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Research Paper

Monoclonal antibodies against DNA-binding tips of DNABII proteins disrupt biofilms *in vitro* and induce bacterial clearance *in vivo*

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ABSTRACT

The vast majority of chronic and recurrent bacterial diseases are attributed to the presence of a recalcitrant biofilm that contributes significantly to pathogenesis. As such, these diseases will require an innovative therapeutic approach. We targeted DNABII proteins, an integral component of extracellular DNA (eDNA) which is universally found as part of the pathogenic biofilm matrix to develop a biofilm disrupting therapeutic. We show that a cocktail of monoclonal antibodies directed against specific epitopes of a DNABII protein is highly effective to disrupt diverse biofilms *in vitro* as well as resolve experimental infection *in vivo*, in both a chinchilla and murine model. Combining this monoclonal antibody cocktail with a traditional antibiotic to kill bacteria newly released from the biofilm due to the action of the antibody cocktail was highly effective. Our results strongly support these monoclonal antibodies as attractive candidates for lead optimization as a therapeutic for resolution of bacterial biofilm diseases.

1. Introduction

The presence of a bacterial biofilm abrogates the ability of antibodies and antimicrobials to effectively eradicate the causative agent of many chronic and recurrent diseases. Given that biofilms are integral to the vast majority of these diseases (Brook, 2016, Dlugaszewska et al., 2015, Subashchandrabose and Mobley, 2015, Dou et al., 2016, Scalise et al., 2015, Hassett et al., 2014, Swords, 2012) knowledge of the key role of biofilms in pathobiology mandates the development of approaches for therapeutic disease resolution. The list of diseases wherein the ability to effectively disrupt bacterial biofilms would be beneficial is lengthy, and ideally this would involve eradication of both the microbes that induce its formation as well as the recalcitrant biofilm matrix. Towards our goal to develop a broad-based therapeutic approach for biofilm resolution, we focused on two common constituents of biofilms produced by multiple human pathogens, extracellular DNA (eDNA) and a family of bacterial DNA-binding proteins called the DNABII family.

Biofilms are communities of bacteria adhered to a surface with division of labor (*e.g.* altered gene expression), intercellular communication (*e.g.* quorum sensing), and the creation of a self-made extracellular polymeric substance (EPS) that enshrouds and protects resident bacteria (Flemming and Wingender, 2010). Often biofilms arise to weather stressful conditions, *e.g.* resistance to immune clearance and antibiotics (in excess of 1000-fold greater than needed to eliminate free-living bacteria). Key to the biofilm's protection is the extrapolymeric substance or EPS that constitutes the biofilm matrix. While the molecular makeup of the EPS varies among bacterial species, extracellular DNA (eDNA) is a common component (Flemming and Wingender, 2010, Fong and Yildiz, 2015). Indeed, DNase can prevent biofilm formation by multiple pathogenic species, but does not effectively treat pre-formed biofilms despite the abundance of eDNA in mature biofilms (Flemming and Wingender, 2010). In association with eDNA is the DNABII family of proteins which serve as lynchpin proteins, positioned at the vertices of crossed strands of eDNA within the biofilm matrix, thus contributing to the structural stability of the biofilm matrix (Goodman et al., 2011, Idicula et al., 2016, Devaraj et al., 2015, Gustave et al., 2013).

The DNABII family is ubiquitous among eubacteria and has been studied for almost 40 years as an intracellular architectural element. This family is one of multiple nucleoid-associated proteins (NAPs) that maintain the structure and function of bacterial chromatin (Swinger and Rice, 2004). Recently, multiple labs showed that these proteins are also abundant extracellularly (Goodman et al., 2011, Stinson et al., 1998, Lunsford et al., 1996, Gao, 2000, Boleij et al., 2009). The DNABII family members include integration host factor (IHF) which is a heterodimer of IHFA and IHFB and histone-like protein (HU), which is a hetero- or homodimer of each subunit. IHF and HU have a conserved sequence homology and as a result, a conserved architecture. This

