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Development of a universal CTL-based vaccine for influenza

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In pursuit of better influenza vaccines, many strategies are being studied worldwide. An attractive alternative is the generation of a broadly cross-reactive vaccine based on the induction of cytotoxic T-lymphocytes (CTL) directed against conserved internal antigens of influenza A virus. The feasibility of this approach using recombinant viral vectors has recently been demonstrated in mice and humans by several research groups. However, similar results might also be achieved through immunization with viral proteins expressed in a prokaryotic system formulated with the appropriate adjuvants and delivery systems. This approach would be much simpler and less expensive. Recent results from several laboratories seem to confirm this is as a valid option to be considered.

Control of Seasonal and Pandemic Influenza

Seasonal influenza is an acute respiratory illness caused by influenza virus. This disease has a strong impact on public health worldwide causing, annually, 3 to 5 million cases of severe illness and between 250,000 and 500,000 deaths, mainly among children, elderly and immune-suppressed individuals (http://www.who.int/mediacentre/factsheets/fs211/en/). The best way to fight the impact of this disease is to vaccinate the population. Available vaccines are mostly inactivated ones, with a smaller proportion of live attenuated vaccines. Inactivated vaccines are produced mainly in embryonated chicken eggs and to a lesser extent in cell culture.[1,2] Influenza vaccines induce protection in immunized individuals through the generation of neutralizing antibodies, mainly directed against the viral envelope glycoprotein hemagglutinin (HA). Virus antigenic variants arise constantly due to the high variability of the gene encoding the HA. Given the high rate of antigenic variation of the HA, antibodies that neutralize a subtype are often ineffective to neutralize other subtypes, and consequently the strains included in seasonal vaccines must be constantly updated. Vaccine efficiency severely diminishes when new strains emerge with antigenic changes in the virus envelope proteins, and situations where the antigenic matching between vaccine strains and the new circulating ones is sub-optimal are not exceptional. On rare occasions, completely new pandemic influenza variants arise, to which most of the human population has not been exposed. Under these circumstances, population immunity is low or null, allowing for an accelerated transmission of the new strain worldwide, which can have devastating consequences in terms of human lives. A recent analysis of the effectiveness of influenza vaccines holds that the immunity generated during certain seasons is at best moderate, when not significantly low or absent. In the ideal situation when antigenic matching between vaccines and circulating strains is optimal, average effectiveness was 69%.[3] During an outbreak of a pandemic strain there is a risk that the development of a vaccine for the emerging strain be too slow and, when available, come too late.

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Improving the Performance of Current Vaccines

Much effort has been devoted to the improvement of current vaccines using different strategies, such as exploring new routes of vaccine administration, like oral,[4] intranasal,[5] or intradermal immunization,[6] new delivery systems like micro-needles[7] or addition of adjuvants which might allow for an increase in the humoral and cellular responses,[8,9] and a significant decrease in the dose of protective antigen needed.[10] It has also been reported that variations in the method of inactivation can significantly improve vaccine efficacy.[11,12]

Development of New Generation Vaccines

The main line of work in this field is focused on generating totally new vaccines using genetic engineering techniques. Conspicuous examples of this are: expression of the viral HA by means of recombinant vectors,[13-15] production of virus-like particles (VLP) of influenza containing the influenza proteins HA and Matrix1 (M1),[16] production of recombinant HA subunit vaccines in insect cells through the baculovirus system,[17] and production of recombinant HA or its fusion with flagellin in *Escherichia coli*.[18,19] DNA vaccines and peptide based vaccines have already been assayed in humans with promising results.[20,21] While many of these strategies proved to be very efficient and in some cases induced significant increases in cross-reactive immune responses, they did not completely solve the problems derived from the high antigenic variability of influenza virus.

