Influenza Vaccination:

THE EFFECT OF DOSE AND AGE ON THE ANTIBODY RESPONSE

A methodological evaluation of serological vaccination studies

Influenza vaccinatie: het effect van dosering en leeftijd op de antilichaam productie. Een methodologische evaluatie van serologische vaccinatie studies

PROEFSCHRIFT

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Voor Ingrid, Ronit, Daphna
en vier grootouders.

"Uit louter ambitie of plichtsbesef
kan niets voortkomen dat echt waardevol is"

Albert Einstein

Preface

During several years, I have been working as a Clinical Research Scientist at Duphar B.V., a Dutch-based international pharmaceutical company.
Duphar B.V. has a 40-year history of being one of the largest influenza vaccine manufacturers in Europe. Working in the field of influenza, I expected to be involved in the research and development of new influenza vaccines some day in the future. The recent worldwide increase in interest for the development of innovative influenza vaccines prompted me to consider in depth the most efficient methodical approach to prove the safety and efficacy of any new influenza vaccine. From my own experience in the field of irritable bowel syndrome I learned, that the availability of an innovative compound, hope for its favourable characteristics in man, researchers' enthusiasm, operating standard procedures to ensure good clinical practice (GCP) studies and large investments are not sufficient ingredients for the succesful development of a novel compound. Pharmaceuticals, whether new chemical entities, biologicals or vaccines can only be succesfully developed for use in man, if in addition to these ingredients, appropriate methods are available to attain to favourable conclusions, which must form the basis for any registration application.

Considering the development of new influenza vaccines, some fundamental questions need to be addressed, some of which are listed below:

1) What antigen-specific and cross-reactive immunological responses should they minimally induce?

2) What type of clinical studies (field-, artificial challenge- and serological studies) would be required to prove the new vaccine's efficacy and/or its superiority to the existing influenza vaccines?

3) Which parameters in serological and/or efficacy studies are the most appropriate markers to assess vaccine efficacy?

4) What criteria could be used as relevant yardsticks to make a final assessment of a new influenza vaccine?

These and similar methodological questions were the rationale behind the work presented in this thesis. For competitive reasons, the strategic consequences of this work for future influenza vaccine development are not discussed. Some proposals, however, are presented to define internationally accepted consensus criteria to quantitatively evaluate serological influenza vaccination studies.

Obviously, the studies presented in this thesis could only be accomplished by the efforts of literally hundreds of people, such as the study participants, the investigators, co-authors, own and consultant statisticians, members of Duphar's influenza

project group, laboratory technicians, secretaries, data-handlers, library staff, editor, Duphar's European National Organisations, colleagues at the Clinical Research Department, the Department of Biotechnology, the International Medical Department, the Adverse Drug Reaction Unit, the Department of Information Management and, last but not least, the Department of Virology of the Erasmus University. I am grateful to all these people for their contribution to the work contained in this thesis.

A few people have played key roles in the accomplishment of this thesis.

In september 1987, when I decided to spent all my time and energy in the field of influenza, Cees Wit, then at the position of chairman of the influenza project group and manager International Operations put his faith in me and took care that the required funds for the influenza research project were made available. Cees, your trust in my professionalism at a time that I needed this badly, has contributed to a large extent to the initiation of the work for this thesis.

Prof. Masurel, dear Nic, you have actually taken the initiative for this thesis. As a result of a scientific argument we had about influenza vaccine doses, you invited me and, more importantly, stimulated me to undertake this work together with you. I am very grateful for the many, many stimulating and pleasant discussions about our favoured topic of influenza and the feelings of friendship and trust we have developed over the last four years. I feel very proud that I am your last PhD student, before you will retire. I hope you will be given many years in reasonable good health to enjoy your retirement.

Prof. van Strik, dear Roel, you, together with Arend Heyting and subsequently Alec Vardy and Paul Koopman have provided me with the basic knowledge on the principles of clinical research methodology. Your stimulating course in Columbus, Ohio in 1984 in which we simulated a clinical development programme for an imaginary compound did not only provide me with some basic knowledge, but triggered my enthusiasm for the methodological aspects of clinical research. This enthusiasm has been the main driving force for the work presented in this thesis. Your comments and hard work needed to finalize this thesis in time is also highly appreciated.

Dr. Beyer, dear Walter, as co-author of this thesis, you know better than anyone else, that the work which is presented in this thesis does not represent the end of our work, but rather a starting point to elaborate some of the ideas we have developed during the preparations of this thesis.
Only your constant optimism, your stimulating suggestions and your hard work has made it possible to complete this thesis at all. I enjoyed our close cooperation very much and I hope, that we will be able to continue our synergetic work in the future.

Hadassa and Wijnandien, do not underestimate your contributions to the succesful completion of this thesis. Without good friends and supporting colleagues, life would have been much harder.

Ingrid, Ronit and Daphna. The completion of this thesis has really been a family affair! I could enjoy even the most hectic times during the preparation of the manuscript because of our continuous team spirit.

Introduction

The medical and economical burden associated with annual influenza activity is well known and well documented (1-19).
In the USA, as many as 20.000 to 40.000 deaths each year are attributed to influenza and its complications with 80% to 90% of these occurring amongst those older than 65 years of age (20,21). The influenza-associated annual economic burden is estimated at 3-5 billion dollars (22). The registered excess mortality data associated with influenza even underestimate the actual influenza-associated deaths as shown by Glezen et al.(23), Marine (24) and Sprenger (25). Influenza-associated morbidity and mortality are not only confined to so-called epidemic years but occur each year (26). For The Netherlands for example, Sprenger has shown (25), that an average of more than 2000 elderly over 60 years died each year between 1967-1989 due to influenza-associated morbidity.

In the influenza season 1989/90, 26.000 people died in association with influenza morbidity in the United Kingdom (10) and 4100 in The Netherlands (27). The influenza virus causing the epidemic (A/Shanghai/11/87 H3N2) was almost identical to one of the vaccine strains in that year (28). Because of the close antigenic match between the epidemic and vaccine strain, it is reasonable to assume that many of these influenza deaths in 1989/90 could have been prevented.

Despite the yearly recommendations by public health authorities for immunization of persons at risk of influenza infections (29,30), vaccination rates of only 20% to 30% of these are reported in the literature to be actually vaccinated in any year (11,21,31,32). From the total influenza vaccine market in 10 European countries (table 1, 33) and assuming that all doses were given to high risk subjects (estimated as 20% of the total population, 34), the vaccination rate amongst the high risk patients is shown to vary between 15% and 63% (mean 41%) in the influenza season 1990/1991.

Table 1. INFLUENZA VACCINATION RATES - EUROPE 1990/91 Vaccination rate (%)

Country	Inhabitants (million)	High-risk (million)*	Doses of vaccine produced (33) (million)	Total population	High risk population**
France	56.0	11.2	7.1	12.7	63.4
Belgium	10.0	2.0	0.9	9.6	45.0
Austria	7.8	1.6	0.2	3.0	14.7
United Kingdom	56.0	11.2	4.3	7.7	38.4
West-Germany	61.0	12.2	3.0	4.9	24.6
Switzerland	6.8	1.4	0.3	4.4	21.4
The Netherlands	15.0	3.0	0.9	6.2	31.2
Portugal	10.0	2.0	0.5	5.1	25.4
Spain	39.6	7.9	3.5	8.8	44.3
Italy	55.0	11.0	5.5	10.0	50.0

* 20% total population high-risk for influenza infections (34)
** best case scenario: assuming all doses for high-risk patients

These data clearly demonstrate that an effective implementation of recommended vaccination policies has not been achieved so far.

Because influenza vaccines are among the least used vaccines available (35), it has been stated by Baum (32) that "we are not doing nearly enough to prevent epidemics that already have killed, and can in the fututre continue to kill, many more people than AIDS has thus far". Therefore, Ghendon (35) points out that "a more effective application of the recommended influenza immunization policy represents the least that medicine should do toward the control of influenza at present".

We can only speculate about the reasons for these low vaccination rates amongst the high-risk populations. Physicians and patients may not be completely aware that influenza causes so much morbidity and mortality even in non-epidemic years. Lack of administrative means for physicians to identify and motivate patients to have their annual influenza immunization may also contribute to the underuse of the vaccine. In addition, Nicholson et al. (36) have shown, that the major reasons for patients not to be immunized if offered a vaccination are disbelief about vaccine efficacy and the fear for systemic reactions. Both arguments imply a negative attitude towards influenza vaccines.

Educational public campaigns to inform the medical profession and the public on the facts about influenza and its prevention, as well as active strategies to achieve target vaccination rates of 60% to 80% for high-risk patients (37,38) have resulted in a considerable impact on vaccine usage in France, the United Kingdom and some states of the USA (39). During the influenza epidemic of 1989/90 in the USA, Mostow (40) has shown its effectiveness to control the impact of the epidemic in the state of Colorado.

Apparently both the scientific community and public health authorities in many European countries, have failed so far to convince the medical profession and the patients on the good reasons for their recommendations and to motivate them to participate in annual immunization programmes.

Indeed, there is ample proof of vaccine efficacy for high-risk patients. Studies by Barker and Mullooly (41), Patriarca et al. (42), Gross et al. (43), Saah et al. (44), Sérié et al. (45), Feery et al. (46), and Howells et al. (47), have shown considerable reductions in the incidence of influenza-associated bronchopneumonia, admissions to hospital, and death among elderly subjects (31).
Also an unpublished study by Patriarca et al. (cited by Arden et al.(48)) in nursing home residents confirm these data. Influenza vaccines are sometimes more effective in reducing influenza-complications, hospitalization and mortality than in preventing infections (49), particularly in the disabled. Both physicians and high-risk patients must be aware of this fact to fully appreciate the need to use the influenza vaccine.

Based on existing safety surveillance systems for medicines run by health authorities in many countries and by the influenza vaccine manufacturers, there are no objective data to justify a subjective fear of serious adverse reactions following influenza vaccination. The most common minor adverse effect associated with influenza vaccinations is a sore arm at the site of injection, which may last for one or two days which occasionally can cause some inconvenience. Taken all available efficacy and adverse effects data together, there is a very favourable benefit/risk ratio for influenza vaccines.

Besides the favourable benefit/risk ratio, current influenza vaccines have also some limitations. First, as noted earlier, protection against infections may be less than desirable in the disabled (46,48-53). This may be attributable to a declined antibody response in these patients after immunizations (54). Second, intramuscularly administered inactivated influenza vaccines induce only low levels of local IgA antibodies (35,55), which are considered to play an important role in the protection against infections (56-59). Third, it is uncertain whether inactivated vaccines induce cell-mediated immunity in humans (35), which has been shown to play an important role in the recovery from infections (56,60-67). Fourth, due to the continuous drift of influenza viruses, immunizations need to be repeated every year, because influenza vaccines are optimally effective if there is a good antigenic match between the vaccine strain and the circulating, epidemic strain (35,68-69), such as in the 1989/90 season. Finally, because influenza viruses for vaccine production are grown in embryonated eggs, the production time of influenza vaccines from seed virus to the final (distributed) product requires six to eight months.

If a potential relevant antigenic change is identified by the WHO surveillance system for influenza viruses some time before the influenza season, there is a great chance that not enough vaccine can be produced in time to accomodate the need. In 1986 such a situation occurred when the A/Singapore/6/86 (H1N1) strain, which was significantly different from the previously circulating A/Chile/1/83 (H1N1) strain, was identified in July and recommended to be included in the vaccine by the WHO in August (70). Only in the second half of December 1986, less than 50% of the usual amount of vaccine with the new strain became available for use (33).

As illustrated for the season 1989/90, where a close antigenic match has been demonstrated between epidemic and vaccine strains, the potential limitations of the currently available inactivated vaccines, as outline above, should never be a reason to limit their usage according to the recommended vaccination policies. At the same time it may be argued that there is a need for better vaccines (71) or alternatively, more flexible production methods for the current vaccines. This latter could be achieved by using cell culture techniques with suitable cell lines, similar to the monkey kidney cells which are used for the production of polio vaccines (72).

Many approaches for alternative modalities for the prevention of influenza are being investigated. Adjuvants (73,74), intranasally administered live vaccines (75-82), purified neuraminidase vaccines (83-85), liposomes (86-88), ISCOMS (89-91), CTB-conjugated inactivated vaccines (92), synthetic peptides (93) and recombinant vaccinia viruses (94) are all being investigated (95,96) to further improve our ability to control the annual impact of influenza. In addition, possibilities to use an oral vaccine formulation are also being explored (97-99). Because of the existence of safe and effective influenza vaccines, it may be expected that new vaccines will only be licensed for routine use after they have been proven to be safe and at least as effective as the current vaccines. The ultimate proof of improved efficacy for each new vaccine or any other preventive modality should come from comparative clinical studies with the existing vaccine (76).

Further, it may be expected that well designed comparative clinical studies to establish the most appropriate dose for standard clinical use, will be required. Field studies are the most direct way to assess vaccine efficacy. However, comparative field trials with different vaccines and doses are very difficult to perform and need large groups of subjects and are,as a consequence, very costly. Therefore, many of the clinical data, including the dose-range studies, required for the evaluation of new influenza vaccines, will be derived from comparative serological studies because of the established correlation between antibody titres and protection against infections (100,101).

The antibody response in serological studies, however, might be affected by age (48,102), health condition (54), antigenic dose (chapter 3), pre-immunization antibody titre (chapters 2,4,5), priming history (chapter 2) and vaccination history (chapters 2,6).
A detailed evaluation of these confounding factors might be helpful in designing clinical studies and clinical development programmes in the search for alternative influenza vaccines. Furthermore, we wanted to evaluate the "usual" experimental conditions for serological studies, such as study designs, serological techniques and parameters, study populations, statistical procedures and criteria to interprete the sero-response differences between different treatment groups and between different age groups.

In Chapters 2 and 3 of this thesis, we reviewed the effects of age and dose respectively, on the seroresponse as reported in the literature. These chapters describe a large amount of variation in experimental conditions and point to some methodological limitations often found in serological studies. In Chapter 4, the results of 5 of our own serological studies, comparing doses of 10, 15 and 20 μg HA in healthy young volunteers, are presented.

Chapter 5 describes the results of a serological, placebo-controlled dose-response study in young healthy volunteers and elderly, nursing-home residents in which doses of upto 60 μg HA of each of three influenza antigens were evaluated.

In Chapter 6, a detailed protocol is presented for an influenza live virus challenge experiment in young healthy volunteers, which was designed to study the effect of repeated influenza vaccinations on the immunity to infections. Although the study has not been carried out for logistical reasons, the study protocol is still presented as an example to demonstrate the details of such a study, including the use of a power calculation and a clinical assessment scale as yardstick to measure influenza morbidity.

In Chapter 7 finally, some of the major findings of this thesis, relevant for the interpretation of future serological studies with current and new influenza vaccines are discussed.

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2

Antibody induction by influenza vaccines in the elderly: a review of the literature

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Review

Antibody induction by influenza vaccines in the elderly: a review of the literature

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Conflicting results have been reported concerning the association between high age and response to influenza vaccines. Some authors have found a reduced response in aged subjects, others have found no difference or even better results as compared with younger control subjects. Seventeen papers were selected from international literature published in the period 1968–1988 for a review of the anti-haemagglutinin-IgG sero-response following vaccination: among 30 cases in which vaccine components could be studied independently, ten revealed a better immune response in young subjects than in the elderly, four found more favourable results in the elderly, and 16 could not detect any significant between-group-differences, the latter most probably because of a high type-2-error. Nine of these 16 cases tended to favour young subjects. These results were relativated by the finding that each paper had at least one of three methodological limitations: (1) the failure to exclude subjects with illnesses or using drugs influencing the immune system, (2) the failure to exclude subjects with previous vaccinations against influenza, (3) the failure to exclude subjects with high prevaccination antibody titres. The direction of these biases is such that failure to address any one issue will lead to an underestimate of the response of aged subjects. In view of the failure to control these biases, it was not surprising that the papers reviewed presented a heterogeneous picture. Thus, the association between high age per se and response to influenza vaccines, if any, has not yet been established. Suggestions are made for future studies in which admission criteria should control health state and previous exposure to influenza antigens.

Introduction

The proportion of people older than 60 years within the total population of developed countries is rapidly growing; the control of disorders related to higher age will increasingly become a focus of the entire health system. Human influenza viruses, naturally occurring during pandemics (influenza A) and epidemics (influenza A and B) cause significant excess morbidity and mortality in the elderly[1,2], not only in those with underlying age-related chronic diseases, but also in apparently healthy subjects[3,4]. Thus, prevention of influenza virus infections in the aged would substantially contribute to longevity and state of health. However, active immunization with currently available whole virus, split and subunit vaccines has several draw-backs: the effectiveness of these vaccines is dependent upon the continuously occurring antigenic drift or suddenly occurring antigenic shift of the membrane proteins of the influenza viruses, and they usually stimulate a short-life antibody response only. These facts have led to the recommendation to repeat vaccination annually. Moreover, published reports suggest, to various degrees, a limited efficacy of vaccines to protect aged subjects from influenza infection.

In principle, there are three ways to determine the efficacy of a given vaccine in human beings: (1) the experimental trial, (2) the field trial and (3) the immune response study.

1 In experimental trials, pathogenic effects are recorded in a vaccinated and unvaccinated group after challenge with a live pathogen. Experimental trials have been performed in healthy children and young adult volunteers with both wild or attenuated influenza strains (see Refs 5 and 6). It is evident that such a study design is impossible in aged populations.

2 In field trials, the challenge occurs by exposition of subjects to the natural field pathogen in the environment. Well-known general and methodological problems of field trials with naturally occurring influenza viruses include unpredictability of the time of its occurrence, antigenic differences between the vaccine strains and epidemic viruses, possible differences in the exposure rates of groups to be compared, and possible misdiagnoses of cases, if not laboratory-confirmed, because of the broad variety of other respiratory pathogens causing influenza-like symptoms. Special ethical problems arise in the aged. Since the benefits of influenza vaccines have been established in the sixties[7], ethical reasons have limited the performance of field trials because of the risk to unvaccinated control subjects. As a consequence, most of the studies dealing with influenza-associated morbidity and mortality of vaccinated and unvaccinated aged populations are retrospective and/or suffer from a variety of serious methodological flaws.

Strassburg *et al.*[8] reviewed 17 papers reporting influenza outbreaks in the aged and the effectiveness of influenza vaccination, during a period from 1967/68 to

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1982/83 and found important differences in study populations, vaccines, methodology and, as a consequence, levels of vaccine effectiveness. Despite these restrictions, they estimated average effectiveness rates for morbidity and mortality, respectively. While reduction of mortality in vaccinated subjects, as compared to unvaccinated controls, was calculated as being 67%, reduction of morbidity was found to be much lower (23%). However, experimental and field trials in young, healthy adults in various epidemiological settings, using various vaccines, have repeatedly revealed much higher rates for reduction of morbidity: 60–90% (for review see Ref. 9). Although comparison of such values should be made very carefully, it could appear that young subjects are better protected than the aged. Among the factors which may contribute to a possibly impaired protection rate in the aged, a direct, age-related impairment of the immunological response to vaccines is to be discussed.
3 The immune response study determines immunological changes after vaccination (mostly antibody production), which are thought to be associated with protection against the pathogen. The various methodological and ethical problems associated with field trials may be avoided. It is essential, of course, that the affirming variables for assessing the protective properties of the vaccine have been sufficiently established by experimental trials and field observations with regard to natural infection.

It is well known that serum IgG antibody against the viral haemagglutinin (HA) plays a major role in protection against influenza[10,11]: in high concentrations, it provides resistance to acquisition of infection (total protection). In lower concentrations, it prevents or ameliorates disease after infection in a substantial number of cases (partial protection). Many authors define a quantitative protection threshold, i.e. a pre-exposition titre beyond which it is highly unlikely to acquire an infection or to develop illness. Thus, anti-HA serum antibody is a predictor of protection and a measure of vaccine efficacy, which makes immune response studies a good and practical alternative to field trials.

In the present paper, available studies dealing with the anti-HA serum antibody response in elderly subjects upon immunization with inactivated influenza vaccine, are reviewed in an attempt to obtain insight into the correlation between high age and sero-response. The collected information should stimulate further research which may lead to improved policies for the use of current vaccines in the elderly or to the development of new types of vaccines.

For reasons discussed later we did not apply quantitative methods for literature review to construct statistical summaries and comparisons of studies (meta-analysis[12]) but chose a traditional narrative approach (qualitative tally), though realizing its limitations[13]. However, we have attempted to obey the strict criteria for meta-analyses as established by Sachs *et al.*[14].

Materials and methods

Criteria for selection of papers and source of literature

Age of study subjects. According to WHO practice[15], the following terminology for chronological age is commonly used: 45–59 years, middle age; 60–74 years, elderly; 75–89 years, aged; 90 or more years, very old. However, chronological age is not always a good indicator of biological age, i.e. the decline in the physiological ability to react appropriately to environmental stimuli[16]. The process of biological aging is heterogeneous and occurs at different rates in individuals. For reasons of convenience and in spite of its arbitrary character, we used the terms 'elderly', 'aged' and 'very old' synonymously and selected papers dealing with persons predominantly above 60 years of age (aged groups), and control subjects younger than 60 years of age (young groups).

Biological predictor of protection, vaccines, study design and laboratory tests. As outlined above, the production of serum antibody against viral haemagglutinin was chosen as a predictor and measure of vaccine efficacy. The induction of this antibody is strongly induced, apart from natural infection, by immunization with inactivated (whole virus, split or subunit) vaccines, but less so by experimental live vaccines whose protective effects may predominantly be caused by stimulation of local immunity[17]. We did not include papers on these latter vaccines.

Any study design was accepted which included the drawing of two blood specimens, one before and a second after vaccination. Booster immunizations were not included. Any laboratory test detecting antibody against viral haemagglutinin was accepted. Tests dealing with antibodies directed against other viral proteins (neuraminidase, core) or measuring cellular immunity were not included, in view of their unknown association with protection.

Year of performance of the studies. The impure and highly reactogenic influenza vaccines used during the 40s, 50s and 60s were of variable potency. It did not seem appropriate for us to include this early vaccine period in our survey. At the end of the 60s, a new generation of highly purified, hardly reactogenic and potent whole-virus vaccines became available[18,19], followed by split and subunit vaccines in the 70s. Coincidentally, in 1968 a new epidemiological situation occurred with the pandemic emergence of the influenza A-H3N2 subtype. Therefore, we only included studies performed from 1968 onwards.

Source of literature. English-language papers mentioned in the monthly 'Influenza Bibliography' of the Medical Research Council and the WHO World Influenza Centre, published by the Medical Research Council Library, National Institute for Medical Research, Mill Hill, London, UK, and the databases of the library computer system of the Medical Faculty of the Erasmus University Rotterdam were searched for the combination of the keywords 'influenza' and 'vaccination'. From these sources, papers were selected using the following criteria: age stratification of study subjects, type of vaccines, study design, laboratory tests, and year of performance of the study. The search was finished in December 1988.

Serological response and statistical calculations

Serological response may be expressed using one or more of the following parameters: (1) the mean fold increase (MFI), i.e. the difference between the logarithm

of mean titres of post- and prevaccination sera, (2) the protection rate (PR), i.e. the proportion of subjects exceeding a given protection threshold after vaccination, (3) the response rate (RR), i.e. the proportion of subjects who show an at least four-fold titre increase[20,21] after vaccination.

The quantities corresponding with these parameters were either taken directly from the selected papers, or recalculated from their original data if given, or derived from appropriate tables or figures as presented in the original papers. For calculation of the protection rate, the protection threshold as given by the authors was used, or, if not available in the paper, a titre of 36–40[11,22,23] when a standard microtitre haemagglutination inhibition technique had been performed. As outlined later, previous antibody against the vaccine component is a factor strongly influencing the production of new homologous antibody upon vaccination. While the protection rate is based on previously unprotected subjects, it has been attempted, for the two other parameters (MFI and RR), to retrospectively stratify the prevaccination state of the study groups as unprotected (including seronegative and low seropositive subjects) or protected subjects prior to vaccination, and to recalculate the quantities for unprotected subjects only. All recalculations are reported in the Tables.

For statistical analysis, the absolute differences between the seroresponse parameters of aged and young subjects were recalculated, and, for the protection and response rates, also the significance level (χ^2-test), and the type-2-error which is the probability of concluding that a difference does not exist, when it does in reality. By lack of original data, the significance level of differences between the MFI-values of aged and young groups could not be recalculated and was taken from the original papers if given.

Criteria for the detection of selection biases in the literature

Several factors may influence the production of antibodies after vaccination: health state of the study subjects, intake of drugs at the time of vaccination, and history of exposition to influenza antigens prior to vaccination. Ignoring these factors introduces substantial biases into a trial. Therefore, we examined the selected papers for addressing and correcting these factors.

Health state and use of drugs. Many acute and chronic illnesses (such as viral infections, malnutrition, renal and bone-marrow diseases etc.) and various drugs (such as analgesics, hormones, antimetabolites etc.) are known to impair the functions of the humoral immune system and should therefore lead to exclusion from immune response trials[24].

Prevaccination antibody. Specific or cross-reacting antibody against a given influenza strain present in high concentrations before vaccination with this strain, may suppress or impair the production of new antibody. According to this 'law of initial values'[25] or 'negative feedback'[26], the response to a given vaccine strain is inversely related to the specific prevaccination antibody titre. Possibly, already circulating antibody combines with the vaccine to mask its recognition by immunocompetent cells, or, alternatively, switches off new immunoglobulin synthesis by a more central mechanism[26]. Therefore, when performing vaccination trials, one should carefully describe the prevaccination state of the study populations and analyse the data for different prevaccination classes, or exclude persons with high prevaccination titres.

History of previous vaccinations. Vaccinations with heterologous influenza viruses in the past, even if they are no longer serologically detectable at the moment of the ungoing study (i.e. in the absence of prevaccination antibodies) may unpredictably influence the antibody production of the vaccine studied: sometimes a booster-effect is observed, sometimes a suppressive effect[27]. Subjects with previous vaccination should therefore be excluded.

Results

Papers to be reviewed, and data on study design

The sources revealed 1805 titles for the combination of the keywords 'influenza' and 'vaccination'. Only 20 papers met all our inclusion criteria. Many more papers dealt with influenza vaccination trials in the elderly but had not included control groups consisting of younger subjects. From the 20 selected papers, three[28–30] were excluded because the anti-HA-IgG ELISA used did not discriminate between the different vaccine components.

Data of seven studies published in the period 1968–88 are presented in *Table 1* in chronological order (years of performance of the trial, or, if this information had not been given, year of publication)[19,31–46]. Most of the studies (10) were carried out in the United States of America. Only 10 studies (not shown) gave information about the seasonal relationship of the vaccination trial to natural influenza epidemics. Trials performed during or shortly after epidemics with considerable impact would make it difficult to decide whether titre rises were due to the vaccine or to the natural virus.

The period between collection of the pre- and postvaccination sera was usually 21–42 days (not shown); two studies[41,44] chose a shorter interval (14–15 days) which might be suboptimal, and two other papers[39,43] did not give any information in that respect. As a technique for anti-HA-antibody determination, usually the haemagglutination inhibition test was performed, with the exception of Ref. 33 (neutralization test).

As in many studies bi- or trivalent vaccines had been used, the seroresponse to, in total, 30 vaccine components could be studied separately. When using various vaccine types[34] or various dosages[19,42], the data on seroresponse were pooled, either by the authors themselves or by us, as there was a similar stratification among the study group of aged subjects and control groups. From 1967 onwards, the antigenic contents of commercial vaccines has been estimated by comparison with an international influenza A standard preparation and expressed in chicken cell agglutination (CCA) units (IU, international units). With the development of split and subunit vaccines, this technique became impracticable (see extremely high dosages in Refs. 38 and 39!) and was replaced by the single-radial immuno-diffusion test (μg haemagglutinin, HA) by the WHO in 1978 (for review see Ref. 47).

Table 1 Papers to be reviewed, and data on study designs

Study[a,f,g]	Year[b]	Place[c]	Vaccines used		
			Strain[d]	Type[e]	Dose
Gwaltney[31]	1968	USA	A/Hong Kong/2/68 (H3N2)	WV	400 CCA
Marine[39]	1968	USA	A/Aichi/2/68 (H3N2)	WV	400 CCA
Fulk[33]	1968	USA	A/Aichi/2/68 (H3N2)	WV	400 CCA
Mostow[79]	1969	USA	A/Japan/170/62 (H2N2)	WV	300/3000 CCA
			A/Aichi/2/68 (H3N2)		300/3000 CCA
Cromwell[34]	1969	USA	A/Aichi/2/68 (H3N2)	WV/SP	400 CCA
Marine[35]	1971	USA	A/Aichi/2/68 (H3N2)	WV	1000 CCA
			A/Japan/305/57 (H2N2)		1000 CCA
Howells[36]	1971	UK	A/Hong Kong/2/68 (H3N2)	WV	400 CCA
			B/Victoria/70		200 CCA
Ruben[37]	1972	USA	A/Aichi/2/68 (H3N2)	SU	700 CCA
McKenzie[38]	1973	AUS	A/England/42/72 (H3N2)	SU	16 000 CCA
Feery[76]	1976	AUS	B/Hong Kong/8/73	SU	8 000 CCA
			A/Port Chalmers/1/73 (H3N2)		16 000 CCA
Sarateanu[40]	1977	FRG	B/Hong Kong/8/73	WV	360 CCA
			A/Victoria/3/75 (H3N2)		400 CCA
			A/New Jersey/8/76 (H1N1)		400 CCA
Hannoun[41]	1977	FR	A/New Jersey/8/76 (H1N1)	WV	200 CCA
Beare[42]	1978	UK	A/New Jersey/8/76 (H1N1)	WV	4–61 μg
Phair[43]	1978	USA	A/New Jersey/8/76 (H1N1)	SU	200 CCA
			A/Victoria/3/75 (H3N2)		200 CCA
Feery[44]	1979	AUS	B/Hong Kong/8/73	SU	250 CCA
			A/USSR/90/77 (H1N1)		250 CCA
			A/Texas/1/77 (H3N2)		250 CCA
Gross[45]	1984	USA	A/Philippines/2/82 (H3N2)	SP	15 μg
			A/Chile/1/83 (H1N1)		15 μg
			B/USSR/100/83		15 μg
Gross[46]	1985	USA	A/Philippines/2/82 (H3N2)	SP	15 μg
			A/Chile/1/83 (H1N1)		15 μg
			B/USSR/100/83		15 μg

[a]First author and reference number of paper
[b]Year of performance of the study or, if not given, year of publication of paper
[c]Country of performance of the study
[d]Abbreviated WHO-nomenclature of the virus strain used in the vaccine
[e]Vaccine type (WV, whole-virion; SU, subunit; NG, data not given)
[f]Vaccine doses (CCA, chicken cell agglutination units; μg, microgram HA)
[g]Comment on study design. See appendix

Data on populations studied

Table 2 presents data to characterize the size, age range and prevaccination titres of the studied groups of aged and young subjects. The total numbers of groups varied from 10[40] and 11[33] up to 437 and 203[34], for elderly and young groups, respectively. The age distribution for the elderly usually reached from 60–70 up to 101 years of age; the young groups consisted of subjects either of a particular narrow age cohort (children[33], young adults[32,41]) or a very broad age range (< 65 years). Three studies[31,37,45] did not report any age range. The first one characterized the aged group only by the term 'elderly population', the latter two gave high mean ages (80 years, 71–74 years). Another paper[33] presented an age range also including persons younger than 60 years but the text suggested that those subjects had been few.

The elderly populations were usually recruited from old people's homes or geriatric institutions which was apparently reflected by the sex distribution: in all studies which gave data on that point, more female than male subjects had been included (not shown).

For 20 vaccine components, the prevaccination state of aged and young groups could be compared. For 10 strains, the elderly had a higher mean titre prior to vaccination; for four strains these values were equivocal, and in six instances the younger groups showed higher mean titres than the elderly.

Serological results and statistical calculations

Table 3 shows the results of the serological tests performed with pre- and postvaccination sera, and some basic statistical calculations.

MFI-values for aged and young groups were present in all but one paper; transformed to a logarithmic scale, the MFI varied in a wide range between 0.2–1.4 for aged and 0.2–1.6 for young groups. For 15 of the 30 vaccine components, the authors themselves had given the significance level of the between-group differences for the MFI-values, which in none of these 15 cases was smaller than 5%. The significance level for the remaining papers could not be recalculated by us because of missing raw data in the papers.

The protection and response rates were also heterogenous, reaching from 16 to 93% for aged, and from 21 to 100% for control groups. Between-group differences of protection rates could be calculated for 12 vaccine components, and of response rates for 16 vaccine components. Differences were only small for six protection rates and seven response rates and were not significant on a 5% level, but they had a high type-2-error (51–97%). In these cases, interpretation is difficult as it is not possible to decide whether (1) there really were no differences between age groups or, (2) there were differences, however, the studies were too insensitive (too small sample sizes) to detect these.

Table 2 Data on the study group composition of 17 selected papers

Study[a] (Ref.)	Strain	Aged			Young		
		No	Age range	S1[b]	No	Age range	S1[b]
31	A/HK/68	80	NG[c]	1.1	53	NG	0.8
32	A/HK/68	31	67–99	1.3	23	23–25	0.6
33	A/HK/68	16	50–87	NG	11	6–14	NG
19	A/JAP/62	297	>65	1.5	545	<65	1.7
	A/HK/68	293		0.7	512		0.7
34	A/HK/68	437	NG	NG	203	NG	NG
35	A/HK/68	40	80–101	NG	70	6–31	NG
	A/JAP/57	36	NG	NG	90	NG	NG
36	A/HK/68R	134	>60	1.0	107	<65	1.3
	B/VIC/70			0.8			1.0
37	A/HK/68	57	NG	1.8	17	NG	0.9
38	A/ENG/72	69	60–79	1.1	231	15–59	1.0
39	B/HK/73	61	68–93	1.5	52	21–60	1.3
	A/PC/73			2.3			2.0
40	B/HK/73	10	>60	0.3	67	2–59	0.6
	A/VIC/75			0.7			0.7
	A/NJ/76			1.0			0.2
41	A/NJ/76	73	69–94	1.4	57	18–20	0.3
42	A/NJ/76	84	≥65	1.9	372	13–65	NG
43	A/NJ/76	72	60–95	1.4	20	<45	NG
	A/VIC/75			1.2			NG
44	B/HK/73	45	66–100	NG	47	25–64	NG
	A/USSR/77			NG			NG
	A/TEX/77			NG			NG
45	A/PHIL/82	25	NG	1.3	21	NG	1.3
	A/CHIL/83			1.3			1.5
	B/USSR/83			1.4			1.5
46	A/PHIL/82	56	>65	1.3	45	<65	1.3
	A/CHIL/83			1.8			1.4
	B/USSR/83			1.7			1.5

[a]Comment on group composition. See appendix
[b]Prevaccination mean titre of the entire group (decadic logarithm)
[c]Data not given (NG)

There were also significant between-group differences, 6 for the protection rate and 9 for the response rate, associated with a low (<1–5%, 8 cases) or intermediate (16–42%, 6 cases) type-2-error. For some vaccine components, a more favourable immune response for the young groups was revealed (significantly negative differences: 2 for PR and 8 for RR). However, also contrary results could be found (significantly positive differences: 4 for PR and 1 for RR).

Thus, a heterogeneous picture could be obtained, with more insignificant and significant results in favour of young subjects, which suggests a slight tendency.

An attempt to combine the pattern of MFI, PR and RR per vaccine component to obtain a tendency per paper is shown in the last column of *Table 3*. Where the absolute differences of all given seroresponse measures had the same sign, this sign was used to indicate a tendency (either '−' for a better immune response of younger groups, or '+' for the alternative case). Where one of the given seroresponse measures was significant on the 5% level, the sign was doubled (either '−−' or '++'). Where the different seroresponse measures showed insignificant differences with different signs, this was expressed as '0' (no tendency detected).

Table 4 presents the 30 cases assessed in this way. For 16 cases, a 'significant' difference between the age groups was not detected (sum of cases indicated as '−', '+' and '0'). However, most of these (9 cases) suggest a slight tendency in favour of young age groups. This is supported by 10 cases with a clear tendency in favour of young groups, *versus* only four in favour of aged groups. The distribution of these results according to types or subtypes of virus strains does not reveal additional information for influenza A-H1N1, A-H2N2 and influenza B, because of the small number of cases per type/subtype. For influenza A-H3N2, results are contradictory, presenting both 3 'significant' cases in favour of young groups, and of aged groups, respectively.

