UNIWERSYTET MARII CURIE-SKŁODOWSKIEJ W LUBLINIE
Wydział Biologii i Biotechnologii



**Review of PhD thesis by Ewelina Łakomiec titled "Structures and immunochemical properties of endotoxin derived oligosaccharides and neoglycoconjugates as vaccine components"**

The PhD thesis submitted to review was completed and written in the Laboratory of Microbial Immunochemistry and Vaccines, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw. Dr. hab. Wojciech Jachymek is the supervisor of the PhD thesis.

The PhD thesis presented for evaluation comprises 117 pages, including 10 pages of the appendix containing six tables, amino acid and nucleotide sequences, and a map of the pET SUMO vector. The main body of the thesis is illustrated with 36 figures. They are compiled carefully and illustrate the text well, making it easier to understand. The PhD thesis contains another 9 tables. Placement thereof in the main part of the doctoral thesis proves that they contain relevant results documenting the achievements of the PhD Student. The dissertation also contains a list of abbreviations, tables and figures, and the index. The PhD thesis has a very concise form, without extensive descriptions of the experiments performed. With this form, Mrs. E. Łakomiec was concise enough to describe her achievements on just over 80 pages.

The PhD thesis has a typical formal structure and does not differ from the scheme accepted for experimental work. Mrs. Ewelina Łakomiec's doctoral dissertation contains the following chapters: two **Abstracts** (Polish and English version), **Abbreviations**, **Introduction**, **Aim of the work**, **Materials and Methods,** a description of the **Results,** and a comprehensive **Discussion** of the results obtained. The dissertation ends with a list of **Cited literature** (192 items) and the **List of figures and tables.** The PhD thesis is written in English and contains 1.5 page of summary in Polish. A list of scientific achievements of Mrs E. Łakomiec is enclosed in the thesis.

The first scientific descriptions of preventive vaccination and vaccines were published by Edward Jenner at the turn of the eighteenth and nineteenth centuries. A majority of the vaccine formulations developed and widely used increase human immunity against viruses. The effectiveness of vaccination can be proved by practical elimination of smallpox (*Variola vera*) or the enormous

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progress in the fight against tuberculosis as well as whooping cough - until recently (about 50 years ago) a very dangerous disease of young children.

Currently, when drug resistance or even a multiple-drug resistance of pathogenic bacteria is increasingly widespread, the use of antibacterial vaccines proves to be one of the few alternative ways of reducing the risk of bacterial infections.

Newly development subunit-containing vaccines are based on non-toxic antigens isolated from pathogenic bacteria. Antigens that have high immunogenicity and high specificity and are representatives of a pathogen or a group of pathogens are selected. Investigations on glycoconjugate-based vaccines are conducted by researchers from the Laboratory of Microbial Immunochemistry and Vaccines, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, PAS in Wroclaw, and Mrs. Łakomiec's PhD thesis addresses these issues. The title "Structures and immunochemical properties of endotoxin derived oligosaccharides and neoglycoconjugates as vaccine components" is very general and, even though it specifies the issues dealt with by the PhD Student, to get a precise idea on what subject exactly Mrs Ewelina Łakomiec worked it is necessary to go back to the **Aim of the work** chapter. In this section, we can learn that the work concerns the preparation and determination of the antigenic properties of synthesized glycoconjugates based on LPS core oligosaccharides type R1 obtained from *Escherichia coli* and inactivated tetanus toxoid (TTd) or the C-terminal fragment of *Clostridium difficile* toxin B. The first protein carrier mentioned was/is relatively often used when making subunit vaccines. Glycoconjugates based on TTd generally provide strong immune responses to the coupled oligosaccharidic portion and simultaneously causes the appearance of antibodies inactivating the toxin secreted by *C. tetani*. The properties of *Clostridium difficile* toxins A and B have been tested less frequently. Based on the available literature data, Mrs. E. Łakomiec concluded that the most suitable carrier for the planned glycoconjugates would be ensured by the C-terminal fragment of *Clostridium difficile* toxin B, which contains receptor-binding domains (RB) and this fragment is devoid of cytotoxic properties towards eukaryotic cells. Having the knowledge and tools of genetic engineering, the PhD Student obtained the designed carrier protein, i.e. a fusion protein containing the above-mentioned fragment of toxin B linked with the SUMO (small ubiquitin-like modifier) polypeptide, which is often used in overexpression of proteins in *E. coli* cells. Oligosaccharide R1, i.e. a linker between the O-specific chain and lipid A in a many enterobacterial species (e.g. some strains of *E. coli, K. pneumoniae*), was selected as a sugar component of the glycoconjugate. The choice was based on the fact that over 90% of clinical isolates of *E. coli* pathogenic strains synthesize this type of core oligosaccharide. The R1 oligosaccharide preparation obtained by depilidation of LPS (LOS) isolated from *E. coli* type R1 was a mixture containing approximately 99 % of a variously modified (with phosphate, pyrophosphate,

