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Soluble recombinant influenza vaccines

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Soluble, recombinant forms of influenza A virus haemagglutinin and neuraminidase have been produced in cells of lower eukaryotes, and shown in a mouse model to induce complete protective immunity against a lethal virus challenge. Soluble neuraminidase, produced in a baculovirus system, consisted of tetramers, dimers and monomers. Only the tetramers were enzymatically active. The immunogenicity decreased very considerably in the order tetra > di > mono. Therefore, we fused the head part of the neuraminidase gene to a tetramerizing leucine zipper sequence; the resulting product was enzymatically active, tetrameric neuraminidase. The protective immunity induced by this engineered neuraminidase, however, remained fairly strain-specific. A third influenza A virus protein, the M2 protein, has only 23 amino acids exposed on the outer membrane surface. This extracellular part, M2e, has been remarkably conserved in all human influenza A strains since 1933. By fusing the M2e sequence to hepatitis B virus core protein, we could obtain highly immunogenic particles that induced complete, strain-independent, long-lasting protection in mice against a lethal viral challenge. Native M2 is a tetrameric protein and this conformation of the M2e part can also be mimicked by fusing this sequence to a tetramerizing leucine zipper. The potential of the resulting protein as a vaccine candidate remains to be evaluated.

Keywords: influenza; neuraminidase; M2 protein; hepatitis B virus core; universal vaccine

1. INTRODUCTION

The high variability of the antigenic properties of the major influenza surface glycoproteins haemagglutinin and neuraminidase has been a major obstacle to controlling the viral disease by vaccination. We have shown before by nucleotide sequencing of viral genes that 'drift' corresponds to a limited number of mostly single nucleotide changes (Min Jou *et al.* 1980; Verhoeyen *et al.* 1980). These results confirmed, at the genetic level, previous data obtained by partial amino-acid determination and by serology. Furthermore, we found that the nucleotide sequence of the human influenza A/Aichi/68 haemagglutinin gene closely resembled the corresponding gene sequence derived from A/duck/Ukraine/63 (Fang *et al.* 1981). As the former corresponded to an early isolate of the 1968 H3N2 pandemic, these results provided almost proof that a 'shift' phenomenon is due to the introduction of a gene derived from the animal reservoir in a human virus strain.

We then started to explore whether this basic genetic information could help in the development of improved influenza vaccines. Immunization of mice with purified, recombinant, secreted haemagglutinin indeed provided complete protection against a lethal challenge with mouse-adapted virus (Vanlandschoot *et al.* 1993; Saelens *et al.* 1999). But this was only true when the homologous virus strain was used as a challenge, and various attempts to broaden the specificity of protection largely failed. Nevertheless, it may be noted that such recombinant vaccines, made in lower eukaryotes, offer many additional advantages, such as cost, the possibility of rapidly adapting to new virus strain specifications, independence from uncertain supplies (e.g. embryonated eggs), etc.

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2. NEURAMINIDASE-BASED VACCINE

The second immunodominant glycoprotein on the viral surface is the tetrameric neuraminidase. Although in a strict sense antibodies against neuraminidase are not neutralizing, they interfere with spreading of the virus and prevent morbidity *in vivo* (Johansson *et al.* 1993). As the gene drifts independently from that of the haemagglutinin (Kilbourne *et al.* 1990), a neuraminidase-based vaccine can be valuable either as such or in combination with a classical haemagglutinin-based vaccine.

Neuraminidase is a type II protein. Hence, in order to obtain a secreted, recombinant product, we replaced the N-terminal, transmembrane coding part by the haemagglutinin type I signal sequence information (Deroo *et al.* 1996; Martinet *et al.* 1997). Immunization of mice with the purified product conferred complete protection against a potentially lethal viral challenge and this protection was transferable by serum. However, efficient protection was only obtained against a challenge virus bearing the homologous subtype neuraminidase. We tried to enhance the response against conserved parts of the molecule by mutating immunodominant epitopes, but were unable to substantially broaden the specificity (Martinet *et al.* 1998).

Soluble, recombinant neuraminidase, for example produced in a baculovirus/*Sf9* cell system, is a mixture of tetrameric, dimeric and small amounts of monomeric

