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New Developments in Viral Vaccine Technologies

Guest Editors
Rainer Ulrich, Berlin
George P. Lomonossoff, Norwich
Detlev H. Krüger, Berlin

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Intervirology

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Editorial

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The development of efficient vaccines requires an interdisciplinary collaboration between physicians, epidemiologists, researchers in basic virology and immunology and experts in vaccine development. In addition, this cooperation should include not only research groups but also representatives of the World Health Organization, admittance authorities and pharmaceutical companies. The need to develop such a wide-ranging collaboration formed the background for the organisation of the latest International Workshop 'Virus-Like Particles as Vaccines', held for the third time at the Charité Medical School in Berlin in September 2001.

In contrast to the previous two workshops held in 1995 and 1998 [1, 2], which mainly focussed on the development of different virus-like particle carriers as potential sources of novel vaccines, the recent workshop was specifically dedicated to the creation and evaluation of vaccines against important viral pathogens such as hepatitis B virus (HBV), human immunodeficiency virus (HIV), human papilloma virus (HPV) and hantaviruses. These pathogens were selected not only because of their importance but also because of the fact that virus-like particles (VLPs) represent plausible vaccine candidates to combat infections by these viruses. This is confirmed by the fact that the first clinical trials for VLP-based vaccines against HIV, HPV, Norwalk virus and parvovirus infections have been completed (see Lechner et al.). However, in addition to VLPs (see contributions of Lechner et al., Menne et al., Wakabayashi et al., Sasnauskas et al., Kazaks et al., Sällberg et al. and Brown et al.), other vaccination strategies such as those based on the use of inactivated viruses (see Norley et al. and Cho et al.), live attenuated viruses (see Norley et al.), recombinant viruses (see Chatterji et al.), recombinant proteins (see Khattak et al.), synthetic peptides (see Norley et al.) and naked DNA vaccines (see El Kholy et al., Bojak et al., Norley et al. and Michel et al.) are covered by this issue, since the comparative evaluation of different approaches will be extremely important in the development of effective vaccines.

As an advance on the previous meetings, the recent workshop enabled the generation of a more complex and complete picture of the nature of the viral infections mentioned above and the basic knowledge which will be necessary for vaccine development. Consequently, in this issue of *Intervirology,* original papers specifically about development of vaccines against HBV, HIV, HPV and hantaviruses are introduced by reviews summarizing recent knowledge regarding the molecular biology, pathogenicity, epidemiology and immunology of these virus infections (see Pumpens et al., Marcus et al., Streeck and Ulrich et al.).

This issue covers selected presentations, most of which were delivered at the workshop in Berlin. The first two papers consist of general overviews of the history and perspectives of vaccine development (Hilleman) and the value of VLPs in vaccine development and treatment of allergy (Lechner et al.). The latter contribution also describes a unique principle of modular VLPs based on specific attachment sites (lysine) on the carrier and (engineered) single free cysteines on the antigen.

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Contributions dealing with new insights into HBV vaccine development are introduced by an update of recent knowledge regarding the molecular epidemiology and immunology of HBV infections (Pumpens et al.). A new approach to establishing an in vivo model for HBV infection based on recombinant HBV genomes containing substitutions of the preS1 of HBV by the corresponding segments of woodchuck hepatitis virus is described by Hourioux et al. The use of the woodchuck model also demonstrated the feasibility of a combination therapy of antiviral treatment with 1-(2-fluoro-5-methyl-β-*L*-arabinofuranosyl)-uracil and therapeutic vaccination with woodchuck hepatitis virus surface antigen for the control of chronic HBV infections in humans (Menne et al.). El Kholy et al. investigated the B cell immunogenicity of two HBV epitopes, namely the linear 'e2' epitope and the conformational 'a' epitope, fused to hsp73-binding or non-hsp-binding simian virus 40-T-antigen sequences when delivered as DNA vaccines. Because of the differences in immunogenicity, the authors concluded that the prerequisites that have to be fulfilled for optimal immunogenicity differ strikingly between individual epitopes. This is an important observation, since it might be true not only for HBV but for all other epitope-based vaccines.

Besides HBV, HIV represents one of the major targets for vaccine development. The epidemiology of HIV infections as well as strategies for therapy and vaccination are reviewed by Marcus et al. A number of HIV vaccine trials are summarized by Norley et al., which suggest that the 'traditional' types of vaccine are either ineffective or inappropriate for use in humans. An attractive alternative is the naked DNA vaccine approach, which can be improved in its efficiency by codon usage modification to induce a strong and long-lasting humoral and cellular immunity (Bojak et al.).

Cervical carcinoma represents one of the most frequent cancers in women worldwide. The biology of papillomaviruses, including viral structure, tropism, function of viral proteins, analysis of the immune response and search for the viral receptor, is covered by Streeck. To analyse the T cell responses to the HPV-16 E7 protein, a variety of different assays such as chromium release, ELISPOT, tetramer staining and intracellular IFN-γ assay were compared (Michel et al.). Wakabayashi et al. compared the vaccine efficacy of two types of chimeric HPV VLPs (L1-E7 or L1/L2-E7). Whereas L1-E7 VLPs are more efficient in tumor prevention than L1/L2-E7 VLPs, the L1-E7 VLPs are more limited with respect to their insertion capacity and yield in insect cells. The exciting properties of a yeast expression system allowing the formation of VLPs derived from the major capsid protein of various human and non-human polyomaviruses are described by Sasnauskas et al.

Hantaviruses have attracted more interest in the public and scientific community since the discovery of the hantavirus pulmonary syndrome, with a case fatality index of up to 60%, in the USA in 1993. The review of Ulrich et al. briefly summarizes the state of the art in hantavirus research and raises selected questions which it will be necessary to address in the future. Cho et al. tested two different inactivated hantavirus vaccines, Hantavax and a bivalent Hantaan-Puumala virus vaccine, in human volunteers, demonstrating them to be immunogenic and well tolerated. Nevertheless, the authors state that these vaccines need further improvements for the induction of a long-lasting immunity. To develop an edible potential hantavirus vaccine, Khattak et al. generated transgenic tobacco and potato plants expressing a Puumala virus nucleocapsid protein which induced humoral and mucosal immune responses in rabbits and mice. Kazaks et al. highlight the advantages of using HBV core mosaic particles to accomodate larger pieces of foreign proteins.

Sällberg et al. presented promising data about a potential malaria vaccine based on chimeric HBV core particles. A new approach to coupling foreign epitopes on the surface of cowpea mosaic virus has been developed by Chatterji et al., extending the potential use of VLPs. In a similar vein, Brown et al. report that VLPs are useful not only for the presentation of foreign epitopes, but also for the encapsidation of nucleic acids and antiviral drugs.

Knowledge of the intimate interplay between a virus and the immune system of the infected host is not only necessary to understand its pathogenicity but also forms a vital background for rational vaccine development. From the aspect of the virus, genetic events like mutation, genetic drift and recombination lead to the emergence of virus variants and eventually quasispecies populations in infected humans. Consequently, the understanding of the evolution of viruses and their underlying mechanisms might have an impact on the development of vaccines and antiviral drugs. This idea should be further explored at a next workshop.

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Intervirology

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Overview of the Needs and Realities for Developing New and Improved Vaccines in the 21st Century

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Key Words
Vaccines · New and improved vaccines · Vaccines for the 2000s · Vaccine technology

Abstract
The science of present day vaccinology is based on the pioneering discoveries of the late 18th and late 19th centuries and the technologic breakthroughs of the past 60 years. The driving force for the development of new vaccines resides in technologic feasibility, public need and economic incentive for translating the basic knowledge into a product. Past efforts by government to define which particular vaccines to develop were mostly irrelevant to the realistic choices which were made. There is a vast array of viral, bacterial, parasitic and fungal disease agents against which preventative vaccines may be developed, and to this may be added cancer and certain amyloidoses such as Alzheimer's and 'mad cow' diseases. The proven past for vaccines has relied on live, killed, protein and polysaccharide antigens plus the single example of recombinant-expressed hepatitis B vaccine. The validity of redirection of vaccinology to exploration of simplified vaccines such as recombinant vectored and DNA preparations and reductionist vaccines based on peptides of contrived epitope composition remains to be proved. Reductionism imposes vastly increased complexity and difficulty on vaccine development and might not be capable of achievement. The challenge in the 21st century will involve new and uncertain pathways toward worthwhile accomplishments.

Background

The first scientific probes into preventive vaccination were made in 1796 by Edward Jenner [1], who prevented smallpox through prior infection with the antigenically related cowpox virus. New discoveries in the last quarter of the 19th century were made principally by Louis Pasteur, Robert Koch, Emil von Behring and Paul Ehrlich, and these discoveries laid the scientific foundations for rational/empirical vaccinology [2–5]. The demands for vaccines to protect the soldiers in World War II marked the beginning of a transition to the modern era of vaccine science, which began in 1950 [3]. Presently, there are vaccines against 26 disease agents licensed for general use in the USA (table 1) [6]. Some are live and some are killed. Some, such as BCG, anthrax and rotavirus, are in need of improvement.

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Table 1. Abbreviated list of vaccines against bacterial and viral disease agents licensed in the USA

Bacterial	Viral
Diphtheria	Poliovirus (live and killed)
Tetanus	Measles
Pertussis (acellular)	Mumps
Botulinum toxin A	Rubella
Lyme disease (OspA)	Varicella
Plague	Yellow fever
Pneumococcus (and conjugate)[1]	Adenovirus
Meningococcus (and conjugate)[1]	Hepatitis A
Haemophilus influenzae (and conjugate)	Hepatitis B
Tuberculosis (BCG)	Influenza
Typhoid fever (live)	Rabies
Typhoid fever Vi	Rotavirus (withdrawn)
Cholera	
Anthrax	

Based on the Jordan Report 2000 [6].
[1] Recent licensure.

Table 2. Numerical ranking of new vaccine priorities, 1985

Rank	Vaccine	Composition	License status 2001
1	hepatitis B	recombinant	yes
2	respiratory syncytial	live	–
3	*Haemophilus influenzae*	conjugate	yes
4	influenza A, B	pure subunit	–
5	influenza A, B	live	–
6	respiratory syncytial	subunit	–
7	varicella	live	yes
8	no designation		
9	parainfluenza	subunit	–
10	streptococcus B	conjugate	–
11	cytomegalovirus	live	–
12	herpes simplex	glycoprotein	–
13	cytomegalovirus	glycoprotein	–
14	rotavirus	live bovine	yes[1]
15	pertussis	acellular	yes
16	rotavirus	live human	–
17	herpes simplex	live	–
18	gonorrhea	miscellaneous	–
19	no designation		
20	hepatitis A	live	–
21	hepatitis A	killed	yes
22	coccidiosis	killed	–

This ranking was based on complex decision analysis formulae, from the Institute of Medicine, US National Academy of Sciences. Abbreviated combination of Tables 1.1 and 9.8 from *New Vaccine Development: Establishing Priorities* [7]. Numbers 8 and 19 were not included in Table 1.1.
[1] Withdrawn.

Systems for Defining Priorities in Vaccine Development

During the early 1980s, the US National Institutes of Health became interested in defining a rank order of priority for the pursuit of prominent vaccine possibilities. The Institute of Medicine of the US National Academy of Sciences was engaged to study the matter and to prepare a report [7], which was published in 1985. Decision analysis methods were used in which a series of calculations were applied to arbitrary estimates of expected costs and benefits for each of 20 vaccine targets.

The rank order of importance of live, killed, subunit or conjugate vaccines against the 20 viral, bacterial and parasitic agents that were considered is summarized in table 2. The rank order of importance bore no relationship to conventional cost-benefit analyses used in decision-making for the application of existing vaccines. Further, the ranking was biased in part by the already advanced state of development of some of the candidates. Most importantly, only 6 of the 20 designated vaccines had achieved licensure by the end of 1999, and there was little correlation between rank order and license attainment.

Late in the 1990s, the Institute of Medicine was asked again (table 3) to evaluate and rank vaccine possibilities [8], this time based on the cost per year that would be incurred per quality-adjusted life year gained by the development and use of a particular vaccine, in the categories of (1) cost effective, (2) <USD 10,000, (3) >USD 10,000 to <USD 100,000, and (4) >USD 100,000. The chosen vaccine candidates were antiviral, bacterial, fungal, parasitic and cancer. Three were therapeutic and the remainder were prophylactic. Three of the diseases were autoimmune and the rest were infectious. This report, like the previous one, provided a rank order of priority which was of limited value because of reliance on estimates of costs and on subjective judgments with apparent divorcement from probabilities of accomplishment.

In 1990, the United Nations Infants and Children's Emergency Fund (UNICEF) assembled a group of knowledgeable scientists to define the qualities and attributes of vaccines (table 4) which would be required for maximal acceptability for application in the mostly poor and undeveloped nations of the world. The report [9], called the Declaration of New York, 1990, was brief and consisted

Table 3. Vaccine priorities, 2000, based on quality-adjusted life years

Basis for selection – cost per QALY							
saves money		<USD 10,000		>USD 10,000 to <USD 100,000		>USD 100,000	
Cytomegalovirus	PxV	chlamydia	PxV	parainfluenza	PxV	Lyme Disease	PxV
Influenza 1–5 years	PxV	*Helicobacter*	PxV	rotavirus	PxV	coccidiosis	PxV
Insulin-dependent diabetes	RxV	hepatitis C	PxV	group A streptococcus	PxV	enterotoxic *Escherichia coli*	PxV
Multiple sclerosis	RxV	herpes simplex	PxV				
Rheumatoid arthritis	RxV	papillomavirus	RxV	group B streptococcus	PxV	Epstein-Barr	PxV
Group B streptococcus	PxV	melanoma	PxV			histoplasma	PxV
Pneumococcus	PxV	tuberculosis	PxV			meningococcus	PxV
		gonorrhea	PxV			shigella	PxV
		respiratory syncytial virus					

From *Vaccines for the 21st Century* [8] (abbreviated and rearranged). QALY = Quality-adjusted life year; PxV = preventative vaccine; RxV = therapeutic vaccine.

Table 4. Elements of the Declaration of New York, 1990 (restated)

Simplicity in vaccine delivery as by oral fed, nasal or transdermal immunization
Immunization in early life
New vaccines protecting against a broad spectrum of diseases
Fewest doses and fewest injections, as by combination, high potency, optimal regimen
Safe and nontoxic (vector delivery, purified antigens, optimal attenuation)
Long duration of immunity, with anamnestic memory
Cheap and affordable in relation to resources; low cost and high benefit
Stability of product in storage and outside the cold chain

of a 'wish list' of desirable attributes of vaccines. Collectively, the call was for vaccines of easy delivery and high-level effectiveness when given in early life. Briefly stated, the vaccines would cover a broad spectrum of disease agents and would be of high potency while requiring few doses. The immunity would be long lasting, with memory. The vaccines would be affordable and of low cost and high benefit. They would be stable in storage and in transit.

These three studies are presented here to illustrate, collectively, the diversity of viewpoints that may be generated by different scientific review groups. The reader, to be sure, will make his/her own judgment of the value of each of the three reports. To the author (who was a member), the two Institute of Medicine reports exemplify what can be created with committee consensus but with little relevance to what drives the actual pursuit of vaccine development in the real world. The Declaration of New York, on the contrary, has been of value in identifying what needs to be achieved for practical universal immunization in a worldwide program. It serves as an ideal, yet to be attained, and the desirable attributes that are designated would apply equally well to vaccines for use in the developed and undeveloped nations.

Redefining the Vaccine Enterprise at a Time of Transition

If one were asked what vaccines might be needed for the 21st century, the list for consideration might be very long and the answer might approach 'all of them'. Actual pursuit of vaccines, however, is based primarily on technical feasibility and economic considerations. In the past, essentially all vaccines were pioneered and developed [3, 4, 10] by pharmaceutical companies in developed countries where market size, risk and profitability are the real and necessary factors for consideration. Assertions and policies of the World Health Assembly and the World Health Organization now presume that prevention of disease by vaccines is a basic human right [11, 12]. That, together with the proactive stance of the Global Alliance for Vaccines and Immunization [13], with financial assistance being given by the Gates Foundation and contributions by individual governments and others, may be changing the focus of vaccine development to include dis-

Table 5. Immune system: components and functions

Innate immune system	Pattern recognition and activation
Adaptive immune system	Delayed specific recognition, activation and response
Components	
B lymphocytes	humoral immunity
T lymphocytes	
Cytotoxic T cells	cell-mediated immunity
Th cells	cytokine modulation
Th2	facilitate humoral (type 2) response
Th1	facilitate cellular (type 1) response
Antigen-presenting cells	
MHC-I	endogenous (class 1) presentation
MHC-II	exogenous (class 2) presentation
Crossovers for both in bone marrow-derived professional antigen-presenting cells	
Memory lymphocytes with long-term survival are retained and available for quick differentiation into effector cells (anamnestic response)	

Table 6. Immune system: appropriate and inappropriate responses

Imperative to avoid excess type 1 or type 2 immune responses; 'appropriate' balance needed
Antibodies reduce viral load and prevent reinfection
Cytotoxic T cells (killer) destroy infected host cells
Direct contact – apoptosis or lysis
Nonlytic cytokine elaboration
Examples of adverse responses
Excessively high-titer live measles vaccine producing excessive humoral immune response and causing immunosuppression and immunodeficiency
Inappropriate killed measles vaccine causes 'atypical measles' upon subsequent measles exposure in nature
Inappropriate killed RSV vaccine causes severe pneumonitis and increases deaths upon subsequent RSV exposure

RSV = Respiratory syncytial virus.

eases which are of greater importance to the developing world [14, 15] than to the developed nations. For some vaccines, there is a major movement to extend the kinds and scope of use to teenagers and adults. Three diseases of major importance to the entire world, namely AIDS, tuberculosis and malaria, have been determined to present the most urgent need, with the priority that these be the first of the difficult vaccines to be achieved.

The limitations of space and time necessitate that the points made in this paper be supported by selective, illustrative examples rather than by extensive presentation and detailed discussion, which may be found elsewhere.

Immune Response

The game of infectious disease prevention through appropriate vaccines is that of equipping the host to prevail in its battle against infective agents subsequently encountered [for a review, see ref. 16]. In all of vaccinology, the most important, though sometimes the most misunderstood or neglected, component of the vaccine endeavor is the need to accommodate the mechanisms for recognition and signaling messages in the immune system, which is a capricious and demanding entity of immense complexity. Pattern (chemical) recognition by the innate immune system gives an immediate response. The specific adaptive immune response is more complex and is slow to generate.

The principal components of the immune system (table 5) [16] are the B and T lymphocytes and the bone marrow-derived antigen-presenting cells (dendritic cells, macrophages) that process and display fragments of proteins in MHC-1 or MHC receptors on their cell surfaces, where they recognize, signal and activate T and B cells of complementary specificity. The MHC receptors in which antigen fragments are displayed are morphologically diverse and restricting (allelic), allowing or not allowing a particular antigen to be bound in the groove of a particular MHC molecule. Antigen presentation by antigen-presenting cells must carry the specificity of the disease agent but with binding also by costimulatory ligands. B lymphocytes elicit specific antibodies, and cytotoxic T cells are the killer cells of cell-mediated immunity. T helper cells (Th) are of two kinds and secrete different cytokines. Th2 cells facilitate a type 2 humoral or antibody response, and Th1 cells facilitate a type 1 cellular response. Following completion of the immune response, long-term memory cells are retained as partially differentiated lymphocytes which are available for quick differentiation into active effector cells in an anamnestic response upon subsequent exposure to the complementary antigen.

The different effector cell responses fulfill different needs (table 6). Antibodies neutralize infectious agents, reduce viral load and prevent reinfection. Cytotoxic (killer) T cells destroy infected host cells by direct contact or disrupt viral replication in them by specific cytokine secretion [17–19]. It is important that the immune re-

Table 7. Selected vaccine targets for which there are no licensed products or existing products are in need of improvement

Bacteria	Virus	Parasitic
Tuberculosis (improved)	*HIV-1 & 2*	*Malaria*
Leprosy	Herpes simplex	Schistosomiasis
Meningococcus B	Cytomegalovirus	Leishmaniasis
Nontypeable *Haemophilus influenzae*	Papillomavirus	Amebiasis
Streptococcus A & B	HTLV-1 (leukemia)	*Fungi*
Staphylococcus	Rotavirus (improved)[1]	Coccidiomycosis
Pseudomonas	Norwalk agent	Histoplasmosis
Enterotoxic *Escherichia coli*	Hepatitis C, E, G	Candidiasis
Shigella	Influenza (live)	
Helicobacter	Parainfluenza	*Other*
Chlamydia	Respiratory syncytial	Cancer
Gonorrhea	Dengue	Conformational:
Syphilis	Equine encephalitis	Amyloidosis-
Mycoplasma	West Nile encephalitis	Alzheimer's disease

Based in part on the Jordan Report 2000 [6].
[1] Licensed but withdrawn.

spons be 'appropriately' balanced since excessive polarization to a type 2 or a type 1 response may reduce efficacy and may lead to immune dysfunction. As an example, use of live measles vaccine of an excessively high infectivity titer has been shown to induce type 2 polarization with an excessive humoral response, giving rise to suppression of cytotoxic T cells (immunodeficiency) and sometimes resulting in death from opportunistic infections [20–22]. Formalin-killed measles vaccine of inappropriate composition has been shown to lead to severe atypical measles in children upon subsequent exposure to wild measles virus in nature [22–24]. Inappropriate killed respiratory syncytial virus vaccine conditioned babies to severe pneumonitis and increased deaths upon exposure to wild virus in nature [22, 25, 26].

Targets for New or Improved Vaccines

Table 7 lists 35 infectious disease entities for which acceptable vaccines do not exist and that are worthy of consideration for vaccine development. The list includes viruses and bacteria that are of etiologic importance in cancer. The most important vaccine targets at the present time include improved tuberculosis, AIDS (HIV) and malaria preparations.

Existing BCG vaccines [27], in spite of their long-term use, are highly variable with respect to protective efficacy, which can vary from 0 to 80% [28]. The major problem for vaccine development lies in the lack of definitive knowledge of which antigens tuberculosis bacilli secrete or display on their cell surface. Importantly, there is no reliable definition of the correlates of protection. Research struggles with an inadequate database, but the emerging definition of the genomics and proteomics of the organism will help in opening the way to a more rational approach to the problem [29, 30].

HIV agents that cause AIDS [31–36] are hypervariable with respect to surface antigens, and certain of the core antigens are also variable. Antibodies are not significantly effective against the disease, and the progressive loss of cell-mediated immunity opens the door to collapse of the immune system and to overwhelming opportunistic infections. After many years of failure, utilization of conserved antigens of HIV that are presented in vector prime/boost regimens and are directed at achievement of cell-mediated immunity show greater promise [35, 36].

The malaria parasite [37–40] undergoes multiple stages in its life cycle with extreme antigenic diversity in the merozoite phase. A rational target for attack may be in the liver stage, where expression of highly conserved antigens may predominate.

Vaccines arising from the present focus on cell-mediated as well as humoral immunity and future developments will be aided by definition of the genome and proteome of the relevant parasite. There is a commonality between all three diseases discussed above, with respect to the need to identify conserved antigens (mostly protein)

Table 8. The amyloidoses

Amyloidosis
Protein conformational disease assuming ordered aggregation with beta sheet replacement of alpha helices
Cerebral amyloidosis – Alzheimer's disease
Precursor amyloid precursor protein is truncated to amyloid beta protein (Aβ)
Aβ protein misfolds to aggregates and fibrils
Immunization of transgenic mice with Aβ vaccine reversed memory and learning deficits
Prion spongiform encephalopathy
Scrapie of sheep, Creutzfeldt-Jakob disease and kuru of humans, mad cow disease
Misfolding of native prion protein PrP^{c}, giving rise to 'infectious' PrP^{sc}

and to offer them in the context of achieving cell-mediated immunity which will destroy the infected host cells outright. Antibodies may aid in reducing parasite load, but are otherwise of limited effectiveness. Importantly, we have no example of an effective therapeutic vaccine against an infectious disease agent, and there is no basis for belief that this approach will provide any significant solutions in the foreseeable future. Because of this, prophylactic vaccines, given before exposure to the parasite, must prevail.

Cancer and the Amyloidoses

Effective vaccines against cancer were long considered to be beyond the realms of possibility, but are now believed by some to be highly probable. In a sense, both cancer and the amyloidoses are chronic diseases, and effective vaccines will need to be therapeutic. Success rests on the hope for identification of antigens that are unique to the relevant tumor or amyloid and against which selected immune responses may be directed that are not achieved in the natural disease.

Cancer

The basis for pursuit of vaccines against nonviral cancers [41–45] lies in the fact that tumor cells do present antigens which are recognizable by the immune system and that are not found in significant amounts on normal cells. A common approach has been to isolate and identify unique antigens [46] and to present them to the immune system by a variety of delivery systems. Vaccination with antigens that mimic [47] the specificity of undefined tumor immunogens may expand the horizons of possibility. Pulsing of antigens onto dendritic cells or delivery by recombinant DNA vectors may facilitate the development of the needed cellular immune response. Prime/boosting procedures are commonly employed. Immune modulators such as GM-CSF or IL-2 may increase the chance of achieving a strong immune response. An indolent immune system that is tolerant to antigen recognition or that is lacking in functioning components needed for a vigorous immune response may present a substantial barrier to immunotherapy in cancers. Irrespective of the complexity of the initiative, very numerous and diverse probes are being taken to clinical trials in human beings. Typically, claims for 'great promise' are made, but none has passed the acid test of licensure.

Amyloidosis

Amyloidosis [48] is considered to be a complex of protein conformational diseases [49, 50] in which different nascent cellular proteins that are chromosomally encoded fail to fold correctly and assume, instead, an ordered aggregation in which beta sheets replace most of the normal alpha helices [51] (table 8). Based on studies of amyloid formation in yeast [52, 53], four different models for fibril assembly have been proposed, the most recent being that of nucleated conformational conversion which proceeds following formation of oligomeric intermediates. Amyloid proteins have a fibrous form, are insoluble and may deposit out in tissues and organs throughout the body. The most commonly discussed amyloid disease of humans is that of cerebral amyloidosis, or Alzheimer's disease [48], which derives from misfolding of the normal amyloid beta protein, Aβ [54, 55]. The significance of Aβ amyloidosis in relation to vaccines can be seen in recent studies in transgenic mouse models for Alzheimer's disease in which the mice overexpress a mutant amyloid beta precursor protein, APP, or presenilin 1. Amyloid beta protein consists of 40 or 42 residues that are released through proteolytic cleavage of APP. As in humans, amyloid beta protein appears in aggregates and fibrils in plaques in the brains of affected mice in association with learning deficits [56]. Most importantly, it was found in two studies [55, 57, 58] that mice which were immunized with Aβ protein developed antibodies that bound with Aβ proteins in the brain and reversed Alzheimer's disease-like memory and learning deficits in the affected mice. The mode of action of the vaccine was not by reduction of plaques but rather by affecting some aspect of Aβ protein metabolism. These findings are quite astounding and have opened the door to possible control of Alzheimer's disease in humans.

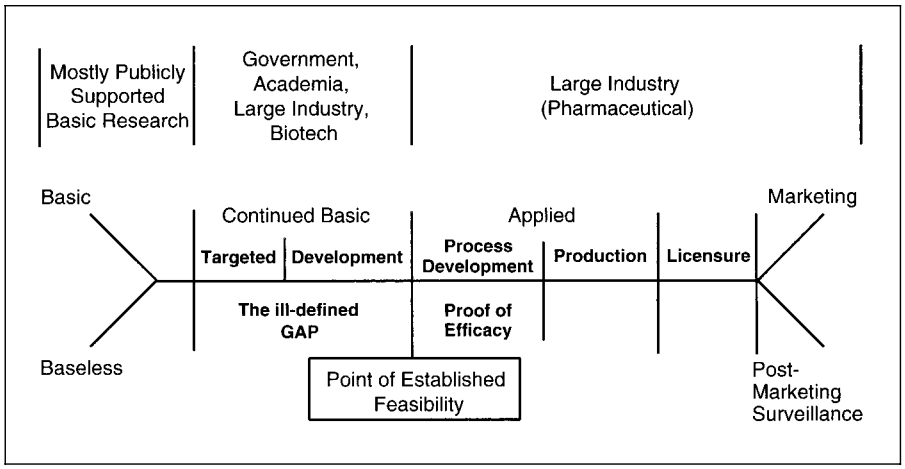

Fig. 1. The Biologics Enterprise. Usual flow of activities from basic discovery through to the product marketed.

The amyloid precursor protein in the Alzheimer model is different from the prion protein of the spongiform encephalopathies [48]. In prion diseases [59, 60], native cellular prion protein (PrP^{c}) may misfold in the presence of a template of 'infectious' misfolded prion scrapie protein, PrP^{sc}, giving rise to insoluble fibrils which appear in plaques in the brain. The 'infectious' entity is a self-replicating peptide and is without nucleic acid [60, 61]. Such autocatalysis has been shown for other proteins as well [62]. Prions cause fatal neurodegenerative diseases [63, 64], including scrapie of sheep, Creutzfeldt-Jakob disease of humans and bovine spongiform encephalopathy of cattle, commonly referred to as 'mad cow disease' [65], which is transmissible to humans. Sheep scrapie prion protein is normally noninfectious for human beings but possibly may transmit by oral feeding of 'infected' beef, giving rise to the Creutzfeldt-Jakob syndrome. An alternative possibility is being investigated based on a concept of spontaneous mutation of protein of cattle [66]. It appears possible that the debate on the origins of mad cow disease may become as complicated and convoluted as that regarding the origin of the AIDS virus. It may be hoped that the findings with Alzheimer amyloid protein will provide clues for the treatment of Creutzfeldt-Jakob and 'mad cow' diseases of humans.

Biologics Enterprise

The Biologics Enterprise in the United States is a complex of entities that receive private or public funding and follow a prescribed course of events (fig. 1) [5, 36, 67]. Basic research discovery is carried out principally by academia. According to most recent concepts of the contract between science and society [5, 67, 68], government is charged with the responsibility to cover the gap between basic and applied research, taking product possibilities through targeted and basic research to the point of technical feasibility for development by industry [69]. In practice, industry may engage heavily in basic research itself, and biotech companies may make a particular vaccine or vaccines central to their programs. Large industrial pharmaceutical companies, however, assume major risk and enter commercial development at the point of proved feasibility, expecting to commit hundreds of millions of dollars to bring a product to licensure and market. Whatever the situation, the gap between basic and applied research [67, 69] is the most difficult to close technically and the most important deterrent to commercialization until bridged.

Basics in the Determination of New Vaccine Development

Decision for corporate entry into development and production of a new vaccine (table 9) is a serious consideration [5, 47, 67], since it requires an assumption of risk and commitment of large capital investment. Decision-making is complicated and is based on technical, market and business considerations. Such engagement must have societal importance while assuming a reasonable return to the corporate shareholders for their investment. Decisions are based on calculations and judgments [47] which, if favorable, allow a new product to be created.

Table 9. Some basics for commercial new vaccine development – many are researched but few are licensed

Technical aspects	Market aspects	Business aspects
Proven feasibility	Size and geography	Patent protection
Development costs	First or me too	Exclusivity
Process simplicity	Affordable or	Manufacturing costs
Approvability	Reimbursable	Capital investment
Time to licensure	Recommended or	Return on investment
Safety and efficacy	Mandated	Liability
Protection, duration	Dosing – number of shots	Distribution costs
Ease of administration	Favorable forecast	Costs and recapture
	Target populations	

In-house development and out-house licensing-in are driven by the same economic determinations of risk and return on capital.

Table 10. Technologic evolution by era, new vaccine research and development

Period	Dominant technologies	Facilitators
Rational empiricism Late 17th to 21st centuries	live attenuated viruses and bacteria killed whole organisms proteins and polysaccharides extracted or secreted recombinant expression	adjuvant (alum) alum adjuvant
Transition to simplification Late 20th century	recombinant expression recombinant vectors virus, bacterium, replicon, DNA	adjuvant, modulators particulation, liposomes, VLP targeted to APC by ligands dermal or muscular injection
Reductionism and delivery	synthetic peptides epitopes	avoidance of added coupling, adjuvants and immune modulators
Challenges for the 21st century	delivery oral fed – r plant tissue mucosal transcutaneous pulsed onto dendritic cells	

VLP = Virus-like particles; APC = antigen-presenting cells; r = recombinant.

Technologic Evolution

The era from Jenner to the present (18th until the 21st century) has been dominated by rational empiricism (table 10) [3–5, 16, 19]. The technologies of the era consisted of live and killed microorganisms, extracted or released proteins and polysaccharides (some of which required conjugation) and recombinant-expressed protein. Live organisms [70] served as the ideal in eliciting host responses, especially when combined, as in the measles, mumps, rubella vaccine [71, 72]. Whole killed organisms preserved the integrity of components of live microbes, frozen in form through cross-linking by formaldehyde.

At a time of transition toward simplification of vaccine approaches, the needs of the time led to the development of two human hepatitis B vaccines that were licensed in 1981 and 1986, respectively [73–75]. The vaccine consisted of the outer membrane proteins of a noncultivable virus. The *first* of the two vaccines [73] consisted of surface antigen extracted and purified from the plasma of

human carriers of the infection. The *second* vaccine [74, 75] came with the breakthrough development of the first recombinant-expressed vaccine. There have been no recombinant viral vaccine encores to the present time.

Proof of the principle of recombinant expression soon led to the development of probes of recombinant live viral and bacterial vector vaccines [76, 77]. Certain vectors, such as canary pox and alpha virus replicons, do not replicate in the human host. The ultimate in noninfectious vectors came into being with the recombinant plasmid DNA vaccine [78]. To date, no recombinant vector vaccine has attained licensure.

A hangover from many decades of effort has led to the now popular pursuit of immunogens that are referred to as peptide vaccines and that represent the ultimate in reductionism [79]. Practical accomplishment of this legacy of the 20th century will be a major challenge to the ingenuity of the 21st century. During this period, means for noninvasive vaccine delivery, as by the transdermal, mucosal or oral route, will also be pursued [80, 81].

Table 11. Comparison of technologies being applied to experimental viral vaccines and to licensed viral vaccines as revealed in the Jordan Report, 1998 [82]

Technology	Number of vaccines	
	licensed products	clinical probes
Live attenuated	10	35
Killed		
Whole	4	36
Split[1]	1	
Protein[2]		
Plasma-derived	1	
r expressed (yeast)	1[2]	
r expressed	0[3]	63
r vector	0	56
r DNA	0	21

r = Recombinant.
1 Ether split influenza.
2 Hepatitis B.
3 Nothing since hepatitis B.

Overview of Simplification and Reductionism

Simplification

It is clear that new and practicable approaches to vaccines for the 21st century must be discovered in order to achieve practical resolution of the problem of control of the plethora of uncontrolled diseases in humans. Such endeavor must occur in a global context for all the world's people.

The degree to which past and future approaches (table 11) are being applied to the future is revealed in the 1998 Jordan Report [82] of the US National Institutes of Health. A summary was made by the author [16] to compare the technologies applied for the licensed vaccines of the present with those of probes being taken to clinical tests. It can be seen in table 11 that 71 of the 211 clinical probes (34%) utilized the killed and live vaccine approaches. By contrast, 140 of the probes (66%) involved recombinant expression, vector or DNA vaccines. There is but a single example of recombinant-expressed vaccine, that against hepatitis B, to lend credibility to such an approach. It remains to be determined whether the pursuit of subunit vaccines is but a gamble of the present and future which can only be judged in terms of licensure of products during the next decade. To be sure, all are being hailed as promising, but the reality lies in demonstrated accomplishment. Vaccines against viruses such as hepatitis C, papillomavirus and cancers offer little or no alternative choice. It could well develop that the length of the expressed proteins, as contrasted with truncated peptides, may hold the clue to what may or may not be successful.

Reductionism

It has been a long-term aspiration of reductionists [46, 79, 83–86] in vaccinology to shuck off the last vestiges of whole-organism and protein vaccines of the past era of rational empiricism and to replace them with synthetic vaccines of defined chemical composition. Simplified synthetic vaccines consisting of minimal collections of epitopes are commonly referred to as peptide preparations. Such vaccines ideally consist of collections of epitopes alone, with or without adjuvants or immune modulators.

To achieve success, synthetic peptide vaccines must rely on identification and incorporation of pertinent and acceptable B cell, cytotoxic T cell and Th1 and Th2 immunologic determinants. B cell epitopes are mostly discontinuous, relying on conformational configuration to retain their immunologic specificity. Presentation of such globular peptides may require presentation of multiple epitope arrays by conjugation to a central protein matrix. A minority of linear epitopes can be recognized by B cells, but all T cells, both cytotoxic and helper, recognize epitopes in linear form. The selected epitopes for a vaccine

Table 12. Examples of tools used to assist antigen/epitope discovery and characterization

Genomic sequencing of pathogenic organisms
Group specificity for variable types
Predictive binding to MHC receptors
Computer-assisted determinations
Proteomic expression and screening
Rapid throughput assay
Combinational and peptide libraries
Immune cell binding; specific stimulation
Phage
Transfection of fragments of DNA and peptide display
Monoclonal antibody identification of epitopes
Search for epitopes by overlapping peptide analysis
Characterization of peptide fragments (epitopes) eluted from MHC receptors
Search for mimetopes, especially for polysaccharides

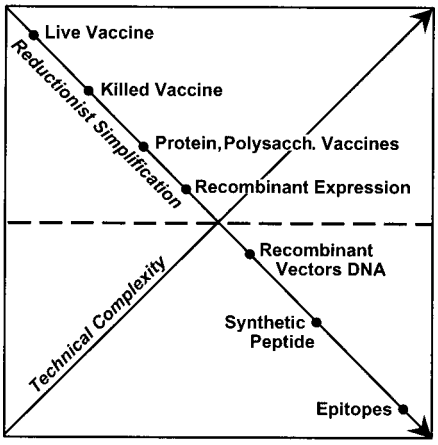

Fig. 2. Simplification and reduction create complexity.

must retain the specificity required to evoke appropriate immune responses. In addition, they must be selected to accommodate the restricted binding sites on the highly polymorphic MHC receptor proteins, overcoming allelic (polymorphic) restrictions in the human population. The actual numbers of specific epitopes that are able to achieve the requirements for MHC presentation in ordinary vaccines may be quite small, and overcoming such problems with a synthetic vaccine may be impossible. In addition, the B and T lymphocytes which are effective in an immune response must be capable of creating the long-lived memory cells needed for late anamnestic recall responses. Finally, the vehicle for epitope delivery needs to be defined.

It is fortunate at this time, both for peptide and ordinary vaccines, that so many technologies for epitope identification exist (table 12). These represent state-of-the-art methodologies based on genomics, proteomics, combinational chemistry and DNA and peptide libraries, yielding antigens for tests in rapid throughput assays, with computer-assisted analyses [87–89].

As shown in figure 2, it seems a verifiable reality that simplification and reductionism may be inversely proportional to accomplishment because of the complexity and difficulty involved in their technical pursuit. Accomplishing a large immune response using less complex antigens, may be reaching a roadblock at the point of feasibility. This may be a warning reality for the future.

Table 13. Vaccinology and societal responsibility

Social contract between science and society – Gibbons [68]	
Produce reliable knowledge	
Communicate discoveries to society – transparent and socially robust	
Players in the enterprise	
Academia	discovers
Government	funds
Industry	translates knowledge to products (value)
Health biology, now heavily funded, needs to attain:	
Procedures and products in return for public support	
Efficiency by:	
Elimination of nonproductive redundancy	
Exercise of selective choice	
Prudent dedication to targeted gap-filling research, making product development feasible	
Elimination of foolish pursuits; responsibility and accountability	
Defining the WHAT of vaccine composition and the HOW of delivery	
Pursuit of affordable and deliverable vaccines for the whole world population	
Oral fed, mucosal or transdermal vaccination	

Vaccinology and Societal Responsibility

After the issue drifted along for many decades, Gibbons [68] has recently given voice to the social contract between science and society (table 13). According to the

present understanding [5], science is expected to produce reliable knowledge and to communicate its discoveries to society in a pursuit that is socially robust and that is both transparent and participated in by society itself. The players in the contract, as applied to vaccines, are academia, government and industry. In this arrangement, academia mainly discovers, government funds and industry translates the knowledge into products once feasibility has been established [69].

The present is a time [5, 67] when US public (governmental) support for basic research in health biology is riding high, but with increasing demands for efficiency and effectiveness in developing procedures and products that provide a return to the public for its support. As to vaccine research, this may be a time for critical review to eliminate nonproductive redundancy, to exercise selective choice, to fix responsibility with accountability and to offer prudent dedication to the targeted research needed to span the bridge between basic knowledge and the feasibility needed for commercial translation into useful products. It may be a time also for a reality check on what is real and feasible, eliminating that which is foolish, since the vaccine enterprise will be held increasingly responsible and accountable to the public for prudent spending. The continuous high level of annual growth of the budget until 2003 is of serious concern, since the long-term commitments for individual grants will leave a dearth of money for new grants when the inevitable slowdown of annual growth begins after 2003 [90]. Most important for vaccine research is the need to identify which immunodeterminants to deliver in vaccines and how to deliver them. The guiding theme for all of this may reside in accomplishment of the tenets of the Declaration of New York. These may be difficult to achieve, but the development of multispecific vaccines that can be given orally, transdermally or mucosally may be the holy grail of future endeavor.

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Virus-Like Particles as a Modular System for Novel Vaccines

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Key Words
Virus-like particles · Vaccine · Antibody response · Self antigen · Adjuvant · Tolerance · Therapy

Abstract
Induction of protective immune responses with recombinant antigens is a major challenge for the vaccine industry. Here we present a molecular assembly system that renders antigens of choice highly repetitive. Using this method, efficient antibody responses may be induced in the absence of adjuvants resulting in protection from viral infection and allergic reactions.

Principles of Virus-Like Particle-Based Vaccines

The most potent vaccines used in the past consisted of attenuated or inactivated forms of whole pathogens. Attenuated pathogens especially are commonly excellent inducers of T cell as well as antibody responses, but there is always a slight risk of reversion to a more aggressive phenotype. A lot of effort has been made to develop similarly potent but safer vaccines. However, noninfectious subunits of pathogens such as proteins, peptides or sugars are poorly immunogenic per se and have to be formulated with immune-stimulating adjuvants. Unfortunately, many potent adjuvants cause considerable side effects such as toxicity of inflammation and are not allowed for human use. Thus, novel effective and well-tolerated adjuvants are sorely needed [1–3].

It is known that B cells are efficiently activated by repetitive structures which lead to cross-linking of B cell receptors on the cell surface [4, 5]. The presentation of an antigen in a highly ordered, repetitive array normally provokes strong antibody responses and can even break B cell tolerance to self antigens, whereas the same antigen presented as a monomer is normally nonimmunogenic [6]. Examples of such repetitive antigenic arrays are type 1 pili of bacteria and the protein capsids or coats of most viruses. For instance, the capsid of hepatitis B virus consists of 180 or 240 identical core protein subunits which can be recombinantly expressed and self-assemble into noninfectious virus-like particles (VLP) [7, 8]. Two subunits each form a dimer which protrudes as a spike on the capsid also known as the major immunodominant region.

Strong antibody responses are generated against these well-exposed and repetitive domains, which would make them very suitable for the introduction of foreign antigens for vaccination [9]. However, insertion of foreign sequences, especially peptides longer than 15 amino acids, into this immunodominant region of the capsid protein often interferes with correct VLP formation [8].

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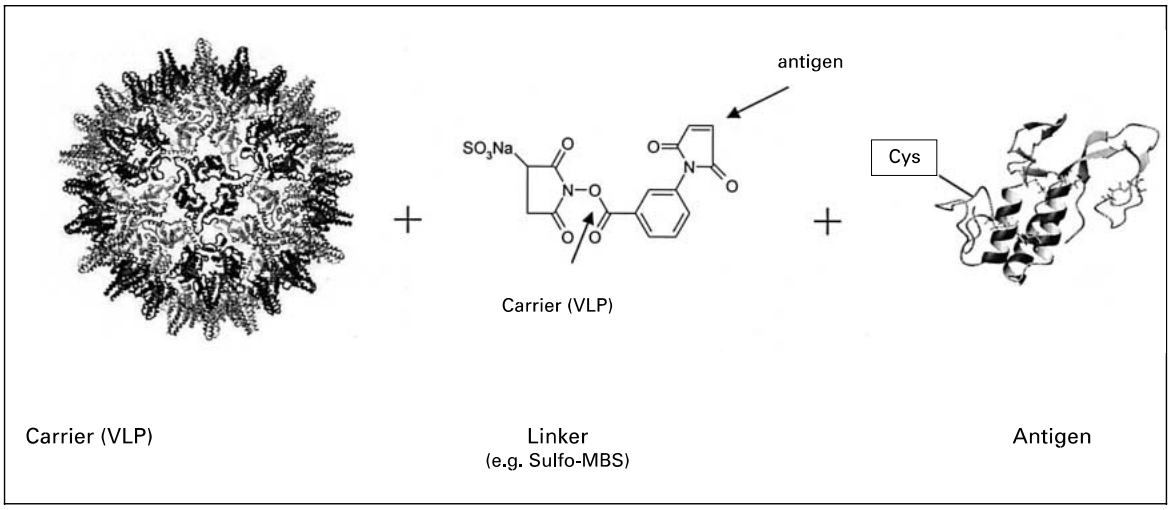
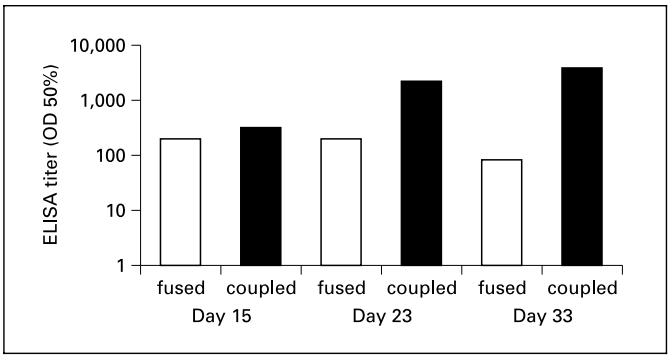


Fig. 1. Schematic representation of a modular system to attach an antigen containing a free cysteine (Cys) to a VLP (HBc) using a bifunctional cross-linker. Sulfo-MBS = Sulfo-m-maleimidobenzoyl-N-hydroxysuccinimide ester.

A Modular System to Render Any Antigen of Choice Strongly Immunogenic

We have generated noninfectious VLP and bacterial type 1 pili that contain one or several lysines in their immunoreactive region. On the antigen of choice, a linker with a cysteine is introduced which allows covalent linkage to the highly repetitive carrier using bifunctional cross-linkers. Since free cysteines in proteins are usually rare, coupling occurs via the genetically introduced additional cysteine (fig. 1). This modular system circumvents the problems of self-assembly often faced with antigens directly fused to the carrier and ensures that the antigens are optimally exposed in a repetitive manner on the surface of the carrier. In this way, modified sugars, peptides or whole proteins can be rendered highly immunogenic. Modified coats from several viruses can be used as VLP carriers, such as, for example, those from hepatitis B virus, polyoma virus, papillomavirus or RNA phage viruses. The use of specific attachment sites (lysine on the carrier and a single free cysteine on the antigen) and bifunctional cross-linkers ensures that the antigen of choice is coupled to the VLP in an oriented fashion. This is of great importance, since antigens need to be presented in an ordered fashion for efficient B cell receptor cross-linking. Moreover, it facilitates the generation of antibodies against certain exposed domains or peptide stretches. Antigens coupled to VLP induce strong and long-lasting B as well as T cell responses in the absence of adjuvants. Another way to display antigens in a repetitive manner on VLP is the use of biotin-streptavidin. VLP are biotinylated and assembled with a chosen antigen which has been expressed as a fusion protein with streptavidin [10]. Such vaccine preparations have been shown to be efficient. However, these vaccines may sometimes be difficult to produce, since fusion to streptavidin can interfere with proper refolding of the antigen. Hence, presentation of full-length antigens with this system may be difficult in many cases.

To demonstrate the strong immunogenicity of antigens coupled to VLP, a peptide of the M2 protein from influenza virus was either genetically fused to the N terminus of the hepatitis B capsid protein (HBc) or chemically linked to a lysine introduced into the immunodominant tip of HBc. As already reported by others [11], the fusion of the M2 peptide to HBc in this case did not interfere with VLP formation and was immunogenic. Both VLP vaccines induced M2-specific antibodies in mice after injection in the absence of adjuvants, but the response was much stronger using the coupled form of the antigen [12] (fig. 2). The lower response induced by genetically fused M2 peptide can most likely be explained by poorer accessibility to membrane-bound immunoglobulins on B cells. Fused M2 is partly buried within the particle, while the coupled M2 peptide is maximally exposed on the VLP. Similarly, mice immunized twice with the M2 peptide coupled to HBc in the absence of adjuvants were protected from a lethal challenge with influenza virus, whereas the antibody titers generated by the M2 peptide fused to HBc were not sufficient to protect the mice.



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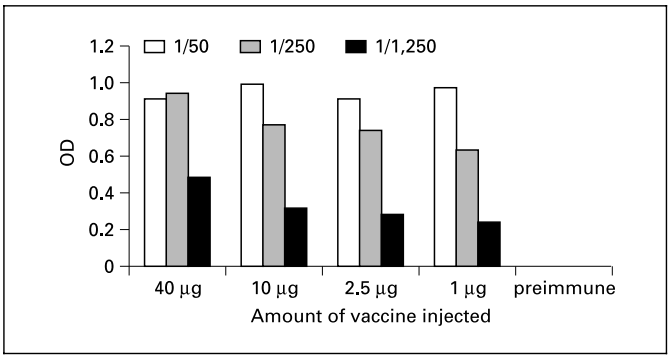

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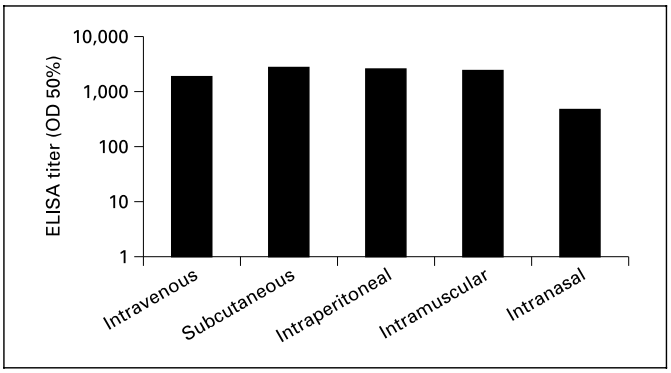

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Fig. 2. A peptide derived from the influenza protein M2 was either genetically fused to the N terminus of HBc (fused) or chemically linked to HBc (coupled). Mice were immunized twice, on day 0 and day 15, with 20 µg of protein in saline. M2 peptide-specific antibody titers in serum were determined at different time points. Average values of 3 mice each are shown. OD = Optical density.

Fig. 3. Mice were vaccinated subcutaneously on day 0 and day 14 with different amounts of a vaccine consisting of an HIV-derived peptide coupled to VLP. Peptide-specific antibodies in serum were measured on day 21. Results are shown as optical density (OD; at 450 nm) for individual serum dilutions. Average values of 2 mice each are shown. 1 µg of vaccine was sufficient to induce strong antibody responses.

Fig. 4. Induction of peptide-specific IgG antibodies in serum by VLP administered by different routes. Mice were vaccinated by different routes on day 0 and day 14 with 10 µg of vaccine consisting of influenza M2 peptide coupled to VLP in saline. M2 peptide-specific antibody titers were determined on day 21. Average values of 2 mice each are shown. OD = Optical density.

Not only peptides but also entire proteins can be coupled to VLP. For this purpose, a linker containing a free cysteine is fused to the protein, which enables directed coupling to the carrier.

VLP-Based Vaccines Are Safe and Effective in Low Amounts

Antigens coupled to VLP induce strong and long-lasting B as well as T cell responses. These vaccines are well tolerated and safe because they are noninfectious and are immunogenic without the use of irritating adjuvants. In human studies, it has been shown that human papillomavirus VLP are similarly immunogenic when given at a sufficient dose intramuscularly without adjuvants as in combination with alum or MF59, indicating that adjuvants are not necessarily needed for VLP vaccines [13].

We found that in mice, as little as 1 µg of a vaccine consisting of a peptide coupled to a VLP in saline induced high serum IgG against the coupled peptide (fig. 3). The same was true for proteins coupled to VLP.

VLP administered by different routes reproducibly resulted in good antibody titers in mice. VLP-based vaccines are similarly effective in inducing serum IgG when given intravenously, subcutaneously, intramuscularly, intraperitoneally and, to a somewhat lesser degree, intranasally (all without adjuvants) (fig. 4).

In addition, intranasal application of VLP-based vaccines induced antigen-specific mucosal IgA in the lung (and possibly other sites [14]), which could be important for mucosal protection against certain pathogens (fig. 5). This type of vaccine is effective in the absence of cholera toxin subunit adjuvant, therefore reducing the risk of mucosal irritation and other unwanted side effects.

VLP-Based Vaccines for the Treatment of Allergy

Similarly high titers of IgG antibodies are obtained against antigens which have been coupled to VLP and to bacterial type 1 pili. Type 1 pili are made of a helical array of densely packed FimA proteins which display several accessible lysines and are therefore suitable for coupling of antigens in a repetitive fashion. However, the IgG isotypes obtained with pili as a carrier were predominantly of the IgG1 isotype, indicative of a T helper type 2 (Th2) immune response, whereas VLP were strong inducers of specific IgG2a antibodies, indicative of a T helper type 1

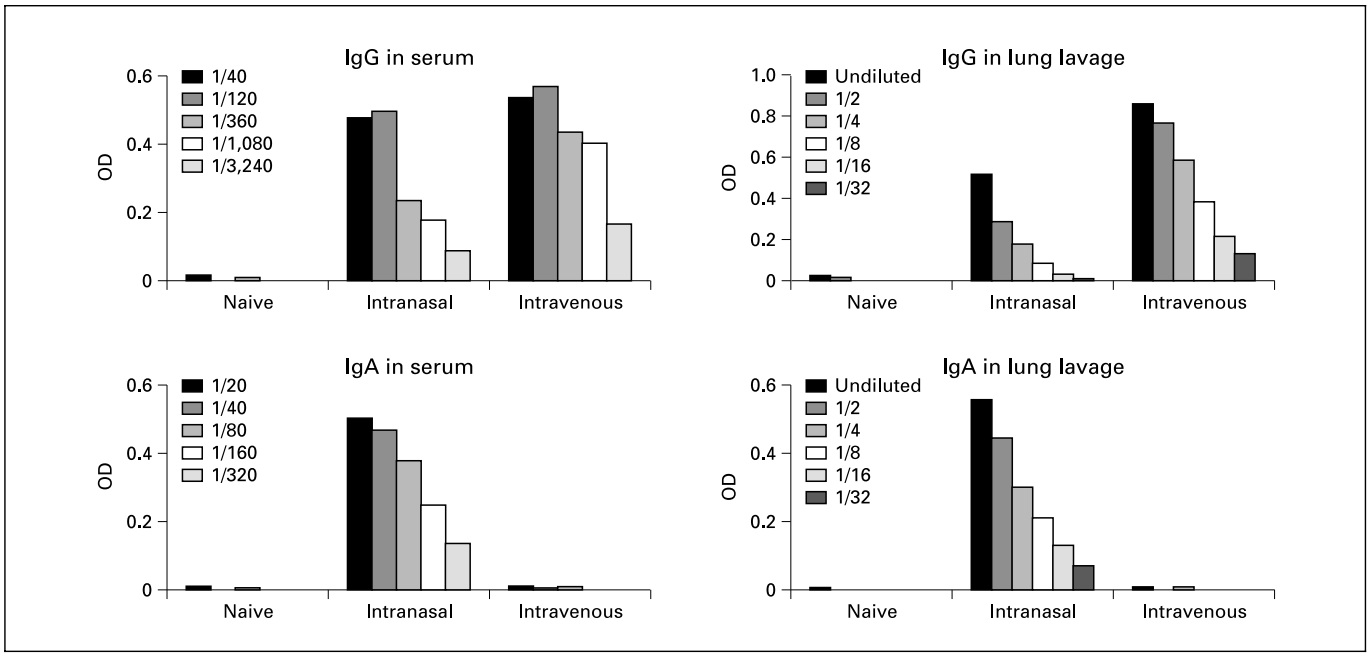

Fig. 5. Induction of mucosal IgA by intranasal vaccination. Mice were vaccinated intranasally on day 0 and day 14 with 10 μg of vaccine consisting of M2 peptide coupled to VLP in saline. M2 peptide-specific IgG and IgA were determined on day 21 in serum and lung lavage samples (1 ml/mouse). Results are shown as optical density (OD; at 450 nm) for individual serum or lavage sample dilutions. Average values of 2 mice each are shown. Only mice vaccinated intranasally showed specific IgA in serum and lung.

(Th1) response. Neither of the carriers induced IgE antibodies in mice. The difference in antibody isotypes generated by different carriers could be exploited for the development of specific vaccines. Allergy is characterized by a Th2 immune response, leading to the secretion of cytokines such as IL-4 and IL-5 and the production of allergen-specific IgE as well as IgG1. By coupling an allergen to a Th1-driving carrier, the allergic response may be reversed by vaccination, finally leading to the production of nonallergenic antibodies and Th1 cytokines such as IFN-γ and/or suppression of Th2 responses. Treatment of mice sensitized to the house dust mite allergen Der p 1 with Ty VLP fused to a Der p 1 peptide led to decreased IL-5 production [15]. Moreover, coupling of a Der p 1-derived peptide to an RNA phage-derived VLP induced a strong IgG2a response against the peptide but no detectable IgE (fig. 6). To test the hypothesis that vaccination with allergens bound to VLP may be able to cure allergies, we coupled the immunodominant allergen of been venom, phospholipase A2 (PLA2), to a VLP carrier and treated mice who had been sensitized to PLA2. Only vaccinated but not untreated mice made high levels of IgG2a antibodies to PLA2, indicative of a Th1 response, and were protected from an anaphylactogenic challenge with PLA2 [Lechner, unpubl. results]. This approach could possibly be used for desensitization of allergic patients. Certain allergies can be treated by repeated injection of low doses of allergen (e.g. bee venom, house dust mite extract, birch pollen).

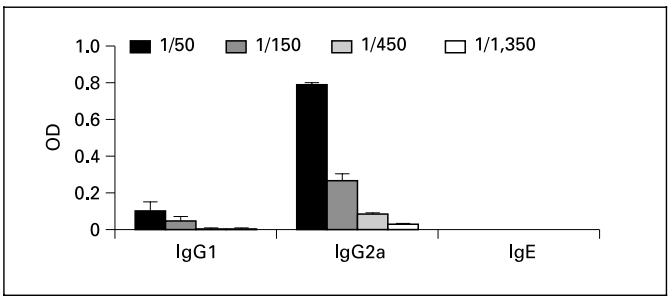

Fig. 6. Induction of allergen-specific IgG2a antibodies. Mice were vaccinated twice on day 0 and day 14 with 10 μg of Der p 1 peptide (amino acids 117–138) coupled to VLP in saline. Peptide-specific antibody isotypes in serum were analyzed on day 21. For IgE determination by ELISA, sera were pretreated with protein G. Optical densities (OD; at 450 nm) are shown for individual serum dilutions. Average values of 3 mice are shown. Whereas IgG2a antibodies were readily detected, no specific IgE was found.

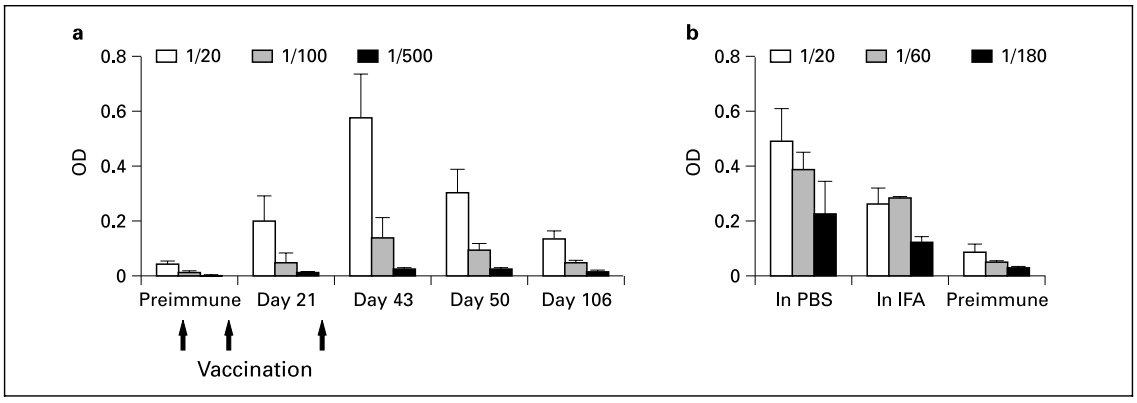

Fig. 7. TNF-α protein-specific antibodies in vaccinated mice. **a** Mice were vaccinated on day 0, 14 and 33 with 30 μg of vaccine consisting of TNF-α peptide (amino acids 3–21) coupled to type 1 pili. TNF-α protein-specific antibodies were analyzed by ELISA at different time points. **b** Mice were vaccinated on day 0 and day 14 with 50 μg of TNF-α peptide coupled to HBc VLP. The vaccine was injected subcutaneously either in phosphate-buffered saline (PBS) or in Freund's incomplete adjuvant (IFA), and the sera were analyzed on day 21. Optical densities (OD; at 450 nm) are shown for individual serum dilutions. Average values of 3 mice are shown.

This desensitization procedure is very costly and time-consuming but generally effective. Vaccination with immunodominant allergens coupled to VLP should hasten the desensitization procedure, thus making it affordable and more convenient for a greater number of patients.

VLP-Based Vaccines in Immunotherapy

In the past few years, substantial progress has been made in the field of immunotherapy using specific antibodies for the treatment of chronic diseases or cancer. For example, TNF-α-binding antibodies proved very effective for the treatment of rheumatoid arthritis and other chronic inflammatory diseases. An antibody against CD20 used in cancer therapy and an antibody against IgE for the treatment of allergies are other examples of immunotherapeutic antibodies [16, 17]. Some disadvantages of therapeutic antibody treatment are the high production costs and the eventual development of anti-antibodies leading to side effects following injection [18]. If the patient could make these specific antibodies him/herself, one would expect that they would be well tolerated since they would be entirely 'self', as opposed to some currently tested therapeutic antibodies which are humanized but not fully of human origin. VLP-based vaccines are able to break tolerance to 'self' because the repetitiveness of the presented antigens induces very strong B cell activation [6]. In addition, potent T helper epitopes are provided by the carrier, which is of great importance since tolerance or 'ignorance' at the B cell level can more easily be overcome than tolerance at the T cell level (T cells against self antigens are often severely anergized or deleted) [19, 20]. If designed appropriately, vaccines consisting of self antigens coupled to VLP should be able to directly induce similar antibodies in the patient to those currently being applied in immunotherapy. In addition, vaccination will be cheaper and possibly also more long-lasting than antibody injection. We tested this approach in mice using peptides derived from TNF-α which were chemically coupled to VLP. Specific antibody titers to TNF-α protein were induced after three injections and then decreased approximately 4-fold by day 106. Surprisingly, titers obtained with the vaccine in Freund's incomplete adjuvant were not superior to the ones obtained with the vaccine in saline (fig. 7). Others have found similar results using the biotin-streptavidin system to attach the TNF-α peptide to VLP [10]. In addition, it was shown that the induced antibodies were protective in a model of rheumatoid arthritis in mice. These promising results demonstrate that VLP-based vaccination with self antigens may be used for the treatment of chronic diseases and in certain cases could replace costly immunotherapy with antibodies. For vaccination against self antigens, it is of course important that a careful evaluation of potential side effects has been made. Not all self antigens would be equally suited for such an approach, but experiences from clinical trials with therapeutic antibodies have shown that certain antigens can be

targeted for a prolonged time without serious side effects. Ideally, therapeutic vaccination induces high but relatively transient antibody responses which may be boosted if needed. In mice, the half-life of specific antibody titers both against self as well as foreign antigens ranged between 30 and 100 days, depending on the antigen and the dosage used. We have conducted a small pilot study in humans to test the immunogenicity of VLP carriers and the reversibility of the antibody response. For this purpose, an RNA phage-derived VLP was injected three times and VLP-specific antibody titers were followed over time. From this study, it was estimated that after each injection, the half-life of the antibody titer in the serum was in the same range as that observed in mice. From these results and the ones obtained in mice, it can be concluded that the reversibility of therapeutic vaccines should not be of significant concern.

Conclusions

First human vaccine trials have been completed with VLP derived from papilloma virus, Norwalk virus, parvovirus and HIV and recombinant hybrid Ty VLP [12, 21–24]. In general, these vaccines were immunogenic and very well tolerated. The extraordinary immunogenicity of VLP can be transferred to any antigen of choice by attaching it to the VLP carrier in a repetitive and ordered manner. Thus, attaching antigens to VLP gives a viral fingerprint to the antigen and renders them as immunogenic as the underlying viral surface. These encouraging results suggest that vaccines based on antigens coupled to VLP could soon become invaluable tools both for disease prevention and therapy.

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Molecular Epidemiology and Immunology of Hepatitis B Virus Infection –An Update

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Key Words
Hepatitis B virus · Epidemiology · Immunology · Genotypes · Subtypes · Viral proteins · Epitopes · Antigenicity · Immunogenicity · Virus-like particles

Abstract
Hepatitis B virus (HBV) continues to be one of the most important viral pathogens in humans. This review provides an update on the molecular epidemiology and immunology of HBV infection. DNA sequencing has allowed replacement of the initial serotypic classification of HBV strains by a more systematic genotype system that currently consists of 7 members (genotypes A–G). More recently, sequence analysis of virus isolates from many individual patients has revealed the occurrence of certain mutational hot spots in the genome, some of which appear to correlate with the patient's immunological and/or disease status; however, cause and effect are not always easily discernible. This holds particularly for the issue of whether virus variants exist that have, per se, an increased pathogenic potential; due to the scarcity of appropriate experimental in vivo models, such hypotheses are difficult to prove. Similarly, because of the compact organization of the HBV genome, almost every single mutation may have pleiotropic phenotypic effects. Nonetheless, there is accumulating evidence that at least some frequently observed mutations are causally related to viral escape from selective pressures, such as the presence of antibodies against dominant B cell epitopes, or drugs that inhibit the viral reverse transcriptase; possibly, this is also true for the cellular immune response. Therefore, despite the availability of an effective prophylactic vaccine, further extensive efforts are required to monitor the emergence of vaccination- and therapy-resistant HBV variants and to prevent their spread in the general population.

Introduction

Despite unceasing efforts of the medical community, hepatitis B remains, besides hepatitis C, the most serious type of viral hepatitis and one of the major problems of global public health. According to the latest World Health Organization fact sheets, 'of the 2 billion people who have been infected with the hepatitis B virus (HBV), more than 350 million have chronic infections. These chronically infected persons are at high risk of death from cirrhosis of the liver and liver cancer, diseases that kill about 1 million persons each year' [1]. The risk of death from HBV-related liver cancer or cirrhosis is approximately 25% for chronically infected persons. Moreover, recent findings

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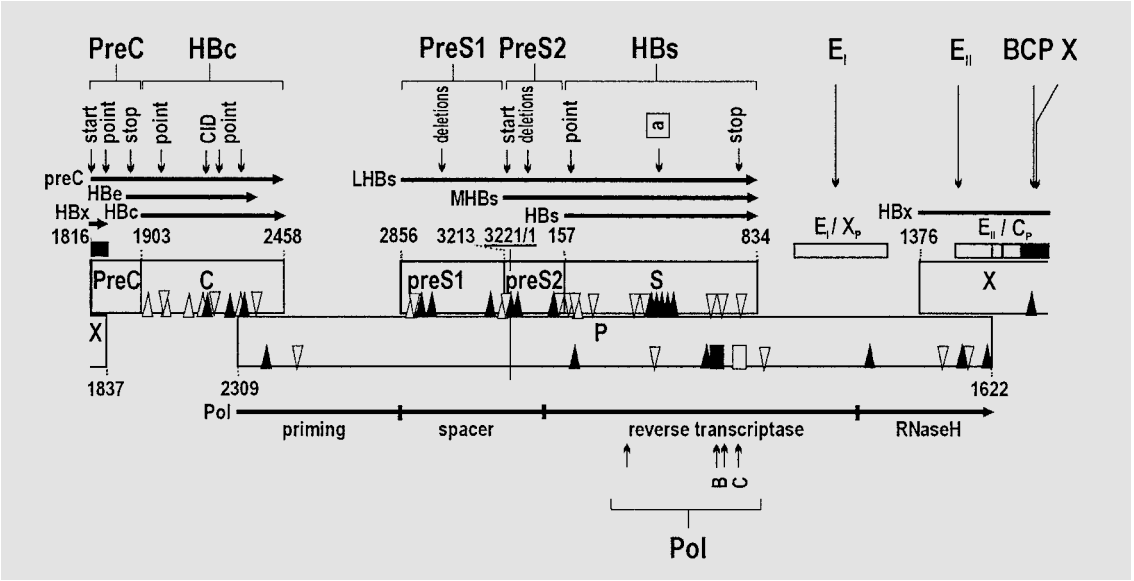

Fig. 1. Location of immunological epitopes and major mutation clusters on the genetic map of HBV. The HBV genome of the genotype A, 3,221 nt in length, with the unique *Eco*RI site as an nt numbering start point, is chosen for illustration. For clarity, the HBV genome is linearized at the start of the gene C, namely at the preC start codon. The four overlapping genes C, P, S and X are shown with the numbers indicating their boundaries. Protein products of these genes are shown as horizontal arrows. Promoter and enhancer regions are depicted as bars, with the basal core promoter in black. Immunological epitopes are compiled from previous reports [28, 184] and shown as triangles: open = T; filled = B; open inverted = CTL. Polymerase domains are shown as rectangles: filled = B (binding); open = C (catalytic). Positions of mutation clusters are depicted by vertical arrows. Main classes of mutations are coupled by brackets.

suggest a role of HBV infection in the pathogenesis of carotid arteriosclerosis, at least in the Japanese population [2]. Not only is HBV the only DNA virus among the human hepatitis viruses, it is also the only type causing chronic hepatitis for which an efficient prophylactic vaccine is available.

HBV belongs to the *Orthohepadnavirus* genus of the *Hepadnaviridae* family, which is related to the large order of *Retroid* viruses. Within a size of only about 3.2 kb, its compact, partially double-stranded DNA genome is extremely small; it bears four highly overlapping open reading frames (ORFs), i.e. S, C, P and X, which encode at least 8 proteins (fig. 1). There are surface proteins (LHBs, MHBs, SHBs or simply HBs) encoded by the S gene, three are core-derived proteins (preC, HBc, HBe) encoded by the C gene, and the two proteins Pol and HBx are encoded by the P and X genes, respectively. Extensive systematic reviews of the molecular biology of HBV have been published recently [3–5].

HBV is transmitted by exposure to infected blood or body fluids. Worldwide, most HBV infections occur perinatally, by household child-to-child and sexual contacts and from unsafe injections and transfusions. In Western Europe and North America, sexual activity and intravenous drug abuse during young adulthood, as well as the exposure of professional health workers to contaminated blood products are the main causes of HBV transmission.

The prevalence of HBV varies tremendously in different parts of the world, with a much higher incidence in the Eastern than in the Western Hemisphere. In the developing world, i.e. sub-Saharan Africa, most of Asia and the Pacific, most people become infected with HBV during childhood. Since young HBV-infected children usually develop chronic infection, 8–10% of the population in this region are chronically infected [1]. High rates of chronic HBV infection are also found in the Amazonian region and the southern parts of Eastern and Central Europe. In the Middle East and Indian subcontinent, about 5% of the population are chronically infected. In Western Europe and North America, the chronic carrier rate is at or below 1% [1, 6].

Table 1. Geographical distribution of HBV genotypes [compiled from ref. 178–181] and prognosis of specified course of viral mutations and hepatic disease [based on ref. 22, 26, 51, 182, 183]

Genotype	Geographical distribution area	Specific prognosis on the basis of established features		
		mutations	course of disease	typical sequelae
A	Northern Europe, sub-Saharan Africa	preC – rare	more inflammation and fibrosis than in other, T1858-containing genotypes	chronization
B	Eastern Asia, Far East	BCP – less than in genotype C	less inflammation than in genotype C	HCC
C	Eastern Asia, Far East	BCP – more than in genotype B	more inflammation than in genotype B	cirrhosis, HCC
D	Mediterranean, Near and Middle East, South Asia	preC – often	severe after transplantation	NS
E	Western sub-Saharan Africa	NS	NS	NS
F	Central and South America	preC – only in the strains with T1858	NS	NS
G	southern France; Georgia, USA	NS	NS	NS

BCP = Basal core promoter; NS = no established specificity; HCC = hepatocellular carcinoma.

Epidemiology of HBV Genotypes

Initially, serological grouping revealed a basic set of HBV subtypes, namely *adw, ayw, adr* and *ayr*, representing the immunological properties of the HBs as a combination of a common immunodominant determinant *a* with at least two mutually exclusive subdeterminants, i.e. *d*, or *y*, and *w* or *r*. In total, 9 serotypes *(ayw1*, *ayw2*, *ayw3, ayw4, ayr, adw2, adw4, adrq–, adrq+)* were described. DNA sequencing formed the basis for a genetic nomenclature of HBV [7–9] that allowed more accurate investigation of the geographical distribution of HBV genotypes (table 1). It is important to note that these genotypes do not directly correspond to the serotypes mentioned above [for the origin and evolution of the HBV genotypes, see ref. 10].

Recent findings of infection in Old and New World primates in the wild (chimpanzee, gibbon, orangutan and woolly monkey) suggest the existence of a set of species-specific HBV variants that do not phylogenetically cluster with the established human HBV genotypes [11–17]. However, except for the clearly divergent woolly monkey HBV, it is still controversial whether these primate HBVs do circulate in nature. Contrary to earlier belief, there is also evidence for recombination between HBV genomes [18–20], although the mechanism is unclear and, in contrast to retroviruses, most likely not directly related to the normal mode of HBV replication.

The HBV genotypes play a significant [21–25], but not always clearly established role in the appearance and distribution of mutations. Less is known about the clinical importance of the HBV genotypes per se for the outcome of liver disease, although clinical differences between the HBV genotypes B and C have been found [26], and genotype A has been suggested to be connected with a higher chronicity rate [27]. A brief summary of this topic is given in table 1.

Immunology of HBV Infection

HBV is preferentially hepatotropic but not directly cytopathic for infected hepatocytes. Both the cellular and the humoral arms of the immune response are essential for viral clearance; the cellular response is also believed to be responsible for disease pathogenesis and thus the clinical manifestations of HBV infection [for comprehensive reviews, see ref. 28–33]. Resolution of infection in acute hepatitis B is typically associated with a vigorous, polyclonal, multispecific CD4+ T helper and CD8+ cytotoxic

T lymphocyte (CTL) response. Infected hepatocytes are recognized and destroyed by HBV-specific CTLs directed against the HBV proteins, peptides of which are bound to class I human leukocyte antigens (HLA) and presented on the cell surface.

Antibodies against surface proteins (anti-HBs, as well as anti-preS1 and anti-preS2), produced with the help of the CD4+ T helper cells, ensure humoral immunity and prevent (re)infection. While killing of infected cells was previously considered the most important pathway for virus elimination, experiments in transgenic HBV-producing mice [34] led to the concept that clearance during human HBV infection may be primarily due to a non-cytopathic mechanism in which HBV-specific CTLs, mediated mainly by inflammatory cytokines such as IFN-γ and TNF-α [35], abolish HBV gene expression and replication. Strong evidence favouring an important role for this non-cytopathic clearance mechanism was recently obtained in chimpanzees [36] and human patients [37–40].

The recent development of HLA-peptide tetrameric complexes able to directly visualize HBV-specific CD8+ cells has allowed for a direct correlation of their frequency with liver damage [41]. Unexpectedly, the frequency of intrahepatic CD8+ cells specific for the immunodominant HBV core CTL epitope 18–27 in chronically HBV-infected patients was much higher in individuals who had low viral replication and no liver inflammation than in those with high replication and liver inflammation [37]. Hence, inhibition of viral replication can be independent of liver damage. This supports the view that the central immune events, i.e. virus-specific immune responses and reduction of viral replication, occur before symptomatic disease in hepatitis patients [38–40], and that IFN-γ, TNF-α and IFN-α/β play a central role in the early non-cytopathic downregulation of HBV replication without accompanying liver damage [42].

It remains clear, though, that patients who fail to mount a vigorous immune response in the acute hepatitis B phase develop chronic infection. In these cases, the persisting ineffective immune response and/or deployment of HBV mutants appear to be responsible for liver damage and, likely, initiating the process of hepatic fibrosis [29, 32]. Decreased telomerase activity of lymphocytes in chronic hepatitis B patients may be partly responsible for the immunosuppressive condition in such patients [43]. Weak or inadequate T cell and CTL responses to the core-derived proteins may be the most important factors in chronic hepatitis B [31]. Mutations in the latter may contribute strongly to the weakening of such immune responses [44, 45]. In general, as a consequence of several combined escape mutations, HBV could become completely invisible to both the humoral and the cellular response.

Epidemiology of HBV Mutants

The mutability of HBV appears now to be much higher than was expected in the early 1980s. Theoretically, the RNA-dependent replication of HBV may lead to 2×10^{-4} base substitutions per site per year, which is four orders of magnitude greater than in genuine DNA viruses, but less than in RNA viruses [46]. Long deletions are emerging, however, by other mechanisms [for discussion, see ref. 32]. The peak production of progeny virions has been estimated to be around 10^{13} virions per day per patient, such that every possible single mutation could, theoretically, be generated every day [47].

Nonetheless, the HBV genome appears to be extremely stable during the HBe-positive phase when the immune response (inflammation) is weak, with an average of 1.6 mutations over about 25 years; by contrast, an average of 20 mutations were found after development of hepatitis and/or loss of the HBe protein, i.e. in the presence of a strong host immune response. Although no association with clinical outcome was obvious [48], this strongly supports the idea that the virus population does respond to the selective pressure of the immune response. It should be emphasized that HBV mutants may coexist with wild-type viruses in the majority of cases where such mutants are found. Such mixed infections can hardly be mimicked experimentally, and their consequences for the biological fitness of individual virus mutants, as well as the clinical consequences, are largely unexplored.

Under the influence of the natural immune response as well as of vaccination and antiviral therapy, mutations can emerge in genomic regulatory elements and all four viral ORFs, enabling the virus to escape these selective pressures (for more details, see recent reviews [25, 32, 49–52]). The location of the most studied HBV mutations on the genomic map of HBV is shown in figure 1. In general, the enhancer II/core promoter (E_{II}/C_P), preC stop codon (preC) and enhancer I/X promoter (E_I/X_P) mutants appear to be associated with disease severity and progression. Mutations in the core antigen contribute strongly to immune escape at the T helper and CTL level. Surface antigen mutants allow for escape from humoral immune responses and reduce the effectiveness of diagnostics and vaccination. Polymerase mutants are generally of the

therapy escape type, and they may render the virus resistant to chemotherapeutic treatment. It should be noted, however, that owing to the overlapping arrangement of ORFs and regulatory elements on the HBV genome, almost every single mutation will influence more than one function of the corresponding nucleotide (nt) sequence; this severely complicates clear-cut interpretations as to what benefit a specific mutation confers on the virus. At least in some cases, however, the corresponding phenotypes are strongly suggestive of a specific escape mechanism.

Core Promoter (E_{II}/C_P) and PreC Mutants

The most characteristic feature of this group of HBV mutants is a decreased or abolished production of the preC protein and its secreted processing product HBe, either by influencing transcription or by nonsense mutations in the preC ORF. A recent detailed analysis shows that the HBe-negative mutants are more common than previously suspected, and their prevalence is increasing over time [25]. These mutants are present worldwide, with marked variations in prevalence across different geographical regions. The median prevalence of the HBe-negative mutants among patients with chronic HBV infection drops from 33% in the Mediterranean to 15% in Asia Pacific and 14% in the USA and Northern Europe. The preC mutants are absolutely prevalent in the Mediterranean, while the core promoter mutants dominate in Asia [25]. An exhaustive review of the diagnosis and treatment of the preC mutants has recently been published [52].

E_{II}/C_P Mutants

Synthesis of both pregenomic and the 5′-terminally extended precore RNA is controlled by the E_{II}/C_P region; hence, mutations in this region can influence the ratio of the two transcript classes. The basal core promoter sequence (nt 1744–1804) is the most important of the numerous regulatory elements that have been mapped within the E_{II}/C_P region. The BCP mutations at nt 1762 (A→T) and 1764 (G→A) are most frequently observed in HBV-infected patients with chronic hepatitis, hepatocellular carcinoma and fulminant hepatitis, but are rare in asymptomatic HBe-positive carriers, in immunosuppressed patients and in carriers without HBV markers [53]. Additional mutations at nt 1653 (C→T) and 1753 (T→C/A/G) may have further association with progression of liver disease to cirrhosis in patients chronically infected with genotype C [54]. A mutation at nt 1752 (A→C) was reported also in conjunction with the 1762 and 1764 mutations and appeared to be associated with liver damage [22].

PreC Mutants

The frequent preC mutation at nt 1896 (G→A) leads to premature termination of the preC protein at codon 28 of the 29-amino acid (aa) preC region, and thus to elimination of the HBe protein [55, 56]. This mutation also affects the RNA stem-loop structure of the encapsidation signal ε, which acts at the level of pregenomic RNA while preC RNA is not encapsidated [57]. The mutation is rarely found in genotype A (and some strains of genotype F), which contains a C at the pairing position 1858 (instead of T in other HBV genotypes), because the loss of a base pair destabilizes the ε stem-loop unless a stabilizing complementary mutation at nt 1858 (C→T) occurs. Simultaneously with the preC mutation at nt 1896, a 1899 (G→A) mutation, which also improves the stability of the stem-loop, may be observed [58, 59]. It is present also in a high proportion of patients with fulminant hepatitis B. However, the implicated importance of the preC mutants in the prognosis of disease remains controversial [32, 49, 50]. Less common preC mutations are: start codon mutations at nt 1814 or 1815, a nonsense mutation at nt 1874, a missense mutation at nt 1862 and frameshift mutations [59]. Together, these data imply that preC and HBe are nonessential gene products, at least under certain conditions. However, the potential advantages for the virus of a decreased or abolished preC or HBe production will remain obscure as long as the normal functions of these proteins are not really clear.

Care Mutants

Though also not finally settled, there is evidence suggesting that aggressive hepatitis B cases are connected with mutations others than those abolishing the HBe protein. First candidates for enhanced HBV virulence are mutations in the HBV core-derived proteins, most prominently in the central immunodominant region of the HBc protein. Such mutations frequently accompany preC mutations and may decrease HBV recognition by cytotoxic T cells. The appearance of the core mutants, in concert with the preC mutations, may significantly diminish the response to IFN-α therapy (see recent reviews [51, 52]).

Substitutions within Crucial HBc Sites

First experimental data supporting the idea that HBc mutants may alter the host immune response and modulate the clinical course of HBV infection appeared in 1991 [60]. Two generally accepted facts are that (1) mutations are clustered within immunologically relevant epitopes [61–64], and (2) mutant clones in patients with active liver disease outnumber the mutant clones in asymptomatic carriers [64–66].

Severe liver damage in patients with chronic active hepatitis was attributed to changes clustering in three regions, i.e. codons 48–60, 84–101 and 147–155 [62]. Mutations within the region 84–101, which overlaps the CTL epitope, accumulated during periods with frequent hepatitis exacerbation [64]. Numerous unique mutations were found in the region 48–60 in fulminant [67–69] and severe exacerbation hepatitis B patients [64–67, 70–72]. In addition, mutation clustering regions at aa 21–34 [73] and 31–49 [74] were detected.

