Influenza Vaccines for the Future
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Influenza vaccines have a short but illustrious history

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Abstract

Isolation of the causative virus of influenza in 1933, followed by the discovery of embryonated hen eggs as a substrate, quickly led to the formulation of vaccines. Virus-containing allantoic fluid was inactivated with formalin. The phenomenon of antigenic drift of the virus HA was soon recognized and, as WHO began to coordinate the world influenza surveillance, it became easier for manufacturers to select an up-to-date virus. Influenza vaccines remain unique in that the virus strain composition is reviewed yearly but modern attempts are being made to free manufacturers from this yolk by investigating internal virus proteins including M2e and NP as "universal" vaccines covering all virus sub types. Recent technical innovations have been the use of Vero and MDCK cells as the virus cell substrate, the testing of two new adjuvants and the exploration of new presentations to the nose or epidermal layers as DNA or antigen mixtures. The international investment into public health measures for a global human outbreak of avian H5N1 influenza is leading to enhanced production of conventional vaccine and to a new research searchlight on T cell epitope vaccines, viral live attenuated carriers of influenza proteins and even more innovative substrates to cultivate virus, including plant cells.

Introduction

When the influenza A virus first emerged from a presumed avian reservoir at the end of the ice age 10 000 or so years ago, there was a distinct difficulty in finding new human victims. For example, at that time, only a few hundred settlers were in the London region near the Royal London Hospital, now a community of four million people. At that time a traveler would have to walk a 100 miles to find another small settlement, perhaps at Stonehenge near Salisbury.

Nowadays we have a truly global community of six billion people, linked so that two million people are moving each day by plane, while perhaps 10 million are journeying in their homelands. Influenza, like all viruses, is opportunistic. In 1918, it had the unprecedented opportunity to spread at the end of the first global war. Ten million soldiers began the move home-

wards and every steamship was packed as they fanned out from France to England, Europe, the US, Canada, Australia, India and SE Asia [1–3]. How perfect for a virus spread by aerosol droplets, close contact and contamination of towels, cups and every day utensils. A virgin population, which had never before encountered the avian virus (H1N1), was on the stage of this theatre of infection. Perhaps a billion people were infected in the next 18 months, and 50–60 million died, making this by far the biggest outbreak of infectious diseases ever recorded, with an impact many times greater than the so-called bubonic plague outbreaks in Medieval Europe. However, more than 2 billion people survived. The overall mortality was around 1%, although in a few semi-closed societies of hunter-gatherers in the Arctic, the mortality from the disease and subsequent starvation as young hunters died and husky dogs attacked and ate the survivors exceeded 90% [4–7]. While most people in the world were infected, we are forced to view the innate protective power of our immune system with awe [8, 9]. We are equipped with 100 000 genes, 7 million years of evolution and 80 000 years of specialization since our emergence from Africa. In contrast, influenza is a miniscule eight-gene vehicle. A recent study [10] of the reproductive number (R0) of the 1918 virus suggests that, unexpectedly, it may have been quite low, not exceeding three persons infected with a single case. This would place pandemic influenza not far above the lowly group of viruses such as small pox and SARS and not reaching the heights that measles has attained. But this unexpected theoretical analysis, if it is not flawed, gives us more practical opportunities to break a chain of infection of a pandemic with antivirals, hygiene and vaccines [11–13].

The new world of the 21st century, although harboring in some countries a few old-fashioned attitudes, akin to "influenza and pneumonia is the old person's friend" nevertheless has the capability for the first time to defend itself against Mother Nature and her threat of influenza. For the first time in history, intense surveillance by the World Health Organization, early identification of a new pandemic influenza virus by molecular diagnostics, application of vaccination and antiviral chemoprophylaxis and possible quarantine and masks, could actually prevent a pandemic arising. For the expressed intention of WHO and the world community of infectious disease researchers is to deflect the first wave of the first pandemic of the 21st century. In this endeavor, our huge resources of natural innate immunity, assisted by new vaccines, will help us. But the formulation of the vaccines and their stockpiling alongside anti-neuraminidase (NI) antivirals will need significant investment of time and money and this has started with a 3 billion euro investment from the USA and EU.

Most recently [14], Baroness Findlay of Glandaff put the epidemiology of influenza H5N1 situation succinctly in the House of Lords Report of Pandemic Influenza "We believe the risk of a pandemic of human-to-human transmissible virus is to be taken very seriously. We believe that it may not happen in the very short time. To explain why we came to this stance; we

believe that the problem, if it does emerge is more likely to emerge in Asia. Asia is where fire fighting must be done today". The Baroness had just heard the background science, that China alone holds 700 million domestic ducks, a possible Trojan Horse of virus persistence, which approximates to 70% of the worlds domestic duck population. Expert evidence from FAO had summarized that China, Indonesia and Vietnam represented the core of the problem, but only 160 million dollars were available at that point in 2005/2006 to help, and bio-security is not imposed strictly, while veterinary services are haphazard.

We are not the first generation of virologists to recognize the influenza pandemic threat, but we are the first to have the knowledge of the avian reservoir and the tools to deal with the problem in a scientific manner. The world capacity for influenza vaccine today of 300 million doses did not arrive by accident: it came to us from the hard work and dedication of four generations of dedicated scientists and doctors. The intention here is to give just tribute to these pioneers and their new discoveries. Using the vaccine methods developed over six decades we can for the first time confront influenza as it emerges, surround it and actually prevent a pandemic. We no longer need to be passive observers at a theatre of infection. Churchill coined the phrase "Give us the tools and we will finish the job". Well, we now have them and we will. Such is the essence and spirit of this chapter.

A snapshot of the first six decades of influenza virology

The serendipitous discovery of infection of ferrets, which produce clinical signs, and the cross infection of a student from a ferret was the first technology foundation stone [9]. Ferrets are used today as a key model to investigate new vaccines.

The two most important technologies, which form the granite like foundation of influenza vaccine research, are the hemagglutination inhibition test (Fig. 1) and the cultivation of virus in embryonated hen's eggs (Fig. 2), first reported in 1941 and 1946 respectively [15, 16].

If one adds two other vital scientific observations, that of Hobson et al. [17] who correlated a HI titer of 40 with protective efficacy, in volunteers in 1972 and then the discovery of a single-radial diffusion for standardization of the hemagglutinin (HA) content of vaccines by Schild in 1973, it is quite apparent that the technologies are all now well tried and tested [18]. The elucidation of the structure of the fragmented influenza genome [19] has quickly led to techniques, genetic reassortment and correlation of functions with certain genes (Fig. 3). From a practical viewpoint, some old much passaged viruses such as A/PR/8/34 (H1N1) grew to extraordinary infectious titers in the egg allantoic cavity, exceeding a new wild-type virus by 100-fold or more. Why not create a reassortant in the laboratory with six replicative genes of A/PR/8/34 to give high replication while having the two new HA

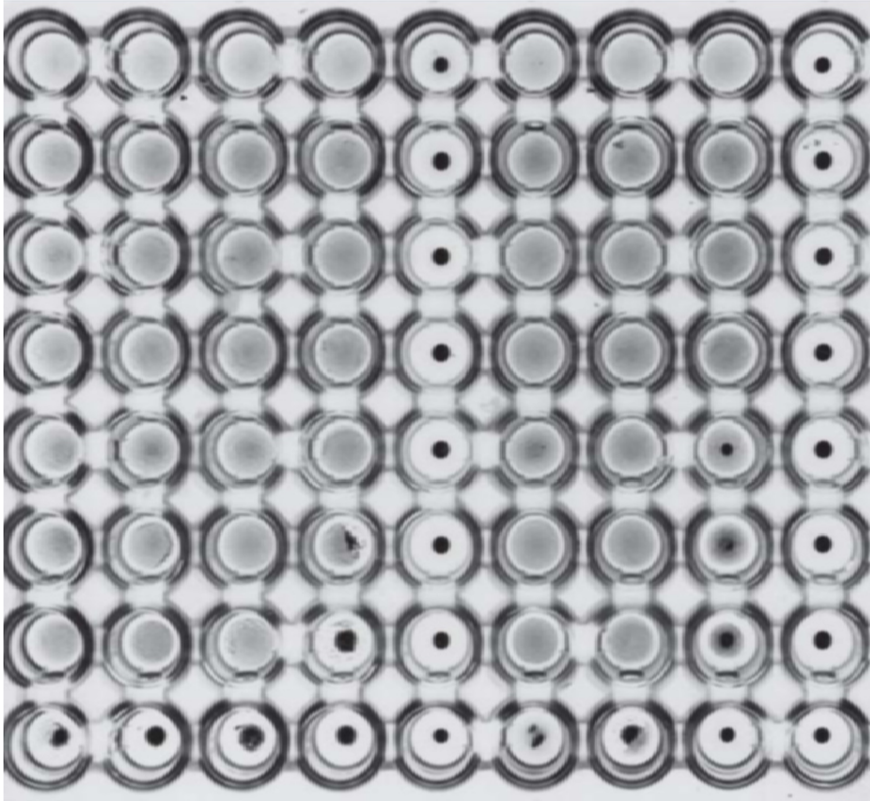

Figure 1. The classic hemagglutination inhibition test
The test depends upon interaction of eight HA units of virus that would normally agglutinate 0.5% turkey red blood cells. Pre-incubation of this standard virus with dilutions of serum antibody abrogate the agglutinating property of the virus (vertical rows 5 and 9). No antibody is detectable in rows 1, 2, 3, 4, 6, 7, 8.

