Learning from the First Pandemic of the Twenty-First Century

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Abstract The response to the first influenza pandemic of the twenty-first century was facilitated by years of preparation for a possible pandemic caused by the avian influenza H5N1. The threat of an H5N1 pandemic had led to an increase in manufacturing capacity, to the development of influenza vaccines made in cell culture instead of eggs, to the development of innovative adjuvants and to the establishment of clear rules to license pandemic vaccines. Most of these tools have been used and validated by the H1N1 pandemic. The main lesson learned is that oil-in-water adjuvants can be safely used in large scale and in all ages and conditions, including pregnant women. Adjuvants increase the titer of the antibody responses and broaden the epitopes recognized by antibodies so that they can neutralize also drifted viruses. In addition, they induce long lasting B- and T-memory cells. A further advantage of the use of adjuvants is the ability to use lower doses of vaccine, thus multiplying the manufacturing capacity up to fourfold. Cell-based vaccines have been established as a new technology to produce influenza vaccines.

Both adjuvants and cell cultures are expected to change not only the way we will address future pandemics but also the way we approach seasonal influenza, changing a field that has been stagnant for too many decades.

1 Of Birds and Humans: The Lessons Learned

Since 1580 at least ten influenza pandemics have occurred, with an average of one pandemic every 42 years. Analysis of the most recent and more accurate data predicts one pandemic every 30 years. The last pandemic was in 1968, 40 years ago. Therefore, common sense and mathematical models predicted that we had to be prepared for a new pandemic.

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During the past 12 years, all the events that were expected to happen before a pandemic did happen, and all these events indicated avian influenza viruses as the most likely cause of the next pandemic. First, a new virus carrying a hemagglutinin (HA) with a new antigenic specificity (H5) that had never been isolated in humans before jumped from chicken into men in Hong Kong and killed six people in 1997. This early outbreak was contained by culling chickens in the Hong Kong area. The virus momentarily disappeared, but it was not dead at all: it was successfully breeding, multiplying, and expanding in birds in South East Asia [1], until it suddenly blew up again in humans in 2003 and 2004 in Vietnam, Thailand, Indonesia, China, etc. The virus had clearly escaped any control and was so widespread that culling hundreds of millions of chickens in the areas of outbreak had provided only a temporary relief and not been able to limit the spread of the virus. The virus in fact was spreading to the rest of Asia and outside, to Turkey, Egypt, and Africa through migratory birds as vectors. Concomitantly with this geographic spreading, the H5N1 virus, like all influenza viruses, underwent antigenic drift, and today many genetically and antigenically distinct clades and subclades of the virus have been identified. As of today, more than 440 cases have been reported since 2003, with an overall mortality rate higher than 60%. The appearance and the worldwide spreading of the swine-origin H1N1 virus have not stopped the transmission of the H5N1 virus from birds to humans. As recently as 2009, 47 new cases have been reported in Egypt (36 cases, 4 deaths), in China (seven cases, four deaths), and in Vietnam (four cases, all fatal) [2]. All human cases of H5N1 infection derived from close contacts with poultry. Although in a few cases close contacts among people may have caused the infection, till now the H5N1 virus has not adapted itself to humans and does not seem to represent an immediate threat, even less now with the appearance of the H1N1 virus of swine origin. One cannot, however, underscore the risk of the potential recombination of the two viruses as they both circulate in the same areas.

At the beginning of 2009, the threat of pandemic due to avian influenza viruses appeared very high, based both on the number of cases occurring in several countries and the very high fatality rate. Although the H5N1 virus was expected to be the most likely cause of the pandemic, other viruses such as the H9N2 and the H7N7 were also under strict observation because, despite the fewer cases they caused, they had the potential to kill the human host as in the case of the H7N7 virus [3, 4]. Because only H1, H2, and H3 viruses had been thus far reported from humans, it was reasonably supposed that human beings were immunologically naïve toward virus strains bearing novel HAs, there being the consequent intrinsic risk of high mortality in case of adaptation to humans. All these (and other) considerations prompted many academic laboratories, biotech companies and vaccine manufacturers to develop a plethora of vaccines potentially active against avian influenza viruses. More commonly, these vaccines are prepared in eggs, using the same technology used to manufacture seasonal influenza vaccines. The vaccines consist of whole inactivated virus, detergent-split virus, or purified HA and neuraminidase (NA) subunits. All these vaccines have been widely tested both in animal models and in extensive clinical studies. Some of them have also been approved in

Europe for a prepandemic use or for a pandemic use through a mock-up application which turned out instrumental for fast approval of vaccines against the pandemic H1N1 virus. Other approaches to the development of vaccines against avian influenza have been represented by live-attenuated vaccines, use of virus-like particles produced in baculovirus, vaccines based on conserved proteins such as the external domain of the M2 protein, and others, which, however, have been less extensively investigated in both the preclinical and clinical settings.

The results of many of the preclinical and clinical studies with H5N1-based vaccines have been discussed in details in recent chapters and reviews [5–7]. The question which now needs to be asked is: which lessons did we learn from the experience with vaccines against avian influenza? A corollary logical question is then: can we apply at least part of this learning to the development and use of the vaccine against the pandemic H1N1 influenza? And against the seasonal influenza in general? In the sections below, we attempt to analyze the new knowledge acquired and discuss how this knowledge could (and should) be exploited for the development of better vaccines against influenza and toward a better use of existing or novel influenza vaccines.

1.1 The Need of Adjuvants

A critical lesson learned from the trials of avian influenza vaccines is the importance of adjuvants, particularly of oil-in-water adjuvants in enhancing the quantity and in shaping the quality of protective antibody responses.

Adjuvants represent the best known way to enhance the immunogenicity of vaccines. Most vaccines which are licensed worldwide contain adjuvants. The influenza vaccine is one of the very few vaccines which are given without adjuvants. This is very likely because the individuals are already immunologically experienced with influenza antigens, thanks to previous annual vaccinations and/or thanks to previous contacts (clinically overt or not) with the influenza virus. In such a context, the vaccination acts through the expansion of an already existing pool of memory cells without any need for further "help" from an adjuvant.

