

COMMENTARY

## Potential protective immunogenicity of tetanus toxoid, diphtheria toxoid and Cross Reacting Material 197 (CRM197) when used as carrier proteins in glycoconjugates

Michael Bröker

GSK Vaccines GmbH, Marburg, Germany

### ABSTRACT

When tetanus toxoid (TT), diphtheria toxoid (DT) or Cross Reacting Material 197 (CRM197), a non-toxic diphtheria toxin mutant protein, are used as carrier proteins in glycoconjugate vaccines, these carriers induce a protein specific antibody response as measured by *in vitro* assays. Here, it was evaluated whether or not glycoconjugates based on TT, DT or CRM197 can induce a protective immune response as measured by potency tests according to the European Pharmacopoeia. It could be shown, that the conjugate carriers TT and DT can induce a protective immune response against a lethal challenge by toxins in animals, while glycoconjugates based on CRM197 failed to induce a protective immune response. Opportunities for new applications of glycoconjugates are discussed.

### ARTICLE HISTORY

Received 4 August 2015  
Accepted 19 August 2015

### KEYWORDS

carrier proteins; Cross Reacting Material 197; combination vaccines; diphtheria toxoid; glycoconjugates; protective immunity; tetanus toxoid

### Introduction

During the last 30 years, conjugate vaccines have been developed to protect against encapsulated bacteria like *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae* and *Neisseria meningitidis*. Compared to plain polysaccharide vaccines, these conjugates have significant advantages as they are principally immunogenic in children below the age of 2 years, they can induce immunological memory and induce a booster effect after repeated vaccination, and they do not induce hyporesponsiveness.

While plain polysaccharides induce a T cell independent immune response, coupling of the polysaccharide antigens to a protein renders a T cell dependent immune response, one of the advantages of conjugates.

Implementation of Hib conjugate vaccine which was the first human conjugate vaccine to be developed in the 1990s has significantly reduced the burden of Hib disease and there are only a few countries left where this vaccine has not been introduced into the vaccinations calendar for infants and young children. The next conjugate which has been developed was the heptavalent pneumococcal conjugate vaccine and now 2 additional vaccines are available, a 10-valent and a 13-valent vaccine. These pneumococcal conjugate vaccines have contributed to a significant decrease in pneumococcal disease where the vaccines have been in broad use.<sup>1</sup> With the reduction of the burden of disease caused by *H. influenzae* and *S. pneumoniae*, the relative importance of disease caused by *N. meningitidis* increased. Conjugates developed against invasive meningococcal disease (IMD) have been shown to be highly effective and nowadays, monovalent serogroup C, serogroup A and quadrivalent serogroups ACWY vaccines are available and have been introduced into the vaccination calendar in many countries depending on the given epidemiological situation in the countries.<sup>2</sup>

Common to all the conjugate vaccines is that the polysaccharide of the encapsulated bacteria is the main immunological parameter for induction of protective immunity. Vaccines have been developed either using the polysaccharide antigen in its natural length or in a chemically downsized form (oligosaccharides). The polysaccharide is then chemically coupled (conjugated) to a carrier protein. Principally, every protein carrying human T cell epitopes and able to convert the T cell independent polysaccharide specific immune response to a T helper cell response could be used. However, in practice, only a few proteins have been used as carrier proteins in conjugate vaccines. The most used carrier proteins are tetanus toxoid (TT), diphtheria toxoid (DT) and the non-toxic diphtheria mutant protein Cross Reacting Material 197 (CRM197). CRM197 differs from diphtheria toxin by only one amino acid exchange at position 52, rendering the toxin to a protein with high reduction of enzymatic activity, but remaining similar immunological properties as diphtheria toxin and/or the toxoid.<sup>3</sup>

Notably, it is well known that the carrier proteins as part of the glycoconjugates themselves are immunogenic and induce a specific anti-carrier antibody response. However, in the SmPCs and leaflets of the conjugate vaccines it is clearly stated that the use of these vaccines must not be assessed as vaccination to help protect against tetanus or diphtheria. The cause for this warning is that the carrier specific immune response accompanying the evaluation of polysaccharide specific immune responses is carried out only by *in vitro* methods (like ELISA, toxin binding assays or cell culture toxin neutralization assays), but the potential protectivity has not been measured *in vivo*. During the production process, vaccines containing (unconjugated) TT and DT and foreseen to immunize against tetanus

and diphtheria are evaluated in animals (mice or guinea pigs respectively) according to the European Pharmacopoeia (Ph. Eur.) for their capability to protect against a lethal challenge by tetanus toxin or diphtheria toxin respectively and the end-point taken is paralysis or death (potency tests). In this report, some marketed glycoconjugate vaccines with the focus on meningococcal vaccines were chosen to evaluate if the DT, TT or CRM197 when used as carrier proteins in these glycoconjugates can protect animals in the routine potency tests against a lethal challenge by toxins.