A Universal Vaccine for Influenza

This line of work is pursuing a "universal" type of vaccine, that is, a vaccine that can protect against almost all known subtypes of influenza, including pandemic strains.[22] In this regard three main strategies are being pursued:

Induction of neutralizing antibodies against highly conserved regions of the HA. The dogma that the influenza virus neutralization is mediated only by antibodies that bind to the globular head of the HA protein has been recently challenged. Several laboratories worldwide have generated broad spectrum human monoclonal antibodies capable of neutralizing the virus by binding to a highly conserved region of the HA (the stem or stalk domain).[23] Some of these monoclonal antibodies are capable of reacting with all the known specificities of HA, and it has been demonstrated that passive transfer of this kind of antibodies to mice and ferrets may protect against a challenge with heterologous strains.[24] However, it is difficult to find the appropriate antigen capable of efficiently inducing such antibodies in vivo after immunization.[25]

An influenza A vaccine based on the ectodomain of the Matrix2 protein. The M2 protein (encoded in the same gene as the M1 protein) is a tetramer, functioning as an ion channel, and is present in very low amounts on the viral particle surface. The N-terminus of this protein (known as ectodomain or M2e) is highly conserved among strains of almost any origin. Based on this, M2e has been postulated as a very good candidate for the development of a universal vaccine. This assumption has been thoroughly demonstrated in pre-clinical models using various strategies.[26,27] It was demonstrated that alveolar macrophages and Fc-receptors are fundamental for anti-M2e IgGs-mediated protection to occur.[28] Currently, the potential of an M2e-based universal vaccine is also being analyzed in human clinical trials.[29,30] Although a vaccine based on the induction of anti-M2e antibodies is very promising, it will probably need to be combined with other conserved influenza antigens, able to elicit an adequate cellular response for a fully protective immunity.

A T-cell vaccine for influenza. The goal of a T-cell vaccine is to induce a strong response of specific CD4 + and CD8 + lymphocytes which may contribute to pathogen clearance by recognition and elimination of infected cells. For several important human infectious diseases, the efficacy of T-cells to induce therapeutic or prophylactic vaccines based on the use of replication-deficient viral vectors has already been established.[31] In the particular case of influenza, the generation of a non-sterilizing cellular based immunity is being sought; an immunity which would substantially decrease morbidity and mortality induced by the infection. Cytotoxic T- lymphocytes (CTL) are very effective in killing target cells by different mechanisms, thus eliminating the viruses from the infected organisms. In the case of influenza, it has been demonstrated that this type of immunity can protect mice from a lethal challenge with influenza A virus.[32]

Candidate Proteins for a T-cell Vaccine for Influenza

Unlike surface glycoproteins, proteins located within the influenza virion such as the NP and M1 are highly conserved due to their functional role during the viral replication cycle. It is well known that while they cannot induce neutralizing antibodies in infected or immunized animals, they are capable of inducing strong cellular immunity. For many years it has been known that mice recovering from infection with a certain subtype of influenza A virus have some protection against lethal challenge with a heterologous strain, and that the immunological basis of this phenomenon is mediated mainly by anti-influenza specific CTLs.[33,34] These CTLs recognize highly conserved amino acid sequences of certain viral proteins, mainly proteins within the virus particle, exposed by the MHC class I pathway on the surface of infected cell.[35-38] Although it has not been clearly established yet, there is some evidence of correlation between CTLs and protection, in humans.[39,40]

The cross-reactivity of polyclonal virus-specific $CD8^+$ T-cell populations (obtained from european subjects), which target cells pulsed with H5N1-derived peptides or NP gene-transfected cells of the same avian influenza virus, demonstrates that human CTL response displays a high degree of cross-reactivity for very diverse influenza virus subtypes.[41]

The concept that a vaccine formulated with a recombinant antigen unable to induce neutralizing antibodies can protect immunized mice from a lethal viral challenge was validated in the early 90s. Earlier experiences with a NP-based T-cell vaccine using purified recombinant NP (rNP) as a vaccine antigen indicated the validity of this concept,[42] which was further confirmed with a genetic vaccine containing a NP gene-carrying plasmid, which was able to protect mice against lethal challenges with an heterologous influenza virus.[43]