Aluminum salts (including alum) are the most utilized vaccine adjuvants worldwide and, until 2009, the only adjuvants admitted for human use in the USA. However, the use of these adjuvants to enhance the immunogenicity of influenza vaccine has consistently failed. Adsorption of influenza virus HA onto aluminum phosphate had been shown to increase the immunogenicity of the vaccine in mice [8]. However, when this aluminum phosphate-adsorbed influenza vaccine was tested in healthy military recruits, it did not enhance the antibody response over a nonadjuvanted vaccine [9]. Despite the failure demonstrated by these studies, during the 1960s and the 1970s, many of the influenza vaccines (whole-virion, split, or subunit) commercially available both in Europe and in the USA were still prepared together with aluminum salts. We had to wait until the 1980s to see the removal of these adjuvants based on the overwhelming evidence that the

adjuvant did not increase the immunogenicity of the vaccine, while it increased its reactigenicity [10–12]. The potential use of aluminum salts has been recently reconsidered for the development of vaccines against the influenza virus A H5N1. Some controversial results have been reported. Indeed, if some enhancement was observed, it was lower than that provided by the oil-in-water adjuvants in dose sparing and in increasing the responsiveness to the vaccine at all ages, including elderly individuals [5–7].

In the 1950s, it had already been shown that the immunogenicity of influenza vaccines could be significantly enhanced by the use of mineral oil adjuvants. These adjuvants allowed significant dose sparing [13], enhancement of the antigen-specific antibody response [14], and persistence of these antibodies, which were still detectable 2–9 years later [15–17]. However, this adjuvant, which was nonmetabolizable and nonexcretable, caused serious adverse events, such as sterile abscesses in almost 3% of the vaccinees, and raised concerns about possible long-term effects. An almost 20-year follow-up of these subjects did not show any increased mortality attributable to the mineral oil adjuvant, not even in those subjects who had had sterile abscesses [18]. Nevertheless, the unacceptably high frequency of local side effects prevented for several years the development of novel, potent oil-based adjuvants. We had to wait until the mid-1990s to see the development of the first oil-in-water adjuvant, referred to as MF59 [19], which was finally licensed for use together with an inactivated subunit influenza vaccine in >65-year-old subjects [20]. The successful approach to the development of a strong and safe adjuvant such as MF59 was to reduce the amount of the oil in the emulsion from 50% to 4–5% and to replace the nonmetabolizable oil with a fully metabolizable, such as squalene, which is a physiological component of the human body, as precursor of cholesterol and adrenal hormones [20].

The very first demonstration of the need for adjuvants for the induction of an optimal response with avian influenza vaccines came in 2001 with the publication in the *Lancet* of the results of a clinical study using the nonpathogenic H5N3 as a source of antigens (at that time the reverse genetics was not available yet and the pathogenic H5N1 strains were lethal for embryonated eggs) and MF59 as the adjuvant [21]. This pioneer study provided most of the useful information available today and paved the way for further vaccine development. In essence, this study showed that the conventional, nonadjuvanted vaccine did not elicit a significant protective antibody response, as compared to the MF59-adjuvanted vaccine. These data were confirmed later by many other groups showing that even increasing the vaccine dosage to 90 μg or more did not induce neutralizing antibodies in the majority of the vaccinees [22]. Instead, the MF59-adjuvanted vaccine allowed three essential features for a pandemic vaccine: (1) dose-sparing not only for H5-based vaccines [21], but also for H9N2 vaccines [23]; (2) broadening of the neutralizing antibody response [24], and (3) induction of strong immunological memory [25–27]. All these features were typical of the oil-in-water adjuvant MF59, and not of adjuvants in general, since alum consistently failed to enhance the neutralizing antibody response to H5N1 at levels comparable to those achieved with MF59

[28, 29]. The very promising data obtained with MF59 prompted other groups to develop oil-in-water adjuvants also based on squalene [30]. One of these, referred to as AS03, is being actively utilized for the development of a vaccine against H5N1 [31] and is part of a vaccine used in various countries against the pandemic H1N1. Another squalene-based emulsion, referred to as AF03, is still at early stages of development [30] and is mainly addressed at the development of H5N1 influenza vaccines [32]. Many of the features exhibited by MF59-adjuvanted avian influenza vaccines were then reproduced also by these other oil-in-water adjuvants (see later).

1.2 Dose-Sparing and Increased Dose Availability

As mentioned earlier, the first consequence of using oil-in-water adjuvants in the formulation of avian influenza vaccine was the possibility to use amounts of antigens lower than 15 μg, the conventional dose of HA used in the seasonal vaccines. This was first demonstrated in adult volunteers immunized with 30, 15, or 7.5 μg of subunit H5N3 vaccine with or without MF59. Indeed, the highest antibody response was observed in the subjects who had received the lowest dose of the vaccine, 7.5 μg [21]. The possibility of dose sparing was then demonstrated also with the H9N2 vaccine. In this study, the levels of specific antibody obtained with 3.75 μg of MF59-adjuvanted vaccine after one single dose were similar to those reached after two doses of 30 μg of nonadjuvanted vaccine. A second dose of MF59-adjuvanted H9N2 vaccine significantly enhanced the level of neutralizing antibodies [23].

Similar data were then obtained by other groups using split H5N1 vaccines formulated with other oil-in-water adjuvants, such as AS03 and AF03. Indeed, doses as low as 3.75 μg or even 1.9 μg of H5N1 vaccine induced significant neutralizing antibody titers in vaccinated volunteers [32, 33].