Selection biases and combinability of serological results

Table 5 addresses three important criteria for group composition which should have been obeyed by the papers: absence of illness and drugs influencing the immune system, no previous influenza vaccinations, and stratification for or exclusion of protective prevaccination titres (or presentation of data in a way that this would be possible in retrospect). The documentation of these data was very incomplete in virtually all papers. In six cases it was stated, at least, that the study populations were clinically healthy. Two other papers reported that it dealt in part with 'chronically ill' subjects which did not lead to their exclusion. Another paper[45] included children and young adults with cystic fibrosis as a control group. Only eight studies stated that the vaccinees, in the course of the study, had not used drugs influencing the immune system. Four papers reported that some of the participants had received other influenza vaccinations in previous years which, however, did not lead to

Table 3 Serological results of 17 selected papers, and basic statistical calculations

Study (Ref.)	Strain	Mean fold increase (MFI)[a,b]						Protection rate (PR)[a,b]							Response rate (RR)[a,b]							
		Aged		Young		Stat		Aged		Young		Stat			Aged		Young		Stat			
		No	MFI	No	MFI	Diff	Sign	No	PR	No	PR	Diff	Sign	T2	No	RR	No	RR	Diff	Sign	T2	Tendency[c]
31	A/HK/68	63	1.1	52	1.2	−0.1	ns[d]	63	67	52	77	−10	ns	78	63	81	52	52	− 9	ns	71	−
32	A/HK/68	31	0.4	23	0.8	−0.4	NG[e]															−
33	A/HK/68	16	0.7	11	1.0	−0.3	ns								16	56	11	91	−35	ns	51	−
19	A/HK/68	293	1.4	512	1.3	0.0	NG								293	82	512	91	− 9	<0.1	5	− −
	A/JAP/62	297	0.5	545	0.9	−0.4	NG								297	48	545	69	−21	<0.1	<1	− −
34	A/HK/68	81	1.2	33	0.8	0.4	NG	73	86	31	52	35	<0.1	4								+ +
35	A/HK/68	40	0.3	70	0.7	−0.4	NG								40	18	70	64	−47	<0.1	<1	− −
	A/JAP/57	36	0.4	90	0.6	−0.2	NG								36	33	90	59	−26	<1	25	− −
36	A/HK/68R	134	0.7	107	0.8	−0.1	NG															−
	B/VIC/70	134	0.2	107	0.5	−0.3	NG															−
37	A/HK/68	24	0.9	13	1.3	−0.4	NG	24	88	13	62	26	ns	55								0
38	A/ENG/72	69	1.5	231	1.4	0.1	ns	54	93	200	78	15	<5	24	54	85	200	75	10	ns	67	+ +
39	B/HK/73	61	0.6	52	0.7	−0.1	ns								61	57	52	77	−20	<5	41	− −
	A/PC/73	61	0.6	52	0.7	−0.1	ns								61	59	52	71	−12	ns	73	−
40	B/HK/73	10	1.2	67	1.3	−0.1	NG															−
	A/VIC/75	10	1.1	67	1.1	0.0	NG															0
	A/NJ/76	10	1.2	67	1.6	−0.4	NG															−
41	A/NJ/76	73	0.9	57	0.9	0.0	ns	32	91	57	29	61	<0.1	<1	73	73	57	42	18	<5	42	+ +
42	A/NJ/76	84	1.1	369	1.4	−0.3	NG	19	89	312	82	8	ns	91	84	77			−13	<1	16	− −
43	A/NJ/76	33	0.9												33	76	20	100	−24	<5	28	− −
	A/VIC/75	56	0.5												56	45	20	100	−55	<0.1	<1	− −
44	B/HK/73	45	0.3	47	0.2	0.0	ns	45	20	47	55	−35	<0.1	5	45	18	47	21	− 6	ns	90	− −
	A/USSR/77	45	0.5	47	0.5	0.0	ns	45	40	47	87	−47	<0.1	<1	45	36	47	38	− 3	ns	95	− −
	A/TEX/77	45	0.6	47	0.6	0.0	ns	45	69	47	68	1	ns	97	45	44	47	43	2	ns	96	0
45	A/PHIL/82	25	0.4	21	0.3	0.1	ns															+
	A/CHIL/83	25	0.5	21	0.2	0.3	ns															+
	B/USSR/83	25	0.4	21	0.4	0.0	ns															0
46	A/PHIL/82	56	0.5	45	0.3	0.2	ns	35	74	26	42	32	<5									+ +
	A/CHIL/83	56	0.2	45	0.2	0.0	ns	16	50	22	59	− 9	ns									−
	B/USSR/83	56	0.2	45	0.4	−0.2	ns	16	63	20	60	3	ns									0

[a] Basic statistics include the absolute difference between the seroresponse measures of aged and young groups (Diff), the significance level of that difference (Sign, %) and, for PR and RR, the type-2-error (T2, %)

[b] Denominator for calculation of MFI, PR, and RR, respectively

[c] Tendency to detect differences in seroresponse between aged and young study groups. −, seroresponse measures higher for young group (− −, including significant differences); +, seroresponse measures higher for aged group (+ +, including significant differences); 0, no tendency

[d] Not significant on the 5%-level (ns)

[e] Data not given (NG)

[f] Comment on calculation of the presented data. See appendix

Table 4 Tendency of 17 selected papers to detect differences between aged and young study groups, subdivided for types and subtypes of 30 vaccine strains

Type/subtype of vaccine strain	Tendency[a]				
	−−	−	0	+	++
A-H1N1	3	2		1	1
A-H2N2	2				
A-H3N2	3	5	3	1	3
B	2	2	2		
Total[b]	10	9	5	2	4

[a] −, seroresponse variables higher for young group (−−, including significant differences); +, seroresponse variables higher for aged group (++, including significant differences); 0, no tendency
[b] Number of vaccine strains

exclusion of those subjects. Only one paper dealt *expressis verbis* with previously unvaccinated subjects. Thus, of the 17 controlled studies, none was free of any bias and 16 failed to address two or three criteria.

As the papers revealed a substantial heterogeneity in view of group composition and distribution of main biases which could not be corrected for, it did not appear to be possible to combine or pool the data on serological response (*Table 3*) in the sense of meta-analysis techniques.

Discussion

Influenza viruses are a major cause of mortality and serious morbidity in the elderly. Therefore, recommendations for the use of influenza vaccines have been given in several countries. Is vaccination really effective in this age-group? It is widely believed that, in the elderly, the ability to produce a sufficient amount of antibody after administration of bacterial and viral vaccines, is decreased, alongside with other age-related changes of the entire immune system. Early support for this impression came from the classical study of Sabin *et al.*[48] describing a lower immune response in aged people to Japanese B encephalitis virus vaccine, as compared to children. This paper has been cited frequently, but the observations of Sabin are based on 20 children and 20 aged individuals only, with a different history of previous expositions to the pathogen. Other support comes from field trials measuring the protection acquired by vaccination, against the naturally occurring pathogens (usually influenza viruses and *Streptococcus pneumoniae*), often suggesting a lower vaccine effectiveness in the elderly as compared to younger subjects. However, such studies usually struggle with methodological flaws[8,49].

Only a few studies have been published which compare the seroresponse of different age groups to vaccines other than influenza. Results, as a working paper of the World Health Organization pointed out, are 'still incomplete and are often contradictory'[15]. Ammann *et al.*[50] in testing a bivalent *Streptococcus pneumoniae* vaccine, found lower absolute antibody titres but a still sufficient protection rate, in a group of 66 elderly persons, as compared to 20 young adults. Musher *et al.*[51] described 10 healthy young adults and five aged patients with chronic pulmonary disease after polyvalent *Streptococcus pneumoniae* vaccination and saw no significant differences in absolute postvaccination antibody titres, but a lower opsonizing activity, in the sera of the elderly. Other papers on *Streptococcus pneumoniae* vaccination not including young control groups, report satisfying antibody development in the aged[52–57]. Solomonova and Vizev[58] detected a slightly delayed antibody production after vaccination with tetanus toxoid in aged subjects as compared to subjects aged 40–60 years. The difference, however, was not significant. In 70 non-dialysed elderly individuals, Denis *et al.*[59] found a profoundly impaired seroresponse to hepatitis B vaccine.

In an attempt to reveal, from the recent literature, the association between high age and the ability to produce specific antibodies after influenza vaccination, we could not detect a clear answer, but a tendency only: for 10 out of 30 single vaccine components, statistical calculations suggest a lower response of the elderly, as compared to younger control subjects. Another 9 cases support this tendency but show insignificant differences between age groups only. On the other hand, some contradictory results exist as well (elderly more favourable than young in 4 cases).

We showed that virtually all studies reviewed by us insufficiently controlled two major groups of biases: differences in health state and differences in previous exposure to influenza antigens. The general direction of these selection biases is such that failure to control these leads to an underestimation of the response of the elderly: in this age group, the prevalence of disease and drug use influencing the immune system, the chance of previous vaccinations, and the mean titres of prevaccination antibody, are generally higher. Neglecting these factors during the selection of the study subjects has introduced a considerable heterogeneity in the study groups of the papers reviewed and has also seriously affected the validity of the described results which may then give an idea about the overall condition of the group studied, but cannot lead to conclusions concerning fundamental age-related changes in the immune system. Although we saw a tendency supporting a decreased seroresponse in

Table 5 Selection biases present in 17 papers

Study (Ref.)	Corrected for		
	Health state[a]	Vacc. history[b]	Prevacc. state[c]
31	No	No	Yes
32	No	No	No
33	Yes	No	No
19	No	No	No
34	Yes	No	No
35	No	No	No
36	No	Yes	No
37	No	No	Yes
38	No	No	Yes
39	No	No	No
40	No	No	No
41	No	No	No
42	No	No	No
43	Yes	No	Yes
44	No	No	No
45	No	No	No
46	No	No	No

[a] Subjects excluded who had diseases or used drugs which may influence immune response
[b] Subjects excluded who had a history of previous influenza vaccinations
[c] Subjects excluded who had high (protecting) prevaccination titres

Table 6 Selection and stratification criteria for influenza vaccination trials

Criterium	Controlled by
Health state	Senieur protocol[24]
Vaccination history	Exclusion of subjects previously immunized with any influenza vaccine
Prevaccination antibody	Exclusion of subjects with high/protective homologous serum antibody prior to vaccination
Priming	Stratification according to priming periods (see *Table 7*)

Table 7 Influenza A prevalence and priming eras

Haemagglutinin subtype	Period of prevalence	Priming period (birth years)
H2	1889–1899	–1877
H3	1890–1917[a]	1878–1905
Hsw	1918–1925	1906–1920
?	1926–1932	1921–1927
H0[b]	1933–1946	1928–1939
H1	1947–1957	1940–1949
H2	1958–1967	1950–1961
H3	1968–[a]	1962–

[a]1908–1918, and 1978– co-circulation of H3 and H1[61]
[b]According to WHO-nomenclature of 1971. In the new nomenclature of 1980, Hsw, H0, and H1 are expressed as H1

the elderly, the high prevalence of biases does not justify any generalization. The association between high age and responsiveness to influenza vaccines, if any, has not yet been established.

In this paper, we have used basic statistical methods analogous to another review on influenza vaccinology: Gross *et al.*[60] reviewed 12 studies on the effects of cancer chemotherapy on the seroresponse to influenza vaccines and found that in eight papers a significantly lower antibody development in subjects on treatment, as compared to untreated control subjects, had been established. Moreover, they detected a high type-2-error in the remaining four studies which could not find any significant effects of chemotherapy which was probably due to insensitivity rather than to the absence of such differences. In contrast to the definite results of Gross *et al.*, our results are more heterogeneous, even taking into account that many papers in our review had also a high type-2-error. The main distinction between the two reviews appears to be the fact that in the studies included by Gross *et al.*, there was usually no major difference in age distribution, and, as a consequence, the two major groups of biases, differences in health state and in previous exposure to influenza antigens, were avoided.

Based on the considerations in the section Materials and methods of this review, we suggest admission criteria for future immune response studies in the elderly as described in *Table 6*. To define the health state of vaccinees, general criteria such as 'apparently healthy' or 'without overt diseases' are insufficient to exclude underlying disease, as pointed out by Ligthart *et al.*[24]. Therefore the strict 'Senieur Protocol: Admission criteria for immunogerontological studies in man' should be used as it includes clearly defined anamnestic and clinical information, laboratory data and drug interference. Subjects previously immunized with any hetero- or homologous influenza vaccines, and subjects who do not clearly remember, are to be excluded. Subjects with high/protective serum antibody prior to vaccination, specifically directed against the vaccine component, are to be excluded. The exclusion threshold may be dependent on the antibody detection method used.

In contrast to the three criteria mentioned above which can be controlled by an appropriate study design, the age-related exposition to different influenza A subtypes is a factor which cannot be controlled. However, it strongly influences the immune response to vaccination even dozens of years later. *Table 7* shows the natural occurrence of haemagglutinin subtypes from 1889 onwards, as has been established by seroepidemiological studies and, from 1933 onwards, by direct isolations in man[61]. In particular, the first influenza A infection during lifetime ('priming') is of interest. It usually occurs at an age between 5 and 15 years and results in a potent immunological memory for the subtype to which the infective strain belongs. Individuals primed by a special subtype, react differently to later homologous or heterologous infections or vaccinations than those primed for another subtype (the doctrine of 'original antigenic sin'[62]). Therefore, age cohorts can be established according to primary infection. *Table 7* presents the distribution of Marine and Thomas[35], based on 687 persons of all ages in the United States in 1971 which has largely been confirmed by Masurel and Andre (1978)[63] who studied a population of 403 subjects in The Netherlands in 1977. The existence of age cohorts with differing natural exposure histories questions the validity of the general definition of 'aged' or 'elderly' as being 60 years of age or more, and suggests the necessity of a more differentiated age stratification according to the priming periods.

We believe that the use of strict selection and stratification criteria is mandatory if one wishes to study ageing *per se*. More knowledge of the latter is needed to develop appropriate strategies to protect elderly who do suffer from diseases and who have an increased risk of acquiring complications during or after influenza infection.

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Literature update

In August 1991, a new literature search was performed for the period January 1989-August 1991 using the databases Medline (National Library of Medicine, Bethesda/ML, USA), Biosis Previes (Philadelphia/PA, USA), Embase (Excerpta Medica, Amsterdam, The Netherlands) and Scisearch (ISI, Philadelphia/PA, USA). Database outputs were checked for duplicates and only unique titles were screened.

The combination of the key-words "influenza" and "vaccination" yielded a total of 1014 titles. A further combination with the key-words "elderly"/"aged" resulted in 146 unique titles from which four were selected to be considered

in more detail for the selection criteria as mentioned in the review. One paper (66) compared the immune response to trivalent inactivated influenza vaccine in young (<60 years of age) and elderly subjects (>60 years of age), but used the single-radial haemolysis technique. The authors did not detect significant differences between the two age groups. In two other papers (67,68), elderly subjects were included in the study, but no young controls. Finally, McElhaney et al. (69) compared IL-2 production of peripheral mononuclear blood cells after whole-virus vaccination in elderly and young subjects and found lower levels for the elderly. None of the papers from the update literature search were further analysed according to the methods used in the published review.

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Influenza vaccination in elderly residents in nursing homes: Immune response to trivalent and monovalent inactivated influenza virus vaccine in the season 1986-87
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68 Gross PA, Quinnan GV, Weksler ME, Setla U, Douglas GR
Relation of chronic disease and immune response to influenza vaccine in the elderly
Vaccine 1989;7:303-308
69 McElhaney JE, Beattie BL, Devine R, Grynoch R, Toth EL, Bloackley RC
Age-related decline in interleukin 2 production in response to influenza vaccine
J.Am.Geriatr.Soc. 1990;38:652-658

Appendix

Comments

Gwaltney *et al.*[31]:
Four study groups were described: 'spray group', 'control group', 'combined group' and 'gun group'. Only the latter two were included here. Data for the neutralization test were not included. PR and RR were read from *Figure 1*.

Marine *et al.*[32]:
MFI-values were read from *Figures 2* and *3*.

Fulk *et al.*[33]:
Groups vaccinated either locally, or parenterally, were described. Only the latter ones were included here. Log_2 values in *Table 1* of the original papers were transformed to log_{10} values.

Mostow *et al.*[19]:
Data for 'prison group (GSP)' and 'school group (OCS)' were pooled to form the young group here. *Table 5*, which presented the seroresponse, gave only percentages without the appropriate denominators. These had partly to be taken from *Table 1* of the original paper, partly to be calculated using a short information, in brackets, in Materials and methods.

Marine and Thomas[35]:
Groups 1 and 2 (birth date 1940–1965), but not group 3 (birth date 1892–1939) of *Table 4* of the original paper were pooled to form the young group here.

Howells *et al.*[36]:
Vaccination trials from 1971 to 1973 were described. Only data for 1971 were included here. The data for the young group came from a different paper[64]. Only seroconversion from negative prevaccination titres to postvaccination titres greater than 10, and mean postvaccination titres were given.

Ruben[37]:
Also Ref. 65 was reviewed (identical experiment). In the original paper, bivalent vaccine (A/Aichi/2/68 and B/Mass/1/71) was described, but no data were presented for the latter strain. It should be mentioned that vaccination of the control group had been performed one year earlier and with a higher vaccine dose.

Mackenzie[38]:
A bivalent vaccine (A/England/42/72 and B/Roma/1/67) was used, but no data were presented for the latter strain. In *Tables III* and *IV* of the original paper, primary and booster vaccination were combined while, in the first two Tables, the effects of both injections could be studied separately. Moreover, the authors included only seroresponders in *Tables III* and *IV* of the original paper.

Sarateanu *et al.*[40]:
Age groups 'I–III' of the original paper were pooled to form the young group.

Hobson *et al.*[26]:
A trivalent vaccine (A/New Jersey/8/76, A/Victoria/75 and B/Hong Kong/2/8/73) was used, but no data were presented for the latter two strains. Calculation of $51:0.5\log_5$ for negative sera. In forming the young group, only data of young persons receiving the identical doses as aged subjects (200 CCA), were used.

The Pandemic Working Group etc.[42]:
The numbers and percentages of *Tables 2, 3* and *4* of the original paper did not fit well. For example, in *Table 3*, 11% of the subjects >65 years had a prevaccination titre greater than 10 but in *Table 4*, 13%.

Feery *et al.*[44]:
The young group was formed only by the group named 'adults' in the original paper; another group named 'young adults' was excluded here because these subjects had received a booster immunization.

Gross *et al.*[45]:
Two more aged groups were vaccinated with higher doses of the trivalent split vaccine, and three more groups with different doses of a whole virus vaccine. The total number of persons was 148, divided into six groups. The mean ages of the groups ranged between 71 and 74 years. The control groups were children and young adults with cystic fibrosis. For our calculations, we used the data of *Table 2* of the original paper. Protection rates could not be recalculated as numbers of protected subjects before vaccination were not given.

Gross *et al.*[46]:
Two more aged groups (C and D in *Table 1* of the original paper) were vaccinated with whole virus vaccine. These data were excluded because of the lack of a young reference group. Data on booster vaccination in the elderly were excluded. The numbers of aged groups vaccinated with split vaccine in *Tables 1* and *2* ($n=56$), and *Table 3* ($n=25$) of the original paper differ. For our calculations, we used the data of *Tables 1* and *2* of the original paper.

3

The effect of vaccine dose on antibody response: a review of the literature

INTRODUCTION

The dose of the influenza vaccine antigens to be administered, is essential for any vaccine policy. A considerable inconsistence on this subject is reflected by the different dose policies in the Western world: In the United States, a dose of 15 µg HA of each of the components is required, in most European countries, however, only 10 µg HA; according to the European Pharmacopae, influenza vaccines should contain 7 - 20 µg HA per strain.

Although influenza vaccines have already been used for many decades, and although many clinical trials have been done to determine the most appropriate dose of vaccine antigen, there still remains confusion on this subject. Table 1 presents some arbitrarily chosen citations from the international literature which illustrate that, for any "opinion" on this matter ("High doses are necessary" - "Low doses are sufficient" - "It does not matter"), one or more publications can be referenced.

Table 1: Arbitrary citations from international literature.

A 'shallow' dose response effect over a wide range of antigen concentrations ... [5-94 µg HA per dose]... was noted for each age group. [1]
The antibody response to the 43 µg HA dose was significantly higher than was the response to the 10 and 4 µg HA doses. [2]
The serum HI antibody responses to vaccine showed a dose-response relationship. [6]
Vaccines containing lower doses ... (6-9 µg) produced ...antibody responses equivalent to those produced by higher doses (15-28, and 19-28, resp.) in all age groups. [4]
The relatively uniform antibody responses observed were attributed to the newer methods of vaccine standardization introduced after the clinical trials in 1976. [16]
An increased antigenic mass of influenza virus HA, above the 9-10 µg ..., did not elicit significantly higher antibody levels, ... than the dose containing 6 µg HA.... It is clear that no advantage is to be gained by increasing HA dosages above that recommended for commercial use. [17]
The lower dose vaccine [10, 10, 10 µg HA] results in an effective serological response. The higher dose vaccine [10, 15, 15 µg HA] has no clinical advantages. [9]
These preliminary observations suggest that large increases in the potency of ... influenza B vaccine may improve the antibody of elderly recipients. [10]
No significant differences in antibody response ...occurred among the vaccine groups. [13]

If a real dose-effect in the required dose-range for influenza vaccines does actually exist, would it be possible, that a great deal of the inconsistency as illustrated in Table 1 is due to the fact that the published studies differ with regard to a great many factors? These would be: vaccine types, vaccine strains, study designs, epidemiological situation, laboratory techniques, criteria for antibody response and statistical procedures used to analyse the obtained data, as was found in the previous chapter for the effect of age on the antibody response. Or, alternatively, if no dose-effect relationship does actually exist, would it be possible, that sometimes significant differences between doses are due to methodological artefacts, or due to just chance?

The purpose of this chapter is to review the recent international literature with regard to the effect of antigen dose on the antibody response. It should be noted that, as is true for each literature review, there may be an unavoidable publication bias, which could possibly affect the conclusions drawn from the review.

Materials and Methods

1. Source of literature, and selection of papers.

The databases Biosis Previes (Philadelphia/PA, USA), Medline (National Library of Medicine, Bethesda/ML, USA), and Embase (Excerpta Medica, Amsterdam, The Netherlands) were searched for the combination of the key-words `influenza', `vaccine' (vaccination) and `dose' (doses, dosing, dose, doses) in papers written in English. The search was undertaken in June 1991 and comprised the period January 1978 through June 1991.

The year 1978 was chosen for the following reason: Since 1967, the antigenic contents of vaccines had been estimated by comparison with an international influenza A standard preparation and expressed in chick red blood cell agglutination (CCA) units. This method was not always reliable as it tended to underestimate vaccine contents when whole virus particles were aggregated. Moreover, with the development of split and subunit vaccines, this technique became impractical because it produced extremely high values: free haemagglutinin molecules can agglutinate erythrocytes more effectively than virus-bound haemagglutinin. In 1978, the World Health Organisation replaced the CCA-method by the single-radial immuno-diffusion (SRD) test which expresses antigenic vaccine contents as µg haemagglutinin [18]. Dose-response studies became more reliable [16]. Therefore, serological studies published since 1978 using the SRD method to determine the antigen dose, were selected for this review.

Studies were selected in which at least two doses of an inactivated influenza vaccine (whole-virus, WV; split, SPL; subunit, SU) were administered intramuscularly or deeply intracutaneously. Any study design was accepted which included the sampling of

two blood specimens (one before and a second after immunization), and the detection of antibody against viral haemagglutinin by the haemagglutination inhibition (HI) method; excluded were alternative methods, such as single radial haemolysis or ELISA, as were tests dealing with antibodies directed against other viral proteins (neuraminidase, M-protein) or measuring cellular immunity, in view of their unknown quantitative association with protection.

2. Serological response measures, and statistical analysis.

In this review, the same basic considerations as in Chapter 2 are observed. Protection rate (PR), mean fold-increase (MFI), and response rate (RR) are defined and calculated in the same way. Modifications of the review-procedure are described where appropriate. The formula to calculate confidence intervals with observed rate differences in those cases where there were only two dose groups in a stratification group, are given in Appendix 4. In case of more than two dose groups in a stratification group, a probit-analysis was performed using the SAS-Software Package.

Results

1. Papers reviewed

The literature search, after removal of duplicates, revealed 217 English-written titles from 1978 through 1991, for the chosen combination of keywords. From those, 199 dealt with subjects other than dose-response trials with inactivated influenza vaccines, many of them (46) using experimental live-vaccines. Eighteen papers described dose-response trials with inactivated influenza vaccine. One paper, not selected, presented the serological response using single radial haemolysis, but not the HI technique [17]. The authors found no positive dose-response relationship in the range of 6-24 mg HA for three influenza strains. Two more papers were not included: one [16] was a summary of three papers which are included [4-6], the other [20] described the same trial as in [8] but used the single radial haemolysis method. Fifteen papers met our selection criteria.

Table 2a presents the first authors and the years of publication of the 15 selected papers, as well as the years and places of performance. Most studies were done in the United States of America. The first six trials were performed in 1978 using the A/USSR/77 (H1N1) virus. This virus had been detected in November 1977 in the Sowjet Union and was antigenically closely related to influenza A-H1N1 strains which had circulated 30 years earlier [19].

Table 2a: Characteristics of the 15 publications selected for review.

REF	FIRST AUTHOR YEAR PUBLICATION	YEAR, PLACE of performance	STUDY POPULATIONS						
			SAMPLE SIZE		AGE-RANGE	HEALTH STATE	PRE-VACC	VACC HIST	PRI-MING
			enter	review					
1	Nicholson 1979	1978 GB	1,335	584	12-85	healthy	no	no	yes
2	Gross 1980	1978 USA	146	129	7-25	chron. ill, inst.	yes	yes	yes
3	Masurel 1981	1978 NL	378	269	13-20	healthy	yes	yes	yes
4	Quinnan 1983	1978 USA	517	260-457	16-83		no	yes	yes
5	Cate 1983	1978 USA	292	136-218	20-88	healthy	yes	yes	yes
6	Wright 1983	1978 USA	1,034	131-432	3-25	healthy / chron.ill	yes	no	yes
7	Gross 1982	1980 USA	80	80	3-33	chron. ill, inst.	yes	no	yes
8	Goodeve 1983	GB	119	96	18-19	healthy	yes	no	yes
9	Clarke 1985	1984* GB	100	96	18-61	healthy	no	no	no
10	Arden 1986	1984 USA	50	50	58-99	chron. ill, inst.	yes	no	no
11	Gross 1988	1984 USA	169	147	71-74*	healthy (ambulatory)	no	yes	no
12	Beyer 1986	1985 NL	94	76-84	20-35	healthy	yes	yes	yes
13	Peters 1988	1985 USA	131	129	70-96	chron. ill,non-inst.	yes	yes	no
14	Sullivan 1990	1985 USA	140	140	18-64	healthy	yes	no	no
15	Guarnaccia 1990	1988* USA	30	29	20-50	healthy	no	no	no

1, enter, number of subjects entering the study (total 4,615); review, number of subjects included in the review process (total 2,940; see Appendix 1 for more details).
2-11, addressing health state (chron.ill, chronically ill subjects; inst., institutionalised), pre- vaccination state, history of previous vaccinations, priming periods, administration mode, randomization, blindness (db, double-blind; sb, single-blind), booster vaccinations, period between drawing sera (in weeks), and statistical methods (conv, conventional; adv, advanced).
*, see Appendix 1; —, not applicable; blanc, not given.

2. Characteristics of study populations (Table 2a).

2.1 Size of study populations

A total of 4,615 subjects were entered in the 15 selected studies. For various reasons (e.g. unsufficient data, interference with natural influenza during vaccination campaign, or subgroups which were not offered different vaccine doses), not all subjects from many of the studies could be included in this review. For those cases where more than 5% of the originally entered number of subjects were excluded for our review [1-6,8,11,12], the reasons for exclusion are described in detail in Appendix 1. The data of 64% (2,940 subjects) were included into this review. For four studies [4,5,6,12], a range of numbers of the evaluated subjects is given in Table 2a. Those studies used trivalent vaccines where every single strain required a different exclusion procedure (see for details Appendix 1).

2.2 Age and health state

In nine papers [2,3,6-8,11-13,15], the age range of the subjects was within 30 years, and in six papers [1,4,5,9,10,14], the age range was more than 30 years. Children and adolescents (<15 years of age) were included in five papers [1-3,6,7], and elderly (>65 years of age) in six papers [1,4,5,10,11,13].

Eight papers dealt with 'apparently healthy' volunteers, typically pupils, university students and employees [1,3,5,8,9,12,14,15]. None of these eight papers established the absence of illness in these subjects by clinical or laboratory measures. One study included both healthy and chronically ill subjects [6], and four studies were conducted in chronically ill patients at risk for influenza complications, such as cystic fybrosis [2,7], and various chronical geriatric diseases [10,13].

2.3 Pre-vaccination titres

Of the 15 papers, five did not take into account homologous antibody titres before vaccination [1,4,9,11,15]. In the other studies, pre-vaccination titres were addressed, although in different ways. Two papers described a pre-screening of subjects for low pre-existing antibody titres before intake [8,12]. Two papers retrospecively stratified for pre-vaccination antibody [5,7], three papers excluded all previously seropositive subjects [2,3,6], and two excluded retrospecively all subjects with high (protective) pre-vaccination titres [10,12]. Two studies [13,14] included pre-vaccination titres in a statistical model and adjusted for this factor by regression anaylsis or analysis of covariance.

2.4 Vaccination history

Seven papers [2-5,11-13] gave information about the history of previous vaccination against influenza, but drew different consequences from that information. Study designs varied considerably from exclusion of all previously vaccinated subjects [12] to inclusion of up to 74% [13] or 82% [5] previously vaccinated subjects in the study samples.

2.5 Priming history

All six studies performed in 1978, to test the new influenza A/USSR/77 (H1N1) strain [1-6], addressed correctly the H1N1-priming period by stratifying for age (for discussion see chapter 2, Table 7). The same trials included booster vaccinations 4-6 weeks after the first immunization at least in the unprimed groups (children and young adults), as was done also in study [7] for an influenza B strain (Table 2b). The rationale for boosting was the previously well-established low immunogenicity of a

single dose of a new influenza subtype in unprimed subjects. Booster vaccinations in subjects already primed for that subtype, however, do not enhance further the sero-response after first vaccination. Thus, booster immunization in unprimed and single immunization in primed subjects yield similar post-vaccination antibody titres [21-24].

Of the nine remaining, more recent studies, only three [7,8,12] adressed the priming periods by a restricted prospective age selection of the study participants.

3. Characteristics of study designs (Table 2b)

3.1 Vaccine administration

Most papers did not give information about the mode of vaccine administration. The subcutaneous or intramuscular route was mentioned in two [1,8] and four studies [3,10,12,13], respectively.

Table 2b: Characteristics of the 15 publications selected for review.

REF	STUDY DESIGN						HI ASSAY		PARAMETER					STAT.
	ADM	RAND	BLI.	BOOST	PLA-CEBO	SERA weeks	HAU	ETHER infl.B	prae-GMT	post-GMT	PROTECTION (thresh.)	RES-PONSE	CUM. TITRE	
1	sc	yes	db	yes	no	4	8	—	yes	yes	yes (40)	no	yes	none
2		no*	sb	yes	no	4	4	—	yes	yes	yes (40)	yes	yes	conv
3	im	yes		yes	no	4	3	—	yes	yes	yes (100)	yes	no	conv
4			db	yes	yes	4	4	no	yes	yes	yes (40)	yes	no	conv
5				yes	yes	4	4	no	yes	yes	yes (40)	yes	no	conv
6				yes	yes	4	4	no	yes	yes	yes (40)	no	yes	conv
7		yes	db	yes	no	4	4		yes	yes	yes (40)	yes	no	conv
8	sc	yes	db	no	yes	4	8		yes	yes	yes (40)	yes	yes	none
9		yes		no	no	3	4		yes	yes	yes (40)	no*	yes	none
10	im	yes		no	no	4	4	yes	yes	yes	yes (40)	no	no	conv
11		yes	sb	no	no	3	4		yes	yes	yes (40)	yes	yes	conv
12	im	yes	pb*	no	no	3	3	yes	yes	yes	yes (100)*	yes	no	conv
13	im	yes	db	no	no	4	4	yes	yes	yes	yes (32)	yes	yes	adv
14		yes	db	no	no	3	4	yes	yes	yes	no	no	no	adv
15				no	no	4	4		yes*	yes*	no	no	no	adv

1, enter, number of subjects entering the study (total 4,615); review, number of subjects included in the review process (total 2,940; see Appendix 1 for more details).
2-11, addressing health state (chron.ill, chronically ill subjects; inst., institutionalised), pre- vaccination state, history of previous vaccinations, priming periods, administration mode, randomization, blindness (db, double-blind; sb, single-blind), booster vaccinations, period between drawing sera (in weeks), and statistical methods (conv, conventional; adv, advanced).
*, see Appendix 1; —, not applicable; blanc, not given.

3.2. Randomization

An essential requirement of a dose-comparative study to validate its consecutive statistical analysis is the random allocation, per stratification group, of the different doses to subjects. Ten papers addressed random allocation explicitely, but four other papers did not report on this point [4,5,6,15]. Considering the context and background of the institution, one may assume, however, that randomization had been performed, except for the lowest dose (2.3 mg HA) in paper [6]. One paper [2] did not randomize but studied dose groups using different protocols, and combined the data retrospectively (see Appendix 1).

3.3 Blindness and placebo control

Double- or single-blindness and placebo control are important when scoring for local and systemic adverse effects of the vaccine. For exploring induction of antibody against the vaccine components by objective end points, the firts two items seem to be of less importance. However, a placebo group will be needed when vaccine trials are done during a period where an outbreak of natural influenza could be expected, in order to estimate placebo response rate and adjust for it in the analysis. Indeed, one study (in which no placebo group was included, [1]) reported such an event. Nine studies [1,2,4,7,8,11-14] reported the degree of blindness; and four studies [4-6,8] included a placebo.

3.4 Blood samples

In all studies, blood samples for titre determinations were taken just prior to, and three to four weeks after vaccinations.

4. Techniques and calculations (Table 2b).

4.1 Haemagglutination inhibition test

The haemagglutination inhibition test to detect homologous antibodies against the viral vaccine components was performed in a micro-titre fashion in all studies. However, this test can be performed in many variations (for instance: pre-treatment of sera, incubation periods, sort of erythrocytes, recording of agglutination patterns). In Table 2b, two important issues are adressed.

First, the concentration of the test antigen (measured in haemagglutination units, HAU) affects the outcome of the test: a low concentration [3,12] increases absolute titre values and may detect even small amounts of antibody with a higher chance of false-positive results (high sensivity, low specifity); a high concentration [1,8] results

in a lower sensivity and lower absolute titre values, but in a better reproducibility. Most studies did not mention the amount of HAU used but referred to previous publications which were consulted as well for this review. From this, it can be concluded that, in most cases, an amount of 4 HAU had been used.
Second, the treatment of the influenza B test antigen by ether [25]: Influenza B strains usually show a low avidity (capacity to bind antibody) which may result in low titre values and a low sensivity in the HI test. This problem can be solved by disrupting the virions by ether before performing the HI-test. Studies carried out after 1985 usually used this method [10,12-14].

4.2 Serological parameters

The pre- and post- geometric mean titres (GMT) were the most common parameters to quantitatively describe the antibody response to the vaccine (all papers). All papers except two [14,15], also reported numbers, percentages, or proportions of subjects under and beyond a `protection threshold' which is believed to correlate with protection (see Chapter 4 for discussion). The threshold titre was 32-40 for those studies which used a test-antigen concentration of 4-8 HAU, and 100 and 200 (influenza A and B respectively) in the two studies [3,12] which used 3 HAU in the HI-test. Numbers, percentages, or rates expressing `response' (titre rise $\geq$ 4 fold) were less often given (nine studies). Seven papers presented pre- or post-immunization titres in cumulative tables for discrete titre intervals [1,2,6,8,9,11,13].

4.3 Statistical analysis

The last column of Table 2b provides an indication on the statistical methods used to assess the significance of differences found between dose groups. No formal statistical analysis at all was applied in three studies [1,8,9]. Here, the absolute differences between groups were interpreted without addressing factors such as group size and probability calculations. Other papers [2-7,10-12] used conventional statistical methods such as Chi^2-, Fisher-exact-, Student t-, or Wilcoxon-rank tests where thought appropriate. The three most recent studies [13-15] applied more advanced statistical procedures such as regression-analysis or analysis of variance. None of the published studies presented data indicating the amount of variation within the study groups for the observed values, such as ranges of observed titre values, or 95% confidence intervals with the reported differences in means and rates. A more detailed description and discussion of the various statistical procedures will be presented in Chapter 4.

5. Stratification groups, vaccine types, and strains (Tables 3a and 3b)

Many of the 15 papers included various vaccine types (WV and SU [1], WV and SPL [4-6]) or more than one strain [4-6,9,11,12,14,15]. Subjects were stratified according to

various criteria. For this review, the dose groups were either adopted as reported in the original papers, or, alternatively, restratified according to the following criteria:

- Different vaccine types were pooled where they showed no significant difference in antibody titres, according to the authors.
- Strains of different subtypes/types were never pooled.
- Other stratifications made by the authors were adopted when justified by significant differences in antibody titres between subgroups; otherwise they were pooled.

Table 3a presents the results of this restratification procedure. A total of 41 stratification groups can be derived from the 15 studies. Split (SPL), whole virus (WV) and subunit (SU) vaccines were used in 23, 18, and seven stratification groups, respectively (double scoring possible). Two papers [13,15] did not report the type of their vaccines. Nearly half of the vaccine strains belonged to the influenza A-H1N1 subtype with the A/USSR/92/77 virus as the most frequent single strain (19 and 13 stratification groups, resp.); the remaining strains consisted of influenza B (14 groups) and A-H3N2 (8 groups) viruses. For the new A/USSR/77, and an influenza B strain (B/Singapore/79), the authors made a stratification between primed and unprimed subjects, by age [1,2,4-6], or vaccination history [3], or serological state prior to vaccination [7]. In the same studies, the unprimed subjects and some primed subjects [3,7] received also a booster vaccination.

The size of the stratification groups varied widely (between 29 [15] and 432 subjects [6]). In bi- and trivalent vaccine studies, the same subjects are scored two or three times, separately for each strain. The total number of subjects in Table 3a (4.967) is considerably larger than the actual number of included subjects (2.940). This reasonably assumes that after administration of bi- or trivalent vaccines, there is an independent antibody induction to any of the vaccine antigens in each subject.

Random allocation per stratification group, a prerequisite of a meaningful statistical analysis, was given for studies [8-14]. In studies [3,7], the stratification groups were made retrospectively by the authors based on pre-vaccination titres. No information was available for the stratification groups of papers [1,4-6,15]. Random allocation was not done in study [2] for the highest dose.

Table 3a: Characteristics of 41 stratification groups, derived from 15 studies reviewed.

