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Reviewing the role of peptide rarity in bacterial toxin immunomics

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1. ABSTRACT

In the past decade, renewed efforts have been made toward the development of vaccines against cancers, infectious agents, autoimmune diseases, and allergies. These efforts have led to the accumulation of numerous peptide sequences experimentally validated as epitopes. However, the factors that render a peptide immunogenic and, more generally, the nature of the antigen-antibody recognition process remain unclear. Based on the hypothesis that potential epitopes correspond to rare sequences and/or structures, we analytically review the data on the molecular structure and properties of immunoreactive sequences derived from (or evoked by) *Clostridium tetani*, *Bacillus anthracis*, and *C. botulinum* toxins. A cohesive picture emerges when peptide motifs are absent or scarcely represented in endogenous self proteins as they define a common immune signature of bacterial toxin B-cell immune determinants. Likewise, the scientific literature also shows that the heavy chain third complementarity-determining regions (CDR3s) from antitoxin antibodies are characterized as being formed by rare peptide sequences. The present meta-analysis aims to provide a key to understanding the molecular nature of the immune recognition process and, in turn, to contribute to the development of effective and safe peptide-based diagnostic tools and vaccine applications.

2. INTRODUCTION

Bacterial protein toxins are powerful poisons. They are known to have high activity even at low concentrations. For example, the lethal dose of tetanus toxin (TT) is 4 x 10^{-8} mg (1), while the minimum oral dose of strychnine lethal to humans ranges from 30 to 120 mg (2). Bacterial toxins also have very specific cytotoxic activity: tetanus and botulinum toxins attack only neurons (3, 4), whereas staphylococcal enterotoxins function at the gastrointestinal level (5). Several bacterial toxins are known immunomodulators and act on T-cells and antigen-presenting cells (APCs), leading to the derailing of the host's immune functions (6). In addition, many bacterial toxins, such as colicins and diphtheria toxin, promote cell death (7, 8). Currently, these abilities are being exploited therapeutically in the selective killing of cancer or virally infected cells (9-11).

A number of studies are underway that aim to develop effective, specific and safe antitoxin vaccines (12, 13). However, certain bacterial toxins remain a threat, such as botulinum and anthrax toxins, which have potential use as biological weapons (3). Maternal and neonatal tetanus are significant causes of maternal and neonatal mortality, claiming approximately 180,000 lives worldwide every year

(14), and the numbers are no better when considering the mortality from pertussis, pneumococcus, and other bacterial pathogens (15-18). In addition, despite free universal vaccination (19), severe tetanus it still a cause of concern, despite a protective level of toxin-neutralizing antibodies (20-25). *B. pertussis* infection and reinfection still occurs despite immunization (26), and, in general, susceptibility to diseases preventable by vaccine (19) pose crucial questions that remain unanswered. Also, antitoxin antibody levels vary during the course of an infection (27) and add to the cross-reactivity phenomena (28), possibly indicating diagnostic sensitivity and therapeutic specificity as well as correlations between humoral antitoxin levels and the course of the infection (29). Hence, understanding the peptide targets and the antigen-antibody interactions that occur during bacterial infections is a key priority in further understanding toxin-neutralizing antibodies, the extent of the humoral response, and the course of the infection.

This review presents an analysis of the peptide immunomics of bacterial toxins as part of the search for structural/functional features that can aid in understanding immunological properties of toxins and that will be useful in developing effective diagnostic/therapeutic applications.

3. EXTRACTING INFORMATION FROM IMMUNOPEPTIDOMES AND RELATED DATABANKS USING THE LOW-SIMILARITY CONCEPT

Locating an epitope along an antigen has been (and is) performed almost exclusively by an empirical multistep procedure that includes antigen fragmentation by chemical/enzymic cleavage (or, alternatively, synthesis of antigen fragments), and blotting of the antigen fragments followed by specific immunoassays (30). By applying epitope mapping procedures, the combined effort of a number of laboratories worldwide has led to a large-scale accumulation of epitopic sequences in specific databases, such as the Immune Epitope Database (http://www.immuneepitope.org) (31, 32). These studies have been integrated with bioinformatic analyses for predicting immune epitopes to be used in the design of effective and safe vaccines as well as in diagnoses of bacterial infection and laboratory analyses (33-36). However, *in silico* epitope prediction tools have produced more confusion than conclusive data (37, 38). Notwithstanding the amount of detailed immunological bioinformation, the number of organisms analyzed, the richness of functional notations and correlations, the final results are modest. Two crucial issues in immunology remain unresolved: the molecular/functional definition of an epitope, and the development of a conceptual framework that explains how epitopes are specified and recognized in the course of an immune reaction. In the face of a plethora of methods, rationales, and algorithms formulated to characterize immune response to peptide sequences (36), the rules governing the potential of a peptide to evoke an immune response are unclear (39). Also, although a practically infinite number of antibodies can be generated by molecular gene-rearrangement processes, it is still not clear what causes a paratopic sequence to specifically recognize (and interact with) an antigenic epitopic sequence.