Taken together, all these findings speak in favor of the possibility of significantly increasing the potential coverage of the human population vaccinated against a pandemic due to the significant reduction of the dose necessary to reach protective levels of circulating antibodies. In the absence of specific knowledge of the evolution of the threat of avian influenza, with the spread of the new H1N1 pandemic virus, and the need to still cover against the seasonal influenza, all this has seen in parallel an increased investment of vaccine manufacturers to enhance the capacity of production of both monovalent pandemic vaccines and trivalent seasonal vaccines. From the surveys conducted during summer 2009, it has been estimated that the total capacity worldwide for seasonal trivalent vaccines has increased from 400 to more than 900 million doses per year, with the potential to produce more than four billion doses in case of reduced output of seasonal trivalent vaccines [34]. This would translate into an increased availability of pandemic vaccine even for developing countries although, in this case, other issues (such as cost, storage, and distribution) will have to be solved by the cooperation between the World Health Organization (WHO), the governments, and other nongovernmental organizations [35].

1.3 *Broadening of the Antibody Response to Drifted Influenza Virus Strains*

An ideal vaccine against influenza should contain conserved internal viral proteins (e.g., NP, M1, and M2e) to induce protective immune responses against all the possible drifted (and possibly shifted) variants of the virus. This would avoid the continuous (almost yearly) change in the vaccine composition necessary to adapt the strains used for the vaccine to those which are expected to circulate that year. In addition, the use of conserved internal viral proteins would also induce cell-mediated immune effector mechanisms to complement the antibody response elicited against HA and NA present in the currently used vaccines. Despite various efforts toward this end (see the chapter "Conserved Internal Proteins as Potential Universal Vaccines" by A. Shaw), these vaccines have not turned the corner. However, the data available so far clearly show that inactivated influenza vaccines can confer significant seroprotection against drifted influenza virus strains when they are prepared together with oil-in-water adjuvants.

This was first shown with subunit H5N3 vaccines adjuvanted with MF59 [24]. While the subjects who had been vaccinated with the nonadjuvanted vaccine had no or very poor detectable neutralizing antibody responses against drifted H5N1 virus strains, the majority of the subjects with the MF59-adjuvanted H5N3 (clade 0-like, isolated in 1997) vaccine had protective levels of antibodies against the heterologous clade 1 virus isolated in Vietnam and in Thailand, isolated in 2003–2004. In summary, the data had shown that MF59-adjuvanted vaccines could induce protective immunity against viruses not fully matching the vaccine strain and could cover the antigenic drifts of the virus occurring over 6–7 years at least. This data opened the way to the concept of the prepandemic vaccination, in other terms the possibility to vaccinate before the formal declaration of the pandemic since the data proved that priming with a mismatched virus induced cross-protective immunity. For a rigorous foundation of the prepandemic vaccination approach, it remained to be shown that the immunological memory induced by this priming could also be boosted by a vaccine containing a drifted H5N1 virus. This was formally proven in subsequent studies and is discussed in the next section on adjuvant-driven immunological memory.

Subsequent studies with H5N1 vaccines have amply confirmed these pioneer findings originally obtained with H5N3-based vaccines. We know now that immunization with H5N1 (clade 1) vaccines containing MF59 or other oil-in-water adjuvants such as AS03 or AF03 induces antibodies against a wide panel of drifted strains, for example, those belonging to the subclades 2.1, 2.2, and 2.3 [32, 36–39]. The induction of antibodies against heterovariant virus strains in humans was paralleled by a stronger efficacy of the adjuvanted vaccine in ferrets challenged with various heterovariant H5N1 virus strains [40, 41]. Cross-clade antibody responses have also been reported in subjects vaccinated with whole-virion H5N1 vaccine unadjuvanted [42] or adjuvanted with aluminum hydroxide [43]. As none of these vaccines were tested in the same clinical study, it is difficult to make a direct comparison of the height and the extent of this antibody response.

Antigenic mismatch between the seasonal vaccine virus strains and the circulating virus strains is not a rare event, and it can affect influenza vaccine efficacy and effectiveness [44]. Mismatch is caused by the accumulation of point mutations at antigenic sites on the HA and NA proteins (antigenic drift), which occur between the time that WHO makes its recommendation for vaccine composition and the period of subsequent exposure to the circulating strain. This leads to the appearance of new antigenic determinants. Although occurring in both type A and type B viruses, the antigenic drift occurs more frequently in the influenza A (H3N2) viral subtype [44]. It has been shown that the antigenic drift causes a decrease in vaccine-induced immunogenicity in elderly people [45]. In older subjects with a high ($\geq$80%) postvaccination seroprotection rate against the homologous vaccine strain, the rate of sero-protection against the drifted circulating strains dropped to 4–75%, based on the circulating and on the vaccine strains, and on the age groups [46–48]. In addition, antigenic mismatch can have a strong impact on vaccine effectiveness, as demonstrated by a study for the period 1995–2005, when the vaccine effectiveness among older adults ($\geq$65 years of age) dropped during the seasons with a drifted strain (1997–1998 and 2002–2003) to values below 30% [49].

The finding that MF59-adjuvanted H5N3 vaccine induced neutralizing antibodies also against drifted H5N1 virus strains suggested that the same could take place with the seasonal influenza vaccines. Indeed, this was the case. Several clinical studies have now shown that the seasonal MF59-adjuvanted influenza vaccine induces strong antibody responses against heterovariant strains [46, 48, 50]. Thus, MF59-adjuvanted influenza vaccine provides greater seroprotection in the case of antigenic drift than nonadjuvanted vaccines. For example, significantly ($P < 0.0001$) more older adults receiving MF59-adjuvanted influenza vaccine containing A/Panama/2007/99 (H3N2) were seroprotected against the drifted variant A/Wyoming/3/2003 (H3N2) than those receiving nonadjuvanted split-virus vaccine or nonadjuvanted subunit vaccine (98%, 80% and 76%, respectively) [46]. The enhanced seroprotection against a large panel of drifted H3N2 [48] and B virus strains [50] has been confirmed in other studies in elderly people and more recently also in 6–36 month-old children [51], showing that this wide breadth of cross-protection is a general phenomenon induced by MF59.

