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Comparative Analysis of Six European Influenza Vaccines

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Three split-virion vaccines (Vaxigrip, Begrivac, and Influsplit/Fluarix) and three subunit vaccines containing only viral surface glycoproteins (Influvac, Agrippal, and Fluvirin) available for the 1994–95 season were analysed by biological, molecular, and biochemical methods. Although all vaccines are required by health authorities to contain 15 μg haemagglutinin per dose of each virus strain, there were significant differences in haemagglutination titres among the examined vaccines of both types. The enzymatic activity of neuraminidase was present in all vaccines except Fluvirin. Total protein content was lower for subunit vaccines. Viral nucleoprotein was detected in all split vaccines but to varying levels according to SDS-PAGE and Western blot analyses. The ovalbumin content was low in general but was about tenfold higher for Influvac than for the other vaccines analysed. This protein may induce hypersensitive reactions among persons with severe egg allergy. All three split-virion vaccines were found to contain the matrix protein; however, it was not detected in the subunit vaccines. Differences in influenza antigen variety in currently available vaccines may affect efficacy, whereas differences in concentrations of nonviral compounds such as ovalbumin and endotoxin may lead to different post-vaccination reactogenicity profiles.

Two of the most important features of influenza caused by influenza viruses type A and B are the epidemic nature of the disease and mortality that in a pandemic situation can be devastating. Although the infection itself may seen banal and not pose a serious threat to life, the danger lies in superinfections resulting in pulmonary and cardiac complications. Elderly people and persons with immunologic deficiencies are at an increased risk for severe sequelae from influenza infections. Regular vaccination before the influenza season begins of persons at high risk is currently the most effective measure for reducing the fatal impact of and the substantial economic costs incurred each year by influenza (1, 2).

Influenza viruses, unlike other viruses from which vaccines have been developed, undergo rapid and unpredictable antigenic variation in monomeric haemagglutinin (HA) and neuraminidase (NA), the two surface glycoproteins primarily responsible for the production of neutralising antibodies during infection. On the basis of these surface antigens, influenza A viruses are classified into subtypes. Infection or vaccination with a virus of one subtype confers little or no protection against viruses of other subtypes. Although influenza B viruses have shown more antigenic stability than influenza A viruses, antigenic variation does occur. The antigenic characteristic of the circulating virus strains, documented by the World Health Organization reference centres and the National Health Centres, is the basis for selecting the specific virus variants included in the vaccines available each year. The vaccines usually contain three virus strains (2 influenza type A viruses from subtypes H1N1 and H3N2 and 1 influenza type B virus), representing the prevalent pathogenic influenza viruses circulating in the human population during the previous season.

The vaccines are produced from highly purified, egg-grown viruses. Whole virus, split-virion, and subunit preparations are available. To minimise febrile reactions, only split-virion and subunit preparations should be used for vaccination of children.

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All influenza vaccines registered on the German market are inactivated by a two-step procedure: (1) the disintegration (splitting) of the virus by ether, chloroform, sodium desoxycholat, or other detergents, and, independently, (2) the real inactivation step which consists of formalin or β-propionolactone (BPL) treatment. Before or after the inactivation procedure, the monovalent material is concentrated by high-speed centrifugation or other appropriate methods. In the subunit vaccines the surface antigens are separated from the rest of the viral proteins by a zonal centrifugation step, so that this type of vaccine consists mainly of HA and NA antigens (3). Only a monovalent bulk that is satisfactory with respect to HA antigen content, presence and type of NA antigen, sterility, and viral inactivation may be used in the preparation of the final bulk vaccine.

Three split-virion vaccines (Vaxigrip, Pasteur Mérieux, France; Begrivac, Behring, Germany; and Influsplit/Fluarix, Sächsisches Serumwerk, Germany, and SB Biologicals, Belgium, respectively) and three subunit vaccines (Influvac, Solvay Duphar, The Netherlands; Agrippal S1, Biocine Sclavo, Italy; and Fluvirin, Evans Medical, UK), available for the vaccination season 1994–95, were analysed in this study. Antigens for the 1994–95 vaccine were derived from influenza A/Shangdong/9/39 (H3N2), A/Singapore/6/86 (H1N1), and influenza strain B/Panama/45/90. Vaxigrip contained a different H1N1 strain, i.e. A/Texas/36/91 (4).