Although some unique mutations were attributed to fulminant rather than acute hepatitis B [67, 69], completely identical HBV strains were found to replicate in patients with acute and fulminant hepatitis B [68]; hence, these mutants are, at least, not generally more pathogenic. Evolutionary studies in chronic hepatitis patients over a period of 6–11 years showed that the HBc mutations tended to increase with time and correlate with exacerbation of the disease [75], although interfamilial conservation of HBV core sequences for more than 20 years has been described [76]. Some other investigations, however, failed to detect diagnostically significant changes within the HBc protein, but found various numbers of missense mutations independent of the grade of liver disease [77–79]. The HBc protein can remain highly conserved for more than two decades during the immune tolerant phase of chronic HBV infection, whereas mutations appear typically after seroconversion to the anti-HBe-positive phase. This was observed in asymptomatic anti-HBe-positive carriers [80] but also in some cases of fulminant hepatic failure. Although the total viral load in such patients is drastically reduced, a small number of the newly appearing mutants may contribute to the fulminant outcome.

Recent data suggest a sequential order in the mutational processes, i.e. first HBe clearance and seroconversion to anti-HBe, then emergence of the preC stop mutations preventing HBe generation, followed by core mutations in regions of B cell, T helper and CTL epitopes as well as in the arginine-rich C terminus, thus supporting the idea of immune escape [45, 81–88]. First direct evidence for the responsibility of the HBV core CTL epitope 18–27 mutations (aa changes S21N/A/V and V27A/I) was found in HLA-A2-positive patients with chronic hepatitis B [89]; however, another group of researchers failed to find significant mutations of this epitope in HLA-A2-positive patients with acute exacerbation during chronic infection [90]. Further, analyzing HBe to anti-HBe seroconverters, mutations were found mostly in the T helper epitope 50–69 and in the B cell epitope 74–83 [45]. Remarkably, the absence of serum anti-HBc was not a consequence of mutations within the HBc gene but rather of an aberrant immune reaction of the host [91].

Mutations I97L and P130I/T/S have been reported to be associated with exacerbation of chronic hepatitis [90, 92] and hepatocellular carcinoma [93]. Interestingly, the I97L mutation is responsible for secretion of immature HBV cores [94], but this phenomenon can be offset by the P130T mutation [95]. Low secretion of HBV virions is caused by the naturally occurring P5T and L60V mutations [96].

Deletions within the HBc Molecule

According to recent data, the core internal deletions (CIDs) may be involved in the onset of hepatitis and the subsequent outcome of chronic infection [97]. Historically, the CIDs were found, first of all, in patients with long-standing HBe-positive infection [60, 81, 98–100], and in a familial cluster of chronic carriers with the absence of anti-HBc [10]. However, HBV variants with HBc deletions were also described as inhibiting HBV replication, and not persisting in preference to wild-type HBV under enhanced immune pressure, e.g. in patients with chronic active hepatitis B [102].

A special group of CID mutants is represented by the HBc variants with deletions in the central part of the molecule, which appear and accumulate in long-term immunosuppressed renal transplant recipients [103–105]. Moreover, persistence of these mutants seems to correlate with progressive liver disease [106]. Some of these variants, with relatively short deletions (for example, amino acid 86–93 and 77–93 deletions), retain their self-assembling capability, whereas mutants with longer deletions are unable to self-assemble [107].

Another group of the CID mutants is represented by a specific set of long in-frame internal HBc deletions (at aa 88–135 and 82–122), which were described as responsible for the appearance of defective interfering HBV particles [108, 109]. Genomes carrying such CID variants possess some replication advantages in comparison to wild-type HBV genomes, but they are dependent on complementa-

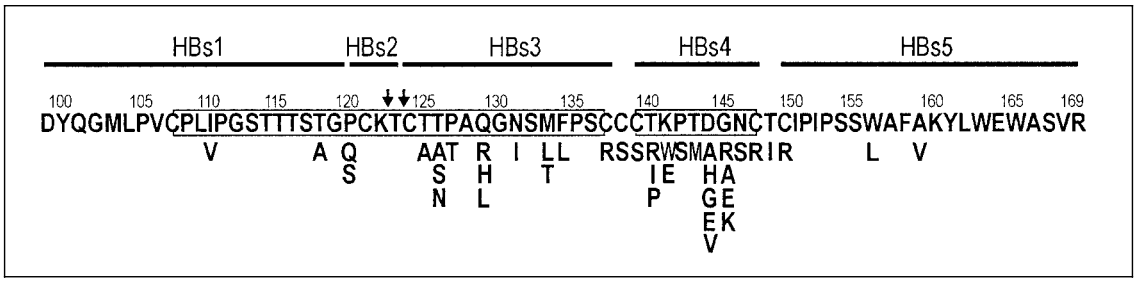

Fig. 2. Location of the vaccine escape mutations in the major hydrophilic domain (aa positions 99–169) of the HBs protein, serotype *adw2*, genotype A (PIR accession: SAVLVD) [185]. Two antigenic loops of the *a* determinant are depicted by boxes. Another tight loop is formed by cysteines 121 and 124. Five antigenic regions are shown in accordance with a previous report [116]. Mutations connected with the escape phenomenon are compiled from other reports [114, 119, 120, 186–191] and shown under the original sequence. Black vertical arrows point to insertion positions of additional aa residues in the HBs2 region.

tion by wild-type HBc protein. No detectable amounts of the CID proteins were found in infected cells [108]. The interference phenomenon is not connected with the synthesis of deleted core proteins, since it was detected also in long out-of-frame deletions within the gene C [110].

Besides HBc deletions, insertions may also occur; for instance, an HBV variant with a 36-nt insertion in the preC region was described that forms an extended HBc protein with an extra 12 aa at the N-terminus [11]. Whether any pathogenic effects are associated with this variant has not been analyzed.

HBs Mutants

The *a*-determinant of the three surface proteins L, M and S is of special importance since it induces virus-neutralizing humoral responses and confers the protection raised by existing HBV vaccines. The *a* determinant is located within the major hydrophilic domain and consists of two loops that protrude from the outer surface of HBV virions and so-called 22-nm HBs particles (fig. 2). The tertiary structure of the *a* determinant is crucial since the vast majority of induced anti-HBs antibodies recognize conformational epitopes.

Vaccine-Induced Mutations

The most frequent vaccine-induced escape mutation is G145R [112], which is poorly detectable in traditional HBs diagnostic assays. The mutation was found in natural isolates, and immune pressure upon vaccination favours its selection. In countries with mass vaccination programmes, for example Taiwan [113], a substantial increase in HBs escape variants of up to 28% has been documented, concurrent with a 10-fold decrease in the HBs carrier rate in children [114]. Generally, escape mutations were found mainly in the aa 139–147 loop, or HBs4 region of the *a* determinant, predominantly at position 145 (G145R) [115]. A summary of mutations found in vaccinees is presented in figure 2. For more detailed information, the reader is referred to several recent reviews on this topic [32, 50, 116–120].

Mutations in Chronic Infection

In contrast to the vaccine-induced escape mutations predominantly found in the aa 137–149 loop of the *a* determinant, the most frequent mutations in patients chronically infected with genotype C were detected within the aa 107–137 loop [115]. Mutations in the CTL epitope encompassing the 29–53 region, especially at aa positions 40 and 47, were found in the chronic patients [115, 121, 122], suggesting that they may contribute to chronicity.

PreS Mutants

Mutations in the preS1 region may include long deletions of up to one half of the entire preS1 region [123], but the hepatocyte-binding site at aa 20–47 always remains conserved. The preS1 mutations destroy the integrity of the S promoter [124–128].

Deletions in the preS2 region have typical aspects of immune escape. Removing T and B cell epitopes [129–132], they may contribute to the generation of hepatocellular carcinoma [133, 134]. Moreover, missense mutations at the start codon of the preS2 region that prevent

the synthesis of the corresponding protein are frequently associated with fulminant hepatitis [135] and hepatocellular carcinoma [93].

In general, the preS mutations affect the ratio of the large, middle and small S proteins, and this could result in the accumulation and retention of these proteins in the endoplasmic reticulum. This may lead to a direct cytopathic effect in hepatocytes, and the appearance of the preS mutants might thus be linked to progression of liver disease [106, 125].

Polymerase Mutants

Chronic Infection

Since the S gene completely overlaps with the P gene, preS and S mutations affect the structure of polymerase. Because the preS region overlaps the non-essential spacer domain of the polymerase (fig. 1), rather long deletions in the preS may remain indifferent with respect to the structure and function of the enzyme. By contrast, the catalytic reverse transcriptase domain coincides with the C-terminal part of the S protein, and changes therein could have drastic effects on polymerase activity. Indeed, this region of the S protein is not a mutational 'hot spot', and no polymerase mutations that could affect the susceptibility of HBV to antiviral nucleoside analogues were found in chronic patients [115]. From the other side, the *a* determinant overlaps the variable linker between the two conserved domains in the polymerase. Nevertheless, in isolates from individuals with anti-HBc reactivity only, an increased variability in the *a* determinant was found that consequently resulted in a similar hypervariable spot in the polymerase which might impair viral replication [136]. Interestingly, a missense mutation in the 5′ region of the polymerase gene resulted in the inability to package pregenomic RNA into core particles [137].

Antiviral Chemotherapy

Upon chemotherapy of hepatitis B with the nucleoside analogues lamivudine and famciclovir, the emergence of two classes of polymerase mutants is almost regularly observed, providing very strong evidence that they are selected by the presence of the drugs. First, the YMDD site in the C (catalytic) domain of the polymerase is subject to changes upon lamivudine therapy [138, 139], similar to the earlier described mutation M→V/I within the analogous site in HIV reverse transcriptase that is associated with lamivudine resistance. The variants M552V and M552I emerged in almost 50% of patients during lamivudine therapy [140], and their frequencies appear to rise even further upon prolonged therapy. Both mutations result in substitutions at aa 195 and 196 in the overlapping S gene. The YMDD mutants have a lower replication efficiency than wild-type virus, and the latter displaces the mutant after cessation of lamivudine treatment. Lamivudine-resistant variants may have an additional L528M substitution in the B (template-binding) domain of the polymerase, often occurring together with the M552V mutation but rarely with the M552I mutation [141]. The additional L528M mutation restores the replication competence of the M552V mutant and increases resistance to various nucleoside analogues [142]. The L528M mutation has no effect on the aa sequence of the S gene. The M539V mutation is also able to confer resistance to lamivudine and other cytosine and thymidine analogues [143]. More distant mutations, L430M and L428V/I, have been reported in genotype C patients in Japan [144]. These mutations were also associated with the basic M552V substitution. The latter remains a central therapeutic problem, since the use of high doses of lamivudine does not prevent its emergence [145]. Interestingly, the HBV subtype may significantly influence the likelihood of the emergence of lamivudine resistance, possibly related to whether or not the aa sequence encoded by the overlapping S gene is affected [24].

The V555I [146] and V542I [147] mutations in the C domain confer resistance of famciclovir. Both mutations cause premature termination of the overlapping HBs proteins. Mild to moderate resistance was found in association with the V521L, P525L and L528M substitutions in the B domain. Although the M552I exchange was not associated with famciclovir resistance, no benefit was found in attempts to add famciclovir in patients with the M552I mutation after lamivudine failure [148]. Fortunately, lamivudine- and famciclovir-resistant viruses may remain sensitive to newer nucleoside analogues such as adefovir and lobucavir. An excellent review on the topic is available [149].

HBx Mutants

The importance of HBx mutants may appear in a new light after the recent discovery of HBx targeting of mitochondrial calcium regulation [150], although the HBx protein has been described as exhibiting numerous activities affecting intracellular transmission, gene transactivation, cell proliferation, DNA repair and apoptosis. Since the X gene overlaps the E_{II}/C_P region, mutations within

the latter may influence the structure of the HBx protein. For example, the mutation at nt 1653 (C→T) leads to the H94Y exchange in the immunodominant epitope of the HBx protein [54]. The E_{II}/C_P point mutations and deletion variants (for example, nt 1770–1777, 1752–1772) may produce truncated HBx protein variants. Such shortening of the HBx protein may have an effect on its transactivating and other properties. C-terminal insertions/deletions in the HBx protein have been described as connected with the appearance of hepatocellular carcinoma [93].

E_I/X_P Mutants

The E_I/X_P region (nt 959–1311) harbours binding sites for many regulatory cellular proteins, including hepatocyte-enriched nuclear factors, and is responsible for IFN-stimulated responses [for a review, see ref. 151]. The strongest decreasing effect on HBV replication was found for mutations which occur in the hepatocyte nuclear factor 3 and 4 binding sites of the E_I core domain [152]. As previously described mutations, the E_I mutations may also serve as an escape mechanisms, switching viral replication from a high to a low level, as is frequently observed during chronic HBV infection.

Prevention and Therapy of Hepatitis B: A Potential Role for VLPs

Safe and effective HBV vaccines based on recombinant HBs virus-like particles have been available since 1986. The yeast-derived HBs vaccine is not only the first, but also the sole approved recombinant vaccine up to now. The World Health Assembly recommended the worldwide introduction of hepatitis B vaccination into national infant immunization programmes by 1997. As of March 2000, 116 countries have added this vaccine to their routine immunization practice. However, the emergence of vaccine escape mutants raises the question as to how the existing vaccines might be improved. A general strategy is to increase the number of targets (B cell, T helper and CTL epitopes), e.g. from other HBV antigens such as the preS and core-derived proteins; hence, one or a few mutations would not allow the virus to escape immune surveillance. For example, the preS-containing Hepacare vaccine was shown to stimulate stronger and more rapid cellular and humoral immune responses and to overcome anti-HBs non-responsiveness [153, 154]. Other preS-containing HBs vaccine candidates demonstrated rapid seroprotection [155] and apparently prevented selection of the determinant *a* escape mutants [156].

Apart from such more conventional recombinant protein vaccines, the unique immunological properties of HBc particles, including the presence of crucial CTL epitopes, may provide an opportunity for the development of novel protective and, in particular, therapeutic HBV vaccines. Numerous studies strongly suggest that for viral clearance, as well as the pathobiological effects of HBV infection, cellular immune responses are of foremost importance. In the mid-1980s, partial protection of chimpanzees by recombinant HBc immunization was documented [157–159]; because there is no evidence for neutralizing anti-HBc antibodies, this protection is probably cell mediated, as corroborated by the complete protection of woodchucks immunized with recombinant woodchuck hepatitis virus core antigen [160–162] or a peptide corresponding to a major immunodominant T cell epitope [163]. While particulate HBc, apart from causing a strong humoral response, predominantly induces T helper type 1-like responses [164], immunization of mice with exogenous HBc protein did not elicit a CD8+ CTL response [165]. However, intracellular expression of the HBc protein by genetic immunization induced both humoral and class I-restricted CTL responses [165–169]. Moreover, genetic immunization with the woodchuck hepatitis virus core gene protected woodchucks against woodchuck hepatitis virus infection [160, 161].

Studies on HBV core CTL epitopes as putative vaccine candidates have been initiated, first of all, on the HLA-A2-restricted CTL epitope aa 18–27 [170]. Recently, it was shown that this sequence also harbours an HLA-B51-restricted CTL epitope, aa 19–27, suggesting that degenerate immunogenicity (e.g. the capacity of a given peptide to be immunogenic in the context of multiple HLA molecules) can occur across HLA supertype boundaries, here the HLA-B7 and HLA-A2 supertypes [171]. Hence, such a single peptide epitope may be effective in a broad and diverse population. In addition, mutated HBc genes from chronic hepatitis patients were recently included into gene immunization studies in mice [172].

Recently, first evidence was obtained in humans that transfer of HBc-reactive T cells from a donor with natural HBV immunity to a chronic HBV carrier resulted in resolution of chronic infection [173].

Together, these data suggest that both exogenously administered and intracellularly produced HBc hold the potential to induce cellular responses that might control, and eventually eliminate, HBV infection. An example is

the Hepacore project initiated by Celltech-Medeva that aims at the development of a therapeutic vaccine based on chimeric HBc virus-like particles carrying the preS1 sequence 20–47 inserted into the major immunodominant region of the HBc molecule; both protein and DNA vaccination applications are considered [Page et al., unpubl. data].

The potential of HBc is, however, not restricted to the presentation of HBV-derived epitopes; rather, it can be used as a much more general molecular platform to which DNA and RNA packaging domains, receptors and receptor binding sequences, immunoglobulins, elements recognizing low-molecular-mass substrates and others may be added; hence, many more applications than its use as a carrier in new HBV vaccines can be imagined.

Conclusions/Perspectives

As should be apparent from the above discussion, the interpretation of sequence data with respect to the origin of HBV mutants and their potential pathogenic consequences is still in its infancy, leading not infrequently to controversial conclusions. There is little doubt that viral escape from selective pressure is responsible for various of the observed mutations. This is most directly evident for the polymerase mutants arising during chemotherapy, where resistance against the drug can be phenotypically assessed in cell culture experiments. Nonetheless, the biological fitness of such mutants in a heterogenous population of patients is hard to evaluate by such relatively simple in vitro tests. The coherent patterns of major B and T cell epitopes and overlapping mutational hot spots also strongly supports the notion that the immune system plays a major role in shaping the virus population circulating in a patient. However, the genetic heterogeneity of the human population and their vastly different social and environmental circumstances makes simple generalization rather problematic; this may also explain, at least in part, the different results and interpretations reported in different studies. Perhaps the most controversial issue is whether certain HBV mutants do have, per se, an increased pathogenic potential; notably, at least in the duck HBV model, there is very good experimental evidence that such more pathogenic variants can exist [174]. At present, however, there is no clear-cut evidence in favour of this concept for the human virus, and the question as to whether a certain variant observed in a selected cohort of patients is causing the corresponding disease pattern or, by contrast, is its consequence, remains largely open. More research, clinical and other, that improves our understanding of the complex interplay between virus and host would certainly help. One major advance would be a small mammalian animal model that reproduces human HBV infection, including the chronic carrier state. Encouraging data have recently been obtained with HBV infection of primary tupaia hepatocytes [175]. Novel systems such as adenovirus-derived vectors capable of transducing complete HBV genomes into various cells [176, 177] also hold promise for in vivo experiments, including adaptation of HBV to tupaia. If this could be achieved, more complex problems might become amenable to systematic studies, for instance how the presence of different HBV variants within one patient influences the disease. Work on severely damaged genomes has clearly demonstrated that these different virus populations can interact, e.g. by complementing defects and thus enabling propagation of even severely truncated or otherwise impaired genomes; hence, the problem is, at least, defined.

Thus, a lot remains to be done. The good news, on the other hand, is that the current and potential new improved vaccines, carefully designed in view of the risk potential of emerging virus variants, should provide the key to eradicating HBV from the list of human diseases. Until this goal is reached, however, the development of new efficient treatments for the millions of existing chronic hepatitis B cases remains a most important issue for the next decades.

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In vitro Expression of Human Hepatitis B Virus Genomes Carrying Woodchuck Hepatitis Virus Pre-S1 Sequences

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Key Words
Hepatitis B virus · Woodchuck hepatitis virus · Virus tropism · Pre-S domain · Chimeric virus

Abstract
Many hepatitis B virus (HBV) in vivo experiments are unfeasible because this virus infects only humans. Former studies demonstrated that the pre-S1 domain of the viral envelope protein L determines host range. Therefore, we tried to generate HBV recombinants which might be able to infect woodchucks by exchanging different portions of the pre-S1 encoding gene with homologous parts from the related woodchuck hepatitis virus (WHV) in a cloned HBV genome. In 6 mutants, 11–92 N-terminal HBV pre-S1 codons were replaced by 20–120 codons from WHV. Four mutants carried C-terminal substitutions. The pre-S1 region overlaps with the viral polymerase gene, which is therefore also affected. After transfection of Huh7 cells, the DNA polymerase activity in cytoplasmic nucleocapsids was found to be only slightly affected. All mutants except for the largest C-terminal substitution allowed virion formation. Only the smallest N- and C-terminal substitutions had a wild-type phenotype. The remaining 7 variants allowed virion yields between 5 and 50% of that of the wild type. This demonstrated that substitution of up to 92 pre-S1 codons in an HBV genome with up to 120 codons from WHV pre-S1 was compatible with DNA replication and virion formation. Some recombinant viruses might be able to grow in woodchucks.

The hepatitis B virus (HBV), the prototype member of the virus family Hepadnaviridae, is a major human pathogen frequently causing persistent infections and chronic inflammations of the liver. Worldwide, more than 300 million persons carry this virus and are, as a consequence, at risk of developing liver cirrhosis or hepatocellular carcinoma [1]. While the molecular biology of the virus has been explored to a significant degree [for a review, see ref. 2], mainly by using in vitro systems like cell lines transfected with molecular cloned HBV genomes, a number of important questions concerning virus-host interactions on a tissue or organ level are unanswered due to the lack of suitable experimental in vivo systems; HBV infects only humans and can be transmitted experimentally only to higher primates. Other members of the Hepadnaviridae family [3], such as duck HBV, heron HBV, ground squirrel hepatitis virus, woodchuck hepatitis virus (WHV) and woolly monkey HBV, also display a very narrow host range and liver specificity. They are used as surrogates for in vivo studies. However, avian hepatitis B viruses are quite distinct from HBV in several aspects and therefore their use as a model for HBV is limited.

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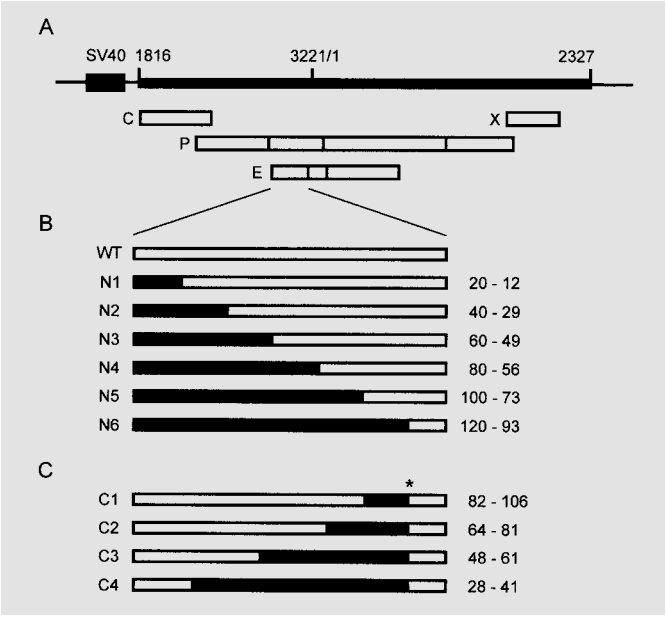

Fig. 1. Map of the parental plasmid pSVHBV1.5 carrying the HBV genome and of the recombinations. **A** A 1.2-mer of the HBV genome, genotype A (black bar), was inserted downstream of an SV40 early promoter (SV40). Numbers indicate nucleotide positions in the HBV genome; nucleotide 1 is the dC residue of the single *Eco*RI site. Open boxes show the HBV genes (C: core gene; P: DNA polymerase gene; E: envelope gene; X: X gene). The vertical bars in the P gene specify the boundaries between the 4 polymerase protein domains: terminal protein, spacer, reverse transcriptase and RNaseH. The vertical bars in the E gene separate the pre-S1, pre-S2 and S domains of the envelope proteins. **B** The mutants of the N series carry 20–120 codons of WHV pre-S1 (black boxes) fused to different positions of the HBV pre-S1 gene (open boxes). Numbers on the right indicate the C-terminal WHV and the N-terminal HBV codon fused in each construct. WT = Wild type. **C** For the mutants of the C series, the different 5′ recombination sites are indicated by the numbers on the right (C-terminal pre-S1 codon of HBV and N-terminal pre-S1 codon of WHV fused). The 3′ recombination site indicated by an asterisk fuses WHV pre-S1 codon 120 to HBV pre-S1 codon 93. The drawings are not to scale.

One determinant of the restriction of hepadnaviruses to single host species probably acts during virus attachment or uptake into the hepatocyte and may be based on a selective interaction between a species-specific virus receptor and a viral surface protein domain. While a virus receptor has been found only for duck HBV to date [4], the potential viral receptor ligand has been mapped for hepadnaviruses to reside in the N-terminal domain of the large envelope protein (L protein) [5]. This domain is called pre-S1 for mammalian and pre-S for avian hepadnaviruses. Exchange of this protein domain between two avian hepadnaviruses [6] or between HBV and woolly monkey HBV [7] by phenotypic mixing of envelope proteins caused the corresponding conversion of the species specificity.

Changing the host range by phenotypic mixing of envelope proteins has the disadvantage that the hepadnaviruses are limited to one round of infections. This limitation can be overcome by recombining foreign pre-S1 sequences into an acceptor HBV genome. However, one potential serious problem with this approach is that alterations in the viral envelope gene also modify the overlapping viral P gene which encodes a multifunctional protein with DNA polymerase activity (fig. 1A). However, the part of the P gene which overlaps with pre-S1 encodes a spacer region separating the terminal protein domain from the DNA polymerase domain and possessing no enzymatic function [8] (fig. 1A).

In this paper, we describe our approach to generating a human HBV carrying portions of the WHV pre-S1 domain by recombination of the viral genomes. We show that the DNA polymerases of the recombinant viruses were active, and demonstrate that recombinant virus was secreted from transiently transfected hepatoma cell lines. The goal is to create a quasi-human virus which is able to grow in woodchucks.

We cloned a 1.2-mer of the HBV genome genotype A [9] into the bacterial plasmid pSV65 3′ of an SV40 early promoter [10], resulting in plasmid pSVHBV1.5 (fig. 1A). The HBV genome copy starts with nucleotide 1816 (numbering of the 3,221-base pair long HBV genome starts with the deoxycytosine residue of the single *Eco*RI site) and is inserted behind the promoter in such a way that the 5′ ends of the majority of the SV40 promoter-driven transcripts correspond to the authentic 5′ end of the HBV RNA pregenome (data not shown). The inserted HBV DNA ends 600 base pairs downstream of the viral polyA site. By PCR, 10 recombinant genomes were constructed in the background of plasmid pSVHBV1.5. In a first series comprising 6 variants (N1–N6, fig. 1B), 20, 40, 60, 80, 100 or 120 codons, respectively, of the WHV pre-S1 sequence (total length: 149 codons [11]) were introduced into the HBV genome. The 5′ recombination sites between the HBV pre-S1 (total length: 119 codons) and the WHV sequence are the start codons of the pre-S1 regions. The 3′ recombination sites were chosen after aligning the pre-S1 amino acid sequences of both viruses (fig. 2). The HBV pre-S1 sequence downstream of amino acid 93 was not exchanged in any construct because this domain was shown to be important for nucleocapsid envelopment [12]. In a second series comprising 4 constructs (C1–C4, fig. 1C, 2), the 3′ recombination site between the WHV and HBV pre-S1 sequences corresponds to the site in N6,

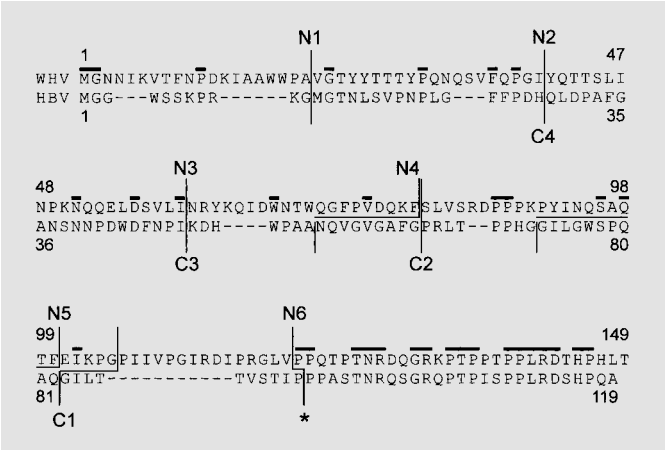

Fig. 2. Alignment of WHV (upper line) and HBV (lower line) pre-S1 amino acid sequences and depiction of the recombination sites. The amino acid sequences were aligned using the computer program blastp. Identical residues are distinguished by bars above the sequence. Dashes indicate gaps. Recombination sites for all 10 mutants are marked by vertical bars. The asterisk indicates the 3′ recombination site in all mutants of the C series. The C-terminal homologous region is important for virion morphogenesis [12] and was not exchanged.

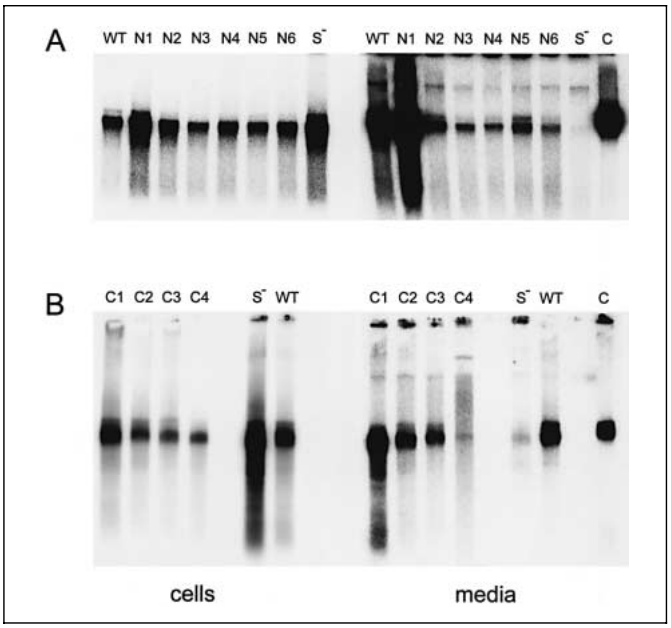

Fig. 3. Detection of DNA polymerase activity and virion formation. Huh7 cells were transiently transfected with the parental HBV construct (wild type; WT) and the indicated recombinants. As a negative control for nucleocapsid envelopment/secretion, a wild-type genome carrying a missense mutation of the start codon for the small envelope protein (S^-) was used. Nucleocapsids from cell lysates (left panels) and virions from culture media (right panels) were immunoprecipitated with anti-HBc and anti-HBs, respectively, and detected by radioactive labeling of the viral genome using the endogenous virus polymerase. The isolated genomes were separated on an agarose gel and depicted by phosphoimaging. **A** N series of mutants. **B** C series of mutants. In lanes labeled 'C', 2 µl of a highly viremic human serum containing approximately 4×10^6 genomes were directly used for the labeling reaction.

but the 5′ recombination sites are varied. The portions generated by PCR were sequenced in all plasmids to exclude unintentional mutations.

These constructs, together with the parental wild-type plasmid pSVHBV1.5, were used for transient transfections of the human hepatoma cell line Huh7 by the calcium phosphate precipitation technique in 10-cm dishes [13]. As a negative control, a point mutant of pSVHBV1.5 (pSVHBV1.5-S^-) carrying an ATG to ACG missense mutation of the start codon for the small surface protein S was used. This mutation is silent in the overlapping P gene but blocks synthesis of the S protein which is essential for envelopment of the nucleocapsid and therefore impedes virion secretion [14]. Five days after transfection, the cleared culture medium and a cleared Nonidet P-40 lysate of the cells were harvested. Nucleocapsids from the cells and virions from the media were immunoprecipitated with rabbit anti-HBc and goat anti-HBs, respectively, bound to protein A sepharose beads and washed with phosphate-buffered saline [13]. The concentrated viral particles were then incubated with 50 µl of a solution containing 50 m*M* Tris-Cl, pH 7.5, 75 m*M* NH_4Cl, 1 m*M* EDTA, 20 m*M* $MgCl_2$, 0.1% (v/v) beta-mercaptoethanol, 0.5% (v/v) Nonidet P-40, 0.4 m*M* deoxyguanosine triphosphate, 0.4 m*M* deoxyadenosine triphosphate, 0.4 m*M* thymidine triphosphate and 10 µCi of alpha-[^{32}P]-deoxycytosine triphosphate (3,000 Ci/mmol) at 37° overnight. During this incubation, the viral DNA polymerase in nucleocapsids and virions elongated the incomplete plus strand of the viral DNA genome. The radioactively labeled genomes were then isolated by the addition of 50 µl of a solution containing 1% (w/v) SDS, 10 m*M* Tris-Cl, pH 7.5, 10 m*M* EDTA, 0.1 mg of tRNA/ml and 0.4 mg of proteinase K/ml, incubation for 30 min at 37°, phenol-chloroform extraction and two ethanol-ammonium acetate precipitations [13]. The viral DNA was separated on a 1% agarose-tris-acetate-EDTA gel. The gel was dried and documented using a phosphoimager.

The N series of mutants displayed viral DNA polymerase activities similar to that of wild type (fig. 3A, left panel), demonstrating that the exchange of up to 92 amino acids in the spacer region of the HBV polymerase, starting with amino acid position 183, with up to 120 ami-

no acids of the WHV polymerase had no or little influence on the enzyme activity. The C series of mutants showed an increasingly negative effect of the increasing WHV proportion on P protein functions (fig. 3B, left panel). Whereas the DNA polymerase of mutant C1 had wild-type activity, the activity dropped slightly from mutant C2 to mutant C4. The reason for the different behavior of the N and C series mutants is not clear.

With the exception of mutant C4, all of the other nine mutants allowed detection of virions in the culture media (fig. 3A, B, right panels). Mutant N1 was comparable to wild type. Mutants N2–N6 showed between 5 and 50% of the virion production of the wild type. For mutants C1–C3, the ratio of intracellular nucleocapsids to secreted virions was near to wild type. Only mutant C4, showing the lowest DNA polymerase activity, failed to produce detectable virions. The S-negative HBV mutants, as expected, generated no virions. The weak band for this mutant (fig. 3B, right panel) is most probably due to low amounts of contaminating naked nucleocapsids present in the culture medium.

This study demonstrated that it was possible to generate recombinant hepatitis B virions carrying up to 120 N-terminal amino acids of the WHV pre-S1 sequence. By analogy to former experiments [6, 7], it seems reasonable to expect that variant N6, for example, might be infectious in woodchucks and could represent a quasi-human virus able to grow in a rodent. Whether the clearly reduced virion yield of this mutant relative to wild type (compare, for example, lane WT with lane N6 in fig. 3, right panel) still allows spread of the infection in the animal has to be elucidated.

During the design of this project, we expected that the most critical point could be a negative influence of the recombinations on polymerase functions. Surprisingly, this effect was rather mild, especially for the N series of mutants. On the other hand, we expected no profound effect of the recombinations on nucleocapsid envelopment and virion release because it has been shown that the HBV pre-S1 sequence 2–92 could be deleted without changing virion yields by transcomplementation [15] and that the WHV pre-S1 sequence could functionally substitute the homologous HBV sequence in phenotypic mixing experiments [16]. Surprisingly, we found that nucleocapsid envelopment and release was affected. This effect was not due to impaired folding, oligomerization or retention of the recombinant L proteins, because the secretion of subviral HBs antigen particles was not correspondingly altered (data not shown). We speculate that the foreign N-terminal WHV sequences in the HBV L proteins of mutants N2–N6 might influence the folding or accessibility of more C-terminal domains of the L protein important for morphogenesis.

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Immunogenic Effects of Woodchuck Hepatitis Virus Surface Antigen Vaccine in Combination with Antiviral Therapy: Breaking of Humoral and Cellular Immune Tolerance in Chronic Woodchuck Hepatitis Virus Infection

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Key Words

Therapeutic vaccination · Drug/antiviral therapy · Lymphocyte proliferation · Immunologic tolerance · In vivo animal models · Woodchuck · Woodchuck hepatitis virus · Hepatitis B

Abstract

Objective: A rational treatment strategy for chronic hepatitis B virus (HBV) infection might involve the modulation of immunity after the reduction of viremia and antigenemia. This strategy was tested in woodchucks chronically infected with the woodchuck hepatitis virus (WHV) by combining antiviral treatment with 1-(2-fluoro-5-methyl-β-*L*-arabinofuranosyl)-uracil (L-FMAU) and therapeutic vaccination with WHV surface antigen (WHsAg). ***Methods:*** Chronic WHV carriers were treated with L-FMAU or placebo for 32 weeks. Half the woodchucks in each group then received four injections of a conventional WHsAg vaccine during the next 16 weeks. ***Results:*** Vaccination alone elicited low-level antibody to WHsAg (anti-WHs) in most carriers but did not affect serum WHV DNA, WHsAg or liver enzyme responses. Carriers treated first with L-FMAU to reduce WHV DNA and WHsAg and then vaccinated developed similar low-level anti-WHs and normalized liver enzymes. Following vaccinations, WHsAg-specific cell-mediated immunity (CMI) was demonstrated in both groups, but was significantly enhanced in carriers treated with L-FMAU, and was broadened to include WHV core antigen (WHcAg) and selected peptide epitopes of WHcAg and WHsAg. Anti-WHs and associated CMI to WHcAg and WHsAg were observed after drug discontinuation in half of the carriers that received L-FMAU alone. ***Conclusions:*** Vaccination with WHsAg following treatment with L-FMAU disrupted virus-specific humoral and cell-mediated immune tolerance in chronic WHV infection and enhanced the immune response profiles beyond those seen with monotherapies alone. The combination therapy resulted in immune response profiles that resembled those observed during resolution of WHV infection. The results in woodchucks demonstrate the feasibility of using such a combination therapy for the control of chronic HBV infection in humans.

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Introduction

Hepatitis B virus (HBV) infection is a major public health problem and is responsible for about 1.2 million deaths per year worldwide. More than 350 million people throughout the world are chronically infected with HBV [1] and are at high risk of developing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Such individuals could benefit immensely from timely and effective antiviral and/or immunotherapeutic treatment. Chronic HBV infection is associated with defects in immunity [reviewed in ref. 2, 3]. The HBV carrier appears immunologically tolerant with a lack of detectable antibodies (anti-HBs) to the viral surface antigen (HBsAg) and hyporesponsive T helper cells to viral antigens [4–6]. In contrast, self-limited HBV infection is characterized by the development of anti-HBs antibodies and appropriate T helper cell responses to HBV [4–6]. The humoral and cellular immune responses that are sometimes detected in the HBV carrier are generally dysfunctional [4–9], in that they contribute more to disease progression than to viral clearance [reviewed in ref. 2] [4, 10, 11].

Current treatment strategies for chronic HBV infection are suboptimal when compared to the curative process observed in self-limited HBV infection. Antiviral approaches to reduce viral load include lamivudine (3TC), interferon-alpha (IFN-α) and their combination [10, 12–16]. Even with such combination therapy, seroconversion to anti-HBs antibody and improvements in endogenous T cell responses to HBV are usually not induced. Cessation of 3TC treatment is frequently associated with viral recrudescence [13, 16]. Prolonged treatment with 3TC leads to the emergence of drug-resistant mutants and an associated increase in viral load [15]. About 70% of HBV patients treated with IFN-α are nonresponsive or only partially responsive [17–19]; the other 30% of patients can clear the virus, but major side effects are frequently observed.

Imunotherapeutic approaches to reducing viral load in chronic HBV carriers include licensed prophylactics, such as HBsAg vaccines and hepatitis B immunoglobulin preparations. About 90% of HBV-naïve vaccine recipients develop strong anti-HBs antibody and T cell responses to HBsAg [20–24]. Vaccination of chronic HBV carriers with HBsAg vaccines, however, elicits only weak and transient anti-HBs antibodies and HBsAg-specific T cell responses in a low proportion of such patients [21, 25, 26]. Sustained reductions in viremia in chronic HBV carriers receiving HBsAg vaccine have been observed in some cases as well [26, 27].

Approaches combining antivirals with immunotherapeutic vaccination included the treatment of HBV carriers with IFN-α before and following vaccination with HBsAg [17–19, 26]. Such combination treatments elicit transient anti-HBs responses in a few patients. In addition, although viremia was reduced in about half of the patients, and HBsAg-specific T cell responses became detectable in about 25%, none of the patients cleared HBsAg from serum.

The above examples clearly indicate that a more potent therapy for chronic HBV infection is needed, i.e. one that involves more pronounced reductions of HBV viremia and HBs antigenemia and sustained seroconversion to anti-HBs antibody and associated T cell responses to HBV antigens, as observed in patients undergoing self-limited HBV infection. One rational strategy to terminate chronic HBV infection would be to first reduce the viral and antigen load by a potent antiviral drug and then to modulate the antiviral immune responses by therapeutic vaccination with HBsAg. The feasibility of such a strategy was tested in the present report using woodchucks as an animal model of human HBV infection.

The Eastern woodchuck *(Marmota monax)* infected with the woodchuck hepatitis virus (WHV) has been used to investigate the basic pathogenesis of acute and chronic HBV infection [reviewed in ref. 3, 28–30]. Chronic WHV carrier woodchucks have been used in the preclinical development of drugs for HBV therapy [31, 32] and for the testing of prophylactic and immunotherapeutic approaches [33–39]. This animal model mimics many of the virologic and immune response features observed in human HBV infection [40–49], and predicts human responses to antiviral agents as well [31].

The present report describes a promising approach to the therapeutic eradication of chronic WHV infection using a combination of an antiviral drug and WHV surface antigen (WHsAg) therapeutic vaccine. Antiviral studies in chronic WHV carrier woodchucks using the new and potent antiviral drug 1-(2-fluoro-5-methyl-β-*L*-arabinofuranosyl)-uracil (L-FMAU) demonstrated that L-FMAU significantly reduces the serum concentration of both WHV DNA and WHsAg and also reduces the levels of covalently closed circular viral DNA in the liver [32]. Conventional alum-adsorbed WHsAg vaccine induces anti-WHs antibody responses and protects woodchucks from WHV challenge [33, 34]. A double-placebo-controlled study has now been performed in chronic WHV carrier woodchucks involving treatment with L-FMAU followed by therapeutic vaccination with a conventional WHsAg vaccine. The results demonstrate that the combi-

nation therapy breaks immunologic tolerance in chronic WHV carriers by inducing virus-specific humoral and cellular immune responses. The immune response profile elicited in carriers during the combination therapy appeared to result from an additive effect of monotherapies and resembled that observed in self-limited WHV infection.

Materials and Methods

Woodchucks

Use of woodchucks born in our laboratory colony (College of Veterinary Medicine, Cornell University, Ithaca, N.Y., USA) was approved by the Cornell University Institutional Animal Care and Use Committee. Ten adult WHV-susceptible woodchucks, approximately 1 year of age, were used to study vaccine safety (n = 3) and immunogenicity (n = 7). Thirty-two chronic WHV carrier woodchucks, 1–2 years of age, were stratified by age and sex into four experimental groups in order to study the influence of antiviral drug treatment on the immune response to the therapeutic vaccination. The WHV carriers had been infected experimentally at 3 days of age with WHV7P1 as described previously [41], and were verified as chronic carriers by detection of persistent WHV viremia and WHs antigenemia from 2–3 months of age.

Drug and Vaccine

The antiviral drug L-FMAU (Clevudine®) was provided by Triangle Pharmaceuticals Inc. (Research Triangle Park, N.C., USA). The subunit vaccine (22-nm-diameter WHsAg particles) was purified by zonal ultracentrifugation from serum of WHV7P1-infected chronic carriers [50], and was therefore homologous to the WHsAg in the WHV inoculum used to infect the present chronic carriers. The particles were formalin inactivated and adsorbed to alum and were not pretreated with enzymes that remove pre-S sequences. Prior to alum adsorption, the purified, formalin-inactivated WHsAg was safety tested in 3 WHV-susceptible woodchucks (50 μg of WHsAg, i.v.) and demonstrated to be free of residual infectious WHV (data not shown). The WHsAg-alum-adsorbed vaccine was tested in 7 WHV-susceptible woodchucks (50 μg, i.m., at 0, 4, 8 and 16 weeks) and confirmed to be immunogenic for the induction of antibodies against WHsAg (anti-WHs) and WHsAg-specific cell-mediated immunity (CMI).

Study Design

Sixteen chronic WHV carrier woodchucks received L-FMAU (L+ carriers; 10 mg/kg/day, one dose orally for 32 weeks) and 16 received saline placebo (L– carriers). Two woodchucks (one in each group) died before the start of the vaccine phase of the study. Eight of the L+ carriers received 50 μg of the WHsAg vaccine (L+V+ carriers; referred to as combination therapy) beginning at week 32 and then again at weeks 36, 40 and 48, while 7 L+ carriers received a saline placebo injection control at these times (L+V– carriers). Eight of the L– carriers received the same course of vaccine (L–V+ carriers), while 7 L– carriers received a saline placebo injection control (L–V– carriers; referred to as double-placebo carrier controls). The immune responses in each experimental group were compared during the placebo-controlled drug treatment phase (weeks 0–32) and during the placebo-controlled vaccine phase (weeks 32–60). Serum samples were obtained throughout the study for measurement of viral markers (WHV DNA and WHsAg) and anti-WHs antibody. CMI was monitored by in vitro proliferation assay using peripheral blood mononuclear cells (PBMC) isolated from whole blood. Most of the woodchucks in each group (63–86%) were monitored at 2- to 4-week intervals. The remainder were tested at intervals of 8–12 weeks.

Assays of Viral Markers, Antibodies and Liver Enzymes

Serum WHV DNA was measured by dot blot hybridization [31, 32, 41] either directly or following polymerase chain reaction amplification (limit of sensitivity: 30 virus genomic equivalents/ml). Serum WHsAg was measured by enzyme-linked immunosorbent assay (ELISA) [51] using a 1/10 or greater dilution of serum (lower sensitivity: circa 30 ng/ml of serum). Anti-WHs antibody was quantified by ELISA [51] in reciprocal standard dilution units (U/ml). Serum activities of the liver enzymes alanine aminotransferase, aspartate aminotransferase and sorbitol dehydrogenase were determined by the Institute of Clinical Pathology (Cornell University) for the biochemical assessment of hepatic injury as described previously [52].

Polyclonal Activators, Viral Antigens and Synthetic Peptides

In vitro stimulators were used at concentrations optimal for woodchuck PBMC cultures as described previously [40, 44–47]. Concanavalin A (8 μg/ml) and lipopolysaccharide (0.5 μg/ml) were purchased from Sigma (St. Louis, Mo., USA). Human recombinant interleukin-2 (100 IU/ml) was obtained from Cetus (Emeryville, Calif., USA). Viral antigen stimulators consisted of native 22-nm WHsAg (0.5 and 2 μg/ml; the same purified antigen used to prepare the vaccine and for the assay of anti-WHs) and recombinant WHV core antigen (rWHcAg; 0.5 and 1 μg/ml). Two synthetic peptides (20 amino acids in length; 10 μg/ml) were purchased from Genosys-Sigma (The Woodlands, Tex., USA), corresponding to amino acid residues 100–119 of WHcAg (C100–119) [44, 46, 47] and amino acid residues 226–245 of WHsAg (S226–245, starting at the N-terminus of the large surface protein and also corresponding to residues 77–96 and 17–36, respectively, of the middle and small surface proteins) [45].

In vitro PBMC Proliferation Assay

The in vitro proliferation assays using woodchuck PBMC were comparable to those performed in human studies [4–6, 10], except that dividing cells were labeled with [2-^{3}H]adenine [46, 47] (37 kBq/well, specific activity 703 GBq/m*M*; Amersham Pharmacia Biotech Inc., Arlington Heights, Ill., USA). Woodchuck PBMC were isolated from whole blood and stimulated in vitro as described elsewhere [44–47]. Counts per minute of PBMC triplicate cultures were averaged and expressed as a stimulation index by dividing the average sample counts per minute in the presence of stimulator by that in the absence of stimulator (7 replicates).

Parameters of Humoral and Cellular Immune Responses

The frequency of responding woodchucks was defined as the percentage of woodchucks in each group that developed positive responses at one or more time points. This was expressed as a cumulative frequency for a given study interval (e.g. drug treatment or vaccine phases of the study). The frequency of positive samples was defined as the percentage of samples testing positive above the assay cutoff during a given study interval.

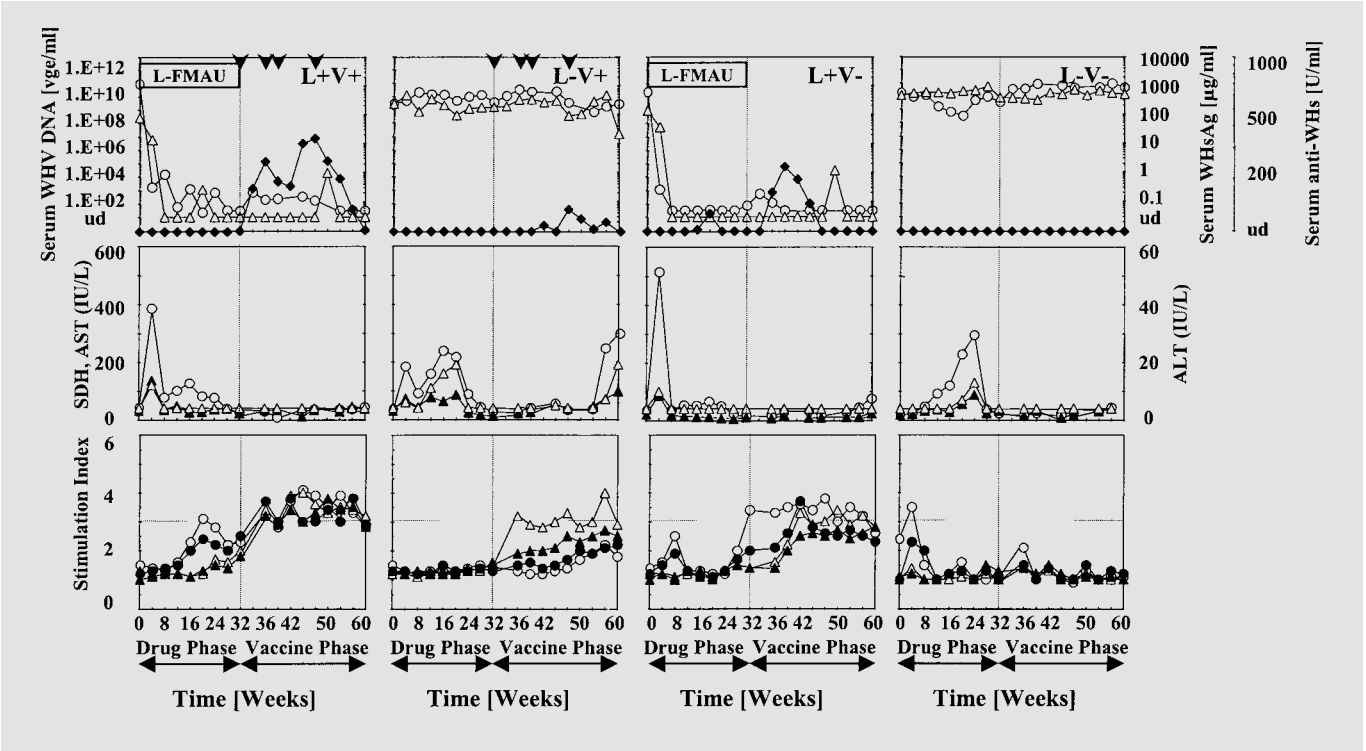


Fig. 1. Kinetics of virological, serum biochemical and immunological responses for representative chronic WHV carrier woodchucks from the four experimental groups in the L-FMAU and WHsAg vaccine study. Experimental groups: L+V+ = L-FMAU/vaccine, woodchuck No. 5437; L–V+ = placebo/vaccine, woodchuck No. 5444; L+V– = L-FMAU/placebo, woodchuck No. 5476; L–V– = double-placebo control, woodchuck No. 5481. Top panels: ○ = serum WHV DNA [virus genomic equivalents (vge)/ml]; △ = serum WHsAg (µg/ml); ◆ = serum anti-WHs antibody response (U/ml). Middle panels: serum biochemical activities of liver enzymes (IU/ml): ○ = sorbitol dehydrogenase (SDH); ▲ = aspartate aminotransferase (AST); △ = alanine aminotransferase (ALT). Bottom panels: vCMI to WHV antigens and selected peptides (stimulation index): ○ = rWHcAg; △ = WHsAg; ● = WHc peptide C100–119; ▲ = WHs peptide S226–245. The positive cutoff for specific vCMI at a stimulation index of 3.1 is marked by a partial, horizontal dotted line. The drug treatment phase (0–32 weeks) and the vaccine and immediate follow-up phases (32–60 weeks) are separated by a vertical dotted line. L-FMAU treatment for 32 weeks is indicated by a horizontal bar. Vertical arrowheads represent the 4 immunizations with 50-µg doses of an alum-adsorbed WHsAg vaccine at weeks 32, 36, 40 and 48.

Statistical Analysis

Mean serum concentrations of WHV DNA and WHsAg were compared by Student's t test in accordance with an F test for equal versus unequal variances. The frequency of responding woodchucks and the frequency of positive samples from the experimental groups were compared by Fisher's test for proportions (one-tailed). p values <0.05 were considered statistically significant.

Results

Overview of Responses to L-FMAU Treatment and Therapeutic Vaccination with WHsAg-Alum Vaccine

Figure 1 shows representative virological, serum biochemical and immunological responses through to week 60 for one chronic WHV carrier woodchuck from each of the four experimental groups in the drug treatment/therapeutic vaccination study. Treatment of WHV carriers with L-FMAU for 32 weeks resulted in prompt and marked reductions in both serum WHV DNA and WHsAg compared with placebo controls. By the end of drug treatment (week 32), significant reductions in serum WHV DNA (8 logs) and WHsAg (2 logs) were evident in most of the L+V+ and L+V– carriers (table 1). Serum WHV DNA and WHsAg remained essentially unchanged in the carriers that did not receive drug (L–V+, L–V–). Serum enzyme responses were variable during the drug treatment phase in the two control carrier groups (L–V+, L–V–), but appeared to undergo some degree of normalization associated with drug treatment (L+V+, L+V–; fig. 1).

After cessation of drug treatment at week 32, the low levels of serum WHV DNA and WHsAg in the drug-treated carriers (L+V–, L+V+) were sustained for at least 2 months in most cases, or increased only slightly above the lowest level (circa 10-fold; fig. 1). In the drug-treated carriers that received vaccine (L+V+), the 4 doses of WHsAg vaccine (weeks 32, 36, 40 and 48) induced frequent anti-WHs antibody responses that were also associated with virus-specific CMI (vCMI) to WHsAg and WHs peptides (fig. 1–3). In carriers that did not receive drug, vaccination also induced anti-WHs antibodies and vCMI to WHsAg, but did not significantly affect serum viral and antigen load compared to double-placebo controls (fig. 1, L–V+ versus L–V–). Serum enzymes were normalized in most of the carriers by week 32 of the study. There were no significant elevations in serum enzymes associated with L-FMAU treatment, withdrawal of L-FMAU or WHsAg vaccination (fig. 1). Thus, the anti-WHs and vCMI responses observed in response to therapeutic vaccination (and to drug withdrawal in some cases) did not lead to hepatic flare reactions involving significant liver injury. Later serum enzyme elevations near or after week 60 of the study (e.g. fig. 1, L–V+, woodchuck No. 5444) were associated with the progression of chronic hepatitis and development of hepatocellular carcinoma (data not shown). We conclude that there were no adverse effects associated with therapeutic vaccination in either the high (L–V+) or low (L+V+) viral and antigen load settings.

Table 1. Mean serum WHV DNA and WHsAg concentrations before and after treatment with L-FMAU for 32 weeks

	Treatment with		p value[a]
	L-FMAU (L+) (n = 16)	placebo (L–) (n = 16)	
Serum WHV DNA, vge/ml			
Week 0	4.95×10^{10}	4.29×10^{10}	NS
Week 32	2.78×10^{2}	1.45×10^{10}	<0.006
p value[b]	<0.001	<0.005	
Serum WHsAg, µg/ml			
Week 0	262.6	258.9	NS
Week 32	3.5	176.7	<0.001
p value[b]	<0.001	NS	

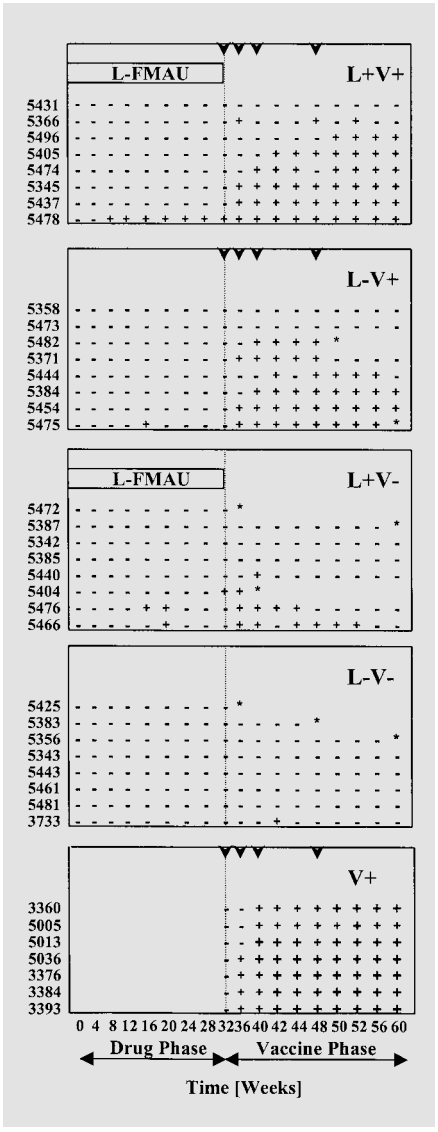
Data are from before the start of drug treatment (week 0) and at the end of drug treatment (week 32). For further reference, the standard deviations of the arithmetic means shown in the table were: serum WHV DNA: week 0: L+, $\pm 2.76 \times 10^{10}$, L–, $\pm 3.09 \times 10^{10}$; week 32: L+, $\pm 4.85 \times 10^{2}$, L–, $\pm 1.81 \times 10^{10}$; serum WHsAg: week 0: L+, ±229.5, L–, ±116.3; week 32: L+, ±5.4, L–, ±117.7. [a]L+ versus L–; [b]week 0 versus week 32. vge = Virus genomic equivalents; NS = not significant.

Anti-WHs Antibody Responses following Therapeutic Vaccinations

The WHsAg vaccine was shown initially to be safe and immunogenic in WHV-susceptible woodchucks. All of the 7 WHV-susceptible woodchucks developed strong anti-WHs antibody responses after their second dose of vaccine, with titers of 2,000 U/ml of serum or higher and an overall frequency of anti-WHs-positive serum samples of 95% (fig. 2, bottom panel). The WHsAg vaccine elicited vCMI to WHsAg and WHs peptides, but not to other viral antigens (data not shown). Thus, the responses observed in vaccinated carriers were specific to the immunization with WHsAg.

The anti-WHs antibody titers induced by therapeutic vaccination of L+V+ and L–V+ carriers were about 10 times lower on average than those observed in the WHV-susceptible woodchucks, and the overall frequencies of anti-WHs-positive samples in the carriers were not as high (fig. 2; $p < 0.01$). Among the double-placebo carrier controls, there were no anti-WHs responses during either phases of the study, except for one sporadic positive sample in woodchuck No. 3733 (fig. 2, L–V–, week 42). L+V+ and L–V+ carriers developed anti-WHs responses most frequently during the vaccine phase. Anti-WHs responses were even observed occasionally without vaccination in carriers that received L-FMAU alone, but mainly after the drug was discontinued (fig. 2, L+V–).

The percentages of vaccinated carriers (L+V+, L–V+) developing anti-WHs antibodies and the frequencies of anti-WHs-positive samples in these groups were significantly higher than in the L–V– control carriers (fig. 2; $p < 0.05$ for both parameters). The L+V+ and L–V+ groups did not differ significantly based on these anti-WHs antibody parameters. The anti-WHs responses induced by therapeutic WHsAg vaccination were similar and sustained through to week 60 in both the high (L–V+) and low (L+V+) viral and antigen load settings. About half of the L+V– carriers developed transient anti-WHs responses, mainly after drug withdrawal at week 32 (fig. 2, L+V–). While the percentage of L+V– carriers with anti-WHs responses did not differ significantly from the other carrier groups, the frequency of anti-WHs-positive samples among the L+V– carriers was intermediate between double-placebo carrier controls (fig. 2, L–V–; $p < 0.01$) and the L+V+ and L–V+ carriers ($p < 0.01$).



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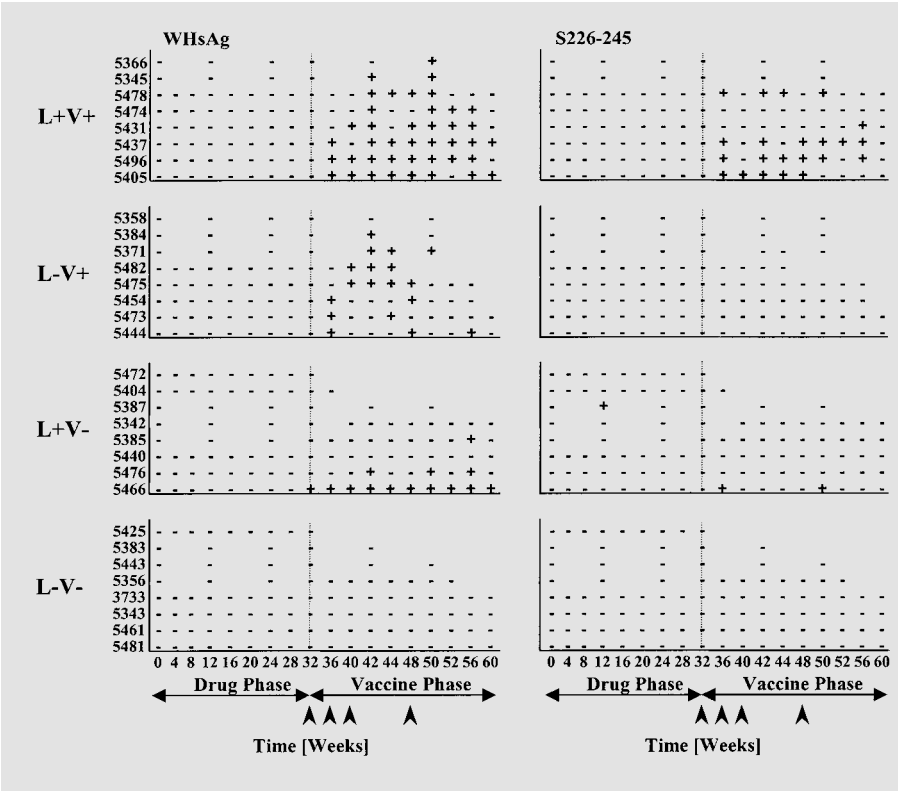

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Fig. 2. Serum anti-WHs antibody responses of chronic WHV carrier woodchucks from the four experimental groups in the L-FMAU and WHsAg vaccine study and of WHV-susceptible woodchucks in the WHsAg vaccine immunogenicity study. L+V+ = L-FMAU/vaccine; L–V+ = placebo/vaccine; L+V– = L-FMAU/placebo; L–V– = double-placebo control; V+ = WHV-susceptible vaccinated woodchucks. – and + represent undetectable (titer values of ≤100 U/ml) or detectable (titer values of >100 U/ml) anti-WHs, respectively. Bold + represents detectable anti-WHs with titer values of ≥2,000 U/ml. An asterisk indicates that the woodchuck died. The respective frequencies of woodchucks developing anti-WHs antibodies and cumulative frequencies of serum samples positive for anti-WHs antibody were: drug phase (weeks 4–32): L+V+, 13 and 11%; L–V+, 13 and 2%; L+V–, 38 and 6%; L–V–, 0%; vaccine phase (weeks 36–60): L+V+, 88 and 67%; L–V+, 75 and 58%; L+V–, 57 and 22%; L–V–, 14 and 2%; V+, 100 and 95%. L-FMAU treatment for 32 weeks is indicated by a horizontal bar. Vertical arrowheads represent the 4 immunizations with 50-microgram doses of an alum-adsorbed WHsAg vaccine at weeks 32, 36, 40 and 48.

Fig. 3. vCMI to WHsAg (left panels) and WHs peptide S226–245 (right panels) of chronic WHV carriers from the four experimental groups in the L-FMAU and WHsAg vaccine study. L+V+ = L-FMAU/vaccine; L–V+ = placebo/vaccine; L+V– = L-FMAU/placebo; L–V– = double-placebo control. – and + represent undetectable (stimulation index values of <3.1) or detectable (stimulation index values of ≥3.1) vCMI to WHsAg and WHs peptide S226–245, respectively. The respective frequencies of woodchucks responding with positive vCMI and cumulative frequencies of PBMC samples positive for vCMI to WHsAg were: drug phase (weeks 4–32): 0% for all groups; vaccine phase (weeks 36–60): L+V+, 100 and 71%; L–V+, 88 and 40%; L+V–, 43 and 28%; L–V–, 0%. Regarding the positive vCMI to WHs peptide S226–245, these frequencies were: drug phase (weeks 4–32): L+V+, L–V+ and L–V–, 0%; L+V–, 13 and 2%; vaccine phase (weeks 36–60): L+V+, 63 and 38%; L–V+, 0%; L+V–, 14 and 4%; L–V–, 0%.

Table 2. Cumulative frequencies of responding woodchucks and of PBMC samples positive to polyclonal activators in experimental groups during the drug treatment and vaccine phases of the study

Study phase	Cumulative frequency of responding woodchucks, %				Cumulative frequency of positive PBMC samples, %			
	L+V+	L–V+	L+V–	L–V–	L+V+	L–V+	L+V–	L–V–
Drug treatment phase	(n = 8)	(n = 8)	(n = 8)	(n = 8)	(n = 54)	(n = 49)	(n = 54)	(n = 54)
ConA	100	100	100	100	100	100	100	100
rIL-2	100	100	100	100	100	100	100	100
LPS	88	100	100	100	69	70	68	63
Vaccine phase	(n = 8)	(n = 8)	(n = 7)	(n = 7)	(n = 58)	(n = 45)	(n = 47)	(n = 46)
ConA	100	100	100	100	100	100	100	98
rIL-2	100	100	100	100	100	100	100	96
LPS	100	100	86	86	72	71	77	65

The drug treatment phase (i.e. L-FMAU or placebo) was from week 0 to week 32; the vaccine phase and follow-up (i.e. vaccine or placebo) was from week 32 to week 60. Note that the cumulative frequencies of responding woodchucks and of positive PBMC samples were not significantly different between any of the experimental groups. L+V+ = L-FMAU/vaccine; L–V+ = placebo/vaccine; L+V– = L-FMAU/placebo; L–V– = double-placebo control; n = number of woodchucks or number of PBMC samples tested; ConA = concanavalin A; rIL-2 = human recombinant interleukin-2; LPS = lipopolysaccharide.

vCMI Responses to WHsAg and WHs Peptide S226–245 following Therapeutic Vaccinations

The anti-WHs responses of carriers correlated with vCMI to WHsAg (fig. 2, 3, left panels, L+V+, L–V+, L+V–). The vCMI to WHsAg was specific to the therapeutic vaccine in that the double-placebo carrier controls (L–V–) had no detectable vCMI to WHsAg during the entire 60-week observation period (fig. 3). Thus, L–V– control carriers remained tolerant to WHsAg at the level of cellular (and humoral) immune responses (fig. 2, 3). Carriers from the experimental groups had no significant vCMI to WHsAg during the first 32 weeks, including those carriers that had achieved marked reductions in viral and antigen load as a result of drug treatment (fig. 1, 3, L+V+ and L+V–). The absence of vCMI during any treatment phase of the study was not due to a general unresponsiveness of carrier PBMC or to drug-induced lymphotoxicity, since samples from all groups of carriers were highly responsive to the polyclonal activators concanavalin A, human recombinant interleukin-2 and lipopolysaccharide (table 2).

Essentially all carriers immunized with the therapeutic WHsAg vaccine (except woodchuck No. 5358, L–V+) had detectable vCMI to WHsAg (fig. 3, left panel). The frequency of PBMC samples positive for WHsAg-specific vCMI in the L–V+ carriers (fig. 3) was significantly higher than in the double-placebo carrier controls ($p < 0.01$), but was significantly less than in the L+V+ carriers ($p < 0.05$). This was related mainly to the reduced detection of vCMI to WHsAg in the L–V+ carriers after the fourth dose of WHsAg vaccine at week 48 (fig. 3). The vCMI to WHsAg in several L+V– carriers observed after discontinuation of drug treatment was significantly less than in the L+V+ group in terms of responder frequency and frequency of positive samples ($p < 0.05$; fig. 3). The frequency of L+V– PBMC samples positive for vCMI to WHsAg was clearly intermediate ($p < 0.01$) between L–V– and L+V+ carriers, but not significantly different from L–V+ carriers.

Interestingly, the frequencies of PBMC samples positive for WHsAg-specific vCMI in the L+V+ carriers (71%) and L–V+ carriers (40%) were significantly higher than those observed in the 7 vaccinated WHV-susceptible woodchucks used in the immunogenicity study (i.e. 23% of PBMC samples positive, $p < 0.01$ versus L+V+, $p < 0.05$ versus L–V+; see below in Discussion). In terms of the overall vCMI response to WHsAg, the trend for frequencies of PBMC samples positive for vCMI to WHsAg, i.e. L+V+ 71%, L–V+ 40%, L+V– 28% and L–V– 0%, suggested that the sustained vCMI to WHsAg in the combination therapy group (L+V+) resulted from a possible additive effect of the monotherapies (L–V+ and L+V–).

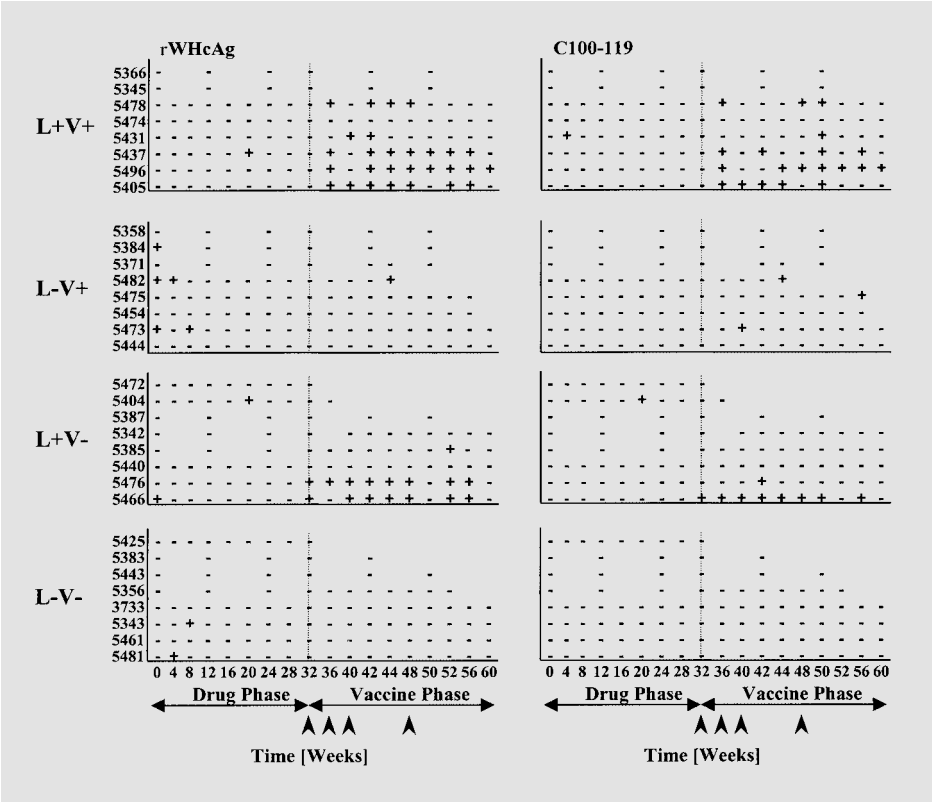

Fig. 4. vCMI to rWHcAg (left panels) and WHc peptide C100–119 (right panels) of chronic WHV carriers from the four experimental groups in the L-FMAU and WHsAg vaccine study. L+V+ = L-FMAU/vaccine; L–V+ = placebo/vaccine; L+V– = L-FMAU/placebo; L–V– = double-placebo control. – and + represent undetectable (stimulation index values of <3.1) or detectable (stimulation index values of ≥ 3.1) vCMI to rWHcAg and WHc peptide C100–119, respectively. The respective frequencies of woodchucks responding with positive vCMI and cumulative frequencies of PBMC samples positive for vCMI to rWHcAg were: drug phase (weeks 4–32): L+V+, 13 and 2%; L–V+, 25 and 4%; L+V–, 38 and 6%; L–V–, 25 and 4%; vaccine phase (weeks 36–60): L+V+, 63 and 48%; L–V+, 13 and 2%; L+V–, 43 and 30%; L–V–, 0%. Regarding the positive vCMI to WHc peptide C100–119, these frequencies were: drug phase (weeks 4–32): L+V+, 13 and 2%; L–V+, 0%; L+V–, 25 and 4%; L–V–, 0%; vaccine phase (weeks 36–60): L+V+, 63 and 35%; L–V+, 38 and 7%; L+V–, 29 and 17%; L–V–, 0%.

WHs peptide S226–245 is recognized by PBMC of woodchucks with acute self-limited WHV infection [45]. Most of the L+V+ carriers developed frequent vCMI to S226–245 following vaccination (fig. 3, right panel; $p < 0.01$ vs. L–V–). Although most of the carriers in the L–V+ group developed frequent vCMI responses to WHsAg (see above and fig. 3, left panel), none of these carriers had detectable vCMI to S226–245 (fig. 3, right panel), but they did have weak responses to other WHs peptides (data not shown). S226–245 was not recognized to a significant extent by L+V– or L–V– carriers. The results suggest that the vCMI response to S226–245 (an S-region epitope) requires vaccination in the presence of a low viral load.

Collateral vCMI Responses to WHcAg and WHc Peptide C100–119 following Therapeutic Vaccinations

Collateral vCMI responses were observed following L-FMAU treatment and therapeutic vaccination with WHsAg. vCMI to rWHcAg and WHc peptide C100–119 was most evident in L+V+ carriers (fig. 4). WHc peptide C100–119 represents an epitope of the WHcAg that is broadly recognized by the PBMC of woodchucks with acute self-limited WHV infection [44, 46, 47] and that was demonstrated to be an immunogen capable of protecting woodchucks against experimental challenge with WHV [44]. The effect of WHsAg vaccine on collateral vCMI appeared to depend at least in part on prior drug treatment and drug withdrawal. For example, discontin-

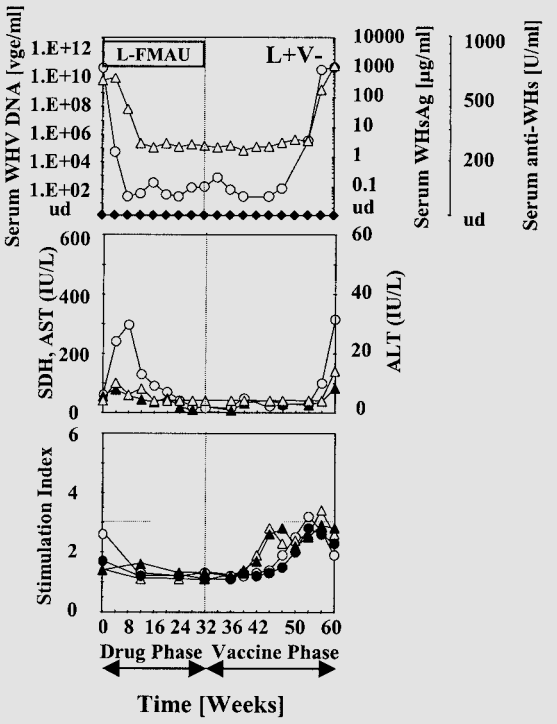

Fig. 5. Kinetics of virological, serum biochemical and immunological responses for one chronic WHV carrier woodchuck with viral recrudescence after discontinuation of L-FMAU treatment (L+V–, woodchuck No. 5385). Top panels: ○ = serum WHV DNA [virus genomic equivalents (vge)/ml]; △ = serum WHsAg (µg/ml); ◆ = serum anti-WHs antibody response (U/ml). Middle panels: serum biochemical activities of liver enzymes (IU/ml): ○ = sorbitol dehydrogenase (SDH); ▲ = aspartate aminotransferase (AST); △ = alanine aminotransferase (ALT). Bottom panels: vCMI to WHV antigens and selected peptides (stimulation index): ○ = rWHcAg; △ = WHsAg; ● = WHc peptide C100–119; ▲ = WHs peptide S226–245. The positive cutoff for specific vCMI at a stimulation index of 3.1 is marked by a partial, horizontal dotted line. The drug treatment phase (0–32 weeks) and the vaccine and immediate follow-up phases (32–60 weeks) are separated by a vertical dotted line. L-FMAU treatment for 32 weeks is indicated by a horizontal bar.

uation of drug treatment was associated coincidentally with sustained vCMI to rWHcAg in 2 of the 7 L+V– carriers and with at least one positive response in another carrier of this group (fig. 4, left panel). vCMI to WHc peptide C100–119 was sustained in one of the carriers and was detected once in another carrier (fig. 4, right panel). In contrast, L–V+ carriers responded to vaccine mainly with WHsAg-specific vCMI, with only a small number of PBMC samples showing sporadic vCMI to rWHcAg and WHc peptide C100–119 (fig. 4). The frequencies of positive PBMC samples for vCMI to rWHcAg and WHc peptide C100–119 were highest in the L+V+ carriers compared to all other carrier groups (fig. 4; $p < 0.05$ to $p < 0.01$). Those same frequencies in L+V– carriers were significantly higher than in the double-placebo carrier controls (fig. 4, L–V–; $p < 0.01$) and significantly less than in the L+V+ carriers ($p < 0.05$ to $p < 0.01$). They were significantly higher for rWHcAg ($p < 0.01$) but not significantly different for WHc peptide C100–119 compared to those in the L–V+ carriers.

Possible Role for Viral Recrudescence following L-FMAU Treatment

Low viral and antigen loads were sustained at or slightly above (circa 10-fold) the lowest levels achieved in most drug-treated (L+) carriers after the drug was discontinued and throughout the vaccine phase. This feature allowed a comparison of the effects of therapeutic vaccination with WHsAg on the responses of carriers with either low (L+V+) or high (L–V+) viral and antigen loads. Occasionally, however, a drug-treated carrier did have recrudescence of serum WHV DNA and WHsAg (fig. 5, L+V–, woodchuck No. 5385). WHV DNA and WHsAg concentrations in serum returned to pre-treatment levels after several months. This appeared to provide an endogenous stimulus for vCMI to WHsAg and rWHcAg, as shown in the case of woodchuck No. 5385. Late and complete viral recrudescence was also associated with elevated serum activities of liver enzymes in these carriers. The lower level of recrudescence (circa 10-fold) in several of the drug-treated carriers immediately after drug treatment was discontinued (fig. 1, L+V+ and L+V–) was not associated with elevated serum enzyme activities. This may have

provided an endogenous stimulusfor the anti-WHs antibody responses and vCMI to WHV antigens observed in some of the L+V– carriers, and for the enhanced immunologic effects observed in L+V+ carriers.

Discussion

High viral and antigen load may contribute to the observed immunologic tolerance in chronic HBV carriers. A rational treatment strategy for chronic HBV infection might therefore involve the modulation of immunity in chronic HBV carriers after the reduction of high viral and antigen load. In the present report, this strategy was tested in chronic WHV carrier woodchucks by combining therapeutic vaccination with WHsAg and antiviral treatment with L-FMAU.

Influence of L-FMAU Treatment on the Immune Responses of Chronic WHV Carriers

L-FMAU treatment of chronic WHV carriers produced marked and sustained reductions in serum WHV DNA and WHsAg (fig. 1, table 1). Reduction of serum viral load during the 32 weeks of drug treatment was insufficient in itself to induce significant seroconversion to anti-WHs antibodies or vCMI in carriers (fig. 1–4). The sporadic detection of anti-WHs antibody in a few L-FMAU-treated carriers during the drug treatment phase was not associated with vCMI to WHsAg. The lack of vCMI in double-placebo carrier controls (L–V–) during this time reflects the specificity of cellular immune tolerance to WHV during chronic WHV infection and during this phase of therapy. Furthermore, no significant changes in the vCMI of placebo-treated carriers were observed with the usual progression of WHV infection and chronic liver disease through to week 60 of the study. Although transient vCMI became detectable in some carriers at the time of increased serum liver enzymes (e.g. fig. 1, L+V+, week 20) or during complete viral recrudescence (fig. 5), serum liver enzymes overall appeared to normalize during and just after drug treatment and throughout most of the vaccine phase of the study (fig. 1). Therefore, the drug and vaccine treatments were not directly associated with the induction of hepatic flare reactions resulting in severe liver injury.

Inhibition of viral replication and WHsAg production by L-FMAU was probably abrogated, in part, when drug treatment was discontinued and this may have provided an appropriate endogenous stimulant for virus-specific humoral and cellular immune responses, also including vCMI to rWHcAg (as in some L+V– carriers). Although the timing of transient anti-WHs antibody responses and vCMI to WHV in L+V– carriers was consistent with an effect of drug withdrawal, with the present study design it is not possible to exclude the possibility that these effects might have occurred if drug treatment had been continued.

In addition to inhibition of WHsAg production, L-FMAU also reduces the expression of WHV core gene products in liver [i.e. WHcAg and WHV e antigen (WHeAg)] [32]. Although there are no established assays for WHeAg, it is reasonable to assume that WHeAg, along with WHcAg, was likely reduced in serum and liver, since L-FMAU produces such a profound inhibition of viral replication, covalently closed circular WHV DNA and mRNA transcription [32]. The WHcAg-specific vCMI elicited in L+V– carriers following drug withdrawal may represent a consequence of the prior reduction of core gene products, which may also be involved in the maintenance of immune tolerance [8, 9]. This could provide a rational explanation for the improved collateral effect of therapeutic vaccination with WHsAg on WHcAg-specific vCMI in carriers that received the combination therapy (L+V+; see below).

Influence of WHsAg Vaccine on the Immune Responses of Chronic WHV Carriers

The immune responses in WHV-susceptible woodchucks demonstrated that the anti-WHs antibodies and WHsAg-specific vCMI responses elicited in chronic carriers (L–V+) were specific to the WHsAg vaccine and not to possible contaminating viral antigens. Therapeutic vaccination of carriers with WHsAg had no effect on serum WHV DNA and WHsAg (fig. 1). Most L–V+ carriers developed anti-WHs antibodies that were associated with vCMI to WHsAg. The overall results for humoral responses of carriers from the four experimental groups indicated that seroconversion to anti-WHs antibody was driven more by WHsAg vaccination than by treatment with and discontinuation of L-FMAU. However, the average antibody titers following therapeutic vaccination were approximately 10–20 times lower than in the uninfected vaccine recipients. The class of antibodies (i.e. IgM or IgG) elicited and their fine epitope specificity and potential immunocomplex composition are unknown. The relatively low antibody levels in vaccinated carriers may be explained, in part, by the presence of excess WHsAg in serum. This is suggested by the observation that some circulating WHsAg was still detectable in most of the treated carriers by the end of drug treatment and

thereafter (table 1). Thus, immunocomplexes may explain the reduced levels of antibody responses.

Collateral vCMI to rWHcAg, including WHc peptide C100–119, was not very frequent in L–V+ carriers when compared to carriers that received the combination therapy (L+V+). This may be explained, in part, by the absence of an effect of WHsAg vaccine on viral and antigen load in these carriers (fig. 1, L–V+). Furthermore, as discussed above, L-FMAU treatment alone followed by withdrawal was associated with an interruption of immunologic tolerance, providing evidence for the presence of endogenous, virus-specific immune responses in some carriers (fig. 1, L+V–) and for the negative influence of high viral and antigen load on these responses in non-drug-treated carriers (L–V+). In fact, the L–V+ carriers vaccinated under conditions of high viral and antigen load may have become refractory to later immunizations, as observed by the absence of WHsAg-specific vCMI after the last vaccination at week 48 (fig. 1, 3).

