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Towards a subunit influenza vaccine prepared by affinity chromatography on immobilized lectin

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Abstract. Disintegration of influenza virions with 0.2% w/v sodium deoxycholate releases haemagglutinin and neuraminidase, which in the presence of detergent are both adsorbed to the lectin from *Crotalaria juncea,* coupled to Sepharose 2B.

The binding is mediated through galactosyl residues on haemagglutinin and neuralminidase, which are completely displaced in 0.2 M lactose and co-elute in a narrow zone.

The immunogenicity of haemagglutinin and neuraminidase in mice is markedly increased after adsorption onto lipid, particles constituting "intralipid" (Kabi Vitrum, Stocholm, Sweden).

Keywords. Influenza subunit vaccine; haemagglutinin; neuraminidase; lectin; *Crotalaria juncea;* affinity chromatography; adjuvant.

Introduction

There are three principal types of influenza vaccine in present use: whole virus vaccine, split vaccine, and subunit vaccine. The advantages most often cited in favour of a subunit vaccine containing only haemagglutinin (HA) and neuraminidase (NA) are the following ones: 1. Lower toxicity leads to fewer local reactions at the site of injection. 2. Since irrelevant material has been eliminated, higher doses of the two relevant immunogens HA and NA can be administered. 3. Inoculation of ostensibly harmless but potentially active nucleic acid is avoided. Although local and systemic reactions against whole virus vaccines purified by zonal centrifugation are rarely serious in adults, clinical symptoms like swelling, pain, and moderate pyrexia are common in children and unprimed recipients (Tyrrell and Smith, 1979). These reactions can be reduced but hardly eliminated in "split" vaccines prepared by disintegration of purified virions with ether or detergents, which are afterwards removed. Split vaccines contain essentially the same elements as whole virus vaccines, and in the same proportions.

There is now ample evidence that the viral constituents of importance in eliciting protective humoral antibody are the surface glycoproteins HA and NA

Abbreviations used: HA, Haemagglutinin; NA, neuraminidase; Dol, Dolichol; Asn, asparagine; Ser, serine; Man, mannose; Glc, glucose; Glc N-Ac, glucose N-acetylamine; Thr, threonine; M_r , molecular weight; TNBP, tri-*n*-butylphosphate; CTAB, Cetyl trimethyl ammonium bromide; SDOC, sodium deoxycholate.

(Schild, 1979), and several experimental and few commercial vaccines based on these purified immunogens have been tried.

Unfortunately, a weak and wavering immune response has been a frequent problem with the subunit vaccines. Reports are conflicting with respect to the level as well as the duration of the immune response to purified HA and NA subunits from different virus strains both in man and experimental animals. Taken together, available evidence strongly suggests that the prospects for subunit influenza vaccines are bleak unless some way to potentiate and stabilise the immunogenicity can be found. Several types of adjuvants have been suggested and a few have been actually tried in man, but to our knowledge none has yet given results that are consistent enough to firmly establish subunit vaccines as being clinically preferable to inactivated or live, attenuated whole virus vaccines.

We have recently had some success with a lipid based adjuvant, "intralipid", originally designed and introduced by Vitrum, as a nutritional aid for intravenous use. As an adjuvant it may be more readily acceptable, since it is cleared for human use although for an entirely different purpose.

Influenza virus HA is synthesized in the infected cell as a single polypeptide chain to which carbohydrate side chains are added at asparagine residues (Klenk, 1980) by the precursor-phosphoryl dolichol pathway (Struck and Lennarz, 1980). Most of the efforts to elucidate the biosynthesis, structure, and function of the two components have been directed towards HA, but the general pattern appears to be similar in NA (Skehel *et al.,* 1980). In HA, assembly of the peptide chain starts in the rough endoplasmatic reticulum. Attachment of carbohydrate begins by the addition of a preformed Dol-P-P-$(GlcNAc)_2$-Man_9-Glc_3unit exclusively to asparagine in the sequence Asn-X-(Ser or Thr). A series of events then follows in which the chronology and cellular locations are not known in detail. After initial attachment of the lipid-carbohydrate precursor, the glucosyl and a variable number of mannosyl residues are removed. During passage through the Golgi membranes the majority of the trimmed carbohydrate chains are processed further by sequential addition of GlcNAc and then galactose. Sometimes one fucosyl residue is put on to one of the Asn-linked GlcNAc residues. Sialic acid is never present in the finished molecule, which is a trimer of M_r 225,000 consisting of identical subunits held together by noncovalent forces (Waterfield *et al.,* 1979).