REF	STRATIF. GROUPS	VACCINE TYPE	STRAIN	PRIMING STATE	BOOSTER	SIZE	DOSAGES (µg HA)
[1]	1	WV	A/USSR/92/77 (H1N1)	unprimed	yes	175	5 9 16 32 47 94
	2	WV	A/USSR/92/77 (H1N1)	primed		128	5 9 16 32 47 94
	3	SU (aq)	A/USSR/92/77 (H1N1)	primed		61	5 18 66
	4	SU (ads)	A/USSR/92/77 (H1N1)	unprimed	yes		
	5	SU (ads)	A/USSR/92/77 (H1N1)	primed		94	3 9 18 33
[2]	6	SPL	A/USSR/92/77 (H1N1)	unprimed	yes	129	1 4 10 43
[3]	7	WV	A/USSR/92/77 (H1N1)	unprimed	yes	124	10 20 40
	8	WV	A/USSR/92/77 (H1N1)	primed	yes	145	10 20 40
[4]	9	WV, SPL	A/USSR/92/77 (H1N1)	unprimed	yes	132	7 20
	10	WV, SPL	A/USSR/92/77 (H1N1)	primed		185	7 20 60
	11	WV, SPL	A/Texas/1/77 (H3N2)			260	7 20
	12	WV, SPL	B/Hong Kong/8/72			260	7 20
[5]	13	WV, SPL	A/USSR/92/77 (H1N1)	unprimed	yes	57	7 20
	14	WV, SPL	A/USSR/92/77 (H1N1)	primed		79	7 20
	15	WV, SPL	A/Texas/1/77 (H3N2)			218	7 20
	16	WV, SPL	B/Hong Kong/8/72			218	7 20
[6]	17	WV, SPL	A/USSR/92/77 (H1N1)	unprimed	yes	432	2.3 7 20
	18	WV, SPL	A/Texas/1/77 (H3N2)			131	2.3 7 20
	19	WV, SPL	B/Hong Kong/8/72			276	2.3 7 20
[7]	20	SPL	B/Singapore/222/79	unprimed	yes	33	7 60
	21	SPL	B/Singapore/222/79	primed	yes	47	7 60
[8]	22	SU	B/Hong Kong/?/73			96	5 10 20 40
[9]	23	SPL	A/Chile/1/83 (H1N1)			96	10 15
	24	SPL	B/USSR/100/83			96	10 15
[10]	25	SPL	B/USSR/100/83			50	15 60
[11]	26	SPL	A/Philippines/2/82 (H3N2)			72	15 30 45
	27	SPL	A/Chile/1/83 (H1N1)			72	15 30 45
	28	SPL	B/USSR/100/83			72	15 30 45
	29	WV	A/Philippines/2/82 (H3N2)			75	15 30 45
	30	WV	A/Chile/1/83 (H1N1)			75	15 30 45
	31	WV	B/USSR/100/83			75	15 30 45
[12]	32	SPL	A/Philippines/2/82 (H3N2)			84	10 15
	33	SPL	A/Chile/1/83 (H1N1)			76	10 15
	34	SPL	B/USSR/100/83			82	10 15
[13]	35		B/USSR/100/83			129	15 60
[14]	36	SU	A/Philippines/2/82 (H3N2)			140	7.5 15 30
	37	SU	A/Chile/1/83 (H1N1)			140	7.5 15 30
	38	SU	B/USSR/100/83			140	7.5 15 30 45
[15]	39		A/Leningrad/360/86 (H3N2)			29	1.5 3 15*
	40		A/Taiwan/1/86 (H1N1)			29	1.5 3 15*
	41		B/Ann Arbor/1/86			29	1.5 3 15*
					Total	4,967	

6. Dose groups (Tables 3a and 3b; Appendix 2).

The last column of Table 3a shows the vaccine doses tested for each of the 41 stratification groups, varying between 1 and 94 µg HA. Some stratification groups contain only two doses, others up to six, resulting in a total of 117 dose groups. In ten stratification groups (30 dose groups), also booster vaccinations were included, either with equal doses of first and second injection [1,4-6], or with a standard second dose regardless of the first dose [2,3,7]. The total antigen dose (sum of first and second immunization, not shown in Table 3a) ranges between 4.6 and 188 µg HA. For conveniance, the doses are devided into four categories (low, ≤10 µg HA; medium, 11-20 µg HA; high, 21- 40 µg HA; very high >40 µg HA). As shown in Table 3b, most frequently, medium doses occur.

Table 3b: Dose-distribution of 87 single dose and 30 booster dose groups.

Dose categories (µg HA)	Number of dose groups		
	single dose	booster doses	
		first	second
≤ 10	25	12	2
11 - 20	37	9	8
21 - 40	12	4	11
> 40	13	5	9
total	87	30	

A complete list of available serological data, including doses, group sizes, and the serological parameters, for all single and booster dose groups in each of the 41 stratification groups is given in Appendix 2. The size of the dose groups varies considerably between 6 and 186, with a mean of 42 and a median of 29.

7. Dose-comparisons (Appendix 2, Appendix 3).

7.1 Dose-comparisons of quantitative parameters

It was not possible to apply statistical procedures on pre-GMT, post-GMT, or MFI values because no reference was made to within-group variances, and the original raw data were not available. Thus, the papers were checked for statistical calculations, done by the authors themselves. In column "Result" of Appendix 3A, "+" (for a higher GMT or MFI value of the group with the higher dose) indicates that the authors

found a significant difference (α<0.05); "-" means the absence of a statistically significant difference, and a blanc describes the cases where the authors did not perform a statistical test. This review-procedure reveals seven significant dose-comparisons out of 31 significance tests for parameters derived from post- vaccination titres.

7.2 Dose-comparisons of dichotomous parameters

Protection and response rates as reported in the papers, were subjected to statistical recalculations. For booster dose groups, comparisons were made separately for first and second vaccination.

In stratification groups containing only two dose groups, the between-dose difference of a rate, and its 95% confidence interval (CI) were calculated (formula see Appendix 4). A difference between protection or response rates of two dose groups was regarded as significant when the lower limit of its CI was above 0. This was defined as a dose-effect on the α-level of 0.05 (Appendix 3B).

In stratification groups containing more than two dose groups, the rates were subjected to a probit-transformation, and slopes were calculated for the regression lines of probit-values on the $_{10}$log doses. A dose-effect within the entire stratification group was considered to be present when a positive slope was significantly larger than 0 (α-level 0.05). In these cases, an ED_{50} (dose which shows an effect in 50% of the subjects) was also calculated (Appendix 3C).

Of 29 stratification groups for which PR-values could be calculated, eight (27%) showed a significant dose effect, either by a CI excluding 0 or by a significantly positive slope. With respect to response rates, in six out of 22 stratification groups (26%), a significantly increasing response rate for increasing doses was detected. In columns SIG of Appendices 3B and 3C, "+" indicates a statistically significantly higher value of the group with the higher dose.

8. Dose-effect and its dependence on priming state and dose range (Tables 4a-c).

Any significant difference between the PR, the RR, the post-GMT or any combination, was taken to indicate the presence of a dose-effect in each stratification group. In 13 out of 41 (32%) stratification groups, significant differences have been found for one or more parameters.

In ten stratification groups with booster vaccinations (nr. 1,4,6-9,13,17,20,21) the dose-effect after the first and the second vaccination could be studied separately (Table 4a). Of seven stratification groups with a dose-effect after the first vaccination, four did not show that effect any more after the second vaccination. In these cases,

for all doses a similar absolute antibody titre was reached after booster vaccination. In unprimed subjects of three stratification groups, a dose-effect after first vaccination, still remained after the second vaccination.

Table 4a: Dose-effects in ten stratification groups with booster vaccinations, according to priming state.

Priming state (μg HA)	Dose-effect	
	first dose	second dose
unprimed	5/ 8 *	3/ 8
primed	2/ 2	-/ 2
total	7/10	3/10

*, first number, significant dose- effect / second number, number of stratification groups.
**, in case of stratification group with booster vaccination: second dose in unprimed subjects; first dose in primed subjects.

As booster immunization in unprimed, and single immunisation in primed subjects yield similar post-vaccination antibody titres [21,22,23,24], the results of primed groups after first vaccination, and of unprimed groups after second vaccination, were chosen to compare stratification groups with and without booster. Table 4b shows the results of this re-arrangement: a total of 11 dose-effects were found in 41 dose-comparisons (27%). The proportion of significant dose-effects in stratification groups with unprimed subjects was 38%, and 24% in stratification groups with primed subjects, or subjects with an undefined priming state.

Table 4b: Dose-effect according to priming state.

Priming state	Booster **	Dose-effect
unprimed	2nd dose	3/ 8* (38%)
primed/undefined	1st dose	8/33 (24%)
total		11/41 (27%)

*, first number, significant dose- effect / second number, number of stratification groups.
**, in case of stratification group with booster vaccination: second dose in unprimed subjects; first dose in primed subjects.

Table 4c describes the distribution of dose-effects according to the difference between the highest and lowest dose within each stratification group (dose range). The proportion of significant dose-effects, not surprisingly, increased with increasing dose ranges, from 0% for small ranges up to 10 μg HA, to 44% for ranges larger than 40 μg HA. More interestingly, some stratification groups which did not show a dose effect, had a very large dose range (for example 5-94, 5-66, 15-60 μg HA, for stratification groups 2, 3, and 13, resp.; Table 5).

Table 4c: Dose-effect according to dose ranges.

Dose-range [μg]	Dose-effect
≤10	-/ 5* (0%)
11-20	2/11 (18%)
21-40	5/16 (31%)
>40	4/ 9 (44%)
total	11/41 (27%)

*, first number, significant dose- effect / second number, number of stratification groups.
**, in case of stratification group with booster vaccination: second dose in unprimed subjects; first dose in primed subjects.

In Table 5, the relevant results of this review are summarized.

9. Dose-effects and ED_{50}-values (Table 5).

The next question was whether an observed dose-effect could be used to calculate a reliable effective dose, like an ED_{50}. Therefore, Table 5 also shows the ED_{50}-values for multiple dose trials with significantly positive slopes, i.e. in four stratification groups (nr. 1,8,17,22). However, the findings were not reliable. The values for protection rates were not within the studied dose range, and there was a huge discrepancy for stratification group 8 between the ED_{50} values for protection rate and response rate. Clearly, the data from the various multiple dose studies are not sufficiently well-behaving to undertake a quantitative meta-analysis of the studies.

Table 5: Significance of dose-comparisons.

REF	SG	BOOSTER	PRIMING STATE	NR. DOSES	DOSE-RANGE (µg HA)	SIGNIFICANCE				ED_{50}	
						GMT	PR	RR	ANY(B)	PR	RR
[1]	1	YES 2nd	unprimed	6	10 -188 (178)		+		+	5	
[1]	2	NO	primed	6	5 - 94 (89)		—		—		
[1]	3	NO	primed	3	5 - 66 (59)		—		—		
[1]	4	YES 2nd	unprimed	3	6 - 66 (60)		—		—		
[1]	5	NO	primed	4	3 - 33 (30)		—		—		
[2]	6	YES 2nd	unprimed	4	21 - 43 (22)	—	—		—		
[3]	7	YES 2nd	unprimed	3	20 - 60 (40)		—		—		
[3]	8	YES 1st	primed	3	10 - 40 (30)		+	+	+	166	17
[4]	9	YES 2nd	unprimed	2	14 - 40 (26)	—	—	—	—		
[4]	10	NO	primed	3	7 - 60 (53)	—	—	+	+		14
[4]	11	NO		2	7 - 20 (13)	—	—	—	—		
[4]	12	NO		2	7 - 20 (13)	—	—	—	—		
[5]	13	YES 2nd	unprimed	2	7 - 20 (13)	—	—		—		
[5]	14	NO	primed	2	7 - 20 (13)	—	—	—	—		
[5]	15	NO		2	7 - 20 (13)	—	—	—	—		
[5]	16	NO		2	7 - 20 (13)	—	—	—	—		
[6]	17	YES 2nd	unprimed	3	4.6- 40 (35.4)	—	+		+		2
[6]	18	NO		3	2.3- 20 (17.7)	—	—		—		
[6]	19	NO		3	2.3- 20 (18.7)	—	—		—		
[7]	20	YES 2nd	unprimed	2	14 - 67 (53)	+	+	—	+		
[7]	21	YES 1st	primed	2	7 - 60 (53)	+	+	—	+		
[8]	22	NO		4	5 - 40 (35)		+	+	+	4	5
[9]	23	NO		2	10 - 15 (5)		—		—		
[9]	24	NO		2	10 - 15 (5)		—		—		
[10]	25	NO		2	15 - 60 (45)	—	—		—		
[11]	26	NO		3	15 - 45 (30)	—		—	—		
[11]	27	NO		3	15 - 45 (30)	+		—	+		
[11]	28	NO		3	15 - 45 (30)	—		—	—		
[11]	29	NO		3	15 - 45 (30)	—		—	—		
[11]	30	NO		3	15 - 45 (30)	—		—	—		
[11]	31	NO		3	15 - 45 (30)	—		—	—		
[12]	32	NO		2	10 - 15 (5)	—	—	—	—		
[12]	33	NO		2	10 - 15 (5)	—	—	—	—		
[12]	34	NO		2	10 - 15 (5)	—	—	—	—		
[13]	35	NO		2	15 - 60 (45)	—	—	—	—		
[14]	36	NO		3	7.5- 30 (22.5)	—			—		
[14]	37	NO		3	7.5- 30 (22.5)	+			+		
[14]	38	NO		4	7.5- 45 (22.5)	—			—		
[15]	39	NO		3	1.5- 15 (14)	+			+		
[15]	40	NO		3	1.5- 15 (14)	+			+		
[15]	41	NO		3	1.5- 15 (14)	—			—		

SG, stratification group; BOOSTER, 1st, first vaccination; 2nd, second vaccination; NR.DOSES, number of doses in a stratification group; DOSE-RANGE, lowest and highest dose, and difference between highest and lowest dose; ANY(B), any dose-effect with regard to booster vaccinations; +, significant dose-effect; -, not significant dose-effect; ED_{50}, median effective dose; blanc, no data available.

Discussion

The different dose requirements for influenza vaccines in various countries indicate a lack of consensus on the "lowest effective dose", despite the great number of studies done to investigate this question. This may be caused by the experimental difficulties to establish such a dose. The lowest effective dose may vary for different target groups, or may be dependent on the selected efficacy parameters to assess dose-effects. Dose-effects on the true efficacy parameters (influenza attack-rate, reduction in influenza-associated morbidity and mortality) can only be derived from field or challenge studies, the latter being only feasible in healthy adults for ethical reasons. Hence, for practical reasons, the choice for the recommended vaccine doses has mainly been based on serological studies where homologous antibody titres are used as surrogate markers of efficacy.

The 15 studies selected for this review, covered doses from 1 to 188 µg HA. We found a fair amount of variation in study designs, study populations, priming and vaccination history of the vaccinees, group sizes, vaccine types, virus strains, laboratory techniques, and statistical procedures for data analysis (Tables 2a-b).

We applied strict post-hoc criteria to each study to form 41 stratification groups as homogeneous as possible, collected the data on response parameters as given in the studies, and performed calculations to detect dose-effects within each stratification group. The dose-response relationship on the entire dose range was, indeed, `shallow' (see Table 1, [1]). After correcting for booster vaccination (Table 4b), in only 11 of 41 stratification groups (27%), a significant dose-effect was shown, i.e. in 73% the absence of a dose-effect could not be excluded. Although far from significantly, in unprimed subjects significant dose-effects were found more frequently than in primed subjects. Other factors possibly influencing the dose- response, such as vaccine types, vaccine strains and subtypes, health state or age of the vaccinees, could not be identified due to insufficient data.

Even if significant positive slopes of dose effect relationships were found, these were not reproducible between studies. Because of the irreproducability and the low frequency of significant dose-response relationships in the stratification groups, particularly if dose differences were smaller than 20 µg HA (Table 4c), our results do not justify the expectation, that a vaccine dose of 15 µg HA/strain would clinically be superior than a dose of 10 µg HA/strain. This conclusion is in line with results by Jennings et al. [17], who found no positive dose-response relationship in the range 6-24 µg HA/strain by using the single radial haemolysis method for antibody titre determinations.

From the presented dose-response literature in this chapter and based on a statement bij Quinnan of the Food and Drug Administration that "from an evaluation of the

scientific issues come the right regulatory decisions" [27], we conclude that there is little scientific basis for the preference of a standard vaccine dose of 15 μg HA/strain over a standard dose of 10 μg HA/strain.

As has been shown, the data from the various multiple dose serological studies as reported in this review are not sufficiently well-behaving to undertake a quantitative meta-analysis. of these studies. The discrepancies between the studies and the low overall frequency of detectable dose-effects in this review should be taken as warning against drawing generalized conclusions from single-study observations.

For the clinical development and evaluation of new influenza vaccines it would be necessary to perform serological studies in a more standardized way, so that formal statistical procedures, such as a meta-analysis can be applied to draw firm conclusions on the dose effects of these vaccines.

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Appendix 1: Comments on the 15 papers reviewed.

Pages, figures, or tables mentioned in this appendix, refer to the original papers. If not described otherwise, titres >10 or >9 have been regarded as 5, and titres >8 as 4, for calculations. Data on heterologous response (i.e. antibodies against virus strains others than vaccine strains) have not been regarded. Data on additional sera drawn after vaccination to measure long-term antibody maintenance (for example, after five months, or after one year) have not been included. Data on booster vaccinations were included.

[1]

Nicholson et al. describe a vaccination trial in 9 groups of volunteers with different age distributions and on different locations, using four different vaccines, two different modes of administration, and many dosages, in part with booster injections for A/USSR/77 (H1N1). After one month, the young age-groups received a booster injection with the same vaccine and dosage as the first vaccination. Of the 1335 subjects entering the study, the authors exclude one group of unknown size because of assumed occurrence of natural influenza during the ungoing trial. Pre- vaccination HI antibody titres (as cumulative percentages) are presented for 980 subjects (Table 1). On page 130 the authors state that they determined the HI antibody responses to vaccination for 972 subjects, but Tables 2-4 present only 938 subjects. No comment is given on the missing subjects. From Tables 2-4, the following distribution of subjects can be derived (for abreviations see paper [1]):

age classes	mode of adm.	WV	aqCTAB	aqTrit	adsTrit
12-25 years	subcutaneous	175	72*	29*	126
	intradermal	44*	39*	-	-
26 years	subcutaneous	128	46*	61	94
	intradermal	48*	26*	50*	-
Totals		395	183	140	220

*, excluded for this review.

According to our own selection criteria, subjects with intradermal administration of the vaccine are excluded. Furthermore, the 29 young subjects receiving aqueous Triton SU vaccine are excluded because only one dosage (9 µg HA) was given in this group. The authors report that they had tested vaccine potency before delivering to and after returning from the study centra. The aqueous CTAB subunit vaccine lost all (low dosage) or much (high dosages) of its detectable HA contents, and data on antibody response were not reliable. For this review, those data are excluded. The remai-

ning 584 subjects form 22 dosage groups in five different dose-response comparisons. No statistical procedures were presented by the authors. The following remarks were interpreted as absence of significant differences between dose groups: "The proportion of these older volunteers" (i.e. 26 years of age or older) " who developed HI titres of >40 after a single vaccination, and the post vaccination geometric mean HI titres (GMT) were similar, irresptective of the type of vaccine, the dose...Although increases of antigen content had little effect on the proportion of volunteers with HI titres of >40 after a second dose of vaccine, a shallow dose-response effect was observed on the GM HI antibody titre...(pag. 130/131).
(Main source for data: Tables 2-4)

[2]

Gross et al. combine two trials in young patients with cystic fibrosis or allergy: one trial with a high vaccine dosage (43 µg, n=48) and a second one with three lower dosages (1, 4 or 10 µg, n=98) which was part of a larger trial. One month after the first vaccination, booster injections were given containing 20 µg HA in all dose groups. The authors included only previously seronegative subjects (confirmed by neutralization and NI-tests) (n=129). Results were presented for two age groups (7-12, and 13- 25 years of age, Table I) which are combined for this review. The significant difference between post-GMT-values (similar to MFI- values because subjects were all seronegative) of 4 and 10 µg HA of the age-stratification 13-25 years is not present for the age- stratification 7-12 years of age. Whether the difference between MFI-values after pooling is significant, cannot be decided.
(Main source for data: Table I.)

[3]

Masurel et al. describe two groups (269 pupils, supposed clinically healthy, receiving either 10, 20, or 40 µg HA of a WV vaccine, and 109 mentally handicapped subjects, receiving one vaccine dosage, 20 µg). We included only the first group in our review. 71 subjects received a booster injection of 20 µg six weeks later. 145 pupils were vaccinated, one year before, with a trivalent vaccine containing 20 µg HA of A/New Jersey/8/76 (H1N1). Table 1 presents stratified data for both previously vaccinated and not previously vaccinated subjects, revealing major differences between them not after first vaccination, but after boosting. Thus, both groups are presented separately. All prevaccination sera were negative (titre <9 = 8).
(Main source for data: Table 1)

[4]

Quinnan et al. describe a complex study design with monovalent and trivalent vaccines from four manufacturers (WV and split) and booster injections for A/USSR/77 (H1N1) with the same vaccine and dosage as the first vaccination. Numbers given in the Tabels do not always correlate with numbers in the text. Table 4 presents the results of 426 subjects receiving vaccine and 91 receiving placebo. There were no titre rises in the placebo subjects, suggesting absence of natural influenza during the performance of the trial. Tables 1 and 2 include data cumulated for all vaccines, despite differences between vaccines (Figures 2 and 3). Stratification for vaccines cannot be done because absence of data. Instead of the mean vaccine dosages in Tables 1 and 2 the numerical dosages (7, 20, 60 µg HA) are used in this review.

Table 1 (A/USSR/77) includes two age groups (16-25, and >26). Stratification has been done, by the authors, for previous vaccination with A/NJ/76 (Table 3), but the numbers cannot be correlated to those in Table 1. Therefore, this stratification is not included here. The authors found no major differences between previously vaccinated and not vaccinated subjects. For the young- age groups (<25 years of age), booster parameters are included. However, there are different numbers after the first and the second vaccination, but the percentages of protected subjects and the GMT-values prior to vaccination are not given for the booster groups. For the calculation, the respective values were adapted from the groups after first vaccination. Table 2 (A/Texas/77 (H3N2) and B/Hong Kong/72) presents, despite differences between ages, only the cumulated data of all ages. The following remarks have been interpreted as absence of significant differences between dose groups: "In all age groups, geometric mean antibody titres were higher in those groups that received the medium dose than in those that received the low dose of vaccine...This dose- response relationship was not evident after vaccination with the high dose, although the number of volunteers who received this dose was small." (pag. 749)
(Main source for data: Tables 1 and 2)

[5]

Cate et al. use a study design similar to [4] with monovalent and trivalent vaccines from four manufacturers (WV and split), three doses (7, 20, 60 µg HA) and booster injections for A/USSR/77 (H1N1) after one month, in two groups of volunteers (age range 20-33 years of age, n=154, and 45-88 years of age, n=138). On page 738, the age range of the first group is wrongly given as 20-23. No information is given about the serology of the placebo groups. Data for 20 and 60 µg HA were pooled by authors as they found no major differences between those dosages. Table 3 presents, according to priming periods, the immune reponse to A/USSR/77 (H1N1) only for 20- to 25-year-old and 55- to 88-year- old subjects. Table 4 gives the protection rates for

all young and older subjects for all three antigens. The usual time between vaccination and post-vaccination blood drawing was four weeks. From the young subjects receiving trivalent vaccine the post- vaccination serum was sampled earlier (after two weeks). Response rates could not be used, as they were based on an unknown number of subjects with a pre-vaccination titre <20. The reponse rates for booster vaccination of Table 3 cannot be used as they seem to be calculated on the titre values after the first vaccination, and not on the pre-vaccination titer values. The following table shows the comparison groups presented by the authors, and the pooling which has been made for this review.

Source	Groups by authors	Stratification groups for review
Table 3	1. A/USSR young unprimed 7 µg WV (n=12)	13-1 A/USSR unprimed 7 µg (n= 29)
	2. A/USSR young unprimed 7 µg SPL (n=17)	pooled with 13-1
	3. A/USSR young unprimed 20 µg WV (n=13)	13-2 A/USSR unprimed 20 µg (n= 28)
	4. A/USSR young unprimed 20 µg SPL (n=15)	pooled with 13-2
	5. A/USSR young primed 7 µg WV (n= 5)	excluded
	6. A/USSR young primed 7 µg SPL (n= 5)	excluded
	7. A/USSR young primed 20 µg WV (n= 3)	excluded
	8. A/USSR young primed 20 µg SPL (n= 6)	excluded
	9. A/USSR older seroneg. 7 µg WV (n= 1)	14-1 A/USSR primed 7 µg (n= 23)
	10. A/USSR older seroneg. 7 µg SPL (n= 4)	pooled with 14-1
	11. A/USSR older seroneg. 20 µg WV (n= 7)	14-2 A/USSR primed 20 µg (n= 56)
	12. A/USSR older seroneg. 20 µg SPL n= 5)	pooled with 14-2
	13. A/USSR older seropos. 7 µg WV (n= 9)	pooled with 14-1
	14. A/USSR older seropos. 7 µg SPL (n= 9)	pooled with 14-1
	15. A/USSR older seropos. 20 µg SPL (n=23)	pooled with 14-2
	16. A/USSR older seropos. 20 µg SPL (n=21)	pooled with 14-2
Table 4	17. A/Tex young 7 µg WV (n=25)	15-1 A/Tex 7 µg (n= 92)
	18. A/Tex young 7 µg SPL (n=28)	pooled with 15-1
	19. A/Tex young 20 µg WV (n=23)	15-2 A/Tex 20 µg (n=126)
	20. A/Tex young 20 µg SPL (n=26)	pooled with 15-2
	21. B/HK young 7 µg WV (n=25)	16-1 B/HK 7 µg (n= 92)
	22. B/HK young 7 µg SPL (n=28)	pooled with 16-1
	23. B/HK young 20 µg WV (n=23)	16-2 B/HK 20 µg (n=126)
	24. B/HK young 20 µg SPL (n=26)	pooled with 16-2
	25. A/Tex older 7 µg WV (n=19)	pooled with 15-1
	26. A/Tex older 7 µg SPL (n=20)	pooled with 15-1
	27. A/Tex older 20 µg WV (n=40)	pooled with 15-2
	28. A/Tex older 20 µg SPL (n=37)	pooled with 15-2
	29. B/HK older 7 µg WV (n=19)	pooled with 16-1
	30. B/HK older 7 µg SPL (n=20)	pooled with 16-1
	31. B/HK older 20 µg WV (n=40)	pooled with 16-2
	32. B/HK older 20 µg SPL (n=37)	pooled with 16-2

(Main source for data: Tables 3 and 4)

[6]

Wright et al. use a study design similar to [4] with monovalent and trivalent whole virus and split vaccines from four manufacturers, three dosages (2.3, 7, 20 µg HA) and booster injections for A/USSR/77 (H1N1). There were 358 children and 676 young adults (healthy and chronically ill, total 1,034, of them 235 receivers of placebo). No information about random allocation of vaccine doses to subjects is given. Whether allocation was really done, can be doubted at least for the lowest dose, as the authors stated: "It may also refelct the fact that the lowest vaccine dose (pediatric), whcih contained ± 2.3 µg of antigen, was selectively given to younger children, ..." Tables 6 and 7 present antibody responses to A/USSR/77 (H1N1), A/Texas/77 and B/Hong Kong/72 of initially seronegative subjects. For our review, data of different age classes (3-6, 7-12, and 13-25 years of age) and two vaccine types were pooled. The authors claim a dose-response relationship for the protection rates of all three vaccine components (Fig. 1), but statistics are not given. A dose-response relationship for GMT- or MFI-values is not discussed.
(Main source for data: Tables 6 and 7)

[7]

Gross et al. conducted this study in cystic fibrosis patients (children, young adults), in part from St. Vicents's Hospital, as was the case in [2]. It can be assumed that some in this study group also participated in that former study, but information about previous vaccination is not given. Two doses (7,60 µg HA) of B/Singapore/79 are compared, given in a trivalent vaccine containing also 7 µg A/Bangkok/1/79 (H3N2) and 7 µg A/Brazil/78 (H1N1). Dosis groups were subdivided into subjects with negative and positive pre-vaccination titres for B/Singapore/79. Authors assume that previously seronegative subjects were unprimed for influenza B which has been confirmed by a neutralization test. After one month most subjects in both dosage groups received a trivalent booster vaccine containing 7 µg HA of each strain.
(Main source for data: Table 2)

[8]

Goodeve et al. do not report the year of their study. After determination of the serostatus of 119 healthy students, four dosages of a B/Hongkong/73 subunit (CTAB) vaccine and placebo were tested. No response occurred in the placebo-group (n=23), thus no natural influenza was present. After four weeks volunteers were challenged with attenuated live virus. Tables 2 and 3 give data on the seroresponse to vaccination. In our review, the protection rates were calculated from Table 2 (thus from subjects with titres>40, i.e. >60), Table 3 gives numbers of subjects with postvaccination titres >40!. GMT of Table 3 are rounded, and MFI calculated from GMT (slight dif-

ferences with `Fold-increase' in Table 3 possible). The authors see "a clear dose-response relationship: for volunteers given 40, 20, 10 or 5 μg HA the g.m.t. or HI antibody at 1 month after immunization was 321.8, 152.6, 137.6 and 86.1, respectively." Unfortunately, no statistical procedures were used to interprete the significance of these findings.
(Main source for data: Tables 2 and 3)

[9]

Clarke et al. do not report the year of performance but used the trivalent vaccine recommended for the season 1984/1985. Two dosages of trivalent split vaccine were administered: A/Philippines/82 (H3N2) 10 versus 10, A/Chile/83 (H1N1) 10 versus 15, B/USSR/83 10 versus 15 μg HA. Thus, as for A/Philippines/82 (H3N2), no dose-response data are available, this strain has not been included in our review. Seroconvertion is defined dependent on serostatus (seronegative subjects reaching a titre >40 after vaccination, seropositive subjects showing a four-fold increase), thus a combination of response and protection rate (Table 1). Those data are not included in our review. Protection rates and GMT are derived from Table 3.
(Main source for data: Table 3)

[10]

Arden et al. vaccinated elderly, chronically ill subjects with trivalent split vaccine (each 15 μg HA of A/Philippines/82 (H3N2), A/Chile/83 (H1N1) and B/USSR/83), and with either placebo or 45 μg HA B/USSR/83, thus a comparison between 15 and 60 μg HA B/USSR/83 was made. GMT values are decribed in the text. The authors tested the difference between the MFI-values for the two dose groups and found P=0.056 by Wilcoxon's rank sum test. This was regarded as not significant by the authors, and in this review. Table 1 presents the protection rates for subjects unprotected before vaccination (<40). The authors used a one- tailed Fisher's exact test and regarded the difference between the protection rates as significant (P<0.05). In a two-tailed fashion, however, this test would give P=0.0816, thus a non- significant difference for α=0.05. The 95%-confidence interval (see Appendix 3) does not exclude 0.
(Main source for data: Table 1)

[11]

Gross et al. compare three dosages of two trivalent vaccines (split, WV), thus 18 groups, in health ambulatory elderly (health state well-defined, mean ages given as 71-74, no age range reported). 56-76% of the subjects were previously vaccinated. Correction for pre-vaccination state is not discussed. The total number of volunteers is given as 148 in the text, but as 147 in Table 1. The numbers for split/Chile-groups

and WV/Chile groups are obviously mixed up in Table 1. The protection rates cannot be calculated because of absence of prevaccination protection numbers. The authors report a significant difference in post- vaccination GMT between groups split/Chile/ 15 µg and split/Chile/45 µg, but when calculating MFI-values, this difference is smaller because the pre-vaccination GMT-values differ in these groups. Whether actual MFI-values differ significantly, cannot be decided here. The 15 µg HA dose groups are compared with a group of cystic fibrosis children and young adults (n=21); these data are not included here.
(Main source for data: Table 1)

[12]

Beyer et al. present data on young healthy students vaccinated with trivalent split vaccine, either 10 or 15 µg HA of each strain. The volumes of both doses differed per syrinx (0.5 versus 0.75), thus it can be assumed that the administration of vaccines was single-blinded but not double-blinded, as is stated in the text. Subjects were pre-tested for low pre-vaccination antibody titres. For calculations, subjects with high (protective) pre-vaccination antibody titres (infl. A >100, infl.B ether-treated > 200) were excluded. Absolute pre- and post-GMT values were recalculated from logarithmated values. Table 4 presents also heterologous response to four other strains, and, as parameters, MFI and protection rates of responders only. These data were not included in this review. (Main source for data: Table 4)

[13]

Peters et al. describe a trial in elderly, some chronically ill, non-institutionalized subjects receiving trivalent vaccine (no information about vaccine type) containing each 15 µg HA of A/Philippines/82 (H3N2) and A/Chile/83 (H1N1), and either 15 µg B/USSR/83 (group I) or 60 µg B/USSR/83 (group III) into the right upper arm and, into the left upper arm, either placebo (group I and III) or 45 µg HA B/USSR/83 (group II). Numbers of subjects for pre- and first post-vaccination sera: Group I 42, group II 44, group III 45-2 = 43. There were significant differences between groups II and III, but not between either groups I and II, or I and III. Thus, groups II and III have been combined here. A second post-vaccination sample (after 5 months) was drawn, data are not presented here. Also data about heterologous response were not included (although they showed a difference between doses). The authors analysed the data by a regression model including age (not dependent), pre-vaccination state (dependent), and previous vaccinations (dependent). (Main source for data: Table 2)

[14]

Sullivan et al. describe healthy university personnel and students receiving a trivalent Triton-split vaccine containing A/Philippines/82 (H3N2), A/Chile/83 (H1N1) and B/USSR/83 with 7.5, 7.5, 7.5, or 15, 15, 15, or 30, 30, 30, or 15, 15, 45 µg HA. The authors combine the data of A/Philippines/82 (H3N2)- and A/Chile/83 (H1N1) of the second and the fourth group. The authors looked at the data on response and protection rates (>32), but did not present these data as they did not find significant differences between dose groups for any strain. The authors have looked also at the influence of age (after adjustment for dosage). (Main source for data: Table 1)

[15]

Guarnaccia et al. evaluated the practice of clinicians who diluted vaccine when immunizing subjects with allergy to egg proteins. In 29 healthy subjects (intake: 30), commercially available trivalent vaccine of unknown type was tested in original volume (0.5 ml), and as 1:5 and 1:10 dilutions. No information about µg HA contents was given. If a contents of 15 µg HA for the original dose is assumed, the dosage groups would be 1.5, 3, and 15 µg HA. No information about study design is provided. No data about response or protection rates are provided. Pre- and 28-day-post-GMT-values are derived from Figures 1-3 according to exp(L*0.07+1.6), where L is the length of bar in mm. According to ANOVA done by authors, post-GMT of high and lower dosages differ significantly for the influenza A strains, but not for influenza B. However, pre- and post- vaccination titres together formed the dependent variable of the analysis; while the dosage was an indepenent variable. It cannot be decided here, whether the differences between the MFI-values for influenza A strains were also significant.
(Main source for data: Figures 1-3)

Appendix 2: Serological data from the 15 dose-response studies *

REF	SG	DG	BOOSTER	DOSE	N	PRE-GMT	POST-GMT	MFI	PRE-PROT	POST-PROT	PR	N RESP	RR
[1]	1	1A	YES 1st	5.0	21	5	23	0.66	0	11	0.52		
[1]	1	2A	YES 1st	9.0	21	5	25	0.70	0	8	0.38		
[1]	1	3A	YES 1st	16.0	51	5	42	0.92	2	24	0.45		
[1]	1	4A	YES 1st	32.0	17	5	21	0.62	0	9	0.53		
[1]	1	5A	YES 1st	47.0	53	5	49	0.99	1	34	0.63		
[1]	1	6A	YES 1st	94.0	12	5	93	1.27	0	9	0.75		
[1]	1	1B	YES 2nd	10.0	21	5	58	1.06	0	14	0.67		
[1]	1	2B	YES 2nd	18.0	21	5	63	1.10	0	14	0.67		
[1]	1	3B	YES 2nd	32.0	51	5	111	1.35	2	48	0.94		
[1]	1	4B	YES 2nd	64.0	17	5	153	1.49	0	13	0.76		
[1]	1	5B	YES 2nd	94.0	53	5	201	1.60	1	51	0.96		
[1]	1	6B	YES 2nd	188.0	12	5	187	1.57	0	12	1.00		
[1]	2	7	NO	5.0	22	10	276	1.44	4	19	0.83		
[1]	2	8	NO	9.0	15	16	250	1.19	4	14	0.91		
[1]	2	9	NO	16.0	26	13	562	1.64	5	26	1.00		
[1]	2	10	NO	32.0	33	10	275	1.44	5	31	0.93		
[1]	2	11	NO	47.0	20	13	465	1.55	4	19	0.94		
[1]	2	12	NO	94.0	12	12	397	1.52	3	11	0.89		
[1]	3	13	NO	5.0	16	13	346	1.43	5	14	0.82		
[1]	3	14	NO	18.0	29	5	343	1.84	3	27	0.92		
[1]	3	15	NO	66.0	16	11	495	1.65	3	16	1.00		
[1]	4	16A	YES1stt	3.0	24	5	34	0.83	0	14	0.58		
[1]	4	17A	YES 1st	9.0	51	5	20	0.60	3	21	0.38		
[1]	4	18A	YES 1st	33.0	51	5	30	0.78	0	26	0.51		
[1]	4	16B	YES 2nd	6.0	24	5	110	1.34	0	22	0.92		
[1]	4	17B	YES 2nd	18.0	51	5	124	1.39	3	43	0.83		
[1]	4	18B	YES 2nd	66.0	51	5	154	1.49	0	43	0.84		
[1]	5	19	NO	3.0	24	13	282	1.34	5	20	0.79		
[1]	5	20	NO	9.0	26	15	530	1.55	9	24	0.88		
[1]	5	21	NO	18.0	16	5	302	1.78	2	14	0.86		
[1]	5	22	NO	33.0	28	19	518	1.44	9	28	1.00		
[2]	6	23A	YES 1st	1.0	6	5	9	0.26	0	0	0.00	2	0.33
[2]	6	24A	YES 1st	4.0	50	5	14	0.45	0	9	0.18	22	0.44
[2]	6	25A	YES 1st	10.0	42	5	22	0.64	0	17	0.40	29	0.69
[2]	6	26A	YES 1st	43.0	31	5	103	1.31	0	27	0.87	28	0.90
[2]	6	23B	YES 2nd	21.0	6	5	45	0.95	0	5	0.83		
[2]	6	24B	YES 2nd	24.0	50	5	38	0.88	0	32	0.64		
[2]	6	25B	YES 2nd	30.0	42	5	54	1.03	0	34	0.81		
[2]	6	26B	YES 2nd	63.0	31	5	148	1.47	0	30	0.97		
[3]	7	27A	YES 1st	10.0	45	8	20	0.40	0	3	0.07	17	0.38
[3]	7	28A	YES 1st	20.0	38	8	34	0.63	0	7	0.18	22	0.58
[3]	7	29A	YES 1st	40.0	41	8	31	0.59	0	7	0.17	19	0.46
[3]	7	27B	YES 2nd	30.0	15	8	266	1.52	0	14	0.93	15	1.00
[3]	7	28B	YES 2nd	40.0	6	8	237	1.47	0	5	0.83	6	1.00
[3]	7	29B	YES 2nd	60.0	10	8	198	1.39	0	9	0.90	10	1.00
[3]	8	30A	YES 1st	10.0	42	8	23	0.46	0	4	0.10	15	0.36

* For legenda, see end of this Appendix. (cont.)

