REVIEW ARTICLE

INTERNATIONAL MICROBIOLOGY (2004) 7:83–94
www.im.microbios.org

INTERNATIONAL MICROBIOLOGY

Carolina Damas Rocha[1,2]
Braulia Costa Caetano[1,2]
Alexandre Vieira Machado[2]
Oscar Bruña-Romero[1,*]

[1]Laboratory of Immunopathology, René Rachou Research Center, Oswaldo Cruz Foundation (FIOCRUZ), Belo Horizonte, Minas Gerais, Brazil
[2]Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Recombinant viruses as tools to induce protective cellular immunity against infectious diseases

Summary. Infections by intracellular pathogens such as viruses, some bacteria and many parasites, are cleared in most cases after activation of specific T cellular immune responses that recognize foreign antigens and eliminate infected cells. Vaccines against those infectious organisms have been traditionally developed by administration of whole live attenuated or inactivated microorganisms. Nowadays, research is focused on the development of subunit vaccines, containing the most immunogenic antigens from the particular pathogen. However, when purified subunit vaccines are administered using traditional immunization protocols, the levels of cellular immunity induced are mostly low and not capable of eliciting complete protection against diseases caused by intracellular microbes. In this review, we present a promising alternative to those traditional protocols, which is the use of recombinant viruses encoding subunit vaccines as immunization tools. Recombinant viruses have several interesting features that make them extremely efficient at inducing immune responses mediated by T-lymphocytes. This cellular immunity has recently been demonstrated to be of key importance for protection against malaria and AIDS, both of which are major targets of the World Health Organization for vaccine development. Thus, this review will focus in particular on the development of new vaccination protocols against these diseases. [**Int Microbiol** 2004; 7(2):83–94]

Key words: adenovirus · vaccinia virus · influenza virus · vaccines · T-lymphocytes

Received 16 January 2004
Accepted 19 February 2004

*Corresponding author:
Oscar Bruña-Romero
Centro de Pesquisas René Rachou-FIOCRUZ
Av. Augusto de Lima 1715
Belo Horizonte, MG 30190-002, Brazil
Tel. +55-3132953566. Fax +55-3132953115
E-mail: oscar@cpqrr.fiocruz.br

Introduction

Immunogenic and protective antigens of the causative agents of malaria (*Plasmodium* spp.) and AIDS (human immunodeficiency virus, HIV), which are two of the major international targets for vaccine development, have been already identified. However, the development of vaccines against those diseases has been hampered in part by the inefficient stimulation of the corresponding protective cellular immune responses to traditional vaccines, which are based on the administration of purified antigens. In many cases, these traditional immunization techniques and protocols do not seem to result in the triggering of sufficient levels of cellular immunity required for the elimination of infected cells.

New vehicles for antigen delivery, immunization adjuvants, and vaccination protocols to improve cellular immunity are currently being tested both in animals and in humans. Among the new vehicles for antigen delivery, some of the most promising are vaccines based on recombinant viruses. These can express the foreign antigens directly inside cells of the host organism, as would happen in natural infection. Antigens so expressed are made available to the intracellular antigen-processing machinery, allowing processing of the antigen and binding of the resulting fragments to major histocompatibility complex (MHC) molecules, favoring their presentation to T-lymphocytes (Fig. 1).

The construction of recombinant viruses requires adaptation of the DNA sequences that encode the antigen for expression in host cells. In many cases, it also requires mul-

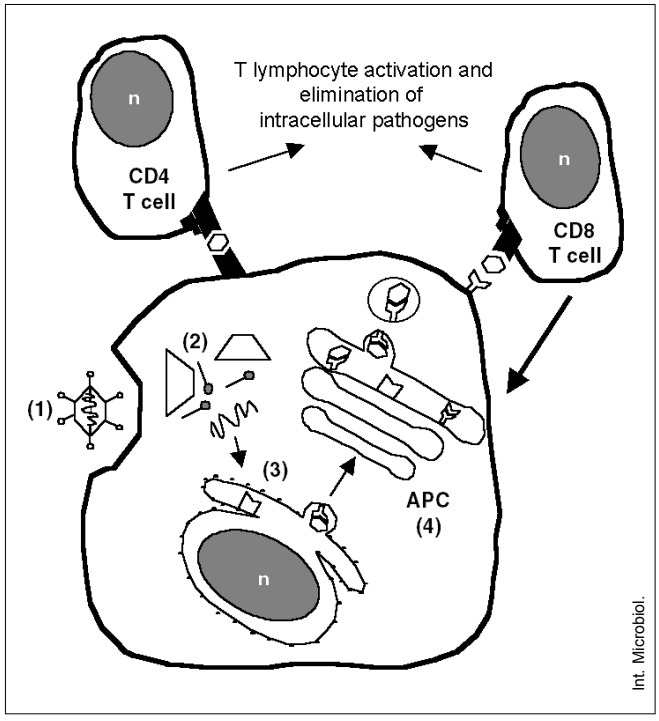

Fig. 1. Immune responses induced by recombinant viruses. Recombinant viruses carrying sequences that code for the desired antigen have several advantages in inducing cellular immune responses (both CD8 and CD4 T lymphocytes) against intracellular pathogens: (1) They infect the cells efficiently by nature. (2) The viral proteins may act as vaccination adjuvants. (3) They express the recombinant products in the cytoplasm of cells of the host organism as the intracellular pathogen would do in a natural infection; fragments of the recombinant products can then be easily loaded onto MHC molecules and presented on the surface of the host cells to T-lymphocytes. (4) They can directly infect antigen-presenting cells, eg. dendritic cells, which favors initiation of the immune responses. n, Cell nucleus.

tiple intracellular recombination steps to incorporate the transgenes into the viral genome. However, although complicated to generate, recombinant viruses have several features that make them excellent immunogens and vehicles for vaccine delivery. First, by nature, viruses have evolved to be the organisms that most efficiently infect cells. In 10 min, more than 95% of the initial viral load is in an intracellular form for some viruses [35]. Second, viral proteins can act as strong immunization adjuvants [36]. Third, viruses can infect directly antigen-presenting cells, thereby avoiding unnecessary cross-presentation steps [4]. In addition, some recombinant viruses can be lyophilized and stored without the need of special refrigeration equipment. As a whole, these and other features have enabled the development of immunization protocols based on a single administration of the viral vectors capable of inducing potent immune responses, which in many cases completely protect against the respective diseases.

