Mini Review

Developments in CRM$_{197}$ Glycoconjugates for Anticancer Vaccines

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Abstract

Glycoconjugate vaccines that utilize cross reactive material 197 (CRM$_{197}$) as an immunogenic carrier have shown clinical success against bacterial pathogens such as *Haemophilus influenzae* type b (Hib), Streptococcus pneumoniae, and Neisseria meningitidis. However, the translation of glycoconjugate vaccine strategies towards cancer has had limited clinical success. Preclinical efforts focused on tumor associated carbohydrate antigen (TACA)-CRM$_{197}$ conjugates include the Globo H, RM2, Thomsen-nouveau (Tn), Thomsen-Friedenreich (TF), and sialylated Thomsen-nouveau (STn) antigens. These collective efforts have shown robust conjugation chemistry and efficacious immune responses. The immune response towards these CRM$_{197}$ conjugates produced Gaussian-like distributions as a function of dose, with a maximum response at dosages in the low to medium range. This phenomenon suggests a tolerogenic effect toward the CRM$_{197}$ immunogen at high doses. An approach to resolve immune tolerance in cancer based glycoconjugate vaccines may be concomitant administration of TACA conjugates. As described by clinical research, the effects of simultaneous administration of antibacterial glycoconjugate vaccines have profound effects on immunological outcome.

Keywords: Cancer, Tumor Associated Carbohydrate Antigens, Glycoconjugates, CRM$_{197}$, Co-administered effects

Abbreviations: KLH: Keyhole Limpet Hemocyanin; DT: Diptheria Toxin; TT: Tetanus Toxoid; CRM$_{197}$: Cross Reactive Material 197; ZPS: Zwitterionic Polysaccharide; APC: Antigen Presenting Cell; Th: T Helper Cell; EF-2: Elongation Factor 2; TACA: Tumor Associated Carbohydrate Antigen; Tn: Thomsen-nouveau; TF: Thomsen-Friedenreich, STn: Sialyl Thomsen-nouveau, MPL-SE: Monophosphoryl Lipid A, C1: α-Galactosylceramide, CFA: Complete Freunds Adjuvant, IFA: Incomplete Freunds Adjuvant, CIES: Carrier Induced Epitope Suppression.

Introduction

The concept of using glycoconjugates to induce immunological responses to a carbohydrate hapten was reported in 1929 by Avery and Goebel [1]. However, glycoconjugate vaccines would not be revisited as therapeutic and prophylactic agents against bacterial infections until the 1970's and early 1980's [2-4]. Prior to these interests in glycoconjugates, licensed antibacterial vaccines consisted of isolated capsular polysaccharides which were efficacious in adults but failed to induce protection in high risk populations such as infants and children [5,6]. Glycoconjugate vaccines consist of a bacterial capsular polysaccharide conjugated to an immunogenic carrier molecule such as keyhole limpet hemocyanin (KLH), diptheria toxin (DT), tetanus toxoid (TT), cross-reactive material 197 (CRM197), as well as others [7-9]. This conjugate vaccine strategy is necessary due to the T cell independent nature of carbohydrates that have nominally elicited low affinity, short lived IgM antibodies with the exception of zwitterionic polysaccharides (ZPSs) [10-13]. Glycoconjugates are able to effectively bind to the MHCI and/or MHCII molecule on antigen presenting cells (APCs). This cellular interaction with the carbohydrate conjugate allows for the development of T cell mediated responses, and consequently leads to helper T cells (Th) which induce antibody isotype switching and immunological memory [14].