Influence of Combination Therapy on the Immune Responses of Chronic WHV Carriers

In carriers that received the combination therapy (L+V+), the anti-WHs antibody responses were not enhanced beyond those in carriers that received vaccine alone (L–V+). The similar anti-WHs antibody responses in L+V+ and L–V+ carriers (fig. 2) did not predict the observed differences in vCMI between both groups. However, additional tests of anti-WHs antibodies may uncover differences in the humoral immune responses between these groups.

The vCMI to WHsAg in L+V+ carriers was rapid and sustained (fig. 1, 3). In fact, these carriers responded sooner to the WHsAg vaccine when compared to the vCMI in WHV-susceptible vaccine recipients, which became detectable mostly after the second and third immunizations (data not shown). This apparent difference in vCMI response may be explained, in part, by the fact that carriers were already primed from the infection. Continual exposure to low levels of WHsAg between vaccinations might facilitate sustained responses in carriers once they are elicited. WHsAg-specific vCMI elicited by vaccination was more sustained in L+V+ carriers with low viral and antigen load than in L–V+ carriers with high viral and antigen load. These differences might also be related to group differences in the trafficking of responding cells.

The collateral enhancement of vCMI to include rWHcAg and WHc peptide C100–119 in L+V+ carriers appeared to be dependent partly on the subsequent discontinuation of drug treatment, as observed during monotherapy in L+V– carriers (fig. 1, 4). The mechanism(s) for induction of collateral vCMI by the therapeutic WHsAg vaccine still remains unknown, but may be related to the drug-induced reductions of core gene product expression as discussed above. In any case, the collateral enhancement of vCMI beyond WHsAg to include rWHcAg, WHc peptide C100–119 and WHs peptide S226–245 appeared to be the result of an additive effect of drug and vaccine. The immunogenic effect of combination therapy was to increasingly modulate the humoral and cellular immune response profiles of carriers toward that observed during resolution of WHV infection. Woodchucks with acute self-limited WHV infection usually clear serum WHV DNA and WHsAg, seroconvert to anti-WHs antibodies and have vigorous, sustained and polyclonal vCMI responses to WHV antigens, at least for WHcAg [40, 42, 44, 46, 47]. vCMI to WHsAg during resolution of WHV infection is less well characterized [45] but seems to be directed against several epitopes of WHsAg [Menne et al., unpubl. results]. These aspects are analogous to the situation in self-limited human HBV infection [4–6].

Implications for the Immunotherapeutic Eradication of Chronic HBV Infection

Therapeutic vaccination of chronic HBV carriers with commercial HBsAg vaccine alone, or before and after treatment with IFN-α, elicited transient anti-HBs antibody responses, associated T cell responses in some patients and modest to sustained reductions in viremia in about 50% of patients, but clearance of HBsAg from serum was not reported in any of these patients [18, 19, 21, 26, 27]. Comparable reductions in viremia were not observed in the chronic WHV carrier woodchucks given vaccine alone (L–V+) in this study. Hervas-Stubbs et al. [35] immunized chronic WHV carriers with a WHsAg-based vaccine using an experimental adjuvant and showed anti-WHs antibody responses, some vCMI to WHsAg and some transient reductions in viremia in a few of the vaccinated carriers. Caution in the use of the therapeutic WHsAg vaccine was recommended, however, since some of the carriers died during the vaccinations. Such adverse effects could have been related to the experimental adjuvant and/or to liver disease present in the woodchucks at entry into the study. In this study, a conventional WHsAg-alum vaccine, homologous to the WHsAg in the infectious inoculum, which was given repeatedly under conditions of high or low viral and antigen load and minimal liver injury, appeared to be safe and immunogenic. There remains ample room for improving

responses to achieve a safe and more effective antiviral state using vaccines alone. Based on the results presented for a conventional WHsAg vaccine, an improved and sustained antiviral state following therapeutic vaccination may only be possible under initial conditions of reduced viral and antigen load. After additional time and with further immunizations, the vaccine responses might then be able to control WHV infection independently of an antiviral drug. This could represent an alternative to lifelong treatment with an antiviral drug, and thereby reduce the possibilities for selection of drug-resistant viral mutants.

Clinical studies in chronic HBV patients have shown that 3TC administered either alone or in combination with IFN-α did not reduce HBsAg [13–17]. Likewise, 3TC alone does not consistently reduce serum levels of WHsAg in chronic WHV carrier woodchucks [31]. In several studies, there were no improvements in T cell responses to HBcAg during treatment of chronic HBV patients with 3TC alone or in combination with IFN-α [16, 17]. However, T cell responses to HBcAg became detectable after discontinuation of drug treatment during viral recrudescence [16, 17]. In the present study in L-FMAU-treated chronic WHV carrier woodchucks, vCMI to WHV was not usually detected during the phase of drug treatment, but was observed on occasion during complete viral recrudescence several months after discontinuation of L-FMAU treatment (fig. 5).

In another study [12], however, chronic HBV patients were reported to develop strong and sustained T cell responses to HBcAg and to several HBc peptides shortly after initiation of 3TC treatment, and general enhancement of T cell responses to mitogen and tetanus toxoid was also observed. Possible explanations for variability in the detectability of vCMI during antiviral treatment may relate to the use of different drugs (3TC versus L-FMAU), to host age during infection and at initiation of chronicity (adult- versus neonatal-acquired chronicity) and/or to levels of serum liver enzymes indicative of liver disease at the start of treatment. Most of the 3TC-treated chronic HBV patients with T cell responses had increased serum liver enzyme levels at the start of treatment, whereas the nonresponders had little or no evidence of preexisting liver disease [12]. The chronic WHV carrier woodchucks in this study were preselected for minimal liver disease on entry into the drug treatment phase. Thus, these carriers may not have had sufficient existing liver injury to unmask vCMI immediately after L-FMAU treatment was initiated. Consistent with this explanation, furthermore, is the observed lack of (vCMI/T cell-dependent) anti-WHs antibody induction in most carriers during treatment with L-FMAU.

The presented results in the woodchuck model of human HBV infection will help to improve the understanding of humoral and cellular immune responses elicited by therapeutic vaccination alone and after sustained reduction of viral and antigen load induced by antiviral therapy. The results will open the way for the development of new strategies for the immunotherapeutic eradication of chronic HBV infection and immunological control of its disease sequelae. Furthermore, the results of this report demonstrate the apparent safety and feasibility of using combined drug treatment and therapeutic vaccination in humans with chronic HBV infection.

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Selective Expression of Immunogenic, Virus-Like Particle-Derived Antibody-Binding Epitopes

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Key Words
Virus-like particles · Protein expression · Stress proteins · DNA vaccination · Antibodies

Abstract
The incorporation of linear and conformational antibody-binding epitopes into polyepitope, chimeric antigens with satisfactory immunogenicity is a challenge. We selectively expressed antigen fragments encoding the linear e2 epitope ($C_{79–149}$) of hepatitis B virus (pre)core antigen (HBc/eAg) and the conformational 'a' epitope ($S_{80–180}$) of hepatitis B surface antigen (HBsAg) in a novel system. The domains were expressed as chimeric antigens containing either heat shock protein (hsp)73-binding simian virus 40 large tumor antigen (e.g. T_{77}) or non-hsp-binding (e.g. T_{60}) sequences at their N-termini. We compared their type of expression with their immunogenicity for B cells (when delivered as a DNA vaccine). The type of expression investigated included their level of expression, the secretion or intracellular expression of the antigen and the stress protein (hsp)-associated versus nonassociated expression. The linear e2 epitope of HBc/eAg was efficiently expressed as an intracellular, hsp73-binding fusion protein, and efficiently primed an HBc/eAg-specific antibody response when delivered in this form. The conformational 'a' epitope of HBsAg most efficiently stimulated B cells as a secreted, non-hsp-associated fusion protein. These data demonstrate that different B cell-stimulating epitopes of vaccine-relevant viral antigens can be selectively isolated and expressed in suitable expression systems, but that the requirements that have to be fulfilled to obtain optimal immunogenicity differ strikingly between individual epitopes.

Introduction

Virus-like particles (VLP) are complex macromolecular structures formed by viral envelope proteins (pseudocapsids) or nucleoproteins (core particles). These particles are potent immunogens because they display a number of features that contribute to their exceptional efficacy as T cell- and B cell-stimulating antigens. These include: (1) a stable package containing hundreds of copies of the same protein antigen that are focused on the site of priming of an immune response; (2) a diverse content of antigenic epitopes present on the particle-forming proteins that can trigger multispecific immune responses, and (3) that synergistic interactions between CD4+ 'helper' T cells and CD8+ 'effector' T cells as well as B cells specifically coprimed by the large variety of different epitopes presented by the VLP can enhance the immunogenicity of many epitopes.

A multitude of antibody-binding epitopes is presented on the surface of VLP. Some of these epitopes are known

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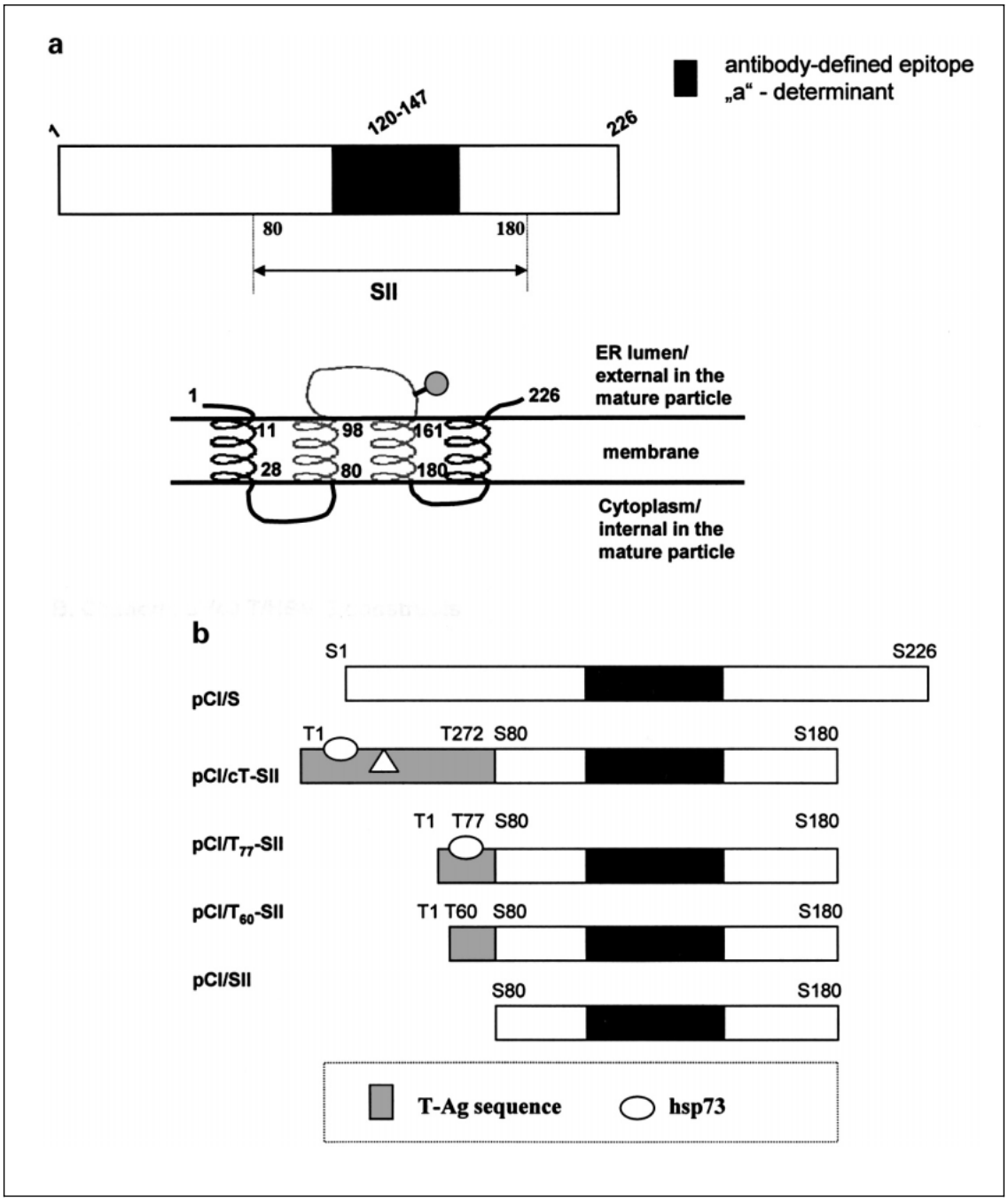

to elicit neutralizing B cell responses. Many of the viral epitopes that elicit protective antibody responses are conformational. These epitopes are therefore preferred targets for vaccines. As the integrity of the conformation of the antigenic epitope is required to induce protective antibody responses, this represents a challenge in vaccinology. Recombinant proteins used as vaccines may lack essential posttranslational modifications or may be subjected to partial denaturation during the production or purification process that can destroy conformational epitopes. DNA vaccines may not encode all the discontinuous sequences required for the correct folding of the conformational epitope. There is therefore an interest in developing expression systems suitable for the production of recombinant,

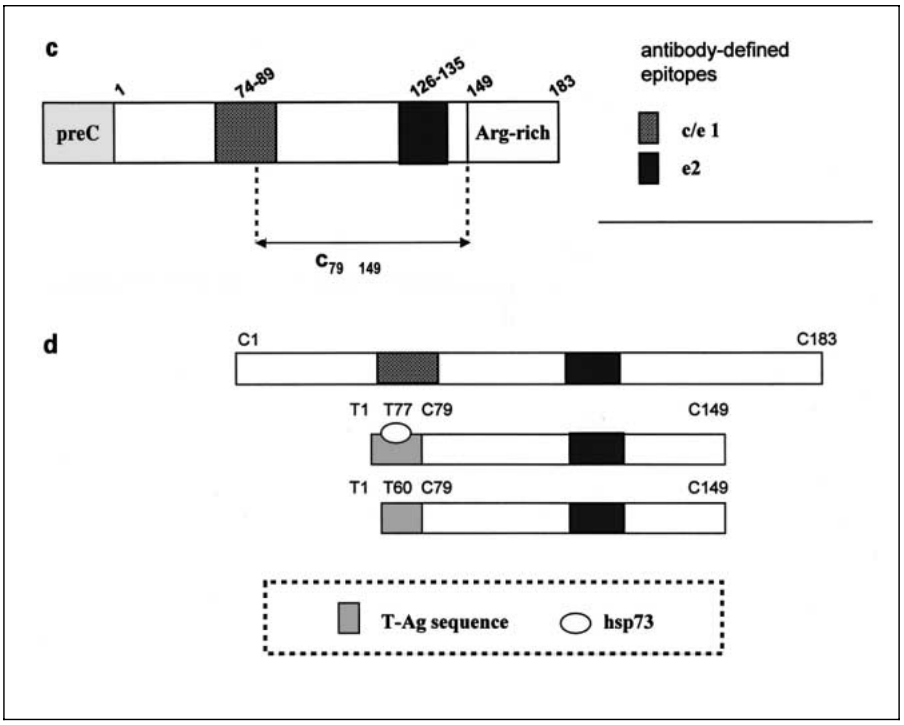

Fig. 1. Expression of HBV surface and core antigen domains using a novel hsp73-binding, SV40 T-Ag-derived expression system. **a** Map of antigenic regions within the HBV small (S) surface antigen. The antibody-defined $S_{120-147}$ 'a' determinant is expressed by the S80–180 fragment. A proposed model of transmembrane interactions of the HBsAg [51] is presented. ER = Endoplasmic reticulum. **b** Construction of hsp73-binding and non-hsp-binding fusion proteins. Fusion fragments consist of the N-terminal hsp73-binding unit, i.e. the SV40 cT_{272} fragment (containing a deletion of the nuclear localization signal sequence) or the T_{77} fragment, or of the non-hsp-binding T_{60} fragment and C-terminal in-frame fusion sequences S_{80-180} (SII). **c** Map of antigenic regions within the HBV core gene. The antibody-defined $C_{126-135}$ 'e2' determinant is selectively expressed by the C79–149 fragment. Arg = Arginine. **d** The C79–149 encoding sequence was cloned in frame to the N-terminal hsp73-binding T_{77} fragment or the non-hsp-binding T_{60} fragment.

vaccine-relevant antigens with minimal but immunogenic conformational antibody-binding epitopes. To critically evaluate this approach, the relative efficacy of priming conformation-sensitive antibody responses can be compared to the relative efficacy of priming responses against linear, antibody-binding epitopes using a similar vaccine delivery technique. The success of this approach would lay the foundations for the design of large chimeric antigens as polyvalent vaccines that contain multiple, immunogenic T cell- or B cell-stimulating determinants.

The Hepatitis B Surface Antigen and the Hepatitis B Core Antigen of the Hepatitis B Virus

The hepatitis B virus (HBV) contains two immunogenic VLP: the hepatitis B surface antigen (HBsAg), which forms porous 20- to 30-nm lipoproteasomes, and the hepatitis B core antigen (HBcAg), which forms 28-nm protein particles.

HBsAg particles secreted in abundance by HBV-infected hepatocytes are present in the plasma of infected individuals as 20- to 30-nm coreless proteoliposomes [1, 2]. Extensive experimental and clinical data are available for HBsAg, the antigen in the commercial vaccine against HBV. Protection against HBV induced by this vaccine is correlated with high levels of serum antibodies specific for HBsAg. Some 100–150 subunits of the 226-residue p24 small surface protein S self-assemble into stable, 20- to 30-nm lipoprotein particles that are very rigid due to multiple inter- and intramolecular disulfide cross-links [3]. The group-specific, immunodominant 'a' determinant is a conformational epitope present on the secreted nonglycosylated p24 and monoglycosylated gp27 small S protein in almost all HBV isolates known. It spans residues 120–147 of the envelope protein and forms a loop between two transmembrane domains (fig. 1a). We selected a sequence encoding residues 80–180 of the small S protein (i.e. the SII fragment), which contains the complete 'a' determinant of HBsAg and the two flanking transmembrane regions.

Immunogenic, icosahedral 30-nm HBcAg particles self-assemble from p21 (21-kD) core (C) proteins of HBV [4]. The N-terminal 144 amino acids (aa) of the C protein are required for its oligomerization into capsids [5]. The 5′ precore AUG within the core gene region directs production of the 25-kD precore protein, which is longer than the HBcAg protein by 29 N-terminal residues. A 19-aa signal sequence that targets this precore protein precursor to the secretory pathway is removed in the endoplasmic reticulum to generate the p22 intermediate protein, from

which the C-terminal 34 residues (aa 150–183) are cleaved to give rise to the secreted p17 protein (i.e. the precore antigen HBeAg or HBe). The in vivo function of HBeAg in the HBV life cycle is unknown, but it plays no role in HBV replication and assembly. Particle formation of HBeAg is prevented by formation of a disulfide bridge between cysteines at position 7 (in the precore region) and 61 (in the core region) [6]. HBcAg and HBeAg thus share a 149-aa sequence but differ structurally (particle versus soluble protein dimer) and with respect to the C-terminal arginine-rich nucleic acid-binding domain (present in HBcAg but not in mutant HBcAg and HBeAg). Most antibody responses to HBcAg and HBeAg are cross-reactive and map to the immunodominant c/e1 and e2 epitopes [4, 7]. We selectively expressed the linear e2 epitope spanning residues 126–135 [4] of HBcAg in a DNA vaccine.

Here, we studied the induction of antibody responses to the conformational HBsAg 'a' determinant and the linear hepatitis B (pre)core antigen (HBc/eAg) e2 epitope determinant by a DNA vaccine based on a novel expression system. This system allowed us to address the following four questions: (1) Can we express a conformational, antibody-binding epitope in the system in immunogenic form? (2) Is the immunogenicity of the conformational, B cell-stimulating epitope superior when the epitope is expressed as a secreted product compared to when it is expressed as an intracellular product? (3) Does the expression of the antigen in association with stress proteins enhance or suppress the immunogenicity of either conformational or linear epitopes? (4) Are the rules that can be defined for the immunogenic expression of antibody-binding epitopes similar for both linear and conformational determinants?

A System for the Stable, Heat Shock Protein-Associated Expression of Protein Fragments

Using a novel system [8], we expressed the protein fragments containing the conformational 'a' determinant of HBsAg or the linear e2 determinant of HBc/eAg as a fusion protein with N-terminal sequences from the large tumor antigen (T-Ag) of simian virus 40 (SV40). The N-terminal residues of varying length we used were either residues 1–60 or 1–77 of the wild-type SV40 T-Ag, or residues 1–272 of the mutant cT-Ag (with a deleted nuclear localization signal).

N-terminal domains of SV40 T-Ag associate with the heat shock protein (hsp)73 through a DnaJ-like structure, i.e. the J domain [9–11]. The integrity of a conserved HPD motif as well as the helical structure of the J domain is required for the association of T-Ag with hsp73 [11]. We found that many mutant T-Ags show stable association with hsp73 [12, 13]. The intact N-terminal J domain of T-Ag was required for stable expression, as the T_{77} fragment (with an intact J domain) but not the T_{60} (with a disrupted J domain) was found to be expressed at readily detectable levels. This correlated with the association with hsp73, confirming that the intact J domain with the conserved HPD motif plays a key role in hsp73 association and stable expression [11].

From these observations, we developed a vector system for the efficient expression of hsp73-associated, chimeric proteins in which the T-Ag-derived J domain is fused N-terminally to different fragments of unrelated antigens. When different sequences from heterologous viral antigens were fused C-terminally to the T-Ag J domain, chimeric, hsp73-binding proteins could be stably expressed at a high level. The conformationally intact J domain of the T-Ag is thus accessible and remains conformationally intact when fused to many different C-terminal fragments of different origins and lengths.

An expression system that can efficiently produce large, chimeric protein antigens in stable association with hsp73 is of interest for new approaches in vaccine designs. To test this option, we cloned different viral antigens (containing antibody- and cytotoxic T-lymphocytes (CTL)-defined epitopes) behind the J domain of the T-Ag to generate DNA vaccines with high, stable, hsp-associated expression of the chimeric antigen. The system greatly facilitated antigen expression and allowed us to design novel DNA vaccine concepts.

Hsp molecules enhance and modulate the immunogenicity of protein and peptide antigens [reviewed in ref. 14, 15] and are thus innate adjuvants [16–19]. Hsp molecules exert their 'adjuvant effect' by binding to surface receptors [20, 21], including CD91, TLR2/4 and/or CD14 [21–24]. This induces migration and maturation (including cytokine/chemokine release) of dendritic cells [19, 25–30]. Hsp molecules can deliver antigens to processing pathways for the MHC class I-restricted presentation of their epitopes, thereby facilitating priming of CTL to peptides from tumor antigens, minor H antigens or viruses [31–39]. Hsp molecules facilitate CD4+ T helper-independent priming of CTL responses to antigens [29, 40]. Hsp73-bound endogenous antigens undergo TAP-independent, endolysosomal processing for MHC class I-restricted epitope presentation [12, 13]. Association of antigens with hsp facilitates cross-priming of CTL [41]

and priming of antibody responses to endogenous antigen by DNA vaccines [8]. Incorporating a hsp as an 'innate adjuvant' into a vaccine formulation is thus an attractive and rational choice to enhance its immunogenicity.

Expression of a Conformationally Intact HBsAg 'a' Determinant

The pCI/SII vector DNA encoding the 80–180 residue fragment of HBsAg without a heterologous N-terminus did not express detectable amounts of the protein and did not prime a humoral anti-HBsAg response. Therefore, alternative ways to express this fragment had to be used. The fragment SII ($S_{80–180}$) of HBsAg was fused in frame behind the hsp73-binding, N-terminal 272-residue fragment of the SV40 T-Ag from which the nuclear localization signal (residues 110–152) had been deleted (fig. 1b) [42]. This generated a vector encoding a cT_{272}-SII fusion protein (pCI/cT-SII). Products of the expected size were immunoprecipitated with the anti-T-Ag monoclonal antibody PAb108 from lysates of radiolabeled, transiently transfected cells (fig. 2a). The two 38- and 40-kD fusion products were coprecipitated with large amounts of hsp73 (fig. 2a). Western blot analyses of the immunoprecipitates with polyclonal anti-hsp73, anti-T or anti-HBsAg antisera confirmed that both bands contained T-Ag-derived and HBsAg-derived sequences, and that the coprecipitated 70-kD protein was hsp73, confirming our previously published data [12]. The vector pCI/cT-SII thus expresses an HBsAg fragment carrying its 'a' determinant as a hsp73-binding fusion protein. The 'a' determinant is a loop between the second and third transmembrane domain of HBsAg that contains a glycosylation site at Asn 146 [2]. Similar to the native 'a' loop, the isolated 'a' loop of HBsAg carried by the cT-SII fusion protein is glycosylated, because its treatment with endoglycosidase H strikingly reduces the 40-kD band at the expense of the 38-kD band. Similar to native LS [2], the cT-SII protein is intracellular but not secreted (fig. 2b).

Binding of hsp73 to the T-Ag depends on the J domain, which comprises the N-terminal 77 residues of the T-Ag [11, 43]. To investigate the role of hsp73 binding to the fusion construct, we designed and expressed two constructs in which the fragment SII ($S_{80–180}$) was fused either to the hsp73-binding 77-residue T_{77} N-terminus (with an intact J domain) or to the non-hsp-binding 60-residue T_{60} N-terminus (with a disrupted J domain) (fig. 1b). Transiently transfected cells expressed both fusion proteins. In contrast to the hsp-associated T_{77}-SII protein, the non-hsp-associated T_{60}-SII protein was secreted (fig. 2). This allowed us to directly compare the immunogenicity of an isolated, antibody-binding epitope delivered either as an intracellular, hsp73-associated protein or as a secreted, non-hsp-associated protein.

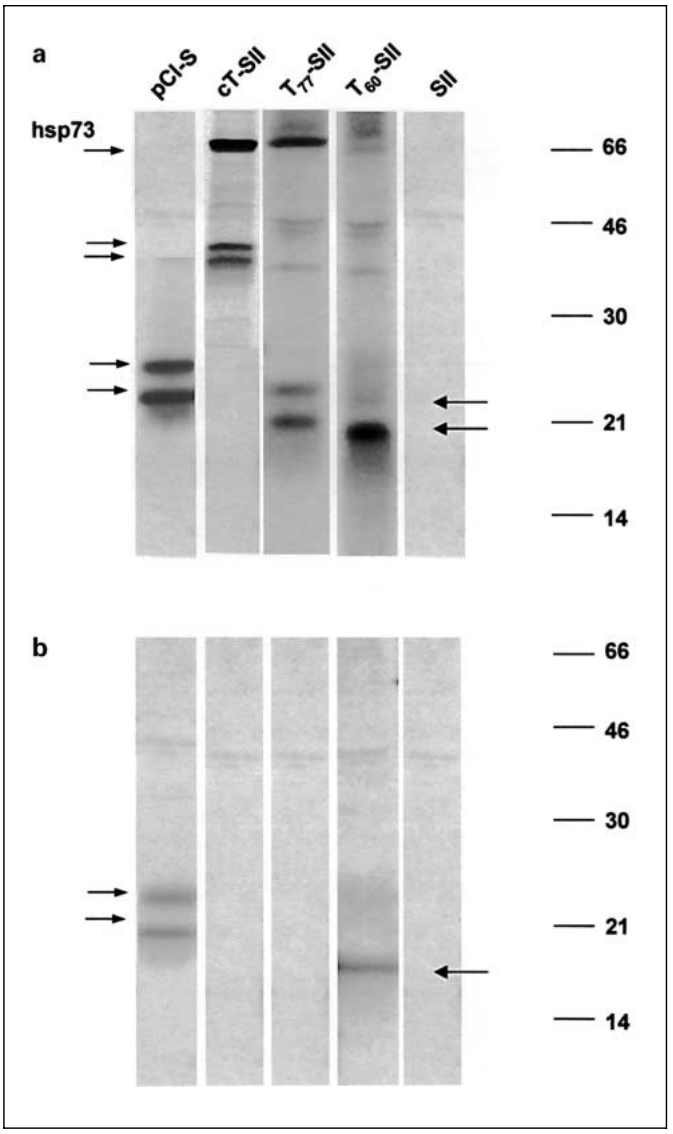

Fig. 2. Expression of wild-type HBV surface antigen or chimeric, surface antigen-encoding fusion antigens. Chicken hepatoma cells (LMH) were transiently transfected with pCI/S, pCI/cT_{272}-SII, pCI/T_{77}-SII, pCI/T_{60}-SII and pCI/SII DNA. Cells were labeled with ^{35}S-methionine. Cell lysates (**a**) and cell culture supernatants (**b**) were immunoprecipitated with HBsAg-specific antiserum (pCI/S, pCI/SII) or with the anti T-Ag monoclonal antibody Pab 108, and processed for SDS-PAGE followed by fluorography of the gels. The positions of glycosylated and nonglycosylated forms of S, cT_{272}-SII, T_{77}-SII and T_{60}-SII and of hsp73 are indicated.

Table 1. DNA vaccination of BALB/c mice induces HBsAg- and HBc/eAg-specific serum antibody responses

Group	DNA vaccine	Serum antibody titer
1	pCI/S	4,170 ± 2,100
2	pCI/T_{272}-SII	80 ± 30
3	pCI/T_{77}-SII	160 ± 40
4	pCI/T_{60}-SII	2,400 ± 450
5	pCI/SII	< 10
6	pCI/C	102,400 ± 25,600
7	T_{77}-e2	12,800 ± 3,200
8	T_{60}-e2	600 ± 100

BALB/c mice were immunized twice with the indicated vectors (100 μg, i.m.), and surface- (mIU/ml, groups 1–5) and core (endpoint titer, groups 6–8)-specific serum antibodies were determined as described previously [52, 53].

Eliciting HBsAg-Specific Antibody Responses by DNA Vaccines Encoding the 'a' Determinant either as an Intracellular, hsp73-Associated Protein or as a Secreted, Non-hsp-Associated Protein

We immunized mice intramuscularly with plasmid DNA vaccines encoding (1) the native (secreted) small (S) HBsAg, (2) the hsp73-associated, intracellular T_{77}-SII fusion protein, or (3) the secreted, non-hsp-associated T_{60}-SII fusion protein. Only serum antibodies specific for the conformational 'a' determinant of native HBsAg are detected by the commercial IMxAUSAB® system (Abbott), which is a competitive ELISA readout. High HBsAg-specific serum antibody responses were induced after inoculation of the pCI/T_{60}-SII DNA vaccine (encoding the secreted, non-hsp-binding $S_{80–180}$ antigen) as efficiently as after injection of the pCI/S DNA vaccine (encoding native HBsAg) (table 1, groups 1–5). Comparable HBsAg-specific serum antibody responses were induced after inoculation of the pCI/T_5-SII or the pCI/T_{45}-SII DNA vaccine (encoding either 5- or 45-residue T-Ag and the $S_{80–180}$ antigen) (data not shown). In contrast, the pCI/T_{77}-SII DNA vaccine (encoding nonsecreted, hsp73-associated $S_{80–180}$) was less potent in stimulating this HBsAg-specific antibody response by two orders of magnitude (table 1). Antibodies binding linear HBsAg determinants or determinants of the T-Ag or hsp73 were very low or undetectable in Western blot analyses. Two important conclusions can thus be drawn from these results: (1) expression of the $S_{80–180}$ fragment as a fusion protein by the pCI/T_{60}-SII and pCI/T_{77}-SII DNA vaccines selectively presents the conformational 'a' determinant of HBsAg to the immune system but does not elicit antibody responses to the T-Ag-derived carrier or the hsp autoantigen, and (2) secreted T_{60}-SII fusion antigen delivered by a DNA vaccine can prime an antibody response to a conformational HBsAg determinant as efficiently as a DNA vaccine encoding native, secreted HBsAg.

Expression of the Linear HBc/eAg e2 Determinant

The pCI/C vector DNA encodes the intracellular core protein HBcAg (fig. 1c). The linear, antibody-binding e2 epitope (encoded by residues 126–135 of the HBcAg) was fused as a C79–149 fragment in frame behind the N-terminal hsp73-binding 77-residue T_{77} N-terminus or the non-hsp-binding 60-residue T_{60} N-terminus (fig. 1d). In transfected cells, the intracellular hsp73-associated T_{77}-e2 fusion protein was efficiently expressed, whereas the non-hsp-associated T_{60}-e2 fusion protein was barely detectable (data not shown). This system confirmed our experience that association with hsp73 greatly facilitates expression of a chimeric protein. The good expression of the T_{77}-e2 fusion protein and the very poor expression of the T_{60}-e2 fusion protein have to be taken into account in the interpretation of the vaccination studies.

Priming HBc/eAg-Specific Antibody Responses by DNA Vaccines Encoding the e2 Determinant as an Intracellular, hsp73-Associated Protein

We immunized mice intramuscularly with plasmid DNA vaccines encoding (1) the native HBcAg (pCI/C), (2) the well-expressed, hsp73-associated, intracellular T_{77}-e2 fusion protein (pCI/T_{77}-e2), or (3) the poorly expressed, non-hsp-associated T_{60}-e2 fusion protein (pCI/T_{60}-e2). High HBV core-specific serum antibody responses were stimulated after injection of the pCI/C (table 1, groups 6–8). Antibody titers induced by the pCI/T_{77}-e2 DNA vaccine were significantly reduced compared to those induced by pCI/C. These findings were expected, as the T_{77}-e2 fusion protein did not express the immunodominant c/e1 epitope [4]. However, e2-specific antibodies were barely detectable after injection of the pCI/T_{60}-e2 plasmid DNA (encoding the poorly expressed T_{60}-e2) (table 1). A very similar pattern of humoral immune responses was observed when either recombinant HBcAg

Table 2. Expression pattern and immunogenicity of HBsAg- and HBc/eAg-derived antibody-binding epitopes

Group	Chimeric antigen[1]	Expression[2]			Potency of priming specific antibody responses when delivered as DNA vaccine[3]
		stable	hsp73-associated	secreted	
1	HBsAg (S)	++	–	++	+++
2	T_{272}-SII	++++	++	–	+
3	T_{77}-SII	++++	+	–	+
4	T_{60}-SII	++	–	+	+++
5	SII	–	–	–	–
6	HBcAg	+++	–	–	+++++
7	T_{77}-e2	+++	+	–	+++
8	T_{60}-e2	+	–	–	+

[1] HBV surface and core antigen encoding genes or gene fragments were cloned into pCI expression constructs.
[2] LMH cells were transiently transfected with the respective expression vectors, immunoprecipitated and processed for SDS-PAGE. Expression levels were determined (1) in Coomassie blue-stained gels and (2) by fluorographic analyses.
[3] BALB/c mice were immunized twice with the indicated vectors (100 μg, i.m.), and surface- and core-specific serum antibodies were determined.

or HBeAg was used as detection antigen in the ELISA. Thus, the linear e2 determinant was accessible for antibody recognition on the respective antigens. We draw the following conclusions from these data: (1) expression of the linear, antibody-binding e2 epitope as an intracellular, hsp-associated fusion protein elicits efficient antibody responses; (2) non-hsp-associated T_{60}-e2 is difficult to express and not immunogenic, demonstrating the essential role of hsp association in expression and/or immunogenicity in this system, and (3) these data thus differ from those presented above for the immunogenic delivery of the conformational 'a' determinant of HBsAg (expressed in the same system) (table 2).

Designing Expression Systems for Enhancing the Immunogenic Delivery of Antibody-Binding Epitopes from VLP

Our experience with the expression of linear and conformational antibody-binding epitopes from the two major antigen systems of HBV and their immunogenicity when delivered by DNA-based vaccines is summarized in table 2.

Expression System

We developed a novel expression system from our previous work with the SV40 T-Ag [12, 13, 42, 44–50]. This system is based on the finding that the N-terminus of the T-Ag carries a J domain that tightly associates with the constitutively expressed, cytosolic stress protein hsp73. Hsp binding is very stable when the T-Ag is mutated (outside the J domain region) or when the J domain is fused to heterologous proteins or their fragments. Apparently, hsp fulfils a number of different functions in this novel expression system: (1) it supports expression of mutant, truncated or chimeric proteins (compare, for example, the expression of T_{77}-e2 and T_{60}-e2); (2) it changes the processing pattern of proteins it has bound for the generation of antigenic, MHC class I-binding peptides [12, 13], and (3) it enhances the immunogenicity of the protein it has bound, facilitating, for example, cross-priming of CTL [41] or priming of antibody responses to intracellular antigens [8].

As this system exploits a physiological feature of chaperone 'help' in protein synthesis, folding and traffic, it is ideally suited for use in DNA-based vaccination.

Expression Level

In the system used, hsp association facilitates expression. This has been our universal experience in expressing more than 40 different fragments from vaccine-relevant antigens. To our knowledge, this is the most efficient system for the expression of mutant, truncated or chimeric fusion proteins. Even proteins (or their fragments) with a very short half-life accumulated to impressive levels in stable transfectants when expressed in this system. The system tolerates expression of chimeric proteins of at least

800 aa. No length restriction has been encountered to date.

Hsp Association

Stable association of the T-Ag-derived J domain with hsp73 seems specific. Stress-induced, cytosolic hsp72 or the endoplasmic reticulum-resident hsp of the 70-kD family do not associate with this viral domain. Stable hsp73 association seems to inhibit secretion of the bound substrate (compare, for example, expression of T_{77}-SII and T_{60}-SII). As hsp association facilitates cross-priming and renders intracellular antigens visible to the B cell system, some type of export of the complexes from the cell is to be expected. The route and mode of this export is obscure.

Expression in Immunogenic Form of Linear versus Conformational Viral Epitopes That Bind Antibodies

The 'a' determinant could be expressed with its intact (cross-reactive) conformation in the system. This is unusual because very few complex antibody-binding determinants have been isolated and expressed in a functional way up to now. For optimal immunogenicity, the antigen fragment had to be expressed as a secreted fusion protein. Hsp association was not essential but actually reduced its immunogenicity for B cells. In secreted form, the T_{60}-SII fusion protein was as effective as the natural, secreted HBsAg particle in priming conformation-specific antibody responses. This may be a special case of the HBsAg fragment chosen for expression that may form stable, secreted, macromolecular complexes similar to those of natural HBsAg particles. The situation was different when the linear e2 epitope was expressed and its immunogenicity was assayed. In this system, hsp association was mandatory for achieving detectable levels of expression and to prime antibody responses. Although the hsp-associated T_{77}-e2 was an intracellular antigen, it was efficiently expressed and immunogenic for B cells. In contrast, only barely detectable levels of the non-hsp-associated T_{60}-e2 antigen were expressed, and low antibody responses could be primed or boosted with this fusion protein. This resembles our published data using the preS domain of the large LS variant of HBsAg fused to the T-Ag J domain [8]. Hence, small linear epitopes are readily expressed in immunogenic form in association with hsp in the system while large, complex, conformational epitopes may each need their own rules governing efficient expression. Interestingly, hsp-associated, antibody-binding epitopes efficiently stimulate a B cell response even when expressed as intracellular antigens. This offers a broad range of potential applications for the design of polyepitope vaccines that combine T cell-stimulating and B cell-stimulating epitopes.

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HIV: Epidemiology and Strategies for Therapy and Vaccination

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Key Words
HIV · AIDS · Epidemiology · Treatment · Vaccine

Abstract
In the decades since its discovery, the acquired immunodeficiency syndrome AIDS has developed into one of the leading causes of death worldwide, with a dramatic situation in many countries of subsaharan Africa. A large body of knowledge has been acquired regarding the molecular biology and replication properties of the causative agent of AIDS, the human immunodeficiency virus (HIV). However, despite these advances the global spread of HIV and especially its spread in developing countries continues almost unabated. During the last decade, drugs inhibiting HIV replication have been introduced into clinical use and highly active antiretroviral therapy (HAART) using a combination of several drugs has been proven to slow or halt the course of the disease in most patients. However, HAART is currently only available in countries of the developed world and the emergence of resistant variants may rapidly lead to a partial loss of this therapeutic arsenal. Thus, the development of a prophylactic vaccine against HIV must be the ultimate goal to control the global pandemic of AIDS. This minireview summarises the state of the epidemic and discusses current treatment strategies as well as future developments. Finally, strategies towards the development of a vaccine against HIV are presented and recent studies using prime-boost regimens to achieve protection from disease in animal models while not preventing infection with the challenge virus are discussed.

Epidemiology of HIV/AIDS

Since AIDS was recognized as a new disease in the beginning of the 1980s and HIV was isolated and identified as a transmissible pathogen causing this disease, HIV has spread all over the world and has already claimed more than 25 million lives. The number of persons currently living with HIV infection worldwide is estimated by the Joint United Nations Programme on HIV/AIDS (UNAIDS) at more than 40 million (fig. 1). About 5 million new infections with HIV occurred in the year 2001 (fig. 2) and about 3 million people died from HIV/AIDS (fig. 3), making HIV infection the single most important cause of death from infectious diseases (for more information, visit www.unaids.org).

If these staggering numbers are broken down according to region, gender and age, it is revealed that meanwhile,

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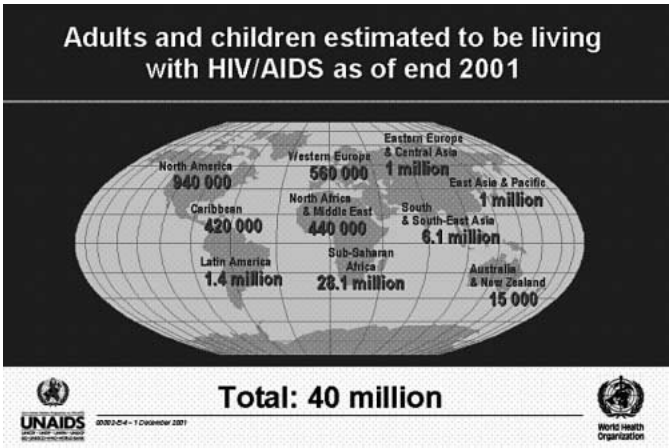

Fig. 1. Number of adults and children estimated to be living with HIV/AIDS as at the end of 2001.

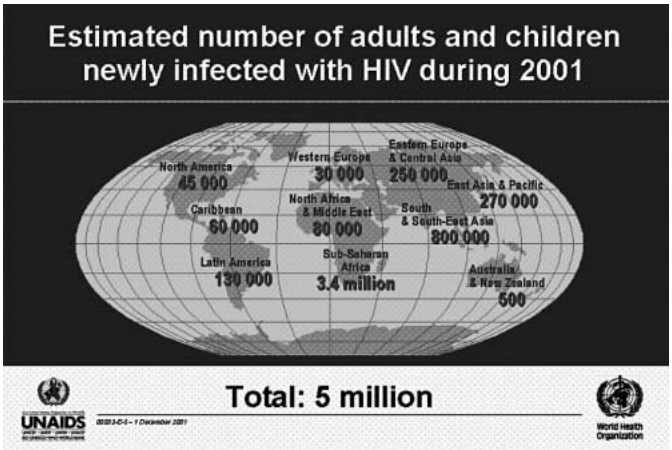

Fig. 2. Estimated number of adults and children newly infected with HIV during 2001.

more than 95% of the approximately 15,000 new infections per day are transmitted in developing countries. More than 60% of infections occur in young people under the age of 25; among these, about 1,700 infections are in children under 15 years of age, mainly through mother-to-child-transmission. Worldwide, 48% of HIV-positive adults are women (table 1).

The most severely affected part of the world is sub-Saharan Africa, with more than two thirds of all infected persons living in this region and 3.4 million new infections occurring in 2001. It is estimated that 2.3 million Africans died of AIDS in 2001, a death toll considerably higher than from famine and wars. There are now 16 countries in sub-Saharan Africa in which more than 10% of those aged 15–49 years are infected with HIV. In some countries and regions in southern Africa, HIV prevalence rates among pregnant women attending antenatal clinics have reached catastrophic proportions, with levels of more than 30%. In urban areas in Botswana, the corresponding figure is 44%; in South Africa's KwaZulu-Natal province, the figure stands at 36%.

The principal mode of transmission of HIV in Africa is unprotected heterosexual intercourse, which is responsible for more than 85% of new infections. About 10% of new infections are due to mother-to-child transmission. Intravenous drug use has not played a large role in dissemination of HIV in Africa so far, but drug abuse is increasing in several countries and the risk of amplification of HIV infections among intravenous drug users, once HIV is introduced into this population, is very high.

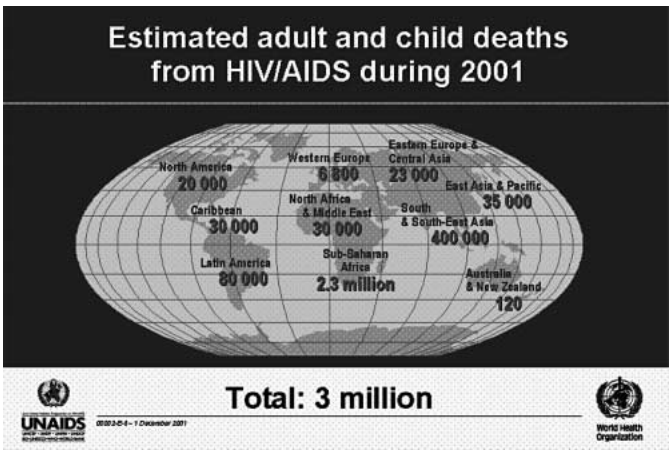

Fig. 3. Estimated number of adult and child deaths from HIV/AIDS during 2001.

Other regions already heavily affected are South Asia and South-East Asia, with about 800,000 new infections in 2001 and an estimated total of 6.1 million persons living with HIV/AIDS, as well as Eastern Europe, with 250,000 additional infections in 2001, bringing the cumulative number in this region to 1 million. As in Africa, in Asia, HIV is spreading mainly by heterosexual contact. However, since the epidemic in many countries is still in an early phase, the commercial sex sector is still playing a large role in the emerging epidemic. This is reflected by a higher proportion of men than women among AIDS and HIV patients. In several countries, intravenous drug users

Table 1. Regional HIV/AIDS statistics and features, end of 2001

	When epidemic started	Adults and children		Adult prevalence rate[1], %	Percentage of HIV-positive adults who are women	Main mode(s) of transmission for those living with HIV/AIDS
		living with HIV/AIDS	newly infected with HIV in 2001			
Sub-Saharan Africa	late 1970s early 1980s	28.1 million	3.4 million	8.4	55	Hetero
North Africa and Middle East	late 1980s	440,000	80,000	0.2	40	Hetero, IDU
South and South-East Asia	late 1980s	6.1 million	800,00	0.6	35	Hetero, IDU
East Asia and Pacific	late 1980s	1 million	270,000	0.1	20	IDU, Hetero, MSM
Latin America	late 1970s early 1980s	1.4 million	130,000	0.5	30	MSM, IDU, Hetero
Caribbean	late 1970s early 1980s	420,000	60,000	2.2	50	Hetero, MSM
Eastern Europe and Central Asia	ealry 1990s	1 million	250,000	0.5	20	IDU
Western Europe	late 1970s early 1980s	560,000	30,000	0.3	25	MSM, IDU
North America	late 1970s early 1980s	940,000	45,000	0.6	20	MSM, IDU, Hetero
Australia and New Zealand	late 1970s early 1980s	15,000	500	0.1	10	MSM
Total		40 million	5 million	1.2	48	

From the UNAIDS report 'AIDS epidemic update – December 2001' (http://www.unaids.org/epidemic–update/report–dec01).
Hetero = Heterosexual transmission; IDU = transmission through injecting drug use. MSM = Male homosexuals.
[1] The proportion of adults (15–49 years of age) living with HIV/AIDS in 2001, using 2001 population numbers.

were among the first to be affected by the spread of HIV. The virus has reached high prevalence levels in this group, and the incidence of HIV infection is still high. Major HIV epidemics among drug users have been experienced in Thailand, Burma, Malaysia, Vietnam, China and India.

In Eastern Europe, the epidemic spread of HIV started only recently. Until the mid-1990s, there were only a few sporadic cases, with the exception of some nosocomial outbreaks of HIV in hospitals, caused by inadequate sterilization procedures. Then, starting in the Ukraine, HIV began to be introduced into the rapidly expanding population of intravenous drug users, resulting in explosive regional epidemics. While the peak of the epidemic among drug users seems to have passed in the Ukraine, most other countries in the region are currently witnessing a phase of almost exponential spread of HIV among intravenous drug users. To make things even more complicated, widespread intravenous drug use is a relatively new and still growing phenomenon in this area. There are very few drug abuse treatment and rehabilitation facilities, and the society as a whole has huge difficulties in coming to terms with this new problem. The majority of drug addicts are young men and adolescents. Heroin is quickly becoming the most popular illegal drug among young people, and lack of knowledge as well as lack of sterile needles and syringes are both contributing to the extensive spread of HIV and hepatitis viruses in this population group.

There is also a considerable risk of sexual transmission of HIV, since the incidence of sexually transmitted infections has increased in the recent past due to greater population mobility, a growing commercial sex industry and a breakdown of the health care system and of traditional public health control strategies.

In Latin America and the Caribbean, there is a complex mix of heterosexual transmission, men having sex with men (MSM) and intravenous drug abuse involved in the spread of HIV. In most Caribbean islands, some countries of Central America and the northern part of the Pacific coast of South America, HIV is mainly transmitted among heterosexuals. Mexico, Costa Rica, Nicaragua, Ecuador, Cuba, Brazil, Uruguay and Chile have experienced HIV epidemics among homo- and bisexual men, while intravenous drug users are heavily affected in Argentina, Uruguay and Brazil.

In North America and the European Union, the original dominance of MSM has been replaced by intravenous drug abuse as the most important risk factor for HIV transmission. Also, the proportion of heterosexually acquired infections has increased in recent years, and in some countries, migrants from regions with a high prevalence of HIV meanwhile form the second largest group of people infected with HIV. However, concerning the decrease of MSM transmission among persons affected by HIV, there is no room for complacency. There have been reports from most of these countries of increasing rates of sexually transmitted infections and outbreaks of syphilis among MSM. The increase in risk behaviour among MSM follows a period of shrinking budgets for prevention and a process of 'normalization' of HIV/AIDS which has been accelerated by improved treatment options. This has weakened the originally very successful model of behavioural change and condom promotion among MSM. Habitualization of condom use, which was achieved at least for sex with non-steady partners, is slowly fading away. If there are no reinvigorated efforts for prevention, we may well see a second wave of the HIV epidemic among homo-/bisexual men in the developed countries in the near future.

Treatment

The benefits of improved treatment so far have been restricted to only a minority of patients, mainly from the richer, developed countries. In those countries, morbidity and mortality from AIDS has dropped dramatically, to about one third of the rates before the introduction of triple combination therapy. Consequently, implementation of antiretroviral therapies in resource-poor settings has been strongly advocated and there has been significant pressure on the pharmaceutical industry to bring down the cost of drugs. However, the implementation of effective and sustained HIV therapy will depend not only on lower prices, although this is an essential prerequisite. In addition, public awareness of the HIV epidemic needs to increase and the level of acceptance of antiretroviral therapy has to be raised in the general population as well as among HIV-infected people. In particular, the need for (life)long treatment on a regular, daily schedule has to be understood and ensured. In the absence of planned and controlled intervention programs, the provision of antiretroviral drugs (even at low prices) to people in resource-poor settings could lead to stigmatization and marginalization of those infected with HIV. Furthermore, this may contribute to the selection of resistant variants due to intermittent drug usage leading to frequent subtherapeutic drug concentrations. Thus, the ethical goal of bringing therapy to people outside the richer countries could in fact prove to be unethical if not achieved as part of a well-planned intervention strategy. Recently, however, it was shown in a rural area of Haiti that such a strategy can be successfully implemented in a poor country, where directly observed HIV therapy was introduced following the model of tuberculosis control efforts [1]. Importantly, the demand for HIV testing in this community has risen since highly active antiretroviral therapy (HAART) was made available, indicating that access to effective therapy may even lessen the AIDS-related stigma and providing strong support for extended treatment programs in resource-poor countries. Programs to prevent or lower mother-to-child transmission of HIV are currently being started in many countries. The rate of infection of newborns by their HIV-positive mothers can be dramatically reduced by a short course of therapy with only a single antiretroviral drug, and programs to prevent mother-to-child transmission may therefore set the stage for more widespread general intervention strategies.

Current antiretroviral therapy always involves a combination of three or more drugs, which need to be given at regular intervals. Initial therapeutic trials in the early 1990s applying inhibitors of the viral reverse transcriptase (RT) as single drugs proved unsuccessful, mainly because of the rapid emergence of resistant escape variants. Once additional drugs became available, combination therapy, usually applying three antiretroviral agents (HAART), dramatically reduced the progression to AIDS in HIV-positive subjects. It should be emphasized, however, that although such treatment can substantially lower the virus load (reaching undetectable levels in many patients), it does not eliminate the virus. Residual virus may persist due to the presence of long-lived infected cells, ongoing low-level replication in privileged sites and delayed reactivation of cells harbouring latent viral genomes.

The current HAART regimen commonly includes either two inhibitors of RT (nucleosidal RT inhibitors as well as non-nucleosidal RT inhibitors) plus one inhibitor of the viral protease or a combination of three different RT inhibitors (usually 2 nucleosidal RT inhibitors and 1 non-nucleosidal RT inhibitor). RT inhibitors block reverse transcription of the incoming viral genome and therefore prevent infection of the cell, while inhibitors of the viral protease prevent the maturation of infectious virions (fig. 4). At the end of 2001, there were 18 licensed

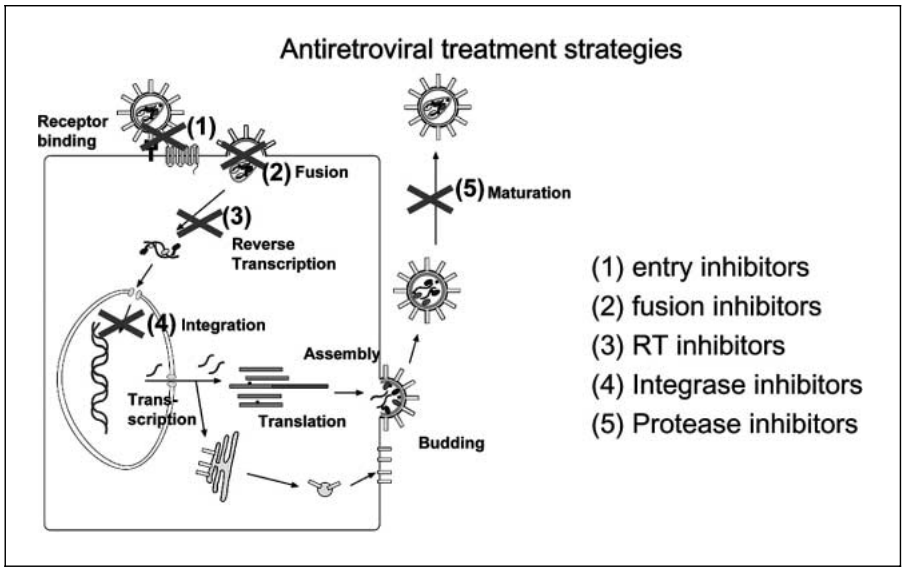

Fig. 4. Antiretroviral treatment strategies.

antiretroviral drugs, which all belong to these classes of agents (for more information, visit http://hivatis.org/trtgdlns.html). Under HAART, a significant suppression of virus load (usually to undetectable levels) can be achieved in most patients. However, significant side effects, including anaemia, neutropenia, allergic reactions and lipodystrophy, can limit the therapeutic options and may require changes in the HAART regimen. Most importantly, emergence of resistant variants has been observed for every drug, and an increasing number of doubly or multi-resistant strains has been detected in patients treated for extended periods of time [2]. Analysis of the prevalence of antiretroviral drug resistance in the USA and UK indicates that up to 50% of infected people harbour HIV variants which are already resistant to at least one antiretroviral drug. Furthermore, transmission of viruses with a more than tenfold resistance to one or more of the antiretroviral agents has been reported for up to 14% of recently infected people [3, 4], and an increasing number of multi-resistant strains is being observed. Therefore, the emergence of drug resistance will be the major and increasing problem in coming years, and a partial loss of the therapeutic arsenal may be anticipated as a consequence of spreading resistance.

The development of resistance requires modification of the capacity of the target enzyme to interact with the inhibitor while preserving its function. The error-prone nature of the viral RT, which lacks a proofreading mechanism, leads to approximately one misincorporation of a nucleotide per replication cycle. Given the very high virus production rate, which has been estimated at 10^9 per day [5], one may assume that every possible point mutation is in fact generated in every HIV-infected person. While most mutations are expected to be either lethal or lead to a selective disadvantage, one may predict that mutations conferring partial resistance to antiviral agents pre-exist in people never treated with the respective agent, and this has in fact been observed. Stochastic events (e.g. activation of an infected cell) and the relative fitness of the respective variant will determine whether a specific variant is selected for and the presence of antiviral agents or a specific immune response (antibodies and/or cytotoxic T cells) will shift the balance. Evolution of drug resistance may therefore best be described as a stepwise process building on a pre-existing partially resistant variant which is selected for upon introduction of the drug and accumulates further mutations, thus increasing its relative resistance and/or fitness. Drug-resistant mutants may persist as the dominant quasi-species or as minor variants after the selective pressure has been removed. These HIV variants are capable of re-emergence once the drug is reintroduced, and treatment failure is the consequence.

Given the evolution and increasing spread of multi-resistant strains, it becomes obvious that novel compounds targeting additional steps in the viral life cycle will be important to maintain successful HIV therapy. Several compounds acting on different steps of the entry pathway have been developed and are currently being tested in early phase I/II trails. The most promising candidates are the so-called fusion and entry inhibitors. These inhibitors

prevent virus attachment (e.g. PRO542), interaction between the envelope protein and chemokine receptors (e.g. AMD3100) or the completion of the fusion process between the viral membrane and the cell membrane (e.g. T20) (fig. 4). Preclinical and clinical assessment of these inhibitors is ongoing and will determine if they possess properties required for drug licensure. The next class of antiretroviral drugs will probably be directed against the viral integrase, the third viral enzyme, which is also essential for HIV replication and serves to integrate the viral genome into a host chromosome (fig. 4). Active lead compounds targeting the integrase have been developed and are currently being analysed.

Vaccines

As stated above, treatment of HIV infection does not achieve eradication of the virus, and the problems of drug resistance may reduce the success rates of HAART in the future. Therefore, a prophylactic vaccine is necessary and urgent. In the absence of a prophylactic vaccine, immune stimulation of HIV-infected people under HAART may also be attempted in order to regain immunological competence and control of the virus (therapeutic vaccination). Studies in monkeys and analysis of HIV-infected long-term survivors indicated that induction of a strong, polyclonal T cell response is most important for efficient control of the virus, while neutralizing antibodies play a role as well. Therefore, induction of a sustained and polyclonal response targeting both arms of the immune system may be essential to achieve protection.

Initial attempts to develop a vaccine mainly involved the viral glycoproteins and were unsuccessful [reviewed in ref. 6]. More recently, several approaches, including DNA vaccines, recombinant viral vectors and virus-like particles, have been developed and are currently being tested in animal experiments. Promising results have been reported for so-called prime-boost regimens. Recently, several groups showed that applying a DNA vaccine cocktail (expression vectors for several viral proteins) followed by boosting with a recombinant viral vector (either replication-defective adenovirus or modified vaccinia virus strain Ankara carrying HIV-specific sequences) can successfully protect monkeys from disease [reviewed in ref. 7] [8, 9]. The animals were challenged with a pathogenic SIV/HIV hybrid virus 6 months after vaccination was completed. Importantly, all monkeys were infected by the challenge virus, but there was no development of disease over a 5-month period in the vaccinated animals, while the control animals succumbed to AIDS during the observation period. Vaccination with another recombinant viral vector expressing several HIV proteins has already been evaluated in the recently completed ALVAC (canary pox vectored HIV vaccine) trial in Uganda. This study used a canary pox vector, which is replication defective in human cells, for vaccination and provided important information on the safety of this vector, as well as regarding the interaction with representatives from the communities, the need for regulatory control mechanisms and how to deal with widespread public and media fear about the risks of participation in such a trial [10].

Another phase I clinical trial applying a DNA vaccine-modified vaccinia virus strain Ankara prime-boost regimen has recently been initiated in Oxford, UK, and in Nairobi, Kenya, by the group of A. McMichael with support from the International AIDS Vaccine Initiative (for further information, visit www.iavi.org and www.oxavi.org). It has been announced that similar vaccination studies using DNA and recombinant adenovirus-based vaccines will be started in the near future by Merck & Co. in cooperation with the HIV Vaccine Trials Network (www.hvtn.org). Another vaccine trial, recently announced by GlaxoSmithKline Biologicals in collaboration with the HIV Vaccine Trials Network, will rely on immunization with several HIV-derived proteins formulated with a novel adjuvant.

These studies will certainly be important steps towards an HIV vaccine, but there are many open questions and obstacles remaining. These include the long-term effectiveness of vaccines, their potential to be protective in humans, the high level of variation and viral escape and in particular the long-term prognosis of those infected despite vaccination. It must be emphasized that none of the vaccines tested to date induced a sterilizing immunity, and all animals subsequently challenged became infected, although there was a clear protective effect regarding the development of disease. Importantly, a recent report provided evidence that at least one vaccinated and subsequently challenged animal showed a delayed onset of immunodeficiency caused by the challenge virus. This could be attributed to a mutation in a cytotoxic T lymphocyte epitope of the challenge virus leading to escape from the protective immune response [11]. The frequency and kinetics of such escape will affect the ultimate usefulness of vaccines that are based on this approach. Although disappointing, this result is not really surprising, since escape from cytotoxic T lymphocyte control has been reported before. It may be predicted, however, that such escape from initial control of replication will increase over time,

which could represent a fundamental challenge to the idea of containing, rather than preventing, HIV infection [12]. Nevertheless, all experimental data suggest that these vaccines slow the course of the disease significantly, which must be considered to be a major step forward compared to the current situation.

Based on other examples, one may predict that the most effective vaccine would be an attenuated virus which replicates in the vaccinated host, but lacks the capacity to induce disease. However, given that such a virus would still have the capacity to integrate into the host's genome and that the possibility of long-term pathogenic effects can never be excluded, such an approach is clearly too dangerous and should not be pursued. It will be important, however, to apply this and other models to understand how protective immunity can be achieved and to use this information to develop vaccination strategies which may succeed in achieving the currently unrealistic goal of sterilizing immunity.

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Vaccine Development Using the Simian Immunodeficiency Virus Model for AIDS

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Key Words
AIDS · Vaccine · Primate · Whole inactivated virus · Lipopeptide · Live attenuated virus

Abstract
Objective: A number of trials in primates using a wide range of putative vaccines based on simian immunodeficiency virus (SIV) have been performed and are summarised here. ***Methods:*** Rhesus macaques and African green monkey (AGMs) were immunised with the test vaccines and challenged with live virus to test the efficacy of the induced or transferred immune responses to protect from infection or disease development. ***Results:*** In initial studies, successful protection from challenge by whole inactivated virus vaccines was subsequently shown to be mediated by immune responses to human cell rather than viral proteins. Passive transfer of neutralising antibodies failed to protect against challenge. The induction of SIV-specific cytotoxic T lymphocytes (CTLs) using lipopeptides also failed to protect from infection, and whereas the frequency of post-infection CTLs (as measured by limiting dilution CTL assay and MHC/tetramer staining) correlated inversely with the cell-associated virus load, no correlation with the plasma virus load was observed. No immunological correlation of protection could be identified in macaques immunised with live attenuated SIV, with sterilising immunity being induced as early as 10 weeks after infection with the attenuated virus. Similarly, whole inactivated virus and passive IgG transfer failed to protect the natural host AGM species from challenge with apathogenic SIVagm, although live attenuated SIVagm afforded some protection despite the lack of overt vaccine virus replication. ***Conclusions:*** 'Traditional' types of vaccine are either ineffective or inappropriate for use in humans. Current efforts are therefore focusing on the rapidly evolving field of genetic vaccines based on vector DNA and recombinant, attenuated viral and bacterial vectors.

This article is dedicated to Harald zur Hausen on the occasion of his retirement as head of the German Cancer Research Center (Deutsches Krebsforschungszentrum) in Heidelberg with gratitude and appreciation for 20 years of leadership.

Introduction

At present, over 40 million people worldwide are living with HIV/AIDS and over 25 million have already succumbed to this modern plague. Although recent advances in the design and production of antiviral drugs have been instrumental in prolonging the life expectancy of most HIV-infected individuals in developed countries, the vast

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majority of those infected or at risk of infection have no access to such expensive treatments. With the continuing failure of education and social efforts to stop the spread of HIV, the only real prospect for combatting the pandemic is the development of an inexpensive and effective vaccine.

The early optimism surrounding the prospects for an AIDS vaccine has been tempered by the poor success of vaccine candidates in inducing protective immune responses in animal models or humans. Due to the enormous logistical, political and economical problems associated with an AIDS vaccine trial in human volunteers and the lack of a clear correlate of protective immunity, it is often necessary to address basic questions of vaccine formulation and efficacy in animal models before progressing to clinical trials. By far the most widely used model involves the infection of Asian macaques with one of the pathogenic simian immunodeficiency virus (SIV) isolates available or, more recently, with a simian/human immunodeficiency virus hybrid virus. These models have been instrumental in expanding our knowledge and understanding of the way in which the immunodeficiency viruses interact with the host immune system, leading to its eventual demise, and of the types of immune responses that are able to limit virus replication.

In this paper, we summarise some of the primate vaccine experiments performed at our institutes and discuss the lessons learned from the results.

Materials and Methods

Details of the materials and methods used to generate the data presented and discussed here can be found in previous publications on SIVmac whole inactivated virus [1, 2], SIVagm whole inactivated virus [3], live attenuated SIVmac [4], live attenuated SIVagm [5] and lipopeptide immunogen [6].

Results

SIVmac Whole Inactivated Virus Vaccine

In an initial study performed as part of a European Community Concerted Action and designed to measure the optimal dose and breadth of protection induced by whole inactivated SIVmac immunogen, groups of rhesus macaques were immunised four times with either 0.5 or 0.1 mg of formalin-inactivated, purified SIVmac produced in human T cells. Following intravenous challenge with live, pathogenic SIVmac also grown in human T cells, all four of the high-dose-treated animals and 1 of the 4 low-dose-treated animals were fully protected from infection [2]. No trace of virus replication could be found serologically, by sensitive co-culture of peripheral blood mononuclear cells (PBMC) or by PCR analysis. Such a high degree of 'sterilising immunity' was obviously very encouraging for the prospects of AIDS vaccine development and, due to concerns about the safety of inactivated virus immunogens, a second experiment was performed to identify the protective component of the immunogen [1]. Taking the approach of starting with material known to be effective (whole virus) and sequentially depleting viral components, four immunogen preparations were prepared: (1) whole virus, (2) outer envelope glycoprotein-depleted ('shaved') virus, (3) viral cores and (4) purified Gag protein. Groups of rhesus macaques were repeatedly immunised with these preparations as a prelude to challenge, inducing a range of antiviral immune responses. However, during the course of the study, results from colleagues in the UK were published suggesting that the protection induced by whole virus vaccines was due, at least in part, to immune reactions specific for human cell proteins incorporated into both the immunogen and challenge virus preparations [7]. To address this alarming possibility, half of the animals in each group receiving the sequentially depleted immunogens were challenged with the 'standard' human cell-grown SIVmac stock and the other half with the same virus passaged once through rhesus PBMC (and therefore devoid of human proteins). Although a total of five animals totally resisted infection with the human cell-derived virus, none were protected from the same virus passaged through rhesus cells (table 1). This demonstrated that, indeed, the vigorous protection induced by the whole inactivated virus immunogens was due primarily to xenogeneic rather than antiviral immune responses. As the induction of xenogeneic responses would have little relevance in humans, there would appear to be little chance of a whole inactivated HIV vaccine inducing protective immunity in humans.

To ensure that the failure of the whole inactivated virus vaccines to protect in the absence of xenogeneic reactions was not an artifact of the SIVmac/rhesus macaque model, a vaccine experiment was performed using the African green monkey (AGM)/SIVagm natural host system [3]. AGMs immunised repeatedly with inactivated SIVagm and challenged with the homologous live virus grown in AGM PBMC were not protected despite the induction of strong humoral antibody responses (table 1). The lack of efficacy of whole virus immunogens was therefore proved in two distinct primate/SIV systems.

Table 1. Summary of experiments using whole inactivated virus vaccines and passive antibody transfer

Immunogen/treatment	Dose	Challenge virus	Number of animals protected
Whole inactivated SIVmac	4 × 0.1 mg	human cell-derived SIVmac	1/4
	4 × 0.5 mg		4/4
Whole inactivated SIVmac	4 × 20 μg Gag equivalent	human cell-derived SIVmac	2/2
		rhesus cell-derived SIVmac	0/2
Envelope-depleted virus	4 × 20 μg Gag equivalent	human cell-derived SIVmac	2/2
		rhesus cell-derived SIVmac	0/2
Viral cores	4 × 20 μg Gag equivalent	human cell-derived SIVmac	1/2
		rhesus cell-derived SIVmac	0/2
Purified Gag protein	4 × 20 μg Gag equivalent	human cell-derived SIVmac	0/2
		rhesus cell-derived SIVmac	0/2
Whole inactivated SIVagm	4 × 0.35 mg	AGM cell-derived SIVagm	0/2
SIVmac-specific IgG	1 × 65 mg/kg body weight	rhesus cell-derived SIVmac	0/2
SIVagm-specific IgG	1 × 500 mg/kg body weight	AGM cell-derived SIVagm	0/2

Passive Antibody Transfer

Whole inactivated virus immunogens are designed to induce strong antibody responses and are not expected to stimulate the production of antiviral cytotoxic T lymphocytes (CTLs). Although the anti-SIV antibody responses induced by such immunogens were unable to protect against infection, it was possible that antibodies induced by live infection would perform better. Uninfected rhesus macaques were therefore injected with high doses of IgG purified from the plasma of SIVmac-infected monkeys shortly before challenge with the homologous virus [1]. Despite conferring high titres of neutralising antibodies in the blood of recipient animals, none were protected from challenge (table 1). Furthermore, AGMs receiving extremely high amounts of anti-SIVagm IgG from infected animals were also unable to resist challenge (table 1) [3]. These results demonstrated that even antibodies produced in response to infection were ineffective alone against infection.

Live Attenuated Virus Vaccines

Historically, live attenuated virus vaccines have been the most successful in combatting a variety of major viral epidemics such as smallpox and polio. The initial demonstration that attenuated forms of SIV were both apparently harmless and also able to induce 'sterilising' immunity to pathogenic SIV challenge was therefore very promising [8, 9]. However, by its very nature, a live attenuated retroviral vaccine would maintain the ability to integrate into the host genome with unknown long-term effects. For this and other reasons, the use of a live attenuated HIV vaccine in humans was generally considered to be too dangerous. Rather, research focussed on identifying the immunologic mechanisms of protection in the hope that based on this knowledge, it would be possible to stimulate the same mechanisms using a safe vaccine.

As part of a second European Community collaborative project designed to determine the breadth of protection and the mechanisms of protective immunity induced by live attenuated SIV, rhesus macaques were infected with the naturally occurring C8 variant of SIVmac32H, which is primarily attenuated by a four-amino acid deletion in the Nef protein. Experiments using SIVs with large Nef deletions indicated that protective immunity required up to 40 weeks to develop, possibly due to the slow production of high-affinity antibodies [10], although C8 was known to be effective as early as 20 weeks. To facilitate the identification of protective immune responses, the C8-infected monkeys at our institute were therefore challenged with pathogenic SIVmac32H at 10 or 20 weeks [4]. Surprisingly, three of the four animals challenged after 10 weeks were nevertheless fully protected from challenge, as were three of four of those challenged at 20 weeks. Analysis of the antiviral immune responses failed to identify a clear correlate of immunity, however.

As the Nef-defective C8 virus was apparently too efficient at inducing protection, a series of SIVmac BK28 mutants containing large deletions in the *nef* gene were selected [11]. These viruses, when used as candidate vaccines, were clearly more attenuated in vivo than C8, and

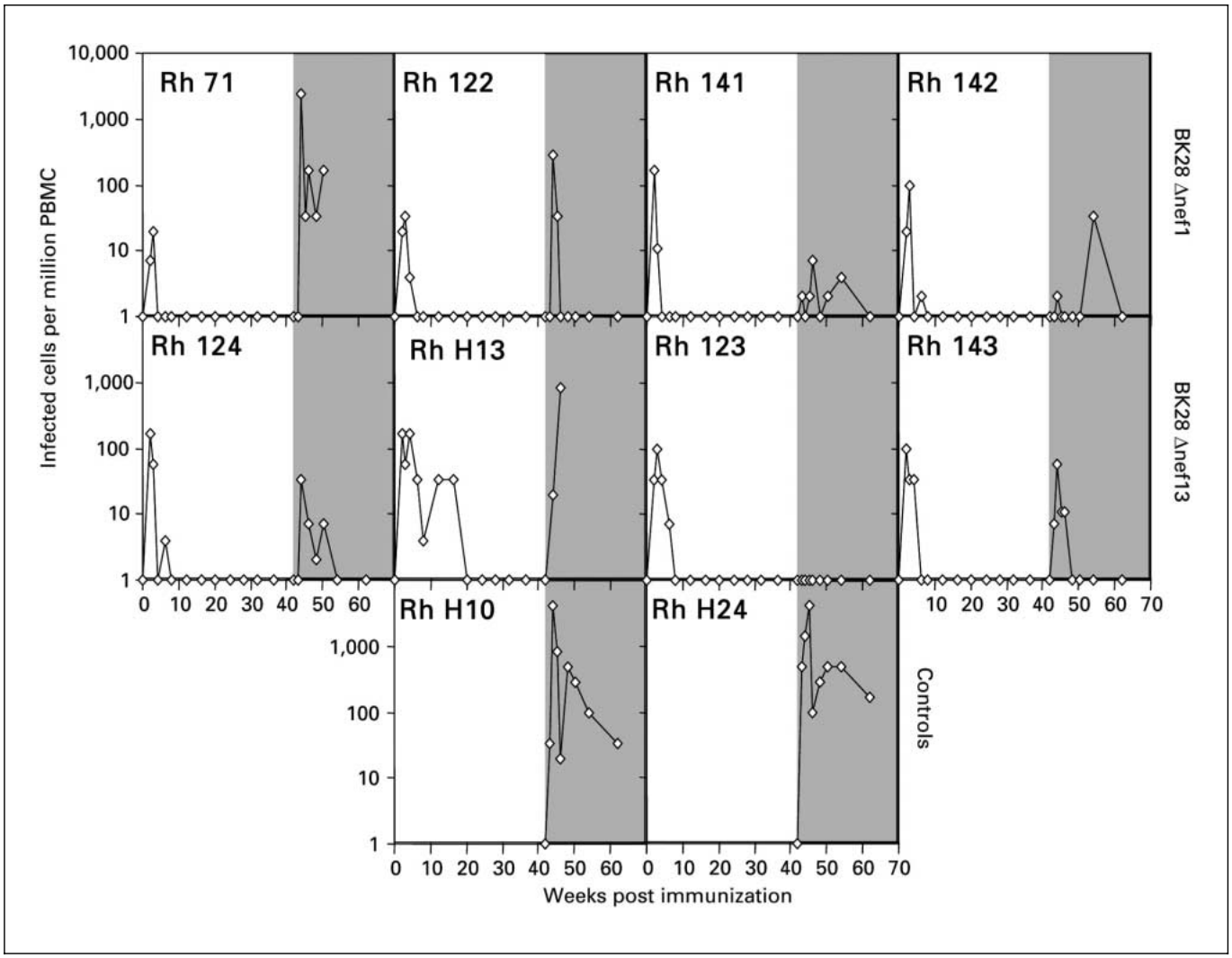

Fig. 1. Cell-associated virus loads in rhesus macaques 'vaccinated' with live attenuated SIVmac mutants (BK28 Δnef1 and BK28 Δnef13) and challenged with a full-length pathogenic wild-type SIVmac32H. All animals except Rh 123 showed a second peak in viral load after challenge (shaded area).

challenge was correspondingly performed after 40 weeks. In this case, however, only one of eight immunised macaques resisted challenge (fig. 1).

In both the C8 and the BK28 Δnef experiments, the balance of protected versus unprotected animals was too biased one way or the other to make clear identification of immune responses associated with protection possible. However, as all 16 animals had been challenged with the same virus stock at the same dose via the same route, it was possible at least to compare the levels of different antiviral antibodies at the time of challenge. Although there was a tendency for protected animals to have higher titres of binding antibodies, high-affinity binding antibodies and homologous neutralising antibodies, there were cases of protected animals with low titres and unprotected animals with high titres (fig. 2). The generation of these antibodies did not therefore appear to be the sole mechanism of protection.

Taking advantage once again of the AGM/SIVagm natural host system, AGMs were 'immunised' with a highly attenuated Nef-deleted mutant of SIVagm [5]. Replication of this virus was barely detectable in vivo and no immune responses could be detected. However, upon challenge with the wild-type SIVagm, rapid anamnestic

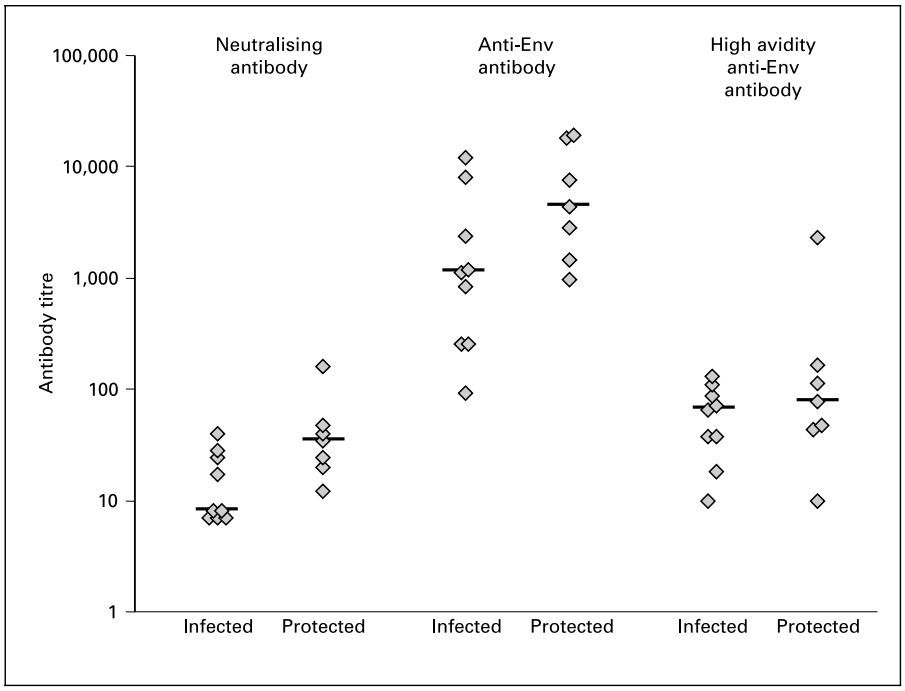

Fig. 2. Comparison of antibody levels and protection on the day of challenge in rhesus macaques previously vaccinated with different live attenuated SIVmac mutants (SIVmac32H C8, BK28 Δnef1 and BK28 Δnef13). All animals were challenged with the same dose, route and virus stock of SIVmac32H. Titres in individual animals are shown, along with the median titre for each group (marked by a horizontal bar). Env = Envelope glycoprotein.

immune responses and suppression or prevention of challenge virus replication were observed. Clearly, the mechanisms of protection induced by live attenuated SIVs are critically dependent on the model system used, the degree of attenuation, the 'aggressiveness' of the challenge virus and the timing of the challenge. This makes identification of general immune correlates of protection (if they exist) very difficult.

Synthetic Peptide Vaccines

The failure of whole inactivated virus vaccines or passive antibody transfer and the lack of correlation between antibody levels and protection in Δnef-immunised macaques indicated that protection requires the stimulation of cellular effector mechanisms, in particular the CTL response, in addition to antibodies. CTLs are generally induced as a result of active infection (e.g. attenuated viruses or live recombinant vectors), although modern technologies such as the use of naked DNA or virus-like particles are overcoming these restrictions. One of the safer methods for inducing CTLs is to use modified synthetic peptides corresponding to known CTL epitopes in the viral proteins [12]. Peptides based on SIVmac CTL epitopes were therefore synthesised, modified by addition of a tripalmitic acid tail ('lipopeptide'), and used to immunise macaques [6]. In addition, multiple antigenic peptides corresponding to neutralising antibody epitopes were used to induce humoral effector mechanisms. As, at the time of the experiment, only one macaque MHC molecule (MaMu-A*01) with a matching SIV CTL epitope (p11C) was known [13], all animals were prescreened and selected for this haplotype.

The peptide immunogens were indeed found to induce antiviral CTLs and neutralising antibodies, albeit at low levels. Although upon challenge with pathogenic SIVmac, no animal remained virus free, a significant anamnestic CTL response was observed with some animals having p11C-specific CTLp accounting for as many as 1 in 20 of their total circulating PBMC, as measured by limiting dilution CTL assay. These extraordinarily high frequencies of CTLs called into question the accuracy or specificity of the assay used. However, with the advent of tetramer technology, it was possible to confirm these data by directly staining and counting p11C-specific CTLs via FACS, with up to 25% of all circulating CD8+ cells binding the tetrameric complexes (fig. 3).

Although the animals were not protected from infection, it was interesting to note that the strength of the CTL response following challenge correlated well with the suppression of the number of infected cells in circulation. However, when compared with the level of viraemia, i.e. the number of SIV RNA copies per millilitre of plasma,

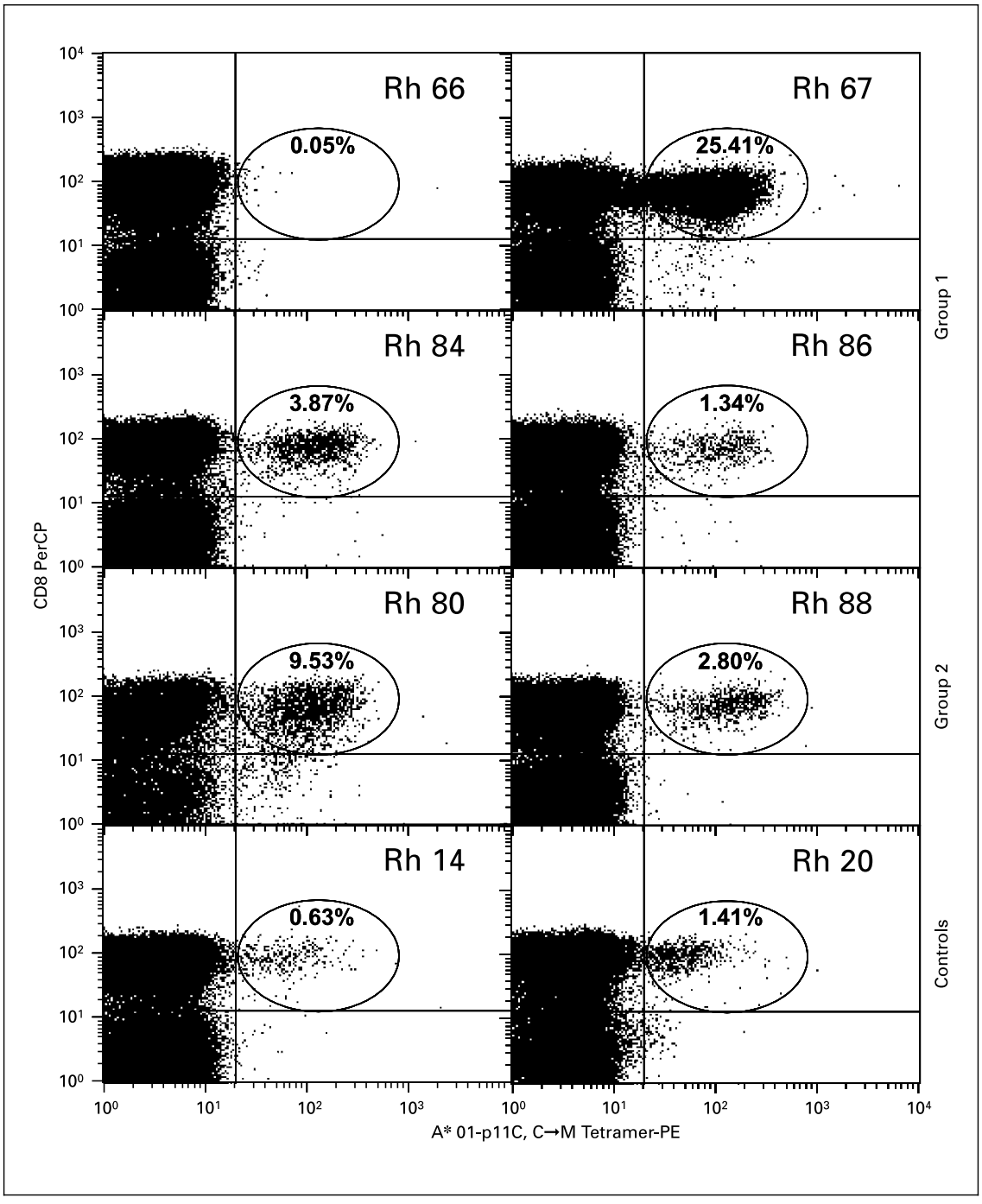

Fig. 3. FACS analyses of PBMC taken 3 weeks after challenge for binding of the Mamu-A*01/p11C,C→M tetramer. Figures within the selected areas refer to the percentage of CD3+CD8+ cells staining positive for the tetramers.

no such correlation was seen [6]. Indeed, the two control animals that had not been immunised appeared to have lower plasma viral loads than the immunised macaques. This suggests that whereas immunisation with lipopeptides corresponding to single CTL epitopes had primed the immune response to react to and eliminate infected cells in circulation, these CTLs were unable to suppress virus replication in general. Indeed, focussing on single epitopes may have compromised the immune system's ability to react effectively to multiple epitopes in different viral proteins.

