Original Article

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Influenza and sudden unexpected death: the possible role of peptide cross-reactivity

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Abstract: This study investigates the hypothesis that cross-reactions may occur between human cardiac proteins and influenza antigens, thus possibly representing the molecular mechanism underlying influenza-associated sudden unexpected death (SUD). Using titin protein as a research model, data were obtained on (1) the occurrence of the titin octapeptide AELLVLLE or its mimic AELLVALE in influenza A virus hemagglutinin (HA) sequences; (2) the immunological potential of AELLVLLE and its mimic AELLVALE; (3) the possible role of the flanking amino acid aa) context of the two octapeptide determinants in eliciting cross-reactivity between the human cardiac titin protein and HA antigens.

Keywords: influenza pathogenicity, sudden unexpected death, titin protein, hemagglutinin antigen, peptide sharing, cross-reactivity, epitope conformers

1 Introduction

Myocardial disorders occur during influenza infection with a varying clinical severity that ranges from imperceptible symptoms to sudden unexpected death (SUD) [1–4]. The association with sudden death is well-known and has been repeatedly described in animal studies [5–7], case reports [8–10], and review papers [11,12]. Most recently, compelling evidence for an association between influenza and SUD has been numerically quantified in a study by Onozuka and Hagihara [13] who reported on registry data for 481,516 out-of-hospital cardiac arrests from 47 prefectures of Japan during influenza seasons between 2005 and 2014.

Disappointingly, the causal mechanistic and molecular links between influenza epidemics and SUD remain unknown, However, it has to be annotated that influenza has been implicated as a potential trigger for autoimmune diseases such as diabetes [14,15], severe pulmonary inflammation in lupus-prone mice [16], thrombocytopenia [14], atherogenesis [17,18], narcolepsy [19–24], brain autoimmunity [24], leukoencephalopathy [25], and neurological disorders and schizophrenia [26]. Moreover, the reports [5–8] that suggest a role of autoimmune processes in the development of influenza lesions in the myocardium are relevant in an immunological context.

Based on the abovementioned data, in this study, the possible immune link was analyzed by posing the following question: Do influenza antigens and human SUD-related proteins share peptides that might underlie autoimmune pathogenic cross-reactions? As a matter of fact, since 2010, this lab reported on an awesome peptide commonality between influenza hemagglutinin (HA) and the human proteome [27–29]. Such a peptide sharing might be potentially involved in neuropsychiatric disorders [28,29]. Instead, to the best of the current knowledge, no data have been reported on cross-reactivity as a possible autoimmune link between influenza infection and SUD. Using titin as a protein that, when altered, may associate with sudden death [30–34], this study specifically explored the peptide commonality between influenza viruses and titin, found viral vs human peptide overlaps that might represent a cross-reactive link to the pathological cardiac sequel, and investigated the structural basis of the potential cross-reactions.

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2 Methods

Sequence analyses were conducted on human titin protein (UniProtKB ID: Q8WZ42, 34,350 amino acids [aa], that is described in detail at https://www.uniprot.org) [35–37] searching for peptide matches with influenza viruses.

The titin primary sequence was manipulated and analyzed as follows. The entire human protein was decomposed in silico to sets of overlapping *n*-mers (*n* from 10 to 7) offset by one residue, ie, MTTQAPTFTQ, TTQAPTFTQP, TQAPTFTQPL, and so on. Four libraries of unique 7-, 8-, 9-, and 10-mers were then created by removing duplicates. Next, for each *n*-mer in the libraries, the entire human proteome was searched for instances of the same *n*-mer. Any such occurrence was termed an overlap (or match). The titin *n*-mers were analyzed for matches in the UniProt/SwissProt database using Peptide Match Protein Information Resource (PIR) program (https://research.bioinformatics.udel.edu/peptidematch) [37]. The resulting data sets were explored, and influenza viruses containing titin peptide matches were manually identified and annotated.

In addition, reference proteomes of influenza A virus, H3N2 subtype (tax ID: 385580), influenza B virus (tax ID: 518987), and influenza C virus (tax ID: 11553) were used to investigate peptide matching at the 5-mer level.