Although almost any viral genome can be manipulated to become recombinant and acquire the capacity of expressing foreign antigens in host cells, not all viruses have the same efficiency in doing so. Certain types have been reported to be more efficient than others in inducing immune responses. Among those, poxviruses, adenoviruses, and influenza viruses are three of the most attractive and efficient vectors that can be used for vaccination purposes. We will describe the features and construction particularities of these three viral vectors, which we have studied for the last several years, as well as the pros and cons of their use.

In the cases in which immunization with one viral vector did not elicit sufficient levels of immunity, researchers have successfully immunized by the sequential administration of two of these vectors. These immunization protocols are the so-called prime-boost protocols. Several seminal studies, first performed in the field of malaria vaccine development [27,34,39,47], have provided much of the present knowledge on these immunization protocols, and most of the new vaccines in development apply this technology, especially when they aim at inducing a strong cellular immune response against a pathogen. Studies that included these potent immunization protocols will also be commented on in this review.

Recombinant poxviruses

Members of the Poxviridae family that infect vertebrates are divided in eight genera: Avipoxvirus, Capripoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suipoxvirus and Yatapoxvirus. The constituents of a genus are genetically and antigenically related and have similar morphologies [12].

Vaccinia virus and other related orthopoxviruses. The Orthopoxvirus is the most studied genus of the Poxviridae family, having as major representatives the variola and vaccinia viruses. Variola, the agent of smallpox, is strictly a human virus. In contrast, vaccinia virus, which is used as a smallpox vaccine, has a wide vertebrate host range and can therefore serve as vaccine vector in a large variety of species. Vaccinia virus replicates in vitro and in vivo in various species of vertebrates from birds to humans. Nowadays, however, vaccinia virus has no natural reservoir and it is considered to be almost exclusively a laboratory virus.

The poxviral genome consists of a linear 130- to 300-kb double-stranded (ds) DNA and is capable of accepting large inserts of foreign DNA, making it an excellent vector for expression of transgenic sequences. The life cycle of

poxviruses is completed within 12–24 h and occurs exclusively within the cytoplasm of the infected cell [38]. A number of features make poxvirus recombinants good candidates as vaccine vectors: (i) the stability of freeze-dried vaccines, its low cost, and the ease of manufacture and administration; (ii) the cytoplasmic site of gene expression; (iii) the ability to induce cellular immune responses against the foreign antigen with long-lasting immunity after a single inoculation; and (iv) the flexibility of the genome, which allows large amounts of the genome to be lost or deleted and foreign DNA to be integrated (over 25 kb) without loss of infectivity.

In 1980, the World Health Organization declared smallpox eradicated and recommended the discontinuation of vaccination with vaccinia. This vaccination, which had been performed for nearly two centuries, consisted of the inoculation of vaccinia virus intradermically. The immune response to vaccinia, although a less virulent virus, promotes cross-protection against smallpox. However, despite the relative safety of this vaccine, some vaccine-related complications occurred during the eradication of smallpox. In young children, eczema vaccinatum and encephalitis were serious albeit infrequent complications. In adults, disseminated or progressive vaccinia infections occurred in individuals with severe immunodeficiency. Although very rare, these complications represented a potential risk.

The 1980s also marked the application of recombinant DNA technology to vaccinia virus. Recombinant gene expression by vaccinia virus was first demonstrated in 1982 [30,41]. These studies provided the means to genetically engineer poxviruses and to develop them as expression vectors and candidate vector vaccines against infectious diseases. However, due to the complications observed during smallpox vaccination, an important safety issue arose because of the possibilities of accidental laboratory infections or side effects of vaccination, especially when using the replicative, e.g. Western Reserve or Copenhagen, strains of the virus.

Two main approaches have been taken in order to enhance the safety of vaccinia virus. One of them consists of deleting the viral genes that are involved in viral replication, host interactions, and/or extracellular virus formation. A second one consists of successive passages of the virus in an unnatural host or in tissue culture, and the isolation of viral variants with more appropriate features.

Multiple deletions in the viral genome have led to highly attenuated vaccinia virus strains, e.g. the NYVAC strain. This strain was derived from the Copenhagen vaccinia virus strain after deletion of 18 open reading frames (ORFs) of the viral genome. Among the ORFs deleted to generate strain NYVAC were genes involved in nucleotide metabolism, host regulatory functions, and viral virulence. The attenuation characteristics of the NYVAC strain were compared in vitro and in vivo with the parental Copenhagen strain as well as with other vaccinia strains. The NYVAC strain was demonstrated to be highly attenuated in many animal studies. It failed to disseminate in immunodeficient mice and also displayed a dramatically reduced ability to replicate on a variety of human tissues, i.e.cultured cells. Despite these highly attenuated characteristics, the NYVAC strain, as a vector, retains the ability to induce strong immune responses to foreign antigens [42].

Successive passages of the virus can also lead to safer vaccinia viruses. The major example is the modified vaccinia virus Ankara (MVA) strain, which was isolated after more than 570 passages in primary chicken embryo fibroblasts. MVA lost the ability to replicate in mammalian cells and, as a consequence, there were no serious complications in about 120,000 recipients, including young children and aged individuals, who received the modified virus. MVA also has the advantage, when compared with replication-competent vaccinia viruses, that it can be used in immunocompromised patients. Moreover, studies using mammalian cells infected with MVA have shown that products of both early and late viral genes are expressed, including foreign antigens, as the block in viral replication occurs during virion assembly [14]. This lack of assembly of MVA makes the chance of spreading to unvaccinated individuals or into the environment very low.