Materials and Methods

Vaccines. Inactivated trivalent influenza vaccines from six manufacturers in Europe, commercially available for the vaccination period 1994–95, were analysed. Two different types of vaccines were included. The three subunit vaccines were Influvac (lot numbers 94G12 and 94G28), Agrippal S1 (lot numbers SV/65 73 4 and SV/65 75 9), and Fluvirin (lot numbers E4249DA1 and E4264AB1); the three split-virion vaccines were Begrivac (lot numbers 636011 and 637021), Influsplit/Fluarix (lot numbers 18207D9 and 18203C9) and Vaxigrip (lot numbers 94G13 and K0632). As indicated, two batches of each brand were used; three or more individual analyses were made from each batch. The samples were stored at 4°C.

Protein Assay. The protein content was measured with the Bio-Rad Protein Kit (Bio-Rad, Germany) following the Bradford method (5). Bovine serum albumin (Serva, Germany) was used as standard. Fifty µl of the vaccine sample were mixed with 750 µl 1M Tris, pH 7.5, and 200 µl dye reagent. The absorbance was measured at 595 nm and fitted to a standard curve for quantification of the protein content.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples were mixed with electrophoresis sample buffer (6% saccharose, 4% SDS, 40 mM Tris, pH 7.5, 0.06% bromphenolblue; 1 vol sample with 1 vol buffer) and heated at 100°C for 5 min. The resolving gel contained 12.5% or 15% acrylamide, 0.35% or 0.42% diallyltartardiamid (DATD), 0.1% SDS, and 375 mM Tris, pH 8.5; the stacking gel contained 3% acrylamid, 0.08% DATD, 0.1% SDS, and 120 mM Tris, pH 8.5. Polymerisation was initiated by adding ammonium persulphate and tetramethyl-ethylendiamin (TEMED). The electrophoresis buffer contained 250 mM Tris, pH 8.5; 192 mM glycine; and 0.1% SDS. Electrophoresis was carried out in the Mini-Protean II System (Bio-Rad) at room temperature applying 80 V for running in the stacking gel and 150 V for migrating in the resolving gel until the leading front reached the end of the gel. Staining of the protein bands was done in a Coomassie Brilliant-Blue solution (1% solution, diluted in a 2:9:9 mixture of acetic acid, methanol, and water) for 10 min; destaining was achieved in a 1:3:6 mixture of acetic acid, methanol, and water. The gel was dried between two thin plastic sheets soaked in a solution containing 4% glycerol, 10% acetic acid, 30% ethanol, and 56% water and then stretched overnight in a frame.

Scanning. The relative content of influenza related proteins was determined by scanning the Coomassie Brilliant-Blue-stained gels with a Hantarex-Cybertech CS1 apparatus (Dalton, The Netherlands). The peak area of the influenza protein was expressed as a percentage of the total peak area of the lane, and the relative protein content was calculated as an equal percentage of the total protein concentration of the sample.