1.4 Induction and Persistence of Immunological Memory

Vaccination with inactivated influenza vaccine without adjuvant works, thanks to an immunological memory, which is acquired with age through clinically overt or asymptomatic infections and is maintained via yearly vaccinations and/or subsequent contacts with the influenza viruses. It is difficult to discriminate which part of the memory is due to the infection and which one is provided by the vaccination. The development of vaccines against the avian H5N1 virus allowed to dissect the priming of the immune response, the induction of the immunological memory, and its persistence over time. In this context, it was possible to discover

the critical role played by adjuvants and, in particular, by the oil-in-water adjuvant MF59, in the induction and persistence of immunological memory against influenza viruses.

Most of the clinical studies carried out so far with H5N1 vaccines have clearly shown their relatively poor immunogenicity, not only because of the need for strong oil-in-water adjuvants, but also because of the necessity of two doses of vaccines to induce protective titers of neutralizing antibodies in the majority of the vaccinees. The question was then to evaluate whether an immunological "signal" could be measured after one single dose of the H5N1 vaccine to formally show that successful priming had taken place, even if antibody titers were generally poor in most of the people. Indeed, after one single immunization with MF59-adjuvanted H5N1 subunit vaccine (clade 1), there was a significant increase in the frequency of HA-specific (using a panel of overlapping peptides spanning the entire length of the HA) central memory CD4+ T cells committed to produce IL-2 (with or without TNF-α), but not IFN-γ. The frequency of these cells did not increase after the second dose of the vaccine 3 weeks later and persisted at frequencies higher than baseline for 6 months, when it increased after a booster dose and was maintained at high levels later on [52]. It is interesting that these CD4+ T cells induced by the MF59-adjuvanted vaccines were mostly directed against epitopes which were conserved among the HA of the various H5N1 clades. Nevertheless, these cells also recognized epitope-containing sequences that varied in the HA of clade 2.1 and of H5N3 virus strains [52]. It is remarkable that a threefold increase in the frequency of H5-specific memory CD4+ T cells after a single dose of MF59-adjuvanted vaccine was predictive of a rise in neutralizing antibody titers above 1:80 after the booster dose 6 months after the first dose and also their persistence over time after the booster dose [52].

The persistence of the immunological memory can be demonstrated clinically by boosting individuals previously immunized with the same or a slightly different (heterovariant) vaccine. This, however, needs to wait for a sufficient long period of time between priming and boosting. The first example of this approach was shown in those subjects who had received the H5N3 vaccine adjuvanted with MF59 or otherwise [21]. Only the subjects previously primed with the adjuvanted vaccine exhibited a fast and strong rise in the titers of anti-H5N3 antibodies when boosted 16 months later with the same vaccine, whereas those who had received the nonadjuvanted vaccine mounted a detectable, but still much lower, response even after being boosted with the adjuvanted vaccine [25].

In order to understand how long the immunological memory at the B-cell level persisted over time and to evaluate the breadth of this memory in terms of cross-reactivity with H5N1 virus strains appeared from 2003 to 2007, these same subjects, and other subjects who had received the MF59-adjuvanted H5N3 vaccine twice [53], were boosted 6–8 years later with an MF59-adjuvanted subunit vaccine based on a clade 1 virus strain. Previously unprimed subjects immunized for the first time with the MF59-adjuvanted vaccine served as a control. One single injection with this vaccine induced a poor rise in the frequency of H5N1-specific

memory B cells in previously unprimed subjects and also in the subjects who had been primed 8 years earlier with the nonadjuvanted H5N3 vaccine. The frequency of these cells increased (doubled) after a second booster dose. On the contrary, the frequency of memory B cells sharply and rapidly increased (up to 12% of all circulating IgG-producing memory B cells) after one single dose of the adjuvanted H5N1 vaccine in the subjects who had been previously primed with the MF59-adjuvanted H5N3 vaccine [27]. This significant and rapid increase in memory B cells was paralleled by a massive production of anti-H5N1 antibodies as detected by hemagglutination inhibition (HI), microneutralization (MN), and single radial hemolysis (SRH). Indeed, only 7 days after the booster dose with MF59-adjuvanted H5N1 clade 1 vaccine, all subjects primed 6–8 years earlier with the adjuvanted H5N3 vaccine had antibody titers that significantly exceeded the "protective" threshold of 1:40, not only against the homologous clade 1 virus strain, but also against other clade 1 strains, and against various strains belonging to the subclades 2.1, 2.2, and 2.3. A second dose of the vaccine did not increase the serum antibody response. This broad neutralizing antibody response persisted at high, protective levels for at least 6 months [26, 27]. Individuals who had been previously primed with the nonadjuvanted H5N3 vaccine mounted an anti-H5N1 antibody response post-boost but with slower kinetics and reaching levels much lower than the subjects primed 6–8 years earlier with the adjuvanted vaccine. Remarkably, all these subjects had post-booster antibody titers to the original priming H5N3 virus, similar to or lower than those detectable against the boosting H5N1 virus [26, 27], strongly suggesting that at least in these conditions — using the MF59 adjuvant in both the priming and boosting vaccine — no original antigenic sin was observed.

These data proved that strong immunological memory is induced upon vaccination with adjuvanted H5N1 influenza vaccines, that it persists for not less than 8 years, and that it can be strongly and rapidly boosted by a heterovariant adjuvanted vaccine. The induction of immunological memory and the possibility of boosting with heterovariant strains have now been shown with other vaccine combinations, using nonadjuvanted split vaccines [54], adjuvanted split vaccines [55], or nonadjuvanted whole-virion vaccines [56]. It is clear from all these data that the best responses are observed when both the priming and the boosting are performed with adjuvanted vaccines. However, it has been reported that the anti-H5N1 response after boosting with an AS03-adjuvanted split H5N1 vaccine can be negatively affected in subjects who had been previously primed with a non-adjuvanted heterovariant vaccine [55]. It is not clear whether this is due to an original antigenic sin. Should this be the case and considering that this was not observed with inactivated subunit vaccine adjuvanted with MF59 [26, 27], one can speculate that differences in the vaccine preparation (i.e., split versus purified subunit) and/or in the adjuvant preparation (i.e., AS03 versus MF59, which, although oil-in-water and squalene-based, contain substantial differences in their formulations – see chapter "Adjuvants for Influenza Vaccines: the Role of Oil-in-Water Adjuvants" by D.T. O'Hagan et al.) affect the antibody response to the influenza vaccine.