We analyze epitopic sequences assuming a five amino acid grouping as a minimal length for an immune unit (39, 40), and applying the concept that only rare pentapeptides have an immunological potential whereas frequent pentapeptides are immunotolerated (39, 41-43). The similarity profile between the antigen pentapeptides and the host proteome (e.g., the pentapeptide sequence identity percentage) is measured by considering the full set of proteins forming the host proteome as a single giant polyprotein and then searching for instances of the same antigen pentapeptide (44). Any occurrence is termed a match. The number of matches is inversely related to the pentapeptide's immune potential *versus* the host proteome. Using this approach, relationships have already been validated between peptide rarity and peptide immunoreactivity in a number of experimental disease models (45-53). This review presents a discussion of the role of peptide rarity in bacterial toxin immunomics.

4. *CLOSTRIDIUM TETANI* TOXIN EPITOPES: A SET OF RARE MOTIFS

A feature of special interest in immunology, bacterial toxins have marked antigenic and immunogenic properties, i.e., they have the capability of inducing an immune response in the host (immunogenicity) and the ability to react specifically with the antibody's paratopic sites (antigenicity) (54, 55). *De facto*, the birth of immunology is considered to have been in 1890, the year in which Behring and Kitasato published their discovery of tetanus antitoxin serum (56). They showed that the antibodies produced by one animal could be used to immunize and cure another. This paper laid the foundation for a rational approach to infectious disease therapy, and the antibody era began. Progressively, the immunological debate shifted toward the exact definition of bacteria-*versus*-host relationships. Translated into molecular terms, the dissection of the peptide-peptide interaction(s) between the bacterial toxin(s) and the host's antibodies became (and remains) a main focus of immunological research.

Using this scientific framework and mining for information on the biological features that define TT immunogenic properties, we explored the TT-sequence identity profile *versus* the human proteome. The resulting sequence-to-sequence profile is displayed in Figure 1. It can be seen that TT heavy chain presents regions formed by pentapeptides repeatedly present in the human proteome alternating to fragments formed by pentapeptides scarcely represented, or absent, in the proteome (Figure 1, panel A), thus clearly showing that numerous TT fragments are formed by consecutive, overlapping, rare pentamers. The wavelike behavior of the TT pentapeptide shared with the human proteome stands out in the magnified toxin segment (TT a.a. 801-1,000) shown in Figure 1, panel B.

Using the data from Figure 1 as a map, experimentally validated TT-derived human B-cell epitopes (57) were annotated along the similarity profile comparing