One of the more recent clinically successful carrier proteins in antibacterial glycoconjugates is CRM$_{197}$ [15]. CRM$_{197}$ is a non-toxic mutant of DT that has a single point mutation at position 52, which substitutes a glutamic acid residue with glycine [16]. The toxicity of DT is generated within a eukaryotic cell in which DT has ADP-ribosyltranferase activity towards elongation factor 2 (EF-2) causing a halt to protein synthesis, and consequently initiates cellular apoptosis. The single point mutation in CRM$_{197}$ limits this cytotoxic mechanism. Aside from being an inherently nontoxic

protein, CRM_{197} also lacks lysine residues within its T cell epitope, meaning chemical conjugation of haptens will not likely effect T cell interactions [17]. There are currently CRM_{197} glycoconjugate vaccines available for *Haemophilus influenzae* type b (Hib) (HbOC(HibTITER), Pfizer, Inc.), *Streptococcus pneumoniae* (Prevnar 13, Pfizer, Inc.), and *Neisseria meningitidis* (Menveo, Novartis Vaccines and Diagnostics, Inc.) [6,15].

The success of these bacterial glycoconjugate vaccines has helped develop similar strategies against certain epithelial cancers. Complementary to bacterial pathogens, cancer cells express a unique carbohydrate fingerprint as a result of genetic mutations that effect glycosyltransferase enzymes and chaperone proteins [18]. These unique tumor-associated carbohydrate antigens (TACAs) have been investigated within many glycoconjugate vaccines in conjunction with other approaches to involve immunological stimulation [7-9]. Unfortunately, these investigations have had limited clinical success as compared to the bacterial glycoconjugate vaccines.

TACA-CRM_{197} Glycoconjugates

One of the first preclinical works on TACA-CRM_{197} conjugates was published by Perico et al. [25] which described the synthesis of the terminal tetrasaccharide (Fucα(1→2) Galβ(1→3)GalNAcβ(1→3) Galα) of the Globo H hexasaccharide [19]. Their linker strategy began with an allyl group at the reducing end which was oxidized to a methyl ester in 3 steps. The resulting methyl ester was then reacted with ethylene diamine which was designed to react with a bifunctional bis-hydroxysuccinimidyl ester of adipic acid. This rather long linker was then used to conjugate to CRM_{197}'s many lysine residues resulting in an 11:1 ratio of hapten to protein. This conjugate, in comparison to CaMBr1-KLH conjugate, exhibited lower titers but was credited to give a more specific response towards the antigen as demonstrated by serum absorption experiments on non-CaMBr1 expressing cell lines [20]. The CRM_{197} conjugate also displayed a dose response curve, highlighting a tolerogenic effect of the immunogen at higher doses. The results of these experiments were promising as they mirrored what has been observed in the clinical studies for bacterial glycoconjugate vaccines containing CRM_{197}.

Wong and coworkers would be the next to publish a series of works that involved TACA-CRM_{197} conjugates [21]. The first one being that of the complete Globo H hexasaccharide (Fucα(1→2) Galβ(1→3)GalNAcβ(1→3)Galα(1→4)Galβ(1→4)Glcβ) moiety. The Globo H hexasaccharide featured a primary amine on the reducing end of the glycan with an alkyl spacer. The free amine was then reacted with a p-nitrophenyl ester homobifunctional linker, which was further reacted with lysine residues on the CRM_{197} protein to form a series of Globo H-CRM_{197} conjugates with epitope ratios of 1.5, 5.1, 9.8, and 15.6. Interestingly, titers induced in mice gave a Gaussian-like response curve with Globo H5.1-CRM_{197} giving the strongest IgG response. Furthermore, this conjugate, in comparison to the Globo H-KLH conjugate, gave equal or higher titer values,

and the induced antibodies were more specific towards the target antigen as demonstrated by glycan array [21].