Avipoxvirus. The Avipoxvirus genus has similar characteristics to all other poxviruses: a large linear dsDNA genome of up to 300 kb, replication in the cytoplasm of infected cells, and, importantly, the capacity to induce immunity in host organisms. Avipoxviruses infect a number of bird species, being named after them, e.g. fowlpox, canarypox or pigeonpox viruses. Wild-type strains can cause serious lesions in their natural host species, but cannot replicate in non-avian species. Attenuated strains of avipoxviruses, produced by successive passages on chicken embryo fibroblasts, have been used as vaccines for chickens and other animals for decades. These attenuated strains are therefore recommended in order to reduce the risk of environmental spread.

Besides being used as vectors for birds, avipoxviruses have also been demonstrated to be efficient vehicles for expression of foreign antigens in mammals. The inoculation of recombinant avipoxviruses into mammalian cells results in expression of the foreign gene in those cells, and inoculation into the mammalian organism results in the induction of protective immunity [52]. These properties, together with the fact that these viruses have lost the ability to replicate in non-

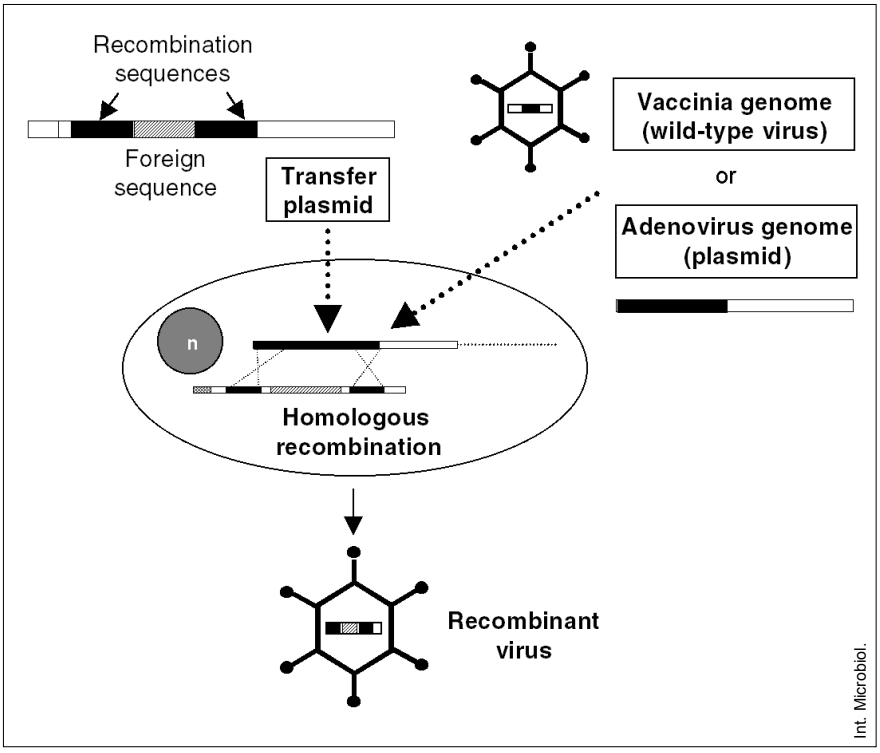

Fig. 2. Construction of a recombinant vaccinia virus and an adenovirus by homologous recombination. Vaccinia virus construction is a helper virus-dependent system, in which a wild-type virus incorporates the desired transgenic sequence into the genome by means of a homologous recombination event with a plasmid vector prepared to transfer that sequence. In contrast, recombinant adenoviruses are mostly generated by homologous recombination using a helper-virus-independent system. Simultaneous transfection of two plasmids, one containing the DNA encoding the desired antigen and one containing the viral genome in a partially deleted non-replicative form, is required. n, Cell nucleus.

avian species, have made avipoxviruses a safe and efficient vector for inoculation of mammals. Attenuated derivatives of fowlpox virus, i.e. TROVAC, and canarypox virus, i.e. ALVAC, have already been tested in a variety of species in the veterinary field and even in human volunteers.

Construction of recombinant poxviruses. The most common methodology for the generation of a recombinant vaccinia virus is homologous recombination. Susceptible cells are transfected with a plasmid containing the gene of interest under the control of a vaccinia virus promoter, a selectable marker gene or antibiotic resistance gene, and flanking portions of nonessential vaccinia virus DNA, i.e. thymidine kinase DNA (Fig. 2). The poxvirus promoters are approximately 30 bp long and occur in three varieties: early, intermediate and late. Some plasmids can combine strong synthetic early/late tandem promoters to achieve a system with both early and high expression levels. Co-transfection of this plasmid and a helper wild-type vaccinia virus leads to homologous recombination between the plasmid and the vaccinia genome [30]. There are a variety of methods for the isolation of recombinant vaccinia viruses, including selection based on bromo-deoxyuridine, antibiotic resistance, detection of a colored marker expression, plaque phenotype, and DNA hybridization. Generally, the recombination occurs into the thymidine kinase locus of the viral genome, leading to the deletion of this gene, a process that also results in the generation of attenuated viral recombinants.

The in vitro ligation of an exogenous gene into the vaccinia virus genome represents an alternative to homologous recombination. This strategy involves cutting the vaccinia virus DNA at a unique restriction endonuclease site, re-ligating the two halves of the genome with the recombinant gene, and finally transfecting the ligated DNA into cells that have been infected with the helper virus. This approach allows the insertion of very large DNA fragments into the viral genome [38].