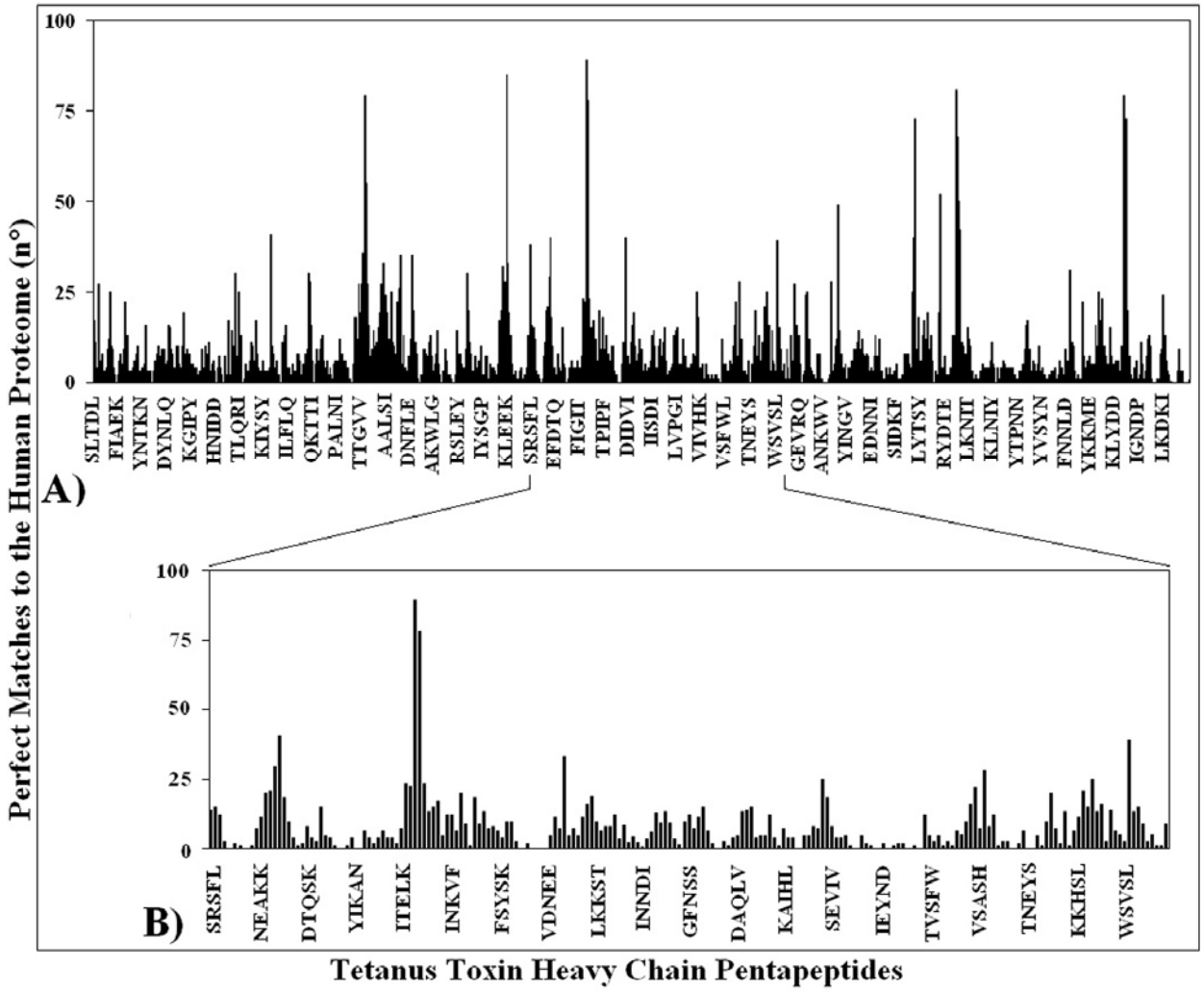

Figure 1. TT similarity profile *versus* the human proteome at the pentapeptide level. A) TT heavy chain sequence aa 458-1,315 (UniProtKB/Swiss-Prot accession: P04958); B) TT heavy chain sequence aa 801-1,000. Columns indicate the number of TT pentapeptide occurrences in the human proteome. For further details see Refs. 46-53.

TT and the human proteome. An analysis of the immunogenicity pattern along this similarity profile is shown in Figure 2. Figure 2, panels A and B, clearly show that the TT epitopes that are immunorecognized by human sera fall into the TT peptide areas formed by the pentapeptide almost uniquely owned by TT and rarely (or never) found in human proteins.

5. ANTI-TETANUS TOXOID ANTIBODY CDR3 SEQUENCES: MIRRORING THE EPITOPIC PEPTIDE RARITY

In response to immunogenic sequences and/or structures, B cells produce antibodies, e.g., proteins able to specifically bind to the triggering immunogenic sequences and/or structures. The molecular immunogen-antibody circuit lies on a random gene rearrangement (the V, D, and J gene segments for the antibody H chain, and the V and J gene segments for the antibody L chain) and on nontemplate insertion/deletion of nucleotides in the joining regions during the gene rearrangement process. The immunogenic features that trigger the sequence of events leading to the acquisition of combinatorial diversity and somatic modification of the antibody V region are not clear. In this conceptual framework, Poulsen *et al.* explored the diversity of the human polyclonal antibody responses against tetanus toxoid by sequencing the heavy chain CDR3s from two healthy volunteers boosted with a tetanus toxoid vaccine (58). Comparison of the data from Poulsen *et al.* to the human proteome using perfect pentapeptide matching reveals a major structural restriction unifying the human polyclonal response against tetanus toxoid: the use of rare pentapeptide fragments (Table 1).