There are two main types of carbohydrate chains. Complex chains contain all the four sugars GlcNAc, mannose, fucose, and terminal galactose, whereas in high mannose chains there are only GlcNAc and a variable number of terminal mannosyl residues. The number of carbohydrate chains seems related to the number of glycosylation sites in the polypeptide chains of a particular virus strain (Klenk, 1980).

The location of glycosylation sites and number of carbohydrate chains in NA have not been as intensively studied as in HA. Total weight of carbohydrate in NA is about 10% compared to 20% in HA (Skehel *et al.,* 1980). The finished NA molecule is a tetramer of four coplanar units with a total M_r of 270,000 (Skehel *et al.,* 1980). Therefore, the terminal sugars galactose and mannose as well as the branched GlcNAc-linked fucose are potentially exploitable for lectin affinity chromatography both of HA and NA.

Experimental

Backed by facilities at the Separation Center of the Biomedical Center for preparation of large amounts of lectin, and with experience in using immobilised lectins for purification of blood group substances and other glycoproteins, we decided about seven years ago to look more closely into the possibility of using lectins to purify intact virus and glycosylated viral subunits on a large scale.

This approach to the preparation of vaccines had already been suggested by Hayman *et al.* (1973) who described the isolation of HA and NA from the strains Ao/Bel/42, X-31, and Rostock Fowl Plague by affinity chromatography on *Lens culinaris* lectin coupled to Sepharose 4B with cyanogen bromide.

Aiming for a large scale method that would have to be scientifically sound, technically feasible, and economically acceptable at the same time, we had to start practically from scratch with all the three parts of the project: a) Disruption of virions; b) Lectin affinity chromatography; and c) Immune potentiation.

Disruption of virions

A multitude of procedures have been described for releasing HA and NA from the outer lipid layer of the influenza virion. They include the use of ethyl ether, either alone (Cromwell *et al.,* 1969), or in combination with detergents (Davenport *et al.,* 1964); tri-*n*-butyl phosphate (TNBP) (Neurath *et al.,* 1970, 1971), as well as cationic, neutral, and anionic detergents. The purification steps following general disruption with a non-selective detergent have involved density gradient centrifugation (Brady and Furminger, 1976), electrophoresis (Laver *et al.,* 1976), both in combination (Laver and Webster, 1976a, b), alcohol precipitation and ultracentrifugation (Janout and Uvizl, 1977), or centrifugation after selective solubilisation with the cationic detergent cetyl trimethyl ammonium bromide (CTAB) (Bachmayer *et al.,* 1976).

The method involves treatment of freshly harvested allantoic fluid with 0.2% w/v of the anionic detergent sodium deoxycholate (SDOC) under gentle stirring for 12 h at room temperature and pH 8.0. This concentration of SDOC is low compared to what other workers have used (Ersson, 1977), but we found it to be the upper limit for preservation of both HA and NA activity in H1N1 strains, which appeared particularly sensitive to SDOC. We observed no turbidity or gel formation with 0.02 M phosphate as present in phosphate buffered saline at this concentration of SDOC. The effect of pH on the efficiency of HA and NA release in 0.2% SDOC varied between virus strains. At pH levels down to 7.4 the H3N2 strains like A/Victoria/3/75 and A/England/42/72 were effectively disrupted, whereas only partial release was obtained from the H1N1 A/USSR strains until pH was increased to 8.0.

Results

Lectin affinity chromatography

Potentially useful lectins for the isolation of HA and NA subunits are those with affinity for mannose, galactose, or fucose. In addition to proper specificity, several other qualities are essential in lectin-based adsorbents to be used for preparation of

