Molecular Immunology 68 (2015) 185–193

Contents lists available at ScienceDirect

Molecular Immunology

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

Investigation of a panel of monoclonal antibodies and polyclonal sera against anthrax toxins resulted in identification of an anti-lethal factor antibody with disease-enhancing characteristics

Parul Kulshreshtha[a], Ashutosh Tiwari[b,1], Priyanka[a,1], Shikha Joon[c], Subrata Sinha[d], Rakesh Bhatnagar[a,*]

[a] Laboratory of Molecular Biology and Genetic Engineering, School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India
[b] Present address: Centre for Biodesign, Translational Health Science and Technology Institute, Gurgaon, India
[c] Structural and Computational Biology Laboratory, Department Of Biotechnology, Netaji Subhas Institute of Technology, New Delhi 110078, India
[d] National Brain Research Center, Manesar, India

ARTICLE INFO

Article history:
Received 18 May 2015
Received in revised form 14 July 2015
Accepted 17 July 2015
Available online 9 September 2015

Keywords:
Polyclonal antibody
Monoclonal antibody
Disease-enhancement
Anthrax

ABSTRACT

Hybridomas were created using spleen of mice that were actively immunized with rLFn (recombinant N-terminal domain of lethal factor). Later on, separate group of mice were immunized with rLFn to obtain a polyclonal control for passive immunization studies of monoclonal antibodies. This led to the identification of one cohort of rLFn-immnized mice that harboured disease-enhancing polyclonal antibodies. At the same time, the monoclonal antibodies secreted by all the hybridomas were being tested. Two hybridomas secreted monoclonal antibodies (H10 and H8) that were cross-reactive with EF (edema factor) and LF (lethal factor), while the other two hybridomas secreted LF-specific antibodies (H7 and H11). Single chain variable fragment (LETscFv) was derived from H10 hybridoma. H11 was found to have disease-enhancing property. Combination of H11 with protective monoclonal antibodies (H8 and H10) reduced its disease enhancing nature. This *in vitro* abrogation of disease-enhancement provides the proof of concept that in polyclonal sera the disease enhancing character of a fraction of antibodies is overshadowed by the protective nature of the rest of the antibodies generated on active immunization.

1. Introduction

The two anthrax toxins, edema toxin and lethal toxin, are derived from the combination of three different factors namely, protective antigen (PA, 83 kDa), edema factor (EF, 89 kDa) and lethal factor (LF, 90 kDa). Anthrax toxins function like the AB type of toxins wherein PA is the B domain of both toxins and LF or EF form the A domain for lethal toxin (LT)/edema toxin (ET), respectively (Collier and Young, 2003). PA binds to the cell surface receptors namely, tumor endothelial marker (TEM) 8 and capillary morphogenesis protein (CMG) 2. TEM8 and CMG2 interact with LDL-receptor related protein (LRP) 6 for endocytosis of EF and LF. On entering the cell, EF acts as a calmodulin-dependent adenylate cyclase (Leppla, 1982) that causes a prolonged increase of cytosolic cyclic adenosine monophosphate (cAMP). LF is a metalloprotease that cleaves most isoforms of mitogen-activated protein kinase kinases (MAPKK/MEK) close to their N-terminus (Pellizzari et al., 1999; Friedlander, 1986). By secreting these two toxins, pathogen ascertains death of the host (Firoved et al., 2005; Firoved et al., 2007; Fang et al., 2006; Smith et al., 1955). Once the systemic levels of the toxins rise, antibiotics alone cannot save the host (Smith et al., 1955). Therefore, toxin-specific inhibitors have to be developed. In this wake, monoclonal antibodies have been developed for the neutralization of toxic effects of anthrax toxins (Chow and Arturo Casadevall, 2012).