In synthesis, Figure 2, panels A and B, and Table 1 indicate that, independently of the constant epitopic nature (Figure 2, panels A and B) or the highly variable polyclonal response (Table 1), experimentally defined tetanus toxin epitopes and anti-tetanus toxoid CDR3 sequences constantly harbor peptide blocks formed by pentapeptides that are rarely, or never, found in the host proteome.

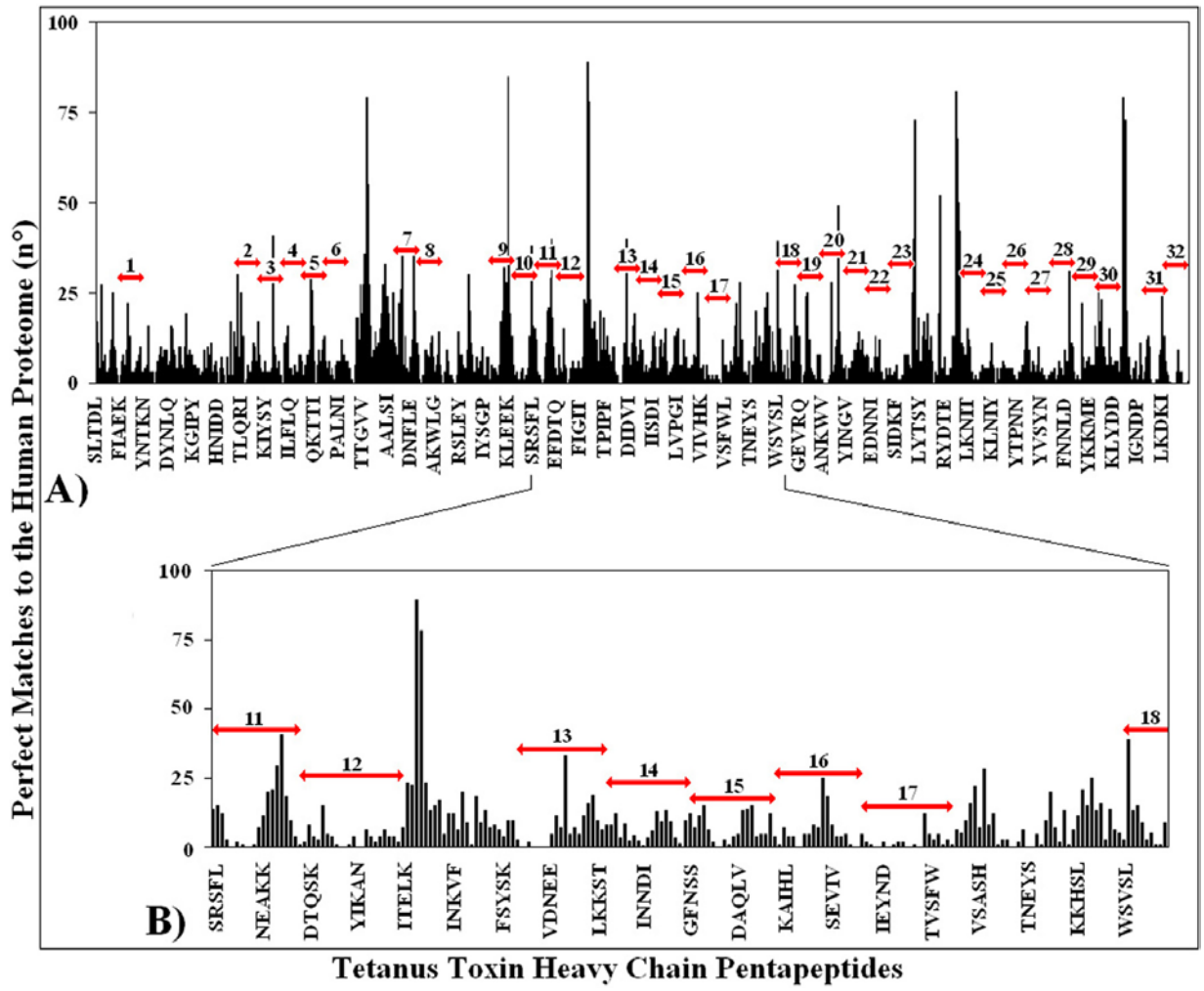

Figure 2. Location of TT epitopes immunorecognized by human sera along the similarity profile of TT *versus* the human proteome. A) TT heavy chain sequence aa 458-1,315 (UniProtKB/Swiss-Prot accession: P04958); B) TT heavy chain sequence aa 801-1,000. TT pentapeptide occurrences in the human proteome is reported in ordinate. Epitope mapping data from Ref. 57. Numbered arrowed red lines indicate the TT epitopic sequences corresponding to IEDB ID: 1) 16155; 2) 51254; 3) 2642; 4) 70166; 5) 7774; 6) 71156; 7) 61408; 8) 56528; 9) 21599; 10) 11711; 11) 60800; 12) 11980; 13) 32546; 14) 33527; 15) 19592; 16) 44007; 17) 29891; 18) 73111; 19) 19469; 20) 34887; 21) 44940; 22) 2066; 23) 45650; 24) 61354; 25) 43280; 26) 44028; 27) 18217; 28) 24113; 29) 39234; 30) 69264; 31) 48697; 32) 31320. IEDB IDs from http://www.immuneepitope.org.

6. *BACILLUS ANTHRACIS* TOXIN: EPITOPES AND CDR3 SEQUENCES

An identical molecular picture appears to characterize the immune interaction between *B. anthracis* toxin and human antibodies. A review of the literature on experimentally validated B-cell epitopes (59-62) found that determinants from the *B. anthracis* protective antigen 63 (PA63) are formed (or contain) peptide fragments that have a low level of similarity to the human host (Table 2). In parallel, Table 3 shows that the heavy chain CDR3 sequences characterizing the humoral immune response against the *B. anthracis* PA63 (63) also have a low level of similarity. That is, all of the analyzed anti-PA63 paratopic sequences have pentapeptides scarcely represented in, or absent from, the human proteome.