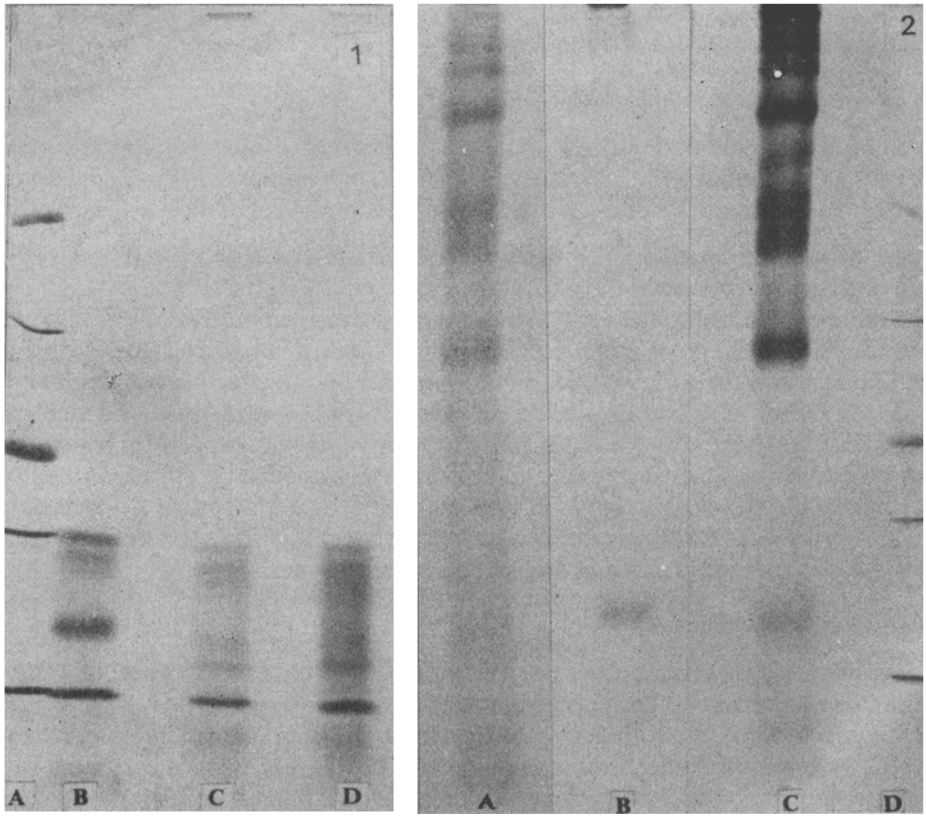
injectable material. The lectin must be non-toxic, and continuous background leakage of lectin, should either lie below the level of immunogenicity, or else the lectin leaking off during desorption should be easily removable. Further, the capacity should be high enough to allow a reasonable volume ratio of adsorbent to vaccine and must be retained through several cycles of adsorption and desorption. Finally, complete desorption with cheaply available carbohydrate should be possible. We found that different lectins of similar sugar specificity could vary considerably in their ability to adsorb and release virions and subunits. Experience with some lectins is summarised in table 1.

Table. 1. Affinity chromatography of virus and subunits on different immobilized lectins.

Lectin	Interacting sugar on HA and NA	Virus material	
		Intact virions	HA+NA
Concanavalin A	Mannose	Strong binding, incomplete release	Binding erratic, strain dependent
Lens culinaris	„	Strong binding, incomplete release	Progressive decrease in binding of NA
Vicia ervilia	„	Good capacity in repeated use	Binding too weak
Crotalaria juncea	Galactose	Strong binding, some progressive decrease in binding of HA and NA	Excellent capacity in repeated use
Lotus tetragonolobus	Fucose	Binding too weak	Binding too weak

For affinity chromatography of HA and NA subunits, the most useful lectin we have tried so far is that from *Crotalaria juncea* (sunn hemp), which has been extensively purified and characterised by Ersson (1977) at the Separation Center. A welcome property of this lectin is its high affinity for lactose, which inhibits haemagglutination about eight times more efficiently than does galactose. This means that HA and NA when adsorbed through their terminal galactosyl residues are very efficiently desorbed by 0.2 M lactose in phosphate buffered saline and eluted in a narrow zone at high concentration.

Pilot experiments showed that the presence of 0.2% SDOC had little effect on the carbohydrate-binding capacity of lectin-Sepharose. Figure 1 shows the pattern obtained after polyacrylamide gradient electrophoresis of the starting material before (lane B) and after (lane C) treatment with SDOC. Lane D represents unadsorbed material passing through the column, but with no residual HA or NA activity. Lanes B and C show that even in the absence of detergent the bulk of allantoic fluid material appears below the lactic dehydrogenase marker of M_r 140,000. Lanes C and D are very similar, suggesting that the adsorbent binds only a small fraction of the protein present in the starting sample. In figure 2 desorbed material from infected (lanes A and C) and non-infected (lane B) allantoic fluid is compared. In the absence of SDOC due to thorough flushing before desorption,



Figures 1 and 2. 1. Polyacrylamide gradient electrophoresis of infected allantoic fluid, Copmassie stain.

Lane A: M_r markers (Pharmacia Fine Chemicals), top to bottom, M_r in thousands: thyroglobulin 669, ferritin 440, catalase 232, lactate dehydrogenase 140, albumin 67.

Lane B: Crude allantoic fluid before SDOC treatment.

Lane C: Crude allantoic fluid after SDOC treatment, as applied to lectin column.