and neuraminidase (NA) genes of the new epidemic virus? This technique proved to be a masterstroke and in the last quarter of a century three laboratories, CSL in Melbourne, NIBSC in London and Ed Kilbourne's laboratory in New York, have rushed each year to produce the new candidate vaccine viruses prefixed IVR-, NIB- and X-, respectively. The almost made-to-order technique of gene reassortment with influenza was also central to producing host range mutants with attenuation genes for live vaccines.

Undoubtedly the simultaneous discovery of the reverse genetics [20, 21] by the three laboratories in New York, Wisconsin and Oxford was a masterstroke in technical advance, which has enabled mutations to be placed, at will, into the genomes of the negative-strand viruses. The conjunction of older and newer techniques with the licensing of the mammalian cell lines from monkey kidney (Vero) [22], dog kidney (MDCK) [23] or human tis-

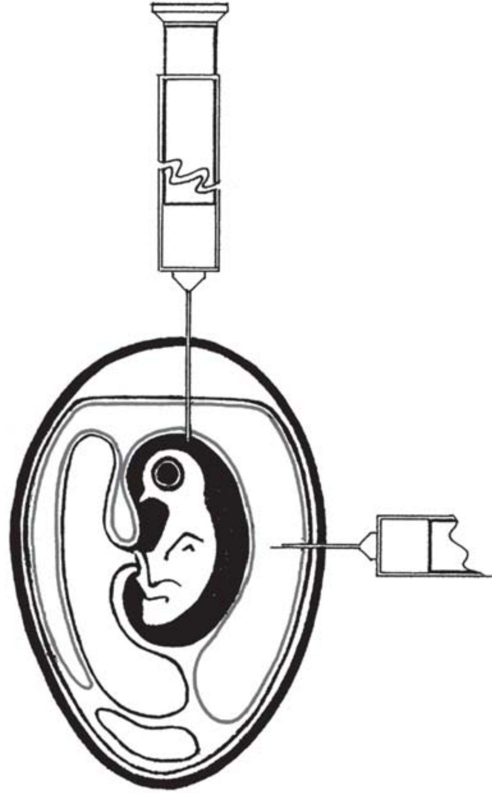

Figure 2. Inoculation of embryonated hens eggs to grow influenza virus for vaccine
Virus is inoculated through the shell of a 10-day-old embryonated hen's egg and more rarely in the research laboratory into the amniotic cavity (top). After 2 days incubation at 37 °C, the clear fluids are removed and titrated for HA by hemagglutination.

sue (PER-6) has led directly to the newly emerging influenza vaccines of the 21st century upon which societies will place such weight in the face of threats of influenza epidemics and pandemics.

The historical steps in killed vaccine development

The first experiments on the attempted immunization of animals were made in the USA by Francis Magill [24] and in England by Andrewes and Smith in 1937 [25]. Mouse lung suspensions or filtrates were used after inactivation with formaldehyde, and it was found relatively easy to protect mice against intranasal infection with influenza. Immunization experiments in man were accelerated when allantonic fluid preparations of virus formed the starting

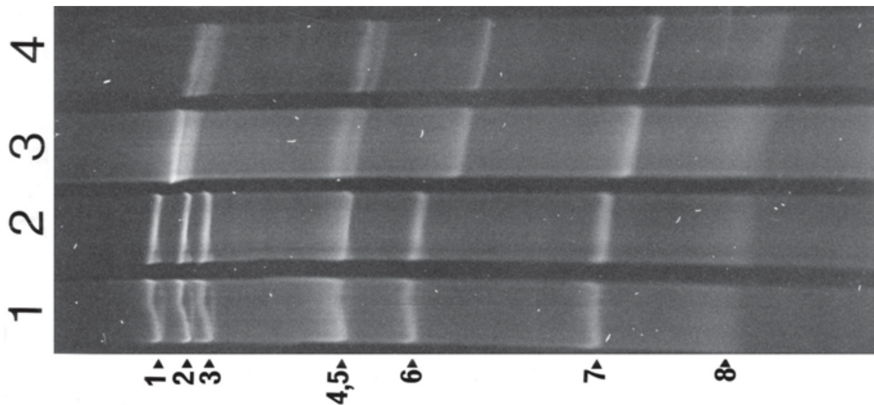

Figure 3. The influenza genome is in eight fragments.
The genome could be labeled with ^{32}P extracted and separated on polyacrylamide gels.

material soon after the technique of allantoic inoculation of fertile hen's eggs was discovered [16]. The first field trial demonstrating short-term protection by inactivated vaccine took place in the United States during a sharp epidemic of influenza in 1943 (Commission Influenza, 1944) [26].

Progress with the development of purer, more potent vaccines has proceeded steadily since those early days and technical advances with ultracentrifugation, by methods producing richer cultures and chemical inactivation avoiding too great a modification of the surface HA and NA antigens have all helped. To avoid the relatively high rate of local and general systemic reactions caused by inactivated whole-virus vaccines, chemical treatment to disrupt the particle and to separate the wanted antigens (HA and NA) from other constituents of the virus has led to a variety of different split or subunit vaccines (Figs 4–6). Ether extraction [27, 28], deoxycholate treatment [29] and treatment with other detergents have been introduced. Some methods have provided subunit vaccines causing fewer clinical side reactions than the older whole-virus particle vaccines, but drawbacks have appeared, including that of reduced antigenicity. Adjuvants of oily emulsions promised potent vaccines with excellent antibody responses, and few reactions were first encountered. But a rare abscess at the site of inoculation caused much distress and this early approach had to be abandoned. In spite of attempts to develop safer materials none have yet been developed commercially until very recently. Thus, after 60 years work, the hope of an ideal inactivated vaccine, free from the induction of clinical reactions and yet potent immunogenically has just been fulfilled with pandemic H5N1 vaccines.

In 1946, a major antigenic deviation of influenza A virus occurred with the appearance of A/CAM/46 (H1N1) virus in Australia. In the USA and Europe outbreaks of influenza occurred early in 1947, which were due to

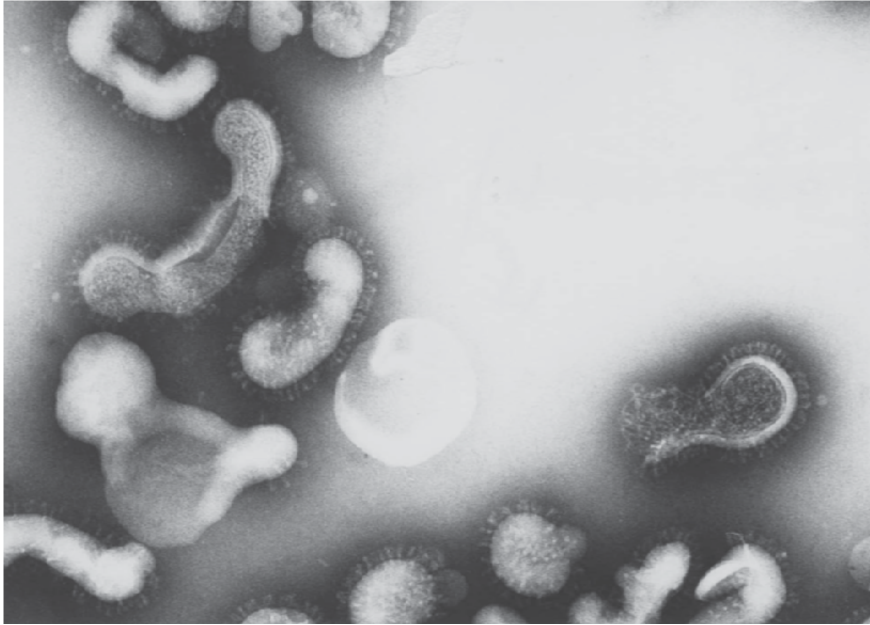

Figure 4. Whole-virus vaccine
Influenza viruses are pleomorphic with a fringe of HA and NA spikes.

the same virus; some communities previously receiving vaccine containing PR8 and Weiss viruses (H0N1 in the old classification and now reclassified H1N1) were attacked. This time the vaccine did not protect against the new virus typified by the prototype A/FM/1/47 (H1N1) [30, 31] and this led to realization of the enormous importance of the updated antigenic make-up of inactivated vaccine.