Immunological potential of shared peptides was analyzed using the Immune Epitope Database (IEDB; www.iedb.org) resource [38]. IEDB offers experimental data characterizing antibody and T cell epitopes studied in human beings and other animal species. Epitopes involved in infectious disease, allergy, autoimmunity, and transplant are also included. This study considered only epitopes that had been experimentally validated as immunopositive in the human host.

PEP-FOLD3 program (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3) [39–41] was used to obtain three-dimensional (3D) conformational structures from linear aa sequences. Starting from a single linear peptide sequence, PEP-FOLD3 runs series of 100 tertiary structure simulations, with each simulation sampling a different region of the conformational space. Then, the program returns an archive of all the models generated. Once generated, models are clustered into 10 best models. In this study, the best model 1 was selected for each analyzed 18-mer peptide.

3 Results and discussion

3.1 Occurrence and conservativeness of a titin octapeptide in influenza A viruses

Sequence analyses of human titin protein vs influenza proteins at the 10-, 9-, 8-, and 7-mer levels show that the longest match is an octapeptide, namely AELLVLLE (Table 1).

Focusing on the AELLVLLE octapeptide and analyzing the influenza viruses involved in the sharing, it was found that the octapeptide AELLVLLE is conserved among HAs from numerous influenza A viruses, mostly H1N1 subtype variants (Table 2), thus conflicting with the high mutation rate of influenza virus [42–45]. To better evaluate AELLVLLE sequence conservativeness, Table 2 summarizes the viral aa sequence context of AELLVLLE, ie, the residues at the NH2 and COOH termini flanking the octapeptide (aa given in small letters in Table 2).

Tab. 1: Quantitative peptide sharing between human cardiac titin and influenza viruses at the 10-, 9-, 8-, and 7-mer levels

10-mer	Sequence	9-mer	Sequence	8-mer	Sequence	7-mer[a]	Sequence[a]
-	-	-	-	1	AELLVLLE	3	SGAAGAA DPKKTGG GEDLKIE

[a]Excluding the two overlapping heptapeptides that form the octapeptide AELLVLLE.

Tab. 2: Distribution and conservativeness of the titin octapeptide AELLVLLE in influenza A virus HAs

aa sequence[a]	Influenza A virus subtypes and variants
iwaynAELLVLLEnqktl	A/Turkey/Wisconsin/1/1966 H9N2 A/Duck/Alberta/60/1976 H12N5
iwtynAELLVLLEnertl	A/USA:Iowa/1943 H1N1 A/USA:Texas/UR06-0195/2007 H1N1 A/Puerto Rico/8/1934 H1N1 A/Wilson-Smith/1933 H1N1 A/China:Nanchang/11/1996 H1N1 A/New Zealand:South Canterbury/35/2000 H1N1 A/USA:Memphis/10/1996 H1N1 A/Brazil/11/1978 H1N1 A/Chile/1/1983 H1N1 A/Henry/1936 H1N1 A/India/6263/1980 H1N1 A/USA:Philadelphia/1935 H1N1 A/Malaysia:Malaya/302/1954 H1N1 A/USA:Albany/12/1951 H1N1 A/USSR/90/1977 H1N1 A/Swine/New Jersey/11/1976 H1N1 A/Kiev/59/1979 H1N1 A/Leningrad/1/1954 H1N1 A/Hickox/1940 H1N1 A/Brevig Mission/1/1918 H1N1
lwaynAELLVLLEnqktl	A/Turkey/Ontario/6118/1968 H8N4
vwtynAELLVLLEnertl	A/Russia:St. Petersburg/8/2006 H1N1 A/Swine/Wisconsin/1/1967 H1N1 A/Shearwater/Australia/1972 H6N5 A/Swine/Indiana/1726/1988 H1N1 A/Duck/Alberta/35/1976 H1N1 A/Swine/Wisconsin/1/1961 H1N1 A/Turkey/Minnesota/1661/1981 H1N1 A/Duck/Australia/749/1980 H1N1 A/Swine/Netherlands/12/1985 H1N1

[a]Shared titin octapeptide is given in capital letters. Five aa at NH2 and COOH termini are given in small letters, and the invariant aa are underlined.
Abbreviations: aa, amino acid; HA, hemagglutinin.