Appendix 2: Serological data from the 15 dose-response studies (continued)

REF	SG	DG	BOOSTER	DOSE	N	PRE-GMT	POST-GMT	MFI	PRE-PROT	POST-PROT	PR	N RESP	RR
[3]	8	31A	YES 1st	20.0	60	8	29	0.56	0	8	0.13	33	0.55
[3]	8	32A	YES 1st	40.0	43	8	43	0.73	0	11	0.26	30	0.70
[3]	8	30B	YES 2nd	30.0	13	8	77	0.98	0	6	0.46	11	0.85
[3]	8	31B	YES 2nd	40.0	14	8	115	1.16	0	8	0.57	12	0.86
[3]	8	32B	YES 2nd	60.0	13	8	133	1.22	0	7	0.54	13	1.00
[4]	9	33A	YES 1st	7.0	69	12	50	0.62	13	40	0.48	43	0.62
[4]	9	34A	YES 1st	20.0	63	14	56	0.60	18	47	0.64	36	0.57
[4]	9	33B	YES 2nd	14.0	48	12	71	0.77	9	39	0.77	37	0.77
[4]	9	34B	YES 2nd	40.0	41	14	96	0.84	12	36	0.83	31	0.76
[4]	10	35	NO	7.0	81	48	129	0.43	49	75	0.81	34	0.42
[4]	10	36	NO	20.0	88	46	170	0.57	53	81	0.80	48	0.55
[4]	10	37	NO	60.0	16	22	89	0.61	4	15	0.92	10	0.62
[4]	11	38	NO	7.0	126	17	63	0.57	35	106	0.78	66	0.52
[4]	11	39	NO	20.0	134	18	65	0.56	40	115	0.80	83	0.62
[4]	12	40	NO	7.0	126	20	50	0.40	40	92	0.60	47	0.37
[4]	12	41	NO	20.0	134	21	75	0.55	51	103	0.63	62	0.46
[5]	13	42A	YES 1st	7.0	29	5	17	0.53	0	6	0.21	13	0.45
[5]	13	43A	YES 1st	20.0	28	5	30	0.78	0	19	0.68	21	0.75
[5]	13	42B	YES 2nd	14.0	29	5	42	0.92	0	24	0.83		
[5]	13	43B	YES 2nd	40.0	28	5	48	0.98	0	25	0.89		
[5]	14	44	NO	7.0	23	11	71	0.96	0	20	0.87	18	0.78
[5]	14	45	NO	20.0	56	11	75	0.72	0	44	0.79	47	0.84
[5]	15	46	NO	7.0	92	32	78	0.77					
[5]	15	47	NO	20.0	126	45	100	0.68					
[5]	16	48	NO	7.0	92	24	62	0.56					
[5]	16	49	NO	20.0	126	45	90	0.56					
[6]	17	50A	YES 1st	2.3	63	5	11	0.34	0	8	0.13		
[6]	17	51A	YES 1st	7.0	183	5	16	0.51	0	46	0.25		
[6]	17	52A	YES 1st	20.0	186	5	29	0.76	0	97	0.52		
[6]	17	50B	YES 2nd	4.6	42	5	39	0.89	0	28	0.67		
[6]	17	51B	YES 2nd	14.0	161	5	39	0.89	0	106	0.66		
[6]	17	52B	YES 2nd	40.0	152	5	56	1.05	0	121	0.80		
[6]	18	53	NO	2.3	13	5	34	0.83	0	7	0.54		
[6]	18	54	NO	7.0	56	5	58	1.06	0	40	0.71		
[6]	18	55	NO	20.0	62	5	84	1.23	0	48	0.77		
[6]	19	56	NO	2.3	31	5	27	0.73	0	12	0.39		
[6]	19	57	NO	7.0	123	5	34	0.83	0	66	0.54		
[6]	19	58	NO	20.0	122	5	42	0.92	0	66	0.54		
[7]	20	59A	YES 1st	7.0	18	5	37	0.87	0	8	0.44	10	0.56
[7]	20	60A	YES 1st	60.0	15	5	97	1.29	0	13	0.87	14	0.93
[7]	20	59B	YES 2nd	14.0	18	5	56	1.05	0	9	0.50	16	0.89
[7]	20	60B	YES 2nd	67.0	12	5	137	1.44	0	12	1.00	12	1.00
[7]	21	61A	YES 1st	7.0	18	15	84	0.75	3	15	0.80	12	0.67
[7]	21	62A	YES 1st	60.0	29	20	170	0.93	8	29	1.00	21	0.72
[7]	21	61B	YES 2nd	14.0	14	15	158	1.02	2	14	1.00	11	0.79

* For legenda, see end of this Appendix. (cont.)

Appendix 2: Serological data from the 15 dose-response studies (continued)

REF	SG	DG	BOOSTER	DOSE	N	PRE-GMT	POST-GMT	MFI	PRE-PROT	POST-PROT	PR	N RESP	RR
[7]	21	62B	YES 2nd	67.0	24	20	137	0.84	7	24	1.00	18	0.75
[8]	22	63	NO	5.0	25	17	87	0.71	8	18	0.59	13	0.52
[8]	22	64	NO	10.0	24	17	138	0.91	4	17	0.65	16	0.67
[8]	22	65	NO	20.0	24	10	153	1.18	2	21	0.86	21	0.88
[8]	22	66	NO	40.0	23	9	322	1.55	2	22	0.95	22	0.96
[9]	23	67	NO	10.0	47	20	900	1.65	18	45	0.93		
[9]	23	68	NO	15.0	49	20	930	1.67	19	47	0.93		
[9]	24	69	NO	10.0	47	10	240	1.38	14	46	0.97		
[9]	24	70	NO	15.0	49	10	200	1.30	13	47	0.94		
[10]	25	71	NO	15.0	25	25	66	0.42	12	19	0.54		
[10]	25	72	NO	60.0	25	19	77	0.61	8	22	0.82		
[11]	26	73	NO	15.0	25	19	43	0.35	64	9	0.36		
[11]	26	74	NO	30.0	23	14	44	0.50	61			10	0.43
[11]	26	75	NO	45.0	24	22	70	0.50		75		12	0.50
[11]	27	76	NO	15.0	25	13	38	0.47		56		10	0.40
[11]	27	77	NO	30.0	23	11	50	0.66		65		12	0.52
[11]	27	78	NO	45.0	24	18	76	0.63		79		13	0.54
[11]	28	79	NO	15.0	25	18	43	0.38		64		6	0.24
[11]	28	80	NO	30.0	23	20	50	0.40		61		7	0.30
[11]	28	81	NO	45.0	24	19	54	0.45		71		9	0.38
[11]	29	82	NO	15.0	24	13	28	0.33		50		9	0.38
[11]	29	83	NO	30.0	26	11	32	0.46		54		13	0.50
[11]	29	84	NO	45.0	25	22	27	0.09		44		5	0.20
[11]	30	85	NO	15.0	24	21	62	0.47		83		10	0.42
[11]	30	86	NO	30.0	26	19	60	0.50		92		14	0.54
[11]	30	87	NO	45.0	25	24	58	0.38		76		10	0.40
[11]	31	88	NO	15.0	24	16	28	0.24		42		4	0.17
[11]	31	89	NO	30.0	26	18	31	0.24		58		6	0.23
[11]	31	90	NO	45.0	25	14	22	0.20		40		4	0.16
[12]	32	91	NO	10.0	45	10	1318	2.10	0	41	0.91	43	0.96
[12]	32	92	NO	15.0	39	11	1622	2.15	0	38	0.97	39	1.00
[12]	33	93	NO	10.0	42	8	490	1.78	0	37	0.88	36	0.86
[12]	33	94	NO	15.0	34	9	407	1.64	0	29	0.85	30	0.88
[12]	34	95	NO	10.0	46	11	1202	2.02	0	42	0.91	45	0.98
[12]	34	96	NO	15.0	36	10	741	1.88	0	34	0.94	35	0.97
[13]	35	97	NO	15.0	42	18	78	0.64	15	34	0.70	21	0.50
[13]	35	98	NO	60.0	87	27	94	0.54	42	69	0.60	44	0.51
[14]	36	99	NO	7.5	35	5	19	0.58					
[14]	36	100	NO	15.0	70	4	18	0.65					
[14]	36	101	NO	30.0	35	5	27	0.73					
[14]	37	102	NO	7.5	35	8	53	0.82					
[14]	37	103	NO	15.0	70	8	70	0.94					
[14]	37	104	NO	30.0	35	11	105	0.98					
[14]	38	105	NO	7.5	35	6	36	0.78					
[14]	38	106	NO	15.0	35	6	30	0.70					

* For legenda, see end of this Appendix. (cont)

Appendix 2: Serological data from the 15 dose-response studies (continued)

REF	SG	DG	BOOSTER	DOSE	N	PRE-GMT	POST-GMT	MFI	PRE-PROT	POST-PROT	PR	N RESP	RR
[14]	38	107	NO	30.0	35	6	37	0.79					
[14]	38	108	NO	45.0	35	6	45	0.88					
[15]	39	109	NO	1.5	10	7	32	0.66					
[15]	39	110	NO	3.0	10	11	73	0.82					
[15]	39	111	NO	15.0	9	14	153	1.04					
[15]	40	112	NO	1.5	10	22	47	0.33					
[15]	40	113	NO	3.0	10	19	104	0.74					
[15]	40	114	NO	15.0	9	26	176	0.83					
[15]	41	115	NO	1.5	10	8	17	0.33					
[15]	41	116	NO	3.0	10	9	23	0.41					
[15]	41	117	NO	15.0	9	9	38	0.63					

Legenda

REF	reference number
SG	stratification group
DG	dose group, or booster dose group
BOOSTER	1st, first vaccination
	2nd, second vaccination
DOSE	dose [µg HA], for booster dose groups sum of two dosages
N	size of dose group for calculation of GMT
PREPROT	number of subjects protected prior to vaccination
POSTPROT	number of subjects protected after vaccination
PR	protection rate: (POSTPROT-PREPROT)/(N-PREPROT)
PREGMT	absolute pre-vaccination geometric mean titre
POSTGMT	absolute post-vaccination geometric mean titre
MFI	logarithmated mean-fold increase: log[POSTGMT/PREGMT]
NRESP	number of responders (subjects with four-fold or higher titre rise)
RR	response rate (NRESP/N)
blanc	missing value

Appendix 3: Dose-comparisons and statistical calculations *

A. Post-geometric mean titres, or mean fold increase.

REF	SG	BOOSTER	PRIMING STATE	DOSE-RANGE	NUMBER OF DOSES	STAT. TEST	RESULT
[1]	1	YES 1st	unprimed	5 - 94	6	NO	
[1]	1	YES 2nd	unprimed	10 -188	6	NO	
[1]	2	NO	primed	5 - 94	6	NO	
[1]	3	NO	primed	5 - 66	3	NO	
[1]	4	YES 1st	unprimed	3 - 33	3	NO	
[1]	4	YES 2nd	unprimed	6 - 66	3	NO	
[1]	5	NO	primed	3 - 33	4	NO	
[2]	6	YES 1st	unprimed	1 - 43	4	YES	+ (4 v. 10 µg, 10 v. 43 µg)
[2]	6	YES 2nd	unprimed	21 - 43	4	YES	—
[3]	7	YES 1st	unprimed	10 - 40	3	NO	
[3]	7	YES 2nd	unprimed	20 - 60	3	NO	
[3]	8	YES 1st	primed	10 - 40	3	NO	
[3]	8	YES 2nd	primed	20 - 60	3	NO	
[4]	9	YES 1st	unprimed	7 - 20	2	YES	—
[4]	9	YES 2nd	unprimed	14 - 40	2	YES	—
[4]	10	NO	primed	7 - 60	3	YES	—
[4]	11	NO		7 - 20	2	YES	—
[4]	12	NO		7 - 20	2	YES	—
[5]	13	YES 1st	unprimed	7 - 20	2	YES	—
[5]	13	YES 2nd	unprimed	7 - 20	2	YES	—
[5]	14	NO	primed	7 - 20	2	YES	—
[5]	15	NO		7 - 20	2	YES	—
[5]	16	NO		7 - 20	2	YES	—
[6]	17	YES 1st	unprimed	2.3- 20	3	YES	—
[6]	17	YES 2nd	unprimed	4.6- 40	3	YES	—
[6]	18	NO		2.3- 20	3	YES	—
[6]	19	NO		2.3- 20	3	YES	—
[7]	20	YES 1st	unprimed	7 - 60	2	YES	+ (7 v. 60 µg)
[7]	20	YES 2nd	unprimed	14 - 67	2	YES	+ (14 v. 67 µg)
[7]	21	YES 1st	primed	7 - 60	2	YES	+ (7 v. 60 µg)
[7]	21	YES 2nd	primed	14 - 67	2	YES	—
[8]	22	NO		5 - 40	4	NO	
[9]	23	NO		10 - 15	2	NO	
[9]	24	NO		10 - 15	2	NO	
[10]	25	NO		15 - 60	2	YES	—
[11]	26	NO		15 - 45	3	YES	—
[11]	27	NO		15 - 45	3	YES	+ (15 v. 45 µg)
[11]	28	NO		15 - 45	3	YES	—
[11]	29	NO		15 - 45	3	YES	—
[11]	30	NO		15 - 45	3	YES	—
[11]	31	NO		15 - 45	3	YES	—
[12]	32	NO		10 - 15	2	YES	—
[12]	33	NO		10 - 15	2	YES	—

* For legenda, see end of this Appendix. (cont.)

Appendix 3: Dose-comparisons and statistical calculations (continued)

A. Geometric mean titres (continued)

REF	SG	BOOSTER	PRIMING STATE	DOSE-RANGE	NUMBER OF DOSES	STAT. TEST	RESULT
[12]	34	NO		10 - 15	2	YES	-
[13]	35	NO		15 - 60	2	YES	-
[14]	36	NO		7.5- 30	3	YES	-
[14]	37	NO		7.5- 30	3	YES	+ (7.5 - 30 µg)
[14]	38	NO		7.5- 45	4	YES	-
[15]	39	NO		1.5- 15	3	YES	+ (1.5 - 15 µg)
[15]	40	NO		1.5- 15	3	YES	+ (1.5 - 15 µg)
[15]	41	NO		1.5- 15	3	YES	-

B. Protection rate and response rate for stratification groups with only two doses

REF	SG	BOOSTER	PRIMING STATE	DOSE-RANGE	PR				RR			
					DIFF	CI lower	CI upper	SIG	DIFF	CI lower	CI upper	SIG
[4]	9	YES 1st	unprimed	7 - 20	0.162	-0.033	0.357	—	-0.052	-0.214	0.110	—
[4]	9	YES 2nd	unprimed	14 - 40	0.058	-0.124	0.240	—	-0.015	-0.180	0.150	—
[4]	11	NO		7 - 20	0.018	-0.088	0.124	—	0.096	-0.019	0.211	—
[4]	12	NO		7 - 20	0.022	-0.118	0.162	—	0.090	-0.035	0.215	—
[5]	13	YES 1st	unprimed	7 - 20	0.472	0.204	0.740	+	0.302	0.057	0.547	+
[5]	13	YES 2nd	unprimed	7 - 20	0.065	-0.096	0.226	—				
[5]	14	NO	primed	7 - 20	-0.084	-0.225	0.057	—	0.057	-0.079	0.193	—
[5]	15	NO 7 - 20			-0.088	-0.220	0.044	—				
[5]	16	NO 7 - 20			-0.003	-0.155	0.149	—				
[7]	20	YES 1st	unprimed	7 - 60	0.422	0.103	0.741	+	0.378	0.091	0.665	+
[7]	20	YES 2nd	unprimed	14 - 67	0.500	0.175	0.825	+	0.111	-0.058	0.280	—
[7]	21	YES 1st	primed	7 - 60	0.200	0.049	0.351	+	0.057	-0.171	0.285	—
[7]	21	YES 2nd	primed	14 - 67	0.000	—	—	—	-0.036	-0.266	0.194	—
[9]	23	NO 10 - 15			0.002	-0.110	0.114	—				
[9]	24	NO 10 - 15			-0.025	-0.108	0.058	—				
[10]	25	NO 15 - 60			0.285	-0.008	0.578	—				
[12]	32	NO 10 - 15			0.063	-0.028	0.154	—	0.044	-0.014	0.102	—
[12]	33	NO 10 - 15			-0.028	-0.168	0.112	—	0.025	-0.115	0.165	—
[12]	34	NO 10 - 15			0.031	-0.072	0.134	—	-0.006	-0.067	0.055	—
[13]	35	NO 15 - 60			-0.104	-0.301	0.093	—	0.006	-0.155	0.167	—

* For legenda, see end of this Appendix. (cont.)

Appendix 3: Dose-comparisons and statistical calculations (continued)

C. Protection rate and response rate for stratification groups with more than two doses

REF	SG	BOOSTER	PRIMING STATE	DOSE-RANGE	NUMBER OF DOSES	PR				RR			
						SLOPE	P	SIG	ED_{50}	SLOPE	P	SIG	ED_{50}
[1]	1	YES 1st	unprimed	5 - 94	6	0.57	0.03	+	15				
[1]	1	YES 2nd	unprimed	10 -188	6	1.30	0.03	+	5				
[1]	2	NO	primed	5 - 94	6	0.32	0.48	—					
[1]	3	NO	primed	5 - 66	3	1.28	0.12	—					
[1]	4	YES 1st	unprimed	3 - 33	3	-0.02	0.94	—					
[1]	4	YES 2nd	unprimed	6 - 66	3	-0.24	0.50	—					
[1]	5	NO	primed	3 - 33	4	0.97	0.07	—					
[2]	6	YES 1st	unprimed	1 - 43	4	2.00	<0.001	+	12	1.29	<0.001	+	4
[2]	6	YES 2nd	unprimed	21 - 43	4	-0.24	0.50	—					
[3]	7	YES 1st	unprimed	10 - 40	3	0.84	0.15	—		0.38	0.40	—	
[3]	7	YES 2nd	unprimed	20 - 60	3	-0.67	0.77	—					
[3]	8	YES 1st	primed	10 - 40	3	1.13	0.04	+	166	1.47	0.002	+	17
[3]	8	YES 2nd	primed	20 - 60	3	0.57	0.73	—		3.95	0.18	—	
[4]	10	NO	primed	7 - 60	3	0.33	0.53	—		0.61	0.05	+	14
[6]	17	YES 1st	unprimed	2.3- 20	3	1.38	<0.001	+	19				
[6]	17	YES 2nd	unprimed	4.6- 40	3	0.55	0.02	+	2				
[6]	18	NO		2.3- 20	3	0.61	0.11	—					
[6]	19	NO		2.3- 20	3	0.29	0.23	—					
[8]	22	NO		5 - 40	4	1.62	0.003	+	4	1.85	<0.001	+	5
[11]	26	NO		15 - 45	3	0.74	0.32	—					
[11]	27	NO		15 - 45	3	0.78	0.30	—					
[11]	28	NO		15 - 45	3	0.80	0.31	—					
[11]	29	NO		15 - 45	3	-0.78	0.62	—					
[11]	30	NO		15 - 45	3	0.03	0.97	—					
[11]	31	NO		15 - 45	3	0.04	0.97	—					

Legenda

DC	dose comparison.
SG	stratification group.
BOOSTER	1st, first vaccination
	2nd, second vaccination
STAT.TEST	statitical test performed by the authors
DIFF	between-dose difference of a rate
CI	confidence interval of difference (lower limit, upper limit)
SIG	statistical significance as defined in the text
SLOPE, P	slope and P-value according to probit-analysis
ED50	50% effective dose
DOSE-DIFF	difference between the highest and the lowest dose of a stratification group
blanc	no data available

Appendix 4: Calculations of confidence intervals for differences in protection and response rates, for trials with two vaccine doses only.

Source: B. Dawson-Saunders and R.G. Trapp [26].

Let n_1 and n_2 be the sizes of the dose groups to be compared, let p_1 and p_2 be the proportions with an effect (protection or response), let p be the pooled proportion,

Then the 95% confidence limits for the "true" difference between the population rates in the two dose groups within a stratification group which contains only these two dose groups, are given by:

(1) $(p_2-p_1) + 1.96 * SQR [p(1-p)(1/n_1+1/n_2)]$

with $p = (n_1p_1+n_2p_2) / (n_1+n_2)$,

and $n_1p_1 \geq 5$ and $n_2p_2 \geq 5$.

(SQR means square root.)

Using the notation of Appendix 2, sample sizes and proportions for the formulas above are given by:

(2) p = (POSTPROT-PREPROT)/(N-PREPROT) = PR

n = N-PREPROT

np = POSTPROT-PREPROT

for protection rates,

and

(3) p = NRESP/N = RR

n = N

np = NRESP

for response rates.

4

Dose-comparative serological studies: Critical evaluation of the parameters to assess the antibody response to influenza vaccination

1. INTRODUCTION

In the previous chapter, 15 papers from the international literature [1-15] were evaluated with respect to dose-effects using the traditional serological parameters such as the post-vaccination geometric mean titre (post-GMT), mean fold titre increase (MFI), protection rate (PR), and response rate (RR). Considerable discrepancies within and between studies were detected with respect to these parameters (for example, see Table 5, Chapter 3). In this chapter, we will critically evaluate these parameters with respect to their ability to detect dose- effects if these really exist, or to establish the absence of dose- effects if these really do not exist. After all, it is reasonable to assume that, by applying inappropriate parameters non-existing dose-effects may be created, or existing dose-effect may be overseen.

To evaluate the appropriateness of the parameters, a data-file was made up from a large collection of vaccination studies. These included groups of subjects as homogenous as possible with respect to confounding factors like previous vaccinations, priming state, age and health state. Conditions and effects of different parameters were exemplified by these data. Doses used in these studies were 10 and either 15 or 20 μg HA. Such narrow dose ranges, in contrast to the wide range reported in the literature, were a consequence of the current dose requirements for commercial influenza vaccines.

We were interested in exploring more objective ways for the interpretation of serological data. Therefore, we evaluated whether a study outcome could be affected by the choice of serological parameters. Since the studies to be discussed in the current chapter represent a selection of all available dose-comparative studies, no final conclusions on the effect of antigen dose on the antibody response should be drawn. The strict selection criteria were applied to demonstrate the methodological principles of serological studies only.

2. Materials and methods

2.1 Sources

In the period 1982-1990, Duphar BV, Weesp, The Netherlands, in cooperation with independent scientific researchers, conducted 35 trials with the influenza subunit vaccine Influvac (R) (see Appendix 1). The aim of these studies was to assess the immunogenicity and reactogenicity of various vaccine components, either as single dose trials (23, including comparisons between different batches, or comparisons to whole virus vaccine) or as comparisons between different doses (dose-comparison trials, 12), mostly in young healthy adults, but in some cases also in nursing-home residents or ambulatory elderly.

Demographic and serological data were available on the computer files of the company. Details of the study designs were extracted from the original trial documents (study protocols, reports of the investigators and medical reports of the company).

2.2 Selection criteria and assumptions

2.2.1. Selection of studies: Participants, laboratorium techniques, vaccine component, and doses.

We included dose-comparison trials conducted in young, healthy adults only. Studies in older populations (nursing home residents, or ambulatory elderly) were not included because, in these groups, the confounding variation with respect to priming state, vaccination history, health state, and drug use was assumed to be greater than in young adults.

Moreover, we included only those studies in which the serological determinations had been done by the National Influenza Centre (NIC), Department of Virology, Erasmus University Rotterdam, The Netherlands, using the haemagglutination-inhibition test as decribed in [16, 17]. This is a highly standardized and stable technique used over the last 20 years.

These selection criteria yielded eight dose-comparison studies. Three studies were not included: two studies (5026/1 and 5026/2, see Appendix 1) will be decribed in detail in Chapter 5 and of a third study (5019 C) not all data were available. Of the remaining five, two studies included an A-H1N1 strain, four an influenza B strain, and five an A-H3N2 strain (Table 1a).

All studies compared a dose of 10 μg HA with either 15 or 20 μg HA.

Table 1a: Some characteristics of five dose-response studies in young adults; antibody determination by NIC-method.

Study	year	Numbers (*)	Vacc. history (**)	Priming state (***)	Vaccine strains	Source
5014	1985	83	77/ 1/5	-/24/ 59	A/Philippines/2/82 (H3N2) A/Chile/1/83 (H1N1) B/USSR/100/83	Palache (1)
5016	1986	68	39/29/-	2/18/ 48	A/Mississippi/1/85 (H3N2) B/Ann Arbor/1/86	Limburg & Palache (2)
5021	1987	50	47/ 2/1	-/10/ 40	A/Leningrad/360/86 (H3N2)	De Jonge & Palache (3)
5028	1988	143	143/ -/-	-/- /143	A/Sichuan/2/87 (H3N2) A/Taiwan/1/86 (H1N1) B/Beying/1/87	Palache & De Jonge (4)
5030	1989	53	50/ 3/-	-/- / 53	A/Shanghai/11/87 (H3N2) B/Yamagata/16/88	De Jonge (5)
	totals	397	356/35/6	2/52/343		

* , numbers of participants completing the study.

** , history of previous vaccinations: not previously vaccinated/previously vaccinated/not known or not recorded.

***, priming state: year of birth 1940-1949/1950-1961/1962 and later.

(1) Palache A.M., Summary medical report. Immunogenicity of two doses of an influenza subunit vaccine. An open randomized study in healthy, adult volunteers. Duphar report June 1985.
(2) Limburg C.M.L.G., Palache A.M., Medical report. Immunogenicity of two doses of an influenza subunit vaccine. An open randomized study in healthy, adult volunteers. Duphar report No. H.201.5016/M July 1985.
(3) De Jonge S., Palache A.M. Medical report. Immunogenicity and reactogenicity of two doses of an influenza subunit vaccine. A double-blind, randomized study in healthy, adult volunteers. Duphar report No. H.201.5021/M July 1987.
(4) Palache A.M., De Jonge S. Immunogenicity and reactogenicity of two doses of an influenza subunit vaccine. A double-blind, randomized study in healthy, adult volunteers. Duphar study protocol No. H.201.5028/1 July 1988.
(5) De Jonge S. Medical report. Immunogenicity and reactogenicity of an influenza subunit vaccine, containing the following strains: A/Shanghai/11/87 (H3N2), B/Yamagata/16/88. A baseline- controlled study in healthy adult volunteers. Duphar report No. H.201.5030/M July 1989.

2.2.2. Selection of subjects: History of previous vaccinations, priming state, and health state

Selection and stratification criteria as described in Chapter 2, Tables 6 and 7, were applied as follows:

Previous vaccinations: The study protocols of the five trials were not uniform with respect to subjects vaccinated against influenza in previous years. While the studies performed in 1988 and 1989, advised to exclude those subjects, this was not true for the studies before 1988. The proportions of previously vaccinated subjects was low

(7% and 6% respectively) for studies 5014 and 5021, but for study 5016 it was 43% of all participants.

Priming periods: The study protocols were not uniform with respect to subjects of different priming periods. Within the first three studies, two subjects were born in the H1 priming period (1940-1949, see Table 7, Chapter 2), and 52 in the H2 priming period (1950-1961). The last two studies consisted exclusively of subjects born in the H3 priming period (1962 and later) to create a collection of serological data from groups of study subjects as homogeneous as possible. To demonstrate the effects of parameters, we excluded all subjects previously vaccinated, those with unknown vaccination record, and those born in 1961 or before, accepting to loose a fair amount of data from two studies (5014 and 5016).

Health state: The study populations, mainly consisting of Dutch students, were 'apparently healthy'. The Senieur protocol [18] to check the health state of the participants, as described in Chapter 2, or a similar clinical or laboratory check-up to exclude subjects with non-overt disease, was not used in any of the studies.

Pre-vaccination titres, at this stage, were not used as a selection criterion.

Table 1b presents the numbers of participants included after these selection criteria had been applied. Of the 397 subjects of Table 1a, 77 were excluded for the following reasons: vaccinated against influenza in previous years (35), previous vaccination not known (6), born before 1962 (and not previously vaccinated;36).

Table 1b: Numbers of subjects of five dose-comparison studies in young, healthy adults born after 1961 without previous influenza vaccinations.

Study	No. subj.	Vaccine strains	No. by dose		
			10µg	15µg	20µg
		Influenza A-H3N2			
5014	55*	A/Philippines/2/82	27		28
5016	32	A/Mississippi/1/85	15	17	
5021	39	A/Leningrad/360/86	22	17	
5028	143	A/Sichuan/2/87	71		72
5030	50	A/Shanghai/11/87	33	17	
totals	319*		168	51	100
		Influenza A-H1N1			
5014	56	A/A/Chile/1/83	27		29
5028	143	A/Taiwan/1/86	71		72
totals		199	98		101
		Influenza B			
5014	56	B/USSR/100/83	27		29
5016	32	B/Ann Arbor/1/86	15	17	
5028	143	B/Beying/1/87	71		72
5030	50	B/Yamagata/16/88	33	17	
totals	281		146	34	101

*, Laboratory determination of anti-A-H3N2-antibodies was not done for one subject (not previously vaccinated, born 1962) because of scarcity of the sera.

2.3 Comparison of study populations and study designs

A total of 320 not previously vaccinated subjects with an age range between 17 and 25 years (belonging to the H3 priming period) in five trials with an influenza A-H3N2 vaccine component, were available for further consideration (Table 2). Overall, there were slightly more male subjects than female, but this was not considered relevant for the evaluation of serological data. Recruitment was done among students and employees of the Erasmus University Rotterdam (5014, 5015, 5021) and a laboratory school in Rotterdam (5030), and volunteers under contract with a research company (Innopharm, Paris;5028).

Table 2: Study populations and study designs.

Study	No. subj.	Age mean (range)	Sex %m : %f	Place *	Study design		Sera **
5014	56	21 (20 – 23)	57 : 43	NL	open	randomized	14-17
5016	32	21 (19 – 24)	38 : 62	NL	open	randomized	15-20
5021	39	20 (17 – 25)	49 : 49***	NL	double-blind	randomized	21
5028	143	22 (18 – 25)	58 : 42	F	double-blind	randomized	21
5030	50	21 (18 – 25)	48 : 52	NL	double-blind	randomized	21-23
total	320	21 (17 – 25)	53 : 47				14-23

* ,NL, Rotterdam, The Netherlands; F, Paris, France.
** ,days between first (pre-vaccination) and second (post-vaccination) blood sampling.
***,sex of one subject not recorded.

Exclusion criteria were allergy to chicken proteins (all studies) and year of birth before 1962 (5026, 5028, 5030). Stated preferences for recruitment, concerned no history of previous vaccinations (all but 5014) and low pre-vaccination antibody titres (5016, included pre-screening).
Vaccination took place in summers or autumns (i.e. absence of naturally occuring influenza viruses), with one exception (5014 during winter 1985; however, there was no natural influenza activity at that time).

In all studies, vaccines were, intramuscularly or subcutaneously, administered to the study participants on the basis of a previously completed, randomized scheme. The first two studies were open as the two doses differed in volumes of injection solution (0.5 ml versus 1.0 ml, and 0.5 ml versus 0.75 ml resp.). In the other three double-blind studies, injections with equal volumes for the different doses were used. In study 5030, the code was broken during the vaccination session. "Because of a slow recruitment rate the study had to be unblinded to ensure at least sufficients numbers on the 10-mg dose level" (ref 5, Table 1a) which were required for registration purposes; allocation was therefore non-random in the last part of this study.

The time between first (pre-vaccination) and second (post- vaccination) blood sampling varied between 14 and 23 days. In all studies, sera were kept frozen at -20°C until analysis. For each study, the determination of homologous pre- and post-vaccination antibodies were done simultaneously and in duplicate by the standard haemagglutination inhibition test of the Dutch National Influenza Centre (NIC) [16, 17], using cholera filtrate and egg- grown homologeous test viruses.

2.4 Character of absolute titre values: Discrete or continuous scale?

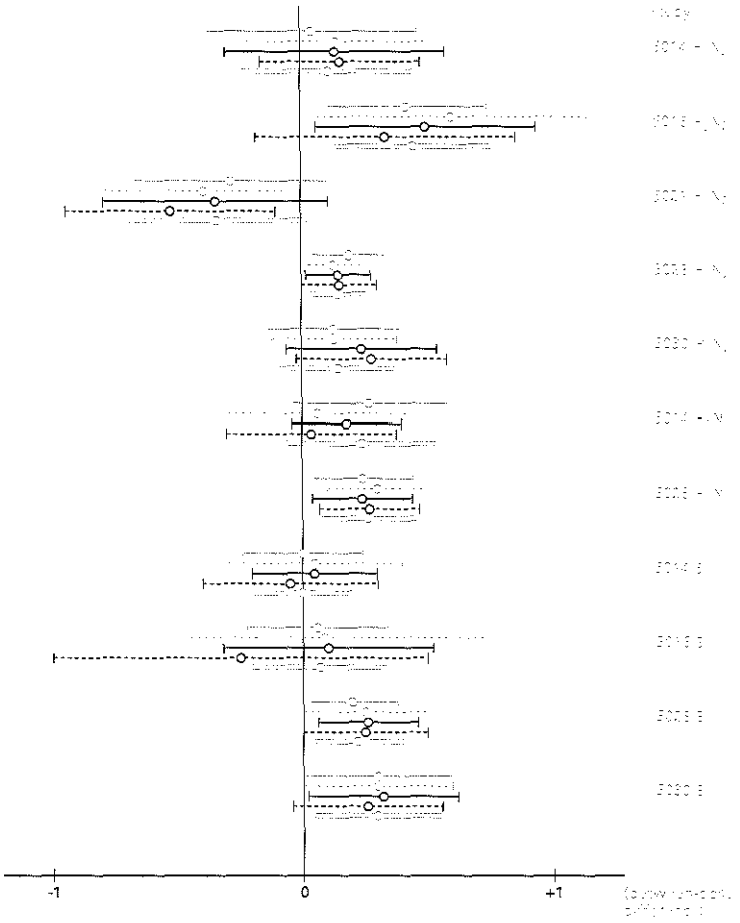
For statistical analyses it is important to know according to which procedure the test outcomes are measured. In the NIC micro- titre plate haemagglutination-inhibition test, the agglutination patterns of 12 two-fold dilutions (from 1:9 to 1:18.432) are studied for each serum. Each dilution is read by a technician using five agglutination categories (4+, complete agglutination; 3+, 2+, 1+, various degrees of incomplete agglutination; 0, no agglutination), and checked by a second technician. The limiting dilution with complete agglutination and one dilution below with incomplete dilution, both determine the end point of agglutination-inhibition of the serum. The actual concentration of the test antigen is assessed simultaneously with the serum determinations. If the antigen concentration is not equal to three units, the end point of the serum is corrected to three units by using a table of correction factors. Each serum is tested in duplicate. The logarithms of reciprocals of the two end point-dilutions per serum are averaged to form the final serum titre. Undetectable high (<1:18.432) and low (>1:9) end points are replaced with arbitrary end points (1:20.000, and 1:4, resp., in this chapter) for calculation purposes.
Thus, a serum HI-antibody titre can virtually reach values between 0.60 and 4.30 on a $_{10}$log scale, and may, for practical purposes, be considered as a quantitative observation on a continuous, numerical scale. It has been shown that the logarithmic transformation of values based on two-fold serum dilutions, results in an approximately normal distribution of the random error of the titre values [19].
In this chapter, titres are therefore presented as logarithms if not mentioned otherwise. For example, the titre of a serum with an end point of 1:100 is usually presented as 2.00; the anti-log titre would be 100. Also GMT and MFI are presented on log-scales.

3. Results

3.1 Quantitative pre- and post-vaccination geometric mean titre (GMT) as measure for a biological dose-effect.

Appendix 2A presents, for each dose group, means and 95% confidence intervals (CI) of pre- and post-vaccination GMT and their difference (mean fold-increase, MFI). The data include studies with different levels of pre-vaccination GMT (0.67 - 0.99 for A-H1N1, 0.99 - 1.78 for A-H3N2, and 1.31 - 1.84 for B). Post-vaccination GMT values exceed 2.00 for all dose groups (with the exception of study 5028 for A-H1N1), and even 3.00 for the 15-µg HA A-H3N2 group of study 5016. The confidence intervals of MFI exclude 0 for all dose groups indicating that all vaccine doses induced a significant antibody rise. Appendix 3A includes a comparison of pre-GMT between two dose groups per study and subtype. All but one of the 95%-confidence intervals include 0 which means that there are no significant differences of the pre-vaccination antibody level between the two dose groups of each study.

Figure 1: Between-dose diferences and 95% CI for five different quantitative parameters.



post-GMT, all subjects included (Appendix 3A)

MFI, idem

post-GMT, excluding subjects with high pre-vaccination titres (Appendig 3B)

MFI, idem

post-GMT, adjusted for pre-vaccination titres by ANCOVA (Appendix 3C)

In the 15 papers reviewed in Chapter 3, five different approaches to estimate a dose-effect from quantitaive pre- and post- vaccination titres were identified:

1. post-GMT without correction for pre-GMT
   - with consecutive t-test [2, 4, 7, 11]
   - without a statistical test [1, 3, 8].

2. MFI
   - with consecutive Wilcoxon's rank sum test [10],
   - without a statistical test [8].

3. post-GMT of subjects with low pre-vaccination titres,
   - without a statistical test [9].

4. MFI of subjects with low pre-vaccination titres
   - with consecutive Wilcoxon's rank sum test [5, 6, 12].

5. post-GMT, corrected for pre-GMT by a statistical model (ANCOVA, analysis of covariance) [13, 14].

In this chapter, these five methods, with consecutive calculation of confidence intervals, are applied to our data. In Appendix 3a- c, the between-dose differences and their 95% CI are shown for each study/subtype and for each method. Calculations are explained in Appendix 4. Figure 1 graphically presents, for each parameter, the mean difference and its 95% CI per dose- comparison; Table 3 summarizes these results. A lower confidence limit >0 means the detection of a dose-effect. As can be seen in Table 3, some studies yield consistent results for all parameters (for example: study 5014 no dose-effect for all three antigens; study 5028 dose-effect for A-H1N1). The interpretation of other studies, however, is clearly affected by the parameter (for example: study 5028 for A-H1N1, 5030 for B). Figure 1 reveals that, in these cases, the lower confidence limit is not far above 0 for all parameters. In such cases, the choice of the parameter will determine whether or not a dose-effect will be shown.

Below we will discuss the advantages and shortcomings of the five parameters investigated.

1. Post-GMT without correction for pre-GMT

Using the raw post-vaccination titres as end points implies the absence of any attempt to correct for the pre-vaccination titre. When performing vaccination trials in previously unprimed or otherwise seronegative subjects, as was the case for studies [2,3] of the previous chapter, the problem of the pre-vaccination titre is, of course, not relevant. The common case, however, is a heterogeneous study population with both seronegative and, to various degrees, seropositive subjects. There exists a linear relationship between pre- and post-vaccination titres, as has been described, to our knowledge, for the first time by Voth et al. [20] and since then by many others. Therefore, the pre-vaccination titre is a confounding factor which, dependent on its actual distribution between the groups to be compared, may mask existing dose-effects, or may suggest dose-effects where none exist in reality. For our data, the line

ar relationship can be demonstrated as well (see Figure 2a, for the H3N2-component of study 5028, as an example). The regression lines for the other components and studies are similar (not shown).

Table 3: Significant dose-effects, as detected by different seroresponse parameters.