Yet other difficulties have become appreciated, one of which is the inappropriate antibody response occurring sometimes after inoculation, when the vaccine induces cross-reacting antibody to heterologous viruses or the first virus in the subtype which the vaccinee first experienced, rather than that appropriate to the specific antigen – HA – of the vaccine virus. This response is probably allied to the phenomenon of "original antigenic sin".

Vaccine purification historical and present

The starting materials for almost all types of inactivated vaccine are allantoic fluids from fertile hen's eggs previously inoculated with a seed culture, the yield of which is enhanced using a recombinant virus, one parent of which is a high yielding laboratory strain (A/PR8/34) and the other acts as the donor

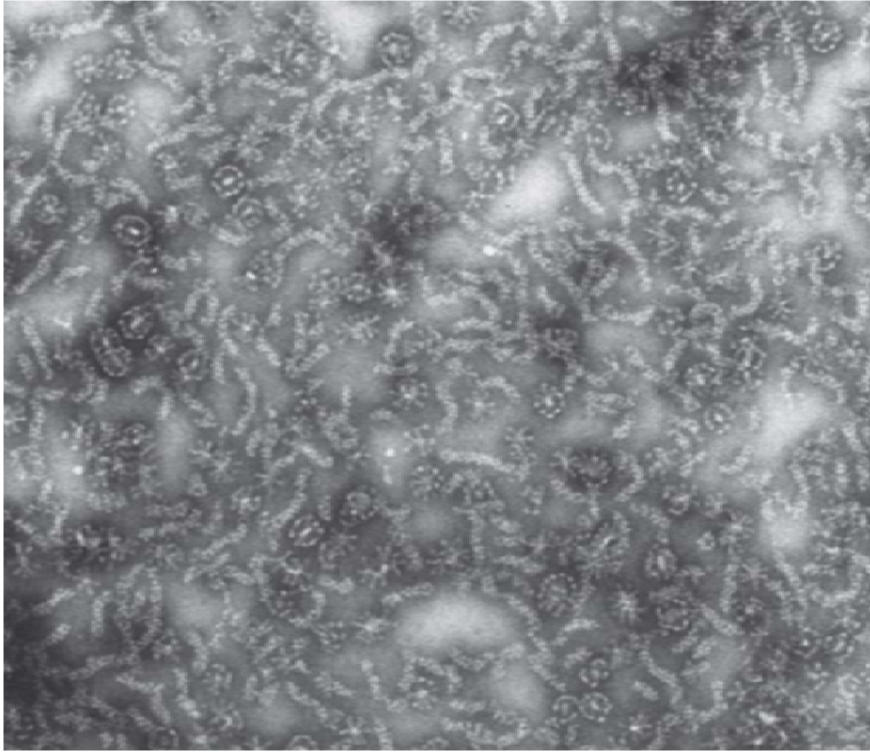

Figure 5. Split influenza virus vaccine
The whole virus is disrupted with detergent, which dissolves the lipid membrane releasing HA, NA and internal NP, seen as "lamb tails".

of the requisite surface HA and NA antigens from a wild-type virus [32]. Purification from unwanted egg material is accomplished by ultracentrifugation on a zonal ultracentrifuge [33]. Whole-virus particles thus separated are inactivated by formalin or β propiolactone, the HA content being as high as possible commensurate with the necessity to avoid febrile reactions after inoculation. Children were sensitive to the older whole-virus vaccines; as many as 30% under 2 years developed fever after 0.25 ml vaccine and up to 8% of 6-year-old children were similarly affected after 0.5 ml [34]. The precise constituent producing the fever was not clearly identified, but the viral proteins were believed to be concerned [35, 36].

Separation of the HA and NA by means of detergents such as Tween 80 or Triton N101 produced split-virus or subunit vaccine and general experience suggested that these materials are less pyrogenic, but less immunogenic, than whole-virus vaccine [37]. This was particularly well demonstrated by studies during the swine influenza campaign in the USA in 1976, when many

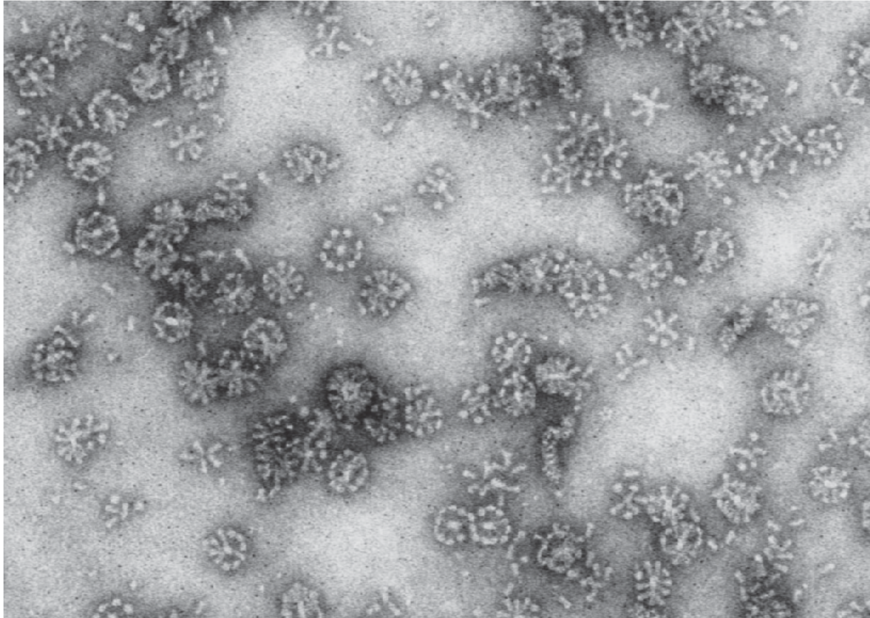

Figure 6. Subunit influenza virus vaccine
The split virus is fractioned in a sucrose gradient and the HA and NA subunits are separated from NP, and M, and standardized by SRD and used for vaccine.

observers reported results, which ultimately led to the recommended use in children of split-type rather than whole-virus vaccines. Such recommendations continue at the present time. In adults, too, the older whole-virus vaccines gave a higher proportion of febrile reactions than split virus [38].

Early progress: The standardization of potency, composition and dosage of inactivated vaccines

Former methods for assays of the potency of inactivated vaccine depended upon measuring the HA activities of the vaccines with erythrocyte suspensions using the Salk pattern technique of Miller and Stanley [15]. In a major technological breakthrough, Schild et al. [18] proposed a method of assay based on single-radial immunodiffusion (SRD) (Fig. 7). The HA antigen content of vaccines was estimated using SRD tests in agarose gels containing specific HI antibodies. The SRD method was modified and refined by Wood et al. [39]. The SRD technique was valid for both whole-virus and split-virus vaccines and was quickly adopted for international use and is still the gold standard. In this test, vaccine virus preparations and refer-

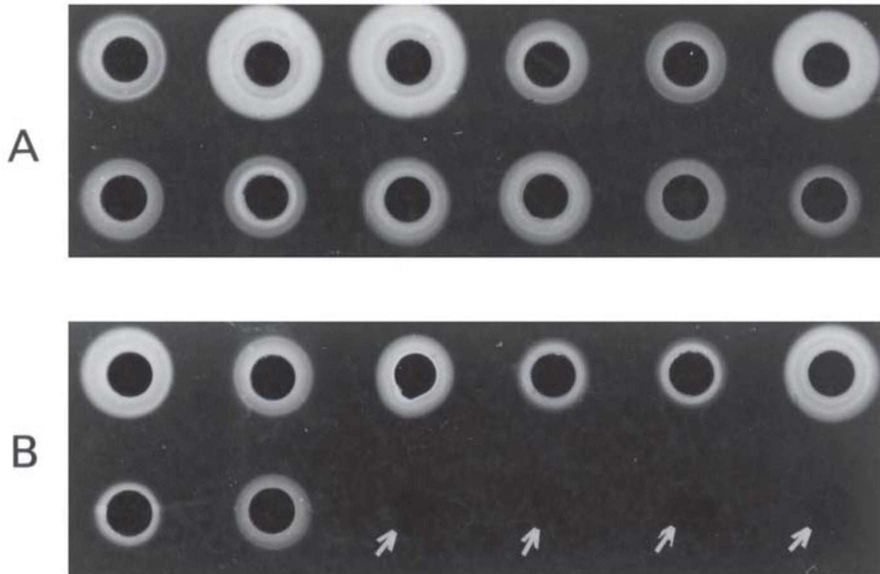

Figure 7. Single-radial diffusion (SRD) test to standardize HA
Vaccine antigen is pipetted into 3-mm wells in an agar plate containing specific anti-HA, -NA and -NP antibodies. After a few hours incubation a zone of precipitation is quantified and the area is proportional to the quantity of HA in the vaccine.

ence antigen calibrated in terms of micrograms of HA are disrupted with detergent and dilutions of the treated antigens are introduced into wells in SRD immunoplates. The size of the precipitation ring obtained for the vaccine is compared with that obtained with a reference antigen of calibrated HA content titrated on the same plate. The vaccine potency is measured in terms of micrograms of HA per vaccine dose. Inactivated influenza vaccines frequently contain two or more virus strains and the HA content of each component (15 μg) is assayed independently.