Western Blot. The gels were made under the same conditions as for Coomassie Brilliant-Blue staining. The transfer to the nitrocellulose membrane (Bio-Rad) was achieved in a Trans-Blot apparatus (Bio-Rad) applying 0.6 mA at room temperature for 1 h. The blot buffer contained 192 mM glycine, 25 mM Tris, 0.1% SDS, 20% methanol, pH 8.75 (all Merck, Germany). The transfer was verified by Ponceau S (Serva) staining, and the blots were blocked in 10% milk powder. Phosphate-buffered saline (PBS), pH 7.4 with 0.1% Tween 20, was used for dilution of samples and for the washing. Primary antibodies used were anti-flu A-NP Mab MCA 400 and anti-flu B-NP Mab MCA 403 (both from Serotec, USA); anti-flu B-HA Mab MCA 402 (Serotec); sheep antiserum raised against A/Taiwan/1/86 and sheep antiserum against A/Beijing/352/89 (both from NIBSC, UK); against-flu B/Panama/45/90 (Centers for Disease Control, USA); against-flu A-M protein Mab MCA 401 (Serotec); and rabbit anti-allantoic fluid (ovalbumin depleted) serum antibodies. Secondary antibodies anti-mouse IgG alkaline phosphatase (AP) conjugated (Promega, USA), anti-rabbit IgG AP conjugated (Promega), and anti-sheep IgG AP conjugated (ICN Biomedicals, USA) and the nitrobluetetrazoliumchloride/5-Bromo-4-chloro-3-indolylphosphate substrate system in 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ (Boehringer Mannheim, Germany) were used for staining.

Ovalbumin Assay. The ovalbumin content in each vaccine was determined by enzyme-linked immunosorbent assay (ELISA). Microtitre plates were coated with goat antichicken egg albumin antibodies, diluted 1:8,000 in 50 mM carbonate buffer, pH 9.6, and postcoated with 2% goat serum in the same carbonate buffer. PBS, pH 7.4 with 0.1% Tween 20, was used for all washings between the incubation steps and as diluting solution. Serial dilutions of the vaccines and ovalbumin reference (Sigma, Germany) were made starting at tenfold for the vaccines and at 20 ng/ml for the ovalbumin standard. After incubation at

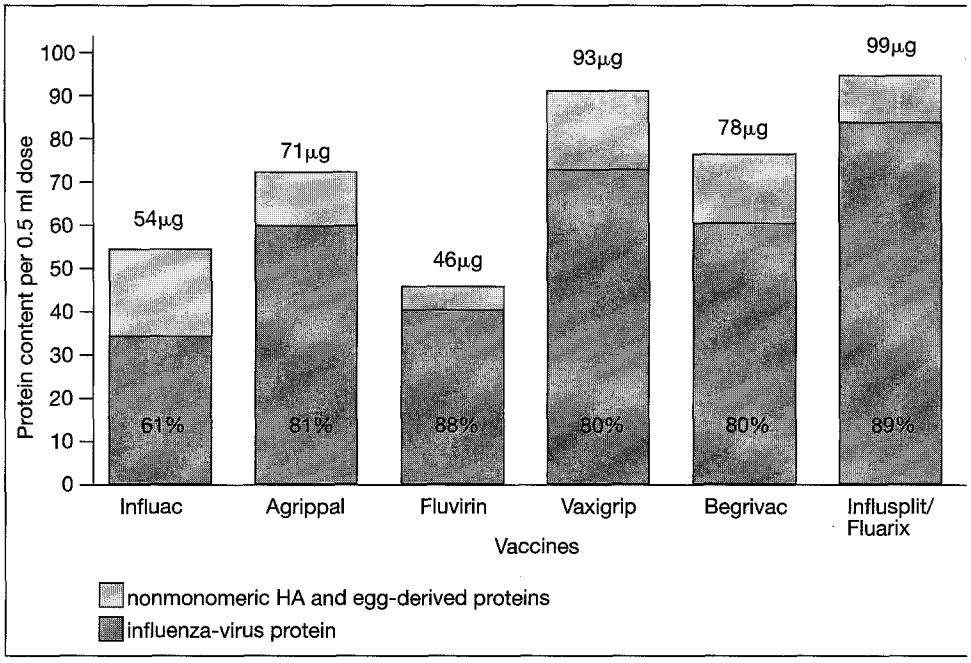

Figure 1: Virus-specific proteins (%) versus total protein content (µg/dose) of the influenza vaccines analysed.

room temperature for 1 h samples were removed and the wells were incubated with rabbit anti-chicken egg albumin antiserum for another hour. For detection, peroxidase-conjugated swine immunoglobulins against rabbit immunoglobulins (DAKO, Denmark) and approximately 5 g/l of the chromogen tetramethylene benzidine dihydrochloride (TMB) diluted in TMB-substrate system (0.1 g/l hydrogen peroxide in acetate buffer) (Behring, Germany; supplementary reagents for Enzygnost/TMB) were used. The absorbances were read at 450/650 nm after stopping the reaction with 0.5 N sulfuric acid and were fitted to the standard curve.

