescence analysis of gut sections from WT and properdin-deficient mice using a mast cell marker, tryptase, confirmed that reactivities of properdin and tryptase overlap for WT, but not properdin-deficient mice (Fig. 4, C–F).

Properdin associates directly with E. coli DH5α

Incubation of formalin-fixed E. coli DH5α with human serum of known properdin concentrations or with the same amount of purified properdin on its own leads to the same patchy reactivity with the monoclonal anti-properdin Ab (Fig. 5, A–C). This association is absent on chelation of serum with EDTA (data not shown). There is no detectable C3 in the properdin preparation (Fig. 5D). To ascertain the physical association between E. coli and properdin, immunoprecipitation was performed using the

![Image](http://www.jimmunol.org/)

**FIGURE 4.** Properdin is produced by mast cells. Immunohistochemical analysis of connective tissue mast cells using goat anti-human properdin (A) or control Ab (B). Both sections were developed with dianamobenzidine tetrahydrochloride (brown stain) and counterstained with toluidine blue (purple stain). Immunofluorescent analysis showing reactivity of properdin (C), mast cell marker tryptase (D), and overlap (E) for gut mucosa (4',6-diamidino-2-phenylindole stained, F) of WT, but not properdin-deficient mice.

buffer conditions. One of three representative experiments is shown (Fig. 2): incubation of serum diluted in PBS/Mg²⁺/Ca²⁺ with the microbe leads to complete conversion of C3 to C3b and iC3b for both WT and properdin-deficient serum. In buffer conditions that restrict activation of complement, either selectively (GVB⁺⁺, GVB⁺⁺EGTA) or globally (GVB⁺⁺EDTA), by contrast, the uncut 113-kDa C3α chain is seen (lanes 2–4 and 6–8, Fig 2). In conditions favoring activation of complement via the alternative pathway (lanes 3 and 7, Fig. 2), properdin-deficient serum shows a clear reduction of the 104-kDa activation product C3b. Further degradation of iC3b to the 40-kDa α-chain product occurs in conditions that preclude C3 binding (EDTA) and in those inhibiting assembly of all recognition molecules to surfaces (PBS⁺⁺). Strips 2 and 1 show properdin-deficient serum (lanes 2 and 4, respectively) and WT serum (lanes 1 and 3, respectively), with properdin-deficient sera containing no detectable C3.

![Image](http://www.jimmunol.org/)

**FIGURE 5.** Properdin associates with E. coli DH5α. A. After incubation of E. coli DH5α with human serum (determined at 27 μg/ml properdin), properdin was detected using a monoclonal anti-human properdin Ab and FITC-coupled anti-mouse Fab Igs. B. After incubation of E. coli DH5α with purified properdin (25 μg/ml stock), bound properdin was detected as above. C. Negative control (addition of the secondary Ab only). Different sera were used and the amount of exogenous properdin was adjusted to the respective serum concentration. This is a representative figure of three such experiments. D. Aliquots of the purified properdin preparation were separated by SDS-PAGE and analyzed for possible C3 content using mouse anti-human C3 (ab11871; Abcam) (strip 1). Strip 2 of the same transfer was developed with mouse anti-human properdin Ab (HYB 039-06; AntibodyShop) and shows the expected 53-kDa band for monomeric properdin. E. Immunoprecipitation of properdin bound to E. coli DH5α (lane 2) and control (lane 1) using monoclonal anti-human properdin Ab. Transferred lysates were developed with polyclonal goat anti-human properdin Ab. The 53-kDa band for properdin is indicated. The secondary Ab (rabbit anti-goat Ig, HRP conjugated) reacts with Igs contained in both immunoprecipitates. The H chain of Ig is indicated.
monoclonal anti-properdin Ab or a control Ab followed by Western blot analysis of immunoprecipitates using a polyclonal anti-properdin Ab. A 53-kDa band for monomeric properdin is detected for the specific immunoprecipitate (Fig. 5E).

Discussion

DH5α is a nonpathogenic E. coli K12 derivative that is able to activate the alternative pathway of complement (20) and was therefore used to test the contribution of properdin toward activation of C3 in properdin-deficient vs WT sera. In conditions using PBS++, the iC3b product seems more abundant in activated WT compared with activated properdin-deficient serum and may reflect the contribution of an intact amplification loop to the alternative pathway for the activation of classical and lectin pathways. Analysis of C3 activation products in sera of WT and properdin-deficient littersmates, using buffer conditions favoring alternative pathway activation, consistently shows impaired processing of C3 in properdin-deficient sera in the 30-min incubation with E. coli DH5α. The difference between properdin-deficient and WT sera is less marked (data not shown). This, along with the fact that Factor B reactivity on Western blot analysis of WT and properdin-deficient sera varies little (data not shown), is consistent with the understanding that properdin provides stabilization of a specific C3-cleaving ability of serum. One variable in this experiment may be the actual serum levels for C3 in properdin-deficient and WT mice, based on a description of elevated C3 levels in heterozygous properdin-deficient persons, due to a proposed lower consumption of C3 (21), but this was not found to be the case.

Properdin-deficient and WT littersmates were subjected to sublethal CLP, a model of subacute peritoneal inflammation provoked by polymicrobial leakage from punctured distal ce-

Discussion

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Disclosures

The authors have no financial conflict of interest.

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