2 Of Pigs and Humans: How to Apply This Learning?

When the scientific community, the public health authorities, the regulatory agencies, and all national and international bodies were actively working on the preparedness plans to counteract the risks of an influenza pandemic caused by avian viruses, and when the discussions on the opportunity and feasibility of prepandemic vaccination with vaccines based on avian virus strains were at their peak, suddenly the alert of cases of influenza infections caused by an A/H1N1 influenza virus of swine origin in Northern America was given in April 2009. Soon the virus started to spread and in a couple of months affected all continents, until the WHO declared the pandemic. In several ways, the event of a pandemic caused by an A/H1N1 virus was unexpected. We were expecting a pandemic due to a non-H1/non-H3 virus. Most of the people were actively working on the development and stockpiling of H5N1-based vaccines because of the very high number of cases in wild and domestic birds in Asia, Europe, and Africa, and in the humans in Asia and Africa, which is an exceptionally high lethality rate. Some people were still pledging to prepare vaccines against other avian viruses, such as H9N2 and H7N7. Second, most of the preparedness focused on virus strains of avian origin, and none at all on strains from pigs. Finally, the entire community was watching at the appearance and evolution of novel influenza strains from Far-East Asia with a westward propagation, while the pandemic originated from the West and exhibited an eastward propagation.

As the virus strain causing the pandemic was an A/H1N1, which has coexisted with humans since the Spanish flu pandemic of 1918, the question was immediately asked as to whether there were similarities between this novel virus that had popped out from North American pigs and the A/H1N1 virus that composes the trivalent seasonal vaccines and whether the seasonal vaccine would have been able to induce antibodies to cross-react with the novel virus. The genetic analysis of the new, pandemic A/H1N1 virus and the prediction of the structure of its HA, inferred by the amino acid sequence, are clearly against this possibility (see the chapter "The Origin and Evolution of H1N1 Pandemic Influenza Viruses" by R.G. Webster et al.). In addition, the very first serological studies confirmed later by comprehensive studies using serum samples from subjects of all ages immunized with seasonal influenza vaccines showed that neutralizing antibodies induced by the seasonal inactivated influenza vaccine poorly recognized the novel A/H1N1 virus, suggesting that novel B-cell epitopes were expressed by this virus. More specifically, such cross-reactive antibodies were undetectable in children below the age of 9, while they were detectable in 12–22% of adults between 18 and 64 years of age and in 5% of older adults. Interestingly, a proportion of older adults had cross-reactive antibodies which preexisted the vaccination with the seasonal vaccines [57]. These findings are in agreement with the epidemiological observation that people older than 65 years are less susceptible to the novel A/H1N1 virus than younger people [58].

All these data suggest that a vaccine against the novel A/H1N1 virus was necessary since most of the people were clearly immunologically naïve (at least based on their neutralizing antibodies). On the other side, the data in the older subjects suggested that some immunological memory could exist between the novel and the seasonal A/H1N1 viruses. The questions then arose as to whether the lessons learned toward preparedness for a pandemic due to avian influenza viruses, such as H5N1 viruses, could be applied to the development and the use of vaccines against the novel A/H1N1 virus. The need for adjuvants and the induction and persistence of immunological memory will be discussed in the next sections.

2.1 Adjuvants and A/H1N1 Vaccines?

When the genetic data of the novel A/H1N1 virus became available and when the first data on the poor cross-reactivity of antibodies between seasonal and pandemic viruses were reported, it was immediately considered that the vaccine against this new virus had to share some key characteristics of the vaccines already developed against the avian H5N1 viruses. For example, because the vaccine was expected to be given to immunologically naïve individuals who had never seen this virus earlier, the vaccine had to contain a strong adjuvant, an oil-in-water adjuvants such as MF59 or AS03, and had to be given twice to reach sustained protective levels of antibodies that met the criteria fixed by regulatory agencies such as the FDA and the EMEA. These expectations, mainly the one related to the double doses required for effective priming, influenced the decision of national authorities on the number of doses required to cover the population included in the national plans of immunization.

It was, therefore, surprising to see the first results of the clinical trials when they were published. Indeed, in contrast to all expectations, the vaccine against the pandemic A/H1N1 virus was immunogenic (i.e., met the regulatory criteria for licensure) even in the absence of adjuvants when the dosage of antigen in the formulation was increased. In addition, and strikingly, one single dose was immunogenic enough to meet these criteria.

In a study carried out in Australia with a split-virion A/H1N1 influenza vaccine (A/California/7/2009) from CSL, 240 subjects aged between 18 and 64 years received twice either 15 or 30 μg of vaccine, 21 days apart. Three weeks after the first dose, 95% and 89% of subjects who had received 15 or 30 μg of vaccine, respectively, had HI antibody titers above 1:40. These percentages became 98% and 96%, respectively, after the second dose. The second dose of vaccine only slightly increased the geometric antibody titers already achieved after the first immunization [59]. It is interesting to note that the initiation of this study (last week of July 2009) coincided with the first pandemic wave in Australia, and one volunteer tested positive for the novel A/H1N1 infection during the 21 days after the first vaccination. In addition, the authors of this clinical study report that 45% of the subjects had received the 2009 seasonal influenza vaccine before being enrolled

in the pandemic vaccine study [59]. It would then be important to understand the potential contribution of natural (subclinical) infection with the pandemic A/H1N1 virus and/or of the prior seasonal influenza infections and/or vaccination with the seasonal influenza vaccine in the priming of an immunological memory that would have been then boosted by the pandemic vaccination. Indeed, almost 27% of the subjects participating in this study had HI antibodies above the level of 1:40 at baseline [59].