With a view to develop therapeutic monoclonal antibodies against anthrax toxins, we developed hybridomas by using spleens of mice immunized with rLFn. After the development of hybridomas, we immunized another group of mice with rLFn to obtain

Abbreviations: AP, alkaline phosphatase; ELISA, enzyme-linked immuno sorbent assay; EF, edema factor; ETNA, edema toxin neutralization assay; FBS, fetal bovine serum; HRP, horse radish peroxidase; domain I of LF, LFn; LTNA, lethal toxin neutralization assay; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PA, protective antigen; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; TMB, 3,3′,5,5′-tetramethylbenzidine; NBT/BCIP, nitroblue tetrazolium dye/ 5-bromo-4-chloro-3-indolyl phosphate.

* Corresponding author. Fax: +91 11 26717040.
E-mail address: rakeshbhatnagar@mail.jnu.ac.in (R. Bhatnagar).

[1] These authors have contributed equally.

http://dx.doi.org/10.1016/j.molimm.2015.07.019
0161-5890/

polyclonal sera to use as a positive control in passive immunization studies. We did a pilot study to standardize the least dilution at which the polyclonal sera was protective. The result of this study was astonishing. It was found that instead of being protective, the sera from one particular group was making the mice die faster than the control mice when challenged with *Bacillus anthracis* Sterne strain, 34F2. At the same time, we were characterizing the monoclonal antibodies as well. It was found that two monoclonal antibodies, H10 and H8, were cross-reactive with LF and EF. We created a single chain variable fragment (LETscfv) which cross-reacted with LF and EF. The other two monoclonal antibodies could recognize only LF (H7 and H11) out of which H11 had disease-enhancing characteristics. The objective of this study was to characterize the disease enhancing nature of H11 antibody alone or in combination with protective monoclonal and single chain antibody.

2. Materials and methods

2.1. Animal maintenance

Balb/c J mice were obtained from National Institute of Nutrition, Hyderabad, India. All mice were maintained in the animal house facility of Jawaharlal Nehru University. The experiments in this study were approved by the animal ethical committee of Jawaharlal Nehru University, Delhi, India. Research workers strictly adhered to the International standards of animal Ethics while performing all the animal experiments.

2.2. Protein purification

rLF, rEF, rPA and rLFn were purified as per the protocol described in our previous studies (Chauhan et al., 2001; Gupta et al., 1998; Kumar et al., 2001; Kulshrestha and Bhatnagar, 2011). Antibody purification was done by standard method described in our previous study (Kulshrestha and Bhatnagar, 2011). LETscFv was purified by in-vitro refolding of inclusion bodies as per the techniques described previously (Lee et al., 2006; Sun et al., 2012). All the proteins were stored at −80 °C until use.

2.3. Active immunization of mice with rLFn and rPA

Six to eight weeks old female Balb/cJ mice, 5 in number, were immunized subcutaneously with rLFn (10 μg or 20 μg/mouse) mixed with the complete Freund's adjuvant (CFA) in 1:1 ratio on day 0 followed by boosters of rLFn mixed with Incomplete Freund's Adjuvant (IFA) in 1:1 ratio on day 15, 42 and 58, the sera was collected on day 0, 14, 28, 57. Control mice, the adjuvant only group, were similarly immunized with PBS mixed with adjuvant (first with CFA and then with IFA) in 1:1 ratio. One group was immunized with rPA (10 μg mixed with CFA/IFA in 1:1 ratio).

2.3.1. Passive immunization study with polyclonal sera

Adjuvant-specific serum, the serum from group of mice immunized with 10 μg of rLFn (Poly anti-10 LFn) and the serum from group of mice immunized with 20 μg of rLFn (Poly anti-20 LFn) was diluted with PBS. For pilot study, Poly anti-20 LFn was diluted hundred times, thousand times and ten thousand times. In this study, the mice ($n = 6$) were immunized i.p. on day 0 with 200 μl of Poly anti-20 LFn (1/100, 1/1000, 1/10,000) or with 200 μL pre-immune sera (1/100). For combination study, adjuvant-specific serum, the polyclonal anti-PA serum (Poly anti-PA), Poly anti-10LFn and Poly anti-20 LFn were diluted 100 times with PBS. The mice were immunized (i.p.) with 200 μL of diluted pre-immune sera or Poly anti-10LFn or Poly anti-20LFn or Poly anti-PA or combination of Poly anti-PA (1/100) and poly anti-10 LFn (1/100) or combination of Poly anti-PA(1/100) and Poly anti-20 LFn (1/100). Twenty four hours after immunization, each mouse was challenged intraperitoneally with 10^8 CFU of live *B. Anthracis*, Sterne strain (pXO^{1+}, pXO^{2-}) vegetative cells as per the model described previously (Shoop et al., 2005).