Recombinant poxviruses as live vaccines. Using some of the techniques mentioned above, recombinant poxviruses were initially engineered to express the protective surface antigen of hepatitis B virus (HBV) [50], hemagglutinin of influenza virus [51], envelope proteins of HIV [23] or the circumsporozoite protein of *Plasmodium knowlesi* [49]. In studies performed using those recombinants, the capacity of vaccinia to induce T-cell responses (CD4+/CD8+) could be shown, making these viruses good candidates as live vaccines against infectious diseases caused by intracellular pathogens. In animals, a replicative recombinant vaccinia virus expressing the rabies virus glycoprotein was responsible for the eradication of rabies in Belgium [3]. In humans, the replication-deficient MVA strain is at present being tested

for immunization against several diseases, and is one of the preferred vehicles for antigen delivery due to its safety profile. Two important diseases for which MVA vaccine research is currently under way in humans are AIDS [11,20] and malaria [37]. Preliminary data obtained from those studies suggest that MVA indeed has the capacity to induce specific T-cells against the corresponding pathogens, as was shown previously in mice and monkeys.

Although these and other reports have highlighted the importance of poxviral vectors as live vaccines, one major drawback of vaccination with recombinant poxviruses, as with other viruses for which humans are natural hosts, has to be mentioned. Some authors observed that the capacity of vaccinia virus to induce an immune response against heterologous proteins could be greatly impaired in recipients that had immunity against vaccinia virus [25]. Since several vaccinia virus strains were used worldwide for years to eradicate smallpox, this may become a serious concern when considering a global vaccination program. Attempts to overcome this limitation include the use of vaccinia virus as booster immunogens in prime-boost protocols combined with other antigenically unrelated vectors that can prepare the immune system for their potent effect. Those protocols are described below.

Recombinant adenoviruses

The Adenoviridae family is divided into two genera: Mastadenovirus and Aviadenovirus. Viruses from the Mastadenovirus genus have the capacity to infect mammals. Adenoviruses are medium-sized (70–100 nm), non-enveloped icosahedral viruses containing a linear, dsDNA genome of around 30–42 kb that does not integrate into the host cell genome. Adenoviruses infect both dividing and resting cells of many cell types, including antigen-presenting cells, a feature that has called much attention upon them both as gene therapy vectors and vaccine delivery vehicles.

There are 49 immunologically distinct adenoviral types (6 subgenera: A–F) that can cause human infections. Adenoviruses most commonly cause respiratory illness. Symptoms range from the common cold syndrome to pneumonia, croup, and bronchitis. Some adenoviruses (eg. serotypes 1, 2, 5, and 6) have been shown to be endemic in regions of the world where they have been studied, and infection is usually acquired during childhood. Adenoviruses have already been used for vaccination purposes in humans. Serotypes 4 and 7 were used for decades to prevent acute respiratory syndrome (ARD) in military troops of the American army, and whether vaccination with those viruses should be maintained in the army vaccination program is a topic of current discussion [22].

In the second half of the 1980s, the first recombinant adenoviruses were constructed, and the adenoviral vectors generated could replicate in many cell types. However, the observation that removal of the E1 region of the viral genome resulted in replication-deficient viruses led to the development of so-called first-generation replication-deficient recombinant vectors. Permissive cell lines, eg. human embryonic kidney 293 cells, which complemented the functions lacking in the deleted viral genomes, enabled the multiplication and purification of these recombinant viruses. Replication-deficient adenoviruses induce self-limited infections in host organisms, representing an enormous advantage in terms of safety, since they allow the recombinant immunogenic products to be expressed for just enough time to induce an immune response.

Since the initial first-generation vectors, researchers have developed new recombinant adenoviruses that contain less or none ("gutless") viral genes [53], in an attempt to limit the immune responses generated against the vectors and to favor their permanence in the host organisms. In addition, several research groups have pursued vector permanence with the aim of attaining high, sustained levels of expression of the transgenes, mainly for gene therapy purposes. However, this may not be necessary, and in some cases could be even prejudicial, when using adenoviruses as vaccines, since the immune responses generated against the vector help to clear the vaccine, avoiding its indefinite persistence in the organism. In addition, immune responses against the vector occur at the same time as those against the recombinant product; and it has been reported that the stimulation of the immune system induced by the former could act as an adjuvant for the latter [36].

Construction of recombinant adenoviruses. Different methodologies were initially used to generate recombinant adenoviruses. To date, however, most recombinants constructed are based on human serotype 5 adenoviruses by a homologous recombination approach performed either in mammalian cells or in bacteria [21,32]. The initial work of Frank Graham and collaborators was seminal in this case [32]. They used two non-infectious bacterial plasmids for this purpose, one containing the desired gene to be expressed inside a mammalian expression cassette and flanked by adenoviral genomic sequences, and a second one containing the serotype 5 adenoviral genome with regions E1 and E3 totally or partially deleted to avoid viral replication and to increase the size of foreign DNA that could be packed in the virion (Fig. 2). After cotransfection of both plasmids, homol-

ogous recombination between them resulted in the generation of a new viral genome with the desired transgene inserted in either one of the deleted regions. Viral particles could then be purified from permissive cell lines that complemented the function of the deleted genes. This methodology has been further improved by the development of new plasmids that allow faster recombinations in bacteria after including site-specific recombination systems such as Cre/loxP or the yeast recombinase FLP-frt system.

Recombinant adenoviruses as live vaccines. The first studies using recombinant adenoviruses as immunization tools were performed in the late 1980s against measles, HIV, HBV, rabies and vesicular stomatitis virus (VSV) [1,10,44,45]. These and subsequent studies showed that adenoviral vectors are extremely efficient in expressing recombinant products when potent promoters, eg. the immediate-early cytomegalovirus promoter, were used to express the transgenes. This efficiency, and the fact that adenoviral vectors infect many cell types, including antigen-presenting cells, revealed them as powerful tools for inducing cellular immune responses. In fact, in several cases, a single immunization with an adenoviral vector was enough to completely protect host organisms against a disease. A recombinant adenovirus expressing the *Plasmodium yoelii* circumsporozoite protein protected up to 40% of mice against experimental malaria after only a single administration [46]. A single inoculation of a recombinant adenovirus expressing herpes simplex virus (HSV) glycoprotein B also protected mice against a lethal challenge with HSV [31].