## Results and discussion

Various conjugate vaccines containing either TT, DT or CRM197 as carrier proteins and present in different amounts of the conjugate vaccines were evaluated in potency tests according to the Ph. Eur. for their capacity to protect mice or guinea pigs after one or 2 injections against a challenge with tetanus toxin or diphtheria toxin respectively. The conjugate vaccines used and their characteristics were as follows: *Menactra*<sup>®</sup> (Sanofi) is a quadrivalent meningococcal conjugate vaccine (Men ACWY\_DT) containing 48  $\mu\text{g}$  of DT per human dose (0.5 ml). *Menveo*<sup>®</sup> (GSK, former Novartis Vaccines) is a quadrivalent meningococcal conjugate vaccine (MenACWY\_CRM) which contains 44  $\mu\text{g}$  of CRM197 per human dose (0.5 ml). *Menjugate*<sup>®</sup> (GSK, former Novartis Vaccines) is a monovalent serogroup C conjugate vaccine (MenC\_CRM) containing 10  $\mu\text{g}$  of CRM197 per human dose (0.5 ml). *Synflorix*<sup>TM</sup> (GSK) is a 10-valent pneumococcal conjugate vaccine including serotype 19F polysaccharide conjugated to 5  $\mu\text{g}$  DT per human dose (0.5 ml), while the polysaccharides of the other serotypes of this vaccine are either conjugated to TT or protein D of nontypable *H. influenzae*. *Menitorix*<sup>TM</sup> (GSK) is a combined vaccine containing the polysaccharide of meningococcal serogroup C and the polysaccharide polyribosyl ribitolphosphate (PRP) of *H. influenzae* type b (MenC\_TT/PRP\_TT) containing 17.5  $\mu\text{g}$  TT per human dose (0.5 ml). While *Menactra*<sup>®</sup> and *Menveo*<sup>®</sup> do not contain adjuvants, *Menjugate*<sup>®</sup>, *Synflorix*<sup>TM</sup> and *Menitorix*<sup>TM</sup> contain aluminum hydroxide or aluminum phosphate. The potency of conjugate vaccines containing DT or CRM197 was evaluated according to the European

Pharmacopoeia (Ph. Eur.) by administration of the vaccine to guinea pigs followed by challenge with diphtheria toxin according to the Ph. Eur. 2.7.6. Assay of diphtheria vaccine (adsorbed) (LD<sub>50</sub> method).<sup>4</sup> The potency of conjugate vaccines containing TT was evaluated according to the Ph. Eur. by administration of the vaccine to mice followed by challenge with tetanus toxin according to the Ph. Eur. 2.7.8. (LD<sub>50</sub> method) Assay of tetanus vaccine (adsorbed).<sup>5</sup>

When a group of immunological naïve mice was immunized with the conjugate vaccine MenC\_TT/PRP\_TT, containing a total amount of 10.5  $\mu\text{g}$  of TT per dose, all mice were protected after one injection against a lethal challenge by tetanus toxin (Table 1, line 1). Protection could also be facilitated by diluted vaccine with a reduced amount of 2.1  $\mu\text{g}$  TT per dose after only one dose of vaccine (Table 1, line 2). In order to evaluate if CRM197 when used as carrier protein can protect against a lethal challenge by diphtheria toxin, MenACWY\_CRM vaccine was evaluated and animals received one dose of vaccine containing 8  $\mu\text{g}$  CRM197. None of the guinea pigs survived the challenge and increase of the CRM197 concentration per dose to 44  $\mu\text{g}$  (the human dose) and applying 2 doses with a time interval of 14 d could not confer protection (Table 1, lines 6 and 7). MenACWY\_CRM is a nonadjuvanted glycoconjugate and next, an aluminum adsorbed MenC\_CRM vaccine was evaluated in the potency test. Neither 3 nor 10  $\mu\text{g}$  of CRM197 per vaccine dose was able to protect guinea pigs against a lethal challenge by diphtheria toxin (Table 1, lines 8 and 9).