2.3.2. Estimation of IgG isotypes for polyclonal sera

Isotype ELISA was carried out by coating each well of microtitre plate with 500 ng/well of rLF protein. After blocking with 2% BSA, a serial 2-fold dilution of pooled sera was used and incubated for 2 h at 37 °C. After washing, goat anti-mouse IgG isotypes-HRP conjugate (IgGl, IgG2a, IgG2b; Sigma) were added at a dilution of l:5000 and incubated at 37 °C for 1 h. Color was developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (BD OptEIA), reaction was stopped with 1 M HCl and absorbance was measured at 450 nm in a microplate ELISA reader (Tecan, Sunrise).

2.4. Creation of Hybridoma

4–6 week old female Balb/c mice were immunized with 20 μg of rLFn. On the accomplishment of high titer serum response to rLFn, mice were sacrificed and the extracted splenocytes were fused with mouse myeloma to obtain hybridoma by method described previously (Kulshrestha and Bhatnagar, 2011). Hybridoma clones were named as H8, H7, H10 and H11. The isotype of the monoclonal antibody was determined by using Isostrip Mouse monoclonal Antibody isotyping kit (Santa Cruz Biotechnology) as per the manufacturer's protocol.

2.5. Creation of single chain variable fragment

H8 and H10 cell lines were grown in Iscove's Modified Dulbecco's Medium (Sigma–Aldrich) Fupplemented with Fetal Bovine Serum in 25 cm^2 flask (Becton and Dickenson). At confluence, hybridoma cells were collected in TRIzol reagent (Invitrogen) for RNA isolation as per manufacturer's instructions. Complementary DNA was amplified as per manufacturer's instruction of first strand cDNA synthesise kit (Omniscript RT kit, Qiagen). The forward and the reverse primers compatible with the pCANTAB5E phagemid vector were used to amplify the V_L (1: 5′-ATTGTGATGACCCAGACT-3′and 2: 5′-TCGACTTGCGGCCGCCCGT-TTKAKYTCCARCTTKGTSCC-3′) and V_H (3: 5′-GCAACTGCGGCCC-AGCCGGCCATGGCCGAGGTGCAGCTKCAGCTGCAGCAG-3′ and 4: 5′-TGARGAGACRGTGACTGARGT-3′) fragments. The linker primers(48:5′-GGTGGTGGTGGGAGCGGGGTGGCACTGGCGGC-GGCGGATCT-3′,49: TCAGTCACYGTCTCYTCAGGTGGTG-GTGGGAGC,50:5 -GTCTGGGTCATCACAATAGATCCGCCGCCGCC-3′) were used to amplify the linker. These primers were utilized to obtain a 750 base pair scFv as per the procedure and PCR conditions described previously (Bose et al., 2003). Total PCR eluate was digested with *SfiI* and *NotI* and cloned in pCANTAB5E vector digested with same restriction enzymes. The ligated DNA was transformed into chemically competent cells of *Escherichia coli* TG1 by standard procedure. The transformed cells were plated on 2XYT medium agar plates containing ampicillin (50 mg/mL), 2% glucose and incubated overnight at 37 °C. The following day, colonies were scraped into 1 mL of 2XYT medium with 20% glycerol and stored at −70 °C. Phages were Rescued and selected by Phage ELISA as per the protocol described previously (Bose et al., 2003). The 750 bp fragment of LETscFv was subcloned in pET22B + expression vector and purified as per the protocol described previously (Bose et al., 2003; Mukhija and Garg, 1999). Selected clones were sequenced by dideoxy DNA sequencing. The sequence was analysed by IgBLAST.