The question still remains open even after a second study with the same split-virion nonadjuvanted vaccine from CSL. This study was carried out in Australia with the same dosages and the same dose regimen in 370 healthy infants and children 6 months to less than 8 years of age [60]. Again, after one single dose of nonadjuvanted split vaccine, 92.2% and 97.7% of children receiving 15 or 30 μg of vaccine, respectively, had HI antibody titers exceeding 1:40. The geometric antibody titers post-first dose ranged between 113 in those below 3 years with the lowest dose and 268 in those above 3 years with the highest dose. Unlike the previous study in adults [59], in the study in children the second dose significantly increased the levels of serum HI antibody titers [60]. This study was carried out (August 2009) in areas in Australia where the notification of the novel A/H1N1 influenza infection had started to decline. In addition, 40% of the infants and children enrolled had been previously vaccinated with the 2009 seasonal influenza vaccine. Finally, even before vaccination with the pandemic vaccine, a high proportion (9.2% to 33.3%) of the infants and children had levels of HI antibodies to the A/H1N1 virus in the ratio of 1:40.

The data from these two studies suggest that this H1N1 vaccine is particularly immunogenic at all ages, including in young children, and more immunogenic than the avian H5N1 vaccines, the seasonal H1N1 vaccines, and the swine H1N1 vaccines developed during the 1970s and used only in the USA. For the H5N1 vaccine, two doses were required to obtain a sustained "protective" antibody response at all ages. For the seasonal H1N1 vaccine, two doses are necessary to induce good priming in young children. For the swine H1N1 influenza vaccine of the 1970s, one dose was enough for adults, but two doses were required for children below the age of 9 [61]. The difference between these three vaccines and the pandemic one tested in Australia is that the H5N1 virus never circulated in areas where the vaccines were tested and there is no H5N1 vaccination ongoing. Similarly, the swine H1N1 virus of the 1970s did not circulate outside New Jersey. Furthermore, the H1N1 virus that appeared in 1918 disappeared in 1957. This means that the <24-year-old subjects who required two doses of vaccine had never been exposed to the H1N1 virus, which would reappear in 1997, after the study with the A/New Jersey H1N1 vaccine [61]. In addition, during the 1970s, seasonal influenza vaccination was not recommended in children and was poorly implemented even in adults. The seasonal H1N1 virus tends to circulate less than the H3N2 virus, depending on the seasons. Instead, the novel A/H1N1 virus was amply circulating during the period of study. To conclude, one cannot rule out that subclinical infection with the virus had happened and that this may have contributed to specific immunological priming that would have then been boosted by the vaccination.

This hypothesis has now been substantiated by a cross-sectional study carried out in the UK, which shows a high prevalence of anti-novel H1N1 antibodies during the first wave of infection [62] Unfortunately, in these clinical studies with the vaccine against the novel A/H1N1 virus, serum samples were not taken earlier than 21 days post-first dose to evaluate the kinetics of the antibody response, as performed, for example, in studies aimed at investigating the immunological memory induced by vaccinations with H5-based vaccines several years earlier [26, 27].

In a study carried out from July to August 2009 in China, 2,200 subjects received 7.5, 15, or 30 μg of a split-virion A/California/7/2009 H1N1 vaccine produced by Hualan Biological Bacterin Company and formulated with or without alum as an adjuvant [63]. Again, a single 15-μg administration without adjuvant was sufficient to induce HI antibody titers above 1:40 in 74.5% of subjects between 3 and 11 years of age, in 97% of those between 12 and 60 years of age, and in 79% of those 61 years of age or older. The GMTs were lower in the youngest group (3–11 years) compared with the older groups. As expected by the previous experience with H5N1 vaccines, the addition of alum did not influence the antibody response. It is interesting that, like in the Australian studies, a second dose of vaccine did not affect the HI antibody titers in the subjects 12 years of age or older, while it significantly enhanced the response in the younger group (3–11 years). It should be noted that the frequency of subjects with antibody titers higher than 1:40 before immunization was much lower than that found in the Australian trial, ranging between 1% and 6% [63]. Similar results were obtained in a much larger (>12,000 subjects) multicenter, double-blind, randomized, placebo-controlled study carried out from August to September 2009 in China, using the same formulations with or without alum, plus two whole-virion formulations containing 5 or 10 μg of HA plus alum [64]. Essentially, this larger trial reported immunogenicity data very similar to those of the first, smaller study in terms of seroprotection rates at baseline by age groups, seroprotection rates after the first and the second dose, and as GMT in the younger compared with the older groups after the first and the second dose. Interestingly, in this study, the addition of alum to the vaccine formulations clearly suppressed the antibody response in comparison with the same nonadjuvanted formulation. There is no evidence of an ongoing wave of pandemic at the time when these two studies were carried out. However, there was no information on the status of previous immunizations with seasonal vaccines or on the status of previous influenza infections, mainly in consideration of the high rates of asymptomatic infections.

Results not different from these reported from China were also obtained with a single dose of 6 μg of HA of a split-virion vaccine produced in Hungary and adjuvanted with aluminum phosphate, and given in August 2009 to 203 adults and 152 elderly individuals. The immunogenicity of this vaccine was not affected when it was given at the same time with a trivalent inactivated seasonal vaccine [65].

The effect of previous vaccination on the immune response to the pandemic vaccine before vaccination has been very well demonstrated in >18-year-old subjects who received a single dose of a split-virion vaccine from Sanofi-Pasteur in two randomized, placebo-controlled studies carried out in the USA during the

first half of August 2009 (>800 adults/elderly). This effect was less, or not at all, evident in children below the age of 9 (>400 children) [66]. In these studies, seroprotection rates (HI titers above 1:40) were consistently higher than 90% in adults and elderly individuals who received 7.5, 15, or 30 μg of HA. However, these frequencies went down to 69% and 75% in children between 3 and 9 years and to 45% and 50% in 6–35-month-old children immunized with 7.5 or 15 μg of HA, respectively [66]. These studies strongly suggest that previous priming with seasonal vaccine may improve the immune responsiveness to subsequent vaccination with the pandemic A/H1N1 vaccine. Indeed, the antibody response was much lower in young children expressed both as seroprotection/seroconversion and as GMT. It is very likely that a second dose of vaccine would have significantly increased the immune response to vaccination. Unfortunately, the results of the second immunization were not reported.