7. *CLOSTRIDIUM BOTULINUM* TOXIN: EPITOPES AND CDR3 SEQUENCES

The B-cell epitopes mapped onto the heavy chain of botulinum neurotoxin (BoNT), serotypes A and B (64-68), were analyzed using the low-similarity criterion. The data obtained are displayed in Table 4, showing that, once more, almost all of the BoNT determinants consist of motifs that are rare or absent in the human proteome. That is, the BoNT-derived B-cell epitope repertoire experimentally validated by Atassi *et al.* (64-68) is specularly characterized by the same motif rarity found in tetanus and anthrax toxin-derived B-cell epitopes.

The characterization of the epitopes listed in Table 4 further supports the concept that a low level of similarity to the human proteins represents the molecular basis of the

Table 1. Human anti-tetanus toxoid antibody repertoire from 2 healthy subjects boosted with a tetanus toxoid vaccine: similarity level to the human proteome of heavy chain CDR3 sequences at the pentapeptide level

Subject 1		**Subject 2**	
Heavy chain CDR3 sequences[1,2]	Matches[3]	Heavy chain CDR3 sequences[1,2]	Matches[3]
arrpgwaatraaGAFDI	1	ardgvitlggvIELRWydp	0
aRDFYSgtyrsfdy	1	aragsswslrPTTFDy	1
ararllfcsgGRCDMds	0	ardyygsGSHYYfdy	0
ardrgritlfgevilrAGWFDs	0	arlgiaaRHYFYgvdv	0
arvfggtrlYYALNv	1	arylgstrGYYYMdv	0
ardygGTRHYyalda	0	atGVTMDy	1
trcrggtrTYYYMdv	0	akglifgVAAYYfdy	1
arhldsYDVFTgynlggymdv	0	akglifgvPAYYFds	0
arhldsYDVFNgynlggymdv	1	akdlilgvPHYYFds	0
vsaprdtstiaarfNRYFFdt	1	arrydFWSGFldy	0
asaprdtstiaarfNRYFFdf	1	arrhycssTSCYDafdi	0
ardrggTRHHYymdv	0	arRIAIFsvvlrsgwfdp	0
ararrtysgydSAFDY	1	arlpKHYIAeavt	0
arvsgwgpRGGIYfdy	0	aringnvtifgMILPRgwfdp	0
arivGYNWKgegnfdy	0	aringyvtvfgMILPRgwfdp	0
ardvrRRFGEflrpfdl	1	aRINGNvtifgmvlprgwfdp	0
arsvvpatRAFDF	3	aRDSAPlsrrgalgi	5
artvaslgTAFDY	4	akgrkQWLVPdfds	0
arivGTHGFdy	1	akylsgGYAIDv	1
aRTMGVvlpfdy	1	vkyLWGGYaidv	0
ararllfcsgGRCDMds	0		
vkrrrQWLVNssfdf	2		

