Vaccine 36 (2018) 3764–3771

Contents lists available at ScienceDirect

Vaccine

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

Cooperative binding of anti-tetanus toxin monoclonal antibodies: Implications for designing an efficient biclonal preparation to prevent tetanus toxin intoxication

Ivana Lukic [a], Ana Filipovic [a], Aleksandra Inic-Kanada [b], Emilija Marinkovic [a], Radmila Miljkovic [a], Marijana Stojanovic [a,*]

[a] Department of Research and Development, Institute of Virology, Vaccines and Sera – TORLAK, Vojvode Stepe 458, 11152 Belgrade, Serbia
[b] OCUVAC – LBCE, Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Kinderspitalgasse 15, 1090 Vienna, Austria

ARTICLE INFO

Article history:
Received 23 January 2018
Received in revised form 7 May 2018
Accepted 11 May 2018

Keywords:
Antibodies
Cooperative effect
Protection
Tetanus toxin

ABSTRACT

Oligoclonal combinations of several monoclonal antibodies (MAbs) are being considered for the treatment of various infectious pathologies. These combinations are less sensitive to antigen structural changes than individual MAbs; at the same time, their characteristics can be more efficiently controlled than those of polyclonal antibodies. The main goal of this study was to evaluate the binding characteristics of six biclonal equimolar preparations (BEP) of tetanus toxin (TeNT)-specific MAbs and to investigate how the MAb combination influences the BEPs' protective capacity. We show that a combination of TeNT-specific MAbs, which not only bind TeNT but also exert positive cooperative effects, results in a BEP with superior binding characteristics and protective capacity, when compared with the individual component MAbs. Furthermore, we show that a MAb with only partial protective capacity but positive effects on the binding of the other BEP component can be used as a valuable constituent of the BEP.

1. Introduction

Tetanus is a severe and often fatal disease that can develop after exposure to the tetanus toxin (TeNT),[1] a neurotoxin produced by the anaerobic bacterium *Clostridium tetani.* Mandatory vaccination against tetanus was introduced worldwide and was a crucial measure that led to a significant decline of cases during the second half of the 20th century [1]. Nevertheless, the prevalence of the disease is not negligible, particularly in the developing world [2]. Tetanus cases are mostly reported in elderly patients, as immunity to tetanus disappears gradually over time [3]. In addition, tetanus outbreaks related to injuries seen during natural disasters such as earthquakes and tsunamis have been documented by the WHO [4].

TeNT intoxications can be efficiently treated with various polyclonal antibody (PoAb)[2]-based therapies [4,5]. The WHO recommends treatment with human immunoglobulin preparations [6]. However, polyclonal preparations of animal origin, consisting of TeNT-binding ($F(ab)_2$) fragments, are still used when human-derived preparations are not available. The manufacture and use of animal-derived therapeutic products has several problems: (i) a long immunization procedure, (ii) batch-to-batch variation in the therapeutic efficacy [7], (iii) potential patient hypersensitivity, and (iv) the risk of acquiring certain zoonosis [8]. In contrast, human-derived products significantly reduce the risk of reactogenicity. They also carry certain difficulties, such as a requirement for intensive pathogen-focused control and a tedious large-scale production process.

Currently, monoclonal antibodies (MAbs)[3] are considered the reagent of choice for tetanus prevention/treatment [9]. It has already been shown that a high affinity toward TeNT does not automatically result in a protective effect against TeNT intoxication, since high affinity is only one of the requirements [10–12]. To be considered protective, an antibody must not only recognize TeNT with sufficiently high affinity but also prevent the initial step in TeNT intoxication (i.e., the interaction of TeNT with gangliosides exposed on the surface of neurons) [13]. This is in line with recommendations for MAbs, which are expected to provide protection against botulinum toxin, a molecule that closely resembles TeNT, both structurally

* Corresponding author.
E-mail address: mstojanovic@torlak.rs (M. Stojanovic).

[1] TeNT – tetanus toxin.
[2] PoAbs – polyclonal antibodies.
[3] MAb – monoclonal antibody.

https://doi.org/10.1016/j.vaccine.2018.05.058
0264-410X/

and functionally [14]. MAb-based therapeutic products have the advantage over PoAbs of being highly purified and well-characterized, and they are generally associated with a lower risk of side effects due to cross-reactivity.

However, the homogenous specificity of single MAb-based preparations can also be a problem. Oligoclonal MAb preparations are less sensitive to structural changes in the antigen (conformational, glycosylation-associated, alterations in the primary structure) than the corresponding single MAb-based preparations [15]. In addition, their characteristics can be more efficiently controlled than PoAb preparations [16,17]. Further, the presence of multiple MAbs that can simultaneously bind one antigen allows for better opsonization and, consequently, faster removal of the target antigen [18–20].

When more than one MAb binds simultaneously to a flexible antigen such as a protein, a phenomenon known as a cooperative binding can be observed. Positive cooperative binding is one of the main reasons for the superiority of oligoclonal combinations over single MAb-based preparations, assuming that two MAbs can bind to non-overlapping epitopes of the same antigen [17]. The binding of the first MAb induces a conformational change that affects the epitope of the second MAb, resulting in stronger binding to its paratope. The idea of using well-defined oligoclonal MAb-based preparations instead of single MAbs has already been considered for the treatment of infections caused by *Clostridium botulinum* [21–23] and *Bacillus anthracis* [24]. In both cases, better protection was achieved by using oligoclonal preparations than single MAbs.

The aims of this study were to evaluate the binding characteristics and protective capacity of TeNT-specific MAb-based biclonal equimolar preparations (BEPs),[4] to determine how these binding characteristics correlate with the observed *in vivo* effects, and to investigate whether these preparations possess any advantages over single MAb-based preparations.

