Bystander Hemolysis of PNH erythrocytes induced by yeast is an artifact as it occurs only with yeast in the presence of CFH-Depleted NHS. Complement activation factors fragment pathways, recruit and activate inflammatory cells at the site of complement activation and act as the nidus for assembly of the lytic membrane attack complex on the surface of pathogens. Complement activation is a well-coordinated physiological response that is highly regulated by various fluid-phase as well as membrane-bound regulatory proteins to prevent host tissue damage. Loss of membrane-bound complement regulatory proteins CD59 and CD95 in paroxysmal nocturnal hemoglobinuria (PNH) leads to hemolysis of PNH erythrocytes.

Hemolysis in PNH is chronic due to continuous alternative pathway (AP) activation through tick-over, but brisk hemolysis (paroxysm) can occur during a “triggering” event such as infection. While the mechanism(s) by which infections cause paroxysm are unclear, one possible explanation is that PNH red blood cells (RBCs) may be subject to bystander lysis as a direct result of pathogen-dependent complement activation. This hypothesis however has not been formally tested and is based largely on studies performed under non-physiological serum conditions.

Accordingly, using normal human sera, PNH erythrocytes, and a panel of clinically relevant bacterial strains, we undertook the studies herein to evaluate whether activation of complement by pathogens leads to enhanced hemolysis and/or complement fragment deposition on PNH erythrocytes. Moreover, we also investigated the mechanism of the bystander lysis induced by Streptococci (paroxysm). We found no evidence that PNH erythrocytes are susceptible to pathogen-induced bystander lysis under physiological serum conditions. Instead, we found that a subtle change in complement factor H (CFH) concentration can lead to hemolysis of PNH erythrocytes and furthermore, that this hemolysis is caused by AP dysregulation as demonstrated by inhibition of a monoclonal antibody against complement factor D (CFD).

**METHODS**

**Experiments with Bacteria**

10 µL PNH erythrocytes (5×10^6/mL in GVB) and 10 µL bacteria in logarithmic growth phase (5×10^6/mL in GVB) were added to 80 µL ABO blood group compatible normal human serum (NHS). Reactions were incubated at 37°C for 1 h and supernatants and cell pellets were separated by differential centrifugation. Absorbance of supernatants was measured at 405 nm. Erythrocytes and/or bacteria recovered from pellets were labeled with FITC-conjugated anti-C4d or anti-C3c antibody and cell surface fluorescence was examined by flow cytometry. PNH erythrocytes were identified by flow cytometry with allophycocyanin-conjugated anti-C5b9 antibody (Fig. 2).

**Bacteria**:

*Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Haemophilus influenzae* type b strain Eagan, *Pseudomonas aeruginosa*, *Escherichia coli*, *Neisseria meningitidis A* and *Neisseria meningitidis B*.

Experiments with Yeast

Assays were performed in GVB buffer with Saccharomyces cerevisiae (1×10⁶ CFU/mL), erythrocytes from PNH patient B (5×10^6/mL), and 2% to 80% NHS and/or CFH-depleted NHS supplemented in some experiments with purified CFH or NHS. Transwell permeable barriers were used in some reactions to separate fluid-phase activation by yeast from solid-phase activation on erythrocyte surfaces (Fig. 3).

**RESULTS**

**Activation of Complement by Bacteria Leads to Bacterial Lysis or Opsonization**

**PNH Erythrocytes Show No Increase in Complement-Mediated Hemolysis or Opsonization When Co-incubated with Bacteria**

**Conclusions**

- **Bystander hemolysis of PNH erythrocytes is not induced by a panel of bacterial strains despite evident complement activation on the bacterial surfaces.**
- **Bystander hemolysis of PNH erythrocytes induced by yeast is an artifact as it occurs only with artificially low serum concentrations and high yeast densities.**
- **Bystander hemolysis requires direct contact between yeast and PNH erythrocytes, indicating that hemolysis is not elicited by activation products in the fluid phase.**
- **Bystander hemolysis is readily suppressed by exogenous CFH, suggesting that this hemolysis in reduced serum concentrations may depend on the resulting dilution of soluble negative regulators.**
- **Subtle reductions in CFH concentration are sufficient to elicit PNH erythrocyte hemolysis, suggesting that perturbation of soluble regulators during infection in vivo is a likely mechanism for "paroxysms."**
- **Lastly, AP inhibition could protect PNH erythrocytes from lysis during "paroxysm," indicating potential therapeutic utility of AP inhibitors."