On the whole, two unexpected data emerge from Tables 1 and 2. First, one out of the 34,343 titin octapeptides occurs in the HA antigen from numerous influenza A virus subtypes and variants. This datum is unexpected since, neglecting aa frequency and protein length, the theoretical probability for two proteins to share one octapeptide approximates one out of 20^8, ie, the probability is 0.0000000000390625. It is an infinitesimal number that, in practice, is equal to zero.

The second unexpected datum is the conservativeness of the AELLVLLE sequence. Such a conservativeness is well documented in Table 2 by the fact that the short N and C termini flanking the conserved octapeptide have a high level of aa variations, with only five invariant aa positions (aa are given in small letters and underlined in Table 2), in this way generating four different aa contexts (ie, 18-mer iwaynAELLVLLEnqktl, iwtynAELLVLLEnertl, lwaynAELLVLLEnqktl, and vwtynAELLVLLEnertl) for the conserved AELLVLLE sequence.

Moreover, Table 2 summarizes that the titin octapeptide is present in numerous influenza A subtypes, including a low pathogenic avian influenza A subtype such as H9N2 [46,47], but not in influenza B and C viruses or in other influenza A virus subtypes such as H3N2, a subtype that dominated recent influenza epidemics [48].

3.2 Occurrence and conservativeness of the titin octapeptide mimic AELLVALE in influenza A virus H3N2 subtype

To better define the peptide sharing with influenza A, B, and C viruses, sequence matching analyses were extended to AELLVLLE subsequences and the octapeptide was further dissected into 7-, 6-, and 5-mer peptides offset by one residue each other, ie, AELLV, ELLVL, LLVLL, and LVLLE. Then, each of the 7-, 6-, and 5-mer peptides was analyzed for occurrences in reference proteomes for influenza A H3N2 subtype, influenza B, and influenza C (Section 2).

It was found that no matching occurs in influenza B and C proteomes and only one titin pentamer (AELLV) is shared with the HA (UniProtKB: D1LNT4_I63A3) from influenza A virus, subtype H3N2 (tax ID: 385580). Of note, the HA pentapeptide AELLV is followed by the tripeptide "ALE". Said otherwise, it was noticed that a sequence AELLVALE that mimics the titin AELLVLLE peptide (with a Leu substituted by an Ala, see the A underlined in the mimic sequence) is present in influenza A virus HA, subtype H3N2.

Hence, search for matching was extended to the mimic peptide AELLVALE. Results are shown in Table 3 that summarizes that the mimic AELLVALE characterizes influenza A HAs from nine different subtypes. Moreover, Table 3 summarizes that, at difference from the AELLVLLE octapeptide, the conserved peptide mimic AELLVALE is flanked by likewise highly conserved NH2 and COOH termini, ie, the 18-mer lwsynAELLVALEnqht is a conserved sequence among the different influenza A virus subtypes and variants as listed in Table 3.

Tab. 3: Occurrences of peptide mimic AELLVALE in influenza A virus HAs, subtypes H3N2, H3N8, H4N2, H4N4, H4N5, H4N6, H4N8, H14N5, and H14N6

aa sequence[a]	Influenza A virus subtype
lwsynAELLVALEnqhti	Aichi/2/1968 H3N2
	Bangkok/1/1979 H3N2
	Beijing/353/1989 H3N2
	England/321/1977 H3N2
	Hong Kong/1/1968 H3N2
	Hong Kong/5/1983 H3N2
	Memphis/1/1971 H3N2
	Memphis/101/1972 H3N2
	Memphis/102/1972 H3N2
	Memphis/110/1976 H3N2
	Memphis/18/1978 H3N2
	Memphis/2/1978 H3N2
	Memphis/4/1980 H3N2
	Memphis/6/1986 H3N2
	NT/60/1968 H3N2
	Port Chalmers/1/1973 H3N2
	Swine/Colorado/1/1977 H3N2
	Swine/Ukkel/1/1984 H3N2
	Udorn/307/1972 H3N2
	Uruguay/716/2007 H3N2
	Victori3/1975 H3N2
	X-31 H3N2
	Duck/Ukraine/1/1963 H3N8
	Duck/Hokkaido/8/1980 H3N8
	Equine/Santiago/1/1985 H3N8
	Equine/Kentucky/2/1986 H3N8
	Equine/Tennessee/5/1986 H3N8
	Equine/Kentucky/1/1987 H3N8
	Equine/Jillin/1/1989 H3N8
	Duck/Hokkaido/21/1982 H3N8
	Duck/Memphis/928/1974 H3N8