Shortly after Globo H, Wong et al. would publish another hexasaccharide, the RM2 antigen (GalNAcβ(1→4)(NeuAcα(2→3)) Galβ(1→3)(NeuAcα(2→6))GlcNAcβ(1→3)Galβ) which has been discovered on prostate cancer cells, and its expression is closely associated with the prostate cancer staging Gleason grading system [22]. Similar to the Globo H approach, a 5-carbon spacer with a terminal amine was placed on the reducing end of the hapten. The terminal amine was first reacted with an N-hydroxysulfosuccinimide (sulfo-NHS) containing ester featuring an internal disulfide bond which was reduced after the formation of the RM2 antigen containing amide. Concurrently, the CRM_{197} was reacted with the N-(ε-maleimidocaproyloxy) sulfosuccinimide ester (Sulfo-EMCS) heterobifunctional linker which resulted in a thiol reactive maleimide functional group on the protein. Conjugation of the hapten to CRM_{197} resulted in epitope ratios of 1.0, 3.0, 4.7, and 10. Furthermore, the immune response observed for these constructs displayed a Gaussian curve with a ratio of 4.7 being the most effective.

Highly desirable specificity was also demonstrated on a glycan array. In comparison, a series of mucin related CRM_{197} conjugates were developed by Ye and coworkers featuring the Thomsen-nouveau (Tn) antigen (GalNAcα), the Thomsen-Friedenreich (TF) antigen (Galβ(1→3)GalNAcα), and the sialylated Thomsen-nouveau (STn) antigen (Neu5Acα(2→6)GalNAcα) [23-25]. The studies that featured the Tn and TF antigens focused on the modifications of the 2-N-acetyl moiety in each of the hapten structures which either displayed the natural N-acetyl structure, the propionyl, fluoroacetyl, difluoroacetyl, or trifluoroacetyl substituents [23,24].

These analogues contained an O-allylic group at the reducing end which was oxidized by ozone in methanol to produce an aldehyde intermediate. The aldehyde was then treated with the protein CRM_{197} with aldehyde reactive lysine residues to form the conjugate through reductive amination. Conjugation of the Tn hapten led to glycoconjugates with hapten ratios ranging from 7.3 to 12.7 whereas the TF hapten led to hapten ratios between 4.0 to 11.2. Interestingly, the immune response was most prominent with the N-fluoroacetyl Tn–CRM_{197} and N-fluoroacetyl TF–CRM_{197} constructs within their respective groups. These studies were extended to the STn antigen with both N-acetyl groups on the STn structure modified to contain the N-fluoroacetyl functionality [25]. Similar trends were observed as the modification of the antigen enhanced both the antibody and cellular immune response to the natural antigen.

Immune Tolerance Toward TACA-CRM_{197} Glycoconjugates

A direct comparison on the efficacy of these vaccine candidates can be rather difficult due to the number of variables present in each

individual study such as the different adjuvants chosen. In these studies, a number of adjuvants were used such as monophosphoryl lipid A (MPL-SE) [20], an analogue of α-galactosylceramide (C1) named C34 [21-25], and a combination of complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) [25]. The adjuvant C34 was used for the majority of the studies due to potent immune activating effects. As an analogue of C1, C34 targets the CD1d receptor on dendritic cells which has downstream effects to promote T cell activation, proliferation, IL-4 production, IFN-γ production, and an enhanced cytotoxicity [21]. These effects, stemmed from the CD1d-glycolipid-T cell receptor complex, promote a Th1 skew in T cell populations which are proposedly favored for anticancer responses. Another variable is the antigen loading within these glycoconjugates. Dose response curves for many successful vaccines created a Gaussian distribution with the most effective doses being within the low to medium range [26].

This was shown with Perico and coworkers as their CRM_{197} conjugate gave good results at a dose of 2.5 μg rather than 0.5 μg or 12.5 μg suggesting a tolerogenic effect of the conjugate [19,20]. Similarly, Wong and coworkers demonstrated a Gaussian distribution of immunological responses when comparing the hapten to protein ratio [21,22]. Best results were observed with ratios of 5.1 and 4.7 rather than lower or higher ratios. These results suggest that there is a "Goldilocks" dosage for these glycoconjugates which also implies an immune tolerance at a high enough dose. The phenomenon involved with these observed dose response curves has been described as carrier induced epitope suppression (CIES). CEIS involves a pre-existing immunity to the carrier which holds potential to suppress the immune response. These mechanisms may involve pre-existing antibodies that can bind to the conjugate causing steric hinderance, promotion of anti-carrier specific B cells over anti-hapten B cells, competition for resources when anti-carrier B cells over populate anti-hapten B cells thus reducing hapten specific B cells from T cell help, and the production of regulatory T cells by the carrier [27,28]. This phenomenon is clearly observed in trials pertaining to anti-bacterial glycoconjugate vaccines where vaccines are concomitantly administered or serially administered [29,30].