Study	Parameter							
	Post-GMT	MFI (high pre-vacc. excluded)	Post-GMT	MFI	Post-GMT adjusted	PR100	PR300 adjusted	RR
5014 H3N2								
5016 H3N2	DE	DE	DE		DE		DE	DE
5021 H3N2				-DE				
5028 H3N2	DE		DE	DE	DE	DE	DE	
5030 H3N2								
5014 H1N1								
5028 H1N1	DE	DE	DE	DE	DE	DE		DE
5014 B								
5016 B								
5028 B	DE	DE	DE	DE	DE	DE	DE	
5030 B	DE	DE	DE		DE		DE	

DE, lower limit of 95% CI above or equal to 0: significant positive dose-effect.
-DE, uper limit of 95% CI under or equal to 0: significant negative dose-effect.

Figure 2a: Linear relationship between pre-vaccination titres and post-vaccination titres.

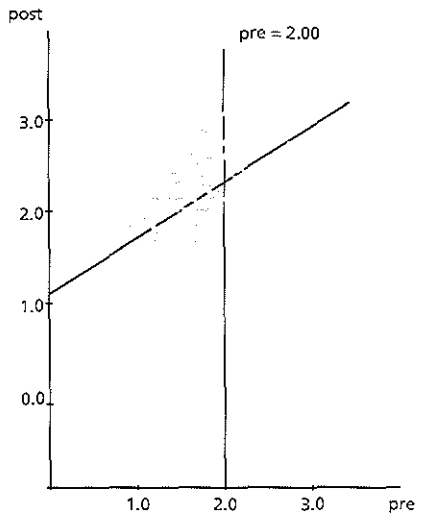
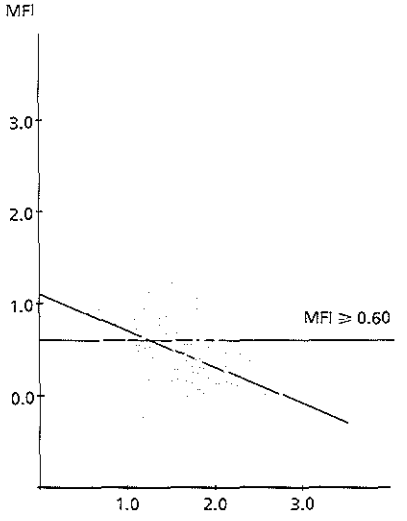


Figure 2b: Linear relationship between pre-vaccination titres and MFI-values.



2. Mean fold titre increase (MFI)

A common approach to correct the post-vaccination titre for variations in pre-vaccination titre is the calculation of the MFI and its comparison between dose-groups. In our data (see Appendix 3, Figure 1), MFI-values behaved sometimes very similar to post-GMT values (for example study 5030 for A- H3N2), but, in other cases, showed large differences with respect to mean or CI-range (for example study 5016 for A-H3N2 and B). The dose-effect for A-H3N2 in study 5028, detected by post-GMT, was absent for MFI.

It can be shown that the correction for the pre-vaccination titre when using MFI, is less than optimal. The linear relationship between pre- and post-vaccination titre in one individual can generally be described as:

(1) $T_{post} = a + b*T_{pre}$

where T_{pre} = pre-vaccination titre
T_{post} = post-vaccination titre
a = y-intercept
b = slope of regression line

The MFI on log-scale, however, is simply the difference between post- and pre- vaccination titre, thus

(2) $MFI = T_{post} - T_{pre}$, or

Combination with (1) gives:

(3) $MFI = a + (b-1)*T_{pre}$

An adjustment for pre-vaccination titre by using MFI, would be optimal only in the special case that truly b=1. Usually, however, the slope of the regression line is different from 1 (see Figure 2b as an example). As a consequence, MFI itself is still dependent on pre-vaccination titre, as is demonstrated in Figure 2b. MFI is, therefore, a less appropriate parameter since it ignores the actual relationship between pre- and post vaccination titres.

3. and 4. Post-GMT and MFI of subjects with low pre-vaccination titres.

Some authors, realizing the relationship between pre- and post- vaccination titres, have proposed to stratify for pre-vaccination titre and to exclude all seropositive [6] or, at least, highly seropositive [5, 12] subjects from analysis, and to compare GMT or MFI only for the more homogeneous subjects with low pre- immunization titres.

It was not relevant to analyse our data for previously seronegative subjects as there were not many (in study 5030 only one for A-H3N2). For demonstration only, we followed the method of paper [12]: HI-assay according to NIC, only including subjects with pre- vaccination titres smaller than 2.00 (log 100) for influenza A, or smaller than 2.30 (log 200) for influenza B. These thresholds were equal to the assumed protection thresholds in the studies.

The effects of the exclusion of subjects with high pre- vaccination titres were different for the three subtypes/types: A total of 70 subjects with pre- vaccination titres of >100 were excluded for A-H3N2 (22%), 4 for A-H1N1 (2%), and 33 subjects with pre-vaccination titres of >200 for B (12%). In Figure 1a, the points at the right of the vertical line (log pre-titre 2.0) represent the number of subjects being excluded. Dose-effects as detected by CI-ranges of dose-comparisons for post-GMT and MFI after exclusion (Figure 1) were quite different from those as detected by the same parameters without exclusion of high-seropositive subjects. As expected, CI-ranges were larger in the former cases, reflecting a loss of test-power. For example, the post-GMT yielded a dose-effect in study 5030 for the B antigen and for MFI, the dose-effect in study 5016 for A-H3N2 disappeared, but there was a "dose-effect" in study 5021 for A-H3N2 in favour of the lower 10-mg dose. The disadvantages of this approach (loss of information and test-power, absence of a rationale for the exclusion threshold, and using the MFI, which was shown to provide an inaccurate correction for pre-vaccination titre), are obvious.

5. Post-GMT, adjusted for pre-GMT by a mathematical model.

The draw-backs described in the previous section were avoided in papers [13,14] where the pre-vaccination titre was used as a co-variate in the ANCOVA analysis. Other co-variates were, in [13], previous vaccinations of the subjects, and, in [14], age. We may demonstrate the principles of this approach by using an ANCOVA model [21] with antigen dose as independent variable, pre- vaccination titre as confounding factor, and post-vaccination titre as dependent variable, applied for each study separately. Other possibly confounding factors (age, previous vaccinations, priming state, and, incompletely, health state) had already been addressed during the selection procedure. This procedure resulted in post-vaccination titres, corrected for pre-vaccination titres (see Appendix 4 for calculations). The CI-ranges for mean differences between post-GMT when comparing dose groups, were smaller than for uncorrected post-GMT. All decisions about dose-effects were clear-cut. Again, studies 5016 and 5028 would appear to show significant dose-effects.

We have demonstrated that the interpretation of the serological data is dependent on the chosen endpoint parameters. For example, if study 5021 was analysed using only the MFI-values of subjects with pre-vaccination titres of <100 and <200, respecti-

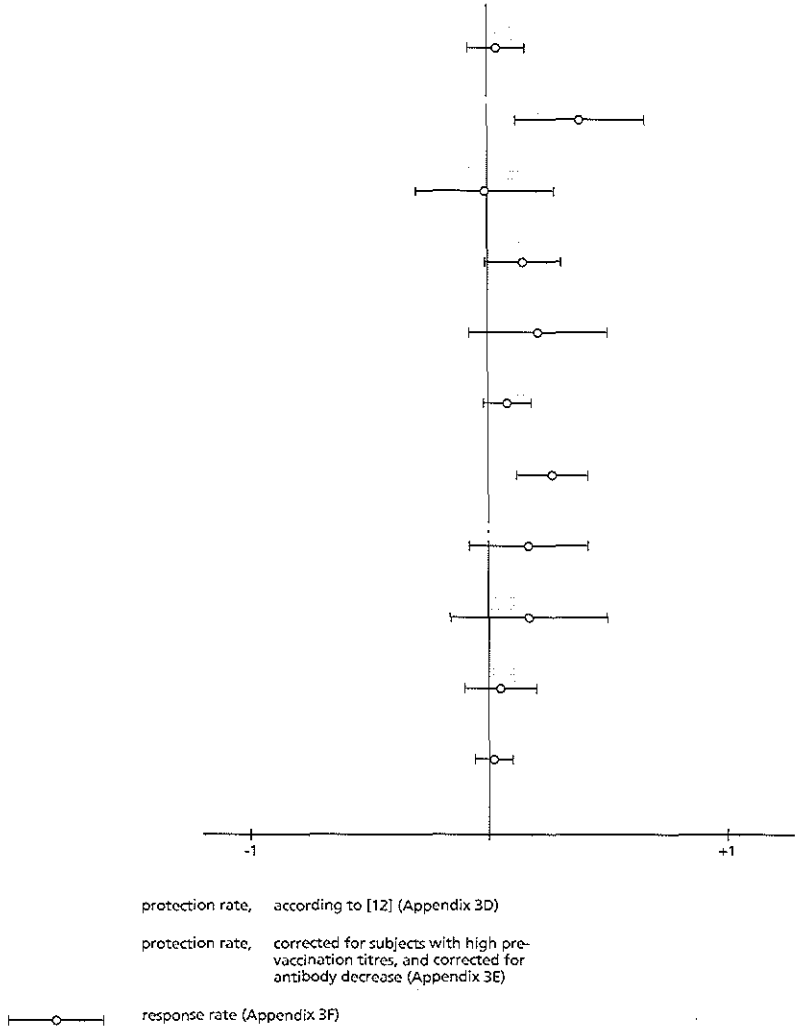
vely, the conclusion would have been that a dose of 15 µg HA was `statistically significantly' inferior to a dose of 10 µg HA, which is evidently not plausible. Dose-effects in two other studies would have been missed (5016 for A-H3N2, 5030 for B). For theoretical and practical reasons, we would suggest that the use of covariance analysis methods which involve all studied subjects and correct for confounding factors such as the pre-vaccination titres, would represent the best approach for the analysis and interpretation of serological vaccination studies.

3.2 Protection rate as measure for a clinically relevant dose-effect

Until now we have been discussing the presence or absence of a biological or immunological dose-effect on absolute numerical titres which may, however, not be clinically relevant. For example, in study 5016 for A-H3N2, the 10-µg dose yielded a post-GMT value of 2.91 (log 813), and the 15-µg dose 3.34 (log 2,188); the difference was significant before and after adjustment for pre-vaccination titre. But if the clinically important minimum antibody level which protects a subject from infection with influenza, was much lower than both post-GMT values and if the proportion of subjects exceeding this minimum threshold was similar in both dose groups, then the detected biological dose-effect would have no clinical implication. This consideration leads to the concept of a titre threshold associated with protection.

In most serological studies, the analysis of antibody response includes the proportion of subjects with titres above 32-40 [1,2,4-11,13]. Very often this threshold titre level is referred to as the `protective titre', which is a misleading terminology since it ignores the fact that these titre values have been shown to represent only the 50% protective level (PL_{50}; [22, 23]), i.e. for an individual with such antibody threshold titre value, there is still a 50% chance to acquire influenza. The threshold titre value of 100 in papers [3,12], based on the NIC assay, represents a slightly higher "protective level" of approximately 60% (see Appendix 4).
Appendix 3D and Figure 3 present differences of protection rates with respect to a `protection' threshold according to paper [12] (100 for influenza A, 200 for influenza B). The protection rates are the ratios of the number of subjects exceeding the threshold after vaccination and the total number of subjects, both after substraction of the number of subjects exceeding the threshold prior to vaccination. The dose-effects for the three antigens of study 5028 for example, as detected by the adjusted post-GMT parameters, remain stable whereas the between-dose differences of study 5016 for A-H3N2 and of study 5030 for B decreased drastically. It seems therefore, that for these latter two cases, a biological dose-effect would have no clinical relevance.

Figure 3: Between-dose differences and 95% CI for three different dichotomous parameters.



There are three arguments to criticize the current use of the "protective titre values" to assess vaccine efficacy:

a. the `protective' level is fixed at too low a value
b. there is no correction for antibody decrease in time
c. there is no appropriate adjustment for pre-vaccination titre.

a. 'Protective' level is fixed at too low a value

Although the ED_{50} (50% effective dose) is a useful parameter to compare relative

potencies of different compounds in pharmacology studies, we feel that this is an inadequate parameter to measure vaccine efficacy.
It would be more appropriate to use, for example, a 90% protective level (PL_{90}), like for the assessment of serological hepatitis B vaccine efficacy studies, where anti- HBs values that exceed 10 S/N units by radio-immuno assay are indicative of immunity to hepatitis B infections [24].

b. No correction for antibody decrease in time

Because the ultimate aim of influenza immunization is to protect vaccinees against influenza infections, which usually will occur between 1-5 months after vaccination, vaccinations should ideally yield antibody titres, which will still be at the PL_{90} at six months after vaccination. Thus, a constant should be added to the PL_{90} which represents the antibody decrease over six months.

c. No appropriate correction for pre-vaccination titre

In statistical terms, the introduction of a protective level means the transformation of a continuous numerical scale (titre) to a dichotomous scale (below or above protective titre). The loss of information associated with this transformation, although acceptable as the procedure is intended to focus on the clinically relevant information, entails loss of power. By deleting subjects with pre-vaccination antibody titres beyond the threshold titre when calculating the protection rate, the statistical power again decreases. This loss is undesirable. Therefore, the protection rate should be calculated preferably by adjusting for pre- vaccination titre by appropriate statistical methods.

We developed an approximation procedure to compute a PL_{90} and another to compensate for antibody decrease in time for the NIC assay. This resulted in a threshold of 2.48 (log 300). We adjusted for pre-vaccination titre by logistic regression (assumptions and methods see Appendix 4). We believe that this approximation is close to a clinically meaningful threshold titre level. Table 3 and Figure 3 reveal that one of the five studies with a significant dose-effect for adjusted post-GMT, did not reach a significant, i.e. clinically relevant dose-effect (5028 for A-H1N1).

3.3 Other traditional serological parameters: qualitative response rate, cumulative tables.

The proportion of subjects with a four-fold or more titre rise (response rate) is an other frequently used parameter in serological studies [2-5,7,8,11-13]. Formally, it is a transformation of a continuous numerical scale (MFI-values) to a dichotomous scale with a threshold of 0.60 (log 4, marked in Figure 1b). Figure 3 gives also the between-dose differences and 95% CI for the unadjusted response rate, as usual in the litera-

ture. For two of the studies, this parameter detected a significant dose-effect (5016 for H3N2, and 5028 H1N1).

Originally, the fourfold increase was taken to account for the assay imprecision, due to factors such as the random error caused by instrumental variations, the degree of unpredictability in the assay reaction mechanisms, enviromental influence, contributions from the analysts [30]. As the HI-test in its early form dealt with clear-cut two-fold dilutions, the difference of only one dilution step between pre- and post-vaccination sera of a subject was assumed to be covered in part by the assay imprecision, but a difference of two or more dilutions was assumed to exceed the assay imprecision. Thus, the response rate is meant to express the proportion of titre rises which cannot be explained by the random error of the assay.

It is essential for any measurement that the problem of assay imprecision is addressed. However, the concept of four-fold or more titre rise is too conservative for the current HI-assay. We estimated the intra-assay error from duplicate determinations per serum (see Appendix 4) and calculated the factor beyond which there is a 5% chance that a difference between two sera may occur as a result only of the random error of the assay. This factor ranged between 1.1 and 2.4. Thus, in all cases a factor of 4 would have been too high to appropriately account for assay imprecision.

Moreover, a rate arbitrarily taking account of assay imprecision, calculated separately from biologically and clinically relevant markers, is of little value. We feel that measures like response rate and similar parameters (seroconversion rate, response rate of unprotected subjects, protection rate of responders etc.) should better be avoided. A preferable approach would be to calculate the adjusted post-vaccination GMT according to a plausible statistical model as described before. Concerning the protection rate, the threshold titre value should be established taking into account the pre-vaccination titre and the actual assay error (SD) during laboratory proceedings.

A different, frequently used, traditional approach from the times when statistical methods had not yet widely been introduced in medical research, is the presentation of cumulative frequency tables on a number of titre intervals [1,2,6,8,9,11,13]. Table 4 demonstrates this method in a form adopted from [2]. From this presentation, it is sometimes easy to conclude the absence of a dose-effect, if the distributions for the two dose groups are similar (for example, in study 5030 for A-H3N2, or 5014 for B). However, when differences seem to occur, as in study 5014 for A-H3N2, or 5016 for A-H3N2, is it a matter of subjective interpretation rather than a decision based on objective criteria. The calculation of confidence intervals for the between-dose differences is clearly superior. If one wishes to get an overview of the distribution shape of the raw data before statistical calculations, a graphic plot should be preferred.

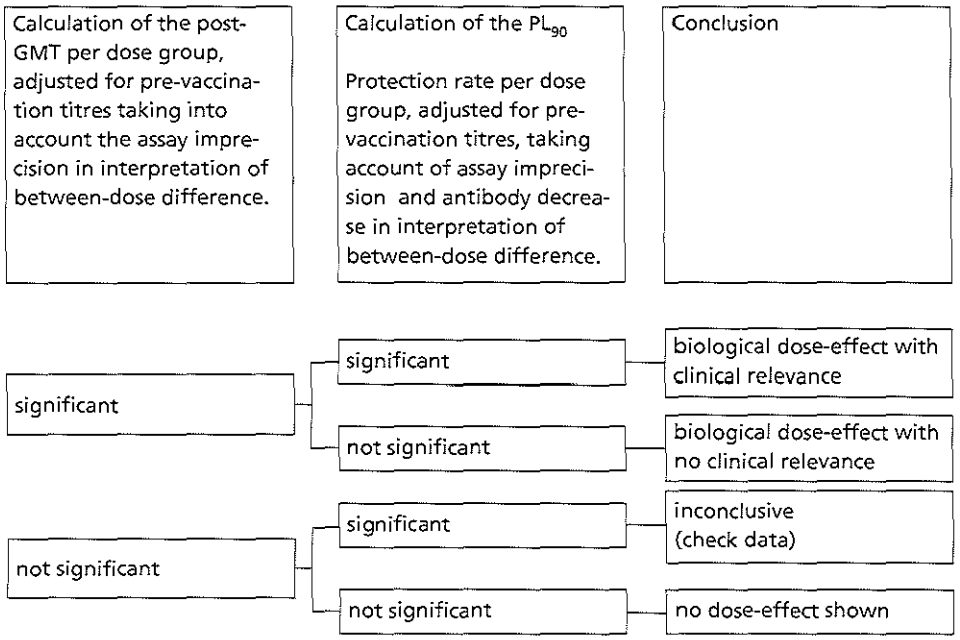
Table 4. Cumulative distribution of adjusted post-vaccination titres.

STUDY	DOSE (µg HA)	subj. No.	Intervals of post vaccination titres ≥9	≥18	≥36	≥72	≥144	≥288	≥576	≥1152	≥2304	≥4608	≥9216	≥18432
5014 H3N2	10	27	96	96	93	78	63	44	26	7	4	0	0	0
5014 H3N2	20	28	96	96	96	89	68	57	32	14	11	4	0	0
5016 H3N2	10	15	100	100	100	100	87	67	47	20	20	7	0	0
5016 H3N2	15	17	100	100	100	100	100	100	100	59	24	12	0	0
5021 H3N2	10	22	95	95	82	64	45	41	27	0	0	0	0	0
5021 H3N2	15	17	100	100	65	53	24	6	0	0	0	0	0	0
5028 H3N2	10	71	94	73	31	13	4	0	0	0	0	0	0	0
5028 H3N2	20	72	99	92	51	18	6	0	0	0	0	0	0	0
5030 H3N2	10	33	100	94	64	36	18	6	0	0	0	0	0	0
5030 H3N2	15	17	100	88	76	59	29	6	0	0	0	0	0	0
5014 H1N1	10	27	93	93	93	93	89	78	44	33	7	0	0	0
5014 H1N1	20	29	100	100	100	97	97	97	76	31	7	3	0	0
5028 H1N1	10	71	79	70	56	48	27	8	3	0	0	0	0	0
5028 H1N1	20	72	92	90	78	56	40	15	6	0	0	0	0	0
5014 B	10	27	96	96	96	89	52	19	11	0	0	0	0	0
5014 B	20	29	100	100	97	83	52	17	3	0	0	0	0	0
5016 B	10	15	100	100	100	100	100	87	80	47	13	0	0	0
5016 B	15	17	100	100	100	100	100	100	71	53	18	12	0	0
5028 B	10	71	96	96	93	77	54	31	14	3	0	0	0	0
5028 B	20	72	99	97	97	93	69	42	29	8	3	0	0	0
5030 B	10	33	100	100	97	97	85	55	21	9	3	0	0	0
5030 B	15	17	100	100	100	100	94	94	53	29	12	0	0	0

3.4 Combined interpretation of parameters

Some authors evaluate many parameters from the same data but then become unsure on how to interprete them when in a dose-comparison a significant difference is observed for some parameters but not for others. The above considerations in mind, we suggest the following to be included in the protocol at the design of the study: (Figure 4):

Figure 4: Decision algorithm for the use of seroresponse parameters in interpretation of difference between two dose-groups



-Calculation of the post-GMT and its between-dose difference, adjusted for pre-vaccination titres, with 95% confidence limits.

If the difference is significant (lower limit of 95% confidence interval equal to or larger than zero), then a biological dose-effect has been established. Now one should look at the clinical relevance of this finding:

- Calculation of the protection rates in each dose group and its between-dose difference, taking account of pre-vaccination titres and assay imprecision, and based on a 90% protective level with correction for antibody decrease over six months. If the difference is significant, then it has been shown that the biological dose-effect has also clinical relevance, and vice versa.

It may be sensible to calculate the protection rates even in case of non-significant between-dose differences of the post-GMT values, to check the findings. The between-dose difference of the protection rate should also be non-significant. If it is not, the raw data should be checked for unusual distributions. In paper [12] such a

case has been described for non-significant post-GMT differences (after exclusion of subjects with high pre-vaccination titres) and significant PR differences (threshold 100). A plot of the raw data (Figure 1 in paper [12]) revealed that the `significant' difference was due only to a unfavourable random distribution of some post-vaccination titres around the protection threshold.

4. Discussion

Many of the clinical data to characterize influenza vaccines are derived from serological studies in which HI-antibody titres are used as surrogate markers of efficacy. There are two criteria for the acceptance of a surrogate marker in clinical research: how far is the use of a surrogate marker necessary and how appropriate is the selected marker [26]. Indeed, the evidence of efficacy of two widely used classes of drugs in prevention of cardiovascular disease, angiotensin converting enzyme inhibitors and calcium antagonists, is based entirely on trials in which blood pressure reduction was accepted as a surrogate measure of cardiovascular events. On the other hand, lack of an identified relevant marker to predict the clinical course of infection with the human immunodeficiency virus (HIV) is one of the current problems for the development of anti-HIV therapies [38,39].

Although the true efficacy parameters of influenza vaccine (prevention of illness and reduction of morbidity and mortality) can and has been measured in large population studies during influenza epidemics, the uncertainty of time and place of such events and of the antigenic match between the vaccine and epidemic strains, make such studies extremely difficult, complex, and costly. Therefore the use of a surrogate marker of efficacy is generally accepted in the case of influenza vaccination efficacy studies.

Evidence for the appropriateness of the HI-antibody titre as surrogate marker for influenza vaccine efficacy comes from several field or artificial challenge studies [22,27-29] in which a quantitative correlation between homologous serum HI-antibody titre and protection against illness have been shown. A recent long-term study by Davies and Grilli [23], however, has shown, that a similar level of antibody produced in response to vaccination or natural infection was associated with different infection rates in favour of the antibody induced by natural infection. Therefore, they concluded that not only the antibody titre per se, but also the circumstances of its induction affects its value as a predictor of immunity.

Local IgA [30-32] and heterologous, cross-reactive antibodies [33,34] are also shown to positively affect protection against illness, and cellular immunity to enhance recovery from infection [35]. In contrast to serum HI-antibody, however, for these parameters, no quantitative correlation with protection has yet been demonstrated in man [36]. Hence, at present, homologous serum HI-antibody titre is the only surrogate marker for influenza vaccines' protective efficacy [30,36] for which consistent experimental evidence is available.

Although serum HI-antibody titre has consistently been shown to correlate with protection against illness, a correlation with reduction of clinical symptoms has not been proven. For example, Davies and Grilli [23] found that "although increasing amount of relevant antibody was associated with lower infection rates, the proportion of

those infected who had clinical symptoms did not decrease with increasing antibody".

As we have shown in the previous chapters, serological studies intrinsically show much variation in the outcome of serological variables. Therefore, we suggest, that not only average figures (GMT, PL_{90}) are presented in study reports and publications, but also actual ranges and 95% confidence intervals. This will make not only the assessments of study results easier, but also will enable proper meta-analysis of series of similar studies to be performed.

We have shown, that MFI and response rates are inappropriate measures of vaccine efficacy from serological vaccination studies. Hence, we propose, that these parameters should not be used anymore to assess vaccine efficacy and/or assess the relative efficacy of different treatment groups. Due to the established correlation between pre- and post-vaccination antibody, we suggest, that post-vaccination antibody will be adjusted for pre-vaccination antibody in the statistical analysis of serological studies.

For the serological assessment of the relative efficacy of vaccine types, vaccine doses or other vaccination regimens in various study populations, we would propose to compare the proportion of subjects converting from pre-immunization titres lower than the 'modified' PL_{90} (unprotected and suboptimal protected) to post-immunization titres at or above the 'modified' PL_{90} (protected at least up to six months post-immunization). We will refer to this proportion as the "Protection-Rate". Only if the 95% confidence limits of the protection rate difference between treatment groups do not have different signs, such difference should be considered as a clinically relevant difference.

In our opinion, the use of a convention such as outlined above offers the possibility to objectively evaluate vaccine-treatment differences. This advantage outweighs the potential disadvantages associated with the intrinsic variability of the true PL_{90} and modification factor used for the 6-month antibody decline rate for each individual study and study population.

Because the formulated proposals for a method to assess serological vaccination efficacy studies differ fundamentally from current practice, we would propose, that an "International Collaborative Group for Influenza Research" be created to discuss these proposals and to determine by convention, the most appropriate values for the PL90 and for a modification factor for a 6-month antibody-decline rate. Such group could also decide to set up guidelines for good prospective clinical studies, including considerations of power calculations to achieve sufficiently narrow confidence intervals. These studies should include artificial challenge studies in young, healthy volunteers, to prospectively establish the biological relevant cut-off values and assess the appropriateness of such factors.

In our opinion, chapters 2-4 of this thesis show convincingly the urgent need to reach a general consensus for the methods to evaluate influenza vaccine efficacy from serological studies. Our proposals for some consensus rules are meant to serve as catalyst for consensus discussions only.

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Appendix 1

Duphar trials (35) with influenza subunit vaccine Influvac®

Study	Year	Participants	Vaccine			Doses (µg HA)	NIC-method
			A-H3N2	A-H1N1	B		
5007	1982	young healthy adults	yes	yes	yes	10	
5008	1982	young healthy adults	yes	yes	yes	10	
5009	1983	young healthy adults	yes			10	
5010	1983	young healthy adults	yes	yes	yes	10	
5011	1984	young healthy adults		yes		10	
5012	1984	young healthy adults			yes	10	
5013	1984	young healthy adults	yes	yes	yes	10	
5014	1985	young healthy adults	yes	yes	yes	10 20	yes
5014 A	1985	young healthy adults	yes	yes	yes	10	yes
5015	1985	young healthy adults	yes	yes	yes	10	yes
5016	1986	young healthy adults	yes		yes	10 15	yes
5017/1	1986	young healthy adults	yes	yes	yes	10 20	
5017/2	1987	young healthy adults	yes	yes	yes	10	
5017/3	1988	young healthy adults	yes	yes	yes	10	
5018	1986	young healthy adults	yes	yes	yes	10	yes
5019 A	1986	young healthy adults	yes	yes	yes	10	yes
5019 B	1986	young healthy adults		yes		15	yes
5019 C	1986	young healthy adults		yes		10 15	yes
5020	1986	young healthy adults		yes		15	yes
5021	1987	young healthy adults	yes			10 15	yes
5023/1	1987	ambulatory elderly	yes	yes	yes	10	
5023/2	1988	ambulatory elderly	yes	yes	yes	10	
5025	1988	young healthy adults	yes		yes	7 10 15	
5026/1	1988	young healthy adults	yes	yes	yes	0 10 20 60	yes
5026/2	1988	young healthy adults	yes	yes	yes	0 10 20 60	yes
5027/1	1988	nursing home residents	yes	yes	yes	0 10 20 60	yes
5027/2	1988	nursing home residents	yes	yes	yes	0 10 20 60	yes
5028	1988	young healthy adults	yes	yes	yes	10 20	yes
5030	1989	young healthy adults	yes		yes	10 15	yes
5032	1989	young healthy adults	yes	yes	yes	10	
5033	1989	ambulatory elderly	yes	yes	yes	10	
5035	1989	nursing home residents	yes	yes	yes	10	
5036	1990	young healthy adults	yes			10	yes
5037	1990	young healthy adults	yes	yes	yes	10	
5038	1990	nursing home residents	yes	yes	yes	10	

Appendix 2.
Serological data for 11 dose groups from five dose-comparison studies.
GMT and MFI presented on log-scale.

A. Pre- and post-GMT, and MFI-values (with 95% confidence intervals), including all subjects.

STUDY	DOSE	NO subj.	PRE-GMT	POST-GMT	MFI
5014 H3N2	10	27	1.10 (0.87 - 1.33)	2.77 (2.46 - 3.09)	1.68 (1.42 - 1.93)
5014 H3N2	20	28	0.99 (0.81 - 1.17)	2.81 (2.52 - 3.11)	1.82 (1.56 - 2.09)
5016 H3N2	10	15	1.61 (1.18 - 2.03)	2.91 (2.61 - 3.21)	1.30 (0.85 - 1.75)
5016 H3N2	15	17	1.44 (1.13 - 1.76)	3.34 (3.17 - 3.50)	1.90 (1.55 - 2.24)
5021 H3N2	10	22	1.44 (1.17 - 1.71)	2.60 (2.32 - 2.89)	1.17 (0.84 - 1.49)
5021 H3N2	15	17	1.54 (1.34 - 1.75)	2.33 (2.06 - 2.59)	0.78 (0.60 - 0.96)
5028 H3N2	10	71	1.67 (1.54 - 1.79)	2.08 (1.97 - 2.19)	0.41 (0.31 - 0.51)
5028 H3N2	20	72	1.75 (1.64 - 1.86)	2.28 (2.17 - 2.38)	0.53 (0.45 - 0.61)
5030 H3N2	10	33	1.78 (1.67 - 1.89)	2.47 (2.31 - 2.63)	0.69 (0.54 - 0.83)
5030 H3N2	15	17	1.78 (1.61 - 1.95)	2.60 (2.37 - 2.82)	0.82 (0.59 - 1.05)
5014 H1N1	10	27	0.78 (0.66 - 0.89)	2.72 (2.44 - 3.01)	1.95 (1.65 - 2.25)
5014 H1N1	20	28	0.99 (0.83 - 1.14)	3.00 (2.86 - 3.13)	2.01 (1.82 - 2.20)
5028 H1N1	10	71	0.74 (0.65 - 0.83)	1.71 (1.55 - 1.86)	0.97 (0.81 - 1.14)
5028 H1N1	20	72	0.67 (0.60 - 0.73)	1.95 (1.81 - 2.09)	1.29 (1.15 - 1.43)
5014 B	10	27	1.43 (1.16 - 1.70)	2.36 (2.17 - 2.56)	0.93 (0.66 - 1.20)
5014 B	20	28	1.40 (1.17 - 1.62)	2.36 (2.23 - 2.49)	0.96 (0.73 - 1.19)
5016 B	10	15	1.84 (1.39 - 2.29)	2.91 (2.71 - 3.12)	1.07 (0.53 - 1.61)
5016 B	15	17	1.82 (1.43 - 2.20)	2.99 (2.80 - 3.18)	1.18 (0.75 - 1.60)
5028 B	10	71	1.34 (1.18 - 1.51)	2.40 (2.26 - 2.53)	1.05 (0.89 - 1.22)
5028 B	20	72	1.31 (1.15 - 1.47)	2.61 (2.49 - 2.74)	1.30 (1.13 - 1.48)
5030 B	10	33	1.47 (1.24 - 1.71)	2.93 (2.75 - 3.11)	1.46 (1.28 - 1.64)
5030 B	15	17	1.48 (1.18 - 1.77)	3.23 (2.99 - 3.48)	1.76 (1.50 - 2.01)

Appendix 2.
Serological data for 11 dose groups from five dose-comparison studies. (continued)

B. Pre- and post-GMT, and MFI-values (with 95% confidence intervals), including only subjects with pre-vaccination titre ≤ 2.00 (log 100) for influenza A or ≤ 2.30 (log 200) for influenza B.

STUDY	DOSE	NO	PRE-GMT	POST-GMT	MFI
5014 H3N2	10	24	0.97 (0.77 - 1.17)	2.68 (2.34 - 3.01)	1.71 (1.42 - 2.00)
5014 H3N2	20	28	0.99 (0.81 - 1.17)	2.81 (2.52 - 3.11)	1.82 (1.56 - 2.09)
5016 H3N2	10	9	1.10 (0.70 - 1.50)	2.88 (2.39 - 3.38)	1.79 (1.36 - 2.22)
5016 H3N2	15	13	1.21 (0.92 - 1.49)	3.38 (3.17 - 3.60)	2.18 (1.88 - 2.48)
5021 H3N2	10	18	1.22 (1.02 - 1.43)	2.55 (2.21 - 2.89)	1.32 (0.98 - 1.67)
5021 H3N2	15	14	1.42 (1.23 - 1.60)	2.21 (1.93 - 2.50)	0.80 (0.57 - 1.02)
5028 H3N2	10	54	1.47 (1.36 - 1.58)	1.97 (1.84 - 2.09)	0.50 (0.38 - 0.62)
5028 H3N2	20	50	1.52 (1.42 - 1.62)	2.16 (2.04 - 2.29)	0.65 (0.56 - 0.73)
5030 H3N2	10	26	1.67 (1.57 - 1.77)	2.35 (2.18 - 2.52)	0.68 (0.50 - 0.86)
5030 H3N2	15	13	1.64 (1.50 - 1.78)	2.60 (2.32 - 2.88)	0.96 (0.73 - 1.19)
5014 H1N1	10	27	0.78 (0.66 - 0.89)	2.72 (2.44 - 3.01)	1.95 (1.65 - 2.25)
5014 H1N1	20	29	0.99 (0.83 - 1.14)	3.00 (2.86 - 3.13)	2.01 (1.82 - 2.20)
5028 H1N1	10	68	0.67 (0.61 - 0.73)	1.69 (1.53 - 1.85)	1.02 (0.85 - 1.18)
5028 H1N1	20	71	0.64 (0.60 - 0.68)	1.94 (1.80 - 2.08)	1.30 (1.17 - 1.44)
5014 B	10	22	1.21 (0.97 - 1.45)	2.30 (2.07 - 2.53)	1.09 (0.80 - 1.38)
5014 B	20	27	1.32 (1.10 - 1.53)	2.35 (2.21 - 2.48)	1.03 (0.81 - 1.25)
5016 B	10	8	1.22 (0.73 - 1.70)	2.92 (2.51 - 3.33)	1.70 (0.92 - 2.49)
5016 B	15	13	1.54 (1.17 - 1.91)	3.02 (2.76 - 3.27)	1.48 (1.06 - 1.89)
5028 B	10	63	1.20 (1.05 - 1.35)	2.34 (2.20 - 2.49)	1.14 (0.97 - 1.31)
5028 B	20	67	1.21 (1.07 - 1.36)	2.60 (2.46 - 2.73)	1.38 (1.20 - 1.56)
5030 B	10	31	1.39 (1.18 - 1.59)	2.90 (2.71 - 3.09)	1.51 (1.34 - 1.69)
5030 B	15	17	1.48 (1.18 - 1.77)	3.23 (2.99 - 3.48)	1.76 (1.50 - 2.01)

Appendix 2.
Serological data for 11 dose groups from five dose-comparison studies. (continued)

C. Post-GMT (with 95% confidence intervals),
adjusted for pre-vaccination titres by ANCOVA.

STUDY	DOSE	NO	POST-GMT
5014 H3N2	10	27	2.38 (2.12 - 2.63)
5014 H3N2	20	28	2.50 (2.24 - 2.77)
5016 H3N2	10	15	2.78 (2.49 - 3.07)
5016 H3N2	15	17	3.23 (3.07 - 3.40)
5021 H3N2	10	22	2.20 (1.93 - 2.48)
5021 H3N2	15	17	1.87 (1.67 - 2.07)
5028 H3N2	10	71	1.43 (1.34 - 1.51)
5028 H3N2	20	72	1.58 (1.50 - 1.65)
5030 H3N2	10	33	1.74 (1.59 - 1.88)
5030 H3N2	15	17	1.87 (1.66 - 2.09)
5014 H1N1	10	27	2.70 (2.42 - 2.99)
5014 H1N1	20	29	2.95 (2.82 - 3.09)
5028 H1N1	10	71	1.66 (1.50 - 1.81)
5028 H1N1	20	72	1.93 (1.79 - 2.06)
5014 B	10	27	2.18 (2.00 - 2.36)
5014 B	20	29	2.18 (2.06 - 2.31)
5016 B	10	15	2.98 (2.78 - 3.17)
5016 B	15	17	3.06 (2.86 - 3.25)
5028 B	10	71	2.21 (2.08 - 2.33)
5028 B	20	72	2.43 (2.31 - 2.56)
5030 B	10	33	2.50 (2.36 - 2.64)
5030 B	15	17	2.80 (2.60 - 3.01)

Appendix 2.
Serological data for 11 dose groups from five dose-comparison studies. (continued)

D. Protection rate (with 95% confidence intervals),
threshold: - 2.00 (log 100) for influenza A,
- 2.30 (log 200) for influenza B.

STUDY	DOSE	NO	PR	
5014 H3N2	10	24	0.83	(0.68 - 0.98)
5014 H3N2	20	28	0.89	(0.78 - 1.01)
5016 H3N2	10	9	1.00	
5016 H3N2	15	13	1.00	
5021 H3N2	10	18	0.72	(0.52 - 0.93)
5021 H3N2	15	14	0.64	(0.39 - 0.89)
5028 H3N2	10	54	0.48	(0.35 - 0.61)
5028 H3N2	20	50	0.70	(0.57 - 0.83)
5030 H3N2	10	26	0.81	(0.66 - 0.96)
5030 H3N2	15	13	0.92	(0.78 - 1.07)
5014 H1N1	10	27	0.93	(0.83 - 1.02)
5014 H1N1	20	29	0.97	(0.90 - 1.03)
5028 H1N1	10	68	0.29	(0.19 - 0.40)
5028 H1N1	20	71	0.49	(0.38 - 0.61)
5014 B	10	22	0.59	(0.39 - 0.80)
5014 B	20	27	0.59	(0.41 - 0.78)
5016 B	10	8	0.88	(0.65 - 1.10)
5016 B	15	13	1.00	
5028 B	10	63	0.56	(0.43 - 0.68)
5028 B	20	67	0.73	(0.62 - 0.84)
5030 B	10	31	0.87	(0.75 - 0.99)
5030 B	15	17	1.00	

Appendix 2.

