Vaccine 36 (2018) 3756–3763

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development of an opsonophagocytic killing assay for group a streptococcus

Scott Jones [a,*], Nicole J. Moreland [b], Marta Zancolli [a,1], Jeremy Raynes [b], Jacelyn M.S. Loh [b], Pierre R. Smeesters [c,d], Shiranee Sriskandan [e], Jonathan R. Carapetis [f], John D. Fraser [b], David Goldblatt [a]

[a] Immunobiology, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London WC1N 1EH, United Kingdom
[b] Department of Molecular Medicine & Pathology, School of Medical Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand
[c] Molecular Bacteriology Laboratory, Universite´ Libre de Bruxelles and Academic Children Hospital, Brussels, Belgium
[d] Murdoch Children's Research Institute and University of Melbourne, Melbourne, Australia
[e] Faculty of Medicine, Imperial College London, Commonwealth Building, Hammersmith Hospital, Du Cane Road, London W12 0NN, United Kingdom
[f] Telethon Kids Institute, University of Western Australia and Perth Children's Hospital, Perth, Australia

ARTICLE INFO

Article history:
Received 1 March 2018
Received in revised form 17 April 2018
Accepted 11 May 2018

Keywords:
Opsonophagocytic killing assay
Streptococcus pyogenes
GAS vaccines
HL-60 cells
Baby rabbit complement
Functional assay

ABSTRACT

Group A *Streptococcus* (GAS) or *Streptococcus pyogenes* is responsible for an estimated 500,000 deaths worldwide each year. Protection against GAS infection is thought to be mediated by phagocytosis, enhanced by bacteria-specific antibody. There are no licenced GAS vaccines, despite many promising candidates in preclinical and early stage clinical development, the most advanced of which are based on the GAS M-protein. Vaccine progress has been hindered, in part, by the lack of a standardised functional assay suitable for vaccine evaluation. Current assays, developed over 50 years ago, rely on non-immune human whole blood as a source of neutrophils and complement. Variations in complement and neutrophil activity between donors result in variable data that is difficult to interpret. We have developed an opsonophagocytic killing assay (OPKA) for GAS that utilises dimethylformamide (DMF)-differentiated human promyelocytic leukemia cells (HL-60) as a source of neutrophils and baby rabbit complement, thus removing the major sources of variation in current assays. We have standardised the OPKA for several clinically relevant GAS strain types (*emm1*, *emm6* and *emm12*) and have shown antibody-specific killing for each *emm*-type using M-protein specific rabbit antisera. Specificity was demonstrated by pre-incubation of the antisera with homologous M-protein antigens that blocked antibody-specific killing. Additional qualifications of the GAS OPKA, including the assessment of the accuracy, precision, linearity and the lower limit of quantification, were also performed. This GAS OPKA assay has the potential to provide a robust and reproducible platform to accelerate GAS vaccine development.

1. Introduction

Group A *Streptococcus* (GAS) or *Streptococcus pyogenes* is a species of Gram-positive bacteria responsible for a significant worldwide burden of disease [1–4]. Clinical syndromes resulting from GAS infection include superficial infections, such as pyoderma and pharyngitis, severe invasive GAS disease (iGAS), acute rheumatic fever (ARF) and associated rheumatic heart disease (RHD), and post-streptococcal glomerulonephritis. Conservative estimates suggest that more than 33 million people are afflicted with severe GAS disease that results in roughly 500,000 deaths each year, with RHD responsible for two thirds of this mortality [5].

Despite the substantial morbidity and mortality GAS infection poses, there are currently no licenced vaccines against GAS [6,7]. Epidemiological data reveals that the majority of infections occur within the ages of 1–15, suggesting that immunity to GAS develops with age [8,9]. First identified by Rebecca Lancefield, the M-protein is a major GAS virulence factor that comprises a hypervariable N-terminus, which forms the basis for GAS strain typing (known as *emm*-typing), and a conserved C-terminus [10,11]. The M-protein has multiple functions, including an ability to subvert phagocytosis by binding multiple host proteins [11,12]. Antibodies specific to the M protein have long been known produce a protective immune response and as such the M protein is being extensively investigated as a vaccine antigen [10,13]. M-protein based vaccines are

* Corresponding author.
E-mail address: scott.jones@ucl.ac.uk (S. Jones).
[1] Current address: Theradex (Europe) Ltd, 2nd Floor The Pinnacle, Station Way, Crawley, West Sussex RH10 1JH, United Kingdom.

https://doi.org/10.1016/j.vaccine.2018.05.056
0264-410X/

generally composed of either the hypervariable N-terminal region of multiple *emm*-types or the conserved C-terminal region and have been shown in early stage clinical trials to be safe and well tolerated [10,14–18]. Non-M-protein based vaccines are also in development, though none have yet been tested in human trials. These tend to comprise a combination of conserved GAS antigens that have been identified through reverse vaccinology or studies on GAS virulence [19–21]. Such antigens include C5a peptidase, serine esterase, GAS carbohydrate, fibronectin-binding protein, serum opacity factor, SpyCep and T-antigens [6,7,14,22–25].

The progress of GAS vaccine development has been hindered, in part, by the lack of a reliable and fully standardised functional assay that assesses immunity to GAS. The bactericidal assay used most widely to assess the immunogenicity of GAS vaccine antigens is known as the Lancefield assay [13,26–29]. The Lancefield assay measures the growth and survival of GAS in fresh human blood from an immune individual (direct) or non-immune whole blood supplemented with serum from an immune individual or animal immunised with an antigen of interest (indirect) [28,29]. Natural variations in complement and neutrophil activity that exist between individuals often result in variable data that is difficult to interpret, and does not allow for comparison of results between laboratories [21,30]. In addition, widespread natural exposure to GAS renders identifying a non-immune donor (i.e. an individual with minimal reactivity to the bacterium) laborious [24,29].