ethanolpyrophosphate residues) core nonasaccharide. A decasaccharide also modified with various phosphate-containing substituents was a small admixture. Compared with nonasacharide, decasaccharide additionally comprises a terminal glucosamine (GlcN), which provides the oligosaccharide with zwitterion properties. Such a distribution of electric charges in the molecules indicates their high immunogenic potential. With suitable equipment (ESI-MS, NMR spectrometer), the PhD Student very precisely characterized various glycoforms of both nona- and decasaccharide from R1 type core preparation. Thus, the results of studies conducted earlier by Vinogradowa and co-workers were complemented. This well-written fragment contains a small inaccuracy. While interpreting the chromatographic separation of the R1OS preparation, the PhD Student summed up the analysis of the first fraction obtained with the following phrase, "Fraction I consisted of Kdo monomers dimers and trimers ...". Looking at the structure of the R1core and taking into account the conditions of the hydrolysis leading to the release of the oligosaccharides from LOS, it does not seem possible that the dimers and trimers of Kdo could be obtained.

To obtain the glycoconjugate, Mrs. Ewelina Łakomiec used the commercially available tetanus toxoid which, when thoroughly cleaned by ion exchange chromatography, can be directly used for synthesis. The second carrier protein, which was a fusion protein as mentioned above, was obtained in Ludwik Hirszfeld Institute. Using suitable commercially available kits, Mrs. Ewelina Łakomiec prepared a genetic construct (vector), which was expressed in *E. coli* cells resulting in overproduction of the designed protein. Purification of the protein was done by ion exchange chromatography. I wonder why the PhD Student did not use, and did not even mention in the dissertation the possibility of using affinity chromatography with solid phase having an affinity for histidine molecules. To be honest, it is necessary to note that the method of protein purification is a matter of choice. It is important that the final product should be of adequate quality.

The procedure of mild oxidation of oligosaccharides and isolation thereof from the reaction mixture was a further time-consuming step in the preparation of the glycoconjugates. After the above-mentioned preparation steps, the PhD Student carried out synthesis and subsequent purification of the synthesized neoglycoconjugates. These steps of experiments require precision, patience, accuracy, and experience in laboratory procedures. All these procedures were carried out to obtain three glycoproteins (R1Z-TTd, R1-TTd and R1Z-TBd), which were used to formulate vaccines for immunization of rabbits. I have a question to this section of the PhD thesis, why the PhD Student did not synthesize a glycoconjugate using the R1 and TBd substrates. In no part of the doctor thesis, have I found an answer.

Mrs. Ewelina Łakomiec described two preparations obtained (R1-TTd and R1Z-TTd) using size exclusion chromatography and mass spectrometry. The description of glycoconjugate R1Z-TBd

is not documented with mass spectrometry analysis; moreover, the description of the results of the size exclusion chromatography is limited to the peak recognized (on the basis of retention time) as the condensation product. The intense signal with a retention time of 17.8 min was completely omitted in the discussion. The characteristics of the R1Z-TBd and TBd preparations by polyacrylamide gel electrophoresis combined with detection of specific material by using anti-TBd, anti-SMR1, and anti-R1Z-TTd antibodies cannot be considered as successful. The immunoblots presented do not show distinct/sharp bands. Instead, more strongly stained areas are visible on a background of strands extending along the entire gel (Figures 25). It should also be noted that electrophoretic separations of the same preparations (TBd) shown in Figures 17 and 25 greatly differ in quality. The PhD Student did not explain these differences as well as the lack of analysis of R1Z-TBd by MS.