1Heavy chain CDR3 amino acid sequences are given in 1-letter code 2Only the lowest similarity pentapeptide in each anti-tetanus toxoid antibody heavy chain CDR3 sequence is capitalized 3Anti-tetanus toxoid antibody heavy chain CDR3 sequences described in Poulsen et al. (58) were dissected into consecutive overlapping pentapeptides shifted by one amino acid. Each pentapeptide was analyzed for the number of occurrences in the human proteome (46-53). Any such occurrence was termed a match. The similarity level corresponds to the number of matches. Low-similarity level is numerically quantified as less than or equal to 5 matches to the human proteome

Table 2. Anthrax PA63-derived B-cell epitopes immunorecognized by human antibodies: similarity analysis to the human proteome at the pentapeptide level

EDB ID[1]	Aa position	Epitopic Sequences[2,3]	Matches[4]	Refs.
68833	338-346	vhasFFDIG	0	59
26841	610-629	iklnakmnilirDKRFHydrn	0	60, 61
118794	625-635	fhyDRNNIavga	1	62
118752	633-644	aVGADEsvvkea	2	62
118887	657-668	llniDKDIRkil	0	62
118963	721-732	PNYKVnvyavtk	0	62
118754	729-740	avTKENTiinps	2	62

[1]Anthrax PA63 epitope IEDB ID from www.immuneepitope.org [2]Amino acid sequences are given in 1-letter code [3] Only the lowest similarity pentapeptide in each B-cell epitope is capitalized [4]Epitopic sequences were dissected into overlapping pentapeptides that were analyzed for the number of exact matches to the human proteome (see legend to Table 1)

Table 3. Similarity analysis to the human proteome of anti-anthrax PA63 antibody variable regions

Heavy chain CDR3 sequences[1, 2]	Matches[3]
gpgppnqsRRVTMivlppprwfdp	0
tedivlgvaakpHAHFDy	0
eRWTGIldy	0
dmygggGYFFAk	1
vtsaiavtsTRWYIdl	0
dkdyfisgSYYNWfdp	0
wdYVWESyrgkafdi	0
apqydlwtgpLYGMDv	0
gDMVTGdpgdy	2
qssnWEDYFqh	0
erlTGILDy	5
ADYAGgrrfdl	2

[1]Amino acid sequences are given in 1-letter code [2]Only the lowest similarity pentapeptide in each B-cell epitope is capitalized [3]Heavy chain CDR3 sequences described by Reason *et al.* (63) were dissected into overlapping pentapeptides that were analyzed for the number of exact matches to the human proteome (see legend to Table 1)

immune response. The relationship between the rarity of the pentapeptide and the BoNT epitope is clearly evident in Figure 3, which shows the location of immunodominant reactive regions of BoNT/A, /B (64, 67, 68) along the similarity profile to the human proteome of a few BoNT linear determinants from Table 4.

Analysis of the CDR3 variable domains of anti-BoNT human IgM antibodies described by Adekar *et al.* (69) provides further proof-of-concept of the link between immunoreactivity and peptide rarity. Table 5 shows that a low-similarity sequence score marks the heavy chain and