Haemagglutination Test. Starting with a 100-fold predilution of the samples, a further serial twofold dilution of the vaccines was made with saline (1:100 up to 1:12,800) in a microtitre plate (U-form, Nunc, Germany). Twenty-five µl PBS, pH 7.2, containing 1% of packed chicken red blood cells was added to each well, vortexed for even distribution, and incubated at room temperature. After 30 min when the chicken erythrocytes had formed one point at the bottom of the well with negative control, the haemagglutination pattern was recorded. The titre was defined as the reciprocal value of the last dilution where agglutination of the red blood cells was clearly visible (6).

Neuraminidase Assay. The enzymatic activity of neuraminidase contained in the vaccines was determined by an EIA according to the method of Lambre (7). Briefly, microtitre plates were coated with 150 µl of a 50 µg/ml fetuin (Sigma) solution in 0.1 M carbonate/bicarbonate buffer pH 9.6 overnight at 4°C and subsequently washed five times with PBS, pH 7.4, 1% Tween 20. The neuraminidase reference (*Vibrio cholerae*, 2 U/ml, Sigma) and the samples were prediluted 160-fold in Eppendorf tubes with PBS, pH 5.5, containing 1% bovine serum albumin (BSA) and 4 mM $CaCl_2$. Further serial dilutions of the prediluted vaccines and the reference on 100 µl basis in the microtitre plate were done and incubated at 37°C for 1 h. After washing five times (with PBS, pH 7.4, 1% Tween 20), a 1/1,000 peroxidase-labelled Peanut Agglutinin (Sigma) solution (in PBS, pH 7.4, with 1% BSA) was added to each well and incubated at room temperature for another hour. After subsequent washing 100 µl substrate was adding to each well. TMB/DMSO (dimethyl-sulfoxid) solution (252.3 mg TMB/25 ml DMSO) and 30% H_2O_2 (all from Merck), prepared in citrate buffer with pH 5.8, was used for substrate. The staining was stopped after 30 min by adding 3 M H_3PO_4. The absorbance was measured at 450 nm (reference 690 nm). A sigmoid curve was obtained for the standard

Table 1: Individual virus protein content of the influenza vaccines analysed.

Vaccine	HA protein (µg per 0.5 ml dose)	NA/NP protein (µg per 0.5 ml dose)	M protein (µg per 0.5 ml dose)	% virus protein per total vaccine protein
Influvac	33	–	–	61
Agrippal	58	–	–	81
Fluvirin	37	4	–	88
Vaxigrip	45	22	7	80
Begrivac	38	18	1	80
Influsplit/Fluarix	56	27	6	89

HA, haemagglutinin; NA, neuraminidase; NP, nucleoprotein; M, matrix.

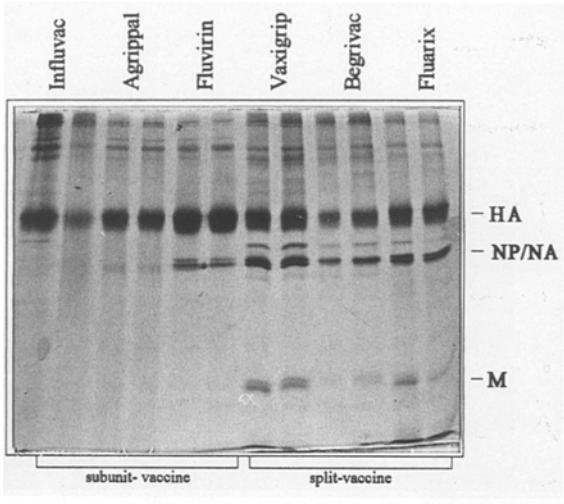

Figure 2: Comparison of total protein content. Coomassie gel showing six vaccines. Two lots of each vaccine were separated by nonreducing 15% SDS-PAGE. Total protein loaded was 7.5 µg. Eighty V in the stacking gel and 150 V in the resolving gel were used until the leading front reached the end of the gel. Coomassie stain clearly detected haemagglutinin (HA) monomer (80 kDa), HA polymer (225 kDa), nucleoprotein (NP) and neuraminidase (NA) protein (both 60 kDa), and matrix (M) protein (30 kDa).