The hybrid carrier protein (TBd) advantages has been discussed in detail. According to PhD Student, this protein is safe to use and has high potential for induction of specific antibodies. This statement has been proved by the results of cross-reactivity of anti-R1Z-TBd with a wide range of antigens (Table 6 and Fig.29). The PhD Student thought that the fusion protein should be modified to increase its immunogenicity. However, it should be noted that preparative purification of the protein (TBD) is not a simple task and, as can be seen from the text of the PhD thesis, the neoglycoconjugate analysis caused many troubles. Moreover, the antibodies obtained from anti-R1Z-TBd sera do not inactivate the toxin B of *Clostridium difficile*.

In the discussion of the results of her research, the PhD Student mentioned that further investigation should focus on decasaccharide. Its zwitterion properties give a great opportunity to obtain a vaccine by synthesis of a polysaccharide composed of decasaccharide repeating units. Experiments show that such a polymer could have good immunogenic properties and would not require a carrier protein for its biological activity

Mrs. E. Łakomiec is not limited to her laboratory duties, but on the basis of the results obtained she made plans for further research. Such an attitude characterizes a good scientist.

Another issue that has been raised in the thesis is the unexpected cross-reactivity of the anti-R1Z-TTd and anti-R1Z-TBd sera with LOS from *B. pretusis* strain 186. After extensive studies including STD NMR, Mrs. E. Łakomiec concluded that a possibility of asymptomatic infection of immunized rabbits with bacteria of the species *Bordetella bronchiseptica* should be considered to explain the experimental results. These bacteria have the same core and trisaccharide subunit within LPS as *B. pertussis* 186. Such an explanation of the observed phenomena has its justification in the fact that the sera used in the study came from animals reared in very well controlled conditions, but the conditions were not axenic. In the context of the issue, I wonder how it was possible that while

testing glycoconjugate preparations to obtain vaccines against enterobacteria the PhD Student discovered the cross-reactions of the *B. pertussis* strain 186 lipooligosacccharide with anti-R1Z-TTd and with anti-R1Z-TBd sera respectively.

In her studies on the anti-R1-TTd and anti-R1Z-TBd sera, Mrs. E. Łakomiec confirmed earlier reports provided by Professor C. Ługowski that the anti-R1 sera react with O-specific polysaccharide of *K. pneumoniae* O3 and O5. This is an interesting observation, and explanation thereof would require further studies but this problem is beyond the main topic of the thesis.

Despite the few comments on the submitted doctoral dissertation, I think Mrs. Ewelina Łakomiec has thoroughly performed the tasks specified in the **Aim of the work** section. The PhD Student showed her great knowledge and an ability of inference on the basis of the results obtained. The PhD thesis is another contribution of IITD PAN scientists and PhD students in the development of immunochemistry and immunology.

In writing the thesis, the PhD Student did not avoid a number of minor errors, inaccuracies, and inconsistencies. These have been marked in the copy of the work sent for review and could be forwarded to Mrs. E. Łakomiec. In this review, I would like to cite only a few inaccuracies/errors:

- Materials and Methods Section (p. 50); Electrophoresis was conducted at a constant voltage of 96 V, and not at a "constant current of 96 V".
- Discussion Section (p. 86, lines 5 to 7 from the bottom): The fusion protein containing the RB domain of *C. difficile* toxin B and the SUMO polypeptide from the vector was expressed in *E. coli*, but it was not "co-expression" of two proteins as could be inferred from the text.
- There are two designations for decasaccharide: R1Z and R1ZW (the latter is particularly frequent in the figures).

I claim that the objective of the PhD work has been achieved and the PhD thesis written by Mrs. Ewelina Łakomiec meets the requirements of this type of work. Therefore, I recommend the Scientific Board of the Institute of Immunology and Experimental Therapy in Wroclaw to proceed all procedural steps to confer Mrs. Ewelina Łakomiec a PhD degree. I recommend Mrs. Ewelina Łakomiec's doctoral dissertation for an appropriate prize.

Lublin, 03.10.2016

prof. Adam Choma