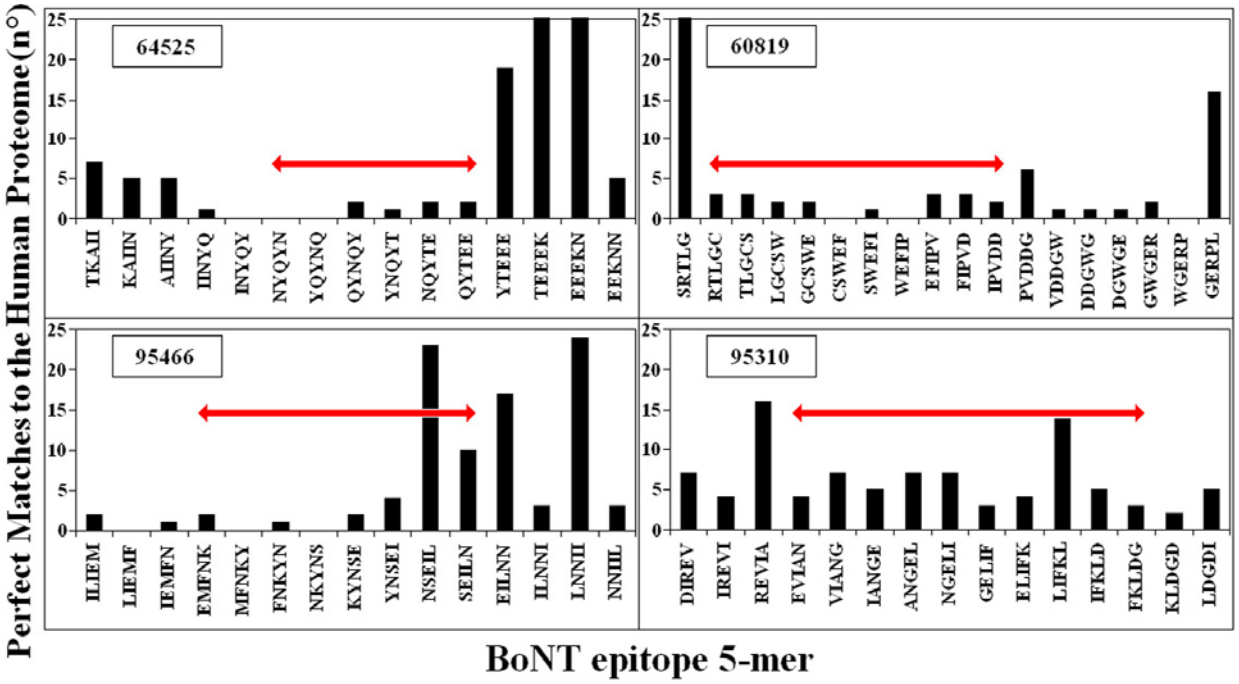

Figure 3. Pentapeptide similarity profile to the human proteome of BoNT epitopic sequences. The boxed number in each panel refers to the epitope IEDB ID. Location of immunodominant reactive regions of BoNT/A, /B epitopes (64, 67, 68) are indicated by double arrowed lines in red. Further details in the legends to Table 4.

light chain (kappa or lambda) variable domains of 5A and 70A IgM antibodies that are able to react with BoNT/A, /B.

Interestingly, the IgM antibody sequences described in Table 5 are encoded by un-mutated germ-line DNA sequences, e.g., the described IgM antibodies are natural antibodies. In the context of the present review, this indicates the possibility that the natural antibody repertoire recurs to low-similarity sequences in building its first-line immune defense. Moreover, it is worth of noting that in Tables 1 to 5 only the lowest similarity pentapeptide in each epitope is shown in capital letters. Actually, almost all of the pentapeptides forming the epitopic sequences are rare motifs (e.g., they have less than or equal to five total matches to the human proteome) (41-43).

8. PERSPECTIVES

The present review explores data from the literature on the link between low-similarity peptide sequences and bacterial toxin immunogenicity. Specifically, we analyze the scientific literature on the peptide-peptide interactions occurring between *C. tetani*, *B. anthracis*, *C. botulinum* toxins and human antibodies using the hypothesis advanced by Kanduc (39, 41-43) that a low level of sequence similarity to the host proteome modulates and shapes the immune repertoire. The data converges toward a scenario in which rare peptide motifs are the chief players in immunoreactivity. Peptide fragments of toxins practically unknown to the human proteome induce human antibody CDRs formed by rare peptide motifs. Pathogen and host appear linked by a same immunological language based on rare peptide words (41).

As additional evidence of the low-similarity hypothesis, the present review adds to an accumulating body of knowledge documenting that almost all of the experimentally validated immunogenic epitope sequences, irrespective of their antigenic nature or associated pathology in infectious diseases (45, 50-53, 70, 71) as well as in cancer (47, 48, 72-75), autoimmunity (48, 76), and allergy (77), are characterized by motifs that are scarcely represented in the host organism. Here, the low-similarity concept is further supported by a number of scientific reports experimentally demonstrating that the immune stimulus (the antigens) and the immune response (the antibodies), meet and integrate on a common ground: rare sequence usage.

Scientifically, the low-similarity hypothesis may help solve the self-nonself protein debate that still enshrouds immunology (39, 43). Clinically, analysis and application of the low-similarity concept might lead to the development of more specific therapeutics, vaccines, and diagnostics for emerging and re-emerging infectious agents, potential bioterrorism agents, cancer, and autoimmune diseases (42, 78). However, most importantly, with this type of information at hand, immune interventions, void of collateral side effects, may be a possibility. In fact, a low level of sequence similarity to the host offers the possibility of uniquely targeting the infectious agent or the tumor cell (79), improving on the current immunotherapeutic protocols that also attack normal host molecules and structures.