Genetic Vaccines

Much of the current effort to develop AIDS vaccines focuses on the use of 'genetic vaccines' alone or in combination. The simplest and most versatile form of genetic vaccine consists of purified plasmid DNA coding for the virus gene or gene fragment of interest under the control of a strong eukaryotic promoter. Immunisation with such constructs leads to stimulation of both humoral and cellular immune responses similar to those induced by infection itself but without the associated dangers [14]. Although rapid progress has been made in improving the immunogenicity of such 'naked DNA' vaccines, the best results are often achieved by using DNA to prime the immune response followed by administration of the same genes expressed in recombinant vectors. For example, our group is currently performing a study as part of a European Union-funded collaborative project to assess the immunogenicity and protective efficacy of multigenic combination genetic vaccines. In our part of the study, macaques have been primed by plasmid DNA corresponding to the SIV *gag, pol, env, tat, rev* and *nef* genes and will receive one boost with the same genes expressed in replication-defective recombinant modified vaccinia Ankara constructs [15]. After a further two immunisations with all genes expressed by replication-defective recombinant Semliki Forest virus [16], the macaques will receive a mucosal challenge with SIV. Pooling the data of all participant laboratories should enable the identification of the optimal immunisation strategy and the correlate(s) of protective immunity.

Discussion

The vaccine experiments summarised here describe advances and setbacks experienced in our own laboratory but in many ways are representative of progress in the field of AIDS vaccine development in general. The early optimism stimulated by the cloning, expression and immunogenicity of HIV proteins [17] was dampened by the relatively poor capacity to protect in animal models or even in human volunteers. As described here, classical vaccines such as whole inactivated virus or live attenuated virus, while conferring a high degree of protection in the macaque model, were either ineffective in a more relevant setting or potentially too dangerous to be used in humans. Indeed, the cautions taken the live attenuated vaccines were shown to have been justified by the observation that the vaccine viruses themselves can induce rapid AIDS development in newborn macaques [18] and eventually even in adult monkeys [19]. Nevertheless, protection by such vaccines is prolonged and vigorous, and identification of the immune correlates remains a high priority in many groups, including our own.

The recent advances made in the use of genetic vaccines to stimulate both arms of the immune response have rekindled the early optimism concerning the prospects for an AIDS vaccine. A first-generation vaccine is now expected to 'only' suppress viral loads to the point that prevents (or at least delays) disease development and renders the vaccinee non-infectious for others. Such a vaccine would have an enormous impact on the spread of HIV and the lives of those at risk of infection. Different types of genetic vaccines are becoming increasingly successful in achieving this degree of vaccine effect in the animal model, and clinical trials using corresponding HIV immunogens are planned or are already in progress. We can only hope that such trials prove to be successful and that an effective AIDS vaccine will become available to those most in need in the not too distant future.

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Impact of Codon Usage Modification on T Cell Immunogenicity and Longevity of HIV-1 Gag-Specific DNA Vaccines

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Key Words
HIV · Synthetic genes · DNA vaccine · T cell immunogenicity

Abstract
In this study, we analyzed the in vitro expression, potency and longevity of immune responses induced in a Balb/c mouse model by a synthetic HIV-1 *gag* gene exhibiting a codon usage that was adapted to that of highly expressed mammalian genes *(syngag).* In contrast to a vector containing the wild-type (wt) *gag* gene, the syngag construct enabled highly efficient Gag expression in both human and rodent cell lines in complete absence of Rev and Rev-responsive element. Immunization of Balb/c mice with the wt gag plasmid DNA induced only weak and inconsistent humoral immune responses. Mice vaccinated by syngag but not wt gag developed substantial and highly consistent Gag-specific antibody titers showing a clear T helper 1 polarization even with low doses of DNA. Moreover, vaccinated mice developed a strong Gag-specific cellular immune response, including cytotoxic T cells, which was not observed in wt gag-immunized animals. Both humoral and cellular immunity were efficient and lasted for more than 20 weeks. Furthermore, the induction of the humoral as well as the cellular immune response was independent of the immunization route (intramuscular or subcutaneous). These results clearly show the advantages of codon-optimized genes with respect to the expression and immunogenicity of plasmid DNA constructs, making them promising vaccine candidates for further studies.

Introduction

Infection of humans by human immunodeficiency virus type-1 (HIV-1) leads in the vast majority of reported cases to immunodeficiency, severe clinical symptoms and death often within less than 10 years. The development of a prophylactic and therapeutic HIV-1 vaccine therefore remains one of the most desirable objectives of research aimed at controlling the current AIDS epidemic. Abundant clinical evidence suggests that, besides neutralizing antibodies, other effectors of the cellular immune response such as cytotoxic T lymphocytes (CTLs) may be of importance in controlling HIV-1 infection [1–3].

A strong antiviral cytotoxic activity has been shown to correlate temporally with the clearance of viremia in primary infection [3–6], and it could be further demonstrated that Gag-specific T helper cell and CTL responses

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correlates inversely with the viral load [7, 8]. In addition, a decline of Gag-specific CTL precursors was shown to coincide with a drop in CD4, increasing viral load and disease progression in chronically infected persons [9]. Furthermore, evidence from studies in long-term nonprogressing individuals suggests that the quality of epitope recognition by the individual's CTLs may account for a long-lasting control of viral replication [10, 11]. In this regard, Gag-derived immunogens seem to be very useful for vaccine development and immunotherapeutic interventions because the protein is relatively conserved among diverse HIV-1 subtypes, and broad cross-clade CTL recognition directed against Gag-specific targets has been documented [12–14].

Over the last decade, the direct injection of naked DNA has been shown to be a promising approach to inducing humoral and cellular immune responses [15–17]. There seem to be potential advantages of using plasmid DNA for immunization compared to traditional protein vaccination due to the induction of strong T helper 1 (Th1) and CTL responses, prolonged antigen expression and long-lived effector activity [17–19]. Plasmids expressing nonoptimized HIV-1-derived genes have recently been shown to induce humoral and cellular immune responses in rodents [16, 18], nonhuman primates [20–22] and in phase I studies in humans [23, 24]. However, in most of these studies, both the titers of induced antibodies and specific CTLs were transient and low. Several factors are suggested to be essential for the efficacy of a DNA expression vector, e.g. the quality of foreign gene expression unit, composition of DNA backbone and gene-regulatory elements [17]. It has also been shown that the route and method of immunization can be important modulators of DNA vaccination [17, 25].

We recently reported the construction of a synthetic *gag* gene exhibiting a codon usage that was adapted to that of highly expressed mammalian genes. This resulted in a Rev/Rev-responsive element (RRE)-independent, stable and increased expression of Gag and the induction of strong humoral and cellular immune responses following DNA immunization of mice compared to wild-type (wt) gag constructs [26–28].

In the present study, we compared the expression rates and immunogenicity of vectors containing a wt or a codon-optimized *gag* gene in different cell lines of mammalian origin. Furthermore, we characterized more closely the induction of immune responses by the superior synthetic DNA vaccine construct with respect to (1) dose dependency, (2) influence of the route of delivery and (3) the longevity of humoral and cellular immunity.

Materials and Methods

Plasmid Constructs

The construction and cloning of UTRgagRRE and the p-syngag plasmid has been described previously in detail [26]. The plasmid pCsRevsg25-GFP (termed p-Rev in this report), expressing Rev fused to green fluorescent protein (GFP), was kindly provided by Dr. Marcus Neumann (GSF, Munich, Germany).

Virus-Like Particles and Synthetic Peptides

Virus-like particles (VLPs) were produced as described in detail previously [29]. The p24(CA)-derived 9-mer peptide A9I (AMQMLKETI) and the 10-mer peptides E10F (EPFRDYVDRF) and R10I (RGPGRAFVTI) were purchased from Toplab (Martinsried, Germany). Recombinant p24 was purchased from Mikrogen (Munich, Germany).

Cell Lines and Transfections

The H-2^d mastocytoma cell line P815 (TIB 64) and the H-2^d B-lymphoma cell line A20 (TIB 208) were obtained from the American Type Culture Collection (Rockville, Md., USA). P815 and A20 cells were propagated in RPMI medium supplemented with 5% (v/v) heat-inactivated FCS, 50 μ*M* 2-mercaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin. CHO (Chinese hamster ovary cells), H1299 (human lung carcinoma cells) and C2C12 (mouse muscle cells) were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% FCS, 2 m*M* *L*-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. All mammalian cell lines were maintained in a humidified atmosphere with 7% CO_2 at 37°.

The cells were transfected by the calcium coprecipitation technique as described previously [26]. Briefly, 1.5×10^6 C2C12 cells or 3×10^6 H1299 or baby hamster kidney cells were seeded on 100-mm-diameter culture dishes, incubated for 24 h and then transfected with 45 μg of different Nucleobond AX (Macherey-Nagel, Düren, Germany) purified plasmid constructs. Sixteen hours after transfection, the cell culture supernatant was replaced by fresh medium. Cells and supernatants were harvested 48 h after transfection.

Immunoblotting and p24 Capture Assay

Total cell lysates were prepared 48 h after transfection using a triple-detergent buffer system (RIPA) which was supplemented with a cocktail of protease inhibitors (Complete™ Mini Kit, Boehringer Mannheim GmbH, Mannheim, Germany). Immunoblotting and p24 capture assay were performed as described previously in detail [27].

Vaccination of Mice

Female Balb/c mice (Charles River, Sulzfeld, Germany) were housed under specific pathogen-free conditions and injected at the age of 8–12 weeks. Mice were immunized with the indicated plasmid or VLP concentrations by intramuscular saline injection with 50 μl each in both tibialis anterior muscles or by subcutaneous saline injection with 200 μl at the base of tail, followed by indicated booster immunizations with the same doses.

Evaluation of Antibody Responses

Serum was recovered from mice by tail bleeding at the indicated time points after the booster injection. Anti-Gag antibodies were quantified by an end-point dilution ELISA assay (in duplicate) on

samples from individual animals as described before [27]. The reported titers correspond to the reciprocal of the highest serum dilution that gave an optical density value three times higher than the corresponding dilution of a nonimmune system.

Determination of Cytokines in Supernatants

Spleens were recovered under sterile conditions from mice 5 days after the booster injection and the obtained splenic single-cell suspensions were seeded at 2×10^6 cells/ml in RPMI-1640 medium containing 10% heat-inactivated FCS and 1% penicillin-streptomycin (Gibco), in the presence or absence of Gag protein (10 μg/ml). After 48 h of culture, cytokine levels were determined from the precleared supernatant using a commercial ELISA assay following the manufacturer's instructions (Becton Dickinson).

Intracellular IFN-γ Staining and FACS Analysis

IFN-γ expression by CD8+ cells was detected by intracellular staining. Splenocytes were stimulated with 10 μ*M* peptide in RPMI medium or medium alone as negative control for 6 h including brefeldin A (5 mg/ml) for the whole incubation time. Cells were harvested and transferred to U-bottomed microtiter plates (Greiner, Frickenhausen, Germany), washed twice in FACS buffer I [PBS without Mg^{2+}/Ca^{2+}, 1% FCS, 0.1% (w/v) sodium azide], incubated (10 min, 4°) with purified 2.4G2 antibody to block nonspecific binding of antibody to receptors for the invariant region of immunoglobulin (FcR), washed with staining buffer, surface stained with anti-CD4-FITC (catalog No. 553047, BD, Heidelberg, Germany) and anti-CD8-APC (catalog No. 553035, BD), washed twice with FACS buffer II [PBS without Mg^{2+}/Ca^{2+}, 0.1% (w/v) sodium azide], resuspended in 100 μl of Cytofix/Cytoperm (4% paraformaldehyde, 1% saponin) solution for 20 min at 4° and washed twice in 200 μl of Perm/Wash (PBS without Mg^{2+}/Ca^{2+}, 0.1% saponin) solution. Fixed and permeabilized cells were resuspended in 100 μl of Perm/Wash solution and stained for 25 min at 4° with anti-IFN-γ-phycoerythrin (catalog No. 554412, BD) or the corresponding phycoerythrin-conjugated rat IgG1 isotype control antibody. Cells were washed twice in Perm/Wash solution and suspended in FACS buffer I. Cells were analyzed by flow cytometry using a FACS Calibur and CellQuest software (BD). 3×10^4 CD8+ lymphocytes were analyzed.

ELISPOT Assay

96-well multiscreen MAHA-S45 plates (Millipore, Eschborn, Germany) were coated with 50 μl of anti-mouse IFN-γ antibody (catalog No. 554431, BD, Heidelberg, Germany; 1/500 PBS without Mg^{2+}/Ca^{2+}). After incubation overnight at 4°, wells were washed 6 times with blocking buffer (PBS without Mg^{2+}/Ca^{2+}, 10% FCS) and blocked with 200 μl of blocking buffer for 1 h at 37°. 100 μl containing 10^6 isolated spleen cells in RPMI medium and 100 μl of RPMI with 0.2 μg peptide or medium as negative control. After 24 h of incubation at 37°, plates were washed 6 times with washing buffer (PBS without Mg^{2+}/Ca^{2+}, 0.05% Tween 20). Then, wells were incubated with 100 μl of biotinylated anti-mouse IFN-γ antibody (catalog No. 554410, BD; 1/500 in PBS without Mg^{2+}/Ca^{2+}, 0.05% Tween 20, 10% FCS) at room temperature for 2 h and subsequently washed 6 times with washing buffer. Then, 100 μl of streptavidin-alkaline phosphatase (catalog No. 1089161, Roche, Mannheim, Germany; 1/5,000 in PBS without Mg^{2+}/Ca^{2+}, 0.05% Tween 20, 10% FCS) was added and incubated for 1 h at room temperature. Wells were washed 10 times with washing buffer. 50 μl of nitro-blue-tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (catalog No. 49008620, Roche; diluted according to manufacturer's instructions) were added to each well. After incubation at room temperature for about 15–30 min, the reaction was stopped by discarding the substrate solution and washing the plates under running water. The plates were then air dried and colored spots were counted.

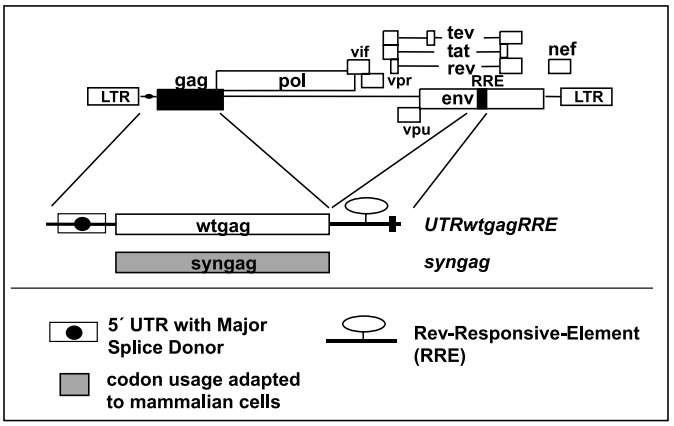

Fig. 1. Schematic representation of the wt and synthetic HIV *gag*-containing expression plasmids used. The wt gag reading frame was fused to the 5′ UTR containing a splice donor and the RRE containing a 3′ splice acceptor. LTR = Long terminal repeats.

CTL Assay

CTL assay by measuring ^{51}Cr release has been described before [27]. The percentage specific release was calculated as [(experimental release – spontaneous release)/(total release – spontaneous release)] × 100. Total counts were measured after adding 1% Triton X-100 to the labeled target cells.

Results

Codon Usage Optimization Enables Increased and Rev-Independent HIV-1 Gag Expression in vitro

The construction of the syngag expression vector p-syngag and the wt gag expression vector UTRwtgagRRE has been previously described in detail [26] (fig. 1). As already reported [27], Gag expression from wt gag without any cis-acting elements is extremely low or even undetectable after transfection of mammalian cells. By adding the authentic untranslated region (UTR) localized at the 5′ end of the HIV-1 *gag* gene, the resulting UTRwtgagRRE gene was expressed in human H1299, mouse C2C12 and hamster CHO cells (fig. 2). Cotransfection with the Rev expression vector p-Rev increased the Gag expression 7-fold in H1299 cells but had no significant influence on the expression in the rodent cell lines, sug-

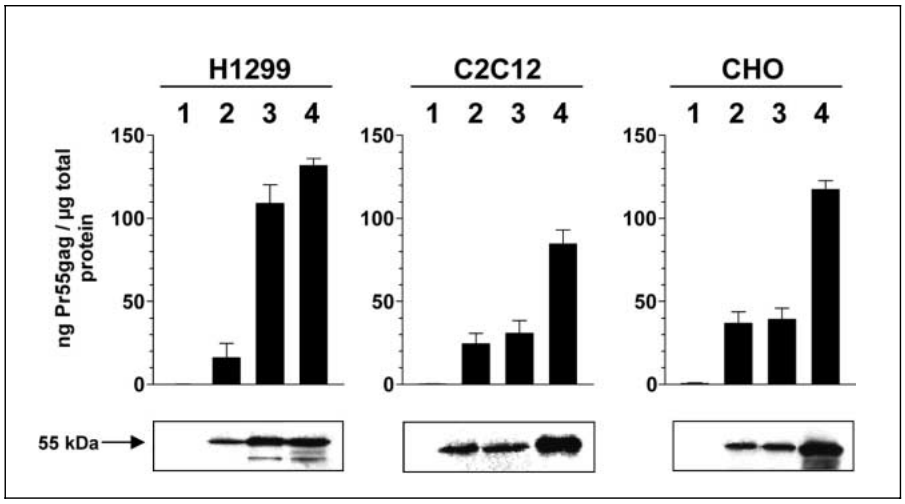

Fig. 2. Expression of HIV gag in different mammalian cell lines. H1299, C2C12 and CHO cells were transiently transfected by calcium phosphate precipitation with (1) pCDNA3, (2) UTRwtgagRRE, (3) UTRwtgagRRE + pRev and (4) syngag. Cells were harvested after 48 h, then 100 µg of total protein of cell lysates were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with a p24-specific antibody. The content of Gag protein in the cell lysates was measured by a p24 capture ELISA using purified Gag for standardization. Bars are the mean of triplicate determinations.

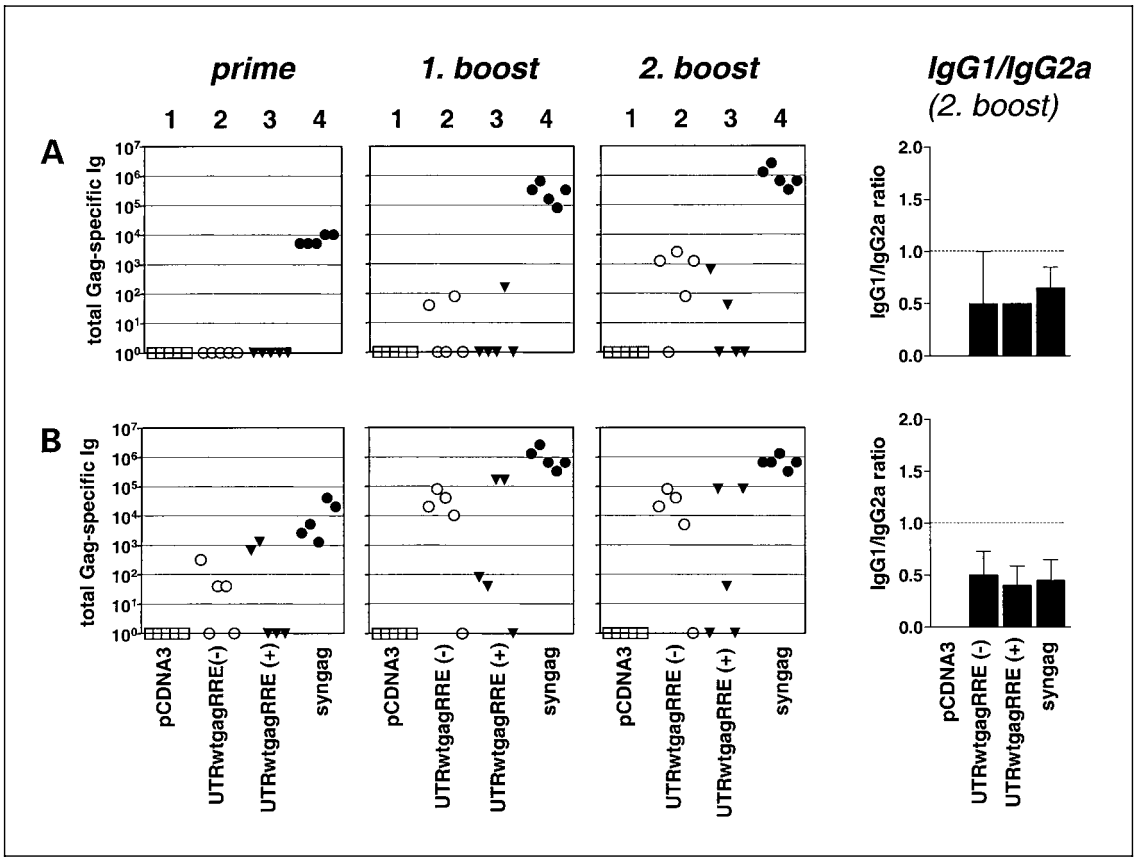

Fig. 3. Humoral immune response after intramuscular immunization of Balb/c mice. Mice were immunized and boostered with 20 µg (**A**) or 100 µg (**B**) of total plasmid DNA of (1) pCDNA33, (2) UTRwtgagRRE + pCDNA33 (2:1), (3) UTRwtgagRRE + pRev (2:1) and (4) syngag + pCDNA33 (2:1). Mice were boostered at weeks 3 and 6 and bled 1 week after the initial and booster immunizations. Each symbol represents the value of one mouse for anti-Gag-specific immunoglobulin antibodies as determined by end-point dilution ELISA assay. At the right, IgG1 to IgG2a ratios 1 week after the second booster immunization are shown. Bars represent the group mean (n = 5) for anti-Gag titers.

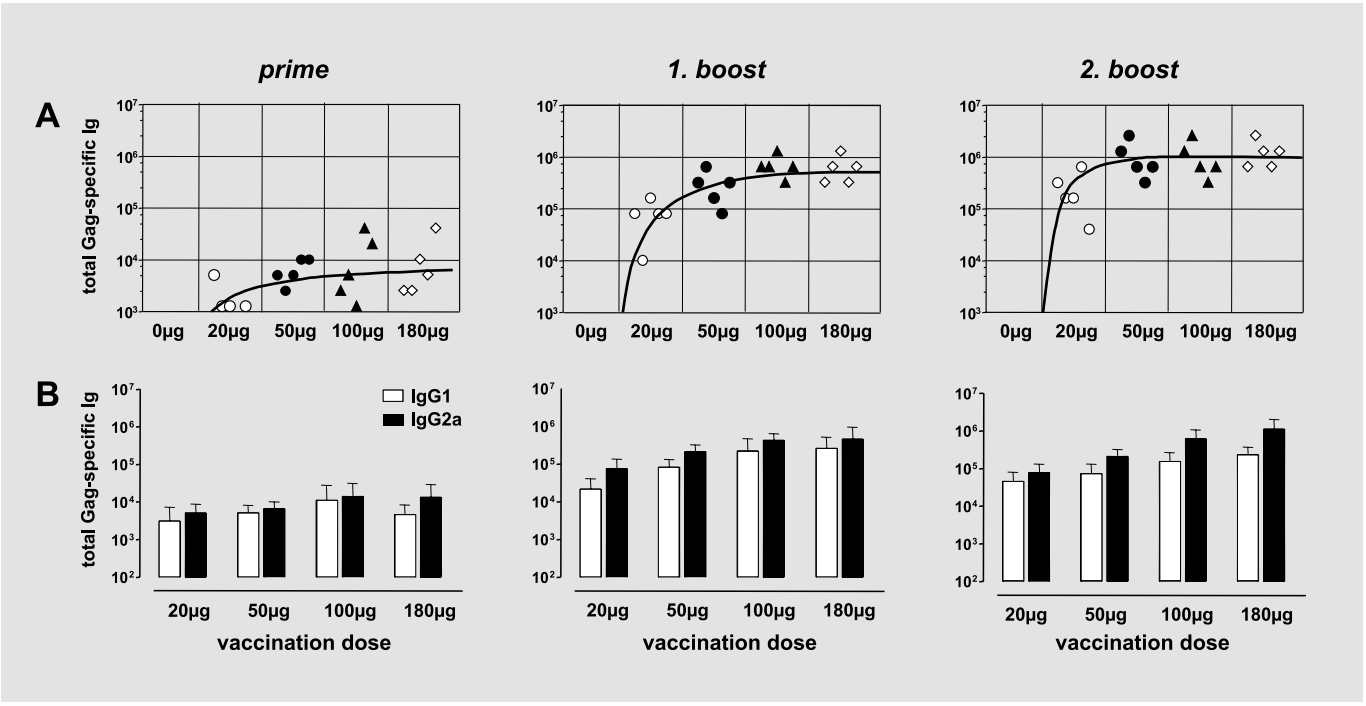

Fig. 4. Dose dependency of the humoral immune response after immunization with syngag plasmid DNA. Balb/c mice were intramuscularly immunized and boostered twice at weeks 3 and 6 with the indicated dose of syngag plasmid DNA. Anti-Gag-specific immunoglobulin titers (**A**) and IgG1 and IgG2a titers (**B**) were determined at weeks 2, 5 and 7. Bars in **B** represent the group mean (n = 5) for anti-Gag titers.

gesting that cell type-specific factors may contribute to the observed Rev responsiveness of UTRwtgagRRE. The highest expression rates were observed after transfection with the codon-optimized syngag vector in all three cell lines, with a 3- to 9-fold higher expression yield compared to the UTRwtgagRRE construct without Rev and a similar ratio in H1299 cells compared to the UTRwtgagRRE plus Rev. This strongly suggests that the adaptation of the gag codon usage to that of highly expressed mammalian genes allows highly efficient Gag expression independent of Rev and cis-acting regulatory elements.

Syngag Plasmid DNA Induces an Increased Humoral Immune Response

To compare the capacity of the different constructs to induce a humoral immune response, we immunized 5 Balb/c mice intramuscularly with 20 or 100 µg of a mix of p-UTRwtgagRRE/pCDNA3, p-UTRwtgagRRE/p-Rev or p-syngag/pCDNA3 and boosted them twice, at weeks 3 and 6. A control group was immunized with equal amounts of pCDNA3 plasmid DNA. Total immunoglobulin and IgG1/IgG2a titers were determined by ELISA (fig. 3). Vaccination with 20 µg of p-UTRwtgagRRE induced only low antibody titers, while using a dose of 100 µg increased the titers significantly, but some mice failed to develop an immune response. The coadministration of p-UTRwtgag and p-Rev neither induced higher nor more consistent antibody titers within the tested group of mice than p-UTRwtgagRRE alone. This finding is in agreement with our previous observation from expression studies (fig. 2), suggesting that Rev/RRE is not functional in rodent cells. Conversely, syngag plasmid DNA induced a substantial and in all mice comparable Gag-specific antibody response even with the low dose of 20 µg already after the initial immunization. Gag-specific antibody responses of all mice, irrespectively of the plasmid construct used, showed a clear Th1 polarization characterized by high titers of IgG2a, resulting in an IgG1/IgG2a ratio <1. No Gag-specific antibody response was detectable at any time point in the sera of control mice.

To evaluate the minimal dose inducing a substantial Gag-specific antibody response and to clarify the question of whether the antibody titers can be further enhanced by escalating plasmid DNA amounts, 5 Balb/c mice were

immunized intramuscularly with 20, 50, 100 or 180 μg of syngag plasmid DNA and boosted twice at weeks 3 and 6. Nonimmunized mice were used as controls. Total immunoglobulin and IgG1/IgG2a titers were determined by ELISA (fig. 4). Almost all mice showed substantial Gag-specific humoral immune responses 1 week after the initial immunization; after the first booster immunization, all mice within each group showed a consistent increase in Gag-specific antibody titers, whereas a second booster immunization revealed only a weak impact on overall Gag-specific titers. The first booster immunization increased the induced antibody titers by 50- to 100-fold, the second boost only 2- to 5-fold, underlining the necessity of at least one booster immunization to obtain substantial antibody titers. Optimal titers were obtained using DNA doses of 50–100 μg, reaching a plateau at ≥100 μg. Irrespective of the DNA dose used and the time point of bleeding, all mice showed a clearly Th1-polarized immune response (fig. 4).

Codon Usage Optimization Enables the Induction of Cellular Immune Responses

Balb/c mice were immunized and boostered twice intramuscularly with 100 μg of p-syngag or UTRwtgag-RRE/Rev plasmid DNA. One week after the last booster immunization, splenic cells were prepared, specifically restimulated in a 5-day mixed lymphocyte tumor cell culture and tested for cytotoxic activity in a standard ^{51}Cr release assay. The p24(CA)-derived 9-mer and 10-mer peptides A9I (AMQMLKETI) and E10F (EPFRDYV-DRF), known to be murine H-2^d-restricted p24 CTL epitopes [30, 31], were used for stimulation and readout; the 10-mer peptide R10I (RGPGRAFVTI), known to be a murine H-2^d-restricted V3/IIIB-specific CTL epitope [32, 33], was used as negative control peptide. In p-syngag-immunized mice, substantial numbers of Gag-specific CTLs were detectable after stimulation with the A9I peptide and, at a slightly reduced extent, after stimulation with E10F (fig. 5A). No CTL priming was observed using the V3/IIIB control peptide R10I, ensuring the specificity of the assay. In contrast, the mixture of the UTRwt-gagRRE and Rev constructs did not induce a Gag-specific cellular immune response, thus underlining the superiority of the codon-optimized gene. Accordingly, in attempts to further characterize the immunogenicity of Gag-based DNA vaccines, we focussed on codon-optimized p-syngag. The induction of a strong cellular immune response was then characterized in more detail by measuring the specific induction of IFN-γ using different assays. For that purpose, spleen cells were stimulated with VLPs (1 or 5 μg), and supernatants were harvested and analyzed for their IFN-γ content after 48 h (fig. 5B). Stimulation with VLPs induced dramatically high IFN-γ titers (up to 17,000 pg/ml) with a relatively high background in pCDNA3-immunized mice, probably due to per se mitogenic properties of the VLPs. A very clear result without any background was seen after a more specific stimulation with the described panel of peptides; A9I again stimulated better than the weaker E10F epitope, whereas the control peptide R10I did not induce any IFN-γ at all. To assess Th2 differentiation, ELISA was performed from aliquots of the same cell culture supernatants to quantify the concentrations of secreted IL-4 and IL-5. In all groups of immunized and nonimmunized mice, no IL-4 or IL-5 secretion was detectable from the supernatants of specifically restimulated as well as nonstimulated splenocytes (data not shown). To quantify the number of IFN-γ-producing cells, an ELISPOT assay was performed; spleen cells were stimulated for 24 h with the most potent A9I peptide and thereafter analyzed. Significant Gag-specific numbers of IFN-γ spots were only detectable in the case of stimulated p-syngag-immunized mice (fig. 5C). Spleen cells were furthermore analyzed to quantify the number of CD8+ lymphocytes producing IFN-γ and were therefore stimulated with the A9I peptide for 6 h in the presence of brefeldin A, surface stained for CD4 and CD8 and thereafter labeled for intracellular IFN-γ. Analysis of the CD8+ lymphocytes showed a strong Gag-specific IFN-γ induction in p-syngag-immunized mice compared to control mice vaccinated with empty pCDNA3 vector (fig. 5D). There was no increase in IFN-γ production by other CD8+ cells detectable, underlining the specificity of the peptide stimulation.

Intramuscular and Subcutaneous Immunization Induce Comparable Immune Responses

Due to the fact that the route and method of DNA delivery are discussed as being important parameters concerning the aimed optimization of DNA vaccination protocols, we compared intramuscular and subcutaneous injection of p-syngag. Mice immunized and boostered twice developed substantial Gag-specific antibody titers with a clear Th1 polarization irrespective of the immunization route used (fig. 6). Nevertheless, subcutaneous immunization showed a delayed increase in antibody induction. However, after the second boost, there was no significant difference between the titers of intramuscularly and subcutaneously vaccinated mice. Furthermore, by both immunization routes, a substantial and comparable CTL activity was detectable in spleen cells isolated 1 week after

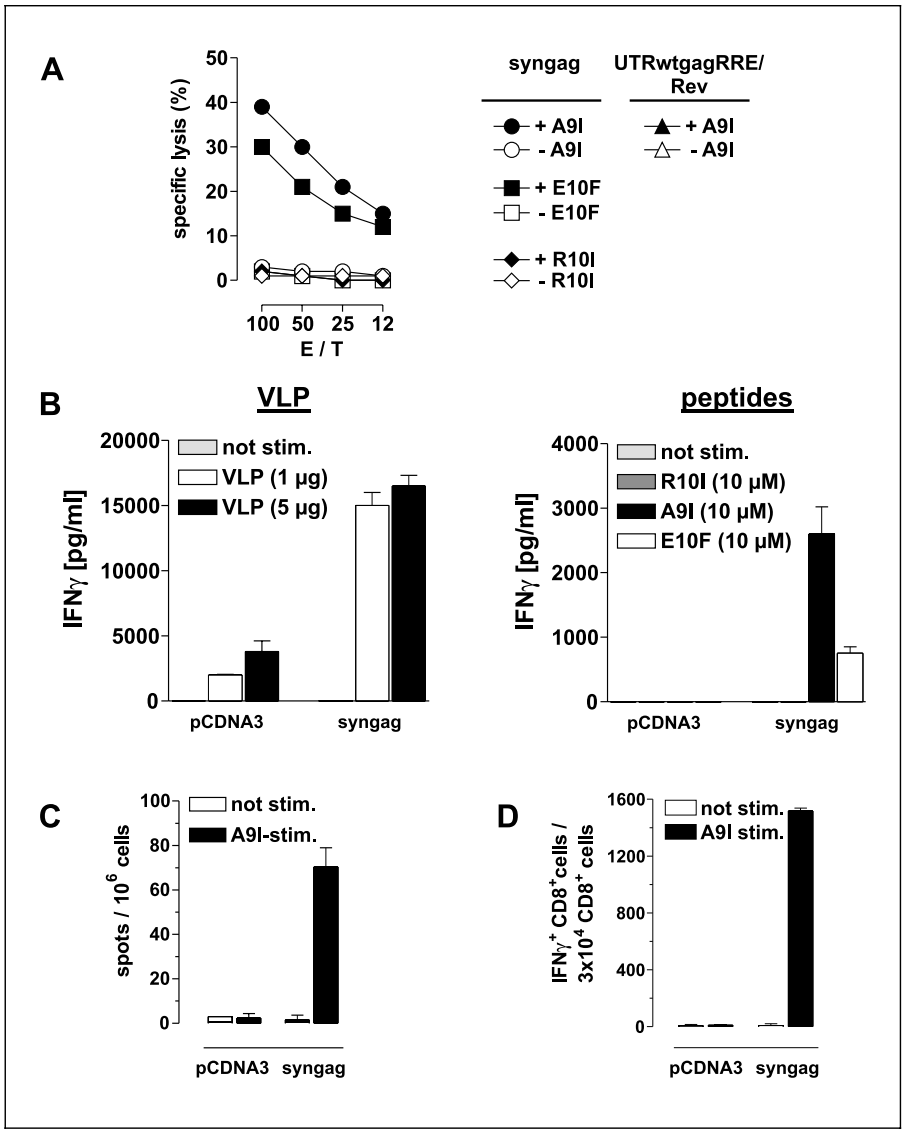

Fig. 5. Cellular immune responses after immunization with syngag plasmid DNA. Balb/c mice were intramuscularly immunized and boostered with 100 µg of syngag or UTRwtgagRRE/Rev plasmid DNA. One week after the second booster, immunized spleen cells were isolated and tested for Gag-specific CTL activity (**A**) and IFN-γ production (**B**–**D**). **A** Spleen cells were stimulated with A20 cells pulsed with the indicated peptide (irradiated with 20,000 rads) for 5 days. Cytotoxic response was read against P815 cells pulsed with the indicated peptide and untreated P815-negative target cells in a standard ^{51}Cr release assay. The data shown are the mean values of triplicate cultures with a standard error less than 15% of the mean. E/T = Effector-target ratio. **B** Spleen cells of mice immunized with pCDNA3 or with syngag plasmid DNA were stimulated with VLPs or the indicated peptides; unstimulated spleen cells served as negative controls. After 48 h, supernatants were harvested and tested for IFN-γ by a commercial ELISA. The bars represent the mean values of triplicate cultures. **C** Spleen cells of mice immunized with pCDNA3 or with syngag plasmid DNA were stimulated with 10 µ*M* A9I peptide. After 24 h, IFN-γ production was determined using a commercial ELISPOT assay. The bars represent the mean values of triplicate cultures. **D** Spleen cells of mice immunized with pCDNA3 or with syngag plasmid DNA were stimulated with 10 µ*M* A9I peptide for 6 h in the presence of brefeldin A; spleen cells not stimulated with peptide served as negative controls. Cells were surface stained for CD8 and intracellularly stained for IFN-γ. 30,000 CD8+ cells of gated lymphocytes were tested for IFN-γ production.

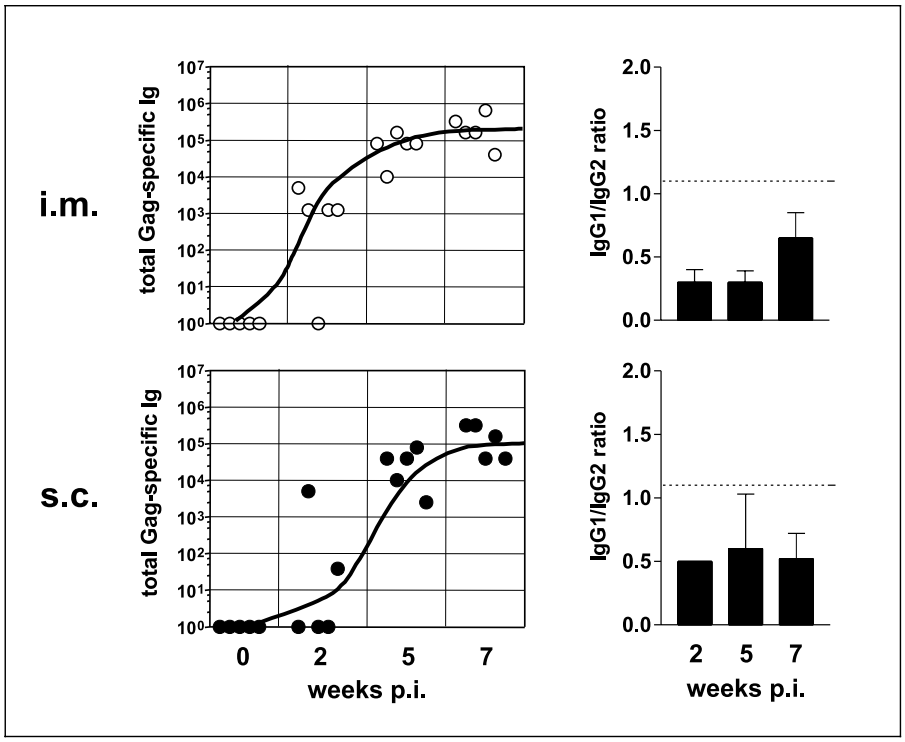

Fig. 6. Influence of immunization route on the Gag-specific humoral immune response. Balb/c mice were immunized with 50 μg of syngag plasmid DNA either by intramuscular (i.m.) or subcutaneous (s.c.) immunization, boostered twice at weeks 3 and 6 and then bled at weeks 2, 5 and 7. Each symbol represents the value of one mouse, while each bar represents the mean values of one group (n = 5) for anti-Gag-specific antibodies as determined by endpoint dilution ELISA assay. p.i. = Postimmunization.

the second booster immunization and tested in a standard ^{51}Cr release assay against A9I peptide-pulsed P815 cells (fig. 7). Conversely, IFN-γ release into cell supernatant after 48 h of stimulation with VLPs (5 or 10 μg) or recombinant p24 protein revealed a significant reduction in the case of subcutaneous immunized mice compared to intramuscular injection. Thus, aside from cytokine release, both immunization routes induce comparable humoral and cellular immune responses.

Immune Responses Induced by p-syngag Are Long Lasting

In order to analyze the progression and longevity of humoral and cellular immune responses, we immunized Balb/c mice with 50 μg of syngag DNA plasmid and boosted them twice. At weeks 2, 5, 7 and 20 after the initial immunization, mice were bled and tested for Gag-specific immunoglobulins. Antibody titers reached their maximum at week 7 and decreased only slightly until week 20 (fig. 8A). Throughout the whole observation period, the antibody response showed a clear Th1 polarization (fig. 8B). Spleen cells isolated at weeks 7 and 20 after the initial plasmid DNA injection and tested in a standard ^{51}Cr release assay against A9I peptide-pulsed P815 cells showed substantial CTL activity (fig. 8C). The lysis capability was reduced only slightly by week 20 compared to week 7. These results clearly demonstrate that vaccination with p-syngag efficiently induces long-lasting humoral as well as cellular immune responses in mice.

Discussion

As we and others were able to show previously, the use of wt gag DNA plasmids for immunization studies is highly limited both for reasons of safety and efficacy. This is primarily due to the Rev/RRE dependency of late HIV-1 gene expression including the wt gag gene. In the absence of Rev, wt gag mRNA is unstable and characterized by a short nuclear half-life, both of which account for a weak or even undetectable Gag expression. Accordingly, previous attempts to use wt gag for plasmid DNA vaccination induced only insufficient or no immunogenicity. We were recently able to show that the Rev-dependent expression from the wt gag gene is the result of a concerted action of multiple cis-acting sequences, involving intragenic AU-rich sequence clusters and the 5′ UTR including the major splice donor site as well as a 3′ RRE [26]. Although the coadministration of p-Rev with UTRwtgagRRE can enhance the expression of Gag in human H1299 cells, there

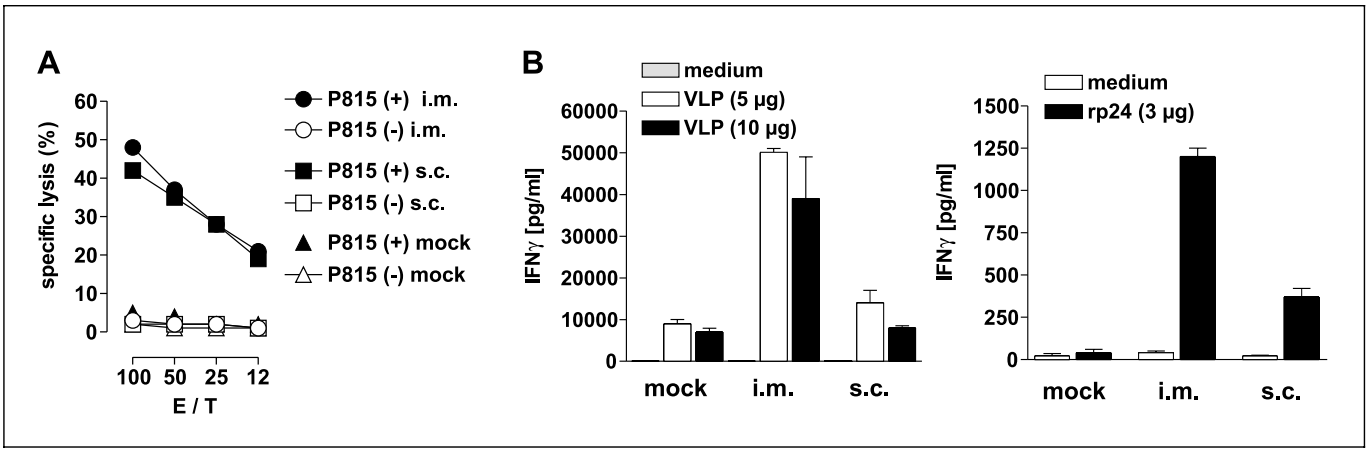

Fig. 7. Influence of immunization route on the Gag-specific cellular immune response. Balb/c mice were immunized with 50 µg of syngag plasmid DNA either by intramuscular (i.m.) or subcutaneous (s.c.) immunization and boostered twice at weeks 3 and 6. One week after the second booster immunization, spleen cells were isolated and tested for Gag-specific CTL activity (**A**) and IFN-γ production (**B**). **A** Spleen cells of immunized and nonimmunized mice were stimulated with A20 cells pulsed with the A9I peptide (irradiated with 20,000 rads) for 5 days. Cytotoxic response was read against P815 cells pulsed with A9I peptide and untreated P815-negative target cells in a standard ^{51}Cr release assay. Data shown are the mean values of triplicate cultures with a standard error less than 15% of the mean. E/T = Effector-target ratio. **B** Spleen cells of immunized and nonimmunized mice were stimulated with VLPs or recombinant p24; unstimulated spleen cells served as negative controls. After 48 h, supernatants were harvested and tested for IFN-γ by a commercial ELISA. The bars represent the mean values of triplicate cultures.

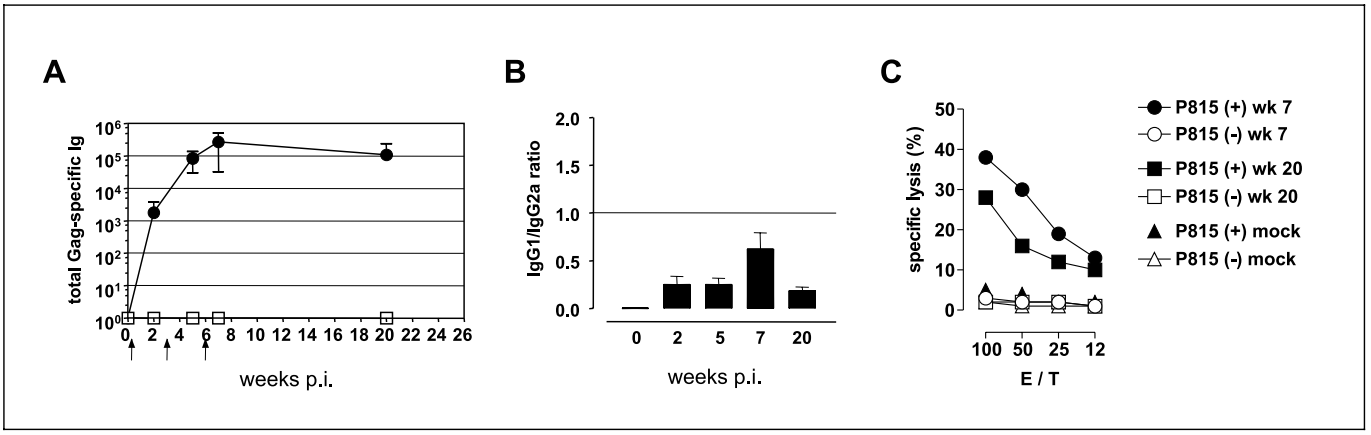

Fig. 8. Kinetics and strength of humoral and cellular immune response induced by intramuscular immunization with syngag plasmid DNA. Balb/c mice were immunized with 50 µg of syngag plasmid DNA and boostered twice at weeks 3 and 6 (time points indicated by arrows). Anti-Gag-specific immunoglobulin titers (**A**) and IgG1 and IgG2a titers (**B**) of immunized and nonimmunized mice were determined at the indicated time points. Symbols and bars represent the group mean (n = 5) for anti-Gag titers. p.i. = Postimmunization. **C** Spleen cells of immunized and nonimmunized mice were stimulated with A20 cells pulsed with the A9I peptide (irradiated with 20,000 rads) for 5 days. Cytotoxic responses were read against P815 cells pulsed with A9I peptide and untreated P815-negative target cells in a standard ^{51}Cr release assay. The data shown are the mean values of triplicate cultures with a standard error less than 15% of the mean. E/T = Effector-target ratio.

is no detectable effect in rodent cell lines, especially in mouse cells. This suggests that a lack of specific cellular [34, 35] and viral factors [36, 37] may be responsible for defects in Gag protein production and the lack of virion assembly in murine cells [27]. Hence, it was not surprising that a mixture of p-UTRwtgagRRE and p-Rev did not induce better humoral immune responses than the UTRwtgagRRE construct alone. However, suggesting that the coadministration of Rev could be successful in humans, there is still a limitation in use due to the high-risk potential of regulatory viral proteins and cis-acting sites within 5′ UTR that entirely overlap the HIV-1 RNA packaging signal [38]. Moreover, a multicomponent plasmid DNA containing gagpol and rev, for example, would be expected to have a highly reduced transfection capability due its size. The utilization of synthetic genes with a codon usage adapted to that of highly expressed genes of mammals represents an effective means to bypass complex viral regulation and, at the same time, to increase immunogenicity and safety profiles of current HIV-specific DNA vaccines. Accordingly, the resulting syngag construct was shown to be expressed independently of Rev/RRE, probably due to the destruction of inhibitory AT-rich sequences of wt gag, and its expression therefore was dramatically enhanced in vitro [26, 28].

Moreover, modification of codon usage within the gag gene resulted in an efficient induction not only of humoral immune responses but also in the efficient priming of Gag-specific CTLs. In contrast, p-UTRwtgagRRE with or without p-Rev was only capable of inducing weak and inconsistent antibody responses and no CTL activity. The improved immunogenicity of *syngag*- compared to *wt gag*-based vaccine vectors obviously correlated with the more efficient in vitro Gag protein expression observed in cultures of murine C2C12 cells. Alternative explanations suggesting that CpG islets randomly generated by increasing the overall CpG content within the synthetic gene could be excluded. Single point mutations destroying CpG motifs without altering the amino acid sequence did not alter the immunogenicity of the tested gag candidate DNA vaccines [27].

Even doses as low as 20 μg per intramuscular injection were sufficient to induce high and consistent antibody titers after a single booster immunization. These humoral responses displayed a clear Th1 bias characterized by an IgG1 to IgG2a ratio clearly less than 1. Furthermore, an initial vaccination with 100 μg of p-syngag was sufficient to induce Gag-specific antibody titers of about 1,000; further booster immunizations increased those titers to >1,000,000. Mice immunized and boostered twice with 100 μg of p-syngag developed strong cytotoxic activity against two known p24 CTL epitopes in Balb/c mice. This CTL response was highly Gag specific, as shown by the missing activity against target cells pulsed with an irrelevant peptide. Furthermore, spleen cells of p-syngag-immunized mice produced high amounts of IFN-γ, the key mediator of Th1-biased cellular immunity, after Gag-specific stimulation, verified by intracellular IFN-γ staining, ELISPOT assays and analysis of cell supernatants. A substantial release of Th2-associated cytokines like IL-4 or IL-5 was not detectable (data not shown).

A number of studies have shown that the method of DNA administration can influence both the strength and nature of immune responses [17]. In the present study, the capacity to induce an efficient immune response was comparable between intramuscular and subcutaneous vaccination. Both routes of plasmid DNA delivery induced a Th1-biased immune response with mostly IgG2a isotypes and comparable titers of Gag-specific immunoglobulins, although the increase in titer was slightly delayed in the case of subcutaneous immunization. The induced cellular immune response measured by ^{51}Cr release assay was comparably high between both groups, although spleen cells of subcutaneously vaccinated mice showed significantly decreased IFN-γ release.

It is worth noting that intramuscular vaccination with p-syngag induces both long-lasting humoral and cellular immune responses. Antibody titers as well as the cytotoxic capacity were nearly unchanged even after 5 months, thus underlining the induction of prolonged memory cell responses.

In sum, these findings confirm and considerably extend data of several groups clearly demonstrating that vaccine vectors exploiting codon optimization of genes to enhance expression yields elicit enhanced humoral and cellular responses compared to vector constructs encoding the corresponding wt gene [39–41]. Due to the obvious advantages of synthetic genes regarding safety profiles and immunogenicity, many current protocols to prevent or treat infectious diseases such as HIV infections or malaria rely on optimized DNA sequences. The advantages of synthetic genes are utilized not only in the context of DNA vaccines. Accordingly, optimized DNA sequences are exploited to foster expression of, for example, viral or prarasitic antigens in cell culture or to enhance the immunogenicity and safety of viral and bacterial vectors such as recombinant vaccinia viruses (e.g. modified vaccinia virus Ankara), alpha viruses (Semliki Forest virus, Venezuelan equine encephalitis virus) or recombinant salmonella or listeria. Of note, one of the most promi-

nent HIV vaccine projects worldwide (EuroVac, www.eurovac.net) utilizes synthetic genes encoding gagpolnef and env polygenes derived from a primary Asian clade C-based HIV-1 isolate for antigen delivery via the above-mentioned plasmid, viral and bacterial vectors. The strain underlying the engineered gene constructs has been selected carefully from amongst now several hundred virus isolates and is representative for the clade C HIV-1 epidemic in China [42]. Preclinical efficacy trials have already proven the potential of the above-mentioned DNA candidate vaccines in a prime boost strategy together with some of the mentioned delivery systems in a nonhuman primate SHIV 89.6P challenge model [in preparation]. Also, based on these promising results, clinical phase I trials in Europe comparing different prime boost strategies in combination with synthetic gene-based DNA vaccines are scheduled for the first quarter of 2003.

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A Short Introduction to Papillomavirus Biology

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Key Words

Papillomavirus · Human papillomavirus · Cervical carcinoma · Capsid proteins · Immortalization · Virus-like particles · Pseudovirion · POD · Receptor · Neutralization

Abstract

In this report, the tropism of papillomaviruses, the structure of virions, the function of viral proteins and the use of pseudovirions for the analysis of the immune response against papillomaviruses and the search for the viral receptor are briefly described.

Papillomaviruses are non-enveloped DNA viruses of higher vertebrates [for a detailed description of the biology of papillomaviruses, see ref. 1]. They are strictly species specific and replicate exclusively in epithelia. To produce a persisting infection, papillomaviruses have to invade basal cells, the only cells capable of dividing in the epithelium. Viral replication is linked to the differentiation program of developing keratinocytes. Synthesis of viral DNA and expression of early genes occurs in cells of the stratum germinativum, and some infected cells may persist here for many years. Synthesis of capsid proteins and assembly of virions occurs only in the terminally differentiated keratinocytes, and viral particles are released from dead cells.

Tropism

In addition to their species specificity and epitheliotropism, some papillomaviruses have a tendency to preferentially infect particular regions of the body. Among the human papillomaviruses (HPV), for example, HPV-1 is associated with deep plantar warts on the feet and HPV-2 with common warts on the hands. Warts (papillomas) arise due to increased cell proliferation stimulated by HPV infection and prolonged retention of the superficial epithelial layers. A large group of HPVs preferentially infect the genital mucosa. Some of these viruses (HPV-6, -11) are associated with condyloma, while others (e.g. HPV-16, -18) are more or less strongly (e.g. HPV-33, -35) associated with metastatic genital cancer. The genital HPVs are sexually transmitted, and the risk of being infected has been estimated to be of the order of 4%/HPV type/lifetime sexual partner [2]. Oral and non-melanoma skin cancer have also been found to be associated with HPVs. Fortunately, our immune system can cope well with HPVs, and most infections pass subclinically. The incidence of infected individuals who develop a genital cancer is less than 0.1%, and additional risk factors, probably some weaknesses of the immune system, are involved. However, since the rate of HPV infection among sexually active women may be as high as 25–40% [3], cervical carcinoma is one of the most frequent forms of cancer in women worldwide. This is why the development of HPV vaccines is urgently needed.

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Virions

Virions isolated from warts are visualized by electron microscopy as spherical particles of icosahedral symmetry and about 55 nm in diameter. The building block of the capsid is the capsomere, a star-shaped pentamer of the major capsid protein, L1. Within capsids, capsomeres are highly cross-linked by disulfide bonds between L1 molecules, and the cysteine residues involved have been identified [4]. A complete viral capsid consists of 360 L1 molecules and some copies of a minor capsid protein, L2, possibly 12 per virion. The capsid accomodates a single molecule of circular double-stranded DNA of about 8,000 bp, associated with histones to form a minichromosome. The genetic organization of all papillomaviruses is very similar. The viral genome carries a non-coding control region and encodes six non-structural, early expressed (E) proteins, in addition to the two late (L) capsid proteins.

Early Proteins

The E1 and E2 proteins are required for replication and maintenance of the viral genome. Full-length and shorter forms of E2 also function as transcriptional activator and repressor, respectively. There is no E3 protein. The E4 protein is encoded in the early region but expressed late in infection. It has been observed to induce the collapse of the cellular cytoskeleton, suggesting that it may facilitate the escape of virus from infected cells. E5 is a small highly hydrophobic protein with some oncogenic potential. It integrates into membranes and activates specific growth factor receptors. E6 and E7 encode the papillomavirus major oncoproteins. Together they can immortalize primary human fibroblasts and keratinocytes. The E6 protein of the high-risk papillomaviruses induces the degradation of p53, the gatekeeper of the cell cycle. Too low levels of p53 promote, of course, uncontrolled growth by preventing the repair of DNA damage or the induction of apoptosis. E6 proteins of low-risk papillomaviruses do not affect p53 levels. The E7 protein binds the retinoblastoma tumor suppressor (pRB) and the related p107 and p130 proteins. These proteins control the activity of the essential transcription factor E2F. E7 liberates active E2F from an inactive pRB-E2F complex by targetting pRB. E2F then drives the cell into S phase and proliferation. The E7 proteins of the high-risk HPVs have high binding constants for pRB and promote its proteolysis, whereas the low-risk HPV E7 proteins bind to the pRB proteins with much lower efficiency. E6 and E7 interact with other proteins but the exact physiologic consequences of these interactions remain to be determined.

Late Proteins

The L1 major capsid proteins spontaneously assemble into virus-like particles (VLPs) when synthesized in recombinant expression systems. The minor capsid protein L2 is not required for the assembly, but it is integrated into VLPs when coexpressed together with L1 [5]. Although VLPs are similar to virions according to cryoelectron microscopy, biochemical analyses have shown that the L1 proteins in VLPs exhibit less disulfide bonding and more exposed C-termini than in virions. Encapsidation of heterologous DNA generating pseudovirions induces conformational shifts in L1 which make its organization more like that in virions [6]. X-ray crystallographic analysis of small HPV-16 VLPs has recently shown that the folding of L1 within capsomeres closely resembles VP1 of polyomaviruses [7].

Although the L2 protein is only a minor component of the viral capsid, it is critical for the infectivity of viruses and pseudovirions [8]. It may facilitate the uptake by cells [9] and somehow helps the infecting DNA to reach the nucleus. Recent studies have shown that L2 seems to play an essential role in viral morphogenesis. It associates with nuclear subdomains called PODs or ND10 [10] and induces specific changes in their protein composition [11]. It also attracts the L1 protein, suggesting that the viral particles may be assembled at these nuclear substructures.

Pseudovirions

Since no efficient cell culture system for the propagation of papillomaviruses is available, VLPs and pseudovirions have been used to analyze the early steps of papillomavirus infection. HeLa cells were shown to bind approximately 2×10^4 VLPs per cell with a dissociation constant of about 100 pM [12]. Proteoglycans carrying heparan sulfate were recently identified as primary papillomavirus receptors using VLPs of HPV-11 [13] and pseudovirions of HPV-16 and HPV-33 [14]. Since proteoglycans are attached to the surface of many types of cells, the high specificity of papillomavirus infection is probably due rather to host regulatory proteins than to receptor interactions.

Immunology

VLPs and pseudovirions have also been extremely useful for the analysis of the immune response to papillomavirus infections. The major neutralizing antigens have been identified on L1, and they are conformational and type specific [15]. Cross-neutralization can therefore only be partially achieved between very closely related types of virus [16]. In contrast, a broadly cross-reactive epitope has been identified on the minor capsid protein L2 [17]. Whereas neutralizing antibodies can prevent infection, T cell responses to early proteins, e.g. E6 and E7, are expected to be critical for viral clearance, and they seem to be type specific as well [18]. However, the relative importance of B and T cell responses to viral determinants responsible for viral regression still remain uncertain.

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T Cell Response to Human Papillomavirus 16 E7 in Mice: Comparison of Cr Release Assay, Intracellular IFN-γ Production, ELISPOT and Tetramer Staining

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Key Words
DNA immunization · HPV 16 E7 · Tetramer staining · ELISPOT · Cr release · Intracellular IFN-γ

Abstract
Successful vaccination against infections by high-risk papillomaviruses aiming at the prevention of cervical cancer most likely requires the induction of neutralizing antibodies and human papillomavirus (HPV)-specific T cells directed against early viral proteins such as E7. Whereas the technology for detection of antibodies is well established, measurement of T cells is more cumbersome and standardization of assays is difficult. By using chromium release assay, ELISPOT, tetramer staining and intracellular IFN-γ assay, we compared the levels of HPV 16 E7-specific T cells obtained after immunization of C57BL/6 mice with different DNA expression vectors. We found that all four assays gave highly comparable results. ELISPOT can be recommended for future studies as it indicates the presence of activated (i.e. IFN-γ-secreting) T cells in a quantitative manner and combines high sensitivity with relatively low T cell demand.

Introduction

Persistent infection by high-risk human papillomaviruses (HPVs) is the main risk factor for the development of cervical cancer. Approximately half a million cases are diagnosed worldwide each year, representing the third most frequent malignant disease in women on a global scale [1]. Considerable efforts are currently being undertaken to develop efficient vaccination strategies aimed at the reduction of the incidence of cervical cancer. The overall plurality of HPVs is also demonstrated in the various cancer-associated HPV types [2]. As there is limited immunological reactivity across types [3], the development of 'the cervical cancer vaccine' is an extremely difficult task. Despite this drawback, there are efforts towards the development of various vaccines against HPV 16, the most common type found in cervical cancer, which accounts for more than 50% of cases worldwide [4]. Based upon the natural history of this disease (infection through sexual contact, development of cancer as a late and rare consequence of persistent infection), the following vaccination scenarios are currently being discussed and suitable vaccines are under development.

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Treatment of Precursor Lesions of Cervical Cancer

Cervical cancer arises through a spectrum of histologically well-defined precursor lesions (cervical intraepithelial neoplasias, CIN) [5]. High-grade CIN is considered a bona fide premalignant tumor with an estimated progression rate of 15% if left untreated [6]. Such lesions are detected during cytology screening programs, diagnosed by colposcopy and histology and are usually removed surgically or by destructive laser therapy or cryotherapy [7]. There is, however, a certain degree of morbidity associated with such treatments that also sometimes may lead to infertility. Since many of the affected women are still at a reproductive age, alternative methods of treatment are being looked for. HPV-specific immune therapy is a valuable option since T cell responses seem to play an important role in controlling this disease [for review, see ref. 8]. The most logical target molecules are the early viral proteins that are expressed during malignant progression of the infected cells, i.e. E6 and E7. In fact, the presence of these molecules appears to be a sine qua non for continuous cell proliferation [9]. The most intensively studied protein is E7, of which several T helper and cytotoxic T cell (CTL) epitopes have been identified both in experimental animals and in humans [10–22]. A variety of experimental vaccines have been analyzed in preclinical studies to verify the concept of an HPV-specific antitumor immune response [for reviews, see ref. 23, 24]. Thus, growth of HPV 16-transformed cells in syngeneic mice was prevented by prior immunization and, under certain circumstances, even existing tumors were induced to regress. The vaccines were administered either (1) as a purified protein (in combination with an appropriate adjuvant or fused to another protein for improved targeting into the MHC class I pathway), (2) as peptides that represent the CTL epitope(s) matching the HLA type of the experimental system, or (3) as expression plasmids injected either as naked DNA or delivered through viral or bacterial vectors [25–28]. Initial clinical trials are ongoing to provide proof of principle that treatment of precursor lesions of cervical (vulval or anal) cancer can be cured immunologically [for reviews, see ref. 23, 29–31]. The possibility of treatment not only of precursor lesions but also of fully developed cervical cancer by HPV-specific immune therapy is also discussed. Although it is unlikely that the immune system can handle a large tumor by itself, there is optimism that patients could benefit in terms of prolonged disease-free intervals and improved quality of life when this treatment is given as an adjunct to the standard therapy.

Postexposure Prevention

Follow-up studies have demonstrated that persistent infection is the prerequisite for development of a high-grade CIN and that infection often precedes clinical symptoms by several years [32, 33]. Therefore, it is assumed that there is a window for cervical cancer prophylaxis even in a postexposure situation. Since the cells express early viral proteins during persistent infection, it is expected that infection can be cleared by the aid of CTLs before the onset of disease. According to current discussions, it seems likely that testing for HPV infection (via detection of the viral DNA) will be included as a complementary measure into cytology screening programs [34]. Women repeatedly found to be HPV positive but devoid of a clinically visible (and therefore treatable) lesion would be eligible for this type of vaccination. Yet, it would be reasonable to include a 'prophylactic component' into such a vaccine protocol since (1) the usually young women are still at risk for new HPV infections that may reinitiate the multistep carcinogenesis and (2) the previous natural infection may not confer sufficient protection [35]. Development of prophylactic vaccines that aim at protection from HPV infection is fairly advanced. The most promising candidates are virus-like particles (VLPs), which consist only of the structural viral proteins (or even only of the major capsid protein L1) and assemble during expression by recombinant vectors in the appropriate host cells (e.g. baculovirus in insect cells). Preclinical data obtained in animals as well as first results from clinical trials demonstrate that VLPs or L1-specific expression plasmids [36, 37] are highly immunogenic, i.e. they induce high-titer neutralizing antibodies even in the absence of adjuvant [38] [for a review, see ref. 30]. In animal experiments studying papillomavirus infections in their natural host (e.g. the canine oral papillomavirus in dogs), such antibodies were previously shown to confer protection against experimental challenge.

Preexposure Prevention

There are considerable efforts both by companies and nonprofit organizations to develop prophylactic vaccines for prevention of primary infection. In order to be successful, the respective programs must be designed for immunization of children before onset of sexual activity and both females and males must be vaccinated. For the reasons discussed above, VLP vaccines are most likely to be the first products on the market. Some researchers, however, question the efficacy of such vaccines since complete protection ('sterilizing immunity') may not be achieved. It is therefore conceivable that, in addition to

neutralizing antibodies, a CTL response needs to be induced within a preexposure vaccination scheme in case there is some escape from the primary immune attack.

Current State of Knowledge

We are working on several aspects of HPV-specific immune therapy and our data obtained so far can be summarized as follows. Chimeric VLPs consisting of HPV 16 L1 proteins fused at their C-terminally truncated portion to the first 60 amino acids of the HPV 16 E7 protein (HPV 16 L1/$E7_{1-60}$) were shown to induce both neutralizing antibodies [39] and an E7- and L1-specific CTL response in a preclinical system [40, 41] [Öhlschläger et al., unpubl. data]. Furthermore, the growth of HPV 16-transformed mouse cells in a syngeneic immunocompetent host (C57BL/6 mice) was inhibited both in a prophylactic and therapeutic vaccination protocol. Similar effects were observed when chimeric VLPs containing only the 55 N-terminal amino acids of the HPV E7 protein (HPV 16 L1/$E7_{1-55}$) were tested in mouse immunization experiments [41]. HPV 16 L1/$E7_{1-55}$ chimeric VLPs are currently being evaluated for treatment of patients with HPV 16-positive CIN (www.medigene.de). Among the preventive vaccination strategies, the use of empty VLPs has been proven to be very efficient in inducing neutralizing antibodies; thus, different studies have already reached the status of clinical phase II. In contrast, most therapeutic vaccination strategies against HPV are still at a less advanced stage, although many different approaches have been undertaken so far (e.g. peptide vaccines, ex vivo pulsed dendritic cells, different viral and nonviral vector systems) [for reviews, see ref. 23, 29].

One reason for the head start of prophylactic vaccination strategies is based on the more straightforward and easy-to-standardize type of assays that are required to measure the immune response. In brief, a powerful prophylactic vaccine needs to induce neutralizing antibodies against the viral capsid proteins (HPV L1 and/or L2 proteins). Antibody titers obtained by VLP-specific ELISA have been shown to correlate well with neutralizing activity [42]. Since ELISA systems allow a standardized, high-throughout screening of humoral immune responses, evaluation of the outcome of a prophylactic vaccine is, at least at this early stage, possible with reasonable costs and effort.

The conditions change completely when it comes to therapeutic vaccination strategies; eradication of virus-infected and virus-transformed cells is obtained by priming cytotoxic T cell responses against the early HPV proteins E7 and/or E6. These viral proteins are sufficient for the induction and maintenance of the transformed state of infected cells in vitro and are the only viral proteins that are expressed at high levels during persistent infections. Therefore, E6 and E7 can be regarded as tumor markers for HPV-transformed cells. For this reason, most therapeutic vaccination strategies aim to induce CTL responses against E7 and E6. Unfortunately, measuring CTL responses is much more complex than measuring serum antibody titers. Consequently, therapeutic vaccination studies suffer from relatively high costs and demands for laboratory equipment and labor.

Having this in mind, we were interested in comparing different assay systems to determine E7-specific T cell responses (in particular, CD8+ CTL responses) in a mouse model system. Following different standard strategies for the measurement of CD8+ T cells, we intended to find differences in the readout of each assay and to set up a future standard protocol to substitute the very labor-intensive chromium release assay. The E7-specific immune response was induced by vaccination with naked plasmid DNA encoding for either wild-type or recombinant E7 proteins and it was analyzed at different time points after vaccination, either ex vivo or after one to two rounds of in vitro restimulation.

Materials and Methods

Cloning of E7 Genes for DNA Vaccination

The various recombinant E7 genes were cloned in the eukaryotic expression vector pcDNA3.1 as described earlier [43, 44].

Purification of Plasmid DNA for Vaccination

Plasmids used in DNA vaccination were purified from *Escherichia coli* DH5α by alkali lysis and CsCI density gradient centrifugation. The supercoiled plasmid DNA was resuspended in H_2O and stored at –20° until used in the DNA vaccination experiments.

Mice

C57BL/6 mice from Charles River WIGA (Sulzfeld, Germany) were kept under conventional conditions and on a standard diet at the animal facilities of the Deutsches Krebsforschungszentrum (German Cancer Research Center).

Cell Lines and Culture Conditions

RMA cells [45] and RMA-E7 transfected cells [46] derived from C57BL/6 mice were cultured in RPMI-1640 supplemented with 5–10% FCS, penicillin-streptomycin and glutamine. Additionally, 0.8 mg/ml G418 was given to the cultured RMA-E7 cells for stable expression of the E7 transgene.

DNA Vaccination of Mice

Six-to-eight-week-old female C57BL/6 mice were treated by bilateral injection of 50 µl of 10 µ*M* cardiotoxin into each musculus tibialis anterior 5–6 days prior to DNA injection. For vaccination, 50 µl

of plasmid DNA (1 μg/μl in PBS) were injected into each pretreated muscle. Ten days later, mice were killed to set up spleen cell cultures out of the spleen.

In vitro Restimulation of CTL Lines

1–2 × 10^6 γ-irradiated (100 Gy) RMA-E7 cells were added to cultures of 2 × 10^7 isolated spleen cells that had been pretreated with ACT (17 m*M* Tris-HCl, 0.16 *M* NH_4Cl, pH 7.2) to deplete erythrocytes. Cells were grown in 10 ml of αMEM (Sigma, Deisenhofen, Germany) supplemented with 10% FCS, β-mercaptoethanol, glutamine and penicillin-streptomycin. Effector cells were expanded in 2 ml of the above-mentioned medium with an additional 2.5% (v/v) supernatant of a concanavalin-A-induced rat spleen cell culture together with 1–2 × 10^5 γ-irradiated (100 Gy) RMA-E7 transfectants plus 5 × 10^6 γ-irradiated (33 Gy) syngeneic feeder cells. Cultures were grown at 37° in 7.5% CO_2.

^{51}Cr Release Assay

Subconfluent growing RMA and RMA-E7 cells were trypsinized, washed once in PBS and resuspended in serum-free RPMI medium with glutamine and antibiotics (target cells). 2 × 10^6 target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 60–80 min in a volume of 100–150 μl of the above-mentioned medium at 37°. Cells were washed 3 times by centrifugation at 200 *g* for 3 min and resuspended in 10 ml of RPMI plus 5% FCS, glutamine and antibiotics. To pulse RMA cells with peptide, 100 ng/ml E7-derived antigenic peptide ^{49}RAHYNIVTF^{57} [47] was added to the suspension and incubated at 37° for 20–30 min. Target cells were distributed in a 96-well round-bottomed plate (5 × 10^3 cells/well) together with titrated numbers of effector cells. Maximum release and spontaneous release of target cells was determined by the addition of either 5% Triton X-100 or RPMI medium alone, respectively. Following a 4.5-hour incubation at 37° in 5% CO_2, the assay plate was centrifuged for 3 min at 200 *g*, then 50 μl of culture supernatant was transferred onto a solid-phase scintillation plate (LumaPlate-96, Packard, Dreieich, Germany) and measured in a microplate scintillation counter (1450 Microbeta Plus, Wallac, Turku, Finland). Specific lysis was calculated as follows:

(experimental ^{51}Cr release – spontaneous ^{51}Cr release)/
(maximum ^{51}Cr release – spontaneous ^{51}Cr release) × 100%.

FACS Staining with MHC Class I Tetramers

HPV 16 $E7_{49–57}$ peptide-loaded D^b tetramers conjugated with phycoerythrin were kindly provided by Ton N.M. Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands) [44]. Ten days after immunization, 10^6 erythrocyte-depleted spleen cells were stained in 96-well plates using 1/15 diluted tetramers together with 1/100 diluted fluorescein isothiocyanate (FITC)-anti-mouse CD8a (clone 53-6.7, BD Pharmingen) in FACS medium (PBS, pH 7.5, 3% FCS, 0.02% NaN_3). After incubation for 30 min at 4°, cells were washed 5 times with 200 μl of FACS medium and subsequently centrifuged at 200 *g* for 2 min. Prior to FACS analysis, cells were resuspended in 400 μl of FACS medium containing 5 μl of 7-AAD (BD Pharmingen). 10^5 lymphocytes in living gate (7-AAD negative) were acquired. Tetramer-positive and CD8+ cells are shown as a percentage of total CD8+ cells.

FACS Staining of IFN-γ Production

T cells were isolated 10 days after immunization and optionally restimulated in vitro with RMA-E7 cells as described above. 2.5 × 10^4 RMA-E7 cells, RMA cells pulsed with 100 n*M* $E7_{49–57}$ peptide and untreated RMA cells were seeded separately in 50 μl of RPMI plus 5% FCS and antibiotics in 96-well round-bottomed plates and incubated for 15 h at 37° in 5% CO_2. Cultured T cells were transferred into 15-ml tubes and sedimented at 250 *g* for 5 min. After resuspension in 1 ml of αMEM, 200,000 cells were added to the wells of the stimulator cells and then centrifuged (1 min, 250 *g*, 4°). Following incubation at 37° in 6% CO_2 for 1 h, cytokine secretion was inhibited by adding 2.5 μ*M* monensin (Sigma) to each well. After 5 h, cells were centrifuged (2 min, 250 *g*, 4°) and subsequently resuspended thoroughly in 100 μl of Cytofix/Cytoperm (BD Pharmingen) and incubated for 20 min at 4°. Following centrifugation as described above and washing twice with 100 μl of 1 × Perm/Wash solution (BD Pharmingen), cells were stained in 20 μl of 1 × Perm/Wash solution containing 1/50 phycoerythrin-conjugated anti-mouse IFN-γ (clone XMG1.2, BD Pharmingen), 1/100 Cy-chrome-conjugated anti-mouse CD4 (clone H129.19, BD Pharmingen) and 1/100 FITC-conjugated anti-mouse CD8 antibody (clone 53-6.7, BD Pharmingen) for 30 min on ice. After two further washing steps using 100 μl of 1 × Perm/Wash, cells were resuspended in 130 μl of PBS-0.5% BSA and analysed by FACS. 10,000 events of a gate for CD8+ lymphocytes of the living (determined by forward scatter/side scatter) cell population were acquired. IFN-γ and CD8+ cells are shown as a percentage of the total CD8+ cells.

IFN-γ ELISPOT

96-well MultiScreen-HA sterile plates (MAHAS4510, Millipore) were equilibrated with PBS and coated with 150–200 ng of anti-mouse IFN-γ capture antibody (clone R4-6A2, BD Pharmingen) in 100 μl of PBS overnight at 4°. After blocking for 2 h with 100 μl of medium (RPMI, 5% FCS and antibiotics) at 37°, splenocytes were seeded as triplicates in a serial dilution ranging from 400,000 to 12,500 cells/well in 100 μl of medium. One of each triplicate dilution was left either untreated (background control), received 200 ng of pokeweed mitogen (Sigma) in 20 μl of medium (positive control) or received 40 pmol of $E7_{49–57}$ peptide in 20 μl of medium (test sample). The plates were incubated for 18–20 h at 37°. Cells were removed by washing three times with PBS and 0.05% Tween 20 and once with PBS. Following the addition of 100 μl of PBS containing 200 ng of biotinylated anti-mouse IFN-γ antibody (clone XMG1.2, BD Pharmingen) per well, plates were kept at 4° overnight. The wells were washed three times with PBS and filled with 100 μl of 1/1,000 streptavidin-alkaline phosphatase (BD Pharmingen) in PBS. After 2 h at room temperature, wells were washed three times with PBS and developed for 2–15 min with 100 μl of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium liquid substrate system (Sigma). The reaction was stopped by rinsing the wells with water. Spots were quantified in an ELISPOT Reader (Zeiss-Vison C, Zeiss, Oberkochen, Germany). Wells with medium instead of T cells and wells without capture antibody were run in parallel as background controls.

Results and Discussion

We have analyzed the E7-specific T cell response following immunization with a series of expression clones that have been assayed by us in earlier studies [43, 44] (table 1). All clones are based on the pcDNA3.1(–) (Invi-

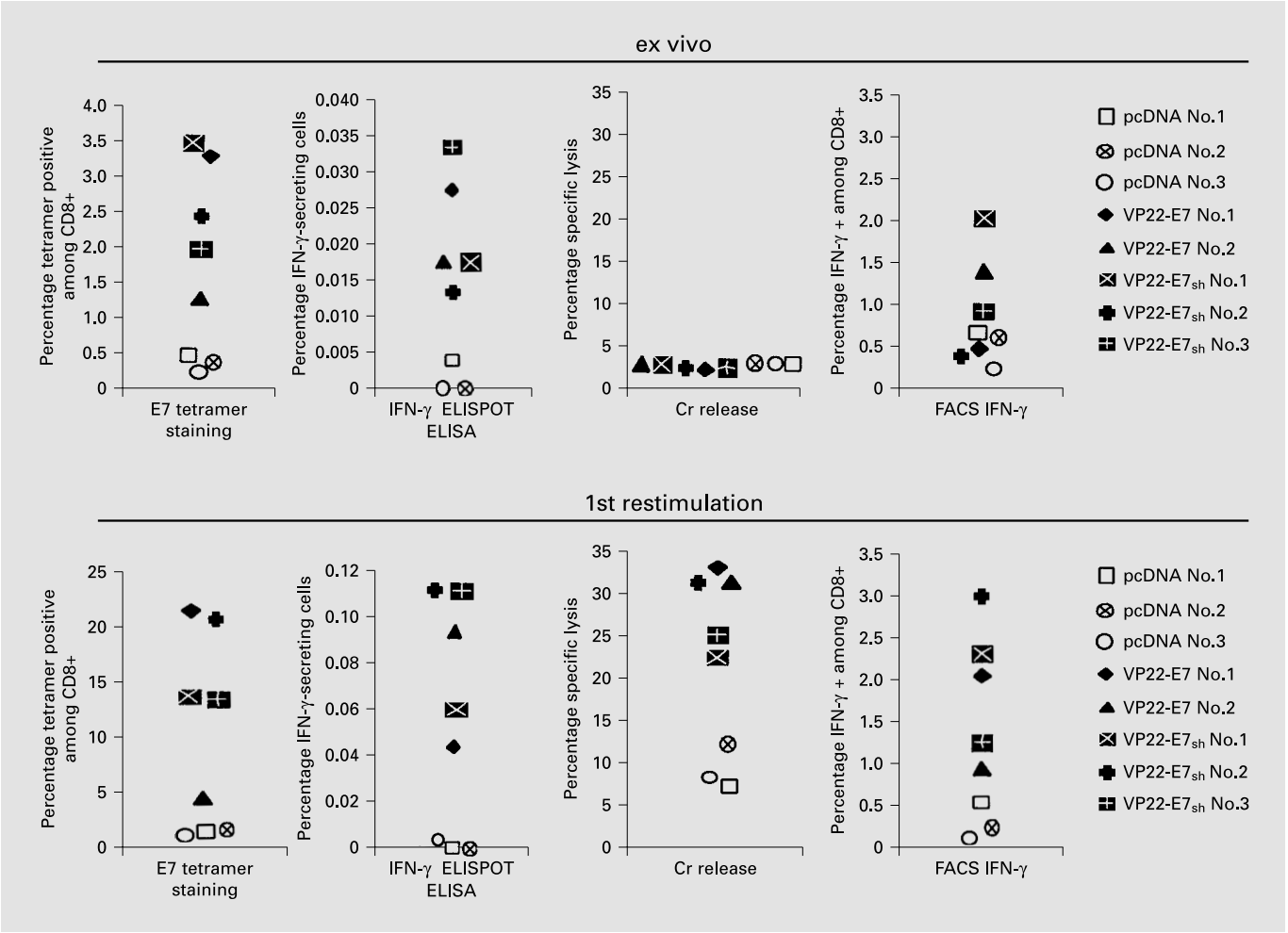


Fig. 1. E7-specific T cell responses in immunized mice ex vivo and after one round of restimulation. Results from the mice immunized in the first experiment are shown (table 1). Tetramer staining, IFN-γ ELISPOT, Cr release assay and intracellular IFN-γ staining were performed with lymphocytes that were isolated from the spleen and used either ex vivo or after one round of E7-specific in vitro restimulation. The reactivity of each vaccinated mouse is shown with the same symbol in all four assays.

trogen, Groningen, The Netherlands) background and contain the HPV 16 E7 gene in different modifications (complete 98 amino acids, amino acids 1–60, 152 amino acids comprising a rearranged E7 gene: $E7_{SH}$ [43]) with or without different heterologous N- or C-terminal sequences (VP22, the secretion signal peptide of Igκ, translocation motif) that modify the intercellular transport of the protein and were previously shown to modify the immunogenicity of E7 [44]. Here, we performed a comparison of different assays, i.e., in addition to the chromium release assay used routinely in our laboratory, tetramer staining, intracellular IFN-γ production measured by FACS analysis and detection of secreted IFN-γ by ELISPOT. The first experiment was designed to find out which assay is most suitable for the analysis of E7-specific CTL activity of DNA-immunized C57BL/6 mice ex vivo, i.e. without additional restimulation of the lymphocytes in vitro prior to the assay. A total of 8 mice were immunized, 3 of which received the empty vector, while the others received either the E7 (n = 2) or the VP22-$E7_{SH}$ expression clone (n = 3) (table 1). In the ex vivo analysis, tetramer staining and (to a lesser degree) the ELISPOT revealed a clear difference between all mice that were immunized with the E7-encoding DNA versus those that received the empty vector (ratio of the means: 20 and 6.6; fig. 1). In contrast, two of the mice immunized with the

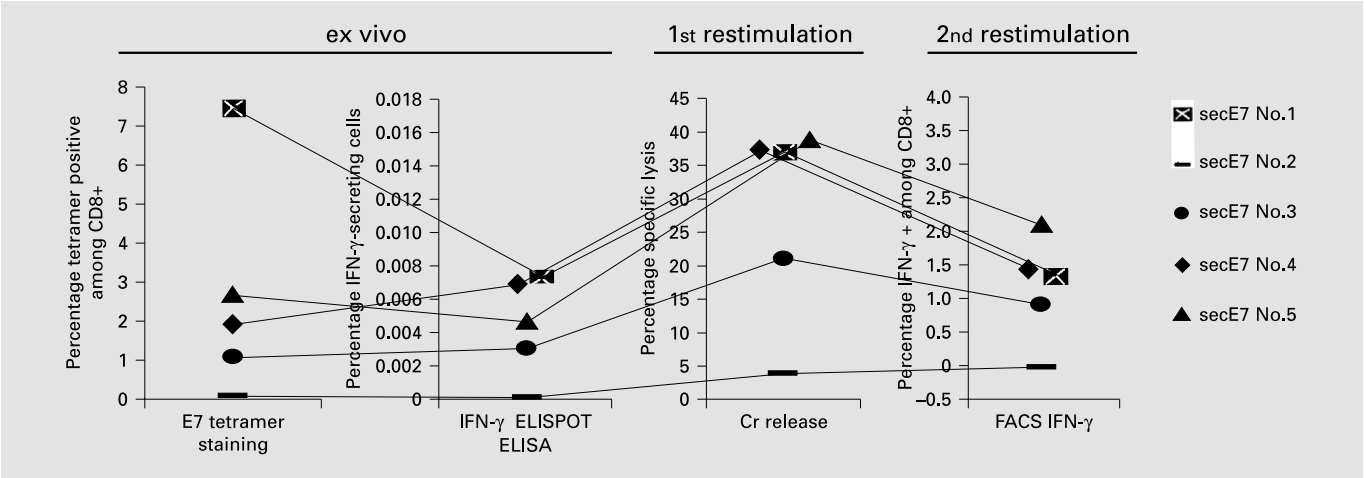


Fig. 2. E7-specific T cell responses in DNA-vaccinated mice with four different assays. Results from the subgroup of mice that were vaccinated with the secE7 vector in the second experiment (table 2) are shown. Tetramer staining and IFN-γ ELISPOT were performed simultaneously with ex vivo isolated lymphocytes from the spleen. Additionally, a fraction of the lymphocytes was analyzed by Cr release assay after one round of E7-specific in vitro restimulation and by intracellular IFN-γ staining after a further round of in vitro restimulation. The reactivity of the individual mice is presented by the same symbol in all four assays.