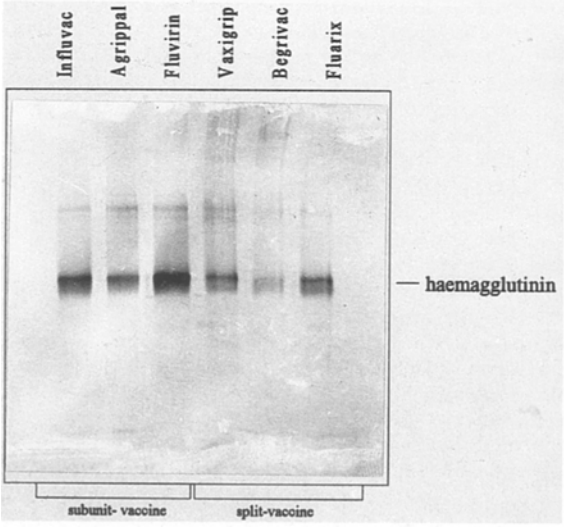

Figure 3: Western blot showing haemagglutinin. The first lot of each vaccine was used. After SDS-PAGE the proteins (1 µg per lane) were transferred by electroblotting to a nitrocellulose membrane. The detection of the three haemagglutinins (H1, H3, HA/B) was accomplished by the use of a mixture of sheep serum anti-A/Taiwan/1/86, sheep serum anti-A/Beijing/352/89, and sheep serum anti-B/Panama/45/90 (1:1:1). Staining was performed by secondary antibodies conjugated with alkaline phosphatase and nitrobluetetrazoliumchloride/5-Bromo-4-chloro-3-indolylphosphate as substrate. In all vaccines haemagglutinin was detectable in different concentrations.

neuraminidase. The linear part of this curve was used for calculation of the neuraminidase activity of the samples.

Endotoxin Assay. Endotoxin was measured quantitatively using the LAL chromogenic kinetic method (Biowhittaker, USA).

Results

The overall amount of protein was lower in the subunit vaccines (Influvac, Agrippal, and Fluvirin) than in the split-virion vaccines (Vaxigrip, Begrivac, and Influsplit/Fluarix). Influsplit/Fluarix and Vaxigrip contained the highest total protein load with 99 and 93 µg per 0.5 ml dose, respectively. Fluvirin had the lowest protein content with 46 µg/dose (Figure 1).

The relative content of monomeric haemagglutinin (HA), matrix (M) protein, neuraminidase (NA), and nucleoprotein (NP) of the vaccines as measured by the protein gel scanning procedure is shown in Table 1. Since influenza nucleoprotein and neuraminidase have virtually the same molecular weight (~ 60 kD), they usually comigrate in nonreducing gels; it was therefore impossible to quantify these two proteins in a specific way.

The HA content ranged between 33 µg and 58 µg per dose for the various brands of vaccine tested (Table 1). Differences in concentrations of this viral protein were also noticed between individual batches of the same vaccine (Influvac and Begrivac; Figure 2).

For Influvac and Agrippal the amount of NA/NP and M proteins was below or at the detection limit. Fluvirin had only a low level of NA/NP proteins and a nondetectable amount of matrix proteins. The portion of "rest proteins" (i.e. nonmonomeric HA and other contaminant proteins) was highest for Influvac at 39.6% of total protein content and Fluarix has the lowest detectable content in egg-derived proteins (Figure 1).