HA dosage of vaccines and relationship to HI antibody response

It has been known for many years that the serological response to inactivated vaccine depends on the previous experience of the recipient to infection by viruses of the same subtype of influenza A virus as that present in the vaccine. Whereas a single subcutaneous injection of (H1N1) vaccine gave as good a response as two doses prior to 1957, the advent of the new pandemic A/Asian (H2N2) virus produced a different effect. Thus, Holland et al. [40] demonstrated that two doses at an interval of 2 or more weeks produced a better response to one dose and in this regard the vaccine induced immune

response was much inferior to that noted before the change in virus subtype. Such an experience was again noted during the first year of circulation of A/Hong Kong (H3N2) virus and also when the A/New Jersey/76 (Hsw1N1) vaccine was used in children and young adults. Also, in the circumstances of 1977–1978, when most persons under 25 years of age had no previous antibody to the re-circulating H1N1 virus, a two-dose regimen for children and young adults produced a more satisfactory response than a single injection [41]. The contrast between the effects of a single dose of vaccine in persons infected with H1N1 viruses at least 20 years earlier was very striking. These data have immediate relevance today in terms of H5N1 vaccine.

Several factors are of importance in the determination of the quantity and the precise composition of the antibody response to the surface antigens of the virus present in inactivated vaccine. First and foremost, the quantities of HI and NI antibodies induced by vaccine are broadly related to the quantity of antigen present in a single dose. Secondly, the precise composition of the antibodies formed in response to influenza A virus is important. Thus, reinforcement of previously acquired antibodies by the orientation of the B lymphocyte response to the first infection by the particular subtype of virus experienced in childhood or later may take precedence over the strain-specific antibody response to the vaccine virus. Thirdly, the precise response is influenced by the route by which the vaccine is presented to the body's immune system.

First then, several earlier studies reported a graded relationship between the quantity of antigen inoculated and the antibody response that results. This was so in the study of Mostow et al. [42], who gave increasing doses of vaccine in a single injection containing 300–4600 chick cell agglutination units (CCA) containing A/Japan/57 (H2N2) virus groups of volunteers. The serum HI response was tested with four different H2N2 viruses isolated 1962–1967 and also the homologous virus. With more than a 10-fold increase in HA from the least to the highest dose, the geometric mean titer (GMT) of antibody increased only 5-fold. Similar results were obtained by Potter et al. [43], who inoculated student volunteers with vaccines ranging in dosage from 5 to 400 International Units and containing A/Port Chalmers/73 (H3N2) virus. The vaccine was a surface-antigen detergent-treated material [44] adsorbed to aluminum hydroxide gel. GMT HI serum titers increased against homologous virus from 8- to 174-fold with the increase in dose of vaccine HA. Three other H3N2 strains and A/Singapore/57 (H2N2) virus were also tested and all three H3N2 viruses showed graded HI antibody responses proportional in magnitude to increase in antigen dose, as did the homologous virus.

The Pandemic Working Group of the MRC Committee on Influenza Vaccine [45] gave graded doses of whole-virus vaccine containing the A/New Jersery/76 (Hsw1N1) strain to groups of volunteers in 1976. Those less than 44 years of age, who did not possess significant serum HI antibody to the virus before immunization, showed a post-vaccination antibody titer ranging from 64 to 148 GMT with a nearly 8-fold increase in dose from 8 to

61 μg HA. Above this age, in those 45 to 64 with pre-existing Hsw1 antibody, there was an increase in antibody titer from 7 to 36 times (GMT) with a change in HA from 4 to 61 μg. Thus, the effect of increasing the potency of this vaccine on the antibody response was much greater in those sera, which indicated that they had been exposed to the antigen, presumably by infection with a related virus, than in those with no such exposure. Both whole and detergent-split-virus vaccines showed a relatively poor HI response in volunteers less than 25 years of age whose initial serum had no significant amount of pre-vaccination or post-infection HI antibody. In this group of subjects two doses of vaccine gave a better antibody response than did one, but the resultant post-vaccination GMT was half that obtained with a single dose in the vaccinees over 25 years of age.

These examples underline the practical importance of a considerable degree of antigenic drift within a subtype comprising HI antibody response. Also, the recall of antibodies induced by previous infection illustrates the general rule that an up-to-date monovalent vaccine reinforces antibodies against former members of the subtype, while also inducing specific antibodies to the vaccine virus. This was clearly shown by direct comparison of monovalent and polyvalent vaccines such as the MRC Committee on Influenza Vaccine's trials [46–49].

The quantitative dose response already described for HI is also found with NI antibody, but is less consistent. Thus, Potter et al. [50] noted that there was a 2- to 6-fold increase in NI antibody as vaccine potency was increased from 5 to 400 IU HA. Yet the trial of A/New Jersey/76 (Hsw1N1) vaccine conducted by the Pandemic Working Group of the MRC Influenza Vaccine Committee [45] found only a slight increase in NI antibody after an increased dose from 100 to 200 IU using 100 IU HA in the vaccine. Nicholson et al. [41] gave a whole-virus vaccine of the A/USSR/77 (H1N1) virus, which ranged in potency up to 6-fold, and found, in those under 25, a 3-fold increase in NI antibody. However, in those over 25 years of age an increase in dose of vaccine had a less constant effect on NI antibody formation. One possible reason for the variation in the effect of different vaccines on the NI antibody is the lack of consistency in the NA content [51]; however, another possibility may be that immunological priming to the HA in the vaccine can in some way suppress the immunogenicity of the NA antigen, which may be physically associated with the HA.

The second important variable in the immune response to inactivated vaccine arises from the relative amounts of cross-reactive and strain-specific antibodies that are generated. The differentiation of these requires special techniques such as SRD and the adsorption studies. Webster et al. [52] compared, in adults, the response to an A/Port Chalmers/73 (H3N2) subunit vaccine to homologous and heterologous H3N2 viruses. Most of the antibody was cross-reactive with A/Hong Kong/68 virus but when higher doses of the vaccines were employed, strain-specific A/Port Chalmers/73 antibody was produced in addition to that against heterologous virus. Oxford et al.

[53, 54] compared whole- and split-virus vaccines containing A/Victoria/75 or A/Scotland/74 viruses and using single-radial-hemolysis and adsorption techniques showed that in an immunized adult, cross-reactive antibody was induced much more frequently than specific antibody against homologous virus. They showed the same phenomenon in adults during infection with A/Port Chalmers/73 virus, who frequently also developed antibody rises to A/Hong Kong antigens from 1968. Oxford et al. [54] used similar techniques to analyze sera from children aged 3–6 years immunized with a surface-antigen vaccine containing A/Victoria/75 (H3N2) antigens. Most children produced a strain-specific serum antibody to the vaccine antigens, whereas adults similarly vaccinated tended to produce antibody cross-reacting with all variants of the H3N2 subtype tested. Post-epidemic sera from those of various ages recently infected by A/Texas/77 –like strain showed cross-reactive antibody in adults but in contrast mostly strain-specific responses in children. Strain-specific antibody is considered to be more protective.