Table 4. BoNT/A, /B, heavy chain toxin epitopes recognized by human antibodies: sequence similarity to the human proteome at the pentapeptide level

BoNT/A				
IEDB ID[1]	Aa position	Epitopic Sequence[2, 3]	Matches[4]	Refs.
2772	449-467	alndlciKVNNWdlffsps	0	64
11800	491-509	eenisldllQQYYltfnfd	0	64
44880	519-537	nlssdiigqlELMPNierf	1	64
44218	533-551	nierfpngkkyeldKYTMF	0	64
34612	547-565	kytMFHYLraqefehgksr	0	64
10975	589-607	dyvkkvnkateaaMFLGWv	0	64
64328	631-649	tiiipYIGPAlnignmlyk	1	64
57988	659-677	sgavILLEFipeiaipvlg	1	64
28105	673-691	ipvlgtfalvSYIANkvlt	2	64
65481	715-733	tnwlakvNTQIDlirkkmk	1	64
64525	743-761	tkaiinYQYNQyteeeknn	0	64
58886	771-789	sklnesinkAMINInkfln	1	64
44491	785-803	nkflnqcsvsylMNSMIpy	0	64,65
4528	813-831	aslkdallkYIYDNrgtli	2	64
53998	827-845	rgtliGQVDRlkdkvnntl	1	64
74279	869-887	yikniintsilnlrYESNH	0	64,66
32595	911-929	knqlQLFNlesskievilk	0	64
14730	925-943	evilknaIVYNSmyenfst	1	64,66
19297	981-999	GEIIWtlqdtqeikqrvvf	0	64-66
52274	995-1013	qrvvfkySQMINisdyinr	0	64,66
45130	1051-1069	nnimfkldgcrdtHRYIWi	0	64-66
34622	1121-1139	kyvdvnnvgiRGYMYlkgp	0	65
74737	1135-1153	ylkgprGSVMTtniylnss	0	64
43525	1177-1195	ndrVYINVvvknkeyrlat	0	64,66
26220	1247-1265	igfigfHQFNNiaklvasn	0	64
60819	1275-1296	srtlgcsWEFIPvddgwgerpl	0	64,65
BoNT/B				
IEDB ID[1]	Aa position	Epitopic Sequence[2, 3]	Matches[4]	Refs.
43826	638-656	nfenafeiagasILLEFip	1	67,68
2806	722-740	alnyqaqaleeIIKYRyni	0	67,68
75691	736-754	yRYNIYsekeksninidfn	0	67,68
27724	764-782	inqaidninnFINGCsvsy	0	67,68
7097	778-796	csvsylmKKMIPlaveklI	0	67,68
64383	834-852	timpfdlSIYTNdtiliem	0	67,68
95466	848-866	iliemfNKYNSellnniil	0	67,68
64843	974-992	tlidingktksvFFEYNir	0	67,68
95310	1030-1048	direviangelifKLDGDi	2	67,68
73893	1058-1076	yfsifntelsQSNIEeryk	1	67,68

[1]BoNT/A, /B, heavy chain toxin epitope IEDB ID from www.immuneepitope.org [2]Amino acid sequences are given in 1-letter code [3]Only the lowest similarity pentapeptide in each B- cell epitope is capitalized [4]Epitopic sequences were dissected into overlapping pentapeptides that were analyzed for the number of exact matches to the human proteome (see legend to Table 1)

Table 5. 5A and 70A antibody CDR3 variable domains: sequence similarity to the human proteome at the pentapeptide level

IgM antibody chain[1]	**CDR3 sequence[2,3]**	**Matches[4]**
5A mu	araalnprgyfdwllHYYYGmdv	0
5A kappa	qqYYSTPpt	3
70A mu	araplsvGFWSGyspyyfdy	0
70A lambda	ssytsSSTWV	2

[1]See ref. 69 for further details [2]Amino acid sequences are given in 1-letter code [3]Only the lowest similarity pentapeptide in each paratope is capitalized [4]Paratopic sequences were dissected into overlapping pentapeptides that were analyzed for the number of exact matches to the human proteome (see legend to Table 1)

9. CONTRIBUTIONS

GN and GC have been involved in data analysis. DK conceived, designed and wrote the review. All authors have read and approved the final manuscript.

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