Lane D: Material not adsorbed on lectin column, devoid of HA and NA activity.

2. Polyacrylamide gradient electrophoresis of material desorbed after passage of infected and non-infected allantoic fluid.

Lane A: Material desorbed with lactose after passage of infected allantoic fluid, PAS-Schiff stain.

Lane B: Material desorbed with lactose after passage of non-infected allantoic fluid, Coomassive stain.

Lane C: Same material as in lane A, but stained with Coomassie.

Lane D: Molecular weight markers (see figure 1, lane A).

most bands stained for proteins in lane C lie above the ferritin marker of M_r 440,000. Almost all appear to be glycoproteins as demonstrated by an aliquot run stained with PAS-Schiff in lane A. The presence of several bands is expected, since it has been generally found that oligomeric aggregates of HA and NA tend to form by hydrophobic association after the removal of detergent.

Lane B is almost empty, sharing a conspicuous band with lane C at around M_r 90,000. The tentative conclusion is, that most of the material released from the

lectin-Sepharose by lactose, after passage of infected allantoic fluid treated with SDOC, is indeed of viral origin.

Lectin toxicity and stability of immobilised lectin

Two conditions essential to the acceptance of lectin chromatography in providing injectable preparations for human use are a lack of lectin toxicity and a lectin content per dose that does not elicit unacceptably high levels of anti-lectin antibody.

The *Crotalaria juncea* lectin is not toxic in mice. No LD-50 could be established even with doses corresponding to 10 mg/kg body weight.

In order to determine the ratio to be expected between viral subunits and background lectin, leakage of lectin labelled with ^{125}I was measured under different conditions and during various stages of the chromatographic procedure. There is some release of lectin when the crude mixture of allantoic fluid and detergent passes through. Apart from a slight progressive decrease in capacity to be expected from this, our main concern was of course the amount of lectin appearing with the subunits in the desorption zone. Preliminary results indicate that a vaccine dose containing 15 µg HA may contain about 0.07 µg lectin, although optimum conditions for production runs have not yet been established.

Immunisation experiments

We realised at an early stage of our efforts that an efficient adjuvant suitable for human use was likely to be required for a practical subunit vaccine to be justified. During discussions about the contribution of hydrophobic bonding in chromatographic systems the hydrophobic tails of viral HA and NA was a recurrent topic. Our attention was drawn to a remarkably stable soybean oil emulsion introduced into the market as an intravenous nutrient called "Intralipid" and cleared for human use. Although stabilised with lecithin, the particles differed from liposomes in being essentially homogeneous rather than consisting of a lipid bilayer surrounding a water phase. Since the soybean oil droplets forming an oil-in-water emulsion were about equal to influenza virions in size and could be expected to have a hydrophobic surface, we decided to make a mixture of Intralipid and viral subunits, inject it into mice, and look at the immune response.

A similar approach was taken by Almeida *et al.* (1975) who describe the preparation of "virosomes" consisting of influenza viral HA and NA adsorbed to the surface of unilamellar liposomes presumably by hydrophobic bonding and looking remarkably like viral particles in the electron microscope. However, apparently no later data have been published on the practical use of these virosomes.

Preliminary results with intralipid as an adjuvant in immunisation of mice with influenza HA and NA are very encouraging indeed. Parameters have not yet been optimised, but some figures are given in table 2.

The future

The main challenges are to secure a steady supply of lectin, to establish optimum chromatographic conditions, and to optimise and stabilise the adjuvant effect. Also, there is no guarantee that Intralipid is as immunologically harmless in humans as it appears to be in mice after conjugation with viral immunogens. Finally,

Table 2. Haemagglutination inhibition titrations of sera from mice immunized with whole virus or HA + NA subunits with and without addition of "intralipid".

Material injected	µg protein per dose (0, 2 ml) before dilution	Antigenic extinction limit [a]	
		Three weeks after first injection	One week[b] after second injection
Current divalent whole virus vaccine without intralipid (A/Vic/375+ B/HK/8/73B)	ND[c]	1 : 150	1 : 1400
Whole virus vaccine + intralipid	ND	1 : 18 600	1 : 49 000
A/Vic HA + NA subunits without intralipid	130	1 : 390	ND
HA + NA subunits in 2% intralipid	130	1 : 25 700	1 : 178 000

[a] The dilution of injected material causing detectable antibody formation in 50% of injected animals

[b] Second injection was given after first bleeding

[c] Not determined

the crucial question of how efficiently the vaccine protects against infection in man remains to be answered.

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