A major obstacle for the elicitation of such potent immune responses when adenoviruses are administered to humans is the presence, in a large percentage of the human population, of pre-existing immunity against the vector, because of naturally occurring infections. With the aim of developing new adenoviral tools that would avoid pre-existing immunity against adenovirus, several human and simian serotypes have been recently examined as possible vehicles for vaccine delivery. Human serotypes 2, 5 and 7 have already been used in humans and would be excellent candidates for vaccination provided that a simple blood test indicated that the individual had not been pre-exposed to at least one of these serotypes. New promising lines of research include canine and chimpanzee-derived adenovirus vectors to avoid pre-existing immunity against human viruses. For example, Pinto et al. have recently shown that AdC6 and AdC68, two simian recombinant adenoviruses expressing HIV gag, displayed a potent capacity to induce immune responses, even when used sequentially in prime-boost vaccination protocols [43].

Recombinant influenza viruses

The causative agents of the flu, influenza A viruses, are enveloped negative-strand RNA viruses belonging to the Orthomyxoviridae family. The genome consists of eight single-stranded RNA segments, each encoding a single protein, except three of the segments (NS, M and PB1), which encode two proteins. Influenza viruses have several features that render them very promising candidates to be used as antigen-delivery vectors [15]. First, they do not integrate into the host genome and do not last for long periods in the host organism, which consequently reduces the risk of potential vector-related oncogenesis. In addition, the existence of multiple influenza subtypes and variants allows multiple immunizations to be performed. Finally, there are attenuated viruses, such as cold-adapted influenza strains, that are currently under study with the aim of generating live attenuated influenza vaccines [24,33].

Construction of recombinant influenza viruses. The segmented nature of the influenza genome and the essentially monocistronic nature of each segment has hampered the development of recombinant viral vectors based on influenza viruses. The development of reverse genetics techniques during the last decade [40], however, has opened the door to construction of recombinant influenza viruses able to express heterologous sequences. Reverse genetics can be used in helper-dependent or helper-independent systems (Fig. 3). For the helper-dependent generation of recombinant viruses (Fig. 3A), a bicistronic segment encoding a viral protein plus the desired antigen together with four more plasmids encoding each of four viral structural proteins (required to generate the proteins that boost the formation of the new viruses) are cotransfected into permissive cell lines. Simultaneous infection of the cells with a wild-type influenza virus supplies the remaining elements necessary for the formation of recombinant viruses. Recombinants so generated have to be then selected, a process carried out in permissive cell lines, eg. MDBK. These cells take advantage of selection features included in the recombinant bicistronic segment, ie. the presence of a particular neuraminidase (NA) functional only in those cells, to favor the growth of recombinant viruses. When using a helper-independent viral system (Fig. 3B), a minimum of 12 plasmids have to be transfected into the same cell in order to provide the necessary elements to generate the new viruses. However, in this case no selection is required since the viral particles can only be generated when the recombinant bicistronic segment is included in the viral particle.

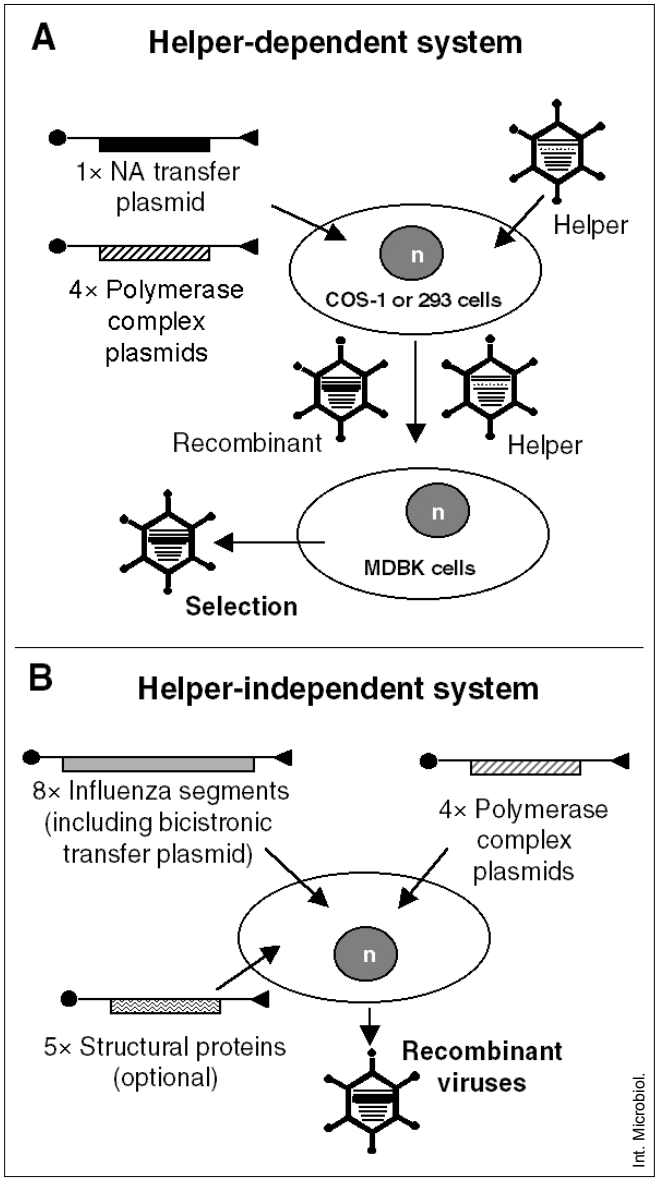

Fig. 3. Recombinant influenza viruses can be generated by helper-virus-dependent or independent methods. (**A**) Five plasmids have to be co-transfected: one encoding the recombinant bicistronic segment or fusion protein, and four encoding the structural proteins that would boost the formation of new recombinant viral particles. The helper virus provides the rest of required elements. However, by using this method, selection of the recombinant viruses in permissive cells is required. (**B**) Using the second method implies co-transfection of up to 17 plasmids to provide the necessary elements to generate recombinant viruses. A major advantage in this case is that no selection of recombinants is required. n, Cell nucleus.