2. Material and methods

2.1. TeNT and anti-TeNT MAbs

TeNT was purified using hydrophobic chromatography, as described in our previous work [13]. The source of TeNT was a supernatant obtained after filtration of a *C. tetani* culture through a 0.2-μm filter, part of the standard TTd vaccine manufacturing process at the Institute of Virology, Vaccine and Sera – Torlak (Belgrade, Serbia).

Four murine TeNT-specific MAbs, designated as MAb33, MAb39, MAb51 and MAb71, were selected for this study. The selected MAbs belong to the same IgG subclass, have similar circulatory half-lives, possess sufficient affinity for TeNT to permit its neutralization in solution, and are all capable of providing a certain degree of protection against TeNT intoxication [13,25]. These MAbs were used to prepare six BEPs: MAb33/MAb39, MAb33/MAb51, MAb33/MAb71, MAb39/MAb51, MAb39/MAb71 and MAb51/MAb71.

2.2. Determination of the additivity indices for selected anti-TeNT MAbs

Determination of the additivity indices (AIs)[5] for selected MAb pairs was carried out by measuring their simultaneous binding to TeNT by ELISA, as described below. First, MaxiSorp plates (Nunc; Roskilde, Denmark) were coated with TeNT (1 μg mL^{-1}, 50 μL per well, 4 °C, overnight), the selected MAbs were biotin-labeled (MAb-B),[6] and their saturating concentrations were determined by testing their binding to the TeNT-coated plates at various concentrations.

After determining the saturating concentrations, microtiter plates (MaxiSorp, Nunc) were coated with TeNT (1 μg mL^{-1}, 50 μL per well, 4 °C, overnight), blocked with BSA/PBS (1% w/v) for 2 h at a room temperature (RT), and washed with Tween 20/PBS (0.05% v/v, 4 × 200 μL per well). The MAb-containing samples were then added to the wells (50 μL per well) and incubated for 1 h at RT, followed by another washing step (0.05% Tween 20/PBS; 4 × 200 μL per well). The extrAvidin-peroxidase/OPD system (Sigma-Aldrich, Germany) was used for reaction visualization. The reaction was stopped by the addition of 2 M H_2SO_4 (50 μL per well), and the absorbance was read at 492/620 nm ($A_{492/620}$). Two types of samples were prepared: 1) samples containing only one MAb at its saturating concentration, and 2) samples containing two out of four selected MAbs, each at their saturating concentrations.

The AI for the defined MAb pair was calculated according to the following equation: $AI = \{[A_{MAb1+MAb2} - (A_{MAb1} + A_{MAb2})/2]/(A_{MAb1} + A_{MAb2})/2\} \times 100$, where $A_{MAb1+MAb2}$ represents the $A_{492/620}$ value for the sample containing two MAbs, each at saturating concentration, while A_{MAb1} and A_{MAb2} represents the $A_{492/620}$ value for the samples containing only one MAb at saturating concentration [26]. By convention, an AI value below 20 implies that the two MAbs recognize the same or closely linked epitopes and cannot bind the antigen simultaneously due to steric hindrance. For MAbs with AI values between 20 and 40, simultaneous binding is possible, but a certain degree of steric hindrance may still occur. An AI>40 implies that the two MAbs can bind to the antigen simultaneously without any obstacles [26].

2.3. Cooperative binding of anti-TeNT MAbs

The mutual influence of selected MAbs after binding to TeNT was evaluated through pair-wise analyses. The MAb being tested (its biotin-labeled form, MAb-B, in concentrations ranging from 0.03 μg mL^{-1} to 1 μg mL^{-1}) was incubated (1h at RT) with TeNT (0.5 μg mL^{-1}) or with TeNT that had been pre-incubated (1h at RT) with one of the three other selected MAbs (0.5 μg mL^{-1} TeNT + MAb at 0.5 μg mL^{-1}; TeNT/MAb). After incubation, a saturated solution of ammonium sulfate (pH = 7) was added (up to a final saturation of 40%) to the TeNT/MAb/MAb-B samples, which were incubated overnight at 4 °C. To separate precipitated immunocomplexes, the samples were centrifuged at 20,000 × *g* for 30 min.

The concentration of free MAb-B in the supernatant was determined by direct ELISA, as follows: MAb-B-containing supernatants (50 μl per well) were added to MaxiSorp plates (Nunc) and incubated for 1 h at RT. Plates were then blocked with 1% BSA/PBS w/v (200 μl per well, 2 h at RT) and washed with 0.05% Tween 20/PBS v/v (4 × 200 μl per well). Reactions were visualized by means of the extrAvidin-peroxidase/OPD system, and the absorbance was read at 492/620 nm ($A_{492/620}$). Serial dilutions of the MAb-B (at concentrations ranging from 1 μg mL^{-1} to 0.03 μg mL^{-1}) were directly adsorbed to microtiter plates after being processed in an identical fashion (blocking with 1% BSA in PBS, washing) to generate a standard curve.

The binding data are presented as Klotz plots, with the percentage of bound TeNT plotted against free MAb-B concentrations in logarithmic form. The concentrations of free MAb33-B, MAb39-B, MAb51-B and MAb71-B in solution, when 50% of TeNT was bound ($MAb33_{f,50\%}$, $MAb39_{f,50\%}$, $MAb51_{f,50\%}$, and $MAb71_{f,50\%}$, respectively),

[4] BEP – biclonal equimolar preparation.
[5] AI – additivity index.
[6] MAb-B – biotin-labeled MAb.