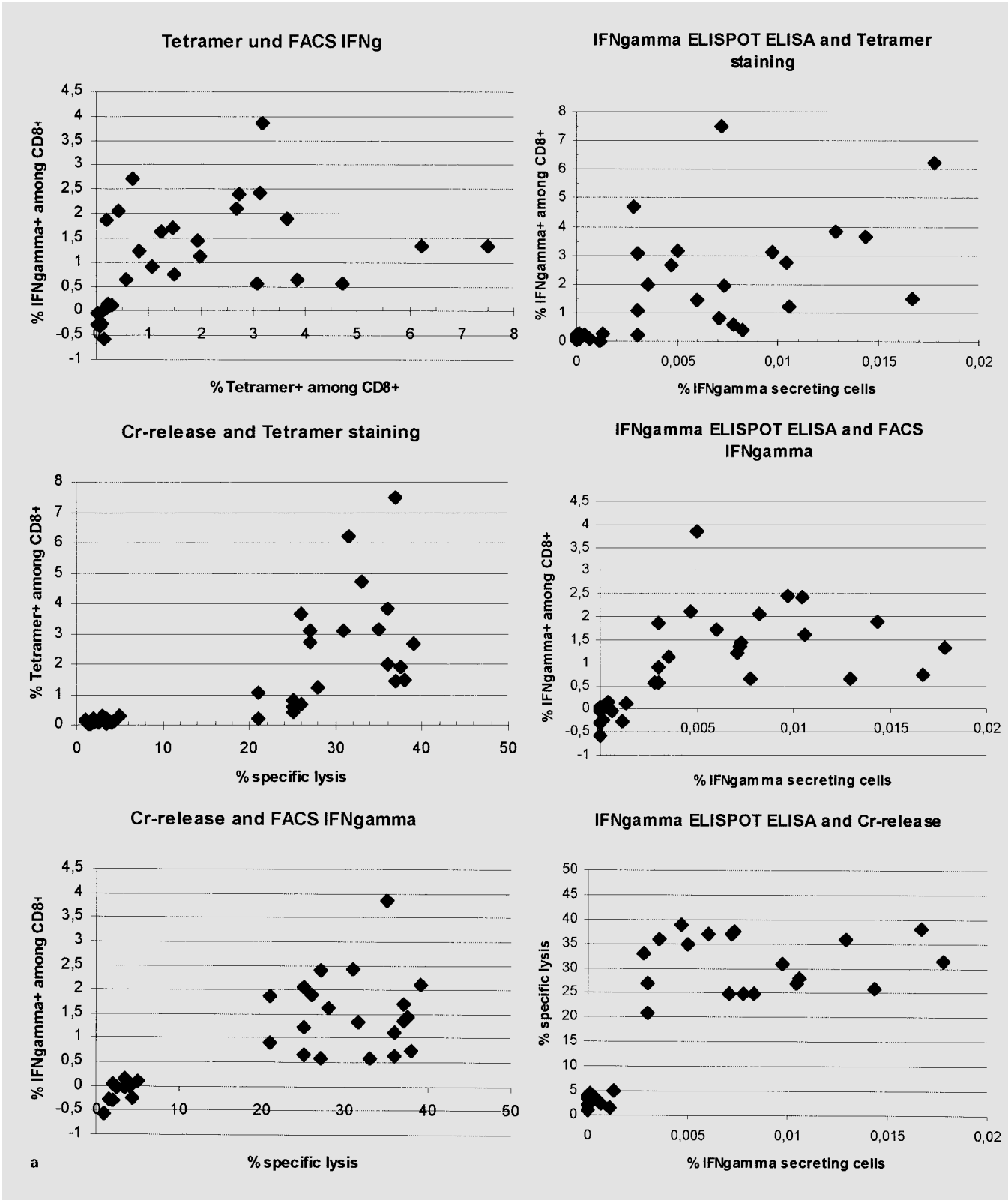
Table 1. Constructs used for DNA vaccination experiments

Insert	Number of mice	
	1st experiment	2nd experiment
–	3	2
E7	–	5
VP22-E7	2	5
VP22-E7$_{SH}$	3	–
VP22-E7$_{1–60}$	–	5
VP22-ΔC-E7	–	5
SecE7	–	5
SecE7-TLM	–	6

The indicated number of mice were immunized with the corresponding recombinant E7 expression vectors (pcDNA3.1) in two separate experiments. The various E7 fusion genes were known from earlier studies to have a different potential in inducing an E7-specific CTL response after DNA vaccination [43, 44].

E7-encoding vectors did not have reactions above background in the intracellular IFN-γ assay (ratio of the means: 2.1). No CTL activity was detected by the ^{51}Cr release assay when the lymphocytes were tested ex vivo. Upon one round of in vitro restimulation of the splenocytes, all E7-immunized mice had reactions clearly above those of the controls (fig. 1), the ratios of means ranging from 3.1 (Cr release assay) to 80 (tetramer staining). It is noteworthy that the relatively low yet significant difference between E7- and vector control-immunized mice resulted from the high background of about 10% specific lysis following the in vitro restimulation. Next, we wanted to test whether the different T cell assays actually produce comparable results following immunization of C57BL/6 mice and whether repeated in vitro restimulation of the splenocytes changes the overall profile of the T cell response. Thirty-three animals were injected with the different expression vectors (table 1) as described in Materials and Methods. After 10 days, mice were sacrificed and spleen cells were tested for E7-specific activity ex vivo (tetramer staining, ELISPOT) and after a first (^{51}Cr release) and second restimulation (intracellular IFN-γ staining). When the results were plotted as positive or negative, all but one mice scored the same in all assays (table 2). One animal (injected with VP22-E7$_{1–60}$) was positive by ELISPOT and negative by tetramer staining, and the positive result was confirmed by Cr release assay and detection of intracellular IFN-γ after in vitro restimulation (table 2). Furthermore, there was a good correlation of the reactivity of individual mice when tested by the different assays (fig. 2, 3).

In our hands, the ELISPOT appears to be the most sensitive assay, allowing the detection of functionally active, i.e. IFN-γ secreting, T cells even without in vitro restimu-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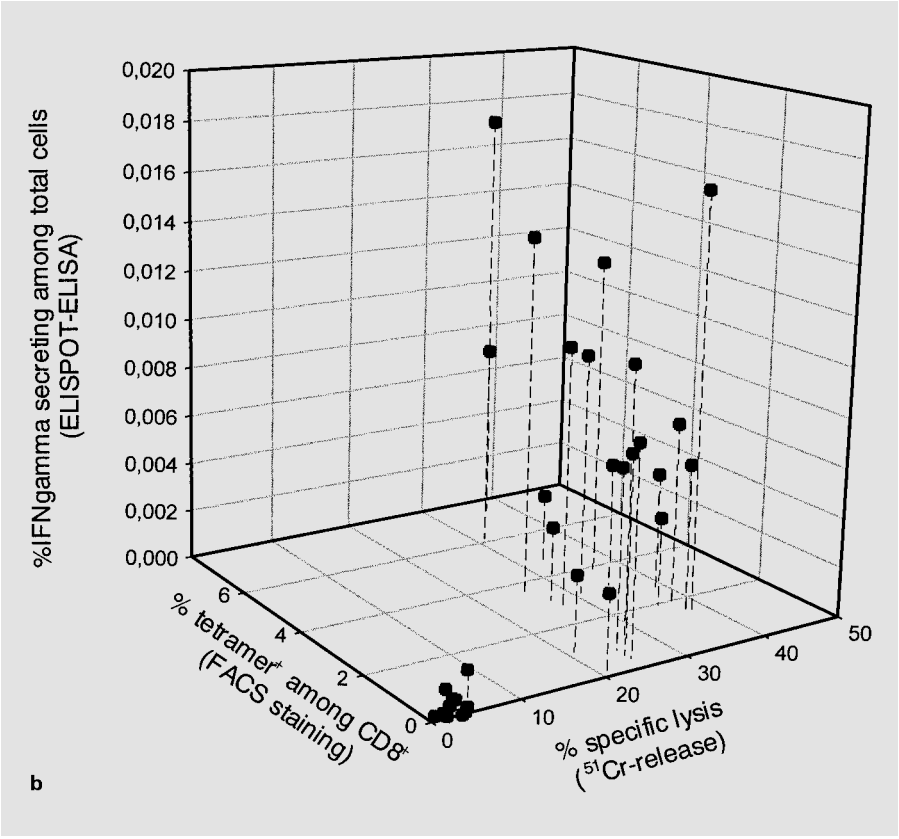

Fig. 3. Comparison of E7-specific T cell responses using different assays. The responses of the individual mice immunized in the second experiment are shown in all six two-by-two comparisons of the four assays (tetramer staining, IFN-γ ELISPOT, Cr release assay and intracellular IFN-γ assay) (**a**) and in a direct comparison of the Cr release assay, tetramer staining and ELISPOT (**b**).

Table 2. Comparison of different assays for an E7-specific CTL response (qualitative readout)

Assay	Cells	Cutoff	Vector						Total
			E7	secE7	secE7-TLM	VP22-E7	VP22-ΔC-E7	VP22-$E7_{1–60}$	
Tetramer	ex vivo	0.4%	0/5	4/5	3/6	5/5	5/5	3/5*	20/31
ELISPOT	ex vivo	0.002%	0/5	4/5	3/6	5/5	4/4[1]	4/5	20/30[1]
Cr release	1st restimulation	15% lysis	0/5	4/5	3/6	5/5	5/5	4/5	21/31
IFN-γ FACS	2nd restimulation	0.4%	0/5	4/5	3/6	5/5	5/5	4/5	21/31

E7-specific CTL-positive mice are indicated as a fraction of the total number of mice in the individual groups tested in the second experiment (for details, see text). Using the cutoff values as indicated for each assay, the E7-specific T cell responses were scored as positive or negative for each mouse in all four assays. The cutoff values were arbitrarily set as follows: 0.4% tetramer-positive cells among total CD8+ cells (tetramer staining): 0.002% IFN-γ-secreting cells among total cells (ELISPOT); 15% specific lysis of E7-expressing target cells above the specific lysis of control target cells at an effector to target ratio of 20:1 (Cr release assay); 0.4% IFN-γ-producing cells among total CD8+ cells (intracellular IFN-γ staining using FACS). Except for one mouse (*), all responses were identical in all four assays. Tetramer = Tetramer staining using murine H2 D^b MHC I tetramers carrying the $E7_{49–57}$ epitope; ELISPOT = INF-γ ELISPOT; Cr release = ^{51}Cr release assay; IFN-γ FACS = intracellular IFN-γ staining using FACS.

[1] Due to technical problems, lymphocytes of one mouse were not tested by ELISPOT.

lation of the lymphocytes. As the intracellular IFN-γ assay is less sensitive under ex vivo conditions, it offers the advantage of simultaneous characterization of the cells (e.g. by CD4 staining). Identification of T cell receptor-positive T cells after tetramer staining also permits a further characterization of these cells in a most timely fashion (2–3 h). Yet the presence of a specific T cell receptor on the surface of CD8+ cells does not necessarily define their functionality. This is particularly important when antigens are investigated that have the potential to induce peripheral tolerance inducing T cell anergy as described for the HPV 16 E7 protein [48]. The ^{51}Cr release assay best defines functionally active CTLs, as it measures the lysis of antigen-presenting target cells. Compared to the other assays, however, it is of low sensitivity (often becoming positive only after repeated restimulation) and provides only semiquantitative information.

We conclude from our data that the ex vivo analysis of splenocytes (tetramer staining together with ELISPOT) yields sufficiently reliable data to determine an E7-specific CTL response in mice, thus permitting completion of the analysis in a timely fashion. In particular, there is no need to perform the labor-intensive cell-killing assay, which requires special precautions due to the radioactively labeled compounds involved, at least not in routine testing, e.g. when different antigen preparations are analyzed for their immunogenicity. It would be helpful to substitute the chromium release assay in studies with human samples to avoid the sophisticated generation of autologous target cells. Obviously, a similar comparative study needs to be conducted to verify whether our data also apply when lymphocytes of human donors are analyzed. Our data further suggest that the ex vivo assays (tetramer staining and ELISPOT) can provide quantitative information when different antigens or immunization protocols are compared. Tetramer staining has certain advantages in terms of the time required for completion of the assay, but it depends upon the availability of defined reagents (MHC-peptide complexes), which may prove to be inconvenient for the analysis of human samples, as often the appropriate T cell epitopes are not known for a given MHC haplotype.

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Comparison of Human Papillomavirus Type 16 L1 Chimeric Virus-Like Particles versus L1/L2 Chimeric Virus-Like Particles in Tumor Prevention

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Key Words
Virus-like particle · Vaccine · Human papillomavirus type 16 · Cytotoxic T cell

Abstract
Chimeric human papillomavirus (HPV) virus-like particles (cVLPs) with the HPV16 E7 antigen fused to either the major capsid protein, L1, or the minor capsid protein, L2, have been used independently to protect against the formation of HPV-induced tumors in animal models. However, the advantages and disadvantages of both types of particles with respect to production and vaccine efficacy have never been analyzed. Therefore, in this study, we compared cVLPs with the HPV16 E7 antigen fused to L1 versus cVLPs with E7 fused to L2 with respect to their ability to protect mice from tumor challenge. The first 57 amino acids of E7 were used to overcome the size limitation and limited VLP production imposed by inserting polypeptides into L1 cVLPs. C57BL/6 mice were immunized with the above cVLPs at various doses. Tumor challenge was then performed with HPV16 E7-positive TC-1 cells. HPV16 L1-$E7_{(1–57)}$ was superior to HPV16 L1/L2-$E7_{(1–57)}$ in eliciting tumor protection at equivalent doses, although both types of particles were able to protect mice. Both cVLPs induced a specific cytotoxic T lymphocyte (CTL) response to the H2-D^b-restricted E7 peptide ($E7_{49–57}$) as determined by an ELISPOT assay and tetramer staining; however, immunization with the L1-$E7_{(1–57)}$ cVLPs resulted in twofold higher CTL precursor frequencies. Our results demonstrate that cVLPs with the antigen fused to L1 are a more efficient vaccine with respect to tumor prevention than cVLPs with the antigen fused to L2. At the same time, however, L1 cVLPs are limited by the size of the antigen that can be incorporated and in the amount of cVLP that can be obtained from cultures when compared to L1/L2 cVLPs. This balances out their superior ability to induce protective immunity.

Introduction

Human papillomaviruses (HPV) are considered to be the causative agent in the development of cervical cancer. More than 99% of cervical cancers are positive for HPV, with HPV16 being the most frequently found type [1]. In the United States and other industrialized countries, the incidence of cervical cancer has decreased dramatically

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since the advent of Pap smears and early treatment of precancerous lesions [2]. Unfortunately, in lower socioeconomic groups and in third world nations, where the majority of cervical cancers occur, most women do not have access to yearly Pap smears and/or treatment [2]. In these cases, a preventive and/or therapeutic HPV vaccine would be most helpful.

Presently, the best candidate for vaccine development is the papillomavirus virus-like particle (VLP). VLPs can be obtained by overexpression of the major capsid protein L1 alone, or by coexpression with the minor capsid protein L2 [3, 4]. HPV VLPs mimic infectious virions in structure and morphology and in their ability to induce neutralizing antibodies [3, 5]. Addition of L2 to L1 VLPs not only provides an increase in the number of neutralizing antibodies but also an increase in particle yield [4, 6, 7]. Several animal studies have shown protective effects after immunization with papillomavirus VLP-based vaccines. These studies have included both L1 VLP vaccines and L1/L2 VLP vaccines [8–11]. A phase I clinical study using an HPV16 VLP composed only of the L1 major structural viral protein has already shown promising results for the generation of neutralizing antibodies in humans [12].

Others have gone a step further and produced chimeric VLPs (cVLPs). cVLPs are made by incorporation of heterologous proteins or peptides as fusions behind either the major or minor capsid proteins [13–15]. The cVLPs deliver the nonstructural virus proteins to the MHC class I processing pathway, induce CD8+ MHC class I-restricted cytotoxic T lymphocytes (CTLs) and protect mice against challenge with E7-transformed tumor cells [14, 15]. cVLPs can also potentially be used as a therapeutic vaccine, leading to regression of established tumors [16, 17].

There are approximately 360 L1 molecules and 12 L2 molecules per VLP, a ratio of 30:1 [4]. The L1 protein can only accommodate a polypeptide of up to 60 amino acids without disrupting the three-dimensional structure of the VLP [13], whereas the L2 protein can certainly accommodate a polypeptide of up to 391 amino acids [15]. Assuming that an L1 cVLP delivers 30 times more antigen to an antigen-presenting cell (APC) than an L1/L2 cVLP, we hypothesized that an L1 fusion would result in more efficient priming of T cells at an equivalent dose compared to an L2 fusion. If, however, both cVLPs are equivalent in inducing T cell responses to the incorporated antigen, fusion to L2 would be more beneficial, since L2 can incorporate larger polypeptides, and thus more potential antigenic epitopes, without disrupting the VLP tertiary structure [15].

In this study, we compared the vaccine efficacy of an HPV16 L1 cVLP with the first 57 amino acids of the HPV16 E7 oncoprotein fused to the L1 protein, HPV16 L1-$E7_{(1–57)}$, to that of an HPV16 L1/L2 cVLP with the same polypeptide fused to the L2 protein, HPV16 L1/L2-$E7_{(1–57)}$. Mice immunized with either the HPV16 L1-$E7_{(1–57)}$ cVLP or the HPV16 L1/L2-$E7_{(1–57)}$ cVLP were challenged with E7-expressing murine tumor cells to determine the extent of protection elicited by both of the cVLPs. Additionally, the HPV16 E7-specific CTL precursor frequency was measured by interferon-γ (IFN-γ) ELISPOT and tetramer binding analyses in cVLP-immunized mice.

Materials and Methods

Cell Lines and Antibodies

The TC-1 cell line was a gift from T.C. Wu (Johns Hopkins Medical Institutions, Baltimore, Md., USA). TC-1 cells are mouse lung epithelial cells transformed by HPV16 E6/E7 plus activated c-Ha-*ras* [18]. TC-1 cells were cultured in RPMI medium (Biowhittaker, Walkersville, Md., USA) supplemented with 10% fetal bovine serum, 2 m*M* *L*-glutamine, 50 μg/ml kanamycin, 50 m*M* 2-mercaptoethanol, 1 m*M* sodium pyruvate, 2 m*M* nonessential amino acids, 0.4 mg/ml G418 and 0.2 mg/ml hygromycin. Cells were grown at 37° with 5% CO_2. Anti-HPV16 L1 (Camvir-1) was purchased from BD Pharmingen (San Diego, Calif., USA). Antibodies H16.V5 (mouse IgG2b) and H16.E70 (mouse IgG2b) were a generous gift from Dr. Neil Christensen (Penn State University, Hershey, Pa., USA). Antibody 8F (mouse anti-HPV16 E7) was a generous gift from Dr. Robert Tindle (Royal Children's Hospital, Queensland, Australia). Anti-HPV16 L2 polyclonal rabbit antibody was a gift from Dr. John T. Schiller (National Institutes of Health, Bethesda, Md., USA).

Animals

C57BL/6 (H2-D^b) female mice, 4–5 weeks of age, were purchased from Taconic (Germantown, N.Y., USA). All mice were approximately 6–8 weeks of age at the time of tumor challenge. Mice were housed under specific pathogen-free conditions.

Virus-Like Particles

Two HPV16 cVLPs were produced: HPV16 L1-$E7_{(1–57)}$ and HPV16 L1/L2-$E7_{(1–57)}$. $E7_{(1–57)}$ was PCR amplified from a plasmid containing the open reading frame of HPV16 E7, pZero-HPV16E7, using the following primers: forward, 5′-ATCATGCATGGAGATACACCTACATTG-3′, and reverse, 5′-TTCAAAAGGTTACAATATTGTAATGGGC-3′. The forward primer added an *Eco*RV site (underlined) in front of the start codon and the reverse primer added a translational stop codon (underlined) after amino acid 57. The PCR product was cloned into the *Eco*RV site of pBluescript II (Stratagene, La Jolla, Calif., USA) and was sequenced. $E7_{(1–57)}$ was subcloned into plasmid 16L1ΔC#155 in which the last 34 C-terminal amino acids of L1 had been deleted [13]. The resulting L1ΔC-$E7_{(1–57)}$ fusion was then subcloned into the baculovirus transfer vector pFastBac1 (Invitrogen Life Technologies, Carlsbad, Calif., USA) under the polyhe-

dron promoter. To construct the L2-$E7_{(1–57)}$ fusion, HPV16 $E7_{(1–57)}$ was subcloned behind the HPV16 L2 gene in which the stop codon had been deleted by PCR, forming the plasmid HPV16 L2-$E7_{(1–57)}$ in pBluescript II. The L2-E7 fusion was subcloned into pFastbac DUAL (Invitrogen Life Technologies) under the p10 baculovirus promoter with wild-type (wt) HPV16 L1 cloned into a second multicloning site under the baculovirus polyhedron promoter. Both of the final constructs were sequenced to confirm that no unintended mutations had been introduced. Recombinant HPV16 L1-$E7_{(1–57)}$ and HPV16 L1/L2-$E7_{(1–57)}$ baculoviruses were generated using the Bac-to-Bac system (Invitrogen Life Technologies) according to the manufacturer's instructions.

HPV16 L1/L2 VLPs, HPV16 L1-$E7_{(1–57)}$ cVLPs and HPV16 L1/L2-$E7_{(1–57)}$ cVLPs were produced in *Trichoplusia ni* (High Five) cells by infection with recombinant baculoviruses at a multiplicity of infection of 5–10. VLPs were purified from cells after 3 days by sucrose and CsCl gradient centrifugation, quantified and examined by electron microscopy, Western blot and ELISA as previously described [13, 15].

Electron Microscopy

Transmission electron microscopy was performed by adsorbing 20-μl VLP samples to carbon-coated copper grids and staining with 2% uranyl acetate. Specimens were examined with a Zeiss EM 900 electron microscope at 75 kV.

VLP ELISA

Purified cVLPs and wt L1/L2 VLPs (250 ng/well) were used to coat 96-well Maxisorp ELISA plates (Nunc, Naperville, Ill., USA). The conformation-dependent antibodies H16.V5 and H16.E70 detected intact VLPs. Bound antibody was detected by the addition of peroxidase-labeled goat anti-mouse IgG antibody (Biosource, Camarillo, Calif., USA), followed by the addition of *o*-phenylenediamine substrate in 0.05 *M* phosphate citrate buffer (Sigma, St. Louis, Mo., USA).

Immunization and Tumor Challenge

For in vitro experiments, two mice were immunized subcutaneously with 50 μg of HPV16 L1-$E7_{(1–57)}$ and two mice with 50 μg of HPV16 L1/L2-$E7_{(1–57)}$. Mice were boosted on day 12 with another 50 μg of cVLPs. Control mice were injected with PBS. Mice were sacrificed 7 days later, after which the spleens and inguinal and axillary lymph nodes were removed. For in vitro stimulation, cells were stimulated for 5 days in 24-well tissue culture plates with 5 μg/ml RAHYNIVTF peptide ($E7_{(49–57)}$) [19].

For tumor challenge experiments, mice (groups of 10) were immunized subcutaneously with HPV16 L1-$E7_{(1–57)}$ or HPV16 L1/L2-$E7_{(1–57)}$ at the following concentrations: 90, 30, 10, 3, 1, 0.3 or 0.1 μg or PBS alone. Tumor challenge was performed 12 days later with 5×10^4 TC-1 tumor cells suspended in 100 μl of Hanks' balanced salt solution. Tumor diameter was measured two times a week along three axes and tumor volume was calculated (diameter 1 × diameter 2 × diameter 3). Mice were sacrificed when the tumor burden reached >1,500 mm^3 or when tumors broke through the skin surface.

ELISPOT Assay

The number of peptide-specific IFN-γ-producing cells was measured as follows. Multiscreen HA plates (Millipore, Bedford, Mass., USA) were coated with 5 μg/ml anti-mouse IFN-γ antibody (Pharmingen) in PBS at 4° overnight. Plates were washed with PBS-0.5% Tween 20 and blocked with culture medium. Freshly isolated splenocytes or stimulated splenocytes were added at 1×10^6 cells/well in triplicate followed by three twofold dilutions in medium containing 10 IU of interleukin-2 and 10 μg/ml RAHYNIVTF peptide. After 24 h of incubation at 37° in 5% CO_2, plates were washed with PBS-Tween and incubated for 2 h at room temperature with 2.5 μg/ml biotinylated anti-mouse IFN-γ antibody (Pharmingen). After extensive washing, 1.25 μg/ml avidin-alkaline-phosphatase (Sigma) was added to the wells for 2 h. Plates were developed by the addition of BCIP-NBT substrate (Promega, Madison, Wisc., USA) for 15 min. Tap water was used to stop the reaction and the plates were dried in air overnight. Individual spots were counted with a dissecting microscope. A positive response was defined as a value greater than the average of PBS-immunized mice ± 3 SD.

Tetramer Analysis

H2-D^b tetramers labeled with phycoerythrin and containing the HPV $16E7_{(49–57)}$ peptide RAHYNIVTF were obtained from the National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, Ga., USA). Splenocytes from immunized and control mice were cultured for 5 days in 24-well plates with 5 μg/ml RAHYNIVTF peptide. CD8+ T cells were enriched using MACS cell sorting (Miltenyi Biotec, Auburn, Calif., USA) prior to tetramer staining. One million CD8+-enriched splenocytes were incubated for 1 h with 20 μl of 1/100 diluted tetramer and 2 μg of FITC-labeled anti-mouse CD8 antibody (Pharmingen) in PBS-0.5% BSA-2 m*M* EDTA. Cells were washed four times and analyzed by flow cytometry for the percentage of double-positive cells. A positive response was defined as a value greater than the average of nonimmunized mice ± 3 SD.

Statistical Analysis

Data were subjected to Kaplan-Meier survival analysis comparing survival curves with the log-rank test. Tumor incidence data were analyzed in 2 × 2 contingency tables by a two-tailed Fisher's exact test. In both cases, a p value <0.05 was considered statistically significant.

Results

Generation of cVLPs

To make a comparison between an L1 cVLP and an L1/L2 cVLP carrying the identical inserted heterologous polypeptide, DNA encoding $E7_{(1–57)}$ was cloned in frame behind either the L1 major capsid protein or the L2 minor capsid protein (fig. 1). The first 57 amino acids of HPV16 E7 were chosen as the length of inserted protein to be used for production of the two types of cVLPs for the following reasons. First, there is a size limitation for inserts when producing fusions to the L1 capsid protein. A maximum of 60 amino acids can be inserted behind a C-terminal truncated L1 without disrupting VLP assembly [13]. An attempt was made to produce HPV16 L1-$E7_{(1–60)}$ cVLPs; however, the VLP yield was not sufficient to produce an adequate amount for in vivo immunization experiments.

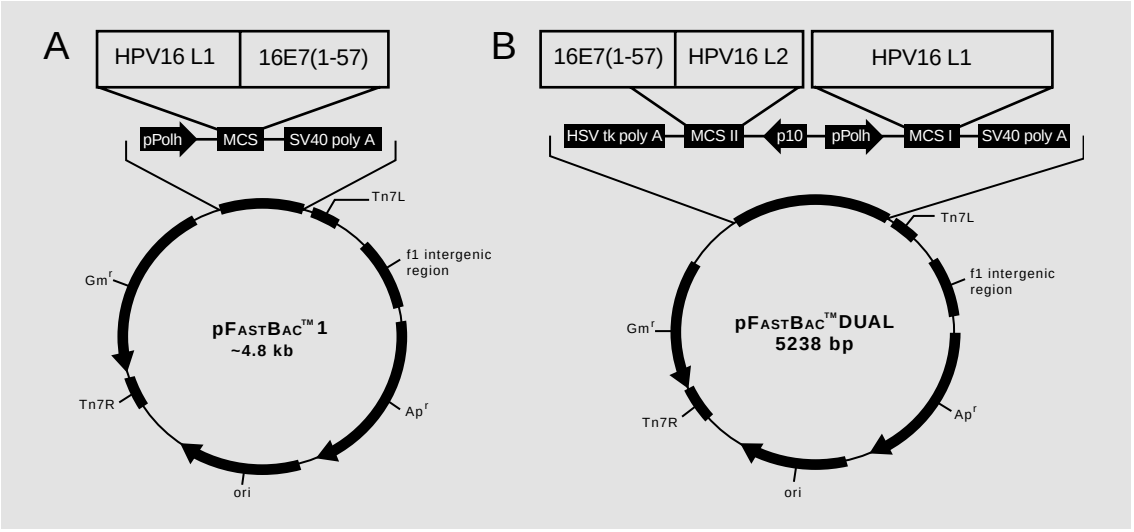

Fig. 1. Construction of HPV16 L1-E7$_{(1–57)}$ and HPV16 L1/L2- E7$_{(1–57)}$ cVLPs. **A** E7$_{(1–57)}$ was PCR amplified and cloned behind HPV16 L1ΔC. The resulting construct was subcloned into the baculovirus transfer vector pFastBacI under the control of the polyhedron promoter. **B** E7$_{(1–57)}$ was PCR amplified and cloned behind HPV16 L2. The resulting chimeric L2-E7 construct was cloned into the second multicloning site of pFastBac DUAL under the control of the minor p10 promoter. wt HPV16 L1 was cloned into the first multicloning site under the polyhedron promoter. The final construct allowed expression of both the L1 protein and the L2-E7 fusion protein in the same baculovirus-infected cell.

Secondly, amino acids 49–57 from the E7 protein encode the murine H2-D^b-binding epitope, which is the major tumor rejection epitope for HPV16 E7-transformed tumor cells [19].

The protein yield of HPV16 L1-E7$_{(1–57)}$ cVLPs was significantly lower than that of HPV16 L1/L2-E7$_{(1–57)}$ cVLPs. Two milligrams of HPV16 L1ΔC-E7$_{(1–57)}$ cVLP were obtained from a total of 12 liters of cultured insect cells. Conversely, 4 mg of HPV16 L1/L2-E7$_{(1–57)}$ cVLPs were obtained from a 1-liter culture of insect cells. Therefore, to make an equivalent amount of L1 cVLP containing a maximum insert length requires 24 times more culture material compared to an L1/L2 cVLP. Though the yield of HPV16 L1-E7$_{(1–57)}$ cVLPs was much less than that of HPV16 L1/L2-E7$_{(1–57)}$ cVLPs, sufficient amounts could be produced to perform the necessary experiments.

To confirm that insertion of E7$_{(1–57)}$ did not lead to irregularly shaped VLPs, electron microscopy was performed. Both the L1-E7$_{(1–57)}$ and L1/L2-E7$_{(1–57)}$ cVLPs were morphologically indistinguishable from wt L1/L2 VLPs and were approximately 55 nm in diameter (fig. 2A). The presence of L1, L2 and E7 proteins was determined by Western blot analysis of the VLP preparations. For HPV16 L1-E7$_{(1–57)}$, only antibodies recognizing L1 or E7 reacted with a band migrating at approximately 70 kD, which is a slightly higher molecular weight than that of the wt L1 protein, as expected (fig. 2B). HPV16 L1/L2-E7$_{(1–57)}$ was positive for all three antibodies (fig. 2B). L2 and E7 co-migrated at approximately 100 kD, with a minor band below 75 kD potentially representing some degradation of the proteins. The L1 protein contained in the HPV16 L1/L2-E7$_{(1–57)}$ cVLPs was detected as the wt L1 55-kD protein (fig. 2B). Antibodies that recognize conformationally dependant epitopes of intact HPV16 VLPs were used in an ELISA assay to confirm that the cVLPs were also conformationally indistinguishable from their wt VLP counterpart (fig. 3). Both HPV16 L1-E7$_{(1–57)}$ cVLPs and HPV16 L1/L2-E7$_{(1–57)}$ cVLPs reacted with H16.V5 and H16.E70 monoclonal antibodies with similar affinity to that of wt HPV16 L1/L2 VLPs. These results support the conclusion that the cVLPs contained the appropriate proteins and were morphologically and conformationally identical to wt HPV16 L1/L2 VLPs except for the inserted E7 polypeptide.

Cell-Mediated Immunity Induced by L1-E7$_{(1–57)}$ and L1/L2-E7$_{(1–57)}$ cVLPs

A dose titration was performed in vivo with both types of cVLPs to determine to what extent the cVLPs were able to induce cell-mediated immunity against HPV16 E7-

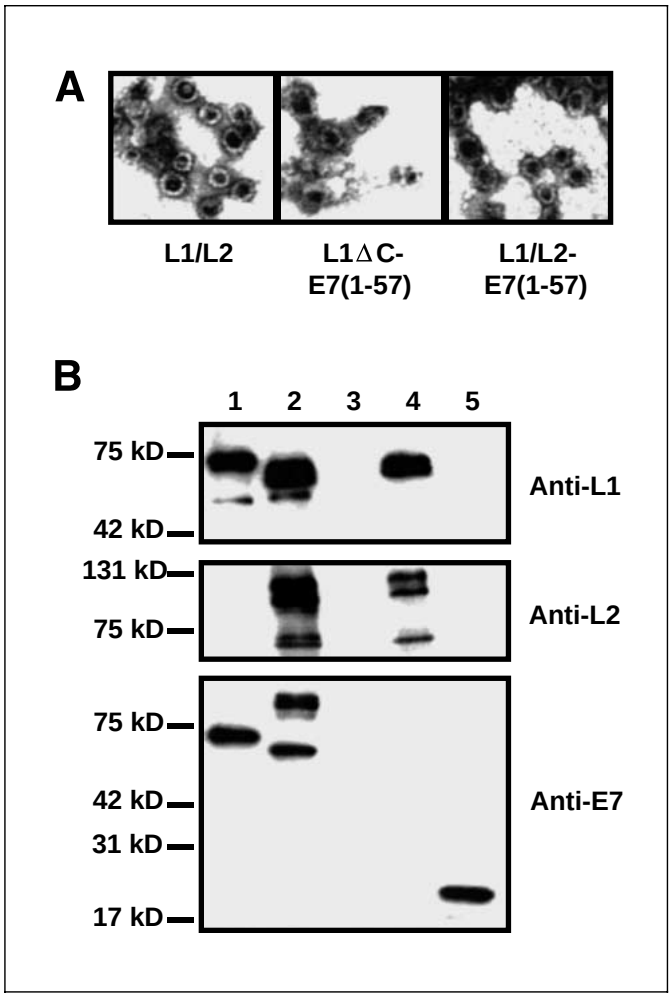

Fig. 2. Electron microscopy and immunoblot analysis of cVLPs. **A** CsCl-purified wt HPV16 L1/L2 VLPs, L1-E7$_{(1–57)}$ cVLPs and L1/L2-E7$_{(1–57)}$ cVLPs were analyzed by electron microscopy for retention of 55-nm icosahedral structure. Bar = 100 nm. **B** Western blot analysis of VLP preparations. L1-E7$_{(1–57)}$ cVLPs (lane 1), L1/L2-E7$_{(1–57)}$ cVLPs (lane 2), wt baculovirus-infected Hi-5 cells (lane 3), wt HPV16 L1/L2 VLPs (lane 4) and insect cell-purified His-tagged E7 protein (lane 5) were separated by SDS-PAGE, blotted onto nitrocellulose membranes and probed with antibodies to HPV16 L1, HPV16 L2 or HPV16 E7. Reactivity was detected by enhanced chemiluminescence.

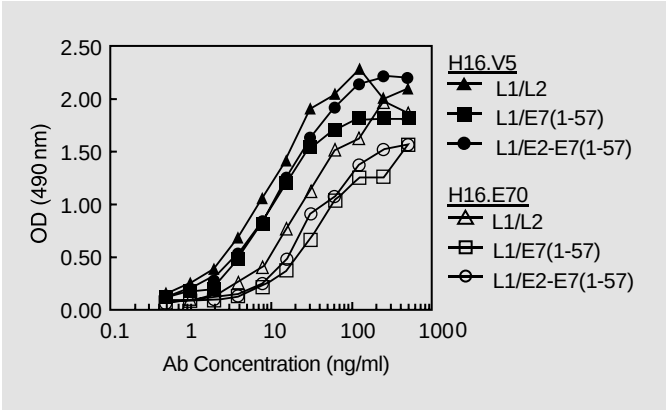

Fig. 3. cVLPs retain conformational B cell epitopes similar to wt VLPs. Conformational-dependent monoclonal antibodies H16.V5 and H16.E70 were used to confirm the presence of L1 epitopes on L1-E7$_{(1–57)}$ cVLPs and L1/L2-E7$_{(1–57)}$ cVLPs. Dilutions of antibody (Ab) were tested for reactivity against purified VLPs in an ELISA assay. Reactivity was detected by the addition of a peroxidase-labeled anti-mouse antibody followed by addition of substrate. Colorimetric changes were measured at 490 nm. OD = Optical density.

transformed tumors. Mice (groups of 10) were immunized with a single dose of 0.1, 0.3, 1, 3, 10, 30 or 90 μg of cVLPs or a PBS control. Mice were then challenged with E7-expressing TC-1 tumor cells to examine E7-specific antitumor immunity. Because the cells used to induce tumors are transformed by HPV16 E6/E7 and do not express either the L1 or L2 proteins, only immunization with E7-containing cVLPs, and not wt L1/L2 VLPs, protects mice from tumor challenge [15]. Immunization with 90 or 30 μg of HPV16 L1-E7$_{(1–57)}$ and 90 μg of HPV16 L1/L2-E7$_{(1–57)}$ resulted in significantly more tumor-free mice compared to PBS-vaccinated control mice (table 1). A significant survival advantage was seen in the mice immunized with 3 μg or more of HPV16 L1-E7$_{(1–57)}$ cVLPs (fig. 4). One mouse in the group immunized with 90 μg of HPV16 L1-E7$_{(1–57)}$ had a small tumor that did not progress for the duration of the experiment, resulting in 90% survival overall seen in this group. In contrast, a survival advantage was only seen in the HPV16 L1/L2-E7$_{(1–57)}$ cVLP-immunized mice that had received the 90-μg dose. In the naive group, 9/10 mice developed tumors; however, 1 mouse in this group died fro unrelated causes (table 1, fig. 4).

Both ELISPOT and tetramer analysis were performed to confirm the specificity of the CTL response to the E7 peptide by both cVLPs. Splenocytes from vaccinated and control mice were tested in an ELISPOT assay for specific IFN-γ release upon stimulation with the H2-D^b-binding E7$_{(49–57)}$ peptide, and for binding of E7-specific T cells to H2-D^b-RAHYNIVTF tetramer complexes. In freshly isolated splenocytes from HPV16 L1-E7$_{(1–57)}$ cVLP-immunized mice, there were twice as many E7-specific IFN-γ-secreting cells than in those from the control mice (table 2). When splenocytes were stimulated in vitro, there

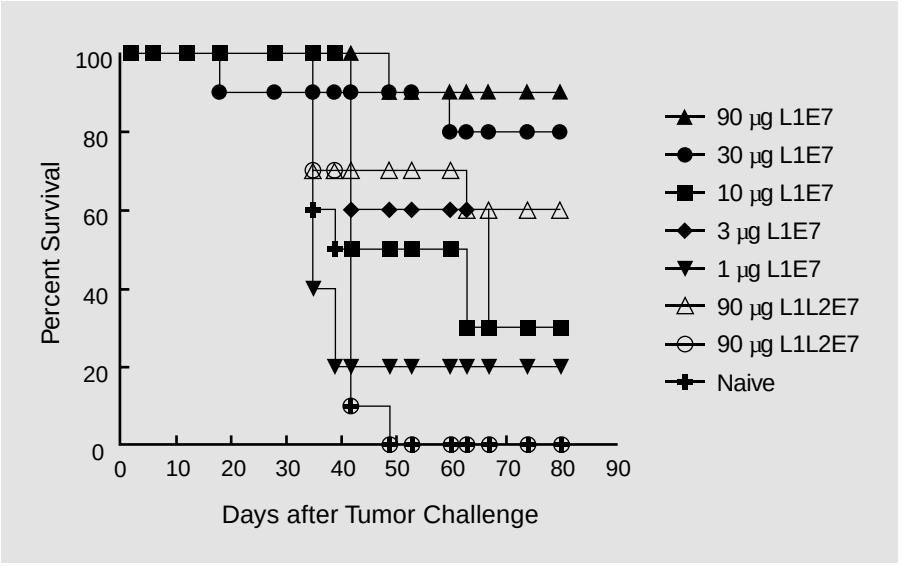

Fig. 4. Long-term survival of mice immunized with HPV16 L1-$E7_{(1–57)}$ cVLPs or HPV16 L1/L2-$E7_{(1–57)}$ cVLPs. C57BL/6 mice (10 animals/group) were immunized subcutaneously with various doses of cVLPs or PBS. Twelve days later, mice were challenged subcutaneously with TC-1 tumor cells. Tumor occurrence and volume were assessed 2 times a week. Mice were sacrificed when tumor volume reached >1,500 mm^3 or when tumors broke through the skin surface. p values were calculated with the log-rank test for each dose compared to the naive group. Only those doses of cVLPs that resulted in a significant survival advantage and the highest dose that did not result in a survival advantage are shown. p values are as follows: 90 µg of L1-$E7_{(1–57)}$, p = 0.0001; 30 µg of L1-$E7_{(1–57)}$, p = 0.0001; 10 µg of L1-$E7_{(1–57)}$, p = 0.001; 3 µg of L1-$E7_{(1–57)}$, p = 0.0005; 1 µg of L1-$E7_{(1–57)}$, p = 0.75; 90 µg of L1/L2-$E7_{(1–57)}$, p = 0.003; 30 µg of L1-$E7_{(1–57)}$, p = 0.6.

Table 1. In vivo dose response and effect of immunization with L1-$E7_{(1–57)}$ VLPs versus L1/L2-$E7_{(1–57)}$ VLPs

Vaccine group	Tumor-free animals	p value
PBS	1/10	reference
90 µg L1-E7	8/10	0.0055
30 µg L1-E7	8/10	0.0055
10 µg L1-E7	3/10	0.582
3 µg L1-E7	3/10	0.582
1 µg L1-E7	2/10	1.0
0.3 µg L1-E7	2/10	1.0
0.1 µg L1-E7	1/10	1.526
90 µg L1/L2-E7	6/10	0.05
30 µg L1/L2-E7	0/10	1.0
10 µg L1/L2-E7	2/10	1.0
3 µg L1/L2-E7	1/10	1.526
1 µg L1/L2-E7	2/10	1.0
0.3 µg L1/L2-E7	1/10	1.526
0.1 µg L1/L2-E7	1/10	1.526

p values were determined by a two-tailed Fisher's exact test comparing tumor incidence in PBS-vaccinated control animals to that in each vaccine group. PBS = Phosphate-buffered saline.

Table 2. Analysis of $E7_{(49-57)}$ CTL frequencies by ELISPOT and H2-D^b-RAHYNIVTF tetramer staining

Vaccine	Mouse No.	ELISPOT		Tetramer staining	
		fresh	following stimulation	fresh	following stimulation
PBS	1	10	33	0.04	0.19
	2	17	46	0.05	0.25
L1-$E7_{(1–57)}$	3	**28**	**378**	0.06	**0.85**
	4	**31**	**438**	0.05	**0.73**
L1/L2-$E7_{(1–57)}$	5	**25**	**210**	0.05	**0.44**
	6	14	**202**	0.09	**0.43**

Values for the ELISPOT assay represent the number of spots per 10^6 splenocytes following stimulation with RAHYNIVTF peptide in freshly isolated splenocytes and in splenocyte cultures stimulated for 5 days. Values for tetramer staining represent the percentage of H2-D^b-RAHYNIVTF tetramer-positive CD8+ T cells in freshly isolated splenocytes and in splenocyte cultures stimulated for 5 days. Figures in bold represent positive responses. PBS = Phosphate-buffered saline.

were 10 times as many specific IFN-γ-secreting cells than in the control mice. In the HPV16 L1/L2-E7$_{(1–57)}$ cVLP-immunized mice, the number of IFN-γ-secreting cells was half that of HPV16 L1-E7$_{(1–57)}$ cVLP-immunized mice. Tetramer binding analysis of in vitro stimulated splenocytes showed a 3.6- and 2.0-fold increase in tetramer-positive CD8+ T cells in HPV16 L1-E7$_{(1–57)}$ cVLP- and HPV16 L1/L2-E7$_{(1–57)}$ cVLP-immunized mice, respectively (table 2). Taken together, these data suggest that immunization with a lower dose of HPV16 L1-E7$_{(1–57)}$ cVLP is equivalent to the effects seen with a high dose of HPV16 L1/L2-E7$_{(1–57)}$ cVLPs.

Discussion

This study has shown that heterologous protein fusion to the major capsid protein, L1, is more effective in delivering antigenic epitopes than fusion to the minor capsid protein, L2. This is consistent with the 30:1 ratio of L1 to L2 molecules in the HPV16 VLP [4]. An L1-E7 fusion would theoretically result in 360 copies of the E7 antigen being delivered to the cytosol of an APC, compared to the delivery of 12 copies of the antigen by an L2-E7 fusion. Although both HPV16 L1-E7$_{(1–57)}$ cVLPs and HPV16 L1/L2-E7$_{(1–57)}$ cVLPs could elicit E7-specific T cells, immunization with HPV16 L1-E7$_{(1–57)}$ cVLPs was more effective in protecting mice from tumor development and for overall survival after tumor challenge.

Müller et al. [13] showed that the construct HPV16 L1ΔC-E7$_{(1–60)}$ could produce a significant amount of capsid but the number of particles was approximately two orders of magnitude less than HPV16 L1ΔC-E7$_{(1–55)}$ cVLPs or wt VLPs. In our laboratory, an attempt was made to produce HPV16 L1ΔC-E7$_{(1–60)}$ cVLPs in order to compare them with HPV16 L1/L2-E7$_{(1–60)}$ cVLPs that were also constructed in our laboratory. However, we were unable to reproduce the protein production described by Müller et al. [13] and Schafer et al. [17] that would yield enough VLP for the planned experiments. Therefore, to facilitate VLP assembly and production, we chose to incorporate a slightly smaller insert while including the presence of the murine E7 CTL epitope.

As indicated in the results, the yield of L1 cVLPs was very poor compared to that of L1/L2 cVLPs. Presumably, the inefficient VLP assembly we encountered is due to the presence of the inserted polypeptide, since L1ΔC VLP formation is similar to wt L1 VLP formation (data not shown) [13]. This is important because even though a smaller dose of the L1 cVLP is needed for tumor suppression, the immunization efficiency for tumor protection is offset by a decrease in VLP production efficiency.

The numbers in this study are too small to state that one needs exactly 30 times less L1-E7 cVLP than L1/L2-E7 cVLP to induce an equivalent immune response against the E7 protein. Theoretically, a 3-μg dose of L1-E7$_{(1–57)}$ should be equivalent to a 90-μg dose of L1/L2-E7$_{(1–57)}$ in terms of the amount of E7 delivered to the immune system by the VLPs. The 3-μg dose of L1-E7$_{(1–57)}$ was not able to result in a significant amount of tumor-free animals; however, the overall survival of the group was significant ($p = 0.0005$) compared to that seen with the 90-μg dose of L1/L2-E7$_{(1–57)}$ ($p = 0.003$). These data suggest that the limiting factor for L1/L2 cVLPs may simply be the total amount of E7 antigen that is delivered to the immune system for processing and presentation to T cells. A single APC would have to take up 30 L1/L2-E7 cVLPs for every one L1-E7 cVLP to receive equal amounts of E7 peptides that would become available for presentation on the cell surface by MHC class I molecules. Because multiple T cell receptors must be engaged on a T cell in order to become activated [20], a single L1/L2 cVLP carrying 12 molecules of heterologous protein may not supply a sufficient dose for optimal presentation of multiple MHC-peptide complexes by an APC.

Whether an L1 cVLP or an L1/L2 cVLP is used for vaccination purposes, it is clear that both types of cVLPs are able to induce specific cell-mediated immunity in mice. The advantage of an L1 cVLP is that each VLP delivers 360 copies of the antigen to an APC. The disadvantage is that the length of polypeptide that can be accommodated limits the number of T cell epitopes that can be supplied. One way around this is by constructing cVLPs that contain multiple CTL epitopes. Liu et al. [21] demonstrated that bovine papillomavirus L1 VLPs carrying a string of four CTL epitopes (48-amino acid insert) can be used to deliver the epitopes to the immune system. Although their cVLPs showed some loss of icosahedral structure and the addition of different immunodominant B cell epitopes, the VLPs were still able to induce CTL responses. However, they too had difficulty with VLP assembly and capsid protein yields [21]. Addition of polypeptides to the L2 protein appears to be more forgiving when it comes to VLP assembly and VLP integrity. This is likely because the addition of extra protein to L2 does not interfere with how the L1 proteins interact with each other. Therefore, when making cVLPs, one can deliver a limited number of foreign epitopes with L1. If a large protein is desired for delivery (greater than 50 amino acids), L2

would be the fusion protein of choice. Since both types of cVLP can be used as antigen delivery vehicles, the difficulties with VLP assembly and yield of product for immunization purposes offset the increase in vaccine efficacy with L1 cVLPs.

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Generation of Recombinant Virus-Like Particles of Human and Non-Human Polyomaviruses in Yeast *Saccharomyces cerevisiae*

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Key Words
Polyomavirus · Simian virus 40 · Avian, murine and human polyomavirus · Capsid protein VP1 · Expression · Yeast · Virus-like particles

Abstract
Objectives: Non-viral methods of gene transfer have been preferred in gene therapy approaches for several reasons, particularly for their safety, simplicity and convenience in introducing heterologous DNA into cells. Polyomavirus virus-like particles (VLPs) represent a promising carrier for encapsidation of foreign nucleic acids for gene therapy. For the development of such gene delivery systems as well as for providing reagents for improving virus diagnostics, an efficient yeast expression system for the generation of different polyomavirus VLPs was established. ***Methods:*** A galactose-inducible *Saccharomyces cerevisiae* yeast expression system was used. Formation of empty VLPs was confirmed by cesium chloride ultracentrifugation, agarose gel electrophoresis and electron microscopy. Cross-reactivity of the major capsid proteins (VP1) of different polyomaviruses was analyzed by Western blot using rabbit and mice sera raised against the VP1 proteins. ***Results:*** VP1 of polyomaviruses from humans (JC polyomavirus and serotypes AS and SB of BK polyomavirus), rhesus monkeys (simian virus 40), hamsters (hamster polyomavirus), mice (murine polyomavirus) and birds (budgerigar fledgling disease virus) were expressed at high levels in yeast. Empty VLPs formed by all yeast-expressed VP1 proteins were dissociated into pentamers and reassociated into VLPs by defined ion and pH conditions. Different patterns of cross-reactivity of the VP1 proteins with heterologous mice and rabbit sera were observed. ***Conclusion:*** The developed heterologous yeast expression system is suitable for high-level production of polyomavirus VLPs. Yeast-derived VLPs are generally free of toxins, host cell DNA and proteins. These VLPs might be useful for the generation of new diagnostical tools, gene delivery systems and antiviral vaccines.

Introduction

Polyomaviruses are nonenveloped double-stranded DNA viruses. Thirteen members of the Polyomaviridae family have been identified so far, with the majority infecting mammalian species [1]. Only two viruses infecting birds have been identified: budgerigar fledgling poly-

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omavirus (BFPyV) [2], previously designated budgerigar fledgling disease virus [1], and a recently discovered goose polyomavirus [3].

Most of these viruses display a narrow host range and do not efficiently infect other species. In addition, polyomaviruses exhibit a high level of tissue tropism due to a combination of receptor specificity and a need for intracellular effector proteins. The initial impetus for studies of these viruses was their ability to malignantly transform cells and their potential to induce tumors.

Murine polyomavirus (MPyV), discovered in 1953, is able to cause a variety of different types of tumors in newborn mice [1, 4, 5]. It emerged in a surprising fashion in the course of experiments aimed at demonstrating a viral agent in mouse leukemia. Newborn mice injected with cell-free extracts of leukemic tissues developed, instead of leukemia, salivary gland carcinomas and a range of other solid tumors. The agent responsible for this action was given the name 'polyoma' for its ability to induce multiple tumors [5]. The virus has also been shown to induce other pathologies, including a runting syndrome [5–7], a myeloproliferative disease [8] and polyarteritis [9], as well as effects in enhancing autoimmune disease [10], depending in part on the particular virus strain and host genetic background.

Simian virus 40 (SV-40), isolated in 1960 from rhesus monkey kidney cell cultures used for production of poliovirus vaccines, has been demonstrated to be oncogenic for newborn hamsters. Recently, SV-40 T antigen sequences have been detected in some cancers, leading to a renewed interest in a potential role of SV-40 infection in the development of some human tumors [11, 12].

Two polyomaviruses of humans have been described, i.e. JC polyomavirus (JCPyV) and BK polyomavirus (BKPyV). JCPyV was originally isolated from patients with progressive multifocal leukoencephalopathy (PML). It establishes a persistent infection in its human host and may cause, in immunocompromised individuals and AIDS patients, the fatal brain disease PML [13]. Interestingly, recent investigations suggested that JCPyV could be used as a marker for investigations of human migration [14]. BKPyV was first isolated from the urine of an immunosuppressed renal transplant recipient and is associated with hemorrhagic cystitis in bone marrow transplant recipients [15, for a review, see ref. 16]. Both human viruses induce tumors in experimental animals; transgenic mice expressing BKPyV and JCPyV develop hepatocellular carcinoma and renal tumors, and primitive neuroectodermal tumors and neurologic illness, respectively [17].

Hamster polyomavirus (HaPyV) was originally isolated from spontaneously occurring hair follicle epithelioma of the Syrian hamster [18–20]. Other mammalian polyomaviruses include bovine polyomavirus, rabbit polyomavirus, a rat polyomavirus and Kilham virus, an additional polyomavirus of mice.

Most members of the Polyomaviridae family have capsids similar in size that are constructed from three viral capsid proteins, VP1, VP2 and VP3. Only avian polyomavirus BFPyV incorporates agnoprotein 1a into the virus particle as a fourth structural protein, VP4 [21, 22]. The major capsid protein VP1 of all polyomaviruses comprises approximately 80% of total virus capsid proteins. Because of its biological functions, e.g. interaction with the cellular receptor, encapsidation and intracellular transport of the viral genome, it is highly conserved between all polyomaviruses [23] (fig. 1). Previously, the assembly of VP1 originating from different polyomaviruses into virus-like particles (VLPs) using different eukaryotic and prokaryotic expression systems was demonstrated [24–35]. Recently, we generated HaPyV VP1-derived VLPs in the yeast *Saccharomyces cerevisiae* [36], which can be used as a carrier for the presentation of foreign epitopes on their surface [37]. Here, we describe the use of this yeast system for high-level expression and self-assembly of VP1 originating from different polyomaviruses, e.g. human polyomaviruses BKPyV (antigenic variants SB and AS) and JCPyV, SV-40, murine polyomavirus MPyV and avian polyomavirus BFPyV. These yeast-expressed recombinant VLPs might be useful for the generation of new diagnostical tools and antiviral vaccines.

Materials and Methods

Generation of Yeast Expression Plasmids

All DNA manipulations were performed according to standard procedures. Enzymes and kits for DNA manipulations were purchased from Fermentas AB (Vilnius, Lithuania). VP1-encoding DNA of the BKPyV strains AS and SB were amplified directly from urine samples from which these strains were originally isolated [38–40] (accession numbers: M23122, Z19536). The template DNAs for PCR amplification of VP1-encoding sequences of JCPyV [41] (accession number: U61771), SV-40 [30] (accession number: J02400), MPyV and BFPyV [26, 27] (accession numbers: J02289, AF118150) were kindly provided by Profs. D. Chang, H. Handa and R.A. Consigli, respectively.

The primers used for amplification of the VP1-encoding sequences incorporated *Xba*I or *Bcu*I sites for subcloning into the yeast vector pFX7, a unique ATG initiation codon in the forward primer and a stop TAA codon in the reverse primer (table 1).

The PCR amplification products were cloned into *Xba*I site of yeast expression vector pFX7 [36], allowing the selection of yeast

```
                                10         20         30         40         50
BKPyV (AS) VP1       1 M--APTKRKG E---CPGAAP KKPKEPVQVP KLLIKGGVEV LEVKTGVDAI
BKPyV (SB) VP1       1 M--APTKRKG E---CPGAAP KKPKEPVQVP KLLIKGGVEV LEVKTGVDAI
JCPyV VP1            1 --MAPTKRKG E--------- --RKDPVQVP KLLIRGGVEV LEVKTGVDSI
SV40  VP1            1 MKMAPTKRKG S---CPGAAP KKPKEPVQVP KLVIKGGIEV LGVKTGVDSF
HaPyV VP1            1 --MAPKRKSG ASSRCANPCG KPCPKPANVP KLIMRGGVGV LDLVTGEDSI
MPyV  VP1            1 --MAPKRKSG VS-KCETKCT KACPRPAPVP KLLIKGGMEV LDLVTGPDSV
BFPyV VP1            1 M---SQKGKG S---CP---- ----RPQQVP RLLVKGGIEV LDVKSGPDSI

                                60         70         80         90        100
BKPyV (AS) VP1      51 TEVECFLNPE MGDP------ --DDNLRGYS QHLSAENAFE SDSPDRKMLP
BKPyV (SB) VP1      51 TEVECFLNPE MGDP------ --DDNLRGYS LKLTAENAFD SDSPDKKMLP
JCPyV VP1           51 TEVECFLTPE MGDP------ --DEHLRGFS KSISISDTFE SDSPNRDMLP
SV40 VP1            51 TEVECFLNPQ MGNP------ --DEHQKGLS KSLAAEKQFT DDSPDKEQLP
HaPyV VP1           51 TQIEAYLNPR MGQNKPGTG- -TDGQYYGFS QSIKVNSSLT ADEVKANQLP
MPyV VP1            51 TEIEAFLNPR MGQPPTPESL TEGGQYYGWS RGINLATSDT EDSPENNTLP
BFPyV VP1           51 TTIEAYLQPR PGQKN----- -------GYS TVITVQAEGY QDAPHSTEVP

                               110        120        130        140        150
BKPyV (AS) VP1     101 CYSTARIPLP NLNEDLTCGN LLMWEAVTVK TEVIGITSML NLHA---GSQ
BKPyV (SB) VP1     101 CYSTARIPLP NLNEDLTCGN LLMWEAVTVK TEVIGITSML NLHA---GSQ
JCPyV VP1          101 CYSVARIPLP NLNEDLTCGN ILMWEAVTLK TEVLGVTTLM NVHS---NGQ
SV40 VP1           101 CYSVARIPLP NLNEDLTCGN ILMWEAVTVK TEVIGVTAML NLHS---GTQ
HaPyV VP1          101 YYSMAKIQLP TLNEDLTCDT LQMWEAVSVK TEVVGVGSLL NVHGYGSRSE
MPyV VP1           101 TWSMAKLQLP MLNEDLTCDT LQMWEAVSVK TEVVGSGSLL DVHGFNKPTD
BFPyV VP1          101 CYSCARIPLP TINDDITCPT LLMWEAVSVK TEVVGVSSIL NMHS--GAFR

                               160        170        180        190        200
BKPyV (AS) VP1     151 KVHENGGGKP VQGSNFHFFA VGGDPLEMQG VLMNYRTKYP QG------TI
BKPyV (SB) VP1     151 KVHENGGGKP VQGSNFHFFA VGGDPLEMQG VLMNYRTKYP QG------TI
JCPyV VP1          151 ATHDNGAGKP VQGTSFHFFS VGGEALELQG VVFNYRTKYP DG------TI
SV40 VP1           151 KTHENGAGKP IQGSNFHFFA VGGEPLELQG VLANYRTKYP AQ------TV
HaPyV VP1          151 TKDI-GISKP VEGTTYHMFA VGGEPLDLQG LVQNYNANY- EAAIVSIKTV
MPyV VP1           151 TVNTKVISTP VEGSQYHVFA VGGEPLDLQG LVTDARTKYK EEGVVTIKTI
BFPyV VP1          151 AFNGYGGGFT ICGPRIHFFS VGGEPLDLQA CMQNSKTVYP APLIGP----

                               210        220        230        240        250
BKPyV (AS) VP1     201 TPKNPTAQSQ VMNTDHKAYL DKNNAYPVEC WIPDPSRNEN TRYFGTYTGG
BKPyV (SB) VP1     201 TPKNPTAQSQ VMNTDHKAYL DKNNAYPVEC WIPDPSRNEN TRYFGTYTGG
JCPyV VP1          201 FPKNATVQSQ VMNTEHKAYL DKNKAYPVEC WVPDPTRNEN TRYFGTLTGG
SV40 VP1           201 TPKNATVDSQ QMNTDHKAVL DKDNAYPVEC WVPDPSKNEN TRYFGTYTGG
HaPyV VP1          201 TGKAMTSTNQ VLDPTAKAKL DKDGRYPIEI WGPDPSKNEN SRYYGNFTGG
MPyV VP1           201 TKKDMVNKDQ VLNPISKAKL DKDGMYPVEI WHPDPAKNEN TRYFGNYTGG
BFPyV VP1          201 GEGEPRETAQ VLDTGYKARL DKDGLYPIEC WCPDPAKNEN TRYYGNLTGG

                               260        270        280        290        300
BKPyV (AS) VP1     251 ENVPPVLHVT NTATTVLLDE QGVGPLCKAD SLYVSAADIC GLFTNSS-GT
BKPyV (SB) VP1     251 ENVPPVLHVT NTATTVLLDE QGVGPLCKAD SLYVSAADIC GLFTNSS-GT
JCPyV VP1          251 ENVPPVLHIT NTATTVLLDE FGVGPLCKGD NLYLSAVDVC GMFTNRS-GS
SV40 VP1           251 ENVPPVLHIT NTATTVLLDE QGVGPLCKAD SLYVSAVDIC GLFTNTS-GT
HaPyV VP1          251 TGTPPVMQFT NTLTTVLLDE NGVGPLCKGD GLYLSAADVM GWYIEYNSAG
MPyV VP1           251 TTTPPVLQFT NTLTTVLLDE NGVGPLCKGE GLYLSCVDIM GRRVTRNYDV
BFPyV VP1          251 PETPPVLAFT NTTTTILLDE NGVGPLCKGD GLFLSAADVA GTYVDQR-GR

                               310        320        330        340        350
BKPyV (AS) VP1     301 QQWRGLARYF KIRLRKRSVK NPYPISFLLS DLINRRTQKV DGQPMYGMES
BKPyV (SB) VP1     301 QQWRGLARYF KIRLRKRSVK NPYPISFLLS DLINRRTQKV DGQPMYGMES
JCPyV VP1          301 QQWRGLSRYF KVQLRKRRVK NPYPISFLLT DLINRRTPKV DGQPMYGMDA
SV40 VP1           301 QQWKGLPRYF KITLRKRSVK NPYPISFLLS DLINRRTQRV DGQPMIGMSS
HaPyV VP1          301 WHWRGLPRYF NVTLRKRWVK NPYPVTSLLA SLYNNMLPTI EGQPMEGEAA
MPyV VP1           301 HHWRGLPRYF KITLRKRWVK NPYPMASLIS SLFNNMLPQV QGQPMEGENT
BFPyV VP1          301 QYWRGLPRYF SIQLRKRNVR NPYPVSGLLN SLFNDLMPRM TGQSMQGSDA

                               360        370        380        390        400
BKPyV (AS) VP1     351 QVEEVRVFDG TEQLPGDPDM IRYIDRQGQL QTKMV----- .......
BKPyV (SB) VP1     351 QVEEVRVFDG TEQLPGDPDM IRYIDRQGQL QTKMV----- .......
JCPyV VP1          351 QIEEVRVFEG TEELPGDPDM MRYVDRYGQL QTKML----- .......
SV40 VP1           351 QVEEVRVYED TEELPGDPDM IRYIDEFGQT TTRMQ----- .......
HaPyV VP1          351 QVEEVRIYEG TEAVPGDPDV NRFIDKYGQQ HTKPPAKPAN .......
MPyV VP1           351 QVEEVRVYDG TEPVPGDPDM TRYVDRFGKT KT---VFPGN .......
BFPyV VP1          351 QVEEVRVYEG MEGLAPEIDM ---------- ----PPKAPR .......
```

Table 1. Primers used for PCR amplification of VP1-encoding sequences of BKPyV, JCPyV, SV-40 and murine and avian polyomavirus

Viral origin of VP1	Forward primer	Reverse primer
BKPyV (AS and SB)	5′-gtg **tct aga** ata ATG gcc cca acc aaa aga aaa g-3′	5′-gtg **tct aga** TTA aac cat ttt tgt ttg caa ttc-3′
JCPyV	5′-gtg **act agt** ata ATG gcc cca aca aaa aga aaa g-3′	5′-gtg **act agt** TTA cag cat ttt tgt ctg caa ctg-3′
SV-40	5′-gtg **tct aga** ata ATG aag atg gcc cca aca aaa ag-3′	5′-gtg **tct aga** TTA ctg cat tct agt tct ggt ttc-3′
MPyV	5′-aga **act agt** ata ATG gct cca aaa aga aaa agc gg-3′	5′-aga **act agt** TTA att tcc agg aaa tac agt-3′
BFPyV	5′-aga **act agt** aca ATG tcc caa aaa gga aaa gga-3′	5′-aga **act agt** TTA gcg ggg agc ttt ggg ggg cat-3′

The start and stop codons are underlined and the sites for restriction with *Xba*I and *Bcu*I are given in bold.

transformants by permitting resistance to formaldehyde [42]. The sequences of the inserted VP1-encoding PCR products were verified by DNA sequencing. The generation of plasmid pFX-VP1-Ha (previously designated pFX7-VP1-12), encoding the authentic VP1 of HaPyV, has been described previously [36].

Expression and Purification of VP1-Derived VLPs

The pFX7-derived expression plasmids pFX-VP1-AS, pFX-VP1-SB, pFX-VP1-JC, pFX-VP1-SV, pFX-VP1-Ha, pFX-VP1-Py and pFX-VP1-Av, encoding the VP1 proteins of BKPyV-AS, BKPyV-SB, JCPyV, SV-40, HaPyV, MPyV and BFPyV, respectively, were transformed into *S. cerevisiae* AH22 derivative *(ura3 leu2 his4).* This strain was selected after comparing the expression of HaPyV VP1 in many *S. cerevisiae* strains derived from different genetic lines (*S. cerevisiae* DC5, FH4C and AH22 and some industrial wild-type strains) because it was found to be the most suitable in terms of expression and VLP formation. The original plasmid pFX7 (without any insert) was used as a control.

Yeast transformation, cultivation and recombinant protein purification were carried out as described previously [36, 37]. After disruption of the cells, yeast lysate was centrifuged at 3,000 *g* for 10 min at 4°. The supernatant was collected and loaded onto a chilled 30% sucrose cushion in centrifuge tubes and ultracentrifuged at 100,000 *g* for 3 h at 4°. The supernatant was discarded and the pellet resuspended in a small volume (4 ml) of chilled disruption buffer. The suspension was then loaded on top of a chilled CsCl gradient ranging from 1.23 to 1.38 g/ml and centrifuged at 100,000 *g* for 48 h. Fractions of 1 ml were collected and subjected to SDS-PAGE analysis. Fractions containing a protein corresponding to the molecular weight of VP1 (approximately 35–45 kD) were pooled, diluted with 1.31 g/ml CsCl and recentrifuged on a second CsCl gradient. Fractions were collected and those containing VP1 were pooled and dialyzed against 10 m*M* Tris-HCl, pH 7.2, and 100 m*M* NaCl for electron microscopic and Western blot analysis. The buoyant density of the fractions was determined with a refractometer.

Fig. 1. Alignment of VP1 protein amino acid sequences of human (BKPyV-AS, M23122; BKPyV-SB, Z19536; JCPyV, U61771), primate (SV-40, J02400), rodent (HaPyV, AJ006015; MPyV, J62289) and avian (BFPyV, AF118150) polyomaviruses.

SDS-PAGE and Western Blot Analysis

Samples boiled in sample buffer were applied to a 12% SDS-PAGE and run in SDS-Tris-glycine buffer. Proteins were stained by the addition of Coomassie brilliant blue. After SDS-PAGE, proteins were transferred to nitrocellulose membrane Hybond™ ECL (Amersham Life Science, Buckinghamshire, UK). After blocking for 1 h in 5% dry milk-PBS-0.1% Tween 20, (PBS-phosphate-buffered saline, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 1,000 ml of H_2O, pH 7.4), the filters were incubated overnight in the antibody solution [mouse serum raised against yeast-expressed BKPyV-AS VP1, diluted 1/1,000; mouse serum against yeast-expressed JCPyV VP1, diluted 1/1,500; rabbit serum against baculovirus-expressed JCPyV VP1, diluted 1/1,000; rabbit serum against SV-40 virions, diluted 1/1,000; rabbit serum against *Escherichia coli*-expressed truncated HaPyV VP1, amino acids 29–320 [23], diluted 1/1,000; rabbit serum against MPyV VP1, diluted 1/2,000; rabbit serum against VP1 of B-lymphotropic polyomavirus (LPyV) diluted 1/2,000]. For detection of specific antibody binding, peroxidase-labeled anti-mouse or anti-rabbit immune globulin conjugate (Sigma, St. Louis, Mo., USA) was used. The blots were stained with TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Fermentas AB, Vilnius, Lithuania).

Detection of Formation of VLPs by Electron Microscopy

CsCl gradient fractions containing recombinant VP1 were placed on 400-mesh carbon-coated palladium grids. The samples were stained with 2% aqueous uranyl acetate solution and examined with a JEM-100S electron microscope.

Nucleic Acid Contamination and Stability of VP1-Derived VLPs

Dialyzed VP1-derived VLPs purified by cesium chloride gradient centrifugation were treated with pancreatic DNase I and RNase A (Fermentas) and again centrifuged in a cesium chloride gradient. Subsequently, 100 μg of particles were lysed with 25 m*M* EDTA-1% SDS, deproteinized with a phenol-chloroform mix and precipitated with 2.5 volumes of ethanol, using glycogen as carrier. The samples were dissolved in 25 μl of TE (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8) buffer and analyzed in a 1.5% agarose gel. 0.5 μg of RNA or 0.5 μg of DNA were added to control samples before deproteinization.

The dissociation of yeast-derived VP1 particles was investigated by addition of 10 m*M* EDTA-10 m*M* DTT (dithiothreitol) in 10 m*M* Tris-HCl, pH 9, at 37° for 60 min. After incubation, electron microscopy analysis was carried out.

Results

Previously, we demonstrated the highly efficient generation of HaPyV VP1-derived VLPs in the yeast *S. cerevisiae* AH22 using the pFX7-derived expression plasmid pFX-VP1-Ha and subsequently expressed the human polyomaviruses BKPyV and JCPyV [36, 38]. We have now further extended these studies by inserting the VP1-encoding sequences of SV-40, murine polyomavirus MPyV and avian polyomavirus BFPyV into this expression plasmid (to produce pFX-VP1-AS, pFX-VP1-SB, pFX-VP1-JC, pFX-VP1-SV, pFX-VP1-Py and pFX-VP1-Av) and introduced the recombinant plasmids into the yeast *S. cerevisiae* AH22.

Upon galactose induction of the yeast *GAL10-PYK1* hybrid promoter, the recombinant polyomavirus VP1 proteins were purified by ultracentrifugation of crude yeast lysates through a sucrose cushion and subsequently on a cesium chloride gradient. SDS-PAGE analysis revealed additional protein bands of approximately 35–45 kD for all constructs (fig. 2A). In general, these protein bands correspond to the expected molecular weights of the different polyomavirus VP1 proteins (table 2). Although the putative amino acid sequence of BKPyV-AS VP1 is of an identical length to that of BKPyV-SB and differs by only five amino acids within the BC loop [39], the observed molecular weight of AS VP1 was significantly higher than that of SB (fig. 2, lane 2). Direct sequence analysis of the VP1-encoding DNA sequences of BKPyV-AS and -SB from yeast cells amplified by PCR using primers based on flanking plasmid sequences indicated correct termini of the AS and SB VP1-encoding sequences (data not shown). In addition, N-terminal amino acid sequencing of the yeast-expressed AS and SB VP1 proteins revealed the predicted N-terminal sequences [38].

As expected, the VP1 derivatives of BKPyV-AS (fig. 2B, lane 1), BKPyV-SB (fig. 2B, lane 2), JCPyV (fig. 2C, D, lane 3), SV-40 (fig. 2E, lane 4), HaPyV (fig. 2F, lane 5), MPyV (fig. 2G, lane 6) and BFPyV (fig. 2H, lane 7) reacted with the homologous mice and rabbit sera raised against BKPyV-AS VP1, JCPyV VP1, SV-40 virions, HaPyV VP1, MPyV VP1 and BFPyV VP1.

To investigate the assembly competence of the different VP1 proteins, they were purified by CsCl gradient centrifugation. For all constructs, the peak of VP1 proteins were detected in fractions corresponding to buoyant densities of 1.29–1.31 g/ml, suggesting the formation of empty VLPs. In addition, a minor fraction of the VP1 proteins was found at a density of approximately 1.33–1.34 g/ml. The formation of VLPs for all VP1 proteins was confirmed by negative staining electron microscopy (fig. 3). As previously observed for yeast-expressed HaPyV VP1-derived VLPs [36], the particles formed by all the various VP1 proteins were heterogeneous in size, with diameters of 45–50 nm. HaPyV and MPyV VP1-derived VLPs were found to be the most homogeneous in size.

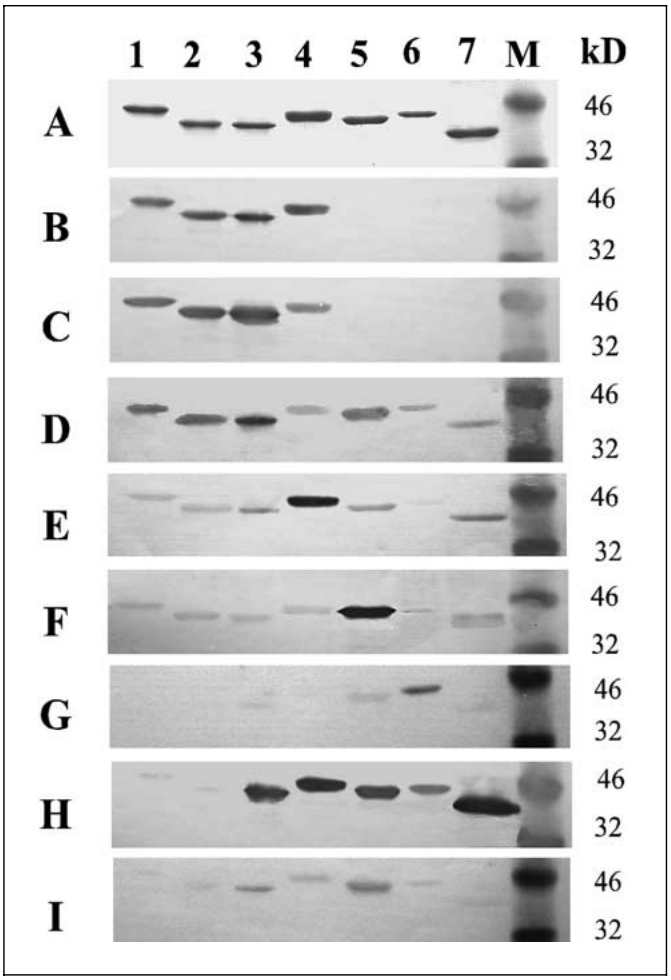

Fig. 2. SDS-PAGE and Western blot analysis of CsCl-ultracentrifugation-purified yeast-expressed VP1 proteins of different polyomaviruses. 2 μg of purified VP1 proteins of BKPyV-AS (lane 1), BKPyV-SB (lane 2), JCPyV (lane 3), SV-40 (lane 4), HaPyV (lane 5), MPyV (lane 6) and BFPyV (lane 7) were separated in a 12% SDS-PAGE and stained with Coomassie brilliant blue (**A**). Separated proteins were analyzed by Western blot using mouse anti-BKPyV-AS VP1 serum (**B**), mouse anti-JCPyV VP1 serum (**C**), rabbit anti-JCPyV VP1 serum (**D**), rabbit anti-SV-40 virus serum (**E**), rabbit anti-HaPyV (amino acids 29–320) serum (**F**), rabbit anti-MPyV VP1 serum (**G**), rabbit anti-BFPyV serum (**H**) and rabbit anti-LPyV serum (**I**). M = Prestained protein molecular weight markers: ovalbumin, 46 kD, and carbonic anhydrase, 32 kD (Fermentas).

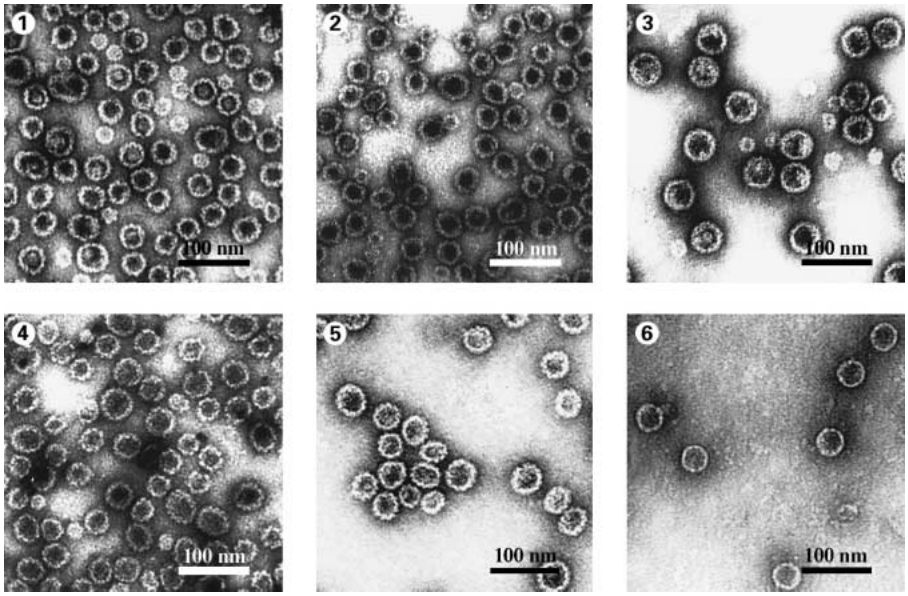

Fig. 3. Detection of yeast-expressed VP1-derived VLPs of BKPyV-AS (1), BKPyV-SB (2), JCPyV (3), SV-40 (4), MPyV (5) and BFPyV (6) by negative stain electron microscopy.

Table 2. Characterization of the yeast-expressed polyomavirus VP1 proteins

Number in SDS-PAGE (fig. 2)	Viral origin of VP1	Predicted molecular weight, kD	Formation of VLPs	Yield of VLPs from 1 liter of yeast culture, mg	DNA contamination
1	BKPyV (AS)	39.98	+	37 ± 5	–
2	BKPyV (SB)	39.99	+	39 ± 5	–
3	JCPyV	39.46	+	35 ± 4	–
4	SV-40	40.03	+	30 ± 4	–
5	HaPyV	41.74	+	40 ± 5	–
6	MPyV	42.46	+	30 ± 4	–
7	BFPyV	37.22	+	5 ± 2	–

Molecular weight was predicted by a computer program (http://www.expasy.ch/tools/). The formation of VLPs was assessed by negative stain electron microscopy. DNA contamination was determined by agarose gel electrophoresis. The values for the yield of VLPs are the average from three independent experiments.

Although VLPs of these sizes dominated, a small fraction of smaller VLPs, 20–25 nm in diameter, was observed for all VP1 proteins (fig. 3).