Tab. 3: Continued

aa sequence[a]	Influenza A virus subtype
	Equine/Algiers/1972 H3N8
	Equine/Fontainebleau/1976 H3N8
	Equine/New Market/1976 H3N8
	Equine/Romania/1980 H3N8
	Equine/Suffolk/1989 H3N8
	Equine/Miami/1/1963 H3N8
	Turkey/Minnesota/833/1980 H4N2
	Grey teal/Australia/2/1979 H4N4
	Seal/Massachusetts/133/1982 H4N5
	Duck/Czechoslovakia/1956 H4N6
	Ruddy Turnstone/New Jersey/47/1985 H4N6
	Budgerigar/Hokkaido/1/1977 H4N6
	Chicken/Alabama/1/1975 H4N8
	Mallard/Astrakhan/263/1982 H14N5
	Mallard/Astrakhan/244/1982 H14N6

[a]Shared titin peptide mimic is given in capital letters. Five aa at NH2 and COOH termini are given in small letters, and the invariant aa are underlined.
Abbreviations: aa, amino acid; HA, hemagglutinin.

3.3 Immunological potential of the AELLVLLE peptide and its mimic AELLVALE

Subsequently, exploration of IEDB (www.iedb.org) [38] showed that the titin AELLVLLE peptide and its mimic AELLVALE have an immunological potential. Indeed, the octapeptides AELLVLLE and AELLVALE are present in influenza HA epitopes experimentally validated and cataloged as immunopositive in the human host [49–61] (Table 4).

Tab. 4: Immunopositive influenza virus HA epitope sequences containing the octapeptide AELLVLLE or its mimic AELLVALE

IEDB ID[a]	Epitope[b]	References[c]
31200	kidlwsynAELLVALE	49, 50
31201	kidlwsynAELLVALEnqhti	49
50489	qdlekyvedtkidlwsynAELLVALEnqhtidltds	49
62654	synAELLVALEnqhti	49
96007	wtynAELLVLLEnertld	51-54
129015	iwtynAELLVLLEnert	51, 55, 56
129078	kidlwsynAELLVALEn	56
130384	ynAELLVALEnqhtidl	56
143423	kidlwsynAELLVALEnqht	*[c]
151075	ynAELLVLLEnertl	57, 58
152823	nAELLVLLEnqktldehdan	*[c]
152915	qiqdvwaynAELLVLLEnqk	*[c]
188707	ldiwtynAELLVLLEnertl	59
238618	idiwtynAELLVLLEnertdfhds	60
538701	kvddgfldiwtynAELLVLLEnert	61

[a]Epitopes are listed according to the IEDB ID number. Epitope details are available at www.immuneepitope.org/[38].
[b]Peptides AELLVLLE and the mimic AELLVALE are given in capital letters.
[c]Asterisk refers to IEDB submission (www.immuneepitope.org) [38].
Abbreviations: IEDB, Immune Epitope Database; HA, hemagglutinin.

3.4 Viral sequence contexts of the two octapeptide determinants AELLVLLE and AELLVALE: the "same peptide–different conformational structures" issue

To understand how the two determinants AELLVLLE and AELLVALE might be involved in potential cross-reactive responses between the human cardiac titin protein and influenza A HA antigens, the role of the short 5 aa regions flanking the NH2 and COOH termini was investigated. Indeed, a main point that emerges by comparing Tables 2 and 3 is that the determinant AELLVLLE is flanked by aa sequences that vary (Table 2), whereas the peptide mimic AELLVALE lies in a highly conserved aa frame (Table 3).

This point might be of relevance in specifying the immunoreactivity of the two octapeptide immunodeterminants. In fact, already in 1984, Kabsch and Sander [62,63] demonstrated that the structure of a short peptide within a protein strongly depends on the sequence context. That is to say that the peptide interactions with other parts of a protein dictate the peptide conformation so that identical peptide segments can assume different conformations in different proteins. Immunologically, the "same peptide–different conformational structures" issue is of utmost importance since it can underlie a humoral immune response formed by a constellation of antibodies with different specificities to the many conformers of a peptide [64–66].