Using a cell culture-derived subunit A/H1N1 vaccine from Novartis, it was possible to show in a study carried out in adults in the UK at the end of July that one dose of vaccine was sufficient to induce seroprotection in 72% and 52% by HI and in 76% and 67% by MN in subjects receiving 3.75 or 7.5 μg of HA without adjuvant, respectively. These percentages increased to >90% by HI and to 100% by MN in the subjects immunized with the same dosages of vaccine in the presence of the oil-in-water adjuvant MF59 [67]. An important finding of this study was that these antibody titers and seroprotection rates were reached just 2 weeks after the vaccination. As expected, a second dose of the vaccine increased the immunogenicity parameters. In a randomized study carried out in Costa Rica in 3–17-year-old children, both unadjuvanted (15 and 30 μg) and MF59-adjuvanted (7.5 μg) egg-derived A/H1N1 vaccines from Novartis met the criteria for immunogenicity. The vaccine with low antigen and adjuvant was clearly more immunogenic after one single dose than the higher dosages without adjuvant, in the younger age group (3–8 years of age) [68]. Seroprotection rates by HI higher than 98% were also reported after one single dose in 18–60-year-old adults vaccinated in Germany with a split-virion vaccine from GSK given without adjuvant or adjuvanted with the AS03, squalene-based adjuvant [69].

2.2 *Immunological Memory: Priming by Previous Influenza Infection/Vaccination*

As mentioned above, the results of these trials are surprising. On the basis of the poor antigenic similarities between seasonal and pandemic A/H1N1 viruses and the poor cross-reactivity between the two viruses, it was expected that more than one priming dose would have to be administered, mainly in young children, and that strong adjuvants, such as MF59 or other oil-in-water emulsions, would be needed.

One hypothesis that could explain these findings is that a certain level of cross-priming takes place through natural infections (clinically overt or asymptomatic) or through vaccination with trivalent seasonal vaccines that contain the A/H1N1 virus component. These hypotheses are clearly motivated not only by the results of the

clinical studies in the USA with the Sanofi-Pasteur vaccine [66] but also by the Australian trials carried out during the eve of the A/H1N1 pandemic in a population that had largely received the seasonal influenza vaccines [59, 60].

This hypothesis has been now formally proven in ferrets. Animals immunized with two doses, 1 month apart, of seasonal trivalent inactivated vaccine with or without MF59 did not mount any detectable antibody response against the novel A/H1N1 virus, either by HI or by MN. HI and MN antibodies became detectable in the ferrets that had received the seasonal influenza vaccine first followed by the nonadjuvanted A/H1N1 vaccine 1 month later. Intermediate antibody titers were achieved with one single dose of MF59-adjuvanted A/H1N1 vaccine. However, the strongest HI and MN antibody response was detected in those ferrets first primed with the seasonal vaccines (better if adjuvanted with MF59) followed by the A/H1N1 vaccine adjuvanted with MF59 [70]. A striking finding of this study was that this antibody response was mirrored by the decrease in the A/H1N1 viral load in the upper and lower respiratory tract. Indeed, if two doses of the seasonal vaccine were totally unable to affect the viral load in the lungs and in the throats of the ferrets, previous priming with seasonal vaccine followed by the nonadjuvanted A/H1N1 or a single immunization with the adjuvanted vaccine in unprimed animals significantly reduced the viral load in the lungs. However, previous priming with the MF59-adjuvanted seasonal vaccine followed by vaccination with the MF59-adjuvanted A/H1N1 vaccine totally prevented the viral colonization not only in the lower, but also in the upper respiratory tracts [70].

A few conclusions can be drawn from this study. First, a previous priming via vaccination (or very likely via previous clinically overt or asymptomatic influenza infection) significantly enhances the immunogenicity and the efficacy of the A/H1N1 vaccine. Second, this priming is not necessarily evident through the detection of cross-reacting antibodies. It is likely that this priming takes place through cross-reactive CD4+ T cells primed by the seasonal vaccination that provide help to B cells to produce antibodies to the A/H1N1 virus after boosting with this vaccine. It is known that seasonal and novel A/H1N1 viruses share several CD8+ T cell epitopes [71]. The same can easily be the case for CD4+ epitopes. Another, not mutually exclusive, hypothesis is that low-affinity, cross-protective memory B cells or high-affinity, but rare, memory B cells primed by seasonal vaccination are further expanded by the adjuvanted 2009 A/H1N1 vaccine. The known effect of MF59 in inducing CD4+ T cells and memory B cells can be in favor of these hypotheses [27, 52]. These hypotheses, however, are difficult to address in ferrets but could be approached in well-designed clinical trials, ideally in populations who are immunologically naïve to influenza, such as young children. Finally, the best immunogenicity and efficacy of the A/H1N1 vaccine (prevention of viral infection both in the lung and in the upper respiratory tract) is observed when all vaccines are given in the presence of MF59. This finding suggests that if nonadjuvanted H1N1 vaccines are immunogenic enough to meet all the criteria required for licensure of the vaccines, the use of adjuvants, and of MF59, in particular, can dramatically affect the quality of the immune response, thereby improving the efficacy of the vaccine.

2.3 *Shaping of the Repertoire of the Influenza B-Cell Epitopes by Adjuvants*

The progress in the understanding of the mechanisms of action of certain families of adjuvants has tremendously boosted the research in a field which, until very recently, has remained very empirical and mostly confined to the mere observation of in vitro and in vivo effects. The discovery that several adjuvant families exert their action through binding to toll-like receptors and that the most utilized adjuvants, the aluminum salts, work via the inflammasome using pathways involving IL-1β [72] has paved the way for further development of novel adjuvants, with the ultimate goal of evoking the most appropriate immune response depending on the targeted vaccine.