The need for improved assays to measure GAS vaccine efficiency has been highlighted as a priority by the international vaccine community and was recently recognized by the Coalition to Accelerate New Vaccines Against Streptococcus (CANVAS) [6,14,18,24]. CANVAS is an Australian and New Zealand government-sponsored GAS vaccine development programme that aims to overcome key developmental hurdles for GAS vaccines. The coalition was formed in response to the unacceptably high rates of ARF and RHD in Indigenous populations in Australia and New Zealand [31,32]. To overcome the issues of variability associated with the traditional Lancefield methods, we have developed a GAS opsonophagocytic killing assay (OPKA) that uses an exogenous source of both complement and phagocytic cells. This assay is adapted from a validated pneumococcal OPKA which makes use of baby rabbit complement (BRC) as the exogenous source of complement and dimethylformamide (DMF)-differentiated human promyelocytic leukemia cells (HL-60) as the exogenous source of phagocytic cells [33–36]. Using high titre anti-M protein rabbit sera and human intravenous immunoglobulin, we have developed and optimised an OPKA for a number of clinically relevant GAS strains.

2. Materials and methods

2.1. Bacterial strains

The following GAS strains were used in this study: emm1 (strains 43, 02–12 and GAS05134), emm12 (strains 611020, 611,025 and GAS09437) and emm6 (strain GASOPA6_02). These emm-types were selected because they belong to distinct *emm*-protein clusters in the recently described *emm* cluster typing system [37]. Additional strain details are listed in Table 1. Working stocks of bacterial strains were generated by streaking a fleck of frozen master stocks onto a horse blood agar plate (Fisher Scientific, Leicestershire, UK), which was incubated overnight at 37 °C, 5% CO_2. Todd-Hewitt Broth (THB; Sigma-Aldrich Company Ltd, Dorset, UK) was inoculated with a single colony and grown at 37 °C, 5% CO_2 to an OD_{600} (optical density at 600 nm) of 0.6–0.7; considered to be the late-log phase of growth. Once the required OD_{600} was reached, cultures were mixed 1:1 with STGG medium (3% w/v tryptone soya broth (Thermo Fisher Scientific Oxoid Ltd, Basingstoke, UK), 0.5% w/v D-glucose (Sigma-Aldrich Company Ltd), 10% v/v glycerol (Sigma-Aldrich Company Ltd) in sterile, pyrogen-free water (Baxter Healthcare UK, Newbury, UK) and stored in 0.5 ml aliquots at −80 °C until required.

2.2. Antisera

Two sets of sera were used to optimise the GAS OPKA: human intravenous immunoglobulin (IVIg; Euglobulin®; Baxter, Berkshire, UK) and rabbit anti-full-length M-protein sera. To generate M-proteins for rabbit vaccinations *emm*1 (SF370) and *emm*6 (MGAS10394) were amplified from genomic DNA. The primer pairs ctagGGATCCaacggtgatggtaatcctagg and ctagGAATTCctgtctcttagtttccttcattgg were used for *emm1*, and cgcGGATCCagagtgtttcctagggggacg and ctagGAATTCctgtctcttagtttccttcattgg for *emm6*. The genes were cloned into pET32a3c sequences and the sequences were confirmed by Sanger sequencing. For *emm*12 (HKU16) the gene was synthesised (Life technologies) and inserted into pET151/D-TOPO. Each of the three proteins were expressed with the signal sequence and transmembrane regions removed (M1, aaN42-Q449; M6, aaR43-T358; M12, aaD42-Q540) in BL21(DE3) pLysS *E. coli* and purified by immobilised-metal affinity chromatography (IMAC) as previously described [37,38]. The His_6 tag was cleaved from the recombinant M proteins with 5 μg/ml 3C-His_6 protease (M1 and M6) or a 1:100 ratio of rTEV-His_6 protease to M protein (M12) for 16 h at 4 °C. The M-proteins were separated from the cleaved His_6 tag and the rTEV-His_6 protease by IMAC and further purified by gel filtration. Female New Zealand White rabbits were immunised subcutaneously with 200 μg of the purified, recombinant M1, M6 or M12 proteins in Incomplete Freund's adjuvant (Sigma-Aldrich) at 0, 2 and 4 weeks prior to exsanguination. The reactivity of immune sera to the recombinant M proteins was confirmed by ELISA (endpoint titre >100,000).

2.3. HL-60 cells

Master stocks of HL-60 cells were prepared from frozen commercial stock (ATCC Standards UK, Middlesex, UK). Working stocks of HL-60 cells were differentiated into neutrophil-like cells by culture in 0.8% DMF (Sigma-Aldrich Company Ltd) in M2 medium

Table 1
GAS Strains. Summary table of the GAS strains used in this study with additional details of M-clusters, clinical syndrome association, country of origin and year of isolation.

M-type	M-cluster	Strain ID	Clinical syndrome	Country of origin	Year of isolation
1	A-C3	43	Pharyngitis	UK	2009
		02-12	Pharyngitis	Brazil	2012
		GAS05134	Acute rheumatic fever	New Zealand	2005
12	A-C4	611,020	Pharyngitis	Australia	2011
		611,025	Pharyngitis	Australia	2011
		GAS09437	Acute rheumatic fever	New Zealand	2009
6	Single protein M-cluster	GASOPA6_02	Pharyngitis	UK	2009