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conserved architecture enables them to not only bind to and bend DNA (achieved by the insertion of two antiparallel β-ribbons into the DNA minor groove that cause the DNA to bend), but also show enhanced affinity to pre-bent DNA structures such as cruciforms or Holliday junctions (Swinger and Rice, 2004). These lynchpin proteins are present in the biofilms produced by multiple human pathogens (Goodman et al., 2011). Further, when biofilms are exposed to polyclonal rabbit antiserum directed against IHF isolated from *Escherichia coli* (anti-IHF$_{E.coli}$), complete collapse of the biofilm occurs with release of resident bacteria (Brockson et al., 2014). These observations suggested that the DNABII proteins and eDNA might serve as universal biofilm constituents that not only contribute to structural integrity, but could also provide a mechanism for multispecies interaction and facilitate the development of mixed microbial biofilm consortia as typically exist in nature (Goodman et al., 2011).

Using nontypeable *Haemophilus influenzae* (NTHI) as a model organism to dissect the mechanism(s) responsible for the observed complete biofilm collapse, we have shown *in vitro* that anti-IHF$_{E.coli}$ captures DNABII proteins when they are in an 'off' state within the culture medium (*e.g.* when they are not in association with eDNA of the biofilm EPS) (Brockson et al., 2014). This action induces an equilibrium shift that results in removal of additional DNABII proteins from the biofilm matrix (*e.g.* those that are in an 'on' state or associated with eDNA of the biofilm EPS), resulting in structural collapse of the biofilm matrix with release of the resident bacteria. These newly released bacteria were not killed by the action of anti-IHF$_{E.coli}$, however they were 4–8 fold more sensitive to the killing action of multiple antibiotics (Brockson et al., 2014). We have also already shown that this outcome is rapid, specific and dose-dependent but does not require direct contact between anti-IHF$_{E.coli}$ antibodies and the NTHI biofilm (Brockson et al., 2014). Moreover, this mechanism (which is characterized as 'disruption') was distinct from the 'dispersal' of an NTHI biofilm induced by exposure to antibodies directed against the Type IV twitching pilus which mediates a distinct 'top-down' dispersal of the biofilm that is linked to expression of the quorum signaling molecule AI-2 (Novotny et al., 2015b).

To then determine if antibodies with similar biofilm disruption functionality could be induced *in vivo*, we conducted a study which showed that active immunization with IHF [now isolated specifically from NTHI (IHF$_{NTHI}$)], induced the formation of antibodies that disrupted biofilms formed by NTHI in the middle ears of chinchillas. This disruption leads to significantly more rapid resolution of experimental disease with eradication of mucosal biofilms (Goodman et al., 2011, Brockson et al., 2014). *Via* extensive epitope mapping efforts, combined with additional pre-clinical evaluation in the same chinchilla model of experimental otitis media (Goodman et al., 2011, Brockson et al., 2014), we found that DNABII proteins that are naturally associated with eDNA within the bacterial biofilm (as they are found in the disease state), do not induce a protective immune response, as binding to DNA obscures the protective epitopes within the DNABII protein. Pre-clinical studies using native protein (with no bound eDNA) *versus* that which was pre-complexed to DNA as comparative immunogens revealed that the typically obscured DNA-binding tip regions of the DNABII proteins served as the protective epitopes. We then showed that polyclonal rabbit antibodies directed against focused 20-residue peptides which mimicked these specific predicted protective domains within the DNA-binding tips of IHF$_{NTHI}$, were equally effective as polyclonal antisera directed against the whole native IHF$_{E.coli}$ protein in terms of their ability to disrupt biofilms *in vitro* (Goodman et al., 2011, Brockson et al., 2014).

