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Note

Development of a high-throughput opsonophagocytic assay for the determination of functional antibody activity against *Streptococcus pyogenes* using bioluminescence

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ABSTRACT

The lack of standardised protocols for the assessment of functional antibodies has hindered *Streptococcus pyogenes* research and the development of vaccines. A robust, high throughput opsonophagocytic bactericidal assay to determine protective antibodies in human and rabbit serum has been developed that utilises bioluminescence as a rapid read out.

Group A *Streptococcus* (GAS) or *Streptococcus pyogenes* is a major human pathogen that causes a range of diseases, from minor localised skin infections and pharyngitis to severe conditions such as streptococcal toxic shock syndrome and necrotising fasciitis (Carapetis et al., 2005; Cunningham, 2008; Walker et al., 2014). Recurring pharyngitis and skin infections have been linked to the development of acute rheumatic fever (ARF) and rheumatic heart disease (RHD), autoimmune conditions with high mortality rates (Carapetis et al., 2016; Cunningham, 2016; Martin et al., 2015). Much of the GAS disease burden exists in resource poor settings, which can make efficient treatment and control difficult. This has fuelled efforts to develop a GAS vaccine with several candidates in various stages of development (Moreland et al., 2014; Steer et al., 2009; Steer et al., 2016). However, progress is slowed by the lack of a robust assay to assess the protective capacity of vaccine antisera. The two main types of immunoassay currently used are enzyme-linked immunosorbent assays (ELISAs) and functional bactericidal assays. While ELISAs are highly standardised, reproducible and allow for a high-throughput, they are limited by the fact that the measured immune response does not distinguish between functional and non-functional antibodies. Existing functional bactericidal assays to determine protective antibodies are based on the classical Lancefield assay (Lancefield, 1959). These indirect bactericidal assays are low throughput, labour-intensive and require fresh human whole blood as a source of phagocytes, resulting in high variability between experiments (Tsoi et al., 2015).

Here we show the development of a robust, high-throughput opsonophagocytic assay that reflects protective capacity by determining functional antibodies. The assay overcomes the challenges of previous assays by using rabbit peripheral blood leukocytes (PBLs) as the source of phagocytes and an exogenous source of complement, thereby mimicking *in vitro* the *in vivo* process of host effector cell-mediated killing following bacterial opsonisation with specific antibodies. Furthermore, our assay introduces bioluminescence as a rapid, high throughput detection method of bacterial survival.

To obtain specific antiserum for assay development, female New Zealand White rabbits were immunised subcutaneously with 50 μg recombinant M1 protein in Incomplete Freund's adjuvant (Sigma-Aldrich) at 0, 2, 4, and 6 weeks. Recombinant M1 protein was cloned from genomic DNA of the GAS strain SF370 using the primers ctagGGATCCaacggtgatggtaatcctagg and ctagGAATTCctgtctcttagtttccttcattgg. Protein was expressed in *E. coli* BL21(λDE3)pLysS using the expression vector pET32a3c, and purified by nickel-affinity chromatography as previously described (Young et al., 2014). M1 antiserum was obtained two weeks after the final injection and reactivity against recombinant M1 confirmed by ELISA (Fig. 1A). Affinity sepharose was made by coupling recombinant M1 protein to CNBr-Activated Sepharose 4B (GE Healthcare) according to manufacturer's instructions. M1 antiserum was depleted by overnight end-over-end rotation at 4 °C of antiserum mixed with affinity

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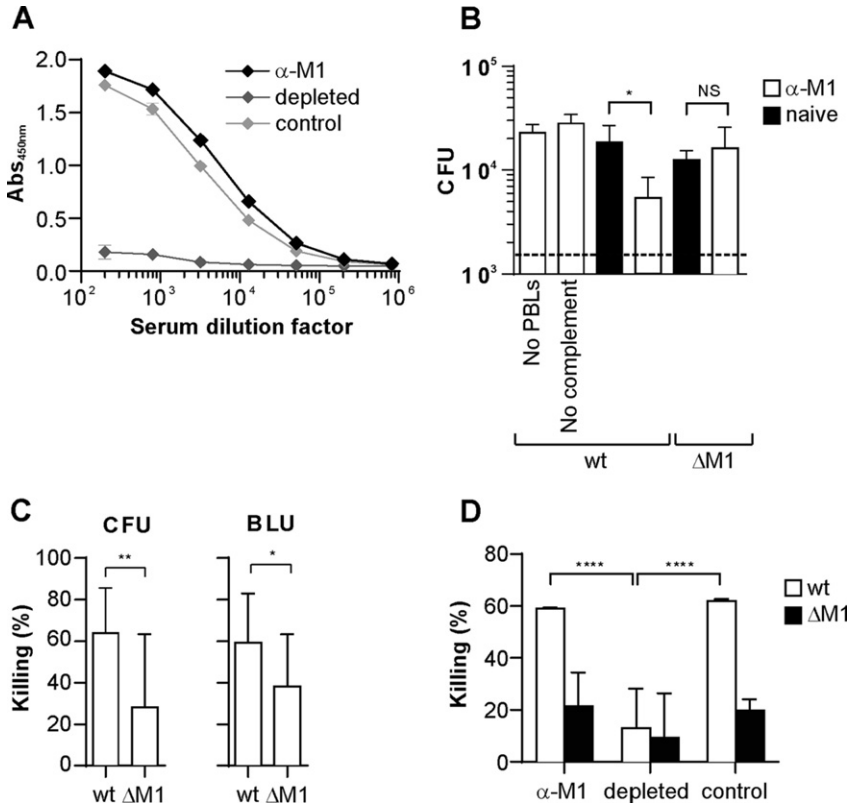