The first approaches to generate recombinant influenza viruses used chimeric hemagglutinin (HA) [26] or NA [8] carrying short foreign sequences. Such strategies enabled the construction of influenza viruses bearing epitopes of *Plasmodium yoelli* [27] or *Plasmodium falciparum* [34] malaria parasites inserted into HA. Recombinant influenza viruses carrying T-cell epitopes of lymphochoriomeningitis virus (LCMV) virus or HSV-2 in the stalk of NA were also generated using this methodology [2,9]. However, these strategies suffered from the small size of the sequences that could be inserted into HA or NA, up to 12 or 28 amino acids respectively.

In order to express longer foreign sequences, several attempts were made to generate bicistronic constructs encoding NA or NS proteins together with the desired foreign antigen. To obtain individual proteins, the IRES element of the heavy chain of human immunoglobulin (BiP) was used as a separator of the coding sequences. In contrast to other separating elements tested before, BiP was functional in such bicistronic constructs [16].

Another strategy to express longer foreign sequences by recombinant influenza viruses was based on the replication and packaging of a ninth independent pseudo-vRNA segment by a wild-type influenza virus. A handicap of this strategy consisted of the loss of this non-essential additional segment after a few passages. However, the use of 3´-promoter mutants with higher replication levels enabled this ninth segment to be retained in the viral progeny for more than 10 passages [28].

Finally, bicistronic influenza segments could also be constructed based on the observation of Flick and Hobom, who demonstrated that the influenza polymerase complex can recognize internally located 5´and 3´promoters [13]. A pseudo-viral segment containing two genes flanked by the 3´and 5´non-coding sequences and separated by a duplicated 3´-promoter could be replicated and transcribed following two modes, after interaction of the single 5′-promoter sequence with either of the two 3′-promoter sequences. As a result, individual proteins could be expressed by using this simplified technique. Following this strategy, Vieira-Machado et al. explored the possibility of rescuing transfectant influenza A viruses harboring a bicistronic NA segment with a relatively long foreign gene, ie. the CAT protein, with a total length of 220 amino acids [29]. Recombinant influenza viruses harboring this bicistronic segment could be generated, and the synthesis of the CAT protein was demonstrated. Moreover, these recombinant viruses have been found to be genetically stable in vitro and in vivo upon replication in the pulmonary tissue of infected mice.

Recombinant influenza viruses as live vaccines. One of the first diseases for which influenza recombinants were generated and used as vaccine delivery vectors was malaria. B- and/or T-cell epitopes from the circumsporozoite protein (CS) of murine [27,47] and human [34] *Plasmodium* parasites were inserted into influenza recombinants. CD8+

T-cell epitopes from CS inserted into either NA or HA proteins displayed the same efficiency in inducing a specific immune response. Against AIDS, recombinant influenza viruses encoding the V3 loop epitope from HIV-1 strain IIIB also induced specific CD8+ T-cells [19]. Although specific, most immune responses induced by recombinant influenza viruses are of low magnitude compared to other viral vectors and specifically to recombinant adenovirus. However, immunizations with influenza recombinants have been shown to be extremely efficient in priming immune responses that will be further boosted by a different viral vector, ie. a vaccinia virus. Their use as priming agents in prime-boost protocols is discussed below.

Recombinant viral vectors as vaccination tools in prime-boost protocols

In many cases, sufficient levels of cellular immunity against a recombinant antigen could not be achieved after a single immunization with vaccinia, adenovirus or influenza vectors. Therefore, with the aim of enhancing the levels of T-cell immunity induced during the primary immune response (Fig. 4A), researchers developed the so-called prime-boost immunization protocols, by analogy with previous works in the field of humoral immunity. As with antibodies, where each of the two or three initial administrations of a purified antigen were capable of increasing the previous levels of specific antibodies generated against it by reactivation of the memory B-cell population, researchers hypothesized that re-exposure of the immune system to the same recombinant antigens expressed by the viral vectors should recall the memory T-cell responses induced during the primary exposure. These memory lymphocytes would proliferate much faster and even acquire much higher affinity for the antigens than in previous encounters. Nevertheless, this analogy to the situation with antibodies turned out to be wrong. A major limitation of the viruses as vaccine delivery vehicles was revealed: subsequent administrations of the same virus were cleared faster from the organism due to immune responses induced against the vector itself. As a consequence, the immune responses induced against the recombinant products were barely improved compared to those induced by the priming immunizations [18,39,54] (Fig. 4B).The limitation by the anti-vector immune responses to the re-expression of the antigen in the host organisms did not seem to make the same virus an optimal boost for the primary immune response. To avoid this problem, a different viral vector expressing a common antigen was subsequently administered (Fig. 4C). The results