As a matter of fact, not all adjuvants exert their action in the same manner. For example, there are adjuvants that neither interact with toll-like receptors nor follow the inflammasome pathway. One of these adjuvants is the oil-in-water MF59, which exerts its immunopotentiating effects at local (muscle) level and then at the level of draining lymph nodes, without interacting with toll-like receptors or with inflammasome [73]. We have mentioned several times earlier the effects of the adjuvants on the enhancement of the immune response to seasonal and pandemic (avian and swine) influenza vaccines. One question that still remains unanswered is through which mechanisms MF59 broadens the immune response when it favors the production of antibodies that are able to neutralize not only the homologous virus strain present in the vaccine, but also a large panel of virus strains that underwent antigenic drift in their HA, sometimes over a large period of time [26, 27, 48]. One simplistic hypothesis would be that this is due to the larger amount of antibodies induced by MF59, which would now be able to cross-neutralize drifted virus strains. Another hypothesis is that MF59 affects the quality of the immune response by inducing antibodies against epitopes in the vaccine antigens that otherwise would have not been recognized if the vaccine was without adjuvants or with other adjuvants.

To answer this question and to elucidate if and how MF59 affected the antibody repertoire against influenza antigens, serum samples from subjects vaccinated with plain, with aluminum hydroxide-adjuvanted, or with MF59-adjuvanted H5N1 vaccines were analyzed by whole-genome fragment phage display libraries (GFPDL) followed by surface plasmon resonance technologies. The results obtained were striking [74]. While sera from subjects vaccinated with nonadjuvanted or with aluminum-adjuvanted vaccines mostly recognized fragments of the HA2 region, the oil-in-water adjuvant MF59 induced epitope-spreading from HA2 to HA1 and allowed the appearance of antibodies to neuraminidase. Moreover, a nearly 20-fold increase in the frequency of HA1/HA2 specific phage clones was observed in sera after MF59-adjuvanted vaccine administration when compared with responses after the administration of unadjuvanted or alum-adjuvanted H5N1 vaccines. Additionally, MF59-adjuvanted vaccines induced a two- to threefold increase in the frequency of antibodies reactive with properly folded HA1 (28-319), a fragment that absorbed most neutralizing activity in immune sera [74]. It is important to note that

this fragment was recognized by cross-reacting neutralizing monoclonal antibodies and by sera from immune subjects who had recovered from a natural infection with the H5N1 virus [75]. The adjuvant-dependent increased binding to conformational HA1 epitopes correlated with broadening of cross clade neutralization and predicted improved in vivo protection. Finally, antibodies against potentially protective epitopes in the C-terminal of neuraminidase, close to the sialic acid binding enzymatic site, were also induced primarily following vaccination with MF59-adjuvanted vaccine, but not with plain nor with alum-adjuvanted vaccines [74].

These data clearly show that MF59 profoundly shapes the repertoire of the B-cell epitopes recognized by protective antibodies that are not only directed against HA but also against the NA. Remarkably, this is not an effect merely linked to the quantity of antibodies induced and would not be detected by the conventional serological assays used to evaluate the immunogenicity of influenza vaccine and, ultimately, to license them. As a direct consequence of these findings, it is very likely that the same principle applies to all influenza vaccines, including the vaccine against the novel pandemic A/H1N1 virus. These analyses are now in progress with a special focus on the priming of the B-cell repertoire (for example in young children) as compared with the boosting of this repertoire (for example at older ages).

3 Conclusions: Rethinking Influenza

The threat of avian influenza and the reality of the influenza pandemic due to a virus of swine origin have had a tremendous impact on the field of influenza in general. It has boosted a striking technological progress. The reverse genetics has been developed which has allowed the preparation of virus seeds suitable for the preparation of vaccines [76]. Vaccines have been produced and licensed using in vitro cell cultures instead of the conventional embryonated eggs [77]. The use of pseudoparticles has permitted a rapid and safe evaluation of neutralizing and cross-neutralizing antibodies against wide panels of virus strains [38]. The role of adjuvants in the preparation of stronger influenza vaccines is being better understood and has pushed various vaccine manufacturers to develop their own adjuvants for influenza vaccines after the original introduction of MF59 in the influenza vaccine arena in 1997 (this chapter and chapter “Adjuvants for Influenza Vaccines: the Role of Oil-in-Water Adjuvants” by D.T. O’Hagan et al.).

This influenza pandemic is teaching us a lot on the gaps and the needs that still remain in the field of influenza vaccine development and in the field of influenza vaccination. These needs will force us to completely rethink influenza as a whole, from the understanding of the virus biology and evolution (could we predict the appearance of an A/H1N1 pandemic virus? From pigs? From North America?) to the vaccine preparation (more attention to novel delivery systems, to internal conserved proteins, etc.), from the methodologies to appropriately analyze the protective immune response evoked by the different vaccines in different age groups (more emphasis on cell-mediated immunity, on the priming of the immune

response in younger ages, on the persistence of memory, and on counteracting the waning of the immune responsiveness in the elderly) to a more precise understanding of the epidemiology in developing countries (in the tropics, influenza does not exhibit the seasonal peaks of transmission as in temperate climates), from the present use of the influenza vaccines, which is oriented toward the elderly, to a broader, universal use of these vaccines [78].

A last word should be added concerning the safety of influenza vaccines and of adjuvanted influenza vaccines in particular. Thanks to the need to implement the pandemic vaccination in a large proportion of the world, important clinical research has been undertaken, with the intrinsic risk of observing a high rate of coincidental side effects, to quantify the baseline risk of acquiring a large panel of diseases (chronic, neurological, autoimmune, etc.) in various populations [79–81]. The information available so far on the use of the pandemic A/H1N1 vaccines in several million individuals worldwide strongly speaks in favor of the safety of these vaccines. This very good safety also applies to vaccines adjuvanted with MF59 or with AS03, which represent the vaccines mostly utilized in Europe. This information is particularly important due to the particular risks caused by the pandemic A/H1N1 infection in some populations such as children [82] and pregnant women [83]. The safety of these adjuvants, for example, MF59, has been shown in these groups of people as well [84, 85]. More data are being reported from the experience in some countries such as the UK [86], and further data will become available in the next months. It is hoped that through this experience and learning, vaccine adjuvants will become more and more useful in the development of other novel vaccines.

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