Serological data for 11 dose groups from five dose-comparison studies. (continued)

E. Response rate (with 95% confidence intervals).

STUDY	DOSE	NO	RR	
5014 H3N2	10	27	0.93	(0.83 - 1.02)
5014 H3N2	20	28	0.96	(0.90 - 1.03)
5016 H3N2	10	15	0.60	(0.35 - 0.85)
5016 H3N2	15	17	1.00	
5021 H3N2	10	22	0.73	(0.54 - 0.91)
5021 H3N2	15	17	0.71	(0.49 - 0.92)
5028 H3N2	10	71	0.30	(0.19 - 0.40)
5028 H3N2	20	72	0.44	(0.33 - 0.56)
5030 H3N2	10	33	0.49	(0.31 - 0.66)
5030 H3N2	15	17	0.71	(0.49 - 0.92)
5014 H1N1	10	27	0.93	(0.83 - 1.02)
5014 H1N1	20	29	1.00	
5028 H1N1	10	71	0.61	(0.49 - 0.72)
5028 H1N1	20	72	0.88	(0.80 - 0.95)
5014 B	10	27	0.56	(0.37 - 0.74)
5014 B	20	29	0.72	(0.56 - 0.89)
5016 B	10	15	0.53	(0.28 - 0.79)
5016 B	15	17	0.71	(0.49 - 0.92)
5028 B	10	71	0.70	(0.60 - 0.81)
5028 B	20	72	0.75	(0.65 - 0.85)
5030 B	10	33	0.97	(0.91 - 1.03)
5030 B	15	17	1.00	

Appendix 3.
Between-dose comparisons for 11 dose groups from five dose-comparison studies.

A. Pre-GMT, post-GMT, MFI. Including all subjects (95% CI).

STUDY	DOSE-COMPARISON	PRE-GMT DIFFERENCE	POST-GMT DIFFERENCE	MFI DIFFERENCE
5014 H3N2	20 v. 10	-0.11 (-0.39 - 0.17)	0.04 (-0.38 - 0.46)	0.15 (-0.21 - 0.51)
5016 H3N2	15 v. 10	-0.17 (-0.66 - 0.33)	0.43 (0.11 - 0.75)	0.59 (0.06 - 1.13)
5021 H3N2	15 v. 10	0.10 (-0.24 - 0.45)	-0.28 (-0.66 - 0.11)	-0.38 (-0.77 - 0.01)
5028 H3N2	20 v. 10	0.08 (-0.09 - 0.24)	0.20 (0.05 - 0.35)	0.12 (-0.01 - 0.25)
5030 H3N2	15 v. 10	-0.01 (-0.20 - 0.18)	0.13 (-0.14 - 0.40)	0.14 (-0.12 - 0.39)
5014 H1N1	20 v. 10	0.21 (0.02 - 0.40)	0.27 (-0.03 - 0.58)	0.06 (-0.29 - 0.41)
5028 H1N1	20 v. 10	-0.07 (-0.18 - 0.04)	0.24 (0.04 - 0.45)	0.32 (0.10 - 0.53)
5014 B	20 v. 10	-0.03 (-0.38 - 0.31)	0.00 (-0.23 - 0.23)	0.03 (-0.32 - 0.38)
5016 B	15 v. 10	-0.03 (-0.59 - 0.54)	0.08 (-0.19 - 0.35)	0.10 (-0.55 - 0.75)
5028 B	20 v. 10	-0.03 (-0.26 - 0.19)	0.22 (0.03 - 0.40)	0.25 (0.01 - 0.49)
5030 B	15 v. 10	0.00 (-0.38 - 0.38)	0.30 (0.00 - 0.60)	0.30 (0.00 - 0.60)

B. Pre-GMT, post-GMT, MFI (excluding subjects with high pre-vaccination titres).

STUDY	DOSE-COMPARISON	PRE-GMT DIFFERENCE	POST-GMT DIFFERENCE	MFI DIFFERENCE
5014 H3N2	20 v. 10	0.02 (-0.24 - 0.28)	0.13 (-0.30 - 0.57)	0.11 (-0.27 - 0.49)
5016 H3N2	15 v. 10	0.11 (-0.34 - 0.56)	0.50 (0.06 - 0.94)	0.39 (-0.08 - 0.86)
5021 H3N2	15 v. 10	0.19 (-0.08 - 0.47)	-0.34 (-0.78 - 0.11)	-0.53 (-0.95 - -0.11)
5028 H3N2	20 v. 10	0.05 (-0.10 - 0.20)	0.20 (0.02 - 0.37)	0.15 (0.00 - 0.30)
5030 H3N2	15 v. 10	-0.03 (-0.19 - 0.13)	0.25 (-0.05 - 0.56)	0.28 (-0.01 - 0.57)
5014 H1N1	20 v. 10	0.21 (0.03 - 0.40)	0.27 (-0.03 - 0.57)	0.06 (-0.28 - 0.41)
5028 H1N1	20 v. 10	-0.03 (-0.10 - 0.04)	0.25 (0.04 - 0.46)	0.29 (0.08 - 0.49)
5014 B	20 v. 10	0.10 (-0.21 - 0.42)	0.04 (-0.20 - 0.29)	-0.06 (-0.41 - 0.29)
5016 B	15 v. 10	0.32 (-0.24 - 0.89)	0.10 (-0.32 - 0.52)	-0.23 (-0.97 - 0.51)
5028 B	20 v. 10	0.01 (-0.19 - 0.22)	0.25 (0.06 - 0.45)	0.24 (0.00 - 0.49)
5030 B	15 v. 10	0.09 (-0.25 - 0.43)	0.33 (0.03 - 0.64)	0.24 (-0.05 - 0.53)

C. post-GMT (adjusted for pre-vaccination titres by ANCOVA).

STUDY	DOSE-COMPARISON	PRE-GMT DIFFERENCE
5014 H3N2	20 v. 10	0.12 (-0.23 - 0.48)
5016 H3N2	15 v. 10	0.45 (0.14 - 0.76)
5021 H3N2	15 v. 10	-0.33 (-0.68 - 0.02)
5028 H3N2	20 v. 10	0.15 (0.04 - 0.26)
5030 H3N2	15 v. 10	0.13 (-0.11 - 0.38)
5014 H1N1	20 v. 10	0.25 (-0.05 - 0.55)
5028 H1N1	20 v. 10	0.27 (0.07 - 0.47)
5014 B	20 v. 10	0.00 (-0.21 - 0.21)
5016 B	15 v. 10	0.08 (-0.19 - 0.35)
5028 B	20 v. 10	0.23 (0.05 - 0.40)
5030 B	15 v. 10	0.30 (0.07 - 0.53)

Appendix 3.
Between-dose comparisons for 11 dose groups from five dose-comparison studies (continued).

D. Protection rate
threshold:- 2.00 (log 100) for influenza A,
- 2.30 (log 200) for influenza B.

STUDY	DOSE-COM-PARISON	PR DIFFERENCE
5014	20 v. 10	0.06 (-0.13 - 0.25)
5016	15 v. 10	0.00
5021	15 v. 10	-0.08 (-0.40 - 0.24)
5028	20 v. 10	0.22 (0.03 - 0.41)
5030	15 v. 10	0.12 (-0.12 - 0.35)
5014	20 v. 10	0.04 (-0.08 - 0.16)
5028	20 v. 10	0.20 (0.04 - 0.36)
5014	20 v. 10	0.00 (-0.28 - 0.28)
5016	15 v. 10	0.12 (-0.06 - 0.31)
5028	20 v. 10	0.18 (0.01 - 0.34)
5030	15 v. 10	0.13 (-0.03 - 0.29)

E. Protection rate
threshold 2.48 (log 300)
adjusted for pre-vaccination titres by logistic regression.

STUDY	DOSE-COM-PARISON	PR DIFFERENCE
5014	20 v. 10	0.06 (-0.13 - 0.25)
5014	20 v. 10	0.04 (-0.09 - 0.17)
5016	15 v. 10	0.23 (0.01 - 0.45)
5021	15 v. 10	-0.17 (-0.46 - 0.12)
5028	20 v. 10	0.09 (0.00 - 0.18)
5030	15 v. 10	0.10 (-0.15 - 0.36)
5014	20 v. 10	0.20 (-0.03 - 0.42)
5028	20 v. 10	0.07 (-0.04 - 0.17)
5014	20 v. 10	0.00 (-0.12 - 0.11)
5016	15 v. 10	0.18 (-0.32 - 0.68)
5028	20 v. 10	0.10 (0.03 - 0.17)
5030	15 v. 10	0.19 (0.00 - 0.39)

Appendix 3.
Between-dose comparisons for 11 dose groups from five dose-comparison studies (continued).

F. Response rate

STUDY	DOSE-COM-PARISON	RR DIFFERENCE
5014	20 v. 10	0.04 (-0.08 - 0.16)
5016	15 v. 10	0.40 (0.13 - 0.67)
5021	15 v. 10	-0.02 (-0.31 - 0.26)
5028	20 v. 10	0.15 (-0.01 - 0.31)
5030	15 v. 10	0.22 (-0.07 - 0.51)
5014	15 v. 10	0.07 (-0.02 - 0.17)
5028	15 v. 10	0.27 (0.12 - 0.41)
5014	15 v. 10	0.17 (-0.08 - 0.42)
5016	20 v. 10	0.17 (-0.16 - 0.51)
5028	15 v. 10	0.05 (-0.10 - 0.19)
5030	20 v. 10	0.03 (-0.05 - 0.11)

Appendix 4
Estimations

A Estimation of PL_{90} threshold with correction for antibody decrease.

The following considerations are based on results published by Wesselius-De Casparis et al. [34] and Masurel et al. [42] since their HI-assay was the same as in our study.
From Table 3 in [34], the correlation between pre-infection antibody titre and rate of protection can be estimated from 95 subjects, with the following results concerning an influenza A strain:

Pre-infection titre	relative protection rate
titre	$_{10}$ log titre
≤ 49	≤ 1.69	0.00
≥ 50	≥ 1.70	0.36
≥ 100	≥ 2.00	0.63
≥ 150	≥ 2.18	1.00

Apparently, a threshold titre of 150 would cover the requirements of a PL_{90}.

The decrease of antibody titres in time can be estimated from Table 4 of [42] where, for 36 subgroeps differing in age, vaccine type, and virus subtype/type, the antibody titres before and 1, 6, and 12 months after vaccination are given. The mean decrease during six months, for all subgroups, was 0.09 ($_{10}$log), the most unfavourable case was 0.30.

Thus, a conservative treshold estimate would be derived as follows:

	PL_{90}	decrease 6 months	combined
$_{10}$log titre	2.18	0.30	2.48
titre	150	2	300

Appendix 4
Estimations (continued)

B. Estimation of within-batch error

STUDY	No. sera (N)*	GMT 1st	GMT 2nd	Repeatability SD	FACTOR
5014 H3N2	164	1.88	1.89	0.12	1.5
5016 H3N2	136	2.33	2.35	0.09	1.3
5021 H3N2	90	1.96	1.96	0.03	1.1
5028 H3N2	273	1.89	1.98	0.21	2.0
5030 H3N2	106	2.14	2.17	0.06	1.2
5014 H1N1	166	1.81	1.85	0.15	1.6
5028 H1N1	262	1.28	1.30	0.15	1.6
5014 B	166	1.80	1.88	0.15	1.6
5016 B	136	2.46	2.44	0.09	1.3
5028 B	273	1.86	1.99	0.27	2.4
5030 B	106	2.22	2.26	0.12	1.5
total	1878				

* Nr. of subjects are not selected according to the criteria as described in Table 1b, but are the total number of studies included. Each subject account for two sera (pre and post-vaccination).

Repeatability SD was calculated from duplicate determinations det_1 and det_2 as:
$SD^2 = \sum(det_1-det_2)^2*0.5/N$

with N = number of duplicate determinations.

The titre of a single serum is $0.5*(det_1+det_2)$, and normal distribution of random error assumed, the 95% CI for the titre would be $0.5*(det_1+det_2)$ DE $\pm 1.96*SD/\sqrt{2}$. The factor was calculated as $_{10}$exponent of $1.96*SD/\sqrt{2}$.

Antibody response after influenza immunization with various vaccine doses. A double-blind, placebo-controlled, multi-centre, dose-response study in elderly, nursing-home residents and young volunteers

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Summary

We have investigated the dose effect (0, 10, 20 and 60 μg HA) of influenza subunit vaccine on the antibody response in nursing-home residents and young controls. The vaccine antigens were: A/Taiwan/1/86(H1N1), A/Sichuan/2/87(H3N2) and B/Beijing/1/87. For the influenza B antigen, the post-GMT and the '% protective titre' increased significantly both in the young controls and nursing-home residents. No dose effect was observed for the A/Taiwan, and a minor dose effect for A/Sichuan. All vaccine doses were well tolerated by both groups.

We conclude from our data, that higher vaccine doses may result in a better antibody response against some antigens but not against others. Therefore, in general, increasing the vaccine dose is no adequate alternative to improve the antibody response.

INTRODUCTION

Worldwide, annual immunizations are recommended for individuals, including nursing-home residents, being at high-risk of serious complications or death caused by influenza infections (1,2). Although various authors have convincingly shown that influenza vaccinations modify influenza by the reduction of influenza- and pneumonia-associated hospitalizations (3-6), vaccination studies with inactivated influenza vaccines have shown disappointing protection against infections in nursing-home residents (7-9). These modest results are generally considered to be due to an age-associated declined immune response following a standard dose of vaccination (7,10), although this assumption is disputed by several authors (11,12).

In an attempt to increase the immune response in the elderly and hence to enhance the protection against influenza infections, several authors have investigated the effect of high-dose influenza B virus vaccines on the humoral response in ambulatory elderly, nursing-home residents and young controls (13-15).

Since these studies did not yield a consistent answer as to whether an increased dose would be beneficial or not, we performed a double-blind, placebo-controlled, randomized, multi-centre, dose-response study in elderly, nursing-home residents and young controls. We investigated the antibody response following doses of 10, 20 and 60 μg HA/strain trivalent subunit vaccine.

MATERIALS AND METHODS

1. Study population

We immunized 282 young volunteers (mean age 21 years;range 17-47) and 262 elderly (mean age 80; range 68-99) nursing-home residents with placebo (saline) or trivalent influenza subunit vaccine of various concentrations, between October and December 1988. For both age groups, all treatment groups were comparable with regard to their age and sex distribution.

Each age group was studied in different study centres. The young volunteers were university students from Rotterdam/The Netherlands (centre 1, N=140) and Paris/France (centre 2, N=142). The elderly were recruited either in 20 nursing-homes spread throughout the western part of The Netherlands (centre 3, N=122) or in a single nursing-home in Tel Aviv/Israel (centre 4, N=140). Fifty-eight subjects from centre 4 had been immunized against influenza at least once during the five years preceding the current study. All other participants had not been vaccinated against influenza during this period. Patients taking immunosuppressive medications such as corticosteroids and cytostatics were excluded. Informed consent prior to the study was given by all participants except for nursing-home residents with dementia. In the Israeli group, relatives of subjects with dementia gave informed consent, whereas in the Dutch group, subjects with dementia were excluded from the study. The study was approved by local ethics committees.

2. Vaccines

Experimental trivalent influenza subunit vaccines (Duphar B.V., The Netherlands) contained 10, 20 or 60 μg HA for each of the following antigens: B/Beijing/1/87, A/Taiwan/1/86 (H1N1) and A/Sichuan/2/87 (H3N2). For each antigen, the different dosages were drawn from the same production lot. Each treatment group received a 0.5 ml intramuscular injection of saline or the experimental trivalent vaccine.
The experimental vaccines were produced according to the manufacturer's standard procedures for its commercially available subunit vaccine.

3. Study design

Vaccinations were given on a double-blind basis according to a treatment randomization scheme. From each volunteer, a 10 ml blood sample was drawn just prior to vaccination and again three weeks thereafter.

4. Laboratory investigations

Sera were separated after blood collection, kept frozen at -20°C, and transported from each study centre to the Department of Virology, Rotterdam, The Netherlands for laboratory investigation. On arrival in Rotterdam, all sera were recoded according to a new randomization scheme in order to achieve blindness in the antibody determinations. Each day, the pre- and post-immunisation sera of 12 subjects from each study centre were analysed. Influenza virus strains for titrations were propagated in embryonated 12-day-old chicken eggs. Because of the low avidity of the influenza B viruses, infectious egg fluids of this strain were treated with ether according to Berlin et al. (16) and the aqueous phase was used in the serological tests. Pre- and post-immunization haemagglutinin-inhibition (HI) titres were simultaneously determined in duplicate by standard methods (17). Titres were expressed as the reciprocals of the dilution showing 50% haemagglutination inhibition with 3 haemagglutination units of the antigen. From the results of the determinations per serum and per antigen, the geometric means were used for further calculations. Negative titres (<9) were arbitrarily regarded as 5. With the method used, protection against infection is assumed to be associated with a HI-titre of ≥100 for influenza A strains (18,19). For ether-treated influenza B strains, no such protection threshold is known. As was done earlier (19), for this study, a titre threshold of 200 was chosen.

5. Clinical investigations

Each participant received a standard symptom questionnaire for the evaluation of reactogenicity of the vaccines during the first 48 hours after vaccination. Symptoms were divided into local reactions such as: redness, swelling, itching or pain on the site

of injection and systemic reactions such as: fever, headache, sweating or malaise. In the elderly group, the same assessments were made by the nursing-home physicians rather than by the subjects themselves.

6. Statistical analysis

As stated in Section 4 above, the HI-antibody titre per subject, time point (pre-/post-immunization) and antigen was calculated as the geometric mean of the two corresponding laboratory determinations. These titre values were used to derive three further variables for statistical analysis; 1) the geometric mean titre (GMT), representing the geometric means of the individual titre values; 2) the proportion of subjects with HI-antibody titres above the assumed protective level ("% protective titre"); 3) the geometric mean fold increase (MFI) and 4) the proportion of subjects with a ≥4-fold titre increase (% responders).

The titre values and mean-fold increases were subjected to a logarithmic transformation and analysed using analysis of variance and covariance (PROC GLM in SAS (20). The "% protective titre" and % responders were subjected to a logit transformation and analysed using logistic regression (PROC LOGISTIC in SAS).

For the analysis of the vaccinated groups, the transformed variables were related by an additive, linear model to the factors age group, centre within age group, dose, interaction between dose and age group, and interaction between dose and centre within age group. In addition, the analyses of post-immunization titre and "% protective titre" included pre-immunization titre as a covariate, since it is recognized that pre-immunization titre has an influence on post-immunization values (21). Because mean-fold increase and % responders "correct" for pre-immunization titre, no covariate was included in the analyses of these variables.

The analyses performed provide for tests of statistical significance of the contribution of the factors listed above to the model describing the response variable. They also provide coefficients so that the value of the response variable can be predicted from an equation including those factors determined as significant.

The analysis of the placebo group followed the methods outlined above, but included only age group and centre (and pre-immunization titre where appropriate) in the model.

In the description of results, the following convention is used. Borderline statistically significant indicates a p-value between 0.10 and 0.05; statistically significant indicates a p-value between 0.05 and 0.01; and highly statistically significant indicates a p-value less than 0.01.

Since the GMT and "% protective titre" are considered clinically the most meaningful markers for efficacy, and the analysis of the other variables showed qualitatively similar results as the GMT and "% protective titre", only these latter parameters are discussed in this paper. Other parameters, such as "protection-rate", response-rate and seroconversion rate, as defined by Beyer et al. (11), were calculated and analysed, but again qualitatively similar results were obtained and are therefore not presented in this paper.

Since increased antibody titres would result in an extended period of effective protection against influenza infections (22,23), we have also analysed the frequency distribution of subjects within discrete post-immunization titre intervals for all antigens. This analysis did not reveal additional relevant dose effects and is therefore also not presented in this paper.

RESULTS

1. Pre- and post-immunization antibody status of placebo treated groups

Table 1 presents the pre- and post-immunization GMT and "% protective titre" of the placebo-treated groups in the four study centres for the three vaccine antigens. Although some highly statistically significant study centre differences within each age category are apparent, there was virtually no antibody titre rise in each of the placebo groups, so that no placebo response corrections were needed in the analysis of the actively immunized groups.

TABLE 1 Pre- and post-immunization antibody status of placebo treated subjects

Group		Number	GMT pre/post vaccination			"%-protective titre" pre/post vaccination		
			B/B	A/T	A/S	B/B	A/T	A/S
Young	Centre 1	34	33/29	9/9	83/91	9/6	3/3	41/44
	Centre 2	36	23/25	6/7	47/48	6/8	3/3	25/31
	Total	70	27/27	8/8	62/66	7/7	3/3	33/37
Elderly	Centre 1	30	22/22	5/6	71/76	7/13	0/0	43/40
	Centre 2	35	85/82	11/11	95/87	23/29	6/3	57/63
	Total	65	45/45	8/8	100/100	15/22	3/2	51/52

2. Pre-vaccination antibody status

Table 2 presents pre-vaccination GMT and "% protective titre" for the four centres and the three vaccine-antigens for the actively immunized subjects.
The average pre-vaccination GMT against the B/B antigen was approximately 30 for both age-groups. However, statistically significant study centre differences within each age group were revealed. Within the young age group, the participants of centre 1 (students Rotterdam) had a highly statistically significant higher pre-vaccination GMT than those of centre 2 (students Paris). The nursing-home residents in Tel Aviv (centre 4) had a statistically significantly higher pre-vaccination GMT than their Dutch counterparts (centre 3). These centre differences were not reflected by the "% protective titre" values which were 10% on average.

TABLE 2: Pre-vaccination antibody status, according to age and study centre.

Group	Number	Pre-vaccination GMT			Pre-vaccination "% protective titre"		
		B/B	A/T	A/S	B/B	A/T	A/S
Young Centre 1	105	41	9	70	8	3	35
Centre 2	107	22	8	52	10	4	32
Young total	212	30	8	61	9	3	33
Elderly Centre 3	92	24	6	65	10	0	37
Centre 4	105	38	9	99	12	7	49
Elderly total	197	31	8	82	11	4	43
All participants	409	30	8	70	10	3	38

Antibodies against the A/T strain were low on average (post-vaccination GMT = 8, "% protective titre" = 3%). The study centre differences for this antigen are considered irrelevant.
The proportion of subjects with protective antibody titres against the A/S strain was much greater than against the other two antigens. Both, age and centre differences were obvious. On average, the nursing-home residents had a significantly higher pre-immunization GMT (82) than the students (61). Also the pre-immunization GMT centre differences within study populations were highly statistically significant (GMT young: 70, centre 1; 52, centre 2; GMT nursing-home residents: 65, centre 3; 99, centre 4).

The generally higher antibody level of centre 4 could not be explained by the pre-immunization titres of the 58 previously vaccinated subjects in this study centre.

When comparing the dose groups with regard to their pre-vaccination antibody status, no relevant differences could be seen (see Table 3, columns "pre-GMT" and "pre-"% protective titre"").

3. Antibody-response after active immunization in both age groups

Table 3 presents the HI-antibody response expressed by post-vaccination GMT and "%-protective titre", after active immunization with 10, 20 or 60 µg HA per vaccine antigen, for both age groups.

For the B/Beijing antigen, a highly significant dose effect on the post-vaccination GMT could be demonstrated for both age groups. After correction for pre-vaccination titres, there was a GMT increase of 1.4 fold when increasing the dose from 10 µg to 20 µg, and of 2.1 fold when increasing the dose from 10 µg to 60 µg, in both age

groups. The positive dose-response relation was also expressed by the corrected "%-protective titre" parameter which rose 9% (young) or 15% (nursing-home residents) when increasing the dose from 10 μg to 20 μg. The additional gain when increasing the dose from 20 μg to 60 μg (4% and 8%, respectively), was small. In all dose groups, the young subjects showed a better response than the nursing-home residents. However, this highly statistically significant difference in response between the two populations, was due to the higher values in all dose groups of centre 1 (Rotterdam) compared to centre 2 (Paris; GMT 1.8-fold; "%-protective titre" 14%-28%). The response in centre 2 (young) and centres 3 and 4 (nursing-home residents) did not differ significantly.

TABLE 3: Antibody response to vaccination, according to age and dose.

Dose group (μg HA)	Young							Elderly						
	Number	pre-GMT	post-GMT		pre-%-prot	post-%-prot		Number	pre-GMT	post-GMT		pre-%-prot	post-%-prot	
			act.	pred.		act.	pred.			act.	pred.		act.	pred.
B/Beijing														
10	70	31	376	362	7	73	78	67	28	223	237	10	52	55
20	70	30	516	512	11	81	87	64	36	349	335	14	67	70
60	72	29	724	765	8	83	91	66	30	513	500	9	77	78
A/Taiwan														
10	70	8	121	}	1	59	}	67	7	40	}	1	33	}
20	70	10	150	}154	7	64	}69	64	7	40	}53	2	33	}41
60	72	8	153	}	1	68	}	66	10	61	}	8	42	}
A/Sichuan														
10	70	67	233	217	37	81	}	67	76	188	197	42	72	}
20	70	58	180	183	34	70	}81	64	85	267	256	44	75	}83
60	72	57	304	316	39	82	}	66	84	315	306	44	80	}

Pre-vaccination titre values used in predictions: B/Beijing : 30; A/Taiwan : 10; A/Sichuan 60 (young), 80 (elderly) .

In contrast to the influenza B strain, the A/Taiwan antigen induced a poor antibody response, particularly in the nursing-home residents and did not show a dose effect in either age group. 1.3-1.5-fold greater post-vaccination GMT values and 9% greater "%-protective titre" values were observed in the 60 μg groups compared to the 10 μg groups. For the predicted values the dose effect was even less.
The young age group responded significantly better in all dose groups than the nursing-home residents: 2.9-fold greater post-vaccination GMT values and 28% greater post-immunization "%-protective titre" values.

In the nursing-home residents, there was a statistically significant effect of dose on the antibody response against the A/Sichuan antigen. After correction for pre-immunization titres, there was a 1.3-fold GMT increase when increasing the dose from

10 μg to 20 μg and a 1.6-fold increase when increasing the dose from 10 μg to 60 μg. The dose effect in post-immunization GMT was not reflected in the post-"%-protective titre" parameter, for which the predicted values were 83 for all dose groups. However, statistically significant centre differences between the nursing-home resident populations were found (Table 4).
Surprisingly, in the young age group, the 20 μg dose group had a lower response (predicted GMT, 183) than the 10 μg dose group (predicted GMT, 217). As the 60 μg dose induced a higher antibody response (predicted GMT, 316) than the 10 μg, a U-shaped dose-response curve with a 'minimum' at 34-40 μg was calculated. This phenomenon could not be explained by differences between centres as it was seen in both study centres (Rotterdam and Paris, Table 4).

4. Centre differences in the antibody-response to vaccination

In Table 3, the antibody response after active vaccination was shown for the total of both study populations, but not for the four study centres. As indicated, however, there were marked and even highly statistically significant differences between centres 1 and 2 of the young population, as well as between centres 3 and 4 of the nursing-home residents. Despite the observed centre differences for the young for the B/Beijing antigen, the dose response curves for both centres were similar. Hence, the centre differences did not affect the overall conclusion of a positive dose-response relationship.

Table 4: Differences in antibody response to vaccination between study centres, for A/Sichuan.

Group		10 μg HA			20 μg HA			60 μg HA		
		number	postGMT	post%	number	postGMT	post%	number	postGMT	post%
Young	Centre 1	35	280	89	35	217	74	35	394	86
	Centre 2	35	195	74	35	149	66	37	238	78
Elderly	Centre 3	31	146	61	30	285	67	31	333	81
	Centre 4	36	234	81	34	251	82	35	300	80

In contrast, the dose response curves in both nursing-home study centres for the A/Sichuan antigen were not similar. Table 4 represents the differences in antibody response between study centres for this antigen. The GMT increase associated with a dose increase from 10 μg to 20 μg was 2.0 (centre 3) and 1.1 (centre 4). A dose increase from 20 μg to 60 μg resulted in a 1.2-fold increase in post-immunization GMT for both study centres. Table 4 also shows that the statistically significant increase in GMT (centre 3) at dose 20 μg compared to 10 μg is not associated with a significant increase in "%-protective titre" (61% and 67%, respectively). By increasing the dose to 60 μg, the "%-protective titre" was increased to 81%, a value which was found for all dose groups in the Tel Aviv study centre.

5. Adverse reactions

Table 5 represents the reported local and systemic adverse reactions following immunization in all dose groups. A clear dose-response relationship in the young is demonstrated for the local reactions (predominantly "pain on contact") but not for the systemic reactions. In all dose groups, only 11%-16% (placebo) of subjects who reported adverse reactions considered these moderate/severe inconvenient. The high incidence of reported reactions may be somewhat exaggerated, since medical students are "trained" to observe very accurately (20% local reactions after placebo).
In striking contrast to the young, in the nursing-home residents no dose-relationship was found for any reactions. Since the assessments were made by the investigators, the low incidence of adverse reactions may be due to underreporting. Thus, although the figures presented may be either over- or underestimates, the results clearly provide evidence of the tolerance and safety of high doses of the subunit vaccine (total 180 μg HA) in elderly, nursing-home residents.

Table 5: Reported adverse effects to immunization, according to age and dose.

Dose group (μg HA)	Young			Elderly		
	Number	Local	Syst.	Number	Local	Syst.
0	70	20%	11%	65	3%	1%
10	70	44%	27%	67	1%	0%
20	70	51%	17%	64	5%	2%
60	72	79%	18%	66	6%	0%

DISCUSSION

The current study was performed to investigate whether a relevant increased antibody response in elderly, nursing-home residents can be induced by using increased vaccine dosages, so that this important high-risk population can be more effectively protected by immunization against influenza infections (7-9).
Our data indicate that the issue cannot be addressed for the trivalent vaccine as a whole, but rather should be addressed for each of the vaccine antigens separately. Moreover, the pronounced study centre differences within both populations as found in this large and relatively standardized study, underscore the intrinsic variability of serological influenza vaccination studies, which warrant caution with the interpretation and extrapolation of individual study results. As has been shown previously from a literature review (11), this variability may often preclude inter-study comparisons of different vaccination studies.

The most consistent dose effect in this study was shown for the B/Beijing antigen (Table 3). By increasing the vaccine dose from 10 to 20 and 60 μg HA, the GMT increased approximately 1.4 and 2.1 times, respectively, in both populations.
In contrast to the B/Beijing antigen, we found a lack of a dose effect on the antibody response against the A/Taiwan antigen for both study populations. Increasing the vaccine dose up to 60 μg HA did not improve the antibody response. Although twice as many of the young controls reached assumed protective titre levels compared to the nursing-home residents, the flatness of the dose-response curve was very similar for both populations. Hence, the low immune response for the A/Taiwan (H1N1) antigen could not be improved by the use of a higher vaccine dose, as was hypothesized by Gross et al. (12). The low antibody response for this antigen has also been found by Iorio et al. (24) and Gross et al. (12).

No firm conclusion on the effect of vaccine dose on the antibody response against the A/Sichuan antigen in nursing-home residents can be drawn from this study, due to statistically significant study centre differences and inconsistent findings. In both study centres of the young volunteers, we found a U-shaped dose-response curve, for which we could not find an explanation. The difference in response following immunizations with 10 and 60 μg HA vaccine doses is not considered relevant.

Comparing the antibody response between the two study populations, this study shows a consistent statistically significant difference for the A/Taiwan, but not for the A/Sichuan and B/Beijing antigens. The better response in the young for the B/Beijing antigen was attributed to the higher response values in one of the two young study centres (centre 1). Such strain differences in the effect of age and/or disease on the immune response may also have contributed to the heterogeneous picture which emerged from a literature survey on this subject (11).

Although various dose-response influenza vaccination trials have been published (25-37) and have shown relatively flat dose-response curves, only a few of those studies have been done in ambulatory elderly and nursing-home residents, which have shown inconsistent data (13-15). Gross et al. (13), who studied the immune response of vaccine doses between 15-45 μg HA in ambulatory elderly subjects, did not find increasing antibody responses with increasing doses.
Similar findings were reported by Peters et al. (14), who compared the immune response of 15 and 60 μg HA of the B/USSR/100/83 antigen in non-institutionalized elderly. In contrast, Arden et al. (15), who also investigated the immune response following immunization with 15 and 60 μg HA of the B/USSR/100/83 antigen, reported an improved antibody response in nursing-home residents. The differences between the studies by Peters et al. (14) and Arden et al. (15) are of interest, since both studied the same doses of the same antigen. Therefore, it is tempting to speculate that the differences in study results are due to differences in study populations, one being ambulatory elderly and the other nursing-home residents. If this were true, our data are in general agreement with Arden et al. (15), in that the seroresponse in nursing-home residents can be increased by increasing the dose for the B antigen in the vaccine.
Our data suggest, however, that a major improvement can be achieved by increasing the dose to 20 μg HA and that only marginal further improvements can be achieved by a further dose increase, a finding which cannot be compared to Arden et al., since they did not study doses between 15 and 60 μg HA. In contrast to Arden et al., who found a relatively rapid decline of the improved titre levels, we have no information regarding the duration of the improved immune response for the B/Beijing antigen, since no late serum samples were obtained in our study.

For both influenza A antigens, our data do not yield compelling evidence to justify the expectation of improved vaccine efficacy for nursing-home residents, if the vaccine dose would be increased up to 60 μg HA, although our data were inconsistent for the A/Sichuan antigen.

Although a total dose of 180 ugHA subunit vaccine was generally well tolerated by the young and elderly vaccinees in this study, a finding consistent with other reports (13-15;38) and increased vaccine doses may yield some better immune responses in certain cases, the overall conclusion from our study is that the problem of the relatively low vaccine efficacy in nursing-home residents (7-9) to protect against influenza infections cannot be adequately solved by increasing the vaccine dose, even up to levels of 60 μg HA/strain. Therefore, the current search for new promising alternative influenza vaccines (39,40), such as adjuvants (41,42), liposomes (43,44), ISCOMS (45) and CTB-conjugated vaccines (46) should be continued. Despite the need for more protective influenza vaccines for nursing-home residents, doubts concerning the capacity of the currently available vaccines to reduce influenza associated morbidity

and mortality are unjustified (3-6) . There is evidence, that much is to be gained if the existing vaccines are more widely used in nursing-home residents (47) as recommended by some public health authorities (48).

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Study protocol for influenza live-virus challenge study in young adults

1. General Introduction

In Chapter 4, we have argued that the increase in PL90 (90% protective titre value) should be considered as the clinically relevant surrogate marker for the assessment of influenza vaccine efficacy from serological studies. This has been based on the fact, that a quantitative correlation between protection against infections and homologous HI-antibody titres has been experimentally demonstrated (1-6).

At the same time, however, it was noted, that other immunological parameters have been shown to contribute to the protection against infections (local IgA antibody titres; cross-reactive antibodies) or to recover from infections (cytotoxic T-cell activity). However, no quantitative correlation between these parameters and their clinical effects in man has been established (7) so that these parameters cannot be used as surrogate markers of efficacy for the quantitative assessment of influenza vaccine efficacy. Still, many influenza scientists feel, that vaccines inducing the whole range of immunological responses are to be considered superior to the current, intramuscularly administered, inactivated influenza vaccines (7-9). These theoretical considerations have been the basis for the development of live influenza vaccines (10-18).

As noted by Clements et al. (15), "new candidate influenza vaccine must be compared with conventional inactivated vaccine to determine whether the former offers advantages over the latter". The vast amount of clinical studies, including comparative trials with the promising live influenza vaccines, have failed so far to show convincingly the clinical superiority of these theoretically advantageous vaccines. From a recent serological vaccine comparative study, Powers et al. (19) concluded that in elderly persons, live attenuated influenza A virus vaccines do not offer an advantage over inactivated virus vaccine in inducing serum or secretory antibodies or local immunologic memory. In addition, Clover et al. (20) found that trivalent inactivated vaccine provided statistically significant better heterotypic protection than did attenuated cold recombinant (CR) vaccine for children aged 10-18 years (infection rate 0 vs 24%), whereas in the younger children (3-9 years), there was no significant difference in between the CR and inactivated vaccine (19% vs 26%).

The history of influenza live-virus vaccine development since 1943 (8) clearly demonstrates the intrisic problems of the development of alternative influenza vaccines. It clearly shows, that immunological and theoretical advantages cannot easily be extrapolated to prove clinical advantages. Or, as Ghendon (7) has summarized the state of the art for 15 years of modern influenza live-vaccine development "it is possible to say that the inference that live intranasal vaccination may provide better and more durable protection than inactivated intramuscular vaccination remains unproven, and the factors responsible for protection should be defined more clearly".

Although intensive research on live-virus influenza vaccines has not (yet) resulted in a live-virus vaccine for routine immunizations in western Europe and the USA, it has yielded a vast experience in artificial virus challenge experiments. Such studies are in principle suited for the validation and quantitation of immunological surrogate markers for influenza vaccine efficacy in man; whether prevention of infections or reduction of morbidity. As illustrated by the live-vaccine development history, these quantitative correlations should be established before definite conclusions on the relative clinical efficacy of different vaccine types or vaccine doses can be drawn based on these immunological markers.

Artificial challenge studies are also suited to assess the effect of different vaccination regimens for inactivated vaccines. For example, in a long-term influenza vaccination trial in young healthy adults, we found lower post-vaccination HI-antibody titres in multivaccinated subjects than in not previously vaccinated subjects (de Jonge S, Palache AM, Vardy A; Duphar report 56638/7M/88). Similar results were found by us in two studies in ambulatory elderly subjects and nursing home residents (de Jonge S, Palache AM, Vardy A; Duphar report 56638/8M/88,1988; de Jonge S, Palache AM, Vardy A; Duphar report 56638/21M/90,1990; Sprenger MJW, Masurel N; long-term influenza vaccination study in nursing-home residents; data not published). Although the phenomenon of a lower HI-antibody titre after repeated immunizations has been known for 15 years (21), conclusive answers to the potential clinical relevance of these findings are not readily available. At the FDA meeting (21), there seemed to be a consensus that vaccine efficacy is not reduced by repeated immunizations.