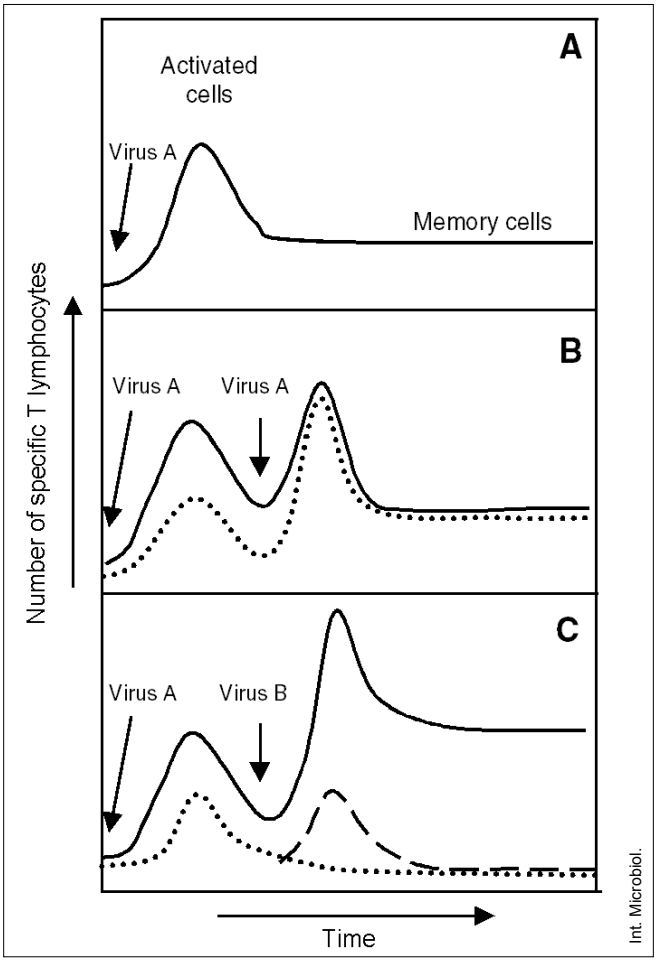

Fig. 4. Kinetics of an immune response after a single immunization with a viral vector or after a prime-boost immunization protocol. (A) Induction of a primary immune response. Starting from a certain number of specific naïve circulating precursors, stimulation caused by the presence of the antigen (delivered by recombinant virus A) in the organism activates those precursors, resulting in their proliferation up to a certain level (maximum number of activated/effector lymphocytes). Immediately after this plateau is reached, activated lymphocytes begin to die because of lack of antigenic stimulus or cytokines. However, long-lived memory T-cells with the same specificity remain in the organism and will respond much faster and with much more avidity upon a second encounter with the same antigen. (B) When the antigen is presented to the immune system a second time by means of a homologous prime-boost immunization protocol using the same recombinant viral vector (virus A + virus A), the immune response against the vector itself (dotted line) increases rapidly, impeding an optimal re-stimulation of the immune response against the recombinant antigen (continuous line). (C) Heterologous prime-boost protocols using viral vectors overcome this limitation. When a different viral vector (virus B) is used to re-stimulate the primary immune response (virus A), the immune response against the previous vector does not interfere with the new one, and thus the immune response against the recombinant product can be fully re-stimulated.

of several studies on malaria vaccine development by Zavala, Nussenzweig and collaborators [5,18,27,34,39,47] showed that greatly enhanced immune responses could be obtained when two viral vectors were used following the

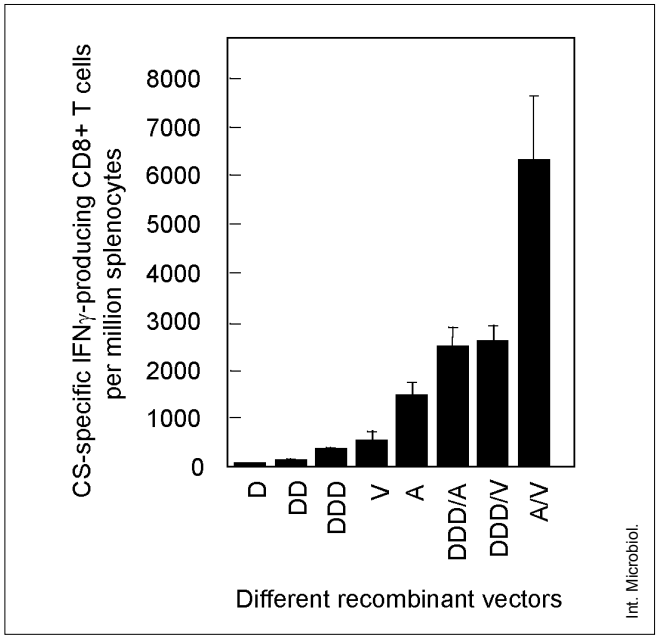

Fig. 5. *Plasmodium yoelii* circumsporozoite (CS)-specific IFNγ-producing CD8+ T-cells induced in animals immunized with different recombinant vectors encoding the CS protein. Animals were immunized with individual vectors or sequential combinations in the so-called prime-boost protocols as indicated. D, DNA plasmid immunization

same prime-boost immunization protocol. For example, a vaccinia virus expressing *Plasmodium yoelii* CS was able to increase more than 20-fold the number of CS-specific IFNγ-producing CD8+ T-cells induced by an influenza recombinant virus [27]. The sequence of administration of the vectors seemed to be of enormous importance. In fact, the immune responses induced by the vaccinia virus administered first could not be enhanced by the later administration of the influenza recombinant vector. Moreover, infection of mice with recombinant adenovirus, influenza virus, or an attenuated cold-adapted influenza virus, expressing all or parts of the CS protein of *Plasmodium* spp., were strongly enhanced by the subsequent administration of vaccinia recombinants derived either from the WR replicative or from the replication-deficient MVA strains. As an example, Fig. 5 shows representative results of our previous and of ongoing work using recombinant vectors, eg. DNA vaccines and viral vectors, encoding the CS protein of the murine malaria parasite *Plasmodium yoelii*. The number of IFNγ-producing CD8+ T-cells induced in mice immunized with the different delivery vectors, regardless of their plasmidic or viral nature, is substantially enhanced when prime-boost protocols are used to immunize the animals. However, Fig. 5 shows that a prime-boost protocol using exclusively viral vectors induces much greater immune responses than combinations of plasmid DNA vaccines and viral vectors. In all these studies, protection against malaria also increased after the boost, and elicited sterile protection against malaria in 100% of the animals when they were primed with a recombinant adenovirus or a cold-adapted influenza virus and boosted with recombinant WR or MVA vaccinia viruses respectively [5,18].