To investigate a potential DNA contamination of VLPs, agarose gel electrophoresis was carried out. After ultracentrifugation twice in cesium chloride, we could detect only traces of DNA contamination in the main fraction of all VLPs (1.29–1.31 g/ml). This DNA contamination was removed after dialysis and subsequent nuclease treatment. Only in the minor fraction (density 1.33–1.34 g/ml) were traces of DNA contamination observed even after nuclease treatment. There were no obvious differences in the DNA contamination between VLPs originating from the various VP1 proteins (data not shown).

The yield of purified VLPs derived from almost all VP1 proteins, except those originating from BFPyV, ranged from 30 to 40 mg per liter of the induction medium (table 2). A much lower yield (5 mg per liter of medium) was obtained for BFPyV VP1-derived VLPs.

All VLPs are unstable in 10 m*M* EDTA-10 m*M* DTT-Tris-HCl, pH 9; under such conditions, VLPs were very efficiently dissociated into pentamers. During dialysis against 0.1 m*M* $CaCl_2$-0.1 *M* sodium acetate, pH 6, pen-

tamers reassociated into particles different in size (data not shown).

Because of the strong similarity between the amino acid sequences of the various VP1 proteins (fig. 1), one may expect a strong cross-reactivity between VP1 proteins of different polyomaviruses. Consequently, preexisting VP1-cross-reactive antibodies may interfere with the efficiency of VLPs in gene transfer. Therefore, we studied the cross-reactivity of the yeast-expressed polyomavirus VP1 proteins with the heterologous sera in Western blots. A high cross-reactivity with heterologous mice or rabbit sera among all the different VP1 proteins was observed in the Western blot. Interestingly, sera raised against VP1 proteins of human polyomaviruses BKPyV (strain AS) and JCPyV reacted strongly with VP1 of JCPyV and BKPyV (strain SB) (fig. 2B–D, lanes 1–3) and SV-40 (fig. 2B–D, lane 4). In contrast, the rabbit sera raised against SV-40 virions, HaPyV VP1 (amino acids 29–320) and MPyV VP1 detected the corresponding homologous proteins very strongly (fig. 2E, lane 4, fig. 2F, lane 5, fig. 2G, lane 6) but the VP1 proteins of the other polyomaviruses only very weakly (fig. 2E, lanes 1–3, 5–7, fig. 2F, lanes 1–4, 6, 7). A rabbit serum raised against LPyV VP1 reacted with VP1 proteins of JCPyV (fig. 2I, lane 3) and HaPyV (fig. 2I, lane 5); however, very weak signals were observed with BKPyV, SV-40, MPyV and BFPyV VP1 (fig. 2I, lanes 1, 2, 4, 6, 7). Surprisingly, the anti-BFPyV-VP1-specific serum also reacted with VP1 proteins of mammalian polyomaviruses, namely JCPyV (fig. 2H, lane 3), SV-40 (fig. 2H, lane 4), HaPyV (fig. 2H, lane 5) and MPyV (fig. 2H, lane 6), but not with BKPyV VP1 (fig. 2H, lanes 1, 2).

Discussion

A large number of recent studies have reported the detection of SV-40 nucleotide sequences in a variety of unrelated human cancers, including pediatric and adult brain tumors, pleural mesotheliomas, bronchopulmonary carcinomas and osteosarcomas. These observations imply that SV-40 is circulating in human communities by person-to-person transmission and that the virus is capable of wide dissemination in the infected individual. Human polyomaviruses BKPyV and JCPyV cause persistent infections in the human host which may cause, under conditions of immunosuppression, complications in transplantation due to virus reactivation and the fatal brain disease PML, respectively. Infections with these viruses might also contribute to other complications that are known to arise in immunocompromised patients, e.g. respiratory tract disease and tumors [15, 16]. There is evidence to suggest that these viruses are dangerous for people; therefore, monitoring of polyomavirus infections would be beneficial. There is thus a need for highly sensitive and specific serological assays for past exposure to BKPyV, JCPyV and SV-40 as well as markers for patients at risk or showing early signs of complications due to polyomavirus reactivation. VLPs provide important means of producing antigens for such study.

Another potential application of polyomavirus-derived VLPs involves gene therapy approaches. Recently, many laboratories have demonstrated the capabilities of polyomavirus VLPs for in vitro packaging and delivery of DNA into human cells [28, 29, 34, 43–48]. The possibility of modifying the surface of VLPs by insertion of foreign sequences [37, 49] may allow construction of VLPs for cell type-specific targeting of therapeutic genes.

For these purposes, highly efficient expression systems allowing the generation of pure, toxin contamination-free VLPs are required. Currently, baculovirus and bacterial expression systems for generation of VP1 are predominant [24, 25, 27–34, 43–47], and only a few successful cases of expression in yeast have been described till now [35, 36, 38].

The yeast system offers several advantages which should be of relevance for synthesis of such VLPs. Yeast expression systems for the biotechnological preparation of products for human use are well established; moreover, the yeast *S. cerevisiae* has been attributed to the GRAS (generally recognized as safe) microorganisms and is described to be free of toxins hazardous for humans. At present, the yeast *S. cerevisiae* is used for the production of recombinant hepatitis B virus subunit vaccines (Engerix, Glaxo SmithKline, Munich, Germany; Gen H-B-Vax, Aventis Pasteur MSD GmbH, Leimen, Germany; Chiron Behring GmbH, Marburg, Germany).

The purpose of the present study was to develop a universal expression system suitable for the generation of VLPs of different polyomaviruses. In the study presented, we cloned the entire VP1-encoding sequence of different polyomaviruses into the pFX7 plasmid. Previously, this plasmid has been used for successful yeast expression of HaPyV VP1-derived VLPs, mumps nucleoprotein and hantavirus nucleocapsid proteins [36, 50, 51].

In this study, we have demonstrated that the yeast *S. cerevisiae* is a suitable host for a high-level production of VP1 from different polyomaviruses. For the development of this expression system, we used the vector pFX7, which contains a dominant selective marker – resistance

to formaldehyde [42]. This vector allowed us to test different yeast strains independently of their genotype, including wild-type ones. In this way, we easily succeeded in selecting the *S. cerevisiae* AH22 derivative strain as being the best suited for generation of polyomavirus-derived VLPs. On the other hand, our study with different yeast strains revealed that not all *S. cerevisiae* strains were suitable for a high-level production of polyomavirus VLPs (data not shown). These observations are in agreement with the results obtained by Palkova et al. [35] for MPyV VP1, where an inhibition of yeast growth was observed under induction conditions. This supports the idea that the expression level and formation of VLPs depend on the genetic background of the yeast. In our experiments, no growth inhibition of the *S. cerevisiae* AH22 derivative strain expressing the above-mentioned VP1 proteins of different polyomaviruses was observed. The expression level of most polyomavirus VP1 derivatives in *S. cerevisiae* on the whole was high, and their yield was 30–40 mg/liter of culture (table 2). Such high yields of a specific protein will be most valuable in future for a potential large-scale production. The lowest yield was observed for BFPyV VP1-derived VLPs. After induction in yeast lysates, the expression level of avian polyomavirus VP1 was comparable to the level of VP1 of other polyomaviruses; however, formation of VLPs was not as effective as that of mammalian polyomavirus VP1. It is possible that for the efficient formation of BFPyV VP1 VLPs, specific host- or virus-coded proteins are required, or, on the contrary, some host proteins could interfere with the formation of VLPs in the heterologous host cells. Exclusively pFX7-VP1-Av-transformed yeast cells adhere to each other after the induction of BFPyV VP1 protein synthesis, forming macroscopic clumps which settle rapidly from suspension in liquid culture. Under induction conditions, the behavior of these yeast cells simulates that of yeast cells carrying a mutation of the *FLO1* gene, which controls yeast flocculation. Such significant physiological changes in yeast cells suggest the possibility of BFPyV VP1 interactions with yeast cell structures. Such interactions could influence yeast physiology and the formation of VLPs. The interactions of MPyV VP1 with yeast cell structures and growth inhibition were observed earlier by Palkova et al. [35]; however, no changes concerning flocculation were described.

Recently, An et al. [33] demonstrated that avian polyomavirus VP1 did not assemble into VLPs when expressed in the baculovirus system in vivo, but could assemble into capsid-like particles in vitro after purification of capsomeres. This failure of in vivo assembly may be due to the greater complexity, compared to mammalian polyomaviruses, of the capsid of avian polyomavirus, which incorporates an additional protein VP4 (agnoprotein 1a). The role of this protein in virus particle formation is not clearly defined yet. So, currently yeast is a unique system for in vivo production of avian polyomavirus VLPs.

Our Western blot analysis with different polyclonal antibodies exhibited high cross-reactivity between human (BKPyV, JCPyV) and primate (SV-40) polyomavirus VP1. Previously, cross-reactivity of VP1 among polyomaviruses was described for sera raised against SV-40 and MPyV [52]. Recently, rabbit sera raised against JCPyV VP1 and SV-40 were found to cross-react with HaPyV VP1. Moreover, the major cross-reactive region was mapped between amino acids 320 and 384 of HaPyV VP1 [23, 53]. More precise mapping using a PepScan and recombinant phage fr coat protein/VP1 fusions allowed the definition of at least two major epitopes in this region [23; Voronkova et al., unpubl. data]. The lack of this C-terminal region in the antigen used for generation of the HaPyV VP1-specific rabbit serum may have caused the low level of cross-reactivity with VP1 of other polyomaviruses observed by us. Whereas monospecific rabbit sera raised against phage fr coat protein fusions with HaPyV VP1 amino acids 333–384 and 351–384 were strongly reactive in ELISA with yeast-expressed BKPyV and JCPyV VP1 [Voronkova et al., unpubl. data], only 1 out of 14 sera from different pet hamsters naturally infected by an HaPyV-like virus reacted with these antigens [54]. This different level of cross-reactivity may be due to differences in the antibody responses induced by natural polyomavirus infection and in laboratory animals immunized with recombinant VP1 proteins. Even between sera raised against yeast- and baculovirus-expressed VP1 of JCPyV, a different cross-reactivity pattern was observed, probably caused by peculiarities of the VLPs formed in the two expression systems. Surprisingly, a strong cross-reactivity of anti-BFPyV VP1 with JCPyV, SV-40, MPyV and HaPyV VP1 was observed, but there was no cross-reactivity with BKPyV VP1. The low cross-reactivity of the mice sera raised against BKPyV and JCPyV VP1 with rodent polyomavirus VP1 proteins makes rodent polyomavirus VP1 VLPs a prospective tool for the development of gene transfer systems for humans. However, additional investigations to study sera of naturally polyomavirus-infected humans are required to prove this conclusion.

Samples of virions from mouse cells infected with polyomavirus and of recombinant MPyV VP1 from in-

sect cells contained a subpopulation of particles that harbored fragments of host chromosomal DNA and histones [47]. Purification of the VLPs in a cesium chloride gradient revealed a buoyant density of 1.29–1.31 g/ml for all particles. The buoyant density in cesium chloride gradients (1.29–1.31 g/ml) of yeast-expressed VP1 particles corresponded to the values observed for empty particles from SV-40 and other polyomaviruses and baculovirus-expressed VP1 particles of JCPyV [19]. In contrast, the density of DNA-containing capsids was determined to be 1.34 g/ml [19, 55]. In our studies, such fractions comprised only a minor part of particles; empty particles dominated in all studied VLPs. Yeast cells likely do not incorporate host DNA into VLPs because of the cytoplasmic location of VLPs in the yeast expression system described herein [36]. Furthermore, in contrast to insect cells, no host DNA degradation occurs in yeast cells during the short induction and VP1 synthesis period. However, VP1 protein could bind small DNA fragments of host DNA during cell disruption and purification. Our results contradict the observations of Palkova et al. [35] concerning the presence of yeast DNA fragments in yeast-derived murine polyomavirus VP1 VLPs. According to our observations, twofold ultracentrifugation in CsCl and nuclease treatment remove any traces of DNA detectable in agarose gels. Only in the minor fraction with a density of 1.33–1.34 g/ml could we detect traces of DNA which were resistant to the action of RNase.

Our results suggest that the yeast *S. cerevisiae* represents a universal system for high-yield generation of different polyomavirus VLPs. Many lines of evidence suggest that yeast-derived heterologous proteins are free of toxic contamination and are excellent tools for developing vaccines, diagnostics and gene delivery systems [50, 51].

In conclusion, the polyomavirus VLPs described here represent useful tools for the development of new virus detection systems and vaccines against human polyomaviruses. In addition, these VLPs may allow the construction of new gene delivery systems and of chimeric particles displaying foreign antigens on their surface as previously described for HaPyV and MPyV [37, 49].

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Emerging Viruses: The Case 'Hantavirus'

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Key Words
Hantavirus · Bunyaviridae · Emerging viruses · Rodents · Evolution · Pathogenesis · Vaccine

Abstract
This review briefly summarises the recent knowledge about hantavirus infections and raises particular problems in hantavirus research that need further investigation. The following questions are addressed: (i) are hantaviruses distributed worldwide and what leads to new outbreaks, (ii) what is known about hantavirus evolution, (iii) how can hantavirus species be defined, (iv) what are the determinants of hantavirus pathogenesis in humans, and (v) what problems are associated with the development of new vaccines and antiviral therapeutics.

Introduction

Human hantavirus (HV) infections are known to cause renal failure. Since the description of a hantavirus cardiopulmonary syndrome in 1993, HV have attracted even more interest not only in the scientific community but also in the public. Recently, a couple of reviews about HV, dealing with their genomic structure and replication, evolution, rodent reservoirs and transmission to humans, pathogenesis, antiviral therapies and vaccines, have been published [1, 2]. The objective of this review is to briefly summarise the recent knowledge about HV infections, but more to critically discuss the state of the art and to raise particular questions that need further investigation. These questions are represented by more theoretical topics like species definition but also applied topics like the development of vaccines and the mechanisms behind the induction of protective immunity (table 1).

When looking at HV research from a more historical perspective, infections in humans caused by HV were already described during World War 1, many years before the isolation of the prototype virus Hantaan (HTNV) by Ho-Wang Lee et al. [3] and the definition of the disease as 'haemorrhagic fever with renal syndrome' (HFRS). The main effort to find the causative agent of this type of disease, carrying various names, including 'Feld-Nephritis' in Germany, was initiated after the Korean Conflict in the fifties of the previous century when soldiers of the UN troops became infected by an unknown agent resulting in Korean haemorrhagic fever, a well-known disease in Asia. Numerous attempts were undertaken in the 1980s to identify HV in America [for a review, see ref. 4]. Moreover, retrospective studies in the USA, which had started after the first occurrence of the hantavirus cardiopulmonary syndrome (HCPS) in 1993, demonstrated that New World HV were already present in the rodent population long before the first description of HCPS. However, HV are much older, as indicated by a Chinese medical account of an HFRS-like disease dating to about AD 960 [5].

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Table 1. Summary of selected questions in HV research addressed in this paper

Section	Question
Are HV Distributed Worldwide and What Leads to New Outbreaks?	Are HV truly distributed worldwide? What could be factors responsible for emergence of HV?
Evolution of HV	What is the importance of different evolutionary factors (mutation, natural selection, genetic drift, recombination, gene flow) in HV evolution? What is the natural relevance of genetic interactions between HV?
Problems of Species Definition	How can a new distinct HV species be defined?
Pathogenesis of HV Infections in Humans	What are determinants from the aspect of the virus/infected person of the virulence and clinical severity of human infections? What is the basis for the probably different severity of DOBV-induced human infections in Europe?
Vaccines and Antiviral Therapy	What is the role of cytotoxic T cell responses in protection and immunopathology? Could a potential vaccine shown to protect rodents also be able to induce protective immunity in humans?

Are HV Distributed Worldwide and What Leads to New Outbreaks?

In general, HV seem to be distributed worldwide, including Asia, Europe and the Americas. However, there are regions, namely Africa and Australia, where only very limited information about HV-infected rodents and human HV infections is available so far. Thus, only very few reports illuminate the presence of HV-specific antibodies in humans in Central and East African countries [6, 7]. As early as 1982, Gajdusek [8] had reported HTNV-reactive human sera not only in Asian countries, like India and Iran, but also in Alaska, Bolivia and Central Africa. Although other bunyaviruses are endemic in Australia, to our knowledge there is only one report showing the presence of HV-specific antibodies in sera of wild-trapped rodents [9].

However, even for Europe, the distribution of different HV species is far from clear. One previous explanation for the worldwide distribution of HV was the putative presence of Seoul virus (SEOV) infections due to migration of SEOV-infected rats, especially in ports around the world. However, again this requires additional proof. To date, we and others have not been able to verify any human infection caused by SEOV in Europe [10–13]. The previous claim of European SEOV infections may have been caused by the lack of specific assays capable of distinguishing among HV species. Thus, presently available neutralisation assays confirmed the presence of Puumala virus (PUUV) and the SEOV-related Dobrava virus (DOBV) (see below) as the major causes of human infections in Europe. On the other hand, HV infections imported from Asia to Europe cannot be excluded as a cause of earlier described SEOV infections.

One major issue, also for the prognosis of potential new outbreaks of infections, is to figure out the reasons for the emergence (or re-emergence) of viruses [14]. Interestingly, investigations connected with the outbreak of HCPS in the USA in 1993 demonstrated a correlation with an increase in rodent population density possibly due to an El Nino Southern Oscillation and the resulting increase in the food resources for rodents on the one hand, and the ratio of HV-infected rodents and the occurrence of the first recognised human infections on the other. As discussed for other emerging infectious agents, too, transmission of HV to humans could also be enabled by changes in the human lifestyle. HV infections in rodents may have emerged due to human-induced landscape alterations and/or climatic changes influencing population dynamics of HV reservoir hosts, with disease consequences for humans.

Evolution of HV

According to the Committee of Virus Taxonomy, HV represent a separate genus *Hantavirus* in the Bunyaviridae family. Like other virus families causing haemorrhag-

ic fevers, e.g. Filoviridae and Arenaviridae, bunyaviruses possess a negative-stranded RNA genome. Bunyaviridae and Arenaviridae contain three- and two-segment genomes, respectively, but Filoviridae possess non-segmented genomes. In contrast to the other genera of the Bunyaviridae, HV are transmitted to humans not by arthropod vectors but from persistently infected rodents. In the future, studies of the evolutionary relationship of bunyaviruses to other negative-stranded RNA viruses would be a fascinating topic.

Keeping in mind the theory of co-evolution and co-speciation of HV and their respective rodent hosts on the one hand and the migration of rodents well documented by fossil records starting in Eurasia and Northern America already at the end of the Mesozoic period/in the early Neozoic period (Tertiary, Paleocene) on the other hand, there is a large gap in the knowledge of HV evolution, raising questions about the monophyletic origin of the *Hantavirus* genus, its phylogenetic relationships and the time of divergence. The general problems of molecular clock approaches (see below) make it difficult to estimate a date for divergence of the most recent common ancestor of HV.

When reconstructing the radiation of HV, an interesting question is which random and non-random evolutionary factors are involved and to what extent. Natural selection is the most important non-random evolutionary factor resulting in radiation and host adaptation. The natural hosts of most known HV are rodents of the family Muridae, in which these viruses cause a persistent but asymptomatic infection. Phylogenetic studies using HV sequences have been undertaken, demonstrating clear division into three well-differentiated clades corresponding to viruses parasitic on three subfamilies (Murinae, Arvicolinae and Sigmodontinae) of the rodent family Muridae [15–17]. In rooted trees of *M* (medium) and *L* (large) segments of the HV genome, the viruses with hosts belonging to Arvicolinae and Sigmodontinae formed a sister group separated from those with hosts in Murinae [15]. This phylogeny corresponded with phylogenetic trees of muroid pancreatic ribonuclease genes [18] and mitochondrial cytochrome *b* genes [15, 19]; Arvicolinae and Sigmodontinae clustered together as sister taxa, with Murinae outside.

The congruence in branching patterns and consistency in the extent of divergence between the HV phylogenetic tree and that of the virus hosts' lineages supports the hypothesis that co-speciation has been a prominent feature in HV evolution. This, in turn, suggests that HV have co-evolved with their rodent hosts at least since the common ancestor of the three subfamilies of Muridae, which probably occurred about 50 million years ago [15]. However, the detection of the Thottapalayam virus (TPMV) in a shrew (Insectivora: Soricidae: *Suncus murinus*) suggested that the HV have co-evolved with mammals since before the divergence of insectivores and rodents about 136 million years ago [20, 21]. Inasmuch as TPMV has been isolated on a single occasion from a single shrew, it remains less than clear that TPMV is truly a shrew-specific HV and not another rodent-borne member of the genus *Hantavirus.* Further investigation to resolve the role of non-murid mammals as carriers of HV is highly desirable (see below).

The zoogeographic pattern of the HV populations parallels the natural distribution of their respective rodent carriers, supporting the hypothesis of co-evolution. While the natural distribution of the Murinae is restricted to the Old World and that of Sigmodontinae is restricted to the New World, Arvicolinae are naturally found in both the New World and the Old World [22]. Accordingly, a group of closely related HV (Tula, Isla Vista, Bloodland Lake and Prospect Hill viruses) whose hosts belong to the genus *Microtus* (subfamily Arvicolinae), inhabiting both the Old World and the New World, represents the only known group of HV possessing both Old World and New World representatives.

Among randomly acting factors in the evolution of HV one would expect variation of the mutation rate, genetic drift and differences in the population history. Assuming that HV have co-evolved with their specific rodent reservoir hosts, Hughes and Friedman [15] estimated a substitution rate of $2.4–2.7 \times 10^{-7}$ synonymous substitutions per nucleotide per year. Sironen et al. [23] estimated the mutation rate for PUUV at about 0.7×10^{-7} to 2.2×10^{-6}. Although considerably lower than mutation rates in other RNA viruses, these rates are about two orders of magnitude faster than typical mutation rates in mammals [24]. Under these circumstances, saturation of synonymous sites in the virus genome should have occurred much more rapidly than in the host species genome, leading to errors when phylogenetic reconstructions are based upon nucleotide sequences rather than amino acid sequences, a common practice in studies of HV phylogenetics. Furthermore, an evolutionary rate shift has been detected in different RNA viruses (HIV, influenza A virus), indicating adaptive molecular evolution [25]. Recently, a high frequency of amino acid residue charge changes was observed in the hypervariable region of the nucleocapsid protein between the three HV subfamilies [15]. Since this region includes epitopes for host anti-

bodies, such changes may represent adaptation to host-specific characteristics of the immune response.

Recombination events like reassortment and intragenic recombination have been reported under in vivo and in vitro conditions [26–29, Klempa et al., submitted]. As their probability seems to be quite low, the importance of recombination events for HV evolution is uncertain. Analogous to the codon usage limitation in the expression of human RNA virus genes [30], one might also expect a rodent-adapted codon usage in HV proteins; however, to our knowledge, this has not yet been studied systematically.

The cyclic population dynamics of rodents makes it very likely that population bottleneck events and genetic drift play an important role [31–33]. Accordingly, the unique features of the genome of the DOBV-like Saaremaa virus, isolated on an Estonian island in the Baltic Sea (see below), might be due to separation and genetic drift. Therefore, characterisation of Saaremaa-like viruses from mainland Estonia would be very conclusive.

Besides the well-supported concept of co-evolution, host switch events and spillover infections have been reported for additional rodent species like *Ondatra zibethicus* [34], *Lemmus sibiricus* [35], *Apodemus sylvaticus* [36] and *Arvicola terrestris* [37]. Spillover infections have been documented in numerous studies in the New World, with detection of *Microtus*-borne viruses in *Peromyscus* and *Sigmodon, Peromyscus*-borne viruses in *Reithrodontomys,* chipmunks and *Mus, Reithrodontomys*-borne viruses in voles and woodrats, *Oryzomys*-borne viruses in *Sigmodon* and a number of unpublished examples of similar observations in South America [38–44; Hjelle, unpubl. data]. There are even reports about probable HV infections of moose [45], bats [46], carnivores (red fox [47], domestic cat and dog [48, 49]) and insectivores (shrew [17, 50]). Further investigations are needed to verify if these species contain only HV-specific antibodies due to a limited infection (spillover) or whether the viruses can be replicated and transmitted within these host species.

Problems of Species Definition

The definition of HV species (as for virus species in general) is a difficult matter and still under discussion. The biospecies concept developed by Ernst Mayr and others is well established in zoology. In contrast to typological species definitions which are based on morphological characters, the biospecies concept is based on the fact that species are separated by a genetically mediated inability to produce fertile infants. Therefore, the sympatric occurrence of distinct animal species without any genetic interaction under natural conditions proved the presence of distinct species (sympatric test in zoology). Consequently, when transferring this definition to virology, this would mean that the separation of viruses by their occurrence in different rodent species makes intragenic recombination and reassortment very unlikely, suggesting the presence of distinct virus species. In line with this assumption, only for closely related New World HV strains within the same species (Sin Nombre virus; SNV) has genetic reassortment been observed in vivo [26, 27].

The current problem of the HV species definition is reflected by the official recommendation of the International Committee on Taxonomy of Viruses [51], representing a mixture of typological characters (e.g. at least a 7% amino acid divergence between species in the nucleocapsid protein and glycoprotein precursor and at least a fourfold difference in two-way virus cross-neutralisation) and a biospecies concept-based part (absence of genetic reassortment in nature, unique ecological niche for each virus species, i.e. a close association of a virus species with a specific rodent reservoir host).

In contrast, the definition by Monroe et al. [52] provided a more generalised biological point of view similar to the biospecies concept in defining HV species as the 'lowest taxonomic unit that is geographically and ecologically contained, genetically (reproductively) isolated, phylogenetically distinct, and self-sufficient'. Nevertheless, this type of definition also leaves several problems, resulting also from uncertainties in recent rodent taxonomy [53]. It is by no means always possible to ascertain that a single virus strain and the host in which it is found are in a true association and not associated through a spillover infection from the true host [54]. This problem becomes especially important in regions in which there are many closely related rodent species. In one trapping session, for example, investigators captured three HV-infected Sigmodontine rodents (a *Peromyscus leucopus* and two *Sigmodon hispidus*), all of which proved to be infected with the rare vole-borne strain Bloodland Lake virus [Hjelle, unpubl. data]. Without additional data that later linked this virus to its true host, *Microtus ochrogaster,* it would have been possible to misidentify even the correct subfamily of its natural host. Making a clear association between a virus and its host at the level of the host species can be difficult in some cases, and associating virus and host at the subspecies level is more difficult still.

An additional problem is related to the time axis for the species definition, which is reflected in palaeontology

in the term 'chronospecies'. The definition of a distinct HV species raises the question: What could be the consequence of detection of footprints of previous recombination events for the definition of an HV species? The occurrence of DOBV-like virus strains in two different hosts, *Apodemus flavicollis* (DOBV-Af lineage) and *Apodemus agrarius* (DOBV-Aa lineage) is still difficult to reflect in virus taxonomy. It has been proposed that the only DOBV-Aa virus strain which could be isolated so far, Saaremaa, represents a unique virus species [53]. However, the relatively high nucleotide and amino acid sequence identities of DOBV-Af and DOBV-Aa lineages and a recent description of probable reassortment within the DOBV species leading to Saaremaa virus [87] have strengthened the doubt as to whether this virus strain really represents a distinct species. On the other hand, results from a majority of anti-DOBV sera in two-way cross-neutralisation studies have suggested that a South-East European DOBV-Af strain and the Saaremaa virus are different serotypes [55]. Consequently, it should be carefully proven whether the terms 'species', 'serotype' and 'genotype' can be used synonymously in the HV taxonomy.

Pathogenesis of HV Infections in Humans

The pathogenicity of HV is determined by the interaction between the virus and the human host. Consequently, when asking what are the determinants of pathogenicity of HV for humans, there are two sides to the coin: From the virus aspect, one can ask what makes an HV pathogenic for humans and what is the reason that another HV is not pathogenic for humans? From the host aspect, it is unclear what makes some infected people ill and others not. In other words, there is an interplay between the pathogenetic markers of the virus and the genetic background of the human host. It is interesting to see the spectrum of asymptomatic infections and symptomatic infections with renal and pulmonary dysfunction of different severity. In some HCPS cases, renal dysfunction has also been seen, and in some HFRS cases, additional pulmonary dysfunction has been observed. When analysing the pathogenicity of HV, one should keep in mind that HV – in contrast to other viruses pathogenic for humans which are thought to be strongly adapted to humans, like human herpesviruses – are mainly adapted to their respective rodent host but not to humans, as they are dead-end hosts. Therefore, further investigations into whether person-to-person transmission, as reported for Andes virus (ANDV) [56], is only exceptional, should have a strong impact on the understanding of the pathogenicity of this specific virus. ANDV is also uniquely able to induce lethal infections in hamsters [57].

The cellular entry site of HV seems to distinguish between HV pathogenic for humans and those which are non-pathogenic. Pathogenic viruses have been found to interact with the $\alpha_v\beta_3$ integrin receptor, whereas HV that are thought to be non-pathogenic seem to use another integrin receptor [58]. It has been recently suggested that transgenic mice expressing human β_3 integrins may provide animal models of HV pathogenesis and have the potential to radically improve the ability to investigate HV disease [58]. Jin et al. [59] demonstrated that HTNV enters cells via the clathrin-coated pit pathway and uses low pH-dependent intracellular compartments for infectious entry. Additional investigations are needed to verify whether, as found for other viruses, co-receptors or alternative receptors are used for receptor-mediated entry of HV. Such investigations may also solve the question of how mice can be susceptible to experimental HV infection. Consequently, regions in the viral glycoproteins might represent markers for pathogenicity which may yet be identified. Indeed, the exchange of as few as one amino acid residue in the glycoprotein of HTNV can significantly alter the pathogenicity of the virus in a mouse encephalitis model [60, 61]. Cell culture adaptation of PUUV resulting in a reduction/loss of infectivity for rodents was shown likely to be due to nucleotide changes in the 3′ noncoding region of the viral S segment [62]. Moreover, Arvicolinae-associated HV like PUUV and Tula virus as well as all Sigmodontinae-associated HV studied to date possess a second putative reading frame on their S genome segment which might play a role in pathogenicity or alternatively in the adaptation of the virus to the rodent host. In other members of the family Bunyaviridae, an analogously located reading frame, 'NSs', may have a role in inhibiting the type I interferon response and decreasing virulence in the host [63]. However, there is no evidence for the expression of an ORF2-derived protein in PUUV-infected cells in cell culture [Lundkvist, pers. commun.]. In addition, the presence of a quasi-species population in an infected patient might influence the pathogenicity of HV infection.

With respect to the pathogenicity mechanism of HV infections, there is increasing evidence that it is mediated not by a direct cytopathic effect of the virus but by indirect ways like immunopathology. Thus, we have found an up-regulation of MHC class I molecules in dendritic and endothelial cells after HTNV infection [64]. Several re-

ports have demonstrated the presence of HV-specific cytotoxic T lymphocytes in HV-infected HFRS and HCPS patients [65, 66]. Increased levels of cytokines after HV infection have been observed in vitro and in vivo. Thus, the production of TNF-α can be related to symptoms observed in both HFRS and HCPS [67]. The potential influence of the genetic background of humans on the clinical outcome of HV infections has been demonstrated. For PUUV infections, a correlation between certain HLA haplotypes and TNF-α alleles and the severity of nephropathia epidemica, a mild form of HFRS in Europe, has been observed [68].

Previously, we have speculated about reasons for the different clinical severity of DOBV-caused HFRS in Europe; mild HFRS in Central and Eastern Europe might be due to infection by members of the DOBV-Aa lineage, whereas severe HFRS cases in South and South-East Europe were shown to be caused by DOBV-Af infections. However, there are several questions requiring further investigations to prove this hypothesis. In parts of Central Europe, both DOBV hosts, *A. agrarius* and *A. flavicollis,* are present. Moreover, a sympatric occurrence of DOBV-Aa and DOBV-Af has been reported for Slovakia [13, 87] and Slovenia [69]. However, at least in Slovakia, a much lower infection rate of *A. flavicollis* by DOBV-Af than of *A. agrarius* by DOBV-Aa seems to exist. In addition, HTNV-like virus infections of humans in South Germany, where *A. agrarius* is not prevalent, might be due to transmission from other rodent reservoir hosts or imported HV infections from other parts of Europe or Asia. Further studies are needed to verify these speculations.

Vaccines and Antiviral Therapy

The major mode of transmission of HV to humans is by aerosolised excreta of HV-infected rodents. Therefore, the main way to prevent human HV infections is to block virus transmission from rodents to humans. Although this transmission might be reduced by prevention of exposure, groups of people at risk due to their profession (forest workers, hunters, employees of horse farms, muskrat hunters, farmers, mammalogists, animal trappers, soldiers) or occupation would profit from antiviral measures, supporting the necessity to develop antiviral vaccines and chemotherapy [1].

To develop highly efficient and safe vaccines, the knowledge of protection mechanisms and viral components involved in their induction is an important precondition. As expected for an enveloped virus and demonstrated by numerous studies, protection can be obtained in vitro and in vivo by glycoprotein-specific antibody-mediated virus neutralisation. In contrast, it is believed that nucleocapsid protein provides a protective immunity by induction of cellular immune responses. However, glycoprotein-specific cellular immune responses as well as humoral responses against N protein could also be involved. Thus far, few studies have shed light on the possible role of cellular immune responses in protective immunity to HV. Adversely, cellular immune responses could participate in the pathogenesis of the capillary leak leading to haemorrhages.

One major obstacle for the evaluation of candidate HV vaccines is the availability of suitable animal models. Although several animal models have been established for different HV species, like suckling mice, hamsters, deer mice, Mongolian gerbils and bank voles, these models do not reflect natural HV infection in humans and do not represent disease models. Nevertheless, they have provided very useful information about desirable characteristics of vaccine candidates. The development of an intranasal virus challenge model in bank voles as a more natural infection scheme has been started [de Carvalho Nicacio and Lundkvist, pers. commun.]. Interestingly, equivalent experiments with the SNV model showed that very low-passage, deer mouse-adapted viral preparations that routinely infected deer mice by the intramuscular route were unable to infect deer mice by the intranasal route, a finding that suggests that SNV is transmitted percutaneously and not intranasally in nature [70]. Recent experiments used hamster and cynomolgus macaque disease models for ANDV and PUUV infections, respectively, which better reflect the clinical features of human infections [71, 72]. In addition, newly established HV infection models based on immunologically well-characterised mice may allow more precise identification of mechanisms of protection [73, 74].

As with other viruses, the development of HV vaccines and antiviral therapies is still based on a trial-and-error scheme, not a rational-based design. This is reflected by the fact that, except for attenuated live virus vaccines, a large number of vaccine approaches used for other viruses were also tested in the development of potential HV vaccines [1, 75]. Interestingly, efficacy studies with an inactivated HTNV vaccine (Hantavax) in regions where HV are endemic demonstrated a reduction in the number of HFRS cases [76]. Nevertheless, the vaccine requires optimisation in terms of induction of a long-lasting neutralising immune response [77]. To overcome the necessity of

high-level safety containment for production of HV vaccines, different recombinant approaches have been followed. Although highly protective in animal models, a recombinant vaccinia virus containing the S and M segments of HTNV proved to be inefficient in clinical trials with vaccinia virus-immune volunteers [78]. Virus-like particles based on hepatitis B virus core, recombinant HV proteins generated in *Escherichia coli,* insect cells and the yeast *Saccharomyces cerevisiae,* Sindbis virus constructs and naked DNA vaccines induced protective immune responses in animal models, but require further characterisation before use in humans [1, 75, 79]. Genetic vaccines are under development and have been tested, largely in hamster models [71, 80, 81], although some laboratories have also used native host species [82]. Recently, we showed that within the M genome of SNV, there are multiple independent epitopes capable of protecting deer mice from challenge. These epitopes were largely confined to G1 and could confer protection in the face of a modest or no neutralisation response [82].

Besides prevention of HV infections by vaccination, the development of efficient antiviral therapies, especially for severe clinical human cases, is very important. So far, the only drug in use for humans is ribavirin. Antiviral activity of ribavirin has been demonstrated in vitro and in vivo. Whereas in a prospective, randomised, double-blind, concurrent and placebo-controlled clinical trial of ribavirin therapy in HFRS patients in the People's Republic of China a significant reduction in fatality as well as in the risk of entering the oliguric phase and of experiencing haemorrhage was observed [83], a recent study in HCPS patients did not suggest an appreciable effect [84]; however, from this uncontrolled experience, it seems difficult to determine whether ribavirin would work in a patient who received the drug early in the course of the disease. Recently, an in vitro inhibitory effect of bovine lactoferrin on SEOV-infected Vero E6 cells was reported which is likely caused by adsorption inhibition [85]. Although first efforts to study the HV RNA-dependent RNA polymerase were initiated [86], so far no in vitro assays to study inhibitors of the enzyme or rational structure-based drug design have been established.

Outlook

Although the distribution of HV in Europe, Asia and the Americas is documented, further efforts are necessary to characterise the presence of HV in other specific regions of the world, like Africa and Australia. Keeping in mind the outbreak of HCPS at the beginning of the 1990s in the USA, further efforts should be made to monitor HV in rodent populations and to characterise so far unknown HV which may represent newly emerging pathogens for humans. These investigations also require long-term studies of rodent population dynamics in different climates of the world.

The accumulation of knowledge about pathogenicity mechanisms of HV infections and HV diversity should allow the development of more efficient vaccines. It is a very interesting question how random and non-random evolutionary factors, like mutation, genetic drift, gene flow/recombination, separation and natural selection, would be able to determine the evolution of HV. The theory of co-evolution of HV and their rodent reservoir hosts requires additional investigation, especially of the genetics of the rodent hosts. Investigations focussed on pathogenicity mechanisms would profit from the future development of reverse genetics systems for HV. On the other hand, further studies about the genetic background of humans infected with HV in correlation with the severity of the clinical outcome are needed. Studies on the structure and function of the viral RNA-dependent RNA polymerase and in vitro functional assays are needed to establish rational drug design for antiviral therapy.

The development of suitable animal models should not only allow us to estimate the protective potential of candidate vaccines, but also to further characterise immune mechanisms involved in protection. This would enable a more rational-based design of HV vaccines by separating protective and immunopathological epitopes in vaccine-relevant HV proteins. In addition, the newly established infection models in monkeys and hamsters may allow in vivo studies of the hantaviral pathogenicity mechanisms which cannot be undertaken in cell culture. Investigations of candidate HV vaccines in primate models are needed to answer the question of whether a rodent-proven vaccine might be able to induce a protective immune response in humans.

However, as we move forward with the quest to identify increasingly suitable models for disease and protection from disease, it is crucial that we do not lose sight of the value of establishing infection models for HV that use the native reservoir hosts, even if those models are not generally associated with disease. It is only through the experimental use of the native hosts, with all of their difficulties and unpredictability, that we will begin to decipher some of the most vexing problems in HV evolution, ecology, replication and epidemiology. Such questions as whether DOBV-Af is or is not intrinsically better adapted for

A. flavicollis than is DOBV-Aa, how HV are transmitted in nature, how they establish persistent infection and overwinter in the face of immune attack (even though their genomes remain highly stable) and what features of the hantavirus genome confer host and tissue tropism will never be addressed without increased utilisation of provocative infection models based upon native host species.

The interdisciplinary investigations mentioned here would profit from a close collaboration of specialists from different research fields, such as virology, zoology, historical biogeography, genetics, evolutionary biology, bioinformatics, immunology and clinical medicine.

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Review of an Inactivated Vaccine against Hantaviruses

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Key Words
Haemorrhagic fever with renal syndrome · Hantavirus · Neutralizing antibody

Abstract
Objective: Hantaviruses cause haemorrhagic fever with renal syndrome and result in severe morbidity and mortality in humans. Safe and effective vaccines are needed to reduce the incidence of human illness. In this study, the immune response to an inactivated hantavirus vaccine was measured in 64 human volunteers for Hantavax® and 10 human volunteers for a Hantaan-Puumala virus combination vaccine at high risk of infection by virtue of their residence and occupation. ***Methods:*** A serum sample was obtained from each volunteer before the initial vaccination (day 0), 30 days after each inoculation and 1 year after the initial dose. All sera were kept at –20° until tested. IgG-specific antibody titres were tested by ELISA and immunofluorescence assay (IFA). Neutralizing antibody titres were determined by a plaque reduction neutralizing test. ***Results:*** Thirty days after vaccination, 79 and 62% of the subjects had developed a significant hantavirus antibody titre as measured by IFA and ELISA, respectively. Seroconversion rates increased to 97% 1 month after the booster dose. Neutralizing antibody titres paralleled this trend, with 13% of vaccine recipients producing neutralizing antibody 1 month after the first dose and 75% of vaccine recipients responding 1 month after boosting. Antibody titres had declined by 1 year, however, with only 37 and 43% of sera found to be positive by IFA and ELISA, respectively. Re-vaccination at this time produced a vigorous anamnestic response, with 94 and 100% of vaccine recipients yielding positive antibody titres. Only 50% of the sampled population, however, produced neutralizing antibodies following the booster dose 1 year later. ***Conclusions:*** The vaccine was well tolerated and there were no apparent differences in the responses in human subjects. However, further improvement of this vaccine is necessary in order to induce a longer-lasting humoral immune response.

Introduction

The clinical syndrome known as haemorrhagic fever with renal syndrome (HFRS) has been recognized among the important emerging and re-emerging viral diseases with high fatality worldwide. HFRS caused by the Hantaan (HTNV), Seoul, Puumala (PUUV) and Belgrade/

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Table 1. Consumption of HFRS vaccine and number of HFRS patients in Korea

Year	Dose of vaccine, ml				HFRS patients
	hospital clinics	public health center	army	total	
1990	0	0	0	0	1,043
1991	35,000	115,000	33,000	183,000	1,234
1992	122,000	493,000	145,000	760,000	1,167
1993	118,000	707,000	133,000	958,000	1,293
1994	107,000	660,000	142,000	909,000	1,041
1995	118,000	640,000	232,000	990,000	782
1996	105,000	460,000	155,000	720,000	687
1997	108,000	360,000	162,000	630,000	415
1998	70,000	310,000	160,000	540,000	750
Total	783,000	3,745,000	1,162,000	5,690,000	8,412

Dobrava viruses are the oldest diseases, first being recognized in 1913. However, hantavirus pulmonary syndrome caused by Sin Nombre, New York and other novel hantaviruses are new diseases which were first recognized in 1993 in North and South America [1].

HTNV causes severe symptoms with a mortality of 5–10%, whereas hantavirus pulmonary syndrome infection can result in a mortality rate in excess of 60%. HFRS has been an important public health problem in Korea since the onset of the Korean war. Clearly, there is an urgent need to develop an effective vaccine against these viruses. In 1984, the World Health Organization (WHO) recommended the development of an effective inactivated vaccine against HFRS as soon as possible. As a result, Lee and An [2] developed an inactivated vaccine against HTNV infection.

This HTNV vaccine is prepared from the HTNV strain ROK 84/105 grown in suckling mice brain and has been shown to induce protein immunity in mice and humans. It is currently produced commercially under the name Hantavax® and licensed for use in the Republic of Korea. A vaccination compaign was started in 1991, and the number of HFRS patients had decreased significantly in 1995, 3 years after the start of the vaccination program. Table 1 shows the relationship between consumption of Hantavax and the number of HFRS patients from 1991 to 1998 in Korea. Recently, Lee et al. [3] developed a formalin-inactivated suckling hamster brain-derived HTNV-PUUV combination vaccine.

This study aimed to assess antibody responses to the inactivated virus vaccine Hantavax and the HTNV-PUUV combination vaccine in humans. The induction of anti-hantavirus antibodies was measured with a view to optimizing antibody level after third booster doses. In addition, the safety and efficacy of both hantavirus vaccines were evaluated.

Materials and Methods

Hantavirus Vaccine

The seed strain of HTNV virus used to prepare the Hantavax vaccine (ROK 84/105) was obtained from the blood of an HFRS patient [4]. Virus was isolated by direct inoculation onto Vero E6 cells. Hantavax was made according to the modified method of Japanese encephalitis vaccine [5] with HTNV strain ROK 84/105 by inoculation into a 1-day-old ICR (Institute of Cancer Research) mouse brain.

HTNV-PUUV combination vaccine was prepared from formalin-inactivated suckling hamster brain-derived vaccine.

Volunteers

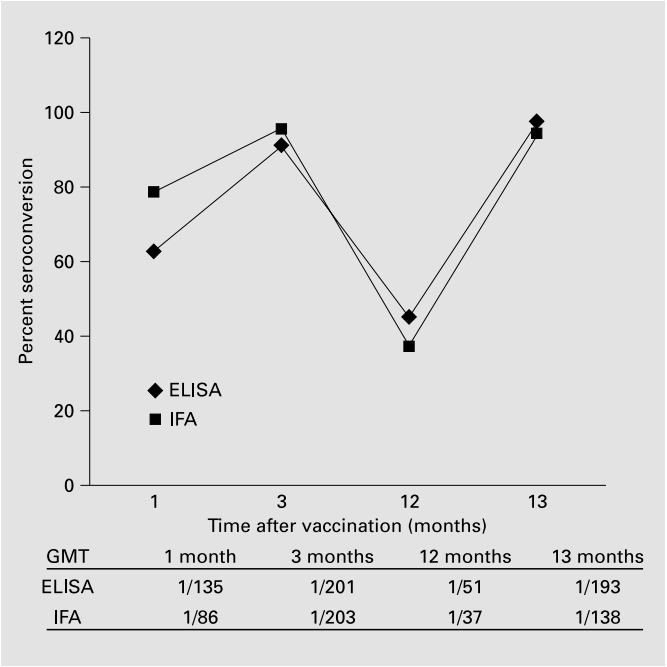
The immune responses of 64 volunteers for Hantavax and 10 volunteers for the HTNV-PUUV combination vaccine were analyzed. Consent was obtained from all the vaccine recipients prior to entry into the study. All participants were interviewed in order to ascertain age, history of allergy, occupation, sex, prior vaccination and previous medical history of HFRS. Those interviewees with a previous history of HFRS were excluded from further consideration.

Vaccination and Follow-Up

Each person received a 0.5-ml dose (5,120 ELISA units/ml) of vaccine by intramuscular injection, followed by a second, similar dose 1 month later for Hantavax. For the HTNV-PUUV combination vaccine, volunteers were vaccinated 3 times subcutaneously at 1-month intervals with various doses of the vaccine. After vaccination, general symptoms and local reactions among the vaccinated individuals were checked and recorded daily for 5 consecutive days.

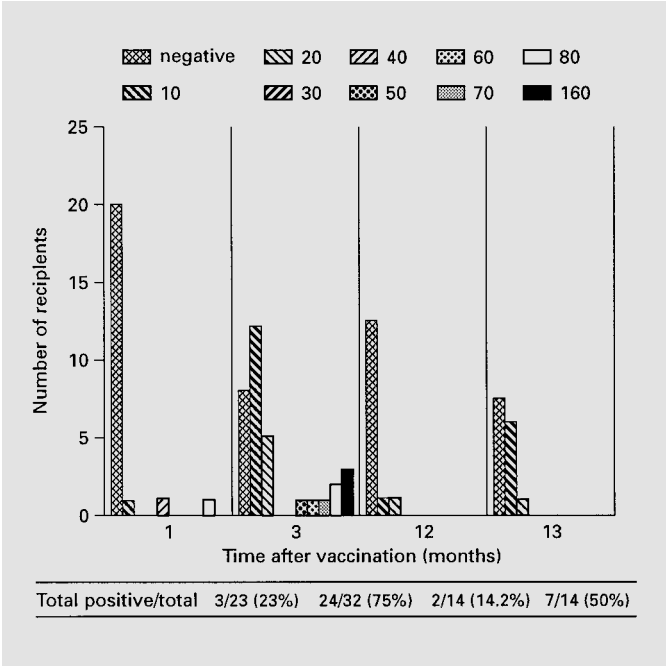
Determination of Antibody Responses

A serum sample (5 ml) was obtained from each volunteer before the initial vaccination (day 0), 30 days after each inoculation and



GMT	1 month	3 months	12 months	13 months
ELISA	1/135	1/201	1/51	1/193
IFA	1/86	1/203	1/37	1/138

Fig. 1. Comparison of anti-HTNV IgG antibody responses measured by ELISA and IFA among 64 volunteers followed for 13 months.



Total positive/total	3/23 (23%)	24/32 (75%)	2/14 (14.2%)	7/14 (50%)

Fig. 2. Neutralizing antibody titres among 64 volunteers expressed as reciprocal GMT values (shown above the figure).

1 year after the initial dose. All sera were kept at –20° until tested. IgG-specific antibody titres were tested by ELISA and immunofluorescence assay (IFA).

Neutralizing antibody titres were determined by a plaque reduction neutralizing test (PRNT) method at each time point, using a dilution series of samples added to a constant amount of virus. Tests were conducted in duplicate by mixing equal volumes of fourfold serially diluted serum with 200 PFU of HTNV (76–118), incubating for 90 min at 37°, then inoculating 0.3 ml onto Vero E6 cell monolayers grown in 6-well plates (Costar, Corning, N.Y., USA). Positive and negative control plates were run in duplicate. Neutralization titres were recorded as the reciprocal of the highest serum dilution neutralizing 50% of the plaques as determined from mean plaque counts on the virus-positive control plates.

Results

Characterization of Antibody Responses

None of the vaccine recipients were positive for anti-HTNV antibodies prior to immunization. The seroconversion rates among the vaccinated volunteers are shown in figure 1. Thirty days after immunization, 51 of 64 individuals (79.7%) were positive by IFA (titre 1/32 by serial dilution); 40 of these sera were determined to be positive also by ELISA (62%). The ELISA titres yielded a greater geometric mean titre (GMT) compared with IFA (1/120 as opposed to 1/32), possibly reflecting either the different epitopes measured by these tests or differences in the affinity of the early IgG antibody responses, or both.

Thirty days after the second dose (day 60 in the protocol), nearly all subjects were seropositive by both assays (62 of 64 individuals), and antibody GMTs were approximately the same using both assay methods (1/203 for IFA, 1/201 for ELISA). Anti-HTNV IgM antibodies were observed in only 8 vaccine recipients (12.5%) by ELISA (data not shown). It is likely that the 30-day sampling interval antibody response may have been transient and low in titre. In natural infections, IgM antibodies may persist for months, but this response may be truncated in the case of non-replicating vaccines.

One year after the initial vaccination, the IFA and ELISA antibody titres had declined significantly to 1/36.8 and 1/51.3, respectively. Only 37.5% of the population tested at 1 year were positive for antibodies as measured by IFA (n = 24); 43.5% were seropositive for HTNV antibodies by ELISA (n = 23). Sample sizes of the population at 1 year were lower due to the difficulties of follow-up.

The volunteers that were bled after 1 year were then boosted with 0.5 ml of Hantavax by intramuscular inoculation. Thirty days after this second booster dose, 16 participants were bled. The IFA seropositivity rate rose sig-

Table 2. IFA and neutralizing antibody titres of HTNV-PUUV combination vaccine vaccinees

Patient No.	Age, years/ sex	History	Vaccine and dose	IFA and neutralizing antibody titres to HTNV, PUUV and Belgrade virus																	
				before vaccination				after first shot (1 month)					after second shot (2 months)					after third shot (3 months)			
				IFA		PRNT		IFA		PRNT			IFA		PRNT			IFA		PRNT	
				HTNV	PUUV	HTNV	PUUV	HTNV	PUUV	HTNV	BELV	PUUV	HTNV	PUUV	HTNV	BELV	PUUV	HTNV	PUUV	HTNV	BELV	PUUV
1	38/M	Hantavax	HTNV-PUUV 0.6 ml	256		40		2,048	512	160	20	40	4,096	128	160	40	320	4,096	512	160	40	320
2	66/M	Hantavax		32				256		20		10	256	32	80		40	512	32	20		40
3	27/M	none						2,048	128	640		160	4,096	128	1,280	10	160	4,096	512	1,280	10	640
4	33/M	none						128		10			1,024	128	20	10	20	2,048	256	20	10	40
5	24/M	none						128	32				128	64			10	512	128	20		20
6	33/F	none	HTNV-PUUV 0.8 ml					32		40	80		64	32	160	80	40	64	64	80	10	80
7	26/F	none						64	32	20			128	32	40	10	20	1,024	128	80	10	40
8	27/M	none						128	32	40		10	256	64	40		40	256	64	40		160
9	28/M	none	HTNV-PUUV 1.0 ml					512	128	20		10	512	64	320	20	320	1,024	128	640	40	320
10	53/F	none						1,024	128	20		40	1,024	64	80	10	160	2,048	256	80	10	160
11	25/F	none	PUUV vaccine 0.5 ml						128		10	80		128		20	320		256		10	640
12	22/F	none							64			10		128			80		128			160
13	54/F	none	Hantavax 0.5 ml					128		40			1,024		160			2,048		160	20	
14	26/F	none						1,024		20			512		80			512		80		

BELV = Belgrade virus.

nificantly to 93.8% and the ELISA antibody seroprevalence was 100%. The GMT of IFA antibody rose to 1/138, which was lower than the response to the first booster at 60 days (GMT of 1/203). The ELISA antibody titres at 13 months (GMT of 1/193.8) were almost equivalent to those after the 60-day booster dose (GMT of 1/201).

The neutralization assay (fig. 2) showed that only 13% of vaccine recipients possessed measurable neutralizing antibodies 30 days after the first inoculation. This proportion increased to 75% following the booster inoculation, with titres in the range of 1/10–1/160. There was a lack of correlation between the PRNT antibody titres and the ELISA titres 2 months after immunization. The serological responses 30 days after the third vaccination (13 months) were even less in agreement, with a significant proportion of volunteers showing high ELISA titres in the absence of neutralizing antibodies.

One year after vaccination, neutralizing antibody titres had declined to the low levels seen prior to vaccination (1/10) in 85.7% of the recipients. Only two individuals showed significant but low titres. After boosting with Hantavax, neutralizing antibody titres rose above 1/10 in only 50% of the subjects tested. This level of immunity was comparable to that seen after the second dose (60 days). Although the seropositivity rate was equivalent after each booster dose, the GMT was much lower at 13 months (fig. 1). In the limited HTNV-PUUV combination vaccine study, 10 vaccine recipients produced relatively high IFA antibodies (1:128–1:2,048) and a medium level of neutralizing antibodies (1:10–1:640) against homologous hantaviruses after the second and third vaccination, as shown in table 2.

Side Effects following Vaccination

No obvious untoward reactions were observed among the vaccinees who received two doses of vaccine by the intramuscular route, as shown in table 3. The most common complaints were mild itching, induration and local swelling at the site of injection, but no serious complaints were registered. The lack of serious side effects indicated that this vaccine appears to be well tolerated in humans.

Discussion

Immunization of volunteers with a novel hantavirus vaccine (Hantavax) resulted in 97% seroconversion by the IFA test and 75% seroconversion by the PRNT fol-

Table 3. Symptoms and frequency of occurrence of local reactions to Hantavax vaccination among 64 volunteers

Reaction	Day of disappearance of symptoms					
	1	2	3	4	5	6
Dizziness	1	1				
Nausea	1	1				
Myalgia	1		1			
Redness	1	1				
Itching	2	1	1			
Swelling	3	2	1			
Induration	2			1	1	
Hyperpigmentation	1	1				
Total	12	7	3	1	1	

lowing two intramuscular doses of vaccine 1 month apart. One year after the initial vaccination, however, these antibody titres had dropped significantly to 37.5 and 21.4%, respectively. A single dose 12 months after the initial vaccination boosted the seropositivity rate to 93.8% as determined by IFA, 100% ELISA and 50% by PRNT. Although the sample size of the population at the 1-year point was smaller due to difficulties in follow-up, the sample population that did participate remained representative of the original group. One disturbing finding, however, was that the neutralizing antibody titres observed 1 year after the previous 2-dose immunization schedule were almost at pre-immunization levels. Notably, a booster dose at the 1-year time point did not elicit a strong neutralizing antibody anamnestic response. It would therefore be necessary to use booster doses every 12 months in order to maintain a minimal protective antibody titre against HTNV in humans. It may also be necessary to consider a different initial vaccination schedule in order to stimulate greater clonal definition of B cells that respond to neutralizing epitopes on the virus. Another possibility would be a re-dosing of the vaccine to produce either greater adjuvancy or an increased presentation of neutralizing epitopes, or both.

The neutralizing epitopes for HTNV are associated with the viral membrane glycoproteins [6]. The stronger IFA and ELISA antibody responses seen 1 year after the first dose may reflect a predominance of antibody production to other epitopes on the glycoprotein, or to the nucleocapsid protein, which is the predominant antigen recognized by IFA and ELISA systems [7]. Antibody has been demonstrated to persist for up to 34 years after natural infection [8]. While it was not possible to evaluate the long-term duration of HFRS antibody in human vaccine recipients, the data 14 months after immunization suggest that antibody wanes to the point of requiring booster doses in order to maintain a protective immune response against HTNV.

Compared to the mouse brain-derived Japanese encephalitis virus vaccine prepared by similar methods, Hantavax showed an immunogenicity approximately equal in extent and duration using a similar delivery schedule. The 75% neutralizing antibody seropositive response rate for the Hantavax vaccine as measured by the plaque neutralization test and passive immunization was approximately equivalent to the 90–100% observed for Japanese encephalitis vaccine recipients after a 2-dose regime over a 30-day period. Nakamura [9] reported that passive protection studies performed with human immune serum in mice for Japanese encephalitis vaccine recipients after a 2-dose regime over a 30-day period revealed a strong correlation between the circulating antibody titres and protection. Similarly, the observed decline in seroprevalence and neutralizing antibodies over the 12-month observation period in individuals receiving Hantavax in the present study roughly approximates the decline observed for Japanese encephalitis vaccine. One difference between the two vaccines is the lower neutralizing antibody response elicited after the 1-year booster dose with Hantavax. Thus, a further evaluation is indicated, for example using a 3-dose schedule, in order to see whether or not a similar enhancement in the protective immune response can be obtained. A long-term, prospective efficacy trial is needed in order to determine whether or not the vaccine has any demonstrable effect in reducing disease in those groups most at risk.

Another major consideration with respect to the utility of Hantavax is the safety and reactogenicity of this mouse brain-derived inactivated vaccine. As with any vaccine produced by these methods, the possibility of adverse reactions exists. This study confirms that the Hantavax vaccine is safe and well tolerated, with minimal reactogenicity in humans following a dose schedule of 2 doses within a 1-month period with a booster dose given at 13 months. The general and local side effects observed in the 64 vaccine recipients receiving two doses of vaccine are shown in table 3. A higher incidence of local reactions than general symptoms was observed. The main local reactions were itching, induration and swelling, but there were no serious complaints and the side affects were usually self-limiting.

The inactivated HTNV vaccine developed in Korea to prevent HFRS was prepared with virus harvested from

Table 4. Comparison of antigenicity of HTNV harvested from different sources

Source of antigen	ELISA antigen U/ml	Antibody titres in guinea pigs	
		IFA	PRNT
Vero cells (inactivated virus)	3,200	1,600	10
Baculovirus-expressed nucleocapsid protein	3,200	800	<10
Adenovirus-expressed G1,G2 glycoproteins	6,400	160	80
Suckling mouse brain (inactivated virus)	12,800	6,400	160

infected suckling mouse brains [10]. The seed strain of virus for this vaccine (ROK 84/105) was obtained from the blood of an HFRS patient in the early acute stages. Virus was isolated by direct inoculation onto Vero E6 cells and subsequent passage in suckling mouse brains. This culture method was chosen because HTNV grown exclusively in Vero E6 and MRC-9 cell lines does not produce sufficient virus yields for vaccine production purposes. In contrast, the yield and immunogenicity of virus from the brain of infected suckling mice is adequate for vaccine production (table 4). The Korean HFRS vaccine Hantavax went into commercial production in 1990. Hantavax meets or exceeds the minimum requirements for biological products (potency test, safety, sterility, pH, concentration of thimerosal, protein concentration, formalin concentration, etc.) comparable to those stipulated by the WHO guidelines for Japanese encephalitis vaccine. After Hantavax was made available in 1991, the total number of hospitalized HFRS patients in South Korea decreased significantly from 1,234 cases in 1991 to 415 cases in 1997, as shown in table 1.

Recently, an efficacy trial of Hantavax in the endemic area of HFRS is under evaluation in a randomized, placebo-controlled field study with vaccinees who received Hantavax twice in 1996 and a booster vaccine in 1997 in Yugoslavia [11]. Twenty-five HFRS patients were documented among a control group of 2,000 non-vaccinees, but none were reported among 2,000 vaccinees.

An HTNV-PUUV combination vaccine could be used after safety tests and a field efficacy trial in the endemic areas of HFRS where these two viruses co-exist in the world. The vaccine is likely to be effective in protecting those recipients who respond to the vaccine against HFRS. The findings of this study have identified areas for future study and could form the basis for development of vaccines against other hantaviruses.

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Characterization of Expression of Puumala Virus Nucleocapsid Protein in Transgenic Plants

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Key Words
Hantavirus · Nucleocapsid protein · Expression · Tobacco · Potato

Abstract
Transgenic plants expressing a foreign gene are a suitable system for the production of relevant immunogens in high amounts that can be used for the development of a new generation of vaccines against a variety of infectious diseases. In the present study, the expression of the nucleocapsid (N) protein of hantavirus serotype Puumala in tobacco and potato plants was investigated. Transgenic tobacco and potato plants were generated and established. These transgenic plants expressed the N protein of Puumala virus strain CG-1820. No major differences were observed when the phenotype and growth rates of transgenic plants were compared to those of normal plants. However, it was found that the leaves of transgenic tobacco plants were more slender and the tubers of transgenic potato plants were smaller than those in normal plants. In order to investigate the distribution of the expression of the foreign gene in transgenic plants, the proteins of leaves and roots of the individual transgenic tobacco and potato plants were examined by Western blot analyses. It was found that all transgenic tobacco and potato plants expressed the N protein in the leaves, whereas transgenic potato plants are able to significantly express the viral proteins also in the tubers and roots. The antigens were expressed at a level of 1 ng of protein/5 μg of dried leaves. The hantaviral recombinant N proteins obtained from transgenic tobacco and potato plants were able to elicit specific humoral and mucosal immune responses when administered intraperitoneally or orally to rabbits and mice. The expression of viral proteins in plants has two major advantages compared to other expression systems: firstly, there is no risk of contamination with mammalian viruses or other pathogens, and secondly, the production of high amounts of antigens is cheap and therefore of great economic interest.

Introduction

Hantaviruses are negative-sense, single-stranded RNA viruses [1] that belong to the Bunyaviridae family. Different serotypes are etiologic agents of a number of diseases with renal and/or pulmonary involvement, ranging from the more benign nephropathia epidemica (lethality 0.1–1%) through hemorrhagic fever with renal syndrome (le-

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thality 3–10%) to the hantavirus pulmonary syndrome (lethality >50%). The individual hantavirus genotypes are carried by specific rodent hosts. Infection of humans occurs by aerosol inhalation of contaminated rodent excretions.

The three-segment genome of hantaviruses codes for the viral RNA-dependent RNA polymerase (L-RNA segment), the glycoproteins G1 and G2 (M-RNA segment) and the viral nucleocapsid protein (S-RNA segment). The viral nucleocapsid protein is an important structural protein and is essential for packaging of the RNA segments and encapsidation [2]. Major antigenic domains of hantaviruses are also located on the nucleocapsid protein [3]. The early immune response is mainly directed against this structural protein, and sera of convalescent patients predominantly contain antibodies against the nucleocapsid protein [4–7]. Furthermore, the viral nucleocapsid proteins, together with the viral glycoproteins G1 and G2, are promising candidates for the development of new vaccination strategies.

Recent studies have shown that proteins expressed in plants are suitable tools for the production of antigens. Bacterial toxins and various viral proteins have been successfully expressed in plants and found to be immunogenic [8–14].

These proteins were able to elicit specific humoral and mucosal immune responses when administered intraperitoneally or orally to animals and to protect the animals against the corresponding viral or bacterial infections. The expression of viral proteins in plants has the following advantages compared to other expression systems: firstly, there is no risk of contamination with mammalian viruses or other pathogens, and secondly, the production of antigens based on expression of the proteins is cheap and therefore of economic interest.

Materials and Methods

Transgenic Tobacco Plants

The transgenic tobacco plants were generated by transformation of *Nicotiana tabacum* cv. SR1 plants as described previously [14]. Briefly, leaves of the plant were immersed in MS liquid medium containing bacterial cells which harbored the recombinant binary plasmid pBinAR-PUU-S encoding the Puumala nucleocapsid (N) protein sequences. After 48 h, the leaves were washed with media containing cefotaxime and plated on selective agar media which contained kanamycin (100 μg/ml), cefotaxime (500 μg/ml), benzaladenine 2 μg/ml and naphthalene acetic acid 0.1 μg/ml. Putative transformed shoots were further grown on kanamycin and analyzed for the expression of Puumala N protein.

Transgenic Potato Plants

The transgenic potato plants were generated by transformation of *Solanum tuberosum* cultivar Desiree tubers as described previously [14]. Briefly, microtubers cut into 1- to 2-mm discs were inoculated with bacterial cells which harbored the recombinant binary plasmid pBinAR-PUU-S encoding the Puumala N protein sequences for 2–3 days. After cocultivation, the discs were washed with medium containing cefotaxime (500 μg/ml). The discs were transferred onto MS medium containing kanamycin (50 μg/ml), cefotaxime (300 μg/ml), GA_3 0.02 μg/ml and zeatin 2 μg/ml. Putative transformed shoots were further grown on kanamycin and analyzed for the expression of Puumala N protein [14].

Sera and Antibodies

Antiserum against recombinant N proteins of Puumala virus strain CG-1820 was induced in New Zealand white rabbits. Recombinant N proteins were generated as described elsewhere [15, 16]. The rabbit antiserum against Puumala virus N protein was able to detect 0.125 ng of recombinant viral N protein.

Immunoblot Analysis

Plant tissues, including leaves and roots, were harvested and dried for 72 h at 50°. Samples of leaf tissue powder (200 μg) were dissolved in lysis buffer [600 μl with 0.001 *M* Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% (w/v) bromophenol blue, pH 8] and heated for 5 min at 95°. The insoluble fraction was removed by centrifugation at 17,000 *g* for 5 min. Proteins were separated by SDS-PAGE and electroblotted on nitrocellulose filters. Transfer efficiency was monitored by ponceau staining (Sigma, Munich, Germany). Filters were blocked for 1 h and incubated with a 1/1,000 dilution of rabbit antiserum. Peroxidase-conjugated antibodies were used to detect interaction of the rabbit antiserum with hantaviral proteins.

Results

Phenotypic Characterization of Transgenic Tobacco Plants Expressing Puumala N Protein

Transgenic tobacco plants were grown in commercial garden mould at room temperature. Under these conditions, the growth rate of transgenic plants was comparable to that of normal tobacco plants. They developed 2–4 leaves per week and grew to a final height of 1.2 m (fig. 1). The growth rate did not differ from that of normal tobacco, but the final height of the normal plants was slightly greater (1.5 m).

The leaves of transgenic tobacco plants were more slender but just as long as normal tobacco leaves. No other differences could be detected.

Phenotypic Characterization of Transgenic Potato Plants Expressing Puumala N Protein

Transgenic potato plants were grown in commercial garden mould at room temperature. Under these condi-

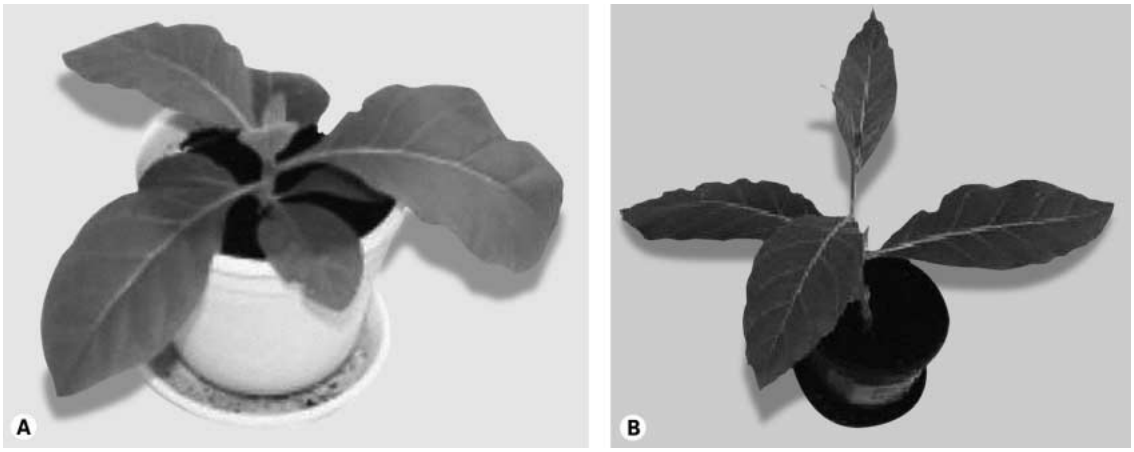

Fig. 1. Transgenic tobacco plant expressing the N protein of Puumala virus CG-1820 (**B**) in comparison to a normal tobacco plant (**A**). Both plants were cultured for 10 weeks under identical conditions. The photographs of both plants were taken at the same scale.

Fig. 2. Transgenic potato plant expressing the N protein of Puumala virus CG-1820 after 3 months of cultivation.

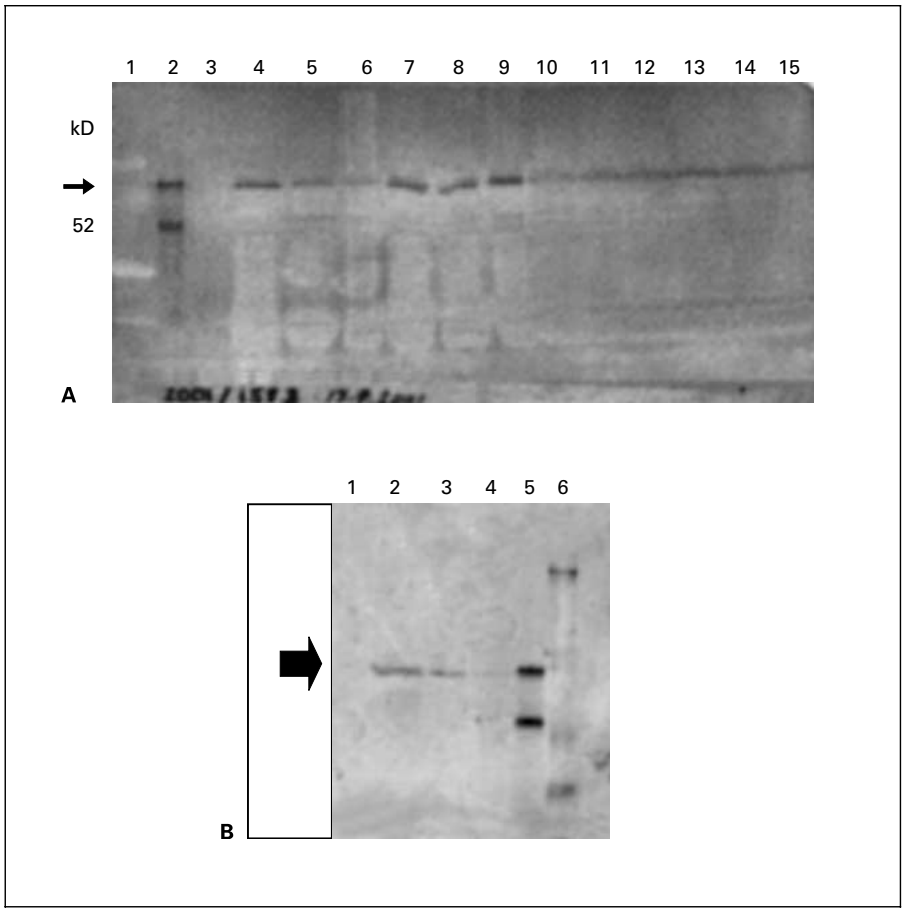

Fig. 3. Western blot analyses of proteins of transgenic plants using rabbit antibodies generated against recombinant Puumala virus N protein. **A** Distribution of the expression of Puumala N protein in the leaves of transgenic tobacco (lanes 3–9) and transgenic potato plants (lanes 10–15). Lane 1: molecular weight marker; lane 2: recombinant N protein of Puumala virus (5 ng). The arrow indicates the position of the Puumala virus N protein. **B** Distribution of the expression of Puumala N protein in the leaves and roots of two transgenic tobacco plants. Lanes 1 and 2: protein extracts of the roots (lane 1) and leaves (lane 2) of a transgenic tobacco plant (NT-PUU-F4-1); lanes 3 and 4: protein extracts of the leaves (lane 3) and roots (lane 4) of a second transgenic tobacco plant (NT-PUU-F4-2); lane 5: recombinant N protein of Puumala virus (5 ng); lane 6: molecular weight marker. The arrow indicates the position of the Puumala virus N protein.

tions, the growth rate of transgenic plants was comparable to that of normal potato plants (fig. 2). They developed 4–6 leaves per week and grew to a final height of 0.8 m (fig. 1). The growth rate did not differ from that of normal potato plants, but the tubers were smaller (average weight of transgenic tubers = 19.8 g, average weight of normal tubers = 30.7 g) and the average number of tubers generated by each transgenic plant was smaller (average number of tubers per transgenic plant = 4.3, average number of tubers per normal plant = 8.0). No other differences could be detected.

Distribution of Expression of Puumala N Protein in Transgenic Tobacco and Potato Plants

In order to investigate the distribution of the expression of Puumala N protein in two transgenic tobacco plants, 200 µg of powdered leaves and roots were analyzed by Western blots. The proteins were separated by SDS-PAGE and blotted onto nitrocellulose. As shown in figure 3, the expression of Puumala N protein differed between the two plants. In plant 1, expression could be detected in the leaves and roots. In contrast, expression of Puumala N protein in plant 2 could only be detected in the leaves.

Similar experiments were performed using leaves and tubers of transgenic potato plants. In all transgenic plants which were examined (n = 6), expression of Puumala N protein could be detected in the leaves and tubers. The amount of expression of the protein was similar, i.e. no major differences could be detected between the transgenic potato plants (data not shown).

Stability of Expression of Puumala N Protein in Different Generations of Transgenic Tobacco Plants

In order to investigate if the expression of Puumala N protein is stable in different generations of transgenic tobacco plants, the leaves of five generations (P, F1, F2, F3 and F4) of plants were analyzed. As shown in figure 4, no differences could be detected. The amount of Puumala N protein expressed in the leaves of these plants was

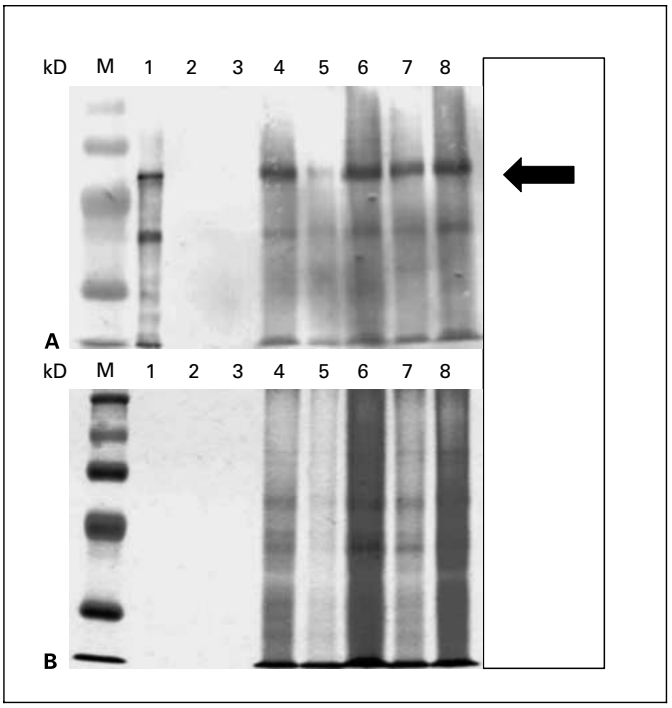

Fig. 4. Stability of expression of Puumala N protein in different generations of transgenic tobacco plants (P, F1, F2, F3 and F4). Lane 1: recombinant N protein of Puumala virus (5 ng); lanes 2 and 3: sample buffer serving as negative control; lanes 4–8: protein extracts of leaves of transgenic tobacco plants (lane 4: NT-PUU-P1; lane 5: NT-PUU-F1-1; lane 6: NT-PUU-F2-1; lane 7: NT-PUU-F3-1; lane 8: NT-PUU-F4-1). M = Molecular weight marker. The arrow indicates the position of the Puumala virus N protein. **A** Western blot analyses of proteins of transgenic plants using rabbit antibodies generated against recombinant Puumala virus N protein. **B** Coomassie staining of the SDS-PAGE.

about the same in each generation. This indicates that integration of the foreign DNA is stable, without any selection pressure, and that the expression is not reduced by silencing mechanisms, at least under the conditions used here.

Discussion

Transgenic plants expressing foreign genes are a suitable system for the production of relevant immunogens in high amounts that can be used for development of a new generation of vaccines against a variety of infectious diseases. In the present study, the expression of the N protein of hantavirus serotype Puumala in tobacco and potato plants was investigated.

The hantaviral recombinant N proteins obtained from transgenic tobacco and potato plants have been shown to be able to elicit specific humoral and mucosal immune responses when administered intraperitoneally in rabbits and mice [14]. Oral immunization of mice is under investigation. Only recently, it was shown that oral immunization of mice with hepatitis B surface antigen expressed in transgenic plants was successful [17]. Hence, the expression of hantaviral antigens in transgenic plants is a suitable approach for the development of a vaccine against hantaviral infections. At present, there is no commercially available hantaviral vaccine. The limiting factor is that it is still not possible to express hantaviral glycoproteins with high manufacturing efficiency. A variety of new generations of hantaviral vaccines are still at an experimental stage [18–20].

The expression of viral proteins in plants has the following major advantages: firstly, there is no risk of contamination with mammalian viruses or other pathogens, and secondly, the production of high amounts of antigens is cheap and therefore of great economic interest. The possibility of antigen expression in high amounts facilitates the characterization of protein interactions in pharmacokinetic studies and in addition enables basic science on the further structural characterization of the viral antigens by crystallization.

Finally, transgenic plants expressing hantaviral antigens could be used for oral field immunizations of rodents which serve as vectors, thereby eradicating the main pathway of transmission of hantaviruses to humans.

Acknowledgment

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Stop Codon Insertion Restores the Particle Formation Ability of Hepatitis B Virus Core-Hantavirus Nucleocapsid Protein Fusions

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Key Words

Virus-like particles · Mosaic hepatitis B virus core particles · Hantavirus · Nucleocapsid protein · Epitopes

Abstract

In recent years, epitopes of various origin have been inserted into the core protein of hepatitis B virus (HBc), allowing the formation of chimeric HBc particles. Although the C-terminus of a C-terminally truncated HBc (HBcΔ) tolerates the insertion of extended foreign sequences, the insertion capacity is still a limiting factor for the construction of multivalent vaccines. Previously, we described a new system to generate HBcΔ mosaic particles based on a read-through mechanism in an *Escherichia coli* suppressor strain [J Gen Virol 1997;78:2049–2053]. Those mosaic particles allowed the insertion of a 114-amino acid (aa)-long segment of a Puumala hantavirus (PUUV) nucleocapsid (N) protein. To study the value and the potential limitations of the mosaic approach in more detail, we investigated the assembly capacity of 'non-mosaic' HBcΔ fusion proteins and the corresponding mosaic constructs carrying 94, 213 and 433 aa of the hantaviral N protein. Whereas the fusion proteins carrying 94, 114, 213 or 433 aa were not assembled into HBcΔ particles, or only at a low yield, the insertion of a stop codon-bearing linker restored the ability to form particles with 94, 114 and 213 foreign aa. The mosaic particles formed exhibited PUUV-N protein antigenicity. Immunization of BALB/c mice with these mosaic particles carrying PUUV-N protein aa 1–114, aa 1–213 and aa 340–433, respectively, induced HBc-specific antibodies, whereas PUUV-N protein-specific antibodies were detected only in mice immunized with particles carrying N-terminal aa 1–114 or aa 1–213 of the N protein. Both the anti-HBc and anti-PUUV antibody responses were IgG1 dominated. In conclusion, stop codon suppression allows the formation of mosaic core particles carrying large-sized and 'problematic', e.g. hydrophobic, hantavirus sequences.

In recent years, the genus *Hantavirus,* a member of the family Bunyaviridae, has attracted increasing interest as a member of the group of 'emerging viruses'. Hantaviruses are zoonotic, rodent-borne human pathogens causing two types of disease, haemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. According to dif-

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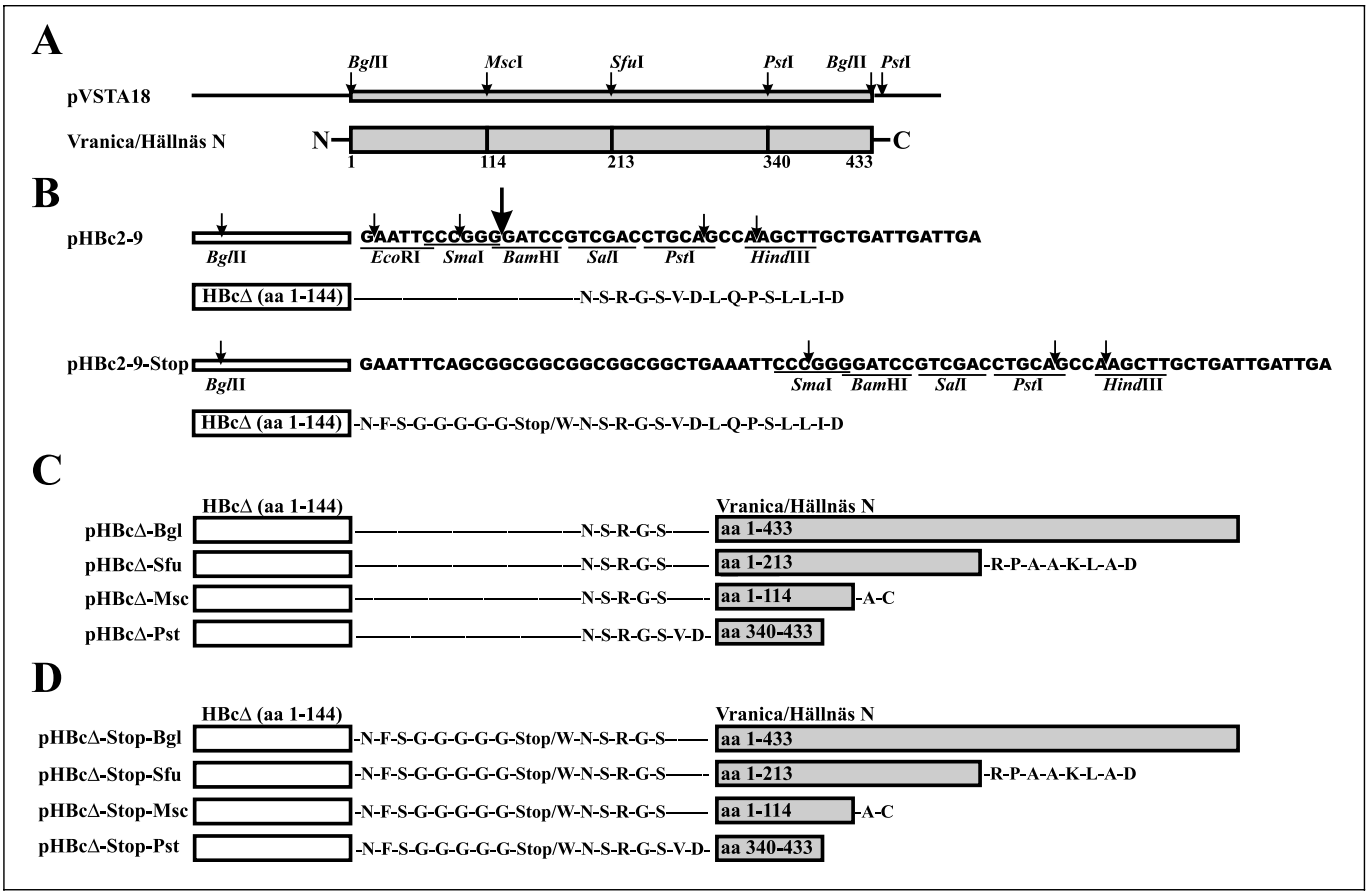

Fig. 1. Schematic presentation of the HBcΔ-PUUV-N fusion and mosaic constructs. The entire N-encoding sequence of Vranica/Hällnäs PUUV was isolated as a *Bgl*II fragment from pVSTA18 (**A**) [23] and cloned into the *Bam*HI-linearized plasmid pHBc2-9 (**B**) [15] to yield pHBcΔ-Bgl (**C**). The plasmids pHBcΔ-Msc [21] and pHBcΔ-Sfu were obtained by subsequent restriction endonuclease-mediated deletion, whereas the plasmid pHBcΔ-Pst was constructed by inserting a *Pst*I fragment isolated from pQE-VST18 [23] into *Pst*I-linearized pHBc2-9 (**C**). The construction of the plasmid pHBc-Msc-Stop126/127 was described recently [21] (re-designated here as pHBcΔ-Stop-Msc; **D**). The plasmids pHBc2-9-Stop (**B**), pHBcΔ-Stop-Sfu and pHBcΔ-Stop-Bgl were generated by substituting the original *Bgl*II-*Sma*I fragment with the similar, but stop linker-carrying fragment from pHBcΔ-Stop-Msc (**D**). The plasmid pHBcΔ-Stop-Pst was obtained by inserting the *Pst*I fragment encoding aa 340–433 of Vranica/Hällnäs N protein from pQE-VST18 into the *Pst*I site of pHBc2-9-Stop (**D**).

ferences in their rodent hosts, nucleotide and resulting amino acid (aa) sequences and cross-neutralization, distinct hantavirus sero-/genotypes have been defined. Whereas serotypes causing hantavirus pulmonary syndrome are mainly distributed in the Americas, haemorrhagic fever with renal syndrome of different severity has been observed in Europe and Asia. Haemorrhagic fever with renal syndrome is caused by various hantavirus serotypes, such as Puumala virus (PUUV), Hantaan virus, Seoul virus, Dobrava virus and the recently isolated Saaremaa virus. In addition, in Europe, the closely PUUV-related serotype Tula virus (TULV) has been detected at several locations [for reviews, see ref. 1, 2]. Although there is some level of cross-protection between more closely related hantaviruses, e.g. Hantaan virus and Seoul virus [3], but also between more distantly related viruses, such as PUUV, Andes virus and Dobrava virus [4], a safe and efficient hantavirus vaccine requires the combination of antigens from several serotypes in a multivalent vaccine.