Recombinant Vector Based T-cell Vaccine for Influenza

Very recently, the use of recombinant adenovirus vectors expressing viral proteins NP and M2 (full length or M2e), showed that a T-cell vaccine against influenza could be extremely effective in mice.[44,45] Furthermore, in a similar approach in a Phase I clinical trial, a modified Vaccinia virus Ankara (MVA) vector, encoding NP and M1, generated potent T-cell CD8+ specific immunity in immunized humans.[46] A Phase II clinical trial, conducted in healthy volunteers showed the efficacy of this vaccine to protect against flu symptoms after a challenge with live virus.[47]

Development of a T-cell Vaccine Based on Adjuvanted rNP Produced in a Prokaryotic System

Although influenza vaccine candidates based on recombinant vectors are very promising, it should be noted that safety trials for this strategy will take a long time before they are approved and massively available in the market. Furthermore, its production involves the handling of sophisticated and expensive technology not available in many developing countries. Therefore, it would be important to develop vaccines able to produce the same results but using simpler and less expensive production systems. Recently published results suggest that a NP based T-cell vaccine against influenza A could also be achieved using the recombinant

protein produced in *E. coli*, which would be a far more simple and inexpensive system. In our laboratory, we have confirmed other group's results, that is that the NP protein can be easily produced and purified in large quantities at low cost in *E. coli*.[48-50] The same appears true for other influenza proteins which are also candidates for a T-cell vaccine, such as M1 and M2.[50,51]

The main limitation of this approach is the difficulty to induce a strong CTL response in animals immunized with an exogenous protein.[31] However, exogenous proteins may induce a CTLs response by the phenomenon known as cross presentation,[52] and it is known that cross-presentation of an exogenous protein can be greatly stimulated with certain adjuvants which favor this process.[53] Recently, several reports confirmed the effectiveness of vaccines formulated with adjuvanted rNP to protect vaccinated animals against a lethal challenge of homologous or heterologous virus. Intranasal administration of cholera toxin combined with rNP protected against multiple viral subtypes.[49] The combination of recombinant NP and M2 formulated as liposomes stimulated a marked increase of specific CTLs and protected the vaccinated mice from a lethal challenge with the H5N1 avian strain.[50] A vaccine formulated with rNP and a TLR3 ligand, induced specific CTLs and protection against lethal challenge from influenza virus.[54] Fortunately, the knowledge on the possibilities of increasing the CTL responses to a recombinant protein is constantly increasing. A priori, there are multiple potential formulations that could lead to the optimization of a T-cell based rNP vaccine for influenza. In the pursuit of such a strategy, our laboratory has recently begun a systematic search of adjuvants able to promote a strong CTL response in animals vaccinated with rNP.