Due to the clinical success of these anti-bacterial glycoconjugate vaccines and because these vaccines are usually administered concomitantly, investigations on vaccine interactions and co-administrative effects have revealed both enhanced and limited efficacy of these vaccines [28]. One such study by Borrow and coworkers involved the co-administration and the sequential administration of TT and CRM_{197} conjugates which showed an enhanced immune response when conjugates were co-administered rather than administered sequentially, suggesting enhanced efficacy of a vaccine in the presence of another carrier [31]. In addition, another study performed by Dagan and coworkers involved the co-administration of a pneumococcal vaccine with an Hib vaccine using only TT as a carrier or both TT and CRM_{197} [29]. When both conjugate vaccines utilized TT a bystander interference effect was observed resulting in decreased efficacy. However, this effect was not observed when both TT and CRM_{197} was used.

Conclusion

Although CRM_{197} has had clinical success in antibacterial glycoconjugate vaccines, CRM_{197} glycoconjugates against cancer has had limited success. Most efforts to increase efficacy of TACA based glycoconjugate vaccines have been focused on creating non-natural analogues of the antigen, creating multivalent displays of a single antigen as well as multivalent displays of different antigens, discovering better adjuvants, and naturally occurring antibody recruiting epitopes [7-9]. However, there are limited studies that have observed co-administration effects of TACA-CRM_{197} conjugates as compared to antibacterial CRM_{197} glycoconjugates.

The co-administrative effects in bacterial glycoconjugate vaccines are still not well understood, but there is consensus that there are both positive and negative effects and that these effects differ in vulnerability [28]. Concomitant vaccine effects may also be translated into preclinical research involved with TACA-CRM_{197} conjugates where the co-administration of TACA conjugates could have a profound effect on observed immunity. Following the few examples herein, using CRM_{197} in combination with another immunogenic carrier may have beneficial effects and enhance immunity towards a single hapten structure and reduce tolerogenic effects observed in TACA-CRM_{197} preclinical studies [19-22]. Such examples could include the combination of KLH, TT, or ZPSs [10-13,32,33].

References

1. Avery Oswald T, Goebel Walther F (1929) Chemo-immunological studies on conjugated carbohydrate-proteins: II. Immunological specificity of synthetic sugar-protein antigens. J Exp Med 50(4): 533-550.

2. Peltol Heikki, Kaythy Helena, Sivonen Aulikki, Makela Helena P (1977) *Haemophilus influenzae* Type b Capsular Polysaccharide Vaccine in Children: A Double-Blind Field Study of 100,000 Vaccinees 3 Months to 5 Years of Age in Finland. Pediatrics 60(5): 730-737.

3. Poltola Heikki, Makela Helena P, Kaythy Helena, Jousimies Hannele, Herva Elja, et al. (1977) Clinical Efficacy of Meningococcus Group A Capsular Polysaccharide Vaccine in Children Three Months to Five Years of Age. N Eng J Med 297(13): 686-691.

4. Schneerson R, Barrera O, Sutton A, Robbins JB (1980) Preparation, characterization, and immunogenicity of Haemophilus influenzae type b polysaccharide-protein conjugates. J Exp Med 152(2): 361-376.

5. BertiFrancesco, Adamo Roberto (2018) Antimicrobial glycoconjugate vaccines: an overview of classic and modern approaches for protein modification. Chem Soc Rev 47: 9015-9025.