The route of vaccination

The influence of the route of immunization with inactivated vaccine has been studied in the past by many observers. The chief alternative to the subcutaneous–intramuscular route is intradermal injection using a reduced amount of vaccine. The advantages of this route are economy and the avoidance of febrile reaction. The principal disadvantage is the fact that the antibody response is less consistent. It was shown by Appleby et al. [55] that the GMT after intradermal vaccine was less than half that obtained with subcutaneous vaccine, and this seemed logical in that only one-tenth of the vaccine dose was given intradermally. McCarroll and Kilbourne [56] found little difference in the antibody responses to intradermal and subcutaneous vaccines in equivalent doses. Tauraso et al. [57] reinvestigated the question using a two-dose regime before the arrival of the A/Hong Kong/68 (H3N2) epidemic. In the equivalent amount of 0.1 ml vaccine, antibodies formed in higher titer after intradermal than subcutaneous vaccine. However, the titers after 0.5 ml vaccine subcutaneously were little different than after intradermal injection of 0.1 ml. It is considered advisable, however, in practice to limit intradermal vaccination to when the vaccine is in short supply or when, in children or the aged, reactions after subcutaneous vaccine might pose problems.

The nasal route of inoculation either by instillation of drops or by spray was first studied in detail by Waldman et al. [58]. Compared with the subcutaneous vaccine in a dose of 0.5 ml, antibodies capable of neutralizing the virus A/Taiwan/64 (H3N2) increased to a greater extent in sputum and nasal secretions after repeated nasal inoculation with a total volume of 3.6 ml vaccine. In contrast, the intranasal vaccine produced a much lower rise in serum antibody, the GMT being only one-sixth that after subcutaneous vaccine. Waldman et al. [59], using an aerosol spray found that a better

serum antibody response occurred with a small-sized particle spray than a larger one, but the nasal antibody response was better after the latter or with nasal drops. Absorption studies showed that a majority of the secretory antibody (IgA) response in nasal secretion was cross-reactive with heterologous viruses (A/Hong Kong/68 H3N2). Phillips et al. [60] compared subcutaneous or intradermal vaccine in nurses with vaccine dropped intranasally. The subcutaneous route produced the best serum antibody rises, and intradermal vaccine was superior to the intranasal route in terms of antibody response. The nasal antibody titers after immunization by either subcutaneous or respiratory routes paralleled those in serum.

The fact that nasal antibodies increase after subcutaneous vaccine [61, 62] is important because the lack of a good response in serum antibody in those given the same vaccine intranasally is a limitation hardly offset by local nasal secretory changes. Challenge of immunized groups of persons by live attenuated virus also supports the view that nasal antibodies play a supplementary role to serum HI antibody [63].

Early quantification of side reactions to vaccines: Whole virus *versus* split and subunit

The field trials of inactivated vaccines in 1976 and 1977 added to knowledge concerning the reactogenicity of different preparations. The split-virus type of vaccine then used unquestionably caused fewer systemic febrile responses in both children and adults. The fact that reactions with whole-virus vaccines used at the time were unpleasantly severe for those without serum antibodies to the vaccine virus before inoculation had not been fully appreciated. In the case of children aged 6–18 in the American trials of A/New Jersey/76 (Hsw1N1) virus, the most potent vaccines caused fever in up to 63% of vaccinees. In the UK, the Pandemic Working Group of the MRC Committee on Influenza Vaccine found that a dose of 61 μg HA (1000 IU) of whole-virus vaccine with the same Hsw1N1 strain produced, in adults, local reactions in 50% and systemic effects in over 60% of volunteers. Even the lower doses of 18–27 μg HA caused local reactions in 50% and systemic effects in 40%. The A/USSR/77 (H1N1) virus vaccine trial in 1978 in Britain showed that adsorbed or aqueous split-virus vaccine produced fewer reactions than did whole virus [51]. After a second dose of the same vaccine, fewer volunteers experienced reactions than seen after the first dose. Later studies of the endotoxin content of various pools of inactivated type A or B vaccines using the limulus lysate test gave no hint of a parallel between the occurrence of general reactions and the endotoxin content [64].

Neurological illness is a recognized sequel to immunization with a variety of vaccines but had not previously been observed with any frequency after influenza virus vaccines. Wells [65] noted the rare instance of Guillain-Barré Syndrome (GBS), which appeared in excess among the persons

recently vaccinated with A/Swine vaccine compared with the numbers in unvaccinated individuals. Of 1098 persons with GBS reported from 1 October 1976 to the 31 January 1977, 532 had received vaccine prior to the onset of neurological symptoms. The overall risk of GBS was calculated as ten cases per million vaccinated. The rate of occurrence during the 10-week swine vaccine period was five to six times greater than in unvaccinated persons. However, the excess in number was greater in the 2nd and 3rd weeks after inoculation than either the 1st or subsequent weeks. As reported by Langmuir [66], GBS was not associated with a particular variety of vaccine or age group. However, that numbers were slightly greater in those aged 25–44 than in middle aged or elderly persons appears to rule out the possibility that the syndrome was, in some way, related to the absence of antibodies to the swine virus before immunization, for most of those over 45 would have been exposed to antigens of this virus many years before. After the swine influenza campaign was terminated, surveillance was continued, and during the period 1978–1979, when 12.5 million doses of ordinary inactivated vaccine were estimated to have been used, the related risk of GBS was 1.4 times the incidence in unvaccinated persons. This risk was regarded as not significant [67]. No clue to the cause of the marginally increased risk of GBS in immunized persons in1976 has yet been obtained, but could be virus strain related.

Advent of the 1968 pandemic virus and use of inactivated vaccines

At the time when A/Hong Kong /68 (H3N2) virus was spreading in Asia, plans were made by the MRC Committee on Influenza Vaccine to protect children in residential schools and other groups in a controlled manner. Inactivated polyvalent vaccine containing two H2N2 viruses (A/England/64 and A/England/66) and a B strain were compared with an H3N2 A/Hong Kong whole or deoxycholate-treated virus vaccine in initial serological trials. Antibody formation even in those without detectable serum HI antibody gave GMTs over 100 in those receiving A/Hong Kong vaccine intramuscularly. However, controlled trials in two boarding schools showed no convincing evidence of protection. In uncontrolled trials in other schools either the polyvalent or the A/Hong Kong vaccine were given or no vaccine at all. There were 12 schools where epidemics of influenza occurred in January and February 1969 but no evidence of protection was found in those receiving A/HK vaccines. The only clue obtained concerning the vaccine failure was first that only one dose of vaccine had been given, and this is known to be inadequate to give a satisfactory antibody response in previously seronegative persons, and secondly, there was an interval between vaccine administration and infection of 2–4 months. These two factors may have combined to explain the absence of protection because of the inadequacy of the antibody response at the time of challenge. It would be

fair to add that others [68, 69] did obtain protection from A/Hong Kong/68 whole-virus vaccine during the first outbreak of influenza due to this virus in the USA. The use of modern adjuvanted H5N1 vaccine in two doses is anticipated to give protective effects.

First studies with live influenza vaccines

The use of living but attenuated virus as an immunizing agent developed slowly from the initial studies of Mawson and Swan [70] in Australia and the USSR. The major difficulty of the lack of a laboratory test to indicate that cultured virus had lost its pathogenicity, while retaining infectivity for man, meant that deliberate intranasal inoculation of volunteers furnished the only way to select a suitable strain for infection without causing clinical reaction. In spite of the widespread adoption of live vaccines selected by this method and given as an intranasal spray in the USSR, little interest was exhibited in most other countries. From 1956 onwards, trials took place in volunteers in England and Wales to provide evidence of safety and immunogenicity of cultured viruses, and the drawback of a reduced infectivity of well-attenuated viruses handicapped progress. The necessity to observe a match between the antigens of epidemic viruses and those present in the vaccine was a further drawback until the technique of reassortment of characters between two strains, one of which was of proven attenuation, was utilized to yield seed viruses with the desirable clinical and antigenic properties. Other disadvantages of live viruses appeared during the intensive researches of the 1980s particularly in the USA and in England [71, 72]. It cannot yet be claimed that the ideal live attenuated virus vaccine has been formulated, but reverse genetics and increased knowledge of virulence genes have now lead to a resurgence of interest.

In the 1980s, genetic studies were intensively pursued in attempts first to define the particular gene or combination of genes, donated by the attenuated virus that confers the property of attenuation upon the reassortant strain. It was found that the biological properties of excreted virus may be altered compared with those of the original virus in the vaccine and the manner of this alteration was also studied genetically. Such work is essential in achieving the goal of an effective and safe vaccine virus for human use. Experimental inoculations were carried out initially in small-scale tests in volunteers under semi-isolation to permit close observation (see below).