Hereupon, protection against a lethal challenge by diphtheria toxin was evaluated for glycoconjugates containing DT as carrier protein. Upon the negative results with even 2 doses of application with MenACWY\_CRM, 2 doses of MenACWY\_DT with 48  $\mu\text{g}$  of DT (the human dose) were evaluated and 80% of the challenged animals survived. After the application of only one dose of MenACWY\_DT, the same protection rate was achieved (Table 1, lines 3 and 4). In order to analyze if the protective capacity of DT as carrier protein is limited to meningococcal vaccines only, a pneumococcal conjugate vaccine was used in which one of the 10 different serotype polysaccharides is conjugated to DT (Pnc\_DT, serotype 18F). Immunization with one dose only and an amount of 5  $\mu\text{g}$  DT

**Table 1.** Protection experiments in mice and guinea pigs to evaluate the immunogenicity of carrier proteins in glycoconjugates against a challenge with tetanus toxin and diphtheria toxin.

Conjugate vaccines	Brand names	Carrier proteins	Vaccine adjuvanted	Amount of carrier protein [ $\mu\text{g}$ ] evaluated per dose	Number of immunizations	Toxin used for challenge	Animals survived / Animals challenged
MenC/Hib	Menitorix	TT	Yes	10.5	1	Tetanus Toxin	8/8
MenC/Hib	Menitorix	TT	Yes	2.1	1	Tetanus Toxin	8/8
MenACWY	Menactra	DT	No	48	2	Diphtheria Toxin	4/5
MenACWY	Menactra	DT	No	48	1	Diphtheria Toxin	4/5
Pnc 18F	Synflorix	DT	Yes	5	1	Diphtheria Toxin	5/5
MenACWY	Menveo	CRM197	No	44	2	Diphtheria Toxin	0/5
MenACWY	Menveo	CRM197	No	8	1	Diphtheria Toxin	0/5
MenC	Menjugate	CRM197	Yes	10	1	Diphtheria Toxin	0/5
MenC	Menjugate	CRM197	Yes	3	1	Diphtheria Toxin	0/5

These tests were carried out in the laboratories of the Quality Control Department of Novartis Vaccines and Diagnostics GmbH in Marburg, Germany, which is routinely carrying out potency tests for vaccines containing diphtheria, tetanus and whole cell pertussis antigens. When diluted vaccine samples were used, the vaccines were diluted with saline directly prior to use. Groups of 5 guinea pigs (Dunkin Hartley) were used for diphtheria tests and groups of 8 mice (NMRI) for tetanus tests. For all challenge tests, positive and negative controls were included. When two injections were applied the time interval was 14 d between the first and the second dose. The challenge with 100 LD<sub>50</sub> diphtheria toxin or 100 LD<sub>50</sub> tetanus toxin was done 2 weeks after the last injection. All vaccinations and all toxin applications were done using the s.c. route.

as carrier per dose resulted in 100% protection against a lethal challenge in the potency test (Table 1, line 5).

Glycoconjugates to protect against various encapsulated bacteria have been developed during the last 3 decades and are in broad use. Antibodies to the polysaccharides and induced by meningococcal, pneumococcal and Hib conjugates have been shown to be bactericidal and help protect against the disease caused by these bacteria. Notably, the SmPCs and package leaflets strictly advise the user to regard these vaccines only as a measure to protect against the bacteria whose polysaccharide is a component of these vaccines, although it is well known that the carrier proteins used in these vaccines like TT, DT and CRM197 are also immunogenic and can induce a carrier specific immune response resulting in an increase of antibody concentration measured by in-vitro assays like ELISA. In this study, it has been evaluated, if glycoconjugate vaccines based of TT, DT or CRM197 can induce a protective immune response measured by potency tests which are used by vaccine manufacturers for the release of vaccine batches of vaccines produced to protect against tetanus or diphtheria. Conjugates based on TT and DT proved to be protective against a lethal challenge by tetanus toxin or diphtheria toxin after only one injection, even in the case that the vaccine has not been adjuvanted (MenACWY\_DT). However, the analogous quadrivalent CRM197-based MenACWY vaccine was not protective after one injection neither after 2 immunizations. Failure of protection was also revealed by an adjuvanted CRM197-based monovalent conjugate (MenC\_CRM), although the amount of carrier protein was double compared to DT based adjuvanted pneumococcal conjugate (10 vs. five  $\mu\text{g}$ ).