Different groups have reported on the protective potential of the nucleocapsid (N) protein of hantaviruses in animal models. Recombinant vaccines have been demonstrated to induce immune responses protecting animals against a subsequent virus challenge. These recombinant vaccines were based on (1) recombinant vaccinia virus or related viruses [5–7], (2) Sindbis virus constructs [8],

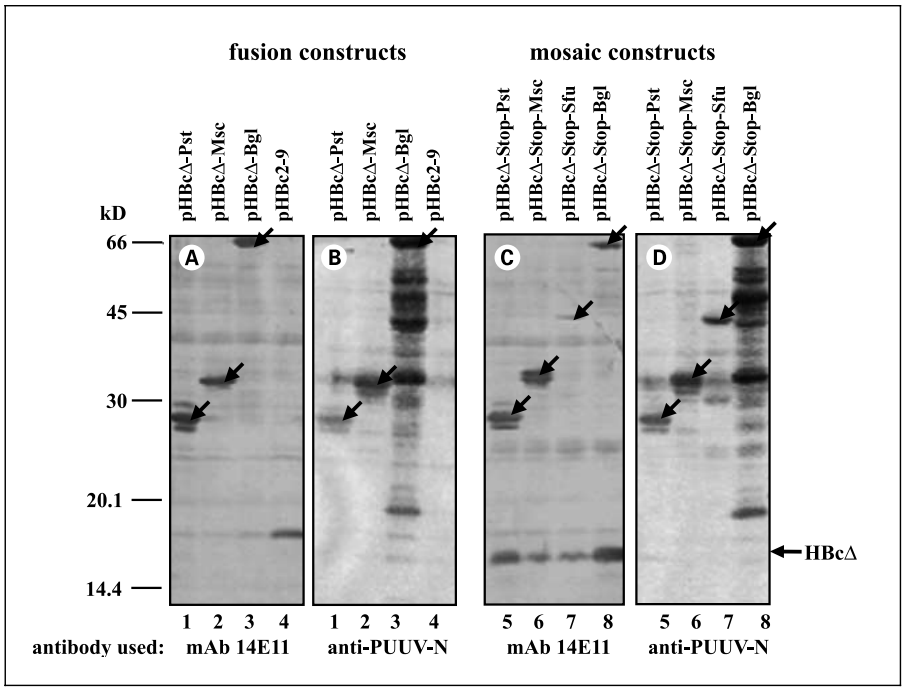

Fig. 2. Detection of purified HBcΔ-PUUV-N fusion (**A**, **B**) and read-through proteins (**C**, **D**) by immunoblot using the HBc-specific mAb 14E11 (**A**, **C**) or a polyclonal rabbit anti-PUUV-CG18-20 N (aa 39–433) serum (**B**, **D**). *E. coli* K802 cells or their opal suppressor derivative conferring pISM3001 were used as hosts for expression of plasmids encoding HBcΔ-PUUV-N fusion and read-through proteins, respectively. To prepare the inoculum for large-scale cultivation, the cells were grown at 37° overnight in LB medium without shaking. The inoculum was diluted 1/50 in tryptophan-free M9 minimal medium supplemented with 1% casamino acids (Difco, Detroit, Mich., USA) and 0.2% glucose and incubated on a rotary shaker (at approximately 130 rpm, 37°) for 18–20 h, and the cells were collected by low-speed centrifugation. Crude lysates were obtained by re-suspending the cell sediments in SDS-PAGE sample buffer containing 2% SDS and 2% 2-mercaptoethanol and heating at 100° for 5 min. The crude lysates of cells transformed by pHBcΔ-Pst (lane 1), pHBcΔ-Msc (lane 2), pHBcΔ-Bgl (lane 3), pHBcΔ-Stop-Pst (lane 5), pHBcΔ-Stop-Msc (lane 6), pHBcΔ-Stop-Sfu (lane 7), pHBcΔ-Stop-Bgl (lane 8) and pHBc2-9 (lane 4) were analysed on a 15% SDS-PAGE and Western blotting according to standard protocols. HBcΔ protein and its derivatives were detected with mouse anti-HBc mAb 14E11 (**A**, **C**) recognizing HBc aa 136–144 (diluted 1:1,000) [39, 40] and rabbit PUUV-CG18-20 N (aa 39–433)-specific serum (diluted 1:500) (**B**, **D**), using anti-mouse and anti-rabbit IgG peroxidase conjugate, respectively (Dako Diagnostika, Hamburg, Germany). The full-sized HBcΔ-PUUV-N fusion or read-through proteins of the expected molecular weights are marked with arrowheads. Molecular weight marker proteins: 66.0 kD (albumin), 45.0 kD (ovalbumin), 30.0 kD (carbonic anhydrase), 20.1 kD (trypsin inhibitor) and 14.4 kD (α-lactalbumin).

(3) DNA vaccination [8–10], or (4) recombinant N proteins expressed in *Escherichia coli*, yeast or insect cells using baculovirus expression vectors [5, 6, 11, 12].

The core protein of hepatitis B virus (HBc), as expressed in heterologous hosts such as *E. coli*, has become one of the favoured particulate carrier moieties improving the humoral immunogenicity of foreign epitopes presented on its surface [13, 14]. Recently, we demonstrated that chimeric HBc particles carrying 45 or 120 N-terminal aa of the N protein of PUUV induced efficient protection against a subsequent challenge with PUUV [15, 16, Koletzki et al., unpubl. data].

In order to develop multivalent vaccines on the basis of HBc particles, a high capacity for incorporation of foreign sequences into HBc without disturbing the particle formation is required. Two different approaches have been established to increase the capacity of HBc particles tolerating foreign insertions: (1) simultaneous insertion of foreign epitopes at different sites of HBc [17–20], and (2) increasing the insertion capacity of a single insertion site by generation of mosaic particles [21]. Due to its supposed high flexibility, the C-terminus of a C-terminally truncated HBc (HBcΔ) tolerates large foreign insertions; nevertheless, a size limitation has been observed [22]. Therefore, we have developed a new method to increase the insertion capacity at the C-terminus of HBcΔ by intro-

Table 1. Characterization of HBcΔ-PUUV-N fusion and read-through proteins

Recombinant protein	PUUV-N insert, aa	Predicted MW, kD	Expression	Solubility	Degradation	Particle formation	Antigenicity of particles	
							HBc	PUUV
HBcΔ	–	16.8	+++	+++	+	+++	5.08	ND
HBcΔ-Pst	340–433	28.2	+++	+	+	+	ND	ND
HBcΔ-Stop-Pst	340–433	29	+++	++	+	+++	4.51	6.00
HBcΔ-Msc	1–114	30.3	+++	+++	+	–	ND	ND
HBcΔ-Stop-Msc	1–114	31.1	+++	+++	+	+++	4.20	6.91
HBcΔ-Sfu	1–213	42.2	–	ND	ND	ND	ND	ND
HBcΔ-Stop -Sfu	1–213	43	+	+++	++	+++	4.20	6.91
HBcΔ-Bgl	1–433	66.2	+++	–	+++	–	ND	ND
HBcΔ-Stop -Bgl	1–433	66.9	+++	(+)	+++	–	ND	ND

Expression was determined by silver staining and Western blotting: +++ = high-level expression, well detectable in stained gels; + = low expression, detectable only in Western blotting; – = no detectable expression. Solubility was determined by Western blotting: +++ = more than 50% of specific protein in soluble form; ++ = 25–50% of protein soluble; + = 10–25% of protein soluble; (+) = about one half of the read-through protein was soluble in 1–2 *M* urea; – = no soluble protein detected. Degradation was also determined by Western blotting: +++ = more than 50% of specific protein in degradation bands; ++ = 25–50% of protein in degradation bands; + = minor degradation. Particle formation was determined by electron microscopy and double-radial immunodiffusion: +++ = particles purified in high amounts useful for immunization; + = particles detected only in total cell lysates; – = no particles detected. Antigenicity of particles was determined by direct ELISA. The values given are the reciprocal endpoint logarithmic titres. The endpoint titre was considered as the highest serum dilution at which the signal was still three times higher than that of a non-immunized mouse. The HBc antigenicity was detected by mouse anti-HBc mAb 13C9 [39, 40]. PUUV-N antigenicity was detected by rabbit polyclonal anti-Vranica/Hällnäs PUUV-N serum. MW = Molecular weight; ND = not determined.

duction of a stop codon linker leading to the simultaneous expression of HBcΔ and a HBcΔ-hantavirus N read-through protein from the same HBc gene in an *E. coli* suppressor strain [21].

We were interested to further study the potential of such mosaic core particles to support the insertion of large-sized sequences. Differently sized segments of the N protein of PUUV were expressed in *E. coli* either as HBcΔ fusion proteins (chimeric particles) or by a read-through mechanism (mosaic particles). Consequently, the ability of chimeric and mosaic constructs to assemble was compared.

For that purpose, the entire N protein-coding region (aa 1–433) of PUUV (strain Vranica/Hällnäs) was isolated from plasmid pVSTA18 (fig. 1A) [23] and inserted into pHBc2-9 encoding a C-terminally truncated HBc (HBcΔ; fig. 1B) [15] to obtain the plasmid pHBcΔ-Bgl (fig. 1C) [21]. In addition, similar plasmids were generated encoding fusions of HBcΔ and differently sized segments of the Vranica/Hällnäs N protein (aa 1–114 [21], aa 1–213, aa 340–433; fig. 1C). In another set of plasmids, HBcΔ- and Vranica/Hällnäs N-encoding sequences were separated by a stop codon linker as previously described for the plasmid pHBc-Msc-Stop126/127 [21] (fig. 1D; redesignated here as pHBcΔ-Stop-Msc).

Analysis of crude lysates of *E. coli* strain K802 cells carrying the expression plasmids pHBcΔ-Pst (fig. 2A, B, lane 1), pHBcΔ-Msc (fig. 2A, B, lane 2) and pHBcΔ-Bgl (fig. 2A, B, lane 3) revealed the synthesis of HBcΔ-PUUV-N fusion proteins of the expected molecular weights (table 1). These fusion proteins reacted in Western blot both with an HBc-specific monoclonal antibody (mAb), 14E11 (fig. 2A, lanes 1–3), and a polyclonal rabbit anti-PUUV-N serum (fig. 2B, lanes 1–3). As expected, the control lysate containing HBcΔ (without PUUV-N; pHBc2-9) reacted only with the HBc-specific antibody but not with the anti-PUUV-N serum (fig. 2A, B, lane 4). In lysates of cells transformed with pHBcΔ-Sfu, no synthesis of the expected full-sized fusion protein could be detected (data not shown). The solubility of all HBcΔ fusion proteins was investigated by comparing the soluble and insoluble cellular fractions by Western blot. The main portion of the HBcΔ-Msc fusion protein was found to be soluble (table 1), confirming our earlier data [21]. In contrast, only a

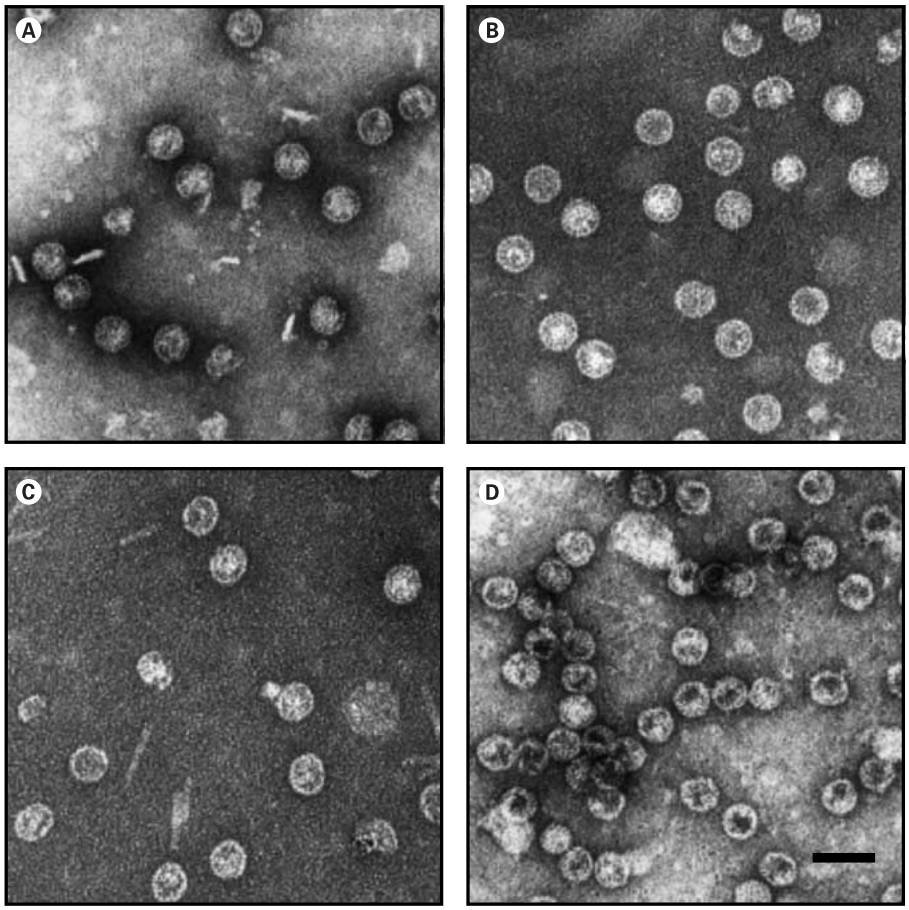

Fig. 3. Demonstration of core particles formed by pHBcΔ-Stop-Msc (**A**), pHBcΔ-Stop-Pst (**B**), pHBcΔ-Stop-Sfu (**C**) and pT31 (HBcΔ; **D**) [41] by negative staining electron microscopy. To analyse the particle-forming ability, 1 g of frozen cells was re-suspended in 1 ml of buffer A (50 m*M* Tris, pH 8.0, 5 m*M* EDTA, pH 8.0, 1 m*M* PMSF). After the addition of 2 ml of buffer A containing 6 mg/ml lysozyme, the samples were incubated for 15 min on ice. After three subsequent cycles of freezing/thawing, 1 ml of buffer A containing 1% Triton X-100, 10 m*M* $MgCl_2$ and 20 μg/ml DNase were added. The samples were mixed by vortexing and then sonicated. The insoluble cell debris was sedimented by centrifugation (25 min, 8,000 rpm at 4°), and the soluble proteins were precipitated with ammonium sulphate (pH 7.0) at 30% saturation (v/v) for 3 h at 4°. The precipitate collected by centrifugation (25 min, 8,000 rpm at 4°) was resuspended in 1 ml of buffer (10 m*M* Tris, pH 8.0, 150 m*M* NaCl) and fractionated using a Sepharose CL-4B column (length 80 cm, diameter 1.5 cm) with an elution speed of 3 ml/h. Fractions with HBc antigenicity in double-radial immunodiffusion and Western blot were selected for further analyses. To detect HBc particles, these samples were adsorbed on carbon-formvar-coated grids and stained with 2% phosphotungstic acid (pH 6.8). The grids were examined with a JEM 100C electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV. ×200,000. Bar = 50 nm.

very small part of the HBcΔ-Pst protein was found in the soluble fraction (table 1). The HBcΔ-Bgl fusion carrying the entire N protein remained insoluble even after treatment with 5 *M* urea (data not shown).

The expression of the corresponding read-through constructs was studied in *E. coli* K802 cells in the presence of the opal suppressor plasmid pISM3001 [24]. It resulted in the simultaneous expression of both HBcΔ (144 aa from HBc plus an additional 8 aa from stop linker; fig. 1D) reactive with mAb 14E11 (fig. 2C, lanes 5–8) and the HBcΔ-PUUV-N read-through proteins reactive with both mAb 14E11 (fig. 2C, lanes 5–8) and the rabbit anti-PUUV-N serum (fig. 2D, lanes 5–8). In general, the expression level of the HBcΔ-PUUV-N read-through proteins was similar to that of the corresponding fusion proteins (table 1). In contrast to the HBcΔ-Sfu fusion construct, which could not be detected by Western blot using mAb 14E11 (data not shown), small amounts of the HBcΔ-Stop-Sfu read-through protein were observed in crude lysates of suppressor cells (fig. 2C, D, lane 7). Inter-

estingly, the solubility of the read-through proteins encoded by pHBcΔ-Stop-Pst and pHBcΔ-Stop-Bgl was significantly higher than that of the corresponding fusion proteins. According to Western blotting data, the major part of the HBcΔ-Stop-Msc and the HBcΔ-Stop-Sfu as well as about half of the HBcΔ-Stop-Pst read-through proteins were found in soluble form (table 1). Even though no HBcΔ-Stop-Bgl protein was detected in supernatant of lysed cells, about one half of it was soluble in 1–2 *M* urea (data not shown). HBcΔ itself is highly soluble (up to 90%); however, its solubility in mosaic particles correlates with the solubility of the read-through proteins. For example, HBcΔ synthesized simultaneously with the HBcΔ-PUUV-N (aa 1–433) read-through protein is also insoluble and did not form particles (table 1).

Particle formation in lysozyme lysates of K802 cells expressing the HBcΔ-PUUV-N fusions alone or the simultaneously synthesized HBcΔ-PUUV-N read-through proteins and the HBcΔ 'helper' was investigated by Ouchterlony's double-radial immunodiffusion with polyclonal human anti-HBc antibodies (table 1). According to previous data [21], the HBcΔ-Msc fusion protein is not self-assembly competent, despite its good solubility. For the HBcΔ-Pst fusion, particle formation was detected only in non-diluted cell lysates. In contrast, supernatants of lysates from *E. coli* cells expressing the HBcΔ-Stop-Pst, HBcΔ-Stop-Msc and HBcΔ-Stop-Sfu read-through proteins formed precipitation bands at dilutions of 1:16, 1:16 and 1:32, respectively, whereas lysates of HBcΔ-expressing cells showed precipitation lines at dilutions of 1:64–1:128 (data not shown).

For purification of HBcΔ particles, cells were lysed by lysozyme treatment and three subsequent freeze/thaw cycles. Particles were concentrated from the supernatants by ammonium sulphate precipitation and applied to a Sepharose CL-4B gel filtration column. Fractions of 3 ml were collected and analysed by SDS-PAGE and Western blot analysis. As a result of the purification of the read-through constructs pHBcΔ-Stop-Pst, pHBcΔ-Stop-Msc and pHBcΔ-Stop-Sfu, large amounts of HBcΔ protein products were collected from columns within fractions 11–15, with a major peak at fractions 12 and 13. Negative staining electron microscopy confirmed the formation of mosaic HBcΔ particles (fig. 3A–C) which were similar in size and shape to particles formed by HBcΔ itself (fig. 3D). Although large amounts of the HBcΔ-Msc fusion protein were purified, no particles were found in the HBcΔ-Msc fusion protein-containing fractions (16–19) by negative staining electron microscopy, which is in line with previous data [21]. Reproducibly, when analysing the HBcΔ-Pst fusion products, no particles were detected after purification, although some particles were observed in lysozyme lysates (data not shown). For both the fusion and read-through variants of HBcΔ carrying the entire Vranica/Hällnäs PUUV-N sequence, immunodiffusion of lysozyme lysates and column chromatography failed to detect HBcΔ particles (table 1).

Table 2. Reactivity of mosaic HBcΔ-PUUV-N particles with PUUV-N- and TULV-N-specific mAbs

mAb	HBcΔ-Stop-Pst (aa 340–433)	HBcΔ-Stop-Sfu (aa 1–213)	HBcΔ-Stop-Msc (aa 1–114)
PUUV			
3G5	–	+	+
5F4	–	–	–
3H9	–	–	–
5E1	–	+	+
2E12	–	+	+
1C12	–	+	+
4C3	–	+	+
5A2	–	–	–
TULV			
3C11	–	+	+
3D3	–	–	–
3F10	–	–	–
6A6	–	–	–
7A4	–	–	–

Microtitre plates were coated with 5 μg/ml of each mAb. 3G5, 5F4, 3H9, 5E1, 2E12, 1C12 and 4C3 are PUUV-N-specific mAbs; 5A2 is a PUUV glycoprotein-specific mAb. 3C11, 3D3, 3F10, 6A6 and 7A4 are TULV-N-specific mAbs [25–27]. + = Reactive; – = no reactivity.

The HBc antigenicity of purified particles analysed in a direct HBc ELISA was found to be very similar between particles formed by the constructs pHBcΔ-Stop-Pst, pHBcΔ-Stop-Msc and pHBcΔ-Stop-Sfu (table 1). The PUUV-N antigenicity was analysed by a polyclonal Vranica/Hällnäs PUUV-N protein-specific rabbit antiserum and a panel of PUUV-N- and TULV-N-specific mAbs [25–27]. The reactivity of the rabbit serum with particles carrying PUUV-N aa 1–114 and aa 1–213 was slightly higher than that with particles carrying aa 340–433 (table 1). In line with these data, the mosaic particles carrying PUUV-N aa 340–433 did not react in the ELISA with any of the PUUV-N- and TULV-N-specific mAbs (table 2). In contrast, the particles carrying the N-terminal segments of PUUV-N reacted with the majority of the PUUV-N-specific mAbs and the cross-reactive TULV-N-specific mAb 3C11. As expected, the mAbs 5F4 and 3H9,

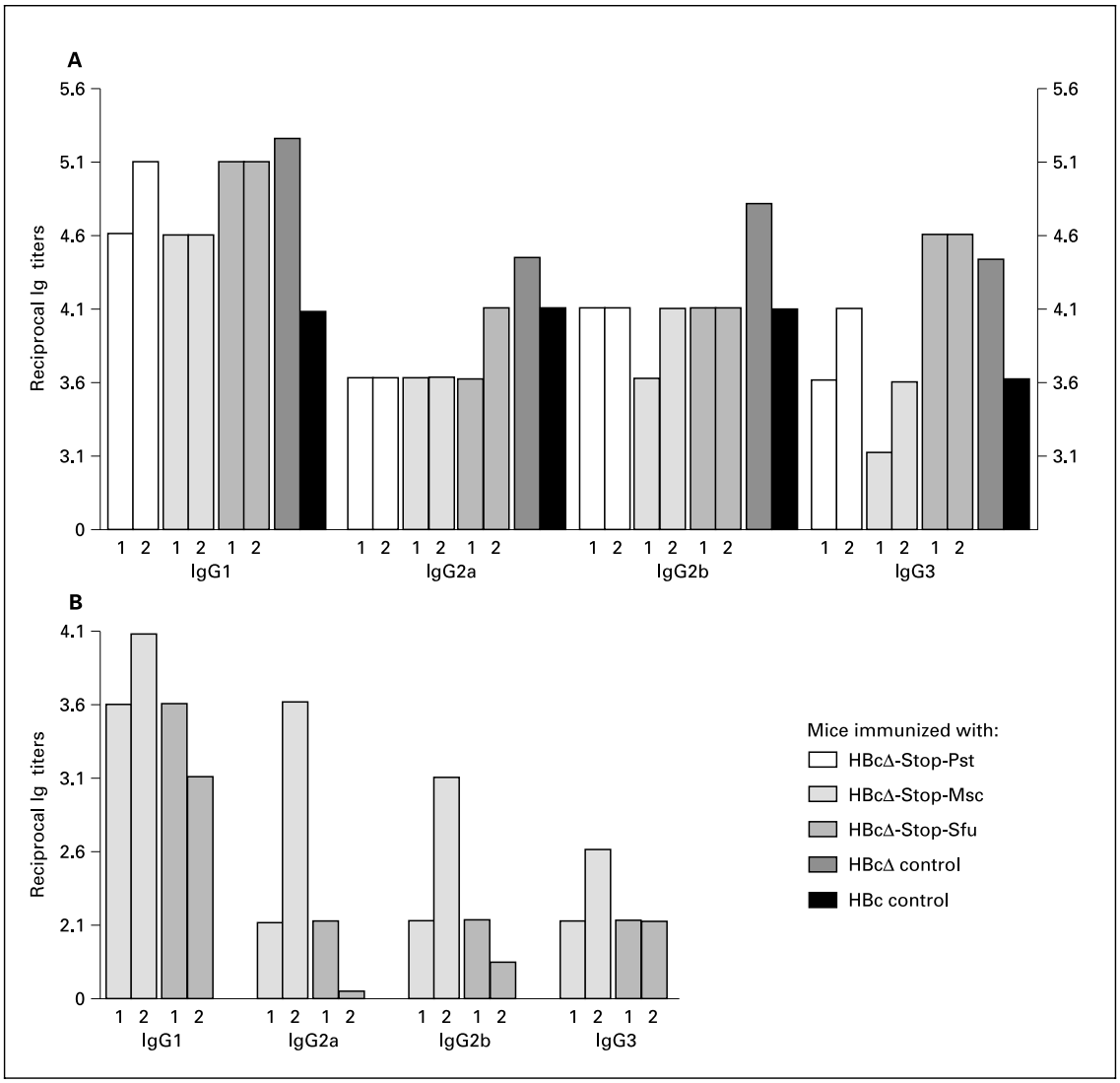

Fig. 4. Immunogenicity of HBcΔ-derived mosaic particles in BALB/c mice. Anti-HBc- (**A**) and anti-PUUV-N-specific (**B**) IgG isotype distribution in murine sera after immunization with mosaic HBcΔ-PUUV-N particles is shown. Six- to eight-week-old female BALB/c mice (breeding colony of the Institute of Microbiology and Virology, Riga, Latvia; 2 per group) were immunized on day 0 with 20 μg (full dose:10 μg i.p. and 10 μg s.c.) of mosaic particles formed by pHBcΔ-Stop-Msc, pHBcΔ-Stop-Pst, pHBcΔ-Stop-Sfu or HBcΔ and HBc dissolved in PBS and mixed with Freund's complete adjuvant (1:1) followed by two booster immunizations in Freund's incomplete adjuvant given on days 10 (half of full dose: 5 μg i.p. and 5 μg s.c.) and 24 (full dose, in the same way). Sera were collected on day 32 after immunization. To analyse the IgG isotype of the anti-HBc (**A**) and anti-PUUV-N antibody response (**B**), 96-well microtitre plates were coated with 10 μg/ml full-length HBc (aa 1–183) and *E. coli*-expressed His_6-tagged Vranica/Hällnäs PUUV-N protein [28], respectively, overnight at 4°. Wells were blocked with 0.5% BSA in PBS for 1 h at room temperature, incubated with serial dilutions of antibodies for 1 h at 37° and processed with the appropriate secondary antibodies conjugated to horseradish peroxidase according to the protocols of the manufacturers. Plates were washed 3 times between incubations with 0.05% Tween-20 in PBS and 3 times with dH_2O. Optical densities were read with an automatic Immunoscan MS reader at 492 nm. IgG isotype analysis was done with a mouse mAb isotyping reagent kit (Sigma, St. Louis, Mo., USA). Antibody titres are expressed as decimal logarithms (lg) from the reciprocal of the highest serum dilution required to yield an optical density value at 492 nm three times that of non-immunized mice. Values below the figures show the number of mice.

recently mapped by synthetic peptides to epitopes between aa 262–284 and 247–263 [25], respectively, did not detect any of the PUUV-N derivatives. Neither the PUUV glycoprotein- nor non-PUUV-cross-reactive TULV-specific antibodies reacted with the mosaic particles (table 2).

To study the immunogenicity of the particles, groups of two female BALB/c mice were immunized intraperitoneally and subcutaneously with 20 μg of purified mosaic HBcΔ-PUUV-N particles derived from the constructs pHBcΔ-Stop-Msc, pHBcΔ-Stop-Pst and pHBcΔ-Stop-Sfu in Freund's complete adjuvant and subsequently boosted with a half-dose of particles in Freund's incomplete adjuvant on days 10 and 24. As a control, mice were immunized with HBcΔ and full-length HBc particles (without any insertion). Sera were collected on day 32 after immunization and analysed in ELISA for HBc- and PUUV-N-specific IgG antibodies of the isotypes 1, 2a, 2b and 3, using full-length HBc and *E. coli*-expressed His_6-tagged Vranica/Hällnäs PUUV-N protein [28], respectively, as antigens (fig. 4).

All sera raised against either the mosaic particles or HBcΔ itself expressed high anti-HBc reciprocal logarithmic endpoint titres in ELISA, reaching 4.6–5.1 for IgG1 (fig. 4A). Except for IgG3, no significant differences in the antibody titres were observed between mice sera immunized with the different kinds of particles. In line with previous findings [Skrastina, unpubl. data], the IgG1 antibody response induced by HBcΔ was stronger than that found in mice immunized with full-sized HBc.

By immunization of mice with mosaic particles formed by the constructs pHBcΔ-Stop-Msc and pHBcΔ-Stop-Sfu, the production of high titres of PUUV-N-specific antibodies, i.e. 3.1–4.1 for IgG1 and 3.6 and lower for the other IgG isotypes, was induced (fig. 4B). Compared to mosaic particles carrying aa 1–213, the antibody response to mosaic particles carrying aa 1–114 of PUUV-N was slightly stronger, especially in mouse No. 2. Interestingly, no N protein-specific antibody response was found after immunization with mosaic particles containing the C-terminal aa 340–433 from the PUUV-N. As expected, no anti-PUUV response was detected in control sera obtained after immunization with HBcΔ (data not shown).

Chimeric HBc particles are considered to be of great interest as potential vaccines for influenza as well as hantavirus and malaria infections [15, 29, 30]. However, one limitation on the use of HBc particles may be their limited capacity to tolerate the insertion of foreign epitopes or protein segments. Indeed, previously we observed a size limitation for foreign sequences derived from the HIV-1 Gag protein in HBcΔ particles when fused C-terminally [22]. Similarly, a size limitation was observed for PUUV-N sequences [21] (this paper). Therefore, we developed an *E. coli* expression system that allows the simultaneous expression and assembly of HBcΔ and HBcΔ-PUUV-N (aa 1–114) read-through protein [21]. Here, we present evidence that the read-through mechanism even allows incorporation of aa 1–213 and aa 340–433 of PUUV-N into HBcΔ mosaic particles, whereas a C-terminal fusion to HBcΔ of these sequences did not give rise to the formation of chimeric HBcΔ particles, or only at a very low level. However, the highly insoluble entire PUUV-N protein could not be integrated into HBcΔ particles even by the use of the read-through mechanism.

The PUUV-N antigenicity, analysed by a rabbit anti-PUUV-N serum, was found to be higher for mosaic particles carrying aa 1–114 or aa 1–213 of PUUV-N than for those carrying aa 340–433. These data are in line with previous data demonstrating that the N-terminal region of PUUV-N is immunodominant [25, 31, 32]. This is also reflected in the reactivity of the N-terminal constructs with the majority of PUUV-N-specific mAbs, which confirmed our recent findings regarding their epitope localization between aa 1–45 and aa 1–80 of PUUV-N [16, 25]. In line with the antigenicity data, the two N-terminal constructs induced PUUV-N-specific antibodies in BALB/c mice, whereas the C-terminal one did not. The high antigenicity and immunogenicity of the mosaic particles carrying the N-terminal segments of PUUV-N is likely due to the immunodominant nature of this region, as detected for N proteins of PUUV and other hantaviruses [25, 31–33]. In contrast, the C-terminal region of PUUV-N has been demonstrated to be responsible for RNA binding [34], which may require an internal non-exposed orientation of this region, resulting in its lower antigenicity and immunogenicity. The immunogenicity of aa 1–114 of PUUV-N was slightly higher, at least in one mouse, which could be due to a lower content of the 213-aa insert within the particle.

Recently, we observed a low level of protection against an homologous virus challenge provided by mosaic HBcΔ particles carrying aa 1–114 of the N protein from Vranica/Hällnäs PUUV behind aa position 144 of HBcΔ [20]. In contrast, chimeric HBcΔ carrying aa 1–120 in the c/e1 loop of HBcΔ induced complete protection in 7 out of 8 animals [Koletzki et al., unpubl. data]. In order to evaluate potential reasons for this difference in protection, IgG isotypes of PUUV-N-specific antibodies were determined. However, both mosaic HBcΔ particles carrying

aa 1–114 (this paper) and chimeric HBcΔ particles carrying aa 1–120 at the c/e1 site [Geldmacher and Skrastina, unpubl. observations] induced an IgG1-dominated antibody response against the PUUV-N protein which may be due to a Th2-dominated response. Although HBc has been reported to elicit primary IgG2a and IgG2b anti-HBc antibodies [35], our results indicate that full-length HBc and HBcΔ-derived particles preferentially induced different Th cell subsets. However, additional investigations, including in vitro assays measuring the cytokine profile of antigen-specific Th cells, are necessary to prove this.

Despite the strong influence of the insertion site on the immune response to the PUUV-N insertion, another potential reason for the low level of protection could be the lower epitope density in mosaic particles. However, neither mosaic nor chimeric particles carrying aa 1–45 of Vranica/Hällnäs PUUV-N at aa 144 of HBcΔ were able to induce any protection in the bank vole model. Conversely, chimeric particles carrying the same insert at the N-terminus or the c/e1 loop of HBcΔ induced some level of protection [16]. Therefore, it is very likely that the insertion site is the main determinant for the low level of protection observed for mosaic particles based on the read-through mechanism. This is in line with data from various investigations suggesting the c/e1 loop region as the most suitable insertion site for foreign sequences [13, 14]. In addition, the insertion of differently sized stop linkers of different primary structure behind aa 144 of HBcΔ did not remarkably alter at least the PUUV-N antigenicity of mosaic HBcΔ particles [20]. Hence, another approach for the construction of mosaic particles, which is based on the co-expression of different HBc monomers from separate plasmids [36], allowing the insertion of the foreign sequence into the c/e1 loop, may be advantageous.

In contrast, one could insert large-sized foreign sequences directly into the c/e1 site to generate chimeric HBcΔ particles because of its high insertion capacity (of up to 240 foreign aa) [37, 38, unpubl. data]. However, we failed to obtain HBcΔ particles when inserting the N-terminal 80 aa of PUUV-N protein into the c/e1 site [unpubl. data]. This failure underlines the potential value of mosaic particles for incorporation of 'problematic', e.g. hydrophobic, sequences.

In summary, stop codon insertion improved or restored the particle formation ability of the HBcΔ-PUUV-N fusions. Stop codon suppression allowed the formation of mosaic HBcΔ particles carrying even up to 213 foreign aa. Immunization of mice with these mosaic particles induced PUUV-N protein-specific antibody responses, confirming at least a partial presence of the PUUV-N sequences on the surface of HBcΔ particles. The improvement of mosaic particles by using a two-plasmid system allowing the insertion of the foreign sequence into the c/e1 loop may improve the potential use of the mosaic strategy.

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A Malaria Vaccine Candidate Based on a Hepatitis B Virus Core Platform

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Key Words
Malaria · Vaccine · Pre-erythrocytic · Hepatitis B core antigen

Abstract
Objective: The recent success of a *Plasmodium falciparum* malaria vaccine consisting of circumsporozoite (CS) protein (CSP) T and B cell epitopes has rekindled interest in the development of a pre-erythrocytic vaccine. Our goal was to design an efficient delivery system for known neutralizing epitopes. ***Methods:*** Well-characterized CSP-specific neutralizing B cell epitopes and a 'universal' T cell epitope were combined with a particulate carrier platform, the hepatitis B core antigen (HBcAg), to produce a novel pre-erythrocytic vaccine candidate. ***Results:*** The vaccine candidate V12.PF3.1 is a potent immunogen in mice, eliciting unprecedented levels (greater than 10^6 titers) of sporozoite-binding antibodies after only two doses. The antisporozoite antibodies are long-lasting and represent all IgG isotypes, and antibody production is not genetically restricted. CSP-specific CD4+ T cells are also primed by V12.PF3.1 immunization in a majority of murine strains. Furthermore, the hybrid HBcAg-CS particles can be produced inexpensively in bacterial expression systems. ***Conclusion:*** These characteristics suggest that V12.PF3.1 represents an efficient and economical *P. falciparum* vaccine candidate for use separately or in combination with other formulations.

D.R.M. was variously affiliated with The Scripps Research Institute, Immune Complex Corporation (currently Apovia Inc.) and the Vaccine Research Institute of San Diego during the performance of these studies.

Introduction

Natural *Plasmodium falciparum* infection does not result in immunity, and partial immunity occurs only after years of recurring infections and illnesses. Therefore, a vaccine must outperform the immune response to the natural infection. this complexity has impeded vaccine development. Although a number of antigens from the various life cycle stages are being pursued as malaria vaccine candidates, the most progress has been made towards the development of a pre-erythrocytic stage vaccine [1]. Sporozoites, which represent the infective stage, are injected into the host by the bite of the mosquito and within minutes leave the circulation and enter hepatocytes. The relatively low antigen load (<100 sporozoites/bite) and

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brief circulation time may explain the lack of protective immunity towards this stage after a natural infection [2]. Studies in the 1980s demonstrated that the sporozoite coat protein, the circumsporozoite (CS) protein (CSP), was the target of protective antibodies [3–5]. Further, the dominant antibody epitope was represented by the CSP central repeat sequences ($NANP_n$ in *P. falciparum*) [6, 7]. Although more recent work indicates that cell-mediated immunity towards CSP epitopes can also confer protection [8–12], the earlier studies demonstrated that antibodies specific for the CSP repeat sequences were protective. These studies led to human clinical trials using recombinant [13] and synthetic [14, 15] forms of the CSP. These antigens formulated in alum were poorly immunogenic in terms of anti-NANP antibody titers determined by direct ELISA or indirect immunofluorescence antibody tests (IFAT) on sporozoites (i.e. IFAT titers of 10^2–10^3). Predictably, the weak immunogenicity was accompanied by limited protection. In an attempt to increase the immunogenicity of the CSP repeat sequences, a virus-like particle consisting of 16 NANP repeats from the CSP of *P. falciparum* fused to the N-terminus of pre-S2 and S sequences of the hepatitis B surface antigen (HBsAg) was developed and tested in a phase I clinical trial. The immunogenicity was suboptimal [16]. A second vaccine based on the use of the HBsAg carrier known as RTS,S consisting of 19 NANP repeats plus the majority of the C-terminus of the CSP [amino acids 207–395 of *P. falciparum* (3D7)] fused to the N-terminus of HBsAg was tested in a human challenge study [17]. The RTS,S vaccine formulated in alum was not protective, but the addition of 3-deacylated-monophosphoryl lipid A (MPL) to the adjuvant elicited protection in 2 of 8 vaccinees with the highest antibody levels [17]. In a more recent phase I clinical trial of RTS,S the use of a more potent adjuvant termed SBAS2 (MPL plus a saponin derivative QS21 in an oil-in-water emulsion) protected 6 of 7 vaccinees challenged 3 weeks after the third dose of vaccine [18]. However, in a rechallenge experiment, only 1 of 5 of the originally protected vaccinees remained protected approximately 6 months after the initial challenge [19]. Similarly, in a field trial of RTS,S/SBAS2 in a malaria-endemic region, high levels of protection were achieved but again appeared to be transient [20].

From the earliest studies using irradiated sporozoites, to the more recent clinical trials with RTS,S, a consistent observation has been the difficulty in eliciting high levels of persisting antibodies to the CSP repeat sequences in all genetic backgrounds, especially with respect to (NANP)n-containing antigens. Rather than relying on ever more potent and inevitably more toxic adjuvant systems, our approach was to use a more efficient carrier platform. The hepatitis B core antigen (HBcAg) is significantly more immunogenic than the HBsAg [21], can be produced in bacterial expression systems, unlike HBsAg, and has been shown to be a highly versatile and efficient carrier platform for a number of pathogen-specific epitopes [22]. For example, immunization with chimeric HBcAg particles containing CSP repeat sequences from the *Plasmodium berghei* and *Plasmodium yoelii* rodent malaria species elicited high-titered anti-CSP repeat antibodies and protected 90–100% of vaccinated mice [23, 24]. Initial attempts to apply the same technology to the design of a CSP repeat-based *P. falciparum* vaccine candidate [23] were not as optimal as the rodent vaccines, which is consistent with the general history of the (NANP)n sequence. Herein, we describe a series of modifications to the HBcAg carrier platform and the selection of malaria CSP sequences necessary to achieve extremely high levels of persisting anti-CSP repeat antibodies, and characterize this new vaccine candidate in murine immunogenicity studies.

Materials and Methods

Mice

Inbred murine strains were obtained from the breeding colony at The Scripps Research Institute and the Jackson Laboratory (Bar Harbor, Me., USA). Female mice 6–8 weeks of age at the initiation of the experiments were used.

Recombinant HBcAg and HBcAg-CS Hybrid Particles and Synthetic Peptides

Recombinant HBcAg subtype ayw [25] and HBcAg-CS2 hybrid particles [23] were produced and purified as previously described and provided by Dr. D. Peterson (Virginia Commonwealth University, Richmond, Va., USA). All other HBcAg-CS hybrid particles were produced in *Escherichia coli* and were provided by A. Birkett (Immune Complex Corporation, currently Apovia Inc., San Diego, Calif., USA). As demonstrated previously, insertion of foreign B cell epitopes into the exposed loop structure of HBcAg is the optimal position for antibody production [25]. Five particles were used in this study: HBcAg-CS2, V1.PF1, V2.PF1, V12.PF1 and V12.PF3.1 (summarized in table 1).

Synthetic peptides corresponding to the 120–140 HBcAg sequence and the CSP-specific $(NANP)_5$, DPNANPNVDPNANPNV and $CSP_{326–345}$ (NF54 isolate) EYLNKIQNSLSTEWSPCSVT sequences were produced by solid-phase peptide synthesis based on 9-fluorenlmethoxy-carbonyl protection on the α-amino group by using manual [27, 28] or automated (Milligen 9050 Plus, Millipore, Bedford, Mass., USA) procedures. All peptides were analyzed by reverse-phase high-performance liquid chromatography.

Table 1. Recombinant HBcAg-CS hybrid particles used in this study

Designation	Insert(s)	T/B epitope	Description	Immunogenicity (anti-NANP titer)
HBcAg-CS2	$(NANP)_4$	B	replacement of loop	20,480
V1.PF1	$(NANP)_4$	B	insertion site 77–78	163,840
V2.PF1	$(NANP)_4$	B	insertion site 78–79	655,360
V12.PF1	$(NANP)_4$	B	insertion site 78–79	2.6×10^6
	+ $CS_{326–345}$	T	C-terminus	
V12.PF3.1	$(NANP)_4$	B	NANPNVDP$(NANP)_3$ insertion site 78–79	10.5×10^6
	+ NVDP	B/T		
	+ $CS_{326–345}$	T	C-terminus	

'V' refers to the HBcAg vector; 'PF' refers to the insert. Immunogenicity is expressed as end-point anti-NANP serum titers determined in an ELISA format. Mice were injected with 20 μg of the HBcAg-CS hybrid particles in CFA and boosted with a 10-μg dose in IFA. In all hybrid particles, the C-terminal HBcAg residues 150–183 are deleted, with the exception of HBcAg-CS2, in which residues 157–183 are deleted. Modified from Milich et al. [26].

Monoclonal Antibodies and ELISA Assays

HBcAg-specific monoclonal antibodies (Mabs) 3105 and 3120 [29] and hepatitis B e antigen-specific Mabs 904 and 905 [30] were supplied by M. Mayumi (Institute for Immunology, Tokyo University, Japan). To detect the (NANP)n sequence on hybrid particles or synthetic peptide, the 2A10 Mab was used, and to detect the NVDPNANP sequence, the 2B6 Mab was used. Dr. E. Nardin (New York University, New York N.Y., USA) provided the CSP-specific Mabs. Anti-HBc and anti-peptide IgG antibodies were measured in pooled murine sera by indirect solid-phase ELISA using HBcAg (0.05 μg/well) and CSP-derived peptides (0.5 μg/well) as solid-phase ligands and goat anti-mouse IgG (or IgG isotype-specific antibodies) as second antibody, and were developed with a peroxidase-labeled swine anti-goat IgG. The data are expressed as the antibody titer representing the reciprocal of the highest dilution of serum that yielded three times the OD_{492} of preimmunization sera.

In vivo Antibody Production and Adjuvants

Mice were immunized for determination of in vivo antibody production by intraperitoneal injection of HBcAg or HBcAg-CS hybrid particles either emulsified in complete Freund's adjuvant (CFA; 50%) or formulated with a series of other adjuvants. Other adjuvants included aluminum hydroxide (AL-OH) or aluminum phosphate ($AL-PO_4$) used at an 8:1 ratio (both from Supefos, Denmark). The alum adjuvants were formulated at the Immune Complex Corporation. The CpG oligonucleotide GCATGACGTTGAGCT and the non-CpG oligonucleotide CCTAGATGTTAGCGT (100 μg/dose) were both made with a thioate backbone (Synthetic Genetics, San Diego, Calif., USA). The Ribi adjuvant system consisted of MPL and synthetic trehalose dicorynomycolate incorporated into a metabolizable oil (squalene) and emulsifier (Tween 80). MPL-SE (RD-529SE) consisted of a synthetic MPL (50 μg) in a stable emulsion of squalene and Tween 80. MPL-AF consisted of aqueous MPL (50 μg). All these MPL-containing adjuvant systems were obtained from Corixa (Hamilton, Mont., USA). Montanide ISA 720 (Seppic, Paris, France) is a water-in-oil adjuvant consisting of squalene and a mannide mono-oleate emulsifier prepared in a 70:30 (w/w) mixture or a 30:70 mixture of adjuvant to antigen.

T Cell IFN-γ Assay

Groups of 3 mice were injected subcutaneously with V12.PF3.1 particles (10 μg) emulsified in CFA in the hind footpads. Ten days later, the mice were sacrificed and the draining lymph nodes were harvested and pooled and single-cell suspensions were prepared. The lymph node cells were cultured (6×10^5/well) with dilutions of a panel of antigens for 48 h, at which point supernatant was removed for the determination of IFNγ by a sandwich ELISA (Pharmingen, San Diego, Calif., USA).

Results

Immunogenicity of a Series of HBcAg-CS Hybrid Particles

The initial attempt to incorporate the $(NANP)_4$ *P. falciparum* repeat sequence into HBcAg particles by deleting the HBcAg loop region (HBcAg-CS2) proved less than optimal. The anti-NANP antibody response was variable and relatively low in 2 of the 3 strains tested (table 1) [23]. Therefore, in an attempt to optimize antibody production to the *P. falciparum* CSP, a series of modifications were made to the HBcAg platform and in the selection of CSP B and T cell epitopes. Inserting the $(NANP)_4$ sequence between HBcAg loop residues 77 and 78 (V1.PF1) minimally improved anti-NANP antibody production as compared to deleting the entire loop region (HBcAg-CS2 particles), without altering the anti-HBc response (table 1). Because the 77–78 insertion site did not appear to be optimal for the $(NANP)_4$ sequence, the $(NANP)_4$ sequence was inserted between HBcAg loop residues 78 and 79 (V2.PF1), which improved anti-NANP immunogenicity 2 to 4-fold as compared to V1.PF1 particles (table 1). The

78–79 insertion site also resulted in a 16-fold increase in the antigenicity (2A10 binding) of the $(NANP)_4$ sequence as compared to V1.PF1 particles (not shown). Whereas the anti-NANP response elicited by the V2.PF1 particles was 2–4 times higher than that of V1.PF1, the anti-HBc response was actually 4–6 times lower as compared to V1.PF1 particles (data not shown). A high anti-NANP/anti-HBc ratio is desirable because anticarrier antibodies are nonfunctional and could potentially inhibit boosting, although no such inhibition occurred in a previous study [23].

A further modification that enhanced antibody production to the $(NANP)_4$ epitope a further 4-fold and increased antigenicity 6-fold was the incorporation of the malaria $CSP_{326–345}$ sequence at the C-terminus of the V12.PF1 hybrid particle. The increased immunogenicity may be explained by the presence of additional T helper cell sites within the $CSP_{326–345}$ sequence [31, 32], although this is unlikely since not all strains recognize the $CSP_{326–345}$ T cell site, or because this sequence may confer increased stability on the hybrid V12.PF1 particle. Stability is an issue because truncation of the HBcAg C-terminus eliminates the nucleic acid binding region but also deletes the C-terminal cysteine at residue 183, which has been suggested to stabilize the HBcAg particle [33, 34]. The $CSP_{326–345}$ sequence contains a Cys^{342} that may be able to participate in inter- or intradimer disulfide bond formation, much like the endogenous HBcAg Cys^{183}. To confirm the effect of CSP Cys^{342} on immunogenicity, V12.PF1 particles were compared with a mutant V12.PF1 ($C^{342}\rightarrow A$) particle. Mice were immunized with 20 μg of each particle in saline, and primary anti-NANP and anti-HBc antibody production was determined. As shown in figure 1, the V12.PF1 ($Cys^{342}\rightarrow A$) mutant was nonimmunogenic in terms of anti-NANP antibody production as compared to the wild-type V12.PF1 particle. Note also that anti-HBc antibody production was not affected by the loss of Cys^{342}. This suggests that the anti-NANP response is more sensitive to destabilization than the anticarrier response. A second mutation placing a cysteine at the C-terminus of the HBcAg carrier, V12.PF1 + Cys^{150} ($C^{342}\rightarrow A$), repaired the defective immunogenicity of V12.PF1 ($C^{342}\rightarrow A$) (fig. 1). Therefore, C-terminal cysteines appear important to immunogenicity and there seems to be some flexibility in the position of disulfide bond formation.

A further modification was the inclusion of a second minor *P. falciparum* B cell epitope that is present in the 5′ repeat region and consists of multiple NVDPNANP repeats in the CSP. This sequence also contains a CD4+ T cell site(s). Particles containing this second B/T cell site, designated V12.PF3.1, bind a second Mab (2B6), which recognizes the NVDPNANP sequence. Immunization with V12.PF3.1 elicited enhanced antibody production to the $(NANP)_n$ sequence as compared to V12.PF1 particles (table 1). Antisporozoite IFAT titers elicited by immunization with V12.PF3.1 were also determined. Four weeks after a single immunization with 20 μg of V12.PF3.1 in CFA, the serum IFAT titer measured on sporozoites was 1.3×10^6, which increased to 5.2×10^6 after a boost. There was a high degree of correlation between the ELISA and IFAT serum titers, indicating that most if not all the anti-NANP and anti-NVDPNANP antibodies recognize

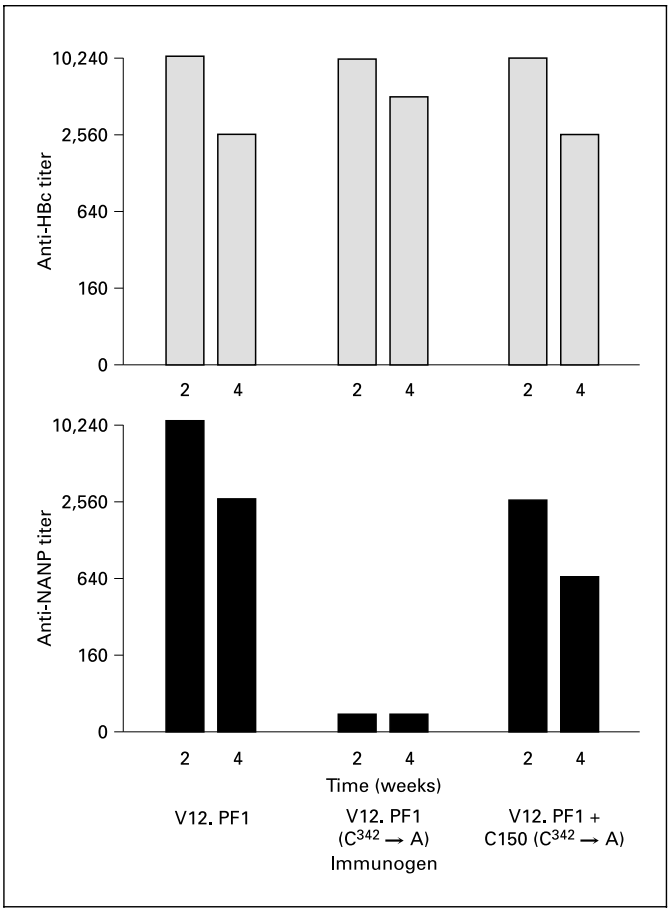

Fig. 1. Effect of C-terminal cysteines on anti-NANP and anti-HBc antibody responses. Groups of 3 Balb/c mice were injected intraperitoneally with 20 μg of the indicated immunogens in saline. Sera were collected before immunization and 2 and 4 weeks after immunization and pooled, and anti-NANP and anti-HBc antibody titers were determined by ELISA. Titers are expressed as a reciprocal of the endpoint serum dilution that yielded 3 times the OD_{492} reading of preimmunization sera [from ref. 26].

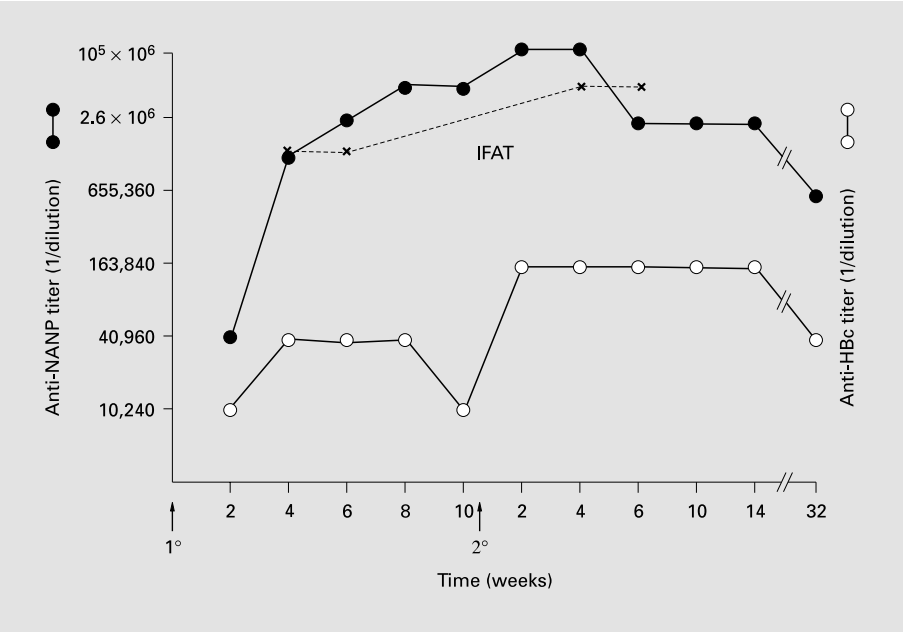

Fig. 2. Kinetics of the humoral immune response to V12.PF3.1 immunization. A group of 5 $(B10 \times B10.S)_{F1}$ mice were immunized intraperitoneally with 20 μg of V12.PF3.1 in CFA and boosted with 10 μg in IFA at week 10. Sera were collected before immunization and at the times indicated and pooled, and anti-NANP and anti-HBc antibody titers were determined by ELISA. Titers are expressed as a reciprocal of the end-point serum dilution that yielded 3 times the OD_{492} reading of preimmunization sera. Antisporozoite antibody titers were determined by IFAT at weeks 4 and 6 after primary (1°) and secondary (2° immunization [from ref. 26].

sporozoites and are potentially functional (fig. 2). In summary, immunization with V12.PF3.1 particles increased serum anti-NANP ELISA and anti-sporozoite IFAT titers by 2–3 orders of magnitude as compared to HBcAg-CS2 particles by virtue of modifications to the HBcAg platform and the selection of *P. falciparum* CSP epitopes. These results prompted us to select the V12.PF3.1 hybrid particles for further characterization as a potential preerythrocytic malaria vaccine candidate.

Kinetics of the Humoral Response to Immunization with V12.PF3.1 Particles

In order to determine the kinetics of a primary and secondary immune response and antibody persistence, $(B10 \times B10.S)_{F1}$ mice were immunized with 20 μg of V12.PF3.1 particles in CFA and boosted with 10 μg in incomplete Freund's adjuvant (IFA) (fig. 2). Immunization with V12.PF3.1 hybrid particles elicited the rapid production of anti-NANP IgG antibodies, reaching a serum ELISA titer of 40,960 within 2 weeks. Anti-NANP serum antibody levels continued to rise from weeks 2 to 4, 4 to 6 and 6 to 8 and plateaued at week 10 at a titer of 5.2×10^6 (fig. 2). This pattern of consistently increasing anti-NANP antibody levels throughout the primary response was not observed with the less immunogenic HBcAg-CS particles and is a correlate of enhanced immunogenicity. Note that the anticarrier antibody response rose and plateaued within 2 weeks of the primary and secondary immunizations. Mice were boosted at week 10, which resulted in a 2-fold increase in anti-NANP titer. The relatively low booster effect is most likely due to the high primary antibody levels. Ideally, the booster injection should be given at a point in time after the primary serum antibody levels have waned. Note also that the IFAT titers measured on sporozoites closely parallel the ELISA titers 4 and 6 weeks after primary and secondary immunizations. Lastly, the anti-NANP antibodies persisted at relatively high levels (655,360) for the entire observation period of 10.5 months after the first immunization (fig. 2).

It was also of interest to examine the IgG isotype distribution of anti-NANP antibodies in the sera of the mice depicted in figure 2. As shown in figure 3, immunization with the V12.PF3.1 particle elicited an anti-NANP response composed of a broad spectrum of IgG isotypes, especially 4 weeks after the boost. However, the kinetics of production of the various IgG isotypes varied. The early primary response (2 weeks) was dominated by the IgG_{2b} and IgG_3 isotypes. Significant IgG_1 and IgG_{2a} anti-NANP antibody production became apparent later in the primary response (10 weeks). Note the extremely high IgG_3 anti-NANP titer at week 10. This predominance of the IgG_3 isotype is not observed in the anti-HBc antibody response and has not been observed with any other B cell epitope inserted into the loop region of HBcAg particles and may be unique to the $(NANP)_n$ repeat sequence in the context of HBcAg.

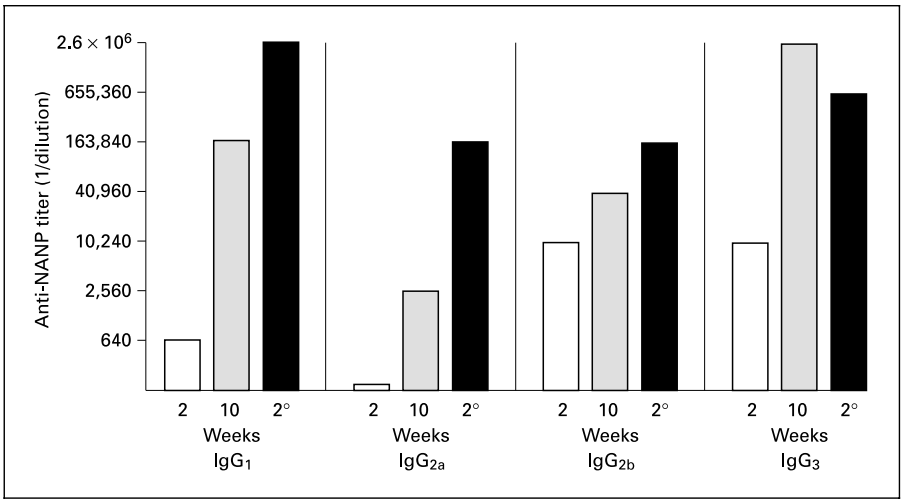

Fig. 3. Kinetics of anti-NANP IgG isotype production after V12.PF3.1 immunization. A group of 5 (B10 × B10.S)$_{F1}$ mice were immunized intraperitoneally with 20 μg of V12.PF3.1 in CFA and boosted with 10 μg in IFA at week 10. Sera were collected before immunization, 2 and 10 weeks after the primary immunization and 4 weeks after the boost (2°). Pooled sera were analyzed for anti-NANP antibodies of the IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 isotypes by ELISA using isotype-specific secondary antibodies. Anti-NANP titers are expressed as a reciprocal of the highest serum dilution to yield 3 times the OD_{492} reading of preimmunization sera [from ref. 26].

Formulation of V12.PF3.1 in Various Adjuvant Systems

Inasmuch as some form of adjuvant system will likely be necessary for human clinical trials, and as the nonmetabolizable mineral oil present in CFA and IFA is not presently permitted in humans, V12.PF3.1 particles were formulated with a number of adjuvant systems and compared in terms of anti-NANP antibody responses. Because these studies were conducted at different times and in some cases in different murine strains, the data have been normalized as a percentage of the anti-NANP response as compared to the response to V12.PF3.1 emulsified in IFA, since IFA was included as a control in each experiment. Groups of 3 mice were immunized with 10 μg of V12.PF3.1 particles in each adjuvant system and the results for sera taken 4 and 6 weeks after a primary immunization are shown (fig. 4). To summarize, AL-PO_4 (100% adsorbed), CpG (100 μg/dose) and MPL-AF (50 μg/dose) all significantly enhanced the anti-NANP antibody response as compared to saline and were 25–50% as effective as IFA. Furthermore, all of the stable emulsion adjuvants tested containing the metabolizable oil squalene (2.5% + MPL or 30% without immunostimulants) performed equally or slightly better than IFA (fig. 4).

Genetic Restriction of the Immune Response to V12.PF3.1 Immunization

Efforts to produce *P. falciparum* vaccine candidates based on CSP repeat sequences have been plagued by low immunogenicity and severe genetic restriction characterized by low responder murine strains. This issue has been addressed effectively by the inclusion of CSP CD4+ T cell epitopes, especially $CSP_{326–345}$ [35]; however, strains differ in responsiveness to $CSP_{326–345}$ as well. One reason the HBcAg was chosen as a carrier platform is because no genetic nonresponder murine strains have been identified for this antigen [21], and humans naturally infected with hepatitis B virus (HBV) universally produce high levels of anti-HBc antibody. To directly examine the issue of MHC-linked restriction of the immune response to V12.PF3.1, B10 H-2 congenic murine strains expressing 7 different H-2 haplotypes but otherwise genetically identical were immunized with an intermediate 5.0-μg dose of V12.PF3.1 in IFA, and their primary anti-NANP responses were compared (fig. 5). Firstly and importantly, no $(NANP)_n$ nonresponder H-2 haplotypes were identified. Secondly, the anti-NANP antibody responses of the 7 H-2 congenic strains were remarkably similar. These results are consistent with previous results using HBcAg as an immunogen in mice and with the serology in natural HBV infection, and suggest that the problem of MHC-linked low to nonresponsiveness may be overcome by the use of the V12.PF3.1 vaccine candidate.

T Cell Specificity of the Immune Response to V12.PF3.1

Although wild-type HBcAg can behave as a T cell-independent antigen in the absence of T cells [21], and this T cell independence can transfer to heterologous B cell epitopes inserted into HBcAg [25, 36], the extremely high levels of anti-NANP antibodies observed after V12.PF3.1 immunization indicate that an efficient CD4+ T helper cell response is operative. To examine the MHC-linked variation and the fine specificity of CD4+ T cell recogni-

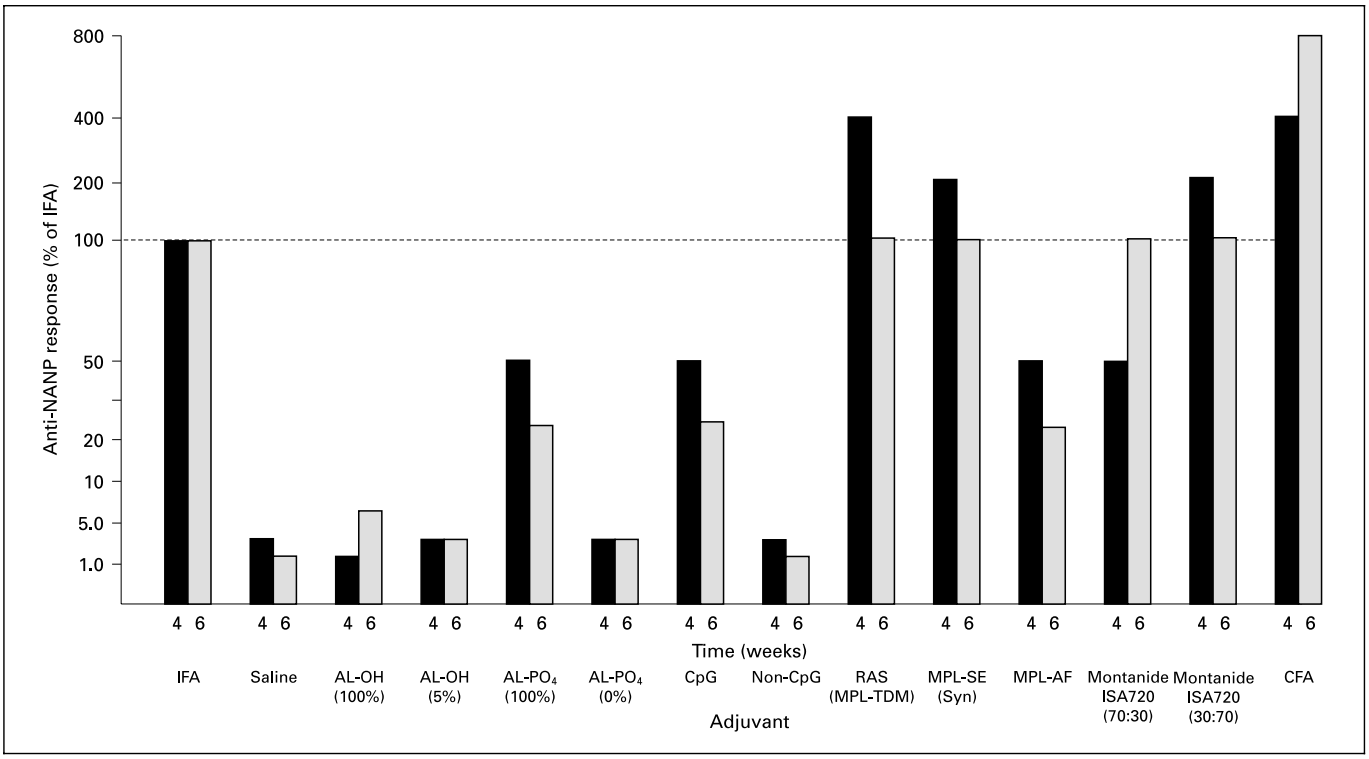

Fig. 4. Effect of formulating V12.PF3.1 in different adjuvants on the anti-NANP antibody response. Groups of 3 mice were immunized intraperitoneally with 10 μg of V12.PF3.1 formulated in the indicated adjuvants or in saline alone. Sera were collected before immunization and 4 and 6 weeks after immunization, and anti-NANP antibody was determined by ELISA. The anti-NANP antibody response was normalized as a percentage of the anti-NANP response achieved with V12.PF3.1 formulated in IFA. Balb/c mice were used, with the exception of the CpG and non-CpG groups, which were $(B10 \times B10.S)_{F1}$ mice, and the Montanide ISA 720 groups, which were C57BL/6 mice. IFA, 50%; AL-OH (100%) = 100% AL-OH adsorbed to V12.PF3.1; AL-OH (5%) = 5% AL-OH adsorbed to V12.PF3.1; AL-PO_4 (100%) = 100 AL-PO_4 adsorbed to V12.PF3.1; AL-PO_4 (0%) = unadsorbed to V12.PF3.1. The alum adjuvants were used at an 8:1 alum to protein ratio. CpG = Oligonucleotide containing the CpG motif (100 μg); non-CpG = control oligonucleotide not containing the CpG motif (100 μg); RAS = the Ribi adjuvant system; MPL-SE (Syn) = a synthetic MPL (50 μg) in a stable emulsion containing 2.5% squalene; MPL-AF = MPL (50 μg) in aqueous solution; Montanide ISA 720 = a water-in-oil adjuvant containing squalene and a mannide mono-oleate emulsifier mixed at a 70:30 or 30:70 (wt/wt) ratio with V12.PF3.1; CFA, 50% [from ref. 26].

tion of V12.PF3.1 particles, the same panel of B10 H-2 congenic strains shown in figure 5 were immunized, and draining lymph node cells were cultured with varying concentrations of a panel of antigens, including wild-type HBcAg, $(NANP)_5$, $CSP_{326–345}$ and c120–140 (a HBcAg-derived peptide). T cell sensitization was measured by IFN-γ production assayed in the supernatant and expressed as the minimum in vitro antigen concentration required to elicit IFN-γ production (fig. 6). The HBcAg was the predominant antigen recognized by the CD4+ T cells of all the strains immunized with V12.PF3.1, and 3 of the strains (B10.S, B10 and B10.RIII) recognized an HBcAg-specific epitope within the 120–140 region of HBcAg. Five of the seven strains also recognized at least one of the two malaria CSP-specific antigens tested. The $H\text{-}2^b$ haplotype is known to be a CD4+ T cell responder to the $(NANP)_n$ sequence, and immunization with V12.PF3.1 did prime $(NANP)_5$-specific T cells in this strain. However, it was somewhat surprising that the V12.PF3.1 particle also elicited $(NANP)_5$-specific T cells in the B10.PL ($H\text{-}2^u$) and B10.BR ($H\text{-}2^k$) strains, which have not been phenotyped as responders [37] to NANP (fig. 6). Perhaps it is the context of the NANPNVDP-$(NANP)_3$ sequence within the HBcAg loop that allows CD4+ T cell priming, and primed T cells are cross-reactive to $(NANP)_5$ in vitro.

It is clear from this analysis that the bulk of the T helper cell function for antibody production to V12.PF3.1 B

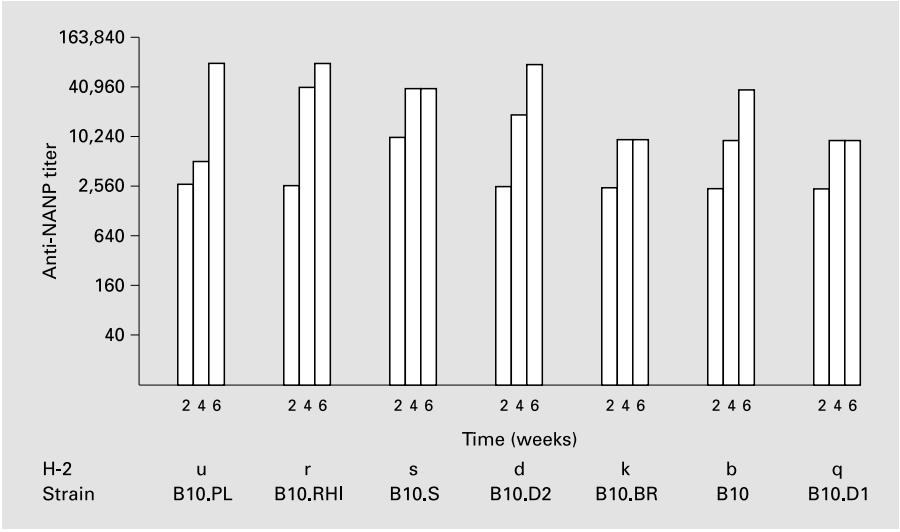

Fig. 5. Effect of MHC haplotype on the anti-NANP antibody response after V12.PF3.1 immunization. Groups of 3 H-2 congenic mice of the indicated strains representing 7 distinct H-2 haplotypes were immunized intraperitoneally with 5 μg of V12.PF3.1 in IFA. Sera were collected before immunization and 2, 4 and 6 weeks after immunization for anti-NANP analysis by ELISA on pooled samples. The anti-NANP antibody titer is expressed as a reciprocal of the highest serum dilution to yield 3 times the OD_{492} reading of preimmunization sera [from ref. 26].

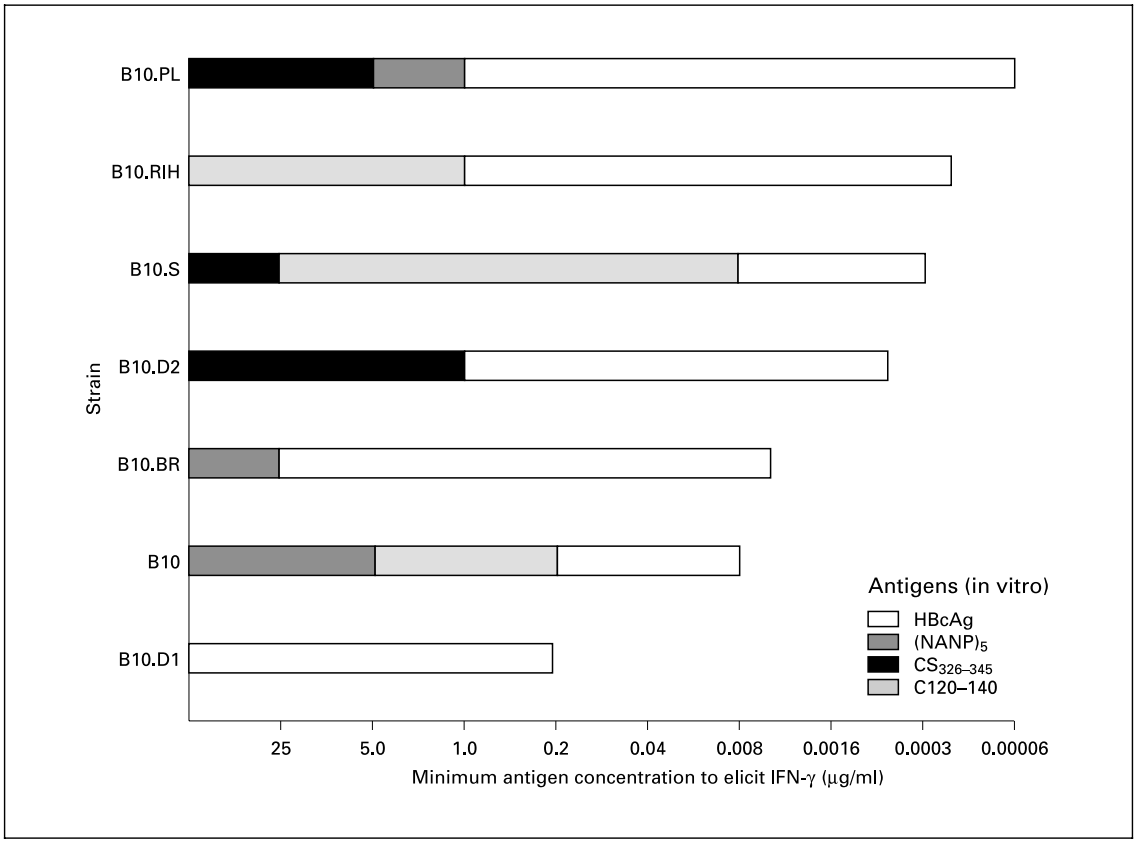

Fig. 6. Fine specificity of T cells primed by V12.PF3.1. Groups of 3 H-2 congenic mice of the indicated strains representing 7 distinct H-2 haplotypes were immunized subcutaneously with 10 μg of V12.PF3.1 in CFA. Ten days after immunization, draining popliteal lymph node cells were harvested and pooled, and 6×10^5 cells were cultured in vitro with varying concentrations (0.00006–25 μg/ml) of a panel of antigens including HBcAg, $(NANP)_5$, $CS_{326–345}$ and an HBcAg-derived peptide C120–140 or medium alone. After 72 h of culture, supernatant was collected and analyzed for IFN-γ by ELISA. The minimum in vitro antigen concentrations required to elicit IFN-γ production (3 times the OD_{492} of the media control) for each antigen is shown [from ref. 26].

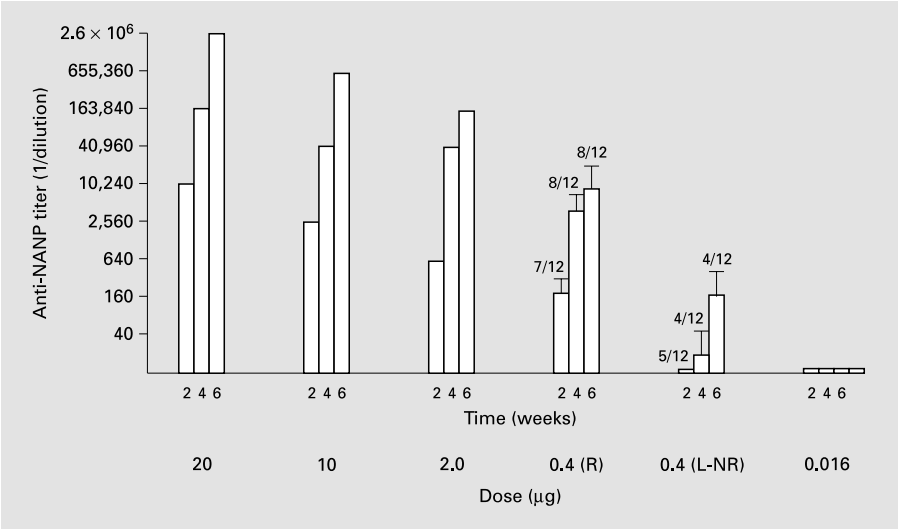

Fig. 7. Effect of V12.PF3.1 immunization dose on anti-NANP antibody production. Groups of 3 Balb/c mice (or 12 Balb/c mice in the case of the 0.4-μg dose) were immunized intraperitoneally with varying doses of V12.PF3.1 (ranging from 0.016 to 20 μg) in IFA. Sera were collected before immunization and 2, 4 and 6 weeks after immunization and anti-NANP antibody titers were determined by ELISA on pooled samples, with the exception of the 0.4-μg dose group, in which sera from individual mice were analyzed. Immunization with the 0.4-μg dose elicited an anti-NANP responder (R) group and a low-to-nonresponder (L-NR) group [modified from ref. 26].

cell epitopes is most likely provided by HBcAg-specific T cells. However, the fact that malaria-specific T cells are also primed by V12.PF3.1 is significant because it is these T cells which will be recalled during an infection with *P. falciparum* in vivo. Because the CSP is expressed in infected hepatocytes, the V12.PF3.1 particle actually represents a liver-stage vaccine candidate as well as a preerythrocytic, prehepatocyte inducer of sporozoite-neutralizing antibodies. In summary, immunization with the V12.PF3.1 particle elicits a broad array of T cell specificities including malaria-specific T cells.

The Effect of V12.PF3.1 Dose on the Humoral Response

To determine the effect of dose on antibody production and to define the limiting dose, groups of Balb/c mice were immunized with a single dose of V12.PF3.1 in IFA ranging from 20 to 0.016 μg, and anti-NANP serum levels were monitored at weeks 2, 4 and 6 (fig. 7). A clear dose effect was observed for doses between 20 and 2.0 μg. A dose of 0.4 μg of V12.PF3.1 appears to be the limiting dose. Note that on a weight basis, the $(NANP)_n$ repeat sequence constitutes approximately 10% of the V12.PF3.1 particle. At the 0.4-μg dose, a responder and a low-to-nonresponder group emerged (fig. 7). At 2 weeks, 7 of 12 mice produced low levels of anti-NANP antibodies and 5 of 12 mice produced none. At 4 weeks, 8 of 12 mice responded and 4 of 12 mice produced no or very low levels of anti-NANP antibodies. By week 6, the anti-NANP mean serum titer was approximately 10,000 in the responder group (8/12), whereas the mean titer in the low-to-nonresponder group (4/12) was approximately 180 (fig. 7). A 0.08-μg dose of V12.PF3.1 elicited no anti-NANP antibody response until week 6 after immunization, at which point a serum ELISA titer of 160 was observed. The 0.016-μg dose of V12.PF3.1 did not elicit detectable anti-NANP antibodies.

Discussion

The sporozoite stage of *P. falciparum* malaria and particularly the CSP have been the target of numerous vaccine design strategies. Because the sporozoite is the infecting agent, is present in the serum in low numbers and neutralizing and/or protective B and T cell epitopes have been identified on the CSP, this focus seems well placed. The recent success of the CSP-based RTS,S/SBAS2 vaccine in clinical trials appears to validate this approach. Although RTS,S/SBAS2 is the most protective malaria vaccine yet tested [18], because of the requirement for potent adjuvants and the rather transient protection period, the developers of this vaccine have stated that 'further optimization ... will be required to induce longer-lasting protective immunity' [19]. We have attempted to optimize the immunogenicity of selected *P. falciparum* CSP-derived B and T cell epitopes by delivering them on an extremely immunogenic and versatile carrier platform, the HBcAg. In murine immunogenicity studies, V12.PF3.1 hybrid particles demonstrated a number of characteristics that suggest that this formulation may represent a viable malaria vaccine candidate: (1) two

doses of V12.PF3.1 particles elicited antisporozoite serum IFAT titers greater than 10^6 – to provide some perspective, antisporozoite IFAT serum titers achieved in rodent and human vaccine studies typically range between 10^3 and 10^5, require more than two doses and are usually quite variable amongst murine strains or human vaccine recipients; (2) high levels of antisporozoite antibodies persist in the serum for at least 10.5 months after only two doses of V12.PF3.1 particles; (3) a broad-spectrum IgG isotype response is elicited by two doses of V12.PF3.1; (4) no genetic nonresponders or low responders to the V12.PF3.1 candidate vaccine were identified among the 7 H-2 haplotypes tested; (5) the V12.PF3.1 particles are compatible with a number of adjuvant systems and do not require potent immunostimulants for efficacy; (6) immunization with V12.PF3.1 particles primed malaria-specific IFN-γ-producing CD4+ T cells in 5 of 7 strains tested; (7) immunization with two doses of similar hybrid HBcAg particles carrying *P. berghei* and *P. yoelii* CSP repeat B cell epitopes conferred 90–100% protection in rodent models [23, 24], and (8) the V12.PF3.1 hybrid particles can be produced in bacterial expression systems and, therefore, the manufacturing is relatively inexpensive. Many of these characteristics are unique to V12.PF3.1 particles, and no existing CSP-based vaccine candidate exemplifies all of them.