Having determined the mechanism of action, and shown the ability to utilize polyclonal antibodies to disrupt biofilms *in vitro* and also induce their formation *via* active immunization *in vivo*, it was important to ascertain the therapeutic potential of monoclonal antibodies in order to move closer to the ability to conduct human trials. Thereby, monoclonal antibodies directed against specific protective domains of the DNABII protein IHF were generated in an effort to develop a lead compound with a very specific epitope as a target. Ideally, this highly focused therapeutic would nonetheless provide broad protection against diverse bacterial pathogens which shared the same or similar targeted epitope. Thereby, in the present study, we extended our observations to determine if highly specific monoclonal antibodies (MAbs) directed against the DNA-binding tip regions of the alpha- and beta-subunits of IHF$_{NTHI}$ could individually disrupt biofilms formed *in vitro* by diverse strains of NTHI. In addition, we tested these MAbs against biofilms formed by four additional human pathogens: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Moraxella catarrhalis*. We also tested cocktails of these two MAbs both *in vitro*, to determine their ability to act synergistically, and *in vivo* using two distinct animal models of experimental upper and lower respiratory tract diseases to determine their ability to also resolve experimental infection. This outcome was achieved as evidenced by eradication of either mucosal biofilms formed by NTHI within the middle ears of chinchillas or classic multicellular aggregates of *P. aeruginosa* present within the lungs of mice.

2. Materials and Methods

2.1. Peptide Design and Synthesis

To identify regions of interest within the alpha and beta subunits of IHF$_{NTHI}$, we used the known crystal structure of IHF from *E. coli* (Rice et al., 1996) along with the deduced amino acid sequence of IHF$_{NTHI}$ to approximate the location of the 20-mer synthetic peptides used for the generation of MAbs (Fig. 1A). Synthesis, purification and sequence confirmation of all synthetic peptides was performed by Ohio Peptide, LLC.

2.2. Generation of Hybridomas, Isolation, Purification and Validation of MAbs

Hybridoma cell lines were generated by Rockland Immunochemical, Inc. Briefly, spleen cells from mice immunized with synthetic peptides IhfA3$_{NTHI}$, IhfA5$_{NTHI}$, IhfB2$_{NTHI}$ or mIhfB4$_{NTHI}$ were fused with Sp2/0-Ag14 myeloma cells. To reduce the risk of overgrowth by non-producer cells and ensure antibody productivity, cell lines were subcloned one or more generations by limiting dilution. All MAbs were of the IgG1 kappa isotype except that to peptide IhfA5 which was IgG2a/IgM kappa isotype.

2.2.1. Culture of Hybridomas

Hybridomas were cultured in medium comprised of DMEM plus 4.5 g glucose/ml (Corning) supplemented with 2 mM L-glutamine (Corning), 100 U penicillin-streptomycin/ml (Corning) and 2 mM sodium pyruvate (Corning). For long-term culture, hybridomas were maintained at a density of 1×10^5 cells/ml in medium supplemented with 10% Superlow IgG fetal bovine serum (FBS; HyClone) and 1×10^6 cells/ml were transitioned to medium with 2.5% FBS for monoclonal antibody production. After 7–10 days in FBS-limited medium, hybridomas were removed from the cell culture supernatant by centrifugation and filtration. MAbs used herein were: IhfA3$_{NTHI}$ (clone 9B10·F2.H3); IhfA5$_{NTHI}$ (clone 14G8.F5.G6); IhfB2$_{NTHI}$ (clone 7 A4.E4.G11) and mIhfB4$_{NTHI}$ (clone 12E6.F8.D12.D5).

2.2.2. Purification of MAbs

Clarified cell culture supernatants were concentrated by centrifugation in Corning Spin-X UF columns (MWCO 30,000) with a final buffer exchange in 20 mM sodium phosphate, pH 7.0. The retentate was then applied to a Hi-Trap Protein G column (GE Healthcare), washed with 20 mM sodium phosphate, pH 7.0 and IgG eluted with 0.1 M glycine-HCl, pH 2.7 subsequently neutralized with 1 M Tris-HCl, pH 9.0. Antibody was dialyzed against Dulbecco's phosphate buffered saline, pH 7.0 for 24 h at 4 °C with D-tube dialyzer MAXI (MWCO 12–14 kDa; Novagen). The concentration of each monoclonal antibody was