In Western blot analysis using three strain-specific anti-HA antibodies, a positive signal for the individual HAs of both subtypes of influenza A virus and for the HA protein of influenza B virus was demonstrated in all six vaccines analysed (data not shown). No differences in migration behaviour of the three HA molecules in the SDS-PAGE electrophoresis could be detected in the Western blot. When a mixture of antibodies against type- and subtype-specific HA was used, the signals were much stronger and could be clearly identified (Figure 3).

Table 2: Compositional differences in the ovalbumin and endotoxin content and in neuraminidase activity among the influenza vaccines analysed.

Vaccine	Ovalbumin content (per 0.5 ml dose)	Endotoxin content (per 0.5 ml dose)	Neuraminidase activity (per 0.5 ml dose)
Influvac	325 ng	0.9 EU	1.5 mU
Agrippal	35 ng	< 0.5 EU	0.4 mU
Fluvirin	10 ng	4.6 EU	not detected
Vaxigrip	11 ng	64.0 EU	0.5 mU
Begrivac	11 ng	1.0 EU	0.5 mU
Influsplit/Fluarix	10 ng	< 0.5 EU	0.6 mU

Probing with the antimatrix protein and antinucleoprotein antibodies showed no specific signal in the three subunit vaccines analysed whereas the split vaccines contained both internal influenza proteins in variable amounts (data not shown).

The concentrations of ovalbumin contained in various vaccine samples differed drastically. The highest ovalbumin content (325 ng/0.5 ml dose), estimated by ELISA, was found in Influvac, followed by Agrippal (35 ng/0.5 ml dose) (Table 2). For all other vaccines tested, the ovalbumin moiety per 0.5 ml dose was around 10 ng.

Only a faint immunoreaction developed for samples of Fluvirin, Vaxigrip, Begrivac, and Influsplit/Fluarix when exposed to anti-allantoic antibodies in Western blots. This reaction, however, was significantly stronger for samples of Influvac, indicating a higher contamination by egg proteins (Figure 4) and corroborating the ELISA results given in Table 2.

The highest endotoxin content was measured in Vaxigrip (64 EU/0.5 ml dose). Agrippal and Influsplit had the lowest level of endotoxin (< 0.5 EU/0.5 ml dose) (Table 2).

All the vaccines except Fluvirin contained variable amounts of active neuraminidase, with Influvac showing the highest enzymatic activity (Table 2). This documented the presence of a second viral membrane antigen in a still biologically functional form.

All vaccine samples tested possessed the capacity to agglutinate chicken red blood cells in a conventional HA test in microtitre plates. The haemagglutination activity of individual brands of vaccines ranged from 1:400 to 1:12,800 (Figure 5).

Discussion

Six commercially available, inactivated influenza vaccines were characterised by molecular and biochemical methods. The data show that the variety and content of viral proteins and contaminants, their enzymatic activity, and their biological properties differ, not only among the subunit and split-virion vaccines, but also among different lots of individual vaccines.

Final vaccine compositions must comply with the regulations laid down in the European Pharmacopoea (8, 9). These regulations differ for split-virion and subunit vaccines. The total protein content of split-virion vaccines should not exceed 100 μg of protein per virus strain included and no more than 300 μg total per dose. The maximum values of proteins in the subunit vaccines are 80 μg of protein per virus strain and 240 μg total per