The current chapter describes the protocol for an influenza virus challenge study which was designed to investigate the protective efficacy of repeated influenza vaccinations in healthy young subjects. The presented protocol is based on a study protocol which has been approved by the Ethics Committee of the Erasmus University, Rotteram, The Netherlands (Beyer WEP, Palache AM; Challenge proef met een levend, gedeeltelijk verzwakt influenza A-H1N1 virus bij frequent eerder en eenmalig tegen influenza A-H1N1 virussen gevaccineerde vrijwilligers, 1989; Palache AM, de Jonge S, Tukker JJ, Sprenger MJW, Masurel N; Influenza challenge study: The effect of multiple influenza immunizations on the protective immunity against a live-virus challenge. A prospectively randomized, double-blind, placebo controlled study in healthy adult volunteers, 1989). The study design is based on a challenge study reported by Sears et al. (22).

Based on a power calculation, we had decided that the study should only be done if at least 44 volunteers in the previously vaccinated group would give informed consent. Unfortunately, this target number could not be reached, so that the study was cancelled. Still, because the study protocol describes in detail both, a clinical assessment scale to quantitatively assess influenza morbidity and a prospective power cal-

culation and demonstrates the importance of the collaborative nature of such challenge studies, we felt it appropriate to present the protocol as an example in this thesis.

STUDY PROTOCOL

INFLUENZA CHALLENGE STUDY

The effect of multiple influenza immunizations on the protective immunity against a live virus challenge.
A prospectively randomized, double-blind, placebo-controlled study in healthy adult volunteers

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Date: November 1989 / March 1990

1. INTRODUCTION

Immunization is the main preventive measure against influenza virus infections. Due to the ever occurring antigenic drift of the influenza viruses, the influenza vaccines, unlike other viral vaccines, must be yearly modified to reflect these antigenic changes and are recommended for annual use by national health authorities of the Western world for high-risk patients (23). The underlying hypothesis for the yearly immunization practice is, that vaccinations will boost adequate antibody responses to the appropriate virus antigens and therefore will protect the immunized individuals against an infection with these viruses. Although other factors than the HA antibody titres have been shown to play a role in protective immunity against influenza infections (24-26), a quantitative correlation has only been shown for serum IgG antibody titres (1-6).

In a series of recent experiments (de Jonge S, Palache AM, Vardy A; Duphar report 56638/7M/88; de Jonge S, Palache AM, Vardy A; Duphar report 56638/8M/88,1988; not published), we studied the effect of repeated influenza vaccinations on the serological response in groups of young volunteers and elderly ambulatory patients.
In both age-categories, we found significantly lower mean post-vaccination HA-antibody titres (GMT) in previously vaccinated subjects, compared to not previously vaccinated subjects, for the H1N1 and B antigens.

Our data are in agreement with serological studies reported by Powers et al. (27), Brandriss et al. (28), Zajac et al. (29) and Ershler et al. (30). In haemodialysis patients, Beyer et al. (31) made similar observations.

Although all these reports point to the same phenomenon, quantitative comparisons between these studies are difficult to make due to the variability in study- and population characteristics and the small treatment group sizes in many of these studies (see Chapters 2 and 3).

It is noteworthy that a negative influence on the immune response to neuraminidase antigens (NA) after prior experience with viral haemagglutinin antigens (HA) in both mice and men have been reported by Kilbourne et al. (32) and Johansson et al. (33).

Three studies have been published addressing the issue of the effect of repeated vaccinations on vaccine efficacy. A study in school boys by Hoskins et al. (34) showed that:

a) the protective effect of inactivated influenza A vaccine was limited to those boys who were vaccinated for the first time with the most up-to-date vaccine strains;

b) revaccination with the same strain did not increase the degree of protection;

c) revaccination with the same strain did not afford protection against subsequent challenge.

Feery et al. (35) also found that a first vaccination did protect, but that a subsequent vaccination did not yield significant protection against influenza.

In contrast to these findings, Keitel et al. (36) reported a somewhat greater efficacy after repeated annual vaccination than after first administration, although vaccine efficacy was only modest in their study. Vaccination induced higher frequencies of antibody titre rise to vaccine components in firstly vaccinated- than in multivaccinated groups, but the mean post-vaccination titres were similar for all strains.

In a ferret model, Potter et al. (37) have tried to reconstruct the "Hoskins phenomenon". They found similar post-vaccination HI-titres against a H3N2 antigen after single or repeated vaccinations and no increased protection from repeated vaccinations in subsequent live-virus challenge experiments.
In addition, virus infections resulted in lower post-vaccination HA antibody titres than immunizations, but were clearly more protective in the challenge experiments. This latter finding is in agreement with a study by Davies and Grilli (38), who conclude that the circumstances of the induction of the antibody affect its value as a predictor of immunity.

From our own and the quoted serological data, we conclude that previous vaccinations against influenza may have an age-independent suppressive effect on the homologous HI-antibody titre against subsequent antigens. The literature does not give a clear-cut answer whether or not this phenomenon is of clinical relevance.

The current study is designed to test two possible hypotheses. First, vaccine efficacy declines after repeated vaccinations due to reduced antibody titres or, alternatively, immunological parameters other than HI-antibody titres "overrule" the declined antibody response, so that vaccine efficacy is stable or even increased, rather than declined after repeated vaccinations.

2. AIMS OF THE STUDY

1. To compare the influenza attack-rates in multiple vaccinated and first vaccinated healthy volunteers.

2. To compare the duration and severity of clinical influenza-like symptoms in multiple vaccinated and first vaccinated healthy volunteers.

3. To study the correlation between HA-antibody titre and influenza attack-rate.

4. To study the correlation between HA-antibody titre, duration and severity of influenza-like symptoms.

5. To generate a hypothesis for the underlying mechanism on the suppressive effect of repeated immunizations on the antibody formation.

6. To assess the correlation between pre-challenge specificity (and/or effector function) of T cells, and the post-challenge attack-rate of influenza illness.

3. STUDY POPULATION

The population for the challenge study will consist of 120 healthy adult volunteers. These subjects will be divided in three groups:

1. The subjects from study H.201.5017 (de Jonge S, Palache AM, Vardy A; Duphar report 56638/7M/88, not published), who have been vaccinated in the past two or three years. The aim is to include 50 subjects in this 'multivac' group. (See section 11.2.)

2. Subjects who have never been vaccinated against influenza in the past and who will receive an active vaccination in this study ('novac' group).

3. Subjects who have never been vaccinated against influenza in the past and who will receive a placebo vaccination in this study ('placebo' group).

All volunteers will receive detailed information during an information meeting about the study procedures, the product Influvac®, and the challenge. Also the fact that their blood will be screened for HIV-antibodies will be mentioned. Subjects who do not want to know the results of this test cannot participate. Written information about the study details will be given during this informative meeting. The written informed consent form will be signed during the vaccination session, one week later.

Volunteers known to be allergic to chicken proteins will be excluded from the study. All volunteers will have a medical examination prior to the start of the challenge. This will consist of routine haematology and biochemistry and a physical examination (see Appendix I). Subjects with chronic disorders, in particular lung, metabolic and circulation disorders will be excluded.

Before the start of the study, two types of serological tests
will be performed:

1) HIV-antibody test, to exclude HIV seropositive subjects. Since little is known yet about the course of an influenza infection in HIV seropositive subjects, it can not be excluded that an influenza infection could trigger the step to a next, less favourable stage of HIV infection.

2) Antibody test against vaccine and challenge virus. For methodological reasons, it is desirable that the subjects of the novac and placebo groups have negative or low titres against the challenge virus. Subjects with a titre > 50 will be excluded from the study. (See also section 8)

4. STUDY PROCEDURE

4.1 Information meeting

One week before the planned vaccination sessions, all potential volunteers will be informed on all relevant aspects of the study, including screening procedures, study procedures and the risk of experciencing an influenza-illness and the possible treaments for influenza-associated symptoms, including amantadine if considered necessary by the study physicians. By the end of the informative meeting, written information will be supplied to those, who in principle agree to participate. The signed information sheet, stating their formal agreement to participation will be collected just prior to vaccination.

4.2 Vaccination

On admission to the study, each participant will be given a code number and issued an individual record form on which all relevant data will be entered.

From all volunteers a venous blood sample (10 ml) will be collected just prior to the vaccination. Subsequently they will receive a 0.5 ml dose of the trivalent vaccine containing 10 µg HA/strain (or placebo) by intramuscular injection into the upper arm.

The administration of vaccine to the novac and placebo groups will be double-blind and prospectively randomized (section 8). Subjects of the novac and placebo groups with pre-vaccination HA-antibody titres ≤ 50 will be selected for further participation. Vaccination will not be given in the arm from which the blood sample was drawn. The reactions to the vaccination will be recorded for the periods 0-24 and 24-48 hours after vaccination by the volunteers in the case record form. The forms will be collected and checked at the second session, three weeks after vaccination.

Three weeks after vaccination a post-vaccination blood sample (10 ml) will be collected from all vaccinated subjects. The titres of the circulating haemagglutinin antibodies against at least the vaccine strains will be determined.

4.3 Pre-challenge medical examination

To exclude as much as possible the risk to include subjects who had a (sub)clinical influenza infection during the period between vaccination and challenge, blood samples will be drawn from all vaccinees at approximately 4-6 weeks intervals.

One to two weeks before the start of the challenge part of the study, the participants will have a medical examination (Appendix I).

Participants will be asked for an additional blood sample of 100 ml some time prior to the challenge (see table 1) for immunological tests (see section 10.3). Participation in the study will not depend on their willingness to have this blood sample taken. A separate consent form will be signed by volunteers who agree to have the immunological tests done.

Table 1 - Schematic flow of procedures

Date	Activity	Assessment
7/8 Nov.	- Information to candidate volunteers	
15/16 Nov.	- informed consent - blood sampling (10ml) - vaccination	HA-antibody titre (pre-vaccination)
6/7 Dec.	- blood sampling (10ml)	HA-antibody titre (post-vaccination)
8/9 Jan.	- blood sampling (30 ml)	HA-antibody titre (natural infection?) Biochemistry/ Haematology HIV-antibodies
	- check inclusion criteria - informed consent - blood sampling (100ml)	Immunology
(22/28 January: pilot-studies to select challenge virus strain).		
12/16 Feb.	- Medical examination - blood sampling (10ml) (Pre-challenge;Natural infection?)	HA-antibody titre
24 Feb.	- Challenge	
3/10 Mar.	- blood sampling (120ml) - Discharge - Payment (f 50,--)	Immunological tests
20 Mar	- blood sampling (10ml) - Payment (f 1250,--)	HA-antibody titre (Post-challenge)

4.4 Challenge study

The challenge study will take place in a hotel, in which no other guests will be present during the first week. The second week there is an option on one floor of the hotel, in case some subjects should stay longer under medical supervision. So it is guaranteed that during the trial the subjects cannot be in contact with other people outside. All medical personnel, personnel working in the hotel and other people present during the study will be vaccinated 6 weeks prior to the study, and their antibody level will be checked prior to the challenge period.

During the first week after the challenge, two physicians will be present in the hotel, 24 hours per day for medical supervision, evaluation of symptoms and to take the necessary samples. These physicians will be independent from the sponsor and the investigators. Also three nurses will be present. During the second week one physician and two nurses will be available. Symptomatic treatment will be given if deemed necessary by the study physician(s). Amantadine will be available for treatment. The medical staff will not be informed on the vaccination history of participating subjects. The nearest hospital will be contacted in advance, so that in emergency situations an adequate treatment can be quickly arranged.
After one week, subjects, who fulfill the criteria for discharge as mentioned in section 10 and appendix 2, are allowed to return home.
Before leaving the hotel, 120 ml blood will be collected for immunological testing (see section 10.3) from those volunteers who gave informed consent for these blood samples.

5. VACCINE TO BE USED

For this study trivalent subunit vaccine will be used.
The batchnumber of the subunit vaccine is C 1901-A.
Also an identical placebo (saline) will be used.
The batchnumber of the placebo is HV 061-089.
The subunit vaccine will be made available in 5 ml ampoules.

The subunit trivalent vaccine will contain per 0.5 ml:

10 µg HA each of:
A/Shanghai/11/87 (H3N2)
A/Taiwan/1/86 (H1N1)
B/Yamagata/16/88

The batch will be controlled for safety and potency by the Quality Assurance Human Vaccine Department of Duphar B.V.

The placebo will consist of saline and will be made available in 5 ml ampoules.

As stated on the package insert of Influvac®, neurological disorders such as encephalo-myelitis and neuritis after influenza vaccination have rarely been reported. An association has not been demonstrated except in the case of the Guillain-Barré Syndrome in the USA mass-vaccination programme of 1976 (A/New Jersey swine vaccine).

6. CHALLENGE VIRUS TO BE USED

Based on the results referred to in the introduction of this protocol and the fact that amantadine is available for effective treatment against influenza A infections, the challenge strain to be used will be of the H1N1 type. A recent isolate, similar to the A/Taiwan/1/86 strain will be selected from one of the following:

1. A virus isolated in SPF eggs at the laboratory of Dr D A J Tyrrell, Common Cold Unit, Salisbury, UK from a clinical specimen provided by Dr P Chakraverty, Central Public Health Laboratory, Colindale, UK. Virus isolation will be done by Prof J S Oxford, The London Hospital Medical College, London, UK. Characterisation of virus will be done by Dr J Wood, NIBSC, London, UK.

2. A virus A/Auckland/3/86 (H1N1) isolated on SPF eggs by Dr Maurie Evered, Commonwealth Serum Laboratories, Victoria, Australia. A virus pool will be produced in SPF eggs by Prof J S Oxford at the Common Cold Unit, Salisbury. Characterisation of virus will be done by Dr J Wood, NIBSC, London, UK.

3. A virus A/Kawasaki/9/86 (H1N1) isolated on chick kidney cells and further passaged five times on chick kidney cells and twice in SPF eggs by Prof H Maassab, University of Michigan, USA. A virus pool will be produced in SPF eggs by Prof J S Oxford at the Common Cold Unit, Salisbury.

4. A virus A/Kawasaki/9/86 (H1N1) isolated on chick kidney cells and further passaged five times on chick kidney cells and once on SPF eggs by Prof H Maassab, University of Michigan, USA. A virus pool has been produced by Dr Louis Potash, Flow Laboratories, McLean, USA.

Safety tests according to WHO guidelines for live Influenza Vaccines (WHO Technical Report Series, 1979, 638) will be carried out for virus preparations (1), (2) and (3) by Prof J S Oxford at the Common Cold Unit, Salisbury, UK. Safety tests on virus (4) have been carried out according to FDA requirements by Dr Louis Potash, Flow Laboratories. Subject to availability and suitability, two potential challenge viruses will be tested for infectivity and virulence in two pilot volunteer studies (Palache AM, de Jonge S, Noury J; Protocol H.201.5031-A for pilot influenza challenge study).

First pilot study: Each virus used to infect five young healthy volunteers with serum HA antibody titres above 100 to A/Taiwan/1/86.

Second pilot study: Each virus used to infect 10 young healthy volunteers with serum HA antibody titres lower than 50 to A/Taiwan/1/86.

A suitable virus will be selected for the challenge study after completion of the pilot studies.

7. MODE OF ADMINISTRATION

The vaccine will be administered by intramuscular injection in the upper arm. Efforts will be made to ensure that one single person vaccinates all volunteers. The challenge will be administered intranasally via a dropping system, the subjects being in a supine position. Each challenge dose will consist of aproximately 10^6 $TCID_{50}$ units.

8. RANDOMIZATION

There will be three different study groups.
The group of volunteers, who have been vaccinated more than twice prior to this study (multivac group; n=50); a group of 35 volunteers who has not been vaccinated against influenza before this study and with pre-vaccination HA-antibody titres ≤ 50 and who will be vaccinated in november 1989 (novac group) and a group of 35 volunteers, who has not been vaccinated before this study and with pre-vaccination HA-antibody titres ≤ 50 and who will be vaccinated with placebo (placebo group).

All subjects of the multivac group will receive active vaccine.
For vaccine or placebo administration to the novac and placebo groups, a prospective randomization scheme in blocks of 6 subjects will be made.

9. SPECIMENS TO BE COLLECTED

Serum samples for determination of antibody titres will be collected at the following times (see table 1 section 4.3):

- Immediately prior to vaccination.
- Three weeks after vaccination.
- Eight weeks after vaccination.
- $\pm$ 10 days prior to start challenge.
- 3 weeks after the challenge.

Sera will be separated as soon as possible after blood collection and these will be kept frozen (-20°C) until titration.

For the immunological tests (see section 10.3), 100 ml blood will be collected 8 weeks prior to the challenge and 120 ml blood will be drawn before discharge from the hotel.

Nasal and throat swabs will be collected once daily after the challenge. From day 5 onwards, two swabs will be taken simultaneously, one of each will be used to determine whether the subjects haved stopped to shed virus.

10. ASSESSMENTS

10.1 Clinical assessments

Clinical assessment of Influenza-illness.

A volunteer will be considered ill if symptoms or physical findings consistent with influenza will be developed within five days after inoculation of the challenge virus.

Illness will be categorized into four classes of symptomatology; febrile illness, systemic illness, upper-respiratory-tract illness and lower-respiratory-tract illness (22). Each of the illness categories are defined as indicated below.

Main symptoms of influenza illness categories (22).

Illness category	Symptoms
Febrile illness	Rectal temperature > 37.7°C
Systemic illness	Myalgia
	Chills
	Sweating
Upper respiratory-tract	Rhinorrhea
	Pharyngitis
Lower respiratory-tract	Cough

Two times a day (8.00 hr, 20.00 hr), a clinical illness assessment form will be completed for each individual. (See appendix II, table 1.) These forms will be completed prior to challenge inoculation up to 10 days after. Based on these completed clinical illness

recording sheets, a scoring system will be used to specify illness in terms of illness categories, duration and severity.
The scoring system to evaluate the occurrence and severity of influenza is described in detail in appendix II.

10.2 Virological assessments

Pre- and post-vaccination blood samples will be analysed simultaneously for antibodies against each of the vaccine-antigens by a routine HI-test (39).

Blood samples taken as control for natural influenza infections, will be analysed only for antibodies against the challenge antigen and the possibly identified local epidemic influenza strain.

The pre- and post challenge blood samples will be analysed simultaneously for antibodies against the challenge antigen and the possibly identified local epidemic influenza strain.
Each day, nasal and throat swabs will be collected. All swabs will be kept at - 70°C and cultured simultaneously for viral analysis.
From day 5 onwards, two swabs will be taken simultaneously, one of each will be used to determine whether the subjects have stopped to shed virus.

10.3 Immunological assessments (in collaboration with Dr.C.Lucas)

McMichael et al. (40) have shown that the elimination-rate of challenge virus in mice is associated with the frequency of MHC-1 restricted T cells. However, no further characterisation on T-cell specificity has been reported by this group. Lucas et al. (unpublished data) have found, that the MHC-II restricted response following influenza infection in mice, is associated with a high frequency of haemagglutinin (H)- and neuraminidase (N)-specific T cells. A clear understanding on the complete inventory of influenza-specific T cells is currently lacking. It is not unlikely that the influenza-illness attack-rate is correlated with the pre-challenge existing nucleoprotein (NP)-H- or N-specific T cells.
In the current study, the frequency of NP-, H- and N-specific CD4 and CD8 cells will be measured in those individuals who consent to the donation of the extra blood samples required for these immunological tests. From each subject, 20 ml blood will be drawn for HLA-typing and 100 ml blood pre- and post challenge. (See table 1 section 4.3.)

11. METHODS OF ANALYSIS

11.1 Laboratory analysis

All serum HA-antibody titres will be determined at the National Influenza Centre, Department of Virology, Erasmus University Rotterdam, The Netherlands (Head: Prof. N. Masurel).
All serum samples will be kept at -20°C until titration. The routine haemagglutination-inhibition test (39) will be used.

Virus shedding will be determined from nasal and throat swabs. Specimens will be transported to Rotterdam and kept in a 50% saccharose solution at -70°C. Virus growth will be quantitatively determined in tertiary monkey kidney cells according to Moritz et al. (41).

11.2 Statistical considerations (see appendix III)

The objectives of this study (section 2) can be divided in three distinct categories:

a) Quantitative comparisons between treatment groups (aims 1 and 2).
b) Correlations between a number of variables and "protective immunity" (aims 3,4 and 6).
c) Scientific hypothesis generation (aim 5).

Group sizes for this study (50, 35, 35; see section 9) are selected for three reasons:

1) The hotel has a maximum capacity of 120 beds for volunteers.
2) Given the restriction of 120 beds, the selected group sizes guarantee an optimal statistical power for detecting treatment group differences.
3) It is anticipated, that it will be possible to recruit 50 of the 86 vaccinees in 1988 (multivac group) for the current study.

The restriction on the group sizes will affect most critically the statistical power for the quantitative group comparisons.

Assuming 80% attack-rate in the placebo-vaccinated group with HA-antibody titre ≤50 and a vaccine efficacy of 70% in firstly vaccinated young healthy volunteers (42, 43), a 57% reduction in vaccine efficacy for the multivac group will be detected with a 80% certainty (power) based on a two-sided test at the 5% significance level.

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Appendix 1
EXAMINATION BY AN INTERNAL PHYSICIAN

Case history and clinical examination:

- General
  Length, weight (recent loss of weight?), temperature (fever, slight rise in temperature?). Serious disorders in the past? Family abnormalities? Consumption of alcohol, nicotine, drugs? Use of medicines? Pregnancy? Previous vaccination against influenza? Chicken protein allergy?
- Circulation
  Pain in the chest (in rest, on exercise?), palpitations, oedema, nocturia, orthopnoea? ECG
- Respiration
  Dyspnoea, common cold, coughing, expectoration, haemoptysis?
- Digestion
  Eating habit, pyrosis, defaecation normal/deviating?
- Urogenital system
  Micturition, urine clear?
- Central nervous system an locomotory system
  Headache, vision, normal relfexes, muscle- or joint pains?
- Skin
  Deviations?

Laboratory:

- Sediment, Hb, Ht, erythrocytes, MCV, leukocytes + differentiation, thrombocytes
- Alkaline phosphatase, urea, SGOT, SGPT, post-prandial glucose
- Urine: protein, glucose, sediment (erythrocytes, leucocytes, granulocytes)
- HIV-antibodies (ELISA)
- Antibodies against influenza A challenge virus (HI)

Exclusion criteria:

1) Infections, auto-immune processes, malignancies, allergies
2) Myocardial infarct, serious circulation-, lung- or metabolism disorders
3) Malnutrition, alcoholism and drug abuse
4) Medicines with known influence on the immune system (anti inflammatory drugs, hormones, analgesics)
5) Deviating laboratory results indicating a state mentioned under 1-4
6) Anti HIV positive
7) Influenza antibody titre > 50 (only for novac and placebo groups)

Appendix 2

CLINICAL ASSESSMENT SCORING SYSTEM

For each illness category, the total daily score (total of two separate scores) will be calculated. All illness categories will be considered as equally important which necessitates to normalize the total daily score for each illness category.

Table 1: Clinical illness recording sheet to be completed two times a day.

		Date Time SCALE		
Temperature	<37,7	37,7-38,2	38,3-38,7	>38,8
	0	1	2	3
	none	mild	moderate	severe
Myalgia	0	1	2	3
Chills	0	1	2	3
Sweating	0	1	2	3
Rhinorrhea	0	1	2	3
Pharyngitis	0	1	2	3
	none	1-2[1)]	3-4	>4
Cough	0	1	2	3

1) Number of cough attacks (confirmed by nurse) Cough-attacks since last observation-time.

This will be achieved by multiplying the total daily score for the temperature with a factor 2, by dividing the total daily score for systemic illness by factor 1.5 and by multiplying the total daily score for lower respiratory illness with a factor 2. After applying these correction factors, the maximum daily score for each illness category will be 2 x 6 = 12, and the maximum total daily score for "total illness" will be 4 x 12 = 48 (table 2).

This latter score will be referred to as the daily illness index (DII). The DII will be used to categorize severity of illness. Table 3 shows the definitions of severity illness categories. The scoring system as described above and outlined in tables 1-3, will be used for both, treatment criteria and data analysis.

Appendix 2 (continued)

Table 2: Calculation of daily illness index devised from table 1 and corrections factors

	Measurement number		
Illness Category	1	2	Total
Temperature (x2)	[ ] (0-6)	[ ] (0-6)	[ ] (0-12)
Systemic illness (:1.5)	[ ] (0-6)	[ ] (0-6)	[ ] (0-12)
Upper respiratory-tract illness	[ ] (0-6)	[ ] (0-6)	[ ] (0-12)
Lower respiratory-tract illness	[ ] (0-6)	[ ] (0-6)	[ ] (0-12)
Total	[ ] (0-24)	[ ] (0-24)	[ ] (0-48)[1]

[1] This figure represents the daily illness-index (DII)

Table 3: Classification of daily illness severity based on "daily illness index", (DII) data (table 2).

Severity	DII-range
Mild	5-16
Moderate	17-32
Severe	33-48

Treatment / discharge criteria

To decide whether to treat symptoms from each of the illness categories, the symptoms of a particular category must be scored > 2 for at least 2 consecutive days. As a rule, treatment will only be initiated if illness index is moderate (DII ≥ 17) for at least two consecutive days, unless particular circumstances warrant earlier treatment. Obviously, the overall responsibility for initiating treatment lies with the responsible physicians. A clear record of treatments should be kept. Patients may be discharged from study location if they have no, or mild illness (DII ≤ 16) for two consecutive days and are not shedding virus. If patients are discharged with mild illness, a follow-up visit by their GP must take place and the result must be communicated to the responsible physician and the sponsor.

Appendix 2 (continued)

Data analysis criteria (see also section 11.2)

A total daily score > 2 for each illness category must be scored for this category for at least 2 consecutive days to be rated as "present" in the analysis.

A DII ≥ 5 for at least two consecutive days must be scored to assess clinical illness "present" in the analysis.
Duration of illness is the total number of days (≥ 2) for which the DII score is equal to or exceeds 5. The comparison of the mean DII curves for each study group will represent the major analysis to assess the effect of vaccination history on severity and duration of disease. Differences in attack-rates will assess the effect of vaccination history on "protective immunity".

Appendix 3
POWER CALCULATION

1. Study design

A total of 120 subjects will be challenged with a live H1N1 virus.
These subject will be divided in three groups:

1. subject vaccinated before receiving vaccine (Multivac group)
2. subjects not vaccinated before receiving vaccine (Novac group)
3. subjects not vaccinated before receiving placebo (Placebo group)

Because of the fact that the multivac group was not randomized simultaneously with the other groups, comparisons concerning this group are only hypothesis generating, rather than hypothesis testing.

2. Aims of the study

The primary aims of the study are to compare the efficacy of the vaccine in order:

1. to test hypotheses about the equality of the placebo and novac group.
2. to generate hypotheses about the equality of the placebo and the multivac group.
3. to generate hypotheses about the equality of the novac and the multivac group.

3. Main response variable

The main response variable is the attack rate, i.e. the rate of subjects having an attack of influenza (see section 11.1; appendix 2).

4. Background information

Power and sample size calculations are done assuming different true attack rates ranging from 60% to 90 %in placebo subjects, different efficacies of the vaccine in novac subjects, i.e. 60 % and 70 %, and different efficacies of the vaccines in multivac subjects ranging from 10 to 60 %.
For the purpose of the calculations it is assumed that we have independent observations from a binomial distribution and the method applied is described in Fleiss et al., Biometrics 1980;36:343-346.

Under the null hypothesis that two treatments have similar effects, the difference in attack rate is zero. Because vaccine treatment is only of interest if it is better than placebo this null hypothesis is tested against an alternative with a one-sided signifi-

Appendix 3 (continued)

cance test at the 5% level of significance. For the comparison of novac subjects against multivac subjects the null hypothesis is tested against an alternative with a two-sided significance test at the 5% level of significance, since differences in both directions are of interest.

5. Results

In Table 1, sample size estimates are given for different values for the power - i.e. the chance of detecting a significant result - for the comparison of placebo subjects against novac subject.

Table 1: Sample size (per treatment group) calculations for the comparison of Firstvac group against Placebo group for different attack rates in placebo subjects and different efficacies for the vaccine in firstvac subjects.

	Vaccine-Efficacy in firstvac group: 70%				Vaccine-Efficacy in firtsvac group: 60%			
Placebo attack rate (%)	Firstvac attack rate (%)	Sample size power 80%	90%	95%	Firstvac attack rate (%)	Sample size power 80%	90%	95%
60	18	21	26	32	24	28	36	44
70	21	16	20	24	28	22	28	33
80	24	13	16	18	32	17	21	25
90	27	10	12	14	36	13	16	19

Estimates are based on a one-sided test with α=0.05

In Table 2 power estimates are given for the comparisons of multivac subjects against novac and placebo subjects for three different distributions of the 120 subjects over the three treatment groups - i.e. all group sizes equal (40 in all three groups), multivac group twice as large (60 against 30 in the other groups) and an intermediate distribution (50 in multivac group and 35 in the other two).

Appendix 3 (continued)

Table 2: Power (%) for different attack rates in placebo group, vaccine efficacies in firstvac and multivac group and sample sizes.

3 Placebo attack rate	2 Firstvac attack rate eff. A: 70% B: 60%		1 Multivac attack rate	1 vs 3 one-sided α= 0.05			1 vs 2A two-sided α= 0.05			1 vs 2B two-sided α= 0.05		
				60/30	40/40	50/35	60/30	40/40	50/35	60/30	40/40	50/35
Multivac effectivity: 10%												
60	18	24	54	<	<	<	89	89	90	<	<	<
70	21	28	63	<	<	<	96	96	97	85	84	86
80	24	32	72	<	<	<	99	99	99	94	94	94
90	27	36	81	<	<	<	100	100	100	98	98	99
Multivac effectivity: 20%												
60	18	24	48	<	<	<	<	75	76	<	<	<
70	21	28	56	<	<	<	86	86	87	<	<	<
80	24	32	64	<	<	<	94	94	95	76	76	78
90	27	36	72	<	<	<	98	98	98	87	87	88
Multivac effectivity: 30%												
60	18	24	42	<	<	<	<	<	<	<	<	<
70	21	28	49	<	<	<	<	<	<	<	<	<
80	24	32	56	<	<	<	77	78	79	<	<	<
90	27	36	63	83	84	84	87	87	88	<	<	<
Multivac effectivity: 40%												
60	18	24	36	<	<	<	<	<	<	<	<	<
70	21	28	42	75	75	76	<	<	<	<	<	<
80	24	32	48	88	88	89	<	<	<	<	<	<
90	27	36	54	97	97	97	<	<	<	<	<	<
Multivac effectivity: 50%												
60	18	24	30	81	80	82	<	<	<	<	<	<
70	21	28	35	91	91	92	<	<	<	<	<	<
80	24	32	40	98	97	98	<	<	<	<	<	<
90	27	36	45	100	100	100	<	<	<	<	<	<
Multivac effectivity: 60%												
60	18	24	24	93	93	94	<	<	<	<	<	<
70	21	28	28	98	98	98	<	<	<	<	<	<
80	24	32	32	100	100	100	<	<	<	<	<	<
90	27	36	36	100	100	100	<	<	<	<	<	<

< : power < 75%

Appendix 3 (continued)

6. Conclusions

Even when conditions are rather unfavourable - i.e. attack rate in placebo subjects 60% and efficacy of the vaccine in novac subjects 60% - 36 subject per group is enough to achieve a power of 90% when testing novac subjects against placebo
When conditions are as assumed - i.e. attack rate in placebo subjects 80 % and efficacy of the vaccine in novac subjects 70% - a group size of 16 is sufficient.

Fifty subjects in the multivac group and 35 subjects in the other two groups give the highest probabilities of detection when compared to the other two distributions of subjects for the comparison of multivac subjects against novac and placebo subjects, although differences are marginal. As seen above, 35 subjects in the placebo and novac group is more than enough for the comparison of placebo against novac.

Only when conditions are very favourable - i.e. attack rate in placebo > 90 % and efficacy of the vaccine in novac subjects > 70% - is there a small range of values for the true efficacy of the vaccine in multivac subjects that has a reasonable power (>75%) for the comparison of multivac subjects against both placebo and novac subjects. This is the case when the efficacy of the vaccine in multivac subjects is about 30%.

Even when conditions are favourable, only quite a large reduction in the efficacy of the vaccine in multivac subjects as compared to novac subjects has a fair chance of being detected.
When conditions are less favourable the situation is worse. The following reductions in efficacy have a power of about 75%:

Attack rate placebo	vaccine efficacy novac	vaccine efficacy reduction multivac
80 %	70 %	57 %
80 %	60 %	71 %
60 %	70 %	71 %
60 %	60 %	>86 %

The chance of detecting differences between the treatment groups is highly dependent on the effectiveness of the virus and the efficacy of the vaccine. Since the total sample size cannot be increased from of a logistical point of view it is essential for this study that these two measures are high. When this turns out not to be the case, only conclusions can be drawn about the comparison of placebo against novac subjects. Even when these two measures turn out to be high, only quite large differences between the multivac and the other subjects have a fair chance of being detected.

Summary and recommendations

SUMMARY

Chapter 2

Many published reports suggest a limited efficacy of vaccines to protect elderly subjects against influenza infections (1-3). This is generally thought to be due to an age-associated decline of the immune system resulting in a reduced antibody response following vaccination. The review on the effect of age on the antibody response to influenza vaccination (chapter 2), however, identified some confounding factors in reported serological studies which confuse the interpretation of the results. Apart from statistical shortcomings, many of the studies did not correct for pre-vaccination antibody status, previous vaccination history, priming age and health state of the study populations. Elderly studies in which the immune response to influenza vaccination have been studied, usually included subjects with underlying disease. Therefore, it is not possible to conclude from these studies, whether the reduced antibody response was solely attributed to age per se or to the, mostly non-specified, health condition of the subjects. We suggest that the use of strict selection and stratification criteria are mandatory if one wishes to study the effect of age per se on the antibody response following influenza vaccinations.Indeed, studies in elderly (4), in which the absence of underlying disease was established by a standarazid protocol, do not suggest that age per se has a negative influence on the immune system. These findings are in agreement with Gross et al. (5), who found that not age, but health condition was a predictor of reduced antibody response. Taken all data together, it can be concluded, that elderly subjects with underlying disease respond less to influenza vaccines than young healthy adults, but also than healthy subjects of similar age. The reduced antibody response in chronically ill elderly subjects should not negatively influence the vaccination policy for healthy elderly, since all elderly over the age of 65 years are at increased risk for complications associated with influenza infections (6). Particularly in light of the consistent reports of vaccine efficacy to reduce influenza-associated morbidity and mortality in high-risk patiens (7-13), including the disabled with a reduced antibody response, strongly indicates the need for an active vaccination policy.

Chapter 3

In an attempt to increase the immune response in disabled elderly and hence to enhance vaccine efficacy to protect these patients against influenza infections, many studies have been done with increased vaccine doses.

In Chapter 3, we have evaluated these serological dose-response vaccination studies. Overall, the inconsistent and shallow dose-response effects, if any, do not justify the expectation that a clinically relevant increased protective efficacy can be achieved in

primed subjects, including the elderly, nursing home residents, if the standard vaccine dose (10-15 μg HA) is increased. Therefore, increasing the standard vaccine dose seems no realistic option to improve vaccine efficacy for the disabled elderly to protect them against influenza infections. Furthermore, we did not find convincing evidence to support the notion, that vaccine doses of 15 μg HA/strain would be clinically superior to a vaccine dose of 10 μg HA/strain.

In Chapter 3, we also evaluated the serological dose-response studies from a methodological point of view. Like for the serological studies in the elderly (chapter 2), we found a lot of variation in study designs, statistical procedures to evaluate the study results and serological parameters and criteria for the interpretation of the study outcome.

Often, retrospective stratification groups were too small to yield meaningful results. We suggest that criteria for stratification groups for serological influenza vaccination studies should be defined prospectively, so that it can be ensured that reasonable group sizes will be included in each of the strata. Using power calculations for the determination of study group sizes would increase the standards of vaccination research.

Chapter 4

In Chapter 4, we have critically evaluated the serological parameters usually assessed for the interpretation of study outcome. We demonstrated that study outcome may be affected by the choice of serological parameter. We argued that the MFI and the response rate (subjects with at least 4-fold titre rises) are inappropriate measures for vaccine efficacy. Hence we suggested that these parameters should not be used anymore as markers of efficacy in serological studies. Since post-vaccination HI-antibody titres are correlated with pre-vaccination titres, we suggested that, on theoretical grounds, it would be necessary to analyse the post-vaccination titres only after appropriate adjustments are made for the pre-vaccination titres. The traditional "protective level", which is used in the analysis of nearly all serological studies (chapter 3), actually represents only a 50%-60% protective level (PL_{50}).

We argued, that this is not a clinically relevant measure of efficacy (in fact misleading) and should be replaced by a PL_{90} value. As a consequence of our critical evaluation, we have suggested a decision algorithm for the objective clinical interpretation of serological vaccination studies. Finally, we suggested not only to present observed mean values for the serological parameters but also ranges and 95% confidence intervals. Treatment differences should be evaluated as statistically significant (α=0.05%) if zero is not included in the 95% confidence interval of the difference.
We believe that an objective decision algorithm will prove advantageous for the

development of new influenza vaccines. Comparative serological studies to evaluate the relative serological efficacy of vaccine types, vaccine doses and vaccination regimens will be easier to interprete in an objective way. Our suggestions for the alternative way to analyse and evaluate serological studies are merely concepts at this point in time, since there are only limited data to support any reasonable figure to determine "the" PL_{90} and "the" decline rate of antibody, six months after vaccination. Although any selected PL_{90} titre value can only represent a reasonable estimate, the use of a standardized, clinically meaningful parameter, is considered of more importance than the "exact" value itself.

Chapter 5

In Chapter 5, a placebo-controlled multicentre dose response study in elderly, nursing home residents and young volunteers with trivalent influenza subunit vaccine was described. Because this study was done in 1988, the analysis was not completely done according to our suggestions as discussed in Chapter 4. Actually, considerations about the statistical analysis of this study, initiated our concepts for the alternative analyses as suggested in Chapter 4. Although all classical serological parameters were analysed, the statistical analysis itself was done according to the suggested alternative method.

Post-vaccination HI antibody titres (GMT) and the proportion of subjects with "protective" post-vaccination titres (%PL_{50}!) were adjusted for pre-immunization HI-antibody titres. In this study, dose-effects differed between strains and study centres, despite the control of some confounding factors in the study design. For the B/Beijing strain, both study populations showed a signaficant dose response relationship for the corrected post-vaccination GMT over the total dose range from 10-60 µg HA.

For the corrected %PL_{50}, there was an increase between the 10 and 20 µg HA dose, whereas only a marginal additional increase was observed by increasing the dose upto 60 µg HA. For the A/Taiwan antigen, there was no dose effect in any of the parameters. Nursing home residents had lower post-vaccination titres than the young for both, the B/Beijing and the A/Taiwan strains. For the A/Sichuan strain, there was a dose-effect in one study centre, but not in the other, again pointing out the intrinsic variability of serological studies.