Hence, the 3D structures of AELLVLLE and its mimic AELLVALE were analyzed as a function of the aa sequence context, ie, of the NH2 and COOH termini. Specifically, molecular modeling was applied to six 18-mer peptides:

- 1) estcaAELLVLLEdtdmt (ie, the 18-mer sequence present in the titin protein),
- 2) iwaynAELLVLLEnqktl
- 3) iwtynAELLVLLEnertl
- 4) lwaynAELLVLLEnqktl
- 5) vwtynAELLVLLEnertl (ie, the 18-mer sequences as described in Table 2)
- 6) lwsynAELLVALEnqhti (ie, the 18-mer sequence as described in Table 3)

Results are illustrated in Figure 1 that shows the different structural conformations of the two octapeptide determinants in the different aa frames listed earlier and obtained by using PEP-FOLD3 program [39–41]. For simplicity, Figure 1 only mentions subtype H3N2 out of the nine HN subtypes characterized by the 18-mer lwsynAELLVALEnqhti sequence.

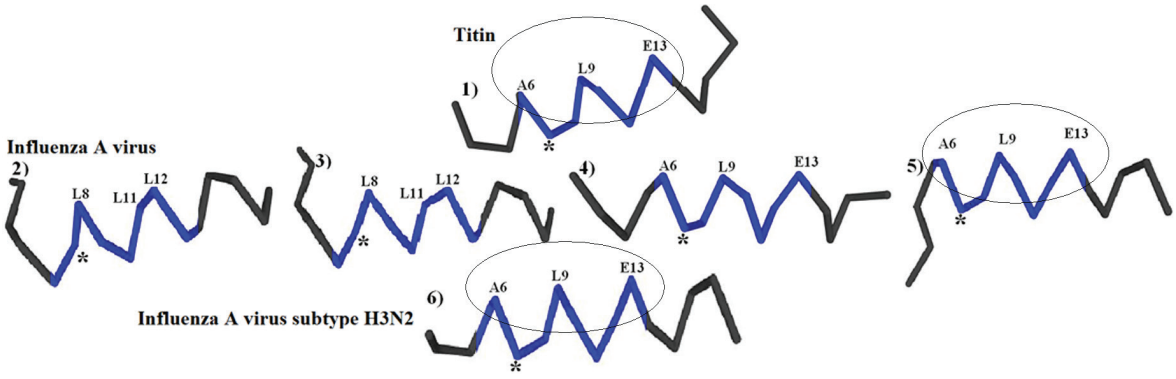

Fig. 1: Structures of octapeptide AELLVLLE and its mimic AELLVALE as a function of the NH2 and COOH aa context. Sequences are given with octapeptide determinants in capital letters, and the aa context, ie, the N and C termini, in small letters: 1) estcaAELLVLLEdtdmt in Titin protein; 2) iwaynAELLVLLEnqktl, 3) iwtynAELLVLLEnertl, 4) lwaynAELLVLLEnqktl, and 5) vwtynAELLVLLEnertl in influenza A virus HA; 6) lwsynAELLVALEnqhti, in influenza A virus H3N2. Structure portions corresponding to AELLVLLE and AELLVALE are given in blue, with similar structures encircled in the ovals. Asterisk indicates the position of the first Glu (E) residue in AELLVLLE and AELLVALE. PEP-FOLD3 [39–41] was used to obtain 3D structures from the 18-mer linear peptide sequences.
Abbreviation: aa, amino acid.

3.5 Context-dependent conformational structures of determinants AELLVLLE and AELLVALE

Figure 1 shows that only viral sequences corresponding to structures 5 and 6 are similar to the titin structure 1, thereby owning the proper 3D configuration to elicit immune responses able to cross-react with the human cardiac titin protein.