Iscomatrix Adjuvanted Influenza Vaccines

Iscomatrix (IMX) adjuvant is an immunostimulating system which also optimizes the process of antigen delivery, and is very efficient in obtaining a strong CTL response after immunization with exogenous protein.[55,56] IMX consists of nanoparticles of about 40–50 nm in diameter with a strong negative charge, generated by the self assembly of phospholipids, cholesterol and saponins.[55] The negative charge of these particles favors their interaction with basic proteins such as the NP.[57] After immunization, the nanoparticles containing the antigen migrate to the draining lymph nodes, where they are captured and internalized by lymphoid resident dendritic cells (DC). IMX favors the process of extracellular antigen translocation from the endosome to the cytosol for proteasomal degradation. The processed peptides can then enter the major histocompatibility complex class I (MHC I) pathway, favoring the mechanism of cross presentation and the generation of CD8+ lymphocytes. The DCs that have taken up the particles are also activated, releasing many cytokines and lymphokines which stimulate the magnitude of the immune response. On the other hand, this system also has a strong stimulating activity on the humoral arm of the immune response.[56] This system has been successful when combined with inactivated influenza vaccines. It has proven very effective in stimulating mucosal immunity by the intranasal route.[58] It has also been effective in decreasing the minimum antigen dose required to obtain protection after pulmonary delivery of an influenza vaccine.[59] In mice, it was shown that IMX greatly improves the efficiency of a commercial vaccine increasing hetero-subtypic protection and CTL response.[60] In humans, a trivalent inactivated influenza vaccine formulated with IMX, elicited a sharp increase in the CTL response compared with those individuals that received the unadjuvanted vaccine.[61,62] Rimmelzwaan et al.[62] found that IMX significantly increases the anti-HA CTL response, but not an anti-NP CTL response. This is different from what happens in vivo, where after infection with influenza virus, the anti NP CTL response is dominant. In our experience, analysis of the values of IgG subtypes and interleukins in the sera of mice that had been immunized with IMX-formulated rNP clearly indicated that the response obtained had a strong Th1 profile.[63] These experiments also showed the generation of high titers of IgG anti-NP. This should be also taken into account, since it has been demonstrated that specific high titers obtained after immunization of mice with rNP contributed to a rapid antibody-dependent elimination of the year 2009 H1N1 pandemic virus strain and that high anti NP titers correlated with an increase in the CD8+ response.[64,65] These results strongly suggest that antibodies induced by immunization with rNP-IMX could also contribute, significantly, to a T-cell vaccine.

Are Adjuvanted Split Virus Vaccines Able to Induce a T Cell Response?

As mentioned previously, Rimmelzwaan et al.[62] found that, contrary to what happens in infected individuals, the formulation of a split trivalent inactivated vaccine with IMX promotes the generation of CTL specific to HA but not NP. Lamere et al.[64] found that the lack of immunological reactivity of the endogenous NP contained in the split trivalent inactivated vaccine in mice, may be slightly improved by adding lipopolysaccharides to the vaccine formulation. In humans, preceding titers of specific anti NP IgG can be boosted in only few cases in subjects immunized with conventional trivalent vaccines. In a similar way, Savard et al.[66] found that NP and M1 proteins present in split virion seasonal flu vaccine, are not immunogenic in immunized mice. However they showed that immunization of mice and ferrets with the same vaccine adjuvanted with papaya mosaic virus nanoparticles triggered a cell-mediated immune response to NP and M1, and long-lasting protection in animals challenged with a heterosubtypic influenza strain. Based on the above mentioned facts, there is some evidence that the endogenous NP contained in split influenza vaccines can be stimulated to produce a CTL response. However, the current processes of vaccine manufacturing are not validated to assess the content of NP in each batch, nor is it certain that the NP complexed with the genomic RNA in whole inactivated virus will be the most suitable antigen. On this basis it is tempting to hypothesize that for the purpose of generating an influenza specific CTL response, it would be more convenient to use recombinant NP, combined with a seasonal subunit vaccine.

Conclusions

In recent years the trend in the field of recombinant vaccines development has been the use of viral vectors when looking for a strong cellular response[31] and purified proteins when looking for a humoral response.[67] The use of purified recombinant proteins for the development of vaccines against infectious agents that require a strong cellular response has been virtually neglected. However, our results and those of other laboratories confirm that it is possible to induce cell-mediated immune responses with purified proteins formulated with the appropriate adjuvants. Such formulations may even be improved by using particulate delivery systems. This type of methodology has been developed extensively in recent years and has also proved to be a very powerful method of inducing cellular immunity.[68-70] In our laboratory we are currently developing delivery systems which include rNP in adjuvant loaded nanoparticles. In our work we have used rNP alone, however it is clear that the inclusion of other proteins with similar properties such as M1 and M2 is desirable. The production of vaccines based on this technology would be inexpensive due to their simplicity, and the technology could be certainly implemented in developing countries, which are now almost completely dependent on external sources of production. A vaccine of this type should be combined with the seasonal vaccine to elicit both robust influenza-specific antibody and CTL responses for maximal protection.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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