Schultz [6] has studied the capacity of pneumococcal conjugates with either TT or DT as carriers. These TT- and DT-based conjugates proved to induce a protective immunity against a lethal challenge with toxins and Schultz concluded that DT used as carrier can be replaced by nontoxic mutant proteins like CRM197. However, in the series of experiments shown in this contribution, it could be shown that such a general statement cannot be made, at least is this not true for the meningococcal MenC\_CRM and MenACWY\_CRM conjugates studied in the series of experiments described here.

Lockyer et al. [7] have analyzed structure-antibody recognition relationship in 9 licensed polysaccharide-tetanus toxoid conjugate vaccines. They found that high ratios of negatively charged conjugated polysaccharides did not interfere with tetanus toxoid tryptophan amino acid side-chain interaction and the recognition of epitopes on the H-chain domain or the holotoxoid was not necessarily hampered by the size of the molecule or extent of polysaccharide loading. It should be noted however, that conjugates did have significantly lower binding to tetanus toxoid specific monoclonal antibodies than did the carrier protein alone and this as well as specific conjugations sites may play a role in their immune response. Thus, the accessibility of key protein epitopes that might serve as epitopes presented to antigen presenting cells may significantly differ among different conjugates. Thus, the immune response to the carriers has to be

evaluated individually for each glycoconjugate, because the different technology and different length and structure of the poly/oligosaccharides can have an impact on the carrier protein's immunogenicity.

Based on the results from these experiments it is warranted to analyze by clinical studies if immunization with TT-, DT-, and CRM197-based conjugates can induce a booster response to TT or DT. If a protective immune response can be verified, this could contribute to a reduction of Td-booster immunizations, e.g. of adolescents or adults. Eventually, combination vaccines for infants and young children and containing TT and DT as carriers for conjugates could be developed without free TT and/or DT and which could nevertheless help protect against TT or DT. For CRM197-based conjugates, it has to be analyzed if concomitant or prior use of a DT containing vaccine or a DT-based conjugate simultaneously applied or given earlier can prime the immune response for a CRM197-based glycoconjugate. Theoretically, a vaccine combination like pertussis-hepatitis B- inactivated polio- meningococcal serogroup C\_TT-conjugate -PRP\_DT (Hib) conjugate could not only protect against pertussis, hepatitis B, poliomyelitis, meningococcal serogroup C IMD and Hib, but also against tetanus and diphtheria. Free valencies for TT and/or DT could be used to add other antigen components which can help protect against additional infectious diseases.

The development of combination vaccines such as the pentavalent DTP-Hib-HBV vaccine (mostly used in emerging countries) or the hexavalent DTaP-Hib-HBV-IPV (mostly used in wealthy countries) have various advantages over the use of monovalent vaccines or combinations with only a few quota: protection against a number of infectious diseases can be achieved very early in life with even less side effects compared to the added -up adverse reactions of multiple single injections. With increasing number of antigens in a given combination vaccine, the risk for negative interference between these components increases and the antigenicity of certain antigens may be compromised. In this contribution, it was shown that some conjugate vaccines have the capacity to protect mice or guinea pigs against a lethal challenge with tetanus toxin or diphtheria toxin, when the conjugates used were based on tetanus toxoid or diphtheria toxoid. The experiments have been carried out according to the European Pharmacopoeia and diphtheria and tetanus vaccine lots are released for human use based on these procedures. Consequently, glycoconjugates passing these tests may be regarded as able to induce a protective immune response to tetanus and diphtheria in yet unprimed human individuals. Various CRM197 based conjugate vaccines were less immunogenic and were not able to confer protection in naïve guinea pigs. Consequently, it is questionable, if these conjugates are able to confer protection in naïve human individuals. However, concomitant immunization with diphtheria toxoid or diphtheria toxoid based conjugates may render CRM197 based conjugates protective. If the concept that conjugates based on diphtheria toxoid or tetanus toxoid is accepted, this would open the development of new combination vaccines without pure diphtheria toxoid and/or tetanus toxoid and these quota may be replaced by other antigens. A new combination

vaccine could have the following feature: PRP\_diphtheria toxoid-MenC\_tetanus toxoid-aP-HBV-IPV. This “pentavalent” vaccine would help protect against Hib, diphtheria, MenC, tetanus, pertussis, hepatitis B and poliomyelitis and thus would stand for a heptavalent vaccine.