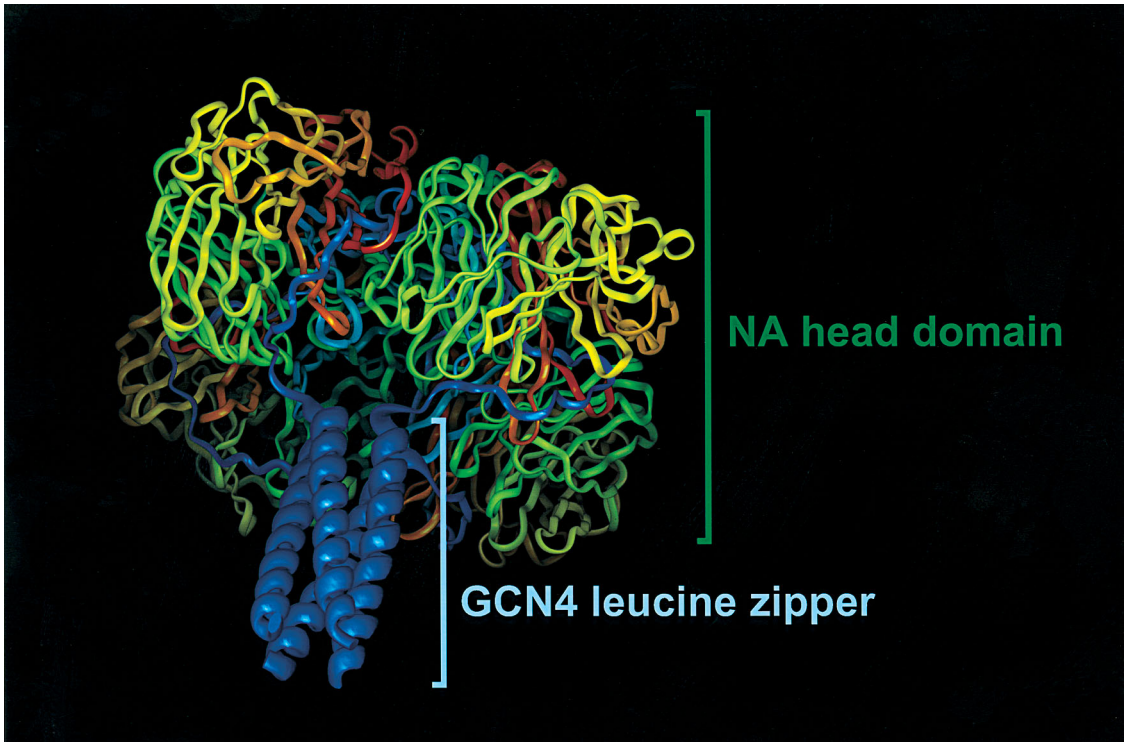

Figure 1. Presumed three-dimensional structure of the chimeric, tetrameric leucine zipper GCN4–neuraminidase based on the known structure of the two constituent proteins.

molecules. Only the first is endowed with enzymatic activity (Deroo *et al.* 1996). On an equal-weight basis, the tetramers were remarkably more antigenic than the dimers, which in turn were considerably better than the monomers. Therefore, we constructed chimeric molecules consisting of the neuraminidase gene (minus the transmembrane part) fused to a tetramer-inducing sequence such as the leucine zipper domain of the yeast transcription factor GCN4 (Harbury *et al.* 1993; T. Deroo, W. Min Jou and W. Fiers, unpublished data). The resulting product (figure 1) was indeed exclusively tetrameric and fully enzymatically active, and constitutes an attractive material for a neuraminidase-based vaccine.

3. M2-BASED VACCINE

M2 protein is the third virus-coded, integral membrane protein. The tetrameric protein is sparingly present on the virus particles, but is abundant on virus-infected cells, where it functions as a selective ion channel (Lamb *et al.* 1985; Zebedee & Lamb 1988; Pinto *et al.* 1992). Drugs like amantadine or rimantadine target the M2 ion channel and inhibit productive infection (Belshe *et al.* 1988). Unlike haemagglutinin and neuraminidase, the extracellular part of the M2 protein (M2e) is not subjected to severe immune selection pressure. Indeed, since the first human virus isolates in 1933, hardly any change has occurred in the 23-amino-acid-long M2e sequence (figure 2), except for an aspartate to glycine mutation in A/PR/8/34 at position 21, and an additional glycine to glutamate at position 16 in some far-eastern Asian isolates in the 1994–1995 period. M2e is only weakly immunogenic but it has been shown that a monoclonal antibody directed against M2e inhibits influenza virus replication in mice (Treanor *et al.* 1990).

Hepatitis B virus (HBV) core particles are easy to produce in *Escherichia coli* and can be readily modified at several positions without loss of their particle-forming properties (Pumpens & Grens 1999). The icosahedral particles consist of 180 or 240 subunits, which are arranged as dimers (Wynne *et al.* 1999). These particles are highly efficient in presenting epitopes to the immune system. We have explored the potential of chimeric M2e–HBV core particles as a universal vaccine against influenza A (Neirynck *et al.* 1999). Immunization was either intraperitoneal together with a mild adjuvant or intranasal without any adjuvant. Both procedures led to appreciable serum antibody titres and provided full protection against a challenge with a lethal dose of a mouse-adapted virus preparation. The protection was long-lasting (half a year in mice) and was based on circulating antibodies, as shown by passive immunization. Most importantly, M2e–HBV could elicit an immune response in mice that protected against a challenge with a different subtype virus. Based on the conservation of the amino-acid sequence of M2e, one may conclude that the protective immunity is universal for all human influenza A strains (the single mutation at position 21 was shown to be irrelevant; the effect of the mutation at position 16 remains to be tested). There are many ways one can explore to improve the antigenicity of the M2e-based vaccine: e.g. one or more copies of M2e can be fused at the N-terminus or inserted internally so that it becomes exposed at the tip of the HBV core spikes; the protamine-like C-terminal region of the HBV core can be replaced by several influenza T-cell epitopes, etc. Also, considering that native M2 protein is a tetramer, one can fuse M2e to the tetramerizing GCN4 leucine zipper sequence or other appropriate multimeric carriers. A number of these possibilities are now being tested.

virus strain (subtype)	2																							24
A/WS/33 (H1N1)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/WSN/33 (H1N1)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/PR/8/34 (H1N1)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	G	S	S	D	
A/Fort Warren/1/50 (H1N1)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/USSR/90/77 (H1N1)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Singapore/1/57 (H2N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Leningrad/134/57 (H2N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Ann Arbor/6/60 (H2N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Udorn/72 (H3N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Port Chalmers/1/73 (H3N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Bangkok/1/79 (H3N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/NY/83 (H3N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Guangdong/39/89 (H3N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Akita/1/94 (H3N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Hebei/19/95 (H3N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	E	C	R	C	N	G	S	S	D	
A/Niigata/137/96 (H3N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	
A/Shiga/25/97 (H3N2)	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	

Figure 2. Representative examples of the extracellular amino-acid sequence of M2 proteins since the first human influenza A isolate in 1933. (These data are a subset of a much more extensive compilation; courtesy of Dr M. De Filette (Laboratory of Molecular Biology, Ghent, Belgium).)

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