6. Micoli Francesca, Costantino Paolo, Adamo Roberto (2018) Potential targets for next generation antimicrobial glycoconjugate vaccines. FEMS Microbiol Rev 42(3): 388-423.

7. Heimburg Molinaro J, Lum M, ijay G, Jain M, Almogren A, et al. (2011) Cancer vaccines and carbohydrate epitopes. Vaccine 29(48): 8802-8826.

8. Yin Zhaojun, Huang Xuefei (2012) Recent Development in Carbohydrate Based Anti-cancer Vaccines. J Carbohydr Chem 31(3): 143-186.

9. Jin Ke-Tao, Lan Huan-Rong, Chen Xiao-Yi, Wang Shi-Bing, Ying Xiao-Jiang, et al. (2019) Recent advances in carbohydrate-based cancer vaccines. Biotechnol Lett 41(6-7): 641-650.

10. De Silva Ravindra, Wang Qianli, Chidley Tristan, Appulage Dananjaya, Andreana Peter R et al. (2009) Immunological Response from and Entirely Carbohydrate Antigen: Design of Synthetic Vaccines Based on Tn-PS A1 Conjugates. J Am Chem Soc 131(28): 9622-9623.

11. De Silva Ravindra, Appulage Dananjaya, Pietraszkiewicz Halina, Bobbitt Kevin R, Media Joe, et al. (2012) The entirely carbohydrate immunogen Tn-PS A1 induces a cancer cell selective immune response and cytokine IL-17. Cancer Immunol Immunother 61(4): 581-585.

12. Trabbic Kevin, Bourgault, Jean-Paul, Shi Mengchao, Clark Matthew, Andreana Peter R (2016) Immunological evaluation of the entirely carbohydrate-based Thomsen-Friedenreich-PS B conjugate. Org Biomol Chem 14(13): 3350-3355.

13. Shi Mengchao, Kleski Kristopher, Trabbic Kevin R, Bourgault Jean-Paul, Andreana Peter R (2016) Sialyl-Tn Polysaccharide A1 as an Entirely Carbohydrate Immunogen: Synthesis and Immunological Evaluation. J Am Chem Soc 138(43): 14264-14272.

14. Feng Danyang, Shaikh Absul Sami, Wang Fengshan (2016) Recent Advance in Tumor-associated Carbohydrate Antigens (TACAs)-based Antitumor Vaccines. ACS Chem Biol 11(4): 850-863.

15. Shinefield Henry R (2010) Overview of the development and current use of CRM197 conjugate vaccines for pediatric use. Vaccine 28(27): 4335-4339.

16. Malito Enrico, Bursulaya Badry, Chen Connie, Lo SurdoPaola, Picchianti Monica, et al. (2012) Structural basis for lack of toxicity of the diptheria toxin mutant CRM197. Proc Natl Acad Sci U S A 109(14): 5229-5234.

17. Jaffe Jake, Wucherer Kristin, Sperry Justin, Zou Qin, Chang Qing, et al. (2019) Effects of Conformational Changes in Peptide–CRM197 Conjugate Vaccines. Bioconjugate Chem 30(1): 47-53.

18. Pinho Salome S, Reis Celso A (2015) Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer 15(9): 540-555.

19. Lay Luigi, Panza Luigi, Poletti Laura, Prosperi Davide, Canevari Silvana, et al. (2001) Improvement of the Synthesis of Immunological Carbohydrate Vaccines Containing the Tumour Associate Antigen CaMBr1. Eur J Org Chem 2001(22): 4331-4336.

20. Perico Maria Elisa, Mezzanzanica Delia, Luison Elena, Alberti Paola, Panza Luigi, et al. (2000) Development of a new vaccine formulation that enhances the immunogenicity of tumor-associated antigen CaMBr1. Cancer Immunol Immunother 49(6): 296-304.