Host-range virus mutants as live vaccines

Multiple cultivation and passage of viruses either in animal hosts such as ferrets and mice, or in developing chick embryos or tissue cultures had been practiced even before the use of temperature-sensitive (*ts*) or cold-adapted

(*ca*) mutants was suggested. Early workers in Britain used the PR8/34 virus as a host range mutant, which, although noninfective for man, has retained animal pathogenicity even after many passages in eggs. As a donor parent with good powers of multiplication in the laboratory, PR8 was mated with various strains of wild-type influenza A viruses to obtain recombinants with up-to-date surface HA and NA antigens. This method was preferable to simple laboratory cultivation because some viruses failed to alter in pathogenicity after as many as 30 serial passes in cultures [73], although other virus strains appeared to become attenuated with only a few passages in eggs.

PR8 virus was chosen also by workers in Belgium who prepared reassortants from a number of viruses, some of which were licensed for human use [74]. To select recombinants with as high proportion of RNA components as possible derived from the host range mutant PR8, Florent et al. [75] used RNA-RNA hybridization to identify gene origins. Later the gene constellation of four of the candidate vaccine viruses were determined and Florent [76] found that some clones of Beare and Hall's [77] recombinants of PR8 and A/Englannd/69 (H3N2) containing five genes from PR8 were satisfactorily attenuated. However, one clone though containing six PR8 genes was nevertheless clinically virulent to volunteers. A further genetic study of PR8 host range recombinants using viruses tested clinically by Beare and Reed [78] was made by Oxford et al. [79]. It was again found that recombinants from PR8 and A/England/69 viruses could contain only the surface HA and NA genes from wild-type virus and yet retain virulence for man.

Additional attempts to stabilize the attenuation of candidate viruses were made both by Beare at the Medical Research Council's laboratories at Salisbury and the RIT workers by rendering the virus resistant to an inhibitor present in normal horse serum. This property was present in the RIT series of recombinants. It seems strange that stabilization has not been pursued since, nor has cultivation of host range mutant viruses, such as PR8, at abnormally low temperatures, such as 25 °C. This method was found by Sabin [80] to be preferable to normal temperatures when attenuating polio viruses, and it was exploited by both workers in the USA and USSR.

Marker tests, which can be equated with attenuation of virulence for man, were sought with relatively variable results. One such test used weanling rats that were inoculated intranasally first with virus and later with cultures of *Haemophilus influenzae*. Virulent virus induces bacteremia and meningitis and using this method Jennings et al. [81] successfully separated a number of reassortant viruses and obtained some correlation with clinical virulence. Yet the host range mutant parent PR8/34 and RIT 4050, which are both attenuated in man, were classed as virulent by the rat.

A new approach at that time used an avian (duck) virus, which was found to have only low pathogenicity for squirrel monkeys inoculated intranasally and was proposed as a donor of attenuation. A reassortant with a virulent humanA/Udorn/72 (H3N2) virus behaved as did the avian parent in the squirrel monkey, although immunizing the latter against the virulent

parent. Clinical trials have suggested that this virus is attenuated for man and is immunogenic, but has not been investigated since [82].

Temperature-sensitive virus mutants as live vaccines

Most work on the development of viruses with restricted multiplication at temperatures above the normal range for cultivation has been affected by Chanock, Murphy and associates at the National Institutes of Health, Bethesda [83]. The technique employed chemically produced mutation in virus RNA by cultivation in the presence of the mutagenic agent 5-fluorouracil. After cultivation and plaquing at 33°, 37° and 38°C, mutant viruses with the requisite temperature sensitivity were obtained. Intranasal inoculation of hamsters confirmed temperature restriction, in that much lower titers of virus were found in the hotter lungs than in the cooler upper respiratory tract.

Spread from inoculated volunteers to adults in contact was not observed and no evidence of a change in virulence was found in viruses recovered from adult recipients of vaccine [84]. But in seronegative children, the A/Hong Kong/68-*ts*-l [E] virus both produced mild febrile reactions and a virus that had lost its properties was recovered from some who were infected.

A second series of *ts*-1a2 was then developed by combining two defective ts viruses, each of which belonged to a different complementation group in respect of the genetic defect. The progeny exhibited greater temperature restriction than the *ts*-1[E] line of viruses. It was termed A/Udorn/72 *ts*-1A2, and it was recombined with three further viruses; wild-type A/Victoria/3/75, A/Alaska/77 (H3N2) and also A/Hong Kong/77 (H1N1). These *ts*-1A2 viruses were highly immunogenic and exhibited temperature restriction of multiplication in cell cultures and reduced replication in the hamster lung. The A/Victoria/3/75-*ts*-1A2 recombinant retained its ts properties after inoculation into doubly seronegative children. Unfortunately, when the A/Alaska/77-*ts*-1A2 virus was similarly tested in a single child after tests in adults had shown genetic stability, the nasal secretions of the vaccinee yielded a ts-positive virus that produced plaques at 39°C even though the child had shown no symptoms or fever. The recombinant 1A2 virus with A/HongKong/77 (H1N1) parent exhibited a capacity to infect 70% of doubly seronegative adults and was attenuated compared with the wild-type parent. Nevertheless, it appeared possible that a virus such as the A/Alaska-*ts*-1A2 might, if transferred to contacts from an inoculated child, result in clinical illness and clinical studies with this particular virus were not pursued.

Cold-adapted virus mutants as live vaccines

Beginning with a strain of H2N2 virus recovered in Ann Arbor, Michigan in 1960 by cultivation of throat washings in tissue cultures at 36°C, Maassab

[85, 86] evolved a virus, A/Ann Arbor/6/60 (H2N2), which has acted as a donor of attenuation to other viruses by genetic reassortment. Earlier passages were made in chick kidney tissue cultures followed by intranasal passages in mice and then a gradual adaptation to lower temperatures, in tissue cultures and in developing hens' eggs inoculated allantoically, led to a virus with good powers of multiplication at 25 °C. The ca variant was found to retain the infectivity of the original strain for both the mouse and ferret, although it produced no deaths in mice and no fever or turbinate lesions in ferrets, whereas the original virus was pathogenic for both species. The virus proved to be temperature sensitive with a shut-off temperature of 37 °C [87]. Recombinants with wild-type viruses of both H2N2 and H3N2 subtypes were prepared, studied in the laboratory and in volunteers and analyzed genetically. The original A/Ann Arbor/6/60 (H2N2) virus was not, however, tested in fully susceptible persons presumably because of the difficulty in that period of finding seronegative adults. A few persons with low titers of serum neutralizing antibodies (1:4 to 1:6) were inoculated and as judged by antibody responses, became infected without undergoing clinical illnesses. More rigorous clinical studies have been pursued with recombinants, in particular those with H3N2 antigens, and details of the results have been bought together and earlier data summarized by Kendal [72]. The donor ca parent has been more recently reassorted with H5N1 genes.

It is clear that infectivity and immunogenicity were fully retained for seronegative adults of whom 111 received H3N2 recombinants. Among those receiving three of four recombinants, clinical reactions were minimal or negligible but with the fourth, derived from the A/Scotland/74 parent, in 4 of 12 volunteers receiving $10^{8.5}$ and in 1 receiving $10^{7.5}$ $TCID_{50}$, there were clinical illnesses. Viruses re-isolated from the vaccinees retained *ts* properties and so did those given recombinants of A/Victoria/75 (H3N2) and A/Alaska/77 (H3N2). However, some loss of *ca* restriction was found in virus re-isolated from volunteers given the A/Scotland/74 recombinant.

Cold-adapted recombinants with A/USSR/77 (H1N1)-like virus have also been studied in adult volunteers and found to be less immunogenic as judged by HI antibody responses. A better response was obtained by Wright et al. [88] in children in Nashville given$10^{6.5}$ $TCID_{50}$ of strain CR 35 (H1N1) and none of 11 children developed adverse clinical reactions even though 8 became infected. All re-isolated viruses retained the ts phenotype. The failure to elicit serum antibody response in adults given this same virus recombinant is puzzling. Using the ELISA enzyme-linked assay, Murphy and others [89] found that by this more sensitive method antibody rises could be demonstrated and the results tallied better with the ability to re-isolate viruses from the inoculated volunteers than did the serum HI responses.

The Leningrad group of workers led by Smorodinstev [90] were the first to obtain a virus indirectly attenuated by cultivation at 25 ºC. The group used strains selected by inoculating volunteers with several viruses derived from cultures repeatedly incubated at 25–26 °C to speed up attenuation. Some

5–7 months were required for the preparation and production of new strains even using genetic recombination to incorporate new surface HA and NA antigens. Although Alexieva et al. [91] found that cold cultivation was not successful in producing reliably attenuated viruses for use in children, the technique was adopted for general use. Genetic studies of the Leningrad viruses are described briefly by Kendal et al. [72] and these parent ca viruses are currently the center of new interest for attenuated H5N1 vaccines.