In adolescence, various countries recommend (booster) immunizations with MenC or MenACWY conjugates and these vaccines can concomitantly be given with Td or Tdap. Principally, a MenA\_diphtheria toxoid-MenC\_diphtheria toxoid-MenW\_tetanus toxoid-MenY\_tetanus toxoid vaccine could replace the concomitant application of Td and a MenA\_diphtheria toxoid-MenC\_diphtheria toxoid-MenW\_tetanus toxoid-MenY\_tetanus toxoid-aP combination is conceivable. Such a combination which offers broader protection by less injections may increase the willingness of adolescents to become vaccinated.

In the current contribution, only a limited number of conjugates (mostly meningococcal vaccines) has been evaluated and the results cannot be assigned to other conjugates. Thus, the protective immunity has to be analyzed for each conjugate and for each combination. The “dual-use” of conjugates may offer new opportunities for vaccination: broader protection by fewer antigens in the vaccine, less interferences, less complicated vaccination schedules, less side effects, broader compliance by vaccinees.

The author is fully aware of the fact that vaccination is a conservative medicine discipline and paradigms in vaccinology are changed much slower than in other medicinal domains. However, this contribution is an offer to discuss the bi-functional characteristics of carrier proteins in glycoconjugates and its potential use for the development of new combination vaccines.

### Disclosure of potential conflicts of interest

MB is an employee of GSK Vaccines GmbH, Marburg, Germany, which is a manufacturer of vaccines including diphtheria and tetanus vaccines and glycoconjugates. When the experiments were carried out and when the manuscript has been prepared, MB was an employee of Novartis Vaccines and Diagnostics GmbH.

### Acknowledgments

The author thanks Heide Reininghaus, Angelika Möbus and Harald Graf for their support in the vaccine release test.

### Declaration

In this contribution, brand names have been used for practical reasons only, and to unequivocally distinguish the products for the reader. It is not the intention of the author to express a preference for any of the vaccine products. Description of the vaccine product profiles made in this paper may be not covered with the SmPSs of the products.

### References

- [1] Vella M, Pace D. Glycoconjugate vaccines: an update. *Exp Opin Biol Ther* 2015; 15(4):529-46; PMID:25496172; <http://dx.doi.org/10.1517/14712598.2015.993375>
- [2] Safadi MA, Bettinger J, Maturana GM, Enwere G, Borrow R. Evolving meningococcal immunization strategies. *Exp Rev Vaccines* 2015; 14(4):505-17; PMID:25494168; <http://dx.doi.org/10.1586/14760584.2015.979799>
- [3] Bröker M, Costantino P, DeTora L, McIntosh ED, Rappuoli R. Biochemical and biological characteristics of Cross-Reacting Material 197 (CRM<sub>197</sub>), a non-toxic mutant of diphtheria toxin: use as a conjugation protein in vaccines and other potential clinical applications. *Biologicals* 2011; 39:195-204; PMID:21715186; <http://dx.doi.org/10.1016/j.biologicals.2011.05.004>
- [4] Assay of Diphtheria Vaccine (Adsorbed), General Method 2.7.6. Ph. Eur. 5<sup>th</sup> Edition, 209-213. Strasbourg. France: Council of Europe, 2004.
- [5] Assay of Tetanus Vaccine (Adsorbed), General Method 2.7.8. Ph. Eur. 5<sup>th</sup> Edition, 214-217. Strasbourg. France: Council of Europe; 2004.
- [6] Schultz D. Pneumococcus polysaccharide conjugates for use as vaccine against tetanus and diphtheria. U.S. Patent No. 20,030,099,672. Publication date: 29 May 2003.
- [7] Lockyer K, Gao F, Derrick JP, Bolgiano B. Structural correlates of carrier protein recognition in tetanus toxoid-conjugated bacterial polysaccharide vaccines. *Vaccine* 2015; 33:1345-52; PMID:25640334; <http://dx.doi.org/10.1016/j.vaccine.2015.01.046>