21. Huang Yen-Lin, Hung Jung-Tung, Cheung Sarah KC, Lee Hsin-Yu, Chu Kuo-Ching, et al. (2013) Carbohydrate-based vaccines with a glycolipid adjuvant for breast cancer. Proc Natl Acad Sci U S A 110(7): 2517-2522.

22. Chuang Hong-Yang, Ren Chien-Tai, Chao Chung-An, Wu Chung-Yi, Shivatare Sachin S, et al. (2013) Synthesis and Vaccine Evaluation of the Tumor-Associated Carbohydrate Antigen RM2 from Prostate Cancer. J Am Chem Soc 135(30): 11140-11150.

23. Song Chengcheng, Sun Shuang, Huo Chang-Xin, Li Qin, Zheng Xiu-Jing, et al. (2016) Synthesis and immunological evaluation of N-acyl modified Tn analogues as anticancer vaccine candidates. Bioorg Med Chem 24(4): 915-920.

24. Sun Shuang; Zheng Xiu-Jing, Huo Chang-Xin, Song Chengcheng, Li Qin, et al. (2016) Synthesis and Evaluation of Glycoconjugates Comprising N-Acyl-Modified Thomsen-Friedenreich Antigens as Anticancer Vaccines. Chem Med Chem 11(10): 1090-1096.

25. Song Chengcheng, Zheng Xiu-Jing, Guo Haili, Cao Yafei, Zhang Fan, et al. (2019) Fluorine-modified sialyl-Tn-CRM197 vaccine elicits a robust immune response. Glycoconj J.

26. Kimura Yasuko, Saito Michiko,Kimata Yukio, Kohno Kenji (2007) Transgenic mice expressing a fully nontoxic diptheria toxin mutant, not CRM197 mutant, acquire immune tolerance against diptheria toxin. J Biochem 142(1): 105-112.

27. Shutze MP, Leclerc C, Jolivet M, Audibert F, Chedid L (1985) Carrier-induced epitopic suppressions, a major issue for future synthetic vaccines. J Immunol 135(4): 2319-2322.

28. Findlow H, Borrow R (2016) Interactions of conjugate vaccines and co-administered vaccines. Hum Vaccin Immunther 12(1): 226-230.

29. Dagan Ron, Eskola Juhani, Leclerc Claude, Leroy Odile (1998) Reduced response to multiple vaccines sharing common protein epitopes that are administered simultaneously to infants. Infect Immun 66(5): 2093-2098.

30. Burrage Moya, Robinson Andrew, Borrow Ray, Andrews Nick, Southern Joanna, et al. (2002) Effect of vaccination with carrier protein on response to meningococcal C conjugate vaccines and value of different immunoassays as predictors of protection. Infect Immun 70(9): 4946-4954.

31. Miller Elizabeth, Andrews Nick, Waight Pauline, Findlow Helen, Ashton Lindsey, et al. (2011) Safety and immunogenicity of coadministering a combined meningococcal serogroup C and Haemophilus influenzae type b conjugate vaccine with 7-valent pneumococcal conjugate vaccine and measles, mumps, and rubella vaccine at 12 months of age. Clin Vaccine Immunol 18(3): 367-372.

32. Ragupathi Govind, Koide Fusataka, Livingston, Philip O, Cho Young Shin, et al. (2006) Preparation and Evaluation of Unimolecular Pentavalent and Hexavalent Antigenic Constructs Targeting Prostate and Breast Cancer: A Synthetic Route to Anticancer Vaccine Candidates. J Am Chem Soc 128(8): 2715-2725.

33. Stergiou Natascha, Gaidzik Nikola, Heimes Anne-Sophie, Dietzen Sarah, Besenius Pol, et al. (2019) Reduced breast tumor growth after immunization with a tumor-restricted MUC1 glycopeptide conjugated to tetanus toxoid. Cancer Immunol Res 7(1): 113-122.