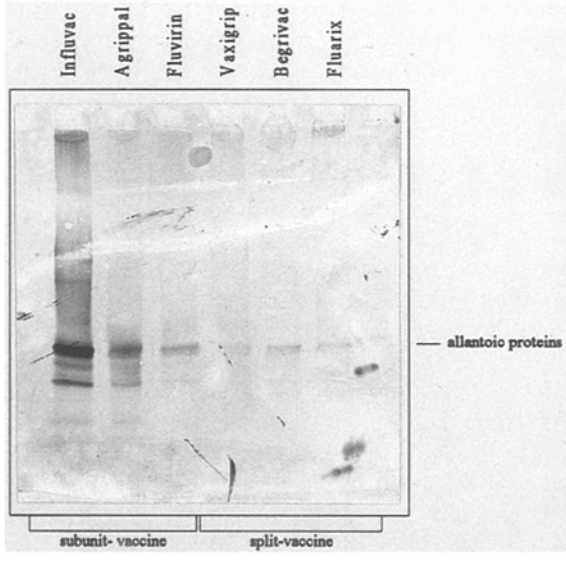

Figure 4: Western blot showing allantoic proteins. Two μg of total protein of vaccines (the first lot of each vaccine) were used. After SDS-PAGE the proteins were transferred by electroblotting to a nitrocellulose membrane. The specific detection of the allantoic protein was accomplished by the use of anti-allantoic protein antibodies. Staining was performed by standard procedures using alkaline phosphatase-linked conjugates and nitrobluetetrazoliumchloride/5-Bromo-4-chloro-3-indolylphosphate as substrate.

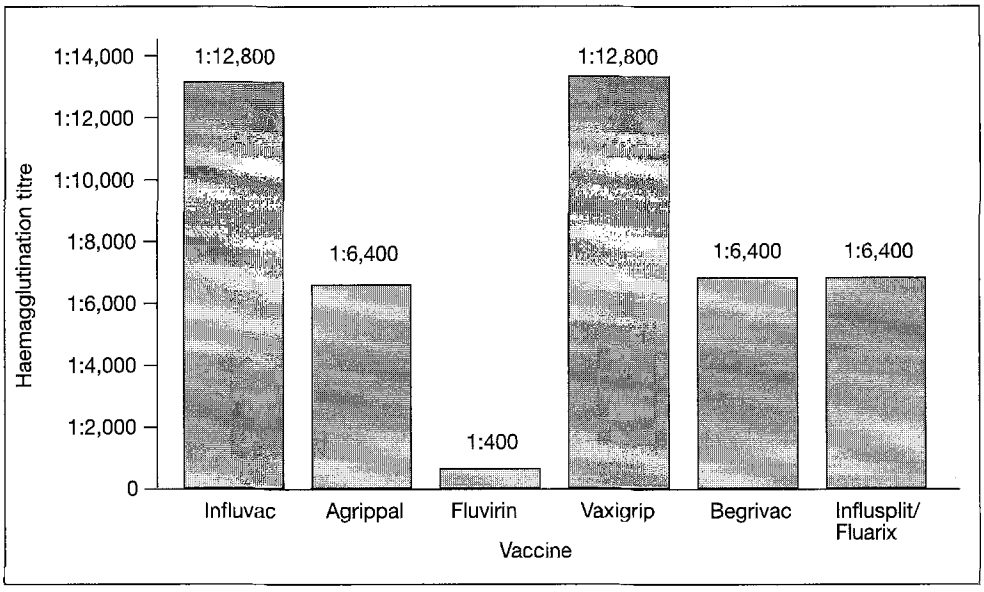

Figure 5: Comparison of haemagglutination properties of the influenza vaccines analysed.

dose. No more than 40 μg of protein other than haemagglutinin per virus strain and no more than 120 μg of total protein other than haemagglutinin per human dose is accepted by the European Pharmacopea for the subunit vaccine.

None of the analysed vaccines exceeded these recommendations. The subunit vaccines had lower total protein content than the split vaccines. This does not correspond to an improved purity profile in subunit vaccines, as is generally believed. The higher total protein content of split vaccines is explained by the presence of internal viral proteins, such as matrix and nucleoproteins, which are either not or barely detectable in subunit vaccines.

According to the scanner analysis of the Coomassie-stained protein gels, Influvac had the lowest content of influenza-specific virus proteins (61% of the total protein content). This vaccine had the highest content of ovalbumin, measured by ELISA. The amount of ovalbumin was more than 20-fold higher than in Fluvirin, Vaxigrip, Begrivac, or Influsplit/Fluarix. Agrippal had about 3.5-fold higher ovalbumin content than these four vaccines. Western blot analysis confirmed the strongest reaction of Influvac and Agrippal vaccines with anti-allantoic antibodies.