Fig. 1. Establishment of a GAS opsonophagocytic assay using bioluminescence. A) ELISA showing reactivity levels of M1 antiserum (α-M1), M1-affinity depleted antiserum (depleted) and antiserum passed over a sepharose control column (control) against recombinant M1 protein. Data shown as mean ± SD. B) Tube-based opsonophagocytic assay of wild-type GAS M1 (wt) and GAS M1 deletion mutant (ΔM1) after incubation with or without isolated PBLs/complement as determined by CFU counts. Dashed line indicates CFU of inoculum. Data combines four independent experiments and is shown as mean ± SD, analysed by Wilcoxon matched-pairs signed rank test, * $P < 0.05$, NS = not significant. C) Comparison of bacterial killing as determined by CFU counts and bioluminescence units (BLU) in a tube-based assay format. Bacterial killing (%) was determined as $[(CFU_{t=3}$ naive serum − $CFU_{t=3}$ immune serum) ÷ $CFU_{t=3}$ naive serum] × 100. For bioluminescence read-out, killing (%) was determined as $[(BLU_{t=3}$ naive serum − $BLU_{t=3}$ immune serum) ÷ $BLU_{t=3}$ naive serum] × 100. Data combines nine independent experiments and is shown as mean ± SD, analysed by paired *t*-test, * $P < 0.05$ and ** $P < 0.01$. D) Plate-based opsonophagocytic assay of GAS M1 wt (wt) and GAS deletion mutant (ΔM1) with M1 specific antiserum (α-M1), M1-affinity depleted antiserum (depleted) and antiserum passed over a sepharose control column (control). Data combines three independent experiments and is shown as mean ± SD, statistically analysed by one-way ANOVA with Tukey'"s multiple comparisons test, *** $P < 0.001$.

sepharose in a 1:1 ratio. Reactivity of the anti-M1 serum by ELISA was abolished after depletion over an M1-affinity column, but was retained when antiserum was passed over a control sepharose column demonstrating the presence of M1-specific antibodies in the serum (Fig. 1A). To standardise complement activity in the experimental set up, all sera used were heat-inactivated for 30 min at 56 °C and exogenous complement activity added with commercial rabbit complement (Sigma-Aldrich).

In the indirect bactericidal assay complement is a source of variability, with complement activity influenced by the health status of the donor as well the anticoagulant, timing and storage conditions used during the blood draw. Besides non-standardised complement activity, human whole blood frequently contains pre-existing anti-GAS antibodies that need to be screened for prior to use (Moreland et al., 2014). Isolating the source of phagocytes, together with the use of exogenous complement, overcomes these problems. To isolate fresh rabbit PBLs, rabbit blood was taken by venepuncture of the ear marginal vein in heparinised vacutainers (BD) and mixed with a 5-fold volume of 3% dextran (molecular weight, 500,000, Sigma-Aldrich), 0.9% NaCl and incubated for 45 min at room temperature (RT). Following dextran erythrocyte sedimentation PBLs contained in the supernatant were pelleted (10 min, 500 ×*g* at RT) and any remaining erythrocytes lysed by resuspension in sterile MilliQ H_2O for 30s followed by immediate restoration of tonicity with an equal volume of 1.8% (w/v) NaCl. PBLs were pelleted (10 min, 500 ×*g* at RT) and resuspended in pre-warmed (37 °C) Hanks' Balanced Salt solution (HBSS) containing 0.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, $HBSS^{++}$ (Gibco) at a final concentration of 5×10^6 cells/ml.

To prepare bacteria for the assays, the GAS strains M1 (strain SF370, ATCC reference 700294) and a M1-protein deletion mutant (ΔM1) (Loh et al., 2013) were cultured in Brain Heart Infusion (BHI) medium (Difco, BD). Overnight cultures were diluted 1:100 in fresh broth and grown to mid-log phase (OD_{600} of 0.4 to 0.6), before addition of 10% sterile glycerol (v/v) and freezing at −80 °C. To enumerate bacteria, several aliquots were defrosted by incubation at 37 °C for 5 min, serially diluted and plated for colony count after incubation over night at 37 °C. To prepare the bacterial inoculum for the experimental set up bacterial aliquots were rapidly defrosted, pelleted and resuspended in $HBSS^{++}$.

Initial opsonophagocytic assays were performed by incubation of 2×10^3 bacteria with 20% serum and 2% rabbit complement for 30 min at RT prior to addition of 4×10^5 isolated lymphocytes made up to a total assay volume of 500 μl with $HBSS^{++}$. Bacterial survival was determined after 3 h incubation at 37 °C with end over end rotation by plating on BHI agar (Difco, BD) and CFU count the next day. A minimum 5-fold increase in bacterial growth was observed with naïve serum over the 3 h incubation period (Fig. 1B). Inclusion of anti-M1 antiserum in the experimental set-up significantly reduced the growth of WT bacteria, but did not affect the growth of GAS ΔM1 (Fig. 1B). When individual components such as the PBLs or complement were left out of the experimental set up, comparable bacterial growth to the naïve serum control was observed (Fig. 1B). These results confirmed that the use of isolated sources of complement (commercial rabbit serum) and phagocytic cells (rabbit leukocytes) provides a solid basis towards the establishment of a more standardised opsonophagocytic assay. Equally important, the removal of whole blood as a source of phagocytes