Chapter 6

Chapter 6 represents a study protocol for a live virus challenge study in young adults. As noted by Ghendon in a recent authorative review (14), "factors responsible for protection against influenza should be defined more clearly". Although animal data can be very helpful, these cannot replace human data to elucidate the immune mechanisms relevant in man, for the protective immunity against influenza infections.

As has been pointed out by Pabst and Gehrke (15), "species differences are of major importance in interpreting the clinical relevance of experiments in animal models on the lung immune system, e.g. antigen uptake and immunostimulation. Therefore, animal experiments serving as models for the situation in humans with respect to aerosol vaccination, uptake of antigen by bronchus-associated lymphoid tissue (BALT), have to be interpreted with great caution, or other species than rabbits or rats should be used". Based on these considerations, we believe, that artificial challenge studies with live influenza viruses represent the best experimental model to study relevant protective immunological factors in man. Further, artificial challenge studies are an important experimental setting to compare the efficacy of different vaccine-types and doses or to evaluate different vaccination regimens. Challenge studies should be done under strict controlled safety conditions. The challenge study protocol has been presented in chapter 6 as an illustration of the usefulness of such a study for fundamental research -or practical purposes. It describes a clinical assessment scale to quantitate influenza illness and presents a power calculation to determine adequate group sizes. It also illustrates the intense collaboration between influenza virologists, immunologists, clinical research scientists and statisticians needed to ensure optimal results from such costly studies.

2. Recommendations

As was discussed in the Introduction (Chapter 1; 7-13), standard influenza vaccine doses are very effective in reducing influenza morbidity and particularly mortality. It was also shown, that in most European countries, less than 50% of the high-risk patients were vaccinated in the influenza season 1990/91 (Chapter 1, Table 1). For the 1989/90 H3N2 (A/Shanghai/11/87) influenza epidemic, 26.000 and 4100 deaths were reported in the United kingdom (14) and The Netherlands (15) respectively, despite the very close antigenic match between the epidemic and the vaccine strain (15). Therefore, it is reasonable to assume, that many of these influenza deaths could have been prevented, if the current vaccines were used more widely. Based on the efficacy of currently available influenza vaccines and the lack of evidence that a dose of 15 µg HA/strain is clinically superior to a dose of 10 µg HA, at least for the influenza A strains, we believe, that an immediate significant improvement of the control of influenza can be achieved by recommending a standard dose of 10 µg HA/strain and to ensure that the subsequent vaccine "overcapacity" will be used to vaccinate a much larger proportion of the high risk patients.

A target vaccination rate of 80% for each of the high-risk groups has been stated as an objective by the Canadian and American Public Health Authorities (16,17) to effectively control the impact of influenza. The succesfull implementation of such a policy should not depend on the difficult and time consuming research efforts to find new influenza vaccines or alternative modalities for the prevention of influenza nor on immediate investments from the influenza manufacturers to increase their maximum production capacity. It should mainly depend on effective public campaigns to inform patients and the medical profession on the need of active influenza vaccination programmes (18,19). A reduction of standard vaccine dose as in fact suggested here for influenza vaccines, has previously been effectuated for the hepatitis B vaccine in 1988, when no evidence was established from clinical studies that the standard dose of 20 IU was superior to a dose of 10 IU.

Discussing the role of regulatory authorities and the requirements for (redundent?) safety tests of biological products, Skinner complained in an critical editorial (20): "nice work for everybody except the patient".

As may be illustrated by the history of live virus vaccine development, we have to be careful, that Skinner's statement should not be applicable for the development of new influenza vaccines.

The intrinsic variability of the influenza viruses contribute to a large extent to the methodological difficulties to establish relative vaccine efficacies of different vaccine types or vaccine doses or vaccination regimens in clinical studies. Factors such as the complexity of conducting field trials, the lack of an in-depth knowledge on the rele-

vant immunological parameters for man for the protection against influenza infections (14), the lack of consensus on study designs, parameters and statistical procedures to evaluate serological studies and the lack of quantitative criteria to objectively judge the outcome of serological studies are obstacles for the development of new influenza vaccines, as has been illustrated for the live virus influenza vaccines (Chapter 6). Due to the complexity of influenza clinical research, as has been illustrated in this thesis, we believe that the most efficient way to make progress in this field of research, is by creating an "International Collaborative Influenza Clinical Research Group" consisting of influenza virologists, immunologists, clinical research methodologists and statisticians, which can coordinate large scale clinical studies, including field and artificial challenge studies in healthy volunteers. Such a group could possibly be financed jointly by funds from national public health institutes and influenza vaccine manufacturers. By this type of synergy, we could possibly achieve a nice piece of work for everybody, including the patient (20).

Before such a group will be actually created and before new influenza vaccines will be available for patients, it may be expected that influenza will have killed many more people. Therefore, physicians and patients at risk of influenza should not wait for new developments to come, but take each year an influenza vaccination as an effective and necessary precaution.

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Samenvatting
Hoofdstuk 2

In veel publicaties wordt de indruk gewekt dat vaccinatie bij oudere mensen slechts een beperkte bescherming biedt tegen een influenza infectie (1-3). Over het algemeen wijt men dit aan de achteruitgang van het immuunsysteem als gevolg van ouderdom, zodat na vaccinatie minder antistoffen worden gevormd. Het literatuur onderzoek naar de samenhang tussen leeftijd en immunoreactie na toediening van influenza-vaccins (hoofdstuk 2) bracht echter een aantal factoren aan het licht betreffende serologische onderzoeken, die de interpretatie van de daaruit voortkomende resultaten kunnen beinvloeden. Naast statistische tekortkomingen werd bij vele onderzoeken geen rekening gehouden met reeds voor de vaccinatie aanwezige antistofgehaltes, de voorgeschiedenis bij vorige vaccinaties, de leeftijd bij vaccinatie en de gezondheidstoestand van de onderzochte personen. Bij onderzoeken naar de immunoreactie na vaccinatie bij ouderen werd met reeds aanwezige ziektes gewoonlijk geen rekening gehouden. Het is dan ook niet mogelijk uit deze onderzoeken conclusies te trekken over de oorzaak van de verminderde immunoreactie: was het de leeftijd of de, meestal niet gespecificeerde, gezondheidstoestand van de onderzochte personen? Wij stellen voor dat, bij onderzoek naar het effect van leeftijd op de mate van immunoreactie na inenting met een influenza-vaccin, het noodzakelijk is om strenge criteria te hanteren bij selectie en stratificatie. Onderzoeken met ouderen (4), waarbij deze door middel van een standaardmethode op aanwezige ziektes werden gecontroleerd, geven dan ook geen aanleiding tot de conclusie dat leeftijd op zichzelf een negatieve invloed heeft op het immuunsysteem. Deze bevindingen zijn in overeenstemming met die van Gross en medewerkers (5), die tot de slotsom kwamen dat niet leeftijd maar gezondheid een aanwijzing geeft voor verminderde immunoreactie. Wanneer alle gegevens op een rijtje worden gezet, kan worden vastgesteld dat van de onderzochte personen, de ouderen met een reeds aanwezige ziekte minder goed reageren op influenza-vaccinatie dan jonge gezonde volwassenen, maar ook dan gezonde personen van vergelijkbare leeftijd. Een verminderde immunoreactie bij chronisch zieke ouderen zou echter geen negatief effect mogen hebben op het vaccinatiebeleid voor gezonde ouderen, daar alle personen boven de 65 jaar een verhoofd risico lopen op complicaties bij influenza infecties (6). Vooral gezien de consequente resultaten in rapporten voor wat betreft de doeltreffendheid van vaccinatie bij het verlagen van het ziekte- en het sterftecijfer bij patiënten met een verhoogd risico (7-13) - personen met een verminderde immunoreactie meegerekend - is een actief vaccinatiebeleid sterk te adviseren.

Hoofdstuk 3

Voor een betere bescherming van chronisch zieke ouderen tegen besmetting met influenza zijn vele onderzoeken verricht naar verhoging van de vaccinatiedoses in een poging de immunoreactie van deze patiënten en zo de effectiviteit van de vaccinatie te verhogen.
In hoofdstuk 3 hebben wij deze serologische onderzoeken naar de samenhang tussen dosis en immunoreactie geëvalueerd. Over het geheel genomen rechtvaardigen de inconsequente en geringe dosis-reactie-effecten, indien al aanwezig, niet de verwachting van een klinisch relevante verbetering van de bescherming van gevaccineerde personen (waaronder ook oudere bewoners van verpleeghuizen) wanneer de standaarddosis van de vaccinatie (10 tot 15 µg HA) wordt verhoogd. Daarom lijkt een verhoging van de standaard vaccinatiedosis geen realistische keus voor de verbetering van de effectiviteit van vaccinaties ter bescherming van chronisch zieke ouderen tegen besmetting met influenza. Tevens hebben wij geen overtuigend bewijs kunnen vinden voor de veronderstelling dat vaccinatiedoses van 15 µg HA/stam klinisch beter zouden zijn dan doses van 10 µg HA/stam.

In hoofdstuk 3 hebben wij tevens de serologische dosis-reactie-onderzoeken vanuit een methodologisch oogpunt geëvalueerd. Evenals bij de serologische onderzoeken bij ouderen (hoofdstuk 2), vonden wij een grote verscheidenheid aan studie opzetten, statistische methoden bij de beoordeling van de resultaten, serologische parameters en de criteria bij de interpretatie van de conclusies van de onderzoeken.

Vaak bleken de achteraf gestratificeerde groepen te klein voor bruikbare resultaten. Wij stellen voor dat de criteria voor de stratificatie van de groepen bij serologisch onderzoek naar vaccinatie tegen influenza van tevoren worden bepaald, zodat redelijke aantallen binnen de stratums zijn gegarandeerd. Berekeningen om het onderscheidingsvermogen van een studie te bepalen ("power calculation") bij het vaststellen van de grootte van de te onderzoeken groepen zou de kwaliteit van het vaccinatie-onderzoek ten goede komen.

Hoofdstuk 4

In hoofdstuk 4 hebben wij de serologische parameters die gewoonlijk worden aangewend bij de interpretatie van de resultaten van een onderzoek, aan een kritische evaluatie onderworpen. Wij hebben aangetoond dat de conclusie die uit een onderzoek wordt getrokken, door de keuze van de serologische parameter kan worden beïnvloed. Wij hebben aangevoerd dat de MFI (gemiddelde titerstijging) en het percentage personen met tenminste een viervoudige titerstijging ongeschikte maten zijn voor het meten van de effectiviteit van vaccinaties. Derhalve hebben wij aangeraden deze parameters niet meer te gebruiken als meetpunten voor doeltreffendheid

binnen serologische onderzoeken. Daar HI-antistoftiters na vaccinatie correleren met de titers voor vaccinatie, stelden wij voor dat het op theoretische gronden noodzakelijk zou zijn de post-vaccinatietiters slechts te analyseren nadat de juiste correcties voor de pre-vaccinatietiters zijn toegepast. Het traditionele "beschermende gehalte" dat bij de analyse van bijna alle serologische onderzoeken wordt gehanteerd (hoofdstuk 3) correspondeert uiteindelijk slechts met een beschermend gehalte van 50%-60% (PL_{50}).
Wij hebben aangevoerd dat dit geen klinisch relevante maat voor doeltreffendheid is (in feite is zij misleidend) en zou moeten worden vervangen door een waarde van PL_{90}. Gebaseerd op onze kritische evaluatie hebben wij een beslissingsalgoritme voorgesteld voor de objectieve interpretatie van serologische vaccinatie-onderzoeken. Tot slot hebben wij voorgesteld niet alleen de gemiddelde waarden aan te geven voor de serologische parameters, maar ook het bereik en de 95%-betrouwbaarheidsintervallen. Verschillen in behandeling zouden als statistisch relevant moeten worden beoordeeld (α = 0,05%) als nul niet wordt opgenomen in het 95%-betrouwbaarheidsinterval over het verschil.

Wij denken dat een objectief beslissingsalgoritme een voordeel zal blijken bij de ontwikkeling van nieuwe influenza-vaccins. Het wordt dan gemakkelijker om de resultaten van vergelijkende serologische onderzoeken naar de relatieve serologische effectiviteit van diverse soorten vaccins, doses en toedieningen objectief te interpreteren. Onze suggesties voor de alternatieve wijze om serologische onderzoeken te analyseren en te evalueren, bevinden zich op dit moment nog maar in de conceptfase, daar het aantal gegevens te beperkt is voor een redelijk cijfer voor de bepaling van de PL_{90} en de daling van het gehalte aan antistoffen na zes maanden. Hoewel iedere geselecteerde PL_{90}-waarde slechts op een redelijke schatting zal zijn gebaseerd, wordt het gebruik van een gestandaardiseerde, klinisch bruikbare parameter belangrijker geacht dan de "exacte" waarde zelf.

Hoofdstuk 5

In hoofdstuk 5 werd een onderzoek naar de samenhang tussen dosis en immunoreactie bij oudere inwoners van verpleeghuizen en jonge vrijwilligers beschreven. In dit onderzoek, dat vanuit diverse onderzoekscentra met, ter controle, placebo's werd uitgevoerd, werd een trivalent subunit vaccin gebruikt. Daar dit onderzoek in 1988 plaatsvond, werd de analyse niet geheel volgens de door ons in hoofdstuk 4 geopperde suggesties uitgevoerd. In feite kregen wij tijdens de beschouwing van de statistische analyse van dit onderzoek het idee van de alternatieve analysemethodes zoals voorgesteld in hoofdstuk 4. Hoewel alle klassieke serologische parameters werden geanalyseerd, is de statistische analyse zelf uitgevoerd volgens de aangevoerde alternatieve methode.

De HI-antistoftiters na vaccinatie (GMT) en het aantal personen met "beschermende" post-vaccinatietiters (%PL_{50}!) werden gecorrigeerd voor de HI-antistoftiters voor vaccinatie. Bij dit onderzoek werden, ondanks de beheersing over een aantal factoren binnen de opzet van het onderzoek, verschillen geconstateerd tussen de dosis-reactieverhoudingen bij de diverse stammen en centra. Bij de B/Beijing-stam was er voor beide leeftijds groepen een duidelijk verband tussen dosis en immunoreactie na correctie van de post-vaccinatie-GMT binnen het gehele dosisbereik van 10 tot 60 µg HA. Voor de gecorrigeerde PL_{50} was er een toename te zien tussen de 10 en 20 µg HA-doses, terwijl er bij verhoging van de dosis tot 60 µg HA slechts een marginale toename werd geconstateerd. Bij het A/Taiwan-antigeen had de verandering van dosis geen enkel effect, voor geen enkele parameter. Zowel bij de B/Beijing-stam als bij de A/Taiwan-stam bleken de bewoners van verpleeghuizen lagere post-vaccinatietiters te hebben dan de jongere personen. Voor wat betreft de A/Sichuan-stam was er in het ene onderzoekscentrum wel, maar in het andere geen veranderende dosis-effect-verhouding, waarmee de intrinsieke variabiliteit van serologische onderzoeken opnieuw werd benadrukt.

Hoofdstuk 6

In hoofdstuk 6 werd een onderzoeksprotocol beschreven voor een immuniteitsonderzoek bij jonge volwassenen waarbij gebruik wordt gemaakt van een levend virus. Zoals Ghendon onlangs opmerkte in een gezaghebbend overzichtsartikel (14), "factoren die van invloed zijn op de bescherming tegen influenza, dienen beter te worden gedefinieerd". Hoewel gegevens van dierproeven zeer goed bruikbaar kunnen zijn, kunnen deze bij het achterhalen van de onderdelen van het immuunsysteem van de mens die bescherming bieden tegen besmetting met influenza, de gegevens van onderzoeken met mensen niet vervangen.

Zoals door Pabst en Gehrke (15) werd geconstateerd, zijn "verschillen tussen (dier)soorten uiterst belangrijk bij de interpretatie van het klinische belang van dierproeven voor het immuunsysteem van de longen, bijvoorbeeld voor de bepaling van de hoeveelheid opgenomen antigenen en voor immunostimulatie. Daarom moeten dierproeven waarbij de dieren als model dienen voor hetgeen er bij de mens gebeurt bij aerosole vaccinatie en opname van antigenen door aan de bronchiën verwant lymfoïd weefsel (BALT), uiterst voorzichtig worden geïnterpreteerd, of er moeten andere diersoorten worden gebruikt dan konijnen en ratten". Op grond van deze overwegingen denken wij dat kunstmatig "challenge"-onderzoek met levende influenzavirussen de beste experimentele methode is bij het bestuderen van de relevante beschermende immunologische eigenschappen van de mens. Verder zijn kunstmatige "challenge"-onderzoeken belangrijk bij vergelijkend onderzoek naar de effectiviteit van verschillende soorten vaccins en doses, of bij de evaluatie van verschillende vaccinatietoedieningen. "Challenge"-onderzoeken moeten onder streng bewaakte, veilige omstandigheden worden uitgevoerd. Het protocol voor zo een onderzoek werd in hoofdstuk 6 opgenomen als voorbeeld van de bruikbaarheid van dergelijk onderzoek voor zowel fudamenteel wetenschappelijke als voor praktisch toepasbare doeleinden. Hierin wordt een klinische graadmeter beschreven om de mate van ziekte door influenza te bepalen en wordt tevens een "power"-berekening getoond voor het vaststellen van de minimaal benodigde omvang van de groepen. Tevens wordt hiermee de nauwe samenwerking tussen influenza-virologen, immunologen, klinische onderzoekswetenschappers en statistici weergegeven die noodzakelijk is voor een optimaal resultaat bij dergelijke kostbare onderzoeken.

2. Aanbevelingen

Zoals reeds werd gesteld in de Inleiding (hoofdstuk 1; 7-13), zijn standaarddoses van influenza-vaccins zeer effectief voor het reduceren van het ziekte- en vooral het sterftecijfer ten gevolge van influenza. Tevens werd opgemerkt dat in de meeste Europese landen minder van 50% van de patiënten met een verhoogd risico binnen het influenza-seizoen 1990-1991 werden gevaccineerd (hoofdstuk 1, tabel 1). Tijdens de H3N2-influenza-epidemie in 1989-1990 (stam A/Shanghai/11/87) werden 26.000 sterfgevallen in het Verenigd Koninkrijk (14) en 4.100 in Nederland (15) gemeld, ondanks de nauwe verwantschap tussen het epidemisch virus en het vaccinvirus (15). Daarom is het redelijk om aan te nemen dat een groot deel van de sterfte door influenza vermeden had kunnen worden als de huidige vaccins meer werden gebruikt. Gezien de effectiviteit van de momenteel beschikbare influenza-vaccins en het gebrek aan bewijs dat een dosis van 15 µg HA/stam klinisch een betere werking heeft dan een dosis van 10 µg HA/stam (in ieder geval voor wat betreft de influenza-A-stammen), denken wij dat influenza veel beter zal kunnen worden beheerst wanneer de standaarddosis van 10 µg HA/stam wordt toegepast en de daaruit voortkomende "overcapaciteit" wordt gebruikt om een groter deel van de patiënten met een verhoogd risico te vaccineren.

Zowel de Canadese als de Amerikaanse instanties voor Volksgezondheid (16, 17) hebben zich als doel gesteld 80% van ieder van de groepen met een verhoogd risico te vaccineren, zodat de invloed van influenza op doeltreffende wijze wordt beperkt. De uitvoering van een dergelijk beleid zou onafhankelijk moeten zijn van de moeizame en tijdrovende en kostbare onderzoeken naar een nieuw vaccin tegen influenza of de ontwikkeling van nieuwe alternatieve mogelijkheden ter voorkoming van influenza, noch zou het af moeten hangen van onmiddellijke investeringen van producenten van influenza-vaccins voor het vergroten van hun maximumcapaciteit. Het zou hoofdzakelijk afhankelijk moeten zijn van effectieve reclamecampagnes om patiënten en medici te informeren over de noodzaak van een actief programma voor vaccinatie tegen influenza (18, 19). De hier, voor influenza-vaccinaties voorgestelde dosisverlaging werd al eerder in praktijk gebracht bij het hepatitis B-vaccin in 1988, nadat uit klinische onderzoeken geen bewijs was voortgekomen dat de standaarddosis van 20 IU beter was dan de dosis van 10 IU.

Bij het bespreken van de rol van regelgevende instanties en de eisen van (overbodige?) veiligheidstests van biologische produkten, beklaagde Skinner in een kritisch redactioneel commentaar (20): "goed werk voor iedereen, behalve voor de patiënt". Zoals wordt aangetoond door de geschiedenis van de ontwikkeling van vaccins met levende virussen, moeten we voorzichtig blijven, zodat Skinners bewering niet van toepassing zou kunnen worden op de ontwikkeling van influenza-vaccins.
De intrinsieke variabiliteit van de influenza-virussen draagt voor een groot deel bij aan de methodologische moeilijkheden bij het bepalen van de relatieve effectiviteit van verschillende soorten vaccins, doses of toedieningen in klinische onderzoeken. Factoren als de complexiteit van het uitvoeren van onderzoeken in praktijk, het gebrek aan grondige kennis van de voor de mens relevante immunologische parameters voor de bescherming tegen besmetting met influenza (14), het ontbreken van consensus over de opzet van onderzoeken, parameters en statistische procedures voor het evalueren van serologische onderzoeken en het gebrek aan kwantitatieve criteria voor een objectieve beoordeling van het resultaat van serologische onderzoeken vormen belemmeringen voor de ontwikkeling van nieuwe influenza-vaccins, zoals werd aangetoond bij de influenza-vaccins met levende virussen (hoofdstuk 6). Door de complexiteit van klinisch influenza-onderzoek, zoals in dit proefschrift werd geïllustreerd, denken wij dat de meest efficiënte manier om vooruitgang te boeken in dit onderzoeksgebied bestaat uit het opzetten van een "Internationaal Samenwerkingsverband voor Klinisch Onderzoek naar Influenza", bestaande uit influenza-virologen, immunologen, klinische onderzoekswetenschappers en statistici. Deze zouden in staat moeten zijn om klinische onderzoeken op grote schaal te coördineren, inclusief veld studies en experimentele "challenge"-onderzoeken, bij gezonde vrijwilligers. Een dergelijke groep zou mogelijk gezamenlijk kunnen worden gefinancierd door nationale gezondheidsinstanties en producenten van vaccins tegen influenza. Met een dergelijke samenwerking zou het wellicht mogelijk zijn voor iedereen goed werk te leveren en ook voor de patiënt (20).

Voordat een dergelijke groep daadwerkelijk wordt opgericht en nieuwe influenza-vaccins voor patiënten beschikbaar zijn, zal influenza nog veel meer slachtoffers hebben geëist. Daarom moeten medici en patiënten die een verhoogd risico lopen op complicaties bij besmetting met influenza niet de nieuwe ontwikkelingen afwachten, maar zich uit effectieve en noodzakelijk voorzorg. ieder jaar laten vaccineren tegen influenza.

Appendix

Safety and tolerance of Influenza subunit vaccine Influvac®

1. Introduction

Concern that vaccination with human influenza strains may cause Guillain-Barre syndrome (GBS) was heightened by a study which showed an increased risk of developing GBS after vaccination with the swine A/New Jersey influenza vaccine in 1976-1977 in the USA (1,2). A recent study to reassess the association between GBS and swine influenza vaccinations by Safranek et al. (17) has confirmed an increased risk of developing GBS during the 6 weeks following vaccination in adults. No increase in relative risk for GBS was noted beyond 6 weeks after vaccination. Studies by Hurwitz et al. (3) and Kaplan et al. (4) in 1981 and 1982 did not reveal an increased risk of developing GBS with the human influenza vaccine formulations used in the period 1979-1981. A recent retrospective study by Roscelli et al. (5) over the period 1980-1988 where data were derived from the US army's mass influenza vaccination program, did also reveal no detectable increase in the incidence of GBS in 5.616.000 active duty army soldiers.

After the introduction of the zonal ultracentrifugation technique in the production process for influenza vaccines in the late sixties (6), the reactogenicity of the vaccines has been reduced dramatically. Although all types of available inactivated influenza vaccines (whole-virus- split- and subunit vaccines) are well tolerated, the American Advisory Committee for Influenza Prevention (ACIP) recommends (7) the use of split vaccines in children because their more favourable side effect profile compared to whole-virus preparations (subunit influenza vaccines are not available in the USA). In Europe, where the influenza subunit vaccines are available, these vaccines should also be included in the recommendations for use in children because of their very favourable side effect profile.

From 1947 onwards, Duphar B.V. in The Netherlands produced whole virus influenza vaccines. Since 1981, it has been the only manufacturer of the highly purified influenza subunit vaccines. During this period, approximately 40 million vaccinations have been carried out with this subunit influenza vaccine (Influvac®).

In the current appendix, we will summarise the safety data as collected by the company's postmarketing surveillance system as well as the tolerance data collected from clinical studies since 1982 with the subunit influenza vaccine.

2. Safety and tolerance of influenza subunit vaccine (Influvac®) from postmarketing surveillance system and clinical studies

2.1 Postmarketing surveillance.

2.1.1.Solvay Duphar's postmarketing surveillance system

In the central organization of Solvay Duphar B.V. in The Netherlands, a special Unit exists to monitor adverse events during therapy with Duphar compounds, including influenza vaccine, reported from all over the world.

Reports of serious events may come from various sources, such as the literature, health authorities, postmarketing surveillance programmes and clinical trials. All potential relevant information is tranferred to this Unit. If an adverse event is reported to the company, a special form is sent to the reporter of the event to obtain as much as possible relevant details of the event and the prevailing circumstances to assess the seriousness and the possible relation with the drug.

Relevant health authorities are informed conform their statutory regulations if an event is considered serious. All information is stored in a central databank.

There are close contacts with large data-bases on patients such as Prescription Event Monitoring, Pharmacists, Disease registries and health insurance companies. These electronic connections allow pharmaco-epidemiological studies, if deemed necessary. The existence of the "Adverse Drug Experience Unit" enables Duphar to closely monitor adverse effects associated with its compounds and to take appropriate steps in case of hazardous events.

The major advantage from the active postmarketing surveillance system by the "Adverse Drug Experience Unit" is the ability to detect real cause-effect relationships, if any, between influenza vaccinations and serious adverse events, even if they occur at too low a frequency to be detected in controlled clinical trials. We prefer to use the term " adverse event" rather than "adverse reaction" because "reaction" may suggest a cause-effect relationship, before such a relationship is established.

An intrinsic weak point of such a systems is the lack of control of serious adverse events being reported in time, so that not all necessary, reliable, data can be obtained to make a formal cause-effect relationship analysis. However, it seems a reasonable assumption, that real serious adverse events will be reported by physicians to health authorities and/or to pharmaceutical companies and that potential serious adverse effects associated with a product, if any, will ultimately be identified.

2.1.2. Safety data from postmarketing surveillance system

Following approximately 40 million vaccinations with influenza subunit vaccine in the last decade, Duphar B.V. received 98 reports of adverse events concerning in total 154 "suspected" signs and symptoms (appendix 1). Of these, 56 mild signs and symptoms were reported from 33 subjects and 98 serious ones from 65 subjects. Nineteen of these 65 subjects died within four weeks after vaccination. Severity scores are assessed according to international regulations. Because of insufficient data, no meaningful cause-effect relationship analysis could be done to confirm or deny such relationship.

Table 1 represents an overview of the number of reported adverse events classified according to a slightly modified COSTART system (8).

Duphar received sofar 25 reports on patients presenting with neurological complaints. Fourteen were Guillain-Barré syndrome-like. Three of these patients died as a consequence. All non Guillain-Barré cases recovered completely. In total more than 40.000.000 vaccinations have been carried out, which yields a relative risk for neurological disorders of 0.5 per million vaccinations. The relative risk for GBS in the total population is 1.1-1.5/million/year (2).

If signs and symptoms of the hemic system, conjunctivitis and asthma are classified as allergic reactions, 33% (26/78) of the reported signs and symptoms may be classified as "allergic reactions".

Table 1. Overview of reported signs and symptoms, according to COSTART system (8).

Symptom categorie	Numbers Symptom	Death
1. Systemic (like fever, malaise)	6	
2. Cardiovascular system (like myocardial infarction, atrial fibrillation, EEG abnormalities, hypertension)	11	4
3. Hemic and lymphatic system * (like thrombocytopenia, urticaria, "allergic reactions")	20	
4. Musculo-sceletal system (like arthritis, myalgia)	4	
5. Nervous system	22	2
- polyneuritis	10	
- headache, vomit, nausea	6	
- paresthesia	2	
- cerebellar syndrome	1	
- paralysis	1	
- Guillain-Barré syndrome	1	
- confusion	1	
6. Respiratory system (like bronchitis, asthma, pneumonia)	8	3
7. Skin and mucosa (like epidermitis, conjunctivitis)	3	
8. Urogenital system (like glomerulitis)	4	
9. Other (gangrene, sepsis)	3	3
	81	12
Not specified	8	7
Total	89	19

* including some symptoms of allergic reactions.

2 Clinical studies

In Duphar's clinical database, there were 17 clinical studies with the subunit influenza vaccine, in which the reactogenicity was evaluated by a standard questionnaire. Reactogenicity data were available for 2.038 subjects. By far, the majority of data were derived from young, healthy volunteers (mostly medical students) after a vaccination dose of 10 µg HA/strain, which has been the standard dose for the vaccine uptill 1991 in most European countries. Because of the relative small number of elderly subjects and/or vaccine doses higher than 10 µg HA, no subgroup analysis was considered relevant. In chapter 5 of this thesis, the effect of vaccine dose on vaccine reactogenicity in young volunteers and elderly, nursing home residents was shown.

After scrutinizing the data of individual studies, we decided to pool all reactogenicity data for an overall analysis.

Both, the incidence of local and systemic reactions was always less in the 24-48 hours reporting period than in the first 24 hours after vaccination.

From the local reactions, "pain on contact" was by far the most frequently reported (N=462, 23%) within the first 24 hour after vaccination. Headache was the most frequently reported systemic reaction (N=170, 8%).
Because of the low incidence of each local and systemic reactions, we present only the observed total incidence of these reactions (table 2). In addition, and more relevant, we analysed the data for the amount of inconvenience caused by the reactions, (table 2).

Table 2. Reactogenicity of influenza subunit vaccine (Influvac)® from 17 clinical studies (N=2.038); 1982-1991.

post-vaccination	0-24 hours	24-48 hours
any local reaction	695 (34%)	480 (24%)
any systemic rection	303 (15%)	197 (10%)
any local and systemic reaction	168 (8%)	99 (5%)
moderate inconvenience	91 (4%)	
severe inconvenience	16 (1%)	

Despite the 34% of subjects reporting any local reaction within the first 24 hours following an influenza subunit vaccination, for 1931 of the 2038 study subjects (95%), the vaccination did cause no or only slight inconvenience, whereas less than 1% reported the vaccination to cause severe inconvenience.

3. Discussion and conclusion.

Since 1976-1977, when an increased incidence of Guillain-Barré syndrome (GBS) associated with the mass use of a swine influenza virus vaccine was noted in the USA (2), fear for severe adverse reactions may have hampered the widespread use of human influenza vaccines in patients at risk of influenza infections. From retrospective epidemiological surveys (3-5) and postmarketing surveillance programmes, no association between human influenza vaccines and the incidence of GBS or other serious neurological disorders has been established. Therefore, fear for neurological adverse reactions following influenza vaccinations should be no justification for a reserved vaccination policy. However, it may be a prudent precaution for patients who have ever developed neurological disorders following an immunization, not to be immunized again.

Also for other reported adverse events, there is no plausible reason to assume a cause-effect relationship with vaccination except probably for the allergic reactions. These might have occurred in subjects allergic against chicken proteins and may be due to residual amounts of chicken proteins in the vaccine. As stated in the package insert, a known allergy against chicken proteins is a contraindication for vaccination.

The reported incidence of local and systemic reactions following influenza vaccinations may be affected by several factors. In the first place, the method of data collection may influence the outcome.

A list with pre-mentioned adverse reactions to be completed by the vaccinees, as used in our case, will represent an overestimate of the true incidence of reactions. In addition, a medical student population in a clinical study setting may be expected to very critically evaluate adverse reactions, which again could result in an overestimation of the real incidence of reactions. Apart from these methodological factors, biological factors such as age and sex (9-11), variation in individual pain perception, vaccine dose and the level of purification of the vaccines may also affect the reported incidence of reactions. Because the intrinsically subjective nature of adverse reactions reported in clinical studies, definite correlations between local and systemic reactions can only be established in placebo-controlled studies.

Such a placebo-controlled study in elderly has recently been published by Margolis et al. (12), who found no significant differences between trivalent influenza split vaccine (15 μg HA/strain) and placebo with respect to any local or systemic symptoms except for one (sore arm), which occurred in 20% of the elderly vaccinees. This is in close agreement with our (not placebo-controlled) studies where "pain on contact" was reported in 462/2.038 (23%) of the subjects.

In light of the amply established efficacy of influenza vaccines and the favourable safety and tolerance profile of these vaccines as shown in this chapter, we conclude that there is a very positive benefit/risk ratio for influenza vaccines, in particular for the subunit vaccine. The incidence of a sore arm following an influenza vaccination is not considered clinically relevant, particularly since it has no serious influence on the degree of inconvenience. We strongly advise that emotional and theoretical safety and tolerance considerations should not bear any negative influence on physicians' or patients' decisions to use influenza vaccines to prevent influenza infections and/or its serious complications. Due to the improved purification techniques used for the influenza vaccine production since the late sixties (6), the negative attitude towards influenza vaccines (13-16) is misplaced today and is a serious threat to our ability to effectively control the annual impact of influenza infections.

We believe that public health authorities and the medical profession together should take initiatives to effectively reverse the negative attitude towards influenza vaccinations and point out the actual very favourable benefit/risk ratio for high-risk patients, including the disabled elderly. Such actions should result in a more effective control of influenza-associated morbidity and mortality with the existing vaccines.

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APPENDIX

Sign/Symptom	Body symptom*	Frequency
Death		7
Fever	Body whole	2
Influenza-like syndr	Body whole	1
Malaise	Body whole	2
Asthenia	Body whole	1
Heart failure	Cardiovascular system	1
Atrial fibrillation	Cardiovascular system	1
EEG abnormality	Cardiovascular system	1
Pulmonary embolia	Cardiovascular system	1
Heart arrest	Cardiovascular system	1
CVA	Cardiovascular system	1
Myocardial infarctio	Cardiovascular system	1
Hemiplegia	Cardiovascular system	1
Chest pain	Cardiovascular system	2
Hypertension	Cardiovascular system	1
Rash	Hemic and Lymphatic system	3
Epistaxis	Hemic and Lymphatic system	1
Urticaria	Hemic and Lymphatic system	3
Allergic reaction	Hemic and Lymphatic system	3
Facial edema	Hemic and Lymphatic system	1
Thrombocytopenia	Hemic and Lymphatic system	2
Purpura	Hemic and Lymphatic system	1
Melaena	Hemic and Lymphatic system	1
Purpura vasculitis	Hemic and Lymphatic system	1
Vasculitis	Hemic and Lymphatic system	1
Petechiae	Hemic and Lymphatic system	3
Arthritis	Musculo-Skeletal system	2
Myalgia	Musculo-Skeletal system	2
Neuropathy	Nervous system	2
Cerebellar syndrome	Nervous system	1
Palpitations	Nervous system	1
Paralysis	Nervous system	1
Guillain-Barré syndrome	Nervous system	1
Paresthesia	Nervous system	1
Confusion	Nervous system	1
Vomit	Nervous system	1
Neuritis	Nervous system	1

* According to COSTART classification system (8).

Sign/Symptom	Body symptom*	Frequency
Headache	Nervous system	1
Polyneuritis	Nervous system	7
Nausea	Nervous system	4
Bronchitis	Respiratory system	1
Cough	Respiratory system	1
Asthma	Respiratory system	5
Respiratory disorder	Respiratory system	1
Conjunctivitis	Skin and Appendages	1
Epidermitis	Skin and Appendages	1
Inflammation	Skin and Appendages	1
Kidney function abn.	Urogenital system	2
Glomerulitis	Urogenital system	1
Nephritis	Urogenital system	1

* According to COSTART classification system (8).

Curriculum Vitae

The author of this thesis has been born on October 10th, 1952 in Amsterdam, The Netherlands. He is married and has two daughters.

After graduation from high school in 1973, he started to study biology and biochemistry at the University of Amsterdam from which he received his MSc degree in 1981.

In January 1981, he started his professional career as a Clinical Research Associate at the Clinicial Research Department of the Dutch pharmaceutical company, Duphar B.V., Weesp, The Netherlands. During his temporary function as secretary of the Internal Clinical Research Review Committee and subsequently as product specialist, responsible for GCP-quality clinical studies, he became particularly interested in methodological aspects of drug development of new chemical entities.

From 1983-1987, he has worked in the gastroenterology clinical group as Clinical Research Associate, Product Specialist and finally as Group Leader. After designing a clinical research programme for the clinical development of an antimuscarinic agent for the treatment of the irritable bowel syndrome and the succesful completion of its first clinical studies (phase I), he switched to the field of influenza.

In 1988, he wrote a clinical research programme to evaluate some methodological aspects of serological vaccination studies. This research programme has been successfully completed and has been the basis for this thesis.

At the moment, he functions as Medical Relations Officer at The International Medical department of Solvay Duphar B.V. and is chairman of an internal Influenza Research Working Group.

The author represents Solvay Duphar B.V. on the board of the "Foundation for Respiratory Virology in particular Influenza". The foundation represents a formal scientific cooperation between Solvay Duphar B.V. and the National Influenza Centre at the Erasmus University, Rotterdam, The Netherlands.

PROFESSIONAL TRAINING

Basic/refresher course for medical department personnel.
One week course given by Clinical Research Services, LTD. (Maxwell; Coventry, UK, 1981).

Statistics for clinical research.
One week intensive course given by statisticians of Duphar and Prof. R. van Strik (Columbus, Ohio, USA, 1984).

Clinical Interpretation of Investigational Data.
Three days course by centre for professional advancement (B. Spilker; The Hague, The Netherlands, 1986).

Strategies for meeting FDA requirements for health products derived from biotechnology research.
Three days cours by centre for professional advancement (J. Fenno; The Hague, The Netherlands, 1988).

Viral infections; prevention and treatment.
A two-day conference by "Forum" (Schild GC, Tyrrell DAJ, Langford D, Shepherd WM, Fiddion AP, Yeo JM; London, UK, 1988).

MEMBERSHIPS

- Dutch Society for Microbiology
- The International Society for Pharmacoepedemiology
- Board member of Dutch "Foundation for Respiratory Virology in particular Influenza" (Chairman Prof. Dr. N. Masurel)

PUBLICATIONS

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