The V12.PF3.1 candidate vaccine is scheduled to be tested in phase I clinical trials in the near future. Is there reason to believe that the immunogenicity data derived from mouse studies will translate to the human immune response to the V12.PF3.1 vaccine candidate? The HBcAg is the most immunogenic structural protein during natural HBV infection. Extremely high levels of anti-HBc antibodies are produced by virtually 100% of infected patients [38]. Even in asymptomatic HBV carriers, antibodies to the HBcAg are produced and are often the only antibodies detected in these patients. Therefore, we expect that the HBcAg carrier used in the V12.PF3.1 vaccine candidate will be a strong immunogen for humans. Further, HBcAg in adjuvant has been demonstrated to be immunogenic and protective in chimpanzee HBV challenge experiments [39–41]. Since anti-HBc antibodies are not virus neutralizing, protection is believed to be cell mediated, possibly through CD4+ T cell cytokine production or intermolecular T cell help for neutralizing anti-envelope antibody production or both [42]. Conceivably, V12.PF3.1 particles may serve as a combination vaccine for *P. falciparum* malaria and HBV. Additionally, malaria-specific T cell sites have been included in the V12.PF3.1 vaccine candidate. The $CSP_{326–345}$ sequence, designated Pf Th/Tc, contains overlapping CD4+ and CD8+ T cell epitopes and has been defined as 'universal' because of its ability to bind multiple DR and DQ human MHC molecules in vitro [43] and because it is recognized by multiple human CD4+ T cell clones derived from sporozoite-immune individuals [44]. Lastly, the dominant CD4+ T cell epitopes recognized by RTS,S/SBAS2 vaccine recipients are contained within the $CSP_{326–345}$ sequence [45]. Although the V12.PF3.1 vaccine candidate has not yet been tested in humans, the T cell components of the vaccine were selected based on their strong immunogenicity in humans.

In rodent models, CSP-based vaccines elicit a number of protective mechanisms including antibody [3, 4], CD4+ T cells [46, 47], CD8+ T cells [8, 9, 48], cytokines [49–51] and nitric oxide [10]. The superiority or sufficiency of any one of these mechanisms has been debated. Immunization with V12.PF3.1 particles elicits three of the five protective mechanisms, which are of course interrelated. The ability of sporozoite-specific antibodies alone to protect against malaria infection has been demonstrated by in vitro neutralization [3, 52], passive antibody transfer in vivo [4, 53] and immunization with isolated B cell epitopes as synthetic peptide vaccines [5] or as recombinant HBcAg constructs [23, 24] in rodent models. A consistent finding in these studies has been that protection is dependent on antisporozoite antibody concentration and the sporozoite challenge dose [2]. Therefore, if sufficiently high levels of persisting antisporozoite antibodies can be produced, antibody-mediated mechanisms may indeed be effective in preventing *P. falciparum* infection in humans. We suggest that the V12.PF3.1 vaccine candidate may at least provide a method for testing this hypothesis. Several intrinsic features of the HBcAg make it a good platform for raising high titers of epitope-specific antibodies. The HBcAg particle is stable, has a regular surface structure exposing multiple copies of the same epitope and has the unique ability to directly bind and activate a high frequency of naive murine and human B cells [54, 55]. Thus, these properties make HBcAg an ideal carrier for naturally nonimmunogenic B cell epitopes.

It has also been debated whether a successful *P. falciparum* vaccine must include protective antigens from all four stages of the parasite life cycle. The V12.PF3.1 vaccine candidate addresses the sporozoite stage and the intrahepatic stage, but not the blood or gametocyte stages. If it turns out that additional stages need to be targeted, the HBcAg platform can be adapted to include additional T and B cell epitopes from other stages, or the V12.PF3.1 particle may be combined with other vaccine formulations.

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Cowpea Mosaic Virus: From the Presentation of Antigenic Peptides to the Display of Active Biomaterials

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Key Words

Plant virus-based chimeras · Cowpea mosaic virus · Human rhinovirus 14 · Chemical coupling of peptides to virus capsids · A kinase anchoring protein · General-purpose surface presentation system

Abstract

The potential of cowpea mosaic virus (CPMV), a plant icosahedral virus, for the presentation of foreign peptides and proteins is reported. The most prominent feature at the virus surface is a region of the smaller of the two coat proteins (S) which has been extensively used for the insertion of foreign peptides. Given the availability of the three-dimensional structure of the native virus and the amenability of foreign peptide-expressing CPMV chimeras to crystallisation, immunological data can be correlated with the conformational state of the foreign insert. The latter is influenced by proteolysis which occurs within the foreign inserts. In an effort to offer an alternative context for peptide expression, extensive exploration of a second region of the S protein is reported with respect to tolerance to small insertions. Moreover, to make CPMV suitable for a wider spectrum of presentation, a technique was developed to allow surface coupling of a peptide which can serve as the anchoring point for a range of proteins. This new approach is also widely applicable for the direct chemical cross-linking of peptides and full-length protein domains to the viral capsid.

Introduction

Cowpea mosaic virus (CPMV) is an icosahedral plant virus with a single-stranded, bipartite RNA genome. RNA-1 encodes the replication machinery and RNA-2 encodes a movement protein and two capsid proteins. The two RNA molecules are separately encapsidated. The capsid is comprised of 60 copies of a large (L) coat protein of 374 amino acid residues and a small (S) coat protein of 213 amino acid residues. The S protein folds into a jelly roll β-barrel whilst the L protein comprises two jelly roll β-barrel domains The surface lattice of the CPMV capsid is analogous to that of animal picornaviruses, with the S protein as the equivalent of VP1 and the two domains of the L protein, the N-terminal C domain and the C-terminal B domain, as the counterparts of VP2 and VP3.

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CPMV, being a plant virus, offers a potentially safe approach for the presentation of epitopes from animal pathogens for the development of inexpensive vaccines. With the availability of infectious cDNA clones and the crystal structure of the virus particles [1, 3], a chimeric virus technology for epitope presentation was developed [4, 5]. Initial insertions were made in the βB-βC loop of the S protein [4, 5]. This loop forms the top of the protrusions around the five-fold axis of the virus particles, which is the most prominent feature on the virus surface. Insertions in this position were successful in generating antibodies against several animal viruses [5–8]. To offer an alternative context for epitope presentation on CPMV capsids, we report the exploration of another surface-exposed loop of CPMV for optimal positioning of a foreign sequence using an epitope from human rhinovirus 14 (HRV-14) as a probe. Two additional insertion positions at the virus surface were tested for the presentation of an A kinase anchoring protein (AKAP)-derived peptide that can serve as the anchor point for other proteins. AKAPs are a family of proteins that are functionally defined for their ability to bind and target cAMP-dependent protein kinase (PKA) to various sub-cellular locations [reviewed in ref. 9]. AKAPs, in general, contain a targeting domain which targets the AKAP to specific sub-cellular locations and an A kinase-binding domain which binds to the regulatory subunit of PKA. The A kinase-binding domain from D-AKAP2, an AKAP with dual specificity (i.e. able to bind both regulatory subunit isoforms of PKA [Burns et al., unpubl. data]) was chosen for the current study. Limitations encountered during virus surface exploration with the D-AKAP2 peptide led to the development of a new technology. Based on the introduction on the virus surface of unique Cys residues which serve as anchoring points, it allows attachment of both peptides and proteins to the virus surface by chemical means. This strategy paves the way to the engineering of CPMV into a general purpose template.

Materials and Methods

Insertions in the βC′-βC″ Loop of S

Vectors for the insertion of foreign sequences within the βC′-βC″ loop of the S protein (which extends from amino acids V42 to W47) were produced by site-directed mutagenesis [10] of a *Bam*HI-*Eco*RI fragment from pCP2, the full-length infectious cDNA clone corresponding to RNA-2 [2]. This fragment, which encompasses the sequence encoding the entire L and S proteins and the 3′ non-coding region of RNA-2, was cloned into bacteriophage M13mp18, to give CP1. By silent mutagenesis of the codon for Val27 of the S protein with oligonucleotide 5′CCATTTTCA*GACGTC*ACAGCAGTAAC3′, an*Aat*II site (shown in italics) was created at position 2735 of the RNA-2 sequence. CP1w*Aat*II thus generated served as the template for further site-directed mutagenesis. Insertion of a linker sequence (shown hereafter in bold) corresponding to the recognition sites for *Sal*I (underlined) and *Sph*I (double underlined) was performed using the following oligonucleotides: 5′CGGCAAAATAACTCCTGTT**GGGTCGACGCATGCA**GGTGATGACAATTGGAATACGCAC3′ for insertion between V42 and G43 (CP1-V42/G43); 5′CGGCAAAATAACTCCTGTTGGT**GGGTCGACGCATGCA**GATGACAATTGGAATACGCAC3′ for insertion between G43 and D44 (CP1-G43/D44); 5′CGGCAAAATAACTCCTGTTGGTGAT**GGGTCGACGCATGCA**GACAATTGGAATACGCAC3′ for insertion between D44 and D45 (CP1-D44/D45); 5′CTCCTGTTGGTGATGAC**GGGTCGACGCATGCA**AATTGGAATACGCACATTTATAATCC3′ for insertion between D45 and N46 (CP1-D45/N46). CP1-V42/G43 and CP1-D44/D45 were subjected to further mutagenesis with oligonucleotide 5′GACAATTGGAATGCGCACATTTATAATCCTCC3′ in order to engineer a *Bsm*I site (underlined with dashes) at position 2800 of RNA-2, thereby generating CP1-V42/G43B and CP1-D44/D45B, respectively. A further set of vectors was produced by mutagenesis of CP1w*Aat*II with oligonucleotides simultaneously introducing the *Bsm*I site (underlined with dashes) at position 2800 of RNA-2 and a *Sal*I (underlined)-*Sph*I (double underlined) linker at the remaining available positions within the βC′-βC″ loop: 5′CAACGGCAAAATAACTCCT**GGGTCGACGCATGCA**GTTGGTGATGACAATTGGAATGCGCACATTTATAATCCTCC3′ for insertion after P41 (CP1-P41/V42b); 5′CTCCTGTTGGTGATGACAAT**GGGTCGACGCATGCA**TGGAATGCGCACATTTATAATCCTCC3′ for insertion after N46 (CP1-N46/W47B); 5′CCTGTTGGTGATGACAATTGG**GGGTCGACGCATGC**GAATGCGCACATTTATAATCCTCC3′ for insertion after W47 (CP1-W47/N48B). Following sequence analysis of recombinants, replicative form DNA was isolated and the *Bam*HI-*Eco*RI fragment exchanged for that of pCP2-*Aat*II, a derivative of pCP2 with the *Aat*II site removed from the pUC portion of the plasmid [8]. A series of pCP2-based clones was thereby created which derive their names from their CP1 counterparts (pCP2-V42/G43 from CP1-V42/G43 etc.). Into vectors harbouring only *Sal*I and *Sph*I sites, a pair of oligonucleotides encoding residues 85–98 from VP1 (NIm-1A epitope) of HRV-14 flanked by *Sal*I and *Sph*I cohesive ends were ligated. Into vectors presenting both a *Sal*I-*Sph*I linker and a *Bsm*I site were ligated annealed oligonucleotides with *Sal*I and *Bsm*I compatible ends and encoding the NIm-1A sequence of HRV-14 plus an appropriate number of native CPMV amino acids.

For the infection of cowpea plants, plasmids pCP1 (encoding the cDNA for RNA-1) and a pCP2-based derivative were linearised and co-inoculated. Recombinant viruses were named after the corresponding pCP2 plasmid, e.g. V42/G43 was derived from pCP2-V42/G43. The suffix -HRV was added when the HRV-14 epitope had been cloned into the modified βC′-βC″ loop. Virus propagation and purification were performed according to the protocol described by van Kammen and de Jaeger [11], modified as previously reported [8]. RT-PCR analysis of RNA extracts from infected plants was performed as described earlier [12].

Insertions in the βE-BF Loop of the C Domain of L

A plasmid with similarities to that described in a previous report [13] was developed for insertions into the βE-βF loop of the C domain of the L protein. pLgEF was generated by PCR-based mutagenesis using oligonucleotides 5′ACCGTTAACACCACTAT-

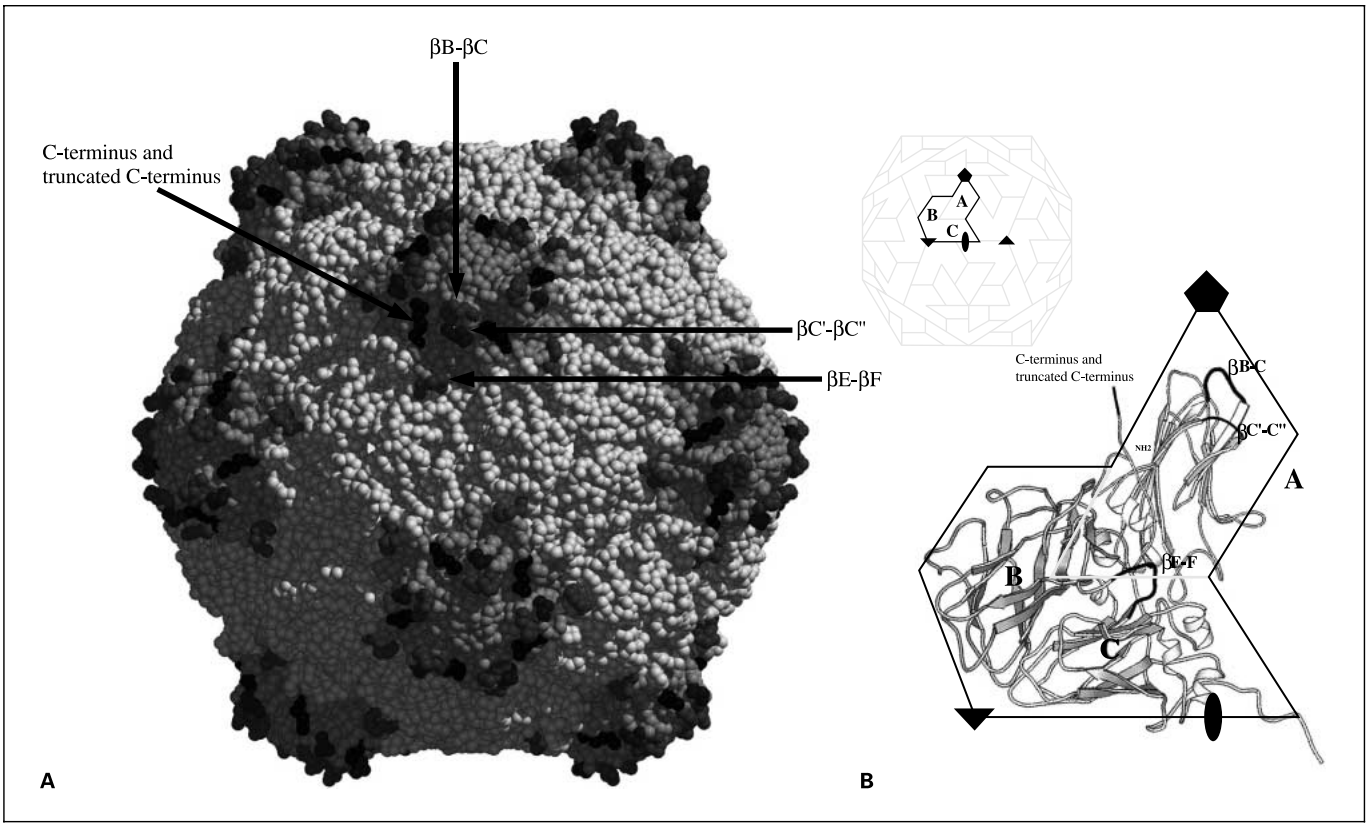

Fig. 1. Insertion sites on the CPMV capsid. **A** Space-filling model of CPMV with the insertion sites highlighted. **B** Upper: schematic diagram of the CPMV capsid with the reference asymmetric unit shown. Lower: ribbon diagram of the two capsid proteins that form an asymmetric unit with the insertion sites labelled.

TTATGGC3′ (*Hpa*I site underlined, mutated nucleotides in bold) and 5′AAGTAT**G**GTAC**C**GATGTTTATACT3′ (*Kpn*I site underlined, mutated nucleotides in bold). The resulting mutations in the coding sequences of Val96-Arg97 and Ser101-Thr102 were either silent or could be restored to the original amino acid sequence when insertions were made between these newly created restriction sites.

Genetic Insertions of the D-AKAP2 Peptide

The D-AKAP2 peptide [Burns et al., unpubl. data; 14] was to be expressed in different locations at the virus surface. Two sets of oligonucleotides were designed to generate the appropriate DNA fragments coding for AKAP. The first set of primers, 5′AGCACTCCTCCTGCTGTGCAAGAA3′ and 5′CTAGAAATGGGTGTGCCTGCTGCATA3′, was used to obtain the DNA sequence encoding the AKAP peptide by PCR from a D-AKAP2-harbouring plasmid [14, 15]. The second set of primers was used to incorporate flanking CPMV sequences and restriction enzyme sites for cloning at the following positions within (A) the S protein (1) βB-βC loop: oligonucleotides 5′TGTATGATAGCTAGCACTCCT3′ and 5′TACTGCTGTGACGTCTGAAAA3′ (*Nhe*I site underlined, *Aat*II site double underlined) were designed to allow insertion of the peptide sequence between residues 22 and 23; (2) the βC′-βC′′ loop: oligonucleotides 5′GCTATGAAGTCGACTGTGCAAGGA3′ and 5′TTGATCAGCATGCGCCTGCTGCAT3′ were used to generate a PCR fragment with *Sal*I (underlined) and *Sph*I (double underlined) compatible ends for introduction of the peptide sequence between residues 44 and 45; PCR primer 5′TTATGCCTGCTGCATAACATCACTC3′ was combined with either primer 5′CTGCTACTGCTGCAGTGCAAGGAA3′ or 5′CTGCTACTGCTGCAGGTGCAAGGAA3′ for cloning between *Pst*I (underlined) and *Stu*I sites at, respectively, (3) the native C-terminus and (4) the truncated C-terminus (following removal of the last 24 residues); and (B) the βE-βF loop of the C domain of the L protein: primers 5′AGGGGTGTGCAAGGAAATACT3′ and 5′AACATCGGTACCATACTTATGCGCCTG3′ were used for cloning between restriction sites *Hpa*I and *Kpn*I (underlined), resulting in insertion of the peptide between G98 and T102 (with K99 and Y100 eliminated and mutation of S101→G).

Each PCR fragment and the appropriate corresponding plasmid were digested with suitable restriction enzymes and ligated according to established procedures [16].

Production of a CPMV Cysteine Mutant

A CPMV chimera containing a single Cys within each asymmetric unit of the viral capsid, inserted between amino acids 98 and 99 in the βE-βF loop of the C domain of L, was created using plasmid pLgEF [17]. This chimera served as the basis for chemical cross-linking of the D-AKAP2 peptide onto the viral capsid using maleimide groups on a cross-linker.

Table 1. Sequences and yield in virus of βC′-βC″ loop-based CPMV vectors and their derivatives encoding the NIm-1A epitope of HRV-14

Virus name	Amino acid sequence of C′-C″ loop	Virus yield, % of wild type
Wild type	KITP*VGDDNW*NTHIYN	
P41/V42B	KITP**GSTHA***VGDDNW*NAHIYN	25
P41/V42B-HRB-(C′-C″)	KITP**GSTKDATGIDNHREAKL**NTHIYN	non-viable
P41/V42B-HRV	KITP**GSTKDATGIDNHREAKL***VGDDNW*NTHIYN	25
V42/G43	KITP*V***GSTHA***GDDNW*NTHIYN	100
V42/G43-HRV	KITP*V***GSTKDATGIDNHREAKLHA***GDDNW*NTHIYN	90
V42/G43B	KITP*V***GSTHA***GDDNW*NAHIYN	100
V42/G43B-HRV	KITP*V***GSTKDATGIDNHREAKL***GDDNW*NTHIYN	80
G43/D44	KITP*VG***GSTHA***DDNW*NTHIYN	90
G43/D44-HRV	KITP*VG***GSTKDATGIDNHREAKLHA***DDNW*NTHIYN	80
D44/D45	KITP*VGD***GSTHA***DNW*NTHIYN	100
D44/D45-HRV	KITP*VGD***GSTKDATGIDNHREAKLHA***DNW*NTHIYN	100
D44/D45$_1$-HRV	KITP*VGD***GSKDATGIDNHREAKLHA***DNW*NTHIYN	100
D44/D45B	KITP*VGD***GSTHA***DNW*NAHIYN	100
D44/D45B-HRV	KITP*VGD***GSTKDATGIDNHREAKL***DNW*NTHIYN	90
D45/N46	KITP*VGDD***GSTHA***NW*NTHIYN	50
D45/N46-HRV	KITP*VGDD***GSTKDATGIDNHREAKLHA***NW*NTHIYN	50
N46-W47B	KITP*VGDDN***GSTHA***W*NAHIYN	50
N46-W47B/HRV	KITP*VGDDN***GSTKDATGIDNHREAKL***W*NTHIYN	50
W47-N48B	KITP*VGDDNW***GSTHA**NAHIYN	non-viable
W47-N48B/HRV	KITP*VGDDNW***GSTKDATGIDNHREAKL**NTHIYN	non-viable

Amino acids forming the βC′-βC″ loop are shown in italics, the *Sal*I-*Sph*I linker is shown in bold, the amino acid mutated for the generation of the *Bsm*I site is underlined and the NIm-1A epitope is double underlined.

Chemical Coupling of a D-AKAP2 Peptide to the Virus Capsid

The synthetic D-AKAP2 peptide sequence had a Cys added to its C-terminus for reaction with the maleimide group of the homobifunctional cross-linker 1,11-bis-maleimidotetraethyleneglycol (Pierce, Rockford, Ill., USA). The maleimide groups at both ends of the cross-linker react with thiol groups to form stable thioether linkages. In the first step of the coupling protocol, the D-AKAP2 peptide was mixed with excess cross-linker (1:100) and incubated at 4° overnight. Unbound cross-linker was removed by passing the reaction mix through a desalting column (PD-10, Pharmacia, Uppsala, Sweden). The fractions containing the maximum protein concentration (measured by absorbance at 280 nm) were pooled and reacted with the CPMV mutant containing a single Cys residue/L protein at a ratio of 50.1 overnight at 4°. The CPMV:D-AKAP2 conjugate was purified by FPLC (Amersham Pharmacia Biotech, Sweden) over a size exclusion column (Superose 6-HR 10/10) and the peak corresponding to virus was collected and analyzed by SDS-PAGE and immunoblotting.

Results

Exploration of the βC′-βC″ Loop of the S Coat Protein

Insertion sites on the CPMV capsid surface and their positions within the viral coat proteins are mapped in figure 1. The βC′-βC″ loop of S was chosen for insertional exploration because the crystallographic structure of CPMV shows that this loop is very well exposed on the viral capsid. A linker sequence encoding GSTHA was cloned into this region in order to provide unique restriction sites *Sal*I and *Sph*I for the practical insertion of foreign sequences. Insertions can be made either between residues GST and HA or between GS and HA, provided that the codon of the first amino acid of the foreign insert is an A. This is illustrated by chimeras D44/D45-HRV and D44/D45$_1$-HRV, respectively, in table 1. Chimera D44/D45$_1$-HRV represented the first attempt to insert an antigenic sequence into the βC′-βC″ loop of the S protein.

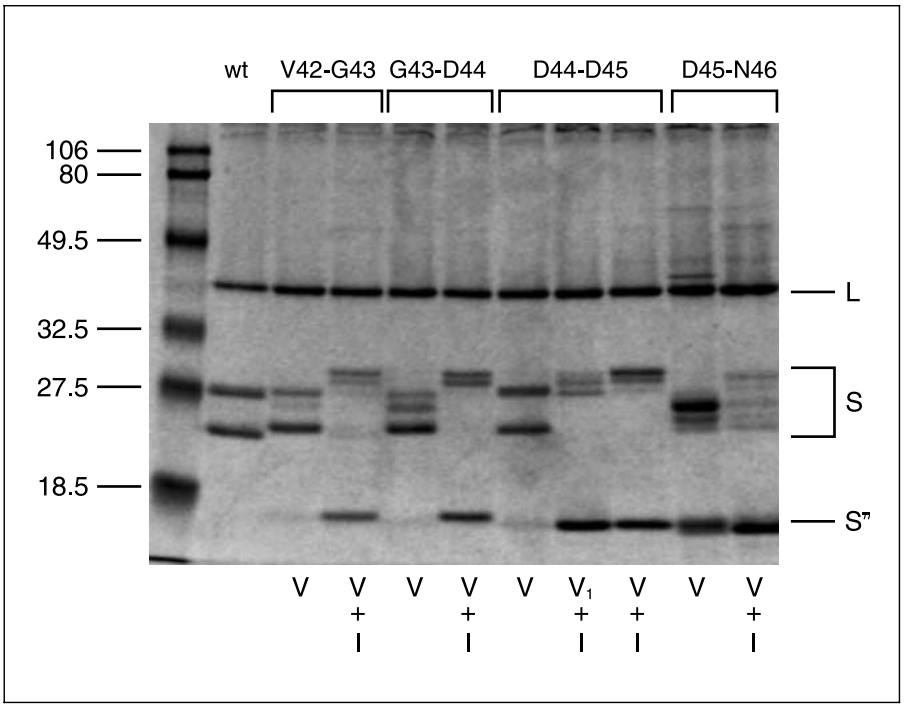

Fig. 2. SDS-PAGE analysis of chimeric CPMV βC′-βC″ loop vectors (V) and of their NIm-1A-harbouring derivatives (V + I). V_1 + I refers to chimera D44/$D45_1$-HRV in table 1. Purified virus samples were denatured by boiling in SDS before loading onto 15% acrylamide-0.4% bisacrylamide gels (4 μg/lane). Staining was done with Coomassie brilliant blue. Insertion positions within the βC′-βC″ loop of S are indicated above the gel. The CPMV coat proteins are identified to the right. The masses (in kD) of the molecular weight markers loaded in the first lane are shown on the left. wt = Wild type.

Table 2. Position of cleavage sites in βC′-βC″ loop-based NIm-1A epitope expressing CPMV chimeras

Name of clone	Amino acid sequence of recombinant βC′-βC″ loop
P41/V42B-HRV-(C′-C″)	non-viable
P41/V42B-HRV	KITP**GSTKDATGIDNHREAKL**↓*VGDDNW*NTHIYN
V42/G43-HRV	KITP*V***GSTKDATGIDNHREAKLHA**↓*GDDNW*NTHIYN
V42/G43B-HRV	KITP*V***GSTKDATGIDNHREAKL**↓*GDDNW*NTHIYN
G43/D44-HRV	KITP*VG***GSTKDATGIDNHREAKLHA**↓*DDNW*NTHIYN
D44/D45-HRV	KITP*VGD***GSTKDATGIDNHREAKLHA**↓*DNW*NTHIYN
D44/$D45_1$-HRV	KITP*VGD***GSKDATGIDNHREAKLHA**↓*DNW*NTHIYN
D44/D45B-HRV	KITP*VGD***GSTKDATGIDNHREAKL**↓*DNW*NTHIYN
D45/N46-HRV	KITP*VGDD***GSTKDATGIDNHREAKLHA**↓*NW*NTHIYN
N46/W47B-HRV	KITP*VGDDN***GSTKDATGIDNHREAKL**↓*W*NTHIYN
W47/N48B-HRV	non-viable

Amino acids forming the βC′-βC″ loop are shown in italics, the *Sal*I-*Sph*I linker is shown in bold and the NIm-1A epitope is double underlined. Arrows indicate the site of cleavage.

As reported earlier, in a preparation of the resulting chimeric virus particles, a proteolytic cleavage was shown to occur between the H and A residues of the linker peptide [12]. In the present study, the NIm-1A sequence was systematically inserted in all the available positions of the βC′-βC″ loop. As an alternative to expressing this epitope surrounded by linker peptide residues, a *Bsm*I site was engineered downstream of the coding sequence for the βC′-βC″ loop. Cloning between restriction sites *Sal*I and *Bsm*I allows a foreign insert to be flanked by linker peptide residues at its N-terminus and directly by native CPMV residues at its C-terminus (see series of B-HRV constructs in table 1). The aim of this investigation was to identify the positions within the βC′-βC″ loop which are most favourable for the expression of additional peptide sequences. In each case, the yield of virus particles was determined in relation to wild-type virus (table 1). Purified virus particles were subsequently subjected to SDS-PAGE analysis. As illustrated in figure 2, the so-called βC′-βC″ vectors, i.e. CPMV particles harbouring the *Sal*I-*Sph*I insert but no additional foreign sequence, have S proteins of a slightly increased size above that of their wild-type counterpart. This effect becomes more pronounced when the NIm-1A epitope, i.e. supplementary 14 amino acids, is added to the βC′-βC″ loop. All S proteins, including the wild type, undergo a proteolytic cleavage which removes the 24 C-terminal amino acids; this phenomenon accounts for the presence of at least two electrophoretic forms of the S protein. In the case of the HRV-14 insert-expressing chimeras, an additional S″ protein is detected. Except for chimera D45/N46, barely any S″ was observed when only the 5-amino acid linker peptide had been inserted into S. By N-terminal protein sequence analysis of the S″ proteins, cleavage sites within HRV-14-expressing βC′-βC″ loops were determined (table 2). Use of the *Sal*I-*Bsm*I cloning strategy results in variation of the position of the cleavage site; the closer the NIm-1A sequence is inserted to the C-terminus of the loop, the further into its own peptide sequence the cleavage occurs. By contrast, the presence of linker peptide residues HA downstream of the NIm-1A sequence (as a result of the *Sal*I and *Sph*I cloning strategy) fixes the cleavage position between His and Ala, independently of the position of insertion within the loop. CPMV chimeras with inserts in extreme positions of the loop, between P41/V42 and W47/N48, multiplied much less efficiently or not at all in plants (table 1). Total replacement of the foreign sequences in chimera P41/V42-HRV-(C′-C″) was unable to produce an infection (table 1). This confirmed similar findings made with insertions into the βB-βC loop of S [4], which had shown that peptide additions to the loop were viable whilst its substitution by a foreign sequence was not.

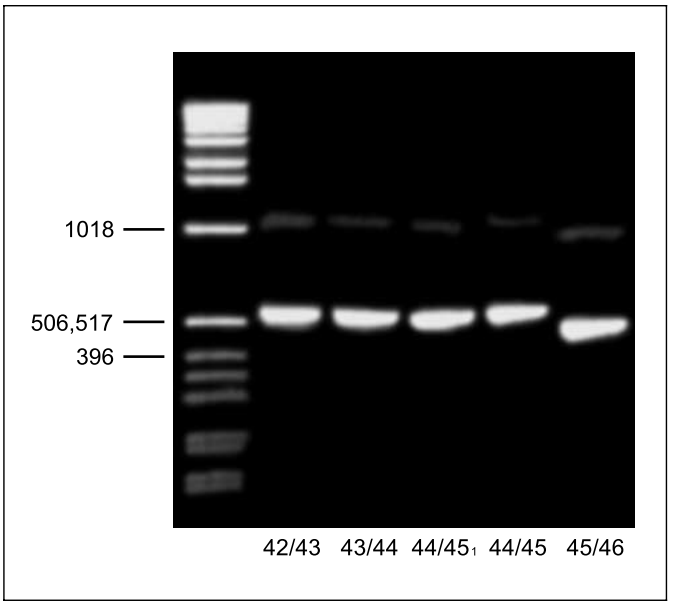

Fig. 3. RT-PCR analysis of RNA extracts from plants infected with βC′-βC″ loop chimeras of CPMV for the NIm-1A epitope. Samples were run on a 2% agarose-TBE (Tris boric acid EDTA buffer) gel and stained with ethidium bromide. HRV epitope-encoding oligonucleotides had been cloned between restriction sites *Sal*I and *Sph*I. The position of insertion in the βC′-βC″ loop is identified below each lane by the numbers of the flanking amino acid residues of the S protein. 44/45$_1$ corresponds to chimera D44/D45$_1$-HRV in table 1. The sizes (in bp) of the DNA standards loaded in the first lane are given on the left.

The genetic stability of NIm-1A harbouring βC′-βC″ loop chimeras was tested by passaging the recombinant viruses 5 times from plant to plant and subsequently analysing the viral genomes by RT-PCR analysis (fig. 3). V42/G43-HRV, G43/D44-HRV and both variants of the D44/D55-HRV construct retained their insert, whilst it was probably lost from D45-N46/HRV. The PCR-amplified fragment encompasses 476 bp from the wild-type sequence (not shown), whilst the size of the corresponding PCR product for all 5 chimeras should consistently be 536 bp. Taken together with the results of the SDS-PAGE gel analysis, which had revealed an anomalous pattern for the S proteins of both D45/N46 and its HRV derivative, these data confirm that insertions into this position result in unstable virus.

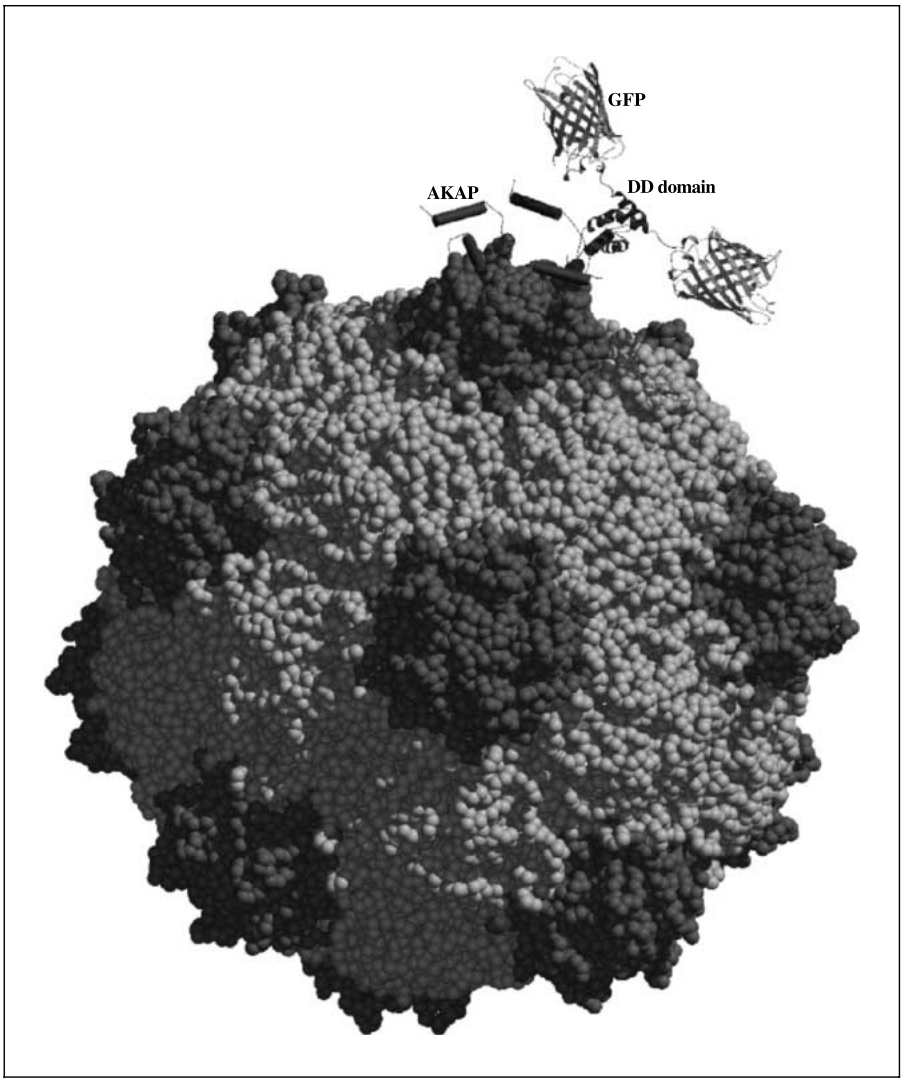

Fig. 4. A model for the presentation by CPMV of green fluorescence protein (GFP) by binding to D-AKAP2 peptides (drawn as cylinders) attached to the virus capsid (in space-filling model).

Attempt to Generate AKAP Chimeras by Genetic Insertion

The D-AKAP2 peptide consists of 27 amino acids (VQGNTDEAQEELAWKIAKMIVSDVMQQ), located near the C-terminus of the intact protein [14], that form an amphipathic helix able to bind with nanomolar affinity to the dimerization/docking (D/D) domain of PKA. Presentation of the D-AKAP2 peptide on the surface of CPMV would produce a general-purpose template for anchoring proteins fused with the D/D domain (fig. 4).

Great effort was made to produce CPMV/D-AKAP2 chimeras by genetic engineering technology. However, no symptoms of infection were detected in plants inoculated with any of the D-AKAP2 chimera clones, despite repeated efforts. Particle bombardment using the Helios™ Gene Gun system (Bio-Rad), which has been demonstrated to be of great efficiency in transfecting cDNA clones (data not shown), was deployed with a similar outcome. Western blot and RT-PCR analysis of asymptomatic leaves showed weak signs of infection inconsistently, and no propagation to a second set of plants was possible.

Coupling of Bioactive Molecules to the CPMV Capsid

Difficulty encountered in presenting the D-AKAP2 peptide by the chimera technology initiated the exploration of another strategy based on attaching the peptide to the virus surface by chemical cross-linking. Lack of accessible, free Cys residues on the exterior surface of wild-type CPMV allowed the introduction of reactive cysteines at

specific positions of the capsid by genetic engineering. These cysteines can serve as the anchor points (fig. 4) for linking foreign peptides and proteins using chemical compounds containing maleimide groups. A chimera harbouring a Cys in the βE-βF loop of the C domain of the L coat protein was used for this work. The Cys chimera of CPMV produced systemic symptoms, and virus yields were comparable to those of the wild-type virus [17]. To allow reaction with the maleimide group of the cross-linker, the synthetic AKAP peptide had a free Cys incorporated at its C-terminus. Following cross-linking, the virus:AKAP conjugate was purified by FPLC, and the virus-specific fraction was collected and analysed by Western blotting using anti-AKAP and anti-CPMV antisera. As shown in figure 5, the L:AKAP conjugate is detected as a 44-kD band using either of the two types of antibodies, while the uncoupled AKAP peptide shows up as a 3.4-kD band with the anti-AKAP serum only. Since no band corresponding to the unmodified L protein could be detected with the anti-CPMV serum, most of the Cys residues at the virus surface were conjugated to the peptide.

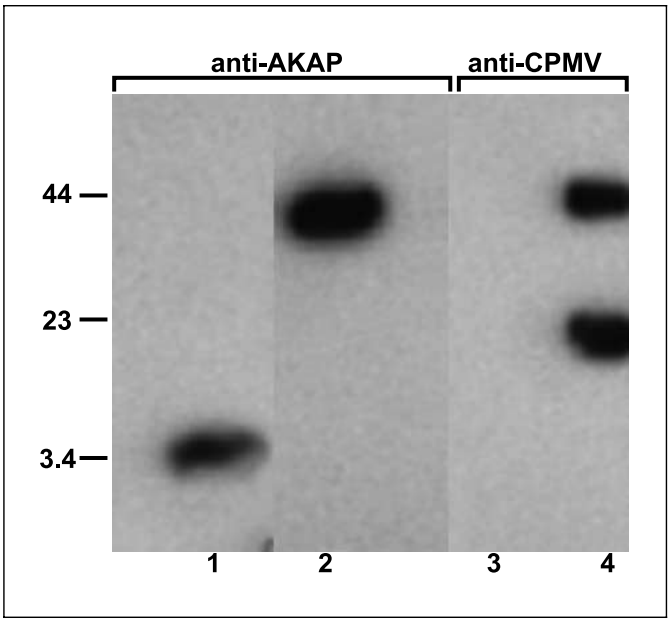

Fig. 5. Western blot analysis of the CPMV:AKAP conjugate. 200 ng of FPLC-purified virus-specific fraction (lanes 3 and 4) or 500 ng of uncoupled AKAP peptide (lanes 1 and 2) were boiled in SDS buffer and resolved under denaturing conditions on a 4–12% acrylamide SDS-PAGE gel before transfer onto nitrocellulose membranes. Probing was with polyclonal antisera specific for either AKAP (diluted 1/5,000 in 1 × PBS) or CPMV (diluted 1/2,000 in 1 × PBS). The molecular weight (in kD) of the protein bands are shown on the left-hand side.

Discussion

Given the problems caused by proteolytic processing of the inserts in the βB-βC loop of the S protein, an alternative insertion site for foreign epitopes at the virus surface was explored. In the βC′-βC″ loop of S, peptide cleavages were again shown to occur, in all positions in which insertion of the NIm-1A epitope did not compromise virus viability. However, this study has led to the identification of (1) three positions within the βC′-βC″ loop (between V42/G43, G43/D44 and D44/D45) which result in wild-type-like yields of recombinant virus and (2) an insertion strategy which leaves the insert intact, by consistently setting the cleavage point within the sequence of a C-terminal linker peptide. These new insertion points can be explored for the future presentation of foreign epitopes. Only the immunological properties of the initial chimera, D44/D45$_1$-HRV, have been analysed and found to reflect the open conformation of the NIm-1A peptide, which results in poor mimicry of the native epitope in HRV-14 [18]. Crystallographic analysis of this chimera had demonstrated that the conformation of the NIm-1A epitope differed from that adopted by the same epitope when it was cleaved upon insertion into the βB-βC loop of S [12].

Cleavage within an insert which had been expressed in the βE-βF loop of the C domain of L has also been reported, though the site was not mapped [13]. All CPMV capsid surface insertions reported so far have resulted in cleavage within the loop to which the foreign sequence had been added, with one exception. When the NIm-1A epitope was placed between amino acids P21 and A22 in the βB-βC loop of S, the recombinant loop remained essentially closed. Although this would seem to be a more favourable position for foreign peptide expression, the chimera CPMV/HRV-L1 had a tendency to form aggregates in solution [12]. The fusion of foreign sequences into surface loops of CPMV will generally result in susceptibility to proteolytic attack. It is reasonable to suggest that this phenomenon is due to the release of plant proteases during virus extraction which cleave those sequences that are vulnerable, such as the heterologous peptides which would not fold compactly with the capsid. Proteolytic processing and inability to accommodate some sequences, as was the case with the D-AKAP2 peptide, constitute limi-

tations to the chimera technology. The presence of a D-AKAP2 peptide at the virus surface would generate a general-purpose presentation platform. A peptide/protein of interest could be fused to the D/D domain of the regulatory subunit of PKA, which would bind with high affinity to D-AKAP2 peptide-coated virus, eliminating the need to produce one CPMV chimera for every display. Failure of genetic insertions of D-AKAP2 to produce viable recombinant virus led to the development of another strategy in which unique Cys residues were positioned on the virus surface by genetic engineering, followed by coupling of the peptide to the virus particles by Cys-mediated chemical cross-linking. This approach can also be exploited to present full-length protein domains, whilst anchoring Cys residues can be inserted in generic positions by oligonucleotide-directed mutagenesis.

The adoption of a multiple-facet approach combining genetic engineering, chemical cross-linking and a peptide-based protein-binding platform extends the use of CPMV from epitope presentation to that of a matrix for the attachment of peptides and proteins.

Acknowledgments

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RNA Bacteriophage Capsid-Mediated Drug Delivery and Epitope Presentation

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Key Words
MS2 virus-like particle · Chimera · Synthetic vaccine · Ricin A chain

Abstract
Objective: To use our knowledge of the three-dimensional structure and self-assembly mechanism of RNA bacteriophage capsids to develop novel virus-like particles (VLPs) for drug delivery and epitope presentation. ***Methods:*** Site-directed mutagenesis of a recombinant MS2 coat protein expression construct has been used to generate translational fusions encompassing short epitope sequences. These chimeric proteins still self-assemble in vivo into $T = 3$ shells with the foreign epitope in an accessible location. Covalent conjugation has also been used to generate RNA stem-loops attached to the toxin, ricin A chain, or to nucleotide-based drugs, that are still capable of stimulating self-assembly of the capsid in vitro. These packaged drugs can then be directed to specific cells in culture by further covalent decoration of the capsids with targeting molecules. ***Results:*** Chimeric VLPs are strongly immunogenic when carrying either B or T cell epitopes, the latter generating cytokine profiles consistent with memory responses. Immune responses to the underlying phage epitopes appear to be proportional to the area of the phage surface accessible. Phage shells effectively protect nucleic acid-based drugs and, for the toxin construct, make cell-specific delivery systems with LD_{50} values in culture sub-nanomolar. ***Conclusion:*** VLP technology has potential for therapeutic and prophylactic intervention in disease.

Introduction

Viruses are natural vectors for the movement of macromolecules, i.e. their nucleic acid genomes, across specific cellular barriers. Cell specificity and avoidance of immunosuppression require defined access to conserved external viral components, such as targeting molecules for cellular receptors, e.g. picornaviral canyons or the neuraminidase of paramyxoviruses, whilst reducing access to the molecules of the immune system. Therefore, viruses can be thought of as the ultimate molecular chemotherapeutic or prophylactic reagents.

The humble RNA bacteriophages have been amongst the most heavily studied viral systems, since they permitted analysis of many detailed cellular processes, such as translation, before the advent of modern molecular biology techniques [1]. They have also been very widely used as laboratory reagents, e.g. in in vivo molecular interaction studies [2], and have provided a major paradigm for the

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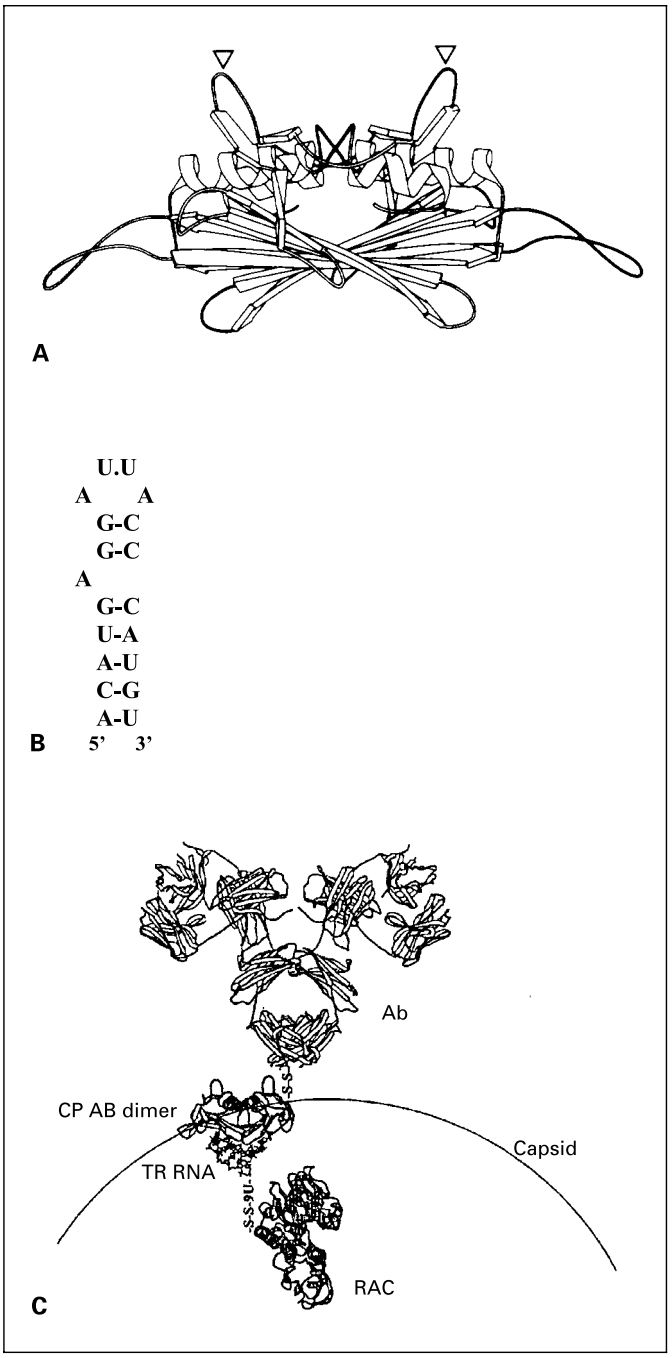


Fig. 1. The structures of the MS2 capsid and its VLPs. **A** A cartoon representation of the three-dimensional structure of the coat protein dimer oriented so that the outer part of the capsid would be towards the top of the page and the RNA towards the bottom. The short loops at the top of each subunit represent the β-hairpins where the foreign epitopes are inserted (marked by arrowheads). **B** The sequence and secondary structure of the translational operator RNA that triggers phage assembly in vitro. **C** A schematic view of an immuno-conjugate RAC VLP. Molecules are images taken from the Protein Data Bank of their X-ray structures. The curve represents the edge of the capsid drawn to scale.

understanding of sequence-specific RNA-protein recognition [3, 4]. This laboratory and our collaborators, together with the Uhlenbeck and Peabody laboratories, have made a major study of the latter aspect. At a specific point in the phage life cycle, replication of phage RNA gives way to the need to package progeny phage particles. The molecular mechanisms of both these events are connected by the same molecular recognition event. Genomic phage RNA encompasses a 19-nucleotide sequence, located at the 5′ end of the replicase cistron, that is capable of folding into a simple stem-loop structure (fig. 1). Translation of the genomic RNA leads to increasing concentrations of coat protein subunits, which exist as non-covalent dimers. These can form a specific recognition complex with the 19-nucleotide stem-loop, sequestering the start codon for the replicase cistron, leading to translational repression and thus a steady reduction in the level of replication [5]. At the same time, the RNA-protein complex formed appears to mark the phage RNA for specific encapsidation by other coat protein subunits [6, 7]. There is a great deal of specificity in this interaction, since in vivo, only homologous phage RNAs are packaged, even in mixed infections [8]. This specificity mirrors in vitro coat protein affinities for differing phage translation operators [9].

The molecular basis for the sequence specificity of the MS2 (R17) translational repression complex has been investigated using sequence variants of the RNA stem-loop (TR) [3] and chemical variants of the bases [10] and backbone residues [11] and by extensive mutagenesis of the phage coat protein [12, 13]. A major addition to our understanding has come from extensive X-ray structural studies that have now made available atomic structures for a number of wild-type bacteriophage capsids [14–16]. We have made significant use of the fact that over-expression of a recombinant coat protein gene results in in vivo self-assembly of $T = 3$ shells lacking phage RNA and the maturation protein [17]. These 'empty' capsids crystallise isomorphously with the wild-type phage particle and can be used to solve the X-ray structures of RNA operators soaked into the crystals [18]. The RNA fragments penetrate both the crystal lattice, via solvent channels, and the centre of the phage shells, via the 14-ångström pores at capsid symmetry axes, where each coat protein dimer in the capsid can then make a sequence-specific complex with a TR RNA, allowing the details of the molecular recognition to be determined by a simple difference map. This technique has now been applied to the wild-type TR operator [18], a high-affinity sequence variant [19] and a number of sequence and chemical variants [20–22], as

well as a series of RNA aptamers that appear to break the consensus rules determined by simple sequence variation experiments [23, 24]. A number of RNA-protein complexes containing protein mutants have also been studied [25]. The result is the most extensive structural database to date for a specific RNA-protein complex.

Previously, we took advantage of this extensive basic knowledge of bacteriophages to modify them for (1) the presentation of foreign epitopes [17, 26] and (2) cell-specific drug delivery [27]. In the former application, short foreign epitopes (9–27 amino acids in length) were translationally fused in frame with the coat protein such that expression and capsid assembly would result in the chimera being displayed at the tip of the N-terminal β-hairpin of the protein subunit. These are the most radially distal sites in the mature capsid [16]. The position of insertion is critical. An insertion one or two amino acid residues away from the tip results in chimeric proteins that no longer assemble [28].

The second application makes use of the TR RNA to trigger specific encapsidation. Using chemical synthesis, it is possible to prepare covalent conjugates between the TR RNA and potential drug molecules, such as the plant toxin ricin A chain (RAC). These species can still trigger the self-assembly reaction, leading to protective encapsidation of the drug. These drug packages can then be targeted to specific cell types by covalent decoration of the outside capsid surface with ligands for receptor-mediated endocytosis (RME).

Here, we describe some of our latest results with applications of these virus-like particles (VLPs) based on RNA bacteriophage capsids.

Materials and Methods

Expression and Synthesis of Macromolecules Used

MS2 phage coat protein and its chimeras were purified as in vivo assembled, largely RNA-free $T = 3$ shells from an *Escherichia coli* recombinant expression construct as described previously [17]. RNAs were synthesized by solid-phase phosphoramidite chemistry, deprotected, purified and characterised as described previously [29]. RAC variants were expressed following the procedures of Wales et al. [30]. Anti-DF3 monoclonal antibody (mAb) was a gift from Nissin Inc., Cambridge, Mass., USA.

Construction of Synthetic Virions

Synthetic virions (SVs) were constructed by reassembly of acid-disassembled recombinant phage coat protein in the presence of TR RNA or its drug conjugates. Packaged molecules were then purified by size-exclusion chromatography before the capsids were covalently cross-linked to targeting ligands, then again purified by size-exclusion chromatography. Characterisation of the resultant SVs and intermediates included the use of SDS-PAGE, Western blotting and transmission electron microscopy (TEM) and followed published procedures [27].

Table 1. Accessibility of MS2 epitopes in chimeric VLPs

Sample	Size of insert, amino acids	Percentage retained by IgG column
MS2/WT	0	100
MS2/HA	11	60
MS2/IgE	12	36
MS2/Mal	14	24
MS2/L1	22	16
MS2/L2	22	15
MS2/HIV	26	7.5

Results

Presentation and Accessibility of B Cell Epitopes

One feature that might limit the effectiveness of chimeric VLPs in epitope presentation is the response elicited to carrier epitopes rather than to the inserts they carry. Strong responses to such common epitopes would limit the number of exposures to the carrier that it would be possible to administer. RNA phage chimeras have an advantage compared to filamentous and comovirus systems because every subunit is chimeric [31]. Thus, each subunit carries the foreign epitope as the most radially distal feature of the capsid, and hence is the most accessible to molecules of the immune system. The practical consequence of this organisation can be seen during immuno-affinity purification of chimeric VLPs. Table 1 shows that as the size of the inserted epitope increases, from 11 to 26 amino acids in length, the amount of the chimeric VLP recognised by immobilised polyclonal anti-MS2 coat protein IgGs decreases. This suggests that slightly larger inserts would completely cover the underlying carrier epitopes.

In the drug delivery application [27], targeting molecules are covalently attached to the outside of the phage capsid. This modification reaction is rarely close to stoichiometric, suggesting that in these applications, carrier epitopes would induce unwanted immune responses. However, we have shown that covalent modification of surface lysines on the phage coat protein with short PEG chains (PEG3000) is reasonably efficient. If such PEGylated viruses are then exposed to anti-MS2 IgGs, as above, they partition into two clear sub-groups. The first of these is not bound at all by the antibodies, whilst the second

Table 2. Effect of adjuvant and nature of immune response with chimeric VLPs

a Effect of adjuvant

Sample	Reciprocal titres	
	minus alhydrogel	plus alhydrogel
MS2/WT	5,300 ± 100	2,500 ± 100
MS2/HA	4,500 ± 100	1,500 ± 100
KLH	600 ± 100	2,800 ± 100
KLH-HA	900 ± 100	4,900 ± 100

b Nature of immune response

Sample	IgM, %	IgG, %
MS2/WT plus	1.2 ± 0.4	83.0 ± 2.0
MS2/WT minus	0.8 ± 0.2	87.0 ± 3.0
MS2/HA plus	1.3 ± 0.4	88.0 ± 3.0
MS2/HA minus	0.6 ± 0.2	84.0 ± 1.0
KLH-HA plus	0.3 ± 0.1	81.0 ± 1.0
KLH-HA minus	0.2 ± 0.1	83.0 ± 3.0

group is. Using PEG molecules derivatised with fluorescein (Shearwater Corp., Huntsville, Ala., USA), it is possible to determine the levels of PEG attachment in these two groups. The former has >90% of its subunits modified, whilst the latter contains on average <30% PEGylated subunits (data not shown). These results suggest that it would be possible to generate 'stealth' VLPs, masked from immune recognition by a layer of PEG and targeted to specific cells by humanised ligands for RME.

Other important criteria for an epitope presentation VLP are the nature of the immune response that is elicited and whether the system needs adjuvant to be effective. As table 2 clearly shows, MS2 VLPs show significantly stronger responses in the absence of adjuvant, whereas KLH and its equivalent conjugates show the reverse behavior. Primary immune responses to bacteriophage (φX174) are reported to result in the production of IgM for a few days followed by a rise in the levels of IgG. Re-administration of the phage results in production of mostly IgG with transient IgM, possibly associated with a memory response [32]. If significant levels of IgA are observed, this is usually taken as a sign that the immunogen and/or adjuvant have caused damage to mucosal membranes. We followed the time course of Ig isotype responses to immunisation with MS2 VLPs and KLH (key hole limpet hemocyanin) conjugates, with and without adjuvant, using specific secondary antibodies. IgA was undetectable in all the regimes used. IgG represented the bulk of the immune response (>80%) at all times. IgM was detectable 21 days after immunisation, but only in the MS2-HA chimera was there any increase with time.

Presentation and Accessibility of T Cell Epitopes

Another putative role of VLPs in epitope presentation is to display T cell epitopes. We tested this idea with the MS2 coat protein by fusing it with a putatively protective malaria T cell epitope. T1 is a 24-amino acid sequence (LTMSNVKNVSQTNFKSLLRNLGVS) found in the N-terminal non-repeat region of the 200-kD immuno-dominant liver stage antigen-1 (LSA-1) of the malaria parasite *Plasmodium falciparum,* which is responsible for at least 3 million human deaths each year. Recognition of LSA-1 T1 by inhabitants of geographically distinct malaria-endemic regions has been suggested as a surrogate marker of acquired resistance, and it is therefore of prime importance as a malaria vaccine candidate [33].

We generated an MS2-T1 chimera by insertion into the β-hairpin loop as above [17]. Chimera expression was approximately 40% of wild-type levels. BALB/c mice were then immunised with wild-type or chimeric MS2 protein by primary (day 0) and secondary (day 37) subcutaneous inoculation of 50 μg of total protein resuspended in PBS and emulsified in Freund's adjuvant [34]. Controls received adjuvant alone or were naive. On day 51 (14 days after boosting), single-cell suspensions of splenocytes from mice were restimulated ex vivo with homologous peptide or T and B cell mitogens and assayed for proliferation and production of type 1 and 2 cytokines and their corresponding Ig isotypes. BALB/c (H-2^d) mice were used, as the T1 peptide contains a putative MHC class I $H2K^d$ binding epitope [35]. Unlike most vaccine candidate antigens, LSA-1 has no known homologue among the murine malarias; hence, its immunogenicity in preclinical studies must by necessity be examined in mice of an appropriate genetic background.

Cellular proliferation increased between days 3 and 6 after stimulation of ex vivo, suggesting that this response was peptide specific and not due to any residual *E. coli*-derived mitogenic contamination following protein purification. Splenocytes derived from MS2-T1-immunised mice elicited significant LSA-1 T1-specific proliferation [34].

The wild-type MS2 capsid appeared to elicit both humoral and cellular immune responses, observed as a predominance of the type 2 cytokines interleukin (IL)-4 and IL-10 but with a mixed profile of Ig isotypes. In contrast, the LSA-1 chimera stimulated a type 1-polarised

response, with significant up-regulation of IL-12, interferon (IFN)-γ and tumour necrosis factor-α and down-regulation of IL-4 and IL-10 [34]. The predominant MS2-T1-specific antibody class following recombinant T1 immunisation was the type 1-regulated IgG2a, suggesting that a polarised response is elicited in vivo.

The pronounced production of IFN-γ by splenocytes from MS2-T1-immunised mice upon T1 peptide stimulation has particular significance, since this is recognised as a major host defence mechanism against liver stage malaria [36]. The predicted CD8+ T cell epitope for BALB/c mice within the LSA-1 T1 peptide sequence most probably elicited the IFN-γ production by MS2-T1-immunised splenocytes ex vivo, as, when the cells were separated into specific T cell fractions, the major source of IFN-γ was the CD8+ T cell population (fig. 2 and data not shown).

We believe that CD8+ T cell release of IFN-γ is a critical component of immunity induced by liver stage malaria, and that successful vaccination of humans with vaccines designed to elicit protective immunity will require induction of specific CD8+ T cells that home to the liver [33]. Moreover, these data not only indicate that further evaluation of MS2 coat protein as a vector for malaria T cell epitopes is merited but also further validate RNA phage capsid display of immunogenic peptides.

These data for the presentation of both B and T cell epitopes suggest that phage VLPs would be a useful system for defined epitope presentation.

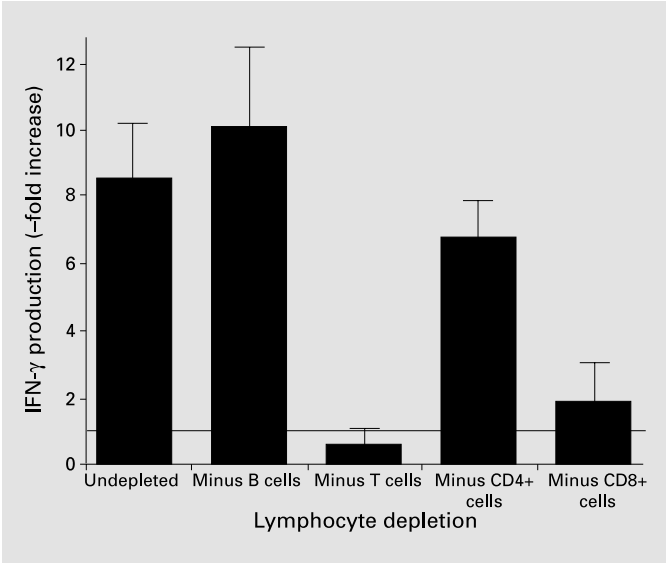


Fig. 2. Cytokine production after challenge by MS2-T1 chimera. IFN-γ production by splenocytes from mice immunised with MS2-T1 following ex vivo restimulation with LSA-1 T1 peptide (25 μg/ml), as previously described [34]. Lymphocyte subset depletion was performed by immunomagnetic cell sorting to >98% purity. Data are -fold increases in cytokine secretion over that of splenocytes from identically immunised mice not restimulated ex vivo (by convention, value = 1.0, depicted as a horizontal line). Data are representative of four similar experiments.

Targeting and Toxicity in Drug Delivery

There are a growing number of potential therapeutic molecules that are larger than the traditional small-molecular-weight, orally available drugs. These include oligonucleotides, such as anti-sense reagents, peptides and natural products. Clinical application of such species presents a number of important challenges, such as the control of unwanted side effects, prevention of immune responses, access to the molecular targets and control of pharmacokinetics, to name but a few. In principle, targeted delivery strategies offer elegant methods to overcome many of these difficulties. For instance immuno-conjugates have been developed to target toxic species to cell surface markers of particular cell types [37, 38].

VLPs offer an even more refined version of the same approach but with significant technical advantages. In the MS2 bacteriophage system, we have developed an approach we describe as the synthetic virion (SV) [27]. Macromolecular drug candidates are encapsidated specifically and efficiently inside a protective coat protein shell by first covalently linking them to the RNA operator that triggers in vitro capsid assembly from disassembled coat protein dimers. These drug packages can then be directed toward specific cells by covalent decoration of the outside surface with ligands for RME. Such SVs can inherently carry large drug loads, protect the cargo from interaction with molecules from the immune system and control the pharmaco-kinetics of quite disparate molecular entities, and are readily amenable to combination drug therapy. They also offer a significant advantage over many smaller targeting systems in that arrays of targeting ligands generate chelate and local concentration effects that significantly enhance their uptake at the target. These features mimic those of natural viruses, hence the term SV. One major drawback is the potential immune response that such SVs could elicit. However, the speed at which ligands for RME are often cleared from the bloodstream and the possibility of using PEGylation, as described above, to create stealth SVs, offer clear directions for overcoming or avoiding problems in these areas. SVs therefore offer clear and potentially important features not readily accessible to liposomes, which are their major equivalent competitors in cell-specific drug delivery.

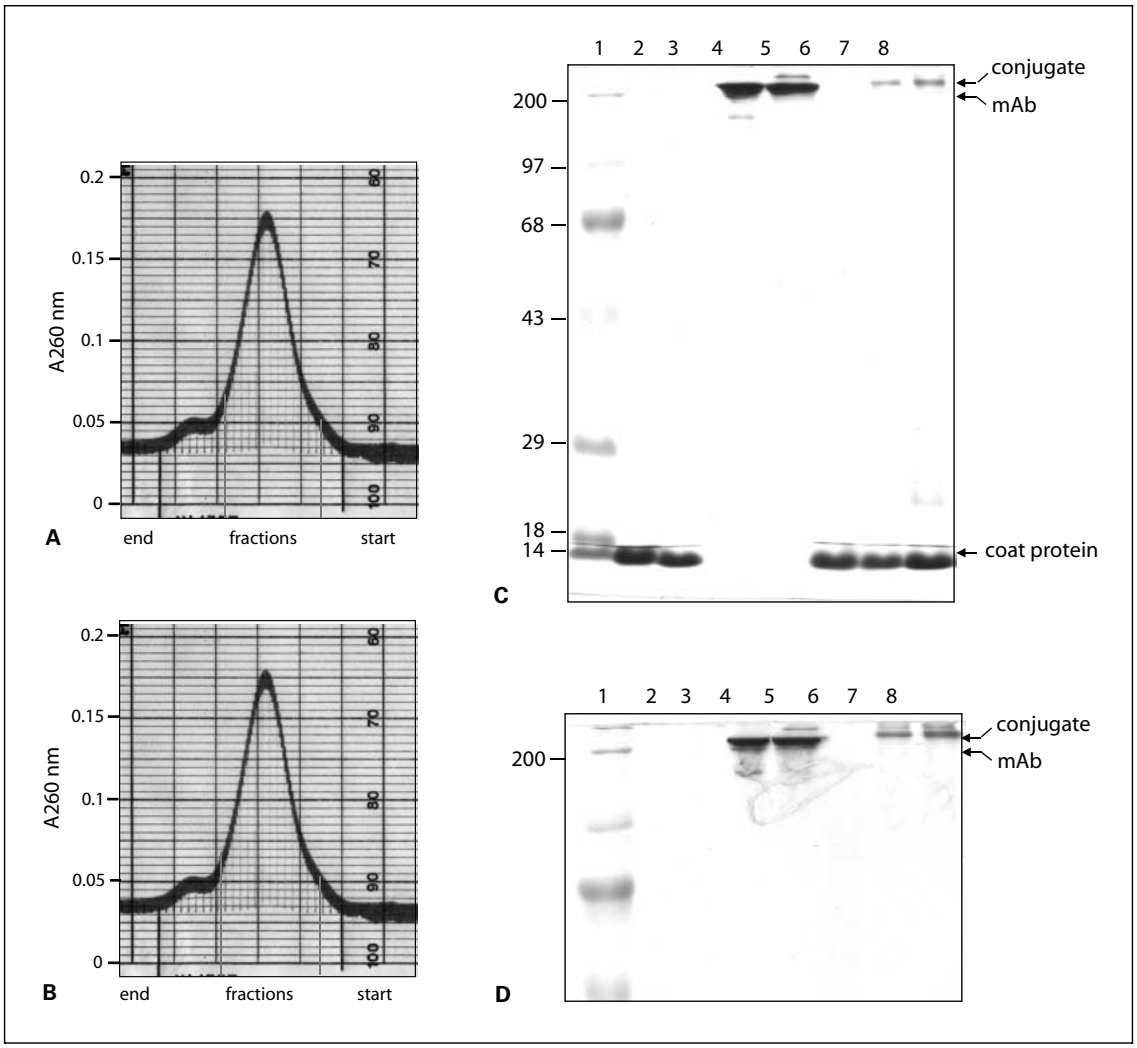


Fig. 3. Characterisation of immuno-conjugate SVs. **A** Elution profile of 5fdU/anti-DF3-SV from a Sepharose CL4B column. **B** Elution profile of anti-DF3-capsid from a Sepharose CL4B column. **C** 10% (w/v) SDS-PAGE gel stained with Coomassie blue. Lane 1: protein molecular weight markers (sizes in kD); lane 2: 8 pmol of capsid; lane 3: 8 pmol of capsid-SATA; lane 4: 13 pmol of anti-DF3; lane 5: 13 pmol of anti DF3-SMCC; lane 6: encapsulated 5fdU-TR-5fdU oligonucleotide (8 pmol of capsid); lane 7: 7 pmol of capsid-anti DF3; lane 8; 7 pmol of 5fdU/anti-DF3-SV. **D** Same as **C**, except that the gel was run for longer to resolve the SV complex from the mAb.

Formation of Immuno-Conjugate SVs

Our previous SV constructs employed transferrin (Tfn) as a covalently attached targeting ligand, cross-linked to the capsid with the heterobifunctional reagent sulpho-SMCC (sulfo-succinimidyl(4-/iodoacetyl)aminobenzoate). Introducing surface thiol groups via lysine modification with the reagent SATA (*N*-succinimidyl *S*-acetylthioacetate) (Pierce) facilitated this linkage. In principle, however, SVs could be targeted to specific cell types by a very wide variety of molecules. In order to illustrate this point, we have constructed an SV equivalent of immuno-conjugates by covalent attachment of mAbs. The mAb chosen for these experiments was directed against a cell surface antigen associated with human breast tumours.

The DF3 antigen is a glycoprotein present on apical borders of secretory mammary epithelial cells, in the cytosol of less differentiated malignant cells, and can be detected in milk [39]. DF3 expression correlates with the degree of tumour differentiation and increases when breast carcinoma cells are induced to differentiate [40]. Immunoassays have demonstrated that DF3 antigen levels are significantly elevated in the plasma of patients with metastatic breast

cancers and this has provided a useful marker for monitoring the clinical course of breast cancer patients [41]. The DF3 antigen in human breast tumours and milk is comprised of a mucin-like glycoprotein of 310 kD, which was later characterised as a core protein of 160 kD [42, 43].

Before constructing the SV with the mAb as a targeting ligand, it was necessary to compare different cross-linking strategies to determine the best conditions for conjugate formation. In order to conserve precious anti-DF3 mAb, these trials were conducted with commercially available anti-Tfn mAb. Three different cross-linking conditions were compared, using SATA, SMCC, sulpho-SMCC and SPDP [*N*-succinimidyl 3-(2-pyridyldithio)propionate) (all from Pierce) in different combinations. The mAb is approximately twice the size of the Tfn ligand used previously, so there were concerns about the size of the resultant SV, its solubility and whether it would aggregate during construction or purification. Therefore, it was decided to use a lower molar ratio of targeting ligand to capsid (5:1 vs. 10:1) than used previously for Tfn. The cross-linking studies showed that there were differences in efficiency with the differing approaches and that the same protocol as that used for Tfn, namely SATA activation of capsids followed by reaction with SMCC-modified mAb, yielded the highest levels of derivatisation (data not shown) [44].

These cross-linking conditions were applied to the anti-DF3 mAb and capsids that had been soaked with the TR RNA carrying poly-5-fluoro-2′-deoxy-uridine (5fdU) extensions (see below). The SVs were then purified by size-exclusion chromatography and characterised. In order to determine whether the mAb had been attached to the capsids successfully, the SV samples were analysed by non-reducing SDS-PAGE. The 5fdU/anti-DF3-SV sample contained coat protein and a high-molecular-weight species (fig. 3C, lane 8), which was also present in the mAb-capsid conjugate control (lane 7). On a gel run longer for better resolution, this high-molecular-weight band in the conjugate samples (fig. 3D, lanes 7, 8) could clearly be resolved from the mAb bands (lane 5). Western blotting with anti-capsid antiserum demonstrated that the SV and capsid-anti-DF3 mAb samples (fig. 3 C, D, lanes 6, 7) contained coat protein monomer and dimer, possibly as a di-sulphide-linked species, as well as a high-molecular-weight conjugate that was not present in the sample containing just the encapsidated oligonucleotide (lane 5). Probing with anti-mouse-fluorescein isothiocyanate-labelled antiserum detected this species but not the coat protein, confirming that it contained the anti-DF3 antibody as a conjugate (data not shown). TEM of the resultant SV samples suggested that they retained their normal capsid structure.

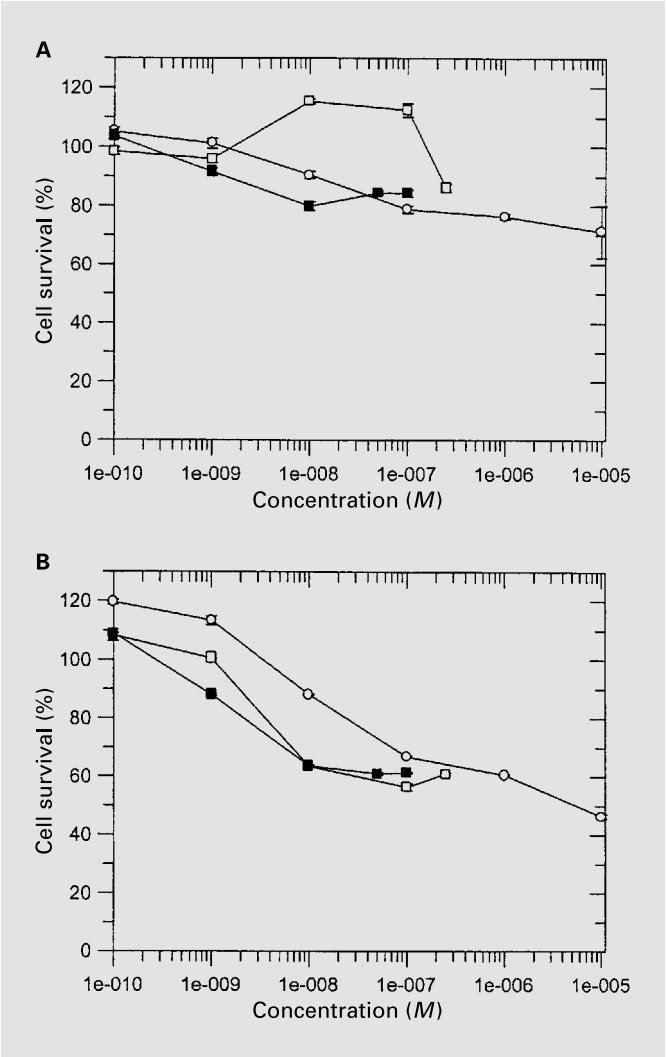
Encapsidation and Protection of Nucleotide-Based Drugs

Previously, we have shown that it is possible to soak crystals of largely RNA-free MS2 $T = 3$ capsids produced in *E. coli* from a recombinant coat protein expression vector with RNAs encompassing the TR RNA stem-loop [18–25]. These RNAs are able to penetrate both the solvent channels within the crystals and the approximately 14-ångström pores located at the particle symmetry axes. Once inside the capsids, the TRs bind to every coat protein dimer in the shell, facilitating determination of the X-ray structures of the sequence-specific RNA-protein complexes. We reasoned that it might be possible to use this route rather than in vitro self-assembly to prepare encapsidated nucleic acid-based drugs such as antisense reagents or anti-metabolites.

In order to test this hypothesis, we chose the nucleotide analogue 5-fluorouridine (5fU). This is a well-characterised reagent, familiar to physicians and readily available, yet it suffers from rapid clearance from the circulatory system, resulting in high-dose regimes and unpleasant side effects [45]. A TR oligonucleotide derivative was synthesised via our standard methods [29], incorporating 5′ and 3′ extensions of five 5fdU using commercially available 5fdU phosphoramidites. This was soaked into empty capsids in solution. These encapsidated 5fdU-capsids were then conjugated to anti-DF3 mAb as described above.

The cytotoxicity of the 5fdU/anti-DF3-SVs was tested against ZR-75-1 breast carcinoma cells, which express the DF3 antigen at high levels. The 5fdU/anti-DF3-SVs were slightly more toxic than the free 5fdU oligonucleotide at the lower concentrations (fig. 4A), suggesting that the SV enhanced the toxicity of the oligonucleotide by protecting it from degradation by nucleases in the serum or by enhancing uptake by the ZR-75-1 cells. They were considerably more toxic than the encapsulated oligonucleotide which had been mixed with DF3 mAb but not covalently linked to it, supporting the idea that the mAb was effective at increasing the level of uptake.

In Caov-3 cells (fig. 4B), which do not over-express the DF3 antigen, there was very little difference between the 5fdU/anti-DF3-SVs and the control packaged oligonucleotide, suggesting that the targeting mAb had no targeting effect. Both the capsid-protected samples were more toxic than the free oligonucleotide across the concentration range, suggesting that encapsulation of the oligonucleotide conferred some protection from degradation to the oligonucleotide and that there was non-specific uptake of capsids in these control cell lines.



Improving Toxicity

Previously, RAC carrying the endoplasmic reticulum retention sequence, -KDEL-, at the C-terminus was shown to be considerably more toxic than the native molecule, the vast majority of which is re-exported from the cell [30]. MS2-TR-RAC(KDEL) particles were generated using the methods described previously [27]. These were then used to produce SVs containing human Tfn as the external targeting ligand for RME. The SVs were partially purified over Sephadex G150. The elution profile of the G150 column contained four peaks, of which the fastest to elute was the Tfn-SV, as judged by SDS-PAGE, TEM and Western blotting. Addition of Fe^{2+} ions to the eluted Tfn-SVs was carried out as described previously [27, 46].

The cytotoxicity of the Tfn-SVs was then tested against HL60 cells, which express the Tfn receptor. Figure 5 shows that the Tfn-SVs containing RAC(KDEL) were significantly more toxic than either RAC(KDEL) or RAC alone, as expected, with an LD_{50} of approximately 5×10^{-11} *M*. The RAC(KDEL) Tfn-SVs were roughly 30 times more cytotoxic than the equivalent RAC SV lacking the KDEL sequence. The cytotoxicity of immuno-com-

Fig. 4. Cytotoxicity of 5fdU/anti-DF3-SVs. ZR-75-1 (**A**) and Caov-3 (**B**) cells (1×10^4 cells/well) were treated with dilutions of 5fdU/anti-DF3-SVs or control reagents. After 69 h, the cells were assayed for dehydrogenase activity. ○ = 5fdU-TR-5fdU oligonucleotide; ■ = 5fdU/anti-DF3-SV; □ = encapsulated 5fdU-TR-5fdU oligonucleotide plus anti-DF3 mAb. Data points represent triplicate determinations. The error bars indicate the standard deviation of the data from the mean.

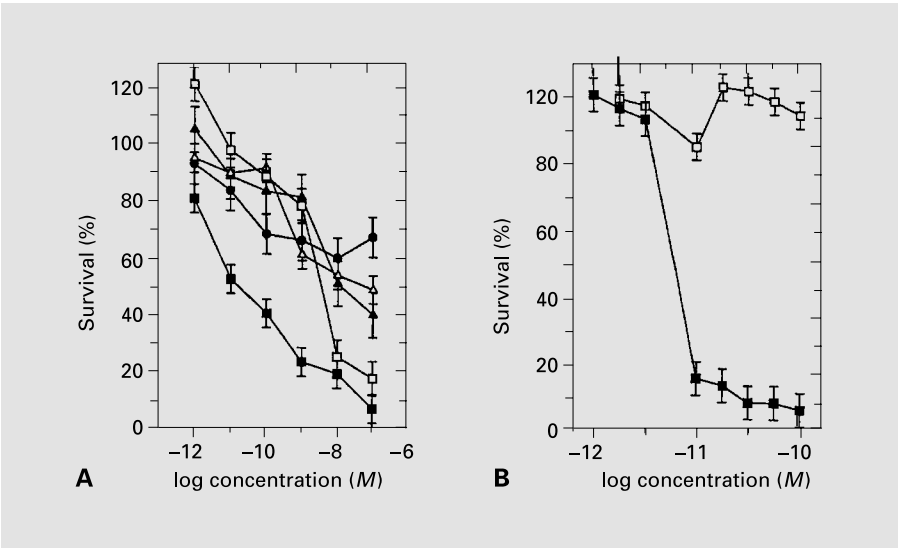


Fig. 5. Cytotoxicity of RAC(KDEL) SVs. **A** Comparison of MS2-TR-RAC-Tfn-SV and MS2-TR-RAC(KDEL)-Tfn-SV cytotoxicity on HL60 cells expressing the Tfn receptor. ■ = MS2-TR-RAC(KDEL)-Tfn; □ = MS2-TR-RAC-Tfn; Δ = RAC(KDEL) only; ● = MS2-TR-RAC(KDEL) plus non-cross-linked Tfn. **B** Cytotoxicity of immuno-complexed MS2-TR-RAC(KDEL)-Tfn SV on Pu518 cells, which express the Ig Fc receptor but not the Tfn receptor. ■ = MS2-TR-RAC(KDEL)-Tfn plus anti-MS2 antibodies; □ = MS2-TR-RAC(KDEL)-Tfn only.

plexed Tfn-SV containing RAC(KDEL) was also tested on Pu518 cells, which express the Fc receptor, as described previously [27]. Despite the presence of attached Tfn ligands, the immuno-complexed material was significantly more toxic than the Tfn-SV alone, having an LD_{50} of approximately 2.4×10^{-12} *M*, whereas the non-cognate SV was not toxic in the concentration range tested (fig. 5B).

Discussion

Our experiments suggest that VLPs based on RNA bacteriophage capsids are readily produced, carrying foreign epitopes as the most radially distal feature of a protein particle lacking genetic material. These particles have useful properties as immunogens and do not need adjuvants to provoke strong responses. Previously, we showed that the responses generated are sensitive to the constrained conformation of the inserts [17]. The use of larger insets or PEGylation, or a combination of these approaches, may overcome a major problem associated with protein carriers of foreign epitopes, namely the generation of an immune response directed against the carrier. Further work is now required to formulate the best approach for the use of such VLPs as synthetic vaccines.

In an alternative application of phage capsids, we have shown their utility in the efficient packaging of large proteins or nucleic acids as potential drug delivery vehicles. These drug packages can then be directed towards specific cells via covalent decoration with ligands for RME. All targeted therapies require control of the avidity and discrimination of their interactions with cellular targets in order to minimise inappropriate interactions. The chelate effects of VLPs are very useful in this respect, and here we have reported the generation of an immuno-conjugate VLP. A similar construct for polyoma VLPs has recently been reported [46]. The toxicity of SVs can be improved by increasing the potency of the drug delivered, in this case a toxin variant. In other experiments not reported here, we have shown that reagents known to increase the permeability of the endosomal membrane also have the same effect [Wu et al., in preparation]. The 5fdU experiment described here shows how the SV system can easily be adapted to delivery versions of traditional low-molecular-weight drug molecules. The interpretation of the experiment shown in figure 4 is complicated, because nuclease action on the oligonucleotide releases monomeric 5fdU, which is also toxic to the cells. However, we have also shown that antisense oligodeoxynucleotides can be delivered via SVs [Wu et al., unpubl. results].

In principle, the use of the phage system might permit a form of phage display of targeting epitopes in the β-hairpin analogous to those in filamentous systems [47]. We have generated such genetic fusions and inserted them into a live phage cloning system. However, recombination rapidly results in the deletion of the inserted region. Recently, van Meerten et al. [48] showed that this instability is linked to the structure of the genomic RNA and that stable inserts can be generated with appropriate substitution to preserve elements of the RNA secondary structure.

The work to date with drug delivery suggests that RNA phages offer simple and highly flexible systems for the development of targeted drug delivery. A problem associated with a protein-based drug delivery system is the likely immune response to the carrier. The PEGylation experiments described above suggest an obvious route to mitigate this problem and create stealth SVs.

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