Although current influenza vaccines contain only a small quantity of egg proteins, they may induce immediate hypersensitive reactions among persons with severe egg allergy (10). In none of the vaccines tested in our study did the ovalbumin content surpass the maximum values for egg proteins (1 μg/0.5 ml dose) recommended by the European Pharmacopoea (8, 9).

Endotoxin contamination was highest in Vaxigrip (64 EU/0.5 ml dose) and lowest in Agrippal and Influsplit/Fluarix. The bacterial endotoxins in the other vaccines were low (0.9–4.6 EU/0.5 ml dose). The European Pharmacopoea standard (100 EU/0.5 ml dose) was satisfied in all cases.

According to the manufacturers, the amount of haemagglutinin antigen present in each vaccine dose for each strain is 15 μg as measured by the single radial diffusion (SRD) test. In comparing the biological properties of haemagglutination on red blood cells, Fluvirin had a four- to fivefold lower haemagglutination titre than the other vaccines. This low titre cannot be interpreted as a sign of lower immunogenicity, nor does it reflect the actual amount of the haemagglutinin antigen. There is no direct correlation between 15 μg of HA measured by SRD, HA amount as determined by gel scanning, and haemagglutination titre. According to electron microscopy analysis, the morphology of these antigens differs regardless of vaccine type (11). The subunit vaccines Influvac and Fluvirin showed a high degree of homogeneity in the immunologically essential HA molecules, present as characteristic rosettes, when compared with the split-virion vaccines. The formation of HA rosettes results potentially in the maximum available intermolecular accessibility for epitope presentation, although it is not known whether they have any appreciable lifetime as integral rosettes once administered (11).

All vaccines except Fluvirin possessed measurable enzymatic activities of neuraminidase. Neuraminidase activity is not mentioned in the European Pharmacopoea recommendations for subunit influenza vaccines. The presence of active neuraminidase can be taken as a sign of gentle preparation of the vaccines. Whether this active enzyme can induce side-effects upon injection is not known.

In contrast to findings on subunit vaccines, influenza virus nucleo- and matrix proteins were present in all three split-virion vaccines to varying levels according to SDS-PAGE and Western blot analyses. Our results showing small amounts of nucleoprotein in one subunit vaccine (Fluvirin) correlate with the SDS-PAGE results of analysis of the same vaccine obtained by Renfrey and Watts (11). We found no reaction with anti-NP antibodies in the Western blot, probably due to the very small amounts of NP in Fluvirin compared to the NP levels in split vaccines. All split vaccines contain very little matrix protein compared to the MP of whole viruses. Renfrey and Watts also found low content of this protein in other split vaccines (Fluzone and MFV-Ject; 11).

Nucleo- and matrix proteins play an important role in the induction of specific cytotoxic T-cell (CTL) and humoral immune responses in mouse models after application of a new influenza-DNA vaccine (12). It was recently suggested that live attenuated vaccines and inactivated vaccines be coadministered in order to improve the influenza-specific CTL response in the elderly (13). Our findings also suggest that split inactivated vaccines have the potential to induce such a CTL response since HA and preserved internal proteins are both present. In a recent paper by Justewicz et al. (14), it was shown in a mouse model that the antigenic organisation of the haemagglutinin molecule substantially affects the immune response. When HA was embedded in the lipid envelope, as it is in a live virus, the response of the B lymphocytes in eliciting protective antibodies as well as the B cell memory was advantageous compared to purified glycoprotein spike preparations.

The antigenic form in the two types of vaccines is different in that HA is present on viral membrane fragments in split-virion vaccines and as isolated HA in subunit vaccines (14). This difference could have direct bearings on efficacy and should be taken into account.

The efficacy of the vaccines analysed, the level of protection they offer against clinical disease, the frequency and intensity of undesirable side-effects after vaccination, and the epidemiological significance of the molecular and biochemical criteria can only be evaluated in clinical trials.

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