Usually, preliminary studies were made in the USSR in 18–21-year-old seronegative adults who receive virus twice at intervals of 10–14 days administrated by nasal spray. Viruses were attenuated by passage for varying periods at 25 °C and both donor viruses and recombinants proved temperature sensitive. In 1961–1964, when H2N2 viruses were circulating, 5165 children aged from 1 to 6 years received the ca A/Leningrad/57 (H2N2) virus. Some febrile reactions occurred but only in less than 1% of the children. Further studies of recombinants with H3N2 or H1N1 antigens and the same Leningrad H2N2 parent after 47 serial passages under cold conditions of cultivation (25 °C) were conducted in children, half of whom had no detectable serum antibody to the vaccine strain. No reactions occurred and over 90% of the children responded with antibody production. It is clear from the earlier papers by Alexieva et al. [91, 92] that intranasal administration of children aged 7–15 years were too reactogenic and that this is the reason why the peroral route has been chosen for routine administration in the USSR.

A Japanese virus recovered in 1957, (A/Okuda/57(H2N2), was found to be attenuated for children and served as a donor of attenuation both in Japan and in England. Japanese workers, Zhilova et al. [92] developed a recombinant virus (KO-1) from ultraviolet-irradiated A/Okuda/57 and wild-type A/Kumamoto/22/76 (H3N2). Serial passaging in eggs, in the presence of normal horse serum was followed by plaque purification and later clinical tests in a few children. The M (membrane) gene was found to have been donated by the Okuda parent. From reassortants with other human viruses, a candidate WRL 105 virus was selected and underwent clinical trials without harmful clinical effects [93] but has been little investigated since that time.

Mammalian cell culture vaccines

Cultivation of influenza viruses in mammalian cells rather than eggs initially encouraged two manufactures to invest in cell culture fermenters for vaccine production [22, 23]. Many more groups are now researching these technologies. Capacity can be increased to cope with a surge in demand for a pandemic virus vaccine. Moreover, the final vaccine has the theoretical advantage of the absence of egg proteins. The cell culture vaccine virus is also easier to purify. Where clinical isolates of influenza viruses are culti-

vated in mammalian cells and eggs in parallel, different antigenic variants may be selected [94]. The biological variants have amino acid substitutions in the receptor binding site in proximity to an antigenic site on the HA, and an amino acid change in this region can alter antigenicity. Of the two virus subpopulations that can be selected, the virus which is grown on MDCK (or Vero) cells rather than in eggs appears more closely related to the wild-type clinical virus. There is some indication that cell-grown virus vaccines offer greater protection in animal models than the corresponding egg-grown vaccine. These are all powerful arguments in favor of the new generation of influenza vaccines being cultivated currently in Vero [22] or MDCK [23] or Per 6 cells.

Unlike historical vaccines: could newly developed 21st century vaccines induce protection across the different virus subtypes?

There are 16 known subtypes of the HA of influenza A virus. Only three subtypes have caused pandemics in humans, H1, H2 and H3, while H5, H7 and H9, predominantly circulating in birds have crossed the species barrier into humans and caused human outbreaks. We do not know whether these latter three subtypes could mutate into human-to-human transmitters and thereby acquire pandemic potential. At present H5N1 is causing considerable concern in SE Asia. An important question therefore is whether a vaccine could be engineered to give so-called heterotypic or cross-subtype immunity to protect against all these potentially pandemic viruses. It is well known that the internal proteins of influenza A virus such as M1, M2 and NP are shared by all influenza A viruses. These internally situated proteins are certainly immunogenic (particular NP) but could the immunity induced, either T cell or antibody, be broadly reacting?

To back up the central core of this approach, it has been known for 40 years that mice infected with an influenza A (H1N1) virus would later resist a lethal challenge from an influenza A (H3N2) virus. Given the lack of genetic and antigenic relatedness between the H1 and H3 proteins, or indeed the corresponding N1 and N2 proteins, this strong cross-immunity was attributed to an internal protein such as NP or M. However, it has been difficult to construct a solid database and there has been a lingering doubt about this so-called cross-protective immunity. Most virologists deduced, virtually by elimination, that a cross-reactive portion of the HA (HA2) could have provided the cross protection. Furthermore, this cross protection is particularly seen in the mouse model, leading some to conclude that the mouse recognized cross protection epitopes that perhaps humans did not.

Fundamental studies to correlate the genetics and immunology of NP and M established the cytotoxic T cell response to portions of these proteins. However, the work clearly showed that M2 could be a cross-reactive immunogen, although a relatively weak one [95]. The M2 protein is an integral

membrane protein of influenza A viruses that is expressed at the plasma membrane of virus-infected cells and is also present in small amounts on virions. The important extracellular domain, potentially targeted by antibodies and T cells, is conserved by virtually all influenza A viruses. Even the 1918 pandemic virus differs only in one amino acid. The first indication that the M2 was immunologically active was the observation that an anti-M2 monoclonal antibody reduced the spread of virus cell culture. Not unexpectedly, the antibody reacted with the extracellular domain of M2. Even more excitingly, the antibody reduced the replication of virus in mouse lungs. Immunization studies with M2 constructs, however, have given more mixed results. Immunization of mice with DNA plasmid of M1 and M2 gene gave protection mainly *via* T helper cell activity. An alternative approach utilized a hepatitis B core and M2 fusion protein. The cross protection resided in antibodies, although M2-specific antibodies did not neutralize the virus *in vitro*. Presumably, protection was mediated by an indirect mechanism such as complement-mediated cytotoxicity or antibody-dependant cytotoxicity. However, the protection induced in the mouse model was considerably less than that induced by a conventional sub unit HA/NA vaccine.

It could be argued that weak heterotypic immunity may be present already in the community and that this is helping to prevent the emergence of chicken influenza A (H5N1) in SE Asia [96]. Certainly with evidence of tens of millions of domestic birds infected since late 2003 in 13 countries in SE Asia, with only a handful of human infections and only human-to-human transmission in family groups, there is a possibility that the unique co-circulation since 1977 of two influenza A viruses (H1N1 and H3N2) may have enhanced heterotypic immunity in most communities, which in turn abrogates the emergence of chicken influenza A (H5N1) into humans. It would be foolhardy, though, to take this argument to a fuller conclusion and relax preparations for a new pandemic influenza A virus.

The historical use of volunteers to study influenza and vaccines

At present, with the unprecedented research investment into influenza vaccines, there are new discoveries of adjuvants and vaccine formulations to be tested as well as fundamentals of virus transmission, infectiousness and pathogenicity. The ultimate test is in influenza-infected volunteers. This specialized work was initiated over 60 years ago.

During the great pandemic of 1918, when the precise nature of the causative microbe of the Spanish influenza had not been established, a group of American scientists asked for young volunteers from the army and navy. The quest was to probe the nature of the microbe that was already causing devastation in their own country and where, by 1919, 500,000 young people were to die. However, this was not the first study into the precise nature of the microbe. The infection had first been documented a year earlier as a her-

ald wave in the great city-sized military base and encampment of Etaples [6, 12]. Here the British Army constructed the largest establishment [97] in its history, where 100,000 newly recruited soldiers each day intermingled with thousands of wounded soldiers, pigs and, in the nearby villages and markets, with ducks, domestic chickens and geese. These are now recognized as the necessary biological features of an epicenter for the creation of a pandemic virus. We surmise, in retrospect, that an avian virus from a silently infected goose or duck could have crossed species either to a pig or to a soldier, already infected with a human strain of influenza. This is the mixing bowl hypothesis. Indeed, common epidemic influenza was known to be circulating in the winter of 1916–1917 in Etaples. Another factor in Etaples could have been the hundreds of tons of gas of 25 varieties contaminating the landscape of the nearby Somme battlefield, as well as many of the wounded soldiers brought by the night trains into the 12 hospitals on site and causing respiratory distress. A group of pathologists there and at Abbeville, led by G Gibson, raised the question of the nature of the microbe. Could it be a Gram-negative bacterium such as *H. influenzae*, already described by Pfeiffer as the cause of the previous influenza pandemic of 1889? Or could it be a virus? Viruses were rather unknown entities at that time but had been identified by their filter-passing nature. So Gibson's experiment was quite simply to take sputum from a soldier victim and filter it through a Berkefield candle filter, which would hold back any known bacterium but allow the passage of the much smaller ultrafilterable virus. But what then? Gibson had not even considered that a human volunteer would receive the filtrate. In fact, he gave it to a series of macaques and, inadvertently, to himself. He died and the macaques became ill. His premature discovery of new virus influenza has lain undiscovered and hitherto unquoted in the archives of the First World War [98].