In fact, in line with the Kabsch and Sander's "same sequence–different structure" principle [49,50], Figure 1 shows that the four varying frames as described in Table 2 generate four different AELLVLLE conformers (Figure 1, structures 2, 3, 4, and 5). Among them, structure 5 is similar in shape and charge to the AELLVLLE conformation defined by the human titin context (Figure 1, structure 1). Instead, structures 2, 3, and 4 would be excluded from participating to possible cross-reactions with titin because of the hydrophobic 3D surface characterized by L8, L11, and L12 residues in structures 2 and 3, and possibly by the V10 smoothed spike in structure 4. Instead, the physicochemical features of structures 1 and 5 as given by the succession of A6, L9, and E13 residues are also found in structure 6, where the mimic AELLVALE allocates in the unvarying 18-mer sequence lwsynAELLVALEnqhti present in influenza A virus H3N2 and other eight influenza A subtypes (Table 3).

In short, only determinant conformers 1, 5, and 6 are structurally similar so as to represent the main target for possible cross-reactions between the human cardiac titin protein and influenza HA antigen. This datum merits attention especially in analyzing the recent 2018 influenza epidemic that essentially consisted of A(H1N1)pdm09 and A(H3N2) infections [67] and was heavily burdened by SUD cases [68]. Actually, influenza A(H1N1)pdm09 HA sequenced in 400 samples collected in the period January–May 2018 (Table S1) was marked by the presence of the 18-mer iwtynAELLVLLEnertl corresponding to structure 3 that is dissimilar in shape and charge from the titin structure 1 (Figure 1). This would exclude a possible role of influenza A(H1N1) pdm09 infection in inducing autoimmune cross-reactions with the human cardiac titin protein. Instead, a potential cross-reactive role is likely for influenza A virus H3N2 that hosts the 18-mer lwsynAELLVALEnqhti sequence structurally corresponding to a conformer (Figure 1, structure 6) that is similar to that of titin (Figure 1, structure 1).

4 Conclusions

Immunological phenomena are complex, and it is well-known that the generation of immune responses (immunogenicity) and antigen recognition by antibodies (antigenicity) are influenced by a plethora of cellular and humoral factors (ie, T cells and cytokines) under the constraint of physicochemical conditions such as pH, antigen concentration, ionic strength, hydrophilicity/hydrophobicity, and epitope accessibility [69]. Hence, the structural data graphically exposed in this study cannot be assumed as absolute certainty but, rather, are preliminary to further research aimed at mathematically defining epitope structures by the analysis of, for example, root-mean-square deviation of atomic positions and, as well, at determining the stability of the various peptide conformations. In addition, studies of biochemical factors such as glycosylation and proteolysis have to be conducted for taking into consideration the structural conformer as an optimal epitope [70].

Of cogent importance for defining future research, it has also to be noted that the structures selected by using PEP-FOLD3 [39–41] as shown in Figure 1 are not static and do not provide a definitive representation of the 18-mer sequences since each sequence actually "resonates" among multiple dynamic status, the stability of which is dictated by the stereochemical properties of amide linkages, double bonds, SH groups, et alia. In the case in point, such a resonance among multiple dynamic configurations is enhanced by the presence of aromatic aa (W, Y) in the sequence contexts shown in Figure 1.

In addition, it deserves notice the fact that the present study is restricted to human cardiac titin, a protein essential for both mechanical and signaling functions of the heart [30–34,71]. De facto, the number of human proteins crucial for cardiac functions is highest [72,73] and warrants extensive further analyses. In this regard,

the data offered in this study do no more than to indicate the vastity of the potential cross-reactivity network connecting influenza infection and cardiovascular diseases.

Given these caveats, the conclusion of this study is that the data discussed might help our understanding of what has been and is still considered as a mysterious enigmatic phenomenon, ie, the influenza-associated pathogenicity [74,75,76].

Finally, from a biochemical point of view, it is important to annotate that the unexpected and apparently unexplainable viral vs human peptide matching most possibly derives from the central role played by viruses in the evolutionary origin of the eukaryotic nucleus, as described by the viral eukaryogenesis hypothesis [77–79].

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Supplementary Table S1: List of sequence entries of influenza A(H1N1)pdm09 HA derived from 400 samples collected in the period January–May 2018. Data obtained from NCBI using the keywords "influenza A(H1N1)pdm09 HA 2018 complete sequence"

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Abbreviation: HA, hemagglutinin.