Several studies in other fields confirmed the capacity of poxviruses as powerful boosting agents of primary immune responses induced by other viral vectors. Thus, vaccinia viruses have been used extensively as boosters of the immune responses induced by other recombinant vectors encoding HIV proteins. A vaccinia recombinant encoding HIV-1 env protein was able to increase between five- and six-fold the immune response induced by a recombinant influenza virus encoding the CD8+ T-cell epitope contained in the V3 loop of this protein [19]. Moreover, even higher specific cellular immune responses directed against the same protein could be detected when the prime immunization with the recombinant influenza virus was followed by a boost immunization with a recombinant MVA virus [17,19], including at sites where the immune response against the virus is fundamental for protection, such as lymph nodes that drain genital and rectal tracks.

Recently, it has been shown that vaccinia virus may not be the only powerful boosting vector. Thus, one of the most promising recent approaches for AIDS vaccine development using viral vectors has been the immunization of monkeys with DNA vaccines as priming agents followed by adenovirus or a MVA recombinant as boosters [6,7,48]. In these studies, the adenoviral vector seemed to induce a better boost compared to MVA, eliciting high levels of protection against infection with a pathogenic simian-human hybrid AIDS virus

in all immunized animals. However, further studies are needed to confirm this conclusion.

Prime-boost protocols have also been demonstrated to be extremely useful to overcome some of the problems of pre-existing immunity to viral vectors. Thus, using a different vector, eg. DNA vaccines [54], to prime the immune responses to an antigen also encoded by a recombinant adenovirus or a recombinant MVA virus, these vectors were capable of providing a strong boost to the initial immune response in animals with pre-existing immunity against them.

In conclusion, viral vectors are promising tools to induce cellular immune responses against diseases caused by intracellular parasites. Since vaccination against these diseases will be difficult to achieve by immunization with purified antigens or even DNA vaccines, prime-boost immunization protocols using recombinant viral vectors that encode protective antigens alone or in combination with any of the other antigen-delivery systems have a great potential to accomplish this goal. The ongoing work in this field is providing new perspectives as to how to deal with infections caused by intracellular microbes, and, hopefully, one of the clinical trials under way will soon result in the first human vaccination program to include recombinant vectors as immunizing agents.

Acknowledgements. This work was supported by research grants of the PAPES III and PDTIS programs from the FIOCRUZ foundation of the Brazilian Ministry of Health and a research grant from the Fundação de Amparo á Pesquisa do estado de Minas Gerais (FAPEMIG). Support of the authors includes a CAPES fellowship to CDR and CNPq fellowships to BCC and AV-M. OB-R is a CNPq/FIOCRUZ visiting scientist.

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Uso de virus recombinantes para inducir inmunidad celular contra enfermedades infecciosas

Resumen. Las infecciones por patógenos intracelulares como virus, algunas bacterias y numerosos parásitos son neutralizadas en la mayoría de los casos tras la activación de respuestas inmunitarias específicas mediadas por linfocitos T, que reconocen los antígenos extraños y eliminan las células infectadas. Las vacunas contra estos organismos infecciosos se han basado tradicionalmente en la administración de organismos enteros atenuados o inactivados. Actualmente la investigación se centra en el desarrollo de vacunas compuestas por subunidades que contengan los antígenos más inmunogénicos para cada patógeno particular. No obstante, si se administran vacunas de subunidades purificadas mediante los protocolos tradicionales de inmunización, los niveles inducidos de inmunidad celular son en su mayoría bajos e incapaces de generar una protección completa contra las enfermedades causadas por los microorganismos intracelulares. En esta revisión presentamos una prometedora alternativa a estos métodos tradicionales mediante el uso de virus recombinantes cuyo genoma codifica las subunidades de vacuna. Los virus recombinantes reúnen varias características que los hacen muy eficientes para inducir las respuestas inmunitarias mediadas por los linfocitos T. Recientemente se ha demostrado que la inmunidad celular es crucial en la protección contra la malaria y el SIDA, que son un objetivo prioritario de la Organización Mundial de la Salud en cuanto al desarrollo de vacunas. Por lo tanto, esta revisión se centra especialmente en el desarrollo de nuevos protocolos de vacunación contra estas dos enfermedades. [**Int Microbiol** 2004; **7**(2):83–94]

Palabras clave: adenovirus · virus vaccinia · virus de la gripe · vacunas · linfocitos T

O uso vírus recombinantes na indução de imunidade celular protetora contra doenças infecciosas

Resumo. As infecções causadas por patógenos intracelulares como os vírus, algumas bactérias e muitos parasitas terminam, em muitos casos, após ativação de respostas imunes de células T específicas, as quais reconhecem os antígenos estranhos e eliminam as células infectadas. As vacinas contra esses microorganismos infecciosos foram tradicionalmente desenvolvidas através da administração dos micróbios completos vivos ou atenuados. Hoje em dia, a pesquisa em vacinas de subunidade contendo os antígenos mais imunogênicos desses patógenos é a principal escolha para as doenças contra as quais não existem vacinas. Porém, quando as vacinas de subunidade são administradas seguindo os protocolos tradicionais de imunização, os níveis de imunidade celular induzidos são comumente baixos e incapazes de gerar proteção completa contra as doenças causadas pelos microorganismos intracelulares. Nesta revisão apresentamos uma alternativa promissora aos protocolos tradicionais, que é o uso como ferramentas de vacinação de vetores virais recombinantes que codificam vacinas de subunidade. Os vírus recombinantes têm várias características interessantes que os tornam extremamente eficientes na indução de respostas imunes mediadas por linfócitos T. Durante os últimos anos, observou-se que esta imunidade celular tem importância fundamental para a proteção contra duas doenças que são objetivos primordiais da Organização Mundial da Saúde: a malária e a AIDS. Por esses motivos esta revisão focaliza especialmente o desenvolvimento de novos protocolos de vacinação contra estas duas doenças. [**Int Microbiol** 2004; 7(2):83–94]

Palavras chave: adenovírus · vírus vaccinia · vírus da gripe · vacinas · linfócitos T