Meanwhile, in the USA, a more vigorous decision had been taken, and army and navy volunteers were infected intranasally with filtered material from Spanish influenza victims. Some volunteers were placed 0.5 m from dying servicemen, who coughed in their faces. The incredible result of this heroic endeavor is that not a single volunteer became ill, whereas all around the USA their companions were dying. It is more than possible that the volunteers had already been subclinically infected in the early summer outbreak of 1918, which was less virulent than the autumn virus and would be expected to give cross immunity.

The MRC common cold and influenza quarantine unit in Salisbury (UK)

As soon as the Second World War was over, the Medical Research Council in the UK established the Common Cold Unit in Salisbury at the Harvard Hospital. The hospital was a donation from the USA to cope with expected

bomb casualties from London. In the event, this fully equipped multi-building facility was used as an acute surgical hospital for servicemen. With Christopher Andrewes as its first chief scientist, the unit recruited volunteers to unravel the virological mysteries of respiratory disease. For the next 40 years, a small team of virologists and clinicians infected volunteers and discovered the first human coronavirus, the common cold virus, and were the first to describe the clinical effects of interferons. Essentially similar units were set up in the USA and Russia.

Estimates of vaccine protection obtained in the past by deliberate challenge in quarantine units

The considerable difficulties encountered in mounting field trials led to experiments in which immunized volunteers were subjected to deliberate inoculation with live virus either in the form of attenuated strain or modified wild-type strain. This protocol was suggested by Henle et al. [99], who immunized a group of children with inactivated influenza A (H1N1) virus vaccine and then inoculated them with egg-cultured virus of the same subtype but recently isolated, by inhalation of an aerosol. High rates of infection (75%) were produced in 28 unimmunized children of whom 10 became ill. Those receiving vaccine either escaped subsequent infection or developed serological changes; only 1 child of the 42 children thus challenged became ill. Although this study illustrated the outstanding success of the immunized protocol, there are probably few observers today who would be prepared to submit their children to a similar risk of deliberately induced illness. Such a risk is, of course, experienced during epidemics and Bell et al. [100] undertook a similar experiment in adult volunteers some of whom were immunized with a single dose of inactivated A/Japan/305/57 (H2N2) virus vaccine soon after the A/Asian epidemic began. The volunteers were isolated before being given intranasally pooled nasopharyngeal washings from patients with influenza and this caused clinical illness in 87% of volunteers previously given a placebo. As 50% of the vaccinated volunteers developed fever after challenge in this experiment, the single injection of inactivated vaccine proved relatively ineffective, presumably because of its inadequate immunogenicity.

The information obtained by deliberate challenge of immunized volunteers has been explored in the past using modified attenuated virus strains. Beare et al. [73] did this in their comparison of inactivated or live influenza B vaccines in which a challenge from the live virus B strain was used to assess the comparative efficacy of the two vaccines. Re-inoculation with live virus was resisted better by those receiving the same material a month previously than by those injected with inactivated vaccine.

Couch [101] has reported a number of trials in volunteers after inactivated vaccine using a low dose of an essentially unmodified H3N2 virus

that had received one or two passages in human embryonic kidney culture. It was first established by Greenberg et al. [102] that previous infection by homotypic H3N2 virus gave protection against deliberate exposure for up to 4 years after the original infection. Comparison of inactivated vaccine (A/HongKong/68 (H3N2) given intranasally or subcutaneously showed that following challenge with live virus only those who had developed a serum antibody response after vaccine by either route resisted infection.

In a further trial of an anti-NA inactivated vaccine made from an Heq1N2 virus, it was shown that a reduced frequency of illness and a reduced titer of virus in nasal wash specimens resulted following live H3N2 virus challenge compared with the findings in control subjects. The number of those who contracted infection was also reduced somewhat by the inactivated NA vaccine, thus supporting the suggestion of Schulman et al. [103] that NA antibody, although incapable of neutralizing viral infectivity, could limit the extent of viral replication. Beutner et al. [104] also immunized children with an NA-specific vaccine and noted that antibody to NA had a role protecting against illness rather than against infection. Slepushkin et al. [105] and Monto and Kendal [106] came to similar conclusions with regard to NA vaccine and the clinical evidence of protection from illness.

A series of experiments on volunteers, designed to obtain evidence of protection from vaccines containing viruses that were homotypic or heterologous to the challenge virus, is important in relation to the determination of the best composition of inactivated vaccine. Potter et al. [43] gave one of four inactivated monovalent H3N2 virus vaccines to groups of students, measured their pre- and post-immunization antibodies by HI and NI tests and later challenged all the groups with a live intranasal H3N2 virus (WRL 105). This virus was antigenically nearest to the A/Port Chalmers/73 virus and vaccine from this latter strain and also that containing A/Scotland/74 virus gave better protection against infection than earlier H3N2 virus vaccines; the result thus correlated with the induced HI antibody titers.

Larson and others [107] also challenged the immunity produced by inactivated vaccine made from A/Port Chalmers/73 (H3N2) virus with that from a strain developed by the Pasteur Institute [108]. This virus (30c) with an antigen closely similar to A/England/72 (H3N2) was selected in the laboratory by a method analogous to natural selection by antigenic drift, and thus represents the first human attempt to anticipate antigen variation in nature. Challenge of those immunized with one or the other vaccines showed that protection by the heterologous 30c virus was about one-quarter as effective as that produced by the homologous A/Port Chalmers/73 virus.

Experiences related by Couch also confirm [101] that antibody effective against the homologous HA of the challenging virus is more protective than that formed by heterologous antigen. Protection was also compared after inactivated vaccine by intranasal or subcutaneous routes, which showed that the important mediator of immunity was the serum IgG content of anti-HA rather than the respiratory secretion content of specific IgA.

Figure 8. A volunteer room at the Common Cold and Influenza Unit, Harvard Hospital, Salisbury in the 1980s. Volunteers would stay for 2 weeks in this country-placed unit to be infected and carefully studied for clinical symptoms.

A new quarantine unit in London

We have established a new quarantine unit, based in London (www.retro-screen.com), but very much centered upon the experience and ethos of the Common Cold Unit of the past [109]. In a series of experiments over the past 2 years, we have infected over 250 young volunteers with influenza A (H3N2), influenza B and influenza (H1N1) virus and more recently respiratory syncytial virus, and now have fully characterized virus pools [110]. In the USA, a quarantine unit had already been established in Virginia and also at Baylor, and pioneered work into the new NA inhibitors of influenza using an influenza A virus isolated in 1991 [111]. So far our own unit has focused on evaluating new influenza vaccines [112]. We use groups of 20 young volunteers and quarantine them in a student hostel or hotel along with clinicians and scientists (Fig. 8). The MRC Common Cold Unit was rooted strongly in the post-war era with deck chairs, free run rabbits, coun-

try walks, afternoon cream teas and two-course English meals. Our new unit reflects a more diverse community, so chicken tikka is as common on the menu as roast lamb and baked potatoes, but the wish of many of the volunteers is the same: to contribute to knowledge.

Conclusion

Influenza A virus has a proven record as a "bioterrorist" virus but driven not in Churchill's words by the "evil forces of perverted science" but by the vast unfathomable laws of nature and emergence, re-emergence, and resurgence of natural disease. Information from the human genome project, whereby a significant proportion of the 30 000 active genes are already known to be involved in innate and acquired immunity, provides reassurance that the immune system will continue to provide some protection against new viruses.

Gauguin in his last great painting "Who are we, where have we come from, where are we going?" asks crucial questions about the future of humankind. But it was the medieval painter Breugel who asked the major question, yet to be answered in the 21st century. His medieval painting "The Triumph of Death" shows a horseman on a white charger scything at random and gathering souls during an outbreak of *Pasteurella pestis* in medieval times. The question haunting the painting is "why do some persons survive while others die". Even in 1918 in most communities 99% of persons infected with the virus survived. But why did some die and exactly how were they killed by such a minute and fragile form of life that we know as the orthomyxovirus influenza? Was the immune reaction and ensuing cytokine storm overwhelming, or was virus replication in the endothelial cells of the air sacs more important?

An extraordinary clear message is emerging, which tells us to build our public health infrastructure and continue and expand our epidemiological vigilance and surveillance against all these infectious viruses and bacteria. For pandemic influenza, every country needs a detailed and practical plan and a supply of antiviral drugs and new vaccines at hand. We would then be "at the end of the beginning" as regards protection of all citizens. Influenza was the 20th century's weapon of mass destruction. Nature is the greatest bioterrorist of our world and emerging viruses could do for us all, as easily and as quickly, or even more so, than the Great Influenza of 1918, except for the fact that we now have the ammunition to fight back: knowledge of virus transmission and effective antivirals and vaccines.

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